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Regenerative Medicine and Tissue Engineering

Edited by José A. Andrades

REGENERATIVE MEDICINE AND TISSUE ENGINEERING

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Notice

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Meet the editor

José A. Andrades completed his doctorate in Cellular Biology by the University of Málaga (Spain). He has been a Post-Doctoral fellow and Visiting Professor at Children's Hospital Los Angeles, University of Southern California (Los Angeles, CA), as well as at the Skeletal Research Center, Case Western Reserve University (Cleveland, OH). He currently has a faculty position at the Department of Cell Biology, Genetics and Physiology (Laboratory of Bioengineering and Tissue Regeneration) at the University of Málaga. Dr. Andrades leads grants, and is author of scientific publications, focused on the study of the chondro-osteo-tendinogenesis by using mesenchymal stem cells, growth factors with specific molecular domains, and different biomaterials, for skeletal tissue engineering. The group has developed patents on cellular procedures that allow regeneration in bone/articular cartilage lesions of live animals. Currently, Dr. Andrades is coordinator of the Cellular Therapy Program NACRE (New Approaches for Cartilage Regeneration) in the CIBER-BBN. He belongs to the TerCel Network, and to the NanomedSpain, in which consortia develops collaborations with several groups of physicians for the clinical translation of their studies.

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Preface

Few events in science have captured the same level of sustained interest and imagination of the nonscientific community as Stem Cells, Tissue Engineering, and Regenerative Medicine. Each of us, scientist or not, is related to someone who has diabetes, arthritis, cardiac failure, Parkinson's, Alzheimer's, or other debilitating diseases. The fundamental concept of Tissue Engineering and Regenerative Medicine is appealing to scientists, physicians, and lay people alike: to heal tissue or organ defects that the current medical practice deems difficult or impossible to cure.

The term "stem cell" appeared in the scientific literature as early as 1868 in the work of the eminent German biologist Ernst Haeckel. Haeckel, a supporter of Darwinian evolution, developed a number of phylogenetic trees to represent the evolution of organisms from common ancestors and called these trees *Stammbaume* ("stem trees"). In this context, he used the term *Stammzelle* ("stem cell") to describe the ancestor unicellular organism from which he presumed all multicellular organisms evolved. In 1868, Ernst Neumann suggested that hematopoiesis occurs in bone marrow. He used the term "stem cell" to refer to the common precursor of the blood system in 1912. The debate about the existence of a common hematopoietic stem cell continued for several decades until definitive evidence was provided in 1961 by two Canadian scientists, James Till and Ernest McCulloch. In a quite astonishing discovery, Kazutoshi Takahashi and Shinya Yamanaka of Kyoto University in Japan in 2006 for the first time turned adult mouse skin fibroblast cells into pluripotent cells, and these are now referred to as induced pluripotent stem (iPS) cells. The discovery of iPS cells turned the field of nuclear reprogramming upside down. This work was extended and further confirmed by several groups that generated iPS cells from individuals with various neurodegenerative diseases, raising the hope of cell replacement therapy and making personalized medicine a reality. Although Yamanaka's technologies can generate living animals, we do not know the molecular mechanisms underlying these two strategies. The potential of iPS cell technology in biology and medicine is enormous; however, it is still in its infancy, and there are many challenges to overcome before various applications can be used successfully.

The term "tissue engineering" was first used by Eugene Bell of MIT in 1984, and later was also used extensively by Wolter and Meyer in 1984. Tissue engineering combines cells, engineering, and materials methods with suitable biochemical and physiochemical factors to improve or replace biologic functions. In other words, it deals with the repair or replacement of portions of or whole tissues such as bone, cartilage, tendon, blood vessels, bladder, skin, and artificial organs. According to Robert Langer and Joseph Vacanti, it "applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ." Powerful developments in

the multidisciplinary field of tissue engineering have yielded a novel set of tissue replacement parts and implementation strategies. Scientific advances in biomaterials, stem cells, growth and differentiation factors, and biomimetic environments have created unique opportunities to fabricate tissues in the laboratory from combinations of engineered extracellular matrices (scaffolds), cells, and biologically active molecules.

Regenerative medicine is a new branch of medicine that attempts to change the course of chronic disease, in many instances regenerating failing organ systems lost due to age, disease, damage, or congenital defects. The term "regenerative medicine" was first referred to in 1992 by Leland Kaiser and then popularly used by William Haselstine of Human Genome Sciences. The term regenerative medicine is often used synonymously with tissue engineering, although those involved in regenerative medicine place more emphasis on the use of stem cells to treat diseases using cell therapies or transplantation methods. This field holds the promise of regenerating damaged tissues and organs in the body by stimulating previously irreparable organs to heal themselves. Regenerative medicine also empowers scientists to grow tissues and organs in the laboratory and safely implant them when the body cannot heal itself. This area is rapidly becoming one of the most promising treatment options for patients suffering from tissue failure.

The selected articles of this book of *Regenerative Medicine and Tissue Engineering* fairly reflect the state of the art of these two disciplines at this time as well as their therapeutic application. It covers numerous topics, such as stem cells, cell culture, polymer synthesis, novel biomaterials, drug delivery, therapeutics, and the creation of tissues and organs.

This text consists of 33 chapters, grouped into 4 sections. Most of the chapters are written by experts in the field from academia and industry. The goal is to have this book serve as a reference for graduate students, post-docs, teachers, scientists and physicians, and as an explanatory analysis for executives in biotech and pharmaceutical companies. I hope that this compendium is of great benefit to you in your work, and also will provide a prologue to the field for both newcomers and those already active in the field.

Many people have contributed to making our involvement in this project possible. We are extremely thankful to all of the contributors to this book, without whose commitment this book would not have been possible. Many people have had a hand in the preparation of this book. We thank our readers, who have made our hours putting together this volume worth it. We are indebted to the staff of INTECH open science, and in particular Danijela Duric for her generosity in giving time and effort throughout the editing of this book. This book is dedicated to our patients and to the memory of all experimental animals who contribute daily with their donation to develop of the science we make.

José A. Andrades
University of Málaga
Spain

To my mother María Luisa
To the memory of my father Julián
To my wife Eva

Section 1

Stem Cells in Regenerative Medicine

Chapter 1

Placenta-Derived Stem Cells as a Source for Treatment of Lung and Liver Disease in Cystic Fibrosis

Annalucia Carbone, Stefano Castellani,
Valentina Paracchini, Sante Di Gioia,
Carla Colombo and Massimo Conese

Additional information is available at the end of the chapter

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1. Introduction

In the first part of this chapter we will summarize the main clinical aspects of cystic fibrosis as well as the pathophysiology of lung and liver diseases, with particular reference to the role of airway and biliary duct epithelia, where the cystic fibrosis gene is expressed. In the second part we will describe the main features of placenta-derived stem cells and their potential use for the treatment of lung and liver diseases in cystic fibrosis.

1.1. Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease of epithelia in the lung, liver, pancreas, small intestine, reproductive organs, sweat glands and other fluid-transporting tissues [1, 2]. In Caucasians the disease affects about 1 in 2500 live births and is the most common eventually lethal genetic disease [3]. The cause of CF is different mutations in the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene, the product of which is a protein expressed in the apical membrane of most epithelia. This membrane protein is a cyclic AMP (cAMP) regulated chloride (Cl^-)-channel involved in different regulatory processes of the cell, *e.g.* both transcellular and paracellular ion and water transport [1, 4].

Chronic progressive obstructive lung disease and pancreatic insufficiency are the main clinical symptoms of CF, where pulmonary disease is the major cause (95%) of morbidity and mortality [5]. However, liver disease is also increasing as the life span of these individuals becomes longer.

The succession of events leading from the defective CFTR to the clinical symptoms is not completely understood. However, it is obvious that the abnormal ion transport with hyper-absorption of Na^+ and impaired Cl^- and $HCO3^-$ secretion in airway epithelial cells and cholangiocytes leads to a disturbance of the fluid lining the airways and the bile ducts [6-10].

1.1.1. The CFTR gene

The *CFTR* gene was identified in 1989 and this has sharply accelerated the research on CF. The gene, which is situated on the long arm of human chromosome 7 (7q31.2), spans approximately 250 kilobases (kb) of nucleotide sequences together with its promoter and regulatory regions. The 27 exons form a 6.5 kb long coding sequence, which is capable of encoding a protein of 1480 amino acids [11].

The *CFTR* gene product is not limited to the cells of epithelial origin. In fact, *CFTR* mRNA transcripts and/or CFTR protein have been demonstrated in lung fibroblasts, blood cells, hematopoietic stem/progenitor stem cells (HSPC), alveolar macrophages, and smooth muscle cells [12-14]. In addition to its typical plasma membrane location, CFTR was also found in membranous organelles such as lysosomes of alveolar macrophages [15] and in both apical and basolateral membrane of the sweat duct [16].

Although over 1,900 different mutations in the *CFTR* gene are known (Cystic Fibrosis Mutation Database, http://www.genet.sickkids.on.ca/cftr/Home.html), approximately 66% of the patients worldwide carry the F508del mutation (a deletion of three nucleotides that results in a loss of phenylalanine at position 508 of the CFTR protein) with somewhat higher prevalence in Western Europe and USA [17]. This type of mutation causes an incorrectly assembled CFTR protein resulting in endoplasmatic reticulum (ER) retention and degradation of the protein [18] as well as defective regulation [19]. Patients homozygous for F508del usually have more pronounced clinical manifestations compared to heterozygotes and genotypes without F508del [20-22] although these differences are highly variable [23].

1.1.2. The CFTR protein

Based on the amino-acid sequence and its structure, CFTR is identified as a member of the superfamily of ATP-binding cassette (ABC) transporters. However, among the thousands of ABC family members, only CFTR is an ion channel [24, 25]. ABC transporters are ubiquitous in the entire animal kingdom due to their role in coupling transport to ATP hydrolysis. They also are involved in many genetic diseases [26]. Like other ABC transporters CFTR contains two membrane-spanning domains (MSDs), two hydrophilic nucleotide-binding domains (NBDs) located at the cytoplasmic site of the protein, and, as a unique feature among ABC transporters, a regulatory domain (R domain) located between NBD1 and MSD2. The R domain contains several consensus phosphorylation sites for protein kinases A (PKA) and C (PKC) [27]. The opening and closing of the CFTR Cl^- channel is tightly controlled by the balance of kinase and phosphatase activity within the cell and by cellular ATP levels [28]. Activation of PKA causes the phosphorylation of multiple serine residues within the R domain leading to conformational changes in this domain [29] relieving its inhibitory functions on CFTR

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channel gating [30]. Once the R domain is phosphorylated, channel opening requires binding of cytosolic ATP. NBD1-NBD2 dimerization induces channel opening, whereas ATP hydrolysis at the NBD2 induces dimer disruption and channel closure [24, 31, 32]. Finally, channel activity is terminated by protein phosphatases that dephosphorylate the R domain and return CFTR to its quiescent state [28].

Besides its cAMP-induced chloride channel function, CFTR is reported to have important regulatory functions on other ion channels and transporters. Below some of these interactions are presented: $HCO3^-$ is conducted from the cell into the lumen [33] through reciprocal regulatory interactions between CFTR and the SLC26 chloride/bicarbonate exchanger [34] and loss of this mechanism contributes to both airway and pancreatic-duct disease in CF [33, 35]. CFTR enhances ATP release by a separate channel [36], not yet identified [37]. This CFTR mediated release, although debated, is thought to be stimulated by hypotonic challenge to strengthen autocrine control of cell volume regulation through a purinergic receptor-dependent signalling mechanism [36, 37]. Furthermore, transport of glutathione is directly mediated by CFTR, which is essential for control of oxidative stress [38]. The interaction between CFTR and epithelial sodium channel (ENaC) is of crucial importance for lung disease development (see below). CFTR downregulates calcium-activated chloride channels (CaCC) [39], and stimulates outwardly rectifying chloride channels [40]. Other channels regulated are the volume-regulated anion channel [41] and ATP-sensitive K_{ATP} channels such as inwardly rectifying outer medullary potassium channels [42].

Regulatory sites on NBD1 interact with several of the above processes. For example, NBD1 contains a CFTR-specific regulatory site that downregulates ENaC. This regulatory site is also needed for CFTR-mediated interactions with other transporting membrane proteins [1, 43]. Several studies also have identified a short stretch of amino acids (-DTRL-) at the COOH terminal end, forming a PDZ binding domain [1, 44]. This PDZ binding domain interacts with different PDZ-domain-containing proteins, anchors CFTR to the cytoskeleton and stimulates the channel activities through downstream signaling elements [44, 45].

2. The airway epithelium

The airway epithelium is a target for potentially noxious substances and pathogens. It plays a critical role in maintaining a sterile undamaged airway and also separates the connective tissue as well as the smooth muscle from the airway luminal contents. In addition to its barrier function, the airway epithelium has a regulated fluid and ion transport together with a secretory function, although its function is mainly absorptive [46]. It can produce mucus, and can release mediators of the immune system such as lysozyme, lactoferrin, mucous glycoprotein, immunoglobulins, chemokines, cytokines, lectins and β-defensin (cationic antimicrobial peptides) [47, 48].

Furthermore, the airway epithelium produces antioxidants such as glutathione and ascorbic acid [49]. Aside from these protective functions it also regulates the airway physiology via

production of smooth muscle relaxant factors such as prostaglandin E_2, nitric oxide and enzymes, which catabolize smooth muscle contractile agonists [50, 51].

In normal human airways the surface epithelium is on average 50 μm thick and rests on a basement membrane. The epithelium in the major bronchi and proximal bronchioles is ciliated pseudostratified with the main cell types: ciliated and secretory columnar cells, and underlying basal cells. In addition, immune cells, inflammatory cells and phagocytic cells migrate to and remain within the epithelium [52].

More distally, in the terminal bronchioles, the epithelium changes towards a simple ciliated columnar and, finally, to simple cuboidal epithelium with ciliated and non-ciliated cells (Clara cells) [53]. In addition brush cells (columnar with microvilli only) have been identified in the respiratory tract from nose to alveoli [54]. Scattered along the respiratory tree, various progenitor niches are present in the airway epithelium [55].

It has been widely accepted that acinar gland serous cells are the predominant site for CFTR expression in the human large airways, arguing for a dominant role of submucosal glands in the volume regulation of airway surface liquid (ASL) and CF [56-59]. However, these findings have later been debated. It has been demonstrated that normal (but not the F508del) surface airway epithelia express CFTR in every ciliated cell, also in glandular ducts, with decreased expression towards the distal airways. This suggests a key role for the superficial epithelium in the initiation of ASL volume depletion and as the site for early disease [60]. It also supports a role for CFTR in regulating glandular secretion homeostasis, but predominantly in the submucosal ducts rather than in the serous acini as was earlier proposed.

2.1. Ion and water transport in airway epithelium

Net vectorial fluid transport depends critically on ENaC and CFTR operating in concert with the paracellular and transcellular pathways [61].

Fluid absorption is mainly controlled by the transport of Na^+ through apical ENaC, which is also the dominant basal ion transport process. *Fluid secretion* is regulated by cell-to-lumen movement of Cl^-, via CFTR, CaCC and volume regulated chloride channel, and/or $HCO3^-$ via the interactions between CFTR and the SLC26 channel. In both cases the transport occurs along the electrochemical gradient and the movement of counterions likely takes place predominantly through leaky tight junctions [61].

Over the basolateral membrane a Na^+ gradient is maintained by the Na^+-K^+-ATPase, which pumps 3 Na^+ ions out of the cell for every 2 K^+ ions coming in. As a result the intracellular concentration of Na^+ is low (20 mM), whereas the K^+ concentration is high (150 mM) [62]. In addition, the Na^+-K^+-$2Cl^-$ co-transporter moves Cl^- against its electrochemical gradient and accumulates Cl^- inside the cell to be released via apical channels. Secretion of Cl^- is electrically coupled to efflux of K^+ through basolateral K^+ conductance channels [63]. Through the paracellular pathway, Cl^- is absorbed or Na^+ secreted and the water-flow is regulated by diffusion following osmotic gradients.

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The maintenance of the electro-osmotic gradients is dependent on limiting back diffusion. The tightness of the paracellular barrier and the molecular selectivity together contribute to the overall epithelial transport characteristics [64]. In many epithelia the transport of different ions is performed by different cell types, however, in airway epithelia the ciliated cell is responsible for both secreting Cl^- and absorbing Na^+ [65].

2.2. The airway surface liquid

ASL, the fluid covering the airway epithelium, consists of a periciliary layer (PCL), which is a watery layer surrounding the cilia, and of mucus on top of the cilia. Mucus is produced mainly by the submucosal glands, while a small amount is produced by the goblet cells. In normal airways PCL height is defined as the length of an outstretched cilium (~6 μm) [66], whereas the ASL layer (mucus plus PCL) varies in thickness of 20-150 μm for different species (20-58 μm in humans) [67]. ASL is the first line of defense against inhaled pathogens and is important for mucociliary clearance. It contains *e.g.*, mucins, phospholipids, albumin, lactoferrin, lysozyme, proteases, defensins and other peptides, ions and water [68], see also paragraph 2.1. The composition, volume and physical properties of the ASL depend manly on secretions of the airway submucosal glands and the absorptive properties of the surface epithelial cells. Regulation of the balance between absorption and secretion determines the net transport of ions across the epithelium through transcellular and paracellular pathways and, thus the mass of salt on an epithelial surface [69].

2.3. Pathogenesis of CF lung disease

The lung of CF patients is normal at birth, but soon after birth an endobronchiolitis ensues with surprisingly few pathogenic bacterial species (*Pseudomonas aeruginosa* in most cases), and which is associated with an intense neutrophilic response localized to the peribronchial and endobronchial spaces [70-72]. The neutrophil-dominated inflammatory response is harmful for the host by causing exaggerated production of inflammatory cytokines and proteases which may sustain infection [73]. CF primarily affects the airways and submucosal glands with sparing of the interstitium and alveolar spaces until late in the disease [74, 75]. The CF lung disease is characterized by a picture of airway epithelial injury [76] and remodeling, such as squamous metaplasia [77], cell hyperproliferation [78], basal and goblet cell hyperplasia, and hypersecretion of mucus due to the inflammatory profile [79-81]. The epithelial regeneration characterized by successive steps of cell adhesion and migration, proliferation, pseudostratification, and terminal differentiation is disturbed and characterized by delayed differentiation, increased proliferation, and altered pro-inflammatory responses [82].

There are several hypotheses about the early pathogenetic steps in the CF lung disease and how defective CFTR leads to the airway disease:

- *The low ASL volume hypothesis* claims that the ASL is isotonic both normally and in CF. CFTR functions both as a Cl^- channel and as an inhibitor of the ENaC. In CF airway epithelia, with an absence of either molecular or functional CFTR, there will be unregulated Na^+ absorption and a decreased capacity to secrete Cl^-. This leads to dehydration of the airway surface, with

a collapsed PCL, concentration of mucins within the mucus layer, and adhesion of mucus to the airway surface [83].

- *The high salt hypothesis* suggests that the ASL normally is hypotonic [84] and provides an optimal environment for defensins. According to this view the ASL in CF patients would have a higher salt concentration than normal because the absorbing function of ENaC depends on the state of CFTR and cannot be activated when CFTR is defective or absent [84].
- *The low pH hypothesis* focuses on the interactions between CFTR and the SLC26 and proposes an acidic ASL. This may compromise the function of airway immune cells and increase toxic oxidant species. Lowering the pH may also eliminate electrostatic repulsive charges between organisms and facilitate "tighter" biofilm formation as well as reduce electrorepulsive forces between bacteria and negatively charged mucins. Furthermore, ciliary beat frequency in bronchial epithelium is reduced when external pH falls [85]. All the above factors may inhibit mucociliary clearance (MCC) and thus elimination of bacteria from the airways [86].
- *The low oxygenation hypothesis* postulates that the oxygen content of the ASL is low, due to build-up of mucus plugs, resulting in enhanced growth of the facultative anaerobic *P.aeruginosa* [87].
- *The defect gland function hypothesis* suggests that the primary defect in CF is reduced fluid secretion by airway submucosal glands and possibly altered secretion of mucous glycoproteins [88].
- *The soluble mediator hypothesis* proposes that signalling molecules within the ASL itself are controlling ASL volume [89]. These molecules are ATP, which is breathing- or shear-stress induced [90], and adenosine. ATP interacts with receptors such as the purinergic P2Y2 receptors and adenosine reacts with the adenosine A2b receptors, that mediate inhibition of ENaC and activation of both CFTR and CaCC [91, 92]. This mechanism is also supposed to include PDZ interactions and cytoskeletal elements [1].

An interesting question is what the role of aquaporins (AQP) is in the production of ASL, compared to paracellular water flow and CFTR. In the epididymis, CFTR appears to regulate AQP-mediated water permeability [93]. In this tissue, CFTR is co-localized with AQP9 in the apical membrane, and this association promotes the activation of AQP9 by cAMP [94]. In a heavily debated study, concerning the clinical benefit of nebulized hypertonic saline in cystic fibrosis, an important role of amiloride-inhibitable AQP water channels in the generation of ASL was proposed [95]. However, although the positive effect of hypertonic saline as such is not disputed, the question whether this effect is mediated by AQP has received conflicting answers [96, 97] and is still open. Recently, it has been found that interleukin (IL)-13 enhances the expression of CFTR but abolishes the expression of AQP in airway epithelial cells [98]. In conclusion, the relation between CFTR and AQP needs further study.

The differences in the proposed hypotheses are due to difficulties in determining the accurate composition of the ASL because of the very small depth of the layer. Among the problems encountered there are difficulties to collect an adequate amount of ASL without disturbing the

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epithelium and inducing secretion from submucosal glands or leakage of interstitial fluid into the lumen, which may modify the composition of the ASL [99].

Furthermore, fluid secretion by submucosal glands differs markedly between mammalian species. For example, in transgenic mice that serve as animal models for CF, the fluid transport in the airways is much less affected than in CF patients [100]. It is also possible that variant forms of ENaC or different regulatory components operate in different systems [101].

3. The biliary duct epithelium

The biliary tree is a complex network of conduits within the liver that begins with the canals of Hering and progressively merges into a system of ducts, which finally deliver bile to the gallbladder and to the intestine. Cholangiocytes are the epithelial cells forming the biliary epithelium which shows a morphological heterogeneity that is strictly associated with a variety of functions performed at the different levels of the biliary tree [102]. Thus, the canals of Hering, located at the ductular-hepatocellular junction, constitute the physiologic link of the biliary tree with the hepatocyte canalicular system and they are the site where a facultative progenitor cell compartment resides; these liver progenitor cells are variably elicited only after liver injury. Given the strong capacity of mature hepatocytes to proliferate, cholangiocyte ability to behave as liver progenitor cells becomes evident only when hepatocellular proliferation is hampered as a result of severe liver damage, as that induced by several toxins or drugs, or occurring under certain conditions, *i.e.* viral hepatitis or non alcoholic steatohepatitis [103]. Cells lining the intrahepatic biliary tree have different functional and morphological specializations: the terminal cholangioles (size <15 μm) have some biological properties such as plasticity (i.e., the ability to undergo limited phenotypic changes) and reactivity (*i.e.*, the ability to participate in the inflammatory reaction to liver damage); interlobular (15-100 μm) and large ducts (100 μm to 800 μm) modulates fluidity and alkalinity of the primary hepatocellular bile.

3.1. Ion and water transport in cholangiocytes

In addition to funnelling bile into the intestine, cholangiocytes are actively involved in bile production. In humans, around 40% of the total bile production is of ductal origin. Cholangiocytes exert a series of reabsorptive and secretory process which dilute and alkalinize the bile during its passage along the biliary tract. Modifications of ductal bile appear to be tightly regulated by the actions of nerves, biliary constituents, and some peptide hormones like secretin [104]. Accordingly to *in vivo* and *in vitro* models, it is possible to distinguish between three different bile flow fractions: 1) the canalicular bile salt-dependent flow that is driven by concentrative secretion of bile acids by the hepatocytes followed by a facilitated efflux of water; 2) the canalicular bile salt-independent flow, which is also created by hepatocytes but through active secretion of both inorganic (bicarbonate) and organic (glutathione) compounds; and 3) the ductal bile flow, that is the bile salt-independent flow contributed by cholangiocytes, mainly through production of a bicarbonate-rich fluid in response to secretin and other regulatory factors. Cl^- secretion into the ductal lumen is the driving force of a chloride/

bicarbonate exchanger that exports HCO_3^- into the bile flowing into the biliary tree. Indeed, this AE (anion exchanger) activity is facilitated by the outside to inside transmembrane gradient of Cl^- at relatively high intracellular concentrations of $HCO3^-$, specially upon secretin stimulation. The AE activity in the liver is operated by AE2/SLC4A2 which is localized not only in the canaliculi but also in the luminal membrane of bile duct cells [105]. Experiments of RNA interference with recombinant adenovirus expressing short/small hairpin RNA have confirmed that AE2/SL4A2 is indeed the main effector of both basal and stimulated Na^+-independent Cl^-/HCO_3^- exchange in rat cholangiocytes [106]. Besides acid/base transporters cholangiocytes possess other ion carriers like those for Cl^-, Na^+, and K^+, which greatly contribute to intracellular pH regulation and bicarbonate secretion. Thus, CFTR had been localized at the apical side, where it plays a role in biliary excretion of bicarbonate [107, 108]. Although bicarbonate permeability through activated CFTR has been shown in several epithelia [109], its main contribution to biliary bicarbonate secretion appears to occur through a coordinated action with AE2/SL4A2 [106, 110, 111]. In addition to CFTR, cholangiocytes possess a dense population of Ca^{2+}-activated Cl^- channels. These channels are responsive to interaction of the purinergic-2 (P2) receptors with nucleotides (mainly ATP or UTP) [112, 113]. The apical fluxes of anions results in increased osmotic forces in the bile duct lumen which in the presence of AQPs contributes to water flux. AE2/SLC4A2 and CFTR colocalize with AQP1 in cholangiocyte intracellular vesicles wich coredistribute to the apical cholangiocyte membrane upon both cAMP and secretin stimulations [114].

3.2. The pathogenesis of CF liver disease

CF is associated with liver disease in almost 30% of all patients. In general, CF-associated liver disease develops during the first decades of life and does not progress rapidly. The diagnostic criteria were initially established by Colombo et al. [115]. Hepatobiliary disease in CF encompass a wide variety of complications, including steatosis, focal biliary cirrhosis (FBC), multilobular biliary cirrhosis (MBC), microgallbladder, distended gallbladder, cholelithiasis, intraheapatic sludge or stones, and cholangiocarcinoma [116]. The pathogenesis of steatosis (fatty liver) is not directly ascribed to the CFTR gene defect but has been attributed to malnutrition, essentially fatty acid deficiency, carnitine or choline deficiency, or insulin resistance [117].

With regarding to the pathogenesis of FBC and MBC, various hypotheses have been proposed [118, 119]:

- *The low chloride secretion hypothesis* proposes that loss of CFTR function leads to blocked biliary ductules with thick periodic acid-Schiff positive material leading to acute and chronic periductal inflammation, bile duct proliferation and increased fibrosis in scattered portal tracts. Hepatic stellate cells (important drivers of hepatic fibrosis) become activated to produce collagen and stimulate the bile duct epithelium to produce the profibrogenic cytokine TGF-β. The progression of FBC to MBC and portal hypertension, which occurs in up to 8% of patients, may take years to decades, and should be viewed as a continuum [120]. Considering CFTR as a driving force for Cl^-/HCO_3^- exchange, the postulated sequence of CF-associated hepatobiliary complications is that loss of functional CFTR protein in the

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apical membrane of cholangiocytes presumably initiates a cascade of abnormal Cl^- and HCO_3^- secretion, decreased bile flow, bile duct plugging by thickened secretions, and cholangiocyte/hepatocyte injury [10].

- *The cholangiocyte damage hypothesis* has been put forward by the studies of Freudenberg et al. in the F508del mouse model for CF [121]. These mice present with increased fecal loss of bile acids and a higher bile salt-to-phospholipid ratio in cell membranes, which was found to be associated with damage to intrahepatic bile ducts determining increased permeability of unconjugated bilirubin into cholangiocytes. They suggest that cholangiocytes injury is caused by a more hydrophobic bile acid pattern and an increased detergency from augmented bile salt-to-phospholipid ratio caused by hyperbilirubinbilia. In addition, lower gallbladder pH values and elevated calcium bilirubinate ion products in bile of CF mice raise the likelihood of supersaturating bile and forming black pigment gallstones [122].
- *The purinergic hypothesis* suggests that CFTR regulates the release of ATP into the bile duct lumen which regulates cholangiocyte secretion via the activation of the purinergic P2Y receptors [123]. Accordingly, Fiorotto et al. [124] have demonstrated that the choleretic effect of ursodeoxycholic acid (UDCA) is mediated via CFTR-dependent ATP secretion.
- *The mechanosensitive pathway hypothesis* indicates that the mechanical effects of fluid flow or shear stress at the apical membrane of biliary epithelial cells results in stimulation of ATP release and Cl^- secretion [123, 125]. The decreased bile flow due to CFTR dysfunction may be associated with alterations in mechanosensitive pathways which exacerbate abnormalities in Cl^- secretion and bile formation [123, 125].
- Finally, *the biliary HCO_3^- umbrella hypothesis* postulates that adequate apical biliary HCO_3^- secretion would appear crucial for protection of cholangiocytes against uncontrolled invasion of protonated bile acid monomers from bile via apical membranes into the cholangiocyte interior, inducing damage and apoptosis [126]. The Cl^-/HCO_3^- exchanger AE2/SLC4A2 and an intact glycocalyx appear to be crucial for the biliary HCO_3^- umbrella [127].

4. Placenta-derived stem cells

The placenta is a highly specialised organ, about 15 to 25 centimetres in diameter, that plays an important role in maintaining normal pregnancy and supporting the normal growth and development of the fetus. It is made up of a fetal and a maternal component: the fetal component include amnion and chorion as well as the chorionic plate, from which chorionic villi extend and make intimate contact with the uterine decidua during pregnancy; the maternal part of the placenta is the decidua basalis and it derived from endometrium.

As reported by Parolini et al. [128], different cell types can be isolated from the regions of the placenta:

- human amniotic epithelial cells (hAEC),
- human amniotic mesenchymal stromal cells (hAMSC),

- human chorionic mesenchymal stromal cells (hCMSC),
- human chorionic trophoblastic cells (hCTC).

In several studies hAEC, hAMSC, and hCMSC have been isolated and characterized for phenotypic and pluripotency molecular markers; moreover, has been demonstrated that these cells display differentiation potential and immunomodulatory effects [129].

hAEC express a pattern of mesenchymal markers while are negative for those of hematopoietic origin ($CD90^+$, $CD73^+$, $CD105^+$, $CD44^+$, $CD29^+$, $CD45^-$, $CD34^-$, $CD14^-$, $HLA\text{-}DR^-$), and these cells are capable to differentiate in vitro into cell types of all 3 germ layers [128]. Like the amniotic epithelial fraction, the human amniotic and chorionic mesenchymal regions display the same pattern of phenotypic markers of bone marrow (BM) MSC, also displaying the expression of pluripotency markers (such as *Oct*-4) and the capability to differentiate toward different lineages including osteogenic, adipogenic, chondrogenic, and vascular/endothelial [128].

Placenta-derived stem cells seems to have a multipotent potential towards other cell types different from mesenchyme cells. hAMSC and hCMSC were shown to differentiate in vitro into a range of neuronal, oligodendrocyte and astrocyte precursors [130-132]. In addition, the use of amniochorionic membrane as a scaffold has been proposed for improving osteogenic differentiation of chorionic membrane-derived cells [133]. Alviano and colleagues reported that hAMSC display the ability to differentiate into endothelial cells in vitro [134]. Recently it has been shown that hAEC can differentiate in vitro in cells with hepatic characteristics, in particular in cells with the ability to differentiate into parenchymal hepatocytes as well as biliary cells that form duct-like three-dimensional structures when cultured on extracellular matrix [135]. hAMSC were demonstrated to differentiate into hepatocyte-like cells as judged by functional and phenotypic markers [136].

As regard the osteogenic and adipogenic differentiation of hAEC and hAMSC, discrepant results have been reported [137, 138], most likely due to the heterogeneous nature of these cell populations and due to the need to isolate the right population of progenitor cells from placental tissues. In this respect, recent efforts have been dedicated to optimizing isolation, culture, and preservation methods for placenta-derived cells; these include a study to determine the quantity and quality of amnion cells after isolation and culture [138], while other studies aimed to define long-term expansion methods to obtain a large cell population for analysis before use in cell-based therapies.

Sources such as amnion tissue offer outstanding possibilities for allogeneic transplantation due to their high differentiation potential and their ability to modulate immune reaction. Limitations, however, concern the reduced replicative potential as a result of progressive telomere erosion, which hampers scalable production and long-term analysis of these cells. The establishment and characterization of human amnion-derived stem cells lines immortalized by ectopic expression of the catalytic subunit of human telomerase (hTERT) resulted in continuously growing stem cells lines that were unaltered concerning surface marker profile, morphology, karyotype, and immunosuppressive capacity with similar or enhanced differentiation potential for up to 87 population doublings [139].

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Interestingly, two groups found a more reliable and unlimited non-animal source for large-scale expansion of hMSC for future allogeneic clinical use: they cultured MSC with animal-free culture supplements such as human platelet lysate (PL), a suitable alternative to fetal calf serum (FCS) showing that these cells exhibit an increased proliferation potential and in vitro life span compared to cells cultured with FCS [140, 141]. On the other hand, it has been demonstrated that phenotypic shift of hAEC in culture is associated with reduced osteogenic differentiation in vitro, therefore different culturing methods may influence cell behavior [137].

In a recent comparative phenotypical study, BM- and placenta-derived mesenchymal cells has been shown that have a very similar morphology, size and cell surface phenotype for characteristics MSC markers [142]; in contrast, differences in proliferation potential have been observed between these two cell types [142]. Another study found different expressions of the chemokine receptors CCR1 and CCR3, which are only present on placenta-derived cells, while the adhesion molecules such as CD56, CD10, and CD49d have been shown to be more highly expressed on placenta-derived mesenchymal cells [143]. On the basis of numerous studies in the literature which clearly show the lack of significant differences between BM- and placenta-derived mesenchymal cells types, and on the basis of the fact that placenta is readily and widely available, a good manufacturing practice-compliant (GMP) reagents and protocols has been established for isolating and expanding human placenta-derived MSC that can be directly translated to the clinical trial setup [144].

4.1. Immunomodulatory features of placenta-derived stem cells

Since the placenta is fundamental for maintaining fetomaternal tolerance during pregnancy, the cells present in placental tissue may have immunomodulatory characteristics; this aspect contributes to make cells from placenta good candidates for possible use in cell therapy approaches, with the possibility of providing cells that display immunological properties that would allow their use in an all-transplantation setting.

It has been demonstrated that cells derived from placenta are negative for the expression of major histocompatibility complex (MHC) class II and for co-stimulatory molecules; all this is reflected as immune tolerance [128, 145]. Furthermore, these cells possess remarkable immunosuppressive properties and can inhibit the proliferation and function of the major immune cell populations, including dendritic cells (DCs), T cells, B cells and natural killer (NK) cells. Most of these studies have been recently summarized in up-to-date reviews [146-148]. Here, we give a brief account of the major findings concerning hAMSC.

Numerous studies showed that amniotic and chorionic membrane-derived cells can suppress the T lymphocyte proliferation induced by alloantigens, mitogens, anti-CD3 and anti-CD28 antibodies in *in vitro* and *in vivo* models [149-152]. The suppression of lymphocyte population was shown to be not dependent on cell death but on decreased proliferation and increased numbers of regulatory T cells [145]. Inhibition of T cell proliferation by placenta-derived stem cells appears to be mediated by both cell–cell interaction [153] and release of soluble factors such as indoleamine 2,3-dioxygenase (IDO), transforming growth factor β (TGF-β), and IL-10 [145, 154, 155]. The immunosuppressive activity of hAMSC on T cells seems to be not only direct but involves also DCs. Indeed, cells derived from the

mesenchymal region of human amnion impaired the differentiation of monocytes into DCs by inhibiting the response of the former to maturation signals, reducing the expression of co-stimulatory molecules and hampering the ability of monocytes to stimulate naive T cell proliferation [156]. The mechanism involved is not known, however, this inhibitory effect might be mediated via soluble factors, like IL-6, and may be dose-dependent, as it has been shown for BM-derived MSCs [157] (Figure 1).

This immune-privileged status of placenta-derived stem cells has been indicated as the cause of lack of rejection in allo- and xeno-transplantation settings. In this regard, several studies examined the fate of amniotic membrane derived stem cells grafts. Wang et al. [158] studied allogeneic GFP^+ mouse intact amniotic epithelium grafts heterotopically transplanted in the eye. Kubo et al. [159] studied xenotransplanted human amniotic membrane in the eye of rats. Several preclinical studies have already reported prolonged survival of human placenta-derived cells after xenogeneic transplantation into immunocompetent animals including swine [152] and bonnet monkeys [128], with no evidence of immunological rejection.

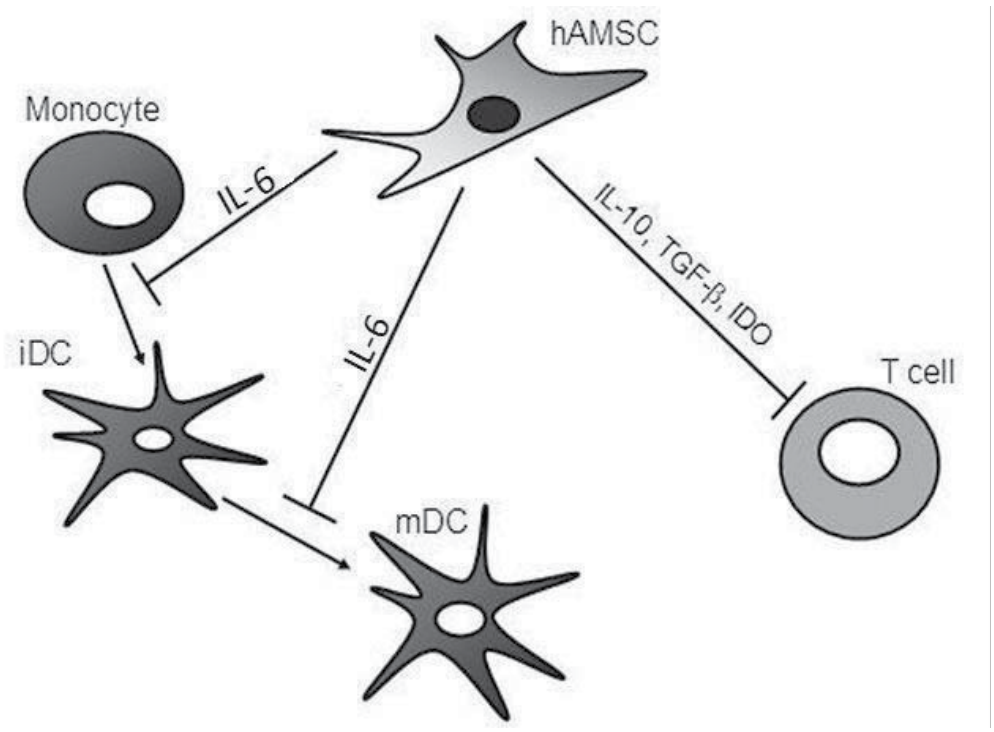

Figure 1. Effects of placenta-derived stem cells on immunocytes. Placenta-derived cells exert immunomodulatory effects both on dendritic cells and T cells. Their inhibitory role is dependent on cell–cell contact and secreted soluble factors. Since most of the studies have focused on hAMSC, this cell type is represented in the scheme. iDC: immature dendritic cell; IDO: indoleamine 2, 3-dioxygenase; IL-6: interleukin-6; IL-10: interleukin-10; mDC: mature dendritic cell; TGF-β: transforming growth factor β.

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4.2. Clinical application of placenta-derived stem cells

More than once century ago, Davis was the first to report the use of the amniotic membrane (AM) to heal skin wounds [160], prompting subsequent applications in the treatment of leg ulcers [161, 162] and burns [163], as well as for applications in ophthalmology [164]. These studies have suggested that placenta-derived stem cells may be useful for treating a range of pathologic conditions, including neurological disorders [165-167], spinal cord injury [128, 168], critical limb ischemia [169], inflammatory bowel diseases [170], and myocardial infarction [171]. Here, we will focus on the potential application of placenta-derived stem cells to lung and liver, the major organs interested by CF.

5. Potential application of placenta-derived stem cells to CF

5.1. Placenta-derived stem cells for lung diseases

The first report demonstrating a therapeutic effect of placenta-derived stem cells in lung diseases is that by Cargnoni and colleagues [172]. In a mouse model of bleomycin-induced lung injury, transplantation of fetal membrane-derived cells resulted in a reduction in the severity of pulmonary fibrosis. This result was obtained when cells were administered either systemically (intravenous or intraperitoneal) or locally (intratracheal) 15 min after intratracheal bleomycin instillation and in two different settings, *i.e.* either using allogeneic or xenogeneic (a mixture of 50% human amnion/chorion mensenchymal stem cells and 50% hAEC) cells. Although the inflammatory score was not decreased, a reduction in the number of infiltrating neutrophils was observed. It is worth noting that that the presence of neutrophils is known to be associated with poor prognosis in idiopathic pulmonary fibrosis in humans [173]. The question arises whether these anti-inflammatory and anti-fibrotic effects may be due to the engraftment of placenta-derived stem cells or to the secretion of soluble factors. In this study allogeneic or xenogeneic cells were detected in the injured lung of transplanted mice, although not in a quantitatively fashion, by means of PCR analysis, and these results are in accordance with those obtained by Bailo and colleagues, who demonstrated microchimerism upon transplantation of human amnion and chorionic cells in neonatal swine and rats [152]. The release of soluble factors has been addressed in a further study. The administration of conditioned medium generated from hAMSC to bleomycin-treated mice determined a reduction in lung fibrosis scores in terms of fibrosis distribution, fibroblast proliferation, collagen deposition and alveolar obliteration [174]. This study support the increasing evidence that MSC isolated from various sources produce bioactive molecules, so that injection of conditioned medium obtained from MSC could be an effective experimental treatment for different tissue injuries [175, 176]. Further studies are therefore warranted to elucidate the mechanisms of action of placenta-derived cells in this model, in particular paracrine factors that act to down-regulate neutrophil recruitment.

It has to be said that the role of exogenous stem cells in pulmonary fibrosis is controversial, meaning that some studies have demonstrated that these cells can act as a potential source of fibroblast, which may accentuate the fibrotic process [177]. Since these findings were obtained

with BM-derived stem cells, it should be further assessed if a similar behaviour is presented by amniotic-derived stem cells. Of note, placenta-derived cells did not exert any profibrotic effect after their transplantation [172].

In vitro studies have so far demonstrated that co-cultures of hAMSC and CF epithelial cells originated from bronchi can elicit CFTR protein expression in 33-50% hAMSC, in front of 6% prior to the co-cultures, and the lower the hAMSC:CFBE41o- ratios the lower the CFTR expression in hAMSC [136]. Indirect co-cultures data indicate that this effect is primarily due to the contact between hAMSC and epithelial cells, and not due to factors acting by a paracrine manner. BM-MSC acquired an airway epithelium phenotype when co-cultured with respiratory epithelial cells and determined a partial resumption of the chloride secretion defect in CF epithelia [178]. Preliminary analysis of the chloride transport defect in co-cultures between CF cells and hAMSC showed a partial correction of the chloride efflux (Carbone et al., unpublished results). Furthermore, since only 6-20% of corrected cells is needed to revert the basic defect in chloride secretion [179], our data showing that 33-50% of hAMSC acquired CFTR expression shed a positive light on the use of amnion MSCs in the CF treatment. Overall, these data point out to a cross talk between amniotic and epithelial cells, for which a critical number of hAMSC is needed. Indeed, in other co-culture systems, developed with MSC and chondrocytes, it has been shown universally that the more chondrocytes the lower the expression of extracellular matrix genes and functional properties of engineered cartilage [180, 181]. Since the cellular interactions between epithelial and mesenchymal cells in monolayer co-culture are likely to be bi-directional, a possible mode of action could be cross talk between cells via gap junctions, which has been observed *in vivo* in the lung between transplanted MSC and resident epithelial cells [182].

Overall, the potential usefulness of placenta-derived stem cells in CF lung disease might be either in the correction of the early basic defect (chloride transport) or in late remodelling events (pulmonary fibrosis).

5.2. Placenta-derived stem cells for liver diseases

Several preclinical studies have reported to date that placenta-derived stem cells can engraft into the liver and perform hepatic functions *in vivo*. Takashima and colleagues [183] showed that after transplantation of human amniotic membrane into the peritoneum of SCID mice, human albumin could be detected in the sera and peritoneal fluid of these animals from day 1 until day 7. Sakuragawa and colleagues [184] showed that the transplantation of hAEC transduced with the β-galactosidase gene into the livers of SCID mice resulted in detection of β-galactosidase-positive cells at 1 week after transplantation, indicating that the transplanted cells had been integrated into the hepatic parenchyma within a few days [184]. More recently, it has been shown that six months after transplantation of hAEC into the livers of SCID/beige mice that had been pretreated with retrorsine, most mature liver genes were expressed at levels comparable to those of authentic human adult livers, including the major CYP genes, other metabolic enzymes, plasma proteins, and hepatocyte-enriched transcription factors and genes encoding hepatic-transporter proteins [185].

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These studies provide compelling evidence in support of the functional hepatic potential of hAEC *in vivo*, thereby supporting the potential of hAEC as a useful tool for liver regeneration in the future.

MSC represent an alternative tool for the establishment of a successful stem-cell-based therapy of liver diseases [186] with preliminary clinical improvements in acute and chronic hepatic diseases [187, 188]. To date, several studies on animal models reported the beneficial effects of MSC in promoting hepatic tissue regeneration [189]. Overall, a number of different mechanisms contribute to the therapeutic effects exerted by MSC, among which their differentiation into functional hepatic cells. However, these studies have not provided definitive evidence that MSC have a capability to differentiate into functional hepatocytes *in vivo* [190]. Rather, the observed improvements could be attributed to the known property of MSC to produce a series of growth factors and cytokines that could suppress inflammatory response, reduce hepatocytes apoptosis, regress liver fibrosis, and enhance hepatocytes functionality [191, 192].

Although numerous studies have reported that BM-derived MSC can reduce carbon tetrachloride (CCl_4)-induced liver fibrosis in mice, the mechanism by which MSC repair the fibrosis is unclear, and the results are controversial [190, 193-197]. One possibility is that MSC differentiate into hepatocytes, because of the *in vivo* niche, and secrete growth factors that promote liver regeneration. Another possibility is that MSC suppress hepatic stellate cells activity and secrete metalloproteinases (MMPs), thereby eliminating deposition of extracellular matrix [198]. It has been demonstrated that fibrosis, infiltration of neutrophils, synthesis of collagen I and α-smooth muscle actin (α-SMA), and expression of inflammatory were all reduced by infusion of isogenic MSC [199]. It is possible that these responses were partly due to the upregulation of cytoglobin expression by hepatic stellate cells, which protect against oxidative stress and controls tissue fibrosis and at the same time inhibits the activation of those cells to become myofibroblasts [200]. Finally, it has been demonstrated that intravenous administration of MSC caused an increase in IL-10 mRNA in the liver and protein in the blood in a CCl_4-induced liver fibrosis rat model [201]. IL-10 is an inhibitor of many cytokines that stimulate liver fibrosis, such as IL-6, TNF-α and TGF-β, all downregulated by the MSC infusion. In addition, IL-10 can suppress tissue inhibitor of metalloproteinase (TIMP)-1 expression and thereby relieve MMP-1 to degrade liver collagen deposits [202, 203].

In a recent study, hAMSC were infused in mice with CCl_4-induced hepatic cirrhosis and exerted various beneficial effects such as reduction of hepatic stellate cell activation, decrease of hepatocyte apoptosis, and reduction of hepatic fibrosis [204]. Infusion of hAMSC also depressed hepatocyte senescence and resulted in engraftment of hAMSC into the host liver as judged by the expression of the hepatocyte-specific markers, human albumin and α-fetoprotein. Finally, a study demonstrated that human AM, when applied as a patch onto the liver surface, reduced progression of experimental biliary fibrosis induced in rats by the biliary duct ligation procedure [205]. Again, a beneficial effect related to the release of soluble factors by the human AM patch has been invoked, since no massive (or at least very low/undetectable) engraftment of AM-derived cells occurred in the host liver.

6. Conclusion

Placenta-derived stem cells are endowed with interesting features that are important for choosing them as a source for approaches aimed to regenerative medicine: immune-privileged status, secretion of biomolecules with anti-scarring and anti-inflammatory properties, and, least but not last, no ethical concerns. Although the AM and AM-derived stem cells have been used in the clinics for over one hundred years, their employment in lung and liver diseases is coming on the stage only in the last few years. Placenta-derived stem cells have been recently more thoroughly characterized for their phenotype, multipotency and expression of pluripotency genes.

In CF, lung disease has been the target first of gene therapy approaches brought to the clinical stage [206, 207], hesitated in a slow progression due to limited efficiency of gene transfer vectors and pathophysiological barriers, and then of stem cell-based experimental treatments in mice [208]. Despite a very low level of engraftment of donor HSPC into the nose and the gut, significant CFTR mRNA expression and a measurable level of correction of the electrophysiological defect were observed after transplantation of wild-type marrow cells into CF mice [209]. It is uncertain whether this effect is due to the presence of CFTR-expressing epithelial cells derived from donor cells or to the paracrine effects of transplanted cells. Other sources, such as umbilical cord blood, embryonic stem cells, and induced pluripotent stem cells are being evaluated [210, 211]. Recent *in vitro* data on the acquisition of CFTR expression by hAMSC indicate placenta-derived stem cells as a possible source for treating the early phases of CF lung disease. Anyhow, caution should be taken when stem cell-based therapies are proposed for an inflammatory disease like that of CF lung, in view of the fact that these cells could be immunosuppressive and/or contribute to the inflammatory process. There is no available information concerning the immunomodulatory effects of placenta-derived stem cells in CF lungs.

Liver fibrosis is a common outcome of a variety of chronic liver diseases following different insults, including the biliary disorder occurring in CF. Orthotopic liver transplantation remains the only viable therapeutic option to treat CF patients with hepatic cirrhosis, and hepatocyte transplantation has never been attempted in this disease. The use of progenitor cell transplantation is emerging as a potential alternative, and several potential sources have been identified for the isolation of these cells [212]. For the treatment of liver cirrhosis, this approach has been performed mainly with BM-derived MSC [213, 214]. Given the drawbacks related to the use of BM-derived MSC (limited frequency, invasive procedure, age and disease state affecting the collection of healthy autologous BM), placenta-derived stem cells could represent a prime candidate for the treatment of liver fibrosis, since they are immunotolerated, can be isolated and produced at high yield, and do not provoke ethical debate. AM and AM-derived stem cells have been demonstrated to halt the progression of liver fibrosis and its evolution towards cirrhosis, but the long-term safety and therapeutic efficacy are not known yet, which warrant further studies. Moreover, optimal therapeutic regimens for clinical application of placenta-derived stem cells, such as optimal doses, transplantation route and interval period for transplantation should be evaluated in detail [215].

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Chapter 2

Isolation of Bone Marrow Stromal Cells: Cellular Composition is Technique-Dependent

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Additional information is available at the end of the chapter

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1. Introduction

Bone marrow contains a colony-forming, fibroblast-like cell population called bone marrow mesenchymal stem cells or bone marrow stromal cells (BMSCs) [1, 2]. Since BMSCs are capable of differentiating into multiple lineages (osteogenic, chondrogenic, adipogenic, neurogenic, and myogenic lineages), they have attracted significant interest as useful somatic stem cells for use in tissue engineering and regenerative medicine [3 - 7]. As BMSCs adhere to tissue culture-treated plastic, they are usually isolated by adherent cultivation of untreated whole bone marrow [8 - 10]. However, this technique may be inefficient for the isolation of BMSCs because untreated bone marrow contains a large proportion of erythrocytes and their presence may interfere with the initial adherence of BMSCs. The removal of unwanted high density blood cells by density gradient centrifugation increases the number of colony-forming units (CFUs) in primary BMSC culture [11]. Removal of erythrocytes by hemolysis treatment is also effective at increasing the number of CFUs [12]. However, recent studies have shown that BMSCs isolated by these techniques are different from those isolated by adherent culture techniques [13]. Since BMSCs consist of a heterogeneous mixture of cells with varying potentials at different stages of differentiation, the characteristics of the cultured cells depend on the initial composition of the cell population [14, 15]. Therefore, the final cellular composition of BMSCs will vary significantly with the isolation technique used. Few studies have focused on the importance of the initial cellular composition of isolated BMSCs. In this chapter, possible differences in the cellular composition of BMSCs isolated from untreated, hemolysed, or density gradient fractionated bone marrow will be discussed. Furthermore, the optimal technique for the isolation of BMSCs for use in tissue engineering and regenerative medicine will be discussed from a clinical point of view.

2. Bone marrow stromal cells

BMSCs are a plastic-adherent, non-hematopoietic cell population residing in the bone marrow [16]. As BMSCs are morphologically similar to skin fibroblasts and can be expanded in a culture medium for fibroblasts, they were initially described as stromal fibroblasts [17], though their differentiation potentials are far different from those of skin fibroblasts [18]. While skin fibroblasts are incapable of differentiating into other cell types, BMSCs are capable of differentiating into cells of multiple mesenchymal tissues such as bone, cartilage, fat, tendon, muscle, and marrow stroma [19]. To emphasize this property, BMSCs are also called mesenchymal stem cells or multipotent mesenchymal stromal cells [20], though they can also differentiate into non-mesenchymal (non-mesodermal) cell types such as neurons [21] and insulin-producing cells [22]. Although BMSCs do not possess totipotencies like embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), they are clinically more useful than these totipotent stem cells because they can be easily isolated from a small volume of bone marrow aspirate and do not require gene transfections to demonstrate their differentiation abilities [23]. Thus, BMSCs have attracted significant interest as potent stem cells for use in tissue engineering and regenerative medicine of various tissues. In fact, clinical studies have shown that BMSCs are useful for the treatment of bone, cartilage, heart, and the central nervous system [24-27]. In addition, BMSCs recently attracted attention as immuno-modulatory cells useful for the treatment of immue diseases such as graft versus host disease (GVHD) [28, 29]. Therefore, clinical use of BMSCs should increase over the next few years.

3. Animal-derived BMSC as a model of human BMSC

BMSCs are present in the bone marrow of humans as well as other animals such as mice, rats, rabbits, dogs, pigs, sheeps, horses, and cows [4, 8, 30 - 35]. As BMSCs seem to be postnatal stem cells that are common among mammalian species, these animals have been used to investigate the origin and *in vivo* functions of BMSCs [36, 37]. In addition, these animal-derived BMSCs are considered useful as a models of human BMSCs because it is not always easy to recruit a sufficient number of human BMSC donors for experimental use. Furthermore, more reliable results can be obtained by using animal-derived BMSCs because experimental animals have uniform genetic backgrounds and are housed under controlled conditions, eliminating behavioral and environmental variations that could influence BMSC properties. In fact, several studies have reported that the characteristics of human BMSCs varied significantly among donors [15, 38, 39], while such variations are not observed in animal-derived BMSCs. Therefore, animal-derived BMSCs are considered to be useful alternatives to human BMSCs for laboratory experimentation. However, it remains unknown which animal's BMSCs offer the best model system to represent human BMSCs. In general, donor animals of BMSCs are chosen based on their costs and availabilities. However, it has been shown that there are a number of characteristic differences in the BMSCs among species [40]. Therefore, it is important to consider species difference in addition to the costs and availabilities when selecting model systems for human BMSCs.

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Considering their costs and availabilities, mice are more attractive candidates than other laboratory animals. However, rat BMSCs are used as a model of human BMSCs in our laboratory because mouse BMSC characteristics differ from those of human BMSCs. For example, mouse BMSCs need the support of feeder cells for their stable growth, while human BMSCs are able to grow in a feeder cell-independent manner [40]. Responses to differentiation stimuli are also different. While human BMSCs are readily induced to differentiate into the osteogenic lineage by dexamethasone, mouse BMSCs are less responsive to dexthamethasone treatment [41]. Although the reasons why mouse BMSCs differ from human BMSCs remain unknown, it has been suggested that mouse BMSCs are very rare in the bone marrow and need support by other cells for their growth and differentiation [40]. On the contrary, rat BMSCs can be easily isolated from bone marrow and they are able to grow without feeder cells, as do human BMSCs [13]. In addition, rat BMSCs are able to differentiate into multiple lineages under induction protocols used for human BMSCs [42]. Therefore, we believe that rat BMSCs offer a more appropriate model of human BMSCs, though fewer reagents and antibodies are available for rat cells than for mouse cells.

4. Isolation of BMSCs

Since BMSCs form adherent colonies in plastic culture vessels, BMSCs are generally obtained from adherent cultures of untreated whole bone marrow [2 - 4]. However, it has been suggested that this technique is inefficient for the isolation of BMSCs because untreated bone marrow contains a large proportion of erythrocytes and their presence may interfere with the initial colony formation of BMSCs [11 - 13]. As human BMSCs are a rare population in the bone marrow (0.01 - 0.1% of whole marrow), it is possible that the efficacy of initial colony formation directly affects the total yield of BMSCs. Inefficient colony formation may also lead to the reduced potentials of BMSCs because previous studies have shown that BMSCs lose their differentiation abilities depending on the duration of *ex vivo* culture [39]. Accordingly, it is important to investigate whether BMSCs can be more efficiently isolated by the removal of unwanted cells. Both density gradient centrifugation and hemolysis (red blood cell lysis) treatment remove erythrocytes for the efficient isolation of the mononuclear cell fraction of bone marrow. Although both these techniques were originally developed for the isolation of white blood cells such as lymphocytes, they can also be used for the isolation of BMSCs because they are contained within the mononuclear cell fraction. In fact, several studies have used either or both of these techniques for the isolation of BMSCs; they reported that BMSCs were more efficiently isolated by these techniques (Table 1) [12, 32, 34, 43].

However, it remains unknown whether BMSCs isolated by these techniques are identical to those isolated from untreated whole bone marrow because BMSCs are composed of heterogeneous cells with varying growth and differentiation potentials [15]. Thus, the cellular composition of BMSC populations could be dependent upon the isolation technique. Although it remains unknown how many different types of cells constitute the BMSC fraction, at least committed osteogenic cells as well as uncommitted stem cells are present when BMSCs are

Target cells	Compared isolation techniques	The most efficient isolation technique	Reference
Human BMSCs	• Hemolysis (red blood cell lysis) • Density gradient centrifugation • Adherent culture of whole bone marrow	Hemolysis	[12] Horn *et al.*, 2008.
Pig BMSCs	• Hemolysis • Dextran sedimentation • Density gradient centrifugation	Hemolysis	[32] Peterbauer-Scherb *et al.*, 2010
Rat BMSCs	• Density gradient centrifugation • Adherent culture of whole bone marrow	Density gradient centrifugation	[43] Polisetti *et al.*, 2010
Equine BMSCs	• Density gradient centrifugation • Adherent culture of whole bone marrow	Density gradient centrifugation	[34] Bourzac et al., 2010

Table 1. Removal of erythrocytes by hemolysis or density gradient centrifugation may enable the efficient isolation of BMSCs.

isolated from untreated whole bone marrow [44]. Changes in the relative sizes of these two cell populations greatly influence the characteristics of BMSCs. In other words, a greater number of committed osteogenic cells makes the BMSC fraction more osteogenic, while a greater number of uncommitted stem cells makes them more stem-cell like. Thus, we investigated differences in the cellular composition of BMSCs isolated from untreated, density-gradient-centrifuged, and hemolysed bone marrow, with a special reference to committed osteogenic cells and uncommitted stem cells. For these experiments, rat bone marrow was used instead of human bone marrow to avoid the influence of variations among donors.

5. The number of committed osteogenic cells contained in BMSCs varies with the isolation technique

Committed osteogenic cells can be defined as a cell population that is capable of forming bone without osteogenic induction. Because of the presence of this cell population, *in vivo* transplantation of untreated whole bone marrow to ectopic sites usually results in the formation of new bone [45]. If this cell population is decreased or lost by the hemolysis or density gradient centrifugation steps, new bone formation may not be observed in the transplants. On the contrary, if this cell population is enriched by these techniques, more significant bone formation should be observed. Therefore, we investigated the *in vivo* bone-forming ability of three

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populations: marrow that was untreated, marrow that was hemolysed with ammonium chloride, and marrow that was fractionated by density-gradient-centrifugation over Ficoll® (Ficoll-treated). As shown in Figure 1A, the percentage of bone-forming transplants (transplants containing ectopic bone/ total transplants) was the lowest in the Ficoll-treated group. The amount of new bone formation, which was scored on a semi-quantitative scale from zero to three (Table 2), was also lowest in the Ficoll-treated group (Figure 1B). The hemolysed group also showed less bone-forming ability than did the untreated group, though its ability was still greater than that of the Ficoll-treated group (Figure 1 A and 1B).

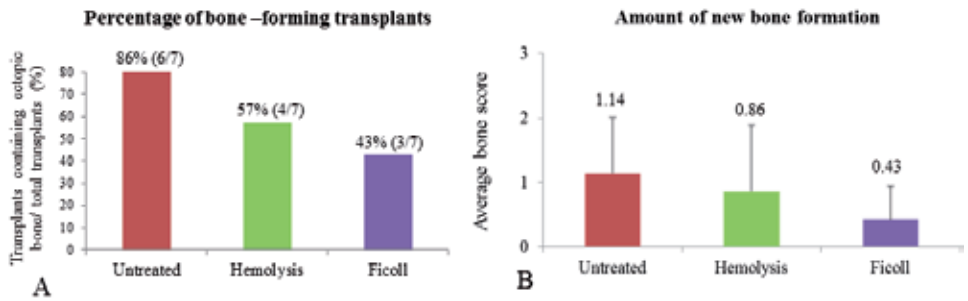

Figure 1. *In vivo* bone-forming ability of untreated, hemolysed, or Ficoll-treated bone marrow. (A) The percentage of bone-forming transplants (transplants containing ectopic bone/ total transplants), which was calculated from the results of seven independent experiments, was greatest in the untreated group, followed by the hemolyzed group, and lowest in the Ficoll-treated group. (B) The amount of new bone formation (total bone score/ total transplants) was greatest in the hemolyzed group, followed by the untreated group, and lowest in the Ficoll-treated group (n = 7). (Modified from Agata et al., 2012 [13] with permission)

	Bone score (Percentage of new bone area in the transplant)
0	No bone evident
1	Bone area < 5%
2	5% < Bone area < 10%
3	Bone area > 10%

Table 2. Bone score of each sample was determined from the percentage of the area containing bone (new bone area/ total area) (Modified from Agata et al., 2012 [13] with permission)

As these results showed that Ficoll-treated bone marrow contains fewer committed osteogenic cells than either untreated or hemolysed bone marrow, we next investigated whether BMSCs isolated from Ficoll-treated bone marrow actually contains lower numbers of committed osteogenic cells. Untreated, hemolysed, or Ficoll-treated rat bone marrow was plated on cell culture dishes, and adherent colony-forming cells were expanded as BMSCs. Although these BMSCs did not show significant differences in their morphology or their expression of cell-surface CD54 and CD90 (Figure 2), they showed a significant difference in the expression of cell-surface alkaline phosphatase (ALP) (Figure 3A). The difference in ALP expression was also confirmed by quantitative ALP assays (Figure 3B).

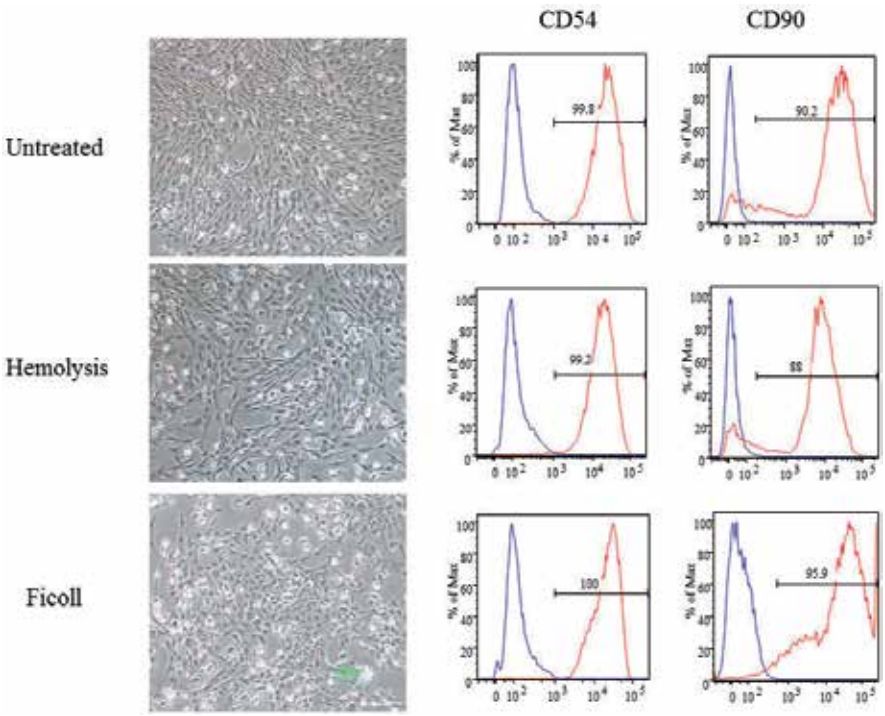

Figure 2. Morphology and expression of cell surface CD54 and CD90 of BMSCs that were isolated from untreated, hemolysed, or Ficoll-treated bone marrows. (Modified from Agata et al., 2012 [13] with permission)

Since these BMSCs were simply cultured in non-induction medium, the expression of cell surface ALP directly indicates the number of committed osteogenic cells contained in each BMSC. Therefore, it can be concluded that BMSCs isolated from Ficoll-treated bone marrow contain lower numbers of committed osteogenic cells than those isolated from untreated or hemolysed bone marrow.

6. The number of uncommitted stem cells contained in BMSCs also varies with the isolation technique

Although it remains unknown whether BMSCs contain committed progenitors of other lineages, their multi-lineage differentiation potentials are mainly attributed to the presence of uncommitted stem cells among heterogeneous BMSC populations. Therefore, it is important to investigate whether the number of uncommitted stem cells contained in BMSCs varies with the isolation techniques. Note, however, that it is difficult to calculate their numbers accurately because no specific markers for uncommitted stem cells are currently available. However, the abundance of these cells in BMSCs populations can be determined by analyzing the responsiveness to differentiation-inducing media (induction media), since uncommitted stem cells are highly responsive to differentiation stimuli. Thus, BMSCs that are rich in these cells show

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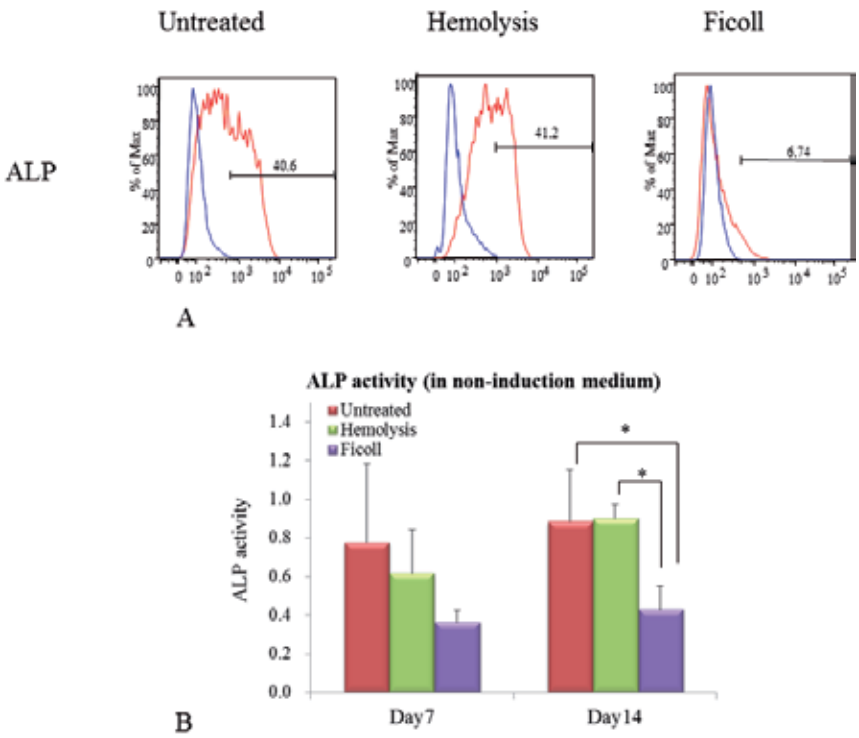

Figure 3. Differences among committed osteogenic cell populations from BMSCs isolated without treatment, or after hemolysis, or Ficoll separation. (A) The expression of cell surface alkaline phosphatase (ALP) of non-induced BMSCs was greatest in the untreated group, followed by the hemolyzed group, and lowest in the Ficoll-treated group. (B) Quantitative ALP assays confirmed the lowest ALP activity in the Ficoll-treated group. Data are presented as the means ± standard deviation (n = 3). *: $P < 0.05$. (Modified from Agata et al., 2012 [13] with permission).

great responsiveness when culture medium is changed from non-induction medium to induction medium. Accordingly, we investigated BMSCs isolated from untreated, hemolysed, or Ficoll-treated bone marrow for their responses to osteogenic induction medium. As shown in Figure 4A, the Ficoll-treated group showed the lowest ALP activity on day seven. However, this group significantly upregulated ALP activity and showed the greatest activity after 14 days of culture in osteogenic medium, though the difference did not reach a statistically significant level.

Since the Ficoll-treated group constantly showed the lowest ALP activity when cultured in non-induction medium (Figure 3B), the ratio of ALP upregulation (ALP activity in osteogenic induction medium/ ALP activity in non-induction medium) was also the greatest in this group. Gene expression analyses of osteopontin and core-binding factor subunit alpha-1 (*Cbfa1*), both of which are indicators of osteogenic differentiation, also showed the greatest responsiveness in the Ficoll-treated group (Figure 3B - 3E). These results indicate that BMSCs isolated from Ficoll-treated bone marrow contain greater numbers or higher concentrations of uncommitted stem cells than those isolated from untreated or hemolysed bone marrow.

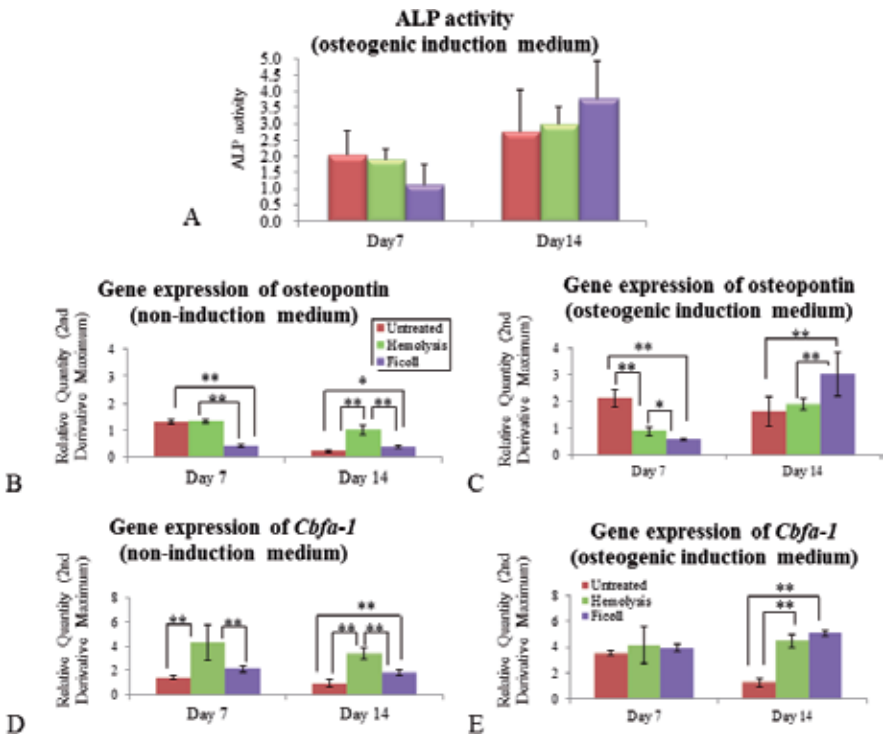

Figure 4. Differences in the responses to osteogenic induction medium among BMSCs isolated from untreated, hemolysed, or Ficoll-treated bone marrow. (A) ALP activities in osteogenic induction medium. (B) Gene expression of osteopontin in non-induction medium. (C) Gene expression of osteopontin in osteogenic induction medium. (D) Gene expression of *Cbfa-1* in non-induction medium. (E) Gene expression of *Cbfa-1* in osteogenic induction medium. Data are presented as the means ± standard deviation (n = 3). *: $P < 0.05$, **: $P < 0.01$. (Modified from Agata et al., 2012 [13] with permission)

7. Potential merits of hemolysis treatment or density gradient centrifugation of bone marrow to isolate BMSCs

Although hemolysis treatment of bone marrow with ammonium chloride primarily removes only erythrocytes from bone marrow, *in vivo* transplantation experiments indicated that some of the committed osteogenic cells contained in bone marrow are lost or damaged during the hemolysis treatment (Figure 1A and 1B). Thus, we hypothesized that BMSCs grown from hemolysed bone marrow might contain lower numbers of committed osteogenic cells and their cellular composition would differ from that of normal BMSCs (BMSCs grown from untreated bone marrow). However, contrary to the hypothesis, flow cytometric analyses revealed that these BMSCs contained equivalent numbers of committed osteogenic cells (Figure 3A). Since these BMSCs showed similar responses to osteogenic induction medium (Figure 4A), they seem to contain similar numbers of uncommitted stem cells as well. Therefore, it is likely that the cellular composition of BMSCs grown from hemolysed bone marrow is relatively close to that of normal BMSCs. As the cell yield in primary culture (harvested cell number after primary culture/ days of primary culture/ initially seeded cell number) was greater in the hemolysis

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group (0.52 in the hemolysed group and 0.44 in the untreated group), it can be concluded that hemolysis treatment of bone marrow is an efficient approach to the isolation of BMSCs.

After centrifugation over Ficoll®, bone marrow is separated into several fractions such as plasma, mononuclear cells, granulocytes, and erythrocytes. Since BMSCs belong to the mononuclear cell fraction in the bone marrow, it is likely that BMSCs are efficiently enriched in this fraction even though this isolate contains significantly lower cell numbers than untreated or hemolysed bone marrow (Figure 5).

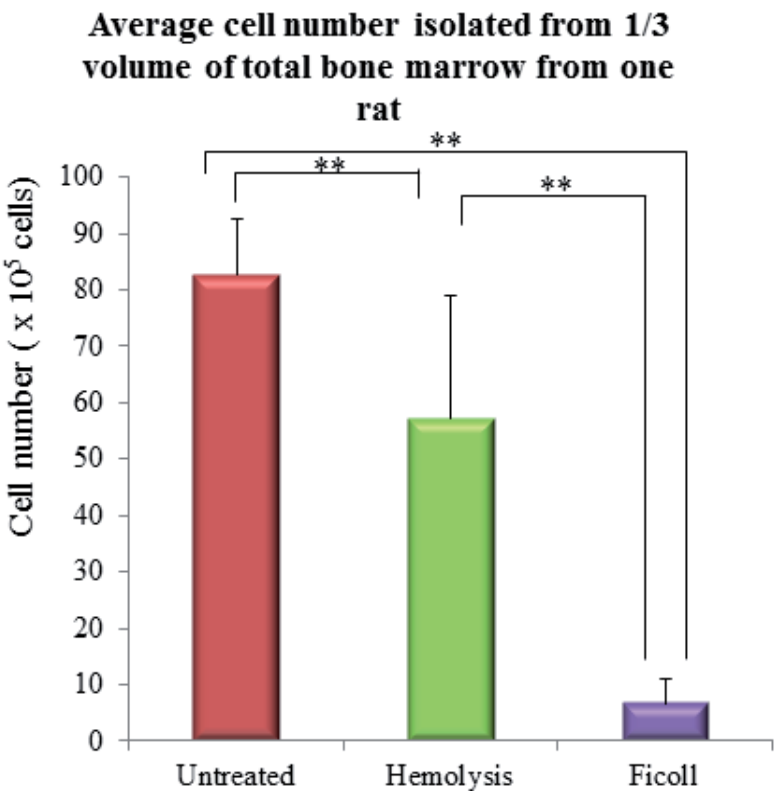

Figure 5. Rat bone marrow was divided into three portions and the suspensions were either hemolyzed, or subjected to Ficoll fractionation, or left without treatment (untreated). Significant differences were observed in the average numbers of cells isolated among the groups. Data are presented as the mean ± standard deviation (n=6). **: $p < 0.01$ (Modified from Agata et al., 2012 [13] with permission).

However, in contrast to expectations, the cell yield in primary culture was the lowest in this group (0.13 in the Ficoll-treated group). In addition, the cellular composition of this group's BMSCs seemed to be different from that of normal BMSCs, because these BMSCs showed significant differences in the percentage of cell-surface ALP-positive cells and the responses to osteogenic induction medium (Figure 3A and 4A), though they showed similarities in the morphologies and the expression of cell-surface CD54 and CD90 (Figure 2). Therefore, it can be concluded that density gradient centrifugation of bone marrow is not an efficient approach to the isolation of BMSCs that possess normal characteristics. However, this technique may be

useful for the isolation of more potent (more primitive) BMSCs because BMSCs grown from Ficoll-treated bone marrow seem to contain greater numbers or higher concentrations of uncommitted stem cells.

8. Conclusion

As the cellular composition of BMSCs varies significantly with the isolation technique, it is important to select an appropriate isolation technique for the purpose that is intended. For example, if BMSCs are used for bone tissue engineering, it might be better to isolate BMSCs by hemolysis, because BMSCs that contain greater numbers of committed osteogenic cells are efficiently obtained by this technique. On the contrary, if BMSCs are used for the stem cell therapies of non-bone diseases such as stroke, it might be better to isolate BMSCs by density gradient centrifugation, because BMSCs obtained by this technique contain greater numbers of uncommitted stem cells. Flow cytometric or magnetic cell sorting with antibodies might also be useful for the isolation of BMSCs for use in stem cell therapies because BMSCs isolated by this technique possess greater multi-lineage potency. However, most of the current clinical studies still use the conventional adherence technique for the isolation of BMSCs because the fact that the characteristics of BMSCs varies with the isolation techniques remains largely unknown. Since the results of clinical studies are greatly affected by the potentials of the BMSCs used, selection of an appropriate isolation technique may lead to a better outcome. Nonetheless, further investigations are required to use these new techniques in clinical studies because available information concerning the safety, feasibility, and efficacy of these techniques is still limited. Furthermore, the cost effectiveness of these techniques should be investigated, since the conventional technique does not require any special reagents. Continuing investigations are important for the establishment of truly reliable new therapies using BMSCs.

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Chapter 3

The ASC: Critical Participants in Paracrine-Mediated Tissue Health and Function

Patricia Zuk

Additional information is available at the end of the chapter

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1. Introduction

1.1. The adipose-derived stem cell — A pluripotent adult stem cell?

In 2001, the journal Tissue Engineering published an article describing the isolation of a population of putative multipotent stem cells from adipose tissue termed Processed Lipoaspirate Cells or PLA cells [1]. Based on isolation methods designed for the harvest of adherent, fibroblastic cells from the adipose stroma capable of adipogenic differentiation in vitro [2], this work by Zuk et al. described the differentiation of their PLA cells toward multiple mesodermal lineages, including fat, bone and cartilage. This ground-breaking article has since been followed by over 3500 studies published and available through PubMed, describing the differentiation capacity of ASCs in a variety of in vitro and in vivo model systems. Early works continued the characterization of PLA cells – now termed ASCs for Adipose-derived Stem Cells - identifying a unique CD "signature" for these cells [3]-[8] and studying their mesodermal differentiation capacity at a molecular and biochemical level [8]. Subsequent studies have since confirmed the ASC's mesodermal differentiation capacity in vitro reporting osteogenic, adipogenic, chondrogenic and skeletal myogenic capacities [9]-[20]. These works have since been expanded into in vivo translational models using a variety of animal systems for bone formation [21]-[25], cartilage [26]-[28], fat [29]-[32] and skeletal muscle [33]-[35]. In addition, recent years have presented some exciting results, expanding ASC potential to add smooth muscle [36], [37] and cardiac myogenesis [38], [39] to the growing list of ASC capacities.

With these increased capacities, it became natural to ask if the ASC possessed pluripotent potential and initial in vitro studies appeared to answer this question, reporting ectodermal [8], and endodermal differentiation [40], [41]. However, the true test of these germ line potentials still lies in the in vivo model. Consistent with the in vitro studies, numerous in vivo

model systems have reported possible ectodermal and endodermal potentials, describing the repair of nervous and epithelial tissues [42], [43], together with hepatic and pancreatic regeneration [44]-[46]. With these in vivo results, combined with earlier in vitro analysis, it becomes easier to conclude that the ASC is an adult pluripotent stem cell population.

1.2. ASC-mediated tissue regeneration: Secretion of soluble factors

Despite the in vivo translational studies above suggesting that ASCs are capable of enhancing tissue healing and regeneration, many of these studies cannot confirm the direct differentiation of the ASC into a specific cell type. For example, while bone regeneration is observed upon implantation of ASCs, very few studies report the presence of the ASC within the newly formed bone. Whether this is an oversight by the research team or an indication that the ASC does not directly form part of the new tissue is unclear. It is entirely possible that the ASC does not directly differentiate into the desired regenerating tissue, but simply directs tissue formation "from the sidelines". Tissue development and healing is incredibly complex and the role of paracrine signaling is still not entirely understood. Therefore, it is possible that ASCs may be intimately involved in tissue regeneration and health through their ability to mediate the host's regenerative capacity using paracrine signaling.

Two arguments can be made in support of this theory. First, in many translational models, it does not appear that the ASC has any difficulty in surviving within the transplantation region for extended periods of time. In addition, the range of tissues capable of engrafting ASCs appears to be quite broad. Initial studies by Nolta and researchers show that systemic administration of human ASCs is followed by multi-organ engraftment in nude mice [47]. In support of this, human ASCs administered via tail vein migrate and home efficiently to multiple tissues (epithelial and endothelial) in irradiated mice [48], [49]. The specific migration of ASCs to injured tissues has also been shown by the Longaker group, who confirm the presence of ASCs specifically in parietal bone defects and their persistence as the defect heals [50]. Second, stem cells like bone marrow MSCs and ASCs are known to secrete numerous factors and cytokines, including VEGF, HGF, NGF, BDNF and multiple interleukins [49], [51]. In fact, Salgado's article calls these factors the "secretome" of ASCs. This secretome may have powerful paracrine effects on the health, repair and function of a tissue and has resulted in an exciting, new theory that proposes the ASC as a mediator of tissue regeneration through the secretion of specific soluble factors. In this regard, the ASC could be used in an incredibly broad range of applications. However, the most popular are reviewed below.

2. The use of ASCs in transplantation — Immunomodulatory and anti-inflammatory actions

Successful transplantation is reliant upon tolerance by the host's immune system. In 2000, human MSCs were transplanted into immunocompetent sheep without significant rejection [52], suggesting that adult stem cells might survive in a xenogeneic environment. Subsequent work with MSCs has described their ability to immunosuppress mixed lymphocyte reactions

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and to suppress stimulated T cell proliferation [53]-[55]. MSCs are also known to inhibit cytotoxic T lymphocyte toxicity [56], [57] and inhibit B cell proliferation by altering the G0/G1 transition [58]. Likewise ASC-mediated immunosuppression has been confirmed through a series of elegant in vitro experiments that describe the suppression of mixed lymphocyte reactions and/or proliferation of key immune cells like the T cell [59]-[63]. Immunosuppression has also been observed in a variety of in vivo model systems (Table 1). For example, reduced inflammatory infiltration and airspace enlargement results from the systemic administration of human ASCs to murine models of emphysema [64]. Moreover, the ASCs are capable of rescuing the suppressive effects of cigarette smoke on bone marrow hematopoietic progenitor function [64]. Experimental autoimmune hearing loss can be treated in mice through the systemic infusion of human ASCs, resulting in protection of hair cells possibly through the production of the anti-inflammatory cytokine IL10 by splenocytes [65] and decreasing the proliferation of antigen-specific Th1 and Th17 cells. Similar immunosuppression and amelioration of disease is reported upon injection of ASCs in models of rheumatoid arthritis [66] and IgA nephropathy [67], resulting in decreased inflammatory markers and Th1 cytokine activity, together with the generation of regulatory T cells capable of suppressing T cell responses. Finally significant anti-inflammatory responses are observed upon the transplantation of allogeneic murine ASCs into dystrophin-deficient mice, decreasing markers of oxidative stress and inflammation, including TNFα and IL6, decreasing production of CD3+ T cells, and enhancing the synthesis of anti-inflammatory IL4 and IL10 [68]. While these studies are supportive of the role for ASCs in modulating immune responses, what remains unknown is the mechanism. One theory proposes that cell-cell contact is required [61]. However, others dispute this finding, suggesting that it is the secretion of soluble factors by the ASC that mediates the eventual reaction by the host's immune system [69]. In support of this, inhibition of prostaglandin E2 production in ASCs by indomethacin can abolish the immunosuppressive properties of ASCs. Alternatively, neutralizing leukemia inhibitory factor has had similar effects [70]. Finally, there are those that suggest a role for IL-6 [55].

The immunosuppressive properties of ASCs may make it possible to use more xenogeneic transplantation model systems without the fear of significant immune reactions in animal hosts. Such models would allow for a more direct study of human ASCs in vivo, thus allowing researchers to more accurately predict what these cells could do clinically. An excellent review of these models can be found in a recent article by Lin et al. [81]. In this article, they present a detailed table outlining many of the recent xenogeneic model systems, such as one by Paul and colleagues [82], who perform a xenogeneic transplantation of human ASCs into myocardial infarcts produced in immunocompetent rats. Histology confirms human ASCs in the infarct region after 6 weeks, with no detectable inflammatory reaction even in the absence of immunosuppressive action. Furthermore, these animals show improvement of cardiac function and reduced infarct size, together with significant improvement in myocardial anti-inflammatory cytokine levels. The success of such xenogeneic transplantation models may be explained, in part, by the immunogenic profile of the ASC. Immunophenotyping of ASCs has not only provided researchers with a CD antigen profile but has confirmed the absence of the HLA-DR antigen on the ASC surface. Divided into classes such as HLA-A, B and C (or MHC

Author and Year (Reference)	ASC type	Disease Model	Inflammatory/Immunosuppressive action
Pinheiro et al. 2012 [68]	human	murine dystrophy	decreased CD3+ve T cells, increased IL-4, IL-10 synthesis
Payne et al. 2012 [71]	human	autoimmune demyelination – IL-4 overexpressing ASCs	increased T cell responses
Zhou et al. 2011 [65]	human	autoimmune hearing loss	secretion of IL-10, decreased proliferation of Th1, Th17 cells
Hyun et al. 2011[67],	mouse	IgA-induced nephropathy	decreased inflammatory markers, decreased Th1 activity
Schweitzer et al. 2011 [64]	human, mouse	emphysema	decreased inflammatory infiltration
Lai 2011 et al. [72]	human	systemic lupus erythamatosis	decreased Th17 production, decrease IL-17 synthesis
Zhou 2011 et al. [66]	human	rheumatoid arthritis	decreased Th1, Th17 proliferation/expansion, increased IL10 synthesis
Kuo 2011 et al. [73]	rat	hind limb allotransplantion	increased Treg proliferation
Gonzalez-Rey et al. 2010 [74], Gonzalez et al. 2009 [75]	human	rheumatoid arthritis	inhibition of CD4+ T cell proliferation, increase in IL-10 producing T cells and monocytes, stimulation of Treg cell development
Cho et al. 2010 [76]	mouse	airway allergic disease	decreased airway inflammation, shift from a Th2 to a Th1-biased immune reponse
Gonzalez-Rey et al. 2009 [77], Gonzalez et al. 2009 [78]	human	experimental colitis	decrease in Th1-driven inflammation, decrease inflammatory cytokines, increased IL-10 activity
Kim et al. 2007 [79]	human	hemorrhagic stroke	decreased brain inflammation markers
Wan et al. 2008 [59]	rat	orthotopic liver transplant	increased IL-2 and IL-10 synthesis
Constatin et al. 2009 [80]	mouse	autoimmune encephalolyelitis (multiple sclerosis)	increased Th2-type shift in cytokine production[80]

Table 1. Immunosuppressive action of ASCs

class I) and HLA-DP, DM and DR (or MHC class II), HLA receptors display proteins on the cell surface for immune surveillance. Of particular interest is the HLA/MHC class II protein, which is found on the surface of antigen-presenting cells and plays critical roles in immunotolerance and transplantation (for reviews see [83], [84]). The absence of this class of HLA protein may allow the ASC to evade the host's immune surveillance machinery. Of additional interest is a recent study by DelaRosa et al. [85], who note that human ASCs have lower susceptibility to natural killer (NK) cell-mediated lysis in comparison to bone marrow MSCs.

This finding may be part of the reason for xenogeneic tolerance of ASCs in that NK-ASC crosstalk does not result in immediate recognition. Continued research in this area is sure to expand the possible uses of ASCs in translational model systems.

3. Vascularization by ASCs in tissue repair

Tissue repair and regeneration is reliant upon vascularization. Newly formed tissues must have sufficient blood flow to maintain their health and support their growth. Early in vitro studies with ASCs suggest the capacity to differentiate into endothelial cells and to form vessel-like structures. For example, using simple in vitro induction conditions, ASCs express typical markers of endothelial cells, such as von Willebrand Factor (vWF) and function as endothelial cells, taking up acetylated LDL and forming tubular structures on Matrigel substrates [40], [41], [86]. Tubule formation, LDL uptake and CD31 expression by ASCs are also found upon in vitro exposure to shear stress [87], [88]. Such evidence provides strong support for the use of ASCs in the induction of vessel formation and some have attempted to isolate the specific ASC subpopulation that might be responsible for endothelial differentiation. For example, Wosnitza et al. postulate that a population of CD31-ve, S100+ve ASCs are capable of endothelial differentiation [89], while CD34-ve ASCs have been observed to undergo differentiation by others [90].

Author and Year (Reference)	ASC type	Secreted Factor
Ribeiro et al. 2012 [91]	human	VEGF, HGF, bFGF, NGF, SCF
li et al. 2012 [92]	human	VEGF, bFGF, SDF1α
Kim et al. 2011 [93]	human	VEGF
Lu et al. 2011 [94]	human	VEGF, HGF, BDNF, NGF
Liu et al. 2011 [95]	rat	HGF
Nie et al. 2011 [96]	rat	VEGF, HGF, bFGF
Salgado et al. 2010 [49]	human	VEGF, HGF, BDNF
Zhu et al. 2010 [97]	human	VEGF
Grewal et al. 2009 [98]	human	VEGF
Rubina et al. 2009 [99]	mouse	VEGF, HGF, bFGF, PDGFB, TGFb
Park et al. 2008 [100]	human	VEGF, HGF, PDGF
Prichard et al. 2008 [101]	rat	VEGF
Kilroy et al. 2007 [102]	human	HGF
Wang et al. 2006 [103]	human	VEGF, HGF, IGF-1
Cao et al. 2005 [41]	human	VEGF, HGF, bFGF, KGF, TGFβ
Rehman et al. 2004 [104]	human	VEGF

Table 2. Growth factor secretion by ASCs

However, the efficacy of ASCs in tissue repair may not be entirely due to their direct differentiation into endothelial lineages, but also to their secretion of paracrine factors capable of

increasing vascularization. In support of this, co-culture of ASCs with postnatal cardiomyocytes results in the formation of stable, branching CD31+ve vessel-like structures that disassemble in the absence of ASCs [99]. Similarly, ASC-conditioned media can induce the formation of vessel-like tubules within Matrigel [105]. More recently, while rat ASCs express Flt-1, CD31 and vascular endothelial cadherin, when injected into a wire injury model in the rat femoral artery, induction of endothelial repair occurs without any observable differentiation of these ASCs into endothelial cells [106]– a finding that can be explained if repair is driven through the production of soluble factors. In the hopes of identifying what angiogenic factors improve a tissue's vasculature, numerous studies have characterized the secretion of growth factors by ASCs (Table 2). Of all of these factors, perhaps the most commonly reported is VEGF, with secretion of this factor being reported under normal culture conditions [98], hypoxic conditions [104] in models of wound healing [96], [107] and cell-assisted lipotransfer [97]. The ability of VEGF to stimulate neoangiogenesis is well known [108]-[110]. Consistent with this, conditioned medium from ASCs, maintained under hypoxic culture conditions in order to increase production of HGF, VEGF and TGFβ, has been found to increase endothelial cell (EC) growth and reduce their apoptosis [104]. In addition, VEGF secretion by ASCs is significantly upregulated in vitro upon metabolic induction of ischemia [111]. However, the role of other secreted factors cannot be ruled out as suppression of HGF production by ASCs through RNA interference significantly impairs ischemic tissue revascularization [112] and SDF-1α from ASCs has been identified as being involved in myocardial vascularization [92]

3.1. Ischemia/ischemia-reperfusion injury

Today, there are several model systems that study the paracrine-mediated vascularization potential of ASCs but some of the most common are: ischemia and ischemia-reperfusion (IR) injuries, wound healing and cardiac infarct treatment. Enhanced angiogenesis within ischemic limbs has been reported following treatment with freshly isolated ASCs (i.e. the stromal vascular fraction) and vessels derived from these cells confirmed [113]. However, the use of such a heterogenous population makes it difficult to confirm direct ASC involvement. Fortunately, there have been numerous studies describing the beneficial use of cultured/purified ASCs in the treatment of ischemia [86], [90], [93], [114]-[117]. Consistent with paracrine action, improved vascularization within ischemic limbs has been associated with increased levels of plasma VEGF [93]. In addition, human ASCs cultured in vitro as spheroids improve neovascularization and limb survival when compared to the implantation of dissociated ASCs – a finding thought to be due to the induction of vascular factors, like HGF, VEGF and bFGF, by the hypoxic conditions of the spheroid [118]. In support of this, decreases in the ability of ASCs to induce reperfusion in ischemic hindlimbs are observed if secretion of HGF by the ASC is inhibited [112]. However, the role of the ASC in angiogenesis may not be restricted to their secretion of established angiogenic factors. Transplantation of ASCs transfected with siRNA to either MMP3 or MMP9 to ischemic hind-limbs results in lower blood flow recovery and higher tissue injury [119], suggesting that ASCs may also promote angiogenesis through their secretion of matrix-remodelling enzymes.

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Whereas prolonged ischemia can cause significant tissue damage, there is evidence now that the reperfusion period is also associated with injury, amplified by the production of reactive oxygen species and inflammatory cascades [120]. Events such as these are a major obstacle to successful tissue transplantation. However, the ASC may ameliorate IR injury through its secretion of pro-angiogenic factors, thus increasing the density of developing capillaries within the reperfused tissue. Consistent with this, a significant increase in pro-angiogenic factors can be confirmed in IR skin flap models treated with ASCs [121]. Long-lasting improvement in cardiac function with increased angiogenesis and vasculogenesis can also be observed in IR in minipigs treated with a trans-endocardial injection of ASCs [122] and a higher number of CD31+ve and vWF+ve cells have been found in models of lung IR followed by ASC injection [123]. While the finding that ASCs can form vessel-like structures in Matrigel in vitro and re-endothelialize carotid injuries in vivo [87], [124] may suggest that the observed angiogenesis is due to differentiation by ASCs, the failure to observe significant ASC engraftment in IR models [122] again suggests that the role of ASCs may be paracrine in nature.

In addition to stimulating angiogenesis, the ASC may also lessen the damaging effects of IR through paracrine secretion of a combination of anti-inflammatory and anti-oxidant factors. The production of oxidative toxins such as free radicals and reactive oxygen species in ischemia and IR is well-established [125]-[128]. The synthesis of enzymatic anti-oxidants, such as superoxide dismutase and glutathione peroxidase, not only can be detected by proteomic analysis in ASC-conditioned media, but this media is able to protect dermal fibroblasts from oxidative damage [129]. Therefore, the ASC may be an excellent candidate for protection against oxidative damage. In support of this, Chen and co-workers, using a model of kidney IR treated with either conditioned medium from ASCs or direct injection of ASCs during reperfusion, find increased clearance of creatinine and urea from blood plasma in ASC/IR groups together with higher levels of the anti-oxidant markers NAD(P)H quinine oxidoreductase, heme-oxygenase 1/HO-1, glutathione peroxidase and glutathione reductase [130]. Increased anti-oxidant marker levels (i.e. NAD(P)H quinine oxidoreductase and HO-1) have also been reported, together with increased eNOS expression and decreased hepatic oxidative stress versus controls upon multiple injections of ASCs in hepatic IR models [131]. These anti-oxidant actions by ASCs are not only likely to protect the reperfused tissue from oxidative damage but may also protect the ASC itself. A recent study by Suga and colleagues suggests that resident ASCs are resistant to ischemia-mediated damage, surviving within ischemic adipose grafts [132]. Moreover, this work specifically postulates that the actions of these resident ASCs may be responsible for the observed increases in vascular density and the number of new adipocytes over time. Therefore, ASCs may be resistant to the toxic environment of ischemic tissues and may retain their functional capacities, thus being able to either differentiate or secrete paracrine factors for critical for angiogenesis.

3.2. Wound healing

Paracrine action is also likely to play a significant role in the beneficial effects of ASCs in wound healing models. ASCs isolated from debrided skin are capable of producing an epithelial layer when seeded into collagen gels, together with a dermis when seeded fibrin gels are co-cultured

with ASC/collagen/epithelial constructs, suggesting that the ASC would be an excellent cell source for healing skin wounds [133]. In support of this, increased collagen density has been reported in full-thickness rat skin grafts injected with ASCs [134] and Lim et al. [135] note improved wound healing rates upon implantation of ASCs. These wound healing rates are significantly higher than in controls treated with ASC extracts, suggesting that production of paracrine factors by viable ASCs are necessary in order to direct the formation of new tissue within the wound. In vitro culture of immortalized keratinocytes or dermal fibroblasts with ASC-conditioned medium results in increased proliferation of these cells, in addition to increased transcription and production of collagen type I, suggesting that secreted ASC-derived factors may ultimately influence keratinocyte-mediated healing in skin grafts [136], [137]. Finally, Jung and colleagues have reported that conditioned medium from ASCs can increase CNI, CNIII and hyaluronic acid synthesis by human dermal fibroblasts and that neutralizing antibodies to TGFβ1 can abolish this effect [138]. However, it is equally likely that improved wound-healing using ASCs is due to their secretion of angiogenic factors, thus improving healing through augmentation of vascularization. As proponents of this theory, Reichenberger et al. [139] and Gao et al. [107] report higher blood flow and skin flap survival, respectively when the flaps are combined with ASCs. In addition, Gao and colleagues report increased capillary density, together with increased expression of VEGF within the dermis in the ASC-treated groups. In support of this, increased VEGF expression and microvascular density is also measured in ASC-treated rat skin grafts [134]. Interestingly, recent studies suggest that AKT/c-myc signaling pathways may mediate increased VEGF secretion in ASCs as injection of constitutively active AKT/v-myc-expressing ASCs promote better wound healing compared to normal controls [140]. How exactly the ASC promotes wound healing is likely to be a combination of increased tissue healing and vascularization as directed by their secretion of specific paracrine factors. In support of this, GFP-labelled ASCs not only secrete the angiogenic factors VEGF, HGF and bFGF in vivo, but co-stain with keratin and CD31 in excisional wound healing models in normal and diabetic rats, possibly undergoing both epithelial and endothelial differentiation [96]. Similar differentiation by human ASCs, implanted into skin wounds via silk/chitosan scaffolds, has also been reported by Altman and colleagues [141]. Therefore, the successful use of ASCs in wound healing models may be due to their paracrine action in promoting angiogenesis by the host and their autocrine action in promoting differentiation in themselves.

3.3. Infarct treatment

In a 2007 study by Fotuhi, freshly isolated ASCs injected into porcine transmural infarcts were shown not to cause arrhythmia, bradycardia or conduction block. Moreover, these ASC-treated hearts required extra-stimuli to induce an arrhythmia, suggesting that ASCs could be used in the treatment of cardiac infarcts [142]. With in vitro studies confirming the cardiomyogenic potential of these stem cells, infarct treatment could be mediated through the differentiation of ASCs into cardiomyocytes. However, there is a debate on whether the ASC contributes directly to cardiac muscle regeneration or supports this event through the production of angiogenic growth factors and cytokines. An example of this debate can be seen in the 2007 article by Zhang et al. [143]. Rabbit ASCs injected into transmural infarcts in hearts three wks

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after occlusion decrease transmural scar and improve left ventricle ejection fraction (LVEF), end-diastolic pressure and myocardial performance relative to saline controls, with ASCs pre-induced with 5-azacytidine for 24 hours giving slightly better results versus untreated controls. When the infarct region is examined histologically, the ASCs form islands of cardiac tissue in and around the scar. However, all infarcts treated with ASCs also show greater capillary density, with the ASCs also differentiating into endothelial cells. Increased capillary densities/angiogenesis have previously been reported using bone marrow mononuclear cells and endothelial progenitors and MSCs are known to cause improvement in cardiac function by incorporating into newly formed capillaries and releasing angiogenic factors [144]. Similar events may also be induced by ASCs. In support of this, mouse ASCs injected into murine infarcts take up residence in the infarct area, with EKGs showing stability of LVEF [145]. Murine ASCs [146] or rat ASCs [147] transplanted into rat infarcts result in significant improvement in heart function and tissue viability. Human ASCs not only increase peri-infarct capillary density in rat infarcts but increase numbers of nerve sprouts [148]. Finally, while Beitnes and co-workers show significant improvement in LVEF, smaller infarct sizes and increased vascularization when human ASCs are injected into infarcts in nude rats, they specifically observe an absence of ASC engraftment [149]. However, it is important to note that ASC engraftment was examined in this study 4 weeks post-transplant. It is possible that the long-term beneficial effects of ASCs on infarct treatment can result from short-term engraftment. In support of this, while transdifferentiation of human ASCs into cardiomyocytes or endothelial cells is also not observed in rat cardiac infarcts, the expression of VEGF, bFGF and SDF-1α can be confirmed in these hearts within the first few days of transplant and improved heart function and vascular density is ultimately observed [92]. Therefore, long-term survival of ASCs within the myocardium may not be necessary for their beneficial effects on cardiac function to be realized. Such a possibility would be extremely exciting if this treatment modality is translated into the clinic.

3.4. Other vascularization systems

In addition to wound healing, infarct treatment and ischemia-reperfusion, there are numerous other vascularization systems that might benefit from the putative angiogenic action of ASCs. Hemodynamic abnormalities may be reversed with the treatment of pulmonary arterial hypertension with ASCs through their augmented expression of HGF for angiogenesis and increased number of small pulmonary arteries [95]. Small-for-size liver injury may be treated through their secretion of VEGF. Inhibition of VEGF secretion by ASCs through RNA interference (RNAi) does not prevent apoptosis of liver sinusoidal endothelial cells in vitro and when cells are transplanted syngeneically results in significant disturbances to graft microcirculation, serum liver functional parameters and graft survival [150]. Finally, at the cosmetic level, cell-assisted lipotransfer fat grafts survive at higher levels, are 35% larger and show increased neoangigogenesis when compared to grafts transplanted without isolated ASCs [151].

4. Neuroprotection by ASCs — Demyelination, stroke, spinal cord injury

Early translational studies do suggest that ASCs can be safely administered to nervous tissue injuries and that functional improvement is noted. Transplanted ASCs have been reported to improve functional deficits following middle cerebral occlusion or ischemic stroke [152]-[154], spinal cord contusion injury [155] and peripheral nerve gaps [156], [157]. Histologic analysis of these injury sites has suggested that ASC differentiation into neurons and/or glial cells may play a role in the functional recovery, with transplanted cells staining positively for MAP2 [153], GFAP, Tuj-1 and an oligodendrocyte marker [155]. However, this functional improvement may be due to paracrine actions on the host more than ASC differentiation, as less then 1% of transplanted ASCs can be found within a spinal contusive injury model, with those remaining appearing to be oligodendrocytes [158]. In addition, extremely low levels ASC differentiation into mature neurons is noted in a model of cerebral cortex injury [159]. However, both of these studies note significant changes in the host tissue with Nakada et al. observing improvements in microvasculature and Zhang et al. measuring increases in host oligodendrocyte formation. Therefore, like wound healing and IR models, ASCs are likely to exert paracrine actions within nervous tissue.

In 2002, Zhao et al. suggested that functional recovery in ischemic brain injury was not due to MSC differentiation but to secreted paracrine factors that act on the host [160]. A similar hypothesis has been put forth by bone marrow MSC groups who have noted increased survival and differentiation of Tuj1+ve neurons and neuroblastoma cells in co-cultures [161] and increased neuronal viability and glial cell differentiation using MSC conditioned media [162]. Consistent with this, ASC/Matrigel constructs implanted into models of mice limb re-innervation stimulate the regeneration of nerves and induce axon growth, likely through the expression numerous neurotrophins [163]. Moreover, enhanced nerve fiber growth is observed if the ASCs are pre-induced toward the neural lineage thus enhancing their production of brain-derived neurotrophic factor (BNDF). BDNF secretion (together with nerve growth factor/NGF and glial cell-derived neurotrophic factor/GDNF) by ASCs pre-differentiated toward a Schwann Cell (SC) phenotype is thought to be the basis for axonal regeneration in sciatic nerve gap models - although these authors speculate that this regeneration is likely due to the neuroprotective function of these three neurotrophins [164]. In support of this, studies using ASC-conditioned media appeared to further strengthen this theory. Protection against cortical and hippocampal volume loss in rats can be achieved through the infusion of ASC-conditioned medium [165]. ASC-conditioned medium containing VEGF, BDNF and NGF is shown to have a protective effect against glutamate excitotoxicity on PC12 cells (a key factor implicated in stroke and neurodegenerative diseases) and increase PC12 viability [94]. Conditioned media from pre-differentiated ASCs infused over one week into a rat model of ischemic stroke 8 days after stroke induction increases the number of CD31+ve cells [166]. Finally, functional deficits in a model of middle cerebral artery occlusion can be dramatically improved using ASC transduced to overexpress BDNF [153].

While these neurotrophic factors may act to protect neurons, ASCs may also play roles in decreasing inflammation and gliosis (i.e. glial cell-mediated scar formation) – two critical

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events that specifically affect healing in the both the central and peripheral nervous system. Systemic transplantation of human ASCs can attenuate cerebral degeneration in rats, reducing both brain atrophy and glial proliferation [79]. Rats implanted with ASC-derived SCs show significant locomotor function recovery compared with untreated ASCs and also reduction in gliosis [152]. Pre-differentiated canine ASCs in Matrigel scaffolds show better functional recovery and reduced fibrosis and inflammation when implanted into spinal cord injuries [167]. Decreased gliosis is also noted upon intrathecal administration of ASCs in a model of IR neuronal damage in rabbits – an event accompanied by increased expression of BDNF within the first 72 hours following ASCs administration [168]. Finally, a possible anti-inflammatory role for ASCs in sciatic nerve repair might be seen in a recent model describing possible immunosuppression of xenogeneic acellular nerve matrices combined with autologous ASCs [169]. Implantation of this construct does not result in host rejection, making it possible that peripheral nerves repair can be accomplished using commercial nerve matrices combined with the patient's own ASCs.

4.1. Controlled release from ASCs — ASCs as a cellular biopump

It is possible that the paracrine action of ASCs may be "fine-tuned" so that the ASC secretes a desired factor, hence turning the ASC into a "cellular biopump". This is not a recent concept as the engineering of numerous cell types to secrete a variety of factors has been reported in the literature for over a decade. In the field of stem cell research, bone marrow MSCs have been modified to secrete various factors, including BMP2 [170], [171], bFGF [172], IFN-β [173] and IL12 [174]. Similar to these studies, ASCs have been engineered for the delivery of BMP4 [175], BMP2 [176], [177], and BMP6 [178] in several bone regeneration models. Delivery of TGFβ2 by ASCs for the induction of chondrogenesis has been reported [179]. Adenovirally-mediated VEGF secretion by ASCs has been used to induce vascular growth in a bone defect model [180] and adipose tissue grafts [181]. Finally, as described above, BDNF delivery by transduced ASCs into a model of middle cerebral artery occlusion improves functional deficits when compared to control ASCs [153].

However, a more exciting idea might be in the engineering of ASCs in the treatment of disease. In 2007, ASCs engineered to express cytosine deaminase were found to decrease the growth of colon carcinoma cells [182]. ASCs have recently been described in the delivery of an oncolytic myxoma virus that will specifically target gliomas [183]. ASC viability is not impacted with transduction and successful cross-infection of gliomablastoma cells is observed upon 3D co-culture with glioblastoma cells, leading to their cell death. More importantly, rat survival is increased with this myxoma virus delivery, with the size of the gliomas significantly decreasing upon injection of transduced ASCs in comparison to non-transduced ASCs controls. Localization of ASCs and increased apoptosis within tumors has also been reported following intravenous or subcutaneous injection of ASCs engineered to express TRAIL, having no effect on the surrounding healthy tissue [184]. Finally, this approach may have far-reaching effects on autoimmune diseases through the delivery of interleukins and interferons. ASCs engineered to overexpress IL4 and administered at the time of T cell priming attenuate autoimmune encephalomyelitis and reduce peripheral T cell responses shifting the host pro-inflammatory

response to an anti-inflammatory one [71]. With the development of inducible viral systems, there is the possibility that the ASC cellular biopump could be controlled not only at the dose level through the number of cells delivered but at the temporal level, giving clinicians more precise control over their therapeutic regimen.

4.2. ASC uses in the clinic

In light of their differentiative capacity and paracrine actions, there is great interest in the use of ASCs within the clinic. As source of regenerative stem cells, the ASC may have no equal. Bone marrow aspirates yield on average $6x10^6$ nucleated cells per ml, of which, only 0.001 to 0.01% are thought to be stem cells [185], [186]. In comparison, approximately three-fold more cells can be obtained per gram adipose tissue [187] [188] with 10% of these cells thought to be stem cells [188], [189]. The abundance of ASCs within adipose tissue, combined with the relative ease of its harvest and isolation also makes the ASC a good choice for clinical work. Patient's could conceivably have their adipose tissue harvested relatively painlessly a few weeks prior to their procedure in a simple outpatient procedure, the ASCs isolated and expanded under good manufacturing protocols and then used for regenerative purposes. With the confirmed absence of HLA/MHC class II proteins and continuing xenogeneic animal models, the patient may not even need to use their own stem cells. Donated allogeneic ASC lines could be used in lieu of autologous cells without the fear of immunorejection or inflammatory complications. Such a situation might be perfect in the case of myocardial infarct treatment where a delay in treatment could have serious consequences.

The first published article using ASCs in a clinical setting was in 2004, in which freshly harvested SVF cells were combined with fibrin glue and used in the repair of a traumatic calvarial injury [190]. Three months after reconstruction, CT scans showed new bone formation within the injury. However, it is important to point out that the cells used in this study were not ASCs, purified through plastic adherence and culture time, but the SVF - a heterogenous mixture of ASCs, endothelial cells, pre-adipocytes, pericytes, fibroblasts and red blood cells. Therefore, it is difficult to attribute the observed healing to the action of the ASC itself. Since that time, other clinical studies using the SVF have been attempted [191] and a review by Casteilla et al. does an excellent job of summarizing these works [192]. It is worth noting that with the exception of some cysts and microcalcifications being observed upon breast reconstruction [193], the use of SVFs clinically has not resulted in any serious complications.

Because of its heterogeneity, clinical studies using purified ASCs have also been performed for the treatment of such disorders as critical limb ischemia and radiation therapy ([194], [195] – for a more comprehensive review, see [192]). Bone regeneration using ASCs has recently been reported in 2009 with the reconstruction of the maxilla being induced using ASC in combination with BMP2 [196]. Bony healing using BMPs has been documented in numerous translational animal models [197]-[201], making this clinical study an exciting addition to the ways bone regeneration and healing can be brought about in the clinic. However, many of these translational models fail to report the appropriate control – the amount of bone being formed just by the BMP itself. The first translational study to combine ASCs and a BMP (i.e. BMP2) failed to measure any significant improvement in bone formation when BMP2 and ASC+BMP2 groups were

compared [197]. Since this study, others have appeared to suggest that BMP2 may not promote the in vivo osteogneic capacity of the ASC [202] but may, in fact, may have a deleterious effect on bone regeneration [203]. Since it is not possible to perform similarly controlled studies clinically, it remains unknown if the addition of ASCs to BMP-treated scaffolds provides any more advantage. However, It is worth noting that, as with the use of SVFs, administration of ASCs into human patients has not been associated with any adverse effects [204].

The first phase I clinical trials using ASCs were not conducted on bone formation or even fat grafting but in the healing of chronic fistulae in Crohn's disease [205]-[210]. In 2005, nine rectovaginal fistulae in four patients were treated with ASCs, purified and cultured for up to one month. Of the eight fistulae examined, six showed complete healing in 8 weeks [206]. These fistulae had previously failed to heal using conventional surgical treatments, thus justifying progression to more comprehensive phase II trials. In 2009, a larger phase II trial using patients with and without Crohn's fistulae were treated with ASCs [211]. As seen with their earlier clinical trial, the majority of Crohn's and non-Crohn's fistulae were healed completely using ASCs in comparison to controls. Currently, there are three phase II clinical trials recruiting for the use of ASCs in Crohn's disease fistulae (Clinicaltrials identifiers: NCT01011244, NCT01157650, NCT00999115, http://clinicaltrials.gov/ct2/results?term=adipose+derived +cells), in addition to one phase III trial (NCT00475410) recently completed [212].

One of the reasons ASCs are considered in the treatment of Crohn's disease is their ability to suppress inflammation. This review includes numerous examples of how the ASC may be capable of suppressing the immune system and recent clinical trials have attempted to take direct advantage of this quality. The treatment of multiple sclerosis (MS) with SVFs, containing ASCs, has been described by Riordan and colleagues in 2009, with the 3 enrolled patients showing improvement in numerous functional categories including balance and coordination [213]. The use of culture expanded ASCs in autoimmune diseases like hearing loss, MS and rheumatoid arthritis was recently discussed in 2011 [214]. Prior to this, ASCs have been proposed as a viable therapy for suppression of graft vs. host disease (GVHD) [215]-[218]. Each of these studies report favorable functional outcomes and propose ASCs, or their SVF counterpart, for the treatment of immune system disorders.

The most obvious application of the ASC clinically should be in breast reconstruction. In the lab, the combination of ASC-containing SVFs with fat grafts through a protocol called cell-assisted lipotransfer has enjoyed success [151]. Clinically, treatment of facial lipoatrophy has been reported [219] and two recent trials overseas has suggested that the ASCs within the SVF are capable of increasing breast volume and improving contour 6 months post-surgery [193], [220]. However, the use of ASCs in breast reconstruction is being pursued carefully in light of recent findings that link stem cells to cancer. Bone marrow MSCs have been found to increase proliferation of breast cancer cell lines [221] and subcutaneous injection of MSCs with tumor cells can favor their growth [222]. Similar to this, ASCs can increase tumorigenesis of established breast cancer lines [223]. In this study, ASCs not only promote the growth of metastatic pleural effusion cells both in vitro and in vivo but the ASC also secretes adipsin and leptin – both of which are known to promote breast cancer growth [224]. Additional work in MSCs has documented their ability to secrete large amounts of IL-6 and the corresponding increase in

the growth of estrogen receptor alpha-positive cell lines [225]. Increased expression of IL4 and IL10 have also been reported by ASCs isolated from breast cancer tissue [226], leading many to speculate that the ASC may be capable of altering the immune environment within the breast, resulting in the "protection" of the cancerous cells. Such a possibility could have far-reaching effects in the development of breast cancer and in its possible reoccurrence if ASCs are used in reconstruction. However, it is encouraging to find that cultured ASCs are resistant to the chemotherapies cisplatin, vincristine or comptothecin and that they still retain their stem cell characteristics [227]. Such findings could make it possible for a more natural reconstruction of the breast if ASCs are found not to contribute to the cancer itself.

4.3. "Paracrines gone wild" — ASCs and adipose disorders

With the proposed paracrine function of ASCs now well accepted, a re-examination of certain disorders and how the ASC might play a role might now be in order. The most obvious of these disorders would be obesity. However, studying the ASC might allow more information into lesser known dysfunctions such as lipedema and rare adipose disorders (RADs) like Dercum's and Madelung's disease. Normal fat has been described as having an anti-inflammatory milieu with adipocytes storing lipid, regulating energy metabolism, and, together with resident macrophages, secreting anti-inflammatory mediators such as IL-10 and adiponectin to protect against the possible development of inflammation-driven obesity [228]-[230]. However, with chronic nutrient overload, existing adipocytes increase their fat storage to become hypertrophic and resident pre-adipocytes (or ASCs) are thought to undergo increased differentiation to increase adipocyte number (i.e. hyperplasia). The hypertrophic adipocytes increase their secretion of "adipokines" - soluble factors known to affect angiogenesis and inflammation [231], [232]. Specifically, these adipocytes shift their adipokine production from anti-inflammatory to inflammatory, producing a series of feedback cascades that ultimately manifests in obesity [232].

Obesity has been recognized since the 1950s as a chronic state of low-level inflammation associated with excess accumulation of adipose tissue [233]. This inflammation is now thought to be a complex response to cellular events, such as hypoxia and oxidative stresses within the adipocyte. Figure 1 outlines the possible interacting events underlying obesity starting with the creation of hypertrophic adipocytes. These adipocytes become too large to be adequately supplied by the existing vasculature in the adipose depot, resulting in localized areas of hypoxia. This hypoxic state induces the production of numerous pro-inflammatory adipokines (e.g. IL1Rα, IL6, IL8, TNFα, MCP-1, leptin) and decreases the secretion of several key anti-inflammatory factors (e.g. IL10, adiponectin). Excellent reviews on these adipokines in obesity can be found in Fain et al. 2010 and Balistreri 2010. In these hypertrophic adipocytes, hypoxia is thought to induce oxidative stress [234], [235]. Oxidative stress is defined as an imbalance in the levels of reaction oxygen species (ROS) relative to the tissue's antioxidant capacity, resulting in the accumulation of oxidative products such as superoxide and hydroxyl radicals, reactive nitrogen species (RNS) and hydrogen peroxide [236]. Excess nutrients and hypertrophic adipocytes can produce ROS through: the nicotinamide dinucleotide phosphate oxidase (NOX) system [237], incomplete mitochondrial respiration due to excess free fatty acids [238]

and endoplasmic reticulum (ER) stress due to excess lipid storage [239]. Both mitochondrial and ER dysfunction have been demonstrated to increase the secretion of inflammatory adipokines [239], [240] and numerous studies in obesity models and obese subjects now exist linking hypoxia, oxidative stress and inflammation (reviewed in [236]). Concomitant with the development of hypertrophic adipocytes, there is a shift within the adipose tissue from M2 macrophages, found in normal adipose tissue, to a more pro-inflammatory M1 macrophage subset [241]-[243]. This shift is likely, in part, a consequence of the production of pro-inflammatory adipokines by adipocytes – such as MCP-1, but this infiltration is also likely to be due to the death of these adipocytes [244]. Consistent with this, "crown-like" structures of macrophages are known to be associated with necrotic adipocytes in obese murine adipose tissue [242]. These macrophages may directly contribute to the production of inflammatory agents within obese adipose tissue [245]. However, they may also augment adipokine production by the adipocyte through possible cross-talk mechanisms. While these mechanisms are unclear at this point, there are many who postulate that adipocyte-macrophage interaction is the key factor in inflammation and resulting obesity [230], [246], [247].

Author & Year (Reference)	Secreted factor
Blaber et al. 2012 [267]	IFNγ, IL8, IL9, IL12, IL17, TNFα
Hsiao et al. 2012 [268]	IL6, IL8, MCP-1, MCSF, RANTES
Bhang et al. 2011 [118]	HIF1α
Salgado et al. 2010 [49]	TNFα, IL6, IL8
Banas et al. 2008 [269]	IL6, IL8, IL1Rα, MCP-1, GMCSF
Kilroy et al. 2007 [102]	IL6, IL8, TNFα, MCSF, GMSCF

MCSF – macrophage colony stimulating factor

GMCSF – granulocyte-macrophage colony stimulating factor

MCP-1 – monocyte chemoattractant protein 1

IFNγ – interferon gamma

TNFα – tumor necrosis factor alpha

IL - interleukin

Table 3. Secretion of Pro-inflammatory Cytokines by ASCs

So obesity results from a complex series of cellular events that ultimately increases the production of inflammatory adipokines within the tissue. These adipokines are known to further increase adipocyte hypertrophy producing a positive feedback system. This feedback system could be augmented further by the secretory activity of non-fat cells – i.e. the pre-adipocyte and even the ASC. Pre-adipocytes and adipocytes secrete many of the same pro-inflammatory factors listed above - with the exception of leptin and adiponectin, factors secreted by the adipocyte (reviewed in [235]). Furthermore, a review of the current literature

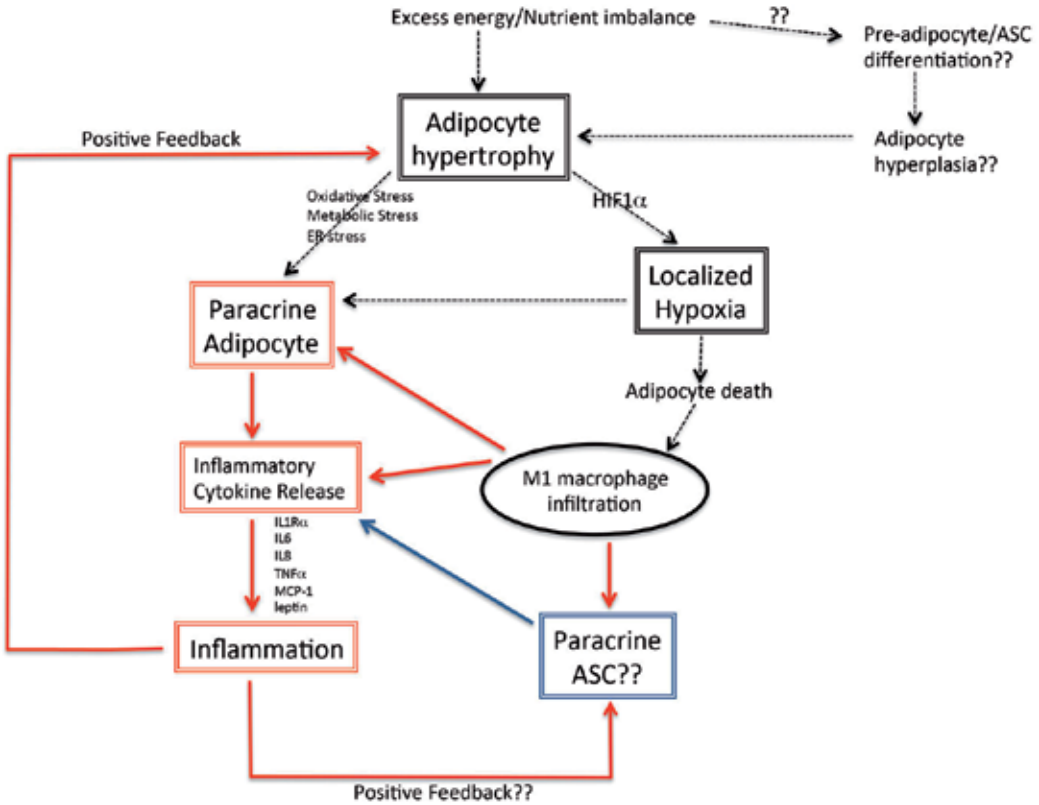

Figure 1. Possible interactions in obesity. Excess energy leads to development of hypertophic adipocytes. Hypertrophic adipocytes lead to the development of cellular stresses and hypoxia, via HIFI1α signaling, which can induce the adipocyte to release numerous pro-inflammatory cytokines. Hypoxia can also result in the death of adipocytes, inducing infiltration by pro-inflamatory/M1 macrophages into the adipose tissue. Paracrine activity by macrophages could affect the release of inflammatory cytokines from the adipocytes. In addition the macrophage may also release these cytokines directly. The resulting inflammation is likely to set up a feedback loop to enhance hypertrophic adipocyte development. The role of the ASC remains unknown in obesity but possible points of interaction could be the differentiation of ASCs, leading to adipocyte hyperplasia and the release of similar pro-inflammatory cytokines. Paracrine activity is shown as solid arrows.

turns up many studies that document the secretion of similar pro-inflammatory factors by ASCs (Table 3). It is possible that the secretion of inflammatory factors, like IL6 or TNFα, by ASCs may play a crucial role in inflammation and the development of obesity. Alternatively, it is possible that inflammation and obesity may result from "defective" ASCs that fail to secrete key anti-inflammatory factors such as IL-10 or have lost their ability to ameliorate oxidative stresses, thus allowing inflammation to go on unchecked. Unfortunately, the effect of inflammation and the ASC is under-represented in today's literature. Those studies that do exist document the inhibition of ASC adipogenesis under inflammatory conditions [248]. This is an interesting finding, as the ASC might be thought of as the logical source for adipocyte hyperplasia observed in obesity. However, if it is the paracrine activity of the ASC that plays a crucial role in the development or maintenance of obesity, then ASC differentiation capacity

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might be sacrificed in the name of maintaining this function. In light of what we know about adipocytes and pre-adipocytes in obesity, more in-depth studies on the ASC are certainly warranted.

A similar argument for ASCs could be made for other adipose disorders such lipedema and rare adipose disorders (RADs) such as Dercum's (aka Adiposa Dolorosa) [249] and Madelung's disease or Multiple Symmetric Lipomatosis (MSL) [250]. Lipedema (LD), or edema of the fat, is defined as the symmetrical accumulation of adipose tissue in the lower extremities [251]. Because the fat may also be painful as the disorder progresses, LD is often described in the same spectrum as Dercum's [252]. While lipedema and obesity share many similarities – leading to the misdiagnosis of lipedema in up to 15% of the population as obesity, there are some significant differences between LD and obesity. Specifically, excess fat accumulates almost exclusively in the lower limbs in LD and this adipose tissue is stubbornly resistant to loss through dieting [253]. LD is almost exclusively seen in women in their 30s or older, suggesting a hormonal component [251]. Despite these differences, the etiology of obesity and LD may share some commonalities, in that LD is thought to be mediated, in part, through hypoxia and the production of inflammatory cytokines (Figure 2). Like obesity, LD is initially characterized by adipocyte hypertrophy and hyperplasia [254], although the reason for this hypertrophy cannot be attributed to nutrient overload and currently remains unknown. This hypertrophy results in hypoxia, which is thought to result in inflammatory adipokine secretion and a putative positive feedback cascade as seen in obesity. Like obesity, LD fat is characterized by macrophage "crowns" in close association with hypertrophic and/or necrotic adipocytes [132]. These macrophages will almost certainly contribute to the inflammatory reactions occurring in LD fat. Furthermore, when examining adipose tissues isolated from Dercum's, similar immune infiltrations in association with perivascular cells and hypertrophic adipocytes are also seen, again, suggesting that LD and Dercum's may be points along the same spectrum [252]. In light of these commonalities with obesity, it would be logical to assume that the ASC would also play some critical role in mediating inflammation in LD or RADs through its production of paracrine factors. Unfortunately, these studies do not exist at this point.

Despite sharing many of the same characteristics, there are some important distinctions between obesity and LD that may also be at work. These distinctions are also likely to be found in RADs like Dercum's and Madelung's disease. Specifically, LD (and possibly Dercum's and Madelung's) is associated with defects in the microvasculature, together with lymphatic dysfunction [252]. Current theories propose that adipocyte hypertrophy leads to hypoxia, which results in increased angiogenesis. However, this angiogenesis is pathologic and the resulting capillaries are said to be "fragile" or "leaky" [255]. In support of this, perivascular cells, indicative of vascular damage, can be found in LD adipose tissue [254] and pathologic angiogenesis producing fragile capillaries have been found in many eye diseases [256], [257]. What produces this pathology is unknown but studies have shown that leptin can increase the number of fenestrations in capillaries [258] and increased plasma VEGF levels can be found in LD patients [259]. Increased plasma VEGF levels can also be found in LD patients [259], so it is possible that paracrine secretion from hypertrophic and hypoxic adipocytes could disrupt angiogenesis within LD adipose tissue. With studies showing ASCs capable of secreting

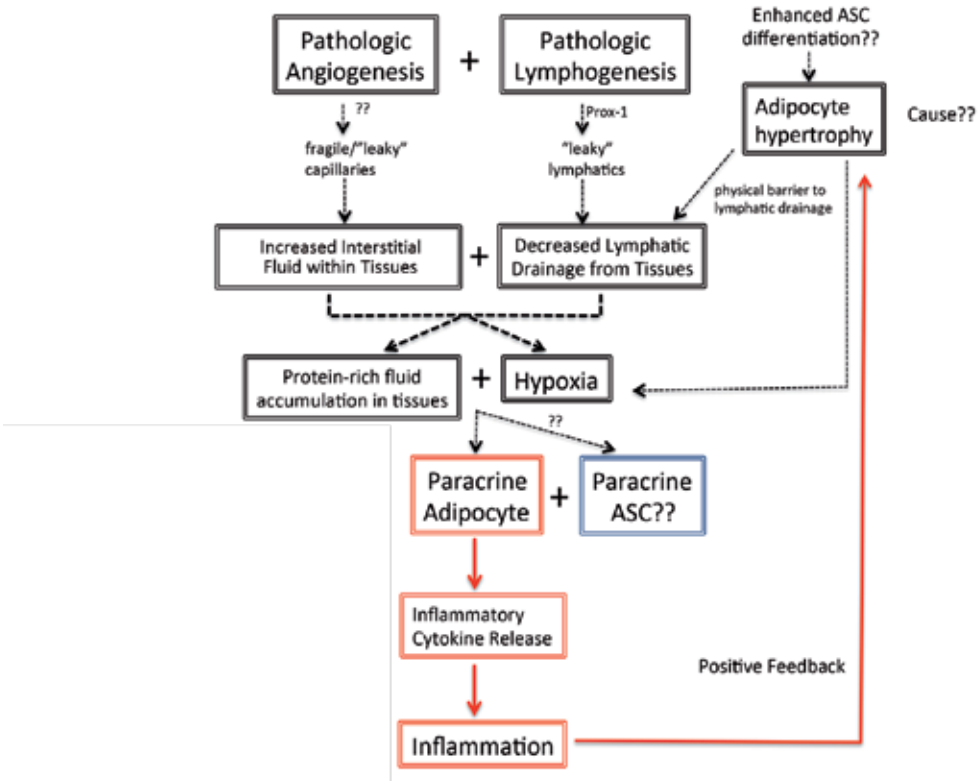

Figure 2. Lipedema. Development of lipedema may have numerous commonalities with obesity starting with the development of hypertrophic adipocytes. However, causation for this is unknown at this time may involve the ASC. As with obesity, adipocyte hypertrophy can lead to the development of hypoxia and the release of inflammatory cytokines from the adipocyte. Possible release of these factors from the ASC due to hypoxia is also shown. In addition, adipocyte hypertrophy is also accompanied by the development of "leaky" capillaries and lymphatics. While the cause of pathologic angiogenesis remains unclear, a role for the gene Prox-1 is though to be involved in lymphatic pathology. Increased filtration from capillaries, combined with poor lypmphatic drainage (due to hypertrophic adipocytes and the the leaking of lymph back from the lymphatic vessel) leads to an accumulation of protein rich fluids within the tissue. Fluid accumulation and hypoxia may induce pro-inflammatory cytokine release. Other mechanisms of obesity (e.g. macrophage infiltration) are also likely to be involved. Paracrine activity is shown as solid arrows.

numerous paracrine factors, including VEGF, and inducing endothelial differentiation and vessel formation, the question of whether the ASC plays a role in this vascular pathology should be asked. The fragile capillaries allow the filtration of protein-rich plasma into the interstitial space, driving the formation of edema [255]. In the early stages of LD, lymphatic drainage can keep up [260]. However with progression of the disorder, lymphatic drainage does decrease as the patient ages [253]. Added to this, the hypertrophic adipocytes are thought to physically restrict fluid drainage and the smaller lymphatic vessels themselves are thought to become "leaky", possibly through the appearance of microaneurysms in these vessels [253]. All of this results in the accumulation of lymph within the adipose tissue. Recent studies now suggest that "lymph can make you fat" [261]. In support of this, adipogenesis in vitro increases

when cells are cultured in the presence of lymph [262], [263]. Furthermore, the removal of axillary lymphs nodes in individuals with breast cancer is frequently associated with increased fat deposition within the arm [263]. More recently, mice heterozygous for a mutation in the Prox1 gene not only exhibit leaky lymphatics, but develop obesity as they age [264]-[266]. What it is in the lymph that enhances adipogenesis is unclear. It simply could be the result of edema causing hypoxia, inflammation and adipocyte hypertrophy – not unlike obesity. Alternatively, factors in the lymph could directly induce the ASC to differentiate or the mature adipocyte to store more fat. Since lymph is interstitial fluid combined with emulsified fats, non-reabsorbed proteins and immunocompetent leukocytes, any of these factors could conceivably alter the behavior of the ASC. As it stands, more studies investigating the exact consequences of lymph accumulation on ASC and adipocyte behavior are needed.

So while the mechanisms may differ at points, at the basis of obesity, LD and RADs is inflammation. How the ASC participates in this inflammation remains to be seen, but the ASC could be used in the treatment of these disorders. If inflammation results in adipocyte hypertrophy, then ameliorating this process could decrease the size and number of these cells. In this regard, the anti-inflammatory, anti-oxidant properties of ASCs could be taken advantage of and enhanced in the hopes of mitigating the damaging effects of inflammation in these adipose disorders. However, before this could be attempted, more information is definitely required on the exact roles the ASC plays in adipose tissue formation and how these roles can go wrong when adipose disorders develop.

5. Conclusion

Since 2001, the number of studies characterizing and utilizing the ASC is truly staggering. It appears that the ASC is even passing the bone marrow MSC as the preferred adult stem cell for regenerative medicine. With its ease of isolation from adipose tissue, its availability within the tissue, its long term viability in culture and its persistence when implanted in vivo, the ASC is not only a great stem cell choice for studying mechanisms in vitro but for how it can regenerate tissues in vivo. In response, the studies using ASCs are incredibly diverse and range from their direct differentiation in regenerating tissues such as bone, muscle, nerve and liver to their indirect use in mediating inflammation, protecting nervous tissue and directing vascularization and wound healing through their production of paracrine factors. Finally, a truly exciting use for the ASC may be based on this paracrine activity, in that ASC appears to be easily engineered for the delivery of key factors capable of regenerating many tissue types and maintaining their health. Only time will tell how far the ASC will go.

Abbreviations

ASC = adipose-derived stem cell; EC = endothelial cell; LD = lipedema; MSC = mesenchymal stem cell; GFAP = glial fibrillary acidic protein; HLA = human leukocyte antigen; IR = ischemia

reperfusion; LVEF = left ventricular ejection fraction; MAP2 = microtubule associated protein-2; MLR = mixed lymphocyte reaction; PLA = processed lipoaspirate; RAD = rare adipose disorder; SVF = stromal vascular fraction; SC = Schwann cell; Tuj-1 = class III beta-tubulin; vWF = von Willebrand factor

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Chapter 4

Dental-Related Stem Cells and Their Potential in Regenerative Medicine

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Additional information is available at the end of the chapter

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1. Introduction

Stem cells have been opening a promising future in clinical therapies because of their two remarkable features known as self-renewal and multi-lineage differentiation. These cells can be classified in terms of their origin (embryonic, prenatal and postnatal stem cells) as well as the differentiation commitments (pleuripotent, multipotent and unipotent). Postnatal stem cells, also known as the adult stem cells (ASCs), normally exist in almost every adult tissues, including bone marrow, skin, neural tissues, and dental epithelium, acting as supportive cells by their regeneration capacity. Among different stem cell types, ASCs seem to be more applicable in stem cell-mediated therapies and regenerative medicine because these cells lack ethical concerns, and possesses less tumorgenic potency than their embryonic counterparts.

Recently, human dental stem cells (DSCs), a subtype of ASCs, have drawn worldwide attention for future therapies due to their both technical and practical superiorities. In addition to having some mesenchymal stem cell (MSC) characteristics, including plastic adherent ability with formation of colonies in vitro, and also immunoprivileged properties, DSCs are easily-accessible cells with higher proliferation capacity than ordinary marrow-derived MSCs. Currently, there are six types of stem/progenitor cells determined in dental-related tissues. 1) dental pulp stem cell (DPSCs), 2) stem cells from human exfoliated deciduous teeth (SHED), 3) periodontal ligament stem cells (PDLSC), 4) stem cells from apical papilla (SCAP) of developing tooth, 5) dental follicle stem/progenitor cells (DFPCs) and 6) gingiva stem cells (GSCs). DPSCs, SHEDs and SCAPs are referred to as dental pulp-related stem cells, and PDLSCs & DFPCs as periodontium-related stem cells [1, 5].

This chapter focuses on different aspects of dental-derived adult stem cells, such as their classification, biological characterization, initiating culture, cultivation systems, cryopreservation and potential applications in tissue engineering and regenerative medicine. The data are organized as three main parts, including: 1) Dental-related stem cell biology: from the classification to the characterization and differentiation potential 2) Dental-related stem cell initiation culture, culture systems and cryopreservation 3) Dental-related stem cell- based tissue regeneration.

2. Dental-related stem cell biology: From the classification to the characterization & differentiation potential

According to the literature, there are several types of stem/progenitor cells existed in dental tissue. In this section, each of these cells will be described in terms of their main characteristics.

2.1. Dental Pulp Stem Cells (DPSCs)

The presence of stem cells in dental pulp tissue primarily have been reported in 1985 by Yamamura [3, 4, 6] (Figure 1). Later on, Caplan et al. have demonstrated that these cells presented osteogenic and chondrogenic potential in vitro, and could also differentiate into dentin, in vivo. In 2000, Gronthos et al. have isolated dental pulp stem cells from adult human dental pulp, which had the ability to regenerate a dentin-pulp-like complex [7]. Interestingly, some recent works have found the presence of stem cells in inflamed pulp with capacity to form mineralized matrix both in vitro and in vivo. These findings make dental pulp as an interesting tissue source of putative stem cells, even in diseased form.[8]

DPSCs are similar to MSCs in some ways: they are of fibroblastic morphology with selective adherence to solid surfaces, having good proliferative potential and capacity to differentiate in vitro, and the ability to repair tissues in vivo. It's interesting to note that DPSCs could differentiate into not only osteoblasts, chondrocytes & adipocytes, but also myocytes, neurons and hepatocytes lineages in vitro [4]. DPSCs are characterized by their negative expression of hematopoietic antigens (*e.g.*, CD45, CD34, CD14), and positive expression of stromal-associated markers (*e.g.*, CD90, CD29, CD73, CD105, CD44) (Table 1). They also express multipotent marker (STRO-1) and extracellular matrix proteins, such as collagen, vimentin, laminin, and fibronectin. [9-11]. Interestingly, some of the pluripotent stem cell markers, such as Oct4, Nanog, Sox2, Klf4, SSEA4 & c-Myc have been reported to express on DPSCs [12-14]. More recently, it was demonstrated that core transcription factor of the reprogramming Oct4, Nanog, Klf4 and c-Myc become significantly down-regulated following the DPSC differentiation [4].

Apart from stemness markers, DPSCs are also shown to express bone markers, such as bone sialoprotein, osteocalcin, alkaline phosphates (ALP), and type I collagen. This indicates their differentiation commitment into bone tissue [15]. On the other hand, the expression of dentin sialophosphoprotein (an odontoblast specific protein precursor) is not present in the cultures of hDPSCs implied that these cells represent an undifferentiated pre-odontogenic phenotype [7, 16].

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From immunological perspective, it has been reported that DPSCs displayed more immnosuppressive activities than the BM-MSCs. This was obvious in inhibiting T cells response in vitro [17].

Based on some investigations, there is a sub-type of DPSCs referred to as "immature dental pulp stems cells" (IDPSCs), which have promising potential in future stem cell researches. IDPSCs were firstly, isolated from pulp tissue of the human exfoliated deciduous as well as permanent teeth [18]. These cells express both embryonic and MSC markers (see part 2.2). It has been indicated that transferring of human IDPSCs (hDPSCs) into mouse blastocysts resulted in formation of human/mouse chimera which was able to retain proliferation and differentiation capacity [19]. Furthermore, hIDPSCs possess the capacity to rapidly reprogrammed into induced pleuripotent stem cells (iPSc) which are able to produce primary hIDPSC-iPSC colonies even under feeder-free conditions [20].

2.2. Dental Stem cells from Human Exfoliated Deciduous teeth (SHED)

In 2003, Miura et al. have reported to isolate a stem cell population from the living pulp remnants of exfoliated deciduous teeth. These authors have termed the cells as stem cells from human exfoliated deciduous teeth (SHED) [21] (Figure 1). These cells which are believed to be of the neural crest origin are heterogeneous fibroblast-like population possessing an extensive proliferating capacity than either DPSCs or BM-MSCs [22]. In terms of surface epitopes, it has been found that they express markers of MSCs (STRO-1, CD146, SSEA4, CD90, CD73, CD 105, CD106 and CD 166) and lack of hematopoietic/endothelial markers (CD34, CD31) (Table 1). Under an appropriate culture conditions, SHED are able to differentiate into the variety of cell types, including neural cells, angiogenic endothelial cells, adipocytes, osteoblasts, and odontoblasts [23-25]. In vivo transplantation of SHED have been reported to result in formation of bone and dentin like-tissue. [18, 21, 26-29]. There are some studies suggested that SHED is different from IDPSCs in terms of expression of stem cell markers (see part 1.1)[18, 30]. Moreover, some research works have been reported that SHED would possess immunomodulatory function as seen in BM-MSCs [28].

2.3. Periodontal Ligament Stem Cells (PDLSC)

Periodontal ligament stem cells (PDLSCs) have first been introduced by Seo et al. [31] (Figure 1). Like MSCs, PDLSCs have been reported to form adherent clonogenic population of fibroblast-like cells in the culture. They express both early MSC markers such as, STRO-1 and CD146, and other MSC and pluripotent makers, such as CD44, CD90, CD105, CD73, CD26, CD10, CD29 and CD166; meanwhile, they have no expression for CD40, CD80, and CD86[31-33] (Table 1). Some investigations have revealed that PDLSCs may be positive for embryonic stem cell markers, as well, including SSEA1, SSEA3, SSEA4, TRA-1-60, TRA-1-81, Oct4, Nanog, Sox2 and Rex1, and ALP [34]. Based on some research works, SSEA4-positive PDLSCs displayed the potential to generate adipocytes, osteoblasts, chondrocytes (from mesodermal layer), neurons (from ectodermal layer), and hepatocytes (from endodermal lineage) *in vitro* [31, 34, 35]. Furthermore, it has been shown that transplantation of PDLSCs

into immunocompromised rodents resulted in the generation of cementum/PDL-like structure and contributes to periodontal tissue repair [31].

PDLSCs show immunomodulatory activity by up-regulation of soluble immunosuppressive factors (TGF-β1, hepatocyte growth factor (HGF) and indoleamine 2, 3-dioxygenase (IDO) in the presence of activated peripheral blood mononuclear cells (PBMNCs). Similar to the DPSCs, PDLSCs are positive for HLA-ABC (MHC class I antigen) while negative for HLA-DR (MHC class II antigen) [32].

2.4. Dental Follicle Progenitor Cells (DFPCs)

In 2005 & 2007, Morsczeck et al. and Kémoun et al., respectively have identified unique undifferentiated lineage committed cells possessing mesenchymal progenitor features in the human dental follicle (Figure 1). The cells were referred to as "dental follicle precursor cells" (DFPCs) [36, 37]. Characteristically, DFPCs, similar to the bone marrow stem cells, are adherent and colony-forming cells. These cells have been reported to express Notch-1, CD13, CD44, CD73, CD105, and STRO-1 [1, 36] (Table 1). Human DFPCs has been believed to consist of precursor cells for cementoblasts, periodontal ligament cells, and osteoblasts. Under appropriate *in vitro* conditions, they are capable of differentiating into osteoblasts, cementoblasts, chondrocytes and adipocytes. Interestingly, although both DFPCs and SHED are of the neural crest origin, their neural differentiation potentials are different under the same culture conditions. It has been reported that SHED possess good differentiation potential than DFPCs in terms of the expression of Pax6 which is a marker of retinal stem cells [27].

2.5. Stem Cells from the Apical Papilla (SCAP)

Stem cells from dental apical papilla (SCAP) were first identified and characterized by Sonoyama et al. in human permanent immature teeth [38] (Figure 1). These authors described the cells as adherent clonogenic cells with mesenchymal stem cell features, which are expressed STRO-1, CD24, CD29, CD73, CD90, CD105, CD106, CD146, CD166, and ALP, and not expressing CD34; CD45; CD18; and CD150. Among these markers, CD24 would be of a specific marker for SCAP since it's not found in the other dental stem cells (Figure 2). Excitingly, some authors have reported that SCAP display higher telomerase expression than both DPSCs and BM-MSCs [38]. Furthermore, SCAP has been shown to positively stain with several neural markers implying their possible origin from the neural crest [39]. In terms of differentiation, SCAP are capable of generating osteoblasts, odontoblasts and adipocytes in vitro. An in vivo study has demonstrated that these cells form hard tissue when being loaded onto hydroxyapatite (HA) and implanted subcutaneously in immunocompromised rats [38-40]. Moreover, SCAP have been reported to possess a significantly higher mineralization potential as well as proliferation rate than DPSCs. This finding might be of some importance for their use in dental and/or bone tissue engineering and regeneration [41].

About the possibility of immunogenicity of SCAPs, an independent study have reported that swine SCAPs are non-immunogenic and suppressed T cells proliferation in vitro [42].

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2.6. Stem Cells derived from Gingiva (GSCs)

The isolation of a stem cell population from gingiva was firstly reported by Zhang et al. in 2009 [43] (Figure 1). These authors derived the cells from the spinous layer of human gingiva and referred to them as gingival stem cells (GSCs). In terms of markers, it has been shown that GSCs are negative for CD45/CD34, but positive for CD29, CD44, CD73, CD90, CD105, CD146, STRO-1 and SSEA4 (Table 1). In addition, extracellular matrix proteins, such as collagen, vimentin, Collagen type-1, and fibronectin have been reported to express in these cells [43, 44]. Like MSCs, GSCs possess a differentiation potential into osteoblasts, adipocytes and chondrocytes in vitro [45]. Moreover, these cells have been found to be able to differentiate along endothelial as well as neural cell lineages. Furthermore, in vivo bone regeneration potential of GSCs was demonstrated by transplantation of GSCs/HA into immunocompromised mice [45]. More importantly, in a comparative study, it was demonstrated that GSCs showed stable phenotypes, maintain normal karyotype and telomerase activity in long-term cultures in comparison with BM-MSC [45].

As with other dental related stem cells, GSCs has been found to display immunomodulatory functions; they inhibit lymphocytes proliferation and express a wide range of immunosuppressive factors, including Interleukin-10 (IL-10), IDO, inducible NO synthase (iNOS), and cyclooxygenase 2 (COX-2) in response to the inflammatory cytokine, IFN- γ [43].

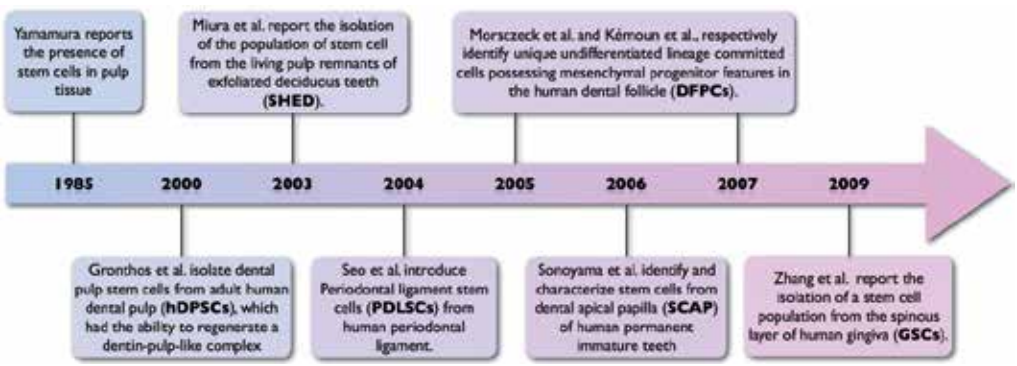

Figure 1. Timeline about the highlights in the history of the isolation of dental-related stem cells (see text).

	DPSCs	SHED	PDLSCs	DFPCs	SCAPs	GSCs
CD (+)	STRO-1	STRO-1	STRO-1	STRO-1	STRO-1	STRO-1
	CD10		CD10	CD10		
	CD13	CD13	CD13	CD13	CD13	CD13
					CD24	
			CD26			
	CD29	CD29	CD29	CD29	CD29	CD29
	CD44	CD44	CD44	CD44	CD44	CD44
	CD59		CD59	CD59		
	CD73	CD73	CD73	CD73	CD73	CD73
	CD90	CD90	CD90	CD90	CD90	CD90
	CD105	CD105	CD105	CD105	CD105	CD105
	CD106	CD106	CD106		CD106	CD106
	CD117					
	CD146	CD146			CD146	CD146
		CD166	CD166		CD166	CD166
CD (-)	CD14	CD14	CD14			
					CD18	
	CD19					
	CD24					
	CD34	CD34	CD34	CD34	CD34	CD34
			CD40			
	CD45	CD45	CD45	CD45	CD45	CD45
			CD80			
			CD86			
					CD150	
	HLA-DR		HLA-DR	HLA-DR		HLA-DR

Table 1. Cell Surface Marker Profiles of dental-related stem cells. DPSC; Dental pulp stem cell, SHED; Stem cells from human exfoliated deciduous teeth, PDLSC; Periodontal ligament stem cells, DFPC, Dental follicle precursor cells, SCAP; Stem cells from dental apical papilla, GSC; Gingival stem cells. [1-4]

3. Dental-related stem cell initiation culture, culture systems and cryopreservation

In dental related stem cell researches, the first step is to isolate cells from tissue sources. The next step is to expand the cells into sufficient number. In some occasion, it may be necessary to preserve the cells for future use since tissue sources would not be available on demand. In this section, we will describe the location of the certain teeth tissue from which the stem cell population can be derived and followed by description of common methods by which the isolation culture can be initiated. At the end, culture systems for the cell propagation as well as the main points regarding issue of cell cryopreservation will be explained.

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3.1. Anatomical location of teeth tissue from which DSCs can be derived

Most of the human DSCs are come from teeth, which are subjected to the orthodontic treatments. Based on the studies, molars and premolars are mostly used for this purpose. Third molars (wisdom teeth) are the most common teeth for extraction in dental clinical practice. In addition, developing wisdom teeth during the adult life are the excellent candidates as the accessible source of developing tissue similar to those in embryonic development. There are a few studies considering the supernumerary teeth derived from other teeth, such as canine, for the isolation of DPSCs [12] In some cases, such as the isolation of DPSCs from inflamed dental pulps, endodontic treatments are used rather than orthodontics surgeries [46]. In any case, considering the precise location of the dental tissues in tooth anatomy is important to achieve certain types of DSCs with minimum cell contaminations. Hence, here is the brief description of the localization for the specific DSCs isolation.

3.1.1. Tissues contained dental pulp-related stem cells (DPSCs, SHED & SCAP)

Isolation of DPSCs following the pulp extraction could be achieved by either through the root or crown of the dental organ. In the case of SHEDs or DPSCs, which are derived from incompleted root teeth, the exposed pulp is accessible from the root without applying any specific procedure [21]. In other situation (extracted permanent or deciduous teeth), dental pulp extraction is accomplished through the dental crown by cutting the cementum-enamel junction (CEJ) using dental instruments, such as pliers (bone forceps), extirpation needle, Gracey curette, dental fissure burs, etc. [6] Moreover, in the case of inflamed teeth, pulp tissues are removed during the endodontic therapies [46] (Figure 2)

The isolation of SCAP is achieved by gentle separation of root apical papilla from the surface of the root with immature apex (i.e. located in the exterior of the root foramen area before the complete eruption of tooth in the oral cavity) during the extraction of third molars [47]. Root maturation results in the elimination of apical papilla; hence, the maturation period of teeth are important for isolation of SCAP. (Figure 2)

3.1.2. Tissues contained periodontium-related stem cells (PDLSCs & DFPCs) & GSCs isolation

PDLSC can be obtained from the middle third of the root surfaces of extracted PDL tissue, which is a soft connective tissue surrounded between the cementum and the inner wall of the alveolar bone socket. It's accomplished by scrapping surface of the middle third of the root [31].

DFPCs can be isolated by dissecting dental follicle from the upside of the dental crown from impacted teeth. Human dental follicle is an ectomesenchymal tissue that is derived from cranial neural crest. This tissue surrounds developing tooth germ and involves in the coordination of tooth eruption and periodontium formation. This tooth germ's tissue can easily be isolated after wisdom tooth extraction by routine orthodontical related surgeries. Impacted teeth, usually third molars, normally fail to erupt through the gum because of their encasement in the jawbone; therefore, routine surgical procedures are required for the extraction. [36, 37]. (Figure 2)

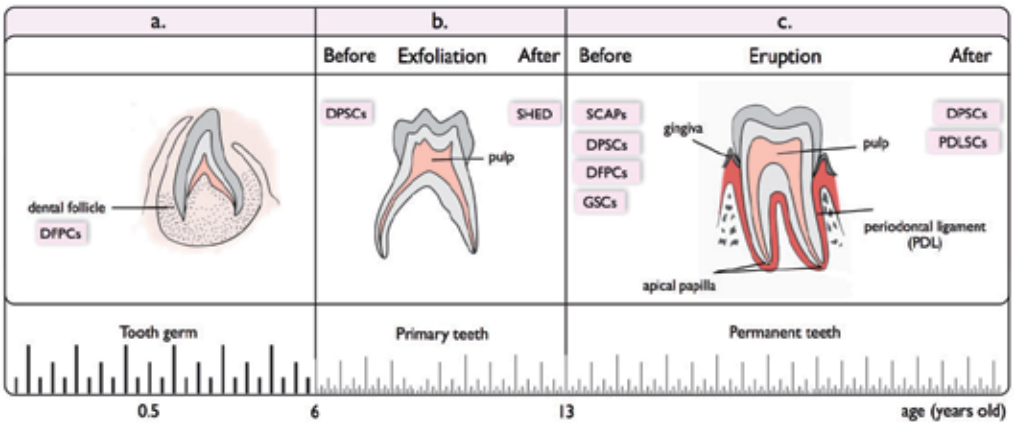

Figure 2. Overall view of dental-related stem cells based on different anatomical locations and stages during the human lifetime in. a. tooth germ, b. primary teeth, c. permanent teeth.

GSCs could be isolated from clinically healthy gingiva, which are obtained as remnant or discarded tissues following routine dental procedures [43]. (Figure 2)

3.2. DSCs culture initiation

In general, dental-related stem cells could isolate by either (1) enzymatic digestion (ED) of tissues or (2) outgrowth (OG) from tissue explant. In the case of enzymatic digestion, after the extraction, tissues are placed into the digestion enzymes, (generally, collagenase type-I & dispase) for about 30-60 minutes at 37 °C to achieve single-cell suspensions. In order to purify DSCs, single-cell suspensions could be subjected into (1) size-sieved isolation (using 3 μm strainer followed by 20 μm strainer), (2) stem cell colony cultivation (single colony culture of stem cells) or (3) magnetic/ fluorescence activated cell sorting (sorting based on surface markers) [48].

In the outgrowth method (OG), tissues are minced into 1-2 mm pieces and placed into the culture dishes to outgrowth [18]. More recently, Lizier and his co-workers established a scaled-up hIDPSCs culture system based on in vitro re-plating of pulp tissue explants followed by 3-4 days expansion [49].

There are some evidences, which suggested different behavior of DSCs according to the ED or OG isolation methods [41, 50, 51]. According to Huang et al. DPSC isolated by ED method (DPSC-ED) from permanent teeth showed higher proliferation rate than those isolated by the OG method (DPSC-OG) [50]. Moreover, STRO-1 & CD34 markers expressed more in DPSC-ED in comparison with DPSC-OG. DPSC-ED derived from deciduous and permanent teeth has been reported to display higher mineralization rate in the defined osteo/odonto medium [51, 52].

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3.3. DSCs culture systems

Following the isolation of dental-related stem cells, the next step is to culture-expand the cells into the multiple copies since in the most strategies related to the cell-based-treatment of tissue defects, the copious amount of regenerating cells is needed. Many researchers have been focused on optimizing effective conditions under which DSCs can efficiently be propagated. On the other hand, differentiation potential of the multiplied cells must be determined because discovering the potential commitments of the cells may lead to better selection of them for future organ-targeted treatments [27, 48, 53]. Due to these considerations, this section opens up a brief overview for different DSC culture systems designed for the cell expansion and differentiation.

3.3.1. Serum free vs. serum rich culture systems

Normally, the isolation and expansion of DSC have depend on a high concentration of serum culture media (10%), which provide better cell adhesion during the initial isolation of the cells. Unfortunately, in long-term cultivation, the high level of serum might lead to spontaneous differentiation or malignant transformation of cells. In addition, use of serum in culture may result in contamination of cell culture with bovine pathogen for instance bovine spongiform encephalopathy (BSE). For these reasons, serum free culture systems are highly recommended [54-57]. In this regards, many attempt has been made to optimize DSC cultivation in serum-free or low serum medium. For example, Karbanova et al. have reported that DPSCs cultivated in low-serum medium exhibit less proliferation rate and different expression of stem cell markers compared to those cultivated in serum rich medium [14]. In contrast, Hirata et al have cultivated the cells in serum free media and have found the same survival rate of the cells as those cultivated in the serum containing medium [58].

In the case of DFPCs, studies indicated that applying serum replacement media didn't affect the expression of connective tissue markers, such as collagen type I and type III, and also neural stem progenitor marker, nestin [59]; however, there is no information about the possible changes in other markers in this regards.

It's interesting to note that SHEDs & PDLSCs cultivated in defined serum free media have been reported to display higher proliferation rate than those cultivated in the medium containing serum. Moreover such cells have found to express comparatively higher "stem-ness" markers [60]. Applying serum free media is one of the critical requirements for the future clinical treatments; therefore, additional works are needed for optimizing conditions to achieve final conclusion.

3.3.2. Neurosphere-forming vs. adherent culture systems

Sphere-forming culture systems are generally applied for neurogenic differentiation of stem cells. This culture system has also been used for DSC cultivation. The idea of applying such a system came from the consideration of neural crest origin of DSCs. It has been well established that neural stem/progenitor cells which are isolated from variety of sources are

grown as neurospheres in defined serum-free culture medium supplemented with EGF and / or bFGF [61-63].

DSC Suspension culture was first suggested by Morita et al. (2007). These authors have cultivated PDLSCs in the sphere culture and found that PDLSC have the ability to form neurospheres in serum-free culture containing epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF) with the ability to differentiate into both neural and mesodermal progeny [64].

In 2008, Sasaki et al. have cultivated DPSCs from the rat incisor in the sphere-culture and found that under these conditions, the cells expressed neural and glial markers. They have also noticed that CD81 positive DPSCs that were localized in odontoblast layer of apical portion of the dental pulp may have more potential to form neurospheres [65] Later on, it was demonstrated that sphere form of SCAP had multi-differentiation potential into mineralized cells, adipocytes and also myocytes under the defined media in vitro. Furthermore, In vivo studies have indicated that sphere SCAP showed more potential to generate mineralized tissues in comparison with the non-sphere SCAP [47].

3.3.3. Co-culture systems

Site-specific tissue interactions are essential for orchestration of proliferation, differentiation and also homeostasis of cells during the tooth germ development. In particular, epithelial-mesenchymal interactions are the most important developmental events, which are involved in reciprocal crosstalk between the ectodermal and mesenchymal tissues. These sequential interactions are critical for tooth morphogenesis and cell differentiation [66]. To imitate these in vivo interactions, co-culture systems have been developed.

Using co-culture systems, some attempts have been made to promote DSCs differentiation in vitro. In this regards different cell types have been examined as co-culture cells. For example, it has been demonstrated that co-culture of DFPCs/SCAP could lead to formation of bone-like structure in vivo and in vitro. This occurs since interaction between the cells stimulate cementogenic/osteogenic differentiation of DFPCs leading to up-regulation of bone intermediate proteins, such as bone morphogenetic protein 2 (BMP2), osteoprotegerin (OPG), bone sialoprotein (BSP) and osteocalcin (OCN), as well as down-regulation of receptor activator of nuclear factor κB ligand (RANKL) [67]. In other experiment, Arakaki et al. have co-cultured DPSCs with dental epithelial cells and found that in such a system, dental epithelial cells secret BMP2 and BMP4, thereby promote odontoblastic differentiation of DPSCs [68]. Interestingly, it has also been reported that co-culture of DPSCs with epithelial cells lead to epithelium invagination, as well [69]. Moreover, Wang et al. have established a co-culture of hDPSCs with osteoblasts and demonstrated that a higher mineralization and up-regulation of osteogenic-related genes in hDPSCs compared with those cultured in the absence of osteoblasts [70]. Furthermore, co-culture of DPSCs with non-dental MSCs, such as amniotic fluid-derived MSCs (AF-MSCs), has been reported to leads to the bone differentiation of AF-MSCs indicating the pre-commitment of DPSCs to induce osteogenic differentiation [71].

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3.4. DSCs cryopreservation

According to the diversity of dental-related stem cells and their remarkable features for cell-mediated therapies and tissue engineering, developing a reliable method for cell banking have become a priority for future use. In this regard, cryopreservation could be established at the levels of teeth (organs), dental tissues or cells. In the case of teeth or dental tissues cryopreservation, minimal processing may needed for banking. There are some evidences which are demonstrated the successful cryopreservation of healthy and diseased teeth as well as dental tissues [72-78]. On the other hand, cryopreservation of DSCs still has been considered as an active area of the researches. There are several parameters which need to be considered prior to an establishment of a successful and more efficient protocol. The parameters which need to be determined include (1) DSCs-cultured passage(s), which leads to high-efficiency recovery post thaw, (2) concentration of cryoprotective agent, (3) cell concentration for high-efficient cryopreservation, (4) storage temperature, (5) the process of cryopreservation, and (6) evaluation of growth, surface markers and differentiation properties of DSCs after post thaw. So far, there have been many researches considering the comparative cryopreservation methods for optimizing the best protocols. More recently, it has been suggested that magnetic cryopreservation of DSCs was much better than conventional slow-freezing procedure in terms of cytotoxicity [76, 79, 80].

4. Dental-related stem cell based tissue regeneration

Although DSCs are newborn in the field of stem cell therapy and tissue engineering, they have opened the promising windows not only in tooth repair and regeneration studies but also in other organs. To date, most of DSCs mediated cell therapies and tissue-engineering studies have been focused on the animal models. However, more recently, a few clinical trial studies have also been accomplished. Meanwhile, the immunogenicity of these cells should be more considered for their allogeneic transplantation.

4.1. DSCs-based tooth engineering and regeneration of dental related tissue

In general, in the field of dentistry, the final goal of tissue engineering is to develop tooth-replacement therapies using the whole-bioengineered-tooth technology. To fulfill this, some authors have conducted the remarkable investigations. In this regard, there is some experiment in which either dissociated tooth germs or mesenchymal and epithelial cells derived from the tooth germs were prepared and loaded on to the prefabricated tooth-shaped scaffolds in order to fabricate a bioengineered tooth. Based on these investigations, tooth germ cells possessed a high potential to form dentin, enamel, pulp, and periodontal tissues in vivo [81-84]. However, using embryonic tooth germ and problems related to immunogenicity of animal transplantation, make this an unfeasible approach to clinical application of tooth regeneration [84].

Alternative cells would be DSCs. Using these cells, some preliminary studies have been accomplished to examine whether DSCs in combination with appropriate scaffold are able to promote regeneration of tooth tissues [85, 86]. For instance, transplantation of PDLSCs

loaded onto HA/TCP have been shown to result in the production of cementum in mice while using gelfoam (collagen based gelatin sponge) as the carrier has been reported to fail creating cementum [31, 87]. Sonoyama and co-workers have reported the creation of a root/periodontal complex being capable of supporting a porcelain crown in swine by applying both SCAP and PDLSCs in HA/TCP as a carrier [38]. In a study on canine model, we have recently succeeded to achieve regeneration on an experimentally-created defect in peridontium using autologous DPSCs loaded onto Bio-Oss scaffolds [88]. Furthermore, Nakashima et al. have reported a successful induction of whole-pulp regeneration after pulpectomy in a dog model using autologous DPSCs loaded onto 3D scaffold of collagen-I & II and SDF-1 (stromal-cell-derived factor-1) as the morphogene [89, 90]. Moreover, a culture system termed as organ-engineering methods using PDLSCs have been developed to generate not only tooth root, but also the surrounding periodontal tissues, including PDL and alveolar bone in mouse model [84]. In addition to the above-mentioned animal studies, there is a published retrospective pilot study in human. According to this trial, autologous transplantation of PDL stem/progenitor cells might provide therapeutic improvement for the periodontal defects without any adverse effects during 32–72 months of follow-up [91].

4.2. DSCs-based tissue engineering and regeneration for other organs

Apart from the potential applications in dental tissue engineering and therapy, DSCs have been opened a dynamic field in repair and regeneration of non-dental tissues. In this context, there are many investigations indicating applicability of dental related stem cells in variety of disease models in the animal. For instance, SHED has been reported to exhibit a potential to improve parkinson's disease in rat by differentiating into dopaminergic neuron-like cells. Based on the different investigations, these cells have also the ability to promote wound healing in mice [92-94]. It has been demonstrated that SHED also contribute to repair of the critical-sized calvarial defects in mice model [95].

Transplantation of DPSCs has been shown to improve alzheimer's and parkinson's disease as well as acute myocardial infarction in a rat model [96-98]. Similarly, transplantation of hIDPSCs in canine model with golden retriever muscular dystrophy (GRMD) resulted in some improvement [99]. In addition, it has been reported that transplantation of tissue-engineered hIDPSC sheet was successfully reconstructed the corneal epithelium in rabbits with total limbal stem cell deficiency (LSCD) [100]. Applying PDLSCs with collagen based gelatin sponge carrier have been found to improve facial wrinkles by generating large amounts of collagen fibers in the mouse indicating the potential capacity of PDLSC in the field of plastic surgery. In the case of GSCs, *Intra-peritoneal (ip)* administration of the cells displayed improvement of inflammation-related tissue destruction in experimental colitis [43].

4.3. Immunogenicity of allogeneic transplantation of DSCs

According to the promising place of DSCs in clinical treatments in future, considering the immunogenicity of DSC transplantation is critical. Although autologous DSCs rather than allogenous ones are preferred for repair and regeneration purposes, several limitations such

as inadequate cell numbers and donor site morbidity carry out problems. Thus, more studies will be needed to evaluate immunogenicity of allogenous DSCs for the future clinical applications. There are some evidences suggested that DSCs, as the mesenchymal stem cells, have immunomodulatory properties both in vitro and in vivo. These studies are almost considering in vitro immunogenicity of DSCs by evaluating the expression of pro/anti-inflammatory mediators, such as MHC classes, TGF-β, ODO, HGF, nitric oxide (NO), prostaglandin, immunosuppressive minor H antigen (HLA-G), and interferon (IFN)-γ as well as effect of DSCs in suppression of T cells proliferation [28, 101, 102].

In addition, in vivo studies also showed DSCs immunomodulatory functions. For instance, SHED transplantation up-regulated the ratio of Treg (regulatory T cells) and Th17 in MRL/ lpr mice model compared to the BM-MSCs; meanwhile, no change in the expression of IL-6 & IL-10 was detected. On the other hand, transplantation of human DPSCs into the rat model didn't initiate the immunologic responses in recipients [103]. Moreover, in another study, it was demonstrated that transplantation of hIDPSCs didn't show the immune reaction in the canine [99].

In spite of existing evidences, which suggested the immunomodulatory effect of DSCs, there are some data proposing that there is different expression of immune receptors, such as toll-like receptors (TLRs) on the cells. Presence of this receptor could affect the immumomodulatory phenotypes of DPSCs and DFPCs [101, 104, 105]; On the other hand, since some studies suggested that BM-MSCs may regain immunogenic property upon differentiation [106], the subject of immunogenicity of DSCs must be examined not only in an undifferentiated state but also in differentiated form.

5. Conclusion

Dental-related tissue contained several types of stem cells collectively referred to as dental stem cells (DSCs). In this stem cell family, there are 6 named member so far recognized and described. These include dental pulp stem cell (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAP) of developing tooth, dental follicle stem/progenitor cells (DFPCs) and gingival stem cells (GSCs). All these easily-accessible stem cells can be derived from dental tissue obtained from both young and adult patients. Furthermore the cells are described as having immunomodulatory function. These characteristics make DSCs a unique source for repair and regeneration of injured tissue. In this context, many studies have so far been conducted on the animal models and the reports together indicated the extensive potential of the cells in tissue repair and regeneration not only in teeth but also in other organs. After all these animal studies, some centers have started clinical trials to examine the cell potential in human diseases. To exploit the extensive regenerating potential of DSCs in clinic, many additional clinical trials must be planed and conducted till therapeutic protocols using these cells become established.

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Chapter 5

Induce Differentiation of Embryonic Stem Cells by Co-Culture System

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Additional information is available at the end of the chapter

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1. Introduction

Stem cells, which are found in all multi-cellular organisms, can divide and differentiate into diverse special cell types and can self-renew to produce more stem cells. In mammals, two main broad types are included, such as embryonic stem (ES) cells and adult stem cells. The former are derived from the inner mass of blastocysts, and the latter have been found in various tissues from adult. In a developing embryo, stem cells can differentiate into all kinds of specialized cells, but also maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body.

ES cells, derived from the inner cell mass of pre-implantation embryos [1], can proliferate in culture and are able to give rise to all derivatives of the three primary germ layers: endoderm, mesoderm and ectoderm. In other words, they can develop into more than 200 cell types of the adult body when given stimulation for a specific cell type. The endoderm is composed of the entire gut tube and the lungs; the ectoderm gives rise to the nervous system and skin; and the mesoderm gives rise to muscle, bone, blood, and so on.

ES cells, being pluripotent cells, make them an excellent candidate as a source of functional differentiated cells for tissue replacement and regenerative medicine and after disease or injury. Using stem cell in regenerative therapy requires specific stimulation or signals for specific differentiation. If implanted directly, ES cells will randomly differentiate into many different types of cells and cause a teratoma eventually. ES cell researchers still face a few of

the hurdles, including differentiating ES cells into specific cells while avoiding transplant immno-rejection [2].

Till to date, mouse embryonic stem (mES) cells and human embryonic stem (hES) cells have been used in researches. They require very different environments in order to maintain an undifferentiated state. Mouse ES cells are cultured on a layer of gelatin as an extracellular matrix and require the presence of feeder cells (STO or SNL) and leukemia inhibitory factor (LIF) [3]. Human ES cells are grown on a feeder layer of mouse embryonic fibroblasts (MEFs) and require the presence of basic fibroblast growth factor (bFGF or FGF-2) [4]. ES cells will rapidly differentiate without optimal culture conditions or genetic manipulation [5].

The multi-lineage differentiation potential of stem cells is not only an opportunity but also a challenge. An undesired cell type may lead to a pathophysiologic state or a non-functional tissue construct once that stem cells differentiate at the wrong time or place. In order to avoid such maladaptive responses, stem cells have evolved elaborate circuitry that triggers them to respond to differentiation cues only in an appropriate biological context. While most of researchers have been focusing on the role of soluble cues (e.g. growth factors and cytokines) in regulating stem cell differentiation, recent evidence demonstrated that the response to these stimuli are strongly modified by adhesive and mechanical cues, and that these microenvironment factors may be used explicitly to control stem cell differentiation in their own right[6]. With these advances in stem cell research, mimicking cellular microenvironment in vitro is becoming increasingly oriented toward to guide stem cell growth and differentiation.

In a living organism, cells are surrounded by peripheral other cells and embedded in an extracellular matrix (ECM) that defines the architecture, signaling, and biomechanics of the cellular microenvironment. As for stem cell, the word "niche" can be in reference to the in vivo and in vitro stem cell microenvironment. In architecture, the word niche refers to a recess, and in ecology it refers to a habitat where an organism can reside and reproduce. So, the grand position of the stem cell in popular concepts of science is appropriately humbled by the cells dwelling in a place where they might awaken with fleas. The concept of a niche as a specialized microenvironment housing stem cells was first proposed by Schofield, although experimental evidence was first provided by invertebrate models. In the gonads of Drosophila melanogaster and Caenorhabditis elegans, the germ stem cells reside at the distal end of a tapered structure, and have been shown to depend upon interactions with somatic cells so that stem-cell features could be maintained [7].

Stem cells are defined by their ability in complex multi-dimensional environment name as niche. Within the niche, several factors are important to regulate stem cell characteristics: (i) cell-cell interactions among stem cells; (ii) interactions between stem cells and neighboring differentiated cells; (iii) interactions between stem cells and extracellular matrix, adhesion molecules, soluble factors (growth factors, cytokines), oxygen tension, and other nature of the environment. Applying for regenerative medicine, specific differentiation of stem cells must be induced in vitro, and then specific graft with sufficient quantity and pure quality could be transplanted back into the patient. In order to archive this purpose, the researchers are trying to replicate the stem cell niche conditions in vitro. However, obviously, it is difficult to mimic the biological complexity of the native cell context in the laboratory under standard 2D culture

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conditions, since much of the complex interplay of mechanical and molecular factors present in vivo is absent in 2D culture status [8]. This is a major limitation to investigate cellular response in vitro. Therefore, we need generate a new culture system that would be "something between a culture dish and the cells", to represent cellular environment in a living organism and be more predictive of in vivo systems [9,10]. In particular, to stimulate stem cell potential and obtain biologically response in vitro, a new environment that are associated with their proliferation, differentiation, and assembly into tissues is desired. The researches should abide by the following premise: the function of the complicated factors is known to play a role during development or remodeling, and cellular responses to environment factors are predictable.

In our researches, we co-cultured ES cells with special cells to induce the specific differentiation. The co-culture system could supply stem cell for physical attachment (mechanical stretch), regulating signals, as well inducing factors such as cytokines (soluble or diffused). All these combined cues determined the differentiation of specific type of cells. The co-culture system recapitulated the combinations of parameters in the native environment to convert "collections of cells" into specific cell phenotypes. Hence, the design of co-culture system is necessarily inspired by stem cells research.

Co-culture system (ES cells and certain cell) supplied the physical, structural, and molecular factors to induce cellular differentiation. It opens several exciting possibilities: (i) establish functional implant which is suitable for transplantation and replace of degenerated tissues, (ii) investigate developmental processes and understand stem cell behavior in a native environment; (iii) avoid using biomaterials in order to escape from immuno-rejection. A variety of parameters were outlined in Fig.1. It included co-culture system and engineered 2D culture environment that influence stem cell behavior (e.g., self-renewal, migration, and differentiation).

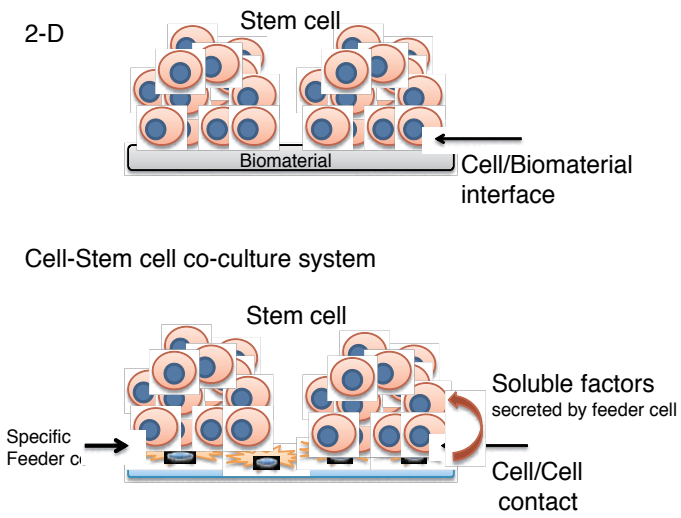

Figure 1. Manipulating the stem cell microenvironment in 2D and cell-stem cell co-culture system. Controllable parameters include matrix properties, cell/cell contact and soluble factors which were secreted by specific feeder cells.

The following section will review several important properties in the design co-culture system to control stem cell differentiation.

2. Interactions with stem cells

2.1. Direct attachment between stem cells and engineered materials

In tissue engineering, either natural or synthetic materials have been investigated for interaction with stem cells and to control their behavior [11]. The benefits to co-culture system include their ability to provide complicated signal to stem cells by physical attachment or chemical excretion. Compared to biological materials, natural or synthetic biomaterials, the former have poor mechanical properties and easily cause immune-response depending on the source of the materials; the later have wide diversity in properties that may be designed according to mechanics, chemistry, and degradation. The toxicity and a limited repertoire of cellular interactions are concerned.

As for the group of natural materials, collagen, matrigel, alginate, and hyaluronic acid (HA) have been used for stem cell researches. Collagen gels have been widely used in stem cell study, including mesenchymal stem cells (MSCs) [12,13] and ES cells [14]. Matrigel consists of a mixture of molecules derived from natural ECM and has been investigated for stem cell culture without feeder cells and inducing differentiation. An improved three-dimension (3D) and serum-free approach was established to differentiate hES cells into functional endothelial cells [16]. Alginate is a seaweed-derived polyanion that forms hydrogels through ionic cross-linking. ES cells have been induced in alginate hydrogels for a variety of applications, typically encapsulated stem cells for transplantation [15]. HA is a polysaccharide found in many tissues and has been modified to form hydrogels. HA hydrogels have been investigated for the culture and growth of undifferentiated human ES cells and MSCs [17, 18].

Synthetic materials were also widely used in stem cell cultures [19]. Materials that degrade through either hydrolytic or enzymatic mechanisms have been synthesized, and the advantage is the tenability and versatility of these physical properties. A hydroxyester has been extensively used in the field of tissue engineering, primarily due to their history of biocompatibility and use in medicine. One composition was seeded with human ES cells for the regeneration of numerous tissues, including vascular and neural structures [20].

2.2. Secreted Soluble factors

Cell and extracellular-matrix components in the stem-cell niche are relatively predictable, although the complexity and integration of these elements is far from known. Soluble mediators of cellular response would also be expected and a number of soluble factors such as growth factors and cytokines are important for stimulate and control the differentiation of stem cells. Adding relative molecules to the culture medium can induce the differentiation. For example, bone marrow stromal cells (BMSCs) have been directed into all kinds of cells [21]. Alternatively, it is advantageous to secrete the molecules directly from

the specific cells. The molecules that can induce differentiation is various, such as basic fibroblast growth factor (FGF), members of the transforming growth factor family (TGF), small molecules such as retinoic acid. Molecule release is typically controlled through diffusion, degradation, or combination of factors. Multiple growth factors have been delivered from the same scaffold based on polymer degradation rates [22]. However, regards to co-culture system, the cells used as basement feeder open up the possibility to control molecule secretion for complex signaling cascades in stem cell differentiation, although the regulating mechanism is difficult to be cleared.

2.3. Mechanical and shape cues to control stem cell differentiation

Using soft lithography techniques, the influence of spatially patterned adhesion molecules on cell differentiation was investigated, such as cell spreading and shape on MSC differentiation, through control the cellular cytoskeleton. MSCs tended to the differentiation of osteoblasts, when they were patterned on larger islands of adhesion ligands, which allowed for cell spreading. On the other hand, MSCs on smaller islands stayed rounded and differentiated into adipocytes [23]. This study indicated that stem cell commitment could be regulated by adhesion molecules and is a consideration in design for inducing the differentiation of stem cells.

During the last decades, much progress has been made in the molecular understanding of early differentiation in stem cells. For example, neural inducer molecules, such as chordin, noggin, and retinoic acid (RA), were identified, and several intracellular mediators of neural differentiation have been characterized. Multiple-step method involving in embryoid body (EB) formation followed by different combination of growth factors was the most common method for inducing differentiation of ES cells [24, 25]. Although the methods can produce a good proportion of different type of specific cells, it has several apparent problems. (i) it is difficult to analyze and control each regulatory step of differentiation in this multiple-step because EBs contains many different kinds of cells, including endoderm, mesoderm, and ectoderm cells; (ii) growth factors have complicated function in vivo. For example, RA, a strong teratogen, is supposed to perturb neural patterning and neuronal identities in EBs as it does in vivo. RA treatment of early embryos causes suppression of forebrain development. It is therefore preferable to avoid RA treatment for therapeutic application unless RA induces the particular type of neurons of one's interest; (iii) in order to avoid infection and rejection, the serum of animal origin should be excluded; (iv) multiple-step method is cost-consuming.

3. Inducing specific differentiation of stem cells using with cell co-culture system

In our research, we introduce an efficient cell co-culture system for in vitro differentiation of specific cell type from ES cells in a serum-free condition that requires neither EBs nor complicated treatment with growth factors.

3.1. Induction of midbrain dopaminergic neurons from primate embryonic stem cells by co-culture with Sertoli cells

In the first study, we have established a new method for generating dopaminergic neurons from primate ES cells by co-culture with Sertoli cells. Neurodegenerative diseases present severe problems due to the limited repair capability of the nervous system [26]. Stem cells have a capacity for unlimited self-renewal, along with the ability to produce multiple different types of terminally differentiated descendants. They are candidate therapeutic tools in neurodegenerative disorders, such as Parkinson's disease, which is characterized by degeneration and death of midbrain neurons that produce dopamine. Transplantation of dopaminergic neurons taken from human fetuses into Parkinson's disease patients shows a remarkable, but inconsistent, ability to replace endogenous degenerated dopaminergic neurons and to ameliorate some of the disease symptoms [27, 28]. However, since treatment of a single Parkinson's disease patient requires dopamine neurons from 6 to 10 human fetuses, replacement therapy is not routinely available. Other sources of dopamine-producing cells, including those from the adrenal medulla or carotid bodies, have been examined for their ability to alleviate Parkinson's symptoms, but these sources are also limited in numbers and/or are not as effective as fetal dopamine neurons [29]. ES cells can proliferate indefinitely and are able to differentiate into cell types of all three germ layers in vivo and in vitro. These unique properties of ES cells make them an excellent candidate for the treatment of Parkinson's disease [30,31].

Our ES cell-Sertoli co-culture system is technically simple, cost-saving, and the induction is efficient and speedy. This protocol only used both of cells and treated without any inducing factors. After 3 weeks of induction, immuno-staining revealed that 90%±9% of the colonies contained tyrosine hydroxylase-positive (TH^+) neurons, and 60% ±7% of the tubulin III-positive (Tuj III^+) neurons were TH^+ (Fig2. A-E). At the same time, the possible roles of Sertoli cell in the differentiation of ES cells into Dopaminergic Neurons were investigated. When cultured on the gelatin-coated dish in the same medium but without Sertoli cells, ES cells differentiated into neurons at a low frequency compared with the rate obtained with ES cells cultured on Sertoli cells (Fig2. F, lanes 1 and 2). This suggested that Sertoli cells had an active role in the promotion of neural differentiation of ES cells. We tested whether direct physical contact between ES cells and Sertoli cells was essential for the induction. ES cells cultured on gelatin-coated dishes and separated from co-cultured Sertoli cells by a 0.22-um filter membrane were still able to induce significant neural differentiation of ES cells (Fig2. F, lane3; Fig2.H). When Sertoli-conditioned medium was used to culture ES cells without Sertoli cells as a feeder layer, neural differentiation was still induced compared with ES cells cultured alone on gelatin-coated dishes (Fig2. F, lane4; Fig2.I)). Together these data suggest that Sertoli cells secrete one or more soluble neural-inducing factors.

Based on the known ability to promote neuronal differentiation [32], GDNF seemed to be a likely candidate for the Sertoli-induced differentiation of primate ES cells. We confirmed the presence of GDNF expression in Sertoli cells by immunohistochemistry (Fig. 2 J, Fig.2 K lane 1) and in the conditioned medium by Western blot analysis (Fig.2 K, lane 2). We therefore tested whether GDNF promoted dopaminergic neuron differentiation of primate ES cells. First, we added GDNF to ES/Sertoli cell co-cultures and compared results with ES cells on a gelatin

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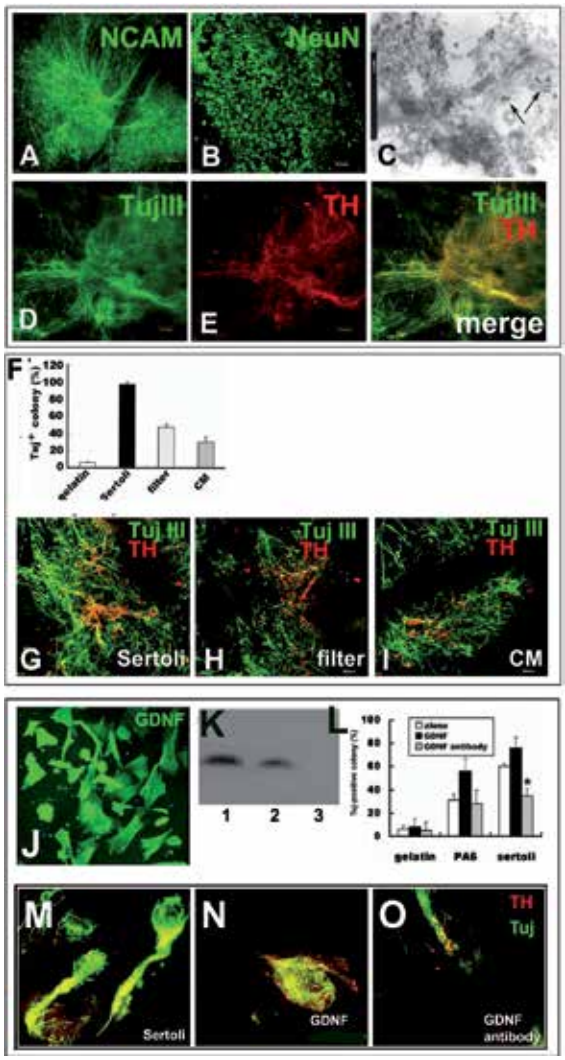

Figure 2. A-E. Sertoli-induced dopaminergic neural differentiation and of monkey embryonic stem cells. Characterization of monkey ES colony induced by Sertoli cells. Expression of NCAM (A), NeuN (B) confirmed the neural identity of cells in ES colony. After 3 weeks, approximately 97% of the ES cell colonies cultured with Sertoli cells were Tuj III$^+$ **(D).** After 3 weeks of culture, immunostaining of Sertoli-induced neurons with anti-TH antibody **(E). (C):** Electron microscopy showed TH ImmunoGold particles (15nm) were associated with small vesicles presumably containing neurotransmitters located at the presynaptic terminal (arrow). **F-I.** The physical and chemical role of Sertoli cells in the induction of dopaminergic neurons. **(F):** Sertoli cells induced the expression of neuronal marker Tuj III in co-cultured monkey embryonic stem (ES) cells even when separated by a filter membrane. CM induced neural differentiation in ES cells cultured on gelatin-coated dish. However the differentiation rate was lower than with direct coculture with Sertoli cells. Tuj III (green) and TH (red) double staining of ES cells when separated from Sertoli cells by a filter **(H)** and cultured in conditioned medium alone **(I).** Scale bar = 50um. **J-O.** The role of GDNF in Sertoli-induced dopaminergic neuron differentiation**. (J):** Immunostaining of GDNF in Sertoli cells. **(K):** GDNF protein was detected by Western blot of lysed Sertoli cells (lane 1) and in conditioned medium (lane 2). However, it was not detected in conditioned medium blocked by anti-GDNF antibody (lane 3). **(L):** In embryonic stem (ES) cells cocultured with conditioned medium derived from Sertoli cells, the TH$^+$ cell percentage was reduced significantly when GDNF was blocked by antibody. TH staining of ES cells cultured on Sertoli cells **(M),** on Sertoli cells treated with extraneous GDNF **(N),** and on blocked conditioned medium **(O).** Scale bars =50um.

coated substrate after 3 weeks in culture. We found that GDNF increased the number of TH^+ cells in co-culture with Sertoli (Fig. 2 L, N) At the same time, we incubated the conditioned medium with GDNF-blocking antibodies and effectively removed GDNF as determined by Western blot (Fig. 2K, lane3). The number of TH^+ cells supported by anti-GDNF-treated conditioned medium was reduced to 35% ±6% (n= 6,000; Fig. 2L), which was significantly less than that induced by Sertoli cells (*p<.05; Fig. 2L), but still more than in colonies grown on gelatin alone. GDNF antibody inhibited the TH^+ neural induction activity of Sertoli cells, as shown by TH staining of ES/Sertoli cell co-cultures and ES cells cultured in conditioned medium blocked by GDNF antibody (Fig. 2 M-O).

Possible roles of Sertoli sells in the differentiation of ES cells into dopaminergic neurons were discussed. Sertoli cells have an active role in the promotion of neural differentiation of ES cells. The mechanism of dopaminergic neuron induction in co-cultures of ES cells with Sertoli cells remains to be understood. First, whether direct physical contact between ES cells and Sertoli cells was essential for the induction was tested through filter membrane to separate ES cells with Sertoli cells. The result showed that Sertoli cells were still able to induce significant neural differentiation of ES cells, indicating that Sertoli cells produce soluble inducing factors. However, Sertoli-conditioned medium could not elicit significant induction. It suggested two possibilities as to the molecular nature of neuron-inducing activity by Sertoli cells. One is that Sertoli cells secrete two different neuron inducing factors, a cell surface-anchored factor and a labile soluble factor. Another might be that the neuron-inducing activity is mediated by secreted factors. At present, we cannot exclude either possibility.

Some factors have been implicated in the regulation of dopaminergic differentiation [33], such as FGF8, Shh, interleukin (IL) 1, IL11, GDNF, and neutralizing antibodies of FGF8 and Shh. Among them, GDNF has the most potent neuroprotective and trophic effects on dopamine neurons in many model systems [34,35,36]. However, it is a large protein and has to be delivered directly to the brain rather than given peripherally. When successfully delivered, GDNF supports the survival and outgrowth of dopamine neurons following transplantation [37]. In addition, GDNF added to cell suspensions of embryonic ventral mesencephalic tissue improves the survival of dopamine neurons following grafting into the degenerative striatum [38]. Other studies have shown that intermittent injections of GDNF in the vicinity of intra-striatal nigral cell suspension grafts have similar effects on improving the survival and/or fiber outgrowth of transplanted dopamine neurons [39,40]. In a previous study [41], neurospheres modified to produce GDNF increased the survival of transplanted dopamine neurons in 6-OHDA-lesioned animals. GDNF is also capable of promoting differentiation of mesencephalic neurospheres towards the neuronal lineage, and more importantly, towards the dopaminergic development indicated by expression of NurrI and Ptx3. Buytaert-Hoefen et al. [42] proved that significant differentiation of dopaminergic neurons were not induced when cultured on PA6 stromal cells alone except for the presence of GDNF or striatal astrocytes. Sertoli cells secrete GDNF and promote the survival of transplanted dopaminergic neurons. In the present study, we showed that GDNF plays a role in dopaminergic neuron differentiation when primate ES cells were co-cultured with Sertoli cells. In our study, extraneous GDNF induced the differentiation of dopaminergic neuron. Sertoli-induced neural differentiation of ES cells

was partly suppressed by a low-dose of anti-GDNF antibody. However, blocking GDNF did not completely inhibit the neural differentiation. We cannot be certain that GDNF activity was completely blocked, although it was not found by Western blot. Therefore, the diminished neuronal differentiation that occurred could have been in response to the remaining, unblocked GDNF. Alternatively, if all of GDNF was blocked, then one or more other factors were present in the conditioned medium and promoted differentiation at a reduced rate. If these factors exist, they are more effective in the presence of GDNF. This confirmed the conclusion of Buytaert-Hoefen et al. [42] that GDNF is required but not necessary for the induction of dopaminergic neurons.

From these results, we can conclude that Sertoli cells may stimulate dopaminergic differentiation by a complex combination of growth factors or other factors, including other unidentified components. Among these, GDNF plays some role, but not a decisive role. It is also possible that the supporting environment provided by Sertoli cells, or an interaction between Sertoli cells and primate ES cells, plays a role in their neuron inducing activity.

In addition, isolated Sertoli cells enable survival and function of co-grafted foreign dopaminergic neurons in rodent models of Parkinson's disease. They also promote regeneration of damaged striatal dopaminergic circuitry in those same Parkinson's disease models [43]. In our study, significant TH^+ cells were found in the degenerative striatum when differentiated primate ES cells were co-transplanted with Sertoli cells. Moreover, 2-month survival of TH^+ neurons derived from ES cells was observed. It is likely that the nutritive support of the Sertoli cells is responsible for this enhanced TH cell survival.

In common, dopaminergic neurons were generated from neural precursor cells amplified from EBs. One group [44] used a lengthy four-step method to produce TH^+ neurons at an efficiency of approximately 7% of the Tuj III^+ neurons. Kawasaki et al. [45] used the term stromal cell-derived inducing activity to describe the dopaminergic neuronal-inducing defect of PA6 cells. The stromal feeder-mediated neural induction had been demonstrated for mouse, primate, and human ES cells [46-48]. In contrast to the previous protocols, our methods does not require growth serum, the formation of EBs, selection of neural precursor cells, retinoic acid, growth factors, or other special treatment. Thus, our method is more suited to detailed analyses of differentiation and transplantation therapy.

3.2. Differentiation of primate ES cells into retinal cells induced by ES cell-derived pigmented cells

In the second study, retinal cells were induced from primate ES cells by co-culturing with ES-derived pigmented cells.

Photoreceptors are the primary sensory neurons residing in the outer nuclear layer (ONL) of the vertebrate retina. Photoreceptor degeneration is a common cause of human visual impairments resulting from light damage, genetic changes and aging. The unfortunate nonrenewable nature of photoreceptors has inspired that these degenerative diseases maybe treatable by transplantation of healthy fetal cells. Previous work has shown that freshly harvested retinal pigment epithelium (RPE) can be effective in rescuing photoreceptors in the

Royal College of Surgeons (RCS) rat, an animal model of indirect photoreceptor degeneration [49]. However, obtaining a sufficient number of suitable donor cells remains a problem.

The isolation of embryonic stem (ES) cells has drawn much attention, given their potential to generate all adult cell types. As ES cells continue to proliferate in an undifferentiated state in vitro, an unlimited stem cell source or its derivatives may be secured. It is also a potential benefit that ES cells may be genetically manipulated to permit the selective differentiation and/or isolation of a specific cell type.

Recently, several laboratories have devised hetero chronic co-culture experiments to characterize the nature of the interactions necessary for the differentiation of retinal cells [50,51]. Early embryonic mouse cells (E12) were shown to differentiate into rod photoreceptor cells with a higher frequency when co-cultured with either postnatal rat retinal cells or with later staged embryonic retinal cells that were competent to generate rod photoreceptors. The results suggested that the differentiation of retinal progenitor cells as rod photoreceptors is influenced by locally diffusible signals in the extra cellular environment that are developmentally regulated during the period of retinal histogenesis.

Although the specific factors that promote differentiation of cells as rod photoreceptors are not known, several classes of molecules have been shown to play a role in the developing retina, for example, small peptide growth factors, Shh, taurine, epidermal growth factor (EGF) and fibroblast growth factor (FGF) [52-54]. The vitamin A derivative retinoic acid has been shown to influence cell fate in the developing nervous system. In particular, all-trans retinoic acid has been shown to alter cell fate decisions in the developing limb bud, hindbrain and inner ear [55,56]. In addition, several studies have demonstrated that all-trans retinoic acid and at least one of the nuclear retinoic acid receptors (RARa) are present in the developing retina (56). RA caused a dose-dependent, specific increase in the number of cells that developed as photoreceptors in culture throughout the period of retinal neurogenesis [52,53].

Retinal pigmented epithelium (RPE) is a neuroectodermal derivative essential for the survival of photoreceptors. It supplies nutrition and provides several trophic factors that help maintain the normal physiology within the neuro sensory retina and photoreceptors [57,58].

In most of studies, differentiation of the retinal progenitors into photoreceptors was infrequent in the absence of co-culture with embryonic retinal tissues [59,60]. Use of the fetal retina involves some ethical and practical consideration, and the cell supply is a problem. Osakada and colleagues [52,53] induced the generation of putative rod and cone photoreceptors from ES cells by stepwise treatments under defined culture conditions. However, the practical steps were complicated and time consuming. In this study, the monolayer of RPE derived from ES cells was used as an inducing feeder layer which could replicate the microenvironment of retina or sub-retinal to successfully induce photoreceptor cells. This is the first time that ES-derived cells are used to induce the differentiation of ES cells. Furthermore, retinoic acid was used to induce the differentiation of photoreceptors. To our knowledge, a few reports have produced photoreceptor cells in uncontaminated culture conditions except for Takahashi's group [52] Osakada [53] and lamba's [60,61,62,] research. The present study demonstrated the generation of photoreceptors from primate ES cells under conditions free of animal–derived substances.

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During the study of ESC-Sertoli co-culture system, one unexpected finding was the appearance of epithelial cells with massive pigmentation from the second week and they grew at a constant rate. After monkey ES cells were cultured on Sertoli cells for 3 weeks, a patch of pigmented cells was mechanically isolated by using a tip after being loosened with trypsin and plated on a gelatin-coated dish without feeder cells in primate ES cell culture medium. The polygonal morphology with a compact cell-cell arrangement was reminiscent of the pigmented epithelium of the eye, and clearly distinct from pigmented melanocytes derived from neural crest (Fig3 A-I). In recent, transplantation of retinal pigment epithelium (RPE) has become a possible therapeutic approach for retinal degeneration. Meanwhile, RPE is a neural ectodermal derivative essential for the survival of photoreceptors. It serves as nutritional cells and provides several trophic factors that help maintain the normal physiology within the neural sensory retina and photoreceptors. Therefore, we investigated whether retinal cells could be induced by co-culturing ES cells together with RPE. At the same time, RA was treated in order to improve the differentiation efficiency. The results showed that after 10-day co-culture of ES cells and these RPE, some ES derivatives became immuno-positive for rhodopsin. RT-PCR analysis demonstrated expression of retina-related gene markers such as Pax6, CRX, IRBP, Rhodopsin, Rhodopsin kinase and Muschx10A. When RA was added, the distinct increase of photoreceptor specific proteins markers was found. Besides, the differentiation of bipolar, horizontal cells was demonstrated by protein and gene expression. RA treatment also altered more retinal cell differentiation (Fig3. J-M).

We demonstrated that pigment epithelial cells can be generated, enriched, and expanded from primate ES cells when ES cells were co-cultured with Sertoli cells. These ES-converted pigment epithelial cells showed development of several of the characteristics of RPE cells and were able to be used to induce the differentiation of retinal cells. For the clinical application, methods for purifying large numbers of lineage specific cells should be developed. In the present study, RPE cells could be easily identified under a dissecting microscope and selectively expanded into a uniform single cell layer.

The RPE cells contain melanin granules, have a characteristic polygonal morphology, and play some critical roles including (i) forming a barrier separating the retina from the blood vessels of the choroid coat; (ii) regulating nutritive substance transport required for retinal progenitors to differentiate into retinal neurons and the maintenance of retinal cells; (iii) regenerating visual pigments; (iv) digesting the shed parts of photoreceptor cells after having phagocytized them [63]. Therefore, we used ES cell- derived RPE cells to provide epigenetic retinal neurons, not only the efficient induction of photoreceptors, but also other retinal cell lineages, such as bipolar, and horizontal cells. It indicated that the secreted/diffusible factors from RPE or direct cell-cell contact were sufficient to induce retinal cells from ES cells.

A monolayer of ESC-derived RPE cells, an inducing feeder layer that replicates the retinal microenvironment and RA were used to successfully induce photoreceptor differentiation. To our knowledge, this is the first time that ESC-derived cells have been used to induce ESC differentiation. ES/RPE co-culture system can serve as a promising method for therapeutic application and basic research on retinal degeneration disease, although we are still far from an established in vitro or in vivo source of retinal cells.

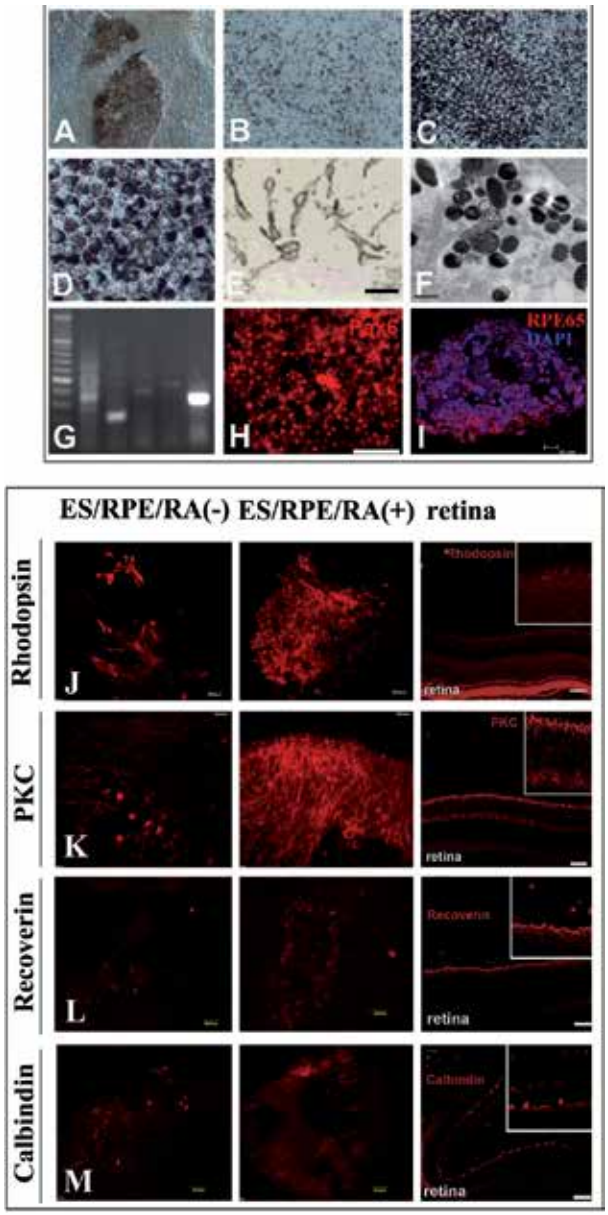

Figure 3. A-I. Analysis of pigmented epithelial cells derived from primate ES cells using LM (A-E), TEM (F), RT-PCR (G), immunofluorescence staining (H-I). (A) LM images showing pigmented cells present in the primate ESC colonies grown at a constant rate on Sertoli feeder cells for 2, 4, and 6 weeks (A,B and C, respectively). The polygonal morphology with a compact cell–cell arrangement (D) is similar to the pigmented epithelium of the eye and clearly distinct from pigmented melanocytes derived from the neural crest (E). (F) TEM images of primate ESC-derived pigmented cells displaying melanin granules. (G) Shown are the RT-PCR-amplified products of RPE cell-specific markers (C1): tyrosinase-related protein-2(Trp-2), RPE65, CRALBP, MertK. (H-I) Immunostaining of RPE cells derived from ES cells with

anti-Pax6 and RPE65 antibody is also shown. J-M. Analysis of differentiated retinal cells induced by ESC-derived RPE cells by immunofluorescence staining. Immunopositive ESC derivatives for retinal cell-specific markers are shown as follows: (J) ES cells were immunopositive for rhodopsin after a 21-day co-culture with RPE cells, and an increased frequency of rhodopsin-positive cells was estimated after RA treatment, and some of them developed into rosettes. The eyes of a db/db mouse (2 weeks) were used as a positive control to identify the positive immunofluorescence staining results and the specificity of retinal antibodies. (K) PKC-positive cells appeared as early as day 3 after starting co-cultures. A drastic increase was observed after RA treatment. Positive control with mouse eyes and PKC antibody is also shown. (L) Recoverin-positive cells appeared after a 10-day o-culture with RPE cells. Expression increased at a constant rate and after RA treatment. Positive control with mouse eyes and recoverin antibody is also shown. (D) After a 10-day co-culture with RPE cells, ES cells became immunopositive for calbindin. Immunopositive ESC derivatives for calbindin are shown at day 2. The number of calbindin-positive cells increased after RA treatment (D2). Positive control with mouse eyes and calbindin antibody is also shown. Scale bars = 50 lm.

3.3. Bone marrow stromal cells as an inducer for cardiomyocyte differentiation from mouse embryonic stem cells

In the third study, bone marrow stromal cells (BMSCs) were used as an inducer to induce cardiomyocyte differentiation from mouse ES cells.

Several studies reported that different feeder layers induced cardiomyocytes from ES cells [64-67]. It proved that the visceral-endoderm-like cell line, END-2 induced mouse P19EC, mouse and human ES cells to aggregate in co-culture and give rise to cultures containing beating areas. For mouse P19 EC cells, it has been demonstrated that a diffusible factor secreted by the END-2 cells is responsible for the induction of cardiomyocyte formation.

Like END-2 cells, various cell types of stem cells remain in a mature body. Among them, bone marrow stem cells (BMSCs) are unique because of rich functional products. A wide array of cytokines including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and insulin growth factor-1 (IGF-1) were detected in the BMSC cultured medium by ELISA [68]. Furthermore, they secreted hepatocyte growth factor (HGF), and transforming growth factors (TGF-beta) [69], which are all potent cardiomyocyte growth and survival factors, or play an important role in proliferation and differentiation of stem cells [70]. IGF-1 can promote angiogenesis in infracted myocardium, reduce the degree of myocardial necrosis, maintain the myocardial structure, stimulate proliferation of cardiac fibroblast, and inhibit matrix degradation; thus preventing ventricular dilatation and reducing load capacity of the heart [71]. After binding to its receptor on ES cells, IGF-1 induces expression of a number of cardiac-specific transcription factors such as the zinc finger GATA proteins and Nkx-2.5, a co-activator of GATA-4. GATA- 4 and Nkx-2.5 are essential for heart development [72]. HGF is a multifunctional factor promoting cell mitosis. It can promote cell survival and regeneration, inhibit the apoptosis of stem cells, and increase the survival rate of transplanted cells. More importantly, HGF enhances growth and favors cell extracellular matrix interactions; the critical steps during myocardial regeneration after infarction. In the heart, high concentration and wide distribution of FGF has been identified from early embryonic stages. Basic FGF plays a vital role in the growth and differentiation of cardiac myocytes. FGF was found to induce DNA synthesis in adult rat myocytes, cells which were considered capable of regeneration [73]. Members of the

TGF-b superfamily play important roles in cardiac development during embryogenesis [74] as well as in various cardiac pathologies [75]. TGF-b1 has been shown to induce cardiac differentiation in vitro in embryonic explants and stem cells [76,77] as well as in adult bone marrow-derived cells [70]. Previous studies of non-conditional global VEGF or VEGF isoform gene deletion have demonstrated embryonic and early postnatal mortality associated with severe cardiac abnormalities and abnormal vascularization; thus establishing that systemic VEGF expression is essential normal myocardial development [78]. From these reports BMSCs are expected to function as inducer for cardiac differentiation from ES cells.

To confirm whether BMSCs are a real cardiac inducer to differentiate ES cells into cardiomyocytes, mouse embryoid bodies (EBs) were co-cultured with rat BMSCs. After about 10 days, areas of rhythmically contracting cells in more solid aggregates became evident with bundle-like structures formed along borders between EB outgrowth and BMSC layer. ESC-derived cardiomyocytes exhibited sarcomeric striations when stained with troponin I (Trop I), organized in separated bundles. Besides, the staining for connexin 43 was detected in cell–cell junctions, which demonstrated that ESC-derived cardiomyocytes were coupled by gap junction in culture (Fig4 A-F). In addition, an improved efficiency of cardiomyocyte differentiation from ESC-BMSC co-culture was found in the serum-free medium: 5-fold up-regulation in the number of beating area compared with the serum medium. Effective cardiac differentiation was also recognized in transfer filter assay and in condition medium obtained from BMSC culture. A clear increase in the expression of cardiac genes and TropI protein confirmed further cardiac differentiation by BMP4 and Retinoic Acid (RA) treatment (Fig4 G,H). These results demonstrate that BMSCs can induce cardiomyocyte differentiation from ES cells through soluble factors and enhance it with BMP4 or RA treatment. Serum-free ESC-BMSC co-culture represents a defined in vitro model for identifying the cardiomyocyte-inducing activity from BMSCs and, in addition, a straightforward experimental system for assessing clinical applications.

In our study, two experiments of separation from BMSCs by the filter and using CM without contact of BMSCs were performed. These results showed that both conditions were still able to induce significant cardiac differentiation (Fig4 I-M). As expected previously, these data have shown that it is due to varied soluble factors secreted by BMSCs, though effective soluble factors have to be determined in future. On the other hand, cardiomyocyte bundles were formed long the edge of EB outgrowth, which was a borderline with BMSC layer. These regular arrangements seemed to be associated with BMSC layer structures. Moreover, in fact, direct cell-to-cell interaction between BMSC and EBs was more effective to induce cardiac differentiation. It may be possible that the supporting environment provided by BMSCs or an interaction between BMSCs and EBs may play a role in their cardiac-inducing activity.

Compared with the inducing effects of low dose cytokines alone or with BMSCs alone, our co-culture with BMSCs supplemented with cytokines could increase the differentiation of cardiomyocytes, which indicated that BMSCs had the ability to promote the induction and proliferation of ESC-derived cardiomyocyte and the addition of low dose cytokines had a synergistic effect on this ability.

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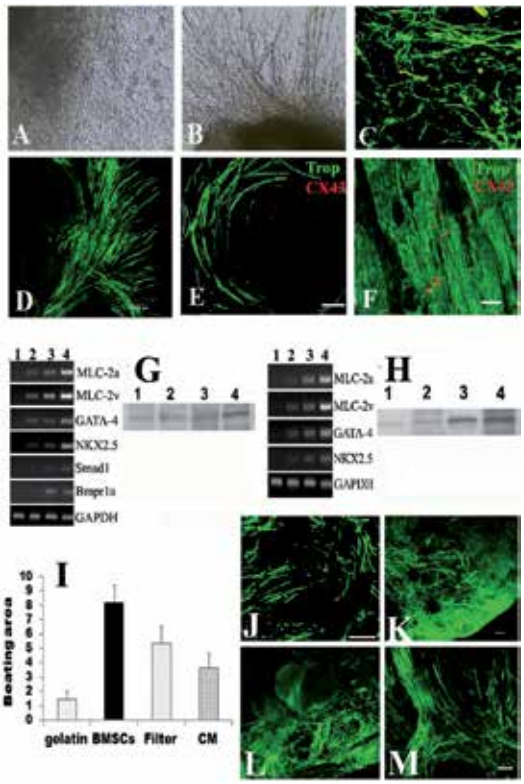

Figure 4. Characterization of cardiomyocyte induced by BMSC co-culture. (A,B) Morphology of serum or non-serum treated EB outgrowth on BMSC layer on day 14. (A) 20% FBS: Multi-angular and flattened cells spread out from EBs. (B) Absence of FBS: fiber-like cells extended radially and formed bundle-like structures each other. Scale Bars = 25um. (C-F) Beating areas stained for Trop I (green) and CX43 (Red). (C) In EBs formed in the presence of FBS, cardiomyocyte fibers are distributed disorderly. (D) Cardiomyocyte fibers are organized regular in EBs formed in the absent of FBS. Radial-morphological-like fibers surrounded or spread from EBs. Beating cells showed spindle, round and tri or multi-angular morphologieswith characteristic organized in separated bundles. (E) CX43 staining among TropI-positive cardiomyocytes showed the presence of gap junctions. (F) The heart of C57BL/6 mice was used as a positive control to identify the positive immunofluorescence staining results and the specificity of retinal antibodies. Bars = 100um. **G-H** Examination of expression changes of cardiomyocytes from EBs after BMP4 and RA treatment. (G) Induction of cardiac-specific genes and BMP signaling molecules in EBs exposed to BMP4, as shown by RT-PCR. Lane1: undifferentiated ES cells; lane2: EB cultured on gelatin-coated dish, FBS(–); lane3: EB co-cultured with BMSCs, FBS(–); lane4: EB co-cultured with BMSCs, FBS(–), and treated with BMP4. The expression of cardiac markers and BMP signals were increased markedly by BMP4 treatment. TropI protein was also detected by Western of lysed EB on gelatin-coated dish (lane1), EB on gelatin-coated dish and treated with BMP4 (lane2); EB co-cultured with BMSCs (lane3); EB co-cultured with BMSCs and treated with BMP4 (lane4). Combined using BMP4 and BMSCs induced significant cardiac differentiation. (H) The effect of RA on the differentiation of cardiomyocytes. (D) RT-PCR analysis revealed that both early cardiac genes, MLC-2a and MLC-2v, were increased in RA-treated EBs. Lane1: undifferentiated ES cells; lane2: EB cultured on gelatin-coated dish, FBS(–); lane3: EB co-cultured with BMSCs, FBS(–); lane4: EB co-cultured with BMSCs, FBS(–), and treated with RA. TropI protein was detected by Western of lysed EB on gelatin-coated dish (lane1), EB on gelatin-coated dish and treated with RA (lane2), EB co-cultured with BMSCs (lane3); EB co-cultured with BMSCs and treated with RA (lane4). Combined using RA and BMSCs significantly induced cardiac differentiation. **I-M.** The physical and chemical role of BMSCs in cardiomyocyte induction. (I) BMSCs induced beating cardiomyocytes in co-cultured ES cells even when separated by a filter membrane. CM induced cardiac differentiation in ES cells cultured on gelatin-coated dish. However the differentiation rate was lower than with direct co-culture with BMSCs. (J-M) EB on gelatin-coated dish (J); EB co-cultured with BMSCs (K); TropI staining of ES cells when separated from BMSCs by a filter (L) and cultured in CM alone (M). Scale Bars = 50um.

4. Conclusion

Cell-to-cell interaction is important to differentiate varied cells or tissues from stem cells and/or in embryogenesis. An important component to the stem cell microenvironment is the surrounding matrix, which includes numerous chemical and biophysical cues. The changing local molecular conditions through selecting specific co-culture system might thereby provide promising method to modulate stem cell differentiation. In our researches, we selected the different kinds of cells as a feeder cell to induce the specific differentiation of stem cells. The molecular basis of induction in co-culture system remains to be understood. We tested some factors that have been implicated in the regulation of differentiation, such as GDNF, bFGF, BMP4, or neutralizing antibodies of GDNF. So far, no significant effect factors on the induction were observed. Interestingly, as showed above, the differentiation efficiency of ES cells in co-culture system is as high as the efficiency in the multiple-step method with lots of growth factors treatment, or even higher than that. The possible explain for this is that supporting environment provided by co-culture basement cells, such as Sertoli, RPE or BMSCs, or an interaction between co-culture basement cells and ES cells, plays a role in their inducing activity.

In conclusion, we established a simple and effective system for the differentiation of specific cells from ES cells. Further study is warranted to establish selection methods, analyze cell functions, and transplanted for degeneration diseases. However, the co-culture system can serve as a promising method for therapeutic applications and basic research on degeneration diseases.

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Chapter 6

Oral and Maxillofacial Tissue Engineering with Adipose-Derived Stem Cells

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Additional information is available at the end of the chapter

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1. Introduction

Oral and maxillofacial tissues are a complex array of bone, cartilage, soft tissue, nerves and vasculature. Damage to these structures, even when minimal, usually leads to noticeable deformities. Therefore, the repair of large segmental bone defects of the jaw or mandible due to trauma, inflammation, or tumor surgery remains a major clinical problem. For many years, simple autogenic, allogenic, or xenogenic bone grafts, or combinations thereof, have been the mainstay for tissue replacement [1]. However, when large bone defects are present, advanced approaches such as free tissue transfer with microvascular reanastomosis of vascularized flaps from distant sites including the fibula, iliac crest, scapula, and radius are needed to repair or regenerate a functionally complex tissue such as maxillofacial tissue [2, 3]. While these procedures have proven to be reliable and effective, they require extended hospitalization, and a secondary donor site with the associated morbidity and complications. As an alternative to current surgical techniques or approaches, developments in tissue engineering using the gene therapy and stem cell biology strive to utilize cells, biomaterial scaffolds and cell signaling factors to regenerate large oral and maxillofacial tissues defect with precise replication of normal body contours. A tissue engineering approach offers several potential benefits, including a decrease in donor site morbidity, a decrease in technical sensitivity of the repair, and the ability to closely mimic the in vivo microenvironment in an attempt to recapitulate normal craniofacial development [1].

Mesenchymal stem cells (MSCs) derived from bone marrow have been used experimentally for tissue engineering applications [4-6]. MSCs can differentiate into several different cell types, such as those that produce bone, cartilage, tendon, and other connective tissues, as well as muscle, adipose, and dermal cells [7-10]. MSCs can be expanded in culture while maintaining their multipotency.

The concept of prefabricated bone engineering with MSCs for large bone defects may play a pivotal role in future therapies. However, bone marrow-derived MSCs have been reported to require selective sera lots and growth factor supplements for culture expansion [11]. Furthermore, traditional bone marrow procurement, particularly in volumes larger than a few milliliters may be painful, frequently requiring general or spinal anesthesia [12-14].

Bone marrow tissue provides the most universal and attractive source of MSCs; however, other tissues such as periosteal [15], muscle [16], synovial membrane [17] and adipose [18-20] tissues also appear to possess MSCs. Particularly, adipose tissue is an important source of stem cells because subcutaneous adipose tissue is an abundant and accessible source of both uncultured stromal vascular fraction (SVF) cells and cultured homogeneous adipose-derived stem cells (ASCs) (21). ASCs obtained from lipoaspirates have multilineage potential and will differentiate into adipogenic, chondrogenic, myogenic, osteogenic, and neurogenic cells [19, 22, 23]. Thus, ASCs have great potential for clinical applications such as the repair of damaged tissues and angiogenic therapy. Injection of human ASCs was recently shown to improve neovascularization in an ischemic hindlimb mouse model and osteoid matrix formation in immunotolerant mice [24-26]. Further, ASCs have been shown to increase the functional capacity of damaged skeletal muscle in vivo [27]. Therefore, these reports suggest that ASCs may also have the potential for use in large bone tissue engineering techniques such as prefabrication. Recently, prefabricated bone engineered with ASCs was reported both with in vivo studies in rat and a clinical human case. Thus, the use of ASCs in maxillofacial tissue reconstruction should be viewed favorably and these novel approaches may have advantages for tissue reconstruction.

In this chapter, the current approaches and the biomaterials used for repair of large bone defects are presented, and the novel approach of prefabricated bone engineering with MSCs and ASCs is introduced.

2. Current therapy for large bone reconstruction

Bone tissue is composed of heterogeneous cell types embedded in a three-dimensional mineralized extracellular matrix. The scaffolds for repair of large bone defects, including autogenous bone grafts or biomaterials, must provide the necessary support for cells to proliferate while maintaining their potential to differentiate, and must possess an architecture suitable for matching the final shape of the newly formed bone [28].

2.1. Autologous bone reconstruction

The current standard of care for repair of critical large bone defects consists of autogenous bone grafting using bone from the rib or iliac crest of the patient. An autologous bone graft is still the ideal material for the repair of craniofacial defects; however, the availability of autologous bone is limited and harvesting can be associated with complications [29]. Vascularized and avascular autogenous bone has a greater osteogenic capacity than any other bone replacement material, as revascularization attracts mesenchymal

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differentiation into osteogenic, chondrogenic and other cell types. Autogenous bone transplants possess an inherent biocompatibility and are therefore more easily incorporated without immunogenic responses [30]. However, the clinical use of autologous bone transplants is limited by considerable donor site morbidity, which increases with the amount of harvested bone. Bleeding, hematomas, infections, and chronic pain are common complications of autologous bone graft harvests [31, 32].

2.2. Allogenic/Xenogenic bone reconstruction

Demineralized bone matrix (DMB) is the de-cellularized and organic component of bone, and is a commercially available osteoinductive and osteoconductive biomaterial. DMB represents a concentrated source of bone morphogenetic proteins (BMPs) and has been used in numerous animals systems since its initial description in 1965 [33]. The widespread use of DMB in humans still remains restricted since the immunologic properties of donor DMB are unknown [34].

With the disadvantages of host morbidity and the limits in suitable harvesting sites and material for autologous grafts, the use of xenografts might be considered for large bone reconstructions, although the histocompatibility issues between the human recipient and animal donor preclude the use of bone xenografts [34]. However, bovine-derived DMB is currently used in oral and maxillofacial surgery [35].

2.3. Synthetic scaffolds for bone reconstruction

A wide variety of synthetic (alloplastic) scaffolds such as ceramics and polymers are used clinically for bone grafting [30]. Ceramics are crystalline, inorganic, nonmetallic minerals that are held together by ionic bonds and usually densified by sintering [36]. Ceramics such as hydroxyapatite and β-tricalcium phosphate (TCP) are currently in use clinically for bone tissue regeneration of large bone defects.

Various synthetic polymer scaffolds exhibit different structural, mechanical and degradation properties that make then suitable for bone tissue engineering [36]. Blending polymers of different molecular weights can achieve both optimal degradation rates and mechanical properties [37]. Some synthetic polymer scaffolds such as polycaprolactone (PCL) scaffold, polylactic acid (PLLA), polyglycolic acid (PGA) and polylactic-co-glycolic acid (PLGA) materials have been approved by the FDA for craniofacial applications or as absorbable sutures and bone pins/screws [36].

2.4. Gene therapy for bone reconstruction

The use of exogenous cytokines and growth factors, which are essential for bone regeneration, promotes cell adhesion, proliferation, migration and osteogenic differentiation [28]. Growth factors such as BMPs, fibroblast growth factors (FGFs), insulin-like growth factors (IGF), vascular endothelial growth factors (VEGF) and platelet-derived growth factors (PDGF) have been used in bone regeneration [28, 36].

Recently the use of combinations of growth factors, such as BMP-2 and NEL-like molecule-1 (NELL-1), was tested in rapid distraction osteogenesis in a rabbit model. The combined

treatment produced significantly greater bone healing compared to single growth factor treatments after four weeks of treatment [38]. However, some reports have cautioned that the clinical use of BMPs and VEGF is in its infancy, and some risks may accompany their use. VEGF is commonly upregulated in various types of tumors to enhance their vascularization, and subcutaneous sarcomas were found in some rats administered recombinant human BMP-7 [39, 40], although no clinical relationship has been established between the use of these growth factors and tumor formation.

2.5. Prefabricated bone engineering for oral and maxillofacial tissue reconstruction

Prefabrication is an interesting area of oral and maxillofacial surgery and plastic and reconstructive surgery, because it represents a bridge between conventional reconstructive surgery and tissue engineering [41, 42]. The purpose of prefabrication is to build a tissue (muscle, bone, skin, or composite) with characteristics as similar as possible to those of the defect that is to be repaired [43]. Conventional osteomyocutaneous flaps do not always meet the requirements for repairing a composite defect. A prefabricated composite flap can be created according to the complex geometry of the defect. Prefabrication of multi-component flaps is a well established procedure in plastic and reconstructive surgery [41]. This concept is based on the revascularization phenomenon directly related to host tissue vascularity [44] and has significantly expanded the frontiers of reconstructive surgery.

Hirase et al. were the first to report the use of prefabricated myocutaneous and osteomyocutaneous tissue in a rat model [45]. Flap prefabrication using conventional bone grafts allows for generation of new types of flaps independent of the vascular anatomy of the bone transplant. However, the donor site morbidity after harvesting of bone for grafting is still a problem. Recently, biomaterials, osteogenic cells and osteoinductive growth factors have been used for generation of vascularized bone tissues in combination with a vascular axis or vascularized flaps. An inflammatory wound healing response as a reaction to the surgical implantation induces vascularization of the scaffolds [31]. Induction of axial vascularization protected the porous biomaterials from bacterial infection and transfer of this vascularized hard tissue as a free flap has been demonstrated [46]. Prefabricated vascularized bone grafts have been used in a clinical setting for mandibular reconstruction following thorough in vivo evaluation in a pig model [47-49]. In these studies, granules of xenogenic bone minerals soaked with recombinant Osteogenic protein-1 were implanted into the latissimus dorsi muscle and the neo-tissue was subsequently transferred to sites of mandibular defects using microsurgical techniques.

3. Mesenchymal stem cells for oral and maxillofacial tissue reconstruction

3.1. Mesenchymal stem cells for bone engineering

The bone marrow is not only the site where hematopoiesis occurs in postnatal life, it is also a reservoir of pluripotent stem cells for mesenchymal tissues [50]. Plated at low densities, single precursor cells derived from bone marrow, and referred to as colony-forming units, give rise

to distinct and heterogeneous colonies. These colonies have been shown to undergo osteogenic, chondrogenic and adipogenic differentiation [51].

Chang and colleagues showed that MSCs can produce ectopic bone generation in a mouse model [52]. A suspension of osteogenically induced MSCs was added to 2% alginate, which was then gelled by mixing with calcium sulfate. The gel was injected subcutaneously on the dorsal side of the experimental animals. Histological examination of the implants revealed signs of endochondrosis with woven bone deposition. The equilibrium modulus of the newly formed bone increased with time up to 678 kPa at 30 weeks, as determined by biomechanical analysis. This value is approximately 1.62% of native bovine cancellous bone. In another study [53] of large mandibular bone defect repair, dog MSCs cultured with ß-TCP to generate osteogenic cells were co-implanted with a titanium plate into a 30 mm segmental mandible defect. Biomechanical tests showed a significant difference between the experimental group (with cells) and the control group (without cells), highlighting the importance of the MSCs in bone formation. Pedicled bone flaps based on collagen I scaffolds, bone marrow stromal cells and a PTFE membrane have been successfully generated using the carotid artery and jugular vein or the saphenous bundle as a vascular axis in a mouse model [54]. The osteogenetic stimulus was supplied by the injection of mouse MSCs cultured in osteogenic medium inside the space delimited by the PTFE membrane. After only 4 weeks islands of bone tissue were present inside the membrane.

3.2. Clinical trials for bone engineering with mesenchymal stem cells

There is some clinical experience with bone reconstruction using expanded MSCs combined with scaffolds. Constructs of expanded autologous MSCs in macroporous hydroxyapatite were used in three patients with large segmental bone defects [55, 56].

Warnke and Terheyden have developed a two stage procedure for mandible reconstruction in humans [57]. This study used prefabrication in the latissimus dorsi muscle with the aim of reconstructing a 70 mm defect in the mandible of a man who underwent a tumor resection years previously. The entire construction of the mandible was built using blocks of Bio-Oss® and MSCs that had been cultured in the presence of BMP-7. The Bio-Oss® and MSCs were placed in a titanium cage, and implanted into the latissimus dorsi of the patient and maintained in situ for 7 weeks. Subsequently, this unit, together with the vascular bundle that supplied it, was removed and re-implanted in the mandible defect by fixation with titanium plates and microvascular sutures connecting the vasculature to the external carotid artery and the cephalic vein.

4. Adipose-derived stem cells for oral and maxillofacial tissue engineering

4.1. Characterization of adipose-derived stem cells

There is a general consensus that SVF cells are a heterogeneous population, and no specific ranges for each subpopulation have been agreed upon formally [21]. In contrast, the Interna-

tional Society for Cell Therapy has provided guidelines for the definition of MSCs, as follows. (1) MSCs must be plastic-adherent when maintained in standard culture conditions. (2) MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface antigens. (3) MSCs must differentiate into osteoblasts, adipocytes and chondroblasts in vitro [58].

SVF cells include preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells (ECs), resident monocytes/macrophages, lymphocytes and ASCs [59]. Although the criteria to define SVF cells remain in contention, the heterogeneous SVF cell population includes putative ASCs (CD31-, CD34+/-, CD45-, CD90+, CD105-, CD146-), endothelial (progenitor) cells (CD31+, CD34+, CD45-, CD90+, CD105-, CD146+), vascular smooth muscle cells or pericytes (CD31-, CD34+/-, CD45-, CD90+, CD105-, CD146+), and hematopoietic cells (CD45+) in uncultured conditions [60]. Cultured ASCs show an extensive proliferative ability in an uncommitted state while retaining their multilineage differentiation potential. ASCs express the mesenchymal stem cell markers CD10, CD13, CD29, CD34, CD44, CD54, CD71, CD90, CD105, CD106, CD117, CD166 and STRO-1. They are negative for the hematopoietic lineage markers CD45, CD14, CD16, CD56, CD61, CD62E, CD104, and CD106 and for the EC markers CD31, CD144, and von Willebrand factor [20, 61, 62]. Morphologically, cultured ASCs are fibroblast-like and preserve their shape after expansion in vitro [20, 63]. The ASC specific surface markers CD29, CD90, and CD166 increase during culture [64]. In later passages, ASC cultures are homogeneous and exhibit fibroblastoid morphology. The composition of the subpopulations, therefore, may change during expansion [65, 66]. Therefore ASCs match the standard criteria for MSCs.

4.2. Differentiation potential of osteogenic cells in vitro and in vivo

Numerous studies have presented results that clearly show that ASCs can differentiate into osteoblasts [20, 59, 63, 67, 68]. ASCs exhibit a time-dependent expression of genes and proteins associated with the osteoblast phenotype, including ALP, Type I Collagen, OPN, ON, RUNX2, BMP-2, BMP-4 and BMP receptors I and II [20, 67, 69, 70]. Additionally, between 2 and 4 weeks of culture, mineralization of the extracellular matrix begins and proceeds via the activity of ALP, an enzyme that hydrolyzes phosphate esters making available inorganic phosphate to form hydroxyapatite [19, 20, 71].

Furthermore, recent reports have shown that ASCs co-cultured with ECs exhibit enhanced osteogenesis [72, 73]. ASCs exhibited increased secretion of alkaline phosphatase and osteocalcin, and an overall increase in osteogenesis in the co-cultured situation compare with other experimental groups. These interactions may be important to regenerate bone in large bone defects since angiogenesis plays a key role in regeneration of large amounts of tissue.

4.3. Fabricated bone engineering with adipose-derived stem cells

To make a functional prefabricated bone, three elements are required: scaffolds to provide a three-dimensional support, growth factors to stimulate neovascularization, and MSCs to give an osteoinductive stimulus. Okuda et al. have reported prefabrication of tissue engineered bone grafts using ASCs in a rat model [74]. ASCs and porous β-TCP as scaffold material were

implanted into the superficial inferior epigastric artery flap. After prefabrication for eight weeks, the prefabricated flaps were elevated and the pedicles were clamped for 4 h; prefabricated tissue was harvested two weeks later. The osteogenic capacity of the prefabricated graft was not significantly different from non-prefabricated grafts examined after two weeks in a rat model. Furthermore, an analysis of angiogenesis suggested that the prefabricated model possessed significantly greater capillary density than the non-prefabricated model.

Recently, repair of a large bony defect using ASCs was clinically reported [75-77] (Table. 1). Mesima°ki and colleagues published a clinical case report of prefabricated bone tissue engineering [77]. The large bony defect was reconstructed with a microvascular flap using autologous ASCs, β-TCP and BMP-2, 36 months after a hemimaxillectomy due to a large keratocyst. After expansion of ASCs and cultivation with β-TCP and BMP-2 in vitro, a titanium cage filled with ASCs and β-TCP was inserted through a vertical incision into a pouch prepared in the patient's left rectus abdominis muscle. The rectus abdominis free flap was raised. Before severing the vascular supply to the muscle, the muscle pouch was carefully opened and the titanium cage was opened. After severing the vessels, the flap was placed in the maxillary defect; the inferior epigastric artery was anastomosed end-to-end to the facial artery and the vein end-to-end to the facial vein.

Clinical reports/trials with ASCs	Design	Results	Ref
Widespread calvarial defect	Autologous SVFs with fibrin glue	Success, follow-up: 3 months after operation	[75]
Large calvarial defect	Implant autologous cultured ASCs with β-TCP	No complications, follow-up: 3 months after operations	[76]
Maxillary reconstruction	Fabricated bone tissue using autologous cultured ASCs with β-TCP and BMP-2	Success, follow-up: 8 months after operations	[77]
Large osseous defect	Autologous ASCs with different scaffolds	Recruiting	NCT01218945 *
Avascular Necrosis of the Femoral Head	Autologous adipose tissue derived MSCs transplantation	Phase 1, 2	NCT01532076 *

Abbreviations: SVF; Stromal Vascular Fraction, ASCs; Adipose-derived Stem Cells, β-TCP; Beta-tricalcium phosphate, MSCs; mesenchymal stem cells, BMP; bone morphogenetic protein

(*Identifier on Clinical trials website: *http://clinicaltrials.gov/ct2/results?term=adipose+derived+cells+bone).

Table 1. Clinical reports/trials for large bony defect using adipose-derived stem cells

5. Future perspective

In the past decade, basic research characterizing ASCs shows that these cells have the potential to regenerate tissue defects such as large bone defects, and clinical studies have examined the potential use of ASCs to reconstruct oral and maxillofacial tissue. Although clinical studies have only just begun, the use of ASCs in the clinical setting is extremely promising because ASCs are a readily available, multipotent, and abundant cell type with the capability to undergo robust osteogenesis. However, further studies, including research to determine the mechanism of osteogenic differentiation and studies to evaluate the safety of ASC usage, will be necessary to realize the potential of ASCs in clinical regenerative medicine of the future.

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Chapter 7

Is the Articular Cartilage Regeneration Approachable Through Mesenchymal Stem Cells Therapies?

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Additional information is available at the end of the chapter

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1. Introduction

Today great hope is set on regenerative medicine in all medical fields. Leland Kaiser introduced the term "Regenerative Medicine" in 1992. He forecasted that a "new branch of medicine will develop that attempts to change the course of chronic diseases and in many instances will regenerate tired and failing organ systems" (Kaiser, 1992). Since then, scientists all over the world try to develop cell-based approaches to regenerate damaged tissues, or even substitute whole organs (Ehnert et al., 2009).

Degenerative disease of articular cartilage (AC), generically known as osteoarthritis (OA), is an irreversible evolution process towards terminal articular failure. Due to its high prevalence on population and its socioeconomic impact, this condition is of great concern, and this way more resources and effort are dedicated to the research on its development. Cartilage tissue engineering seeks to combine cells, biomaterial scaffolds, and bioactive signals to create functional tissue replacements to treat cartilage injuries or osteoarthritis (Song et al., 2004).

Cartilage degenerative disease, OA, is the end stage of several conditions such as trauma, inflammatory diseases, overweight etc. The fatalistic theory that states that it is impossible to recover the cartilage once it has been damaged leads to the assumption that the progression to any form of OA is unavoidable (Fig. 1). The annual incidence of young adults suffering any cartilage injury in UK is 10000 and this figure is continuously increasing (NICE, 2008).

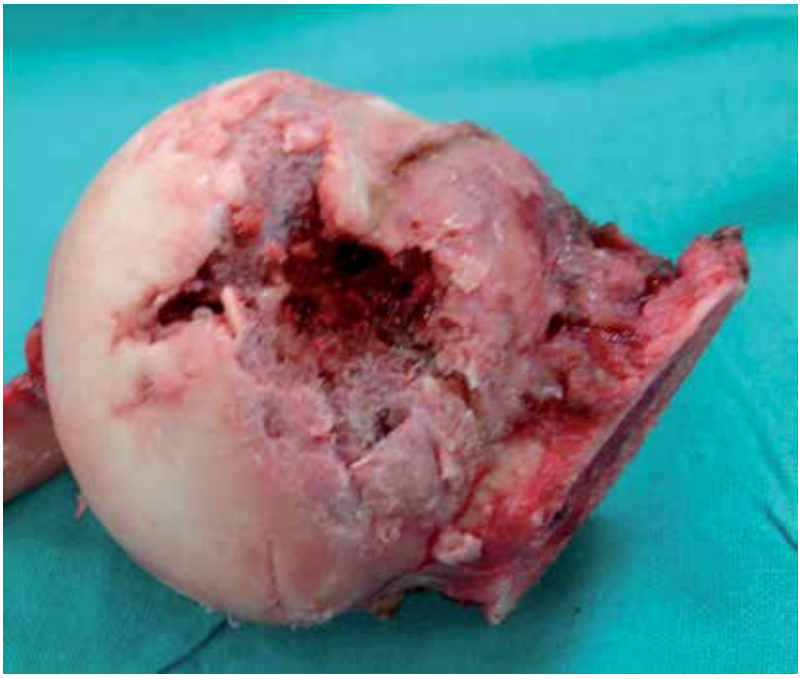

Figure 1. Osteochondral injury in a femoral head from a surgical intervention in our hospital

The new patient does not accept a reduction in his demands and quality of life because of the OA as the culmination of an articular injury during sport activity. On the other hand, a higher risk for developing degenerative disease and obesity, mainly knee arthritis, has been correlated. The symptomatic cohort of pain, swelling, range of motion diminution and loss of quality life can only be partially recovered by a total joint replacement (Fig. 2).

Unfortunately, joint replacement is neither a procedure free of complication nor a forever-realistic solution. It is expected that by the year 2030 the number of total hip or knee replacement implanted annually will be respectively 1.8 and 7 times the current figures (Kurtz et al., 2007). It is doubted that such an economic impact could be ever be afforded. Finding an alternative option to manage these lesions will be a challenge and this must be closer to achieve an almost AC tissue able to bear the requirements of an active long period of life.

AC is a quite simple structure with scarce cellularity within an extracellular matrix (ECM). However, in spite of this simplicity, its structure and performance is very complex.

The ability of the AC to heal these injuries on its own is almost none. The lack of blood supply is the main handicap this tissue has at the time of healing. Without vessels the preliminary inflammatory step of the healing process is not possible. To start with, the dead tissue needs to be removed and the flood of new cells from the vascular stream is essential for this.

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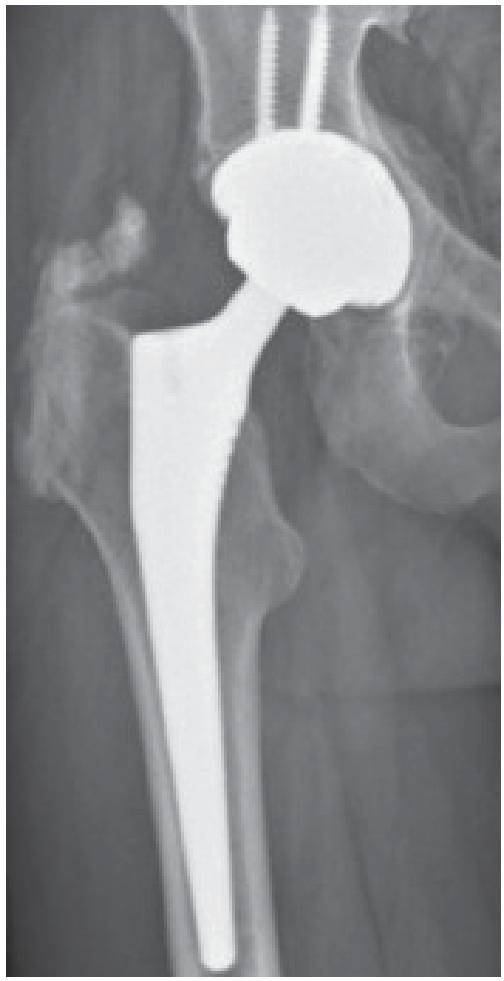

Figure 2. Treatment of a OA by total joint replacement performed in our hospital

Furthermore, the cell mediators will not appear as the granulocytes are not present and the degranulation of these is its source. Secondly, the chondrocytes are well-differentiated cells with a limited capacity to produce ECM. This is sufficient to cover the necessities of a still cartilage turnover but not to cope with the healing of a traumatic defect.

In any case, if the trauma produces a lesion that breaches the subchondral plate, it allows access to the vascular network and the final produced tissue is made of collagen type I, more resistant to tension, instead of type II, more resistant to compression.

1.1. How can this be sorted out?

Several attempts have been made in order to repair the traumatic defect and to achieve a regeneration of the original injured cartilage.

A first group of interventions can be described as "marrow stimulating techniques". Drilling the defect beyond the subchondral plate is its essential and allowing a repair promoted by the bleeding from the subchondral bone creating a "super clot" its rationale (Mithoefer et al., 2005). The star cells here are the mesenchymal stem cells (MSCs) emerging with the hematoma and its subsequent proliferation and differentiation. Unfortunately, the final result is the promotion of a fibrous tissue not durable in time (Steret et al., 2004). Pridie et al. promoted this concept in 1959. Abrasion arthroplasty (Steret et al., 2004), microfracture (Steadman et al., 2003), or Autologous Matrix Induced Chondrogenesis (Gille et al., 2010) have been more recently developed with the same rationale.

In essence, previous treatments have attempted to promote the healing of the damaged reminding nature. That is to say, allowing the flood of blood from the inner areas of the subjacent bone. Unfortunately, this will promote a scar tissue that in the long run will be lost and the joint degenerative process will be stated (Kreuz et al., 2006).

In a second group of interventions, it is proposed the articular defect to be covered by a "replacement technique". In these, the defect is reshaped to a standard cylindrical way and substituted with a plug of osteochondral graft harvested from a non-weight bearing donor site. Using this technique two handicaps can be appreciated: the limited available graft and the morbidity of the donor site. Additionally, the differences in characteristics between the donor and the receiver areas may impede a complete integration. This may be the reason why the clinical results have not been in accordance with the initial enthusiasm (Mishima et al., 2008). Both, auto- and fresh allograft (Gross et al., 2002) have been attempted with the name of mosaicplasty. With the aim of avoiding the donor site morbidity, synthetic reabsorbable scaffolds have been used to fill up the osteochondral defect. A "toast and butter" cylinder, engineered mimicking components, bone and cartilage. In contrast with the graft, this scaffold will have osteoconductive properties instead and eventually resorb in 12 months. The pseudo-cartilage now created will be poorly incorporated and the biomechanics will fail (Yasen et al., 2012).

The third group of proposed interventions are cellular-based. The aim in this alternative option is producing a regeneration of the cartilage mediated by the own patient chondrocytes. This is a two-stage procedure. During the preliminary intervention a biopsy from the cartilage is obtained and then the chondrocytes are isolated and cultured till the number of cells is about fifty folds. For the second intervention, *ex vivo* expanded chondrocytes are implanted in the damaged area. This procedure has evolved with the aim of getting a watertight seal environment in order to receive the chondrocytes and avoid the leakage. First, it was attempted a sheet of periosteum, then a collagen gel and a collagen membrane has been developed. But, are the delivered chondrocytes, the MSCs coming from the subchondral bone or the cells evading from the layer of periosteum the source for promoting the repaired tissue?. In spite of the rationale of this techniques, the autologous chondrocytes implant has not finally reached the expected results, the final tissue obtained was fibrous instead of AC (Tins et al., 2005) and hence, the OA is once more the undesirable expected long-term result (Hunziker, 2002; Temenoff et al., 2000).

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2. Therapeutic interventions without active biologics

2.1. Bone marrow stimulation

First approaches to heal cartilage by *in situ* regeneration date back to 1959. Pridie technique was directed to BM cells recruitment to be used in cartilage defects by drilling small holes into the subchondral BM space underlying the damaged cartilage regions. It was improved later on by reducing the size of the perforations and being then called microfracture technique which is now a frequently performed and well-studied procedure (Steadman et al., 1999). This technique is based on the mechanism of mesengenesis or capacity of the non-differentiated mesenchymal cells in choosing a determined phenotype as a response to inducing or GFs. A non-differentiated cell from the BM can be promoted to different cell types such as osteoblasts, with a later maturing to osteocytes, chondroblasts and chondrocytes, but also to endothelial cells, mesothelial cells, fibroblasts or adipocytes. It is a cell signalling process of local cytokines on local cells. In order to achieve all this, the surgical technique is based on drilling the subchondral plate to get bleeding and a superclot that will become a scaffold and supply cells and proteins, starting this way the physiological cascade of the chondrogenic cell differentiation. Other alternative techniques of BM stimulation to regenerate cartilage would be abrasion chondroplasty and in case the articular surface remained untouched, the retrograde stimulation technique. Cartilage defects are repaired only with fibrous tissue or fibrocartilage when using these methods, probably because the number of chondroprogenitors recruited from the BM is too small to promote the hyaline cartilage repair and results are often followed by degeneration of the repair tissue. This was used as an explanation for the observations of other studies that good short term results may be followed by deterioration starting about 18 months after surgery.

Clinical observations and theoretical considerations pointed towards several possible limitations of marrow stimulation techniques. The non-adhesive properties of the cartilage surface and the softness and shrinking of the superclot can lead to only partial defect filling and facilitate an early loss of repair tissue from the cartilage lesion. To avoid this, the treatment has been recently advanced into a matrix-supported technique in which the performed defect was stabilized in an additional way with a biomaterial. The microfractured lesion is covered with a collagen type I/III scaffold and it is called autologous matrix induced chondrogenesis (AMIC) (Kramer et al., 2006; Steinwachs et al., 2008). This technique has been developed to allow the treatment of larger defects by microfracturing and it is used as alternative treatment to autologous chondrocytes transplantation (ACT).

2.2. Autologous osteochondral transplantation: Mosaicplasty

Autologous osteochondral mosaicplasty, sometimes known as osteoarticular transfer system, OATS, is an effective method for the resurfacing of osteochondral defects of the knee. The technique consists in transplantation of many osteocondral autologous plugs obtained from the periphery of the femoral condyle articular surface, which supports less weight and transferring them to create a durable resurfaced area in the defect (Fig. 3). The procedure shows some advantages regarding other repair techniques, such as the viable hyaline cartilage

transplantation, a relatively short rehabilitation period and the possibility of carrying out the procedure in one only operation.

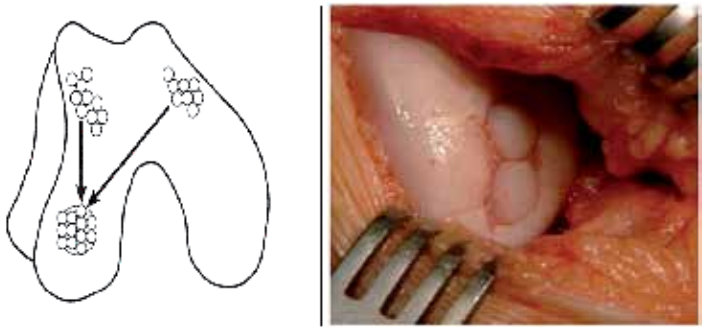

Figure 3. In mosaicplasty cylindrical osteochondral plugs are harvested from nonload-bearing sites in the affected joint and pressed into place within the osteochondral defect, creating an autograft "mosaic" to fill the lesion

However, the OATS limitations are the donor-site morbidity and a limited availability of grafts that can be obtained from the femoropatellar joint or the area adjacent to the intercondylar fossa. Other possible limitations are differences in bearing, thickness and mechanical properties between the donor's and the receiver's cartilages, as well as the graft sinking into the surface due to the support of weight after surgery. Besides, the lack of filling and the possible dead space between cylindrical grafts can limit the repair quality and integrity. Lane et al. transplanted autologous osteochondral grafts into sheep knee joints and reported the lack of integration of the cartilage, which determined the persistence of gaps through the full thickness in all the specimens (Mishima et al., 2008).

2.3. Alogenic osteochondral transplantation

Osteochondral allograft transplantation is a procedure for cartilage resurfacing which involves the transplantation into the defect a cadaveric graft composed of viable, intact AC and its underlying subchondral bone. It is a well known resource, especially for tumour surgery. The defect size, its location and its depth are crucial factors for the suitability of the donor graft. Advantages of using osteochondral allografts are the possibility if achieving a precise architecture of the surface, the immediate transplantation of viable hyaline cartilage in a one-time procedure, the possibility to repair large defects, even half-condyles and the donor-site lack of morbidity. Gross et al. have reported results from fresh allografts in 123 patients with good clinical results in 95% of the patients after five years (Gross et al., 2002). There are different possible allografts. Fresh osteochondral allografts are generally used because both freezing and cryopreservation have proved to reduce the chondrocytes viability. Traditionally grafts have been obtained, kept in lactated Ringer's solution at 4 °C and then transplanted in a week. Another alternative for allografts conservation and implantation is cryopreservation, which involves freezing at a controlled speed of specimens within a nutrients rich medium, a cryoprotector agent (glycerol or dymethil sulfoxide), to minimize the cells freezing and keep

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their viability; finally, there is the possibility of fresh-frozen allografts, with the advantages of lower immunogenic capacity and less transmission of diseases but with lower chondrocyte viability.

2.4. Soft tissues transplantation

Two main theories support the practicing of covering the cartilage defects with soft tissues, such as perichondrium or periosteum. On one hand, the defect has to be covered mechanically and on the other, we know about the presence of pluripotential stem cells in the perichondrium and the periosteum cambium layer. The different factors able to promote these cells differentiation into active chondrocytes still remain unknown.

3. Therapeutic interventions with active biologics

3.1. Autologous chondrocytes implant

The clinical use of the autologous chondrocytes implant (ACI) technique was first reported by Brittberg et al. in 1994, following animal studies which had shown its effectiveness (Grande et al., 1989). In this method, chondrocytes are obtained from a biopsy taken from a non-weight bearing part of the patients cartilage, and are expanded *in vitro*, followed by the injection of a suspension of chondrocytes into cartilage defects, covered with autologous periosteal flap (Fig. 4). This technique premises are based on the capacity of adhesiveness of the cells to certain surfaces, they spread on them and proliferate producing their specific ECM. Although clinical results of the original ACI looked promising (Minas, 2001; Peterson et al., 2000), this procedure has some potential disadvantages, such as leakage of transplanted cells, invasive surgical method, hypertrophy of periosteum (Haddo et al., 2005; Kreuz et al., 2009) and loss of chondrogenic phenotype of expanded chondrocytes in monolayer culture (Benya & Shaffer, 1982). Second generation ACI, named membrane autologous chondrocyte implantation (MACI), has a similar procedure, but a collagen type I and III membrane instead of periosteum. This technique was introduced to improve the ACI problems, and biomaterials such as collagen type I gel (Ochi et al., 2002), hyaluronan-based scaffold (Manfredini et al., 2007) and collagen type I/III membrane (Bartlett et al., 2005) were applied to secure cells in the defect area, to restore chondrogenic phenotype by way of three dimensional cultures (Gigante et al., 2007]) and to replace the periosteum as defect coverage. This is the way MACI technique is created *a posteriori*, by implanting autologous chondrocytes in three dimensional matrices of collagen types I and III, or hyaluronic acid.

At present, only two prospective studies comparing the original and second generation ACI are available (Bartlett et al., 2005; Manfredini et al., 2007) and both studies show no significant differences in the short term clinical results. As for the first generation ACI, the newly regenerated cartilage often consists of fibrous tissue (Horas et al., 2003; Tins et al., 2005), possibly due to the limited number of chondrocytes and their low proliferation potential. Bone overgrowth that causes thinning of the regenerated cartilage and the violation of the tidemark are also of concern. Moreover, this method still sacrifices healthy cartilage. Thus, these aspects

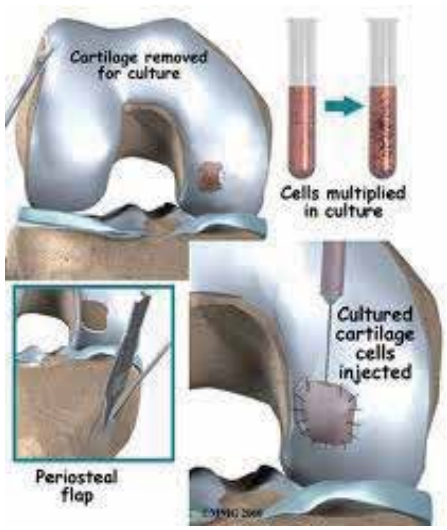

Figure 4. In autologous chondrocytes implantation (ACI) a chondral biopsy is taken from a donor site at the time of clinical examination to be treated with enzymes in the laboratory to obtain chondrocytes cultures that are re-injected under the periosteal flap

limit ACI in the treatment of large defects and may increase the long-term risk of osteoarthritis development.

3.2. Are Mesenchymal Stem Cells what we need?

Stem cells are of particular interest in Regenerative Medicine. They inhere several unique characteristics that distinguish them from other cell types. Besides autograft transplantation and ACI, current therapeutic concepts of cartilage defects include the recruitment of MSCs. Tissue engineering (TE) based on cell and genetic therapy offers some of the most promising strategies of tissue repair, including AC repair. It is the science able to create alive tissue to replace, repair or strengthen ill tissue. Thus, the TE refers to a wide variety of techniques.

The process for using MSCs to produce cartilage tissue comprises four elements: cells, inductor factors, scaffold to deliver the cells and vascular supply to the host area.

From the previous experiences we have learned that we need:

- To minimize the risk and inconveniences of the donor site. In this case, MSCs are easily available from bone marrow, synovial membrane, adipose tissue, etc. So then, we can get a variable number of cells from a different tissue, partially avoiding the donor site secondary complication (Winter et al., 2003). In ACI, this extra procedure adds a risk for infection, inflammatory changes in the joint, pain and rises up the final cost of the treatment (Hunziker, 2002)
- To achieve a minimum number of cells. MSCs have a high proliferation and differentiation potential. In any case, MSCs coming from different tissue have an uneven chondrogenic

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differentiation capacity and it must be related to the special cytokines, growth factor and induction molecules composition of the medium (Claros et al., 2012; Hennig et al., 2007). To induce the differentiation of predifferentiated cells, MSCs, is a more controlled way of driving the process to a cartilage final effector. One of the cons of chondrocyte transplantation is the dedifferentiation process that these cells suffer when they are treated *in vitro* and the limited ability to redifferentiate them (Benz et al., 2002). On the contrary, MSCs are very stable and they do not suffer this dedifferentiation (Najadnik et al., 2010) process and have a high differentiation capacity.

Up to now, MSCs have been used to treat osteochondral defects in two different ways. We have the option of implanting the MSCs once expanded. It is of concern in this case the lack of control on the growth after transplantation and how much exhausted the cells will result after the expansion and how it will influence on the aging of the new induced tissue. The clinical results after implantation of these expanded MSCs have been analysed and were substantially the same compared with those obtained after any marrow stimulating techniques (Wakitani et al., 2002; López-Puerta, 2013). A second option is to implant the MSCs after the induced differentiation to a chondrocyte phenotype. In these circumstances, to assure the stability of the achieved phenotype is of concern as this lineage is able to go on with the complete chondral process and ends in producing final stages of hypertrophy and calcification (Pelttari et al., 2006; Andrades et al., 2010; López-Puerta, 2013). In any case, the final challenge will be to produce a stable cellular lineage that produces an AC tissue that works under compression forces without losing its initial characteristics as time goes by.

Beside the characteristics of MSCs exposed before, these cells have self-renewal potential as well as multilineage differentiation potential (Becerra et al., 2011), including chondrogenesis (Johnstone et al., 1998; Pittenger et al., 1999; Prockop 1997; Sacchetti et al., 2007). MSCs chondrogenesis was first reported by Ashton et al. (1980) and the first ones to describe a defined medium for *in vitro* chondrogenesis of MSCs were Johnstone et al. (1998), who used micromass culture with TGF-β and dexamethasone. Sekiya et al. (2001, 2005) reported that addition to bone BMPs enhanced chondrogenesis under the conditions employed by Johnstone et al. (1998). Nowadays, the micromass culture is widely used to evaluate chondrogenic potential of MSCs *in vitro*. However, this *in vitro* chondrogenesis does not mimic cartilage formation during development. During micromass culture, MSCs increase expressions of both collagen type II (chondrocytes marker) and X (hypertrophic chondrocytes marker) (Barry et al., 2001; Ichinose et al., 2005). Other cytokines such as IGF (Pei et al., 2008) and parathyroid hormone-related peptide (PTHrP) had been tried for better differentiation cocktails, but it is still difficult to obtain *in vitro* MSC-based cartilage formation comparative to native cartilage tissue.

To initiate any regeneration based on MSCs activity, the cells first have to be recruited to the site of damage (Fig. 5). Second step is adhesion to a local matrix followed by activation and extensive proliferation to provide the necessary high numbers of chondroprogenitor cells to build up new tissue. In stem 3, the cells need to switch from expansion to chondrogenic matrix production by induction of chondrogenesis to build up the shock absorbance and gliding characteristics for proper tissue function. The seamless integration with neighbouring cartilage and bone tissue depends on successful crosstalk between new and old tissue, and an instruc-

tional capability to guide neighbouring cells. For durable cartilage regeneration, the tissue eventually needs to regenerate a tidemark, adapt to biomechanical loading and build up a balanced tissue homeostasis. We are going to summarize these steps including talking points taken from our annual meetings in NACRE (New Approaches for Cartilage Regeneration; CIBER-BBN, Spain) consortium, in order to propose strategies for the Regenerative Medicine of AC (Becerra et al., 2010; Andrades et al., in press)

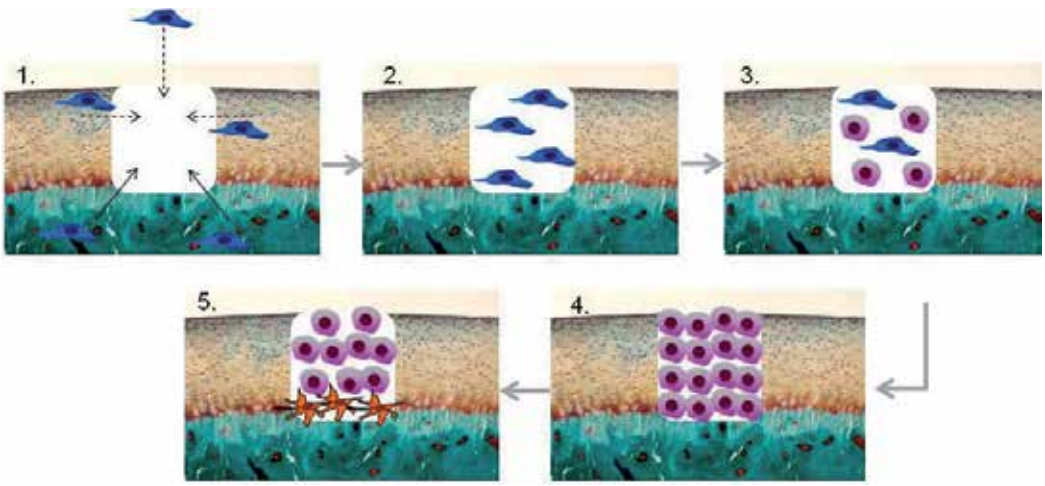

Figure 5. Principal mechanisms for *in vivo* cartilage regeneration. 1) MSCs recruitment from the surrounding tissues such as synovial fluid/membrane, neighbors cartilage, or subchondral bone; 2) MSCs retention by local adhesion, and proliferation; 3) MSCs are induced to chondrogenic differentiation by local factors; 4) chondroblasts and chondrocytes arranged according to the cartilage zones pattern and biomechanical loading; 5) integration of repair tissue interaction with cartilage and subchondral bone

3.2.1. Recruitment of MSCs

Cell migration is a prerequisite for development from conception to adulthood and plays a major role in regeneration of all tissues. Even articular chondrocytes, which are encased in a dense matrix throughout their life, can show cell motility when transferred *in vitro.* A number of studies demonstrated that chondrocytes migrate under the action of different stimuli on or within planar and 3D matrices. Attracting factors include bone morphogentic proteins (BMPs) (Frenkel et al., 1996), insulin-like growth factor-one (IGF-1), transforming growth factor-beta (TGF-β), fibronectin (Andrades et al., 2003: Chang et al., 2003), plateled-derived growth factor (PDGF) (Fujita et al., 2004), fibroblast growth factor (FGF) (Hidaka et al., 2006), fibrin and collagen type I (Kirilak et al., 2006).

But, what are the best factors to attract more MSCs to a cartilage lesion? Recent work has established that MSCs are not largely distinct from chondrocytes regarding the panel of factors capable to attract the cells *in vitro.* All tested factors were further effective in stimulating the migration of MSCs but not fibroblasts (Ozaki et al, 2007). Factors contained in a natural blood clot are highly chemoattractive for MSCs; augmentation of a clot-stabilizing matrix with a

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potent chemoattractive factor like PDGF may be an attractive way to further enhance cell numbers early after microfracturing.

In this sense, a quick release kinetics within hours and days from the biomaterial is desired for this first step of healing. Unfortunately, no 3D *in vitro* model in a biomatrix has yet been applied to test chemoattraction of human MSCs under more natural conditions and no adequate animal model has been used to dissect the factor requirement of progenitor cell recruitment from bone marrow into cartilage defects. Most likely, the presence of synovial fluid and the joint loading initiated pumping mechanism will strongly affect attraction and retention of MSCs. A search for superior factor combinations to enhance and speed up MSC attraction, and the best retention matrix to keep cells local, thus, is an important topic for upcoming studies.

3.2.2. Multiplication of MSCs

Proliferation of MSCs is the second important step to rapidly enhance the cell numbers in the repair tissue. As the replicative lifespan of MSCs is, however, not unlimited and telomerase activity is absent or low (Parsch et al., 2004) this can only ground on the sufficient attraction of initial MSCs numbers to the defect. During embryonal development proliferative chondroprogenitors are densely packed and condensed to an area in which cartilage tissue is forming. The high cell numbers may be needed to deposit the vast amount of ECM characterizing this tissue. Low cellularity of AC is rather a late phenomenon during tissue formation and may have developed in adaptation to its biomechanical competence and the extremely slow turnover of its ECM. Proteoglycan turnover in cartilage is up to 25 years and collagen half-life was estimated to range from several decades up to 400 years (Eyre et al., 2006). Based on the assumption that embryonal traits should best be recapitulated during tissue regeneration, early defect filling tissue should contain densely packed proliferating chondroprogenitors. This would mean that rather hundreds of millions of new cells are needed per cm^3 during this step in the defect to allow for optimal chondrogenesis and rapid ECM production. At a later phase, cell numbers could decline to a density of 5-10 million cells per gram tissue, when homeostasis is the left over task for the cells in the fully regenerated tissue.

These points towards an outstanding need for highly efficient transient induction of proliferation, a typical feature ascribed to adult stem cells. Growth requirements of human MSCs are distinct from those of other species (Kuznetsov et al., 1996) and many factors have been identified as potent mitogens, being PDGF-BB, EGF and TGF-β have been regarded as the most important amongst them (Kuznetsov et al., 1997). As they induced the migration of MSCs and have at the same time the potency to enhance their proliferation, the migration and proliferation steps of MSCs can take place simultaneously *in vivo.* However, whilst nutrients and oxygen are almost unlimited in tissue culture, a rapid supply of cells with O_2 and nutrients may be more restricted in a cartilage defect and depend on the distance to and conditions found within the subchondral bone. Histology of early human cartilage repair tissue demonstrates that indeed cartilage differentiation initiates in contact with subchondral bone (Steck et al., 2009) whilst upper regions remain fibrous for quite a long time and may need to mature over years (Brun et al., 2008). Furthermore, earliest chondrogenesis is often seen in areas where active remodeling of the subchondral bone plate occurs and, thus, enhanced nutrition and a

higher anabolic rate of the cells can take place. Beside strong mitogenic factors used for augmentation of a clot-stabilizing biomaterial, the access to optimal nutrition in the course of tissue remodeling may indeed be a limiting aspect of cartilage regeneration techniques. Enhanced remodeling of microfractured compared with unopened subchondral bone areas is likely, but quantitative and localization-dependent studies have so far not been reported. The stimuli mentioned here could be obtained by direct administration of recombining growth factors in the culture media or via transfer of the respective genes. Thus, the possibility of considering genetic therapy as an applicable measure for the treatment of cartilaginous lesions arises.

4. The recapitulation of AC morphogenesis through MSCs differentiation

In spite of therapeutic strategies mentioned, which offer the patient a temporary relief of symptoms, they do not resolved, in the medium or long term, the disease that affects the joint. In most cases, fibrocartilage is generated that does not provide the necessary structural integrity and this often results in the subsequent replacement of the damaged joint (Mahmoudifar and Doran, 2012).

The main problem with these strategies is that only tries to restore the biomechanical characteristics of tissue but not its physiology. That is why the goal of current research in AC regeneration with MSCs is moving to methods that attempt to recapitulate the morphogenesis of the tissue. The challenge is to develop strategies that will cover the widest possible range of intervening factors including: the dynamics of genes and proteins that control and participate in the chondrogenic process, their spatiotemporal patterns of expression, variations in culture conditions, biomaterials functionalized with effector molecules inducers of chondrogenesis and the inclusion of differentiation enhancers using genetic engineering and others.

4.1. Novel genes and protein: The path to recapitulating AC morphogenesis

As previously mentioned, the AC's ECM is composed mainly of collagen type II fibers and proteoglycans with strong negative charges, aggrecan being the most abundant of them (Han et al, 2011). That is why the detection of both proteins and their corresponding genes has been classically used as markers of chondrogenesis in numerous studies *in vitro* and *in vivo*. However, most modern regenerative strategies should aim at obtaining a more complete and organized ECM that achieves the greatest similarity with the ECM on the original AC. With this objective, have been identified dozens of genes that play a fundamental role in the formation and maintenance of AC, which are potential targets for research in the design of regenerative strategies (Quintana et al., 2009; Bobick et al., 2009; Mahmoudifar and Doran, 2012).

4.1.1. Proteoglycan 4 (PRG4): Biological function and its relationship to OA

A good example is the proteoglycan 4 (PRG4), also known as "superficial zone protein" (SZP) and "lubricin". It is a proteoglycan specifically synthesized by chondrocytes located at the

surface of AC and by synoviocytes. Their functions are the lubrication of articular joints, elastic absorption and energy dissipation of synovial fluid (Jay et al., 2007).

It has been shown that mutant mice *Prg4*$^{-/-}$ have normal joints at birth and during the newborn period but older mice exhibit accumulation of proteinaceous deposits on the cartilage surface, disappearance of the surface zone of flattened chondrocytes, synoviocyte hyperplasia, calcification of structures flanking the ankle joints and, consequently, a total failure of the joint (Coles et al., 2010). These data are consistent with OA degeneration, and have also been observed in veterinary cases of sheep with early OA which has been shown a downregulation of the expression of *PRG4* (Young et al., 2006). In a human clinical example, a case study reported in 2004 a 10 year old boy with Camptodactyly-Arthropathy-Coxa vara-Pericarditis syndrome (CACP) which arises as a result of truncating mutations of this gene. Clinical manifestations of CACP include congenital or early-onset camptodactyly, noninflammatory arthropathy with synovial hyperplasia and progressive coxa vara deformity, all symptoms related with the cartilage's physiology (Choi et al., 2004).

All these studies show that PRG4 is closely related to morphogenesis, maintenance and functioning of the AC as well as defects in its expression are related to the development of OA. That is why in recent years has aroused interest in the field of *in vitro* studies as a potential aim in developing AC regenerative strategies.

4.1.2. PRG4: An in vitro, in vivo and clinical marker of chondrogenesis

Several studies show that this protein participates in the chondrogenic process and that its expression can be induced on *in vitro* chondrogenesis experiments. Recently, has been found an increase in *PRG4* gene expression in cells derived from infrapattelar fat pad (IFP) in response to treatment with TGF-β1 and BMP-7, demonstrating that the *in vitro* induction of this gene expression is possible. Furthermore, it served to demonstrate the suitability of the source cell used (Lee et al., 2008). However, the same research group in a similar characteristics experiment but using human embryonic stem cells (hESC) was able to induce chondrogenesis but failed inducing PRG4 expression, concluding that the induction conditions should be optimized. PRG4 use as a marker allowed the discrimination of both experiments (Nakagawa et al., 2009). It has also been observed that in rat skeletal muscle-derived mesenchymal stem/progenitor cells (MDMSCs) treated with TGF-β1 and BMP-7, PRG4 increased in a time-dependent manner on days 3, 7 and 10. As early as day 3, there was a three-fold increase of the PRG4 detected by ELISA analysis. This was confirmed by immunochemical localization of PRG4 as early as day 3 after treatment with TGF-β1. Even, the mRNA expression of PRG4 was enhanced by the two factors, along or in combination. This work demonstrates that is possible induce *in vitro* not only the increased of PRG4 gene expression, but also the accumulation of the protein in the medium (Andrades et al., 2012).

In clinical setting, there are also studies related with PRG4. Patients with OA tend to form small cartilaginous deposits in the exposed subchondral bone. The histological study of aggregates of patients undergoing total knee replacement, reveals that these aggregates were fibrocartilaginous, positive staining for glucosaminoglycan Safranin-O and type II collagen expression but, more interestingly, of PRG4. In those aggregates embedded in the bone, the

staining was positive for the entire surface while in which protruded to the surface, the PRG4 was detected only in the edge surface as would be observed in normal cartilage. This *in vivo* observation is an excellent example of the cell's genetic response to the environment. Osteochondroprecursors contact with the synovial fluid and physicochemical stimuli inherent to the articular surface is able to modify the spatial distribution patterns of PRG4 expression and thus, the tissue architecture. These results are an invitation to test culture conditions that attempt to emulate not only the biochemical environment, but also mimic the biophysical characteristics of the physiological niche (Zhang et al., 2007) (Fig. 6).

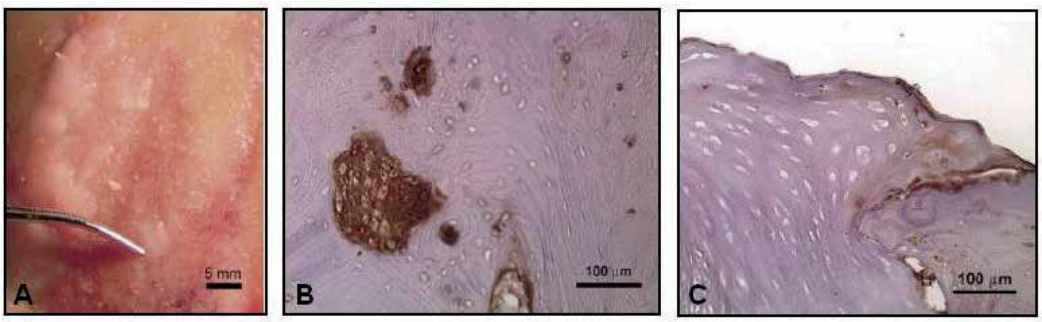

Figure 6. A) Probe pointing to a white spot on the exposed bone surface of an osteoarthritic femoral condyle obtained at the time of total knee arthroplasty. B) Immunohistochemistry for PRG4 to subsurface chondrocyte aggregate in subchondral bone staining fully for PRG4; and C) fibrocartilaginous deposit protruding through the joint surface containing PRG4 in a zone just below the surface. *From the same authors, published by Journal of orthopaedic research: official publication of the Orthopaedic Research Society 25(7): 873–883. Copyright 2007*

It has also been demonstrated the possibility of reversing the decline in the expression of PRG4 in cartilage chondrocytes culture from OA patients. In this study, cartilage explants were obtained from healthy and OA patients and performed monolayer cultures and encapsulated in poly (ethylene glycol) diacrylate scaffold (PEG-DA). The OA cartilage explants have weaker inmunolabeling of PRG4 than healthy cartilage explants. However, the difference was reduced significantly between these 2 samples when were cultured in PEG-DA hydrogels. OA chondrocytes regain the ability to express PRG4 at levels virtually identical to those obtained in chondrocytes from normal cartilage demonstrating the importance of culture conditions in the condrogenic induction (Musumeci et al., 2011).

All these evidences indicate that the inclusion of proteins as PRG4 in experimental designs offers advantages such as:

1. The inclusion of more highly specific markers that allow a better understanding of chondrogenic potential of cell sources, bioactive scaffolds and treatments applied.

2. Implant design that better mimic the characteristics of the AC and try to emulate not only the composition but also tissue architecture.

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3. The ability to understand more deeply the dynamics of diseases that affect the AC like OA, in order to raise regenerative strategies that offer to patients, medium and long term solutions.

4.2. Genetic engineering: Enhance of chondrogenic potencial beyond biochemical signals and functionalized scaffolds

4.2.1. SOX9: The key regulator of the chondrogenic process

Given the low regenerative capacity of the AC, it is vital find ways to increase the chondrogenic potential of bioimplants designed. Today, not only can enhance the biochemical environment biomaterials and implants, but genetic engineering can increase the potential of MSCs used, changing their gene expression patterns from "inside". For this, it is important to consider transcription factors and their intracellular signaling cascades. Perhaps the most studied of these factors is SOX9, considered the key regulator of the chondrogenic process (Bi et al., 1999). The expression of SOX9 is upregulated by members of the FGFs, TGF-βs and BMPs, all of them widely used chondroinducers. In turn, SOX9 is responsible for regulating SOX5, SOX6 and activating the expression of collagens type II, VI, IX and XI, the proteoglycans Aggrecan, Byglican and Perlecan and important binding proteins as COMP (Quintana et al., 2009) (Fig. 7).

On the other hand, during skeletogenesis, SOX9 is responsible for the osteochondroprogenitors differentiation into chondroblasts and not into osteoblasts to direct and indirect repression of RUNX2 (the main regulator of osteogenic differentiation) favoring the endochondral ossification (Zhou et al., 2006).

4.2.2. Transfection of SOX9 as an activator of chodrogenesis

The application of these regulatory genes on regenerative design strategies could be useful to increase both specificity and efficiency of the bioimplants. This opens the possibility of using not only functionalized biocompatible scaffolds, but also cells previously treated to have a higher chondrogenic potential. In a recent study, human MSCs are transfected with a nonviral vector plasmid complexed with SOX9 cDNA in order to induce chondrogenesis. Micromass culture and transplantation into nude mice of control and transfected cells were made. Both procedures showed increased levels of mRNA for COL2A1, Aggrecan and COMP; increased GAG content; alcian blue staining positive and detection of type II collagen and Aggrecan by immunofluorescence, all of the cells transfected with respect to control. Similar results were achieved using a viral vector transfection of SOX9 (Fig. 8). Additionally, this group demonstrated that transfection of the gene, in addition to inducing chondrogenesis, reduces the levels of markers of hypertrophy, osteogenesis and adipogenesis, thereby inhibiting the possibility that the human MSCs to differentiate into these mesenchymal lineages (Venkatesan et al., 2012).

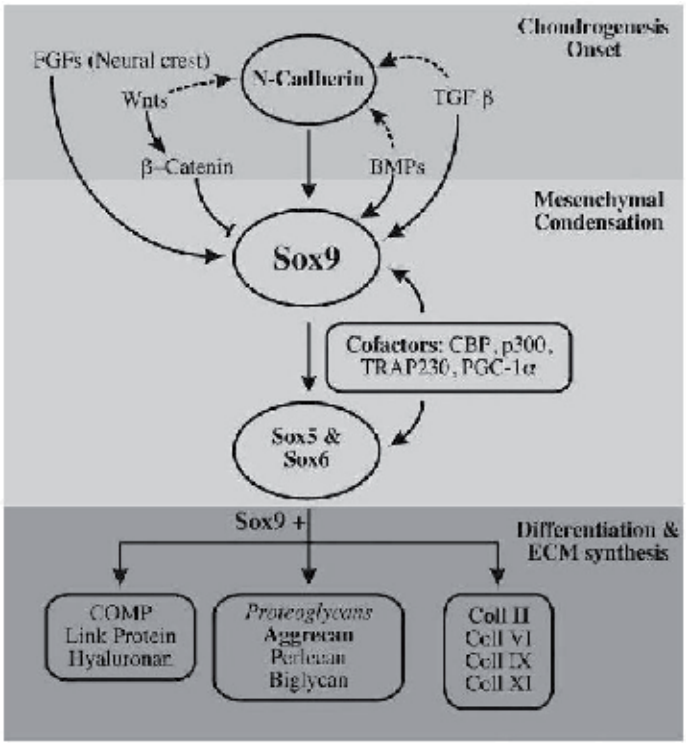

Figure 7. Upstream and downstream regulation of Sox9. From the same authors, published by Tissue engineering. Part B, Reviews 15(1): 29–41. Copyright 2009

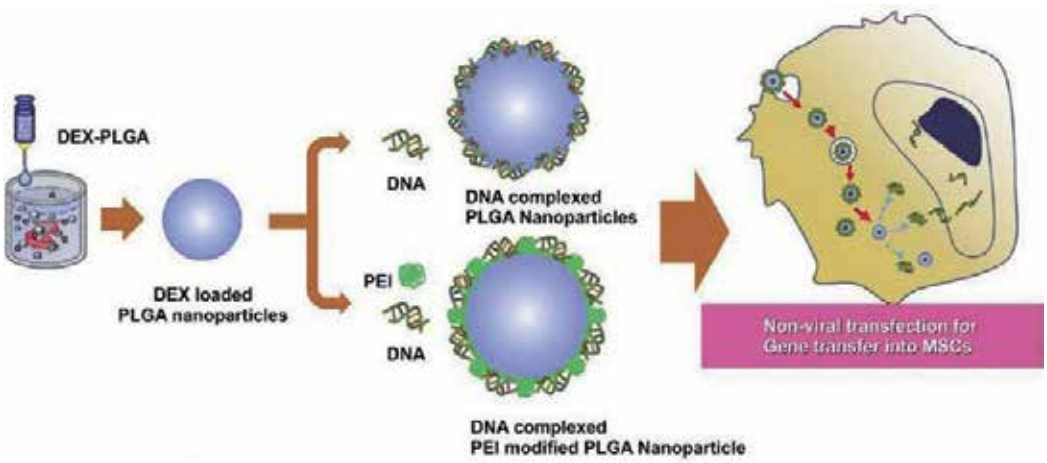

Figure 8. Schematic diagram of SOX9 gene transfection using a modified and non-modified biodegradable nanoparticles, an example of Non-viral transfection. During hMSCs transfection, nanoparticles interact with the negatively charged lipid bilayers and are influxed into endosomes and destabilized, resulting in the release of the transfected genes into the cytosol. *From the same authors, published by Biomaterials 32(1): 268–278. Copyright 2011*

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The possibility of genetically engineering the different types of MSCs used in cartilage regeneration is a promising tool for increasing chondrogenic capacity and, consequently, improving future regeneration bioimplants. A deepest and detailed knowledge of gene regulation involved in the process and their possible clinical utilities will lead the way of successful recapitulation of AC morphogenesis through MSCs differentiation.

5. Commercial and industrial translations in tissue engineering

Applications for TE and biomaterials were originally limited to prosthetic devices and surgical manipulation of tissues but now include development of biomaterial scaffolds, bone/cartilage engineering, tissue-engineered blood vessels and wound healing, among other fields.

As an industry, it could significantly contribute to economic growth if products are successfully commercialized. However, to date, relatively few products have reached the market owing to a variety of barriers, including a lack of funding and regulatory hurdles. Policy interventions, including increased translational government funding, adaptation of policies, and regulatory clarity, would likely improve the general outcomes for the regenerative medicine industry.

The technical challenges of TE are, of course, intellectually and scientifically interesting and can add substantial and previously unattainable knowledge to our understanding of biological systems (Mansbridge et al., 2006). TE models of biological systems can even provide insight into pathologic processes. However, perhaps the major attraction of academic researchers and industrial organizations to this field is the potential of the technology to be readily converted to clinical applications. For this to happen, the technology almost always will be transferred from an academic environment to an industrial organization that will lead the comprehensive translational studies and convert scientific observations into a manufactured product.

As s technology, TE has been shown to be feasible *in vitro* and *in vivo,* but the true demonstration of the potential value of the technology is in its clinical applications. Although the field is still in its infancy, there are already tissue-engineered products on the market, addressing previously unmet clinical needs in wound care and in orthopedics and demonstrating that the attractiveness and motivation of the field is justified. Perhaps one of the next major challenges is demonstration that the technology can lead to commercially feasible products, with manageable investment, product development costs, and time to market and, finally, a revenue generation that justifies the expense. The close connection between new technology, clinically effective treatment, and commercially feasible product is obvious and is no better demonstrated than in TE. All three of these areas, each complex in itself, must be aligned and achieved before TE can be regarded as successful.

5.1. Product development pathway

To appreciate the challenge of developing a tissue-engineered product, it is useful to first understand in general terms the various processes that must be completed (Fig. 9). The

development of a product through to approval, manufacture, and marketing is complex, and most companies (within and outside health care) use a staged process to ensure efficient and effective product development. The general scheme that applies for health care products (devices, biologics, or drugs) is outlined in the figure. These stages encompass all the activities that are required to develop a product through to the market. This integrated product development process can be customized to be appropriate for the development of products addressing repair and regeneration.

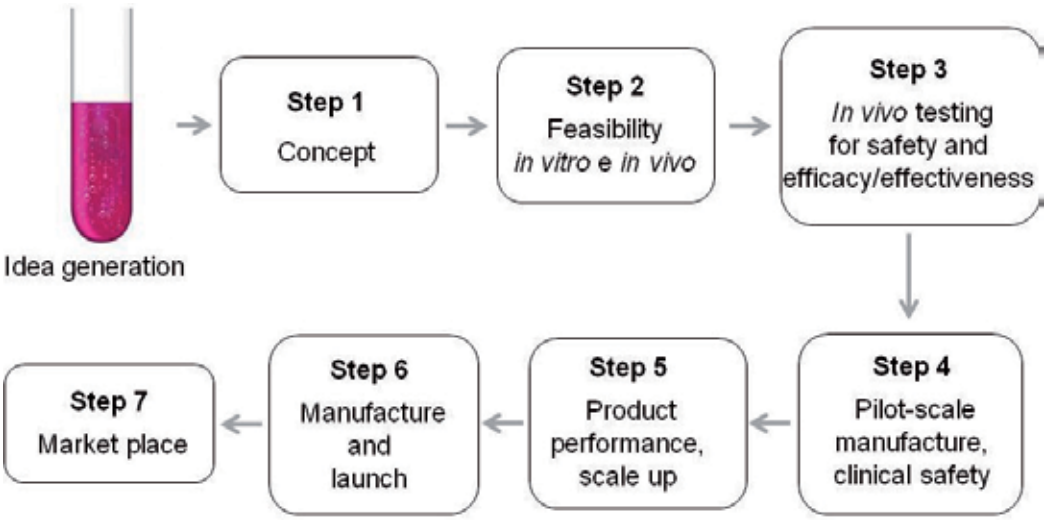

Figure 9. The general product development pathway used to develop tissue-engineered products

Patient protection for an individual product is a critical feature of product development. Developing new patents is costly, the outcome is uncertain, and it must occur near the beginning of product development. The limited time for patent protection (usually 10 years from initial submission) requires that the product development pathway be followed in an efficient manner; otherwise, patent protection will be lost by the time profitability arrives.

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Chapter 8

Adipose Derived Stem Cells: Current State of the Art and Prospective Role in Regenerative Medicine and Tissue Engineering

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Marco Mammana and Bassetto Franco

Additional information is available at the end of the chapter

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1. Introduction

1.1. Adipose tissue: The Good, the Bad, the Ugly

Excessive body fat has been socially recognized for ages as a symbol of wealth and prosperity. Clues of these concepts may be found in arts and literature. In addition, it has been substantially ignored by scientists, anatomists and physicians for many centuries. As a matter of fact only a minimal number of medical reports focused on "fat" have been historically handed down. Nowadays, however, adipose tissue has become a growing point of most interest for researchers and physicians worldwide. Notably, societies and health care systems are facing a severe pandemic rise of obesity and of several associated co-morbidities such as cardiovascular disease, diabetes, metabolic disorders and cancer. Fat and misregulation of adipose-related pathways are recognized as key elements in each of these processes. Importantly, the role of adipose tissue has progressively evolved from being a passive energy store to representing an important endocrine organ that directly modulates metabolism and immunity towards an healthy phenotype or leading to pathologic processes. The investigation of the physiologic-pathologic attitudes of adipose tissue is currently among most relevant scientific targets of researchers, endocrinologists and bariatric surgeons. Beside, in the last fifteen years adipose tissue has been reappraised also for a different reason. In fact, nearly forty years after the identification of bone marrow stem cells, it has been gathering attention for the opportunity to obtain autologous pluripotent adipose-derived stromal stem cells (ADSCs). This population of cells has been extensively investigated and it currently holds out many hopes for prospective

stem cell therapies for the repair and regeneration of various tissues and organs in a large number of different diseases. Thus, over the past years, this field has become a very active and attractive area of clinical and experimental research, providing significant outcomes and reaching important milestones. Today adipose tissue embodies an hot spot of regenerative medicine that may give rise to a new era of active stem cell therapy.

2. Purpose

2.1. Meeting the adipose tissue

Giving the increasing amount of experimental and clinical data regarding adipose tissue and ADSCs, in this chapter we are going to briefly review the concepts and the insights behind the role of adipose tissue in regenerative medicine and tissue engineering. In particular we are going to focus the attention on current cutting edge translational research from bench to bedside, including the investigation of biological properties of ADSCs, the state of art of their manipulation, the latest progresses in their clinical adoption, the development of bio-engineered products and the actual therapeutic prospective opportunities.

3. Basic science background

3.1. The outline and the anatomy of adipose tissue

Adipose tissue is a complex and multi-depot organ, constituted for one third by mature adipocytes and for the other two thirds by a combination of a large variety of other cells. [1] Among represented cell lines are included small blood vessels, nervous cells, fibroblasts and, importantly, adipocyte progenitor cells, also known as preadipocytes or Adipose Derived Stem Cells (ADSCs). Evolution has preserved in mammals two histologically different qualities of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT), which are composed by different types of mature adipocytes [Table 1]. In particular, white adipocytes are spherical, having a diameter ranging between 30 and 70 μm according to the amount of lipid depots, and lipids within the cells are organized in a single large "uni-locular" droplet, the size of which can exceed 50 μm. Thus, the lipid droplet occupies the vast majority of the whole intracellular space, pushing the remaining cytoplasm and nucleus into a thin marginal rim. On the other hand, brown adipocytes are polygonal with a centrally placed nucleus and their cellular size ranges from 20 to 40 μm. They accumulate lipids in smaller "multi-locular" droplets and they are rich of specific mitochondria, containing the protein UCP-1 which is responsible for uncoupling of oxidative phosporylation and production of heat. WAT and BAT are both innervated by noradrenergic fibers of the sympathetic nervous system. As for the vascularization of adipose tissue, white adipocytes are organized in collections of fat lobules, each supplied by a selective arteriole and surrounded by septae of connective tissue. An individual adipocyte is supplied by an adjacent capillary and it is associated to a glycoprotein layer, reticular fibrils, fibroblasts, mastocytes and macrophages. Compared to WAT, BAT provides a more extensive vascular tree, characterized by dense multiple capillaries. The relevant vascularization of the latter in combination with the

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presence of a significantly high number of mitochondria, account for the typical "brown" color. WAT and BAT have also different roles in energy metabolism. Primary function of white adipocytes is to store excess energy as lipid, which is then mobilized in response to metabolic needs. Brown adipocytes, on the other hand, use accumulated lipids primarily as a source of energy released in the form of heat. WAT can be found in several anatomically distinct and separate collections, or "depots." There are two major anatomic subdivisions of these depots, each showing unique anatomic, metabolic, endocrine, paracrine, and autocrine properties: intra-abdominal or visceral adipose tissue and subcutaneous adipose tissue. In addition, WAT can also be found in small amounts of fatty layers surrounding other organs, such as the heart, kidney and genitalia. Intra-peritoneal fat, composed of omental and mesenteric adipose tissue, comprises the vast majority of visceral fat. Importantly, subcutaneous adipose tissue shows different structural features in different anatomical districts. [2] In fact, fat depots in the abdominal area are characterized by the presence of large adipocytes, densely packed together and surrounded by a poor stromal (collagen) network. Instead, in more localized depots (such as throcanteric areas, the sovra-pubic area, arm pits, medial regions of the knees, tights, arms, pectoral and mammary areas) adipocytes present a smaller diameter, a more represented stromal component and a more extensive vascular network. BAT in newborns and children can be found in several body areas. However, while in other small mammals these depots persist during growth, in humans brown adipocytes undergo a morphologic transformation, rapidly accumulating lipids, becoming uni-locular and losing their typical ultrastructural and molecular properties, including mitochondria [Figure 1.]. As a consequence, there are no discrete collections of BAT that can be found in human adults.

	White Adipocyte	**Brown Adipocyte**
Shape	Spherical	Polygonal
Diameter	30-70 μm	20-40 μm
Ultra-structure	One large "unilocular" lipid droplet, cytoplasm and nucleus compressed into a thin visible rim	Multiple smaller "multilocular" droplets, high content of mitochondria, centrally placed nucleus
Innervation	Noradrenergic fibers, confined to capillary wall	Noradrenergic fibers, directly interfacing plasma membrane
Vascularization	Supplied by an adjacent capillary	Richer vascular tree, dense with multiple capillaries
Main function	Store excess energy as lipids	Thermogenesis
Localization	Visceral compartment (intraperitoneal, retroperitoneal, around organs) and subcutaneous compartment	Several areas in newborn, no discrete collections in adult. Probably isolated cells scattered between WAT depots

Table 1. Main differences between White and Brown adipocytes.

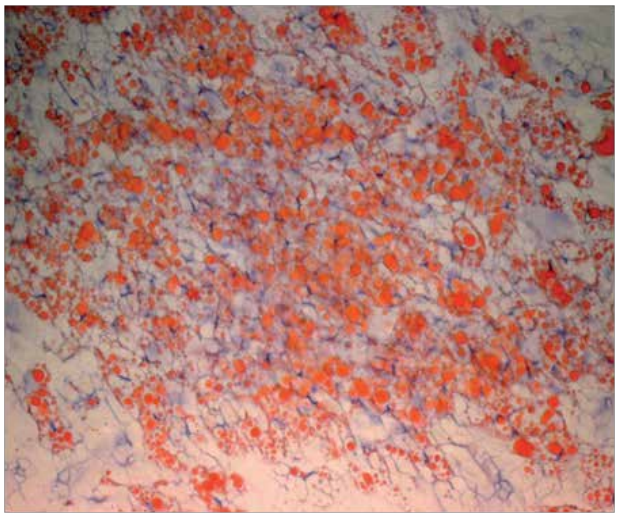

Figure 1. Monolayered culture of adipocytes in vitro with adipogenic medium.

3.2. The living image of adipose derived stem cells

The understanding of biochemical characteristics, molecular/cellular biology, immune-biological characteristics and phenotype of adipose tissue has significantly advanced in the last years. Adipose tissue has shown to consist mostly of cells of mesenchymal origin with few others endothelial cells, smooth muscle cells and pericytes, all showing low levels of cell senescence. Adipose tissue derives from the mesodermal layer of the embryo and develops both during pre-natal and post-natal growth. The microscopic location of the adipogenic progenitor cells in the adult is still controversial. [3] It remains to be proven whether the origin of the cells correlates with endothelial, pericytic or stromal compartments. A large number of surface antigens are in common with endothelial cells, suggesting a common origin. According to some researchers, adipogenic progenitor cells could be released directly by the bone marrow and distributed systemically by blood flow: experimental evidences of bone marrow derived-cells capable of differentiating into adipocytes in vivo have already been described but the contribution of these circulating cells to the overall growth and development of adipose tissue is still under investigation. Mesenchymal stem cells (MSC) were first described as immature cells in the bone marrow, capable to give rise to mesenchymal lineages such as osteoblasts, chondrocytes and adipocytes. [4] MSCs represent a small fraction of nucleated cells of human bone marrow (0.01%-0,0001%). MSCs are defined by three minimal criteria, as established by the International Society for Cellular Therapy in 2005: adherence to plastic dishes, specific surface antigen (CD73+, CD90+, CD105+, CD45-, CD34-, CD14 or CD11b-, CD79- or CD19-, HLA-DR) and in vitro capability to give rise to adipocytes, osteoblasts and chondrocytes. A similar protocol has been used for a long time to isolate adipose tissue progenitors: the resulting immature adherent cells were thus called pre-adipocytes. To obtain these cells fat pads are minced and digested with collagenase, separating an upper layer of floating mature adipocytes

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from a lower layer of pelleted stromal vascular fraction (SVF). [5] The SVF is an heterogeneous cell population of circulating blood cells, fibroblasts, pericytes, endothelial cells and pre-adipocytes. Pre-adipocytes may be isolated from the SVF by plating and washing. This cell population, adopting appropriate differentiating agents, can give rise to mature adipocytes, demonstrating their nature of adipose progenitors. Cell cultures have provided evidence of regenerative capacities in both the heterogeneous stromal vascular fraction (SVF) and in the more homogeneous adipose-derived stem cells (ADSCs). In 2002 pre-adipocytes were better characterized and they were demonstrated to show clear multi-potency potential: thus, they were named Adipose Derived Stem Cells (ADSCs). [6] In particular, ADSCs represent a mesodermal stem cell population with clonal mesodermal, ectodermal, and endodermal potentials capabilities that express multiple CD marker antigens similar to those of other mesenchymal stem cells as those residing in bone marrow. Several investigations have reported a differentiation into adipogenic, osteogenic, chondrogenic and myogenic lineages in vitro by means of specific culture media. In particular, the potential to differentiate into non-mesodermal lineages is exciting. The differentiation into neural precursors, which are of an ectodermal origin, has been described. In addition, evidence of differentiation into hepatocytes, pancreatic islet cells, endothelial cells and other epithelial cells has been provided in different reports. By definition, a stem cell is characterized by the ability to self-renew and to differentiate along multiple lineage pathways. Since the self-renewal of ADSCs has not been fully established yet, it is accepted that some investigators may use the same acronym to mean "adipose-derived stromal cells", in agreement with the statement of the International Society for Cellular Therapy. Indeed, ADSCs present several differences from MSCs at genomic, proteomic and functional levels. For instance, during the earliest rounds of proliferation, ADSCs express the CD34 antigen: the frequency of these cells is much higher (100 to 500 folds higher) than that of MSCs in the bone marrow. In addition, MSCs are probably more committed towards osteoblastic and chondrogenic lineages than ADSCs. Thus, although numerous author use the same term “MSCs” both for cells derived from bone marrow and for those derived from adipose tissue, MSCs and ADSCs are probably two distinct cell populations. A more precise definition of ADSCs, based on their immune-phenotype and/or differentiation capabilities, has not been yet provided. Some authors believe that ADSCs are a heterogeneous group of progenitor cells with differences in their stem cell potential. Thus, ADSCs and SVFs cells represent an autologous alternative to pluri-potent embryonic stem cells with a multi-lineage differentiation potential, a significant therapeutic impact and a critical role in the rapidly expanding fields of tissue engineering and regenerative medicine. Significantly, further investigations are needed to better clarify these aspects. Importantly, the most important characteristics of ADSCs, with a possible interest for clinical applications, comprise their multi-potency, secretory functions and immune-modulatory capabilities.

3.2.1. Differentiation potential of ADSCs

ADSCs, like MSCs, have the ability to differentiate into mesodermal cells, such as adipocytes, fibroblasts, myocytes, osteocytes and chondrocytes, in a process called lineage-specific differentiation. The increasing evidence for the ability of ADSCs to differentiate into cells of non-mesodermal origin such as neurons, endocrine pancreatic cells, hepatocytes, endothelial

cells and cardiac myocytes, is surprising. This process is called “cross-differentiation”. Lineage-specific differentiation can be tracked at a molecular level by the expression of key transcription factors of mature tissues. The earlier stages of differentiation, named "allocation" or "commitment", that drive the ADSCs into the specialized lineage are not completely known yet. In vitro, the differentiation of multi-potent cells into a desirable cell phenotype can be obtained by appropriate culture conditions and stimulation with a cocktail of known differentiating agents [Table 2].

Type of differentiation	Stimulating factors
Adipogenic	Insulin; isobutylmethylxanthine (IBMX) ; dexamethasone; rosiglitazone; indomethacin.
Osteogenic	Dexamethasone; β-glycerophosphate; vitamin D3; bone morphogenetic protein (BMP-2)
Chondrogenic	insulin growth factor (IGF); BMPs; transforming growth factor-β (TGF-β)
Myogenic/cardiomyogenic	Dexamethasone; hydrocortisone; IL-3; IL-6
Vascular/endothelial	Specific environment
Neurogenic	Valproic acid; epidermal growth factor (EGF); fibroblast growth factor (FGF); nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF)
Tendinous	FGF; platelet derived growth factor (PDGF-BB); EGF; TGF-β; IGF-1; BMPs

Table 2. Experimental growth factors used for differentiation of ADSCs in different cell lineages.

- Adipogenic differentiation

ADSCs have an exceptional potential for differentiation into mature adipocytes, which is very promising in developing techniques for repairing soft-tissue defects. [7] Differentiation can be induced by a large variety of substances, including insulin, dexamethasone, rosiglitazone and indomethacin. During differentiation ADSCs, initially showing a fibroblast-like spindle or stellate shape, undergo morphologic changes with the appearance of one or more lipid vacuoles and they begin to express several genes and proteins characterizing the mature adipocyte, including leptin, peroxisome-proliferating activated receptor γ (PPARγ), glucose transporter type 4 (GLUT4) and glycerol-3-phosphate dehydrogenase (GPDH).

- Osteogenic differentiation

Osteogenic differentiation can be induced in vitro by supplementing the culture medium with dexamethasone, β-glycerophosphate and vitamin D3. The acquisition of the osteoblast phenotype is accompanied by expression of specific genes and proteins, including alkaline phosphatase, type I collagen, osteopontin, osteonectin, and Runx2. Osteogenic differentiation may also be obtained by transfection of osteogenic lineage-determining genes (BMP2 and Runx2): this approach has proved to be effective both in vitro and in vivo in a large number

of reports. These experimental findings hold great promise for the use of ADSCs in bone regeneration.

- Chondrogenic differentiation

Insulin growth factor (IGF), bone morphogenetic proteins (BMPs), and transforming growth factor-β (TGF-β) have shown to induce chondrogenic differentiation of ADSCs when added to the culture medium. Chondrogenic differentiation occurs also by seeding ADSCs into polyglycolic acid (PGA) scaffolds, as it was largely demonstrated in several other in vitro models and in vivo in nude mice.

- Differentiation into other lineages

Terminally differentiated myoblasts can be obtained in vitro, showing the ability to form multinucleated myotubules and to shrink/diastole under the influence of atropine. This property of ADSCs is of particular interest for the treatment of genetic muscular dystrophies: preclinical in vivo studies on animal models are currently ongoing. In addition, other studies have focused on the capability of ADSCs to differentiate into cardiomyocytes with a possible application in heart regeneration or repair after an ischemic injury. Furthermore, endothelial regeneration is another important field of research: ADSCs have shown to be able to differentiate into endothelial cells and to secrete several pro-angiogenic factors, like vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). Differentiation into neuron-like cells has also been reported by different authors: ADSCs may acquire a neural-like morphology and they may express several proteins specific for the neuronal phenotype (Neuron Specific Enolase; Neuron Specific Nuclear Protein). Finally, some studies have explored the chance for ADSCs to differentiate into pancreatic islet cells, hepatocytes and epithelial cells with the purpose to find an alternative cellular therapy for diseases such as diabetes mellitus and liver disfunction: data and outcomes are however still preliminary and lacking of strong evidence.

3.2.2. ADSCs as a secretome

Importance of ADSCs does not only reside in their potential to differentiate in mature lineages. Similarly to the original adipose tissue from which they can be isolated, ADSCs have shown to act as a "secretome", accurately regulating proteins and growth factors secreted into the extracellular milieu and having a relevant impact on different organs and systems within the human body [Table 3.]. [8] Trophic effects of ADSCs include stimulation of angiogenesis, hematopoietic support, gene transfer and suppression of inflammation. Indeed ADSCs represent a source of several cytokine/soluble factors regulating the survival and differentiation of various endogenous cells/tissues. A large number of these molecules have been related to the regenerative attitude of ADSCs: among these, we may include hepatocyte growth factor (HGF), granulocyte and macrophage colony stimulating factors, interleukins (ILs) 6, 7, 8 and 11, tumor necrosis factor-alpha (TNF-alpha), vascular endothelial growth factor (VEGF), brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), adipokines and others. Full characterization of the secretory profile of ADSCs, either by immune-enzymatic techniques (ELISA) or by mass spectrometry, is still object of investigation. Several adipokines such as

adiponectin, angiotensin, cathepsin D, penetraxin, pregnancy zone protein and retinol binding protein, as well as stromal cell-derived growth factor (CXCL12) have been found in the conditioned media of ADSCs differentiating towards the adipocyte lineage. ADSCs secrete also oher different well characterized cytokines (GM-CSF, TGF-β, PGE2, IGF-1) and their release can be modulated by exposure to different agents, such as b-FGF and EGF or inflammatory stimula, like lipopolysaccharide (LPS). The role of these and other factors has been investigated by multiple studies regarding one or more possible applications of ADSCs in the field of regenerative medicine. Brain Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF), Glial Derived Neurotrophic Factor (GDNF) are thought to be important molecules secreted by ADSCs mediating neurotrophic effects and modulating in animal models of Parkinson Disease the recovery after hypoxic-ischemic injuries. Hepatocyte Growth Factor (HGF) and Vascular Endothelial Growth factor (VEGF) are the most important factors capable of inducing angiogenesis in areas that have undergone ischemic episodes and their importance is particularly relevant in wound healing. In cardiac regeneration, IGF-1 and VEGF mediate respectively an anti-apoptotic and angiogenic action, to which is attributed the capacity of ADSCs to have beneficial effects when transplanted/injected in different animal models of myocardial infarction/failure. In conclusion, most of ADSCs secreted factors act through mechanisms that mediate protection against cell death or, alternatively, induce cell migration and proliferation. Alternatively, they can indirectly act on the targeted cell populations: by promoting vascularization they can be indirectly linked to an increase of oxygen and nutrients in the affected areas, which may in turn promote local regenerative processes. Indeed, up to now most reports have focused on a limited set of known factors but it is expected that other molecules are responsible for the regenerative effects of ADSCs.

3.2.3. Immunomodulatory properties of ADSCs

The regenerative potential of ADSCs has been related also to their immune-modulatory abilities. ADSCs have been shown to be an immune-privileged site, preventing severe graft-versus-host response after transplantation procedures in vitro and in vivo. A concern of fundamental importance is the interplay between ADSCs and the host tissue, with particular focus on the immune system. Several studies have shown that ADSCs can be used either for autologous or allogenic cell transplants: this feature would be a major advantage for the employment of adipose tissue as a source for cell-based therapies. Furthermore ADSCs seem to act also as modulators of the immune system. The allogenic potential of these cells could be explained by the property of ADSCs to decrease the expression of hematopoietic markers and HLA-DR after subsequent passages. In addition, it has been observed that ADSCs only express HLA class I, but not HLA class II molecules: the latter can only be induced in ADSCs after incubation with IFN-γ. Furthermore, several experiments have proved that ADSCs do not stimulate lymphocyte proliferation and they do not elicit a response by Mixed Lymphocyte Reaction (MLR): in addition, they can also inhibit phyohemagglutinin (PHA)-stimulated lymphocyte proliferation. These immune-suppressive effects are likely mediated by soluble factors, among which PGE-2 seems to be the most important. Notably, the secretion of cytokines by ADSCs can be modulated not only by the inflammatory stimulus but also by the

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surface upon which they are seeded: thus the bio-scaffold/environment provided could be another mechanism to control the immune-modulatory properties of ADSCs.

Main properties of ADSCs	
Differentiation potential	*Into cells of mesodermal origin*: adipocytes, fibroblasts, myocytes, osteocytes, condrocyes *Into cells of non-mesodermal origin*: endothelial cells, neuronal-like cells, pancreatic islet cells, hepatocytes
Secretion of soluble factors (ADSCs "secretome")	Adiponectin, angiotensin, cathepsin D, penetraxin, pregnancy zone protein, retinol binding protein, CXCL12, HGF, GM-CSF, ILs 6 ,7, 8, 11, TNF-α, VEGF, BDNF, NGF, GDNF, IGF-1, TGF-β, FGF-2, PGE2,
Immunomodulatory capabilities	Allogenic cell transplant potential Lack of response by MLR, Inhibition of PHA-stimulated lymphocyte proliferation

Table 3. Synopsis of properties of ADSCs.

4. Manipulation of adipose tissue and ADSCs

4.1. Introduction

Human subcutaneous adipose tissue provides an ideal alternative source of autologous pluripotent stem cells showing several advantages compared with other sources. As a matter of fact it is ubiquitous and commonly easily obtainable in large quantity with minimal invasive harvesting procedures or methods (either liposuction aspirates or subcutaneous adipose tissue fragments), limited patient discomfort and minimal ethical considerations: it may be transplanted safely and efficaciously. The abundance of stem cells available enables the direct therapeutic adoption of primary cells without any need for culture expansion. Moreover, adipose tissue is also uniquely expandable: currently available procedures for cell isolation yield a high amount of stem cells with remarkable properties of stable proliferation and potential differentiation in vitro, being attractive candidates for clinical applications offering protocols that may provide alternative therapeutic solutions in cell-based therapies and tissue engineering to repair or regenerate damaged tissues and organs. The technologies for adipose tissue harvesting, processing, and transplantation have substantially evolved in recent years together with appropriate commercial development and with updated refinements and information regarding extraction, isolation, storage, options for cultures, growth and differentiation, cryopreservation and its effect on survival and proliferation of isolated ADSCs, also related to their adoption in tissue-engineered constructs involving biomaterials and scaffolds. Inconsistencies in literature regarding the handling of ADSCs require more extensive investigations and controls, in particular in the in vitro processing and differences between the regenerative properties of freshly-processed heterogeneous stromal vascular fraction cells and

of culture-expanded relatively homogeneous ADSCs, or the related risk of complications and possible adverse events. There is a need for stronger evidence of the safety, reproducibility and quality of the ADSCs prior to a more extensive use in clinical applications. As a matter of fact, despite the clinical use of adipose tissue grafts and ADSCs worldwide has dramatically increased, questions concerning the safety and efficacy of these treatments are still opened and currently the use of isolated ADSCs for medical indications in a clinical setting has been approved only in selected cases and few countries.

4.2. Origins and delivery of adipose tissue grafts

Adipose tissue have been used for long time for reconstructive purposes through fat grafting or autologous fat transfer, a method according to which fat from the patient is removed from one area of the body ad reinserted into the desired recipient location. [9] Fat grafting has shown to be beneficial as a reconstructive and cosmetic procedure for patients with volume losses to soft tissues due to disease, trauma, congenital defects or aging. Even so, outcomes of these techniques are often unpredictable and rates of graft reabsorption may be disappointing. As a matter of fact, fat tissue is re-vascularized at the transplantation site within 48 hours from the surgical procedure, in the meantime being fed by diffused materials from surrounding free plasma. The survival rates of the graft are dependent on size of transplanted fat particles and on surface area from which these cells could re-establish their blood supply. In order to minimize reabsorption, studies have demonstrated the efficacy of less traumatic methods of harvesting, processing and injecting. Microinjection of fat by means of the "lipostructure technique" known also as Coleman's technique has been adopted by many plastic surgeons. [10] This technique distributes fat grafts in small aliquots by meticulous injection through multiple access sites, from which the graft fans out into various subcutaneous layers. The abundance of stem cells obtainable in many common procedures, such as liposuction and liposculture, enables their direct therapeutic adoption without any need for culture expansion. [11] Even so, precursor cells can be purified by a variety of processes and enzymatic techniques may be adopted to obtain an ADSC-rich stromal vascular fraction (SVF). This issues are currently investigated as adjuvants to free fat transfer in order to increase yield of graft retention (cell-assisted lipotransfer). The ADSCs contained in the stromal vascular fraction have been applied clinically as early as 2004 for the treatment of perianal fistulas in Crohn's disease. [12] However, it is worth pointing out that, even though harvesting and first processing steps overlap, fat grafts SVF cells and ADSCs represent three different therapeutic options. Fat grafts are obtained directly after centrifugation of lipoaspirates. They contain predominantly mature adipocytes and are poor in ADSCs. The stromal vascular fraction, as mentioned above, is obtained by digestion with collagenase of the lipoaspirate sample and a subsequent centrifugation step: its cellular composition is heterogeneous, being rich in ADSCs but containing also circulating blood cells, fibroblasts, pericytes and endothelial cells. The adoption of a pure ADSC population requires plating of the SVF and expansion of the stem cell population and thus, differently from the previous two options, ADSCs cannot be harvested and implanted in a one step-procedure. Even if all these approaches exploit to some extent the regenerative potential of adipose tissue they

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are quite different procedures having also different therapeutic indications. Thus, attention has to be paid in order to avoid confusion. As for harvesting of ADSCs, several factors related to the patient, such as Body Mass Index (BMI) and age, have been analyzed for their impact on cell viability and number. Results are controversial, there is no evidence of a strong correlation of BMI with stem cells viability, number or size. Instead, there seems to be a negative correlation between age and rates of pre-adipocytes proliferation or differentiation, with higher lipolitic activity in the younger population and lower levels of apoptosis. [13] The body region of the donor site is another important variable patient-dependent. The abdomen, according to some studies, seems to be the best harvest site, while medial thigh and knee seem to have the lowest levels of viability of ASDCs. [14] These differences have not been proved in other studies. Effects of infiltration of local anesthetics during harvesting have also been investigated: lidocaine and adrenaline seem to have no effects on adipocyte viability. The method of harvest can affect not only viability of ADSCs but also their level of adhesiveness to extracellular matrix proteins. Standard liposuction allows the harvest of larger volumes of adipose tissue but it might result in up to 90% rate of adipocyte rupture. For this reason this technique is not ideal for fat grafting, while it could be more appropriate for ADSCs harvesting. An equivalent damage to pre-adipocytes has been measured comparing syringe aspiration with fat surgical excision. It is accepted that a larger cannula diameter at harvest correlates with improved cell viability. Partial purification of lipoaspirate can be carried out in the operatory room. The first step is centrifugation, which separates harvested fat into three layers: infra-natant (lowest layer composed of blood, tissue fluid and local anesthetics), middle portion (mostly composed by fatty tissue) and supra-natant (least dense upper layer including lipids). Infra-natant components can be ejected from the base of the syringe, while supra-natant can be poured off and soaked up using absorbent materials. While this technique is the most practical and today commonly used for fat grafting, it may not produce the best fraction of ADSCs possible. Several studies have been conducted on this issue, revealing that gentle centrifugation produces the highest cell viability, while long periods of centrifugation lead to isolation of the most proliferative cell type. When comparing decantation, washing and centrifugation, stem cells concentration results greater in washed lipoaspirates and pellets contained at the bottom of the centrifuged samples contain the highest concentration of stem cells.

4.3. Origins and delivery of ADSCs

Embryonic stem cells have an enormous multilineage potential but many ethical and political issues accompany their use. Therefore researchers have directed their attention on pluripotent adult stem cells. Adult stem cells were initially thought to have the differentiation capacity limited to their tissue of origin, however, as already mentioned above, many studies have now demonstrated that stem cells have the capacity to differentiate into cells of mesodermal, endodermal and ectodermal origin. MSCs from the bone marrow show extensive proliferative capacity and a multilineage differentiation potential into several lineages, including osteoblasts, chondrocytes, adipocytes and myoblasts. However, pain, morbidity and low cell numbers upon harvest represent an obstacle to their extensive clinical application. The

harvesting of adipose tissue, in comparison, is much less expensive than bone marrow. ADSCs can be isolated both from tissue samples and from lipoaspirate with less invasive procedures and are available in greater quantities (5 x 105 stem cells from 400 to 600 mg tissue). [15] ADSCs can be easily cultured and expanded, retaining their stem cell phenotypes and mesenchymal pluripotency still after several passages, features that make them an ideal source of stem cells for clinical applications.

- Isolation and culture of ADSCs

Since Rodbell's description of isolated pre-adipocytes from adipose tissue, a variety of methods have been developed. [16] Today, most laboratories use several common steps to process cells from adipose tissue. These methods include: washing, enzymatic digestion/mechanical disruption, centrifugal separation for isolation of cells which can be used directly, after cryopreservation, or after culture expansion for the generation of ADSCs. Still, despite the extensive use of ADSCs for research purposes, there is no any widely-accepted unique standard protocol for isolating and culturing these cells. For enzymatic digestion most laboratories use collagenases of different subtypes, trypsin, or a mixture of both, at various concentrations with an average incubation time of one hour, at 37°C, in constant shaking. The optimal centrifugation speed is considered to be around 1200g for 5 to 10 minutes. Some additional purification procedures can include filtration through nylon meshes and incubation with an erytrocyte-lysing buffer, usually Krebs Ringer Buffer (KRB) or NH4Cl. This procedure, however, seems to have a negative influence on the growth of ADSCs. Some investigators, after the identification of ADSCs surface immunophenotype, use immune-magnetic beads or flow cytometry to purify the stem cell population directly from the heterogeneous sample, using the CD34+ antigen. The most used culture medium are α-Modified Eagle's Medium (α-MEM), or Dulbecco's Modified Eagle's Medium (DMEM), after addition of fetal bovine/calf serum, (FBS/FCS), L-glutamine, penicillin and streptomycin.

- Cryopreservation of ADSCs

The development of simple but effective storage protocols for adult stem cells will greatly enhance their use and utility in tissue-engineering applications. [17] Cryopreservation is regarded as a promising technique and many studies have focused on this procedure. Other protocols investigated drying (anhydrobiosis) and freeze drying (lyophilization). The majority of in vitro studies agree that cryopreservation of adipocytes in liquid nitrogen, preferably using a set cooling and re-warming protocol, provides the lowest damage to cell viability. These results have been replicated in vivo (murine models) showing that grafts frozen in liquid nitrogen and stored at -35°C had a similar viability and histology compared to fresh tissue: in addition, this method obtained better results than freeze drying and immersion in glycerol. Recently, in order to increase the yield of adipose-derived stem cells post-thawing, the use of cryoprotective agents, such as dimethyl sulphoxide (DMSO) has been examined: samples frozen with DMSO achieved better outcomes than unprotected ones. Thus, cryoprotective agents are now considered as an essential part of any cryopreservation protocol aiming to provide appropriate conditions for the survival of ADSCs and adipocytes.

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4.4. Safety concerns

Inconsistencies in literature regarding the handling of ADSCs require more extensive investigations and controls. In particular, a focus should be placed on in vitro processing as well as differences between the regenerative properties of freshly-processed heterogeneous adipose cells and those of culture-expanded relatively homogeneous ADSCs. Related risks of complications and possible adverse events like fat necrosis, seromas, oncological recurrences, should be accurately considered. In addition, adiponectin is implicated in the pathogenesis of insulin-resistant states, such as obesity and diabetes type 2. In particular, several studies reported that differentiated WAT cells and WAT resident progenitors may promote cancer growth and metastasis by means of a variety of different mechanisms (endocrine, paracrine, autocrine interactions). The main cellular component of WAT are adipocytes, the large cells accumulating tryglicerides in lipid droplets. In particular, in conditions like obesity, adipocytes in WAT may eventually became under oxygenated, leading to hypoxia, increased oxidative stress, recruitment of inflammatory leukocytes and eventually fibrosis. In recent experimental models, some adipokines showed to be able to promote tumor growth along with fatty acids released by adipocytes. High levels of adiponectin have been associated with the development of endometrial carcinoma and breast cancer. Leptin has been identified in regulation of cell proliferation and neo-vascularization in malignant and normal cells of different origins, including lung, gastric, colonic, kidney, leukemic, hematopoietic and epithelial cells. Notably, these molecules can enhance proliferation and survival of malignant cells and/or of tumor vasculature. So far, studies investigating the role of WAT in cancer have predominantly focused on pro-tumorigenic effect of ADSCs. In fact the increased proliferation and survivor of malignant cells may result from the engagement of perivascular ADSCs into angiogenesis and vascular maturation, resulting in improved tumor blood perfusion. Cytokines such as adiponectin, leptin, interleukin-6, and TNF alfa seem to be responsible for a chronic low-grade inflammation. Furthermore, mesenchymal cells are known to suppress the activation of T-killer cells: this finding suggests that also ADSCs may help tumors to evade the host immune response. Thus, adipocytes may be able to produce adipokines and several secretions which could potentially induce cancer reappearance by “fueling” dormant breast cancer cells in tumor bed true “tumor-stroma interaction”: even so, up to now, especially for grafting of adipose tissue after breast cancer treatment, there is no strong clinical evidence or international agreement on this topic. [18-19] Depending on country, the safety of adipose tissue grafting is still a controversial issue. In 2009, the American Society of Plastic surgeons Fat Graft task Force concluded that no reliable studies could confirm definitely the oncologic safety of lipofilling in breast cancer patients. A more accurate point of view is provided by a large multicentric observational study on adipose tissue grafting in patients previously affected by breast cancer: considered parameters included the complication rate of the technique, the risk of modification of mammography and a rigorous long-term clinical/instrumental follow-up. [20] At the moment no studies on the effects of lipotransfer on human cancer breast cells in vivo are available. We cannot provide the definitive proof of the safety of lipofilling in terms of cancer recurrence or distant metastasis, but until then, should be performed in experienced hands, and a cautious oncologic follow-up protocol is advised.

5. Clinical use

5.1. The regenerative cells

The growing interest in this area of research has driven the adoption of adipose tissue and ADSCs in a wide number of clinical situations, medical fields and conditions for the repair and regeneration of acute and chronically damaged tissues, with an increasing number of translational efforts. Clinical trials have been advanced in order to investigate the therapeutic potential and applicability of these cells based on the induction of their properties similar to that observed in BMSCs. An extensive great knowledge concerning the harvesting, characterization and transplantation of ADSCs has been developed. Even so, current literature still lacks of strong evidence about the clinical potential of ADSCs and adipose tissue. In particular this may be due to the fact that human lipoaspirates may significantly differ in purity and molecular phenotype and that many reports have adopted heterogeneous populations of cells providing uncertain results. Remarkably, some problems still affect the correct interpretation of outcomes. One of the most significant issues limiting the interpretation of clinical progression is the lack of standardization in defining ADSCs, since both SVF and ADSCs may be used. [4] Another issue is whether ADSCs operate on tissue regeneration through direct transdifferentiation or paracrine mechanisms based on the secretion of numerous cytokines and growth factors. Thus, standardization of a method and improvement of current preclinical data may allow direct comparison of different results as well as a better definition of clinical potential of ADSCs. Current preclinical and clinical data of such cell-based therapies should include the osteogenic, chondrogenic, adipogenic, muscular, epithelial and neurogenic differentiation of progenitor, endothelial, and mesenchymal stem cells involved. Thus, skin, bone, cartilage, muscle, liver, kidney, cardiac, neural tissue, pancreas represent some of the most prominent clinical targets on which these therapies are focused. ADSCs are commonly adopted in clinical settings in surgical fields such as: cell-enriched lipotransfers, soft tissue augmentations and reconstructions of defects after trauma or oncologic surgery, healing of chronic wounds (phase 1 trials for the healing of recurrent Crohn's fistulae), skin regeneration and rejuvenation (repair of damages induced by aging or radiations), scar remodeling. In addition, they have been adopted in the treatment of cardiovascular disease, metabolic disease and encephalopathy (cerebral infarction) and a wide range of other surgical needs by orthopedic surgeons, oral and maxillofacial surgeons and cardiac surgeons. Indeed, the clinical application of adipose tissue relies on convincing results but the full therapeutic potential of ADSCs may still need further investigation.

5.1.1. The "Lipofilling technique"

Fat graft has been initially adopted to generate adipose tissue in the treatment of contour deformity or volumetric defects. The "lipofilling technique" has been used for many years and it has become rapidly popular especially in aesthetic surgery to improve cosmetic results in facial surgery. In fact it may be considered an ideal filler since it is totally biocompatible, readily available, inexpensive and it enables good aesthetic results. More variable are the application of fat injection in reconstructive surgical treatments. For example in breast reconstruction the

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indication of lipofilling include micromastia, tuberous breasts, Poland syndrome, post-lumpectomy deformity, post-mastectomy deformity, sequelae of post-radiotherapy (every anatomical region previously subjected to radiotherapy is subject to fat injection), refinement of secondary reconstructions after flap or prosthesis reconstruction and nipple reconstruction. In head and neck reconstructive surgeries it has been used to correct Treacher Collins syndrome o other cranio-synostosis. In burns, lipofilling has been adopted to improve the structural features of extracellular matrix in the treatment of burn sequaele, such as pathologic scars, with the aim to restore a more physiologic skin architecture. The lipofilling is also a valuable option to enhance volumes in facial hypotrophies, for example in patients affected by HIV-related lipo-distrophy. In addition, fat injection has proved to be very useful to improve local vascularization and trophism in chronic ulcers, especially vascular or post-traumatic ulcers.

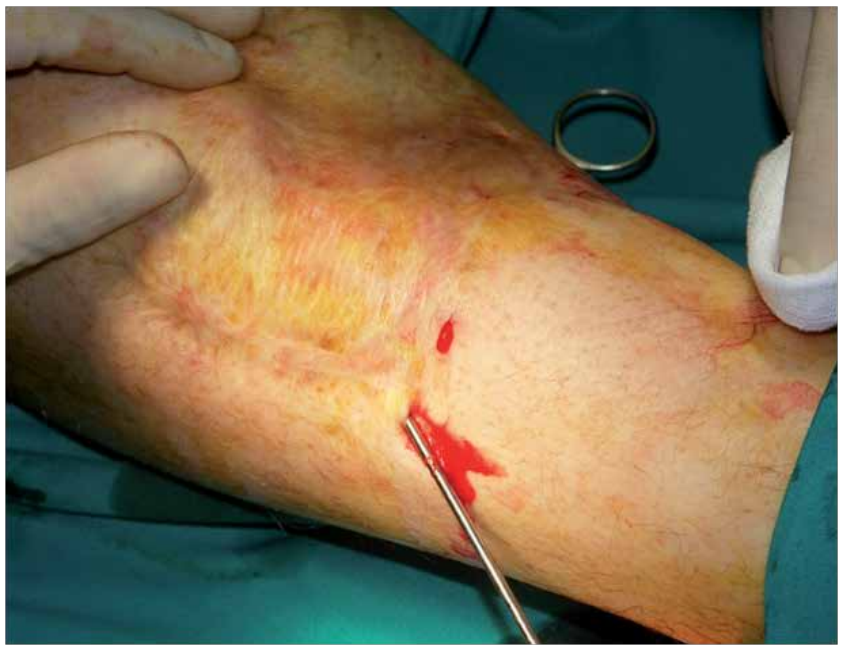

Figure 2. Injection of autologous adipose tissue ("lipofilling technique") in a scar.

5.1.2. Clinical trials with ADSCs

Most of clinical trials on humans are based on previous experiments on animal models. The evidence of the ability of ADSCs to differentiate into cells of non-mesodermal origin has been tested in some models in treatment of several diseases. The ADSC-derived hepatocytes transplanted into nude mice restored liver function and freshly isolated ADSCs could differentiated into hepatocytes after intrasplenic transplantation into nude mice in vivo, supporting their application in clinical setting. [21] However clinical trials are still mostly lacking of

promising results. [4] A recent study showed that the direct injection of ADSCs could restore blood flow in a mouse ischemic hindlimb model, as confirmed by clinical data. [22] The myogenic differentiation of ADSCs may be used in the treatment of muscular diseases such as Duchenne dystrophy and for regenerative cell therapy in heart failure. [23] Other novel potential clinical uses of ADSCs include the treatment of Alzheimer disease, of multiple sclerosis due to the anti-inflammatory effect of ADSCs, of neurogenic bladder and other neurologic disorders. A preliminary study showed that peri-urethral injection of autologous ADSCs acts positively in stress urinary after prostatectomy. Regarding current clinical applications of ADSCs, apart from a phase III trial on the treatment of Crohn's fistula, most clinical trials are in phase I. Beside the use in breast reconstruction, trials are in progress to treat acute myocardial infarction and chronic myocardial ischemia by intracoronary injection of SVF. Other trials are focused on the treatment of cirrhosis and of diabetes I or II. [4] Another trial adopted ADSCs (after purification and expansion) for the management of fistulas associated or not to Crohn's disease: results demonstrated an efficient control of inflammation and an improvement of healing process, most likely due to paracrine action that cells differentiation. Another trial investigated the restoration of volumes in hypotrophic scars after subcutaneous injection of ADSCs. Only two trials have studied the effect of ADSCs on chronic critical limb ischemia: the first adopting intra-muscular injection, the second by intravenous injection in diabetic patients. The literature regarding different clinical trials [Table 4.]demonstrates that ADSCs-based therapies are a concrete opportunity but despite these results, molecular, cellular e biological features of these cells are still uncertain and it is also unclear if regenerative therapy is related to their differentiation potential or paracrine activity: indeed, more appropriate in vivo investigations are necessary.

Pathology	Operating methods	Condition
Stress urinary after prostatectomy	peri-urethral injection of autologous ADSCs	Report of three initial cases
Crohn's fistula	injection into rectal mucosa of autologous of ADSCs with fibrin glue	Phase III
Cirrhosis	intrahepatic arterial administration of autologous SVF	Phase I
Diabetes I	intravenous injection of autologous SVF	Phase I/II
Diabetes II	autologous SVF	Phase I/II
Hypotrophic scars	subcutaneous injection of ADSCs	Phase III
Chronic critical limb ischemia	intra-muscular injection of ADSCs	Phase I
Chronic critical limb ischemia in diabetic patients	intravenous injection of ADSCs	Phase I/II
Myocardial infarction	intracoronary injection of SVF	Phase II/III
Multiple sclerosis	intravenous injection of autologous ADSCs	Phase I/II
Reumathoid arthritis	intrarticular injection of autologous ADSCs	Phase III

Table 4. Clinical trials using adipose-derived stem cells (ADSCs) or stromal vascular fraction (SVF).

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6. Tissue engineering

6.1. Adipose derived bio-products

In the past decade, preclinical and translational efforts have established the future basis for the application of ADSCs from the bench to the bedside. Significantly, ADSCs have been widely used in tissue engineering, organ repair and gene therapy. These multipotent cells, have shown a remarkable plasticity and the ability to differentiate towards different cell lineages with similar yet enhanced properties (their multipotency and proliferative efficiency) in comparison to bone marrow-derived mesenchymal stem cells. [3,6-7,21-24,26] Moreover, ADSCs also show adjuvant angiogenic properties likely related to the secretion of vascular endothelial growth factor. [21] In vitro studies have rapidly increased during the last decade, resembling the need to optimize the variables of the differentiation process cells towards the desired lineage. The efficient use of biomaterials, delivery vehicles and bioreactors has promoted the development of a large variety of novel tissue engineered products for repair and regeneration of various tissues and organs. The use of suitable animal models in an extensive preclinical literature has also established the basis for successful stem cell-based therapies that may implement current therapeutic solutions for several diseases. Thus, a focus of most interest for the scientific community is posed today in the production of safe and reliable cell delivery vehicles/scaffolds useful in applying ADSCs as a therapy as well as in the development of novel suitable in vivo animal models. A large variety of bioengineered products have been developed by means of selected differentiating cultures of ADSCs. [Table 5] Preclinical studies have experimentally reported the adoption of ADSCs in order to develop cells of mesodermal origin as well as cells of non-mesodermal lineage such as neural o neural-like cells for repair of neural traumatic injuries, fibroblast for reconstruction of soft tissue defects, tenocytes or regenerated tendon constructs for optimal musculoskeletal system reconstruction, osteoblasts for bone tissue replacement, chondrogenic lineages and cartilage substitutes for implantation, skeletal muscle cells and subsequent myotube-like formation depicting myogenic differentiation in vivo in muscular dystrophy model. Other reported lineages and engineered tissues that may be obtain through selective differentiation include hepatocytes, pancreatic endocrine cells, cardiomyocytes and vascular endothelial cells. [24] Most relevant transcription factors involved in differentiation into adipocytes, chondrocytes, myocytes and osteocytes are well-known. However, in addition to specific differentiation factors, tridimensional biomaterials are essential to address differentiation of ADSCs to the required cell type and to use them for tissue-engineering purposes. Among investigated effective scaffolds and matrices we may include: type I collagen, hyaluronic, poly lactic-co-glycolic acid (PLGA) and silk fibroin-chitosan. [26] Moreover, the combination with specific growth factors determines the overall outcome of the applied biopolymer.

Tissue	Cell type	Gene	Scaffold	Result
Bone	human ADSCs	BMP-2	-	heal critical sized femoral defects in a nude mouse model
	ADSCs	BMP-2	collagen sponge	increase bone induction in SCID mice
	Autologous SVF	-	bone graft	treat calvarial defects in human
	Autologous ADSCs	-	β-tricalcium phosphate-filled titanium scaffold	create neo-maxilla in human
Cartilage	ADSCs	-	polyglycolic acid scaffolds	exhibit in vitro chondrogenic characteristics
	ADSCs	-	-	improve outcome measures in osteoarthritis in dogs
Endothelia	ADSCs	-	porous polycaprolactone (PCL) scaffold	endothelial differentiation
Tendon	ADSCs	-	decellularized human tendon	recellularize
Nerve	ADSCs	-	hyaluronan membrane and fibrin meshes	differentiate in glial-like and neuronal-like cells

Table 5. Synopsis of current approaches in ADSCs and tissue engineering.

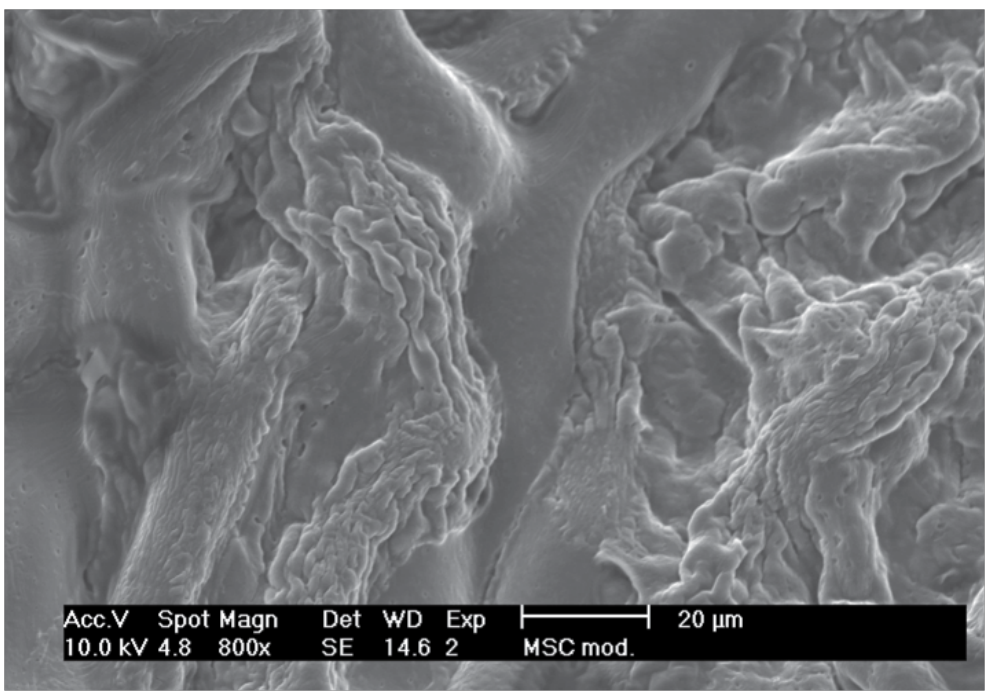

Figure 3. Electron microscopy scanning of ADSCs cultured on a Hyaluronic acid-based biomaterial.

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6.1.1. Bio-engineered bone

There is still a clinical need to generate bone for the repair of large osseous defects, since current strategies are based on non-vascularized bone grafts, suitable only for small defects. As an alternative, progenitor cells might be implanted on biomaterials and differentiated in vivo supporting reconstruction of large bone losses. Osteo-inductive factors include vitamin D3, β-glicerophosphate, acid ascorbic and Bone Morphogenic Proteins (BMPs). [7] Treating ADSCs with recombinant BMP-2 has shown to stimulate osteogenic differentiation: [27] human ADSCs overexpressing BMP-2 could heal critical sized femoral defects in a nude mouse model. Similarly, ADSCs exposed to BMP-2 adenoviral transfection and seeded in collagen sponges increased bone induction in SCID mice. [27-28] These results suggest that transfected stem cells can replace the exogenous addition of growth factors when transplanted in a bio-engineered scaffold. The use of scaffolds is critical in repair of structural tissues such as bone. Demineralized bone matrix, collagen, PLGA, hydroxyapatite and β-tricalcium phosphate scaffolds were reported to be suitable for ADSC-derived osteochondral tissue engineering. Most of clinical trials of osteogenesis in ADSCs rely on murine studies and human trials are based on very limited reports. The first human case involved transplantation of SVF together with bone graft to treat calvarial defects [29] and in another case a neo-maxilla has been created using a β-tricalcium phosphate-filled titanium scaffold associated to cultured ADSCs. [30] Thus, ADSCs-based osteogenesis is possible, however, more adequate evidence is needed in the clinical setting.

6.1.2. Bio-engineered cartilage

ADSCs might be used to generate cartilage for clinical use in the treatment of degenerative joints. The list of potentially useful growth factors for cartilage repair comprises TGFβ, IGF-1, FGFs, EGF and BMPs, transcription factors as SOX9 and signal transduction molecules such as SMADs. Several in vitro studies have shown the chondrogenic differentiation of ADSCs and this feature is confirmed by their ability to generate cartilage in a variety of experimental models. ADSCs seeded into polyglycolic acid (PGA) scaffolds exhibited in vitro chondrogenic characteristics and they could synthesized cartilage extracellular matrix. [23] The great potential of ADSCs in cartilage tissue engineering was also demonstrated in different studies in vivo. Moreover ADSCs have been used recently for treatment of osteoarthritis in dogs [32] and rheumatoid arthritis in human. [33] However, given the lack of evidence, it seems likely that the symptomatic benefits seen in these trials may relate to the anti-inflammatory properties of ADSCs rather than to a real chondrogenic differentiation.

6.1.3. ADSCs and vascular/endothelial tissue engineering

The vascularization of regenerated tissues is an important field of research since it allow the survival of tissue and the differentiated cells. [24] It has been reported that human ADSCs have the potential for endothelial differentiation and they can participate in blood vessel formation by means of the secretion of several pro-angiogenic factors, like vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). [23] This feature makes these cells suitable for regenerative cell therapy, treatment of ischemic disorders and construction of

vascularized grafts in one-step procedure, as it has already been performed in many experiments on animal models. [22] Furthermore, as reminded, the angiogenetic properties of ADSCs have been already investigated in several clinical trials to treat various diseases.

6.1.4. Bio-engineered tendon

Tendon tissue engineering is relatively unexplored due to the difficulty to maintain in vitro preservation of tenocyte phenotype: only recently research has demonstrated the fundamental role of in vitro mechanical stimuli in maintaining the phenotype of tendinous tissues. [34] The main growth factors inducing tendon differentiation include fibroblast growth factor (FGF), platelet-derived growth factor-BB (PDGF-BB), epidermal growth factor (EGF), insulin-like growth factor (IGF)-1 and members of the transforming growth factor-β (TGF-β)/bone morphogenetic proteins (BMPs) family. Several in vivo and in vitro studies have showed the ability of ADSCs to differentiate in tenocytes under specific stimuli and under biomechanical force. [34] Furthermore, recent experiments have focused on the possibility of re-cellularize by means of seeded ADSCs a decellularized human tendon. [35] Thus, an integration of ADSCs, growth factors, mechanical stimuli and biopolymers may provide a solution for the treatment of difficult tendon injuries

6.1.5. ADSCs and neuronal tissue-engineering

Incubation of ADSCs under neuro-inductive conditions (culture medium containing EGF, FGF, NGF and BDNF) has shown the potential to form neurospheres expressing neurospecific markers, including nestin¸ βIII tubulin, S100 and glial fibrillar acidic protein (GFAP). [36] Moreover, seeding of these neurospheres in different scaffolds (hyaluronan based membranes and fibrin glue meshes) demonstrated further differentiation in glial-like and neuronal-like cells. [37] Although these are only preliminary researches, these promising results are of significant clinical interest. ADSCs-induced neural cells may provide beneficial therapeutic effects in treatment of injuries occurring to both the peripheral and central nervous systems such as in the treatment of neurodegenerative states, including Parkinson's disease, Hungtinton's disease, multiple sclerosis and Alzheimer's disease.

7. Prospectives

Regenerative medicine is an evolving field of research and therapeutics in which adipose tissue and ADSCs hold great promise for translational research and future clinical applications in many fields of tissue regeneration with a wide range of potential clinical implications. In the past decade, preclinical data from in vitro studies and pre-clinical animal models has been provided on the reproducibility, safety and efficacy of ADSCs in tissue regeneration or tissue engineering, supporting their use in clinical applications and establishing the basis for a translational application in the bedside: consistently, recent preliminary clinical trials have confirmed positive outcomes. The enhancing effect of ADSCs on autologous repair might enable better clinical outcomes and play a relevant role in healing acute and chronic tissue

damage. Thus, more accurate information regarding optimal management and methods to promote differentiation lineages (among which differentiation factors, cell scaffolds, cell culture conditions) are strongly required. Further translational research, adequate clinical investigation and novel strategies should be promoted and designed to overcome current limitations, encourage future therapeutic implementation and face challenges posed by regenerative medicine.

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Chapter 9

Regulatory Issues in the Therapeutic Use of Stem Cells

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Stem cell tourism

Advances in stem cell research and media publicity of stem cell potential have raised the hopes of patients with severe disabilities and conditions which lack a cure. While stem-cell-based therapies are the clinical standard of care for a few conditions, such as leukemia and more recently for some burns and corneal disorders, stem cell tourism continues to rise worldwide.

Unfortunately, clinics around the world are exploiting patients' hopes by offering supposed stem cell therapies, without credible scientific rationale, oversight or patient protections. Occurring particularly in Asia and South America, treatments which are illegal in most counties are being offered for what are often considered incurable conditions, such as brain tumors, congestive heart failure or chronic obstructive pulmonary disease. In addition, countless other conditions are listed as candidates by these clinics including eye disease or orthopedic injuries or disease. In response to this, the International Society for Stem Cell Research (ISSCR) released "The *Guidelines for the Clinical Translation of Stem Cells"* which called for rigorous standards in the development of stem cell therapies and outlining what needs to be accomplished to move stem cells from promising research to proven treatments[1]. The goal of ISSCR in shining this light on the dangers of stem cell tourism is to ensure that the promise of stem cell research is delivered to patients in a safe, effective and fair manner. A number of professional organizations have also published guidance documents for the responsible conduct in translational stem cell research.

The general public receives information regarding stem cell potential from mainstream media and does not fully understand the risks associated with unproven treatments. In the

most desperate situations, patients may see no other options, or may view the years of continuing research as an obstacle to their potential cure. Yet, untested treatments can be dangerous and years of preclinical and clinical research are required to determine which novel stem-cell based therapies are effective and safe. In one example, brain tumors were discovered in a 9-year old boy who travelled to Russia to receive stem cell treatments to his brain; later it was found that the tumors were the result of cells from at least 2 different donors [2]. Even carefully planned and approved studies can go wrong and have unfortunate results, as in the fatal gene therapy case of Jesse Gelsinger, who received experimental therapy at University of Pennsylvania[3, 4].

Lau et al reported on the clinics around the world that are exploiting patients' hopes by professing to have effective stem cell therapies for seriously ill patients. These therapies often carry a hefty pricetag. However, they occur in counties which have limited oversight and allow treatment to occur in the absence of credible scientific rationale, transparency, oversight, or patient protections [5].

Comprehensive government regulations exist in the US, and several other countries. Below, we describe the U.S. and other government regulations associated with the use of human stem cell and tissues in regenerative medicine.

2. Cell Products must follow FDA regulatory guidelines

2.1. FDA's risk-based approach

To protect the public from risks associated with cell therapies and demonstrate the effectiveness of treatments, the U.S. FDA and other professional societies such as the ISSCR, and the United States Pharmacopia (USP), have established guidelines for therapies using human cellular and tissue-based products (HCT/Ps). The FDA has statutory authority to prevent the spread of communicable diseases granted under Section 361 of the Public Health Service Act (PHS Act, 42 U.S.C. § 264). HCT/Ps are regulated through a risk-based approach outlined predominantly in 21 C.F.R. Part 1271. Some HCT/Ps are regulated solely under Part 1271 while other HCT/Ps are regulated under both Part 1271 and FDA's Federal Food, Drug, and Cosmetic Act (FDCA, premarket and post- market regulation of medical devices and drugs), & section 351 of the PHS Act for biological products. FDA's regulation focuses on three general areas: 1) limiting the risk of transmission of communicable disease from donors to recipients; 2) establishing manufacturing practices that minimize the risk of contamination; 3) requiring an appropriate demonstration of safety and effectiveness for cells and tissues that present greater risks due to their processing or their use [6, 7].

Stem cell therapies show excellent promise for many types of treatments. However, scientific, manufacturing and safety challenges exist. Once the optimal stem cell type is identified for a given treatment (Table 1), there is a requirement to demonstrate the product's safety and efficacy in a clinical setting. Cell therapies must overcome several challenges before they can be considered safe for human use. First, most cell therapies will

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require large numbers of cells. Large cell doses are obtained by increasing cell harvest yields and by increasing ex vivo expansion yields. As cell cultures are expanded over long time periods, they show signs of aging that may be similar to human aging [8, 9]. Lengthy expansion periods can result in ineffective cellular products[10]. Cells may also be manipulated in other manufacturing steps that include cell-selection processes, genetic modifications, or encapsulation with another biological device. Cells that undergo ex vivo manipulation may lose potency, or acquire infectious contaminants, or become transformed / tumorogenic due to the cell culture conditions [11, 12]. Finally, the cells themselves may pose a risk, simply due to the novelty of the therapy and unknowns associated with their behavior in the body.

	Embryonic Stem Cells	***Adult iPS***	***Adult BM-MSCs***	***Adult Adipose MSC***
Ethical concerns	*[56-60]*	*[61-67]*		
Tumorogenic	*[68-72]*	*[59, 73-75]*		*[76-79]*
Scale-Up challenge	*[80-83]*		*[84-86]*	*[87]*
Genetically unstable	*[88, 89]*	*[34, 39, 90-94]*		*[95]*
Immunogenic difficulties	*[96, 97]*	[37, 98]		

Table 1. Scientific and Manufacturing Challenges in Stem Cell Sourcing *(numbers refer to literature references)* Several stem cell types are studied for their potential use in regenerative medicine, including, but not limited to, embryonic stem cells [20-27], inducible pluripotent stem cells [28-41], bone-marrow stem cells [42-46] and adipose-derived stem cells [47-55]. However, there are challenges with all stem cell types. A major concern with clinical application of iPSCs is their tendency to form tumors and cause cancer. Both ESC and iPSCs form teratoma in vivo, a major obstacle to stem-cell based regenerative medicine by the FDA. Also they are ethically controversial since they require genetic engineering using oncogenes. More recently, proteins have been used to generate piPSCs but the conversion efficiency us quite low. Adult derived BM-MSCs or adipose MSC are limited by their expandability.

In 1993, the US FDA began establishing regulatory and guidance documentation for cell therapies with the issuance of Application of Current Statutory Authority to Human Somatic Cell-therapy and Gene-therapy Products [13] which provided a biologics regulatory framework for the use of HCT/Ps. Table 2 provides a list of other key regulatory and guidance documents. The tiered risk-based approach means that products which present a lower perceived risk will be less regulated, while products with a larger perceived risk will undergo more extensive controls and examination. Both will require the cell products to be manufactured following Good Manufacturing Practices (GMP), and Good Tissue Practices (GTP). Additional regulatory requirements will depend on whether the cell product is minimally manipulated or more-than-minimally manipulated.

Guidance for Industry: Guidance for human somatic cell therapy and gene therapy		**1998**
GMPs	GMP	2001
Suitability determination for donors of HCT/Ps; proposed rule	GTP	1999
Current good tissue practice for manufacturers of HCT/Ps; inspection and enforcement; proposed rule		2001
Human cells, tissues and cellular and tissue-based products (HCT/Ps); establishment registration and listing; final rule		2001
Good clinical practice, GCP, ICH E6	GCP	1996
Validation of procedures for processing of human tissues intended for transplantation: final guidance		2002
Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products		2007
Guidance for Industry Potency Tests for Cellular and Gene Therapy Products		2011

Table 2. Key US FDA Regulatory and Guidance Documents. Over the past 15 years, the FDA has provided several guidance documents for HCT/Ps. A few products such as Genezyme's *Carticel* received approval prior to the issuance of these documents and has been grandfathered in. Many of these guidance documents are issued by CBER, the center within FDA that regulates biological products for human use following applicable federal laws, including the Public Health Service Act and the Federal Food, Drug and Cosmetic Act.

Minimal manipulation is defined by the FDA for cells or nonstructural tissue as processing that does not alter relevant biological characteristics of cells or tissues. HCT/Ps that meet 1271 criteria for regulation solely under section 361 of the PHS Act and the regulations in Part 1271 are called "361 HCT/Ps", and are not subject to any premarket review requirements. The Center for Biologics Evaluation and Research (CBER) has jurisdiction over 361 HCT/Ps.

According to 21 CFR 1271.10, minimal manipulation criteria include:

1. The HCT/P is minimally manipulated;

2. The HCT/P is intended for homologous use only, as reflected by the labeling, advertising, or other indications of the manufacturer's objective intent;

3. The manufacture of the HCT/P does not involve the combination of the cell or tissues with another article, except for water, crystalloids, or a sterilizing, preserving, or storage agent, provided that the addition of water, crystalloids, or the sterilizing, preserving, or storage agent does not raise new clinical safety concerns with respect to the HCT/P; and

4. Either:

i. The HCT/P does not have a systemic effect and is not dependent upon the metabolic activity of living cells for its primary function; or

ii. The HCT/P has a systemic effect or is dependent upon the metabolic activity of living cells for its primary function, and:

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a. is for autologous use;

b. is for allogeneic use in a first-degree or second-degree blood relative; or

c. is for reproductive use.

For cells, minimal manipulation means processing that does not alter the relevant biological characteristics of cells or tissues. Examples of products regulated as 361 HCT/Ps include bone marrow or blood transplants and organ transplants.

HCT/Ps that do not meet one or more of the four major criteria, are considered more-than-minimally- manipulated HCT/P. FDA has stated that density-gradient separation, cell selection, centrifugation, and cryopreservation constitute minimal manipulation. All processes that manipulate the cell / tissue product such as cell activation, encapsulation, ex vivo expansion, and gene modifications are considered more-than-minimal manipulations. Most advanced cellular therapies meet criteria for the more-than minimally manipulated category [14]. Finally, it is possible to request an informal jurisdictional determination on the level of manipulation from the Tissue Reference Group (TRG), or submit a formal Request for Designation (RFD) from the Office of Combination Products (OCP). Figure 1 is a schematic of regulatory pathway assessment to determine which guidelines apply to a given HCT/Ps product.

2.2. Manufacturing of HCT/Ps requires GTPs

For HCT/Ps that do not meet the criteria established in Section 1271.10, FDA premarket review is required; this includes obtaining FDA license, approval, or clearance.

All steps in the manufacturing of HCT/Ps will require compliance with Current Good Tissue Practice (cGTPs). cGTPs cover manufacturing facilities and processes. The manufacturing process can be broadly described as 1) procurement of HCT/Ps (donor screening and testing, product recovery), 2) processing of HCT/Ps (tissue or cell recovery /isolation, product handling, product labeling), 3) storage (e.g. cryopreservation), and 4) distribution. Many of these steps are common to GTPs and GMPs with the goal of safe and effective products via well-controlled processes and thorough supporting documentation. Requirements for standard operating procedures (SOPs), labeling controls, and storage requirements also exist.

2.2.1. Procurement

Therapies with HCT/Ps will require a determination of donor eligibility. For the FDA, donor eligibility is determined based on donor screening and testing for relevant communicable disease agents and diseases, and is required for all donors of cells or tissue used in HCT/Ps, with some exceptions listed in C.F.R. Part 1271.90.

As part of clinical or industry compliance with donor testing requirements, procedures to process, store, label, and package cell products also are needed. Hospitals and companies involved in cell/tissue therapeutics manufacturing must establish quality programs which consist of a comprehensive system for manufacturing and tracking HCT/Ps. The quality

program must follow CGTP requirements, and be designed to prevent, detect, and correct deficiencies that may lead to circumstances that increase the risk of introduction, transmission, or spread of communicable diseases.[6, 7]

2.2.2. Processing

The implementation of a Quality Assurance (QA) program includes principles of good manufacturing practice (GMP) and a quality control (QC) system. A QC system is required to ensure safety and efficacy of cell applications. GMP regulations apply to all phases of cell/tissue collection, processing and expansion, and storage. GMP quality practices are required for HCT/Ps to be used for clinical procedures and INDs. A compliant quality program for record and process control is a critical part of a QC system.

A compliant material control program is essential for FDA licensure. During review of new license applications, clinics and companies are asked to provide detailed descriptions of the manufacturing process and documentation of source country for all materials of animal origin. Additionally, for FDA-regulated products intended for administration to humans, companies must minimize any chance that BSE could be introduced into products during the manufacturing process and ensure that all materials are used as intended in the processing and are contamination free. Subsequently, a program for control of materials used in the process is necessary to meet FDA compliance and product safety.

2.2.3. Storage

If the HCT/P product involves cryopreservation, then compliance requires that the process includes an understanding of the shelf-life and how the freezing & storage process affects the HCT/Ps to complete the quality testing program. Banked cells should be stored under conditions shown to be suitable for long-term stability. Cell/tissue stability under the freezing and storage conditions should be validated using cell recovery or viability data. It is expected that establishment of a stability program for a banking process will lead to the development of quality products over a long term storage period and provide confidence that they will be effective in clinical applications.

2.2.4. Distribution testing

For the lot release of patient's cells/tissues for clinical use, standards for in-process and final product quality must be established. Specifically for FDA licensure, companies must submit their facility controls, process controls, and product standards designed with scientific principles to ensure the safety and effectiveness of all HCT/Ps products. This again is based on SOPs and controls for adherence to the cGTP, Current Good Manufacturing Practice (cGMP) and 21 CFR 1271 requirements. Product lot release specifications ensure that all products are produced in a safe and consistent manner and should be effective in clinical applications. In order to meet HCT/Ps regulations, product lot release specification should include testing for cell phenotype to confirm purity, potency, and identity.

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The application for licensure requires that companies demonstrate that the HCT/P product standards and procedures are based on good science, and thorough and extensive data. A comprehensive product characterization program is needed to understand the products and how they may be clinically beneficial. During the application process, the FDA may request that the hospital or company applicant expand on a concept or further explain the rationale/ approach or provide additional data. FDA premarket review and licensing is considered a lengthy and arduous process, however new products applicants may benefit by the recent approvals of several cell-based products (Table 3).

Product **(Company)**	**Condition**	**Cell Type**	**Approval**
Carticel **(Genzyme BioSurgey)**	Articular cartilage damage in the knee	Autologous chondrocytes (adult/ differentiated)	US FDA approval 1997 (grandfathered in)
*Apligraf***(Organogenisis)**	Diabetic foot ulcers and venous leg ulcers	Neonatal foreskin allogeneic keratinocytes and fibroblasts in bovine collagen scaffold	US FDA approval 1998
Provenge **(Dendreon)**	Asypmptomatic or hormone refractory prostate cancer	Autologous dendritic cells (adult/ differentiated)	US FDA approval 2010
Gintuit **(Organogenesis)**	Asypmptomatic or hormone refractory prostate cancer	Autologous dendritic cells (adult/ differentiated)	US FDA approval 2010
La Viv **(Fibrocell Science inc)**	Moderate to severe nasolabial fold wrinkles	Autologous fibroblasts (adult/ differentiated)	US FDA approval 2012
ChondroCelect® (**TiGenix**)	Single symptomatic cartilage defects in the knee	Autologous chondrocytes (adult/ differentiated)	EMEA approval 2009
Prochymal (**Osiris**)	Graft vs. host disease in children who are refractory to steroid therapy post-BMT	Allogeneic mesenchymal stem cells from donor bone marrow	Health Canada/New Zealand grant conditional approval 2012
Hearti-cellgram-AMI (**FCB-Pharmicell**)	Heart repair post-myocardial infarction	Autologous bone marrow-derived mesenchymal stem cells	Korean approval 2011
Cartistem **(Medipost)**	Traumatic and degenerative osteoarthritis	Allogeneic mesenchymal stem cells from donor umbilical cord blood	Korean approval 2012
Cupistem **(Anterogen)**	Anal fistula in Crohn's Disease	Autologous fat-derived 'stem cells'	Korean approval 2012

Table 3. Approved Cell Therapy Products by the U.S. FDA and non-3rd World Countries. Several cell products have received US approval[99] and are in current use for a number of patients. Most US approved products are for autologous use, only Apilgrafs foreskin cells are used allogeneically. Osiris recently received conditional approval for allogeneic use of mesenchymal stem cells in pediatric graft-vs-host disease.

3. Non-U.S. regulatory systems

The European Union, Australia and Canada and other countries have established similar regulatory systems for the use of post-natal human HCT/Ps.

The European Medicines Agency (EMEA) is the regulating body with authorization and supervision of cell therapy products and other "advanced therapy medicinal products" [15]. As of January 2011, the EMA's Committee for Advanced Therapies (CAT) recognized the potential of stem cell therapies and released a reflection paper to work in conjunction with the *Guideline on Human Cell-based Medicinal Products* (EMEA/CHMP/410869/2006) for the Marketing Authorization Application (MA). Both the reflection paper and the guidance detail the quality and manufacturing, non-clinical, and clinical aspects required for MA approval. The quality and manufacturing considerations include starting and raw materials, manufacturing process, quality control, validation of the manufacturing process, development pharmaceutics, traceability and biovigilance, and comparability. Pharmacology and toxicology are the non-clinical development aspects to be considered. From a clinical development standpoint, general aspects, pharmacodynamics, pharmacokinetics, dose finding studies, clinical efficacy, clinical safety,pharmacovigilance, and risk management plans are necessary for approval.

In Australia, HCT/Ps or products (biologicals) are regulated by the Therapeutic Goods Administration (TGA) which is the Australian equivalent to the FDA. Similar to the FDA approach, the TGA's regulatory framework for biological imposes varying levels of regulation on the therapy or product depending on risk, extent of manipulation, and whether the intended use of the biological is its *usual biological function[16].* In order to gain approval a treatment that used a biological, and the biologicals intended use was not its normal function, a hospital or company would be required to submit substantial evidence that the particular therapy or product is safe, effective and of high quality.

In order for a stem cell therapy to be approved by Health Canada it must meet the regulations as stated in the Safety of Human Cells, Tissues and Organs for Transplantation Regulations (CTO Regulations[17]). The CTO Regulations detail requirements to ensure safety in processing; storage; record keeping; distribution; importation; error, accident and adverse reaction investigation and reporting. Requirements for donor screening, testing, and suitability assessment are described in the processing regulations as well as the testing and measurements performed on the products after retrieval or in preparation for use, preservation, or packaging[17].

Health Canada, the FDA equivalent in Canada, is the first approving body in the world to approve a manufactured stem cell based drug intended to treat a systemic disease -acute Graft versus Host Disease (aGvHD) [18]. Osiris Therapeutics of Columbia, Maryland developed Prochymal [remestemcel-L, adult human mesenchymal stem cells (hMSCs) for intravenous infusion], a liquid cell suspension of ex vivo cultured adult MSCs derived from the bone marrow of healthy adult donors. Prochymal is the first stem cell therapy approved for clinical use in patients, specifically pediatric patients. Health Canada required Osiris to continue a Risk Management Plan to demonstrate that the benefits of Prochymal continue to outweigh

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risk, the addition of post-market studies, and maintenance of a treated patient registry for approval[19].

Table 3 provides a list of cell therapy products that have received U.S. FDA approval or other government approval. Despite extensive stem cell research over the past 15 years, most cell products are not stem cell derived. Only Osiris' BM-MSC product and 3 Korean products are stem cell based products.

4. Conclusions

This report examines the different processes involved in HCT/Ps manufacturing and highlights the guidelines that must be followed to obtain FDA or other country specific regulatory approval. Ex vivo expansion, cell selection or gene modification will likely be necessary for most advanced cell and tissue therapies. These modifications increase the risk associated with the treatment and render the product to be regulated under a higher risk category of more-than-minimally-manipulated product. Key to biomanufacturing is the implementation of a QA/QC program including a quality control system and GMP principles which apply to all phases of manufacturing.

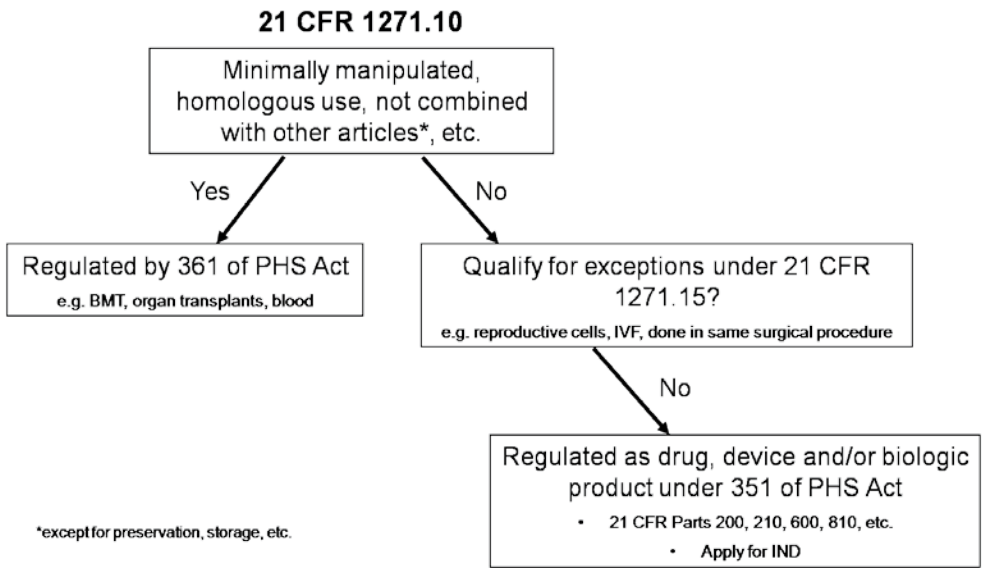

Figure 1. Regulatory Pathway Assessment If an HCT/Ps product is minimally manipulated it is regulated as a "361 HCT/Ps", and it is not subject to any premarket review requirements. However, if the HCT/Ps is more-than-minimally manipulated, and does not qualify for exemptions under 21 CFR 1271.15, it will be regulated as drug, device and/or biologic product under 351 of the PHS Act.

Many counties actively regulate the use of stem cell products, however, there are still a number of areas around the world that have little regulations and unregulated treatments

pose risk to patients and the careful development of the field. The current challenge to deliver safe cell and tissue therapies and curb unregulated treatments may soon apply to gene therapy and other innovative technologies. Early government regulation and active education by a number of professional organizations should reduce the spread of medical tourism and aid in the development of safe and effective treatments in the field of regenerative medicine.

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Section 2

Scaffolds and Matrices

Chapter 10

The Evolution of Three-Dimensional Cell Cultures Towards Unimpeded Regenerative Medicine and Tissue Engineering

Aleksandar Evangelatov and Roumen Pankov

Additional information is available at the end of the chapter

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1. Introduction

The idea that cellular survival and growth could be maintained outside the body was recognized as possible almost hundred years ago when the German zoologist Wilhelm Roux described a successful experiment where he cultured chick neural crest in warm saline for a few days [1]. Nobel Prize winner Alexis Carrel performed numerous experiments clearly showing that tissue explants, including connective tissue and heart tissue, could be cultured in vitro preserving their characteristics for prolonged periods of time [2] supporting the notion that entire organs could be cultured in vitro. A defined synthetic mixture of amino acids, salts, carbohydrates, vitamins and serum was shown to support cells in vitro[3], thus unifying a major variable in cell culturing experiments and providing a possibility for rapid development of this novel method. Since the establishment of the first cell line by Gey et al.[4] in 1951 cell culturing has become one of the most widely used methods with exceptional contribution to the advances in almost all fields of contemporary biology – cell biology, genetics, cell biochemistry, physiology etc. Significant progress in the field made possible numerous achievements that were believed to be the foundations of personalized medicine. Among these is the isolation of the first line of murine stem cells [5, 6] in 1981, followed by establishment of the first human embryonic stem cell lines by Thompson [7].

Current knowledge of cellular behavior is mainly acquired by studies concerning homogenous populations of cells cultured as monolayers. This simplified approach towards understanding the essence of the mechanisms, underlying the processes determining life and death of a cell has undoubtedly provided scientists with enormous amount of knowledge. However, recent advances in the field of three-dimensional cell cultures have revealed a lot of imperfections in

this limiting approach. Growing fibroblasts on flat, rigid surface of the Petri dish or flask results in major differences in adhesion formation and maturation, proliferation, cell signaling, migration and cytoskeletal function, compared to the three-dimensional environment [8-12]. Even changes in stiffness of the two-dimensional (2D) substrate have shown significant difference in fibroblasts response to the environment [13, 14]. Being cultured on 2D surfaces, these non-polar cells are forced to adapt to polar setting, thus significantly changing their response to the environment. One could argue that this culturing method would be ideal for epithelial cells, allowing them to form distinct apical and basal layers. Nevertheless growth in 3D environment allows normal polarization and differentiation of epithelial cells [15] and is a prerequisite for the formation of duct-like structures in vitro [16]. Thereby the emerging differences between conventional, monolayer cell cultures and in vivo cellular behavior led to the increasing number of scientists aiming to provide in vivo-relevant results.

The idea of cell culturing in 3D environment is not a novel one though [17], but until recently the importance of the cell environment – its dimensionality, stiffness, elasticity, composition and remodeling during tissue morphogenesis and disease, remained neglected. Number of studies have shown that knockdown of extracellular matrix (ECM) components like fibronectin, collagen, laminin, aggrecan, etc. lead to lethal phenotypes or severe pathologies during embryonic or post-natal development [18-24]. Furthermore, recent discoveries like the importance of Cdc42 for the appropriate acquisition of apical and basal polarity during morphogenesis or cell specification during tubulogenesis wouldn't have been possible without the use of 3D model systems [25, 26]. Thus the concept of the importance of the surrounding settings emerged and an entirely new scientific direction evolved, focusing on the role of the extracellular matrix and its importance in cell biology.

In the living organism cells are usually embedded in a complex three-dimensional extracellular matrix that is dynamic in its structure[27] and rarely do have the opportunity to attach to planar, rigid substrates. Reciprocal interactions between cells and the ECM facilitate signaling to and from cells and lead to continuous reorganization of the environment [10]. Investigation of the dynamics of the ECM, its structure in different tissues and cellular response to changes in the mechanical properties of the extracellular matrices have shown that cells are not only able to feel and respond to the environment, but also to cause changes to the environment's mechanical properties [14]. These interactions deliver further signals responsible for cell growth and differentiation, survival, migration and reorganization of the resident tissue [28]. In addition when one thinks of cells in the third dimension it has to be considered that cells are not just randomly incorporated in the ECM but form complex 3D structures, characteristic of the specific tissue or organ [29].

The recognition of the importance of dimensionality of cellular environment encouraged the creation of a variety of three-dimensional culturing systems. Essentially they could be divided into two groups – three-dimensional systems made of artificial materials, mimicking the natural components of the matrix and three-dimensional culturing systems based on natural ECM components, or cell-synthesized ECM. This article's focus will be mainly on the later 3D culturing models.

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2. Artificially fabricated 3D scaffolds

Design of artificial 3D environments for cells aims at creating an environment that would mimic the physical properties of the natural extracellular matrix along with the signaling cues it provides for cell development. Such scaffolds should be non-toxic, preferably biodegradable in time and must allow cell attachment and migration as well as diffusion of vital nutrients [30]. Cells should be able to proliferate and differentiate, and subsequently to synthesize and organize their own ECM. In time, eventually, the naturally synthesized and organized extracellular matrix should substitute the artificial scaffold. All those prerequisites mean that the surface should be suitable for cell attachment, have the adequate chemical and mechanical properties to support adhesion, proliferation and differentiation. To our knowledge there are currently several reported methods for fabricating appropriate artificial scaffolds:

- The technique of controlled rate freezing and lypohilization [31] produces suitable extracellular matrices, in spite of the relatively uneven pore sizes. Another drawback of this method is that the produced artificial matrix does not have fibrillar structure which is common for the natural ECM.
- Phase separation of nanofibers [32] yields fibrous matrices, but also produces pores with uneven sizes.
- Formation of porous scaffolds [33]. This method allows the formation of matrices with similar pore sizes, but is limited by the matrix thickness
- Computer-assisted design and manufacturing (CAD/CAM) technologies [34] allow the formation of geometrically complex structures, with precise control over pore sizes, variety of material choices, layering, etc. with the use of some biological materials. A drawback of these technologies is the lack of ability to produce fibrous structures in the nanoscale.
- Nanofiber self assembly of thinner fibrils [35] is a relatively expensive method, but allows the production of thinner than 10nm filaments that subsequently form thicker fibers, organizing structures, similar to the natural extracellular matrix.
- The electrospinning technology [36] is used to produce matrices of fibers in the micro- and nanoscale by employment of numerous polymers including also natural polymers. Even though this technique allows for much more precise tailoring of the biological and mechanical properties of the resulting matrices, it is still limited by the achieved small pore sizes thus limiting cellular migration within the matrix.

All these methods find application in medicinal practice and tissue engineering, but there are still many questions to be answered regarding their safety with long term usage, biodegradability, their influence on cellular signaling, proliferation and differentiation, etc. that are beyond the scope of this review.

3. 3D culturing systems based on natural components of the ECM

Gels present the easiest, most affordable and quickest way to provide cells with a three-dimensional environment that can be further manipulated by variety of methods in order to

modulate their properties. Different types of gels have been utilized during the past few decades with collagen, fibrin, hyaluronan and basement membrane extract (BME) gels being the most frequently employed.

3.1. Collagen gels

Collagens represent the most abundant type of proteins in mammals making up to 35% of the whole body protein content. They are found in fibrillar and non-fibrillar forms and are widely distributed among tissues with a distinct structural, organizational and density variance among tissues and organs. Currently, 28 types of collagen have been described that are known to be products of 49 different α-chain gene products [37]. Collagen gels are one of the first employed 3D culturing systems used to study cellular adhesion and migration in three-dimensional environment [17, 38]. Currently collagen gels have found application in a wide variety of studies in the field of cellular motility [11] and the importance of physical parameters such as density, stiffness and elasticity for cellular adhesion, proliferation, migration and contraction [39]. Fibroblast cells, seeded in collagen gels, have been shown to acquire different morphology when compared to fibroblasts grown on two-dimensional surfaces and appear similar to their counterparts in normal connective tissue. The three-dimensional environment of the gel mimics the in vivo conditions and also causes normal polarization and differentiation of epithelial cells [16]. It has also been developed as a model for studying the progression of prostate and breast cancer [40, 41].

Few types of collagen gels are most widely employed as three-dimensional culturing systems. Pure gels that remain attached to the dish after polymerization are considered as "stressed". The cells plated within this 3D environment usually exert isometric tension. If the gels are released, detached from the bottom of the Petri dish, they contract and represent the so called "relaxed and loaded" gels. If the gels are detached from the dish before cells are being seeded, they represent "relaxed and unloaded" gels. The ease of manipulation of the collagen gel's stiffness provides a useful tool for the evaluation of the role of isometric tension for cell survival and differentiation as well as for the investigation of mechanical features of the environment and it's reorganization during wound healing processes or various pathological conditions [39]. Some interesting features of cell migration in 3D have been established using those different types of collagen gels. Migration in softer substrates, or compliant matrices, results in migratory activity that is independent of the small GTPase Rho [39], thus, the cell acquires an amoeboid phenotype. As tension increases and the extracellular matrix becomes more rigid, the migratory mode of fibroblasts switches over to Rho dependent migration [42] with a mesenchymal phenotype. Moreover, in order for fibroblasts to differentiate to myofibroblasts upon TGFβ stimulation, a typical process during wound healing, increased tension in the extracellular matrix is required [43]. Reorganization of the extracellular matrix also appears to be dependent on the mechanical properties of the substrate as incorporation of fibronectin in the extracellular matrix requires internal tension of the ECM [44].

Collagen gel-based dressings were also used in some of the first attempts to create in vitro equivalents of full thickness skin for regenerative medicine and tissue engineering applications

[45]. Currently there are a few temporary and semi-permanent dressings available as off-the-shelf products, intended for use as bioconstructs for skin reconstruction [46].

3.2. Fibrin gels

Fibrin gels are another type of easily manufactured 3D culturing systems. They are formed as a result of thrombin cleavage of fibrinogen, resulting in a mesh of fibrin fibers. Stressed, relaxed and loaded and relaxed and unloaded types of fibrin gels are also frequently used model systems. In normal physiological conditions fibrin represents the provisional matrix of the clot, formed after wounding. Thus, the defined use of fibrin gels to study cellular invasion and contraction during the processes of wound healing [47] has emerged. Historically, polymerized fibrin gels were one of the first scaffolds used for skin tissue engineering after severe burns or for treatment of chronic wounds [48]. Currently fibrin gels have found further applications in the field of vascular tissue engineering and are being extensively studied especially as a possible resolution of a number of rapidly growing problems related to arterial occlusive diseases [49]. Studies in cardiac tissue engineering and cartilage regeneration and reconstruction have also benefited of the use of this natural, biodegradable scaffold [50, 51].

3.3. Hyaluronan gels

Hyaluronan or hyaluronic acid is widely distributed throughout all tissues glycosaminoglycan. It is an anionic, nonsulfated polysaccharide, formed on the plasma membrane instead of in the Golgi apparatus, with molecular weight varying between 5 kDa and 2,000 kDa. Hyaluronan is a major component of the cartilage, synovial fluid, the extracellular matrix of the skin and has a major role during the developmental processes [52]. Presumably, due to the high abundance of the molecule and its role during development, wound healing and migration, hyaluronan gels became another suitable three-dimensional model for studies in numerous fields.

As a natural component of the cartilage, hyaluronan is being used as a milieu for culturing chondrocytes in vitro. Current research indicates that culturing in tissue-like hyaluronic 3D environment sustains chondrocytes phenotype, leading to increased proliferation, sulphated glycosaminoglycans production as well as collagen type II and aggrecan synthesis and indeed supports chondrogenic differentiation [53]. Additional studies have shown promising results concerning the use of hyaluronan for cartilage repair and as scaffold for regenerative medicine [54-56]. Another intriguing direction of studies is the utilization of three-dimensional hyaluronic acid scaffolds for culturing mesenchymal stem cells. Promising results from culturing stem cells in three-dimensional hyaluronan-based scaffolds have been obtained in regard to the generation of cartilage-like tissue for the use of regenerative medicine [57]. Hyaluronic acid gels have been also investigated as possible 3D scaffolds for culturing cardiomyocytes and hepatocytes in vitro [58, 59]. Therapeutical use of hyaluronan gels has been reported also in the field of adipose tissue engineering [60] as well as in neuromedicine. The use of this biodegradable material has revealed promising results in cell and drug delivery to the central nervous system[61].

3.4. Basement membrane extract gels

Almost 30 years ago the Engelbreth-Holm-Swarm (EHS) mouse sarcoma cell line was found to secrete vast amount of unknown protein mixture. Later it was determined that this mixture was composed of the typical for the basement membrane proteins collagen IV, laminin, entactin and the heparansulfate proteoglycan [62]. Further components like matrix metalloproteinases as well as number of growth factors were also identified in this complex mixture. This protein composite is marketed by BD Biosciences under the trade name Matrigel®, but similar products are available from other sources. Matrigel resembles the complex extracellular matrix found in many tissues and is thus used by cell biologists as a substrate for cell culturing.

Utilization of the basement membrane matrix led to numerous scientific discoveries. For the first time the EHS matrix was used as a substrate to cultivate Sertoli cells, which led to their survival and the differentiation of the accompanying germ cells[63]. The first in vitro myelination was also observed in cultures based on the basement membrane matrix. Carey et al. demonstrated in 1986 that rat Schwann cells cultured in 3D conditions based on basement membrane matrix show increased dendrite outgrowth and myelination [64]. Thus a reliable in vitro model for investigation of nerve regeneration was established.

Another interesting observation was made when epithelial and endothelial cells were cultured on basement membrane matrix. Both cell types show different morphology compared to flat 2D surfaces, but also form specific structures depending on whether cells are cultured on top or within the matrix. Ducts were formed when epithelial cells were cultured on top of the matrix, and acinar-like structures appeared when cells were embedded within the BME gel [65, 66]. Different types of acinar epithelial cells (breast epithelial cells, salivary gland cells, pancreatic and prostate cells) form distinct structures in 3D indeed clearly supporting the importance of the extracellular matrix in cellular differentiation and proliferation. Endothelial cells form capillary-like structures when seeded in lower counts, or monolayers when seeded in higher counts. These observations made possible the development of in vitro models for studying angiogenesis. Tumor-induced angiogenesis is a key prerequisite for neoplastic progression thus angiogenesis suppression is one of the major directions investigated as possible cancer treatment. Vascular endothelial cells form capillary-like structures when plated on 3D basement membrane gels and have provided a suitable model for testing pharmacological substances and screening of chemical agents as angiogenic inhibitors [67].

Basement membrane extract gels provided also another important model system aimed at investigation of cancer invasion and metastasis. Kramer et al. noticed that normal, non-malignant cells polarize on top of the matrix whereas malignant cells invaded the 3D basement membrane [68]. While normal cells attached, polarized and differentiated on top of the matrix and showed almost no migratory tendency, malignant cells exhibited increased invasive and migratory phenotype within the matrix. Formation of long protrusions in the direction of migration and channels of degraded matrix behind the cells were observed. Evidently, malignant cells mimicked their in vivo invasive behaviour since synthesis of proteolitic enzymes and degradation of the basement membrane are key events during tumor metastasis. Continuous investigation led to the development and improvement of invasion and metastasis

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assays [69, 70] providing identical conditions and criteria to measure the invasiveness of tumors and the efficiency of treatment.

Maybe one of the most significant discoveries related to the progress of regenerative medicine and tissue engineering is the possibility to culture stem cells on basement membrane matrix [71]. Normally the basement membrane is the first ECM to be synthesized in the developing embryo [72] hence the logical use for culturing stem cells. Feeder layers of irradiated mouse embryonic fibroblasts were required for long term culturing and limited the large scale production of human embryonic stem cells. Moreover, they present the possibility of viral cross contaminations. Utilization of other extracellular matrix components like fibronectin, collagen I, collagen IV or artificial substrates has failed to support undifferentiated stem cells growth [73], whereas coating of the dish with BME extract retained the undifferentiated state of human embryonic stem cells for up to 30 passages, maintaining their proliferation rate, high telomerase activity, normal karyotype and the expression of the pluripotency markers.

3.5. Cell-derived matrices

The use of three-dimensional gels of collagen, fibrin, basement membrane matrix or glycosaminoglycans represents a significant advance in cell culturing. Those gels provide the much needed dimensionality to bring the environmental conditions closer to the in vivo settings. A major disadvantage of those models though is the fact that they still lack the chemical complexity and spatial organization of the ECM, characteristic for tissues (Figure 1). Indeed cells plated in three-dimensional gels degrade and reorganize to some extent the constituents of their surroundings and also secrete and integrate new components within the existing extracellular matrix [74]. Presumably cells are trying to shape their environment by their liking, but all those processes are believed to keep cells in an "activated" state that is unnatural for healthy tissue. In the quest for creating better model systems that approximate closer to different healthy tissues a few groups have developed during the last few decades affordable methods for creating cell derived three-dimensional cultures and matrices [9, 75-79]. Those methods are based on in vitro preparation of three-dimensional extracellular matrices made by the cells themselves. The result is a naturally synthesized and organized extracellular matrix, providing in vivo-like conditions.

Conducted research, based on these tissue-like cultures has already demonstrated significant differences between cells grown in three-dimensional cell derived matrices and other 3D culturing methods. Ahlfors and Billiar [79] demonstrated that culturing fibroblasts in appropriate conditions induces synthesis of extracellular matrix components, thus resulting in a multilayer culture, with mechanical properties approximating normal tissue. Investigation of the mechanical properties of these cell derived 3D cultures revealed that the resulting matrices are stronger than collagen or fibrin 3D cultures. Moreover cells in naturally synthesized cultures had higher protein synthesis rate than fibroblasts cultured in collagen or fibrin gels, and more importantly, inhibition of collagen synthesis was not observed at later stages of cultivation. The production of matrices was achieved in serum supplemented media as well as in chemically defined media. The second option makes exploitation of this method relatively

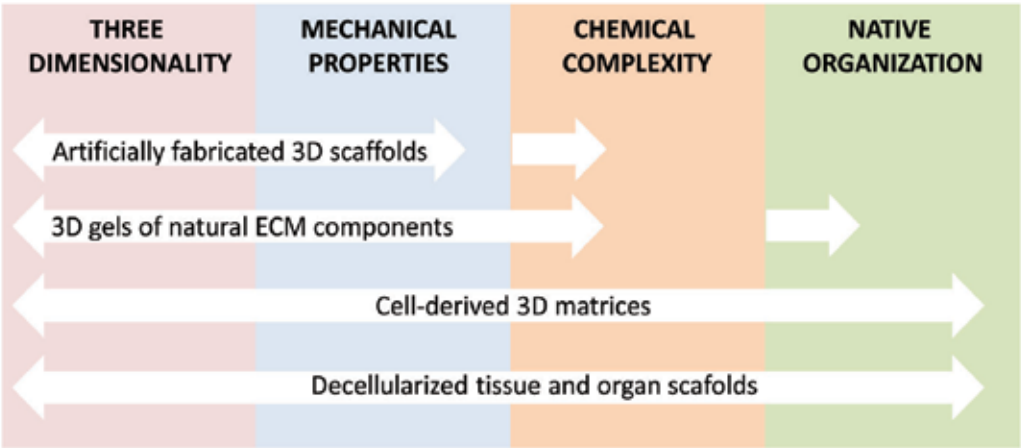

Figure 1. Schematic presentation of the main characteristics of 3D cell culture systems. While artificially fabricated 3D scaffolds can be designed with specific three dimensional organization and desired mechanical properties, they, however, generally lack the chemical complexity of the natural ECM. This limitation can be compensated to a certain extent by incorporation of natural ECM proteins or their peptides (indicated by additional small arrow). 3D gels, made out of natural ECM components have also restricted chemical complexity and in addition their structural organization is a result of spontaneous, rather than cell-directed polymerization. Cell-derived 3D matrices and decellularized tissues and organs meet the general requirements for in vivo-like 3D environment. However, they often pose difficulties in preparation and restrictions in their mass production (see text for details).

expensive, but increases the chances of utilization of such cultures for the needs of tissue engineering by greatly diminishing the possibilities of species or viral cross contamination.

As we have previously discussed, cellular morphology of fibroblasts differs between 2D and 3D conditions. Even though fibroblasts appear to acquire the same morphology when plated on collagen gels and cell derived matrices, there are a number of issues to be considered – fibroblasts attach, proliferate and migrate at much higher rate in cell derived matrices compared to collagen gels [9]. Though dimensionality influences fibroblast morphology one must also take into account the heterogeneity and specific organization of the extracellular matrix which are of significant importance as well. The fact that 3D matrix adhesions were established in cell derived extracellular matrices, but not in fibrin or collagen gels, or 2D surfaces coated with ECM components [9] supports the notion of the importance of structural organization of the environment. Both, the components and their colocalization in these adhesion structures differ from focal and fibrillar adhesions. Formation of such in vivo-like adhesions appears to depend on the heterogeneity and organization of the ECM and their formation does not require de novo protein synthesis during cellular adhesion. Major differences in signal transduction of fibroblasts in cell derived matrices were also discovered. The focal adhesion kinase (FAK), a molecule with exceptionally important role for cellular adhesion in conventional monolayer cultures, appears to be bypassed in in vivo-like conditions [8, 9]. In spite of this, downstream molecules maintain or even augment their activities, resulting in increased proliferation for example. Furthermore culturing of fibroblasts as a cell derived, three-dimensional multilayer culture leads to altered distribution of plasma membrane lipids

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[80-82]. Changes of the localization and properties of plasma membrane cholesterol and sphingomyelin in 3D are a probable cause for lowered oxidative stress, thus supporting the growing number of studies indicating that 2D cultures are an inappropriate, stressful method for cultivation of cells. Moreover the differences in the structural organization of the membrane coupled with the higher content of cholesterol and sphingomyelin, the major lipid components of the lipid rafts, in the plasma membranes of cells in 3D tissue-like environment, probably contribute to the differences in cell signalling.

The major difference between other 3D culturing systems and the cell derived in vivo-like three-dimensional matrices is the fact that their ECM is synthesized and organized naturally by the cells. In vivo, the extracellular matrix main functions are to present proper and specific 3D environment to cells and thus to define boundaries between different tissues. It provides the required elasticity and integrity during tissue and organ development, but is also being degraded and remodelled during both developmental processes and disease. Serving as an adhesive substrate, the ECM directs migratory cells and variations in its components concentrations may act as chemotactic gradient as well as differences in its mechanical properties can serve as durotactic gradient. The extracellular matrix also participates in the accumulation, storage, release and presentation of growth factors to the cells. The synthesis and immobilization of growth factors is spatially and temporally regulated by the ECM and the release of the incorporated ligands is also dependent upon appropriate cell-mediated forces, proteolitic degradation and proper presentation to cells. As a supportive structure, the ECM also participates in the reciprocal mechanical signalling. The transmittion of forces to and by the cells is regulated by their mechanical receptors – the integrins – and is manifested by changes in the intracellular signalling resulting in activation of the cytoskeleton machinery, growth factor production, proliferation rate alteration, etc. [83]. Among all these specific characteristic of the extracellular matrix probably the most important one is the fact that it is a dynamic structure, being constantly remodelled by cells in response to intrinsic signals, depending on the specific periods of organism's development or due to occurring diseases.

Currently cell-derived extracellular matrices are being applied with great success for in vitro investigation of developmental processes [84], tumor cell invasion and the role of the accompanying stroma cells [85], the mechanisms of fibrosis [86], studies of cellular migration [12, 87], as drug screening systems [88], for exploring the processes of wound healing [78] and numerous other applications. Despite the advantages of this model system, there are still a lot of considerable hindrances for its application in regenerative medicine and tissue engineering. Therefore numerous groups have undertaken a "reverse" approach towards meeting the requirements of modern medicine (see below).

3.6. Decellularized tissues and organs as scaffolds for tissue engineering

The need for organ transplantation in modern society far much exceeds the donor availability. Moreover the immunological incompatibility limits further the patient's possibilities for finding a matching donor for the subsequent transplantation. Even if all those criteria are met more often than not, patients are treated with powerful immunosupressants to reduce the chances of transplant rejection.

The aspiring role of the ECM in governing the appropriate behaviour of a number of cell types led to the investigation of the properties and possible use in regenerative medicine of decellularized organs. The diversity of the extracellular matrix, its structure and micro-patterns suggest that specific cell types would overall "feel and perform better" if embedded in their corresponding natural matrix. There is a growing number of research to confirm this view. Culturing of hepatocytes as conventional monolayers, outside of their natural environment, results in loss of specific hepatocyte functions thus limiting the possibilities for their use in regenerative medicine. However, culturing of human hepatocytes in porcine liver-derived extracellular matrix supports albumin secretion and ammonia metabolism as well as restoration of hepatic transport activity [89]. Characteristic extracellular matrix is even required for the differentiation of human or murine embryonic stem cells to pneumocytes [90]. To date a number of ECM derived scaffolds have been described and investigated for use as organ reproducing systems, including heart-derived ECM [91], liver-derived ECM [92] and lung-derived ECM [93].

The process of removal of cells from the organ and obtaining a cell-free scaffold is crucial for preserving the scaffold's qualities and usually includes several stages. Physical methods, involving freezing and thawing, mechanical agitation and sonication, could be considered non-harmful to the remaining biological scaffold and are usually combined with enzymatic and/or chemical methods, depending on the organ that is being decellularized. Treatment with exo-and endonucleases yields better results than physical methods, but is likely to affect the extracellular matrix as well. Harsh chemical treatment with acid or alkaline solutions, especially detergents, either ionic or non-ionic, is known to extract cells from the extracellular matrix, but also severely damages the remaining scaffold. Depending on the organ, its cellular content, overall lipid content, ECM biochemical composition, structure and complexity an appropriate method for decellularization has to be selected. More often than not a combination of methods is used to achieve better results. Successful removal of cells would ideally yield DNA- and cell debris-free extracellular matrix that is not affected or altered by the applied treatments, thus resulting in a minimal or even absent immunological reaction towards the allogenic or xenogenic extracellular matrix. Some protocols have already been developed and successfully used for producing biological scaffolds for heart, liver and lung [91-93].

Decellularization of organs would ultimately provide the most suitable scaffold for reconstruction of an organ – a natural extracellular matrix. Repopulation with cells of the obtained scaffold is a process which also poses some difficulties in organ reconstruction. Reintroduction of cells to such scaffold requires organ-specific types of cells as well as endothelial and epithelial cells for rebuilding of blood vessels, stem or progenitor type of cells to support future cell renewal in the organ and most of all, distinct methods for introduction of the appropriate cell types to their targeted environment. When possible, autologous cells are used since they are less likely to provoke immune response and be rejected. Such cells also present lesser chance of inducing cancer or the possible non-immune toxic reactions caused by immunosupressants [94]. Allogenic cells from matching donors could also be used for regenerative purposes when autologus cells cannot be harvested, or are terminally damaged. Although these cells are not derived from the patient, they have some advantages too. The required cell

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types can be derived from healthy individuals, characterized and maintained until needed, thereby providing the opportunity for faster therapy application. A well known example of such use of allogenic cells is provided by the mesenchymal bone marrow cells.

Often isolation of highly proliferating autologous cells from most organs is an almost impossible task. The use of embryonic stem cells could help solve this problem but the employment of human embryonic stem cells is still bounded up with heated moral debates. In addition it is a quite expensive technology, demanding proven methods for directed differentiation and extensive clinical trials. Therefore the use of adult cells with similar properties emerged as possible solution. Adult stem cells represent autologus cells which are multipotent and capable of self-renew. They have been known to exist in number of tissues like the gonads, intestine, skin and blood, and further data indicates their presence in adipose tissue [95], kidney [96], lung [97] and muscle [98]. In recent years, scientists have tried to identify specific markers to ease the isolation of adult stem cells, but such insignia are yet to be defined for most of them. Furthermore, it is currently evident that adult stem cells are localized in a specific extracellular environment – their niche. Signalling to and regulation of the self-renewal or differentiation processes appears to be tightly linked to the stem cell's niche, suggesting that micro-environmental cues may also be regulating cellular "stemness" [99]. A possible localization of different types of adult stem cells in niches in close proximity also exists [100, 101]. Thus, even though the specific localization of diverse types of adult stem cells have been identified, their isolation and further characterisation has proven to be an extremely difficult task. Label retention techniques and in vivo linage tracing as well as in vitro culturing and transplantation have yielded promising results and have aided the significant advancement in the field, but isolation of adult stem cells is still a major difficulty [99]. Despite the obstacles though, different types of adult stem cells have already found application in regenerative medicine [57, 102-104].

As pointed above, harvesting highly proliferating cells from healthy tissues has rendered a difficult task. Therefore researchers have sought to develop novel technologies that would aid obtaining and multiplication of such cells for further use in regenerative medicine. A promising technique developed just a few years ago is the induction of pluripotency in differentiated somatic cells. In 2006 Takahashi and Yamanaka introduced a method for inducing pluripotency in fibroblast cells by overexpression of four transcript factors – Oct-4, c-Myc, Sox2 and Klf4 [105]. Their results showed that the timed overexpression of these factors is sufficient to convert fibroblasts to embryonic stem cell-like cells, termed induced pluripotent stem cells (iPSC), with many subsequent articles confirming their observations. The iPS cells are able to differentiate to any type of cell, just like embryonic stem cells, but since the progenitor cells are derived from the adult organism their use is liberated from the moral burdens concerning the use of embryonic stem cells. Research indicates that the genetic profile of good quality iPSC and embryonic stem cells is nearly identical, although some articles suggest that there may be some differences, probably attributed to different laboratory practices [106-108]. Further analysis of the whole genome have indeed found 71 differently methylated regions between iPSC and embryonic stem cells and 2,179 between iPSC and fibroblast [109], supporting the hypothesis that probably even though somatic cells are converted to pluripotent cells, there is still a "memory" preserved of the type of the donor cell.

Even though there are a lot of difficulties with the production of iPS cells, already a plethora of articles has demonstrated the ability of iPS cells to differentiate to numerous types of cells, thus providing the ability to direct the in vitro differentiation of iPS cells to the required cell type for the specific therapy. Moreover significant results have been accomplished, namely the generation of adult mice from iPS cells [110] thus confirming the vast capabilities of such cells. Therapeutically, iPS cells have been used together with gene therapy to correct genetic defects in mice, with two studies already showing promising results in the treatment of different types of anaemia [111, 112]. Future efforts in the field of iPS cells though have to be made before a successful iPSC therapy for human patients becomes a reality – research is currently targeted towards the development of methods of pluripotency induction, not relying on viral transduction, therefore lowering the possibility of cancer induction by the transplanted iPS cells as well as improvement of the efficacy of reprogramming. Furthermore the remaining questions of whether the donor cells are completely reprogrammed or retain a "memory" of their differentiated state are still to be answered.

4. Does a perfect 3D system exist?

In vitro 3D models present the opportunity to investigate in depth the molecular mechanisms of the interactions between cells and the extracellular matrix. The increased number of research based on 3D scaffolds, mimicking specific physiological and developmental processes, as well as tissues, bridges the gap between laboratory investigation and unimpeded tissue regeneration. At the same time the increasing number of research has made it clear that variables such as cell source, the extracellular matrix's biochemical and mechanical properties, growth factor cues and developmental stage affect cell behavior and therapy outcome, imposing the need of even more careful reconsideration of yet the slightly interfering environmental factors for the successful outcome of regenerative therapies or tissue engineering.

Development of optimal bioengineered scaffolds requires indepth knowledge of physiology and understanding of cell–cell and cell–ECM interactions. The choice of inappropriate model system could have a negative effect on the study or the therapy outcome. Such an example could be any of the discussed 3D culturing systems. Sometimes even slight changes in experimental design like difference in cell count in collagen gels for example could lead to different migrational patterns [87] or as mentioned, the change of the substrate stiffness can induce different responses of cells to the environment [14]. Still, the obscured causes underlying such different outcomes are yet to be defined. Another example could be the inappropriate use of three-dimensional BME gels. In vivo basement membranes represent thin extracellular structures that underlie endothelial and epithelial structures. Basement membranes also surround muscle, fat and nerve cells. Moreover the composition and the amount of the basement membrane are specific and vary in different tissues and during particular stages of development. Even though introduction of cells to a 3D environment, based on basement membrane matrix, results in numerous morphological and physiological changes, there are a number of discrepancies to be considered and questions to be answered:

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- Contact with the basement membrane is not typical for all cell types, but a lot of cells express receptors for components of the basement membrane matrix. Therefore what would happen with cells that accidentally get caught in such unnatural environment? Would they become apoptotic or would they transform into malignant cells?
- Not all cell types respond to the basement membrane matrix and thus an optimization of the environment is required.
- Differences in basement membrane matrix composition during development imply that cells may respond to the matrix up to a certain stage. Acquisition and synchronization of cells of developmentally correct phases could be difficult and thus could inappropriately selected cells lead to activation of pathologic-like processes?
- The available basement membrane extract is of cancerous origin. Since tumor environment has been shown to be sufficient to promote desmoplastic differentiation [85] is it possible that contact of normal cells with cancerous cell-derived BME could induce their transformation?

All these discrepancies and unanswered questions do not make the BME gels unsuitable model systems, but stress the importance of experimental design. Hakkinen et al. have shown that normal fibroblasts respond unexpectedly in 3D BME gels – they acquire a rounded morphology with short protrusions, possibly for environmental sensing and do not migrate [12] – behavior typical for non-malignant fibroblasts, thus supporting the notion that an adequate model should be chosen for each experimental design. Indeed BME gels provide scientists with a great tool to study malignant cell invasion through the basement membrane [70, 113], with numerous assays developed for investigation of the mechanisms of invasion. Moreover comparison between different three-dimensional scaffolds in terms of cell morphology, adhesion and migration and cytoskeletal organization concluded that indeed careful attention should be paid on the experimental design [12].

Use of other types of 3D gels faces researchers with similar problems. Neither collagen nor fibrin or hyaluronan gels incorporate other components of connective tissue. They are suitable for investigation of a specific response of a specific cell type towards distinctively modulated environments, but are still far away from the complex organization of the native extracellular environment. At the same time the provisional matrix of a blood clot is formed of disorganized fibrin mesh, just like the in vitro 3D gels. Cell-derived matrices on the other hand represent a complex, cell-organized structure based on cell-specific type of expressed matrix components. However, it is possible that in vitro generated cell derived matrices could also have different matrix composition, pore sizes, mechanical and biochemical characteristics than in vivo analogues because of the initially unnatural substrate cells were cultured on. Thus it is important of one to consider the specific characteristic of the tissue in vivo or the nature of the modeled process in order to be able to select an appropriate 3D matrix for in vitro research or for in vitro based preparation for therapies.

Besides looking at the ECM as a 3D structure one must not forget that in vivo cells do have a specific polarity that is usually lost during conventional 2D cultivation, and sometimes even in 3D, if an inappropriate environment has been selected. Fibroblasts for example, lack an

apical and basal organization in vivo. So when placed back in 3D environment after being cultured as 2D monolayers they tend to regain their typical in vivo morphology [9, 74, 114], but the essence of the environment also has to be considered since it could provoke different morphology [12]. Moreover, both the mechanical and the structural state of the environment have to be taken into consideration. Recent studies have shown that gene expression patterns, fibroblast morphology, as well as organization of the extracellular environment differ not only between particular healthy tissues but also compared to tumor stroma [85, 115, 116]. Furthermore it is currently evident that cells do feel and respond in different ways to the environment – fibroblast cells differentiate to myofibroblasts due to changes in the substrate stiffness [86] and begin to remodel the extracellular matrix. Stem cells have the ability to differentiate to osteogenic, myoblastic and neuronal lineages based just on the change on substrate stiffness [13]. Tumor associated stroma alone was shown to be able to induce desmoplastic stroma fibroblast differentiation [85] as well as at later stages of tumor progression, to be more permissive for epithelial invasion [117]. Taking into consideration these differences it is not surprising that the compliance of the substrate and its topography and mechanical features can control cellular behavior. Therefore the transplantation of cells into bioengineered scaffolds for the purposes of regenerative medicine or tissue engineering has to be precisely assessed and executed, since even the small differences in the substrate's composition, organization or stiffness have the potential to alter the donor cells gene expression [85, 118] or to promote tumorigenic transformation [119].

Despite the drawbacks revealed about each type of bioengineered scaffolds they have already found use in regenerative medicine. Fibrin and collagen grafts for example were among the first used in burn patients for skin reconstruction. Study by Chua et al. has shown that use of skin tissue constructs has reduced mortality in patients with 60% of total body area burns from 100% in 1952 to 41,4% in 2003 [120]. The advance in the skin tissue regeneration is accentuated by the high number of currently available off-the-shelf bioengineered skin grafts [46], that could be grouped by several criteria:

- Biomaterial – biological (autologus, allogenic and xenogenic) and synthetic (biodegradable, non-biodegradable)
- Duration of the graft – permanent, semi-permanent, temporary
- Composition regarding cell content – cellularized or acellular
- Anatomical structure – epidermal, dermal, composite

Together with this immense success in the field of burn wounds and the increase in survival rate though, patients are faced with new obstacles. More often than not wounds in surviving patients develop severe fibrosis after healing. The resulting hypertrophic scars present a major discomfort in survivor's life by possibly limiting the range of motion of joints, also resulting in impaired thermal regulation and not at the least a disturbed visual appearance [121].

In spite of the advances made in skin tissue engineering and without undervaluing the accomplished results there is still a lot of progress to be made towards the prefect reconstruction of the skin. There still isn't an available option that makes possible the regeneration of

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hair follicles and sebatious glands, thus allowing for rehabilitation of thermal regulation. Even though the perfect skin graft is still to be created, the available skin models have made a significant impact in in vitro studies regarding skin biology and skin disease progression: engineered skin of different aspects helps in producing more relevant results than 2D cultures, thus leading to reduced use of animals for experimentation. As discussed earlier investigation of cell–cell and cell–extracellular matrix interactions has evolved rapidly due to the use of skin tissue-mimicking models. Wound healing, skin contraction [122] and angiogenesis are all fields benefiting from the use of 3D skin equivalents. Investigation of skin diseases such as melanoma invasion [123, 124], psoriasis [125] and skin blistering disorders [126] is also making significant progress based on engineered skin.

Besides skin tissue engineering biological scaffolds have found use in other sections of tissue engineering. Promising results are obtained with decellularization and recellularization of heart, liver and lungs. After recellularization of a hearth-derived ECM, a macroscopic contraction and pump function of the newly generated heart were observed in vitro [91] – indeed a promising result for the generation of a biological artificial heart. Recellularization of liver [92] and lungs [127] has gone even further and transplantation of the newly formed organs back to animals showed that they are able to sustain their natural functions. Probably one of the highest successes of tissue engineering for regenerative medicine is the successful transplant of trachea in a 30-year old patient [128] based on decellularized tracheal scaffold, reseeded with autologus epithelial and mesenchymal cells thus reducing the possibility of organ rejection and the need of strong immunosupressants. Despite the great success in the field the major disadvantage of these scaffolds still persists – the need of a donor organ for their preparation. Nevertheless the first successful transplantation of entirely bioengineered constructs, a completely new tracheobronchial tube in a patient suffering from tracheal cancer, is already a reality, partly due to the virtue to three-dimensional model systems [129].

Based on the review of decades of research it is currently evident that a perfect 3D system able to fulfill all scientific and medicinal requirements does not exist. On the contrary – a careful and differential approach towards appropriate 3D system selection, experimental or therapeutical design and data interpretation is required for every particular case (Figure 2). Such approach would contribute to the optimal outcome of the specific therapy or the distinct experiment thus leading the way to unimpeded regenerative medicine.

5. Conclusion

The evolution of the cell culturing method has led to the sophistication of culturing procedures with one main goal – to approximate in vitro conditions to in vivo. The advancement of three-dimensional culturing models has allowed for an in depth understanding of cellular behavior and especially the role of the cell's environment has emerged as an important modulator of cellular functions. Current data has made it clear that future development and use of superior three-dimensional cultures should focus not just on the dimensionality of the environment per se, but mainly on its characteristics – biochemical composition, production source and possible

unwanted cell-cueing signals, mechanical properties, etc. thus making 3D culturing, along with regenerative medicine multidisciplinary fields. However, as science advances, more and more questions emerge and await answers in order to confirm the long term safety of the applied methods for human therapy.

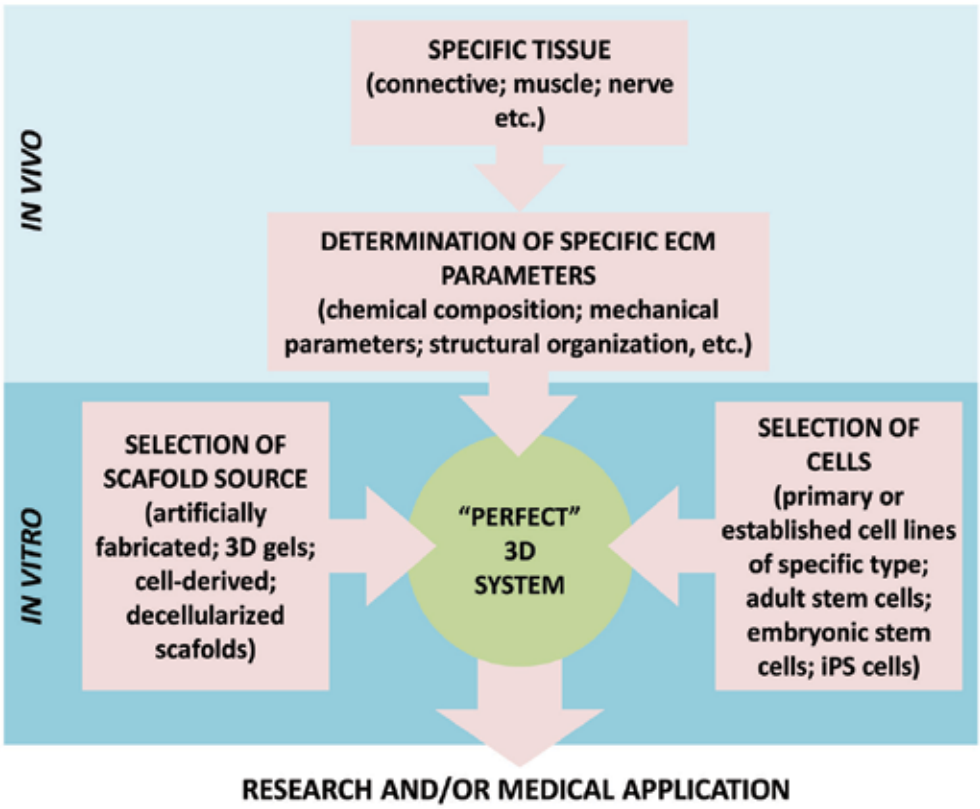

Figure 2. General strategy for design of a "perfect" 3D system. The specific set of ECM characteristics, typical for selected normal tissue can be determined. Based on these studies, a scaffold with matching parameters and suitable cells can be identified and combined in vitro for development of particular, tailored made 3D culture, meeting the needs of a specific research or specific therapy.

During the resolution of the moral issues concerning the use of human embryonic stem cells or donor organs the remaining fields continue to advance rapidly. It appears that isolation of autologus cells, their expansion in vitro and probably stimulation to produce extracellular matrix with the required dimensionality, biochemical and mechanical properties would represent the optimal tissue engineered scaffold for tissue and organ reconstruction. Further advance in the field of regenerative medicine and tissue engineering would possibly see the development of 4D model systems, incorporating time as the forth dimension. Such models would represent not just single stages of organ development or disease, but the chronology of

their maturation/progression, thus providing an opportunity to transfer developmental processes to in vitro conditions.

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Chapter 11

Naturally Derived Biomaterials: Preparation and Application

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Additional information is available at the end of the chapter

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1. Introduction

The success of any implant depends so much on the biomaterial used. Naturally derived biomaterials have been demonstrated to show several advantages compared to synthetic biomaterials. These are biocompatibility, biodegradability and remodeling. Therefore, these biomaterials are usually applied in the repair or replacement of damaged human tissues and organs. The aim of this chapter is to provide a brief knowledge of naturally derived biomaterials as well as methods of preparation and application of them.

Biomaterials can be classified into two main groups: synthetic and natural biomaterials. Synthetic biomaterials are classified as: metals, ceramics, nonbiodegradable polymers, biodegradable polymers… Some synthetic biomaterials are commercialized and applied in clinical treatment such as metal hip, Dacron, plastic intraocular lens… However, synthetic biomaterials have some disadvantages, including their structure and composition is not similar to native tissues/organs, their biocompatibility and their ability to induce tissue remodeling are low. Thus, other biomaterials have been developed that can overcome the disadvantages of synthetic biomaterials. Today, naturally derived biomaterials have been attracting scientist's interest all over the world. Naturally derived biomaterial can be classified into many groups including protein-based biomaterials (collagen, gelatin, silk…), polysaccharide-based biomaterials (cellulose, chitin/chitosan, glucose…) and decellularized tissue-derived biomaterials (decellularized heart valves, blood vessels, liver…). Protein and polysaccharide-based biomaterials can be prepared by two distinct ways. Protein and polysaccharide from living organisms are dissolved by solvents or enzymes. Then, they are precipitated and reconstituted into fibrils. The second way to prepare protein and polysaccharide is removing other elements of living organisms by solvents or enzymes. Decellularized biomaterials are created by

eliminating all cells from native tissues/organs. Physical, chemical and enzymatic approaches are combined to make the effective decellularization protocol.

Because of their advantages, naturally derived biomaterials are usually applied to replace or restore structure and function of damaged tissues/organs. They have ability to adequately support cell adhesion, migration, proliferation and differentiation. In particular, when implanted into a defective area, naturally derived biomaterials can enhance the attachment and migration of cells from the surrounding environment, therefore, induce extracellular matrix formation and promote tissue repair. Some biomaterials are used to acting as drug delivery system and medical devices such as surgical sutures. The silk fiber produced by silkworm or spider has been used as a surgical suture for a long time due to its biodegradable and non-antigenic protein. These silk fibroin nanoparticles are the globules with a fine crystallinity that may offer various possibilities for surface modification and covalent drug attachment. Furthermore, some biomaterials are used to produce environmental friendliness of packaging (such as resorbable chitosan packing) and other products. Some commercial products were made from naturally derived biomaterial such as SIS, Matrigel, Alloderm… In this chapter, we focus on a brief knowledge as well as the methods of preparation and application of naturally derived biomaterials in our researches.

2. Naturally derived biomaterials: Preparation and application

2.1. Protein

2.1.1. Collagen

2.1.1.1. Structure

Collagen is the most abundant protein of connective tissues in all animals. Now, at least sixteen types of collagen have been identified, in which 80-90% of the collagen is types I, II and III. Collagen is secreted by not only fibroblasts but also epithelial cells [1].

The basic structural unit of collagen is a triple helix. Most collagen is fibrillar because of pack of collagen molecules type I, II, III. Contrast, collagen IV forms a two dimensional network which is unique to the basement membranes [1]. Basement membranes have been performed a number of mechanical and biological functions. They provide physical support for tissue because of their tensile strength. They also influence cell proliferation, adhesion, migration, differentiation, polarization, and are thus implicated in biological processes such as development, tissue maintenance, regeneration, and repair, and in various pathological processes such as tumor growth and metastasis [2].

The basement membranes composition varies from one tissue to another. In general, the major constituents of all basement membranes are collagen IV, laminins, nidogen/entactin, and proteoglycans. The functional diversity of basement membranes arises from the molecular diversity of their components, particularly the different collagen IV and laminin isoforms [2].

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2.1.1.2. Preparation

Collagen can be obtained from various sources, in which amniotic membrane (AM) is an attractive source. AM is a thin membrane surrounding the fetus which is filled with amniotic fluid.

The AM consists of an epithelial monolayer, a thick basement membrane, a compact layer, a fibroblast layer and a spongy layer [3]. The innermost layer, nearest to the fetus, is monolayer of epithelial cells anchored on the basement membrane. The collagen component of basement membrane of AM includes types III, IV, V, VII, XVII which similar morphological and ultra-structural basement membrane of skin. Therefore, basement membrane of AM is often used to create skin equivalents. Besides, AM has outstanding properties such as anti-inflammatory, anti-bacterial, anti-fibrosis, anti-scaring as well as low immunogenicity and reasonable mechanical features [3].

AM can be used either with amniotic epithelium (intact) or without it (denuded), fresh or preserved. To remove the amniotic epithelium, the AM is incubated in trypsin-EDTA at 37^{o}C in 30 min and the cells are gently scraped while maintaining the intact basement membrane. H&E staining was performed to confirm removing the amniotic epithelium. Then, the basement membrane can be preserved by drying or glycerol - cryopreservation after γ-sterilization [3, 4, 5].

2.1.1.3. Application

Collagen is commonly used in biomedical applications. The basement membrane of the AM is a typical example. The extracellular matrix components of the basement membrane of the AM are native scaffolds for cell seeding in tissue engineering. AM has been applied in tissue engineering related to eye, skin, cartilage, nerve, especially cancer [3, 6, 7].

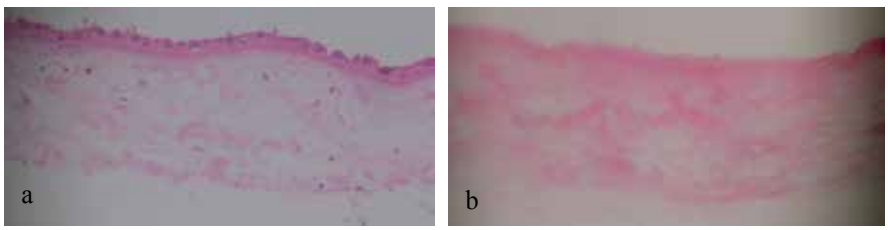

Figure 1. Amniotic membrane with epithelium: intact (a) and denuded (b)

To use AM in creating skin equivalent, AM must be removed the epithelium. Keratinocytes are seeded onto basement membrane which is denuded AM. Briefly, keratinocytes were isolated from intact skin samples by incubation in trypsin-EDTA at 4^{0}C in 18 hours and detached mechanically. Keratinocytes were cultured in serum free medium in 7 days. Medium was chanced every two days [8]. After 3rd passage, cells were subcultured onto AM basement membrane which spreaded on bottom of the insert dishes. The cells were maintained in culture for 7 days when the cells reached confluent. Air-lifting was performed to induce cell

differentiation. After 7 days, the cells formed multi-layers on the AM basement membrane. Cultured keratinocyte sheets were grafted on patients who were defected skin because of injury or burn. The result showed that, the advantages of cultured keratinocyte sheet autograft: the possibility of the grafting area multiplication (50 times after 3 weeks), the diminution of scaring, the relief of pain, the low infection risk, the same effect in compare to the split - thinness autograft.

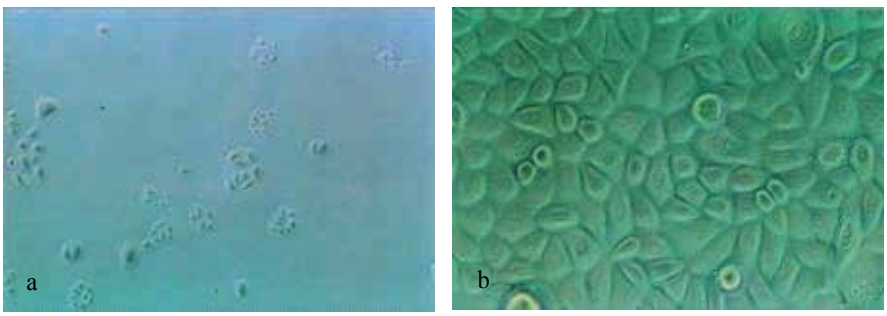

Figure 2. Keratinocytes formed colonies (a) and monolayer onto culture dishes (b) (200X)

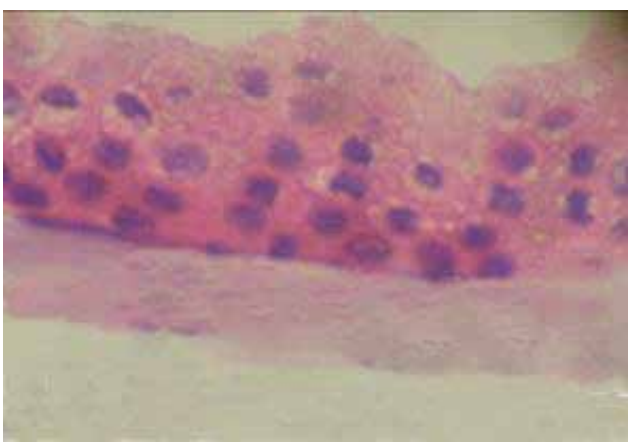

Figure 3. Result of HE staining of the cell sheet, the cells were exposed to the air in seven days. The cells formed 4 – 5 layers onto AM basement membrane (400X).

2.2. Gelatin-Alginate

2.2.1. Structure

Gelatin is obtained by controlling the hydrolysis of collagen, a fibrous insoluble protein which is widely found in nature and is the major component of skin, bone and connective tissue. Characteristic features of gelatin are the high content of the amino acids such as glycine, proline

and hydroxyproline. Structually, gelatin molecules contain repeating sequences of glycine-X-Y triplets, where X and Y are frequently proline and hydroxyproline. These sequences are responsible for the triple helical structure of gelatin and its ability to form gels where helical regions form in the gelatin protein chains immobilizing water [9].

Alginate was first discovered by Edward Stanford in 1883. Since being commercialized in 1927, alginate has now expanded to about 50.000 tonnes per year worldwide; 30% of this tonnage is devoted to the food industry, the rest being used in industrial, pharmaceutical and dental applications [10]. The function of alginates in algae is primarily skeletal, with the gel located in the cell wall and intercellular matrix conferring the strength and flexibility necessary to withstand the force of water in which the seaweed grows [11].

Alginate is a hydrophilic polysaccharide extracted from marine brown algae such as Laminaria hyperborea or soil bacteria such as Azobacter vinelandii and composed of 1,4-linked β-D-mannuronic acid (M) residues and 1,4-linked α-L-guluronic acid (G) in varying proportions, displaying carboxylic acid functionality at the C5 residue. The alginates have broad distributions of molecular weights of 10-1000 kDa depending on source and processing. The relative amount and sequential distribution of homogeneous M-M segments (M-blocks), homogeneous G-G segments (G-blocks) and alternating M-G segments (MG-blocks), which represent the primary structure of alginate, depend on the producing species, and for marine sources, on seasonal and geographical variations.

2.2.2. Preparation

Cross-linked gelatin/alginate was made in two steps. Briefly, 1wt % (w/w) aqueous solution of gelatin and sodium alginate, respectively, was dissolved in double distilled water at 500C for 3 h. Each solution with certain mixing ratios of gelatin and sodium alginate (8G:2A) was stirred for 30min at room temperature, frozen to -700C for 40 h. This soluble sponge was cross-linked with EDC by immersing the soluble sponge in 90% (w/v) aqueous acetone containing 0.3% EDC for 24 h at room temperature, while shaking slowly.

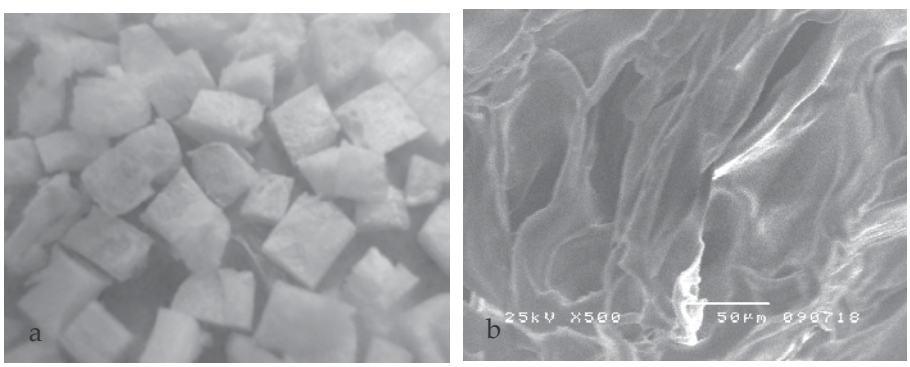

Figure 4. Gelatin-Alginate. Block (a), SEM (b)

The sponges consisting of gelatin from hydrolysis of collagen and alginate-a polysaccharide from Phaeophyta were established by using EDC as a crosslinking agent. The sponges were combined with some natural substances traditional used for burn treatment such as tamanu oil (from nuts of Calophyllum inophyllum); cajeputi oil (from leaves of Meulaleuca leucadendron); madecassol (from extract of Centella asiatica); turmeric and python fat. Data obtained from testing on mice showed that the coordinated sponges have rather good ability on preventing infection and promoting wound healing compared with control. The sponges combined with mixture of cajeputi oil and madecassol have the best potential for burn treatment.

2.2.3. Application

Gelatin has been used in medicine as plasma expander, wound dressing, adhesive, and absorbent pad for surgical use. While collagen, also known to have wide biomedical applications, expresses antigenicity in physiological condition, gelatin is known to have no such antigenicity. Recently, gelatin has shown to exhibit activation of macrophages and high hemostatic effect. Finally, gelatin is practically more convenient than collagen because a concentrated collagen solution is extremely difficult to prepare from the native collagen, and furthermore gelatin is far more economical than the collagen.

Alginate is an effective natural disintegrant, tablet binder and offers an attractive alternative for sustained-release systems. It offers advantages over synthetic polymers as it forms hydrogels under relatively mild pH and temperature and is generally regarded as non-toxic, biocompatible, biodegradable, less expensive and abundantly available in nature; in addition, alginate meets the important requirement of being amenable to sterilization and storage. All these advantages make alginates very useful materials for biomedical applications, especially for controlled delivery of drugs and other biologically active compounds and for the encapsulation of cells. Calcium alginate is a natural haemostat, so alginate based dressings are indicated for bleeding wounds. The gel forming property of alginate helps in removing the dressing without much trauma [12].

Alginate has been much used in medical applications such as wound dressings, scaffolds for hepatocyte culture and surgical or dental impression materials. Alginates are also known to be broken down to simpler glucose type residues and can be totally absorbed.

Alginate has been successfully used as a matrix for the entrapment and/or delivery of biological agents, such as drugs and proteins. In particular proteins can be loaded and released by alginate matrices without loss of their biological activity because of the relatively mild gelation process of alginate. In pharmaceutical formulations, the alginate gel can be prepared prior to use, or it can spontaneously form in situ in physiological fluids, by low pH and/or calcium ions naturally present in the site of administration [13]. Alternatively, the gelling agent can be added either as a part of the formulation or separately administered. The microencapsulation technique has been specifically developed for the oral delivery of proteins, as they are quickly denaturated and degraded in the hostile environment of the stomach.

Among the possible applications of alginate, one of the most promising is for cell immobilization. Alginate gel allows cell suspension to be cultivated in several types of bioreac-

tors to achieve high cell densities [14]. In cell immobilization applications, the main drawback of alginate matrix gels is represented by their high density of network, which limits the cell growth; moreover, cell anchorage, a strict requirement for survival, is limited on alginate gels, because of its hydrophilic nature. PEG copolymers are used to improve their biocompatibility [15].

2.3. Silk

Silks are generally defined as protein polymers that are spun into fibers by Lepidoptera larvae such as silkworms, spiders, scorpions, mites and flies [16]. Silks are fibrous proteins synthesized in specialized epithelial cells that line glands in these organisms. Silk fibroin polymers consist of repetitive protein sequences and provide structural roles in cocoon formation, nest building, traps, web formation, safety lines and egg protection. The most extensively used silk for various applications are those from silkworm silk; Bombyx mori and spider silk; Nephila clavipes.

The domesticated silkworm (B. mori) silk fibroin fibers are about 10–25 μm in diameter. Each fiber consists of core protein covered by a coating protein (sericin) that glues core fibers together. The core protein consists of three chains: heavy chain, light chain and a glycoprotein, P25. The light chain (26 kDa) and heavy chain (390 kDa) which are present in a 1:1 ratio and linked by a single disulfide bond. The disulfide linkage between the Cys-c20 (20th residue from the carboxyl terminus) of the heavy chain and Cys-172 of the light chain holds the fibroin together and a 25 kDa glycoprotein, named P25, is non-covalently linked to these proteins [17]. Light chain is necessary for the secretion of protein from the silk glands. Heavy chain is fiber forming protein and its structure determines properties of silk fiber [18]. Heavy chain is commonly referred as fibroin protein. These proteins are coated with a family of hydrophilic proteins called sericins (20–310 kDa). Silk proteins are particularly promising for these needs due to their unique combination of biocompatibility, biodegradability, self-assembly, mechanical stability, controllable structure and morphology.

Spiders are look like insects and come in to the category of Arthropoda, but they belong to a completely different class of animals, called Arachnida. Spider silk is the result of 400 million years of evolution. Spiders use silk for variety of functions including reproduction as cocoon capsular structures, lines for prey capture, lifeline support (dragline), web construction and adhesion. Spider silk is a biopolymer fiber. Its composition is a mix of an amorphous polymer (which makes the fiber elastic), and chains of two of the simplest proteins (which give it toughness). Out of 20 amino acids, only Glycine and Alanine serve as a primary constituent of silk. The Dragline silk of orbweb spider seems to be most studied in the scientific research. The protein in dragline silk is fibroin (Mass of 200,000-300,000 Daltons) which is a combination of the proteins spidroin 1 (Alanine-rich) and spidroin 2 (Glycine-rich), the exact composition of these proteins depends on species. Fibroin consists of approximately 40% Glycine and 25% Alanine as the major amino acids. The remaining components are mostly glutamine, serine, leucine, valine, proline, tyrosine and arginine [19]. Nephila clavipes can produce seven types of silk from seven different silk glands as shown in depending on needs and environmental

conditions [20]. The superior mechanical properties of dragline spider silks can be used as a template for developing specific structures for various biomaterial needs. Spider silks have not been commercialized in fashion as silkworm silk due to the lack of domestication and lower productivity of spiders.

2.3.1. Preparation

Nanotechnology is becoming a key technology and capable of application in all fields of science and technology. In particular, nanoparticle delivery system significantly improved pharmaceutical treatment of many incurable diseases which require complex treatment regimens, as well as, patients must take multiple medications and need time long-term drug use. Silk Protein is the ideal material for this purpose, because they have many unique features such as highly biocompatible and biodegradable ability, self-restructuring, mechanical stability, easy control and adjustment of the object's structure and shape.

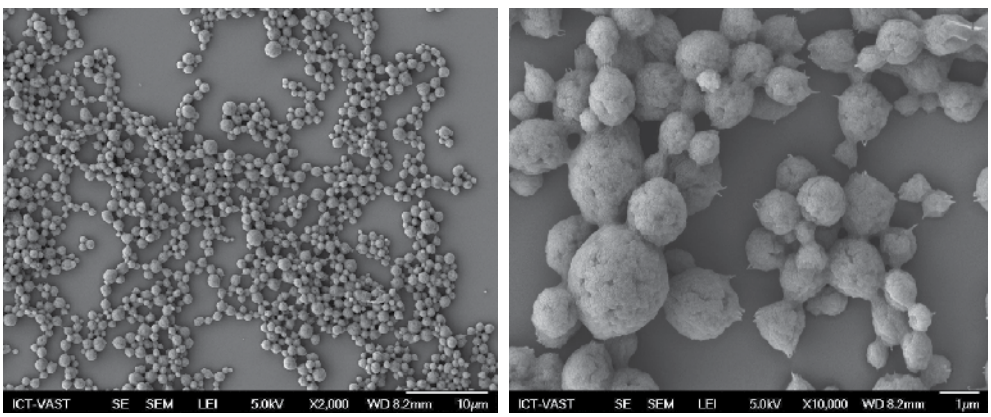

Figure 5. Nanofibroin particles

The cocoon shell of silkworm Bombyx mori was degummed in boiling solution of 0,5% Na2CO3 in 700C for 35 min. Then degummed fiber was dissolved in a mixed solution of calcium chloride, ethanol, and water (CaCl2/C2H5OH/H2O: 1:2:8 mole ratio), at 800C. After the silk fibroin–salts solution was centrifuged at 5000 rpm for 10 min, the supernatant was dialyzed continuously for 72 h against running pure water to remove CaCl2, smaller molecules, and some impurities. The resulting liquid silk fibroin was stored at 40C and used in the following experiments for the preparation of silk fibroin nanoparticles. Spider silk proteins form nanoparticles upon salting out with potassium phosphate. Milk-like silk protein particles

were formed at once and suspended in the mixture comprising water and organic solvent. These protein particles were water insoluble and went down slowly due to the gathering of microparticles. The precipitates of silk protein nanoparticles were collected and purified from the mixture by repeated centrifugation at 20,000 rpm to separate these particles from the solvent. After the research, we have obtained nanoparticles (500 nm-2000 nm) from silk protein can load and delivery of proteins in vitro.

2.3.2. Application

The silk bio-polymer is used in tissue regeneration for treating burn victims and as matrix of wound healing. The silk fibroin peptides are used in cosmetics due to their glossy, flexible, elastic coating power, easy spreading and adhesion characters [21]. Silk powder is touted and relieves from sunburns, due to crystalline structure it reflects UV radiation and as demulcent it acts as protective buffer between skin and environment. The lower micron silk powder is added with hair and massage oils and water dispersible finer grade silk powder is an ingredient of liquid cosmetic preparations.

The silk is used to fight edema, cystitis, impotence, adenosine augmentation therapy, epididymitis and cancer [22]. Silk protein derivative, Serratio peptidase is used as anti-inflammatory, anti-tumefacient for treating acute sinusitis, tonsiloctomy, oral surgery, tooth filling, cleaning and extractions. The silk fibroin is a useful dressing material with the property of non-cytotoxic to the tissues and also in veterinary medication.

Since long, silk fiber is being used as surgical sutures as it does not cause inflammatory reactions and is absorbed after wounds heal. Other promising medical applications are as biodegradable micro tubes for repair of blood vessels and as molded inserts for bone, cartilage and teeth reconstruction [23, 24, 25]. In biomedical and bioengineered field, the use of natural fibre mixed with biodegradable and bio-resorbable polymers can produce joints and bone fixtures to alleviate pain for patients.

Drug delivery is a rapidly developing field in biomedical research. It is interdisciplinary and requires expertise in biotechnology, pharmacology, microbiology, biochemistry, polymer chemistry and materials engineering. Advantages of using such systems include maintenance of drug levels within desired range, fewer administrations, optimal use of the drug, and better patient compliance. The material used for drug delivery should be biocompatible, chemically inert, easily processable and physically and mechanically stable. Biopolymers are of great interest for this kind of application. Silk and silk-like variants are used by some scientists as carriers for drug delivery. Their biocompatibility and ability to form hydrogels in situ makes them attractive candidates for the localized, controlled delivery of therapeutic agents. Their ability to incorporate drugs at room temperature, by simple mixing, and without the use of toxic or denaturing solvents makes them attractive for the delivery of protein or DNA-based therapies [26].

Future applications of silk biomaterials include new generation soft contact lenses that enable greater oxygen permeability, artificial corneas, skin grafts and epilepsy drug permeable devices.

2.4. Fibrin

2.4.1. Structure

The mechanism of fibrin fomation is elucidated primarily from the thrombin-mediated cleavage of fibrinogen. Fibrinogen, the principal protein of blood clotting, is a 340 kDa trinolar protein which presents at high concentration in blood plasma (2 – 4 mg/ml, 6 – 12 μM). Fibrinogen molecule consists of three different pairs of polypeptide chains (Aα, Bβ and γ) cross-linked to each other by 29 disulfide bridges (Fig. 1). The amine-termini (N-termini) of six polypeptide chains are converged in the central of fibrinogen molecule called the E domain. The carboxy-termini (C-termini) of the Bβ chain and γ chain comprise of the distal D domain. The C-termini of Aα chains which are known as globular, depart from the D domain and fold into a conformation that stretches back toward the E domain of fibrinogen [27].

Fibrinogen plays as precursor protein of fibrin in blood clotting. The conversion of fibrinogen to fibrin occurs in 3 ordered steps. In the intinial step, thrombin binds to the central E domain of fibrinogen and slipts off the fibrinopeptides A – FpA (16 amino acid residues) and B – FpB (14 amino acid residues) from N-termini of Aα and Bβ chains, respectively, whereas the γ chains remain unaltered. The cleavage of FpA and FpB results in exposure of "A" and "B" binding sites. Then, the self-assembly proccess will spontaneously occur. The "A" and "B" sites will interact with complementary sites ("a" and "b" sites located in the γ and β chain) at the D domain of other fibrinogen molecules, which results in new fibrin monomers. The fibrin monomers are bound to each other non-covalently (Fig. 2) and assemble in a half-staggered manner into two-stranded protofibrils which continue to aggregate laterally to form fibers branching into a three-dimensional network of fibrin [28, 29]. Finally, fibrin cross-linking is activated by Factor XIII (FXIII) in order to improve the strong and elastic properties, additionally, avoid fibrinolysis in solution [30, 31].

2.4.2. Fabrication of fibrin gel

According to the usage purposes, some methods have been applied to fabricate fibrin gels. Fibrin gels can be conducted either from the separating components including thrombin, fibrinogen and CaCl2, or from serum of patients, which will be mentioned in two following methods, respectively. For manipulation of fibrin gel from separating commercial components, fibrin gels were prepared by combining fibrinogen, NaCl, thrombin, CaCl2. This complex is also supplemented with aprotinin in order to proving a stable fibrin structure and prevents postoperative bleeding. The contents are allowed to gel for 1 hr in standard culture conditions [32]. Furthermore, fibrin gel physical properties can be manipulated by adjusting the fibrinogen and $CaCl_2$ concentration [33, 34], or using different cross linking agents such as enzymes or UV radiation [35]. In terms of autologous fibrin glue, the patient blood is havested and prepared 3 to 4 days before surgery. The plasma is separated from red blood cells by allowing the blood tube to stand vertically for at least 2 hours or centrifuged at 4000 rmp for 5 minutes. The fibrin gel preparation is created by combining plasma with commercial thrombin and calcium at appropriate concentration [36]. Autologous plasma fibrin gel not only

shows an excellent hemostatic agent, but also helps eliminate the risk of viral transmission associated using donor plasma.

2.4.3. Fibrin gel applications

Numerous studies have exploited fibrin function as heamostatic plug, scaffold for cell proliferation and migration, and wound healing, which suggest fibrin potential applications in medical and tissue engineering. Fibrin glue or fibrin sealant is a formulation of fibrinogen and thrombin at very high amounts cobined with calcium and FXIII, used as an adjunct to hemostasis in patients undergoing surgery. Commercial products of fibrin sealant such as Tisseel (Immuno, Vienna, Austria), Beriplast (Behringwerke AG, Marburg/Lahn, FRG), and Biocol (CRTS, Lille, France) have been extensively used in clinical. In addition to fibrin'role in heamostatic, fibrin is also indicated as biological scaffold for cell proliferation, migration and differentiation applied in various tissue engineering. Natural fibrin matrix consists of sites for cellular binding, and has been shown to have excellent effects in cell culture and accelabrate tissue regeneration. In 2000, Ye et al. fabricated and investigated the three-dimensional fibrin scaffold in cardiovascular tissue engineering. In this research, human myofibroblasts (MFBs) from the ascending aorta were cultured in fibrin gel solution. Consequently, the cell growth, high collagen secretation and tissue development were determined. Besides, toxic degradation or inflammatory reactions was not detected in the fibrin gels [37]. In 2003, W. Bensa.ıd and colleagues conducted a research in which they use fibrin glue as a delivery system for human MSCs (HMSCs). The result confirmed a good good HMSCs spreading and proliferation in the fibrin scaffold. Besides, the HMSCs migration out of the fibrin scaffold and appearance of calcium carbonate from the differentiation of HMSCs when implanted in vivo suggest that fibrin gel is a promising delivery system for HMSCs toward bone healing application [38]. Fibrin glue also performs its role in the application of skin grafts to burned areas. Using fibrin glue instead of sutures or pressure dressings in the immediate postoperative period enhances healing, and minimizes scarring [39]. One of commercial fibrin sealant products used for burn treatment is ARTISS fibrin sealant (Baxter International Inc., USA). ARTISS fibrin sealant is indicated to adhere autologous skin grafts to surgically prepared wound beds resulting from burns, for both adults and pedipatients.

3. Polysaccharide

3.1. Cellulose

3.1.1. Structure

Cellulose is the most abundant polymer on Earth, which makes it also the most common organic compound. Annual cellulose synthesis by plants is close to 10^{12} tons. Plants contain approximately 33% cellulose whereas wood contains around 50% and cotton contains 90%. Most of the cellulose is utilised as a raw material in paper production. This equates to approximately 10^{8} tons of pulp produced annually. From this, only 4 million tons are used for further

chemical processing annually. It is quite clear from these values that only a very small fraction of cellulose is used for the production of commodity materials and chemicals [40]. Cellulose, a linear polysaccharide of up to 15,000 D-glucose residues linked by β-(1→4)-glycosidic bonds, is biocompatible and has excellent thermal, mechanical properties. It is considered easily biodegradable, thus less contaminating to the environment.

Cellulose is regarded as a semi-flexible polymer. The relative stiffness and rigidity of the cellulose molecule is mainly due to the intramolecular hydrogen bonding. This property is reflected in its high viscosity in solution, a high tendency to crystallise, and its ability to form fibrillar strands. The chain stiffness property is further favoured by the β-glucosidic linkage that bestows the linear form of the chain. The chair conformation of the pyranose ring also contributes to chain stiffness. This is in contrast to the α-glucosidic bonds of starch [41].

Plants are an attractive cellulose source primarily because they are abundant and there is a preexisting infrastructure in the textile industries for harvesting, retting/pulping (i.e. to treat and isolate micron sized cellulose particles), and product processing. Tunicates are the only animals known to produce cellulose microfibrils. Tunicates are a family of sea animals that have a mantle consisting of cellulose microfibrils embedded in a protein matrix. It is this thick leathery mantle in their mature phase that is used as a source of cellulose microfibrils. Most research has used a class of Tunicates that are commonly known as ''sea squirts'' (*Ascidiacea*), marine invertebrate filter feeders. Several algae species such as green, gray, red, yellow-green… produce cellulose microfibrils in the cell wall. There are considerable differences in cellulose microfibril structure between the various algae species caused by differences in the biosynthesis process. Most cellulose microfibril researchers have used various species of green algae. Bacterial cellulose (BC) is a glucose polymer produced through bacterial fermentation. This macromolecular polymer features the same molecular formula and properties of natural cellulose. A fiber bundle of 40 to 60 nm thick is formed by micro-fibers with a diameter range of 3 to 4 nm. These bundles aggregate randomly to produce a developed structure forming a typical type of nanobiomaterial [42].

Cellulose derivatives and composites offer an excellent biocompatibility, and are considered as promising materials for biochemical engineering for economic and scientific reasons.

- Oxidized cellulose (oxycellulose) is cellulose in which some of the terminal primary alcohol groups of the glucose residues have been converted to carboxyl groups. Therefore, the product is possibly a synthetic polyanhydrocellobiuronide and that contain 25% carboxyl groups are too brittle and too readily soluble to be of use. Those products that have lower carboxyl contents are the most desirable[43].

- Purified microcrystalline cellulose (MCC) is partially depolymerized cellulose prepared by treating α-cellulose, obtained as a pulp from fibrous plant material, with mineral acids. Silicified MCC (SMCC) is manufactured by codrying a suspension of MCC particles and colloidal silicon dioxide such that the dried finished product contains 2% colloidal silicon dioxide. SMCC shows higher bulk density than the common types of MCC. Also, tensile strength of compacts of SMCC is greater than that of the respective MCC and it is most probably a consequence of intersurface interactions of silicon dioxide and MCC [44].

- The esterification can be considered as a typical equilibrium reaction in which an alcohol and acid react to form ester and water. Cellulose is esterified with certain acids such as acetic acid, nitric acid, sulfuric acid, and phosphoric acid. A prerequisite is that the acid used can bring about a strong swelling thus penetrating throughout the cellulose structure. Cellulose acetate phthalate is a partial acetate ester of cellulose that has been reacted with phthalic anhydride. One carboxyl of the phthalic acid is esterified with the cellulose acetate. The finished product contains about 20% acetyl groups and about 35% phthalyl groups [45].

3.1.2. Preparation

The treatments for wood and plants involve the complete or partial removal of matrix materials (hemicellulose, lignin, etc.) and the isolation of individual complete fibers. Fortunicate the treatment involves the isolation of the mantel from the animal and the isolation of individual cellulose fibrils with the removal of the protein matrix. Treatments for algal cellulose sources typically involve culturing methods, and then purifying steps for removal of algal wall matrix material. Bacterial cellulose treatments focus on culturing methods for cellulose microfibrillar growth and then washing to remove the bacteria and other media.

The general processing of engineered BC materials can be considered to occur in four main stages: (1) BC culturing, (2) pellicle management, (3) water removal, and (4) chemical modification. For stage 1, the biosynthesis of BC occurs in culture solutions, generally in a bioreactor, in which bacteria secrete cellulose microfibrils, producing an interwoven web of fibrils that is a hydrogel. The hydrogels are composed of entangled cellulose microfibrils formed from the random motion of the bacteria, contain upwards of 99% water, and are called pellicles. For stage 2, pellicle management refers to any process imparted on the pellicle up until the point of water removal. To remove the bacteria from the pellicles, the pellicles are washed by boiling in a low concentration (2%) NaOH bath for several hours, then it is rinsed under running water for several days. Additional NaOH and NaClO treatments have also been used for further purification of the BC microfibrils. For stage 3, once the pellicle is formed and purified, a sample is cut from the gel-like sheet. Water removal either by evaporation or a combination of pressing and evaporation collapses the gel-network and produces a dense film. For stage 4, chemical modification to the BC microfibril network can be achieved at three points along the engineered BC material processing, (i) during stage 1, (ii) during stage 2, and (iii) after stage 3 (i.e. to dried BC structures or films) [42].

3.1.3. Application

Cellulose is extensively used as a raw material in the paper industry in the production of paper and cardboard products. However, cellulose has shown its versatility in numerous applications.

Natural cellulose spheres are often applied in bioseparation, immobilized reaction, cell suspension culture, and as an adsorbent for sewage treatment. Spherical BC produced from dynamic method is translucent, loose, porous, and has a hydrophilic network structure. Its specific surface area increases with decreasing spherical diameter, so it could be used as a

carrier to adsorb or crosslink various kinds of substances (e.g., enzyme, cell, protein, nucleic acid, and other compounds). Spherical BC may be applied in bioseparation, immobilized reaction, cell suspension culture, and as an adsorbent for sewage treatment. Compared with natural spherical cellulose, the fermentation production of BC spheres is simple, controllable and environment friendly. Moreover, BC sphere can be used repeatedly, expanding their potential applications.

Cellulosic derivatives such as cellulose acetate, cellulose propionate and cellulose acetate-butyrate, cast as membranes, have been reported as useful supports for immobilizing various enzymes such as catalase, alcohol oxidase and glucose oxidase. These supports gave better activity and storage stability for the enzymes. Cellulose ethers are widely used as important excipients for designing matrix tablets. On contact with water, the cellulose ethers start to swell and the hydrogel layer starts to grow around the dry core of the tablet. The hydrogel presents a diffusional barrier for water molecules penetrating into the polymer matrix and the drug molecules being released. Cellulose acetate butyrate microcapsules, as well as cellulose-based microspheres, have been used for the delivery of drugs [46].

Microbial cellulose synthesized by *Acetobacter xylinum* shows considerable potential as a novel wound healing system, resulting from its unique nanostructure. During the process of biosynthesis, various carbon compounds of the nutrition medium are utilized by the bacteria, then polymerized into single, linear β-1,4-glucan chains and finally secreted outside the cells through a linear row of pores located on their outer membrane. Cellulose derived from *Acetobacter xylinum*, as discussed above in the context of wound healing, has also been explored as a potential scaffold material, due to its unusual material properties and degradability. Moreover, bacterial cellulose derived from *Acetobacter xylinum* has an ultrafine network architecture, high hydrophilicity, and mouldability during formation. In addition to the applications discussed, it is also suitable for use in micronerve surgery and as an artificial blood vessel suitable for microsurgery [47].

3.2. Chitin-Chitosan

3.2.1. Structure

Chitin is a white, hard, inelastic, nitrogenous polysaccharide found in the exoskeleton as well as in the internal structure of invertebrates. Chitin is a hydrophobic linear polysaccharide derived from many natural sources including the exoskeleton of arthropods and insects and is the second most abundant natural polysaccharide next to cellulose. Chitin comprises a polysaccharide consisting of (1→4)-β-N-acetyl-D-glucosamine units. Derivatives of chitin may be classified into two categories; in each case, the N-acetyl groups are removed, and the exposed amino function then reacts either with acyl chlorides or anhydrides to give the group NHCOR or is modified by reductive amination to $NHCH_2COOH$ of greatest potential importance are derivatives of both types formed by reaction with bi or polyfunctional reagents, thus carrying sites for further chemical reaction [48]. In practice, such reactions are carried out on native chitin or on incompletely deacetylated chitin, chitosan, so that the resulting polymer contains three types of monomeric units.

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Chitosan is a partially deacetylated derivative of chitin and is the second most abundant biosynthesized material. Structurally, chitosan is a mixture of N-acetyl-D-glucosamine and D-glucosamine [49]. Generally, chitosan is insoluble in neutral or basic conditions, while protonation of free amino groups facilitates solubility of chitosan in dilute acids (pH < 6). In vivo degradation of chitosan is mainly attributed to the effect of lysozyme through hydrolysis of acetylated residues.

Chitosan itself chelates metal ions, especially those of transition metals, and also finds application as a matrix for immobilization of enzymes. Special attention has been given to the chemical modification of chitin, since it has the greatest potential to be fully exploited. Reactions with pure chitin have been carried out mostly in the solid state owing to the lack of solubility in ordinary solvents. A 50% deacetylated chitin has been found to be soluble in water [50]. This water soluble form of chitin is a useful starting material for its smooth modifications, through various reactions in solution phase. Some of the very recently reported chitosan derivatives are enumerated as follows:

- Fully deacetylated chitosan was treated with phthalic anhydride to give N-phthaloyl-chitosan. It was readily soluble in polar organic solvents. Further reactions had been carried out using this new derivative to improve the solubility of chitosan [51].
- To improve water solubility, Sashiwa *et al.* has successfully synthesized dendronized chitosan-sialic acid hybrids by using gallic acid as focal point and tri(ethylene glycol) as spacer arm. The water solubility of these novel derivatives was further improved by N-succinylation of the remaining amine functionality [52].
- Recently, Baba *et al.* have synthesized methylthiocarbamoyl and phenylthiocarbamoyl chitosan derivatives to examine the selectivity toward metal ions from aqueous ammonium nitrate solution [53].
- The synthesis of chitosan hydrogels was carried out by Qu *et al.* by direct grafting of D,L-lactic and/or glycolic acid onto chitosan in the absence of catalysts. They demonstrated that a stronger interaction existed between water and chitosan chains after grafting lactic and/or glycolic acid. The side chains could aggregate and form physical crosslinking, which results in pH sensitive chitosan hydrogels [54].

3.2.2. Preparation

Chitin is easily obtained from crab or shrimp shells and fungal. In the first case, chitin production is associated with food industries such as shrimp canning. In the second case, the production of chitosan–glucan complexes is associated with fermentation processes, similar to those for the production of citric acid from *Aspergillus niger*, *Mucor rouxii*, and *Streptomyces*, which involves alkali treatment yielding chitosan–glucan complexes. The alkali removes the protein and deacetylates chitin simultaneously. Depending on the alkali concentration, some soluble glycans are removed. The processing of crustacean shells mainly involves the removal of proteins and the dissolution of calcium carbonate which is present in crab shells in high concentrations. The resulting chitin is deacetylated in 40% sodium hydroxide at 120^0C for 1–3 h. This treatment produces 70% deacetylated chitosan [55].

3.2.3. Application

Chitin and chitosan are known for their excellent biological properties, among which the biocompatibility with human cells, the ordered regeneration of wounded tissues, the immunoenhancing activity, the induction of immediate hemostasis, the radical scavenging activity, and the antimicrobial activity. Recent studies indicate that chitin and chitosan are most versatile in drug and gene delivery, elaborated diagnostics, devices for selective recognition of tumor cells, and surgical aids ranging from anti-adhesion gels to coated sterile stents.

As a kind of renewable resource, unmodified chitosan has been widely used in many fields such as pharmaceutical, agriculture, food, and biomedical applications. In order to realize the full potential of chitosan and bring a breakthrough in its broader utilization, attempts have been made to modify chitosan to obtain various derivatives. For the tissue repair and regeneration applications, chitosan can be functionalized by chemical reaction, coupling with specific ligands or moieties, combining with biomacromolecules, and crosslinking in the presence or absence of crosslinkers.

Particularly, skin substitute made of chitosan or its derivatives have attracted much attention due to the outstanding characteristics of chitosan, such as biocompatibility, hemostatic activity, antibacterial property, and ability to accelerate the wound-healing process [56].

The design of artificial kidney systems has made possible repetitive hemodialysis and the sustaining life of chronic kidney failure patients. Chitosan membranes have been proposed as an artificial kidney membrane because of their suitable permeability and high tensile strength. The most important part of artificial kidney is the semipermeable membrane and so far made from commercial regenerated cellulose and cuprophane. Since the primary action of the cellulose membrane is that of a sieve, there is little selectivity in the separation of two closely related molecules. These novel membranes need to be developed for better control of transport, ease of formability and inherent blood compatibility.

A series of membranes prepared from chitin and its derivatives improved dialysis properties. One of the most serious problems of using these artificial membranes is surface induced thrombosis, where heparization of blood is needed to prevent clotting, and people who are liable to internal hemorrhage can be dialysed only at great risk. Hence, these are the most challenging problem still to be resolved in the development of membranes which are inherently blood compatible. From these point of views, chitosan is hemostatic, i.e., causes clots [57].

Chitosan has replaced the synthetic polymers in opthalmological applications. Chitosan possesses all the characteristics required for an ideal contact lens; optical clarity, mechanical stability, sufficient optical correction, gas permeability, partially towards oxygen, wettability, and immunologically compatibility. Contact lenses are made from partially depolymerized and purified squid pen chitosan by spin casting technology, and these contact lenses are clear, tough, and possess other required physical properties such as modulus, tensile strength, tear strength, elongation, water content, and oxygen permeability. Antimicrobial and wound healing properties of chitosan along with excellent film forming capability make chitosan suitable for development of ocular bandage lens [58].

The special attention on chitosan has been paid for the repair of articular cartilage. Articular cartilage is particularly vulnerable to injury trama, disease or congenital abnormalities because of its avascular, alypmhatic and aneural nature. Once damaged, it has little capacity for intrinsic repair. Although many repair techniques have been attempted over the past four decades, but none has succeeded to regenerate long-lasting hyaline cartilage tissue to replace defected or damaged cartilage. Recently, preliminary studies on chitosan-GAG composite and its biologically interaction with articular chondrocytes showed promising results. Chitosan and its derivatives are being extensively used for bone tissue engineering and central nervous system also.

The growth of *Escherichia coli* was inhibited in the presence of chitosan. Chitosan also inhibited the growth of *Fusarium*, *Alternaria* and *Helminthosporium*. The cationic amino groups of chitosan probably bind to anionic groups of these microorganisms, resulting in growth inhibition. Extracellular lysozyme activity was enhanced in in vitro cultures of several mammalian cells by treatment with chitin and its derivatives. As a result, connective tissue formation was stimulated, and the self-defence function against microbial infection was enhanced at the cellular level. On the basis of these results, several chitin and chitosan dressing materials have been developed commercially for the healing treatment of human and animal wounds [59].

Chitosan is non-toxic and easily bioabsorbable with gel-forming ability at low pH. Moreover, chitosan has antacid and antiulcer activities which prevent or weaken drug irritation in the stomach. Also, chitosan matrix formulations appear to float and gradually swell in an acid medium. All these interesting properties of chitosan make this natural polymer an ideal candidate for controlled drug release formulations [60].

4. Decellularization

Autologous grafts are “gold standard” for implantation. However, the most disadvantage of autologous is quantity. The number of autograft does not meet needs of patients. Homograft is greater than autograft but they cannot satisfy needs of patient. Many patients must wait for a long time to take a homogenous organ. Xenograft is the greatest but they can evoke serious immune reaction. So, one method developed to process homograft and xenograft is decellularization. Every tissue/organ concludes cells and extracellular matrix. Cells are structure and functional units of tissue/organ but cells are major antigen of tissue/organ. Extracellular matrix is many protein, polysaccharide, protoglycan released by cell. ECM plays an important role in mechanical support, signal transportation, adherence of tissue/organ. Decellularization is a multi-step process to remove all cell components from tissue/organ and leave intact ECM. Many decellularization agents were researched such as physical methods, chemical methods and enzyme methods. Every decellularization agent has specific affections of cell and extracellular matrix. So, these agents are combined to make an effective decellularization process which removes all cell components and reverses maximum ECM. Decellularization effectiveness depends on type of tissue/organ. One agent can be a good detergent for decellularizing one tissue but not for another [61]. Moreover, cell derived ECM can be used as a matrix for cell culture.

Method	Mode of action	Effects on ECM
Physical		
Snap freezing	Intracellular ice crystals disrupt cell membrane	ECM can be disrupted or fracture during rapid freezing
Mechanical force	Pressure can burst cells and tissue removal eliminates cells	Mechanical force can cause damage to ECM
Mechanical agitation	Can cause cell lysis, but more commonly used to facilitate chemical exposure and cellular material removal	Aggressive agitation or sonication can disrupt ECM as the cellular material is removed
Chemical		
Alkaline; acid	Solubilize cytoplasmic components of cells; disrupts nucleic acids	Removes GAGs
Non-ionic detergents		
Triton X-100	Disrupts lipid–lipid and lipid–protein interactions, while leaving protein – protein interactions intact	Mixed results; efficiency dependent on tissue, removes GAGs
Ionic detergents		
Sodium dodecyl sulfate (SDS)	Solubilize cytoplasmic and nuclear cellular membranes; tend to denature proteins	Removes nuclear remnants and cytoplasmic proteins; tends to disrupt native tissue structure, remove GAGs and damage collagen
Sodium deoxycholate		More disruptive to tissue structure than SDS
Triton X-200		Yielded efficient cell removal when used with zwitterionic detergents
Zwitterionic detergents		
CHAPS	Exhibit properties of non-ionic and ionic detergents	Efficient cell removal with ECM disruption similar to that of Triton X-100
Sulfobetaine-10 and -16 (SB-10, SB-16)		Yielded cell removal and mild ECM disruption with Triton X-200
Tri(n-butyl)phosphate	Organic solvent that disrupts protein–protein interactions	Variable cell removal; loss of collagen content, although effect on mechanical properties was minimal
Hypotonic and hypertonic solutions	Cell lysis by osmotic shock	Efficient for cell lysis, but does not effectively remove the cellular remnants
EDTA, EGTA	Chelating agents that bind divalent metallic ions, thereby disrupting cell adhesion to ECM	No isolated exposure, typically used with enzymatic methods (e.g., trypsin)
Enzymatic		
Trypsin	Cleaves peptide bonds on the C-side of Arg and Lys	Prolonged exposure can disrupt ECM structure, removes laminin, fibronectin, elastin, and GAGs
Endonucleases	Catalyze the hydrolysis of the interior bonds of ribonucleotide and deoxyribonucleotide chains	Difficult to remove from the tissue and could invoke an immune response
Exonucleases	Catalyze the hydrolysis of the terminal bonds of ribonucleotide and deoxyribonucleotide chains	

Table 1. Overview of decellularization methods (Thomas W. Gilbert)

Today, many decellularization grafts were applied in clinical treatment such as acellular valve, vascular and some are commercial include: SurgisSIS (porcine small intestinal submicosa), Alloderm (human dermis), ACell (porcine urinary bladder)… Small intestine contains four layers such as mucosa, submucosa, muscularis externa and serosa. Small intestine submucosa (SIS) is a submucosa tissue between mucosa and muscularis externa. SIS is isolated from small intestine by mechanically removing of internal mucosal layer and outer muscular layer. Then, SIS is processed step-by-step with 0.1% peracid acid, 0.05% gentamycin and sterilized using 2500 kRad gamma irradiation. SIS is consisted of collagen, proteoglycan, glycosaminoglycan, glycoprotein and growth factor (VEGF, FGF-2 …). Most of these components can be preserved in extracellular matrix after decellularizing. In tissue engineering, SIS is used as soft tissue grafts such as vascular, skin or used for reconstructing genitourinary, ligament tissue [62].

4.1. Vascular tissue engineering

Atherosclerosis is the most dangerous cardiovascular disease. Atherosclerosis is a condition in which vascular wall is harden, thicken because of fatty lipid accumulation. Atherosclerosis is responsible for millions of death all the world every year. In 2004, seventeen million people passed away because of cardiovascular disease all over the world (29% world total death). More than 7 million people are killed by coronary disease (9.6% world total death) (WHO). Heart American Association, in 2006, coronary disease was responsible for 17.6% death in America. One out of six American died for coronary disease [63].

Today, there are some methods to cure atherosclerosis including drug treatment, coronary artery bypass surgery and angioplasty. However, these methods can not cure this condition completely. More than 30% patients don't have qualified autogenous vascular grafts. Therefore, artificial grafts are made to overcome some current drawbacks.

Artifical graft can be classified into synthetic and biologic scaffold. Synthetic scaffolds include undegradable polymers such as Dacron, ePTFE and degradable polymers such as polylactic acid (PLA) and polyglycolic acid (PGA). Some advantages of synthetic polymers are easy processing, high initial strength, however, they have some disadvantages such as slow recellularization, poor ability to support remodeling. Biologic vascular grafts are either obtained by manipulating native proteins of vascular vessels such as collagen, elastin… or by decellularizing vascular vessels. Decellularized native vascular vessels have some advantages such as slow cost, reduction of graft rejection and immune reaction [64].

In the early time, decellularization studies were tested without cell-seeding. In 1990, Lantz et al use SIS (small intestine submucosa) as small-diameter arterial graft in18 dogs. 48 weeks after surgery, 28 vascular grafts (75%) were patent and tree dogs can survive from 76 to 82 weeks after implantation. This result suggested that small intestinal submucosa can be used in small diameter vascular tissue engineering [65]. In 2001, Hodde et al demonstrated that porcine SIS ECM extract containing vascular endothelial growth factor (VEGF) with concentration of 0.77 ng VEGF/g SIS [66]. In the same year, Nemcova et al isolated and decellularized SIS from bovine and porcine. Nine decellularized SIS grafts were implanted into femoral arteries of five male mongrel dogs. After 9 weeks, eight grafts remained patent and some kinds of cell such

as endothelial cells (ECs), smooth muscle cells (SMCs) invaded into the grafts. No evidence of inflammatory and aneurysmal symptom was observed during the experimental time [67].

However, the main disadvantage of vascular graft is thrombus which ussually occurs immediately in vascular graft lumen after implantation. This process can lead to graft failure and threaten patients' survival. In order to solve this problem, ECs are used as anti-thrombus agent in vascular graft lumen, additionally, SMCs are used to improve mechanical strength of vascular graft. Broschel et al decellularized and recellularized rat iliac arterial grafts. Iliac arterial grafts were decellualrized by glycerin, SDS for 12 hours. Then, decellularized iliac grafts were recellularized with adult rat heart ECs and implanted to femoral arteries of allogeneic rats without systemic anticoagulation injection. After 4 weeks, 2 of 7 control grafts (29%) were patent (decellularized grafts without recellularization) and 8 of 9 (89%) experimental grafts (decellularized grafts with recellularization) maintained blood vessel patent [68]. Consequently, this experiment result proved excellent function of ECs in vascular tissue engineering. Some researchers on vascular tissue engineering seeded autologous vascular cells including ECs, SMCs and fibroblasts to make autologous tissue engineered vascular. At the same time, the appearance of bioreactor systems sped up development of vascular tissue engineering. Niklason developed a pulsatile perfusion bioreactor system in 2001. Bioreactor contained a stirbar, a lid for gas exchange and one (or two) silicone tubing(s). Porous vascular scaffolds were threaded over silicone tubing. Bovine aortic SMCs at the passage 2 or 3 were pipetted onto the outer surface of the scaffolds, then bioreactors were slowly rotated and removed to incubator with 10% CO_2, 100% humidity, and 37°C. Each silicone tubing was linked to a pulsatile perfusion system operated at 165 beats/minute and 260/-30 mmHg. After 8 weeks culture, under nonpulsatile condition, SMC growth was on the outer surface of tubing scaffold and poorly organized. Under pulsatile condition, SMC distribute homogenously in scaffold wall from outer to inner similar to native structure [69].

Nowaday, some decellularized vascular grafts can be obtained from human. Human umbilical veins and arteries can be used in decellularization experiment. Human umbilical arteries were isolated and completely decellularized by Gui et al in 2009. Decellularized umbilical arteries preserved intact collagen matrix and mechanical properties, burst pressure had no signicant change from native form. Decellularized artery graft remained patent after 8 weeks surgery [70]. In 2005, Joel Daniel et al processed human umbilical vein by automated dissection. Human umbilical cord was inserted a mandrel into vein and frozen to -20 and -80°C, human umbilical cord was maintained for 12 hours at least at this temperature. The mandrel was lathed with rotation speed of 2900 rpm, cutting depth of 750 μm. Then, human umbilical vein was decellularized with 1% (w/v) SDS. The result showed that the treated human umbilical vein contained no cell; burst pressure results were 972.8 ± 133.8 mmHg (972/1082), compliance results were 5.7 ± 1.3% over 80 – 120 mmHg. Vascular smooth muscle cells can adhere, proliferate and migrate on the surface of dHUV [71]. Tran Le Bao Ha et al carried out a research on decellularization method for HUVs. HUVs were isolated by manual dissection. HUVs were decellularized by distilled water or NaCl 3M or SDS 1%. The result confirmed that the combination between SDS 1% (24h) and NaCl 3M (24h) showed the best effective on cellular elimination.

http://dx.doi.org/10.5772/55668

Figure 6. Human umbilical cord (a) and human umbilical vien (b)

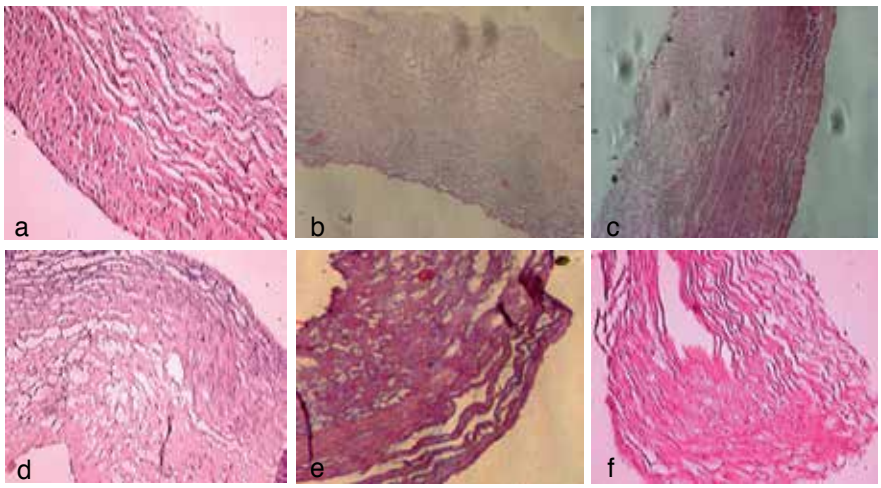

Figure 7. HE staining of HUV with different decellularization methods. Control (a), distilled water (b), NaCl 3M (c), SDS 1% 24h (d), SDS 1% 36h (e), SDS 1% 24. and NaCl 3M 24h (f)

4.2. ECM from cultured cells

One of the most important properties of ECM is its functional diversity. ECM has been reported to support and enhance for adhesion, migration, proliferation of cells as well as to create stem cell niches *in vitro*.. ECM can be harvested from different sources, one of which is from cells under culture condition. When cultured, cells will produce three-dimensional matrix surrounding themselves. A method is described for generating tissue culture surfaces coated with a human fibroblast-derived ECM [72, 73, 74, 75].

For this purpose, human foreskin fibroblasts are isolated, plated and cultured until third passage (see Figure 8). Fibroblasts are maintained in culture medium until reaching 80%

confluency and stimulated to synthesize ECM by culture medium supplemented ascorbic acid. Matrices are denuded of cells and cellular remnants are removed by using Triton X-100, NH_4OH and DNase.

Then, ECM coated culture surfaces are tested by staining with PI to access DNA remnant (see Figure 9), with H&E and PAS in order to characterize component of ECM. The results suggest that fibroblast-derived three-dimensional matrix was determined to be free of cellular constituents and still remain attached to the culture surface. The conducted matrices were washed and covered with PBS; and stored at 4^0C. Under these conditions, biological activity (for example, induction of cell attachment, proliferation…) was reported to be well-preserved for up to 6 months.

In order to evaluate the quality of the ECM, the test of cell rapid attachment ability is performed. Cell proliferation on ECM is also assessed. Results showed that the cultured cells attached and proliferated on ECM coated surfaces faster than on ECM non-coated surfaces.

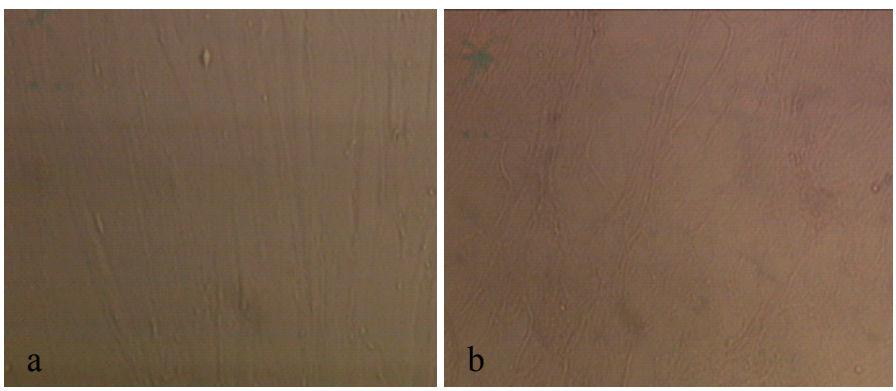

Figure 8. Fibroblasts are in cultured surfaces (a) and are stimulated to synthesize ECM (b)

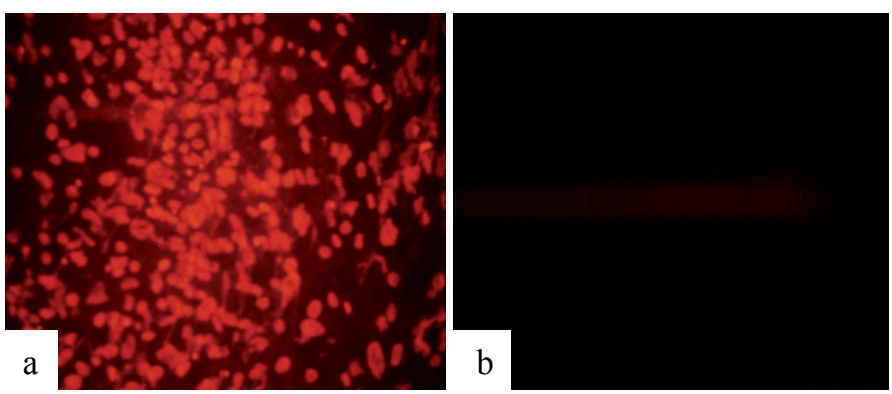

Figure 9. ECM stained PI before (a) and after (b) using DNase

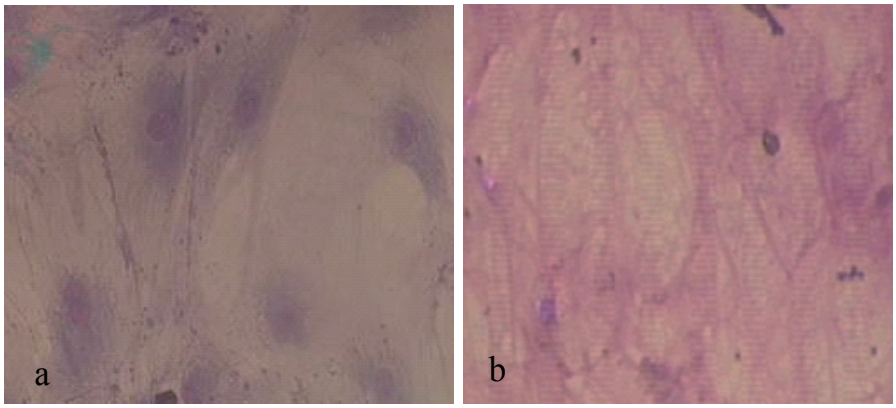

Figure 10. Culture surface before (a) and after (b) is denuded cells

5. Conclusion

In modern society, many diseases have been increasing in human because of pollution, accident, lifestyle... The mutilation in human body leads to expand the needs of replacing tissues/organs. However, the available source of tissues/organs is limited. Creating artificial tissues/organs for replacing damaged, dysfunctional tissues/organs becomes a big discipline on material science. Naturally derived biomaterials have been studied and applied in clinical applications as artificial tissues/organs because they are capable of supporting cell proliferation, biodegradability and remodeling tissues. Although the current results have not completely satisfy the clinical demand, the potential applications of naturally derived biomaterials are still highly considered, therefore, research on this field have now being taken place all over the world.

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Chapter 12

Biomaterials for Cardiac Tissue Engineering

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Cardiovascular diseases

Cardiovascular diseases (CVD) are a leading death cause in developed countries (1 of every 3 deaths in the United States in 2008) [1]. Changes in diet and habits are causing CVD to become major mortality pathologies in developing countries too [2] (they are already responsible for a 30% of the world deaths). This group of diseases constitutes a great burden for the national health systems, consuming great percentages of the health systems budgets. In the particular case of the coronary heart diseases (CHD), 3,8 million men and 3,4 million women die a year worldwide because of them [3]. In the United States 1 of every 6 deaths in 2008 was caused by CHD [1].

The heart is a complex organ that pumps 7000 liters of blood to all the tissues in the body per day [4]. This pumping function precisely determines its anatomy. Heart tissue basically is formed by cardiac myocytes (contractile elements) [5], smooth muscle cells, fibroblasts, blood vessels, nerves and the extracellular matrix components (cardiac interstitium and collagen) [6] organized in a very particular way. Myocytes form muscular fibers with changing orientation across the ventricular wall up to 180º [7]. At the same time, muscular fibers are organized into myocardial laminas 4-6 myocytes thick separated from neighboring laminas by extracellular collagen [8]. The particular arrangement of the ventricular myocytes influence the mechanical and electrical function of the heart and small changes in it can lead to severe changes in these functions [9].

The extracellular matrix (ECM) connects the cells into a 3D architecture allowing the coupling of the forces produced by the myocytes. The anatomical model proposed by Torrent-Guasp [8], which considers the heart one muscle band plied in a double helical loop, explains how the

ventricles contract and get an efficient pumping in every heart beat, achieving an ejection fraction of the 60% when sarcomeres individually contract 15% only [10].

Myocytes are intimately connected, forming a functional syncytium [8]. Each myocardial cell is coupled in average to 9,1 ± 2,2 [11] myocytes, by 99 [12] gap junctions where the transfer of ionic currents takes place. Gap junctions are a specialized form of cell connection; they are formed by a cluster of ionic channels essential to the rapid propagation of the action potential. The action potential is the electrical impulse responsible for the contraction of the cells [13]. A proper electrical coupling of the cells is critical to avoid arrhythmias and reentries and essential for the contraction to spread as a wave front.

Acute myocardial infarction (AMI) occurs when a coronary artery is clogged, in 80% of the cases, by coronary atherosclerosis with superimposed luminal thrombus [14]. This occlusion leaves the downstream zone of the heart without blood supply, what means lack of oxygen, nutrients and metabolites wash for the affected zone. As a consequence, the aerobic metabolism changes to anaerobic glycolysis [14], leading to a decrease in the pH and reduction in the contractile function. Within 20 to 40 minutes without blood supply cells start to die and as times passes more myocardial tissue is compromised. There is also a zone of the heart affected by the infarction, where myocytes remain viable but lower their activity to reduce the metabolism and oxygen consumption to survive under hypoxic conditions; they can recover their contractibility after revascularization [15].

Clinical practices aim to limit the severity and extension of the AMI by rapidly restoring the blood flow (reperfusion), alleviating the oxygen demand [16] and reducing reperfusion injury. This can be done with different treatments or combinations of them. Pharmacological approaches involve the use of anticoagulant therapies and thrombolytic drugs to eliminate the clot. Vasodilatators like nitrates are also used to favor the dilation of the vessels, aspirin to avoid platelet aggregation, betabloqueants to reduce the heart pace, as well as morphine to reduce the pain are employed. Another group of therapies are the percutaneous coronary interventions; they physically reopen the vessel via catheterization. There are different techniques: the regular angioplasty uses a catheter with a balloon that is inflated in the place of the thrombus to reopen the lumen [17], or allows the permanent implantation of a stent in the vessel to keep it open. There is a wide variety of these devices depending on their composition, whether they release drugs or are biodegradable or not, etc [18, 19].

These therapies restore the blood flow to the infarcted zone; but reperfusion therapy is not exempt of risks: it is a complex process that can induce apoptosis by the microenvironmental changes that the recovery of the blood supply induces (formation of free radicals, calcium release, neutrophils, etc.) [20]. So it has to be done carefully and there is always a compromise between limiting the infarction extension due to the time without oxygen and the induced apoptosis due to the reperfusion. Reperfusion done soon after the onset of the ischemia is very advantageous, saving more tissue by restoring the blood flow than the tissue that will be lost because of the toxic substances released in the reperfusion. All the aforementioned treatments basically limit the damage of the acute episode but do not regenerate the damaged tissue and do not avoid the subsequent ventricular remodeling following an AMI.

In the infarcted area there is a great number of dead myocytes, and the host response to the injury consists in activating the inflammatory response and producing cytokines [21]. Thereupon neutrophils, monocytes and macrophages migrate into this area to remove the necrotic tissue [22]. Then, matrix metalloproteases (MMPs) are activated, which have a deleterious effect on the collagen matrix of the heart and in the surrounding coronary vasculature by degrading them [23]. The weakening of the collagen leads to wall thinning and ventricular dilation, as well as mural realignment of myocytes bundles [24]. After the inflammatory phase and the resorption of the necrotic tissue, there is an increase in the deposition of cross-linked collagen in the infarcted area that leads to scar tissue formation. During the remodelling process a change in the collagen composition occurs, the type I collagen fraction is reduced from 80% to 40% and the collagen III is increased [25].

Against what it was thought, this scar is a living tissue with a fibroblast-like cell population nourished by a neovasculature; these cells regulate the collagen turnover of the scar tissue [22]. The scar tissue has a reduced or absent contractility as compared with the original healthy myocardium [26], what leads to a reduction in the overall cardiac function [27].

The remodeling process initially is a compensatory mechanism to overcome the loss of contractile tissue. But with time this adaptative process of overload becomes maladaptative [15]. To compensate the additional effort, the remaining beating tissue hypertrophies trying to overcome the reduction in the cardiac function. This overload leads to myocyte slippage and fibrotic interstitial growth and to a degenerating process that may end in heart failure. The heart remodeling produces in the ventricles a set of anatomical and functional changes, including increased wall stress, slimming of the wall, chamber dilation, increase of the sphericity, and a significant loss of cardiac function.

The ventricular shape change from elliptical to spherical reduces its ejection fraction, because of a change in the apical loop fiber orientation [28]. Another problem caused by the shape change is that the papillary muscles are separated, what leads to regurgitation, contributing to the overload of the heart [24]. Besides, remodeled hearts are more prone to suffer arrhythmias as the membrane potential is altered and because of the interstitial fibrotic growth that may affect conductivity [15].

The end stage of the degeneration is the heart failure, when the heart is unable to pump enough blood to match the metabolic needs of the tissues. Current treatments aim to avoid reaching this point. Pharmacological treatments aspire to reduce the work load and to protect the cardiac tissues from the accumulated harmful substances [29]. Surgical therapy involves different techniques with different objectives: to restore a proper blood flow in areas that lack it (bypass surgery), to restore the normal elliptical geometry (Dor and Batista procedures), to restore the wall stress to normal (Dynamic Cardiomyoplasty), to limit the pathologic dilation, etc [10].

1.2. Cell therapy and cardiac tissue engineering

For many years, the heart has been considered a fully differentiated organ, with no myocyte regeneration after birth [30]. Recently it has been proved that myocytes have a limited regenerative capacity, around 1% of the cells per year at the age of 20 and it is reduced to 0,3%

at the age of 75 [31]. This regenerative capacity is achieved thanks to a small population of cardiac stem cells [32]. Nevertheless, their regenerative capacity is limited and in any case it is not enough to regenerate the heart if it suffers severe damage, like the one provoked by a myocardial infarction. New therapies under development like cell therapy or tissue engineering, aim to boost this limited regenerative potential of the native tissue by employing cells, drugs, factors or patches.

The aim of cardiac cell therapy is to heal the damaged infarcted tissue by the implantation of cells into or onto the pathologic myocardium by different techniques (figure 1 a). In tissue engineering strategies, different types of cells have been combined with materials and with bioactive molecules if necessary to again try to recover the injured tissue. The employed materials will support cells, provide them 3D organization, protect them, stimulate and guide its growth, maintain them in the site of interest, etc.; in sum, they will act as an artificial extracellular matrix during the regeneration process. But the use of materials either injectable, or *ex vivo* conformed (gels –patches- or scaffolds) (figure 1 b) has an additional and important effect: the implantation of a material in the scarred ventricular wall, increases its thickness and by Laplace's law, this increase leads to a reduction in the wall stress. This side-effect could be by itself very positive, even although regeneration did not arrive to happen, to limit ventricular remodeling and improve the quality of life of cardiac patients [29].

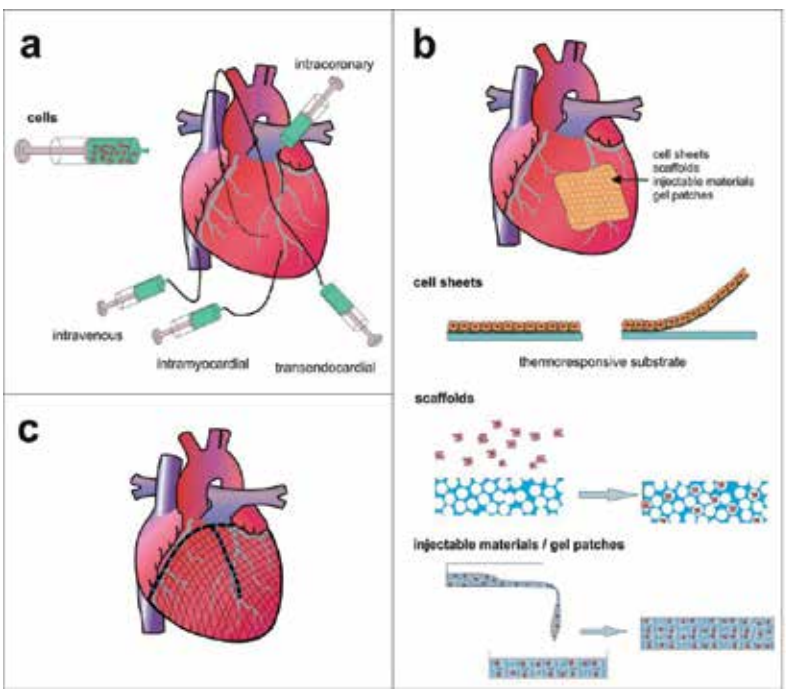

Figure 1. (a) Classical cell therapy in the heart (*freely inspired* in Strauer BE, Kornowski R, *Circulation* 2003; 107: 929-934). (b) Tissue engineering approaches with cell sheets, scaffolds or injectable materials (*freely inspired in* Masuda S *et al*, *Adv. Drug Del. Revs* 2008; 60(2): 277-85). (c) Ventricular restrain device.

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2. Cardiomyoplasty

2.1. Need for cell cardiomyoplasty

Cardiomyoplasty has evolved from "dynamic" to "cellular cardiomyoplasty". The term dynamic cardiomyoplasty is referred to a surgical procedure developed in 1987 [33] to wrap the heart with the latissimus dorsi muscle, aiming to support the heart beating and limit the remodeling. Nevertheless, the obtained results were not as good as expected. With the advances in cell therapy, cellular cardiomyoplasty appeared as a promising therapeutical approach. This name encloses the therapies that use the injection of cells, from different origins, directly into the heart to try to obtain an improvement in the reduced heart function after an ischemic insult (figure 1 a).

The injected cells are envisaged to induce angiogenesis, inhibit apoptosis, help to recover hibernating myocardium, activate endogenous repair mechanisms, and create new contractile tissue that will replace the damaged one. Also they are expected to reverse the remodeling process that provoked ventricular dilation [34]. Many cells have been employed and the initial promising results obtained in animal models made this technique moved very fast to clinical trials, even if the mechanisms involved in the observed improvements were unknown. Unfortunately, the results obtained from the clinical trials were not as good as expected, and some were contradictory between them. One possible contributing cause to this discrepancy is that studies are carried out in young healthy animals, while patients susceptible to receive these treatments normally are aged people and in many cases with other co-morbidities [35].

Different ways to deliver cells into the damaged heart have been explored: intracoronary infusion (with the hope that cells will migrate through the vessels and be hosted in the infarcted area) or directly into the infarcted area either by intramyocardial or endocardial injection [36], as shown in figure 1 a. The advantage of injecting them directly into the infarcted area is that this will ensure that the cells are delivered in the site of interest.

2.2. Related problematic

Many different cell types have been employed in the numerous studies that have been done. Autologous cell sources are interesting because they do not require immunosuppression treatment of the patient and there is no risk of illness transmission. On the contrary, allogenic cells could be ready to use whenever a patient needs them, but would require immunosuppressive therapy after their implantation, and there is always a remaining risk of illness transmission. Another disadvantage is that prior to implantation cells need to be extracted and expanded. This whole process in some cases may take several weeks, limiting its application in the acute state. Besides, autologous cells coming from patients that suffer other conditions like diabetes or are simply aged, may have limited proliferation and attachment [37].

An important aspect of this technique is the low engraftment into the heart tissue of the supplied cells. The retention of the cells in the heart seems to be determined by the cell type and delivery route [38]. It has been estimated that in humans 50-75 min after intracoronary injection of bone marrow cells only 1,3-2,6% of the injected cells remain in the myocardium

[39]; after 2 hours less than 10% of the injected cells survive [32]. Many causes can be advanced: the heart beats, so cells can easily be pumped out of the heart; the solution in which cells are injected has a low viscosity, so cells can be washed away; the mechanical loss of the cells through the injection hole left by the needle, etc [40]. A different contributing cause to the low cell engraftment is that the injured heart is not a cell-friendly environment, type I collagen fibers have been substituted by type III, which has worse properties in terms of adhesion and promoting angiogenesis, what can induce anoikis [4]. Another problem is cell survival itself. The conditions in the infarcted myocardium are very hostile for the cells: hypoxic conditions (studies show that the survival of injected cells decreases towards the center of the scar), cytokines, inflammatory factors, etc., are present in the damaged myocardium, and can negatively affect the survival of the injected cells. Immunological rejection can be another cause reducing cell survival [41].

An interesting approach is to train cells prior to their implantation for them to resist the hostile conditions they will find in the implantation site. For instance, the resistance to hypoxic conditions is key and needs to be improved even for skeletal myoblasts (which are the cells that have better resistance to lack of oxygen). Privation of glutamine reduces the oxygen consumption rate, what has been proved to improve survival of myoblasts when implanted [42].

The fact that most of the cells did not graft into the host myocardium in the studies performed to date, that there is a very limited transdifferentiation of implanted cells into beating cardiomyocytes (the differentiation reported in animals may have been fusion events between native cardiomyocytes and injected cells [41]), and that a wide range of non-myogenic cells also induce an improvement of the ventricular function [36], suggests that the mechanism leading to this enhancement cannot be only myogenesis regenerating the myocardium. The pathways through which cell implantation induces improvements in cardiac function remain to be elucidated, but different events that can take place simultaneously have been proposed. The most remarkable are the induction of angiogenesis (formation of new vessels) and the improvement in the myocardial perfusion, the reduction of the wall stress because of the increase in cell mass [43] and the paracrine effect of the injected cells [32].

2.3. Cell types investigated

As previously said, many cell types from different origins have been employed: embryonic stem cells, mesenchymal stem cells, bone marrow cells, induced pluripotent stem cells, cardiac stem cells, skeletal myoblasts, umbilical cord blood cells and amniotic fluid stem cells, among others. In what follows the use of these cell types is discussed, with the advantages and disadvantages that each one presents for its application in heart regeneration.

Embryonic Stem Cells (ESC)

ESC can be obtained from the inner mass of an embryo in the blastocyst stage. These cells have the capacity of growing undifferentiated indefinitely, and when they differentiate they can form any cell from the three germ layers. But the use of ESC raises ethical issues, requires

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immunosuppression, and has the risk to form theratomes. Their use in clinical trials has been limited because of these ethical considerations and risks [36, 44].

A protocol for ESC differentiation into cardiomyocytes and improving their survival when implanted has been established; when these differentiated cells were implanted in rodent models the heart function was improved [45]. In another study in mice, ESC-derived cardiomyocytes implantation reduced the reactive collagen deposition in the ventricular septum, which is one of the remodeling process hallmarks. Nevertheless, the implanted cells were isolated from the host myocardium by scar tissue, although the implanted cardiomyocytes were able to couple functionally to each other [46].

Induced Pluripotent Stem cells (IPS)

Induced pluripotent stem cells are fibroblasts treated with viral factors to recover their pluripotency. Therefore, IPS do not raise the ethical concerns of the ESC. IPS are very interesting because they can be autologous pluripotent cells. However, their application in clinical trials has been limited precisely for the use of viral vectors that may promote malignancy and act as oncogenes [43], as well as for the intrinsic risk of theratomes inherent to their pluripotency [44].

Adult stem cells

These cells have the advantage of being autologous and can be obtained from different sources like bone marrow or adipose tissue. In addition, they can be expanded *in vitro* and do not raise ethical or immunologic problems [47, 48].

Bone marrow cells (BMC) are easily accessible, can be obtained rapidly and have been reported to have certain plasticity. This property allows them to differentiate *in vivo* into cardiomyocytes [26] (although this fact remains controversial [42]). They can also differentiate into cardiomyocytes *in vitro* by supplementing the medium [49]. Studies in animal models demonstrate that the injection of these cells increases neovasculature improving heart function [42]. But the use of BMC is not exempt of risks: intracoronary administration of them can cause microinfarctions due to their big size and irregular shape, making necessary the use of an alternative way of delivery [50]. In clinical trials, results indicated only temporary benefits or no improvement after cell administration [38, 51]. A strategy to enhance the therapeutic efficacy of BMC is to precondition them: BMC treated with growth factors improve the therapeutic effect when implanted and show greater survival rate [52].

Adipose derived stem cells (ASC) can be obtained in great quantity without culturing them. These cells have been implanted in small animal models of AMI and left ventricular function was improved [48]. The underlying mechanisms are unclear, although the hypothesis of a paracrine effect is considered [53]. Clinical trials are ongoing for the implantation of ASCs: PRECISE and APOLLO [54]. These cells are also are under study at the moment in the RECATABI project [55] as part of a strategy that combines them within a three-dimensional polymer scaffold with a peptide gel filling, to lengthen their positive effect and serve as a mechanical support for the dilated ventricle.

Cardiac Stem Cells (CSC)

CSC are undifferentiated cells found in the heart that can become endothelial cells, smooth muscle cells, and functional cardiomyocytes [36]. In undamaged hearts, these cells seem to contribute to the normal self-renewal of the tissue. CSC can be isolated from biopsies and can be expanded *in vitro* [56], although there is a lack of availability from human origin as they are obtained from biopsies. Human CSC injected in mice hearts after infarction led to functional improvement and to support myocardial regeneration [57]. Currently, autologous cardiosphere-derived cells are being evaluated in the CADUCEUS clinical trial [58].

Skeletal Myoblasts (SM)

SM are cells present in the basal membrane, where they remain in a quiescent state while there is no damage. These cells have better resistance to hypoxic conditions than many other cell types, and can be from autologous origin, but 2 to 3 weeks are necessary to establish and expand myoblasts from skeletal muscle biopsies [36]. These cells are capable to contract; that is the reason why they were expected to attach to the beating cardiomyocytes and contribute to the effective beating by integrating in the working syncytium muscle. Nevertheless, there is no electro-mechanical coupling between the implanted cells and the native cardiomyocytes. This absence of coupling turns the implanted cells into a pro-arrhythmic substrate [44]. The cause for this uncoupling is the lack of the gap junctional protein connexin 43. Therefore, the implantation of a pacemaker or a defibrillator to avoid malignant arrhythmias and sudden death would be necessary when implanting these cells, to obtain a synchronous beating of the heart and the grafted cells [26, 59]. Despite the lack of electro-mechanical coupling of the myoblasts with the host cardiac cells, improvements in the ventricular performance have been observed in animal models, even with a reduced number of grafted cells, suggesting a cytokine-mediated effect [46].

The encouraging preliminary results and its autologous origin made this cell type the first to reach clinical trials. Initial clinical trials carried out with these cells showed symptomatic improvements in the patients, but some of them experienced arrhythmias, making necessary the use of implantable defibrillators [36]. For instance, in the phase II randomized placebo controlled trial MAGIC [60], skeletal myoblasts and a cardioverter defibrillator were implanted during a coronary artery by-pass graft surgery.

Umbilical Cord Blood Cells (UCBC)

UCBC can be easily obtained from the umbilical cord and do not present ethical concerns [42]. These cells have certain plasticity and reduced risk of rejection because they show low immunogenicity [25]. Their injection in animal models has been found to improve their left ventricular function [61].

Amniotic Fluid Stem Cells (AFSC)

Amniotic fluid is extracted for prenatal diagnosis and AFSC are isolated from it. They have many characteristics of ESC and seem to be in an intermediate stage between embryonic and adult stem cells in terms of versatility. Interestingly, these cells do not present ethical concerns and do not present risk of tumorogenicity [62].

Human AFSC have been successfully differentiated into endothelial or cardiac lineages *in vitro*. When these cells were implanted in an immunosupressed rat model, they contributed to attenuate its left ventricular remodeling, to preserve the thickness of the ventricle and to improve cardiac function [63].

3. Cell sheets

The use of cell sheets is based on the fact that when cells are cultured in normal flasks and enzymatically digested to detach them, the adhesive proteins and membrane receptors are disrupted leaving the cell damaged [64]. The alternative is to grow cell sheets and then detach them from the culture surface in a way that keeps the electromechanical connections between the cells and benefits from the fact that cells are kept together by their own deposited ECM, as figure 1 b displays. In that way, cells maintain the adhesion and membrane proteins, as well as the natural pro-survival and maturation environmental cues that the ECM provides [65]. Altogether, this is expected to help them to survive when implanted onto the infarcted myocardium.

Cells can be cultured, for instance, on temperature-responsive poly(N-isopropylacrylamide) (PnIPAAm)-coated plates. PnIPAAm is a hydrophobic polymer at 37ºC, and cells can attach to its surface. When the temperature is lowered, PnIPAAm suffers a transition to a hydrophilic state and this change causes the attachments of the cell monolayer to the surface to disrupt, and the entire cells sheet detaches from the surface [65]. Other materials, such as a thermo-responsive methylcellulose hydrogel, have been used to successfully obtain cell sheets fragments of human amniotic fluid stem cells (hAFSCs) [66]. Results obtained with these cell sheet fragments were superior to those with dissociated cells in terms of heart function, cell retention, proliferation and vascular density. Moreover, cardiomyocyte sheets were found to functionally integrate with the host tissue in a rat myocardial infarct model [67]. New techniques based on patterning with a gelatin stamp the thermo-responsive substrates allow obtaining complex tissue structures with cells having a determined orientation [68].

The muscle mass loss following an infarction is significant, up to 50 g [69], so the amount of cells needed to overcome this loss is obviously not covered with a single sheet of cells. On the other hand, when several layers of cell sheets are superimposed, they are easier to handle. Some groups have tried to obtain thicker grafts by overlapping several monolayer cardiomyocyte sheets, which adhere one to another forming gap junctions and intercellular adhesions within minutes [70]. But this approach poses a problem: as cell sheets lack of vascularization, the maximum thickness that can be achieved by overlapping them is limited to the depth at which diffusion of oxygen and nutrients can take place (a maximum of three cardiomyocyte sheets can be piled up). To try to overcome this problem, three-layer thick cardiomyocyte sheets were implanted in rats at 1-, 2- and 3-day intervals [71]; in the time between transplantations it was assumed that there is enough time for the cell sheet to be vascularized. With this approach constructs of 1 mm were obtained successfully. But anyway, this option is very invasive, so its application in patients might be limited.

A different approach based on the same idea of providing cell-cell connections and ECM to the implanted cells to improve their retention and survival is to implant them as spherical cell-bodies. Human amniotic fluid stem cells (hAFSC) cultured in a methylcellulose hydrogel to form cell aggregates were implanted in immunosuppressed rats as cell-bodies, and cell retention and engraftment were enhanced as compared with disaggregated cells. This enhancement led to functional improvement and limited the progression of heart failure [72].

4. Injectable gels

4.1. Rationale

As previously stated, cell cardiomyoplasty presents problems in terms of cell attachment and survival. Cells usually reside in a determined microenvironment which regulates their fate and function. The surrounding ECM with its chemical and biophysical cues is a key element, so the lack of cell-ECM interaction limits their survival [73]. To try to overcome the problems of cells supply, alternative approaches are considered in current studies. The use of natural or synthetic materials in an injectable format, alone or together with cells (figure 1 b), has been investigated to limit remodeling and improve both cell attachment and survival upon implantation in the heart. Ideally, they should be tailored to be amenable to delivery with minimally invasive catheter based procedures [69]. The injectable materials have to cure or self-assemble rapidly (without the need or the release of toxic components) once delivered in the site of interest. As injected, they adopt the shape of the cavity, and may increase the stiffness and thickness of the ventricular wall [74]. Simulations showed that the injection of non-contractile materials with proper mechanical properties can contribute to limit the stress the ventricular wall withstands, thus helping to limit the remodeling [75].

These materials can help to keep the cells in the site of interest, provide them a 3D environment and also protect them from the hostile environment represented by the cytokines and hypoxic conditions, reactive oxygen species, etc., consequence of the infarcted condition of the site [41]. The injected gels can provide a cell friendly environment that will prevent anoikis [69]; they can also include adhesion motifs and then actively contribute to cell attachment. Moreover, they can be used as a controlled release system providing in a sustained way drugs or growth factors to improve cells survival, integration and proliferation [32]. And in the case of bioactive materials, their degradation products may provide additional chemicals that stimulate cells.

Among others, the ideal injectable material should be biodegradable, have a low immunogenity, be no cytotoxic, non-adhesive and have antithrombogenic properties, adequate mechanical properties, provide stiffness to the scar but at the same time being compliant with the heart beating and transmit properly the mechanical stimuli to the cells, induce angiogenesis or at least not disturb the angiogenic activity after incorporation, be capable of delivering cells and or bioactive molecules [76]. Next, some of the materials investigated for their potential use as injectable ones are described.

4.2. *In situ* gelling biomaterials employed

4.2.1. Natural materials

Fibrin

Fibrin is a natural biopolymer that forms the natural provisory matrix for wound healing. It is FDA approved for many applications and there are different preparations commercially available, but it can also be obtained from autologous origin [77]; it is biocompatible, not toxic, or inflammatory [78]. Besides, some of the degradation products of fibrin have interesting properties, like improving healing promotion or a protective effect against myocardial reperfusion injury [79]. Fibrin contains arginine-glycine-asparagine (RGD), which are known cell adhesion motifs [77]; it is cytoprotective for anoxia and provides a favorable microenvironment for cardiomyogenic differentiation of marrow-derived cardiac stem cells [77]. It can also be used as a controlled release system [80]. In sum, fibrin as a gel is a potential candidate to enhance cell adhesion and survival. To obtain the fibrin, fibrinogen monomers in saline solution are mixed with thrombin and they polymerize forming a 3D net by mechanisms similar to normal clotting *in vivo* [81]. The properties of the network can be tailored by modifying the polymerization process.

A concern about translating the fibrin glue for cardiac tissue engineering into the clinic is the risk of inducing intravascular thrombosis [79]. The concentrations of fibrin amenable to delivery through current percutaneous catheters have been studied, demonstrating the feasibility of using fibrin in a non-invasive injectable application [81]. The injection of fibrin alone was proved to preserve left ventricular geometry and cardiac function in a rat acute MI model [82]. But it has also been combined with many types of cells. As an example, it was employed to deliver bone-marrow derived mesenchymal stem cells, which enhanced cell retention and prevented their redistribution in other organs, improving the beneficial effects of the treatment [81]. Injection of fibrin combined with myoblasts [82], bone marrow stem cells [83] or with autologous endothelial cells [84], improved the results obtained with cells alone.

Chitosan

Chitosan (CHT) is a natural cationic polysaccharide, obtained from the deacetylation of chitin of the mollusks, crustaceans and insects. It is soluble in acidic aqueous solution but after neutralization forms a gel-like precipitate [85]. CHT exhibits numerous positive biological and physicochemical properties: biocompatibility, non immunogenicity, and can be conjugated with various molecules thanks to the amino groups on the polysaccharide backbone [86]. A thermally responsive chitosan-based polymer was capable of scavenging the reactive oxygen species produced by the ischemic conditions and recruit key chemokines for stem cell homing such as SDF-1. As a cell delivery system with adipose-derived mesenchymal stem cells, this material was capable of improving the microenvironment for the cells when injected in the infarcted myocardium of rats, improving their survival and engraftment [87]. Chitosan mixed with collagen has been conjugated with QHREDGS (peptide thought to mediate attachment and survival responses of cardiomyocytes) in the format of a thermoresponsive hydrogel to

improve maturation and metabolic activity of cardiomyocytes [86]. Alginate-chitosan nanoparticles have been loaded with placental growth factor (PlGF) to increase the left-ventricular function and vascular density in rats [88].

Matrigel

Matrigel is a commercial ECM proteins mixture that undergoes a temperature mediated sol-gel transition, and is obtained from the ECM of mouse sarcoma cells [27]; its clinical application is limited precisely by the source from which it is obtained. It has been implanted alone and in combination with mouse ESC [89] or neonatal cardiomyocytes [90] into a mice model of infarcted myocardium. The gel prevented worsening of the cardiac function, but animals receiving both Matrigel and cells maintained more wall thickness and preserved better cardiac function in terms of fractional shortening and regional contractility [91].

Hair keratin

Keratin materials can be obtained from hair, importantly from autologous source. More than 30 growth factors are involved in hair morphogenesis, and the residual of them remains in the keratin, what can be beneficial for cardiac repair. Lyophilized keratin powders have the ability to self-assemble upon addition of water, and form gels. Keratin has been implanted onto infarcted rat hearts, and native cardiomyocytes as well as endothelial cells were able to infiltrate the keratin gel, promoting angiogenesis without inducing inflammation; after 2 months animals exhibited preservation of cardiac function and limited ventricular remodeling [92]. These improvements were attributed to the biomaterial's contribution to the mechanical support to the ventricular wall and the presence of cell binding motifs in it.

Alginate

Alginate is a linear block co-polymer of (1-4)-linked β-D-mannuronate and α-L-guluronate residues obtained from seaweed. It is a negatively charged polysaccharide that gels by the presence of calcium ions and is non-thrombogenic [4]. The properties of this material can be tuned either by changing the concentration of the solutions or by controlling the molecular weight. Greater concentrations will increase mechanical strength but also will increase the solution viscosity and the degradation time of the gel [27].

Alginate has been used as an injectable material in recent and old infarcts in rats, and it was observed that its injection augmented the scar thickness and limited systolic and diastolic dysfunction [93]. It has also been proposed as a controlled delivery system: based on the different binding affinity of alginate to insulin-like growth factor-1 IGF-1 and hepatocyte growth factor HGF, a dual delivery system of these factors was developed [94]. The hydrogel beads protected the proteins from degradation maintaining their bioactivity and increasing the therapeutic effect of the system.

Alginate sustains very low protein adsorption and it does not support mammalian cells attachment [95], but it can be combined with adhesion motifs to improve its attachment properties. Its conjugation with RGD increased the arteriole density in a rodent model of chronic ischemic cardiomyopathy [96]. However, the combination of alginate with RGD and tyrosine–isoleucine– glycine–serine–arginine (YIGSR) reduced the therapeutic effects of the

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hydrogel in terms of scar thickness, left ventricular dilation and function [97]. Another modification of alginate has been the addition of the electrical conducting polymer polypyrrole [98], which increases arteriogenesis and promotes myofibroblasts infiltration.

Hyaluronic acid

Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan prevalent in the extracellular matrix of many tissues. HA plays an important role in homeostasis, transport of nutrients and also mediates the inflammation and repair processes. It is biocompatible, non-immunogenic, biodegradable and has different biological activities depending on its molecular weight. Precisely the low molecular weight degradation products of HA stimulate angiogenesis and endothelial cell proliferation and migration [99]. It can be functionalized to improve its biological development, for example with PEG-SH_4 [100]. Moreover, it is a FDA-approved material for its use in humans in certain applications like dermal and intra-articular injection.

There are already commercially available *in situ* crosslinkable HA-derived hydrogels. Different types of HA hydrogels have been compared with commercial fibrin, poly(vinyl alcohol)-chitosan and elastin hydrogels, in terms of *in vitro* degradation rates and cytotoxicity and *in vivo* degradation, immune response and angiogenic potential [76]. Traut's grafted HA hydrogel and periodate oxidated HA hydrogel, especially the first one, demonstrated to be the most suitable for new artery formation in ischemic myocardium because they were both digested within 2 weeks with low immune response and strong angiogenesis compared with the other examined hydrogels.

HA alone does not support cell adhesion. Cardiosphere-derived cells were delivered using a thiolated hyaluronan-based hydrogel crosslinked with thiol-reactive poly(ethylene glycol) diacrylate and covalently linked or not with thiolated denatured collagen. It was observed that the retention rate achieved with the hydrogel without collagen was similar to that of cells delivered in phosphate buffer saline (PBS), either by a low physical retention or poor cell survival and adhesion of HA [101]. In the *in vivo* study in a mouse model of myocardial infarction, some functional benefits were observed though.

Collagen

Collagen supports growth and survival of cardiomyocytes *in vitro*, and is one of the main components of the ECM in the adult heart [102]. Commercial collagen alone has been implanted in animal models showing improvements in ventricular cardiac function and geometry [103]. In another study in a myocardial infarction model in rats (with ischemia-reperfusion model this time) increased capillarity density and myofibroblasts infiltration after 5 weeks were reported [104].

The therapeutic potential of injectable collagen has been evaluated in combination with different cell types. Bone marrow stem cells were injected via catheter in a swine model in combination with collagen, demonstrating the feasibility of a non-invasive delivery of this system [105]. Collagen was also used as a carrier for mesenchymal stem cells (MSC) transplantation to improve the retention of the cells in the infarcted myocardium [106]. 4

weeks after implantation, rats receiving cells in saline suspension, had the implanted cells in remote organs, whereas in animals receiving the cells with collagen, were detected to a lesser extent in remote organs. However, cardiac function was improved in animals receiving cells in saline and collagen alone but not in the combined collagen MSC group. The mechanisms underlying this negative interaction (controverted in other works) are unknown, but is suggested that collagen may limit oxygen and nutrients diffusion, and compromise cell-cell interactions. In another study, collagen combined with chondroitin 6-sulfate was employed to deliver CD-133+ progenitor cells derived from peripherial blood after expansion *in vitro* [107]. It was expected that the material would improve cell adhesion and survival into ischemic hind limb athymic rats. The collagen increased two-fold the number of cells retained when implanted alone; the implanted material was vascularized and the injected cells added into vascular structures.

Gelatin

It is a non-immunogenic partially degraded product of collagen [108]. It has been injected as a hydrogel in rat infarcted hearts bare or loaded with basic fibroblast growth factor; adding the factor improved arteriogenesis, ventricular remodeling and function [109]. Basic fibroblast growth factor has also been delivered with gelatin microspheres [110], inducing angiogenesis and improving cardiac function. The loaded nanoparticles induced an increase in the blood flow in the infarct border (thanks to stimulated angiogenesis), and as a result left ventricular function was improved.

ECM-derived materials

A different approach is based on decellularized tissues, their digestion and injection. This type of materials has the advantage of containing a physiological proportion of the native components of the ECM [102] and cues for cell-matrix interactions. ECM coming from different tissues has been studied, and apparently the ECM of each tissue has its unique combination of proteins and proteoglycans. This makes of myocardial decellularized matrix, among all other tissues matrices, the best candidate for myocardial repair when it is available [111]. Decellularized porcine myocardial tissue able to self-assemble into a nanofibrous structure similar to collagen *in vitro* at 37°C and deliverable *in vivo* upon catheter injection was tested in rats. It induced endothelial cells and smooth muscle cells migration increasing the arteriole formation at 11 days post-injection [111].

Small intestinal submucosa (SIS) is a dense sheet of acellular extracellular matrix. This material is used in the clinic for accelerated wound healing. SIS supports proliferation, attachment and migration of various cell types and stimulates angiogenesis thanks to the growth factors and binding motifs embedded in the matrix. Two different types of commercial available SIS-derived gels have been studied as an injectable material for cardiac repair in a murine model [112]. The two materials differed in the concentration of basic fibroblast factor, obtaining best result the material richer in this factor. In another work, an emulsion of digested ECM from SIS was injected into infarcted rat hearts, improving cardiac function, increasing neovascularization and promoting cell recruitment [113].

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4.2.2. Synthetic materials

Synthetic materials are made in the laboratory from primary building blocks, so their properties can be tuned to match desired characteristics. Besides, they are free from animal origin components and the risks related therewith.

Thermosensitive hydrogels

This group of materials has temperature-dependant sol to gel transition. The great advantage of this group of materials is the possibility to tune their properties for them to undergo the gelation transition around body temperature [114]. In this way they can be comfortably manipulated and injected and only when they are inside the body they will undergo the transition.

Some of the materials of this group are based on N-isopropylacrylamide (NiPAAm). It is non biodegradable, but copolymerized with degradable polymers becomes biodegradable. For instance, NiPAAm was copolymerized with acrylic acid (AAc) and hydroxyethyl methacrylate-poly(trimethylene carbonate) (HEMAPTMC) [115]. The ratio of each material was adjusted to obtain a hydrogel at 37ºC. It can also be degraded *in vitro* with a mass loss over 85% after 5 months. This material was injected *in vivo* in rats and proved to preserve the area of the left ventricular cavity and contractility. Tissue ingrowth, a thicker left ventricle (LV) wall and greater capillarity density were also found when compared with PBS controls. After 8 weeks, a layer of smooth muscle cells with contractile phenotype was formed next to the remaining material.

Another family of thermoresponsive hydrogels based on polycaprolactone, N-isopropylacrylamide, 2-hydroxyethyl methacrylate and dimethyl-g-butyrolactone acrylate has been developed [116]. Cardiosphere derived cells (CDC) combined with the hydrogel were suitable for myocardial injection and the solutions formed solid gels within 5 s at 37ºC. Hydrogels with different mechanical properties were obtained and it was shown that they influence the fate of the CDC differentiation. Another thermoresponsive material containing biodegradable dextran chain grafted with hydrophobic poly(ε-caprolactone)-2-hydroxylethyl methacrylate (PCL-HEMA) chain and thermoresponsive poly(N isopropylacrylamide) (PNIPAAm) (Dex-PCL-HEMA/PNIPAAm) has been synthesized. It can shift from sol to gel within 30 s and is reversible within the same time frame [117]. It was injected in rabbits, 4-days post-infarction. Histological analyses one month later indicated that the material prevented the scar expansion and thinning of the wall. Left ventricular ejection fraction was increased and it attenuated left ventricular systolic and diastolic dilation.

Poly (Ethylene Glycol) (PEG)

A strategy based on non-biodegradable *in situ* crosslinkable PEG hydrogel has been developed, to provide a permanent support to limit the remodeling [118]. Its therapeutic effects were tested in rat myocardial infarction model at short and long term. Beneficial effects were observed at 4 weeks, but at long term (13 weeks) it was unable to prevent the dilation. Besides, the material injection induced some inflammatory response.

An injectable α-cyclodextrin/poly(ethylene glycol)–b-polycaprolactone-(dodecanedioic acid)-polycaprolactone–poly(ethylene glycol) (MPEG–PCL–MPEG) hydrogel was used to deliver and encapsulate bone marrow stem cells into infarcted myocardium [119]. The CD/MPEG-PCL-MPEG hydrogel alone does not induce angiogenesis, but can serve as a support in the infarcted zone and contribute to inhibit the left ventricular remodeling. One month after the injection of the gel combined with cells, cell retention and survival and the density of vessels were increased when compared with cells injection alone; moreover, the gel was absorbed, ventricular dilation was limited and the ventricular ejection fraction improved.

PEG-based temperature-sensitive hydrogels have also been combined with growth factors or other molecules. VEGF was mixed or conjugated with the aliphatic polyester hydrogel poly(δ-valerolactone)-block-poly(ethylene glycol)-block-poly(δ-valerolactone) (PVL-b-PEG-b-PVL); the sustained VEGF release during the degradation time of the hydrogel translated into an improvement of the myocardial and functional recovery, in dependence of the preparation method [120]. In another work, a metalloproteinase-responsive PEG-based hydrogel was synthesized to be a thymosin β4 (a pro-angiogenic and pro-survival factor) delivering scaffold. It was implanted combined with endothelial and smooth muscle cells derived from human embryonic stem cells (hESC) in rats [121]. The gel provides structural organization and when was loaded with cells and thymosin b4 enhanced more contractile performance than when the hydrogel was only loaded with the factor, because of their paracrine effect. Another PEG-based hydrogel, α-cyclodextrin/MPEG–PCL–MPEG, was tested as a delivery system for erythropoietin (EPO) [122], a hormone that plays a protective role in the infarcted myocardium. Rats treated with this system showed limited cell apoptosis and increased neovasculature formation; also infarct size was reduced and cardiac function improved.

PEG in the format of nanoparticles has also been studied. They can be injected intravenously, circulate in the body for long periods and bind only to desired tissues. Nanoparticles targeting the infarcted myocardium were developed based on the overexpression of angiotensin II type 1 (AT1) receptor in the infarcted heart [123]. The system was formed by a vehicle and a targeter, a ligand specific to AT1 that will make the nanoparticles bind specifically. The vehicle was 142 nm diameter PEGylated liposomes, which could carry therapeutic molecules and release them in a controlled way. This system was proved to target the infarcted heart in mice model, but not the healthy.

Self Assembling Peptides (SAPs)

SAPs are short peptides capable of forming hydrogels at physiological pH and osmolarity [124]. When the SAPs solution is placed in contact with ions or pH is changed, the charges are partially neutralized and a hydrophobic packing takes place forming beta-sheet structures, constituting fibers that build a 3D network if the concentration is high enough. Fibers shape is different depending on the nature of the employed peptides. In the particular case of the RAD16 ionic peptides family (R: arginine, A: alanine, D: aspartate) fibers thicknesses are of 5-10 nm.

Peptides can be combined with cells to encapsulate them within the peptide network [125]. RAD16-I (AcN-RADARADARADARADA-CNH_2) has proved to be a useful synthetic gel

capable of maintaining the cells in the site of interest, and has been used as a delivery system of different types of cells to the heart. On the contrary, when it was implanted alone limited improvements were observed in the infarct area and the remodeling process. RAD16-II (AcN-RARADADARARADADA-CNH2) peptide has been shown to create microenvironments in the infarcted myocardium that are infiltrated with endothelial and smooth muscle cells, suggesting a potential for vascularization [124]. It was also observed that combining RAD16-II with neonatal cardiomyocytes the density of endogenous α-sarcomeric actin positive cells increased.

As stated, SAPs gels can be modified to incorporate growth factors or drugs. The self assembling peptide RAD16-II has been used as a drug vehicle to deliver both platelet derived growth factor and fibroblast growth factor (PDGF-BB and FGF-2) [126]. The first is arteriogenic and the second is angiogenic; their combination targets endothelial cells (EC) and vascular smooth muscle cells (VSMC). Infarct size and cardiomyocyte apoptosis were considerably reduced in rats. The capillary and arterial density was recovered, and cardiac function was almost recovered. This system also induced long-lasting vessel formation. RAD16-II combined with IGF-1, a cytokine that protects and promotes cardiomyocytes growth, has also been used as a delivery system for cardiomyocytes [127]. The addition of IGF-1 acted reducing cell apoptosis and improving systolic function.

5. Preformed gels and scaffolds

5.1. Rationale

An alternative approach in the field of cardiac tissue engineering involves the use of biomaterials to produce patches *ex vivo* and implant them epicardially onto the infarcted tissue, conveniently adapted to its size and shape. These patches can be pre-loaded with cells (incorporated within their pores in the case of microporous scaffolds, or encapsulated in the case of a gel conformed before implantation, as shown in figure 1 b) and growth factors or drugs, and act as a cell supply, a mechanical reinforcement to the infarct scar to avoid ventricular dilation and a drug release system simultaneously.

5.2. Requirements of the scaffolds

In this strategy a key aspect is to find a material that matches the required properties. The material needs also to be cell-friendly, non-cytotoxic and promote cell attachment and proliferation, and it must also be non-immunogenic [128]. The scaffolds should provide a 3D environment to the cells with a porous structure able to guide cardiomyocytes alignment and promote maturation, also induce the development of a contractile phenotype and the electromechanical coupling of the implanted cells among them, and also with the host tissue [129, 32] and need to be easily vascularized [37].

The mechanical properties exhibited by the scaffolds should be adequate to their application in heart tissue engineering. It implies that they should ideally be compliant with contractions

and exhibit non-linear elasticity, as well as be capable to adapt to the shape of the heart in all phases of the heart beat. Anisotropy to mimic the directionally-dependent electrical and mechanical properties of the native myocardium is important too [130]. Besides, the stiffness of the material employed affects to a great extent the phenotype and contractile properties of the neonatal cardiomyocytes [131, 132], and has to be carefully tuned to match physiological conditions. During heart development, the ECM on which cardiomyocytes maturation takes place, stiffen 9 times. An interesting approach to mimic it is the development of materials with time dependant mechanical properties [133]. For instance, hyaluronic acid hydrogels that stiffen with time form more contractile units when compared with cultures in hydrogels without such time-dependant stiffness.

Attending to the type of strategy, three groups can be distinguished, in terms of the nature of the matrices: biologically-derived materials, synthetic (either biodegradable or biostable) materials and decellularized tissues. With the use of biodegradable scaffolds, it is expected that the matrix will degrade as the surrounding tissue is regenerated; the degradation products should not be toxic and metabolized by the body. By using permanent scaffolds, the idea is that they will be infiltrated by the host tissue and contribute to the regeneration, but also act as a permanent mechanical restraint to limit ventricular dilation. The approach of scaffolds derived from decellularized tissue is based on the use of tissues whose cells are removed and the remaining ECM maintains the architecture and mechanical properties similar to those of the native tissue. Obtaining a scaffold matching the desired properties is a hard task, as many different properties are required; thus, materials exhibiting different properties have been mixed in more advanced strategies to obtain a composite that combines them.

5.3. Related problematic

As all the approaches described so far, this one also has some advantages, disadvantages and unsolved problematic. An important disadvantage is that the application of a patch in the heart needs a much more invasive technique than a catheter-delivered system, as it requires a surgical procedure to be implanted. As advantage, the fact that the materials are synthesized and conveniently prepared out of the body can be outlined. It implies that there is no limitation in the preparation procedure and in the use of solvents (if they are properly removed at the end of the fabrication process and do not induce cytotoxicity). Therefore, the range of chemistries and techniques available to obtain scaffolds with different architectures is broadening. Besides, cells can be pre-cultured *in vitro* within them prior to implantation if desired. In addition, the mechanical properties of polymer scaffolds may be tuned to match more closely those of the heart muscle than with gelly biomaterials.

Unlike native myocardium, where the greatest distance between capillaries is around 20 microns [69], scaffolds are not vascularized *a priori*. Then, cells seeded in the scaffolds have their oxygen and nutrients supply limited to their molecular diffusion through the thickness of the scaffold. Given the fact that cardiomyocytes have great consumption rates of nutrients and oxygen, diffusion is insufficient supply for thick constructs. Consequently, to obtain a thick engineered tissue with viable cells through all its thickness, pre-vascularization or improved diffusion throughout the scaffold until it is vascularized is key for the implant to

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succeed. Otherwise, cell density will be concentrated in the external parts and cell viability will be compromised in the center of the scaffold if the distance to the surface is greater than a critical value estimated around 100 microns [134]. For example, the influence of oxygen concentration in cell density and viability in collagen scaffolds has been studied, the former decreasing linearly with the distance to the surface and the latter exponentially [135]. These results indicate that in order to guarantee an appropriate oxygen concentration throughout the scaffold, additional measures need to be taken.

Many attempts have been done in this direction, like the addition of oxygen carriers to the culture medium to simulate the effect of the hemoglobin in the blood. Their addition contributed to improve mass transport and to increase cell density [136]. Another strategy includes the use of scaffolds releasing growth factors to enhance the vascularization process, like basic fibroblast growth factor [137], vascular endothelial growth factor (VEGF) [138] and Thymosin beta-4 [139]. Another approach is the addition of the growth factor platelet derived growth factor BB to the culture medium to protect cardiomyocytes from apoptosis [140]. In a different methodology, channeled scaffolds were produced to simulate the capillary structure of the native tissues and guide endothelial cells growth. The porosity might be adjusted to increase capillary infiltration but it is limited to the maximum size of the pores on which endothelial cells can form vascular structures [141]. An alternative involves the use of decellularized tissues that already provide a native vascular network [142, 143]. The culture of endothelial cells prior to implantation of cardiac myocytes has also been explored [144], and reduced cardiomyocytes apoptosis and necrosis was found. Another possibility is to pre-implant the scaffold to pre-vascularize it prior to its implantation in the final site: alginate scaffolds loaded with angiogenic and pro-survival factors (Matrigel, SDF-1, VEGF and IGF-1) were pre-implanted into the omentum of rats [145]. It proved to be a very interesting *in vivo* "bioreactor", providing to the patch a functional vascular network that maintained the viability of the transplanted cells.

Pre-culturing the scaffolds *in vitro* in bioreactors has also been a considered an option. There are many types of bioreactors (stirring, spinning flasks rotating, perfusion, etc.), but not all of them improve enough the diffusion to lead to uniform cell density and compact tissue formation. As an example, in a study where rotating bioreactors were used to culture polyglycolic acid (PGA) scaffolds [146], functional and interconnected cells only were found in the peripheral parts, where there was a better diffusion of the oxygen. Perfusion bioreactors have been developed to try to reduce diffusional limitations by establishing interstitial flow through the scaffolds in order to allow the formation of thick tissues with uniform cell density throughout them. The effect of culturing scaffolds in perfusion bioreactors was compared with culturing them in spinner flasks [134] or orbital mixed dishes [147]. In both studies results were improved with the perfusion bioreactors; when cultured in the others, high cell density was only found in the outer layers. However, a limitation of perfusion bioreactors is the medium flow rate, because of the hydrodynamic shear the interstitial flow inflicts to the cells, which could maintain them in a rounded morphology or even wash them out if it is too high. This finding led to the combination of the perfusion culture with the use of channeled scaffolds that provided separated compartments for medium flow [148]. Even more, this strategy has been

successfully combined and used simultaneously with a selective pre-seeding of the scaffold in the channels with endothelial cells using a perfusion seeding technique, which provides uniform seeding throughout the entire scaffold without the use of cell carriers [149].

Another step was made when the pulsatile perfusion bioreactor [150] was developed. It was expected that the pulsatile interstitial medium flow would provide mechanical conditioning and improved mass transport, intending that all together would lead to a tissue with better contractile properties. Indeed, scaffolds cultured under these conditions had enhanced contractile properties. A different type of bioreactor, with bidirectional slow flow perfusion obtained with an oscillatory system was tested with culture medium loaded or not with Insuline-like growth factor-I [151]. The advantage of the combined strategies was revealed.

However, despite the great efforts put and the improvements achieved, obtaining vascularized constructs is still an unsolved problem.

5.4. Preparation techniques

Many different techniques have been proposed to obtain 3D porous structures with different topographies and porosities, basically based in phase separation procedures or the use porogen templates to create the pores. Now with the introduction of controlled computer assisted systems, new possibilities are open. Next, a brief description of the main techniques employed to prepare scaffolds for heart tissue engineering is outlined.

The electrospinning technique is based in the application of a high voltage to a polymer melted or in a solution that leads to the formation of ultrathin nonwoven fibers [152], which are projected on a collector giving rise to fiber mats with controlled thicknesses. The fibers diameters can be obtained in the range of the ECM proteins. This technique also allows the preparation of aligned fibers, which can be applied to obtain aligned cardiac cells [153].

The particle leaching technique is based on the use of a porogen that is mixed with a polymer solution or a melted polymer. This porogen is removed after the solvent has been eliminated (solvent casting, freeze extraction) or the polymer has solidified after cooling, leaving empty spaces (pores) with the size and shape of the porogen template (and also small pores for the elimination of the solvent, if used). Porosity and pores interconnection can be tuned by changing the porogen-polymer ratio. Gas foaming avoids the use of solvents and high temperatures, because the pores are obtained by exposition to a high pressure gas followed by a pressure decrease with nucleation and growth of pores. The freeze-drying technique consists in freezing a polymeric solution and then lyophilize it to remove the solvent in the frozen state and obtain a solid porous structure [154]. Different morphologies can be obtained by changing the freezing conditions [155].

Microfluidic patterning consists in forcing a polymer solution through a channeled mould previously obtained with the desired geometry. Once the polymer is consistent, the mould is removed and the scaffold or patterned surface is ready. Selective laser sintering is a technique based in the use of a CO_2 laser to sinterize selectively the powder of a material to form the cross section of each layer of a 3D object.

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Microcontact printing is a technique that allows cell adhesion guidance [156]. It consists in the use of a stamp, with the pattern to be followed by the cells. The stamp is inked with the solution that is expected to promote the adhesion (laminin, ECM proteins, etc.) and then pressed against the substrate to transfer the solution. By loading the solution with growth factors, cell differentiation can also be induced in patterns [157].

5.5. Biomaterials employed as scaffolds

Many different types of materials have been considered for cardiac tissue engineering. According to their origin we can distinguish: biologically-derived materials, decellularized tissues and synthetic materials. Natural materials include collagen, gelatin, fibrin, silk and alginate; and synthetic materials include polyurethane (PU), polylactide acid (PLA), polyglycolic acid (PGA), polycaprolactone (PCL), or polyglicerolsebacate (PGS), among others.

5.5.1. Natural materials

Collagen

There are a number of commercial collagen patches, widely used by clinicians for other purposes, which are now under study as epicardial patches, because it has been reported to be a good substrate for cell attachment and infiltration [158]. They have been combined with different cell types and molecules. Unfortunately, collagen sponges have a great swelling rate and poor mechanical performance in aqueous medium.

Collagen can be used in two formats, as a porous scaffold or as a hydrogel. To obtain the scaffold a collagen solution is lyophilized and then rehydrated and seeded with cells. In the case of hydrogels, a collagen solution is mixed with cells *ex vivo* and then gelled. As a gel entrapping embryonic chick cardiomyocytes [159], it was found to beat and arrange as a highly organized tissue-like when pulses with different frequency were applied.

The potential of collagen scaffolds as an attractant for neovascularization was demonstrated in a study with rats [160]. Collagen sponges implanted in both healthy and cryoinjured hearts were almost absorbed after 2 months, but the remaining structures were populated by new arterioles and capillaries. In another study, collagen has been combined with chondroitin 6-sulfate to obtain porous scaffolds. These scaffolds delivered MSC in the infarcted region in a rat model, promoting neovascularization [161].

The therapeutical potential of collagen as epicardial patch has been compared with injectable approaches. Collagen matrices loaded with mesenchymal stem cells (MSC) [162], and collagen scaffolds loaded with human umbilical cord blood cells (hUCBCs) [25], gave better results than the injection of cells alone in mice. In the MAGNUM phase I clinical trial [163], intrainfarct cell therapy of autologous BMC was combined with collagen scaffolds loaded with BMC. This treatment was found to be safe and contribute to limit left ventricular remodeling by increasing the thickness of the ventricle wall and then reducing the stress of the wall.

Collagen has been modified to incorporate bioactive molecules to improve its biological behavior. Its scaffolds have been modified with RGD [164] and cardiac markers of cardiospheres derived from cardiac progenitor cultured on them were upregulated. Collagen functionalized with interleukin-10 plasmid [165] (an anti-inflammatory plasmid) increased 5 times cell retention and modulated inflammation.

Gelatin

Gelatin is obtained from chemical denatured collagen; it is therefore weaker and degrades faster than it [27]. It has been reported to provoke unspecific inflammatory response upon degrading; at first this can be considered an undesired effect, but for certain applications it might be beneficial for the positive impact that can have on angiogenesis [166]. A commercial gelatin sponge bare or cultured either with fetal or adult rat heart cells was implanted to replace the resected right ventricular outflow tract (ROVT) of rats [167]. After 4 weeks a great inflammation was observed and after 12 weeks the patches had endothelial cells on the endocardial surface. Nonetheless, the authors concluded that a material inducing less inflammatory response is needed.

Fibrin

Fibrin can be used as an injectable gel, but can also be preformed *ex vivo*, which broadens the possibilities of fabrication. For example, SDF-1 (a factor that is up-regulated for a period of time after a myocardial infarction, and contributes to mobilize cells from bone marrow and peripherial blood to the damaged tissue) was covalently bound to a PEGylated fibrin patch [168] and implanted in an AMI mouse model; the SDF-1 loaded patch reduced more significantly the scar area expansion and improved the left ventricular function than the un-loaded patch.

Alginate

Alginate scaffolds obtained by the freeze drying technique have been extensively explored in myocardial regeneration. Loaded with fetal cardiac cells and implanted in infarcted rats, they limited left ventricular dilation [169]. However, cultured with neonatal or fetal cardiomyocytes in static conditions, cell aggregates were formed due to the non-adhesive nature of the alginate [170].

To improve cell adhesion and survival modifications of alginate scaffolds have been investigated. For example, it has been modified to incorporate the adhesion peptide RGD [171], which improved cell adhesion, reduced apoptosis, accelerated tissue regeneration and led to the organization of cardiomyocytes in myofibers *in vitro*, and also with a combination of RGD and the heparin-binding peptide G4SPPRRARVTY (HBP) [172], with better results.

Polysaccharides

Polysaccharide-based scaffolds have also been investigated with myocardial regeneration purposes. The effectiveness of freeze-dried pullulan and dextran patches was compared to mesenchymal stem cells endocardial delivery alone in a rat myocardial infarction model [173], the scaffolds improving the cell engraftment and survival at 1 and 2 months.

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Silk

Because of silk fibroin good mechanical properties, biological performance, and its easy processing to obtain different morphologies, it has generated interest in the tissue engineering field. Silk is produced by some insects like spiders or silkworms, and is considered a non-degradable material by the FDA [174]. Silk fibroin has been combined by chitosan and hyaluronic acid to produce microparticles that were pressed and crosslinked with genipin to obtain cardiac patches [175]. MSC cultured on the composite patches exhibited greater proliferation and cardiomyogenic differentiation than in silk patches.

Recently, non-mulberry silk fibroin from Antheraea mylitta has been investigated as a material for cardiac tissue engineering [176]. It has better mechanical properties than mulberry silk, contains RGD sequences, is non-cytotoxic and induces low level of inflammatory response. When neonatal rat cardiomyocytes were seeded in an Antherea mylitta silk lyophilized scaffold, the results were better than those obtained with a mulberry silk.

Decellularized-tissue derived scaffolds

Decellularized extracellular matrices have been used as scaffolds in many studies and also in preclinical and human clinical applications [177]. The decellularization process consists in a set of washes to remove the cells but maintain as much as possible the architecture, proteins and adhesion molecules. The more aggressive the washes and treatments are, the lower the risk of allogenic immune reaction is, but undesired washout of adhesion proteins and architecture damage can be associated [65].

Decellularized sheets have been tested in combination with fibrin, TGF-beta, and MSC and tested in a nude rat model of infarction with positive results [178]. A patch of urinary bladder-derived extracellular matrix (UBM) was implanted in pigs, as a left ventricular wall replacement after infarction, and compared with a polytetrafluoroethlyene (ePTFE) [177] one. At three months, the results were better with the UBM: it was reabsorbed and a cellularized and vascularized tissue rich in collagen was formed.

Sliced decellularized porous scaffolds of acellular bovine pericardia have been combined with cell sheets from bone marrow stem cells, cultured and implanted in rats replacing the resected infarcted myocardium [179]. The patch pores were filled by cells, new vessels and new muscle fibers, indicating that the graft was integrating. Cardiac function was improved and the dilated left ventricle was restored after implantation. In a revolutionary study entire rat hearts were decellularized, and then re-cellularized with neonatal cardiac cells [180]. The architecture was conserved and the preserved vasculature was perfusable. Seeded cardiomyocytes coupled electromechanically and after 8 days under external electrodes stimulation the re-cellularized heart beat and was capable to pump blood.

5.5.2. Synthetic materials

Synthetic materials are prepared in the laboratory, allowing precise control over their mechanical properties, degradation, morphology and porosity that can be tuned as desired

[181]. However, they may not have as good biological performance as biologically derived materials [4].

Polylactic Acid and Polyglycolic Acid (PLA and PGA)

Polylactic acid is a biocompatible, biodegradable and FDA-approved polymer; it degrades into lactic acid (non-cytotoxic), and has been widely used in patients, for example as sutures. However, its degradation products can induce a slight, undesired, acidification of the microenvironment [65]. Polyglycolic acid is a thermoplastic too; it has also been used in the clinic and degrades into non-toxic products. However, neither PLLA nor PGA exhibit the desired elasticity to match that of native heart tissue. In many studies PLA and PGA have been combined as poly(lactic–co-glycolic acid) (PLGA), or other polyesters, to modify their properties as desired. Electrospun PLGA fibrous membranes with different compositions (having different hydrophobicity and degradation rates) [4] were found to align cardiomyocytes in the direction of the nanofibers, the best results being those of the slightly hydrophobic copolymers. Porous beads of PLGA seeded with human amniotic fluid stem cells (hAFSCs) have been tested as a cell delivery vehicle or "cellularized microscaffold" [182]; after implantation by intramyocardial injection in a rat infarct model, they showed good retention of the cells in the site of interest. PLGA has been treated with laminin [183] to improve its biological development and combined with carbon nanofibers (CNF) to increase its conductivity and cytocompatibility [184]. PLLA-PLGA scaffolds loaded with Matrigel have been co-cultured with endothelial cells, cardiomyocytes and embryonic fibroblasts simultaneously [185], for EC to provide vasculature and act synergically with cardiomyocytes to improve cell survival and proliferation.

Poly (epsilon-caprolactone) (PCL)

Poly(epsilon-caprolactone) is a FDA-approved biocompatible polyester, as PLA and PGA. It is more elastic because of its lower glass transition temperature, and behaves as a rubber at body temperature. Its degradation does not produce acidification because it occurs more slowly [158]. It has been proposed for myocardial regeneration for example in 3D constructs obtained by overlapping electrospun PCL nanofibrous mats (up to 5 layers) on which neonatal cardiomyocytes were cultured [186]. The layers established morphologic and electrical connections between them and exhibited synchronized beating, and no ischemia was found in the center of the constructs.

It is usually combined with PLA, PGA or its copolymer. Poly-glycolide-co-caprolactone (PGCL) biodegradable porous scaffolds have been studied as cell vehicles for bone marrow-derived mononuclear cells (BMMNC) in rat myocardial infarction models [187]. BMNC migrated from the scaffold and neovasculature over the implant was detected; left ventricular function improvement and limitation of the progression of the left ventricular dilation was also observed. Scaffolds made of poly(DL-lactide-*co*-caprolactone) (PLACL), PLGA, and type I collagen [158], cultured with neonatal rat heart cells, have been compared. The composite scaffolds gave better results than controls (collagen and PLGA sponges) in terms of cellularity, contractility and cardiac markers expression (Tn-I and Cx-43). Perfusion culture improved cell density distribution.

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Polyurethanes (PU)

Polyurethanes are synthetic biocompatible materials widely used in the biomedical field. Their mechanical properties and biodegradability can be tuned by changing their composition. PU degrades *in vivo* through hydrolytic chain scission, which is accelerated by the enzymes action and loads, among other factors [188], but with the appropriate composition non-biodegradable polyurethanes can be obtained [189]. This family of polymers can be used to obtain fibrous scaffolds by electrospinning with different mechanical properties depending on the fibers orientation [190] or porous elastic scaffolds [191]. Polyester urethane urea (PEUU) elastic porous scaffolds have been implanted in sub-acute infarctions in rats and were found to promote the formation of smooth muscle bundles, to increase the ventricle thickness and to improve contractile function [192]. Cell attachment on polyurethane-based porous scaffolds can be improved by pre-treating them with laminin [193].

Poly(glycerol sebacate) (PGS)

Poly(glycerol sebacate) is a biocompatible and biodegradable elastomer capable of recovering from deformation. It can be obtained by polycondensation of glycerol and sebacic acid. By changing the synthesis temperature, the properties of the resulting material can be tuned to match the desired mechanical properties. The degradation rates can also be adjusted from fast degradation to nearly inert [194].

By the use of excimer laser microablation, 3D porous PGS scaffolds with anisotropic structural and mechanical properties were obtained [195, 130]. These scaffolds induced neonatal cardiac cells alignment in the absence of external stimuli and matched the mechanical properties of adult rat right ventricle. Moreover, they allowed cell contractility when stimulated. For its interesting mechanical properties, PGS has been coaxially electrospun with gelatin to form a nanofibrous mat with PGS in the core and gelatin in the shell [196] to enhance cell adhesion and proliferation. PGS has been modified to incorporate acrylic groups in different number (to modify its mechanical properties and degradation) and electrospun in combination with gelatin [197].

Acrylate based materials

Acrylate based materials have not been widely exploited for cardiac tissue engineering yet but the interest on them is increasing, for their versatility of processing and variety of properties obtained. For example, scaffolds made of poly(2-hydroxyethyl methacrylate-co-methacrylic acid) (P(HEMA-co-MAA) hydrogel have been obtained by fibers and microspheres templating to obtain spherical pores and parallel channels [198], which allow simultaneously mass transfer and guidance of the cardiomyocyte bundles. Mechanical properties were adjusted intentionally for the elastic modulus to be lower than that of native myocardium in order to make possible the mechanical stimulation of the cells when implanted *in vivo*. In [199], poly(ethyl acrylate) (PEA) scaffolds are filled with HA gel; the scaffolds provide the three-dimensional environment and mechanical properties and the gel may act as an encapsulating medium for the cells and may be also used as a medium for drug or growth factors release. RAD16-I gel may also be used as a filler in PEA scaffolds, where it acts as a diffusion medium and improves cell seeding efficiency (figure 2).

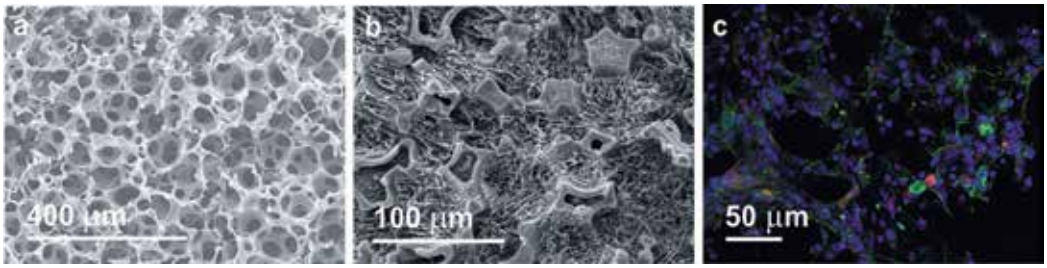

Figure 2. (a) Scanning electron microscopy (SEM) image of poly(ethyl acrylate), PEA, elastomeric membranes with interconnected spherical pores. (b) CryoSEM image (cross section) of a PEA scaffold whose pores are filled with the self-assembling peptide (SAP) gel RAD16-I. (c) Adipose stem cells (nuclei stained in blue and actin cytoskeleton stained in green) seeded in a PEA scaffold with a SAP gel filling. Confocal laser scanning microscopy image of a 50 µm thick internal slice.

5.6. Electrical and mechanical stimulation

Electrical stimulation

External electrical fields have been shown to contribute to the differentiation towards cardiomyocytes of different cell types, such as embryonic stem cells (ESC) [200] or BMSC [201] seeded in collagen scaffolds, and to the development of conductive and contractile properties of neonatal cardiac cells, in this case seeded with Matrigel in a collagen porous scaffold [202]. It has been proposed that the intracellular endogenous reactive oxygen species (ROS) produced when an electric field is applied contribute to the hESC differentiation [203].

In an attempt of optimizing the electrical stimulation parameters [204], it has been determined that the electrode material is very important, and best results have been obtained for carbon electrodes. Amplitude and frequency of the stimulation have also a great influence in the cultured cardiac tissue. Micropatterned electrodes can be of interest as they allow spatial control of the electric field [205].

Polymeric scaffolds limit cardiomyocytes electric communication, what restricts the synchronous beating of the engineered tissue. To improve it, gold nanowires were incorporated to a porous alginate scaffold [206]. Another approach to obtain elastic and electrical conductive scaffolds consisted in impregnating thiol-HEMA/HEMA scaffolds with gold nanoparticles [207]. In both cases even without electrical stimulation the improvement in the scaffold conductivity had positive physiological effects.

Mechanical stimulation

Mechanical stress has a great impact on cell proliferation, ECM formation and hypertrophy (increased cell size), and has been intensively studied in the field of cardiac tissue engineering. Embryonic chick and neonatal rat cardiac myocytes mixed with collagen and mechanically stimulated exhibited hypertrophy and improvement of contractile function [208]. Cardiac myocytes from neonatal rats mixed with collagen I and Matrigel and casted in rings subjected to mechanical stretch [209] showed histological characteristics of adult cardiac tissue. Action potential measurements indicated electrophysiological behavior akin to cardiac tissue.

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Constructs produced by simultaneously electrospinning PU and electrospraying mesenchymal stem cells [210] were cultured in spinner flasks with stretching, which led to cells alignment, cardiac markers increase and ion channels development. Similarly, cells isolated from neonatal rat hearts seeded in chitosan-collagen I channeled porous scaffolds [211] and cultured under high mechanical stimulation induced cell alignment, elongation and the presence of gap junctions connecting the cells. Mechanical stress applied to human cardiac cells cultured in a gelatin scaffold improved cell distribution and proliferation within the scaffold, increased the production of the ECM, and the structure and organization was similar to normal myocardium, likely because the stretching of the scaffold favors nutrients and oxygen exchange improving cell microenvironment [212].

6. Ventricular restraints

After Chachques and Carpentier work [213], it was found that wrapping the heart even with a passive muscle flap had beneficial effects; this finding led to the development of the ventricular restraint therapy [214]. In this approach the aim is not to regenerate the ischemic tissue, but to avoid the progress of the adverse remodeling following a myocardial infarction. It is based on the application of a mechanical restraint (schematized in figure 1 c), which should limit or revert ventricular dilation. A variety of synthetic meshes have been proposed to achieve this goal.

A bilayer membrane with polypropylene in one side to promote tissue ingrowth (or at least limit the ventricular dilation) and with polytetrafluoroethylene in the other side to prevent pericardial adhesions was studied in a chronic infarction model of pig as a restraint [215]. The use of this patch induced improvements once the remodeling process following an infarction had started. The use of a non-biodegradable material is intentional as authors considered that a permanent mechanical reinforcement would be necessary to limit the remodeling.

To determine the extent at which a mechanical restraint is beneficial, a comparative study of two types of restrain was carried out in sheep: a patch over the infarct (non-biodegradable Marlex mesh) or a wrap (non-biodegradable Merseline mesh) [216]. The use of the mesh wrapping the ventricle reduced the remodeling whereas the patch applied over the infarct did not yield considerable improvements when compared with controls (untreated infarcted animals).

Paracor heartnet is a nitinol mesh proposed as a restrain device that is under clinical study in patients with severe dilated cardiomyopathy. In a study, six months after the implantation in 51 patients, results obtained suggested clinical benefits tending to reverse remodeling and that it could consequently be reliably implanted [217]. The PEERLESS-HF trial is the last carried out with this device so far [218]. It proved to be safe and improved patient's quality of life and ventricular dilation; however, no improvement in the peak of VO_2 was produced (which was an end-point of the trial), what led to stop enrollment in the trial. Nevertheless, a new clinical trial is planned. In another study in an animal model, it was shown that the heartnet can alter myocardial blood flow patterns in dilated cardiomyopathy, although it remains unclear if these changes are clinically relevant [219].

Another left ventricular restraint proposed is Acorn Corcap, a polyester mesh that is also being assessed in clinical trials after the positive results obtained in animal models [220]. 5 years after implantation it exhibited safety, a sustained reverse remodeling with a significant reduction in the left ventricular end diastolic volume and a slight increase in the sphericity index [221]. However, in an echocardiographic study using tissue velocity imaging, no improvement in cardiac output was achieved [222].

Limited results obtained with the ventricular restraint therapy can be, among other reasons, because of the absence of tissue regeneration. A more advanced approach combines the ventricular restraint therapy with a regenerative strategy such as patches or scaffolds loaded with cells. For instance, the Acorn Corcap and a collagen matrix loaded with MSC has been implanted in sheeps, and the combination was found to limit the fibrosis produced as foreign body reaction against the Corcap and improve the systolic and diastolic function [223].

7. Concluding remarks

Several therapeutic strategies have been proposed in the last decade to limit the adverse spread of the ischemic tissue and ventricle dilation or even to generate new myocardial tissue. These treatments consist in cellular therapy (so-called cellular cardiomyoplasty) where cells of different origin are implanted by different techniques onto the infarcted ventricle with the hope that cells will contribute to the generation of new contractile tissue to replace the scar, electrically coupled with the host myocardium. But despite the intense efforts and work put in the field, attempts so far have failed. Most of the implanted cells die soon after transplantation due to the fact that the cells cannot withstand the mechanical forces they experience in the host tissue. Mechanisms underlying the slight improvements observed are still undetermined; the paracrine effect is usually considered the way through which cells act, but the precise mechanisms are not completely understood yet. Besides, for this therapeutic approach to evolve to a realistic alternative to conventional treatments, some critical issues are still to be clarified: the way of delivery to maximize cell engraftment and minimize cell loss and death, the ideal cell type to be used, and the optimal time of cell administration (if they are implanted too soon, the inflammatory process kills the implanted cells, but if it is too late, the presence of the fibrotic scar limits their beneficial effects). New strategies already under study envision to improve cell survival by pre-conditioning the cells, pre-treating the host tissue or combining cells with other elements.

A possible way of localizing the appropriate cells in the target diseased tissue is to entrap them in a cell-friendly gelling biomaterial. Besides, gels can incorporate bioactive molecules for their controlled supply, and their preparation procedure (in the case of *in situ* gelling materials) avoids any invasive surgery. The injection of gelly materials alone onto the infarcted myocardium has shown some beneficial effects by itself and contributes somehow to limit the ventricular remodeling, for their slight role as mechanical support. Combining cells with gelly materials contributes, to some extent, to increase the cells residence time in the site of interest, and enhances cells adhesion and survival by providing them a better microenvironment. However, the consistency of these materials is generally too weak to withstand the synchro-

nous contraction of the heart muscle without spreading from their target location, and their mechanical properties are too low to reach significant improvements in terms of containment of the dilated ventricle and post-infarct ventricular dysfunction.

Alternative tissue engineering strategies combine cells with three-dimensional scaffolds or patches to host them and improve their survival, induce the formation of new blood vessels and extracellular matrix and at the same time support the native tissue mechanically. The advantages of using myocardial patches or scaffolds are not only their usually superior mechanical properties, but also their wide versatility in terms of chemistries and morphology. There are many fabrication techniques for the preparation of scaffolds, leading to very different architectures, and these options are broadening with the computer-assisted techniques. Generally, positive results have been obtained by using scaffolds. In studies in which the therapeutic efficiency of a material was compared when used as an injectable gel or as a prefabricated scaffold or patch, the scaffold gave better results. When the scaffolds were loaded with growth factors or adhesion motives, in most of the cases the outcome was better. Mechanical and electrical stimulation are of help for cardiomyocytes to mature within the scaffolds and develop the characteristics and structures typical of cardiac tissue. Unfortunately, the implantation of epicardiac patches is much more invasive than that of injectable gels, and they need to be vascularized to ensure the success of the graft. Many attempts have addressed these questions but a satisfying solution has not been found yet.

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Chapter 13

Treatment of Bone Defects — Allogenic Platelet Gel and Autologous Bone Technique

Dragica Smrke, Primož Rožman, Matjaž Veselko and Borut Gubina

Additional information is available at the end of the chapter

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1. Introduction

Bone defects are a serious illness that can result after a pathological process has destroyed vital components of the bone. Most commonly the causative event is extensive trauma and subsequent infection. It can be also osteomyelitis that destroys the bone and leaves non-vital bone sequesters along the length of the bone. This damage to the bone and soft tissues heals slowly and restitution can be only expected after some time of rest and procedures of debridement.

1.1. Bone defects

Bone defect by definition is a lack of bone tissue in a body area, where bone should normally be. Lack of bone tissue results in a pseudarthrosis, artificial joint that has no physiological importance. In that area, two parts of diseased bone are joined with a fibrous tissue. That area also lacks appropriate vascularization and is usually covered with scarred or fibrotic skin [1].

Bone defects can be treated by various surgical methods. One is always constrained with fibrosis that healed a wound or the site of infection [2]. Often there are factors that impair bone healing like diabetes mellitus [3, 4], immunosuppressive therapy [5, 6], poor locomotory status and others that one has to take in account when a procedure is planned.

There are some common methods of bone defect reconstruction, like decortication, excision and fixation, cancellous bone grafting [7] and the Ilizarov intercalary bone transport method [1]. The application of these methods results in successful final outcomes as far as the bone restitution is concerned.

However, one must consider repeated surgical procedures and often long hospitalization time or frequent outpatient visits for these patients. It is also common for patients to have prolonged ambulatory impairment with suboptimal functional and aesthetic results [8, 9].

1.2. Tissue bioengineering

Tissue engineering involves the restoration of tissue structure or function through the use of living cells. The general process consists of cell isolation and proliferation, followed by a re-implantation procedure in which a scaffold material is used. Cell sources can be autologous or allogenic cells. Autologous cells are usually the better choice, because the allogenic cells could incite immune rejection by the recipient. Mesenchymal stem cells provide a good alternative to cells from mature tissue and have a number of advantages as a cell source for bone and cartilage tissue regeneration [10].

Some authors report that most tissue engineering applications in the head and neck area would probably involve the use of chondrocytes and osteoblasts along with some type of scaffold material because of the importance of initial support and shaping [11].

Theoretically, the ideal bone graft substitutes should be osteogenic, biocompatible, bioabsorbable, able to provide structural support, easy to use clinically and cost-effective. A composite graft combines an osteoconductive matrix with bioactive agents that provide osteoinductive and osteogenic properties [12].

Novel techniques have been studied recently, many involving growth enhancers with varying results. These have been used for healing wounds, ulcers, fractures, and in maxillofacial settings. Such biological enhancers are autologous platelet rich plasma (PRP) in the form of activated platelet gel and recombinant bone morphogenetic proteins (rBMP) [11, 13-23]. An animal study showed enhanced bone growth when autologous bone was combined with platelet-rich plasma [24, 25].

The healing effects of platelet rich gel were attributed to the numerous growth factors (GFs) released by the platelets after activation [19, 26]. Some of those identified are: the platelet derived growth factor (PDGF), TGF-α and β (transforming growth factor alpha and beta), EGF (epidermal growth factor), FGF (fibroblast growth factor), IGF (insulin growth factor), PDEGF (platelet derived epidermal growth factor), PDAF (platelet derived angiogenesis factor), IL-8 (interleukin-8), TNF-α (tumour necrosis factor alpha), CTGR (connective tissue growth factor), GM-CSF (granulocyte macrophage colony stimulating factor), KGF (keratinocyte growth factor), and Ang-2 (angiopoetin), as reviewed by several authors [26, 27]. The inductive potential of platelet gel in tissue regeneration could also be attributed to its significant antimicrobial activity [28].

1.3. Clinical experiments

Recent studies on patients for the regeneration of long bone and foot and ankle defects have provided promising clinical results when using platelets as a source of GFs [10]. Some studies demonstrated that with the use of platelet gel a better and stronger bone yield was achieved

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as compared to reconstruction with conventional methods [29]. X-ray images of treated bones showed increased density early in follow up and in-growth of treated area was enhanced [30].

In the majority of clinical experiments, authors have applied autologous platelets obtained by preoperative apheresis from the peripheral blood of the patient undergoing surgery. However, this may not always be the best solution. In cases of diabetes it has been shown that the release of platelet GFs is decreased in experimental diabetic animals [31]. If allogeneic platelet rich plasma was used as a source of additional GFs, healing of tissues in diabetic patients can considerably improve [32].

Allogenic single donor platelet units are easy to obtain, since they are a standard blood bank product. They are highly standardized in terms of platelet content and residual leukocyte and red blood cell content is low. All of this is due to proven centrifugal forces used for their isolation, temperature of centrifugation, techniques of separation and processing and composition of preservative solution. Also, they are available in large quantities and considered safe. Autologous platelet preparations, on the other hand, are subject to enormous variability, which hinders serious studies of their clinical efficacy [33].

We used for our procedures the standard blood bank platelet concentrates. We prepared a graft composed of allogeneic platelet gel mixed with autologous cancellous bone in order to improve the healing conditions in bone defects, which was successfully demonstrated in our pilot clinical case [34].

In our case study, we showed that the healing potential of the gel GFs obtained from a high number of allogeneic platelets could be combined with the bone forming potential of autologous osteogenic and other stem cells from the cancellous bone. We employed the plasticity of the resulting graft mixture for the modeling and all of this contributed to a successful clinical outcome.

2. Body

2.1. Problem statement

The treatment of bone defects of long bones after injury is still one of the most difficult tasks in reconstructive bone surgery. The golden standard in bone graft surgery is still the use of autologous bone graft [7]. In certain settings, especially in extensive bone defects, this method of treatment could be insufficient and could only pose an additional trauma for the patient.

Numerous authors have reported difficulties when treating defected non-unions, such as extremely long healing time and incorporation of the graft, necrosis of the grafts, and reacutisation of infection [35, 36]. Concomitantly, long-term immobilization contributed to the contractures of the joints and soft tissue, and in the long-term perspective, also to the inferior functional and aesthetic results [37].

Pseudarthroses with certain mid size bone defect are complicated to treat because it is difficult to determine an appropriate treatment method. Smaller size defects can be treated with simple

bone fixation and some debridement. Larger bone defects must be treated with bone transport (Ilizarov method) or transplant of bone graft with vascular pedicle [36].

Reconstruction by vascularised bone transfer along the Ilizarov intercalary bone transport and cancellous bone grafting has been the most widely used method of treatment for large defected nonunions after injury [37, 38]. There have been several modifications of the Ilizarov method, which retain its versatility, stability and mechanics, but these methods also contribute to a high rate of complications [35, 37, 38].

Mid sized defects can be treated with cancellous bone transplant, but many limitations exist with this method. Cancellous bone is of limited availability in human body and sometimes sources have been depleted after repeated surgeries. Often, resorption of transplanted cancellous bone is seen which leads to unsuccessful bone defect bridging [39].

Bone grafts are used to replace a part of the bony defect or to enhance the healing of a fracture. Because of the inability to procure large quantities of autologous bone and the added morbidity for the patient associated with the autograft donor site, new methods of bone transplant materials have emerged in recent years [7].

Substitutes for bone defects have been tested and one of the research tasks is to devise a easily attainable promotor of ingrowth of autologous cancellous bone. Theoretically, the ideal bone graft substitutes should be osteogenic, biocompatible, bioabsorbable, able to provide structural support, easy to use clinically and cost-effective. A composite graft combines an osteoconductive matrix with bioactive agents that provide osteoinductive and osteogenic properties [39].

Synthetic substitutes that provide a scaffold to support or direct bone formation include calcium sulphate, ceramics, calcium phosphate, cements, collagen, bioactive glass and synthetic polymers. These are available in a variety of formulations, including pellets, cement and injectable paste [39, 40].

The functional properties of bone morphogenetic proteins (BMP) 2 and 7, mesenchymal stem cells (MSC), demineralised bone matrix, and biocompatibile ceramics are presented in many papers describing their use in bone defect treatment [41-44]. Bone morphogenetic proteins exhibit an extraordinary power to induce new bone formation de novo without the presence of cancellous bone [45]. With their high cost, limited availability and restricted clinical indications, BMPs are a less attractive option for clinical application.

One of the clinical challenges in long bone defects is the induction of appropriate bone formation, especially in patients with diabetes. Several studies have demonstrated the clinical efficacy of various platelet derived GFs. Recent evidence shows that in diabetic patients platelets are handicapped by decreased expression of growth factors and lower potential for healing fractures [31, 46].

Although there is some evidence that the GFs are released to some extent in the stored platelet concentrates, the majority of GFs remain intact in the platelet granules if they are appropriately stored for up to 5 days [47].

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The safety and efficacy of allogeneic platelets was also shown in our recent pilot case study [34]. Moreover, the preparation of autologous platelet gel requires pre-operative apheresis and blood draws from the patient, and adds to the complexity, risk and cost of surgery [48].

Based on these facts, we were of the opinion that allogeneic platelets constitute a superior alternative to autologous preparations obtained by pre-operative apheresis. Therefore, we used a standard platelet concentrate from the blood bank as a component for the activated platelet gel.

2.2. Application area

Tissue engineering involves the restoration of tissue structure or function through the use of living cells. The general process consists of cell isolation and proliferation, followed by a re-implantation procedure in which a scaffold material is used. Cell sources can be autologous or allogenic cells.

Autologous cells are usually the better choice, because the allogenic cells could incite immune rejection by the recipient. Mesenchymal stem cells are progenitor cells and can be developed in a laboratory along separate cell families. They can be differentiated into more maturated cells like osteoblasts and chondroblasts and chondrocytes. They provide a good alternative to cells from mature tissue and have a number of advantages as a cell source for bone and cartilage tissue regeneration [49].

Here we present the results of a prospective clinical study performed from May 2004 to February 2010 in the University Clinical Centre Ljubljana, Slovenia. We treated defected non-union of long bones with cancellous bone transplantation. We used allogeneic platelets as a source of additional GFs.

We treated 9 consecutive patients (3 female and 6 male), aged from 21 to 73 years (average 45.9 years), each with a defect of a different long bone (3 femoral, 4 tibial, 1 humeral and 1 ulnar). We present patients' size of bone defect, which were classified as mid-size in a Table 1.

Patient	Pseudarthrosis site	Graft volume in mL
1	Femur	16
2	Distal tibia	35
3	Distal tibia	45
4	Femur	15
5	Distal tibia	30
6	Proximal femur	25
7	Humerus	35
8	Ulna	30
9	Distal tibia	25
	Average graft volume	28.5

Table 1. Size of bone defect per patient and average of the group

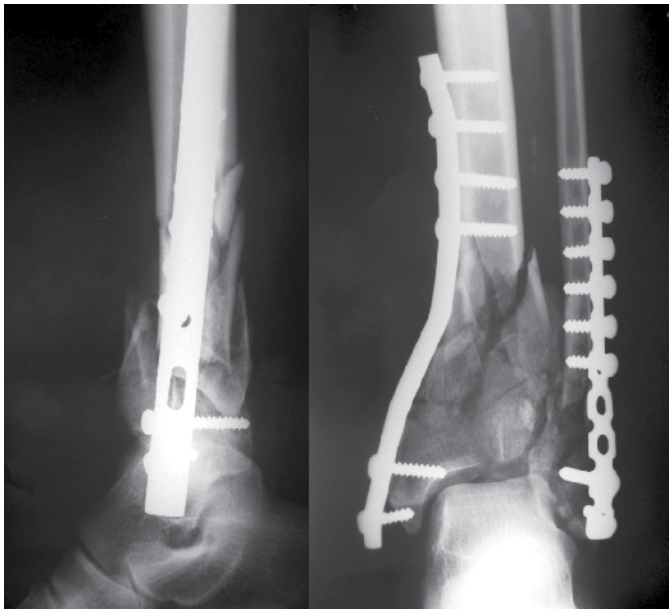

Figure 1. Bone defect of distal tibia (plain X-ray)

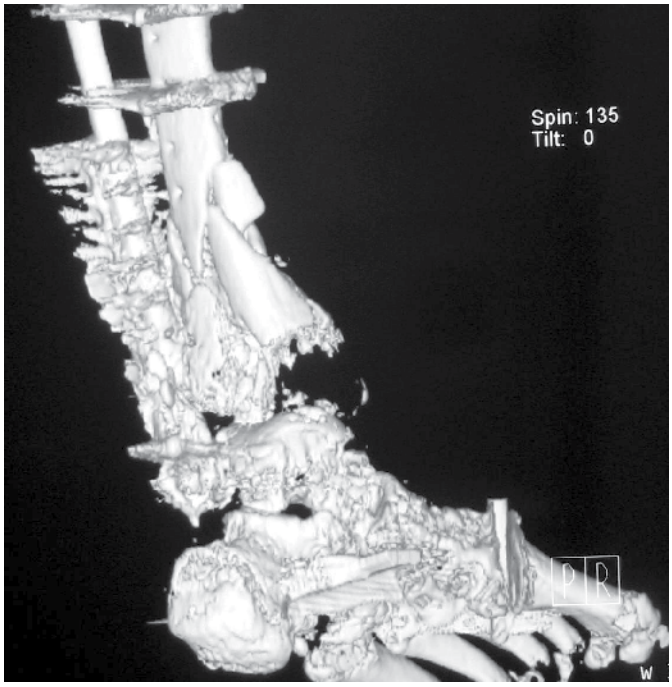

Figure 2. Bone defect of distal tibia CT reconstruction

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They had already been unsuccessfully treated with conventional methods in our or other hospitals. The therapeutic options in these cases had been exhausted. In the Figure 1 and 2, we present an example of a bone defect we treated.

In 2 of the patients, we treated osteomyelitis before applying our treatment. We took additional microbiological samples at the time of operation. Three samples were positive for pathologic bacteria and patients received appropriate antibiotic therapy. After the operation no reacutisation of infection was noted. Two of the patients had diabetes on per oral therapy.

2.3. Research course

In our clinical investigation plan, the primary objective was to establish the potency of allogeneic platelet gel, from our blood bank, added to the transplanted autologous cancellous bone when treating post-traumatic mid-sized bone defect, with a follow-up of one year. The secondary objective was to investigate the healing, safety, handling and tolerance of the method and potential cost benefits.

We noted all the patients' major variables in a protocol, radiologic examinations, and post-operative follow-up for up to one year. As a survey of the immunological side effects of allogeneic platelets, we performed a screening of HLA antibodies class I and human platelet antibodies (HPA) before the implant operation and in the third month after the operation.

2.4. Methods used

We harvested autologous cancellous bone from one or both patients' iliac crests and ground it by hand and instruments until the particles were smaller than 5 mm. It was then stored on a sterile dish with wet gauze for later use.

For preparation of the platelet gel we used a standard allogeneic random single donor platelet concentrate that was ABO and RhD matched, serologically HIV, HBV, HCV and lues-negative, leukocyte depleted, and irradiated. A standard single donor platelet concentrate was prepared from 450 mL of whole blood, containing 70 x 10^9 platelets in 50 mL of citrated plasma, and stored in a plastic bag designed for platelet storage at 20-24ºC on an automatic agitator for up to five days.

We performed leukocyte depletion by using a commercial filter (BioP05 Plus, Fresenius HemoCare, Bad Homburg, Germany) with 10–15% platelet loss post-filtration. We irradiated the platelet concentrate with a cobalt irradiator with 25 Gray. All platelet related procedures, including the bacteriological controls, were performed according to the recommendations for blood banking procedures.

Finally, we prepared a mixture of lightly compressed autologous cancellous bone and an equal volume of allogeneic platelet concentrate with approximately 1.4 x 10^9 platelets per 1 mL (which is around five times higher than the physiological level of platelets in the blood).

We mixed the ingredients and added the fibrin glue components (human thrombin (100 IU/mL) in 40mM $CaCl_2$ (Beriplast P, ZLB Behring, Marburg, Germany)) for the activation of platelets and polymerization of fibrinogen. The implant is presented in the Figure 3. The

mixture achieved the appropriate plasticity in 20 to 30 seconds. The resulting gelatinous graft was shaped according to the defect and implanted.

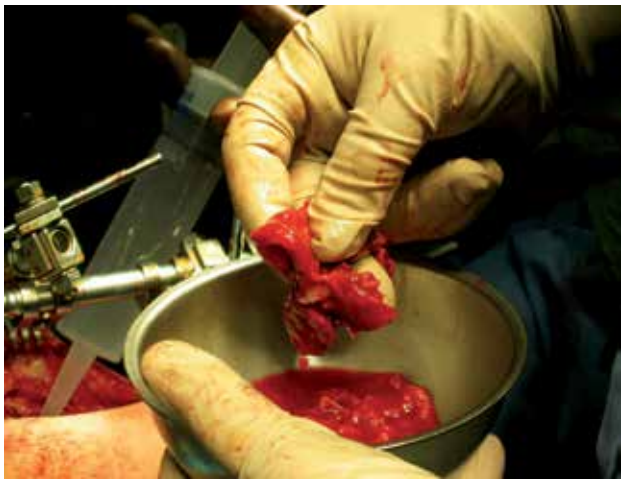

Figure 3. Cancellous bone and platelet rich plasma implant

2.5. Surgical procedure

In all our operations, we approached the bone defects through previous surgical incisions after administering a single dose of prophylactic antibiotic. After debridement of the non-union which is presented in Figure 4, we filled the resulting bone defect with a semi-solid, moldable gelatinous graft, presented in the Figure 5.

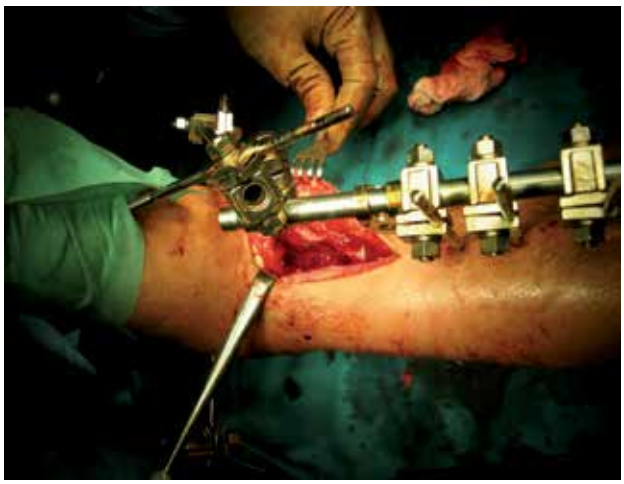

Figure 4. Bone defect at the operation

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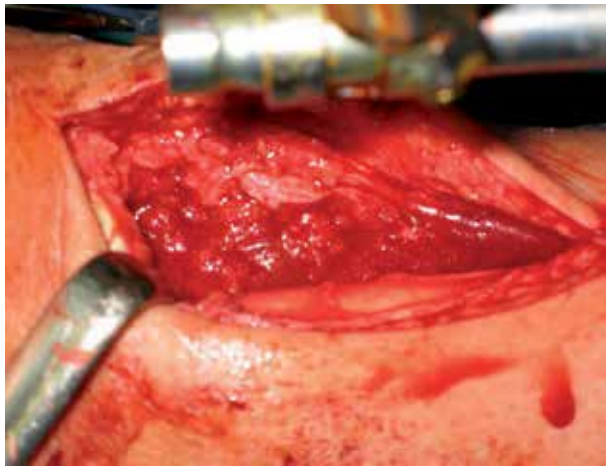

Figure 5. Bone defect filled with implant

We revised the method of fracture fixation and repositioned bone fragments were and fixed them in good alignment. We applied a different fixation method where it was necessary or inadequate and we present the fixation methods in Table 2.

Patient	Pseudarthrosis site	Fixation method
1	Femur	Internal plate
2	Distal tibia	External fixator
3	Distal tibia	Internal plate
4	Femur	Tutor brace
5	Distal tibia	Internal plate
6	Proximal femur	Dynamic hip screw with long plate
7	Humerus	Internal plate
8	Ulna	External fixator
9	Distal tibia	Internal plate

Table 2. Fixation methods used and graft volume per patient

We placed negative pressure suction subcutaneously, away from the graft in order to minimize the removal of GFs. All procedures were carried out within a sterile operation field - aseptic conditions.

In the follow-up protocol, we assessed the general status after the operation, and the bone configuration with X-ray at 2, 4, 6, and 12 months. We assessed bone remodeling at 6 and 12 months by CT scan. We drew blood samples from each patient at week 14 for the identification of anti-HLA/Class I antibodies and anti-HPA antibodies in order to assess potential immune reactions related to the use of allogeneic platelets. We used the standard in-house platelet immuno-fluorescence test (PIFT) and antigen capture ELISA test (PAK-12, GTI, Brookfield, USA) to screen for antibodies.

2.6. Results

We removed the drains on the second or third day after the operation, draining different volumes, on average 250 mL (200 to 450 mL). Immediate post-operative care was uneventful in all cases. We discharged the patients 6-8 days after the operation and they were regularly examined in the outpatients' clinic.

Out of 9 patients, 7 successfully healed their defect with the implant (78%). Figure 6 shows healed bone defect in distal tibia.

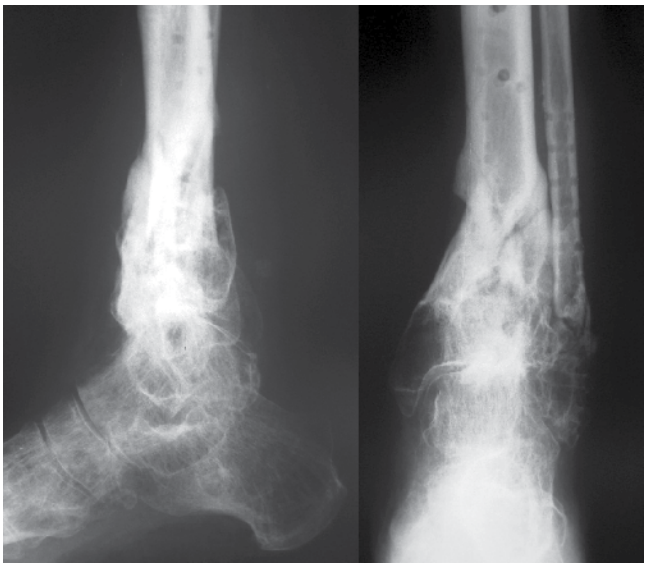

Figure 6. Healed bone defect after grafting and platelet additive

Different healing times are presented in Table 3.

Time in weeks	Minimal	Maximal	Average	Median
Time to appearance of hazy callus	6	24	10	8
Time of partial weight bearing	12	40	22.5	18
Time of free mobility and full weight bearing	16	48	31	31
Time of overall bone healing	16	36	23	24

Table 3. Healing times after the operation for successful cases (time in weeks)

We noted major complications during the treatment in 3 patients (33%): poor incorporation of implant, mental deterioration leading to non-compliance, and radial nerve palsy, which receded (1 patient respectively). Two of these patients had to undergo further surgery. More detailed data of bone healing are displayed in Table 4.

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Patient /sex/age (years)	Time to appearance of hazy callus (weeks)	Time to partial weight bearing in defects (weeks)	Time to free mobility and full weight bearing (weeks)	Extent of graft incorporation	Time to stable bone healing (weeks)	Complications during treatment	Major compli-cation	Final result (12 months)
1/F/63	8	12	40	complete	16	none	no	healed
2/M/50	8	18	32	proximally 1/4, distally complete	24	left hip fracture	no	healed
3/F/49	10	16	-	complete proximally, distally not at all	24	pseudarthrosis, re-operated	yes	failed
4/M/45	24	32	44	both ends 1/2	28	none	no	healed
5/F/45	8	40	48	proximally ½, distally not at all	36	poor compliance, mental disorder, pseudarthrosis, re-operated	yes	failed
6/M/73	6	16	24	complete	24	extremity shortening	no	healed
7/M/33	8	-	16	complete	16	n. radialis paresis	yes	healed
8/M/21	12	-	16	complete	16	none	no	healed
9/M/34	8	18	30	complete	24	none	no	healed

Table 4. Detailed bone healing data

No side effects caused by the implant were observed; no platelet or HLA-class I antibodies were detected in any patient on follow-up.

We observed the survival of the implant to be excellent; most of the volume of the implant was preserved. Of clinical importance are ingrowths of the implant into adjacent bone. This was critical in the case of distal tibia (patient 2, 3, 5, and 9), where we observed diminished incorporation in the distal part, where the metaphysis of the tibia is less vascularized. In the case of femur pseudarthrosis (patient 6), bone quality was insufficient for the implant to regenerate the whole bone circumference, so healing was prolonged.

In theory, allogeneic platelets could have several certain side effects. In order to minimize these, all platelet units in our study were leuko-depleted and irradiated in order to prevent immune and bacteriological side effects, especially alloimmunisation to HLA-Class I and HPA antigens [50]. In fact, there was no evidence of immune reactions or transfusion-transmitted infections following the procedures. There have been no signs of bacterial contamination, which is not strange, based on the recent observation that the platelet gel exhibits significant antimicrobial activity in vitro [28].

The combined autologous/allogeneic graft showed successful incorporation into the defective pseudarthrosis in 7 out of 9 patients, which was confirmed with the CT scans and plain X-ray

film. The problem with the two patients in whom the therapy failed was the poor incorporation of the graft in the distal tibia, where bone healing is compromised through many factors [51].

One of the patients had a deteriorating psychiatric disorder and could not follow instructions later in the study, and one had a poor bone situation arising from previous treatments. The other seven patients with successful outcomes achieved a satisfactory clinical improvement with no side effects related to the procedure.

A bacterial infection did not reoccur in cases where an infection was previously treated. Our treatment has concluded prolonged ongoing hospitalizations and immobilizations for some patients who previously underwent numerous operations and rehabilitations. Only one patient had to be reoperated only once again, because of poor implant ingrowth.

2.7. Further research

As bioengineering techniques improve and become more clinically applicable, so does the field of application expand. In our work we have shown one of the methods to be useful in treating mid-size bone defects.

Further application of platelet rich plasma as a source of growth factors can be used in other settings where tissue defects exist. It is a natural derivative like blood transfusion and can be applied on the part of the body, where natural mechanisms would need some bioengineering support.

Further investigation should be directed into measuring the comparative efficiency of this treatment. It should be compared to golden standard treatment and determine also novel applications in bigger and smaller defects.

3. Conclusion

We showed that adding a platelet gel to a cancellous bone graft can help in retaining grafted bone from resorption and enhances its incorporation into adjacent bone. The standard platelet concentrates from the blood bank did not pose a significant risk for the affected patient. The results indicate good reasons for the application of this method in the treatment of bone defects in long bones.

This is the first report of a prospective clinical study monitoring the use of allogeneic platelets mixed with autologous cancellous bone for the treatment of the non-union of long bones after fractures. Our new method of tissue engineering seems to have the potential to become a widely approved and accepted method of bone tissue replacement in the treatment of the non-union of long bones.

Last, but not least, it is worth noticing that the outdate rate of the platelet units is currently in the range of 8-27% of all prepared platelet units [52] This leads to the conclusion that the successful use of allogeneic platelets would significantly decrease the amount of wasted platelets, which could consequently favorably change the results of blood banking policies.

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Chapter 14

Skeletal Muscle Ventricles (SMVs) and Biomechanical Hearts (BMHs) with a Self-Endothelializing Titanized Blood Contacting Surface

Norbert W. Guldner, Peter Klapproth,
Hangörg Zimmermann and Hans- H. Sievers

Additional information is available at the end of the chapter

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1. Introduction

For most patients with end-stage heart failure (more than 90%) there is no definitive treatment option up to now. This fact is caused on the one hand by a severe shortage in donor hearts, and on the other hand by technical and economic limitations of mechanical cardiac assist devices and artificial hearts. An additional cardiac output of 2-3 litres per minute should give most patients with end-stage heart failure a better quality of life and a longer survival. Latissimus dorsi muscle as a source for muscular blood pumps would have several advantages. Its availability is nearly unlimited, there is no foreign tissue rejection and of course no need for an immune-suppression, less risk of infection and this procedure should be less costly than heart transplantation and the treatment with fully implantable cardiac assist devices and artificial hearts.

Skeletal muscle ventricles (SMVs) and Biomechanical Hearts (BMHs) are experimental muscular blood pumps to support the circulation. They are developed and tested as future treatment option for patients with end stage heart failure. SMVs and BMHs basically have two main limitations: firstly muscle damage after electrical muscle fiber transformation from a fast twitched into a slow twitched non-fatigue muscle and secondly thromboembolic complications from the blood contacting surface especially when a muscular blood pump works on demand. Former investigators developed sack-ventricles within circulation in dogs pumping up to 4 years [1-5].

Figure 1. Boer goats with a weight between 60 and 100 kg and a latissimus dorsi muscle of 300 to 450 g.

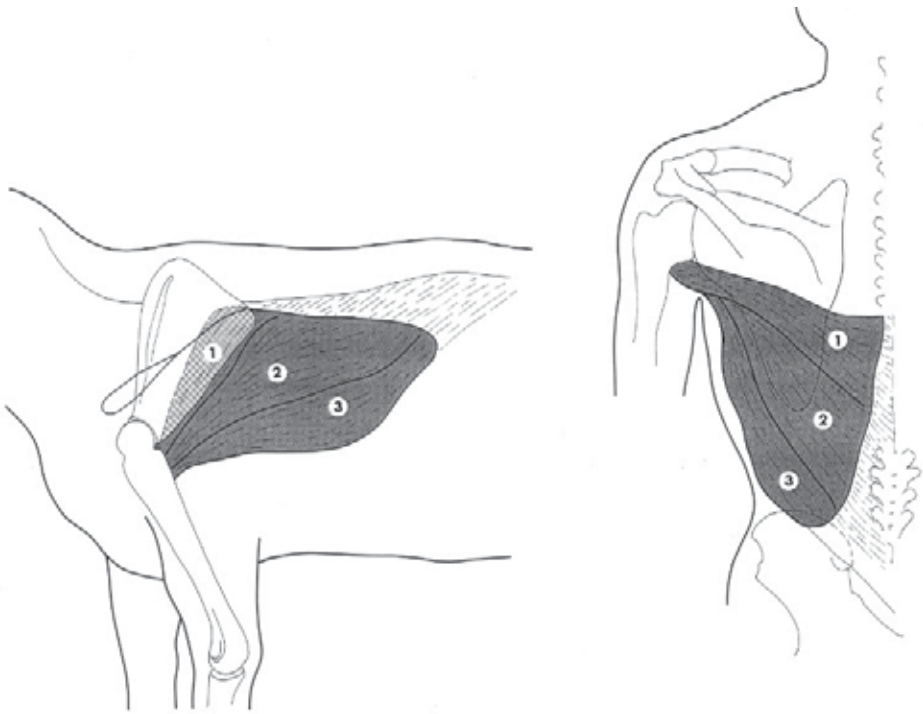

Figure 2. Topography of the latissimus dorsi muscle (LDM) in a big animal (goat) and a human. LDM consists of three parts: Pars transversalis (1), Pars obliqua (2) and Pars lateralis (3), LDMs weight is 300 to 450g in Boer bocks and about 600g in humans.

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2. Training device

To study muscle protection of LDM under different stimulation patterns and drugs to improve muscular power an elastic training device was created, where the LDM was wrapped around and muscle performance could become evaluated [6].

The training device (Figure 3) is made of silicone rubber (Q3, Dow Corning). It consists of a central chamber and two compliant side bladders filled with saline solution. The barrel-shaped central chamber and the side bladders have volumes of 150 mL and 50 mL each, respectively. The side bladders are constructed with a compliance of 1.0 to 1.3 mL/mmHg, simulating the windkessel characteristics of the arterial system in normal subjects with 1.07 mL/mmHg. More technical details can be found elsewhere (Guldner et al.,1994; 2000).

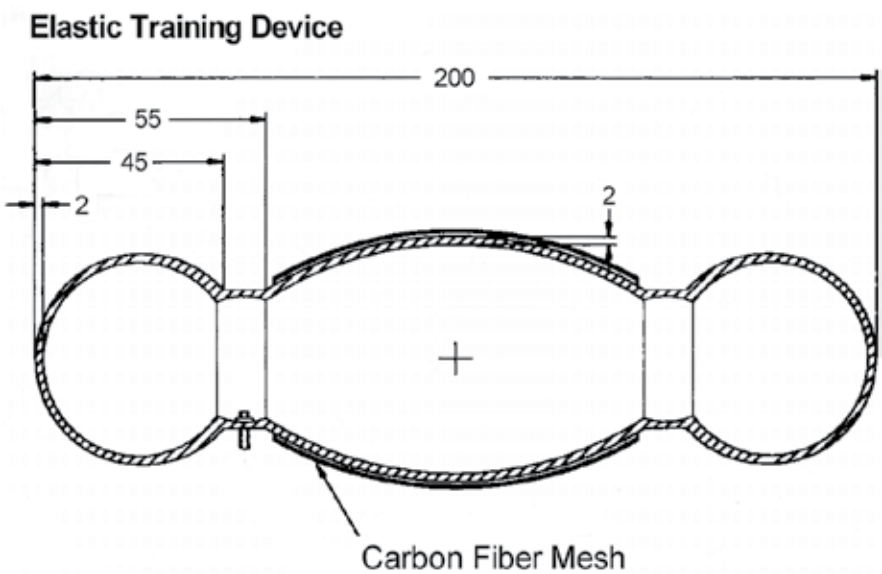

Figure 3. The Frog, an elastic training device, made of silicone, consists of a central pumping chamber which is compressible but not extendible (carbon fibre mesh).

3. Intra-thoracic implantation

Experiments were carried out in adult Boer goats (Figure 1), with a weight of 70±11 kg. They were castrated 4 weeks before the operation to keep them together and avoid injuries between them. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. They were supervised by a representative of the District President of the local society for Prevention of Cruelty to Animals.

The operation was performed under general anaesthesia. Left LDM was dissected free, folded to a double layer, and wrapped around the central chamber of the training device. The SMV was transferred into the thorax and fixed at the thoracic wall.

Commercially available myostimulators were used (Medtronic model 7420/7424 and Telectronics model 7220). An epi-mysial electrode 30mm long (custom-made, Medtronics, Bakken Research Center) was attached to the muscle close to the branches of the nervus thoracodorsalis. On the opposite side of the muscle, an electrode 60mm long (Medtronics SP 5591 – 500-60-NMS) was placed sub-fascially.

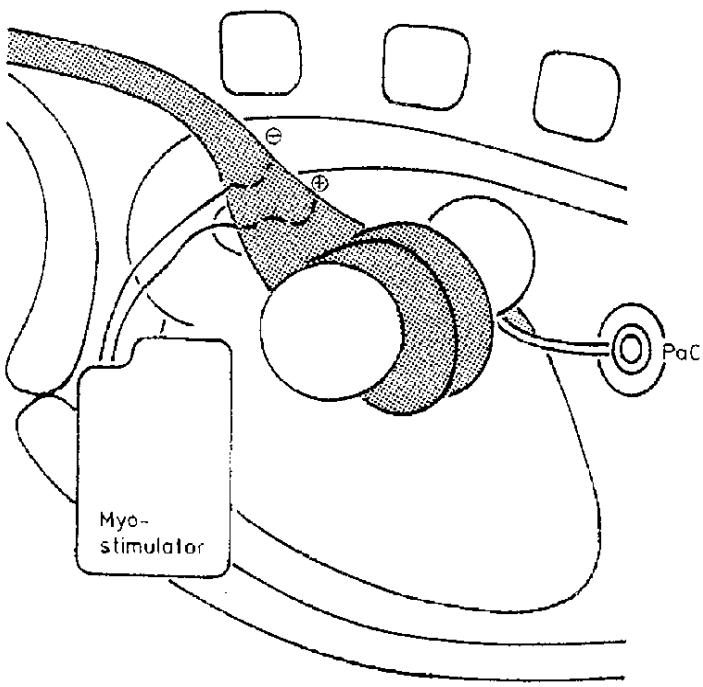

Figure 4. An intra-thoracic skeletal muscle ventricle is wrapped around the Frog: A myostimulator induces muscle contractions via 2 electrodes. Contractions of LDM cause a volume shift from the central chamber into the expanding bladders, with a corresponding pressure increase. This pressure is measured by piercing a subcutaneous vascular access port (PaC).

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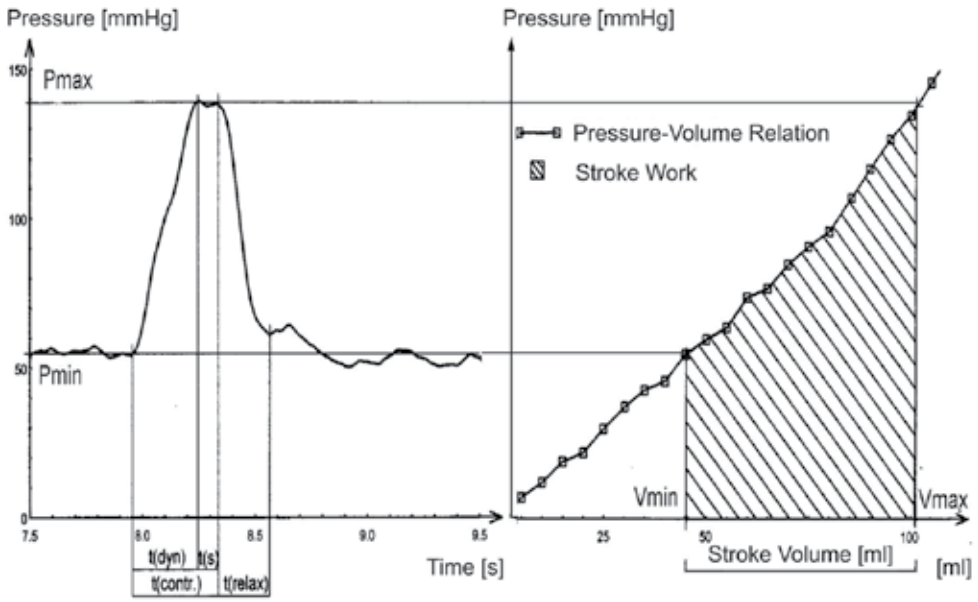

Figure 5. Method of stroke volume and stroke energy determination, relating pressure increase of a ventricle contraction (left) to the compliance curve of the bladders of the Frog, stroke volume is received (right). The area of the stroke volume below the compliance curve represents the stroke work or stroke energy [7].

Stroke volume evaluation is performed relating maximal pressure P_{max} from the pressure curve during muscle contraction to the compliance curve of the Frog's elastic side bladders (Figure 5). Stroke volume multiplied with P_{max} results in stroke work W. Daily energy in KJ/d is to calculate by stroke work W multiplied with the number of SMV contractions per day.

4. Clenbuterol supported dynamic training of skeletal muscle ventricles against systemic load

The profound loss of power that occurs in skeletal muscle after electrical conditioning has been the major limiting factor in its clinical application. This study investigates a 3-fold approach for chronic conditioning of skeletal muscle ventricles combining electrical transformation, dynamic training against systemic load and pharmacological support with clenbuterol.

In 10 adult male goats, SMVs were constructed from latissimus dorsi wrapped around an intrathoracic training device with windkessel characteristics [8]. SMVs were stimulated electrically and trained dynamically by shifting volume against systemic load. Group 1 goats were controls (n=5), and group 2 goats (n=5) were supported with clenbuterol (150μg 3 times a week).

Peak pressure, stroke volume and stroke work per day were significantly improved ($p< 0.007$) in the clebuterol- treated group after 151±2.7 days (Fig. 6). At termination, myosin heavy chains

were totally transformed into myosin heavy chain-I in all SMVs. Other investigators found different functional and histological effects of clenbuterol in dogs [9].

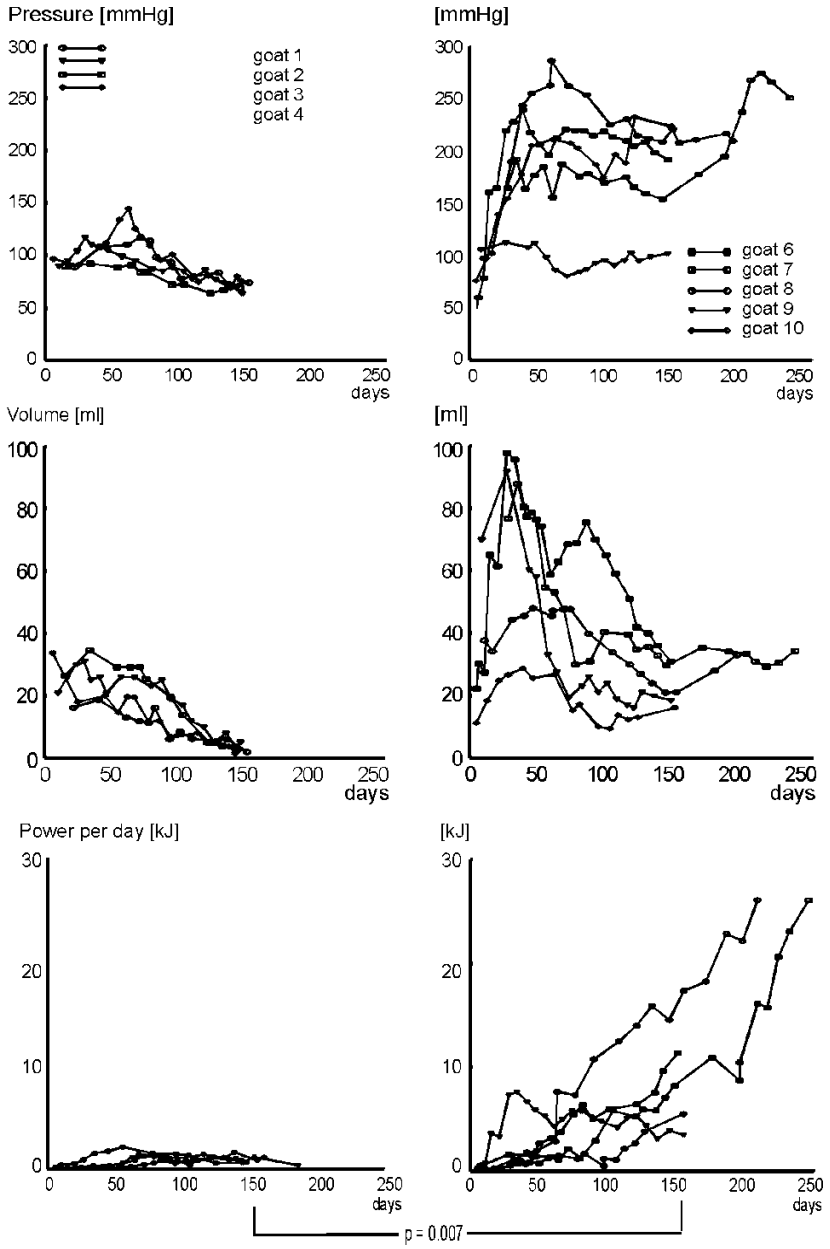

Figure 6. Time course of systolic pressure (top), stroke volume (middle), and power per day (bottom) during dynamic training of SMVs without Clenbuterol n=5 (left) and supported by clenbuterol n=5 (right) against load conditions of 60 to 70 mm Hg [8].

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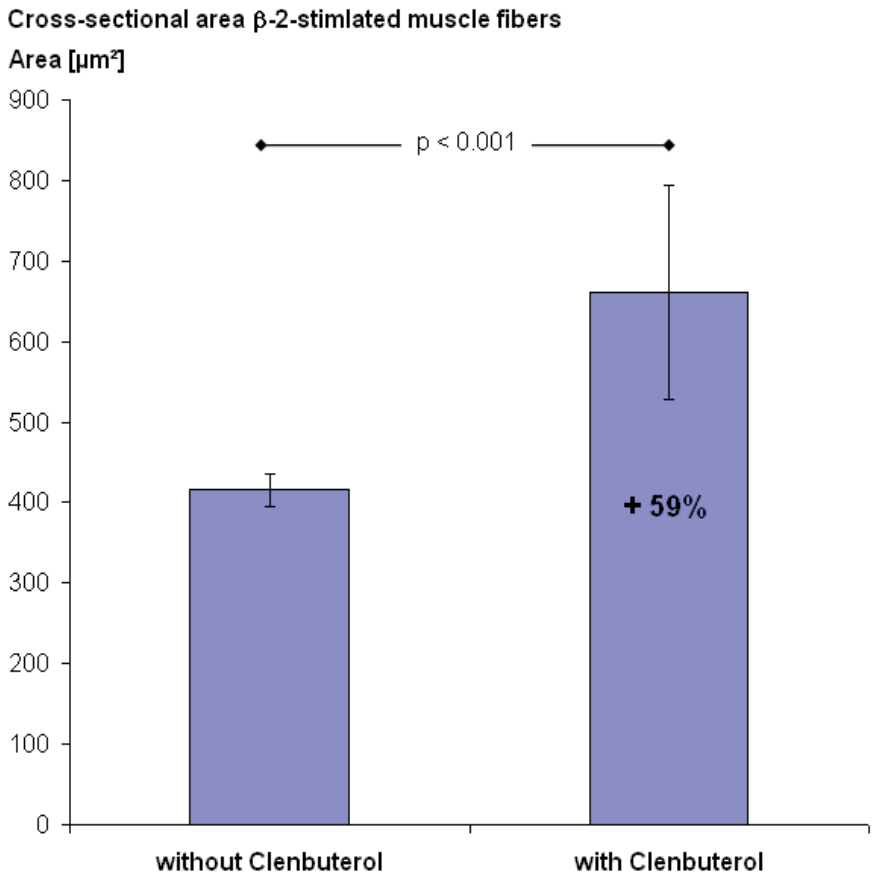

Figure 7. Cross-section areas of clenbuterol treated (β-2-stimulated) muscle fibers were enhanced by 59%.

5. Fibertransformation preserving non-fatiguable Typ IIa fibers

In our experience muscular blood pumps that were active over several months, were highly vulnerable when totally transformed into type I muscle showing severe muscle damage and decreased function. Thus, in our new experimental setting, encouraged by the Liverpool findings of the working group of Salmons and Jarvis [10], fast and fatigue resistant muscle ventricles were performed around the Frog. Pre-stimulation with about 2 Hz proceeded in

situ, to open intramuscular collaterals connecting the proximal with the distal blood supply of the muscle and to increase capillary density. Thereafter we used continuous basic burst stimulation with an average pulses frequency of less than 1 Hz. Intermittent series of pumping within the Frog were performed, measuring pumping capacity in correlation to stimulation frequency. This procedure should be a "model setting" for a pumping on demand. During our first investigations, we could see that intermittent pumping capacity was already manifold after 4 to 5 months of pumping (Figure 8).

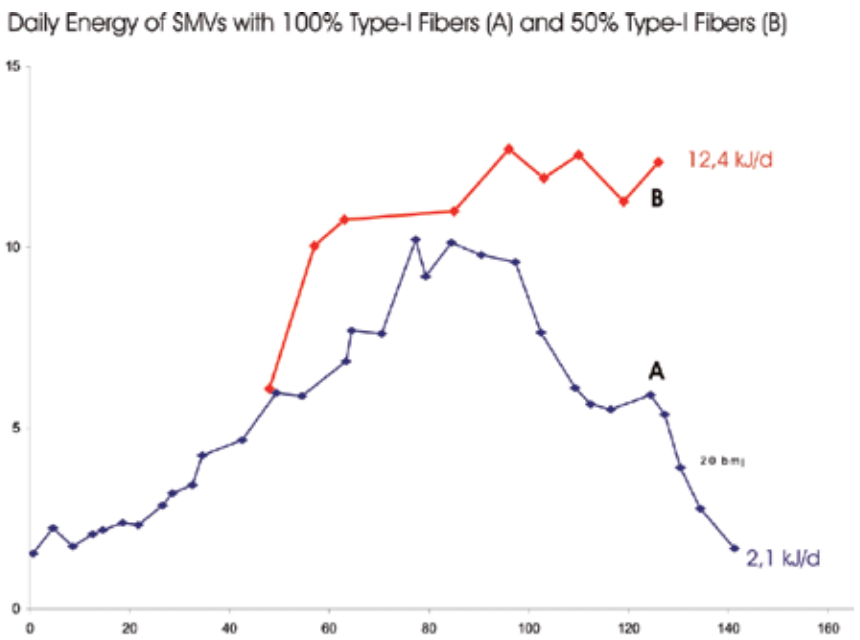

Figure 8. Daily energy of goat A (lower curve) with a mean pulse frequency of 5 Hz, which is massively declined. With 1 Hz stimulation in goat B however (upper curve) a high pumping capacity was observed.

Mean electrical pulse frequency with 5 Hz of group A (n=6) in Fig.9 resulted in a non relevant delivery of daily energy after 200 days of pumping. A 1 Hz stimulation of group B however showed an enhancing pressure and stroke volume and an increasing development of daily energy after 200 days with a well preserved muscular tissue.

Gel electrophoresis for myosin heavy chains MHC I and MHC IIa analysis (Figure 10) of group A with a mean pulse frequency of 5Hz showed a composition of MHC I (bottom of the gel) and MHC IIa (top of the gel) with mainly MHC IIa in the controls (C). In the trained SMVs (T) of group A was 100% MHC-I in all cases. In group B and with 1 Hz stimulation over months MHCIIa was preserved (50% MHC-IIa and 50% MHC-I). The preservation of Type II MHC in group B explains the more powerful contractions and the maintained pressure, stroke volume and daily energy (Figure 9).

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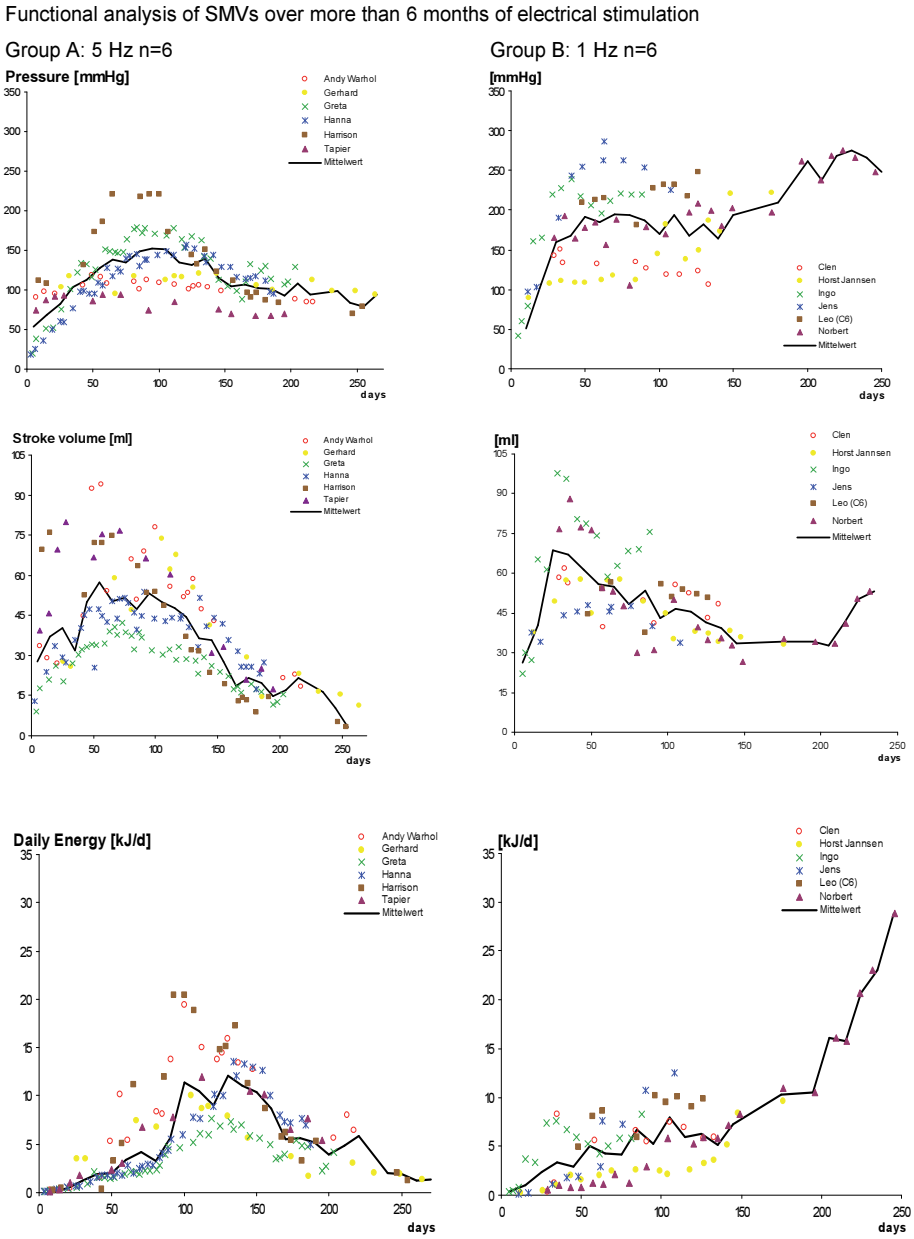

Figure 9. Systolic pressure (top),stroke volume (middle) and daily energy (bottom) of group A (n=6) with a mean pulse frequency of 5 Hz (left) and with 1 Hz in group B (n=6, right).

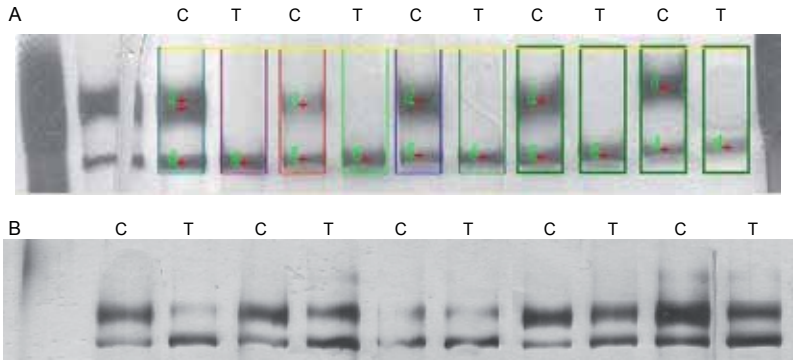

Figure 10. Gel electrophoresis of myosin heavy chains MHC I and MHC IIa from group A with a mean pulse frequency of 5 Hz. Control (C) was the non stimulated contra-lateral LDM. In group B with a mean stimulation frequency of 1 Hz, type IIa MHC is well preserved and about 50% after several months in all stimulated SMVs.

6. Stroke volume of SMVs with 100% type-I-fiber (A) vs. SMVs with 50% type-IIa fiber (B)

As an example one fast, relatively fatigue resistant SMV delivered a maximal pump volume of about 3 L/min. It could be maintained over two minutes. Thereafter it decreased to 1,5 L/min after 5 minutes. This dynamic adaptation of stroke volume per minute in that high level of pumping volume up to 3 L/min was solely possible in the 50% type IIa fibre muscle. 100 % type I fibre ventricles did enhance its pumping capacity however only up to 1 L/min.

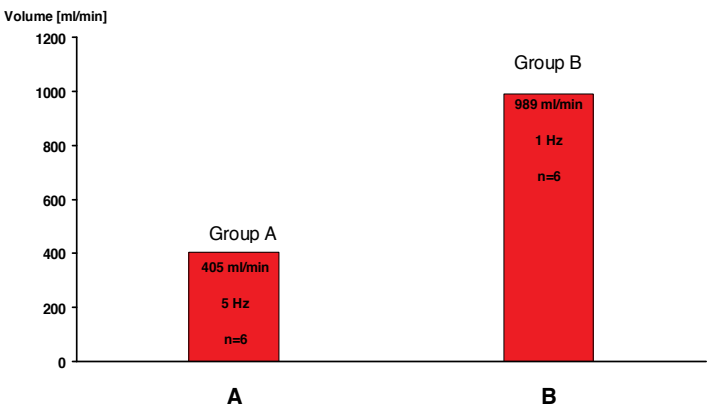

Figure 11. Stroke volumes evaluated in a Frog surrounded by a goat's SMV of a latissimus dorsi muscle of 330g up to 200 days postoperatively. In group A with 100% type I fibres stroke volume per minute was at 405 ml and in the fast, relatively fatigue resistant muscle with about 50% type IIa fibres stroke volume per minute was at 888 ml per minute. This amount of stroke volume per minute could be maintained over months.

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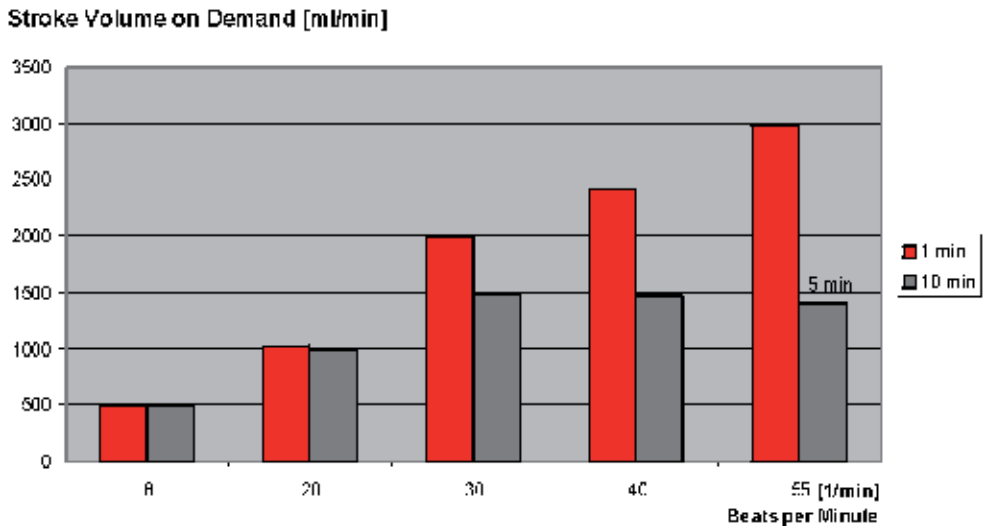

Figure 12. Stroke volumes of an "on demand" setting (see text!) evaluated in a Frog surrounded by a goat's SMV of a latissimus dorsi muscle of 330g 6 weeks postoperatively.

These recent experimental results in SMVs around the Frog were basic to construct a pre-clinical Biomechanical Heart Model on demand, which is described as follows.

7. Valve-less Biomechanical Hearts

Biomechanical Hearts, constructed in adult Boer goats (n=5), are blood pumps, consisting of a pumping chamber with clinically relevant stroke volumes [11]. They can be integrated into the circulation in a one-step operative procedure during pharmacological stimulation with the β-2-stimulator Clenbuterol (5 x150μg/wk). This experimental pumping chamber, made of PTFE, was anastomosed to the descending aorta by two ring armoured PTFE-prostheses (Impra Medica GmbH, München), as shown in Figure 13. The pumping chamber was used mainly for three reasons: firstly, to stabilize the ventricular pump cavity with improved flow characteristics to minimize thrombo-embolic complications; secondly, to prevent muscle damage by overstretch-induced ischemia; and thirdly, to prevent a ventricular chamber rupture.

During surgery, the mean stroke volume of BMHs was 53.8±22.4 ml. One month after surgery, in peripheral pressure, the mean and minimal diastolic pressure of BMH-supported heart cycle differed significantly from unsupported ones (Figure 14). After BMH-supported heart contractions, the subsequent maximal rate of pressure generation, dP/dt_{max} increased by 20.5±8.1% ($p<0.02$). One BMH, catheterized 132 days after surgery, shifted a volume of 34.8 mL per beat and 1.4 L/min with a latissimus dorsi muscle of 330 g (Figure 14, top).

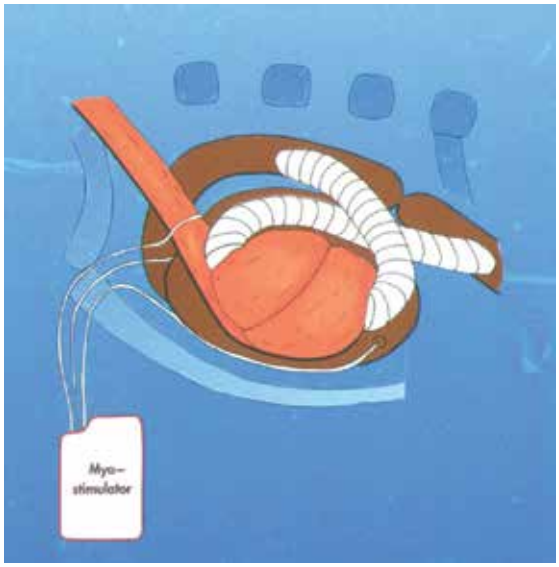

Figure 13. Scheme of an experimental setting in a big animal model in an aorto-aortic configuration. The thoracic aorta is ligated between the two anastomoses. Two muscular stimulation electrodes activate the LDM and an epicardial sensing electrode enables the syncronization with the heart cycle.

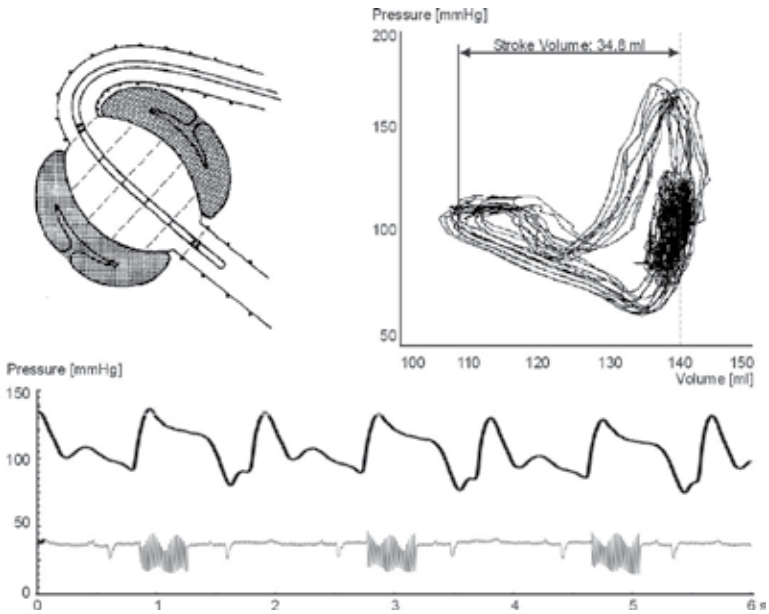

Figure 14. Stroke volume determination with a conductance catheter, placed within the pumping chamber of the BMH (left, top). Pressure-volume-loop of a BMH on postoperative day 132 with a stroke volume of 34.8 ml and an output of 1400ml per minute (right, top). ECG with stimulation bursts, a pressure trace from a peripheral artery where the BMH is in a 1:2 mode and synchronized with the heart (bottom).

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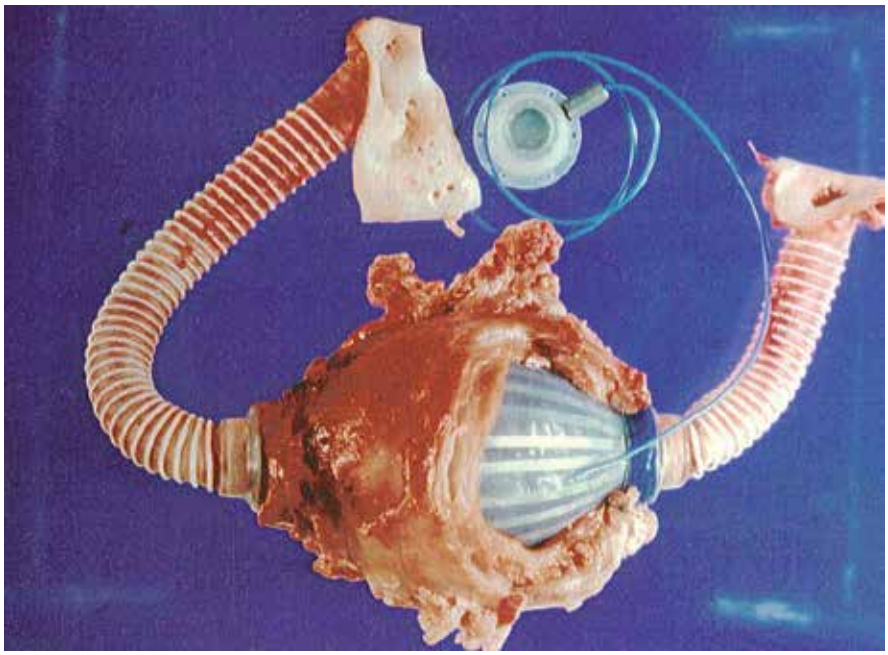

Figure 15. Explanted BMH without valves after 414 days of pumping within a Boer goat. The pumping chamber was made of a double layered polyurethane membrane including steel springs and it was connected to the aorta by ring armoured PTFE prostheses.

8. Hemodynamic evaluations of a valve equipped Biomechanic Heart Model supporting a failing myocardium in goats

As shown previously in goats, valve-less Biomechanical Hearts (BMHs) of a clinically relevant size could be trained effectively in the systemic circulation under support of clenbuterol. Pumping capacity was more than 1 L/min but due to a high pendulum volume no significant flow contribution for the circulation was gained. Thus, the following investigations were performed to evaluate the efficacy of valve-equipped BMHs in comparison to valve-less BMHs. To mimic the clinical situation, this test was performed in failing hearts [12].

Heart failure was induced in adult Boer goats (n=5) by a repeated intra-coronary embolization. A valve-bearing and balloon-equipped pumping chamber was integrated into the descending aorta simulating standardized circulatory BMH support. Circulatory flow was evaluated by a flow meter around the pulmonary artery. Myocardial function was evaluated by a conductance catheter placed in the left heart ventricle (Figure 16).

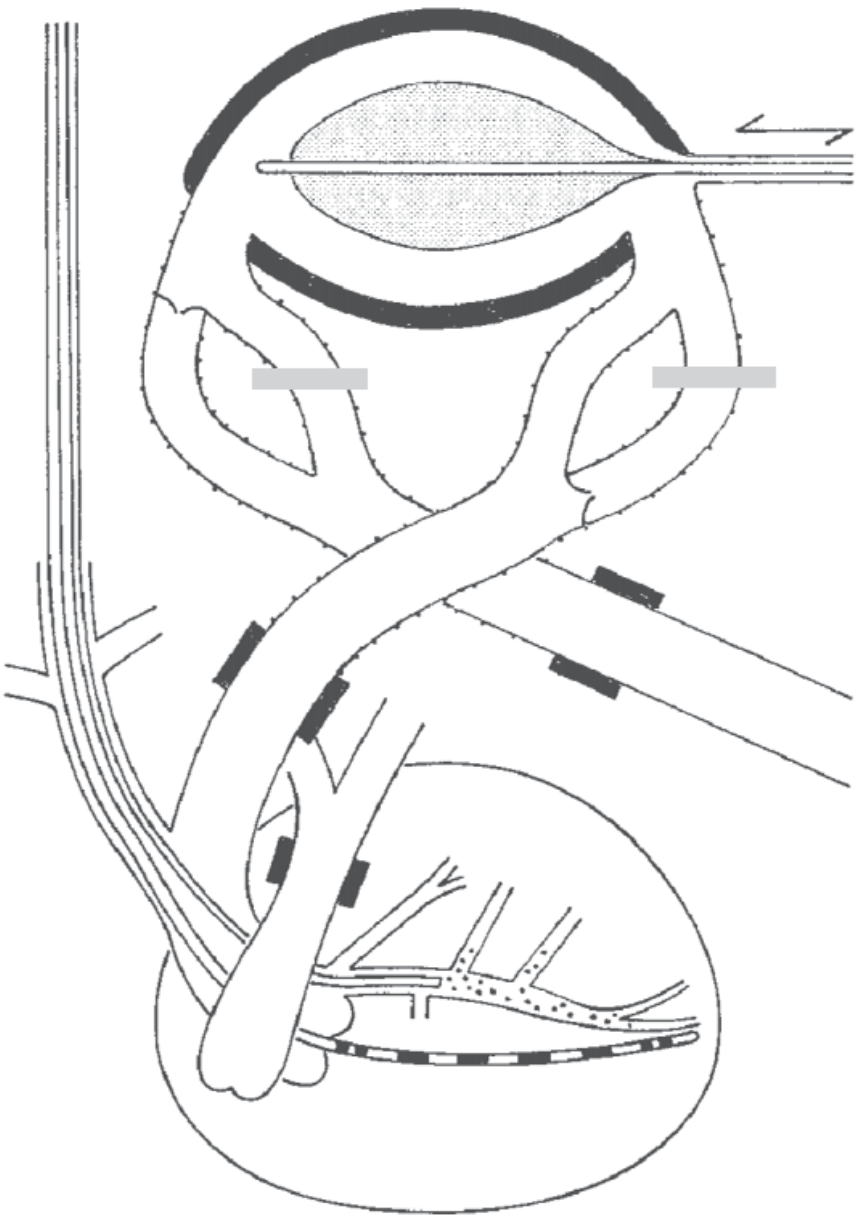

Figure 16. BMH model is made of a stiff polyurethane chamber with an integrated pumping balloon. The dividing and re-uniting vascular prostheses were connected end –to-end with the divided descending aorta. In this setting two of the four prosthetic limbs carried heart valves. Thus, by clamping, no, one or two valves could be integrated into the circulation. The influence of different valve configurations on circulation could be evaluated in supporting a failing heart. Ultrasonic flow probes were placed around the pulmonary artery, aortic arch and the descending aorta. Within the left heart ventricle a conductance catheter was placed, and via a catheter within the left coronary artery an embolization could be induced and a flow wire could be introduced [12].

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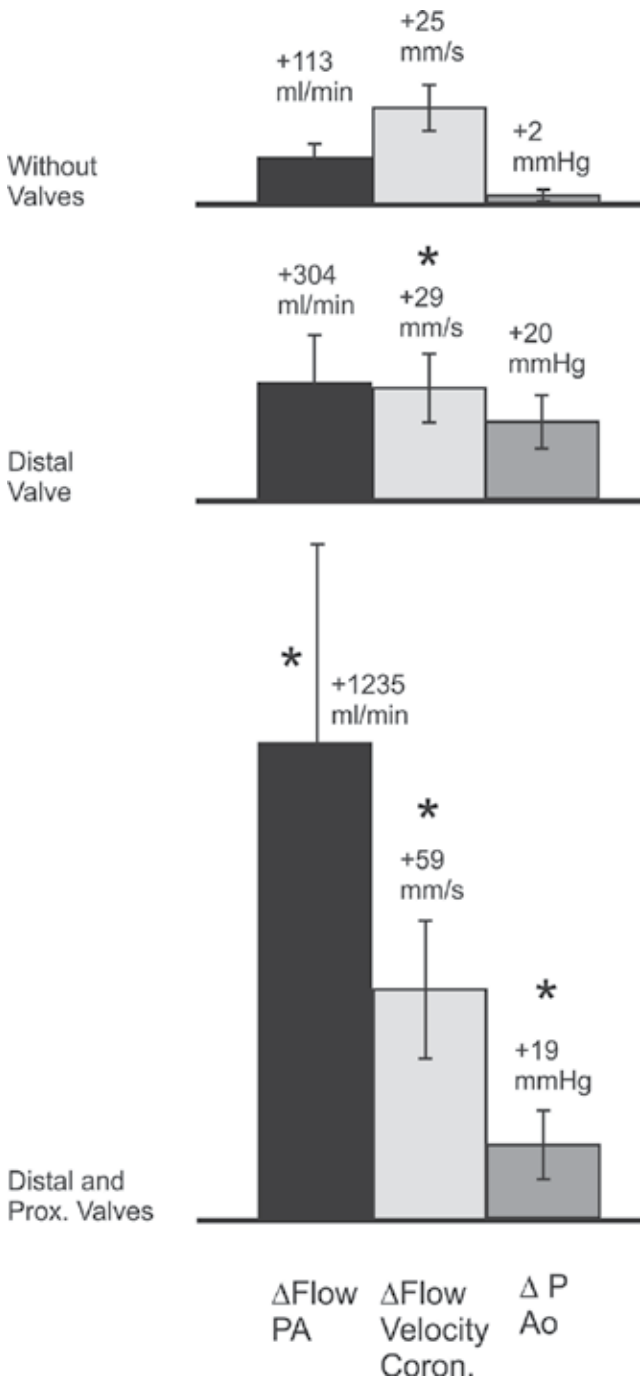

Figure 17. Results of the BMH model described in Fig. 16, without, with a distal and with two valves: mean aortic pressure(P_{Ao}, grey column), mean pulmonary flow (Q_{PA}, black column), mean flow velocity within the left coronary artery (V_C, white column) [12].

Valve-less BMHs offered an additional pulmonary flow of 113± 37 ml/min resp. 5.4±1.8%, those with one distal valve offered 304±126 ml/min resp. 14.5±6%. BMHs equipped with two valves increased the pulmonary blood flow by 1235± 526 ml/min resp. 58±25 % ($p<0.05$), the mean aortic pressure in this setting raised to 19±9 mmHg ($p<0.05$) and the coronary flow velocity to 59±18 mm/sec ($p<0.05$). Corresponding reduction of left ventricle's end-diastolic pressure ranged from 31 to 17 mmHg ($p<0.05$), while the myocardial dp/dt increased by 470±192 mmHg/s resp. 145±48 % ($p<0.05$).

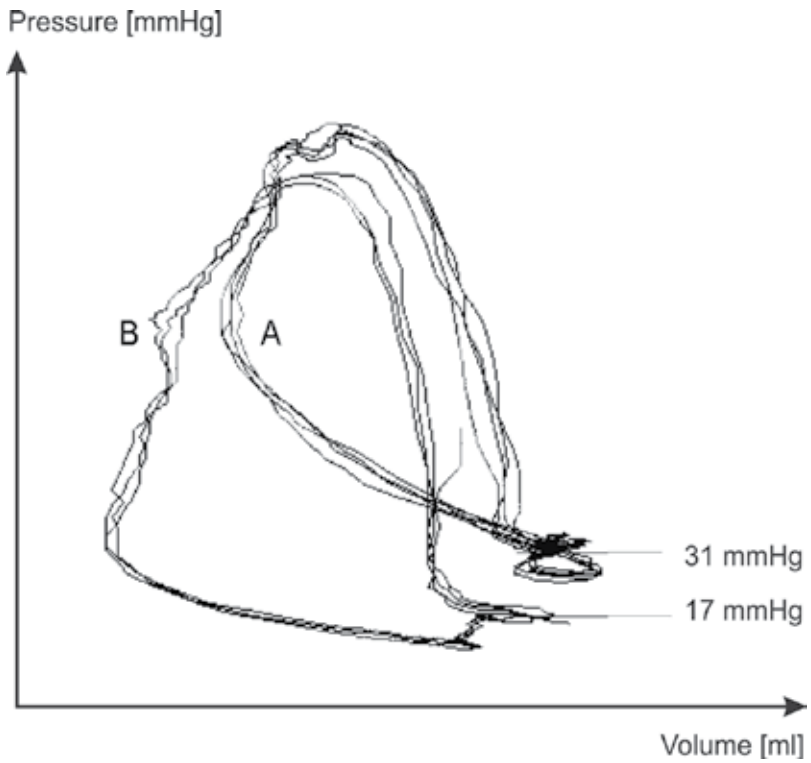

Figure 18. PV- loops from a conductance catheter placed in the left heart ventriclea cavum, without (A) and with an activated (B) double-valved BMH-model as shown in Figure16. It works ECG-triggered in a 1:2 mode with a balloon inflation of 60ml helium gas. The area within a loop represents the left heart ventricles stroke work. During activation of the BMH-model the stroke work of the failing heart ventricle is increased (B) and the end-diastolic pressure (LVEDP) drops from 28 to 14 mmHg [12].

The use of two valves in BMHs is essential for a relevant circulatory support. Unloading and contractility of the left heart ventricle were thus improved significantly. Two-valves-BMHs driven by a sufficient skeletal muscle ventricle may contribute to the therapy of a failing myocardium.

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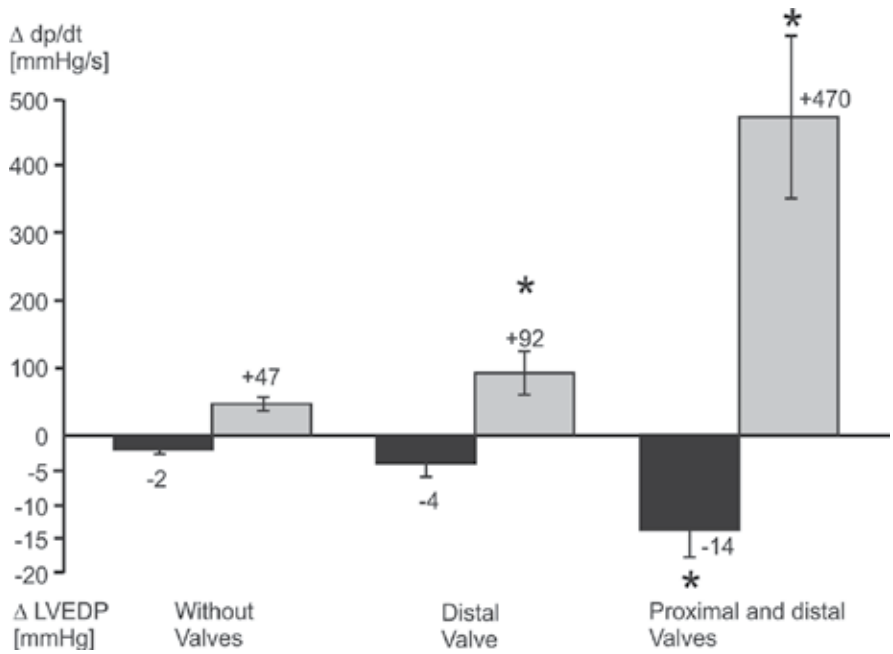

Figure 19. Reduction of the left ventricular end-diastolic pressure (LVEDP, black columns) evaluated as demonstrated in Figure 16 and 18 and an increase of the left ventricular contractility activation of the pumping balloon from the BMH-model in Figure 16, without, with a distal and with a proximal and a distal valve.

9. Valve equipped Biomechanical Hearts

As demonstrated above, efficacy of BMHs on the circulation is dependent on the integration of two heart valve prostheses into the in- and outflow part of the pumping chamber. Valve equipped Biomechanical Hearts were constructed and integrated within circulation in adult Boer goat (n=5), and pharmacological stimulation with the β-2-stimulator Clenbuterol. (5X150μg/wk). This pumping chamber, made of PTFE, was anastomosed to the descending aorta by two ring armoured PTFE-prostheses (Impra Medica GmbH, München). Between these prostheses two porcine glutaraldehyde fixed valve bearing porcine aortic conduits were integrated like shown in Figure 20.

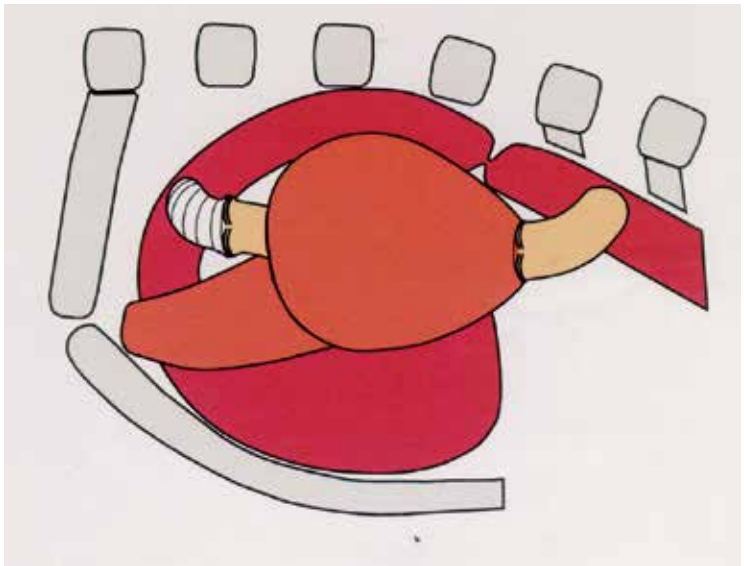

Figure 20. Scheme of an experimental setting from a valve equipped Biomechanical Heart in a big animal model (Boer goats) in an aorto-aortic configuration. The thoracic aorta is ligated between the two anastomoses. Two porcine glutaraldehyde fixed valve bearing porcine aortic conduits were integrated between the connecting PTFE prostheses and the PTFE pump ventricle.

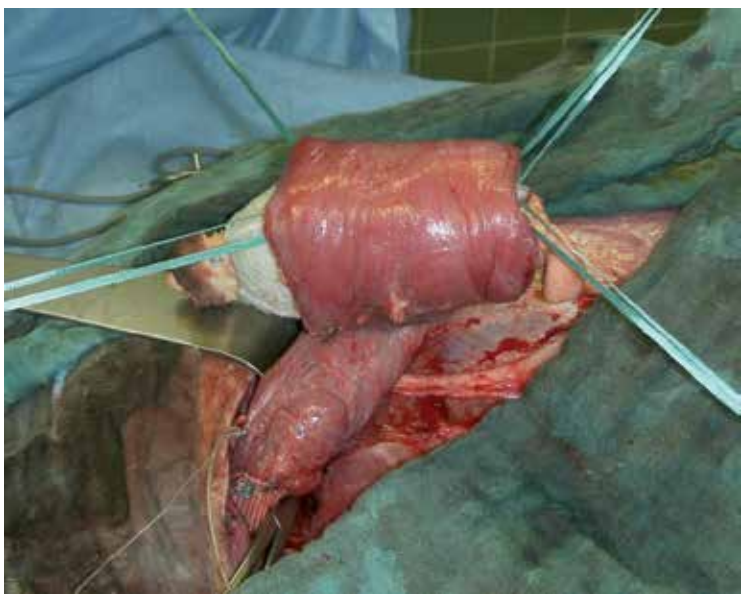

Figure 21. Operative situs of a BMH before transferring it into the cavity of the thorax, with four strings to fix it via the thoracic wall to the inner thoracic surface. Two stimulation electrodes and an artificial muscle tendon made of Dacron for a re-fixation onto the external thoracic wall are visible.

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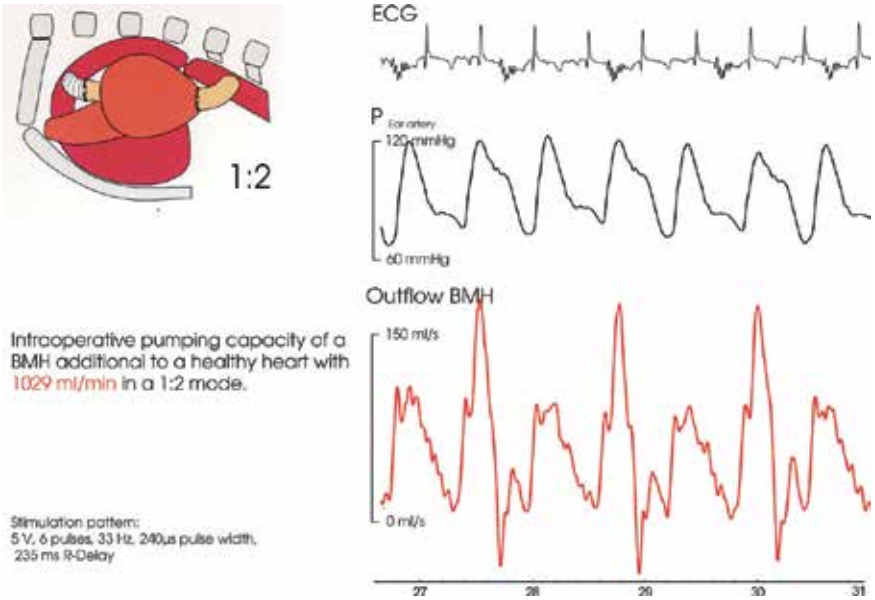

Figure 22. Intraoperative testing of the function of a valve equipped BMH by visualization of burst stimulation within the ECG, a 1:2 support within the arterial pressure curve and the flow curve which was obtained by a flow probe around the distal biologic aortic conduit

	Stroke Volume (L/min)	Type of pumping chamber	Days of Pumping	Days of Survival	MHC- I	Significant Findings and Cause of Death
Larry	1029,6	Dacron	93	93	56 %	Seroma in both pleura
Juan	407,5	Dacron	267	301	95 %	Acute abdomen, total thrombosis of the pumping chamber, sacrification
Ugo	444,5	Dacron	136	182	78 %	Acute abdomen, total thrombosis of the pumping chamber, sacrification
Stanley	474,9	ePTFE (titanized)	225	385	94 %	Hematothorax, bleeding from proximal conduit, no chamber thrombus formation
Pierre	588	ePTFE (titanized)	180	446	100 %	Acute abdomen, intestinal infection, no chamber thrombus formation
Valeri	576,3	ePTFE (titanized)	531	914	--	Infection, sepsis. no chamber thrombus formation

Table 1. Intra- and post-operative data of six experimental valve equipped BMHs in adult Boer Goats

10. Self-endothelializing titanized blood contacting surface for Biomechanical Hearts

Titanium has proven itself as the leading structured metallic biomaterial for 50 years [13]. One reason for this widespread use is the excellent biocompatibility of the metal and its alloys [14]. Theoretically, surface coating of the blood contacting PTFE may open new avenues for improving biocompatibility of this kind of implant material. However, a titanium coating on PTFE seemed not to be possible until now due to the high temperatures needed for commonly used sputtering techniques. Therefore a novel coating method was used [15] for PTFE applying a plasma activated chemical gas deposition (PACVD) at temperatures of 30-35°C.

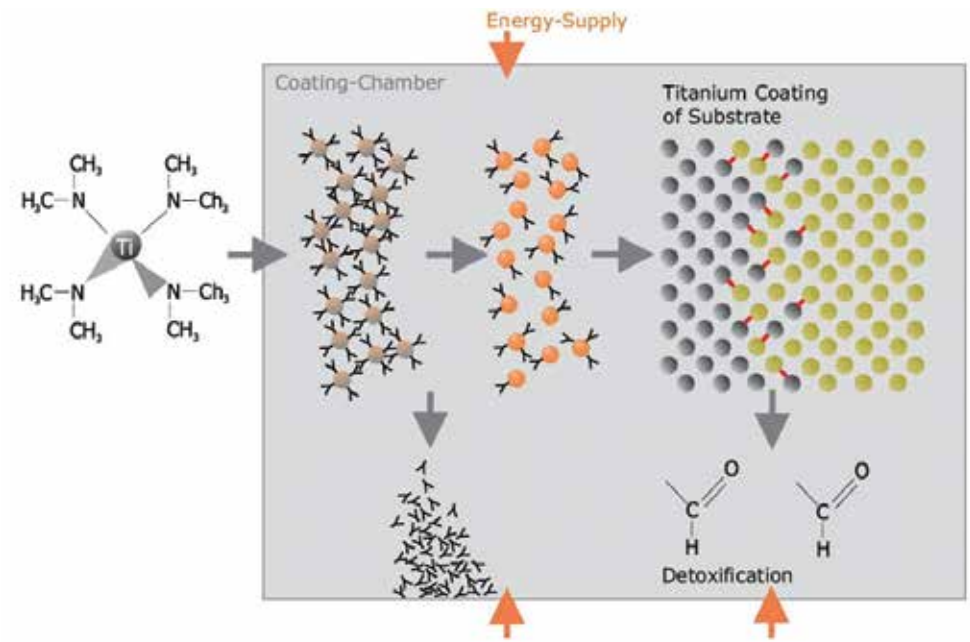

Figure 23. Principle of titanium coating at low temperature (see text).

PACVD is a coating technology (Pfm Titanium GmbH, Nürnberg, Germany, patent number EP 0 897 997 A1) where the so called precursor (Tetrakisdimethylamidotitanium, Ti $[N(CH_3)_2]_4$) is transferred into the gas phase and brought into the reactor by a carrier gas like nitrogen gas [16]. The plasma is able to supply the substrate with high energy while the temperature during deposition can be kept low at about 30-35°C. Within that non-thermal plasma with high electron temperatures but neutrons and ions at room temperature, solely the electrons can follow a quickly changing electrical field with a typical frequency of 13.56 MHz under low pressure plasma as described in detail elsewhere [17]. The precursor, or part of it, reacts with the substrate and creates a layer of 30nm in thickness (Figure 23).

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The blood contacting surface of the PTFE-made pumping chamber was titanized as described above. This titan surface attracts progenitor cells derived from the bone marrow which were delivered into circulation. The seeded cells transform into endothelial cells within time [18]. This kind of self-tissue engineering of the blood contacting surface is mandatory to prevent thrombo-embolism mainly for blood pumps contracting on demand [14].

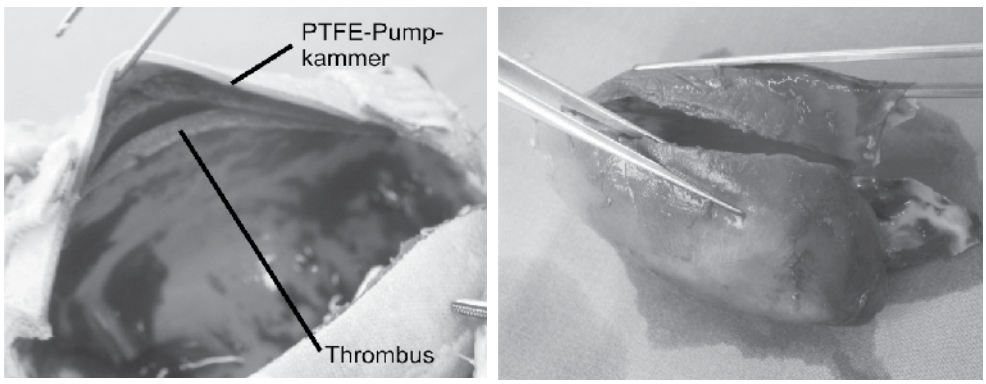

Figure 24. Thrombosis of the total blood contacting surface 8 weeks after implantation (left): Isolated thrombus formation from the PTFE- pumping chamber (right).

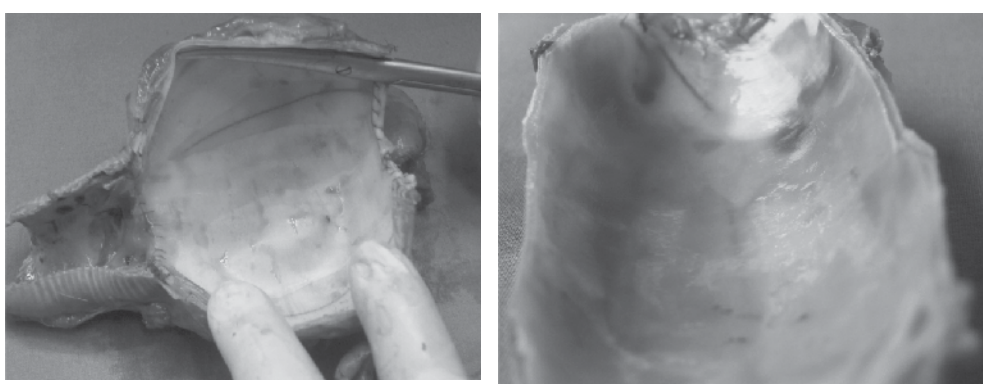

Figure 25. Titanized PTFE pumping chamber 6.5 months after implantation without any thrombus formation. This thin cover could be identified as an endothelial layer by a von Willebrand immun-histological staining.

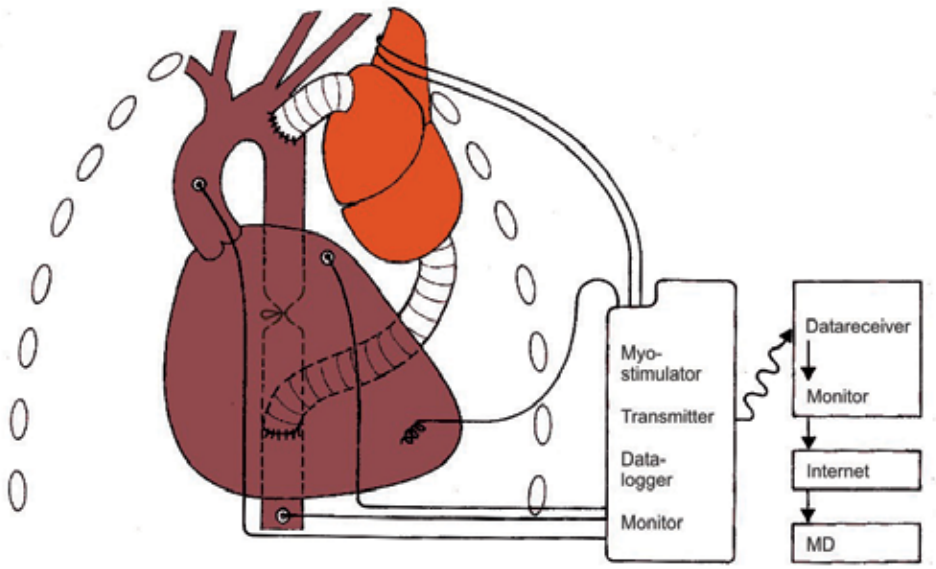

Figure 26. Clinical setting of a BMH in an aorto-aortic configuration

11. Summary and perspectives

A BMH is therapeutically indicated for patients with end-stage heart failure with an additional need of a support with 2-3 litres per minute "on demand", especially for candidates older than 60 years having no chance for heart transplantation. For a severe bi-ventricular myocardial insufficiency however, heart transplantation is the first choice.

Experimental Biomechanical Hearts in Boer goats with autologous skeletal muscle were to construct pumping up to 1.400 mL/min for more than 400 days under support of Clenbuterol. Furthermore was to demonstrate that a BMH model supporting a failing heart in Boer goats could pump about 2 litre blood per minute additionally. BMHs equipped with two valves were most effective in-vivo. Effective circulatory support by counter pulsation was achieved by SMVs elsewhere [19].

Muscle damage and power-loss of a BMH can be avoided by a muscle protective myostimulator applying a closed-loop controlled stimulation and thus maintaining type IIa fibres over years (Microstim GmbH, MyoSen®, Wismar; Germany). A titanized blood contacting surface with endothelialization (Pfm titanium GmbH, Nürnberg; Germany) might prevent thromboembolic complications. In the light of 25 years of systematic progress in basic science of muscle powered cardiac assist this biologic treatment option should become reconsidered as a future surgical treatment option for the therapy of end-stage heart failure [20].

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Chapter 15

Cartilage Tissue Engineering: The Role of Extracellular Matrix (ECM) and Novel Strategies

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Additional information is available at the end of the chapter

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1. Introduction

Articular cartilage is a hyaline cartilage that consists primarily of extracellular matrix with a sparse population of cells, lacking blood vessels, lymphatic vessels and nerves. The only cell type within cartilage is the chondrocyte and has a low level of metabolic activity with little or no cell division and is the responsible for maintaining in a low-turnover state the unique composition and organization of the matrix that was determined during embryonic and postnatal development. The biological and mechanical properties of articular cartilage depend on the interactions between the chondrocytes and the matrix that maintain the tissue. Chondrocytes form the macromolecular framework of the tissue matrix from three classes of molecules: collagens, proteoglycans, and non-collagenous proteins and maintain the extracellular matrix (ECM) by low-turnover replacement of certain matrix proteins [1, 2].

Aggrecan and type II collagen are the most abundant proteins found within the ECM in the articular cartilage and they are linked together by a number of collagen-binding proteins including cartilage oligomeric matrix protein (COMP), chondroadherin and other minor collagens on their surface. Aggrecan is a large aggregating proteoglycan which is in association with hyaluronan (HA) and link protein (LP). These aggregates are responsible for the turgid and they provide the osmotic properties to resist compressive loads and retain water. Also contain a variety of small leucine-rich repeat proteoglycans (SLRPs) as decorin, biglycan, fibromodulin and lumican where they help maintain the integrity of the tissue and modulate its metabolism [3, 4].

2. Alteration in cartilage composition in Osteoarthritis (OA)

The chondrocyte is responsible for both the synthesis and the breakdown of the cartilaginous matrix but the mechanisms that control this balance are poorly understood [4]. The distribution of load across the joint is an important function of the articular cartilage for avoid excessive load affecting both cartilage and bone. It has been demonstrated that articular chondrocytes are able to respond to mechanical injury where biological stimuli such as cytokines and growth and differentiation factors contribute to structural changes in the surrounding cartilage matrix. It has been demonstrated that many non-mechanical and mechanical factors such as load clearly have a role in the initiation and propagation the processes of OA. The OA is the most common joint disease allowing dysfunction and pain. The OA is characterized by changes in chondrocyte metabolism that leads to elevated production of proteolytic enzymes, cartilage damage and loss of joint function. It have been described several mechanisms that can lead to OA, among of these mechanisms are mechanicals, bone changes and changes in the cartilage extracellular matrix [5, 6]

Aging, cartilage senescence and reactive oxygen species (ROS) are normal changes in the musculoskeletal system that contribute to the development of OA, but the mechanisms are poorly understood [5]. Inflammation is considered as a very early event in OA perhaps induced by joint trauma affecting chondrocytes in the cartilage and synovial cells (fibroblasts and macrophages) to produce cytokines as interleukin-1-beta (IL-1β) and tumoral necrosis factor-alpha (TNF-α), and other signaling molecules as proteoglycans to switch to or increase catabolic processes [6]. Obesity has been described as a risk factor for OA by increased mechanical load factors and degenerative knee pain. The mechanisms between obesity and OA are not completly understood but, it has been found the release of fat molecules that can affect the processes in the joint, including adipokines as visfatin and leptin, perhaps affecting the inflammatory response [7, 9]. Malalignment of the knee joint plays an important role in the development of early osteoarthritis changing the center of pressure of articular cartilage and subchondral bone. Varus or valgus malalignment of the lower extremity results in an abnormal load distribution across the medial and lateral tibiofemoral compartment and being increased in patients with knee osteoarthritis and is increased in patients with overweight. However, studies examining the relationship between malalignment and early knee osteoarthritis have produced conflicting results. The association between malalignment and OA changes is based on radiographic changes mainly and different multicenter OA studies [10-12]. Meniscus is an important tissue in the system of the knee. It is function is the load transmission and absortion shock. Complete or partial loss of meniscal tissue alters the biomechanical and biological of the knee joint modifying the pattern of load distribution and the instability of the knee. Meniscal narrowing, cartilage loss and chondral lesions increase the risk of secondary OA with cartilage degeneration. This secondary OA is associated to chondral damage, ligamentous instability, and malalignment with reduction in the shock absorption capacity of the knee [13-15]. Extrussion has been associated with articular changes according to their depth into partial-thickness and full-thickens defects. Partial-thickness lesions are considered less symptomatic with little evidence of progression on osteoarthritis. Full-thickness chondral and osteochondral lesions frequently cause symptoms, and they are considered to predispose to

premature osteoarthritis [16]. Osteochondritis dissecans studies have demonstrated knee joint dysfunction and high prevalence of osteoarthritic change after fragment removal and all the studies take in account the limitation of a small defect size from 1.5 to 4.0 cm^2 as well the zone and the location of the defect in the cartilage [17, 18]. The anterior cruciate ligament (ACL) is the knee ligament most common disrupted. ACL lesion frequently is associated to other ligamentous structures like, menisci, the articular cartilage or subchondral plate [19, 20].

3. Articular cartilage homeostasis

Articular cartilage is composed of four distinct regions and they differ in their collagen fibril orientation: (a) the superficial or tangential zone (200 μm), (b) the middle or transitional zone, (c) the deep or radial zone and (d) the calcified cartilage zone. The superficial zone is composed of thin collagen fibrils in tangential array parallel to surface with a high concentration of decorin and lubricin and a low concentration of aggrecan. The middle zone is composed thicker collagen fibrils more random organized. The deep zone is composed the collagen bundles thickest and arranged in a radial fashion, orthogonal to the surface, and the calcified cartilage zone, located above subchondral bone and the tidemark that persists after growth plate closure and is composed of matrix vesicles, vascularization and innervation from the subchondral bone. The collagen type in the calcified zone surrounding the cells is type X as in the hypertrofic zone of the growth plate [21, 22], [23]. From the superficial to the deep zone, cell density progressively decreases. The chondrocytes in the superficial zone are small and flattened. The chondrocytes in the middle zone are rounded, and the deep zone chondrocytes are grouped in columns or clusters and they are larger and express markers of the hypertrophy as well. Differences in expression of zonal subpopulations may determine the zonal differences in matrix composition and in the mechanical environment [24, 25].

Chondrocytes live at low oxygen tension within the cartilage matrix, ranging from 10% at the surface to less than 1% in the deep zones. *In vitro*, chondrocytes adapt to low oxygen tensions by up-regulating hypoxia-inducible factor-1-alpha (HIF-1α), which stimulate expression of glucose transport via constitutive glucose transporter proteins (GLUTs) and angiogenic factors such as vascular endothelial growth factor (VEGF) as well as a number of genes associated with cartilage anabolism and chondrocyte differentiation [26, 27].

It is no clear how chondrocytes maintain their ECM under normal conditions since they lack access to the vascular system but gene expression and protein synthesis may be activated by injury. The aging may affect the properties of normal cartilage by altering the content, composition and structural organization of collagen and proteoglycans. The normal function of the articular cartilage within the joint is to be elastic and have high tensile strength and these properties depend on the extracellular matrix [28]. The chondrocytes produce, in appropriate amounts, this ECM that consist of structural macromolecules of type II collagen fibers, proteoglycans, non-collagenous proteins and glycoproteins, organized into a highly ordered molecular framework. The collagen matrix gives cartilage its form and tensile strength. Proteoglycans and non-collagenous proteins bind to the collagenous network and help to

stabilize the matrix framework and bind the chondrocytes to the macromolecules of the network. The matrix protects the cells from injury due to normal use of the joint, determines the types and concentrations of molecules that reach the cells and helps to maintain the chondrocyte phenotype [29, 30].

The ECM surrounding the chondrocytes has been divided into zones depending on their distance from the cell. The pericellular matrix is localized immediately around the cell, the territorial matrix is next to pericellular matrix and the most distance is the interterritorial matrix. Each matrix zone is characterized by different types of collagens as shown in figure 1.

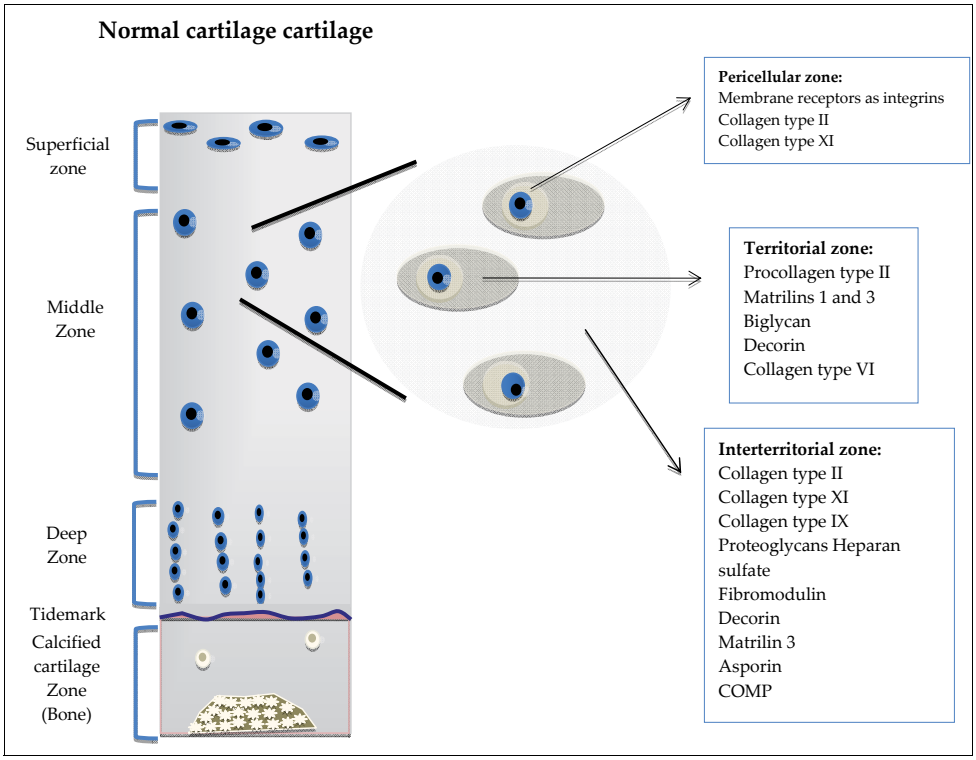

Figure 1. The organization of normal articular cartilage. The organization of chondrocytes is divided in superficial, middle or transitional, deep or radial and calcified cartilage zones with a boundary or tidemark between the first three zones and the calcified zone. The extracellular matrix is divided depending the distance from the chondrocytes. The pericellular zone is the matrix surrounding immediately the chondrocytes. The territorial zone is the next to pericellular zone and the interterritorial zone is the most distant. Every zone has specific characteristics related with the shape of the chondrocyte as well the activity and the expression of different molecules by the cell.

The pericellular matrix is a region surrounding chondrocytes in the articular cartilage where diverse molecules as growth factors have interaction with the receptors expressed on the membrane cell of chondrocyte. This region is rich in proteoglycans as aggrecan, hyaluronan and decorin. Type II, VI and IX are collagen most concentrated in the pericellular network of thin fibrils as fibronectin. Type VI collagen forms part of the matrix immediately surrounding

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the chondrocytes and may help them to attach to the macromolecular framework of the matrix. This pericellular matrix enclosed cells has been termed chondron. The territorial zone contains type VI collagen microfibrils but little or no fibrillar collagen. The interterritorial cartilage matrix is composed of a collagen type II, type XI collagen and type IX collagen integrated in the fibril surface with the non-collagen domain, permitting association with other matrix components and retention of proteoglycans. These collagens give to the cartilage form, tensile stiffness and strength [31-33].

Cartilage contains a variety of proteoglycans that are essential for its normal function. These include aggrecan, decorin, biglycan, fibromodulin and lumican each proteoglycan has several functions determined. The proteoglycans are very important for protecting the collagen network. Other non-collagen molecules as the matrilins and cartilage oligomeric protein (COMP) are also present in the matrix. COMP acts as a catalyst in collagen fibrillogenesis, and interactions between type IX collagen and COMP or matrilin-3 are essential for proper formation and maintenance of the articular cartilage matrix. Perlecan enhances fibril formation, and collagen VI microfibrils connect to collagen II and aggrecan via complexes of matrilin-1 and biglycan or decorin [34].

Throughout life, the cartilage undergoes continual internal remodeling and the chondrocytes replace matrix macromolecules lost through degradation. Therefore normal matrix turnover depends on the ability of chondrocytes to detect alterations in the macromolecular composition and organization of the matrix, including the presence of degraded molecules, and to respond by synthesizing appropriate types and amounts of new molecules. In addition, the matrix acts as a signal transducer for the cells. Loading of the tissue due to use of the joint creates mechanical, electrical, and physicochemical signals that help to direct the synthetic and degradative activity of chondrocytes [22, 35].

4. Extracellular matrix and cell signaling

Chondrocytes respond to the mechanical and biochemical changes in ECM through signaling events by various cell surface growth factor receptors and adhesion molecules. ECM proteins can determine the cell behavior, polarity, migration, differentiation, proliferation and survival by communicating with the intracellular cytoskeleton and transmission of growth factor signals. Integrins and proteoglycans are the major ECM adhesion receptors, which cooperate in signaling events, determining the signaling events, and thus the cell function [36].

Integrins are heterodimeric transmembrane receptors formed of eighteen α subunits and eight β subunits and they are non-covalently assembled into 24 combinations. The integrin dimers bind to different ECM molecules with overlapping binding affinities determining expression patterns and the downstream signaling events in the cell. Integrins respond specifically to the molecular composition and physical properties of the ECM and integrate both mechanical and chemical signals through direct association with the cytoskeleton. Integrins recognize and bind to the Arg-Gly-Asp (RGD) motif that they are attachment sites for integrin mediated cell adhesion. It has been demonstrated that high density of

RGD motifs allows a precise spatial distribution pattern of integrins for specific cellular response among ligand molecules [36, 37].

Integrins can activate several signaling pathways independently and frequently they act synergistically with other growth factor receptors as insulin receptor, type 1 insulin-like growth factor receptor, VEGF receptor, TGF-b receptor, platelet-derived growth factor-b (PDGF-b) receptor and epidermal growth factor (EGF) receptor [37,38].

4.1. Role of proteoglycans in signal regulation

The heparan sulfate proteoglycans (HSPGs) contribute to the organization of the matrix by binding to the many core matrix molecules via HS chains as laminin, fibronectin and collagen. The chondroitin sulphate proteoglycans (CSPGs) as aggrecan, versican, brevican and the small, leucine-rich proteoglycans such as decorin and biglycan also bind to and regulate a number of growth factors, such as members of the TGF family. The hyaluronic acid is a glycosaminoglycan synthesized on the cell surface and is responsible for the gel-like consistency of cartilage by its hydroscopic properties [36, 39].

4.2. Remodelation and degradation of ECM

During normal or pathologic physiology of the cartilage, the ECM must be remodeling and degraded to allow the chondrocytes for processing and deposition of new matrix by specific proteases. There are two well-known families of proteases that are involved in the biology of the ECM, the matrix metalloproteinase (MMP) and the desintegrins and metalloproteinases with thrombospondin motif (ADAMTS) families. The MMP-13 is involved in the cleavage of fibromodulin and type IX collagen and is present and active in the pathological process of cartilage as OA and rheumatoid arthritis. The aggrecanases family's ADAMTS-4 and ADAMTS-5 play an important role in cartilage damage during early OA which cleavage the glycosaminoglycans chains that are the key contributors to the maintenance of the charge density, the osmotic environment and water retain important characteristics of the mechanical properties of the cartilage [40, 41].

5. Alterations of the ECM in the skeletal tissue: Injuries and pathologies

The extracellular matrix has structural and functional relevance, it's a highly organized and assembled macromolecular structure, also provide cellular adhesion environments, activation and inactivation of growth factors and regulatory cytokines. The proteolytic processing of ECM components, results in the production of fragments with biological effects on migration, proliferation and cellular organization.

When any component of the ECM has a disorder, could generate chondrodysplasia, it means alterations in the development and growth of cartilage. Chondrodysplasias are caused by various mutations in genes involved in cartilage development and finally in the formation and growth of the long bones. These mutations also often alter the formation of other tissues.

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Achondrogenesis type II, is a chondrodysplasia classified as collagenopathy type II. In this family are located several chondrodysplasia caused by mutations in the gene for collagen II, which is the most abundant protein in cartilage [42]. These dysplasias are, achondrogenesis type II, hypochondrogenesis, congenital espondiloepiphysial dysplasia and Kniest dysplasia, among others. Collagen II is a homotrimer (three identical chains encoded by the COL2A1 gene located on chromosome 12. This collagen is mainly found in the hyaline cartilage and vitreous humor, so its deficiency is associated with abnormalities of the spine, of the epiphysis and eye problems. Despite their differences these dysplasias share clinical and radiological manifestations, so the axial skeleton is affected more than the limbs, cleft palate, myopia and retinal degeneration [43].

Furthermore, other disorders of matrix components such as collagen IX and XI, which interact with the collagen II to form supramolecular structures, are closely related phenomena.

It is found that the Osteogenesis Imperfecta (OI) is caused by molecular defects of collagen type I[44] and metaphyseal chondrodysplasia Schmid type is caused by errors in collagen type X biosynthesis [45], the latter is characterized by alterations in vertebrae and in the metaphysis of long bones, also show reduction of the area of reserve cartilage in growth plate and in the articular cartilage, alters the contents of bone and there is an atypical distribution of the matrix components of the growth plate.

The cartilage oligomeric matrix protein (COMP) is a member trombospondins family, and its alteration causes pseudoachondroplasia, this disorder shows short limbs and lax ligaments [46], the growth plate is shorter and the area of hypertrophic cartilage is reduced.

Cartilage needs molecular signals for development and maintenance, such as growth factors, which in many cases are regulating the synthesis of the ECM, and may be found active or latent in the extracellular matrix. Bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF-β), growth and differentiation factor 5 (GDF-5), are signals related to the development and growth of cartilage, alterations in these molecules cause some malformations, such as the brachypodism (short limbs) [47].

Cartilage matrix is rich in sulfated proteoglycans and the gene encoding for sulfate transporter called DTDST (Dystrophic Dysplasia Sulfate Transporter) in patients with dystrophic dysplasia was found mutations in this gene, and shown to be deficient cartilage sulfating [48].

Campomelic dysplasia is a rare disease associated with XY individuals who possess varying degrees of sex reversal. SOX-9 is a transcription factor structurally related to the gene SRY (sex-determining region Y) required for testicular development. However, SOX-9 also directly regulates the gene for type II collagen, the main molecule of the cartilage matrix and therefore of chondrocyte differentiation [49, 50, 51].

The inactivation of the gene coding for the mouse gelatinase B, defined the mechanism that controls the final step of the chondrocyte maturation [52]. Gelatinase B is an enzyme present in the extracellular matrix of cartilage and its activity is related to the control of apoptosis of hypertrophic chondrocytes and the vascular tissue. This study hypothesized the existence of chondroclast, these cells of myeloid origin express gelatinase-B and are located in the cartilage/bone region and resorb cartilage matrix.

Based on the above is to emphasize the importance of the extracellular matrix as a modulator of cellular differentiation of chondrocytes, the extracellular components correlate with the differentiation state. That is, collagen I is present at early stages of differentiation and maturation, in mesenchyme and perichondrium; collagen II is on mature cartilage and collagen X is exclusive of hypertrophic cartilage also collagen type I are expressed in terminal stages of chondrocytes [53].

The ECM not only serves as a binder that gives form to tissues in addition to their structural role has physiological functions. The chondrocytes are in the array a series of signals that allows them to gain some cell shape and organization of the cytoskeletal network. Cell morphology that can modulate many physiological functions such as proliferation, differentiation, cell death and gene expression. This transmembrane receptor-mediated would be able to receive the extracellular signal from the ECM and transduce the signal into the cell, triggering a response by the chondrocyte differentiation [54].

Integrins are transmembrane receptor consisting of one α subunit and a β, are only functional to form the α-β heterodimer on the cell membrane. β1 family of integrins are major receptors of ECM molecules and have the ability to allow cell adhesion and simultaneously issuing an intracellular signal to which the cell responds in different ways, as also interact with integrins the cytoskeleton and molecules involved in signal transduction.

It has been shown that integrins interaction with extracellular matrix molecules affects cytoskeleton organization, proliferation, differentiation and gene expression in fibroblasts and epithelial cells.

In addition we have studied the survival and differentiation of chondrocytes, including the deposit in the interstitial matrix of collagen type X could be mediated by integrins [55]. Inhibition of integrin b1 subunit with a neutralizing antibody blocks the deposition of collagen X in the interstitial matrix and growth of the breastbone is decreased. Moreover, the chondrocytes are significantly smaller, show a disorganization of the actin cytoskeleton and show increased apoptosis.

There is also evidence that blocking the β1 subunit of integrins in an in vitro model of differentiation of cartilage inhibits cartilage nodule formation and the synthesis of collagen type II [56].

However, the study of the role of these receptors in the process of chondrocyte differentiation is not yet well established, but it would be of significant importance in determining the relationship of the extracellular matrix to the chondrocyte.

5.1. The extracellular matrix and chondrocyte differentiation in osteoarthritis

Articular cartilage mineralization frequently accompanies and complicates osteoarthritis and aging. Several works has demonstrated that certain features of growth cartilage development are shared in degenerative cartilage. These include chondrocyte proliferation, hypertrophy, matrix mineralization and apoptosis. Development of growth plate is regulated by growth factors signaling and cellular interactions with the extracellular matrix (ECM). Parathyroid

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hormone related protein (PthrP) and Indian Hedgehog (Ihh) are central mediators of endochondral development; PthrP is abundant in synovial fluid of osteoarthritic patient but Ihh expression is diminish in OA cartilage, Fgf-18 is a regulator of chondrocyte proliferation and its intra-synovial application in OA rat results in cartilage generation. Also, Wnt signaling plays an important role in chondrocyte differentiation in growth plate, Wnt-5a promotes chondrocyte prehypertrophy and inhibits chondrocyte hypertrophy unlike Wnt-4 that induces chondrocyte hypertrophy and increases its expression in early stage of osteoarthritis. On the other hand, is pronounced imbalance of cartilage matrix turnover in osteoarthritic cartilage, and results in mayor deposition of collagen type I and X, reduced expression of collagen type II. Thus, the rate of chondrocyte hypertrophy is higher on growth plate and OA articular cartilage than healthy articular cartilage, it recap the signaling in cartilage growth plate. But, although articular and growth plate cartilages share several features, there are one important difference, the rate of cartilage hypertrophy. What is the signal that makes the difference? In the ECM we could find some elements to answer this question.

5.1.1. Alterations in the extracellular matrix of articular cartilage during OA

Traditionally it has been thought that osteoarthritis is a disease of wear or tears consequence of articular cartilage due to aging or following injury. The limited regenerative capacity of cartilage cannot reverse its destruction, it is sometimes triggered by an inflammatory response from the synovial, inflammation occurs when the condition is called osteoarthritis [57]. Until recent years genetic mutations were excluded as a risk factor or predisposition to osteoarthritis. The first genes identified to OA encode components of the extracellular matrix, such as Collagen *COL2A1*, *COL9A2* and *COL11A2*, which were studied in transgenic mouse models [58]. It has been found that the substitution of glycine destabilizes the triple helix structure of collagen type II making it more susceptible to degradation by MMP-13 [59]. Other ECM molecules related to OA are ADAMTS-4 and ADAMTS-5 enzymes which degrade aggrecan, the most abundant proteoglycan in articular cartilage [60]. When aggrecan is degraded, the collagen II is exposed to the DDR-2 enzyme which is able to degrade it [61]. The alteration of the ECM of articular cartilage in the first instance causes cell proliferation and the formation of fibrous tissue that forms a scar in response to injury, there are produced growth factors such as TGF-β could promote chondrocyte hypertrophy, so that recapitulates OA cartilage differentiation mechanisms of the growth plate to form ultimately bone nodules at the edges of articular cartilage called osteophytes [62]. Clearly the importance of ECM in the differentiation of articular cartilage, but there are various growth factors and transcription factors that regulate the maturation and proliferation of chondrocytes in articular cartilage and cartilage growth plate, which also control the expression of many of the components of the ECM, and also direct the skeletal morphogenesis. Genes has recently been determined as Smad-3, Dkk, Wnt4, Mig-6 etc [63- 66], OA generated in murine models, these molecules regulate different cellular processes such as cell proliferation, cell differentiation, cell death, degradation and synthesis of ECM. We can group the molecules according to the governing process: Chondrogenesis, Proliferation, Differentiation and Cell Death. Many of these molecules can be good genetic markers of predisposition to OA, and are fundamental to how to design a strategy for articular cartilage repair.

5.1.2. Differentiation of articular cartilage chondrocyte

Although exists different types of cartilage, they are very similar but have different functions. Articular cartilage and cartilage growth plate are good examples. In general, the molecular mechanisms of chondrocyte differentiation in both cartilages are equivalent. However, for the function of synovial joints is essential that chondrocytes maintenance in prehypertrophic state differentiation, while the longitudinal growth of bone depends on the proliferation and differentiation of chondrocytes in the growth plate to the hypertrophy and bone formation [67, 68]. We can even talk about a model that relates the structure and function of cartilage based on histological and functional differences of both cartilages. Both in the cartilage growth plate and in articular cartilage chondrocytes can be found at various stages of differentiation, but the organization and activity of chondrocytes differ in each stage of both cartilage.

In the growth plate chondrocytes reserves represent an immature state and are organized in tiny rows of small round cells, embedded in an abundant extracellular matrix rich in collagen type II and aggrecan, proliferating chondrocytes are stacked as "coins" several rows forming compact occupying a large area of the growth plate, the first rows are more proliferation activity than the rows deep; prehypertrofic chondrocytes (mature) are larger cells that have exited the cell cycle and express Ihh, a key molecule in cartilage differentiation, these cells secrete and accumulate a large amount of carbohydrates and finally the hypertrophic chondrocytes are cells of highest volume and high alkaline phosphatase activity, the ECM is mainly composed of collagen type X and begins to calcify, some cells degenerate and die by apoptosis leaving the spaces occupied to consolidate osteoblasts and bone tissue. This process is known as endochondral ossification which regulates the growth of bone in terms of cartilage differentiation. It is noteworthy that an important signaling center in this process is the perichondrium, which are very small and flattened cells surrounding the cartilage and expressed PTHrP [69] and Fgf-18 [70], which respectively induce and inhibit the proliferation of chondrocytes, the receiver PPR and PTHrP [71] is expressed in the upper rows, whereas the Fgf-18 receptor and FGF-R3 is found in the deeper cell layers of proliferating chondrocytes. Patch is Ihh receptor and is expressed in the perichondrium, so that Ihh induces the expression of PTHrP and this in turn induces proliferation and expression of Ihh in the growth plate. This regulatory loop promotes the longitudinal growth of the mold of cartilage, but it is necessary that the mold is rigid. For this, the FGF18 inhibits the proliferation of cartilage to regulate expression of Ihh and this result in the differentiation of chondrocyte hypertrophy up. This signaling cascade also occurs during the formation of joint cartilage, where bone formation is more limited as in the secondary ossification centers.

Articular cartilage has apparently different stages of differentiation of chondrocytes, only that which corresponds to the resting chondrocytes have important differences in the composition of the ECM, as the presence of lubricin, the Collagen type IIa the aggrecan, CD44, ASC, [72, 73] these cells are most abundant in the articular cartilage cells for proliferation area are not organized in rows and have very low proliferation rate, making them more similar to the prehypertrophic cartilage, as the rate of is very slow maturation, hypertrophic chondrocytes

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make up a small area of just one or two cell lines the border between cartilage and bone, known as "water mark" (tide mark).

5.2. Endochondral ossification during skeletal development and OA

The joints that separate from each other skeletal elements serve as important signaling centers during skeletal development, and regulate the proliferation and maturation of chondrocytes. It is well known that chondrocyte maturation is crucial for endochondral ossification and to define the final size of each skeletal element. In the end, the processes of the formation of joints and cartilage differentiation of skeletal elements are strongly related. The limb skeletal elements are formed by endochondral ossification, the process begins with the aggregation of mesenchymal cells that form the pre-cartilaginous condensation, this condensation increases the proliferation of chondrocytes and forms a "bar" initial cartilage [74]. It has been proposed that the first step for the formation of the joint is that it inhibits differentiation of prehypertrofic chondrocytes in cells located in the region of the joint prospecting, outside the influence of signals that promote maturation of the cartilage, while neighboring cells continue their differentiation process to form bone hypertrophy and subsequently by endochondral ossification, so contributing to the formation of adjacent skeletal elements [75]. Cells suspected joint region form the interzone, characterized by a highly packed region of flattened cells, these cells produce other types of collagen and collagen type I and III, unlike chondrocytes that produce collagen type II. The interzone also expressed molecules such as Wnt-9a [76] and Bmp antagonists like noggin [77], which remain the property of these cells not chondrogenic. Some cell adhesion molecules such as integrin $\alpha 5\beta 1$ also regulate the formation of joints by controlling the differentiation of chondrocytes [78], whereas other signaling molecules that are expressed in the interzone as Wnt-4, Fgf-18, Gdf (5, 6 and 7) and several members of the Bmp, promote growth and differentiation of adjacent cartilaginous elements [79]. It is likely that different cell types present in a mature synovial joint, including synovial cells, articular chondrocytes and permanent joint capsule cells originate in the interzone. Permanent articular chondrocytes originating from the interzone, are very similar to chondrocytes in the growth plate, and although both cell types are hyaline cartilage and functions have important differences. The most important difference is that articular chondrocytes decrease its maturation toward hypertrophy of chondrocytes unlike the growth plate which we observed a wide region of hypertrophic chondrocytes, as this process allows for the ossification and growth of long bones. Hypertrophic chondrocytes are the highest volume and produce a very specific extracellular matrix rich in collagen type X. The hypertrophy of chondrocytes is followed by apoptosis, the invasion of blood vessels, osteoclasts and other mesenchymal cells from the perichondrium and production of bone matrix. Therefore, the size and fine structure of the long bones depends on the coordinated regulation of proliferation, maturation and hypertrophy of chondrocytes in response to many extracellular signals. The protein Indian hedgehog (Ihh) and peptide related to Thyroid Hormone (PTHrP) play a critical role in these processes, Ihh is pro-

duced by prehypertrophic chondrocytes and induces the expression of PTHrP in the perichondrium which in turn regulates the rate of chondrocytes which exit the cell cycle and continue to hypertrophy [80]. Ihh also stimulates proliferation of chondrocytes and controls the differentiation of mesenchymal cells into osteoblasts in the collar bone. Thus, when the chondrocytes stop expressing Ihh activates the expression of Runx-2 and Runx-3 [81], some transcription factors required for hypertrophy of chondrocytes and differentiation of osteoblasts. On the contrary, in particular FGF-18 [82] expressed in the perichondrium and through its receptor Fgf-R3 expressed in cartilage prehypertrofic cartilage negatively regulates cell proliferation and promotes the hypertrophy of chondrocytes, the constitutive activation of FGFR3 results in dwarfism [83] and may inhibit the formation of joints, this confirms the idea that proliferating chondrocytes may have two possible destinations, become pre-articular chondrocytes or prehypertrophic chondrocytes.

5.3. Control of chondrocyte differentiation and two destinations, Ihh vs Wnt signaling and its role in OA

During the formation of the skeleton some chondrocytes are involved in the growth of long bones and ossification. At this early stage, the GDF-5 signaling is essential for the formation of joints and articular cartilage [84, 85], its expression is delimited in the interzone and begins just before forming the joints, on the other hand, the Bmp-7 is important for the chondrocyte maturation and bone formation and is expressed in the perichondrium of the skeletal elements in formation and growth [86], but not expressed in the perichondrium of the developing cartilage. Although the induction of the joint is initiated by the expression of Wnt-9a in the interzone and the interzone chondrocytes lose their phenotype [76], GDF-5 signaling is essential for the joint and articular cartilage formation. Ihh is another important molecule for skeletal development, Ihh inhibits Wnt-9a expression and is maintained in skeletal growth and endochondral ossification, as when it reaches a certain size decreases the expression of Ihh and thereby activates the expression of Wnt patway induces hypertrophy of chondrocytes and bone formation [87]. It is noteworthy that during the OA Wnt signaling is overactivated [65] and GDF-5 is downregulated, which suggests a recapitulation of endochondral ossification during OA. Furthermore, when the receptor Bmp-RIA is inactivated in mouse generated phenotypes similar to human osteoarthritis and when activated the Wnt pathway by blocking antagonist Dkk [64], reverse the process of articular cartilage destruction and endochondral ossification, this suggests that these pathways permit the maintenance of adult articular cartilage.

5.4. Proliferation, hypertrophy and cell death are activated during OA

Not only in the embryonic stages imbalance of proliferative signals and bring important consequences hypertrophy in articular cartilage, osteoarthritis is a striking example of this imbalance of signals. There are animal models that recapitulate this degenerative joint disease, as in the case of the mutant mice of Smad-3 [63], a molecule that transduces the TGF-β signal. Molecular analysis of these mice shows ectopic expression of type X collagen in the articular

cartilage and increased hypertrophy of chondrocytes; this shows the TGF-β as an inhibitor of differentiation of articular chondrocytes. Similarly, the cancellation of Mig-6 in mice results in early degeneration of joints [66], as evidenced by degradation of articular cartilage, fibrous tissue formation and growth of osteophytes. It is well known that articular cartilage injuries may result in osteoarthritis, fibrous tissue formation is an immediate healing response to a traumatic injury, and the healing is often promoted by TGF-β, which in turn could induce osteophyte formation that recapitulates chondrogenesis and endochondral ossification in adult articular cartilage.

5.5. Why not articular cartilage regenerate

During development are constantly chondrocytes proliferation and differentiation, thus skeletal elements grow in length and ossify, as mentioned earlier, articular cartilage chondrocytes have a low rate of proliferation and differentiation, this makes them different and allows articular cartilage is kept almost throughout life. What keeps the ever-growing cartilage during development is the molecular signals that modulate the rate of growth and differentiation, these signals are regulated by the perichondrium. The perichondrium has progenitor cells that are very useful for cartilage repair, its similar to bone, the periosteum is important for bone repair, such as fractures. While the perichondrium is maintained until adult stages, the perichondrium is disappearing from the stage young individuals, which is why the low capacity of regeneration of cartilage [88].

6. Current methods for cartilage tissue engineering and future perspectives

6.1. Autologous chondrocytes for tissue regeneration

The hyaline articular cartilage is a highly specialized tissue and its main function is to protect the bone from friction in the joints [89, 90], once articular cartilage is damaged their ability to self-repair and regeneration is limited as mentioned above. Cartilage injuries are mainly associated with anterior cruciate ligament, patellar dislocation, followed by a meniscectomy [91]. Osteochondral lesions of the knee are determined mainly by arthroscopic knee surgery [92, 93], which is seen mainly in traumatic injuries, together with abnormal stresses on the knee.

To determine the treatment for the repair and regeneration of articular cartilage injury, have developed different techniques, the techniques described are focused on the repair, reconstruction or regeneration of tissue. The repair methods (drilling or microfracture) support the formation of new tissue fibrocartilaginous [94, 95] while the reconstructive method seeks to fill the defect with allografts (OATS) combining with miniarthrotomy arthroscopy. And finally the regenerative methods that rely on bioengineering techniques to develop a hyaline cartilage tissue graft or autologous chondrocyte cell matrices (Table1).

Ref.	Method	Technique	Results
[96]	Drilling with lavage and debridement	Removal of osteophytes and knee abrasion	
[97]		Perform subchondral drilling of the lamina	Tissue repair and pain relief
[98]		Elimination of subchondral lamina	Significant symptomatic improvementin 75% of patients
[99, 100]	Microfracture	Perforation of the subchondral lamina by arthroscopy, it promotes the release of mesenchymal cells in the lesion, forming a plug of tissue	Avoids necrosis associated with the use of the drill and preserves the subchondral surface. The results observed in the medium term, mainly in young patients, about 20% of patients do not reach after five years.
[101-104]	Chondrogenesis induced stimulation of bone marrow (AMIC)	Followed by a micro abrasion bill and placing a collagen scaffold on the defect, inducing the formation of fibrocartilage by migrating mesenchymal cells and the expression of cytokines and tissue repair	Stimulation of bone marrow has limited mechanical strength and may even degrade the cartilage is repaired with fibrous tissue or fibrocartilage so that there is tissue degeneration.
[105-107]	Mosaicplastyandt ransplantosteoch ondral allograft	Is based on obtaining osteochondral cylinder obtained from areas of low load from the distal femur, which are grafted into the defect	The results are limited in large lesions due to donor site morbidity and healing of the seams in the recipient
[108-110]	Autologous chondrocyte implantation	1st Generation: In this technique, cartilage cells are injected under a cover of periosteum is sutured into the defect. 2nd. Generation: is replaced cover membrane or periosteum biomaterials, which can have different components	It has been reported good results in most patients after 10-20 years after implantation. In the second generation transplants with areas of fibrocartilage, possibly because of low cell density and lack of proliferative capacity. This technique replaces healthy cartilage to regularize the defect.
[111]	Autologous chondrocyte implantation induces extracellular matrix	3rd. Generation: In this technique, autologous chondrocytes cultured on a three-dimensional artificial scaffold	Has been used in the past two decades, with this type of membranes hypertrophy is reduced by 5%, after 3 to 6 months membrane is reabsorbed.

Table 1. Cartilage repair techniques

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Each of these procedures is associated with improvement of these techniques with the use of biomaterials or with the use of growth factors. In the autologous chondrocyte implantation of the second generation is required arthrotomy so this technique becomes more complicated. In order to facilitate and improve the technique and quality of the tissue repair, has developed a method which has proved more effective and easy to implement in the knee joint [112, 113] develop and autologous chondrocyte implantation induced extracellular matrix of the third generation.

6.2. Description of the technique of autologous chondrocyte implantation induced extracellular matrix (third generation).

6.2.1. Obtaining the tissue

This technique is mainly based on the autologous cultured chondrocytes on a biocompatible three-dimensional scaffold which is subsequently implanted into the defect. As in the technique of autologous chondrocyte implantation of the second generation, it requires a prior arthroscopic surgery where a piece of cartilage obtained from a zone of no load of the knee joint (intercondylar notch or the lateral edge of the trochlea) after obtaining the sample fragment is processed to obtain chondrocytes in culture.

6.2.2. Implant preparation

Cartilage fragments are disintegrated mechanically to obtain smallest fragment, is performed subsequent enzymatic digestion to release trapped chondrocytes in the matrix of collagen. Expansion of chondrocytes was performed in 8 weeks. Days before implantation chondrocytes are seeded on a scaffold or membrane [112] Rich in collagen, which is considered a three-dimensional extracellular biomaterial consists mainly of collagen I and III, the scaffold contains glycosaminoglycans, proteoglycans and glycoproteins [111, 114, 115] cells are capable of synthesizing a typical matrix of chondrocytes facilitating cell adhesion and influence the morphology, migration and differentiation of cells.

6.3. Advantages of autologous chondrocyte transplantation induced extracellular matrix (third generation) on the autologous chondrocyte implantation (second generation)

The main advantages of autologous chondrocyte transplantation induced extracellular matrix (third generation) is that no cell loss is not presented hypertrophic tissue growth, requiring only a second incision is a safe procedure for treatment of injuries symptomatic articular cartilage surgery facilitates reducing the operating time and the need for open surgery compared to traditional surgery for autologous chondrocyte implantation (second generation). While in the second generation technique leads to form hyaline cartilage on the surface showing fibrosis and proliferation of small blood vessels (reactive fibrosis), by the use of periosteum, so that in this case it is advisable the use of membrane collagen

7. New proposals for repairing articular cartilage

In recent years they have sought new strategies for cartilage repair, with technological advances have currently been proposed the use of scaffolding or matrix on which cells can grow. Among the scaffolds used in the clinic (Table 2) are those that are based on collagen, hyaluronic acid and fibrin as these provide a substrate normally found in the structure of native articular cartilage. Collagen is a major extracellular matrix protein, exists to provide strength and stability to the connective tissues. At the clinic is used collagen I-III as scaffolds for growing chondrocytes in order to improve the structural and biological properties of the graft [116, 117] this is used as a sponge, foam, gel and membrane form, all these are subject to enzymatic degradation. Hyaluronic acid is another important component of articular cartilage matrix and is a glycosaminoglycan that is involved in homeostasis [118, 119] provides viscoelasticity to synovial fluid, is credited as a lubricant and shock absorbing properties, is essential for the correct structure of proteoglycans in articular cartilage. Between scaffolds containing hyaluronic acid is the Hylaff-11, which is an esterified derivative of hyaluronic acid and is used for growing chondrocytes in three dimensions, has been shown that when using this type of scaffold maintaining the chondrocyte phenotype, so that chondrocytes are capable of producing the proteins and molecules characteristic of a hyaline cartilage [120-122]. Fibrin is a protein involved in blood coagulation, is regarded as a biomaterial for cartilage repair, as can be found in gel form, having an adhesive function that is also biocompatible and biodegradable [123]. However in vivo studies in animals have shown to have low mechanical stability and can also trigger an immune response [124, 125], fibrin because this has only been used clinically to ensure healthy cartilage tissue-engineered the [126-128].

Based on the foregoing and which is being used in the clinic and according to results obtained in patients who have been treated with different biomaterials has been observed that although there is a suitable biomaterial that contributes to the production of extracellular matrix to provide the right conditions for chondrocyte cell differentiation. So it is necessary to propose new biomaterials that help produce extracellular matrix, capable of activating a cascade of signaling that can form a cartilage which has structural properties suitable for tissue repair, as well as having viscoelastic properties and to provide mechanical stability.

8. Cartilage tissue engineering and low scaffold successful

Many advances in the field of cartilage tissue engineering have been closely connected to the improved performance of biomaterials. Successful cartilage tissue engineering relies on four specific criteria: (1) cells, (2) signaling molecules, (3) biomaterials, and the (4) mechanical environment. Furthermore, they should be biocompatible, non-toxic, bioresorbable and highly permeable to facilitate mass transport [139].

The use of scaffolds to support replication of chondrocytes for production of cartilage in vitro has been the most common approach for tissue engineering of cartilage, however, despite the

Ref.	Biomaterial	Component	Method of autologous Chondrocyte transplantation	Results
[126, 129-132]	Carticel	Collagen I-III	2nd and 3rd generation	Three-dimensional multi-layer keeps the chondral phenotype
[113]	Matricel		2nd generation	
[133, 134]	CaReS®	Collagen I hydrogel	2nd generation	It presents a significant functional improvement as well as acting on the levels of pain.
[135-136]	Hyalograft-C	Hyaluronic acid	3rd generation	Maintaining the chondral phenotype, absence of inflammatory response, formation of hyaline cartilage
[137]	Hyalgan®		----------	Indicated for the treatment of osteoarthritis of the knee, improves mobility and reduces pain.
[113]	Tisseel	Fibrin	3rd generation	Fibrin is an integral component of the extracellular matrix induced chondrocytes, so that the new cartilage is well integrated into the underlying subchondral bone. Moderate application of fibrin
[138]	Cartipatch	Alginate Hydrogel-agarose	--------	Hyaline cartilage was observed in eight of the 13 patients treated, clinical improvement at 2 years of treatment

Table 2. Biomaterials most used in the clinic, with different components for the repair of articular cartilage by autologous transplantation method of chondrocytes from second and third generation.

apparent simplicity of cartilage, to our knowledge, tissue engineered cartilage has not been successfully reached so far [140-142].

In theory, a scaffold for tissue engineering should have a three dimensional porous structure forming an interconnected porous network. These structures should be made of biocompatible and biodegradable materials capable to provide mechanical strength, support cells ingrowth, promote cells adhesion, uniform cell spreading, and conserve phenotypes and functional characteristics of transplanted cells [143,144]. Unfortunately, this list of requirements looks too long and hard to accomplish. Probably this is one of the main reasons of why the advances in cartilage engineering have been too slow. But also we should rethink these concepts in order to find shorter and easier pathways to find more efficient and effective tissue engineering methods.

The vast majority of scaffolds used in tissue engineering are solid sponge-like porous structures that are seeded with cells in a culture media. Analyzing this approach from the basic principles for the design of biomaterials, the biomimetism, easily we can find out that this process lacks of this basic concept. In natural tissues, cells grow in a physiological environment which is more like a gel medium than a porous scaffold, they do not form tissues by populating porous structures, but they do it by creating their own ECM starting from a gel-like environment. Following this line, many researchers are proposing the encapsulation of cells in hydrogels instead of using porous scaffolds, looking to improve the biomimetic environment for cells [145,146,147,148].

Besides biomimetism, sponge-like scaffolds provides only a two dimensional surface for cell attachment, although their structure is 3D, cells attach to the walls of the scafflod, thus changing completely the way they are integrated into natural tissues. On the other hand hydrogels are capable to provide a real 3D environment when cells are seeded (encapsulated) into them [149].

8.1. Hydrogels for articular cartilage tissue engineering

Hydrogels are water-swollen, cross-linked polymeric structures [150] that possess unique mechanical and chemical properties that make them very attractive for a variety of biomedical applications; actually there are no other materials capable to display characteristics too close to natural tissues such as Hydrogels. Therefore hydrogels have been considered as a key material in the development of new biomaterials for tissue engineering and artificial organs fabrication.

Their particular properties come from their structure, composed of swollen randomly cross-linked networks of rod-like polymer chains with water filling the interstitial spaces.[151] Water commonly comprises more than 80% of the total volume. The physical properties of hydrogels are determined by the polymer composition and concentration, the cross-linking density between polymer chains [152], polymerization conditions [153], the addition of hydrophobic monomers which may create regions of more dense coiled or entangled chains, the introduction of composite materials such as rubber or glass, the use of cross-linking agents such as glutaraldehyde, and the use of freeze-thawing procedures to induce partial crystallinity [154].

Hydrogels can be classified by the type of crosslinking: covalently or ionic cross-linked, physical gels, or entangled networks [155]. The two first are the most common gels. Physical gels are formed by non-covalent interactions, such as hydrogen bonding, and hydrophobic interactions [156]. Covalently or ionic cross linked gels are considerably more stable than physical gels and once they are formed they may not be re-melted again.

Hydrogels can be obtained from natural or synthetic polymers. Natural hydrogels come from proteins and polypeptides (commonly collagen and gelatin), polysaccharides (i.e. alginate, agarose, hyaluronic acid, fibrin, chitin and chitosan). On the other hand, synthetic polymers come from man-made materials such as polyester (i.e. poly L-lactic and polyglycolic acid, poly ε-caprolactone, polypropylene fumarate), polyethylene oxide, polyethylene glycol, polyvinyl

alcohol, polyurethane, polydiol citrates, polyhydroxyethyl methacrylate, and many others polymers [157].

Although hydrogel scaffolding technologies plays a crucial role in cartilage tissue engineering, several studies has been shown low success cartilage tissue repair. They are unable to generate cartilaginous tissues with similar properties to native cartilage [141-142].

There are a number of reasons for scaffolds failure, we summarized some of them:

1. The scaffold architecture should be designed to mimic the depth-dependent heterogeneity of articular cartilage structure or to generate multiphasic scaffolds to promote the simultaneous growth of bone and cartilage with a stable interface for engineering osteochondral tissue.
2. However, manufacturing scaffolds technologies are limited and no optimum architectures have been produce yet.
3. The study of biological cartilage development is still growing.
4. Not enough knowledge about:
5. the role of chondrocyte ECM and their implications during chondrogenesis.
6. the role of adhesion molecules and signaling pathways during chondrogenesis.
7. Culture chondrocytes *in vitro* and density cells conditions in scaffolds.
8. Dynamic cartilage ECM and their Nanomechanical properties.
9. Chemical variables in cell-scaffolds interactions, among others.

8.2. Trends in hydrogel-scaffolding cartilage repair

However towards designing biomimetic native environments cartilage is still a challenge due articular cartilage is intricately organized and heterogeneous tissue. This tissue reveals a highly defined structural organization that can be subdivided into two domains, the cartilage zones and the organization of the extracellular matrix. In that sense the ECM of articular cartilage is a unique environment with complex heterogeneity and spatial conformation very difficult to mimic. One of the most notable variations in this tissue is the spatial organization of collagen network and cells arrangement. [141]. Moreover cartilage presents different morphology, gene expression, matrix spatial array between cultured populations isolated from distinct cartilage zones [142]. However, intensive researches have been focus on the development of an ideal scaffold material with versatile properties that actively contribute to cartilage repair [158]. In that regard, there have been several attempts trying to recreate the different zones in cartilage by different hydrogel fabrication technologies, giving as a result tridimensional homogeneous structures with little resemblance to the native organization in cartilage, so it is necessary to material scientists thinking in others design hydrogel-scaffolding strategies trying to biomimetic hierarchical structures capable to deliver bioactive molecules such as growth factors with an ideal mechanical response and mediated by adhesive molecules in order to have an integration tissue [159].

Currently strategies in the design of biomimetic cartilage hydrogels are governed by the use of collagen Type I and derived from porcine small intestine submucosa implants. Although the chondrocytes typically lose their phenotype, the gene expression patterns changed when they are removed from their native environment, so give them a proper environment is necessary to keep its phenotype of chondrocytes in different populations to recreate the zonal organization [160]. In addition, biological trials *in vitro* be made taking into account the cell density for each zone [161,162].

According with reference [163] concentrations of 12-25 million cells/cm^2 are needed to increase the matrix production and mechanical properties of human adult chondrocytes under static conditions. Nevertheless, material researches are focus on fabrication of three-dimensional artificial arrays in form of hydrogels using macromolecules present in the cartilage inter-territorial matrix and trying to mimic the distinct cartilage zonal [160]; however, no substantial data of the formation of cartilage are reported.

Others approaches in cartilage tissue engineering are the use of hydrogel culture employed mesenchymal stem cells (MSCs) and the use of bioreactors in order to provide the necessary biochemical and biomechanical stimulations to enhance chondrogenesis [164,165]. Due to the many mentioned limitations related to chondrocyte sources, there is much effort to explore better alternative cell sources. Desirable characteristics for such sources include accessibility, availability, and chondrogenic capacity. Consequently, stem cells such as adult mesenchymal stem cells (MSCs) have emerged as promising cell sources for articular cartilage tissue engineering. Chondrogenic potentials of MSCs from different tissues have also been investigated and compared. Specifically, MSCs from bone marrow are the most popular considering they are easily harvested (via the iliac crest) and have good chondrogenic potential. Many in vitro and in vivo studies have revealed promising results of marrowderived MSCs combined with various biomaterials or growth factors for repairing cartilage defects [164,166]. Recently, Johnson et al. describe the discovery and characterization of kartogenin, a small molecule that induced stem cells to take on the characteristics of chondrocytes and improves joint function and promotes the regeneration of cartilage in vivo in two rodent models of chronic and acute joint [166].

Mechanical stresses are an important factor of chondrocyte function as they stimulate them to increase the synthesis of ECM components. In cartilage culturing processes the main types of mechanical forces currently being investigated are hydrostatic pressure, direct compression, shear environments [167, 168].

Finally, to better recapitulate the ECM environment for cartilage tissue engineering, researchers have to introduce several biological signals, including chondroitin sulfate (CS), hyaluronic acid (HA), and collagen type I and II, into tissue-engineered scaffolds to encourage tissue specificity [169]. CS, hyaluronic acid, and collagen type II have been shown to promote or enhance chondrogenesis of mesenchymal stem cells (MSCs) in hydrogel-based culture systems. In addition to the physical cues of native matrix, cells are exposed to an array of biological cues throughout the ECM that direct cellular behavior.

Cells are constantly interacting with the surrounding ECM, which gives rise to a dynamic transfer of information between the extracellular and intracellular space. In addition, biological trials *in vitro* be made taking into account the cell density for each zone [169].

Tissue engineering should be the best way to achieve successful cartilage regeneration by combining novel biologically inspired scaffolds approaches, nanotechnology, cell sources such as stem cells, chondrogenic factors, and physical stimuli [165].

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Chapter 16

Fabrication of PGA/PLA Scaffold with the Shape of Human Nose

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Additional information is available at the end of the chapter

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1. Introduction

Reconstructive surgery for the repair of nose deformities is challenging [1]. Nasal surgery involves autologous rib or septum cartilage grafts [2] and prosthetic devices [3] for reconstruction and reinforcement of the nasal skeleton. These conventional procedures are associated with donor site morbidity, limited tissue availability, and prosthesis related infection and extrusion [4]. Although tissue engineering is a promising method for repair and reconstruction of cartilage defects [5- 7], engineering cartilage with a delicate three dimensional (3D) structure, such as human nose, remains a great challenge in this field. Since in 1997 Cao *et al.* engineered the cartilage with a shape of human auricle in a nude mouse model [8], many researchers have tried to explore further developments of this tissue engineering system, but few of them have succeeded in *in vitro* regeneration of a cartilage construct with a complete and anatomically refined structure [9].

One major reason leading to the failure of *in vitro* engineering a cartilage construct with sufficient control over shape is the lack of appropriate scaffolds. The optimal scaffold used for engineering a cartilage construct with accurate designed shapes should possess at least three characteristics: good biocompatibility for cell seeding, ease of being processed into a specific shape, and sufficient mechanical strength for retaining the pre-designed shape. Polyglycolic acid (PGA) has proven to be one of the most successful scaffolds for cartilage regeneration [10-12]. Cartilage engineered with the PGA scaffold has structure and composition similar to the native tissue, as demonstrated by histological analysis and cartilage specific matrices [13- 15]. However, the most widely used form of PGA material in cartilage engineering is unwoven fiber mesh, which is difficult to be initially prepared into a complicated 3D structure and would most likely fail to maintain its original architecture during subsequent *in vitro* chondrogenesis due to insufficient mechanical support [14, 16, 17].

To overcome these problems, two crucial issues should be addressed. First, the PGA-based scaffold should be prefabricated into the exact shape of human nose. Second, the mechanical strength of the above-mentioned scaffold should be further enhanced so that it can retain the pre-designed shape.

In order to meet these requirements, in the current study, a computer aided design and manufacturing (CAD/CAM) technique was employed to fabricate a set of negative molds, which was then used to press the PGA fibers into the pre-designed nose structure. Furthermore, the mechanical strength of the scaffold was enhanced by coating the PGA fibers with an optimized amount of PLA.

2. Materials and methods

2.1. Preparation of scaffolds with different PLA contents

10 mg of unwoven PGA fibers (provided by Dong Hua University, Shanghai, China) were compressed into a cylinder shape of 5mm in diameter and 2mm in thickness. A solution of 0.3 % PLA (Sigma, St. Louis, MO, USA) in dichloromethane was evenly dropped onto the PGA scaffold, dried in a 65 ºC oven, and weighed. The PLA mass ratio was calculated according to the formula: PLA%= (final mass-original mass)/final mass×100%. The above procedures were repeated until the predetermined PLA mass ratios of 0%, 10%, 20%, 30%, 40% and 50% were achieved. The scaffolds were examined by SEM (Philips XL-30, Amsterdam, Netherlands) [18].

2.2. Biocompatibility evaluation of the scaffolds

Cell seeding: Chondrocytes were isolated from the articular cartilage of newborn swine (2-3weeks old) as previously described [19]. The harvested chondrocytes were adjusted to a final concentration of 50×10^6 cells/mL, and a 100uL cell suspension was pippeted onto each scaffold. The cell-scaffold constructs were then incubated for 4h at 37ºC with 95% humidity and 5% CO_2 to allow for complete adhesion of the cells to the scaffolds.

Cell adhesion: After 4 hours of incubation, the cell-scaffold constructs were gently transferred into a new 6-well plate. The remaining cells were collected and counted. The cell seeding efficiencies of the scaffolds with different PLA contents were calculated based on the formula: (total cell number- remaining cell number)/ total cell number×100% [14].

2.3. Mold fabrication by CAD/CAM

A patient's normal nose was scanned by CT to obtain the geometric data (Figure 3). These data were further processed by a CAD system to generate both positive and negative of the normal nose, and the resultant data were input into a CAM system (Spectrum 510, Z Corporation) for the fabrication of the resin models by 3D printing. The negative mold was composed of two parts: the anterior part and the posterior part. (Figure 4A)

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2.4. Fabrication of nose shaped scaffold

Two hundred milligrams of unwoven PGA fibers were pressed using the negative mold for over 12 hours. A solution of 0.3 % PLA (Sigma, St. Louis, MO, USA) in dichloromethane was evenly dropped onto the PGA scaffold, dried in a 65 ºC oven, weighed, and pressed again with the negative mold. This procedure was repeated until the final PLA mass ratio of 20% was reached. The edge of the scaffold was carefully trimmed according to the shape of the positive mold.

2.5. Statistical analysis

The differences of cell seeding efficiencies (n=6) among the six PLA content groups were analyzed using the Student's t-test. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. SEM observation of the scaffolds with different PLA contents

PLA/PGA scaffold compositions were visualized under SEM. (Figure 1) The pure PGA scaffold (0% PLA added) appeared as a smooth fiber mesh. In PGA scaffolds supplemented with 10% PLA, the PLA coating can be seen connecting some fibers, particularly at nodes where PGA fibers cross. In the 20% PLA embedded scaffold, most mesh nodes visualized were covered with PLA. 30% PLA scaffold had not only most mesh nodes embedded in PLA, but also the PLA coating was seen covering small portions of the mesh itself, minimally obstructing the porosity of the fiber network. In the 40% PLA embedded scaffold, most of the mesh porosity is obscured by a PLA. In the 50% PLA scaffold, the mesh is almost completely obscured by a PLA sheet.

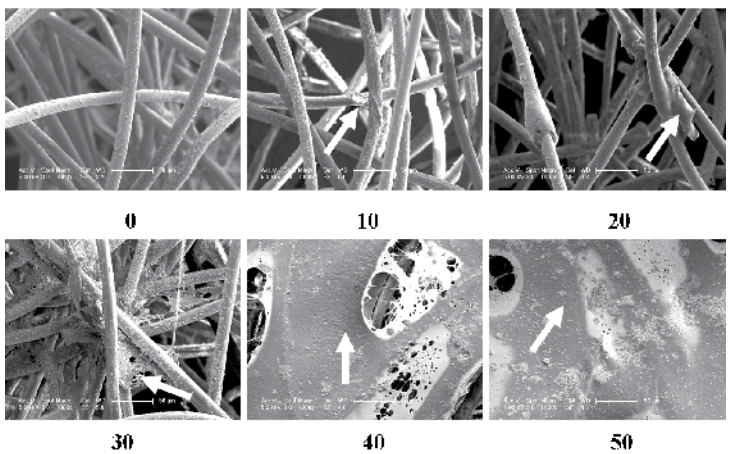

Figure 1. SEM examination. Scaffolds with different PLA contents (0%, 10%, 20%, 30%, 40% and 50%) show different pore structures. The white arrows indicate the coated PLA.

3.2. Evaluation of the biocompatibility of the scaffolds with different PLA contents

Cell seeding efficiencies were performed to evaluate the influence of PLA contents on cell compatibility of the scaffolds. The results showed that the increase in PLA content could lead to the reduction in the ability of the scaffolds to absorb the cell suspensions (Figure 2A). Quantitative analysis (Figure 2B) demonstrated that all the groups with PLA presented significantly lower cell seeding efficiencies compared to the group without PLA ($p<0.05$). If the acceptable cell adhesion rate is defined over 80%, these results indicate that 20% but not 30% is an acceptable PLA amount for preparing the scaffolds in terms of cell seeding efficiency.

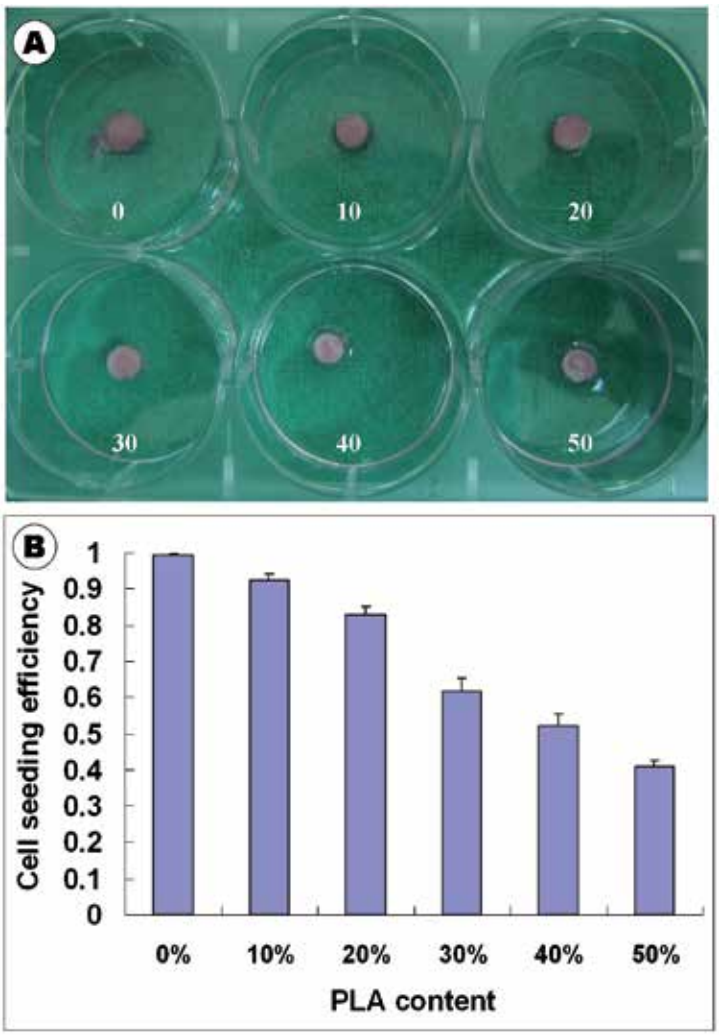

Figure 2. The influences of PLA contents on cell seeding efficiency. (A): Scaffolds with different PLA contents absorb different volumes of the cell suspension. (B): Cell seeding efficiencies decrease with increasing PLA contents in the scaffolds with significant decreases ($p<0.05$).

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3.3. Mold preparation and fabrication of the nose-shaped scaffold

Because good biocompatibility could be achieved in the scaffold with 20% PLA, this formulation was further used for the fabrication of the human nose shaped scaffold. In order to prepare the scaffold into a shape of normal nose, a set of negative molds was produced according to image of the normal nose (Figure 3). The resulting nose-shaped scaffold (Figure 4C) achieved a precise shape compared to its positive mold (Figure 4B). These results indicate that the mold produced by CAD/CAM technology is allowed to accurately fabricate a scaffold into a nose shape.

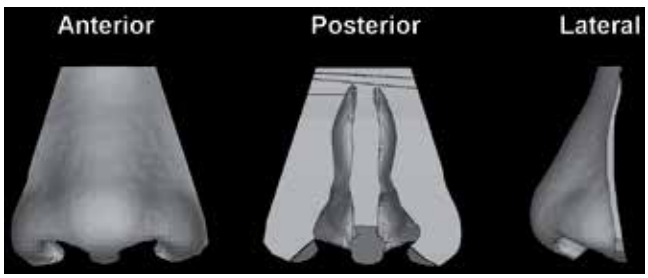

Figure 3. image of a patient's normal nose.

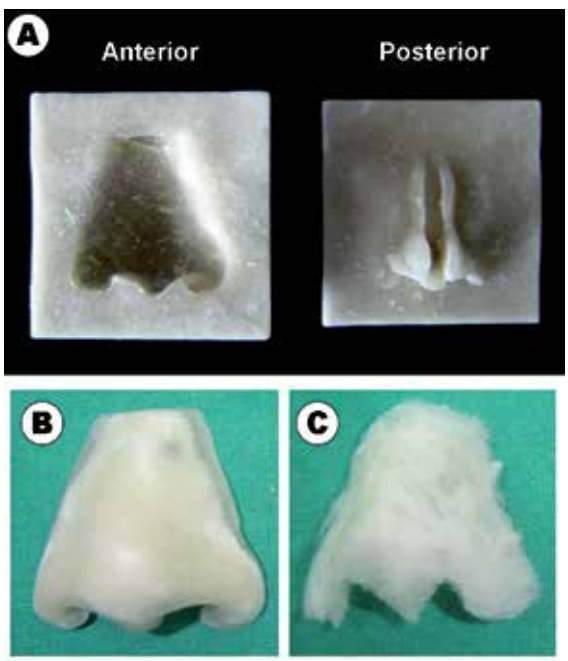

Figure 4. Mold preparation and the fabrication of the nose-shaped scaffolds. (A): The resin negative mold: anterior part and posterior part; (B): The resin positive mold; (C): the nose-shaped PLA/PGA scaffold.

4. Discussions

Despite the rapid progress in cartilage engineering, *in vitro* engineering of cartilage with a fine controlled 3D structure, such as human nose, remains a great challenge due to the lack of appropriate scaffolds. PGA has proven to be one of the most successful scaffolds for cartilage regeneration. However, for *in vitro* engineering of a cartilage with a precise shape, PGA unwoven fibers (the most widely used physical form) still have some drawbacks, such as the difficulties in controlling an accurate shape.

To achieve this, a negative mold corresponding to the desired shape is required. CAD/CAM, as a novel technique, has been widely used for the fabrication of anatomically accurate 3D models [20- 23]. Particularly, this method can accurately perform complicated manipulations of the original 3D data, including Boolean operations, mirror imaging, and scaling [24- 26]. CAD/CAM technique was therefore used in the current study for the production of the negative mold for a human nose. Using this mold, PGA fibers were able to be accurately prepared into the nose-shaped scaffold.

The mechanical strength of PGA scaffold alone is not sufficient for the shape maintenance, and thus PLA coating was used to strengthen its mechanical properties as reported [10, 11, 27]. However, a high amount of PLA in the scaffold would negatively affect cartilage formation because of poor cell compatibility [14]. Therefore, an appropriate PLA content in the scaffold is important for both shape maintenance and biocompatibility. In the current study, we evaluated the effects of six PLA contents on the scaffolds' biocompatibility. According to the current results, although the mechanical strength of the scaffolds increased with increasing PLA content, 20% is an acceptable PLA amount for preparing the scaffolds in terms of cell seeding efficiency.

Finally, aided by CAD/CAM technique, the PGA fibers were prepared into the accurate shape of a human nose. Furthermore, by coating with PLA, the scaffold could obtain sufficient mechanical strength to retain the original shape. These results may provide useful information for future nose reconstructions by *in vitro* engineered cartilage as well as for the engineering of other tissues with complicated 3D structures.

In summary, this study established a method to precisely engineer a PGA/PLA scaffold with the shape of human nose. In future studies, we will also investigate the fate of these scaffolds after cell seeding, especially subcutaneous implantation in an immunocompetent animal model.

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Section 3

Regeneration of Tissues and Organs

Chapter 17

Bone Marrow–Derived Cells Regenerate Structural and Functional Lower Urinary Tracts

Tetsuya Imamura, Osamu Ishizuka and
Osamu Nishizawa

Additional information is available at the end of the chapter

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1. Introduction

In this chapter, we show that with the application of tissue engineering principles, utilization of bone marrow-derived cells has the potential to reconstruct functional lower urinary tracts, which are composed mainly of the urinary bladder [1] and urethra [2]. Patients complain about lower urinary tract dysfunctions that significantly decrease their quality of life. Regenerative medicine provides great hope for the recovery of lost tissue and organ functions. In urology, novel regenerative medicine techniques are being developed for the treatment of irreversibly damaged lower urinary tracts. Notably, injection of autologous cells harvested from adipose tissue into the sphincter and urethra has been attempted clinically to treat urinary incontinence by increasing the urethral closure pressure [3]. Our laboratory has also been vigorously investigating regenerative medicine as a tool to treat irreversibly damaged urinary bladders and urethras.

An important factor in the development of regenerative medicine is selection of the proper source for the regenerative cells and/or tissues. Recently, attempts to use various kinds of cells, such as induced pluripotent stem cells, embryonic stem cells, and mesenchymal cells derived from adipose and oral mucosal tissues, have been reported. Based on the literature, we have considered many sources of cells from which to derive adult somatic stem cells that could regenerate lower urinary tracts [4-7]. Recently, we have focused on two sources of cells with the potential to meet a variety of demands: bone marrow-derived cells [8] and adipose-derived cells [9]. In this chapter, we show that cells derived from bone marrow are an excellent resource for the development of regenerative medicine. These cells are capable of differentiating both in vitro and in vivo along multiple pathways that include striated and smooth muscle [10-16] as well as bone, cartilage, adipose, neural cells, tendon, and connective tissue [17,18]. Also, bone marrow-derived cells, which are easy to grow in culture, produce cytokines and growth

factors that accelerate healing in damaged tissues and inhibit apoptosis and the development of fibrosis [19,20]. However, the operation to harvest the bone marrow cells is generally considered to have higher patient risks compared to harvesting adipose cells. The increased risk for harvesting bone marrow cells for autologous transplantation is especially important for elderly patients with lower urinary tract symptoms (LUTS) and who may have other diseases as well.

Equally important as the sources of cells for regenerative medicine are the survival rates for implanted cells, the differentiation into target cell types, and the structural support that enables the reconstruction within the recipient tissues [21]. The survival, differentiation, and reorganization of the implanted cells are affected by the microenvironment within the recipient tissues. However, our understanding of these microenvironments is currently insufficient to provide clinically effective and reliable resources for regenerative medicine. Thus, to obtain the optimum microenvironment, we need to investigate the utilization of scaffolds, growth factors, and combinations of these materials.

This chapter has three major topics: (1) implantation of allogenic mouse bone marrow-derived cells into freeze-injured urinary bladders, (2) implantation of autologous bone marrow-derived cells into freeze-injured urethral sphincters, and (3) importance of the microenvironment in reconstructing functional lower urinary tracts. We show that bone marrow-derived cells implanted into freeze-injured bladders or urethras differentiate into smooth muscle and striated muscle cells. These cells become organized into layered structures that are associated with the recovery of tissue function. In injured tissues, we have begun to uncover the important roles that may support the differentiation. Our information leading to future studies will enable the development of regenerative medicine in urology and other clinical areas.

2. Reconstruction of functional urinary bladders

Our final goal is to develop treatments for irreversibly damaged urinary bladders resulting from neurogenic bladder associated with brain and spinal cord disease, cystitis, peripheral neuropathy, or radiotherapy-induced injury. To determine the capacity of bone marrow-derived cells to fulfill these goals, we have performed preliminary investigations in allogeneic transplantation by using a mouse freeze-injured urinary bladder model [1].

2.1. Freeze-injured urinary bladder model

Three days prior to implantation, we induce a highly reproducible freeze-injury to the urinary bladders of mice [1]. We apply an iron bar (25 x 3 x 2 mm) chilled by dry ice onto the posterior urinary bladder wall for 30 seconds. Placement of the chilled iron bar causes local freezing of the bladder wall tissues. Within 10 seconds after removal of the bar, the frozen spot thaws due to body and/or room heat and appears to the naked eye similar to the intact normal bladder wall. However, when we monitor blood flow within the capillaries of the frozen area with a charged-couple device (CCD) video microscopy, the local circulation pauses for approximately 20 min after the operation, and then it resumes. It is likely that the freeze-injured urinary

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bladders experience a period of ischemia followed by reperfusion as described by one of the microcirculation dysfunction models [22]. At 3 days after the freeze-injury operation, the wounded area, which occupies approximately one-third of each urinary bladder, is readily identified by the presence of a hematoma. The freeze-injured urinary bladders have both injured and uninjured regions that are easily observed by histology. The smooth muscle layers of the injured regions are disorganized and readily distinguished from the surrounding uninjured regions (Figure 1A).

2.2. Implantation of cultured bone marrow–derived cells

We harvest mouse bone marrow cells by flushing them out from both ends of femurs, and the recovered cells are cultured on collagen-coated dishes for 7 days. During the culture period, we completely replace the 15% fetal bovine serum-containing medium every day and remove non-attached cells. Immediately after plating in dishes, the bone marrow cells consist of heterogeneous, spindle-shaped, round, and polygonal cell types along with red blood cells. At 5 days after seeding, the cells achieve approximately 80% confluence, and at that time we transfect them with the green fluorescence protein (GFP) gene for identification in the recipient tissues. After 7 days of culture (i.e., 2 days after transfection), the adhered proliferating cells are relatively homogenous in spindle shaped appearance, and they expressed GFP. We confirm that the cultured cells do not differentiate into smooth muscle cells during the culture period.

The culture conditions readily promote attachment and proliferation of bone marrow cells. The cultured bone marrow-derived cells contain a variety of stem cell types, such as hematopoietic, mesenchymal, and stromal stem cells [17,18]. Marker proteins on cells can be used to sort the ones that will differentiate into specific target cells [23-27]. However, which of the sorted cells are best for clinical use is unknown [28]. The simplicity of our selection procedure, based only on attachment and proliferation of bone marrow cells on collagen, would be a significant advantage for clinical applications.

On Day 7 of culture, we dissociate the cultured bone marrow-derived cells and allotransplant 2.0×10^6 cells with a 30-guage micro-syringe into the center of the injured region of the 3-day-old freeze-injured urinary bladders. As controls, we inject cell-free solution. The implantation cell number and volume are chosen to avoid further damaging the cells with shear stress or the recipient tissues by bursting the bladder wall. Each operation is performed under a stereomicroscope where we visually confirm the presence of a small swelling, indicating that the implanted cells remain at the site.

2.3. Reconstructed smooth muscle layers

At 14 days after implantation, we estimate the effect of the bone marrow-derived cells. The regions implanted with these cells have numerous alpha-smooth muscle actin (SMA)-positive smooth muscle cells compared to the control regions injected with the cell-free solution (Figure 1B). The cells are organized into distinct smooth muscle layers. In contrast, the few SMA-positive cells present in the regions injected with the cell-free solution are not organized into layers.

At 14 days, SMA mRNA expression in the implanted regions is significantly higher than that in the cell-free injected regions, which supports the immunohistochemical observations [1]. In fact, the expression level of SMA mRNA in the implanted regions is not significantly different from that in the normal urinary bladder. Expression levels of other smooth muscle cell differentiation marker genes in the implanted region are also elevated. In the implanted regions, smooth muscle myosin heavy chain (MHC) and calponin I mRNA expression are significantly higher than in the cell-free injected control regions [1]. There are no significant differences in MHC and calponin I mRNA expression levels when the implanted regions are compared to the normal urinary bladders. Desmin mRNA expression is significantly higher than either control or normal regions. Thus the implanted regions have a larger number of mature smooth muscle cells and developing smooth muscle layers compared to the control regions [29-33]. Collectively, the immunohistochemical and gene expression results show that the implanted regions have formed smooth muscle layers composed of regenerated smooth muscle cells during the 14 days of the study period, while the control regions have only minimal recovery.

2.4. Progress of implanted bone marrow–derived cells

We detect the implanted bone marrow-derived cells in the recipient tissues by the presence of GFP labeling. Some of the GFP-labeled implanted cells are positive for proliferating cell nuclear antigen (PCNA), a marker of proliferating cells. In addition, within

the newly formed smooth muscle layers, some GFP-labeled cells that do not express smooth muscle cell differentiation markers are organized into cord-like structures. The cells may have differentiated into cell types that provide histoarchitectural elements for the blood vascular system [24] or the nervous system [25]. The implanted bone marrow-derived cells may participate in as yet other unknown changes in the various tissues of the urinary bladder.

We perform double staining with the smooth muscle cell differentiation markers and GFP antibody to identify the regenerated smooth muscle cells that are derived from the implanted cells. Both GFP- and SMA-positive cells show that the implanted bone marrow-derived cells differentiate into smooth muscle cells within the injured urinary bladders (Figure 1C). Other implanted GFP-labeled cells are also positive for MHC, desmin, and calponin I. Some of the GFP-labeled, differentiated smooth muscle cells contact each other, and they also contact non-GFP-labeled smooth muscle cells of the host that surround the implanted regions. Collectively, these cells form smooth muscle layers. The differentiation toward smooth muscle cells occurs after implantation because none of the cells expressed detectable levels of the marker proteins while in culture.

2.5. Recovery of bladder contractions

Cystometric investigations at 3 days after injury show that the mice do not have defined regular bladder contractions. The bladder contractions at 14 days after cell-free control injection also remain disrupted. However 14 days after cell implantation, there are distinct regular bladder contractions that are similar to those of normal mice without injury [1]. Thus, cystometric

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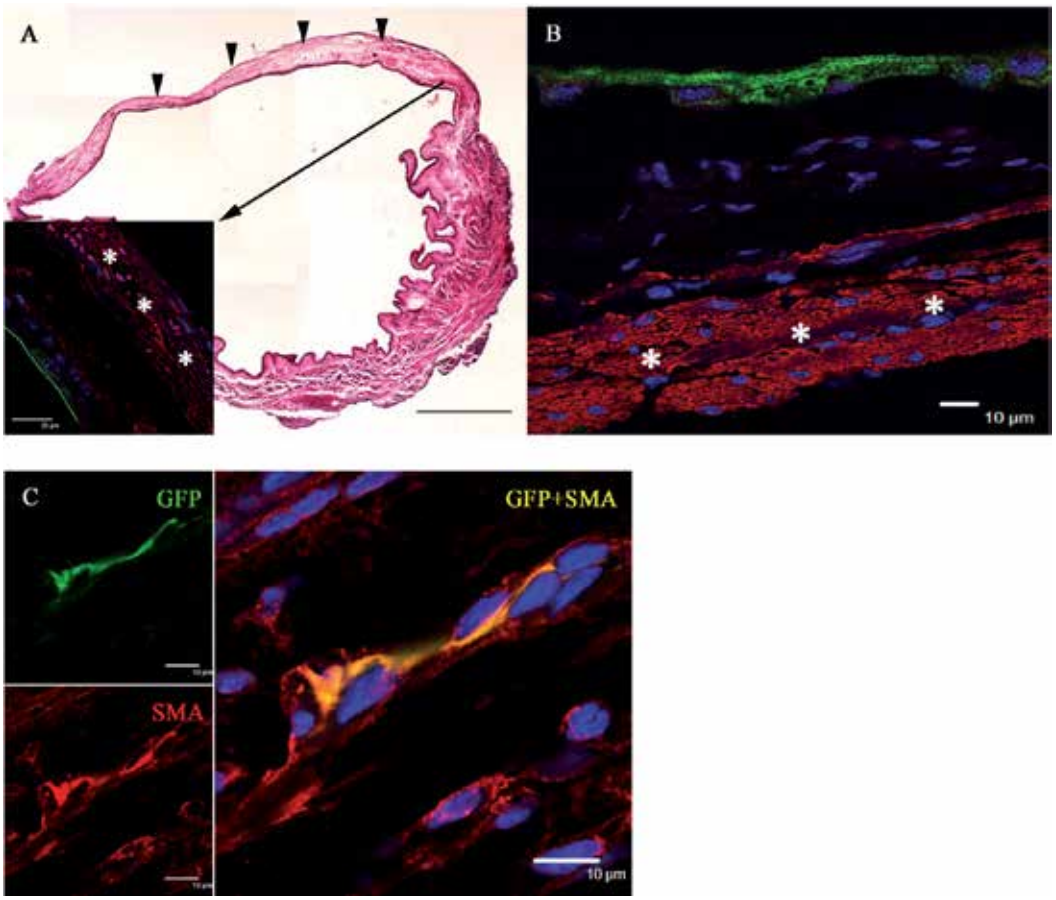

Figure 1. Implantation of bone marrow-derived cells into freeze-injured urinary bladders. (A) At 3 days after operation, the freeze-injured regions (arrowheads and asterisk in inset) lose typical smooth muscle layers. Black bar: 1 cm. (B) At 14 days, the bone marrow-derived cell-implanted regions have numerous SMA-positive smooth muscle cells (red, asterisks) that are organized into layers (green, urothelium; blue, nuclei). (C) The cells detected with GFP antibody (upper left inset, green cells) are simultaneously positive for the smooth muscle marker SMA (bottom left inset, red cells) within the newly formed smooth muscle layers. The merged images (right, yellow cells) show that the implanted GFP-labeled cells have differentiated into cells expressing smooth muscle markers. Blue, nuclei.

investigations indicate that implanted bone marrow-derived cells have the potential to restore some or all normal bladder functions. We believe that the smooth muscle layers reconstructed by the implantation of the cells contribute to the restoration of bladder contractions.

3. Reconstruction of functional urethral sphincters

In clinical diagnosis, urinary incontinence is separated into two major categories: (1) stress urinary incontinence and (2) post-surgical urinary incontinence associated with intrinsic sphincter deficiency (ISD). Stress urinary incontinence is related to urethral hypermobility,

which results from the loss of bladder neck support. This form of urinary incontinence can be improved by surgical therapies to lift the bladder and urethra. In contrast, post-surgical ISD-related urinary incontinence can occur as a result of radical prostatectomy or bladder neck surgery. It is characterized by severely decreased urethral closure pressure due to malfunction of the closure mechanism, and it results in intractable urinary incontinence. Improvement of urethral closure pressure is widely accepted as one of the effective treatments for ISD-related urinary incontinence [34]. In the urinary continence system, the urethral sphincter is composed of both striated and smooth muscle cells and produces urethral closure pressure. Thus, our strategy for relieving ISD-related urinary incontinence is the reconstruction of functional urethral sphincters by the implantation of autologous bone marrow-derived cells [2].

3.1. Freeze–injured urethral sphincter model

For ISD-related urinary incontinence studies, we have developed a rabbit freeze-injured urethral sphincter model [2]. The sphincter, which is located at the internal urethral orifice where it joins the inferior end of the bladder, is sprayed with the liquid nitrogen for 15 sec. The frozen regions are thawed by room and body heat within approximately 20 sec. As an immediate consequence of the freeze and thawing, the wounded internal urethral orifice is flaccid and gapes open.

Seven days later we compare the effect of the urethral freeze/thaw procedure to sham-operated uninjured animals. In sham-operated animals with uninjured urethral sphincters, the internal urethral opening remains tightly closed [2]. The muscle tissues within the intact sphincters are composed of striated muscle containing highly organized myofibrils and smooth muscle cells containing irregularly placed myofibrils. Immunohistochemical analysis shows that the sham-operated urethral sphincters are composed of distinct muscle tissues containing numerous myoglobin- and SMA-positive cells. In contrast, the freeze-injured internal urethral openings remains flaccid (Figure 2A), and the leak point pressure of the injured animals is significantly lower than that of the sham-operated animals. Consistent with these observations, the injured urethral sphincters show reactive changes including loss of muscle mass and relative disorganization of the remaining muscle tissues (Figure 2A). The majority of the striated and smooth muscle cells are lost, and there is a complete absence of most myoglobin- and SMA-positive cells (Figure 1A).

Our ISD-related urinary incontinence model is similar to other models of urinary incontinence with lost striated and smooth muscle and reduced leak point pressures [35-38]. The urinary sphincters of patients with post-surgical urinary incontinence are irreversibly damaged. However, this appears not to be the case in our model. The cell-free treated rabbits show a weak but natural recovery of striated and smooth muscle cells associated with a slight increase of leak point pressure. Rabbits may have inherently different regenerative powers than humans. Additionally, the rabbits are young and in good health, in contrast to patients with ISD-related urinary incontinence, who are typically elderly and not in good general health. In our rabbit model, we intentionally avoided more severe and serious sphincter damage that would have produced irreversible incontinence because of the potential for urethral stricture

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or perforation, followed by death. Thus, our model is considered to be an acute incontinence of relatively short duration [2].

3.2. Implantation of autologous bone marrow–derived cells

To conduct autologous implantation without euthanasia, we harvest bone marrow cells from a femur of each anesthetized animal by the flush out method, which is modified from the technique described by Kushida et al. [39]. Two pediatric bone marrow needles are inserted 2 cm apart into a femur, and then the cells are flushed out with saline pushed through one needle and collected in a tube through the other needle. The harvested bone marrow cells are cultured on type I collagen-coated culture flasks for 10 days. The culture and cell-labeling methods are the same as for mouse bone marrow-derived cells (as above 1.2). During the culture, the cytomorphologic changes are similar to those in the mouse bone marrow-derived cells [2]. At 10 days, the cultured cells express mesenchymal cell marker STRO1 (CD34), but not myoglobin, SMA, or Pax7, which are differentiation markers for striated muscle cells, smooth muscle cells, and myoblast, respectively.

Aging, disease processes, and medications may affect the potential of bone marrow cells for differentiation. Thus, for the purpose of advancing the fundamental research necessary for understanding the basic parameters of autologous bone marrow-derived cell growth, differentiation, and transplantation, we selected young and healthy rabbits. The large size of these animals, in contrast to rats, mice, or other rodents, facilitates the performance of the autologous bone marrow-derived cell-implantation procedures.

Ten days after culture, and 7 days after the freeze-injury operation, we implant $0.5x10^6$ autologous bone marrow-derived cells suspended in 100 μl culture medium. A total of $2.0x10^6$ cells are injected via a 29-gauge syringe needle into the injured regions at the 3-, 6-, 9-, and 12-O'clock positions. For controls, we inject cell-free solution with same manner. The implantation cell number and volume are chosen for the same reasons described above (section 1.2).

3.3. Reconstructed layered muscle structures

At 7 and 14 days after cell-implantation or cell-free control injection, recovery of the urethral sphincters is determined by histology, cytology, and immunohistochemistry [2]. At 7 days after the cell-free control injection, there are few myoglobin-positive striated muscle cells, and few clusters composed of SMA-positive smooth muscle cells. In contrast, at 7 days after cell implantation, there are developing muscle layers composed of myoglobin-positive striated cells, and clusters composed of SMA-positive smooth muscle cells. At that time, the proportions of the myoglobin- and SMA-positive areas in the cell-implanted regions are significantly higher than in the cell-free injected regions [2].

At 14 days after control cell-free injection, the regional composition of cells is similar to the 7-day control regions with relatively few cells expressing myoglobin or SMA [2]. In contrast, at 14 days after cell implantation, the regions have distinctly regenerated muscle layers composed of numerous myoglobin-positive striated and SMA-positive smooth muscle cells that are

similar to the intact urethral sphincters (Figures 2B, C). At that time, the proportion of both myoglobin- and SMA-positive areas are significantly higher than in the control regions.

Bone marrow-derived cells have the unique ability to promote healing activities that can produce cytokines and growth factors that accelerate healing in damaged tissues. While we do not yet know if the implanted cells secrete trophic factors that promote differentiation of endogenous cells, there is the potential that a portion of the regenerated muscle layers are formed in response to trophic factors secreted from the implanted cells.

3.4. Differentiation of implanted bone marrow–derived cells

At 7 and 14 days after implantation, we conduct double staining with GFP antibody in combination with striated muscle cell-, smooth muscle cell-, or myoblast-differentiation marker antibodies [2]. At 7 days, some of the implanted cells identified by the presence of antibody-labeled GFP are simultaneously positive for myoglobin or SMA antibody. These double positive cells show that the implanted autologous cells differentiate into striated or smooth muscle cells. These differentiated cells are widely distributed within the reconstructed muscle layers. At 14 days after implantation, the double-labeled cells appeared to form contacts among themselves, creating layered muscle structures (Figures 2D, E). In addition, the striated- and smooth-muscle differentiated cells contact non-GFP expressing muscle tissues that are presumably derived from the uninjured surrounding tissues. These cells are then integrated into the recovering muscle layers.

At 7 days after cell implantation, a few of the GFP-labeled implanted cells are simultaneously positive for Pax7, suggesting that they have myoblast properties [2]. In the development process to mature muscle, Pax7 acts as transcription factor, and satellite cells and myoblasts both express Pax7, but mature muscle cells do not [40]. Currently we cannot determine if the cells expressing both GFP and Pax7 are presumptive satellite cells or myoblasts. Nevertheless, the implanted cells clearly follow a development process that leads to the differentiation of striated or smooth muscle cells. The number of the cells expressing both GFP and Pax7 on day 14 is distinctly higher than on day 7 [2].

Myoblasts properly differentiate into striated or smooth muscle cells according to the surrounding environment. The greater number of Pax7 cells on day 14 compared to day 7 suggests that the formation rate of differentiated muscle cells may have decreased or even stopped. This suggests that the differentiation process of new striated and smooth muscle cell is under some type of intrinsic regulation. Understanding the controls for differentiation of the implanted cells is very important for further development of regenerative medicine. While the details of this regulation are currently unknown, it is clear that the presence of the myoblasts in the regenerated region may have important long term significance. In the event that the newly differentiated striated and/or smooth muscle tissues and structures spontaneously regress or are lost for other reasons, the presence of the myoblasts could ensure the replacement of the lost cells. Thus, the effects of treatments may be maintained for a long period of time.

We focus only on the implanted cells that maintained expression of GFP after implantation. At 7 days, the majority of both GFP and myoglobin, SMA, or Pax7 double-positive cells are

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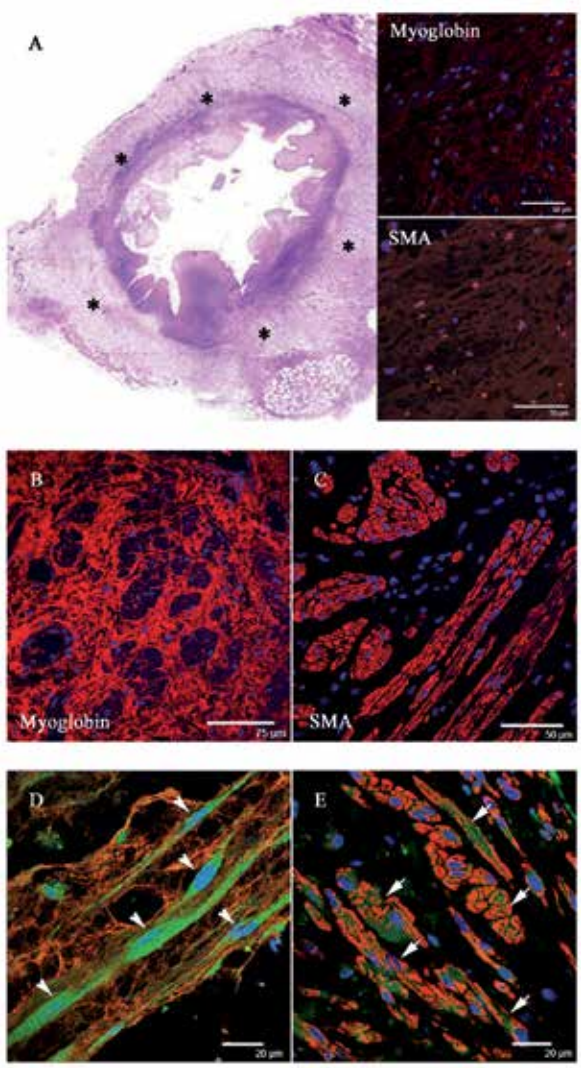

Figure 2. Implantation of autologous bone marrow-derived cells into freeze-injured urethral sphincters. (A) At 7 days after wounding, the urethral orifices are flaccid and gape open due to the loss of the surrounding muscle tissues (asterisks). The injured urethral sphincters lose the majority of the typical striated (upper right inset) and smooth muscle (bottom right inset) cells. Blue, nuclei. (B and C) The 14-day cell-implanted regions have distinct layered muscle structures containing numerous myoglobin- (B) and SMA- (C) positive cells that are similar to intact urethral sphincters. Blue, nuclei. (D and E) The 7-day cell-implanted regions have some GFP-positive cells that are simultaneously positive for myoglobin (D, arrowheads) or SMA (E, arrows) antibody. These cells are in contact with each other in the reconstructed the muscle layers.

mononuclear. While we cannot definitively exclude the possibility of cellular fusion, the findings suggest that the number of these double-positive cells formed by cellular fusion is small. Thus, the GFP-labeled implanted cells differentiate into myoglobin-positive striated muscle cells and SMA-positive smooth muscle cells within the injured regions.

3.5. Recovery of leak point pressure

At 7 days after cell implantation, the leak point pressure of the cell-implantation group, 13.15±2.82 cmH_2O, tends to be higher than the cell-free control group, 8.13±2.43 cmH_2O, but the difference is not statistically significant. At 14 days, the leak point pressure of the cell-implantation group, 17.82±1.31 cmH_2O, is significantly higher than that of the control group, 11.78±3.23 cmH_2O ($P<0.05$) [2]. We do not yet know the leak point pressures of healthy rabbits, and whether or not the cell-implanted rabbits have voluntary control of the restored sphincters. Clinically, while less than 60-65 cmH_2O of (abdominal) leak point pressure is one of the indexes of human stress urinary incontinence, it is not sufficient to diagnose it. Nevertheless, it is clear that increased or a high leak point pressure is helpful to inhibit urine leakage that can occur during physical activity. Therefore, cell therapy using bone marrow-derived cells has a great potential to reduce urinary incontinence and improve the quality of life.

4. Microenvironment

The microenvironment within the damaged recipient tissues affects regeneration of functional tissues [21]. We confirmed that bone marrow-derived cells implanted into uninjured normal tissue do not undergo differentiation and development. In injured tissues, bone marrow-derived cells exhibit significant potential to recover functional tissues. In addition, bone marrow-derived cells have the unique ability to differentiate into target cells and promote healing activities. However, these abilities are expressed only in suitable environments. In this section, we show that important roles are played in freeze-injured urinary bladders by the local microcirculation, large tissue pores, host tissue scaffolding, and expression of growth factor mRNAs that may support the differentiation.

4.1. Microcirculation in the freeze–injured urinary bladders

At 3 days after the freeze-injury of mouse urinary bladders, we observe the wounded areas by CCD video microscopy. Blood capillaries in the intact normal bladder walls have a robust flow of red blood cells with a velocity of 0.26±0.03 mm/s. In contrast, while maintaining a partial microcirculation, blood capillaries within the wounded bladder walls are not as abundant compared to normal bladder walls. Further, the blood flow velocity of the injured regions is 0.12±0.11 mm/s. The mechanism(s) for the reduced flow rate is not known with certainty. Regardless of the reason, the most important finding is that the injured regions are maintained with only a partial microcirculation [21]. The maintenance of at least a minimal microcirculation to provide oxygen and nutrition is likely to be one of the prerequisite factors necessary for successful tissue engineering.

4.2. Structures in the freeze–injured urinary bladders

We observe the intact normal and freeze-injured bladder walls by scanning electron microscopy. The normal bladder walls have smooth muscle cells organized into layers that are

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readily apparent. These layers do not contain any porous spaces that are over 10 μm in diameter (Figure 3A). In contrast, the freeze-injured bladder walls have few typical structures composed of smooth muscle cells, but there are many large porous spaces that are over 10 μm in diameter (Figure 3B).

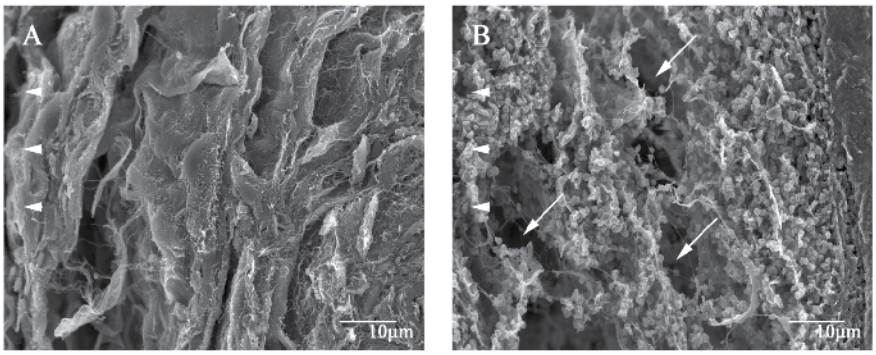

Figure 3. Layered smooth muscle structures within the freeze-injured urinary bladders. (A) The intact bladder walls contained layered structures composed of smooth muscle cells. (B) The freeze-injured regions had many large porous spaces (arrows). Arrowheads: exterior surface of the bladder wall.

By transmission electron microscopy, the normal bladder walls contain spindle-shaped smooth muscle cells with readily apparent nuclei. These cells are arranged in sheets and connected with each other by gap junctions. In contrast, smooth muscle cells in the freeze-injured bladder walls are shrunken, and exhibit blebbing [21]. The chromatin is condensed and nuclear fragmentation is apparent. Also, gap junctions are rarely present between the remaining cells of the smooth muscle layers. Based on the cytological observations, smooth muscle cell death is predominantly due to apoptosis, though we cannot exclude the occurrence of necrosis, especially immediately after the freezing injury.

The freeze-injured bladder walls contain numerous large pores, like those seen by a scanning electron microscopy, that are not present in the normal bladder walls [21]. The origin of these pores is not certain, but may be due to loss of smooth muscle cells that are the principal component of the wall in intact urinary bladders. In fact, the pores within the freeze-injured urinary bladders may be helpful in establishing a high rate of cell implantation and survival. They may also serve as scaffolding for the reconstruction of tissue structures.

4.3. Expression of growth factor mRNAs in the freeze–injured urinary bladders

Using real-time RT-PCR arrays, we estimate that 84 growth factor mRNAs are expressed in the freeze-injured bladders [21]. Nineteen of these exhibit at least a two-fold increase over the intact normal bladders. The most impressive increases are for secreted phosphoprotein 1 (SPP1), inhibin beta-A (INHBA), glial cell line derived neurotrophic factor (GDNF), and transforming growth factor, beta 1 (TGFB1). TGFB1 specifically promotes differentiation of smooth muscle cells from bone marrow-derived cells [41-43]. The others, SPP1 [44,45], INHBA [46-48], and GDNF [49-51], also support differentiation of smooth muscle cells from bone marrow-derived

cells. Moreover, inflammation-related cytokine growth factor mRNAs for interleukins (IL)-6, -11, -1A, -1B, and -18 are upregulated along with angiogenic-associated growth factor mRNAs for epiregulin (EREG), chemokine (C-X-C motif) ligand 1 (CXCL1), teratocarcinoma-derived growth factor (TDGF1), fibroblast growth factor 5 (FGF5), C-fos induced growth factor (FIGF), and vascular endothelial growth factor A (VEGFA) that have the potential to improve microcirculation within the injured regions. In addition to the above growth factors, expression of trefoil factor 1 (TFF1), colony stimulating factor 3 (CSF3), hepatocyte growth factor (HGF), and bone morphogenetic protein 1 (BMP1) mRNAs is also elevated. The roles of these growth factors are unclear, but it is likely that they participate in wound healing.

Collectively, these results show that cells of the urinary bladder respond to freeze injury by enhanced transcription of mRNAs specifically associated with differentiation of smooth muscle cells and wound healing. If translated, expression of these genes can promote growth and development of a suitable physical and biochemical environment. Under these circumstances, the microenvironment within the freeze-injured urinary bladders would promote organization of the developing cells into physiologically functional tissues.

4.4. Uninjured regions in the freeze–injured urinary bladders

It is likely that recovery within the freeze-injured urinary bladders requires participation of the undamaged tissue adjacent to the injured site [1, 21]. In general, the success of implanted undifferentiated cells depends upon the recovery of host cells to provide an appropriate microenvironment at the location of the injury or disease site. These host cells are necessary to support the production of growth factors by the implanted bone marrow-derived cells [52-54]. The absence of a supportive microenvironment in the surrounding host tissues, as might occur in cases of irreversible or chronic diseases and/or injuries of the urinary bladder due to spinal injury or radiation therapy, might prevent or limit the recovery processes associated with the implanted cells.

4.5. Tissue engineering

Tissue engineering consists of three components: (1) undifferentiated cells having the potential to differentiate into specific cell types, (2) scaffolding to support construction of tissue structures, and (3) growth factors to promote differentiation of various and specific cell types. The bone marrow-derived cells are an excellent source of multipotent undifferentiated cells that can develop into smooth muscle cells [1, 14, 55, 56]. The tissue pores that are present three days after freeze-injury operation are likely to provide scaffolding and spaces suitable for colonization by the implanted bone marrow-derived cells. This would optimize the chance for a high rate of cell survival and differentiation [21]. Though we have not actually measured the secretion of growth factors by the surviving cells, at least 19 different growth factor mRNAs are increased three days after the freeze-injury operation. These mRNAs includes growth factor mRNAs for SPP1, INHBA, GDNF, and TGFB1 [21]. If they are translated, they would be able to support the differentiation of the implanted bone marrow-derived cells into smooth muscle cells. Finally, the maintenance of a minimal microcirculation within the injured regions probably supports growth and development of the implanted bone marrow-derived cells [21].

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For all of these reasons, the freeze-injured urinary bladders provide a suitable microenvironment for differentiation and development of the implanted cells.

Recipient tissues do not always have a suitable microenvironment for the implanted cells. Thus, there is a need for new investigations that develop novel combinations of scaffolding and/or growth factors to support tissue engineering of stem-type cells that promote regeneration in severely damaged organs. In many cases, there might not be an adequate scaffold in vivo to support the implanted cells. Under those circumstances, it may be possible to construct scaffolds in vitro using biocompatible materials. To promote appropriate cellular differentiation, growth factors delivered by sustained-release or other drug delivery systems also may be necessary.

5. Conclusion

This chapter shows that bone marrow-derived cells have the potential to be an important cell source for regeneration of lower urinary tracts. The implantation of bone marrow-derived cells can produce functional smooth muscle layers in irreversibly damaged urinary bladders associated with the loss of smooth muscle layers due to injury or disease. Also, the cell implantation can recover functional urethral sphincters that prohibit the inadvertent release of urine. We suggest that to develop the full clinical potential of regenerative medicine, we need a further understanding of the requirements for undifferentiated cell proliferation and targeted differentiation. Moreover, based on tissue engineering principles, knowledge of each unique microenvironment within recipient tissues is necessary.

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Chapter 18

Corneal Endothelial Tissue Bioengineering Using Cultured Human Corneal Endothelial Precursor Cells

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Additional information is available at the end of the chapter

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1. Introduction

The cornea is composed of a multilayered epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium. The corneal endothelium (CE) is a single layer of hexagonal cells that separates the corneal stroma from the aqueous humor of the anterior chamber. Transparency of the cornea is maintained by regulation of stromal hydration through the barrier and pump functions of the CE, and corneal transplantation has long been used to treat corneal endothelial defects. In fact, more than half of the patients who undergo full-thickness corneal transplantation have impairment of visual acuity due to corneal endothelial problems alone and have a normal corneal epithelium (Cosar et al., 2002; Mannis et al., 1981; Rapuano et al., 1990). Corneal transplantation requires a fresh human cornea, but there is a worldwide shortage of donors (Barboza et al., 2007; Cao et al., 2006; Shimazaki et al., 2004; Tuppin et al., 2007).

Stem cells or progenitor cells are defined by a capacity for self-renewal and the ability to generate different types of cells (multipotentiality) that are involved in the formation of mature tissues. In contrast, precursor cells are unipotential cells with limited proliferative capacity. Regenerative stem cells or precursors can be detected by the sphere-forming assay in various adult tissues, including the central nervous system (Nunes et al., 2003), bone marrow (Krause et al., 2001), skin (Kawase et al., 2004; Toma et al., 2001), retina (Coles et al., 2004), corneal epithelium (Mimura et al., 2010a; Yokoo et al., 2008), corneal stroma (Amano et al., 2006; Mimura, 2008a, 2008b; Uchida et al., 2005; Yamagami et al., 2007), and corneal endothelium (Amano et al., 2006; Mimura, 2005a, 2005b, 2005c, 2007, 2010b; Yamagami, 2006, 2007; Yokoo et al., 2005).

Despite the successful isolation and characterization of stem cells from various tissues, relatively few animal studies have been done to investigate the efficacy of stem cell transplantation. A three-dimensional carrier that maintains cell-to-cell interactions is indispensable for tissue engineering using stem cells, but the resulting structural complexity does not allow us to easily perform investigations of stem cell transplantation.

We have isolated precursors with the propensity to develop into corneal endothelial-like cells from the CE of human donor corneas (Yokoo et al., 2005). We have also demonstrated that cultured human corneal endothelial cells (HCECs) and rabbit CE-derived precursors are an effective cell source for treating corneal endothelial defects in a rabbit model (Mimura 2005a, 2005b). Because the number of corneal endothelial cell (CEC) precursors that can be isolated from a native cornea is insufficient for corneal transplantation, establishment of a method for the mass production of precursor cells is required before CEC transplantation can be employed clinically.

In this chapter, we introduce our recent work in the fields of regenerative medicine and tissue engineering for the CE using bipotential precursor cells. We isolated precursors with the propensity to develop into CECs from human CE, and we investigated the distribution and proliferative capacity of precursor cells derived from the central and peripheral regions of the cornea by the sphere-forming assay. We also tested the effect of injecting human corneal endothelial spheres anterior chamber (instead of full-thickness corneal transplantation) in a rabbit model of bullous keratopathy, a condition associated with corneal endothelial defects.

2. Origin and development of the Corneal Endothelium

Neural crest cells, from which the CE is derived (Bahn et al., 1984; Johnston et al., 1979), migrate and differentiate in two waves during corneal development (Liu et al., 1998; Meier et al., 1982). In the first wave, the corneal epithelium is formed by periocular mesenchymal cells of neural crest origin and it synthesizes the primary stroma, after which neural crest cells migrate to the margin of the optic cup and then migrate between the lens and corneal epithelium to contribute to development of the CE and the trabecular meshwork. In the second wave, neural crest cells invade the primary stroma and differentiate into corneal keratocytes.

3. Isolation of sphere colonies from human Corneal Endothelium

3.1. Primary sphere-forming assay

This study was conducted in accordance with the Declaration of Helsinki. Corneas were obtained from the Central Florida Lions Eye Tissue Bank and the Rocky Mountain Lions' Eye Bank at 4 to 10 days after death. The age of the donors was 41 to 78 years. The CE and Descemet's membrane were peeled away in a sheet from the periphery to the center of the inner surface of the cornea with fine forceps, as described previously (Sakai et al., 2002). To

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avoid the inclusion of posterior stromal tissue, we only used endothelium that was smoothly peeled off together with Descemet's membrane. The harvested CE was incubated at 37ºC for 3 hours in basal medium containing 0.02% collagenase (Sigma-Aldrich, St. Louis, MO). This was followed by incubation in 0.2% ethylenediaminetetraacetic acid (EDTA) at 37ºC for 5 minutes, and then dissociation into single cells by trituration with a fire-polished Pasteur pipette. The viability of the isolated CECs was >90%, as shown by trypan blue staining (Wako Pure Chemical Industries, Osaka, Japan). After addition of a trypsin inhibitor (Invitrogen-Gibco), the cells were resuspended in basal medium and the number of cells was counted (Coulter counter; Beckman-Coulter, Hialeah, FL). Neither cytokeratin-3 nor cytokeratin-12 expression was detected, indicating that the cells thus obtained were all CECs without contamination by other corneal cell types.

Half of the cells were labeled with a fluorescent cell tracker (CM-DiI; C-7000; Molecular Probes, Eugene, OR), as described elsewhere (Mimura et al. 2004), to examine sphere formation by reaggregation. DiI-labeled cells and unlabeled cells were mixed and seeded at a density of 1 cell/μL (250 cells/cm^2), 10 cells/μL (2,500 cells/cm^2), 30 cells/μL (7,500 cells/cm^2), or 50 cells/μL (12,500 cells/cm^2) on 60-mm uncoated dishes containing 5 mL of medium for floating culture (Reynolds & Weiss, 1992, 1996) (Fig. 1B). No spheres were generated in the cultures with only 1 viable cell/μL, but numerous spheres were formed at 30 and 50 cells/μL, with some arising from reaggregation as indicated by DiI staining. Spheres were completely DiI-positive or DiI-negative when culture was performed at 10 cells/μL (Fig. 1C), indicating that these spheres were derived from proliferation and not from reaggregation of the dissociated cells.

Incubation was done in a humidified incubator under an atmosphere of 5% CO_2, with 40 ng/mL basic fibroblast growth factor (bFGF) and 20 ng/mL epidermal growth factor (EGF) being added to the medium every other day. To investigate whether the isolated cells were contaminated with corneal epithelial cells, expression of epithelial markers such as keratins K3 and K12 (Irvine et al., 1997; Moll et al., 1982) was assessed by the reverse transcription–polymerase chain reaction (RT-PCR) before the start of culture. Then primary culture was performed and the existence of fibroblast-like cells was investigated to assess contamination by stromal cells. CECs were isolated without contamination by corneal epithelial cells, as demonstrated by RT-PCR analysis of corneal epithelial markers (K3 and K12 genes), as well as the characteristic hexagonal shape of the cells in primary culture (data not shown). Almost complete disaggregation into single cells was achieved, since counting of single, double, and triple cells showed that 99% of all cells were single (Fig. 1B).

After incubation for 5 days, small floating spheres formed. These spheres grew larger after 10 days, while the nonproliferating cells died and were eliminated (Fig. 1D). After 10 days, we only counted cell clusters with a diameter of at least 50 μm, in order to distinguish growing spheres from dying ones. To verify that the increase of colony size was actually due to cell proliferation, we added the thymidine analogue BrdU to cultures at 24 hours before fixation. Then the spheres were stained with an FITC-conjugated anti-BrdU antibody (1:100; Roche Diagnostics, Basel, Switzerland) at room temperature (RT) for 60 minutes in the dark. We found that BrdU labeled most of the cells in each sphere on day 10 (Fig. 1E), indicating that the spheres contained proliferating cells. These results suggested that the sphere colonies arose from single

isolated HCECs and that the sphere-forming cells possess the capacity to proliferate. When the number of spheres obtained was counted after 10 days of culture, we found that 257 ± 83 spheres (mean ± SD, n=8) were generated per dish (50,000 cells). In a typical case, 2.5 ± 104 cells were isolated from a 10-mm piece of corneal tissue, generating approximately 130 spheres after 10 days. These spheres had a diameter of 88.3 ± 15.9 μm (mean ± SD, n=35). The replating efficiency showed a dramatic decline between primary and secondary sphere colonies. When the primary spheres were trypsinized and incubated in serum-free floating culture, secondary colonies were generated (Fig. 1F) at a level of approximately 15 ± 1 (n=3) per dish of 10,000 cells. This suggests that HCECs have the capacity for self-renewal and formation of sphere colonies, but this capacity is limited.

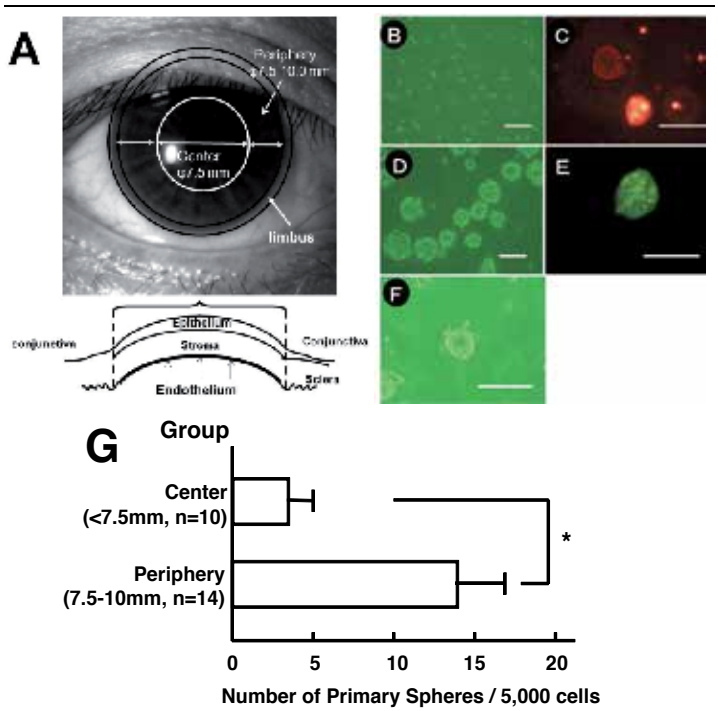

Figure 1. Sphere formation from donor human corneal endothelium. (A) Anterior view of a human cornea and a diagram of the corneal epithelium and stroma. Stromal keratocytes were isolated from specimens obtained from both the peripheral cornea (7.5-10.0 mm in diameter) and the central cornea (7.5 mm in diameter). (B-F) Sphere formation by human corneal endothelial cells (HCECs). After disaggregation into single cells, HCECs were plated at a density of 10 viable cells/μL in basal medium (B). More than 99% of the cells were single cells on day 0. (C) Spheres were completely DiI-positive or DiI-negative after culture at a density of 10 viable cells/μL. (D) The mean (±SD) sphere diameter was 88.3 ± 15.9 μm on day 10. (E) Each sphere colony was labeled with BrdU on day 10. (F) Secondary spheres generated after the dissociation of primary spheres. The replating efficiency was much lower than that of the primary spheres. Scale bar=100 μm. (G) The number of primary spheres obtained was compared between the peripheral and central regions of the cornea. The number of sphere colonies obtained from the peripheral cornea (n=14) after 10 days of culture was significantly higher than that obtained from the central cornea (n=10) (unpaired *t*-test). This experiment was repeated 3 times using different donor corneas, and representative data are shown as the mean ± SD. *P<0.0001. These figures were modified from Yokoo et al. (2005) and Yamagami (2007) with permission.

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3.2. Distribution of sphere colonies derived from human Corneal Endothelial cells

HCECs were obtained from the central cornea (up to 7.5 mm from the center) and the peripheral cornea (from 7.5 to 10 mm) (Fig. 1A). As a result, the number of primary sphere colonies per 5,000 cells (mean ± SD) was significantly higher when peripheral HCECs were used (13.6 ± 3.5 spheres/5,000 cells) than when central HCECs were used (3.3 ± 1.6 spheres/5,000 cells) (Fig. 1G). The rate of sphere formation by HCECs from the peripheral cornea was approximately 4 times that for HCECs from the central cornea in repeated experiments (data not shown).

It has generally been accepted that human CE does not proliferate after birth, but our findings and some previous reports suggest that the CE may undergo slow proliferation *in vivo*. In 2003, Amann et al. demonstrated that paracentral and peripheral HCECs exist at a higher density than central HCECs by specular microscopy and histological observation of donor corneas. The presence of slowly proliferating HCEC precursors in the peripheral cornea could explain this higher cell density at the periphery. Otherwise, the cell density should be uniform throughout the corneal endothelium, because it tends to equalize over time. Another suggestive point is the outcome of Sato's method of anterior–posterior refractive surgery that involves making multiple peripheral and midperipheral incisions in the endothelium and stromal layer from the anterior chamber to treat myopia (Kanai et al., 1982; Kawano et al., 2003). This type of radial keratotomy performed via the anterior chamber leads to a decrease of HCECs many years later, possibly as a result of the corneal incisions causing more rapid cell loss than would occur with normal aging (Kanai et al., 1982; Kawano et al., 2003). It is possible that direct damage to HCEC precursors slows their proliferation, so that replacement of CECs decreases. The third point to consider is the outcome of corneal transplantation for various conditions associated with damage to the cornea, such as bullous keratopathy, keratoconus, and corneal leukoma. In hosts who retain their peripheral CE, such as patients with keratoconus, the grafts survive for much longer than in hosts with loss of the peripheral endothelium, such as patients with bullous keratopathy (Boisjoly et al., 1993; Williams et al., 1992; Yamagami et al., 1996). Keratoconus patients are typically younger than those with bullous keratopathy, so it could be suggested that their peripheral endothelium has greater proliferative potential because of this age difference, but differentiation of CEC precursors from the host cornea augmenting viable cells from the graft may be another reason for the longer survival of grafts after transplantation for keratoconus compared with bullous keratopathy. Therefore, when full-thickness corneal transplantation is done, a larger graft may be preferred for eyes with bullous keratopathy because it can supply more HCEC precursors, whereas a smaller graft may allow the optimum use of host-derived HCEC precursors in patients with keratoconus.

3.3. Characterization of primary spheres derived from human Corneal Endothelium

Immunocytochemical analysis of 10-day spheres was performed as follows. The spheres were fixed with methanol (Wako Pure Chemical Industries) in phosphate-buffered saline (PBS) for 10 minutes, washed in PBS, and incubated for 30 minutes with 3% bovine serum albumin (BSA) in PBS containing 0.3% Triton X20 (BSA/PBST) to block nonspecific staining. Then, the spheres were incubated for 2 hours at RT with the following specific primary antibodies diluted in

BSA/PBST: mouse anti-vimentin monoclonal antibody (mAb) (1:300; Dako, Glostrup, Denmark), mouse anti-nestin mAb (1:200; BD PharMingen, San Diego, CA), rabbit anti-p75 neurotrophin receptor (p75 NTR) polyclonal antibody (pAb) (1:200; Promega Corp., Tokyo, Japan), mouse anti-neurofilament 145 mAb (NFM, 1:400; Chemicon, Temecula, CA), rabbit anti 3-tublin pAb (1:2000; Covance Research Products, Denver, PA), rabbit anti-glial fibrillary acidic protein (GFAP) pAb (1:400; Dako), mouse anti-O4 mAb (1:10; Chemicon), rabbit anti-peripherin pAb (1:100; Chemicon), and mouse anti-α-smooth muscle actin (α-SMA) mAb (1:200; Sigma-Aldrich). As a control, mouse IgG (1:1000; Sigma-Aldrich) or normal rabbit serum (1:1000; Dako) was used instead of the primary antibody. After the spheres were washed in PBS, incubation was done for 1 hour at RT with the appropriate secondary antibody diluted in BSA/PBST. The secondary antibodies were fluorescent-labeled goat anti-mouse IgG (Alexa Fluor 488, 1:200; Molecular Probes) and fluorescent-labeled goat anti-rabbit IgG (Alexa Fluor 594, 1:400; Molecular Probes). Nuclei were counterstained with Hoechst 33342 (1:2000; Molecular Probes). After another wash in PBS, the spheres were examined under a laser scanning confocal microscope (Fluoview; Olympus, Tokyo, Japan). When anti-O4 or anti-p75NTR mAb was used, the permeabilization step was omitted.

Figure 2A shows a bright-field image of a typical sphere colony. Spheres derived from HCECs were not stained by nonimmune mouse IgG (Fig. 2D) or normal rabbit serum (Fig. 2G). Nestin has been used as a marker for the detection of immature neural progenitor cells in multipotential sphere colonies derived from the brain (Gage, 2000), skin (Toma et al., 2001), inner ear (Li et al., 2003), retina (Tropepe et al., 2000), corneal epithelium (Mimura et al., 2010a; Yokoo et al., 2008), corneal stroma (Amano et al., 2006; Mimura 2008a, 2008b; Uchida et al., 2005; Yamagami et al., 2007), and CE (Amano et al., 2006; Mimura 2005a, 2005b, 2005c, 2007, 2010b; Yokoo et al., 2005, Yamagami, 2006, 2007). Expression of α-SMA (a marker of mesenchymal myofibroblasts) and expression of p75 NTR (a marker of neural crest stem cells) was also investigated by immunocytochemistry because HCECs are derived from the neural crest. Cells in the spheres showed immunoreactivity for nestin (Fig. 2B) and for α-SMA (Fig. 2C), but not for p75 NTR (data not shown). Next, the spheres were immunostained for various neural markers. As a result, spheres were found to be positive for an immature neuronal marker (β3-tubulin, Fig. 2E) and an astroglial marker (GFAP, Fig. 2F), but not a mature neuronal marker (NFM), an oligodendroglial marker (O4), or a peripheral nerve neuronal marker (peripherin; data not shown). These findings indicated that spheres isolated from human donor CE contain bipotential precursors that are capable of undergoing differentiation into mesenchymal cells and neuronal cells.

3.4. Secondary sphere formation

To further evaluate the proliferative capacity of HCECs, cells from the primary spheres were passaged under the same conditions as those used for the initial sphere culture. On day 10, primary spheres were treated with 0.05% trypsin/0.02% EDTA and dissociated into single cells, which were added to 24-well culture plates at a density of 10 cells/μL in medium containing primary culture supernatant. These cells were then incubated for a further 10 days in basal medium.

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Secondary spheres were generated from the dissociated primary spheres, but the yield of secondary sphere colonies was lower than after primary culture. Although self-renewal potential was indicated by the ability of cells from individual primary spheres to form secondary spheres, this potential was limited, as evidenced by the failure of sphere formation at the third passage. These results indicated that the precursor cells had a limited proliferative capacity. Photographs of representative secondary spheres are shown in Figure 1F.

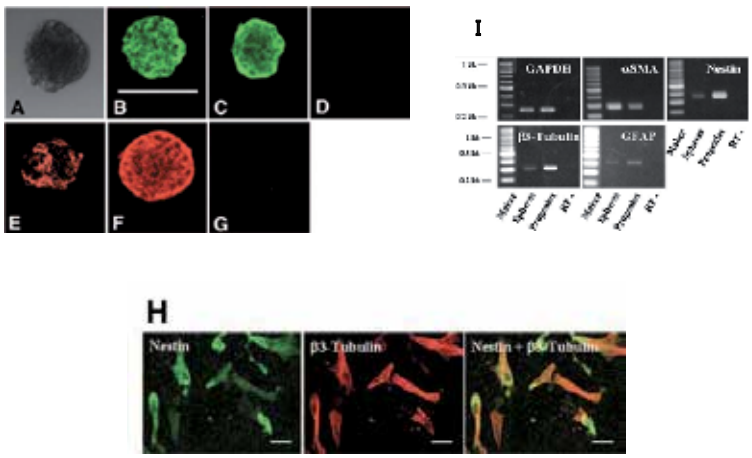

Figure 2. Immunocytochemistry (A-H) and RT-PCR analysis (I) of sphere colonies and their progeny. (A) Bright-field image of a typical sphere colony. (B) Immunostaining of the entire sphere on day 10 identifies cells expressing nestin, a marker of immature cells. (C-G) Spheres show immunostaining for a mesenchymal myofibroblast marker (α-SMA, C), an immature neuronal marker (β3-tubulin, E), and an astroglial cell marker (GFAP, F), indicating that both mesenchymal and neuronal differentiation have occurred. Sphere colonies derived from HCECs are not stained by nonimmunized mouse IgG (D) or normal rabbit serum (G). Differentiated cells derived from primary spheres are double immunostained by nestin and β3-tubulin, indicating that the colonies contain immature (undifferentiated) cells. (I) RT-PCR analysis of cells from spheres and their progeny. GAPDH gene expression is detected in the sphere colonies and their progeny (30 cycles), but not when reverse transcription is omitted. Nestin, α-SMA, β3-tubulin, and GFAP genes are detected in both spheres and their progeny, but not when total RNA is processed without reverse transcription (35 cycles). Scale bars=100μ m (A-G) or 200μ m (H). Figures are modified from Yokoo et al. (2005) with permission.

3.5. Differentiation of sphere colonies

Individual primary spheres (day 10) were transferred to 13 mm glass coverslips coated with 50 μg/ml poly-L-lysine (PLL) and 10 μg/ml fibronectin (BD Biosciences, Billerica, MA) in separate wells, as described previously (Reynolds & Weiss, 1992). To promote differentiation, 1% fatal bovine serum (FBS) was added to the basal medium, and culture was continued for another 7 days. Immunocytochemical examination of spheres and their progeny was performed after 7 days of adherent culture on glass coverslips.

To investigate whether sphere progeny possessed the characteristics of mesenchymal or neural cells, single spheres (day 10) were transferred onto PLL/laminin-coated glass coverslips in medium containing 1% or 15% FBS or onto bovine ECM-coated culture plates in medium containing 15% FBS. Spheres remained adherent to the PLL/laminin-coated glass coverslips,

but cells migrated out from the spheres grown on glass coverslips coated with bovine ECM alone. After 7 days, some of the cells that had migrated from the spheres showed double immunostaining for nestin and β3-tublin (Fig. 2H), as has been reported for human scalp tag–derived cells (Toma et al., 2001). However, there was no staining of cells migrating out of the spheres for α-SMA, p75NTR, NFM, peripherin, GFAP, or O4.

RT-PCR was performed to examine the expression of genes governing the proteins detected by immunocytochemistry in the spheres and their progeny (Fig. 2I). GAPDH mRNA was detected in both spheres and progeny, but not in the control assay without the RT reaction. Expression of nestin,β 3-tublin, GFAP, and α-SMA mRNA was detected in the spheres and adherent progeny after 35 PCR cycles. However, mRNAs for NFM, p75NTR, and peripherin were not found under any cycling conditions. Nestin andβ 3-tublin mRNAs were also detected in HCECs from primary culture.

These findings indicated that spheres isolated from human CE contain bipotential precursors, yielding progeny that display the morphologic characteristics of HCECs. Taken together, these results suggest that precursors from the CE remain close to the tissue of origin and undergo differentiation into CECs. Because precursors should ideally differentiate efficiently to produce their tissue of origin, precursors obtained from the CE may be more appropriate for tissue regeneration or cell transplantation than those derived from the multipotential stem cells.

4. Isolation of precursors from cultured human Corneal Endothelial cells

4.1. Culture of human Corneal Endothelial cells

As mentioned in sections 3.1-3.4, we have isolated precursor cells from human donor corneas (Yamagami et al. 2007; Yokoo et al., 2005). However, the number of precursors that can be isolated from a cornea is insufficient for corneal endothelial regeneration, so establishment of a mass production method for precursor cells is needed before clinical application can be attempted. Accordingly, we isolated spheres from cultured HCECs and investigated whether the cells of these spheres had CE-like functions. We also tested the effect of injecting these spheres into the anterior chamber (instead of full-thickness corneal transplantation) in a rabbit model of bullous keratopathy, representing a state in which corneal endothelial defects exist.

Several groups have established HCEC culture techniques (Chen et al., 2001; Engelmann & Friedl 1989; Miyata et al., 2001; Yue et al. 1989). Various growth factors have been reported to influence the proliferation of cells cultured from human CE, including fibroblast growth factor (Chen et al., 2001; Engelmann 1988, 1989, 1995; Yue et al. 1989; Samples et al., 1991), epidermal growth factor (Chen et al., 2001; Samples et al., 1991; Schultz et al., 1992; Yue et al. 1989), nerve growth factor (Chen et al., 2001), and endothelial cell growth supplement (Blake et al., 1997; Yue et al. 1989). In addition, cell attachment and growth can be supported by seeding cells onto an artificial matrix, such as chondroitin sulfate or laminin (Engelmann et al., 1988), laminin-5 (Yamaguchi et al., 2011), extracellular matrix

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secreted by bovine corneal endothelial cells (Blake et al., 1997; Miyata et al., 2001), or fibronectin/type I collagen coating mix (Joyce & Zhu, 2004).

In our studies, HCECs were isolated and cultured according to the published protocols of Joyce and our laboratory with some modifications (Chen et al., 2001; Joyce & Zhu, 2004; Miyata et al., 2001). Briefly, Descemet's membrane was carefully dissected with the intact CE. After centrifugation, membrane strips were incubated in 0.02% EDTA solution at 37ºC for 1 hour to loosen intercellular junctions. Then isolated cells were plated in 6-well tissue culture plates that had been precoated with undiluted fibronectin/type I collagen coating mix, and incubation was done at 37ºC under a humidified atmosphere with 5% CO_2. After primary cultures reached confluence, cells were subcultured at a 1:4 ratio, and cells from the 4th to 6th passages were used.

4.2. Isolation and characterization of sphere colonies

Cells from the 4th or 5th passages were used in this study. HCECs were incubated in 0.2% EDTA at 37ºC for 5 minutes and then were dissociated into single cells by pipetting with a flame-polished Pasteur pipette. The viability of the isolated HCECs was >90% as shown by trypan blue staining. The sphere-forming assay was used for primary culture (Reynolds & Weiss, 1992). Cells were plated at a density of 10 viable cells/μL (40,000 cells per well or 1,420 cells/cm^2) in the uncoated wells of 60-mm culture dishes. The basal medium was Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with B27, epidermal growth factor (EGF, 20 ng/mL), and basic fibroblast growth factor (bFGF, 20 ng/mL). A methylcellulose gel matrix (1.5%; Wako) was added to the medium to prevent reaggregation of the cells (Gritti et al., 1999; Kawase et al., 2004). To distinguish growing spheres from dying cell clusters, only spheres with a diameter of more than 50 μm were counted. For passaging, primary spheres were harvested on day 7 and treated with 0.5% EDTA for dissociation into single cells, which were plated in 24-well culture plates at a density of 10 cells/μL. Then culture was continued for another 7 days in basal medium containing the methylcellulose gel matrix.

Spheres formed after 7 days of culture (Fig. 3A), while nonproliferating cells were eliminated. Many of the cells in each sphere were BrdU-positive (Fig. 3B), indicating that such cells were proliferating. These findings suggested that the spheres had developed from single HCECs and that the sphere-forming cells displayed proliferative activity. The number of sphere colonies obtained after 7 days of culture was 44 ± 10 per 10,000 cells (mean ± SD). Replating of primary spheres to generate secondary sphere colonies was less efficient, indicating that the cells only had limited self-renewal capacity.

On immunostaining, the spheres were positive for nestin (Fig. 3C), which is a marker of immature cells (Lendahl et al., 1990), and for α-SMA (Fig. 3D), a mesenchymal myofibroblast marker. We previously demonstrated that primary spheres derived from human donor CE express β-III tubulin and GFAP, a mature glial cell marker, as well as nestin and α-SMA (Fig. 2), but β-III tubulin and GFAP were negative in the spheres derived from cultured HCECs.

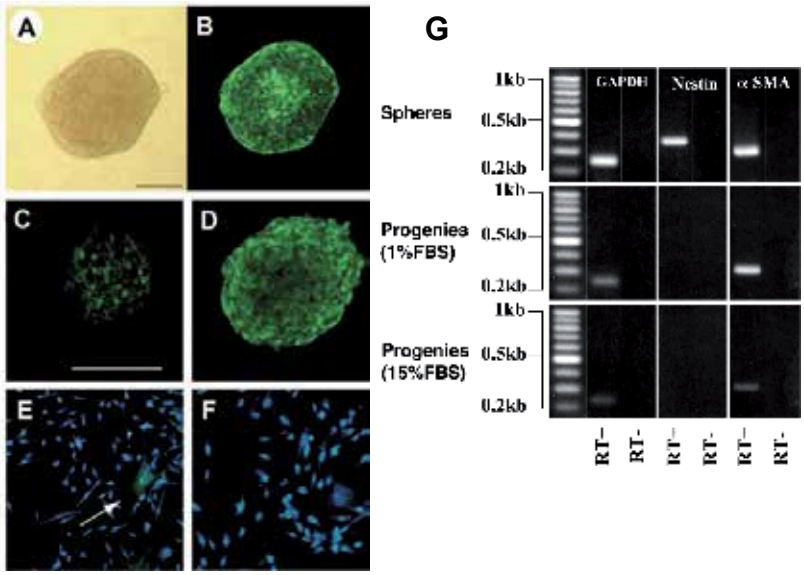

Figure 3. Immunocytochemistry (A-F) and RT-PCR analysis (G) of sphere colonies derived from cultured HCECs were their progeny. Cultured HCECs were disaggregated into single cells and plated at a density of 10 viable cells/μL in basal medium containing a methylcellulose gel matrix to prevent reaggregation. (A) A representative day 7 sphere. (B) Cells in a sphere colony labeled by BrdU on day 7. A total of 44± 10 primary spheres were generated per 10,000 cells (mean ± SD). Scale bar=50 μm. (C-F) A day 7 sphere shows staining for nestin (C) and α-SMA (D). Less than 5% of the sphere progeny cells were stained by the mesenchymal cell marker α-SMA (E, arrow). There is no staining by control IgG (F). Scale bar=100 μm. (G) RT-PCR of spheres and progeny. cDNA was obtained from spheres and from their progeny cultured in 1% FBS or 15% FBS. GAPDH was detected in all samples, except those reacted without reverse transcriptase. Nestin mRNA expression was detected in cultured spheres, but not in their progeny cultured in either 1% or 15% FBS. Both the spheres and progeny were positive forα -SMA mRNA. Figures are modified from Mimura et al. (2005b) with permission.

4.3. Differentiation of sphere colonies

Individual primary spheres (day 7) were transferred to 13-mm glass coverslips coated with 50 μg/mL PLL and 10 μg/mL fibronectin in separate wells (Mimura et al., 2005a). To promote differentiation, 1% or 15% FBS was added to the basal medium, after which culture was continued for another 7 days.

Then the spheres were transferred to PLL/fibronectin-coated glass coverslips in 24-well plates and were cultured in a differentiation medium containing 1% or 15% fetal bovine serum (FBS). After 7 days, many cells were found to have migrated out of the spheres. Fewer than 5% of these cells were α-SMA-positive (Fig. 3E), whether cultured with 1% or 15% FBS. All of these cells were negative for control IgG (Fig. 3F) and for the differentiated epithelial cell marker cytokeratin 3, as well as for nestin, β-III tubulin, and GFAP (not shown). These findings indicated that a single sphere colony could give rise to a small population of mesenchymal cells under clonogenic conditions. Expression of nestin and α-SMA by the spheres, as well as α-SMA expression by their progeny, was confirmed using RT-PCR (Fig. 3G). Positivity for β-III tubulin mRNA was only detected in cultures with 1% FBS.

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Spheres derived from donor CE expressed an immature cell marker (nestin), an immature neuronal marker (β-III tubulin), and a mature glial cell marker (GFAP), while their progeny expressedβ -III tubulin and nestin, but not GFAP. In contrast, the spheres and progeny obtained from cultured HCECs did not express neuronal markers and showed decreased expression of immature cell markers. These findings suggested that the precursors were close in nature to the original tissue and underwent differentiation during culture. Thus, precursors obtained from cultured HCECs may be a more appropriate cell source than cells from donor CE, because precursors that efficiently differentiate into the tissue of origin are ideal for tissue regeneration or cell transplantation.

4.4. Assessing the pump function of cells derived from spheres

The pump function of four collagen sheets seeded with cells derived from HCEC spheres was measured in an Ussing chamber, as reported previously with some modifications (Wigham, 1981, 2000; Hodson & Wigham 1983). The collagen sheets were obtained from the Nippi Biomatrix Research Institute (Tokyo, Japan). Cells from HCEC spheres were suspended at 5.0×10^6 cells in 1.5 mL of culture medium and transferred to circular collagen sheets (10 mm in diameter). Each sheet was placed in one well of a 24-well plate, and the plate was centrifuged at 1,000 rpm (176 g) for 10 minutes to enhance cell attachment. Then the sheets were incubated in culture medium for 2 days, after which nonadherent cells and debris were removed (Fig. 4A). Human donor corneas with the epithelium removed mechanically (n=4), plain collagen sheets (n=4), or HCEC-coated collagen sheets (n=4) were mounted in the Ussing chamber.

Changes of the potential difference (Fig. 4B) and short circuit current (Fig. 4C) were compared between human donor corneas without epithelium and HCEC-coated collagen sheets constructed with cells from spheres. The average potential difference and short circuit current of the HCEC-coated sheets ranged from 81% to 100% at 1, 5, and 10 minutes, corresponding to the results for normal human donor corneas denuded of epithelium. These findings suggested that the cultured HCEC spheres could generate CE-like cells with adequate transport activity.

4.5. Migration and proliferation of spheres on rabbit descemet's membrane

Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Primary HCEC spheres (cultured for 7 days) were labeled with a fluorescent cell tracker (CM-DiI). After the endothelium was gently scraped off four freshly excised rabbit corneas with a sterile cotton swab, HCEC spheres were applied to the posterior surface of each cornea. Then the corneas were placed in 24-well plates and maintained in culture medium for 7 days. HCECs that migrated onto the corneas were detected under a fluorescence microscope, and the area occupied by fluorescent cells migrating from the spheres was measured with the NIH image program (n=10).

Figure 5A shows cells migrating from DiI-labeled spheres on days 1-7. The mean area covered by migrating cells per sphere reached 1.2 ± 0.2 mm^2 on day 7 (Fig. 5B).

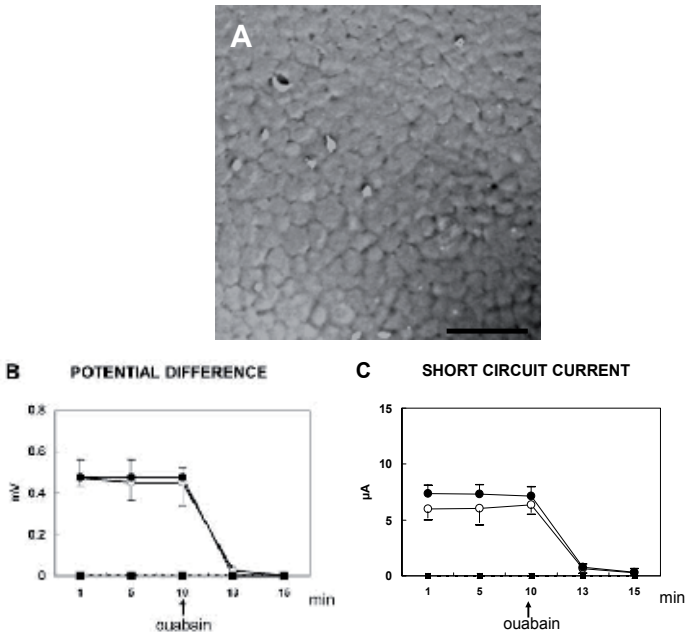

Figure 4. Morphology (A) and transport activity (B, C) of cells from cultured HCEC spheres, modified from Mimura et al. (2005b) with permission. (A) Confluent cells cultured in DMEM containing 10% FBS show the characteristic hexagonal shape of corneal endothelial cells. Changes of the potential difference (B) and short circuit current (C) for human donor corneas without epithelium and HCEC-coated collagen sheets (mean ± SD). The mean potential difference and short circuit current after 1, 5, and 10 minutes ranged from 81% to 100% for the HCEC-coated sheets, similar to the results for human corneas denuded of epithelium, indicating that the HCEC-like cells generated in culture had adequate transport activity. When Na^+ - K^+ ATPase inhibitor ouabain was added to the chamber, the potential difference decreased to 0 mV and the short circuit current declined to 0 μA in all cases.

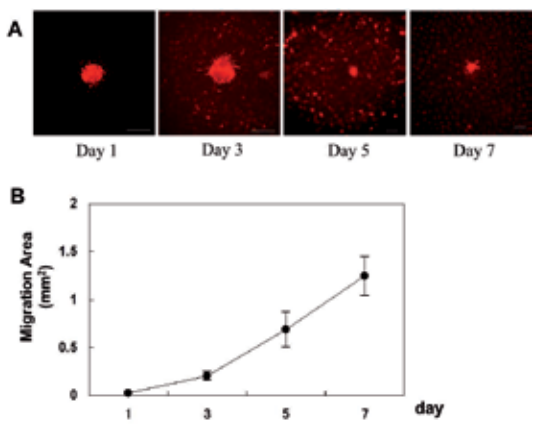

Figure 5. Migration of sphere-derived cells during culture for 7 days, modified from Mimura et al. (2005b) with permission. DiI-labeled spheres were seeded onto the denuded Descemet's membranes of rabbit corneas and cultured for 1 week in a humidified incubator. (A) Representative photographs of cell migration around an adherent DiI-labeled sphere. Scale bar=100 μm. (B) Mean area occupied by cells migrating from the spheres on each day (n=10).

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5. Treatment of bullous keratopathy with precursors derived from cultured spheres

5.1. Cryoinjury and injection of spheres into the anterior chamber

Animals were handled in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. New Zealand White rabbits (weighing 2.0–2.4 kg, n=24) were anesthetized with an intramuscular injection of ketamine hydrochloride (60 mg/kg; Sankyo, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Leverkusen, Germany). To detach the CE from Descemet's membrane, a brass dowel cooled in liquid nitrogen was touched onto the cornea nine times (at the center and at eight peripheral sites). This procedure was repeated twice. Then the anterior chamber was washed three times with PBS through a 1.5-mm paracentesis.

To estimate the number of spheres needed to cover the inner surface of the cornea (Descemet's membrane), DiI-labeled spheres were seeded onto the denuded Descemet's membrane of freshly excised rabbit corneas and the mean area covered per sphere was found to be 1.2 ± 0.2 mm^2 on day 7 (Fig. 5B). Therefore, it was calculated that 75 spheres were needed to cover a cornea. To allow for loss of spheres that failed to adhere, 150 DiI-labeled HCEC spheres or 1.0 × 10^7 HCECs were injected into the anterior chamber of the right eye after cryoinjury. Then the rabbits were maintained in the eyes-down position (Descemet's membrane down) for 24 hours to allow attachment (sphere eyes-down group, n=6). Cryoinjury alone (cryo group, n=6), injection of cultured HCECs with the eyes-down position being maintained for 24 hours (HCEC group, n=6), and injection of spheres in the eyes-up position (sphere eyes-up group, n=6) were also tested as controls. However, injection of cultured HCECs or injecting spheres in the eyes-up position did not reduce corneal edema in our preliminary study (Mimura T, unpublished observation, 2003), so these controls were not used in the present study. Each eye was inspected 2 or 3 times a week and was photographed on postoperative days 7, 14, 21, and 28. Central corneal thickness was measured with an ultrasonic pachymeter having a range of 0 to 1,200 μm (Tomey, Nagoya, Japan) and intraocular pressure was determined with a pneumatic tonometer (model 30 Classic; Mentor O & O, Norwell, MA) at 1, 3, 7, 14, 21, and 28 days after surgery. The average of three readings was obtained each time. One-way analysis of variance and Scheffe's multiple comparison test were used to compare mean values.

5.2. Findings after surgery

Our previous studies had suggested that cultured HCEC precursors have a limited self-renewal capacity and mainly differentiate into HCEC-like cells. Then we investigated the use of precursors derived from cultured HCECs in a rabbit model of corneal endothelial damage. In the cryo and HCEC groups, the mean corneal thickness ranged from 953 ± 182 to 1,200 ± 0 μm (mean ± SD), as shown in Figure 6A. The mean (±SD) corneal thickness of the sphere eyes-up group (704 ± 174 μm) was significantly less than that of the cryo group (1,011 ± 190 μm; P=0.006) and the HCEC group (953 ± 182 μm; P=0.022) after 28 days of observation, but the corneas were still edematous in the eyes-up group (Fig. 6A). In contrast, the corneal thickness decreased rapidly in the sphere eyes-down group, and the corneas were significantly thinner

than in the other three groups after 14 (672 ± 90 μm), 21 (483 ± 84 μm), and 28 (394 ± 26 μm) days (*P*=0.006; Fig. 6A). Representative anterior segment photographs from the cryo group (Fig. 6B), HCEC group (Fig. 6C), and sphere eyes-up group (Fig. 6D) show that the corneas of rabbits from these groups were edematous and displayed stromal opacity. In contrast, corneas from the sphere eyes-down group corneas became clear and the anterior chamber was easily visualized (Fig. 6E). No apparent inflammatory reactions suggesting rejection were observed by slit lamp microscopy throughout the postoperative period. On day 14, the intraocular pressure of the sphere eyes-up group was significantly higher than that of the cryo group (P=0.013). However, there was no increase of intraocular pressure (a possible side effect) on any other day in any group (Table 1).

Injection of spheres in the eyes-down position, but not injection of differentiated cultured HCECs or injection of spheres in the eyes-up position, restored endothelial function and decreased corneal edema in this rabbit model of bullous keratopathy model. These findings suggest that injection of spheres derived from cultured HCECs and maintenance of an eyes-down position for 24 hours may be a potential treatment strategy for corneal endothelial defects that is less invasive compared with conventional full-thickness corneal transplantation.

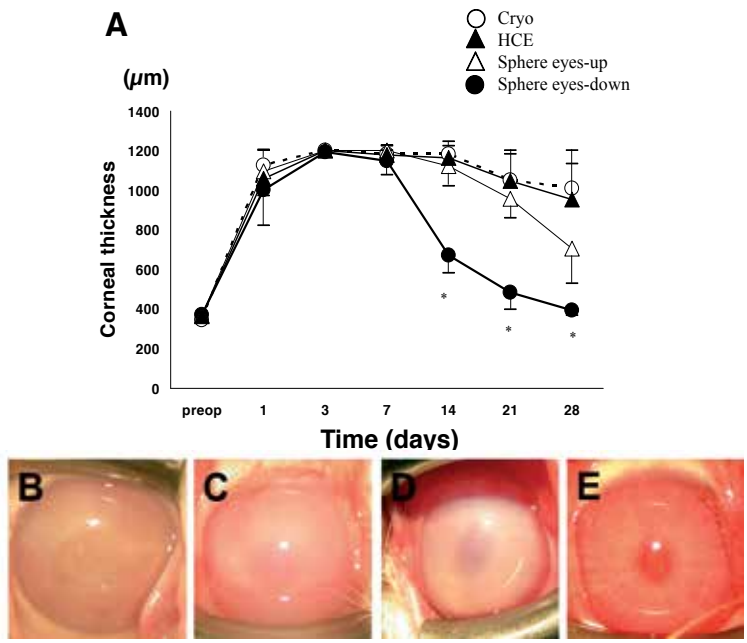

Figure 6. Changes of corneal thickness and other findings in a rabbit model of bullous keratopathy, modified from Mimura et al. (2005b) with permission. (A) Mean corneal thickness decreases gradually in the sphere eyes-down group (closed circles, n=6). It is significantly less than in the cryo group (open circles, n=6), HCEC group (closed triangles, n=6), and sphere eyes-up group (open triangles, n=6) on days 14, 21, and 28 (*P<0.001 by one-way analysis of variance and Scheffe's multiple comparison test). (B–E) Representative photographs of corneas from each group. The cornea is opaque in the cryo group (B), HCEC group (C), and sphere eyes-up group (D), and the anterior chamber is not well visualized. In contrast, there is no corneal opacity in the sphere eyes-down group (E).

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	Days after Surgery				
Group	**0**	**7**	**14**	**21**	**28**
Cryo	12.7 ± 1.9	10.8 ± 1.8	10.5 ± 4.3*	11.0 ± 1.6	10.2 ± 1.5
HCEC	11.8 ± 1.5	11.6 ± 2.8	12.3 ± 4.5	11.8 ± 2.2	11.5 ± 2.8
Sphere (eyes-up)	13.2 ± 2.0	9.9 ± 1.9	17.2 ± 4.0*	13.3 ± 3.4	10.5 ± 3.1
Sphere (eyes-down)	13.5 ± 3.2	8.7 ± 2.1	14.8 ± 2.2	13.7 ± 1.7	11.7 ± 1.9

Table 1. Intraocular pressure in each group after surgery (mm Hg). Data represent the mean ± SD for six rabbits.
*P=0.013 by one-way analysis of variance and Scheffe's multiple comparison test.

5.3. Histologic findings

Examination of hematoxylin & eosin-stained sections revealed that corneas from the cryo group (Fig. 7A), HCEC group (Fig. 7B), and sphere eyes-up group (Fig. 7C) were thickened and no cells could be detected on Descemet's membrane. In contrast, a monolayer of cells had formed on Descemet's membrane in the sphere eyes-down group, and there was no edema and no mononuclear cell infiltration of the posterior stroma (Fig. 7D). In the cryo group (Figs. 8A, 8E), HCEC group (Figs. 8B, 8F), and sphere eyes-up group (Figs. 8C, 8G), no HCECs (Figs. 8A–C) with positive staining for DiI (Figs. 8E–G) were found on Descemet's membrane at the central cornea in flat mount preparations. In contrast, HCEC-like hexagonal cells were detected at this site in the sphere eyes-down group (Fig. 8D). These cells were DiI-positive (Fig. 8H), indicating that they had originated from the injected spheres and not from the host. In the sphere eyes-down group, DiI-negative cells were present in the peripheral cornea, but all cells in the central and paracentral (8 mm in diameter) cornea were DiI-positive. The density of HCECs in the six grafts of the sphere eyes-down group at 28 days after surgery ranged from 2,625 to 2,875 cells/mm^2, with a mean (±SD) value of 2,781 ± 92 cells/mm^2. Before surgery, the density of endothelial cells in the rabbit cornea was from 3,300 to 3,500 cells/mm^2. In the sphere eyes-down group, very few DiI-positive cells were detected in the inferior trabecular meshwork or on the iris, whereas a number of DiI-positive cells were attached at these sites in the HCEC group and the sphere eyes-up groups (data not shown).

Cells adherent to the inner surface of the cornea (Descemet's membrane) were DiI-positive in the sphere eyes-down group, indicating that these were HCECs derived from the injected spheres and not residual host cells. In addition, DiI-positive cells were rarely detected in the trabecular meshwork or on the surface of the iris, so the spheres mainly attached to and spread over the cornea in the eyes-down group. These results suggested that sphere-derived HCECs could restore corneal hydration after sphere transplantation.

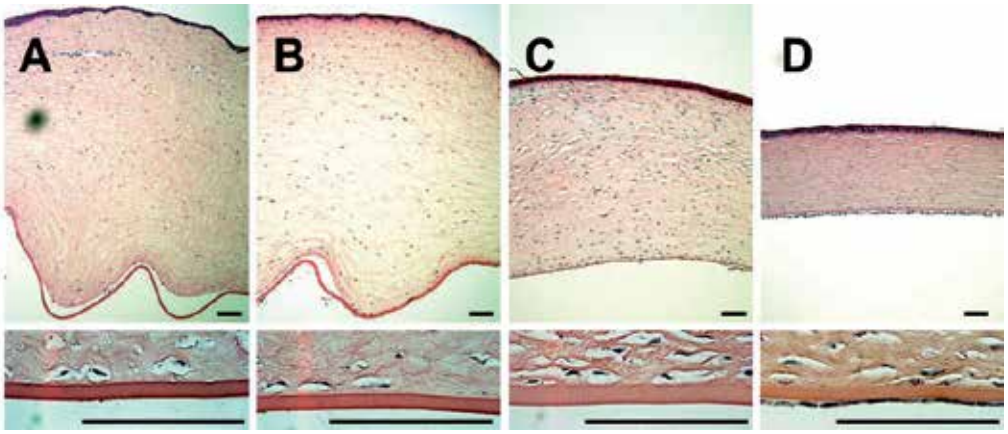

Figure 7. Histologic findings, modified from Mimura et al. (2005b) with permission. In the cryo group (A), HCEC group (B), and sphere eyes-up group (C), stromal edema is prominent and no cells are present on Descemet's membrane at the central cornea. In contrast, a monolayer of cells can be detected on Descemet's membrane in the sphere eyes-down group (D). There is no mononuclear cell infiltration near Descemet's membrane, suggesting no rejection of the xenogeneic cells (D). Scale bar=100 μm, hematoxylin & eosin stain.

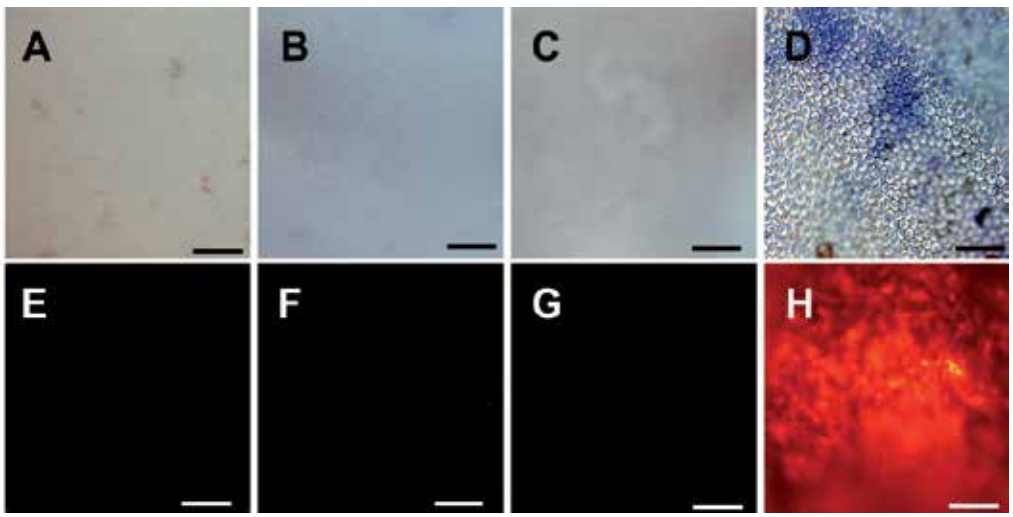

Figure 8. Flat mount preparations with phase-contrast (A–D) and fluorescence (E–H) microscopy, modified from Mimura et al. (2005b) with permission. At the central cornea, there are no cells on Descemet's membrane in the cryo group (A, E), HCEC group (B, F), and sphere eyes-up group (C, G). In contrast, HCEC-like hexagonal cells are present in the sphere eyes-down group (D). These cells are also DiI-positive (H). Scale bar=100 μm.

5.4. Advantages of transplanting CE precursors

For regenerative medicine, amplification of stem cells is required to treat each tissue or organ. Although much attention has been paid to maintaining the undifferentiated nature

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("stemness") of stem cells and promoting their amplification, the molecular mechanisms of stem cell replication and differentiation are still not fully understood. In comparison with amplification of adult stem cells, cultured cells can be used more easily to produce tissue-committed precursors by the sphere-forming assay, as demonstrated in our studies. Similar techniques to produce abundant precursors should be tested for various tissues as a method of obtaining cells for regenerative medicine.

Transplantation of HCEC precursors into the anterior chamber has several advantages over penetrating keratoplasty with a full-thickness donor cornea. For example, complications associated with open-sky surgery (expulsive hemorrhage and the risk of wound dehiscence) are essentially eliminated. In addition, several postoperative complications, such as irregular astigmatism, wound leakage, corneal infection, neovascularization, and persistent epithelial defects, can be avoided when using the combined approach. After conventional full-thickness human corneal allografting with local and/or systemic immunosuppressants, the leading cause of failure is graft rejection (Price et al., 1991; Wilson & Kaufman, 1990). Although there was no apparent inflammatory reaction histologically, we cannot deny the possibility of allograft rejection over the long term because nonadherent cells should migrate out of the anterior chamber. It is noteworthy that injection of HCEC precursors did not improve bullous keratopathy created by scraping off endothelial cells in rabbits (data not shown). This may be because cryoinjury to the cornea, but not endothelial cell scraping, promoted the proliferation and migration of HCEC s that led to recovery of corneal clarity.

6. Conclusion

We demonstrated that the endothelium from the peripheral region of the human cornea contains a higher density of precursors with strong proliferative capacity compared to the central endothelium. These HCEC precursors are able to differentiate into both mesenchymal and neural cells. We have also established a method for mass production by isolation of precursors from cultured HCECs using the sphere-forming assay. Transplantation of spheres into the anterior chamber and short-term maintenance of the eyes-down position was shown to be a simple and effective treatment strategy in our rabbit model of bullous keratopathy. This method of managing corneal endothelial defects may have the potential to replace conventional full-thickness corneal grafting and compensate for the worldwide shortage of donor corneas.

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Chapter 19

Angiogenesis — The Key to Regeneration

Susanne Jung and Johannes Kleinheinz

Additional information is available at the end of the chapter

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1. Introduction

Regenerative concepts are one of the basic ideas of modern biomedical research. Regeneration means origination in stark contrast to substitution. This process aims not to replace or to reconstruct but to restore the physical integrity of cells, tissues and organs by means of the organisms' own repair mechanisms.

Especially the regeneration of neuronal tissues has been in the focus of interest, but these restorative concepts will also become applicable in the treatment of metabolic or degenerative diseases. Gentherapy and tissue engineering procedures are two established areas of research in which regeneration plays a major role and has already proven its significance.

The fundament of regeneration is the tissue's potential to grow, to differentiate and therefore to continually bridge permanently emerging damages. It is a stepless coexistence of build-up and degradation processes in which a plethora of enzymes, signal proteins, ligands and their corresponding receptors on different regulatory levels are involved. These processes concern every part of the body; the least common denominator of all these physiological events that include a transition from single cells to a complex tissue structure is their demand for energy and substrates. It becomes obvious that there can never arise a regenerative course without a functioning vasculature to provide the essential cells and proteins, to ensure the oxygen and nutrient supply and to evacuate accumulating metabolic products. Any regeneration is only able to develop with a simultaneously developing vessel system. The realisation seems to be trivial, but the vital importance of a functional vasculature is not generally considered in regenerative concepts.

On the following pages the role of vasculogenesis and angiogenesis in regeneration is to be described; after a depiction of the angiogenic cascade, the regulation by hypoxia is tracked. From the description of the physiological molecular course of the angiogenic cascade and the interrelation of its key protagonists we trace the impact of macro- and microscopic vascularisation in various regenerative processes and clarify its central position in tissue engineering models.

Finally the role of pluripotent cells in modern tissue engineering concepts is summarized.

Fundamental research with special respect to cell culture, immunohistochemistry, in vitro and in vivo trials, circulation modelling and gene expression profiling provides the scientific basis for this survey.

2. The angiogenic cascade

Angiogenesis is the complex physiological sequence of vasodilatation, degradation of basement membrane, endothelial cell migration, chemotaxis, increasing vascular permeability and eventually endothelial cell proliferation and vessel formation. The fine-tuned balance of vasculo- and angiogenesis is controlled by many growth and transcription factors (Pandya, Dhalla et al., 2006).

2.1. Angiogenesis versus vasculogenesis

Lack of oxygen and nutrients threatens the tissue integrity and viability fundamentally. In these situations of undersupply, the organisms´ reaction is to improve the local perfusion by inducing the growth of the vasculature.

Angiogenesis is one crucial mechanism; it describes the sprouting of a vascular system on the base of pre-existing capillaries via endothelial migration and proliferation. This kind of vascular regeneration is the more common one.

When endothelial progenitor cells are mobilized from the bone marrow to differentiate and proliferate to form new vessel architecture, vasculogenesis takes place. An alternative way to vasculogenesis bears on the potential of local endothelial to differentiate and proliferate without a pre-existing vessel structure (Tepper, Cappla et al., 2005, Hankenson et al., 2005).

Both processes have in common that the smooth frictionless procedure depends on a continuing dynamic crosstalk between endothelial cells and surrounding connective tissue. Other important cell types are monocytes, macrophages, fibroblasts, pericytes or smooth muscle cells (Nyberg, Salo et al., 2008).

The biochemical and morphological roll out of vessel formation is standardized: angiogenesis starts with the vasodilatation of the original vessels followed by the degradation of the basement membrane, migration and proliferation of endothelial cells, their arrangement in luminal structures, loop formation and establishment of new basement membranes (Moulton, Folkman et al., 1998).

2.2. VEGF pathway

The healthy vasculature is one prerequisite of every regenerative process. It its governed by many interacting signalling pathways, the VEGF pathway is considered as one of the most crucial ones; it is certainly the most investigated and understood one (Dyer, Portbury et al., 2010).

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The first observed function of VEGF was its ability to enhance the permeability of tumour vasculature. Later its power as endothelial mitogen was described: VEGF attracts endothelial cells and promotes their differentiation, proliferation and survival. Today the role of VEGF as one of the angiogenic factors to keep up and promote vascular homoeostasis in the organism has become clear.

The striking significance of VEGF becomes obvious considering the fact that the first definitive marker protein on ripening endothelial cells in the yolk sac is the VEGF receptor 2 (VEGFR2 or Flk 1). Under the influence of VEGF A these endothelial progenitors marked by VEGFR 2 form areas of blood islands; these formations are characterized by clusters of initial erythroblasts lined by the endothelial precursors (Park, Afrikanova et al., 2004, Pearson, Sroczynska et al., 2008).

VEGF-A exists in several splice variants, with different characteristics; VEGF120 for example is thought to be an especially diffusible isoform due to the lack of a heparin-binding domain.

Three relevant receptors transmit the signal of specific VEGF binding: VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1) and VEGFR-3 (flt-4). The ligand-receptor interaction leads to cellular response on the base of receptor phosphorylation (Autiero, Waltenberger et al., 2003).

One regulative factor is the appearance of a soluble VEGF receptor, VEGFR-1 (sFlt-1) that acts as a so-called VEGF trap, catching VEGF-A and so inhibiting the initiation of angiogenesis (Maynard, Min et al., 2003).

The VEGF pathway is summarized in the KEGG signalling pathway: in this survey it becomes obvious that the most important receptor on endothelial cells is VEGFR-2 to transmit the angiogenic information. Starting from there several cascades are initiated. Their common outcome is the up regulation of genes that accomplishes endothelial cell proliferation and migration, focal adhesion and cell survival.

Relevant pathways are the calcium-signalling pathway, the MAPK signalling pathway and the arachidonic acid metabolism.

2.3. Role of endothelial cells

Endothelial cells are the cellular key element of angiogenesis and play a significant role in all crucial steps:

Endothelial cells are able to produce and release growth factors.

Endothelial cells express different growth factor receptors on their surface and are regulated by their impact.

Endothelial cells are actively involved in the dissolution of the surrounding matrix.

Simultaneously the migration adhesion and proliferation of endothelial cells continues.

Endothelial cells start to express characteristic integrins to anchor and pull forward the sprouting vessels.

Special endothelial cells, the tip cells secrete matrix metalloproteinases to pave the way and loosen the connective tissue in front of the sprouting vessels´ tip to facilitate the further out growth.

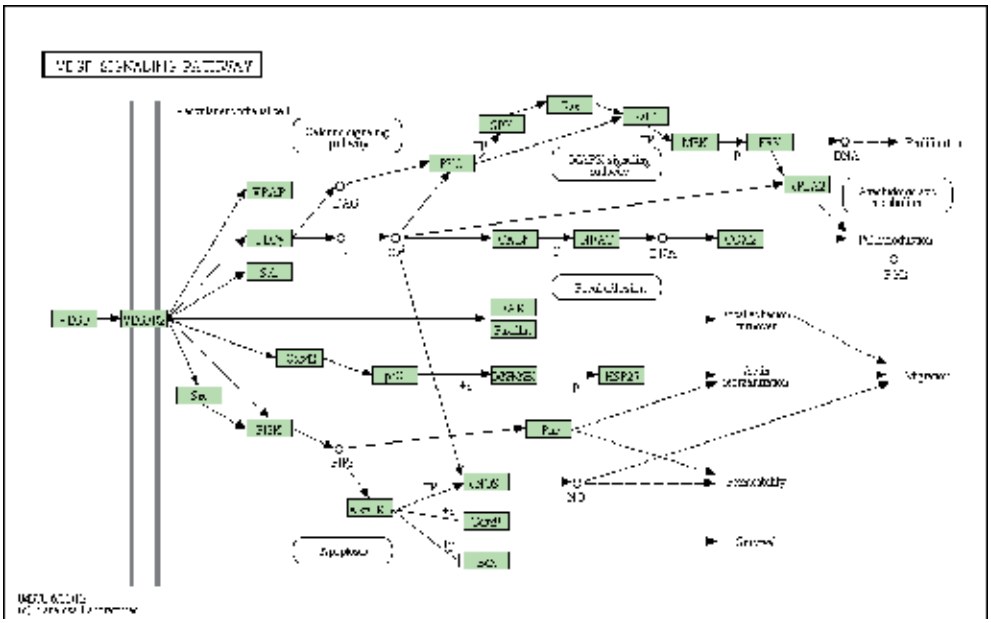

Figure 1. KEGG VEGF signalling pathway (Homo sapiens)

Proliferating endothelial cells are capable of forming three-dimensional structures as tubes and loops, the structural fundament of a functioning circulation.

Considering all these key functions it becomes clearly obvious that only an intact endothelial property can effectively lead to angiogenesis and provides the prerequisite for any regenerative process (Pandya, Dhalla et al., 2006).

These special demands during vascular regeneration are reflected in a significantly increased turn over time. Normally the endothelial turnover is up to hundreds of days. Under angiogenic conditions the turnover time speeds up rapidly to a turnover of under five days, which corresponds with the proliferation of bone marrow cells. This adaptation is of vital importance for the cells to live up to the regenerative demands (Kalluri, 2003).

Other, non-endothelial cells are regulated by VEGF via autocrine control and contribute directly or indirectly to the stimulated processes: monocytes, macrophages, mast cells, dendritic cells, lymphocytes, hematopoietic cells, epithelia, hepatocytes and many others (Breen, 2007).

3. Hypoxia: Master and commander of vascular regeneration

The lack of oxygen immediately threatens the organism's integrity. Few minutes without oxygen supply lead to irreparable damages in the affected organs. The oxygen sensing and the

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quick and efficient induction of regulative measures are among the most sensitive and fine-tuned processes in physiology.

3.1. Physiology

Hypoxia is one of the most potent inductors of angiogenesis. Hypoxia is defined by a deficiency of oxygen that can concern the whole organism or parts of it.

The standard is age dependent and varies from 80-100 mmHg, the formula is paO2=102-(age in years x 0.33).

A disturbance of the oxygen haemostasis can be caused on different levels: the partial pressure of the tidal air, the gaseous exchange in the lung or the peripheral tissues or the binding capacity of the erythrocytes.

One has to differentiate different forms of hypoxia: the hypoxic hypoxia refers to a lack of oxygen caused among others reasons by a low partial pressure in heights or by the inability of the lung tissue to perform the necessary gaseous exchange.

The anaemic hypoxia reposes on a reduced capacity of oxygen transport in the blood, e.g. caused by a reduced content of haemoglobin or a carbon monoxide poisoning.

The ischemic hypoxia is caused by a disturbed perfusion of single organs, e.g. due to an embolic insult.

In histotoxic hypoxia the concerned cells are not able to exploit the present oxygen. It is observed in cyanide or alcohol intoxication.

The pathologies resulting from hypoxia are various: on cellular level an alteration of oxygen tension can lead to endothelial changes, among others.

Systemically persisting hypoxia results in pulmonary hypertension. The aim of the increased perfusion of the pulmonary vessels is an optimal oxygen profit during the gaseous exchange.

On bio molecular level hypoxia interferes with gene expression; via oxygen sensing molecules and their downstream signalling cascade the transcription of genes is promoted that induce an enhanced haematopoiesis and angio- and vasculogenesis.

3.2. Oxygen sensing

Cellular mechanisms of oxygen regulation concern the aerobe glycolysis, the arrest of the cell cycle end the initiation of apoptosis.

Systemic regulation includes the release of erythropoietin from the kidneys, hyperventilation and finally angiogenesis.

The interesting question is: which molecule represents the sensor of a low intracellular oxygen tension?

3.3. HIF-1 alpha

Hypoxia inducible factor 1 alpha (HIF-1 alpha) is the key regulator of cellular and systemic oxygen haemostasis. It has been described in 1995 for the first time by Wang et al. (Wang, Jiang, et al., 1995)

HIF-1 alpha consists of two subunits: the alpha subunit is the virtual oxygen sensor. It is O2-sensitive and very unstable. In presence of oxygen, the alpha subunit is not detectable. Under normoxic conditions a quick ubiquination and immediate proteosomic degradation is observed. Oxygen-dependent enzymes, the prolyl-hydroxylases (PHDs) bind oxygen and couple it to HIF1-alpha. Von Hippel Lindau (VHL) protein attacks this complex and initiates its degradation.

The necessary co-factors for the degradation are oxygen and iron. Under hypoxic conditions HIF-1 alpha cannot be degraded for the lack of the co-factor oxygen and accumulates. The molecules reach the nucleus where the come in contact with the HIF-1 beta subunit.

The beta subunit, the so-called aryl hydrocarbon receptor nuclear translocator, ARNT, is expressed in the nucleus constantly.

Triggered by hypoxia there is a dimerisation of alpha and beta subunit that finally leads to the activation of target genes via binding to so-called hypoxia responsive elements (HREs) (Semenza, 2001).

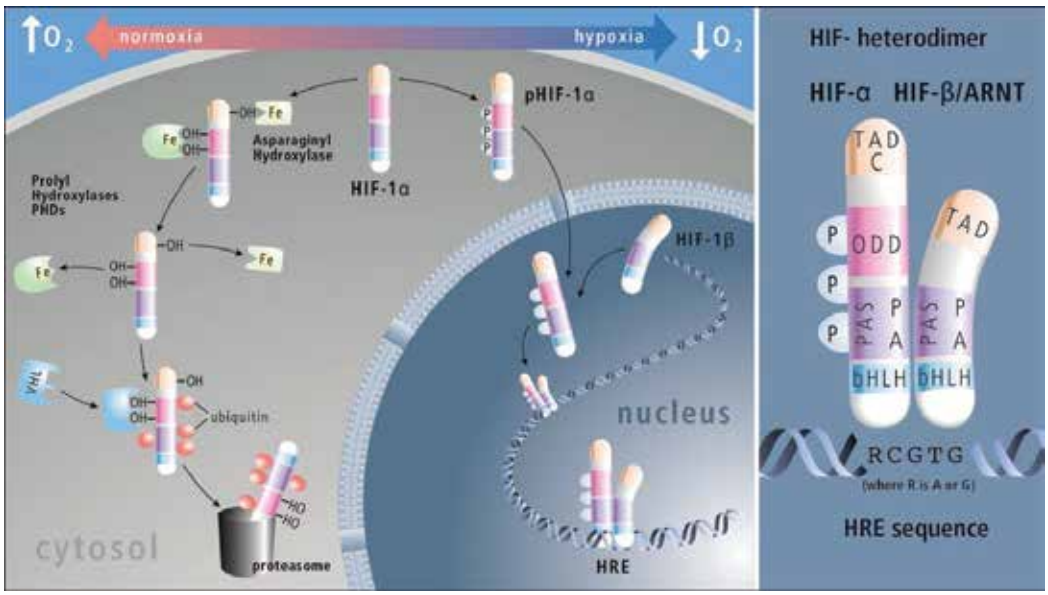

Figure 2. Oxygen sensing (Zagórska, Dulak et al., 2004)

Thus HIF-1 transmits the gene activation that is initiated by the existing hypoxia; many genes – directly or indirectly regulated by hypoxia - are involved in the unleashed cascade that

basically leads to cell differentiation, migration and glycolysis. Next to the famous players VEGF, Flk-1 and Flt-1, EPO, LDH-A, platelet-derived growth factor-ß (PDGF-ß) or basic fibroblast growth factor (bFGF) are involved.

3.4. Target genes

A plethora of target genes are governed by hypoxia via HIF-1. The following tables summarize the most relevant genes according to their function in the context of angio- and vasculogenesis

Extracellular matrix proteins and enzymes	Angiogenic growth factors and cytokines	Cell surface receptors
MMP2	ANGPT1 and 2	CXCR4
FN1	EPO	VEGFR 2
COL5A1	IGF2	TFRC
PLAUR	VEGF	
	TGFA and B	

Table 1. Extracelullar targets

Cytoskeletal proteins	Proapoptotic proteins	Transcription factors	Glucose tranporters and glycolytic enzymes
KRT14	RTP801	DEC1	GLUT1 and 3
KRT18	NIP3	DEC2	ENO1
KRT19	NIX	ETS1	HK1 and 2
VIM		CITED2	LDHA
			TPI
			PGK1

(Metzen, Ratcliffe et al, 2004, Marxsen, Stengel et al., 2004)

Table 2. Intracellular targets

3.5. Clinical relevance

The functionality of HIF-1 alpha, its capacity to transmit the need for oxygen and therefore for more vasculature has been the basic idea of innovative therapeutic concepts.

Recently several drugs have been developed which act as selective HIF prolyl-hydroxylase inhibitors; the inhibited degradation of HIF-1α persuades the system of a severe lack of oxygen and leads to an initiation of counter-measures.

By inhibiting HIF prolyl-hydroxylase, the activity of HIF-1 alpha in the bloodstream is prolonged, which results in an increase in endogenous production of erythropoietin (Bruegge, Jelkmann et al., 2007).

HIF activity is involved in angiogenesis required for cancer tumour growth, so HIF inhibitors are under investigation for anti-cancer effects (Semenza, 2006).

In addition, there have been observations that suggest that HIF pathway is not only a pivotal inductor of neo-angiogenesis but also is relevant in questions of bone regeneration for example in fracture repair.

The mechanism behind this hypothesis postulates the ability of osteoblasts to instrumentalize HIF-1 alpha as oxygen sensor and the corresponding signalling cascade to improve angio- and osteogenesis concurrently; the molecular interconnection is not finally elucidated. A dynamic crosstalk between osteoblasts and endothelial progenitors is assumed.

Therefore the application of HIF activators might improve bone healing by optimizing the angiogenic properties of the wounded bone but more important by inducing bone regeneration itself. Encouraging observations have been made in mouse fracture models where an overexpression of HIF and VEGF in long bones of mice results in pronounced vascularisation. Even a separate cultivation of the special osteoblasts without the corresponding endothelial cells does not affect their proliferation and differentiation (Wan, Gilbert et al., 2008, Wang, Wan et al, 2007).

4. Influence of angiogenesis during bone regeneration

The statements and examples of the preceding sections underlined clearly the impact of angiogenic processes on any tissue regeneration.

One well investigated area of research in this context in the regeneration of bone, e.g. in terms of skeletal development or fracture repair. The vasculatures´ job is to bring oxygen and nutrients to the metabolically active areas, but also to provide the bone with precursors or inflammatory cells. As far as the cytokines are concerned, there are many factors that act as key protagonists in angiogenesis as well as in bone regeneration and remodelling: VEGF, especially its isoforms VEGF120, 164 and 188 play a significant role. But there are other relevant players: bFGF, TGFβ, HIF are among the most potential ones.

VEGF in its isoforms with the corresponding receptors have emerged as the decisive coupling factors between epi- and metaphyseal vascularisation and cartilage development and enchondral ossification. A block of VEGFR-1 and -2 with selective antibodies leads to a reduced VEGF signalling and consecutively to a reduced intramembraneous bone formation in distraction osteogenesis; VEGF in this setting is produced by local inflammatory cells (Jacobsen, Al-Aql et al. 2005).

The angiopoietins Ang-1 and Ang-2, hepatocyte growth factor HGF, platelet-derived growth factor PDGF, the IGF family and the neurotrophins NGFs also have angiogenic properties.

The effect of HIF 1-alpha as stimulator of bone regeneration also has been observed: in a mouse model with increased HIF activity the animals showed significantly higher bone mass. The stimulated HIF activity led to enhanced intramembraneous bone regeneration

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in a mouse distraction model (Wang et al., 2007, Portal-Núñez, Lozano et al., 2012, Hankenson, Dishowitz et al., 2011)

In fracture vascularisation and repair VEGF function is required: here the matrix-bound forms of VEGF are activated by matrix metalloproteinases, enzymes that fulfil many functions during bone and matrix degradation and remodelling.

MMP9, expressed in osteo- and chondroclasts during fracture repair, initiates cartilage resorption. This degradation process releases matrix-bound VEGF from the cartilage matrix and thus stimulates the vascularisation. This callus degradation in addition provides the base for bony fracture repair in contrast to persisting cartilage non-union (Colnot, Thompson 2003).

MMP 13 activates VEGF relase independently: whereas MMP9 depends on osteo- can chondroclast functionality, MMP13 is expressed by hypertrophic osteo- and chondroblasts. Lack of MMP13 interferes with the proteoglycan degradation leading to a reduced permeability of the cartilage matrix for recruited inflammatory cells and sprouting blood vessels. The result is delayed callus resorption and altered vascular invasion (Behonick, Xing et al., 2007).

5. Autologous bone tissue engineering

Established concepts in the management of bony segmental defects or non-union after fracture rest upon the surgical implantation of either autologous bone as free grafts or micro-surgically anastomosed or artificial substitutes.

The concept of autologous bone tissue engineering wants to make available an amount of bone chips or bars that the organism itself is not able to supply without severe consequences. The base of the ideal three dimensional vascularised bone scaffold comprises the presence of a mechanically stable scaffold, seeded with different autologous cell populations and precursors, loaded with growth factors, embedded from the moment of implantation in a functioning vasculature to provide oxygen and nutrients and to remove metabolic by-products. Whereas many demands of this regenerative model can be met during in vitro culture in bioreactors, the vascular continuity remains the big problem to be solved.

As the supply of nutrition and oxygen via diffusion in three-dimensional tissue formations is restricted to an area of 100 μm around the nutritive capillary, resorption and devitalisation in the centre of the implant lead to a loss of mechanical stability.

The improvement of vascularisation therefore is an important demand on bone tissue engineering concepts. As far as the scaffold itself is concerned, there are different aspects to be considered; one decisive factor is the porosity of the material. In in-vitro studies the scaffolds with smaller pores (5-20 μm) come with increased endothelial cell growth and enhanced osteogenesis (Narayan, Venkatraman et al., 2008). In vivo the opposed effect is observed: higher porosity leads to more efficient osteo- and angiogenesis (Santos, Reis et al., 2010).

The modern materials provide the base for successful vascularisation simply by their design. In the structure of biodegradable polymers the negative of a vascular network can be imprinted

and thus provide the architectural structure of an efficient vasculature; the endothelial (progenitor) cells have to populate the form, the structure is pre-fabricated. The predefined geometry has to fulfil special demand to grant for optimal results, so the network should be designed in branches with defined numbers and localization of vertical nodes.

This concept of microfabrication has been upgraded: with CAD/CAM techniques three-dimensional scaffolds can be designed (Ciocca, De Crescenzio et al., 2009). So far these techniques are mainly applied in soft tissue engineering.

The loading of the scaffolds with growth factors is an established concept. The systems of drug delivery and release have become more refined, due to a combination of advanced scaffold materials and bio molecular perception concerning the anigogenic and osteogenic characteristics of the applied factors, their interconnection and vice-versa regulation. Combined application of several interacting growth factors is regarded as one of the pivotal steps towards successful factor application: from a polymeric scaffold a combination of VEGF and PDGF is delivered with defined dose and release kinetics. The advantage is the interaction of VEGF as endothelial mitogen and initiator of angiogenesis whereas PDGF impact on muscle cells and pericytes leads to vessel maturation and stabilization (Richardson, Peters et al., 2001).

In vitro pre-vascularisation of the scaffold often requires the colonisation with a co-culture of osteoblasts and endothelial (progenitor) cells, the duration of the in vitro phase ranges from hours to weeks. Investigations with poly-lactides implanted with a co-culture of endothelial progenitors and osteoblasts resulted in improved osteogenesis and vascularisation. The ischemic necrosis that was observed in the center of a graft that has only been implanted with osteoblast was not shown in the co-cultured scaffold (Yu, Vandevord et al., 2008).

Finally the success of any implant relies on a quick and efficient perfusion. In this context microsurgical techniques are combined with tissue engineering concepts in hybrid approaches that combine the respective advantages (Santos, Reis et al, 2010).

Modern approaches aim to design a custom made scaffold, loaded with autologous cells and growth factors including autologous vessel loops to grant for a spontaneous microvascular supply to support the expanding tissue. The details of this technique will have to be refined, but the first results are promising (Locmic, Stillaert et al., 2007). In autologous bone tissue engineering the combination of different regenerative strategies including tissue support and angiogenesis on various levels of the implant design and prefabrication finally will lead to successful therapeutic concepts.

6. Regenerative concepts

The support of developing vasculature happens on different levels from the (systemic) application of growth factors to the local application of loaded implants.

Next to the selection of growth factors, the colonisation with prefabricated cell populations or the nano-structural design of the implants are decisive factors considering the implant integration and the development of a functional vessel network.

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6.1. Therapeutic angiogenesis

In different clinical applications the angiogenic effect of different growth and transcription factors could be observed. In the context of angio- and osteogenesis, their coupling and the chance of therapeutic intervention, the administration of VEGF is the best investigated one. In fracture healing and bone regeneration therapeutic angiogenesis finds many points of attack. Beside the acute trauma the especially interesting indications considering bony regeneration are non-unions and distraction osteogenesis.

There are several approaches to stimulate angiogenesis and consecutively bone regeneration. The administration of angiogenic factors, VEGF or FGF, is supposed to effect a direct angiogenic up regulation. Another initiator of angiogenesis is HIF; its application or the inhibition of its degradation results in angiogenic effects. Generally these therapies aim to promote angiogenesis, to block anti-angiogenic processes and to bring endothelial progenitor cells to the wounded bone (Hankenson, Dishowitz et al., 2011).

The effects of VEGF as a promoter not only of angiogenesis but also of bone regeneration have been reported in a femur fracture model in mice and in a rabbit radial segmental defect; improved ossification and callus maturation where observed (Street et al, 2002).

Another growth factor with angiogenic and osteogenic characteristics is platelet derived growth factor (PDGF) that acts as mitogen for osteoblasts and up-regulates VEGF expression. In animal models the administration of PDGF came with increased mechanical stability and callus density (Hollinger, Onikepe et al., 2008). In human pilot projects these positive results of PDGF application in combination with fracture stabilization could be verified.

A modern area of research dealing with VEGF as a means of vascular protection and regeneration aims to neuroprotection; VEGF has been reported to protect motor neurons in vitro from hypoxia induced toxicity, reactive oxygen and other degrading factors (Svensson, Peters et al., 2002). In addition, VEGF seems to be able to stimulate growth and development of neuronal stem cells as well as to recruit neuronal progenitor cells (Schaenzer, Wachs et al., 2004). In ALS rat models the protective effect of VEGF in the cerebrospinal fluid has recently been reported, showing a protracted course of disease with delayed paralysis and increased survival time (Storkebaum, Lambrechts et al., 2005).

The potential of angiogenesis as a pivotal factor in many areas of tissue maintenance and regeneration is obvious; in future regenerative medical concepts the manipulation of angiogenesis in parallel with tissue regeneration will be integral part and lead to successful strategies.

The most important growth and transcription factors enhancing vascularisation are summarized in table 3. The variety of different angiogenic factors with similar functions implies the idea of redundancy: the quick and undisturbed succession of events of angiogenesis is too important for the function of the whole organism to take the risk of relying on unique regulators or promoters.

Growth factor	Molecular target	Effects on progenitor cells
VEGF	VEGF receptors expressed on endothelial cells, monocytes, hematopoietic stem cells; stimulates proliferation, migration, and tube formation	Mobilization of EPC Improves survival and differentiation of EPC
Placenta-derived growth factor (PlGF)	VEGF receptor 1 (cross talk with VEGF receptor 2)	Mobilization of hematopoietic stem cells and EPC
Fibroblast growth factor (FGF)	FGF receptors expressed on endothelial cells, smooth muscle cells, and myoblasts; stimulates proliferation	Included in EPC culturing media
Angiopoietin-1	Tie-2 receptor expressed on endothelial cells; enhances vessel maturation and stability	Mobilizes EPC and hematopoietic progenitor cells
Insulin-like growth factor (IGF)	IGF receptor expressed on vascular cells and satellite cells; enhances skeletal muscle regeneration	Included in EPC culturing media
Erythropietin (EPO)	Activates the Epo receptor, which is expressed on hematopoietic stem cells, EPC, endothelial cells, and cardiac myocytes; improves survival	Mobilization of EPC
Hypoxia inducible factor 1 (HIF-1)	Activation of gene expression (eg, VEGF, VEGF receptor 2, erythropoietin, IGF-2, and NO synthase)	

Table 3. Growth and transcription factors stimulating angiogenesis (Losordo, Dimmeler et al., 2004)

6.2. Nanotechnology in regenerative medicine

Modern biomaterials have to meet many requirements: not only do they have to provide mechanic support and stability; they have also to enhance regenerative processes, to be anti-infective and non-inflammatory. The problems of bone implants so far were seen in the poor osseointegration and bone regeneration on the one hand and implant loosening and fracture on the other (Dhillon, Schwarz et al., 2011).

The relevance of nanotechnology in the area of tissue engineering research is reflected by the fact that it has become an independent field of interest in regenerative medicine: the nanomedicine. Nanomedicine deals with implant structures that have (surface) dimensions of fewer than 100 nm. The used materials include fibres and particles, imitating natural bone structure to improve the mechanic and biological properties of the implants.

Figure 3 illustrates the dimension of nanostructures in the context of bone anatomy (Sato, Webster et al., 2004, Khang, Lu et al., 2008).

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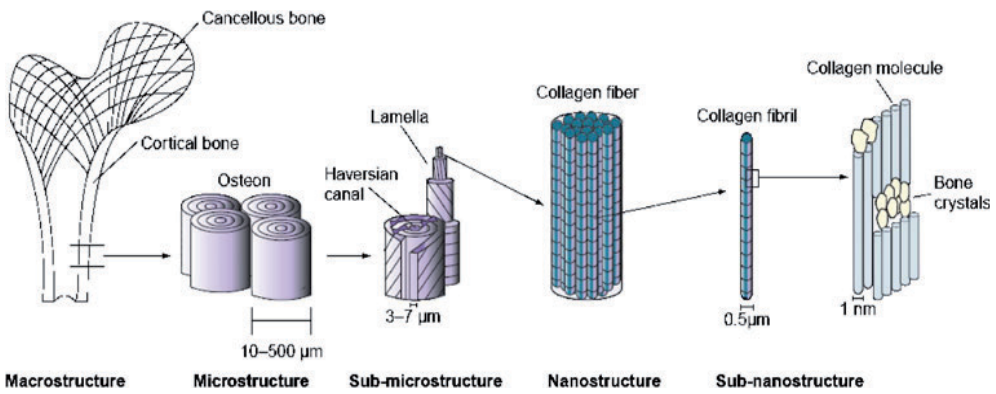

Figure 3. Nanostructure in bone structure (Sato, Webster et al., 2004)

One important task of nanomedicine is to come up with improved, probably intelligent biomaterial. In fact there are two different strategies to modify established materials. One deals with the materials chemistry, the other cares for the surface properties. By individually adapting chemical and physical characteristics the idea scaffold can be designed (Khang, Carpenter et al., 2010).

The established nano-materials in bone regeneration are nano-hydroyapatite, silk and nano structured titanium surfaces. To reach optimal cell adhesion and function there have been efforts to imitate the physiological anisotropy of natural bone. These modifications led to enhanced adhesion and mineralisation (Khang, Lu et al., 2008).

These nano-materials will find their way into clinical practice as far as orthopaedic indications or dental implants are concerned.

These developments are also realized in vascular tissue regeneration: the material surfaces are supposed to promote endothelial cell migration, adhesion and proliferation of vascular graft or stents. In an investigation of polylactide-co-clycolic acid (PLGA) surfaces with spherical surfaces features with ascending diameters a positive correlation of vertical surface feature dimension to cell adhesion and protein adsorption was measured; the optimal dimension was 20 nm (Carpenter, Khang et al., 2008). In special etching techniques, titanium inductively coupled plasma deep etching (TIDE), a linear nano-structured surface pattern is created that allows for increased endothelial cell proliferation compared to smooth titanium surfaces and even to randomly nano-structures titanium surfaces after five days of cultivation (Lu, Rao et al., 2008). Recent concepts deal with mechanical strain applying pulsed or sustained pressure to the implanted scaffolds to meet the demands of physiological vascular tension.

Nanotechnology in current tissue engineering concepts investigates the cell- biomaterial interaction and perfects the surface properties to achieve maximum regenerative support in combination with a prolonged implant lifetime. In the context of angiogenesis the development and integration of nano-materials will be of vital importance in regenerative strategies.

6.3. Critical aspects

Many promising investigations featuring growth factor therapy are performed in cell culture or healthy young animals. The therapeutic use of these developments especially addresses the ageing population suffering from ischemic diseases and vascular degeneration. Special attention in coming investigations has to be shifted to the functionality and regenerative demands of diseased of damages cells and tissues. Therapeutic angiogenic strategies have to be scrutinized under the focus of safety and effectiveness in systems with impaires endogenous endothelial function (Sun, Bai et al., 2009).

Beside the form of application, the definition of the ideal dose of angiogenic growth factor is one the most difficult questions to answer. When dealing with loaded scaffolds one has to define release kinetics. Additionally, in histological investigations, the vasculature that develops under the influence of high doses of VEGF repeatedly showed malformations and an insufficiency in the cell-cell junctions (Zisch, Lutolf 2003).

In most therapeutic concepts the desired effect of VEGF is a local one and aims to improve the formation of nutritive vasculature supporting tissue regeneration in a limited area of tissue as well as a in a limited period of time. VEGF application has to take place locally and only during defined period; considering the fact, the VEGF coming with increased angiogenesis is part of many pathologic processes, e.g. tumour vascularisation or proliferative retinopathy, the control of VEGF effect has to be granted. These demands require a lot of conceptional research considering the therapeutic application of VEGF. The angiogenic and osteogenic effects of VEGF delivered from poly-lactide scaffolds in irradiated osseous defects was illustrated impressively in increased vascularisation and bone formation, the application of a potent growth factor in tumour patients however bears many risks (Kaigler, Wang et al., 2006)

In every therapeutic concept the medical gain and the patients´ profit has to be weighed against the impending costs. Nowadays the production of recombinant VEGF in the desired doses is enormous. For routine clinical application the methods of generation, application and delivery have to be refined (Barralet, Gbureck et al., 2009).

7. Role of stem cells in regenerative medicine

The potency of multilineage differentiation and self-renewal makes stem cells an attractive target for scientific approaches concerning tissue repair and regeneration. The interesting cell populations in this context are embryonic and mesenchymal stem cells.

7.1. Embryonic stem cells

Human embryonic stem cells are capable of self-renewal and differentiation in cells from all germ layers. These regenerative characteristics are very attractive in basic science as well as in potential clinical applications. The pros and cons of embryonic stem cell therapy are discussed controversially.

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Current investigations describe the cells potential to differentiate into vascular cell lines and therefore to support tissue repair via angiogenesis. The difficulty is to control and regulate the cell proliferation and differentiation to avoid spontaneous development of teratoma from undifferentiated embryonic stem cells.

In figure 4 an overview of different culture protocols to induce vascular differentiation from embryonic stem cells is given (Descamps, Emanueli et al., 2012).

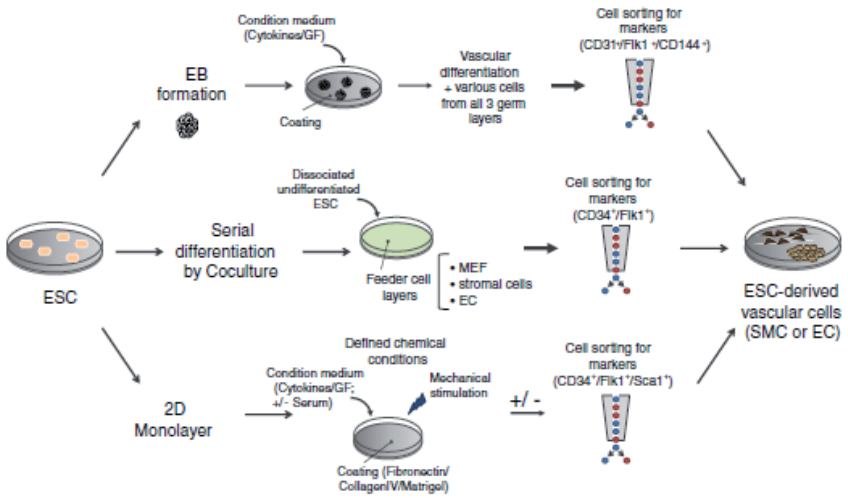

Figure 4. Vascular differentiations

Therapeutic neo-vascularisation is of extraordinary interest in the therapy of cerebral or heart ischemia and regenerative strategies. In mouse models with limb ischemia the implantation of embryonic stem cells – alone or in combination with muscle cells – proved a significantly better perfusion via neo-vascularisation in stark contrast to an implantation of adult endothelial cells (Kane, Xiao et al., 2010).

The positive results in animal models are promising. Yet the pitfalls of stem cell transfer represented by tumorigenicity and immunocompatibility have to be overcome (Descamps, Emanueli et al., 2012).

7.2. Mesenchymal stem cells

In contrast to embryonic stem cells, mesenchymal stem cells are characterized by pluripotency. These adult stem or progenitor cells, harvested from fat, skin or dental pulp are less prone to ethical concerns and directly available for autologous approaches.

Especially in cardiovascular regeneration mesenchymal stem cells hold great promise for further advanced in therapeutic strategies.

In different investigations these cell lines have shown their potential: not only are they able to differentiate into vascular cells, endothelial cells or pericytes, in addition they stimulate the

local cells via paracrine secretion of growth and transcription factors, among others VEGF or IGF. In this context, a secretion of microparticles by the pluripotent cells has been observed; these particles seem to support the regenerative process, the mechanism behind this phenomenon is not understood (Vono, Spinetti et al., 2012).

8. Conclusion

Angiogenesis in vivo and vitro, in physiologic and pathologic processes is a multifactorial process. It includes a plethora of signalling molecules and pathways, dynamically cross-talking cells and innumerable cytokines and growth factors to generate a functional and stable vessel system. This vasculature, however, is one prerequisite of tissue regeneration for it grants a continuous supply of nutrients and oxygen. These considerations found their entry to modern tissue engineering when the vital importance of a vascular network was more and more focussed.

The future of regenerative approaches to bone healing and regeneration will inevitably combine the field-tested strategies of tissue engineering with modern bio molecular techniques in the scientific environment of stem cells and gene therapy.

To define the fragile equilibrium between mechanical properties, tissue support and angiogenic stimulation will be the interest of research in regenerative medicine for the coming years.

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Chapter 20

Engineering of Inflammation-Resistant Osteochondral Cells

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Clinical issue

Osteoblasts and chondrocytes engineered from various sources of stem cells to replace damaged bone or cartilage in an inflamed joint in patients with inflammatory diseases like osteoarthritis or osteoarthrosis need to be made resilient to the impact of the inflamed environment within the joint cavity (Neve, Corrado et al. 2011; van der Kraan and van den Berg 2012). Clinical experience has clearly shown that the engineered osteochondral cells relatively quickly tend to lose their defined cell phenotype (Chen and Tuan 2008; Kastrinaki and Papadaki 2009), while also tending to perpetuate bone loss through activation of osteoclasts, thus yielding a negative bone remodelling volume (Lories 2011; Goldring 2012). The present concept of manipulation of stem cell microRNA levels envisages one route, by which one may obtain engineered osteochondral cells with a better potential of producing bone and cartilage *in vivo*, and to withstand the putative detrimental effect of immune cell (e.g. T-helper cell = Th-cell) influence constituted by a plethora of cytokines and exosome-conveyed microRNAs.

1.2. Previous findings

We have previously shown that osteochondral cells can be derived from human mesenchymal stem cells (hMSCs) by manipulating the levels of a group of microRNAs, i.e. mir-16, mir-24, mir-125b, mir-149, mir-328, and mir-339 (Gordeladze 2009; Gordeladze, Djouad et al. 2009). All

these microRNAs are low in differentiated osteoblasts, but highly expressed in stem cells and differentiated chondrocytes. Especially mir-149 seems to serve as a switch, both stimulating chondrogenesis, while suppressing osteoblastogenesis in differentiating hMSCs. Furthermore, mir-328 and mir-339 are the better determinants of the osteochondral cell phenotypes, since manipulations of their intracellular levels enabled us to reciprocally trans-differentiate osteoblasts to chondrocytes at day 7-14 in 2D and 3D cultures *in vitro* (Gordeladze, Djouad et al. 2009; Gordeladze 2011). Hence, we propose that these six microRNAs, and mir-149, mir-328, and mir-339 in particular, might be able to influence the osteochondral phenotypes in such a way that they are able to maintain their phenotype *in vitro* and *in vivo* for an extended period of time, while under the influence of a Th-cell invaded arthritic luminal space.

1.3. Experimental procedures

The material used in a defined set of experiments consisted of hMSCs differentiated towards osteoblasts in monolayer cultures or in scaffolds (hydroxyapatite or alginate beads, respectively), hPBMCs (human peripheral blood monocytic cells) differentiated towards osteoclasts and grown on bovine dentine slices, exosomes (100,000xg pellets) from activated Th-17 cells, and SCID mice. The cells were transfected with scrambled microRNA, mir-16, mir-24, mir-125b, mir-149, mir-328 or mir-339 pre-microRNAs or antago-microRNAs, using amounts known to suppress or enhance endogenous levels approximately 5 times (Gordeladze 2009). Human MSCs, osteoblasts or chondrocytes were grown in a medium supplemented with chemokines (Interleukin-1 = IL-1, Interleukin-6 = IL-6, and Interleukin-17A = IL-17A, and TNFα), or over-layered with exosomes from Th17 cells, either in the absence or presence of dentine slices over-layered with osteoclasts differentiated from hPBMCs, using RANK-L and M-CSF (Fritz, Brondello et al. 2011). Subsequent to a certain period of time (7-21 days), osteochondral cells were either analysed for phenotypic characteristics (e.g. matrix production, microRNA levels, phenotypic gene expression) or injected (without or with mature osteoclasts) into the tibial muscle of SCID mice. After 14 days of *in vivo* incubation, the *de novo* formed osteochondral tissues were analysed for phenotypic markers of injected cells by Q-PCR, as well as matrix characteristics (e.g. matrix protein immunohistochemistry, hydroxyapatite production, or GAG = glucosaminglycan over DNA ratio).

1.4. Results

These experiments showed the following:

1. Human MSCs, pre-chondrocytes or pre-osteoblasts lost many of their phenotypic characteristics as matrix builders upon exposure to either pre-microRNAs (osteoblasts) or antago-microRNAs (chondrocytes).

2. These cells, when grown in co-culture with differentiated osteoclasts, appeared to enhance osteoclast multi-nucleation, gene expression, and ensuing dentine resorption surface area.

3. By exposing these cells to either artificial, i.e. “inflamed” synovial fluid (growth medium fortified with Th17 cell like profile of cytokines), exosome-like fractions obtained from activated Th17 cells, or certain pre-microRNAs (i.e. mir-222 for chondrocytes or mir-22

for osteoblasts), we observed that the osteochondral phenotypes were disturbed, along with a concomitantly enhanced potential for osteoclast activation.

4. Growing hMSCs in a 3D structure (hydroxyapatite for osteoblasts and soft alginate beads for chondrocytes) also stabilized phenotypic characteristics, though to a lesser degree.

5. Injecting manipulated osteochondral cells with or without a certain number of pre-differentiated osteoclasts into the tibial muscle of SCID mice yielded *de novo* formed bone and cartilage tissues, where more than 95% of the injected cells resided within the newly formed tissues. Parameter evaluation (histology, immuno-histochemistry of collagens and aggrecan, X-ray analysis, GAG/DNA ratio, estimation of total Ca^{2+}-contents, and Q-PCR analyses of marker genes) of the osteochondral cells, as well as Q-PCR analyses of osteoclast marker genes (like the calcitonin receptor, TRAP, cathepsin K, and carbonic anhydrase II) clearly showed that either of the *de novo* formed tissues were not functioning optimally, and that enhanced osteoclast activation prevailed.

6. Interestingly, cells having been transfected with optimal amounts of either pre-microRNAs or antago-microRNAs maintained their phenotypic characteristics within the tibial muscle of SCID mice, even when pre-exposed to the "inflammation"-mimicking medium or exosome-like particles from activated Th17 cells.

7. Polycistronic vector constructs containing the pre- and antago-microRNAs 149, 328, and 339 are now being made to verify the benefit of controlling the endogenous levels of these microRNA species to produce stable osteochondral phenotypes to be implemented in *in vitro* and *in vivo* animal model systems.

1.5. Conclusion

Manipulating pertinent microRNAs in osteoblast or chondrocytes derived from hMSCs (or other sources, such as hADSCs = human adipose tissue derived stem cells) may facilitate differentiation to proper, functional cell phenotypes, which are also resilient to the detrimental impact from inflammatory chemokines and/or microRNAs, proteins or mRNAs transferred from Th-cells (via exosome-like vehicles) to engineered osteoblasts or chondrocytes.

2. Bone remodelling

2.1. Cellular and molecular mechanisms of bone remodelling

Physiological remodelling of bone has proven to be a highly coordinated process constituted by bone resorption following bone formation, and it is necessary for damaged bone to be repaired, and to maintain mineral homeostasis (Mackie, Ahmed et al. 2008; Kular, Tickner et al. 2012; Thompson, Rubin et al. 2012). In addition to the traditional cells localized within the bone tissue (i.e. osteoclasts, osteoblasts, and osteocytes) necessary for completion of multiple bone remodelling cycles, several classes of immune cells have also been implicated in bone turnover and also bone disease states (Chen and Tuan 2008; Lories 2011; Mellis, Itzstein et al.

2011; Sims and Walsh 2012). The two processes of modelling and remodelling ensure proper development and maintenance of the skeletal system. The process of bone modelling is mandatory for growth and mechanically induced adaption of bone (Kular, Tickner et al. 2012; Thompson, Rubin et al. 2012), but it requires that the processes of bone formation and bone resorption (removal of "old" bone with micro-fractures) occur independently, and at distinct locations within the skeleton (Chang, Raggatt et al. 2008; Raggatt and Partridge 2010; Kular, Tickner et al. 2012). These tightly coordinated events require the synchronized activities of multiple cellular participants and compartments, in order to ensure that bone resorption and formation occur sequentially within the same anatomical locations to preserve bone mass with a functional geometry.

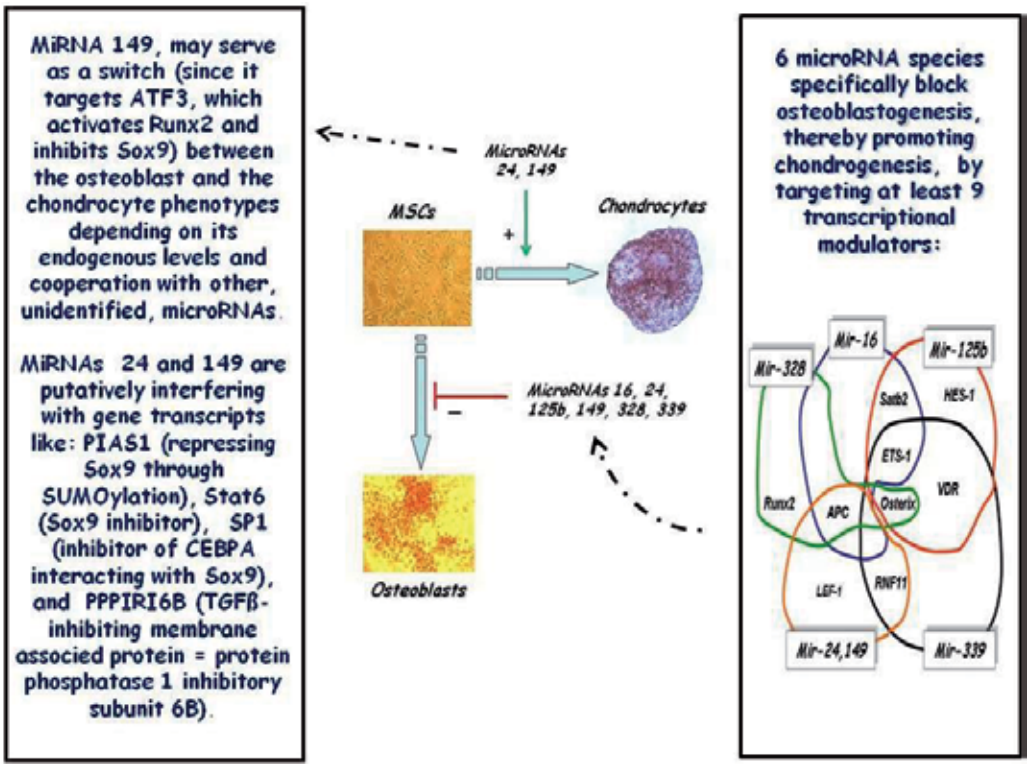

Figure 1. Schematic representation of a previously published microRNA profile distinguishing between osteoblasts and chondrocytes differentiated from human mesenchymal stem cells (hMSCs). Right hand frame: The six microRNA species have been proven to bind to and suppress the transcription of genes encoding nine factors deemed important for osteoblastogenesis, i.e. SATB2, HES-1, ETS-1, RUNX2, APC2, OSTERIX, VDR, LEF-1, and RNF11 (Marie 2008; Gordeladze, Djouad et al. 2009). Left hand frame: Interestingly, the microRNA species mir-24, and especially mir-149, may be regarded as important "switches" between the osteoblast and chondrocyte phenotypes, since they both positively suppress transcription factors (TFs), as predicted by using the MirnaViewer and the Sanger algorithms, necessary for osteoblastogenesis, and at the same time negatively affect factors (like ATF4, PIAS1, STAT6, and PPPIR6B) (Legendre, Dudhia et al. 2003; Komatsu, Mizusaki et al. 2004; Oh, Kido et al. 2007; Suzuki, Osumi et al. 2010), which serve as inhibitors of Sox9 expression, deemed as the most important TF initiating chondrocytogenesis and maintaining the chondrocyte phenotype.

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2.2. Cells being involved in bone remodelling

Osteoblasts - Osteoblasts are specialized bone-forming cells responsible for many of the crucial functions constituting bone remodelling (i.e. expression of osteoclastogenic factors, production of bone matrix proteins, and bone mineralization) (Karsenty 2008). The stage of differentiation acquired by the osteoblasts influences the functional contribution to and impact of these cells on *in vivo* bone remodelling. Immature pre-osteoblasts influence osteoclastogenesis, while mature osteoblasts are responsible for matrix production and mineralization. Osteoblasts develop from pluripotent hMSCs, which exhibit inherent potential to develop into adipocytes, myocytes, chondrocytes, and osteoblasts orchestrated by a series of defined transcription factors (TFs). Osteoblast differentiation has since long been reported to be controlled by RUNX2, but several other TFs are now recognized as equally important for osteoblastogenesis (Franceschi, Xiao et al. 2003). It has been published that 14 different TFs (and/or modulatory proteins), including SATB2, HES-1, ETS-1, LEF-1, SP7 (OSTERIX), RNF11, VDR, and APC2 (a positive regulator of β-catenin of the canonical WNT-pathway) define the differentiating potential of osteoblasts with a statistical extremely high probability ($p = 2\alpha < 5x10^{-13}$) (Gordeladze, Djouad et al. 2009).

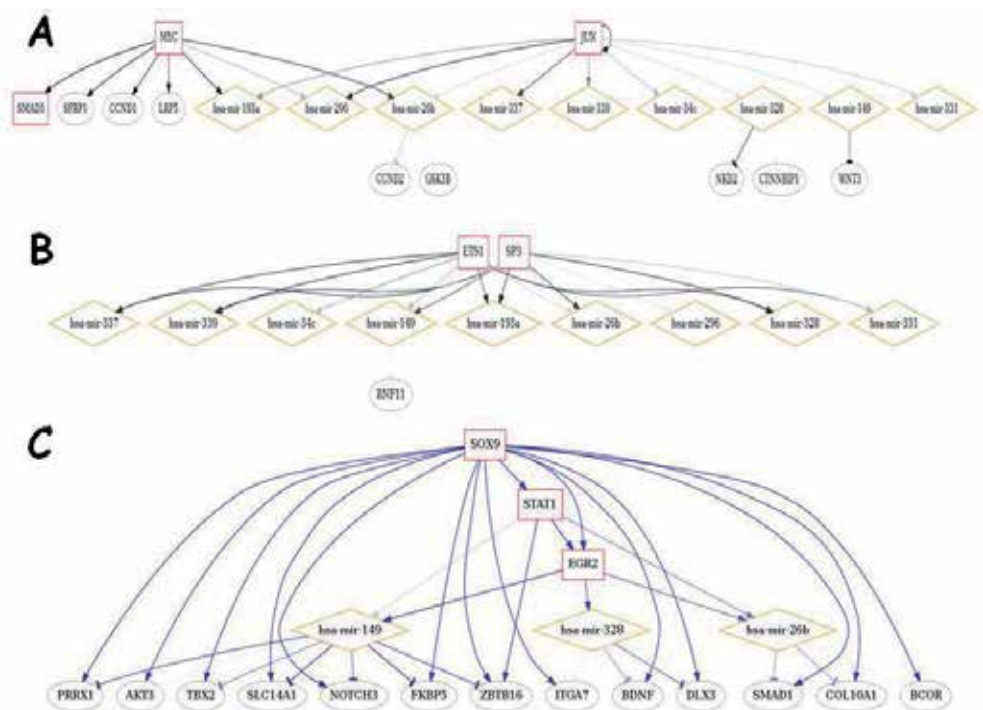

Figure 2. Putative interactions between transcription factors (TFs), microRNA species, and functional genes describing: (A). Hierarchical feed-forward regulation by MYC (Myc proto-oncogene protein) and JUN (transcription factor AP-1) of the canonical WNT-system (important for osteoblastogenesis), including factors like SMAD3 (modulator of TCF/LEF), SFRP1 (secreted frizzled-related protein 1), CCND1 (Cyclin D1), LRP5 (low density lipoprotein receptor-related protein 5), hsa-mir-339, hsa-mir-328, and hsa-mir-149 directly, while CCND2 (cyclin D2), GSK3B (glycogen syn-

thase kinase 3β), NKD2 (naked cuticle 2), CTNNBIP1 (catenin β1), and WNT3 (wingless-type MMTV integration site family, member 3) are affected indirectly. **(B)**. Hierarchical feed-forward regulation by ETS1 (a stimulator of CCN2 = connective tissue growth factor, a mediator of TGFβ1-mediated matrix protein synthesis in osteoblasts) and SP3 by microRNA species including hsa-mir-339, hsa-mir-149, and hsa-mir-328. The TF RNF11, which has now received increasing attention as a major modulator of osteoblastogenesis (Gao, Ganss et al. 2005), is directly affected by hsa-mir-149. **(C)**. Hierarchical (and possibly superior) feed-forward regulation by Sox9 of STAT1- and EGR2-mediated transcription of the micro-RNA species mir-149 and mir-328. EGR2/KROX-2 (a transcription factor stimulated by WNT3) exerts a major impact on mir-149 and mir-328, which directly affect the transcription of genes like PRRX1, AKT3, TBX2, SLC14A1, NOTCH3, FKBP5, BDNF, and DLX3 (as predicted using the MirnaViewer and Sanger algorithms. Of these genes, PRRX1 is a regulator of OSTERIX, AKT3 and RUNX2 (Lu, Beck et al. 2011) where all are mutually involved in osteoblast differentiation, TBX2 is required for osteoblast proliferation, NOTCH3 inhibits osteoblast differentiation(Lin and Hankenson 2011), FKBP5 overexpression is known to ensure osteoblast differentiation from hMSCs, BDNF is involved in endochondral ossification (Yamashiro, Fukunaga et al. 2001; Marie 2008), and DLX3 is well known as an early inducer of osteoblast differentiation co-acting with RUNX2, DLX5, and MSX2 (Heining, Bhushan et al. 2011). This particular scheme of interactions emerged when combining an osteoblast microRNA micro-array with published osteoblast mRNA transcriptomes (Gordeladze 2011). The bioinformatics modulations are performed by using the algorithm Mir@nt@n (Le Bechec, Portales-Casamar et al. 2011) with standard stringent conditions for putative modulations of gene expression. As the reader will notice, a few other microRNA species may also have been selected to be included amongst the six microRNAs constituting the present osteochondral signature.

Osteocytes - During the course of bone formation, a sub-population of osteoblastic cells undergoes terminal differentiation and becomes engulfed within unmineralized osteoid. They are referred to as osteocytes (Palumbo 1986). Following the mineralization phase, these entombed cells form a network which extends throughout the mineralized bone tissue. Osteocytes respond to mechanical load (mechano-stimulation), and their network is believed to integrate the detection bone micro-damage (micro-fractures within the mineralized bone), which accumulates as a result of normal skeletal loading and fatigue (Verborgt, Tatton et al. 2002). Data have been obtained that support the idea that osteocytes initiate and direct the subsequent remodelling process that repairs damaged bone. Furthermore, the osteocytes have been hypothesized to serve as sensors for the nervous system connected to the skeleton, and for instance convey hypothalamus-derived nerve impulses driven by leptin receptors as a feedback regulatory loop controlling bone mass (i.e. bone turnover) within optimal limits (Takarada and Yoneda 2008; Thompson, Rubin et al. 2012; Motyl and Rosen 2012).

T-cells and B-cells - T-lymphocytes and B-lymphocytes serve as central players of the adaptive immune system facilitating the recognition and the destruction of pathogenic microorganisms. Mice, which are devoid of either B- or T-lymphocytes exhibit osteoporosis, indicating that these immune cells are routinely participating in the maintenance of bone homeostasis (Li, Toraldo et al. 2007). Mature B-cells produce ~50% of total bone marrow-derived OPG, which would contribute significantly to the restraint of osteoclast differentiation and activation during normal bone turnover. However, the role of T-cells in regulating bone remodelling during homeostasis is less clear. Based on data that T-cell-deficient CD40 knock-out and CD40L knock-out mice are osteoporotic (Li, Toraldo et al. 2007), it has been proposed that T-cells work cooperatively with B-cells and enhance OPG production via CD40/CD40L co-stimulation (Raggatt and Partridge 2010)

Osteomacs - Osteomacs are defined as tissue macrophages residing on on or within the endosteal and/or periosteal surfaces. *In vitro*, osteomacs are required to ensure full functional differentiation of the osteoblast, i.e. the acquisition of mineralizing properties, which consti-

tutes the late phase of the osteoblasts' life span. *In vivo*, osteomacs form a canopy over mature matrix-producing osteoblasts at sites of bone modelling, which serves as an ideal anatomical location from which to regulate such a process. A depletion of macrophages *in vivo* inevitably leads to a complete loss of endosteal osteomacs and associated osteoblasts, indicating that the osteomacs are needed to sustain osteoblast maturation and mineralizing property (Chang, Raggatt et al. 2008).

Osteoclasts - Osteoclasts are terminally differentiated myeloid derived cells, being adapted to excavate mineralized osteoid (bone matrix). These particular cells exhibit distinct morphological and phenotypic characteristics routinely used as functional markers. They include multinuclearity and expression of tartrate-resistant acid phosphatase (TRAP) and the calcitonin (CT) receptor (Teitelbaum and Ross 2003). M-CSF (macrophage colony-stimulating factor) and RANK-L (receptor activator of NFκ-B ligand) serve as critical cytokines being required for the survival, expansion, and the differentiation of osteoclast precursors (hPBMCs = human peripheral blood monocytic cells) *in vitro* (Lacey, Timms et al. 1998). The requirement of these cytokines for osteoclast induced bone resorption *in vivo*, has been demonstrated in animal model systems lacking functional M-CSF and RANK-L. The molecule OPG-2 (osteoprotegerin-2) serves as a soluble decoy receptor for RANK-L and a negative physiological regulator of osteoclastogenesis, i.e. loss of functional OPG in mice, results in osteoporosis, due to excessive induction (or differentiation) and activation of osteoclasts (Simonet, Lacey et al. 1997). The current paradigm asserts that the RANK-L/OPG expression ratio determines the degree of the osteoclasts' biological activity (i.e. their differentiation and function) (Hofbauer, Khosla et al. 2000). Several TFs, like FOS, MYC, AP-1 (JUN), PU.1, and CREB (Tondravi, McKercher et al. 1997) are deemed necessary to differentiate myeloid cells toward the osteoclast phenotype. MITF (microphthalmia-associated transcription factor) and NFATc1 (nuclear factor of activated T-cells, cytoplasmic 1), which is also important for hMSCs to develop into the osteoblastic lineage (Marie 2008) are also required for osteoclast formation and their expression of osteoclast-specific genes like TRAP (Luchin, Purdom et al. 2000), cathepsin K, and the CT receptor (Matsumoto, Kogawa et al. 2004; Hu, Sharma et al. 2007).

2.3. The process of bone remodelling

Prior to activation, the resting bone surface is covered with bone-lining cells, including pre-osteoblasts intercalated with osteomacs. B-cells are present in the bone marrow and secrete OPG, which suppresses osteoclastogenesis (Raggatt and Partridge 2010).

Activation: The endocrine bone-remodelling signal PTH binds to the PTH receptor on pre-osteoblasts. Damage to the mineralized bone matrix results in localized osteocyte apoptosis, reducing the local TGF-β concentration and its inhibition of osteoclastogenesis (Juppner, Abou-Samra et al. 1991; Heino, Hentunen et al. 2002; Swarthout, D'Alonzo et al. 2002; Verborgt, Tatton et al. 2002; Aguirre, Plotkin et al. 2006; Bonewald 2007; Weitzmann and Pacifici 2007).

Resorption: In response to PTH signalling, monocyte chemotactic protein (MCP-1) is released from osteoblasts and recruits pre-osteoclasts to the bone surface. Additionally, osteoblast expression of OPG is diminished, and production of M-CSF and RANK-L is increased, in order to promote proliferation of osteoclast precursors (hPBMCs) and differentiation of mature

MicroRNA species	16	24	125b	149	328	339
Fold miRNA alteration in *chondrocytes* using *PremiRNAs*	6 ± 2	8 ± 3	6 ± 3	9 ± 3	7 ± 1	8 ± 2
Effect on: Sox9, Coll2a1, Gli3, GAG/DNA	132 ± 17	91 ± 13	108 ± 8	120 ± 18	110 ± 8	129 ± 16
Fold miRNA alteration in *chondrocytes* using *AntagomiRNAs*	0.12 ± 0.03	0.14 ± 0.03	0.13 ± 0.06	0.12 ± 0.05	0.14 ± 0.06	0.11 ± 0.04
Effect on: Sox9, Coll2a1, Gli3, GAG/DNA	18 ± 5	278 ± 35	17 ± 7	191 ± 31	16 ± 5	19 ± 4
Fold miRNA alteration in *osteoblasts* using *PremiRNAs*	9 ± 4	8 ± 3	8 ± 3	7 ± 2	9 ± 2	7 ± 3
Effect (relative to control) on: Runx2, Coll1a1, OC, %Min Surf	13 ± 6	17 ± 5	14 ± 3	15 ± 7	15 ± 6	12 ± 4
Fold miRNA alteration in *osteoblasts* using *AntagomiRNAs*	0.40 ± 0.13	0.38 ± 0.16	0.41 ± 0.14	0.49 ± 0.12	0.43 ± 0.14	0.53 ± 0.17
Effect (relative to control) on: Runx2, Coll1a1, OC, %Min Surf	237 ± 42	192 ± 31	227 ± 22	210 ± 26	198 ± 22	231 ± 32

Figure 3. The impact of pre-microRNA and antago-microRNA species of the osteochondral microRNA signature on the differentiation of osteochondral cells from hMSCs. The following parameters were analysed: endogenous microRNA levels, and average alteration in various differentiation markers. MicroRNA levels and marker gene mRNAs (Collagens, Gli3, Sox9, Osteocalcin, and Runx2) were analysed using the Q-PCR method (Ambion). Estimations of GAG/DNA-ratio (chondrocytes only) were performed using colorimetric methods. Mineralized surface calculations in osteoblast cultures were achieved using the Alizarin red S staining method (Gordeladze, Noel et al. 2008)

osteoclasts. The mature osteoclastic cells become anchored to RGD-binding sites (Arg-Gly-Asp containing proteins), creating a localized micro-environment (i.e. a sealed zone) facilitating the degradation of mineralized bone matrix (Partridge, Jeffrey et al. 1987; Insogna, Sahni et al. 1997; Saftig, Hunziker et al. 1998; Burgess, Qian et al. 1999; McHugh, Hodivala-Dilke et al. 2000; Teitelbaum 2000; Ma, Cain et al. 2001; Yang, Chien et al. 2004; Li, Qin et al. 2007).

Reversal: So called reversal cells engulf and destroy demineralised, undigested collagen from the bone surface. Transition signals are generated for the purpose of halting bone resorption and to stimulate the process of bone formation(Tran Van, Vignery et al. 1982; Kraal, Rep et al. 1987; Heinemann, Siggelkow et al. 2000; Everts, Delaisse et al. 2002; Takahashi, Takahashi et al. 2004; Newby 2008).

Formation: Signalling molecules stimulating bone formation arise from degraded bone matrix, mature osteoclastic cells, and potentially also reversal cells. PTH-binding to and mechano-stimulation of osteocytes reduce the expression and secretion of sclerostin (an inhibitor of BMP-binding to its receptor), allowing Wnt-stimulated bone formation to occur (Fermor and Skerry 1995; Harmey, Hessle et al. 2004; van Bezooijen, Roelen et al. 2004; Li, Zhang et al. 2005; Martin and Sims 2005; Murshed, Harmey et al. 2005; Zhao, Irie et al. 2006; Pederson, Ruan et al. 2008; Robling, Niziolek et al. 2008; Tang, Wu et al. 2009).

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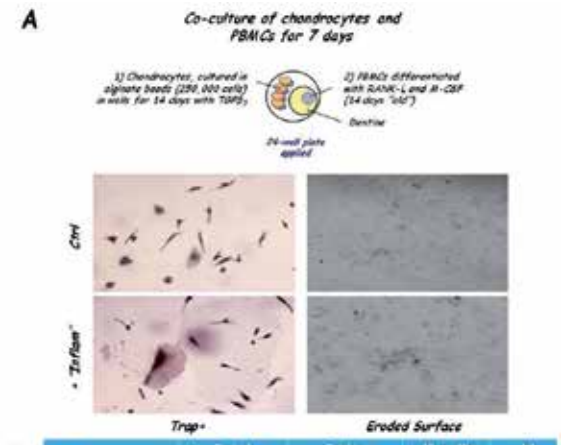
A



B

Parameters	Control (- TNFSM on days 15-21)	+ Cytokines (A)	+ All pro-MiRs (B)	+ (A) + (B)
Sox9	100	45.8	263	163
Collagen2a1 (%)	100	38.3	167	112
Aggrecan	100	45.6	188	134
Runx2	100	234	82	132
Rankl-L	100	287	112	106
OPG	100	34.0	156	133
Chisel score	7.0	3.2	7.9	6.5
Trap+ cells ≥ 3 nucl	12.1	22.1	8.2	13.2
Eroded surface	7.3	18.3	5.8	9.4
Mir-16	100	78.8	457	145
Mir-24	100	67.9	488	178
Mir-125b	100	56.8	512	154
Mir-149	100	112	445	298
Mir-328	100	18.2	565	88.9
Mir-339	100	24.7	512	112

C

Parameters	Control (- RANKL on days 15-21)	+ Cytokines (A)	+ All antagomirs (B)	+ (A) + (B)
Runx2	100	55.7	251	147
Collagen1a1	100	28.5	155	124
Osteocalcin	100	35.9	173	145
Osterix	100	64	182	132
Rankl-L	100	287	112	111
OPG	100	53.3	176	121
Mineralizing surface	47.0	23.1	95.3	72.7
Trap+ cells ≥ 3 nucl	13.3	27.3	14.8	15.1
Eroded surface	12.6	28.7	10.9	11.7
Mir-16	100	112	21.1	112
Mir-24	100	147	31.3	118
Mir-125b	100	118	21.7	114
Mir-149	100	112	18.5	92.2
Mir-328	100	82.9	16.9	117
Mir-339	100	108	17.9	112

Figure 4. A. Co-culture of chondrocytes in alginate beads (or osteoblasts in 2D culture) in standard medium (Gordeladze 2011). Osteochondral cells were differentiated from hMSCs in appropriate medium for 14 days. Then, a dentine slice, with close to 100% enriched peripheral blood monocytes (PBMCs) had been differentiated for 14 days with RANKL-L and M-CSF, was placed adjacent to the osteochondral cells, and both cell types were co-cultured for 7 days. At the end of the experiment, the number of TRAP-positive cells was counted, and the eroded dentine surface (%) was estimated in a reflecting light microscope. **B**. Co-culture of chondrocytes in alginate beads (or osteoblasts in 2D culture) in standard medium (Gordeladze 2011). The subject experimental setting was also applied to differentiating osteoblast. Here, we used the cytokine mixture as described in (B), and antago-microRNAs counteracting the os-

teochondral signature. Variables analysed were much like those for the experiment with chondrocytes. However, here we measured mineralizing surface (Alizarin red S coloration) induced by the osteoblasts in a 2D culture.**C**. Co-culture of chondrocytes in alginate beads (or osteoblasts in 2D culture) in standard medium (Gordeladze 2011). The above mentioned experimental setting was used with chondrocytes differentiated from hMSCs in alginate beads in the absence or presence of cytokines (IL-1, IL-6, IL-17, and TNFα), pre-microRNAs of all microRNA species of the osteochondral signature, or the set of cytokines along with the pre-microRNAs. Parameters analysed were: Q-PCR of transcription factors (TFs) and marker genes expressed by the chondrocytes, clinical score of alginate beads containing chondrocytes embedded in newly synthesized proteoglycan matrix, the number of Trap-positive osteoclasts, and the eroded dentine surface (%).

Termination: Sclerostin expression then fades or ceases, and the process of bone formation comes to a halt. The newly deposited osteoid is subsequently mineralized, the bone surfaces return to their resting state, This is when bone-lining cells intercalate with osteomacs, and the remodelling cycle is concluded (Raggatt and Partridge 2010).

3. Osteoclast differentiation and activation

3.1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease perpetuated by a dysregulated immune system. The cause remains grossly unknown, however both environmental and genetic factors are believed to contribute to RA development (Kochi 2010; Boissier 2011). The disease is characterized by joint inflammation, initially resulting in pain and swelling, and in a majority of the patients, erosion of bone and cartilage takes place (Lories 2011). The goal of current treatment regimens is to control inflammation, as well as to halt or retard the progression of damage of the bone structure of the affected joint(s), as measured by X-ray analysis (Tan and Conaghan 2011). The RA joint, synovial fluid, and synovium contain a large variety of hematopoietic cells, which are situated in direct proximity to the articular cartilage and its underlying bone structures (Carvalheiro, da Silva et al. 2012; Shegarfi, Naddafi et al. 2012). Hence, the synovial fluid with its contents of immune cells and cytokines secreted from the cells in question, undoubtedly are responsible for the joint-destructive process (Iwamoto, Okamoto et al. 2008; Yamada, Nakashima et al. 2008).

3.2. Osteoclast differentiation

Physiological and pathological bone resorption is achieved by the osteoclast. Osteoclasts are large 20 to 100 μm multinucleated cells containing from three to 100 nuclei with many mitochondria, lysosomes, dense granules, vesicles, and an extensive Golgi network required for the synthesis and secretion of factors required to degrade bone matrix and subsequently phagocytose the resorbed products (Holtrop and King 1977). Tartrate-resistant acid phosphatase (TRAP) (Minkin 1982), cathepsin K (Drake, Dodds et al. 1996), the calcitonin (CT) receptor (Hattersley and Chambers 1989), as well as $\alpha v\beta 3$ integrin (Davies, Warwick et al. 1989) are marker gene products of the mature osteoclast (Teitelbaum 2000). The initial event in bone resorption is the attachment of the mature osteoclast to the bone matrix. Cell surface $\alpha v\beta 3$ integrins bind to a plethora of extracellular matrix proteins synthesized and secreted by

osteoblastic cells, including vitronectin, osteopontin, and bone sialoprotein. Arg-Gly-Asp-containing peptides, Arg-Gly-Asp mimetics, and blocking antibodies to $\alpha v\beta 3$ integrins have been shown to inhibit bone resorption, both *in vitro* and *in vivo*, indicating that this integrin plays a pivotal role in osteoclast functioning (Horton, Taylor et al. 1991). Once attached to bone, the osteoclast generates an isolated extracellular, acidic microenvironment between itself and the bone surface by creating a sealing zone structure unique to the osteoclast.

3.3. Osteoclast activation

As described in a previous paragraph, macrophage CSF (M-CSF) and receptor activator of NFκB ligand (RANK-L) are the most important factors known to date to drive osteoclast formation and activity (Lacey, Timms et al. 1998; Quinn, Elliott et al. 1998). M-CSF serves as a survival factor for osteoclast precursors due to up-regulation of Bcl-XL, inhibition of caspase-9 activation (Woo, Kim et al. 2002), and the support of mature osteoclast survival by preventing apoptosis (Fuller, Owens et al. 1993). Exposure to M-CSF also stimulates receptor activator of NFκB (RANK) expression in osteoclast precursor cells, thereby allowing RANK-L to drive formation of mature osteoclasts (Arai, Miyamoto et al. 1999). RANK-L is a trans-membrane protein expressed by activated osteoblasts, synovial fibroblasts, as well as T-cells. It can also be proteolytically cleaved by TNF convertase (TACE) to generate a soluble molecule that has osteoclastic activity at distal sites (Hofbauer, Khosla et al. 2000; Li, Toraldo et al. 2007). RANK-L induced osteoclastogenesis is inhibited by OPG (osteoprotegerin), a soluble decoy receptor for RANKL, which is also produced by a variety of cells including osteoblasts, synovial fibroblasts, B-cells, and T-cells (Simonet, Lacey et al. 1997; Hofbauer, Khosla et al. 2000; Takayanagi, Iizuka et al. 2000; Gillespie 2007; Li, Toraldo et al. 2007). In the arthritic joint, there is a significant macrophage infiltrate, and the extent of the subject infiltration correlates heavily with the extent of joint erosion in both RA patients and animal models with arthritis (Yanni, Whelan et al. 1994). Synovial macrophages isolated from different types of arthritis may differentiate *in vitro* to fully functional osteoclasts subsequent to RANK-L stimulation as well as independently of the RANK/RANK-L signalling pathway, i.e. after short term exposure to TNFα and IL-1α (Adamopoulos, Sabokbar et al. 2006). Stimulatory, co-stimulatory, and/or inhibitory signals may be provided by adjacent T-cell species present in the inflammatory infiltrate.

4. T-cell subsets and their action on osteoclasts

4.1. Th17 cells versus Th1 and Th2 cells

The Th17 lineage has only recently been fully characterized (Adamopoulos and Bowman 2008) and the factors involved in its differentiation are still being identified and sorted out (Hirota, Hashimoto et al. 2007; Parsonage, Filer et al. 2008). When a (CD4+) naïve T cell is activated in the presence of transforming growth factor beta (TGFβ) plus IL-6 in the mouse or TGFβ plus an inflammatory stimulus in the human, the resulting clonal memory T-cell population will be instructed to produce the Th17 signature cytokines IL-17A, IL-17F, IL-22,

and (only in humans) IL-26 (Manel, Unutmaz et al. 2008). The inflammatory stimuli in the human setting can be IL-1β, IL-6, IL-21, and/or IL-23 (Langrish, Chen et al. 2005; Acosta-Rodriguez, Napolitani et al. 2007; Wilson, Boniface et al. 2007; Manel, Unutmaz et al. 2008; Volpe, Servant et al. 2008; Yang, Anderson et al. 2008). IL-17A is the only Th17 signature cytokine presently known to impact osteoclast biology. Synovial residing and synovial fluid derived macrophages are able to differentiate to fully functional bone-resorbing osteoclasts, and Th17- induced synovial macrophage–mediated osteoclast differentiation is believed to represent an important mechanism in bone destruction associated with rheumatoid arthritis (Fujikawa, Sabokbar et al. 1996; Adamopoulos, Sabokbar et al. 2006). Several types of IL-17A antagonists have been applied in a variety of experimental arthritis models to elucidate the efficacy of therapeutic IL-17A neutralization. Polyclonal anti-IL-17A antibody treatment after disease induction in the collagen induced arthritis (CIA) model diminished clinical scores subsequent to 10 days of therapy, compared with controls. Ankle and knee joints displayed reduced synovitis, cartilage destruction, chondrocyte cell death, depletion of proteoglycans, and bone erosion (estimated by both histology and X-ray analyses) (Lubberts, Koenders et al. 2004). Polyclonal anti-IL-17A antibodies also suppressed exacerbation of antigen-induced knee swelling, proteoglycan depletion, and bone erosion in experimental RA model systems upon reintroduction of the antigen (Koenders, Lubberts et al. 2005). The Th1 and Th2 cell lineages, of which the Th1 cell phenotype resembles that of Th17 cells, have interestingly proven to counteract the effect of Th17 cells on osteoclastogenesis, both *in vitro* and in experimental and idiopathic conditions of rheumatoid arthritis (Aarvak, Chabaud et al. 1999).

4.2. T-helper 17 cells in Rheumatoid Arthritis (RA)

A multitude of experimental reports point to Th17 cell mediated inflammation being associated with RA. IL-17A protein is located in both the synovium and the synovial fluid of patients suffering from the disease. It was also demonstrated that a subset of T-cell lines could be expanded *in vitro* from their RA synovium, which expresses both IL-17A and IFNγ [(Aarvak, Chabaud et al. 1999; Pene, Chevalier et al. 2008). Classical IFNγ-only Th1 cells were also present, constituting a clonal species which can be expanded from the RA synovium (Aarvak, Chabaud et al. 1999; Yamada, Nakashima et al. 2008). Exploratory medical studies, however, not only establish the association of IL-17A with disease development, but also implicate IL-17A with poor disease prognosis. The IL-17A message in synovial membrane biopsies was one factor (including TNF, IL-1β, and IL-10) being predictive for subsequent bone erosion and joint damage, as assessed by NMR imaging and X-ray analyses (Kirkham, Lassere et al. 2006). Taken together, these data support the notion that IL-17A is present in the inflamed synovium and that the levels of IL-17A expression correlate with poor prognosis and greater joint destruction. All in all, Th17 cells secrete master factors that can directly and indirectly drive osteoclast differentiation and activation. It is, however, a consensus in the field of T-cell research that signature cytokines of the Th1, Th2, and Treg lineages inhibit Th17 cell development. Therefore, one has construed that osteoclastogenesis is inhibited by the same Th1, Th2, and Treg lineage signature cytokines counteracting the stimulatory potential of the cytokine mixture secreted by the Th17 cells (Adamopoulos, Sabokbar et al. 2006; Adamopoulos and Bowman 2008). Contrastingly, when comparing clonal cells lines,

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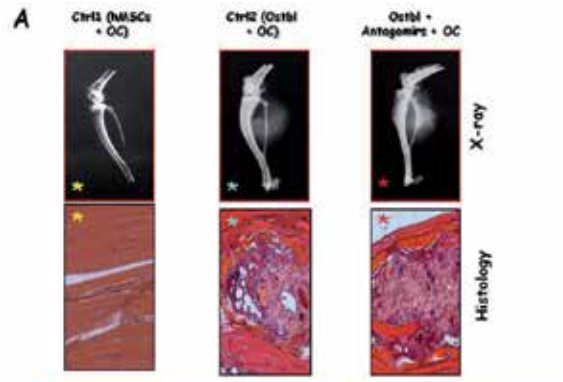
Th1 cells may resemble Th17 cells, questioning the postulate that the Th1 cell lineage consistently will counteract the impact of Th17 cells. In some instances, infiltration by Th1 cells may even potentiate the detrimental effect of Th17 cells on the cartilage and bone in affected joints (Yssel et al., unpublished data).



B

Parameters	Control	Chondrocytes + Osteoclasts	Chondrocytes + Cytokines + Osteoclasts	Chondrocytes + PreMirs + Cytokines + Osteoclasts
Collagen2a1	100	112	48.9	127
Aggrecan	100	96.8	28.7	138
Sox9	100	108	38.5	141
GAG/DNA	100	114	42.7	121
Ca^{2+} contents	Nd	Nd	Nd	Nd
Verified on X-ray	No	No	No	No
Calcitonin receptor	-	100	223	124
TRAP	-	100	312	88.9
Cathepsin K	-	100	288	109
Carbonic anhydrase II	-	100	232	86.9

C

Parameters	Control	Osteoblasts + Osteoclasts	Osteoblasts + Cytokines + Osteoclasts	Osteoblasts + AntagoMirs + Cytokines + Osteoclasts
Collagen1a1	100	113	53.8	98.8
Osteocalcin	100	127	33.6	123
Runx2	100	88.8	27.5	129
Osterix	100	95.9	22.1	112
Ca^{2+} contents	100	97.2	18.9	89.9
Verified on X-ray	Yes	Yes	Yes	Yes
Calcitonin receptor	-	100	267	88.8
TRAP	-	100	361	113
Cathepsin K	-	100	322	97.3
Carbonic anhydrase II	-	100	276	114

Figure 5. A. Effect of microRNA manipulations and cytokines on human osteochondral cells and osteoclasts injected into the tibial muscle of SCID mice. X-ray and tissue histology of the lower leg (*m. tibialis*) from SCID mice. Each tibial muscle was injected with hMSCs (2.5 million cells) and osteoclasts (0.5 million cells). Yellow, blue and red stars indicate

that X-ray analyses and histology (visual parameters) were not significantly altered when cells were manipulated by cytokines (IL-1, IL-6, IL-17, and TNFα). However, molecular parameters were dramatically changed. X-ray analyses and histology were performed on excised legs 21 days subsequent to cell deposition. **B**. Differentiated chondrocytes (21 days) exposed to cytokines (initial 14 days) or pre-microRNAs (initial 14 days) and osteoclasts injected into the tibial muscle of SCID mice. Molecular markers of chondrocytes and osteoclasts (PBMCs differentiated for 10 days) were analysed by Q-PCR or colorimetric methods (GAG = glucosaminoglycan/DNA). Nd = Not determined. All values are given relative to control conditions (chondrocytes alone or chondrocytes + osteoclasts). **C**. Differentiated osteoblasts (21 days) exposed to cytokines (initial 14 days) or antago-microRNAs (initial 14 days) and osteoclasts were injected into the tibial muscle of SCID mice. Molecular markers of osteoblasts and osteoclasts (PBMCs differentiated for 10 days) were analysed by Q-PCR or colorimetric methods (Ca2+-analyses in HCl-extracts). All values are given related to control conditions (osteoblasts alone or osteoblasts + osteoclasts).

5. Cell-to-cell communication through exosomes

Cell-to-cell communication is required to guarantee proper coordination among different cell types within tissues. Cells may communicate by soluble factors (Majka, Janowska-Wieczorek et al. 2001), adhesion molecule mediated cell-to-cell interactions including cytonemes connecting neighbouring cells enabling ligand–receptor-mediated transfer of surface-associated molecules, or by tunnelling nanotubules which establish conduits between cells. These would allow the transfer of both surface molecules and cytoplasmic components (Rustom, Saffrich et al. 2004; Sherer and Mothes 2008). Recently published studies have suggested that cells may also communicate by spherical membrane fragments called micro-vesicles (MVs) or exosomes (Ratajczak, Wysoczynski et al. 2006). MVs or exosomes were since long considered to be inert cellular debris, and the frequently observed vesicles by electron microscopy in the interstitial space of tissues or in blood were considered the consequence of cell damage or a result of dynamic plasma membrane turnover (Siekevitz 1972). It was suggested that the circular plasma membrane fragments released from human cells might possibly result from a specific process, and it was shown that they carried functional membrane enzymes in the same ratio as the membrane of the cells they originated from (De Broe, Wieme et al. 1977). However, only a few recent studies have assigned a defined function to the vesicles/exosomes released to the microenvironment by various cell types. Two distinct processes of shedding vesicles from the cells have been described. MVs may originate from the endosomal membrane compartment that, subsequent to fusion with the plasma membrane, is extruded from the cell surface of activated cells as exosomes (Heijnen, Schiel et al. 1999; Rozmyslowicz, Majka et al. 2003). Otherwise, MVs may take origin by direct budding from the cell plasma membrane as shedding vesicles (Cocucci, Racchetti et al. 2009).

Released MVs may remain in the extracellular space in the proximity of the place of origin, or they may enter into the circulation. One has hypothesized that, under normal healthy conditions and disease states, micro-vesicles contain microRNAs, contributing to biological homeostasis in general (Hunter, Ismail et al. 2008; Gordeladze, Djouad et al. 2009; Gordeladze 2011). It may also be hypothesized that MVs or exosomes, when present in disproportional amounts within a tissue during development of certain diseases, in fact may affect all adjacent cell types, thus altering their phenotypes and main functional properties (Camussi, Deregibus et al. 2010).

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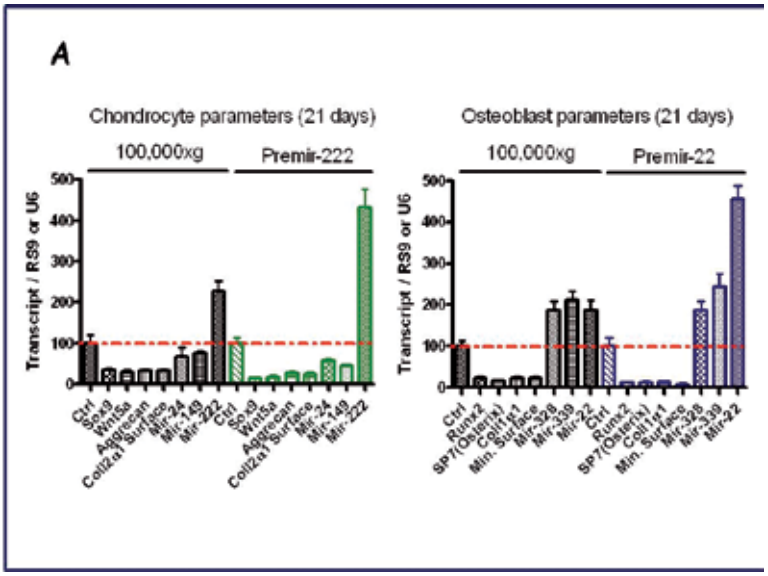

B

Parameters	Control	Osteoblasts + Osteoclasts	Osteoblasts + Exosomes + Osteoclasts	Osteoblasts + AntagoMirs + Exosomes + Osteoclasts
Collagen1a1	100	113	29.9	121
Osteocalcin	100	127	27.1	97.1
Runx2	100	88.8	29.9	119
Osterix	100	95.9	17.9.1	123
Ca^{2+} contents	100	97.2	18.9	90.1
Verified on X-ray	Yes	Yes	Yes	Yes
Calcitonin receptor	-	100	227	112
TRAP	-	100	313	88.6
Cathepsin K	-	100	271	107
Carbonic anhydrase II	-	100	239	107

Figure 6. A. The effect of exosomes-containing 100,000xg fractions of supernatants derived from activated human Th17 cells on human chondrocytes and osteoblasts in the presence and absence of osteoclasts. Chondrocytes (500,000 cells) derived from hMSCs were either exposed to 1 ml volume containing exosomes from 250,000 Th-17 cells or pre-microRNA 222 (which is highly expressed in Th17 cells, but not in chondrocytes) and analysed for marker gene and microRNA expressions using standard Q-PCR methods (Ambion) or immunohistochemistry (aggrecan and collagen2α1 positive surfaces). Osteoblasts (500,000 cells) derived from hMSCs were either exposed to 1 ml volume containing exosomes from 250,000 Th-17 cells or pre-microRNA 22 (which is highly expressed in Th-17 cells, but not in

osteoblasts) and analysed for marker gene and microRNA expressions using standard Q-PCR methods (Ambion) or colorimetric methods (mineralized surface visualized by Alizarin red S). **B**. The effect of exosomes-containing 100,000xg fractions of supernatants derived from activated human Th17 cells on human chondrocytes and osteoblasts in the presence and absence of osteoclasts. Differentiated osteoblasts (21 days) exposed to exosomes from Th17 cells (initial 14 days) or antago-microRNAs (initial 14 days) and osteoclasts were injected into the tibial muscle of SCID mice, and molecular markers of osteoblasts and osteoclasts (PBMCs differentiated for 10 days) were analysed by Q-PCR or colorimetric methods (Ca^{2+}-analyses in HCl-extracts). All values are given related to control conditions (osteoblasts alone or osteoblasts + osteoclasts).

In one publication, it was shown that exosomes from healthy volunteers matched mononuclear cells and contained 420 known mature microRNAs (Hunter, Ismail et al. 2008). Hierarchical clustering of the data sets pointed to significant differences in microRNA expression between peripheral blood mononuclear cells (PBMCs) and plasma micro-vesicles. It was observed that 71 microRNAs co-expressed between micro-vesicles and PBMCs. Prediction of the gene targets and associated biological pathways regulated by the detected microRNAs demonstrated that the majority of these microRNAs expressed in the micro-vesicles from the blood were predicted to regulate the differentiation of blood cells and their metabolic pathways (Hunter, Ismail et al. 2008). Interestingly, a small group of these microRNA species were also predicted to serve as important modulators of immune function. The microRNAs in question were hypothesized to be taken up by adjacent cells, and thus it may be asserted that exosomes from Th17 cells, known to be present in vast numbers in articular fluid of patients with rheumatoid arthritis (Adamopoulos and Bowman 2008; Nakashima and Takayanagi 2009; Raggatt and Partridge 2010; Nakashima, Hayashi et al. 2012) with rheumatoid arthritis, may disturb the phenotypic characteristics of osteochondral cells in inflamed joints.

6. Results and discussion

6.1. How does the osteochondral microRNA profile work?

In Fig. 1, we have presented a microRNA signature, consisting of six microRNA species. These species have been selected amongst microRNAs putatively targeting one or several of 14 transcription factors (TFs) deemed important for osteoblast differentiation from stem cells or precursor cells (Gordeladze, Djouad et al. 2009). It was shown through reporter constructs, that nine of these 14 TFs were targeted by the six microRNAs. Literature studies further indicated that mir-149, and possibly also mir-24, may serve as a switch mechanism instrumental in determining whether stem cells will become osteoblasts or chondrocytes by interfering with activators, inhibitors and associated proteins instrumental in modulating the effects of Sox9 and Runx2 (lee legend to Fig. 1). These two TFs oppose each other when it comes to differentiation of stem cells or progenitor cells to become mature osteoblasts and chondrocytes (Marie 2008; Gordeladze, Djouad et al. 2009).

A bioinformatics study (see Fig. 2, A-C), using the Mir@nt@n algorithm (Le Bechec, Portales-Casamar et al. 2011), showed that the microRNA species mir-149, mir-328, and mir-339 in particular seemed to serve important regulatory roles when looking at some important criteria for osteoblastogenesis. (A) JUN = transcription factor AP-1, known to be mandatory for

osteoblast differentiation (Gordeladze, Djouad et al., 2009), appears to exert a major impact on mir-339, and mir-149 in a hierarchical feed-forward fashion. (B) ETS1 and SP3, two of the 14 TFs constituting the selection criteria for the osteochondral microRNA profile (Gordeladze, Reseland et al., 2011), appeared to serve as inductors of several microRNAs, among which we find the microRNA species mir-149, mir-328, and mir-339. However, ETS1 and SP3 (along with SP1 and SP7 = Osterix) are up-regulated in osteoblastic cells. Hence, the positive control exerted by ETS1 and SP3 may be counterproductive, or maybe not? (C) In a more complicated hierarchical feed-forward network, it can be noted that Sox9, an important regulator of chondrogenesis, actually serves as a master regulator of mir-149 and mir-328, which both are up-regulated in chondrocytes. When modelling the osteochondral microRNA profile with all know factors of the canonical WNT-pathway (using all factors from the KEGG's pathway), a stunningly close interaction between some of the microRNA species of the profile and factors discerning the osteoblast from the chondrocyte phenotypes emerged.

It may therefore be postulated that the presently described osteochondral microRNA profile serves as a discriminator between osteoblasts and chondrocytes, and can be used as such in the process of cell engineering, while the microRNAs published as markers for osteoblasts by G. Stein and his collaborators distinguish osteoblasts from all other cell phenotypes in a comprehensive manner (Li, Hassan et al. 2008; Hassan, Gordon et al. 2010; Zhang, Xie et al. 2011).

6.2. Summary of experiments proving the functionality of the osteochondral microRNA profile

In Fig. 3, we list the results of some experiments where the endogenous levels of each microRNA species of the profile are altered. These experiments indicate that chondrocytes show optimal microRNA profile levels, since some 6-8 fold increase do not alter their characteristics, subsequent to differentiation from hMSCs. A reduction to 12-14% of their endogenous levels shows that mir-16, mir-125b, mir-328, and mir-339 are necessary for chondrogenesis, while mir-24 and mir-149 are not. Contrastingly, enhancing the endogenous levels of all the microRNA species in hMSCs more or less block their differentiation towards the osteoblast lineage. As expected, reducing their levels in hMSCs by a factor of 2-2.5 clearly fortified their phenotype as osteoblasts. This experiment establishes the six microRNAs as instrumental in osteochondral differentiation, while mir-24 and mir-149 play a dual role, being able to simultaneously block osteoblastogenesis and stimulate chondrogenesis. An explanation for this dual role has been sought in the information conveyed in Figs. 3A&B, looking for published data on their known targets, as well as bioinformatics profiles, i.e. positions in feed-forward interaction networks (see legend to Fig. 3).

6.3. Osteochondral cells influence osteoclasts: The impact of cytokines

In Fig. 4A, we depict a co-culture system where hMSCs are differentiated towards osteochondral cells for 2 weeks and co-cultured with differentiated osteoclasts on dentine for a third week. The osteoclasts have been differentiated from hPBMCs for 1 week with RANK-L and

M-CSF. It is clear that chondrocytes do stimulate osteoclasts to resorb bone, and that exposure of the chondrocytes to cytokines ("artificial" synovial fluid from individuals with rheumatoid arthritis = RA, i.e. growth medium containing IL-1, IL-6, IL-17A and TNFα) enhances the osteoclasts' bone resorbing potential.

6.4. MicroRNA manipulation of osteochondral cells: Obliteration of the impact of cytokines

In Figs. 4B&C it is shown that TGFβ_3-stimulated hMSCs to chondrocytes or BMP2-stimulated hMSCs to osteoblasts exposed to cytokines in fact are protected against loss of phenotypes and normalize (i.e. reduce) their ability to enhance osteoblast activity upon normalization of their endogenous microRNA profile. The tables show data for Q-PCR of phenotype marker genes, RANK-L and OPG (counteracting each other), functional markers like clinical score (constituted by chondrocyte bead histology, immunohistochemistry, distance between cells, GAG-contents), mineralizing surface, percentage of TRAP-positive cells containing ≥ 3 nuclei, eroded dentine surface (%), as well as Q-PCR of microRNAs of the osteochondral signature.

6.5. In vivo model of osteochondral cells interacting with osteoclasts

In Fig 5A, X-ray pictures and biopsy histology of tibial muscles of SCID mice are shown. Their muscle tissue has been injected with hMSCs, osteoblasts without and with the presence of differentiated osteoclasts. The osteoblasts were either loaded with antago-microRNAs of the osteochondral signature or not. Additional experiments where hMSCs or osteoblasts had been exposed to cytokines alone or cytokines and antago-microRNAs together are not shown on the figure. A similar experimental matrix was conducted (but not shown) with chondrocytes differentiated from hMSCs. The pictures indicate that mature osteoblasts and osteoclasts are able to build bone within the muscle tissue, and that a suppression of the endogenous levels of the microRNA species of the osteochondral signature lead to enhanced mineralization of osteoid produced.

When chondrocytes (see Fig. 5B) are pre-exposed to osteoblasts, it appears that the cells are able to maintain their net cartilage-building potential, possibly by down-regulating their cytokine-induced osteoclast-activating potential. The same phenomenon is also seen when osteoblasts are injected into the *m. tibialis* of SCID mice.

Cytokines induce a reduction in osteoblast functions, while also enhancing osteoclast activation resulting in a lessened bone deposit, while introduction of antago-microRNAs reverses the action of cytokines towards maintenance of the bone-building capacity of the osteoblast (see Fig. 5C). A special attention to the Q-PCR data on osteoclast specific markers (CT receptor, TRAP, cathepsin K and CA II) analysed in the excised *de novo* bone tissue is warranted. Furthermore, it should be emphasized that the X-ray analyses of the lower legs injected with osteoblasts were corroborated analysing the Ca^{2+}-contents in acid extracts/digests, using standard spectrophotometry.

6.6. The effect of exosomes derived from activated Th17 cells

Lastly, we shall address the hypothesis put forward that exosomes shedded from immune cells may affect the characteristics of osteochondral cells. Th17 cell exosomes-like particles were obtained from 100,000xg centrifugates of cultures of activated Th17 cells (Larsen, Arnaud et al. 2011). The pellets were examined for microRNAs (micro-arrays), and 55 microRNA-species were identified. Of these, hsa-mir-22- and hsa-mir-222 are especially interesting, since the exosome-fraction contained large quantities of these, and because Th17 cell clones also produced large quantities of the same microRNA species, and since osteoblasts and chondrocytes expressed relatively low levels of the mir-22, and mir-222 species, respectively.

Fig. 6A depicts chondrocytes having been differentiated from hMSCs for 2 weeks receiving control medium or control medium + Th17 cells derived exosomes or pre-microRNA mir-222 on days 15-21. Q-PCR of marker genes and microRNA transcripts, as well as collagen2α1-positive surface analyses were conducted and showed that chondrocyte characteristics were suppressed. The same experiment was conducted on hMSCs differentiated towards osteoblasts in the absence or presence of Th17 cell exosomes or pre-microRNA mir-22, and phenotype marker analyses (i.e. Q-PCR of marker gene transcripts and mineralized surface) conducted. Not surprisingly, the osteoblast phenotype was suppressed due to both treatments. This indicates that exosomes from Th17 cells contain microRNAs detrimental to the functioning of mature chondrocytes and osteoblasts, and it may well be so that engineered osteochondral cells, deposited within a joint to replace damaged tissues, should be made resilient to inflammatory cells to maintain their proper phenotypic functioning over time.

One experiment (see Fig. 6B) with osteoblasts, osteoclasts, exosomes and antago-microRNAs of the osteochondral signature was performed on SCID mice, as envisaged earlier. X-rays (not shown) and analyses on excised biopsies from the tibial muscle showed that exposure to Th17 cell exosomes hampered the development of the matrix-producing and mineralizing properties of osteoblasts, while enhancing activating of adjacent osteoclasts. The introduction of antago-microRNAs of the osteochondral signature did, however, reinstall the osteoblasts' ability to produce bone, which was not degraded by osteoclasts. This phenomenon was corroborated through analyses of the Ca^{2+}-contents of the biopsies taken (see also text-Fig. 5B).

6.7. Other results obtained

The present series of experiments also addressed whether certain scaffold materials and their 3D-structures were better suited to produce osteochondral cells with a "true" (*in vivo* like) phenotype being more robust and resilient towards provocations, such as chronic disease states. Whether we used scaffolds, i.e. alginate beads instead of micro-pellets, or hydroxyapatite instead of 2D-growth, really did not matter. However, we did not use promising scaffolds for osteoblast differentiation, like TiO_2 or surfaces with an inverted profile of cellular outer membrane surface nanostructures (Muys, Alkaisi et al. 2006; De Smet, Jaecques et al. 2007; Mazzola, Bemporad et al. 2011).

6.8. General discussion of the findings presented

This report is more about experimental approaches and results speaking for themselves than a lengthy discussion of their implications from a theoretical point of view. A few issues do, nevertheless, deserve attention.

Most reports on microRNA impact on cell differentiation and phenotype development and stability focus on one microRNA species only, or they present a microRNA micro-array showing a bulk of microRNAs being up- and down-regulated subsequent to cell differentiation. Since microRNAs exert different binding kinetics when they bind to different mRNAs at various places (not only to the 3'-UTR, but also to the 5'-UTR region) (Moretti, Thermann et al. 2010), it is reasonable to believe that a minimum effective group of microRNAs is necessary and sufficient to ensure complete differentiation and stability of a certain cell phenotype (Gordeladze, Djouad et al. 2009; Iorio, Casalini et al. 2011; Schoof, Botelho et al. 2012). The osteochondral signature of microRNAs presented in this chapter may serve as such a group, discriminating between cell types, however, several other microRNA species described by G. Stein and collaborators (Li, Hassan et al. 2009; Hassan, Gordon et al. 2010; Zhang, Xie et al. 2011) may serve the same purpose. In fact, our microRNA micro-arrays obtained from osteoblastic cells in different stages of differentiation from hMSCs do overlap extensively to the ones published in recent literature (Lian, Javed et al. 2004).

Which microRNAs are to be construed as the "true" marker for osteoblasts? They may constitute more than the six species presented here, or even other than the ones we have focussed on. The main issue is how they interact with feed-forward and feed-back regulatory loops, in which transcription factors and marker genes are positioned. This issue has been addressed in the bioinformatics exercises outlined in Fig. 2. Here, mir-26b pops up as being important, and this microRNA is known to a network of factors serving as a possible target for RNA-based therapy of bone diseases (Luzi, Marini et al. 2012). The main issue is: which microRNAs out of a smaller group will be necessary and sufficient to determine or alter a certain phenotype? Our experiments with the present osteochondral microRNA signature seem to render one such microRNA group distinguishing between osteoblasts and chondrocytes. A further proof of concept put forward is the fact that these microRNAs can be applied to trans-differentiate osteoblastic cells to chondrocytes and back again, and they are also able to a large extent (some 85%) substitute growth factors (TGFs and BMPs) as mandatory to obtain chondrocytes and osteoblasts from hMSCs or human adipose tissue derived stem cells (hADMSCs) (Gordeladze, JO, unpublished results).

In chapter 2 of the book "Regenerative Medicine and Tissue Engineering: Cells and BioMaterials" (Gordeladze 2011), we introduced a concept of compatibility score between microRNA micro-arrays and transcriptomes obtained from chondrocytes and osteoblasts differentiated from precursor cells of stem cell origin (ref). We still believe that a proper way to ensure "correct" phenotype is to correlate consensus micro-arrays of marker gene transcripts with microRNA micro-arrays; however, such analyses were not performed on the present manipulated biological material. Due to relative meticulous experimentation consuming a lot of

manpower, biological material and costly analyses, it was not performed. However, microarrays analysed at certain intervals during a differentiation process may seem necessary to be able to arrive at the superior microRNA profiles describing a certain cell phenotype to be distinguished from other phenotypes.

One question pertaining to the assurance of a reproducible process of establishing engineered cells with the "correct" phenotype is: Why play with microRNAs, is it not enough to manipulate using growth factors and the 3D-structure, in which the precursor cells are grown? Firstly, it is important to know the interplay between factors able to induce a certain cell phenotype and/or trans-differentiate phenotypes. In this respect, it seems fruitful to concentrate on several microRNAs, and not only on one microRNA species. But, it is also reasonable to play with the expression of certain transcription factors (TFs) being positioned in key places in a regulatory network constituted by TFs, activators, inhibitors or associated proteins, as well as functional marker genes. Sox9, a TF deemed sufficient and necessary for chondrocyte development (see legend to Fig. 2C) may be over-expressed or suppressed to engineer chondrocytes or osteoblasts, since it affects the expression of important microRNAs and marker genes in a hierarchical fashion. Apparently, so do ETS1 and SP3, or MYC and JUN (see legend to Figs. 2B&A).

However, it seems reasonable to believe that, at least during a shorter, critical period of time, externally added growth factors and the selection of biologically "correct" scaffold structures and materials will aid in the process of proper phenotype acquisition for tissue replacement purposes. It has also been pointed out that it is vital, for a proper tissue to be generated, to use cell material which can differentiate into several cell types constituting a certain tissue, or that the engineered cell material is able to recruit cells from the ambiance to produce a correctly functioning tissue *in vivo*. A good example of this seems to be an artificially made tracheal epithelium, using collagen vitrigel-sponge scaffolds containing bFGF as growth factor and chemotactic agent (Tani, Tada et al. 2012).

We are now seeking to produce polycistronic constructs containing pre-microRNAs or antago-microRNAs to be temporarily activated in order to test whether the microRNA-based concept of cell engineering may function in an *in vivo* animal model of rheumatoid arthritis, where articular joints are inflamed and contain immune cells (i.e. Th17 cells). Hence, we will be able to find out whether osteochondral cells may be engineered to withstand the detrimental effect of cytokines and exosomes containing (amongst many bioactive molecules) unwanted microRNA species. Promising small animal models to start with are collagen-induced arthritis in the mouse, and the senescence mouse (Aigner, Rose et al. 2004; Manolagas and Almeida 2007) prior to the testing in larger animals (i.e. sheep model systems) (Gordeladze, Reseland et al. 2009).

Lastly, the present experiments confirm the complexity of bone remodelling in the healthy individual and in patients with diseases affecting cartilage and bone structure. Not only hormones, like active vitamin D (calcitriol) and PTH affect bone turnover, via their dual effects on osteoblasts and osteoclasts. The interaction with the immune system directly interfering with the balance between RANK-L and OPG (Adamopoulos, Sabokbar et al. 2006; Nakashima,

Hayashi et al. 2012), as well as cell-to-cell communication through exosomes, containing a plethora of bioactive molecules, urges new research approaches to unravel the intricate mechanisms ensuring bone and cartilage health before and after the onset of disease states damaging the tissues in question. We have here confirmed that chondrocytes, in fact, do affect osteoclasts directly (Xiong, Onal et al. 2011), and that cytokines obtained from Th-cells, and Th17 cells in particular, are detrimental to the osteochondral phenotypes, with an additional activation of osteoclasts.

The present experimental setting also show that exosomes from Th17 cells may interfere with both the chondrocytic and osteoblastic phenotypes in a negative fashion (i.e. phenotype acquisition and matrix deposition), while also speeding up bone remodelling via over-activation of osteoclasts embedded in the bone adjacent to the cartilage lining. These findings are consistent with the development of cartilage loss and the appearance of osteophytes (newly formed bone in disarray) in arthritic joints due to the progressing autoimmune process characteristic of rheumatoid arthritis (Hayashi, Xu et al. 2012).

7. Summary and future prospects

This particular report entails the use of transient microRNA manipulations to ensure acquisition of proper osteochondral phenotypes when engineering cells to replace damaged bone and cartilage in patients with inflammatory diseases targeting articular joints (in particular rheumatoid arthritis). We have shown that cell engineering, as a research field, needs to take into consideration how osteochondral cells affect osteoclasts directly, and that osteochondral cells may lose their acquired phenotypes upon exposure to cytokines (e.g. IL-1, IL-6, IL-17, and TNFα) or micro-RNA-containing exosome-like particles derived from activated Th17 cells. These detrimental effects can be counteracted by manipulating stem cell microRNA contents (the optimal minimal number and species of microRNAs are yet to be defined).

When refining the search for the minimal number of effective microRNAs, it is recommended that bioinformatics approaches are used along with micro-RNA micro-arrays and marker gene transcriptomes in engineered osteochondral cells, and that maximal compatibility score (Gordeladze 2011) between them are obtained. Assessment of phenotypes obtained should include analyses of how and to which extent these cells affect osteoclasts, and whether altered (i.e. enhanced) remodelling of bone formed within an *in vivo* model system (e.g. calcium deposits in the tibial muscle of SCID mice) of choice.

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Chapter 21

Tissue Engineered Animal Sparing Models for the Study of Joint and Muscle Diseases

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Additional information is available at the end of the chapter

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1. Introduction

The aim of this book chapter is to highlight the fact that regenerative medicine and tissue engineering have important consequences for animal research and can be exploited to develop powerful animal sparing *in vitro* models. The main objective of the authors is to encourage researchers to consider designing, developing, utilizing and exploiting biomimetic alternatives to *in vivo* animal models. This chapter will focus on some of the most popular *in vitro* models that have been developed for skeletal muscle and articular cartilage. Refining these animal-sparing models will advance tissue engineering and regenerative medicine and may replace animal models and significantly reduce our dependence on animals in research. Over the last few decades, a number of different animal models have been developed to support preclinical and clinical research to address hypothesis or curiosity driven research. However, many of the currently available animal models are not suitable models for human disease. 'Evidence-based medicine' refers to the ability to locate, critically appraise and incorporate evidence into clinical practice. This important branch of medicine seeks to assess the strength of evidence of the risks and benefits of treatment (or lack of treatment) and relies on well-established and reliable models to facilitate the research that underpins the decision-making process. Clinical research and evidence-based medicine therefore rely on the availability of reliable model systems that can be used for translational research. Using *in vitro* culture models eliminates some but not all of the problems associated with clinical research with human subjects and animal models of disease. In many cases culture models help to overcome barriers and allow researchers to gain a better understanding of disease pathogenesis, characteristics and responses to treatments. This chapter will focus exclusively on animal sparing *in vitro* models of muscle and joint tissues that have been developed to mimic these musculoskeletal structures. Many of these models are well established and possess similar morphological, histological, biochemical

and molecular characteristics of muscle and joint tissues *in vivo*. These *in vitro* models allow researchers to perform original, hypothesis-or curiosity-driven research without using animals while still accurately mimicking musculoskeletal tissues in real life. *In vitro* models also enable screening for new drugs to treat musculoskeletal diseases in a more efficient and cost-effective way such that real progress is achieved more quickly.

2. Biomimetic models

Tissue engineering is an interdisciplinary field that applies the principles of biology and engineering to the development of functional substitutes for damaged tissue [1]. The loss or failure of an organ or tissue is one of the most frequent, devastating, and costly problems in human health care. By applying the basic principles of engineering and cell biology tissue engineering is helping us to move toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ [2]. The new tissues can be used as test beds in basic research and development and have potential for future use in transplantation and reconstructive surgery [3].

Biomimetics is defined as the study of the structure and function of biological systems as models for the design and engineering of biomaterials. The term biomimetics was coined by Otto Schmitt in the 1950s for the transfer of ideas and analogues from biology to technology [4]. It generally refers to human-made and engineered processes, substances, devices, or systems that imitate and therefore *mimic* natural processes and biological systems. It extends to the study of the formation, structure and function of biological substances and materials (i.e. enzymes, polymers, surfaces) and biological mechanisms or processes (i.e. catalysis, protein synthesis) aimed at synthesizing similar products by artificial mechanisms that mimic natural ones. Therefore, a biomimetic model aims to *mimic* the structure and function of a biological system in its structure or function.

Organ, tissue and cell culture have been used for decades as biomimetic models of cells, tissues and organs. The pioneering work of eminent scientists such as Sydney Ringer, Wilhelm Roux and Ross Harrison from 1880 to the early 1900s helped to establish the principles and methodology of tissue culture. Ringer developed salt solutions containing the chlorides of sodium, potassium, calcium and magnesium suitable for developing organ culture. He used this model to maintain the beating of an isolated animal heart outside of the body. Roux maintained a portion of the medullary plate of an embryonic chicken in a warm saline solution for several days, establishing the principle of tissue culture. Harrison published the first paper that successfully introduced tissue culture to settle the argument of how nerve fibres originated [5]. Although other scientists had examined cells *in vitro* several decades earlier, none of them had successfully manage to culture them and it was Harrison who first successfully overcame basic culture problems and created a technique that other investigators could follow [6]. The first permanent fibroblast-like cell line was developed by Earle in 1943 from subcutaneous mouse tissue [7]. This allowed the development of techniques for generating the first human "transformed" cell line, the HeLa cell, derived from a cervical carcinoma [8]. This resulted in the

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advent of cell and tissue culture. This technique is arguably one of the best and earliest examples of biomimetics and has become one of the foundations of modern biomedical research. It has been used for decades to grow tissues and cells isolated from living organisms. Tissue culture generally refers to the growth of human or animal cells *in vitro*. However, the term tissue culture can also be used to refer to the culturing of pieces of tissue, i.e. explants or whole organs in culture. It is an invaluable tool for the study of cell biology and pathology.

Organ, tissue and cell culture are powerful reductionist techniques that have allowed us to study the function of biological systems. However, they are not suitable models for every type of biological question. For example results from cell culture are often not comparable to those derived from *in vivo* studies using whole animals e.g. in studies of drug action and metabolism since the drugs are metabolized *in vivo* but not *in vitro*. Furthermore, primary or derived culture is rarely able to accurately model the physiological functions of an organ because of the difficulties associated with re-creating neural, circulatory and endocrine signals *in vitro*. Nevertheless, tissue culture remains a powerful technique and has many animal sparing applications.

3. Biomimetic models and their potential for replacing, refining and reducing laboratory animal models in research

The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) based in London, UK recently carried out some research to demonstrate that poor or incomplete reporting of studies that use animals have made it difficult to derive the maximum scientific knowledge from animal research. In addition, over the last few decades many research laboratories in academia and industry have used laboratory animals unnecessarily. A survey published by the National Institutes of Health (NIH) Office of Laboratory Animal Welfare (OLAW)[1], and the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs[2]) has demonstrated that many peer-reviewed scientific publications reporting publicly funded animal research from the UK and US lacked key information on how the study was designed, conducted and analysed, which could limit their value in informing future scientific studies and policy. In response to these findings the NC3Rs has recently published the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines [9], which were specifically introduced to improve reporting of animal experiments. These new guidelines have been published in PLoS Biology and several other prestigious scientific journals. Many biomedical research organisations and international scientific societies have already begun to implement these guidelines. The guidelines were developed by the NC3Rs to improve standards of reporting and ensure that the data from animal experiments can be fully evaluated and utilised. The ARRIVE guidelines consist of a 20-point checklist of the essential information that should be included in publications reporting animal

1 http://grants.nih.gov/grants/olaw/

2 http://www.nc3rs.org.uk/

research. Developed in consultation with the scientific community, including researchers, statisticians, journal editors, and funders, the guidelines request essential information that should be included in publications reporting animal research. Essentially the guidelines are intended to improve reporting of research using animals; guide authors as to the essential information to include in a manuscript, and not be absolutely prescriptive; be flexible to accommodate reporting a wide range of research areas and experimental protocols; promote reproducible, transparent, accurate, comprehensive, concise, logically ordered, well written manuscripts and improve the communication of the research findings to the broader scientific community. The UK's major bioscience funding bodies, including the Medical Research Council, the Biotechnology and Biological Sciences Research Council, Department for Environment, Food and Rural Affairs (Defra[3]), the Natural Environment Research Council (NERC[4]), the European Research Council (ERC[5]) and the Wellcome Trust[6], have all incorporated adherence to the guidelines into a revised version of 'Responsibility in the use of animals in bioscience research: Expectations of the major research council and charitable funding bodies', which was originally published in May 2008.

Although the ARRIVE guidelines are primarily aimed at scientists writing up their research for publication and for those who are involved in peer review and intended to improve standards of reporting data from animal experiments, they have also highlighted the potential of biomimetic and tissue engineered *in vitro* models for replacing, refining and reducing the use of laboratory animals in research.

4. Biomimetic models of muscle

Cells - Skeletal muscle cells can be cultured *in vitro* following their isolation from muscle tissue. The main advantage of *in vitro* cultures of skeletal muscle precursor cells (MPCs) is the faithful recapitulation of the events that occur *in vivo* during development and regeneration whereby mononuclear MPCs fuse together under the correct conditions to form multinucleate myotubes - essentially nascent muscle fibres. Skeletal muscle development occurs in the somites; segments of paraxial mesoderm which form on either side of the neural tube [10], which is further divided into the epaxial and hypaxial dermomyotome [11]. Cells in the dermomyotome migrate and form the myotome, and transiently express the paired box transcription factors Pax 3 and 7, and Muscle Regulatory Factors (MRFs) Myf5, MyoD and Myogenin [12], which regulate myotube formation. The expression of these transcription factors has been shown to be critical for 'normal' muscle formation during development [13, 14] and null mutations of some of these genes can be lethal during the early post-natal period [15, 16]. Whilst the myotome gives rise to the musculature of the trunk, Pax 3+ cells migrate out of the hypaxial

3 http://www.defra.gov.uk/

4 http://www.nerc.ac.uk/

5 http://erc.europa.eu/

6 http://www.wellcome.ac.uk/

dermomyotome to the limb bud where they form the musculature of the limbs [10] under the influence of MRFs.

A subset of Pax 7/3+ cells have been identified in the limb buds and myotome in development, but fail to differentiate to form myotubes [17]. These proliferating precursor cells can adopt a position between the developed myofibre and the basal lamina during later muscle development [17, 18], which strongly indicates that this subset of progenitors form the satellite cells of post-natal skeletal muscle.

Satellite cells are the resident stem cells of skeletal muscle tissue, which provide additional nuclei to a muscle fibre during regeneration, such as may occur following muscle injury, damage or overload. Myonuclear addition is important in order for the muscle to effectively synthesise new proteins and thus adapt to specific stimuli, as well as generally maintain and increase its mass. Satellite cells are so called due to their anatomical location between the sarcolemma and basal lamina of a muscle fibre [19], and this parameter was for some time the only true 'marker' of these cells, achievable via electron microscopy. More recently however, a host of proteins which are expressed by satellite cells have been identified, including Pax 7 [20], Caveolin-1 [21] and myf-5 [22], and thus a molecular signature of these cells is being derived.

Satellite cells lie quiescent on the muscle periphery until becoming activated upon muscle damage/overload. The precise cause of satellite cell activation remain elusive[7]. However the fact that muscle injury leads to activation suggests that muscle damage itself may be the trigger or a factor secreted from the muscle may provide the initial cue [23]. The latter notion has support from the fact that Insulin Like Growth Factor-I (IGF-I) and indeed Hepatocyte Growth Factor (HGF) are released from the muscle following mechanical use or injury [24-29] and have been shown to activate satellite cells when exogenously administered [30-35]. Once activated, satellite cells proliferate extensively and express the transcription factors myf-5 and MyoD [36] as well as proteins specific to the myogenic lineage such as Desmin, before fusing with the damaged/overloaded fibres under the influence of myogenin [37] to provide additional myonuclei. As stem cells, satellite cells have the ability to self-renew and replenish the pool of quiescent cells following activation. The literature suggests that asymmetric distribution of a number of potential proteins is implicated in regulating self-renewal [38-40], causing some daughter cells to express pax 7 and MyoD, and thus commit to the myogenic lineage and undergo fusion, whereas a small portion fail to express MyoD or myf-5 and thus repopulate the satellite cell pool [38, 39, 41].

Isolation and subsequent culture of MPCs (satellite cells when in their anatomical niche) can be conducted either by explant culture, whereby muscle tissue is minced and maintained in culture until MPCs migrate from the tissue [42, 43], or via enzymatic digestion, whereby the tissue is broken down completely to release all of the resident mononuclear cells [44, 45]. Following isolation, careful consideration of the culture techniques should be taken in order to standardise/optimise the desired experiments. Indeed, MPCs are sensitive to the media

7 Neil R.W. Martin and Mark P. Lewis.Satellite cell activation and number following acute and chronic exercise: A mini review. Cellular and Molecular Exercise Physiology 1[1]: e3. doi:10.7457/cmep.v1i1.e3

composition, and will either proliferate or exit the cell cycle and differentiate when exposed to various media compositions [46]. Furthermore, MPCs are sensitive to environmental factors, and will proliferate and fail to differentiate when exposed to low oxygen levels [47, 48] and similarly, serial passaging of MPCs also appears to negatively effect their ability to differentiate [49]. Finally, as the population of cells released from skeletal muscle tissue tends to be mixed in nature (e.g. MPCs, fibroblasts, pericytes, endothelial cells etc.), it is often favourable to further purify the MPCs prior to experimentation either via differential adhesion methods or magnetic separation, a methodology optimise in our laboratory [50], however these cells may contribute to the development of more biomimetic tissue (detailed in Figure 1 below).

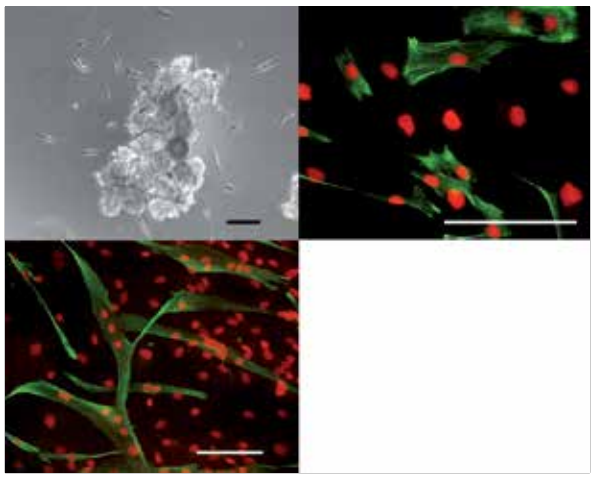

Figure 1. Skeletal muscle precursor cells can be isolated and cultured *in vitro* to recapitulate the events of development and regeneration. (A). Phase contrast photomicrograph of human skeletal muscle explant with MPCs migrating away from the tissue. (B) Immunostaining for Desmin (green) highlights those cells, which are commited to the myogenic lineage. (C) Desmin positive cells form multinucleate myotubes when stimulated to do so. Scale bars = 100μm.

The isolation and characterisation of cells for utilisation in the development of *in vitro* skeletal muscle models is an important stage in the engineering process. However, the consideration of the influence of environmental factors in the creation of a biomimetic model is a necessity and will be explored below.

Environment - The skeletal muscle cell microenvironment (commonly referred to as the niche) supports the development of multi-nucleate myotubes (muscle fibres) in a number of ways: provision of directional cues, provision of topographical cues, availability of growth factors, transmission of mechanical signals and interactions with other cell types. Initial evidence that particular proteins within the skeletal muscle cell niche play critical roles in these processes and hence subsequent success of *in vitro* muscle cell behaviour, came with the establishment of the varying effects of ECM molecules on MPC proliferation and differentiation. Extracting bioactive ECM proteins from skeletal muscle for use as a growth substrate with *in vitro* MPC culture, results in superior proliferation and differentiation compared to standard growth substrates [51]. It is however the identification of interactions between ECM proteins and

muscle cell surface proteins that has provided data regarding the requirements for successful *in vitro* culture of MPCs.

Particularly, integrins and proteins of the Dystrophin Associated Protein (DAP) complex have been demonstrated to play significant roles in MPC-ECM interaction *in vitro*. Indeed, the expression of α v, α v β 3 and α v β 5 integrins is evident in MPCs seeded on ECM substrates such as gelatin, fibronectin and vitronectin [52]. Moreover, blockade of α v integrin using a specific antibody abolishes cell migratory capacity on fibronectin and vitronectin substrates [52]. The effect of ECM matrix components has also been demonstrated to influence the contractile phenotype of engineered skeletal muscle constructs. Increasing the content of ECM protein rich Matrigel, contributed to greater peak force of engineered constructs, whilst utilising growth factor reduced Matrigel did not affect the results [53]. This suggests that ECM matrix composition contributes to skeletal muscle maturation and phenotype to a greater extent than growth factors. These data provide clear evidence as to the importance of the ECM in the successful culture of MPCs *in vitro*.

The primary function of skeletal muscle *in vivo* is to provide vector force, for the controlled movement of the skeleton [54]. This directionality is achieved by the highly organised macro and microstructure of the skeletal muscle fibres and the surrounding ECM[8]. The recapitulation of this organised structure has been achieved through a variety of methodologies *in vitro*, including the use of both synthetic [55-58] and naturally derived polymers [45, 59-63]. These methods have provided both directional and topographical cellular cues, display varying amounts of resemblance and functionality to *in vivo* tissue. It has been suggested however, that the rigidity of synthetic polymers may affect the contractile properties and function of developed myotubes, whilst if the polymer is bio-degradable the alignment of cells may be affected by this process [64]. The use of naturally derived polymers (including fibrin and collagen) has in part been shown to overcome these issues. Particularly in a more biomimetic three-dimensional (3-D) structure, the rapid polymerisation of collagen matrices allows for an even distribution of the cells seeded, whilst the nanostructure allows for multiple attachment sites for the cells [64].

The provision of mechanical signals for the alignment and fusion of MPCs is an essential criterion for developing biomimetic constructs *in vitro*, since skeletal muscle *in vivo* is under continuous passive strain through the myotendinous junction. Such signals have been generated by the polymerisation of a biological substrate to a fixed structure in a variety of *in vitro* skeletal muscle models [45, 53, 65, 66]. The anchored structures provide the necessary passive lines of strain, which provide the required signals to generate an orientated cellular architecture (See Figure 2 below). Indeed, it is clear that the 3-D structure and the presence of passive mechanical signals promote the alignment and fusion of MPCs to a greater extent than in conventional monolayer culture [45]. Furthermore, the morphological structure, gene expression and contractile phenotype are also representative of *in vivo* skeletal muscle [43, 45, 67].

8 Alec S.T. Smith, Rishma Shah, Nigel P. Hunt, Mark P. Lewis. The Role of Connective Tissue and Extracellular Matrix Signaling in Controlling Muscle Development, Function, and Response to Mechanical Forces. Seminars in orthodontics 1 June 2010 (volume 16 issue 2 Pages 135-142 DOI: 10.1053/j.sodo.2010.02.005)

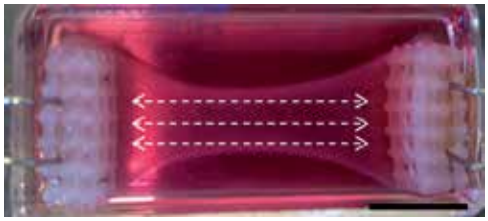

Figure 2. Biomimetic skeletal muscle constructs can be engineered from biological scaffolds. Example model of the typical macroscopic contraction of a type-1 collagen matrix, following 14 days in culture. Note the custom made anchors at either end, which provide predictable lines of longitudinal tension (illustrated by dashed arrows) to promote cell alignment and fusion in a single plane. Scale bar = 10 mm.

A further advantage of using naturally derived polymers is the ability to easily stimulate, both mechanically and electrically, due to the innate mechanical compliance of the polymerised tissue. To this end, numerous investigations have employed such techniques to promote myogenesis and maturation of seeded MPCs [65, 68, 69]. In contrast, the effect of a combined mechanical stimulation protocol, contributed to a reduction in maturation of 3-D seeded myogenic cells compared to monolayer controls [66]. Nevertheless, the effect of increased mechanical signals for the promotion of MPC alignment and fusion, satisfies the desire for a biomimetic.

Skeletal muscle *in vivo* is comprised of myogenic progenitors, non-myogeninc progenitors and fibroblasts. The presence and influence of these non-myogenic populations has not been widely investigated, however, there is evidence to suggest a potential synergistic role in the development and maturation of skeletal muscle *in vitro*. The non-myogenic fibroblast population of cells has been shown to enhance skeletal muscle cell alignment, through contact dependent and independent mechanisms [70]. Data from 3-D skeletal muscle constructs also demonstrates an increased myogenin mRNA expression (marker of terminal differentiation) and increased peak force in a heterogeneous mixture of myogenic and non-myogenic cells, compared to myogenic only cultures [43]. Furthermore the expression of MMP-2 mRNA was greater in the heterogeneous cell population cultures, suggestive of greater matrix remodelling and fusion potential [43]. Together, these data illustrate the requirement of the non-myogenic cell population to recapitulate the *in vivo* skeletal muscle cell niche and associated biological processes. As such the incorporation of a heterogeneous population of cells should be utilised when developing skeletal muscle models *in vitro*.

Consideration of the body of data discussed above, indicates that an argument can be made that the ideal solution for a tissue engineered skeletal muscle construct would embrace the following:

1. Appropriate matrix signals.
2. Induction of alignment.
3. Presence of other cell types.
4. Mechanical stimulation.

Provision of all of the cues simultaneously requires an approach that moves beyond conventional monolayer models. The structure defines function in skeletal muscle *in vivo* and all of the essential elements above can be combined using tissue engineering techniques. There are a number of systems that have moved towards this aim, however important validations for such systems is making comparisons against the *in vivo* tissue, physiological skeletal muscle. The systems described have shown the following features to date:

Parameter	Resemblance to In Vivo Tissue	System and References
Passive longitudinal tension generated by anchor points	Myotendinous junctions	Collagen [43, 45, 53, 67, 68, 71]; Fibrin [53, 60, 65, 66]
ECM	ECM protein composition	Collagen [43, 45, 53, 67, 68, 71]; Fibrin [53]
Morphology of cells	Alignment and fusion of seeded cells in a single plane	Collagen [45, 53]; Fibrin [53, 66]
Active contractile phenotype (vector force)	Excitability and contractility profile	Fibrin [60, 62, 65];
Passive contractile phenotype (vector force)	Cell-matrix passive contractile interaction	Collagen [43, 45]
Myosin Heavy Chain (MYH) expression	Increase in MYH-7 mRNA (slow genotype)	Collagen [67]
Responses to stimulation (mechanical and electrical)	Increases in gene and protein expression similar to that with exercise	Collagen [68, 71] Fibrin [65]

Table 1. Comparison of current biomimetic skeletal muscle models of *in vivo* skeletal muscle tissue.

There can now be a high degree of confidence that the aforementioned systems do recreate a significant aspect of *in vivo* skeletal muscle biology. Their use to-date with respect to providing a pre-clinical test bed for exercise, genetic, pharmaceutical and nutritional therapies is limited, however the system are now at a stage where such investigations are warranted.

5. Animal models of cartilage for arthritis research

Experimental models of degenerative joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) have been in existence for several decades [72]. Animal models of arthritis have been used to understand elements of the arthritic disease process in human patients [73]. They are powerful tools for studying pathologic changes in articular cartilage and bone in great detail, and can be used to evaluate mechanisms of erosive processes [73]. Animal models of arthritis are also used to evaluate potential anti-arthritic drugs for clinical use in human patients [74-76]. The capacity for predicting efficacy in human disease is one of the most important criteria in the selection of animal models [74]. The use of animals has been indis-

pensable to the investigation of the aetiology, pathophysiology, and treatment of various forms of juvenile arthritis [77]. Animal models of rheumatoid arthritis (RA) are also well established and have a proven track record of predictability [74, 78]. These include rat adjuvant arthritis [79], rat and mouse type II collagen arthritis [80-82], and antigen-induced arthritis in several species. Many animal species are currently used in OA research [83]. Figure 3 summarises the main procedures and disadvantages of animal models of OA.

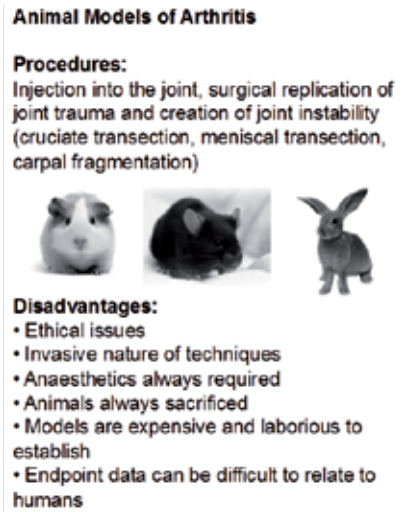

Figure 3. Procedures and major disadvantages of animal models of arthritis.

The animal models of OA include laboratory animals: mice, rats, guinea pigs, rabbits; farm animals: sheep, goats; and companion animals: dogs, cats, horses. Animal models of OA roughly fall into five categories; firstly, spontaneous OA, which naturally occurs in the knee joints of animals, such as guinea pigs and dogs, and has a similar pathogenesis to human OA [84, 85]. Secondly, the surgical creation of joint instability, for example anterior cruciate ligament transection (ACLT) in dogs [86], meniscal tear model in rats [87], and collateral ligament transection in horses [88]. Thirdly, the surgical replication of joint trauma, for example the canine groove model [89], and carpal chip fragmentation in horses [90]. Fourthly, injection into the joint, for example papain [91], sodium mono-iodoacetate [92] and collagenase [93]. The final category is the knockout model, which deletes certain genes in mice resulting in the development of OA-like degenerative joint disease. For example the deletion of genes that code for type IX collagen [94], or the double deletion of biglycan and fibromodulin [95].

6. Biomimetic models of cartilage

Biomimetic models of articular cartilage were developed specifically for use in preclinical and clinical research long before the advent of tissue engineering and regenerative medicine, and

the realisation that the development of these models is an elementary form of tissue engineering. One of the major advantages of articular cartilage is the fact that it is a relatively simple tissue consisting of a fairly homogeneous extracellular matrix and a single cell type. Also, cartilage is avascular, aneural and alymphatic [96, 97], a fact that was disputed for centuries until modern histological techniques were applied to study the tissue [97]. This unique property overcomes many of the obstacles that are involved in culturing other vascularised and innervated tissues. The following sections will discuss the most popular 2- and 3-dimensional models of cartilage.

6.1. 2-Dimensional monolayer models of chondrocyes

Two-dimensional *in vitro* models of chondrogenesis [98, 99] and methods for isolating and cultivating primary bovine chondrocytes were established in the early 1960's [100]. Methods have been published for human [101] and rabbit [102] cartilage. Primary chondrocytes [103-105] and SV-40 transformed chondrocyte-like cell lines [106] in monolayer culture are routinely used for basic research [107]. These models have been useful for studying the cell biology of cartilage and responses to drugs, pro-inflammatory cytokines, growth factors, nutrients and nutraceuticals. Chondrocytes are also able to survive freezing and cryopreserved cells retain the capacity to proliferate and synthesise ECM [108-111]. Although 2-D culture of chondrocytes is widely used (see Figure 4), this method suffers from several major weaknesses: 1). The cells de-differentiate to fibroblast-like cells after 4-5 passages; 2] They do not synthesise a genuine ECM. Early studies carried out in the 1960's showed that high-density culture preserves the chondrocyte phenotype; when cultured chondrocytes attain a certain cell density they re-acquire their rounded shape, stop dividing, and re-synthesize collagens and proteoglycans. Clusters of chondrocytes synthesize more proteoglycans than isolated chondrocytes [112]. If chondrocytes are actively engaged in ECM synthesis they do not concurrently synthesize DNA and so cannot divide and proliferate [112]. Interaction between chondrocytes is important for maintaining ECM synthesis and failure of interaction between chondrocytes leads to cell proliferation, a fact that has been exploited for expanding small numbers of isolated chondrocytes from limited amounts of clinical material.

Although chondrocyte survival and phenotype are regulated by culture conditions, the expression of the chondrogenic transcription factor Sox9 is of crucial importance [113]. The unique phenotype of the chondrocyte requires sustained expression of Sox9. This transcription factor plays an important role in the normal skeletal development and regulate the expression of other genes involved in chondrogenesis [114, 115].

6.2. 3-Dimensional cartilage culture systems — Alginate beads

Alginic acid, also called algin or alginate, is an anionic polysaccharide that is widely distributed in the cell walls of brown algae. When extracted in granular or powdered forms, alginate is capable of absorbing 200-300 times its own weight in water. Due to its biocompatibility and simple gelation with divalent cations such as Ca^{2+}, alginate is widely used for cell immobilization and encapsulation. Therefore, alginate beads offer an ideal substrate for developing support matrices for 3-dimensional chondrocyte culture (see Figure 5). Immobilization of cells

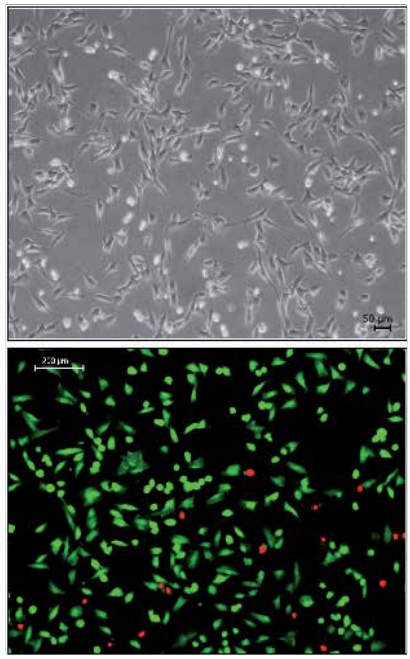

Figure 4. Dimensional cultures of primary chondrocytes. This culture system is widely used for basic research on chondrocyte biology and for drug toxicity testing.

along with macromolecules and biomaterials in alginate gels has become a well-established technology. Alginate beads are used in many biomedical and industrial applications. Cells immobilized in alginate gels maintain their differentiated phenotype during long-term culture due to the 3-dimensional environment of the gel network. In tissue engineering applications immobilized cells or tissue explants can be used as bioartificial organs as the alginate gel may function as a protective barrier towards physical stress and to avoid immunological reactions with the host. Chondrocytes can be encapsulated and maintained in calcium alginate beads or gels in 3-dimensional culture [116]. Avian and mammalian chondrocytes cultured in "semi-solid" and "hollow" alginate beads exhibit a spherical shape as opposed to the fibroblastic morphology that is observed in monolayer culture [116]. The encapsulation methodology is suitable for the culture of chondrocytes in single beads, in multiwell dishes, or mass culture. Human and bovine adult articular chondrocytes have also been cultured in alginate beads and studies have shown that they retain their spherical shape and typical chondrocyte-like appearance for at least 5 weeks [117]. Alginate culture has also been used for cultivating intervertebral disc cells [118], nucleus pulposus and annulus fibrosus cells [119] and chondrocyte cell lines [120]. Aggrecan appears to be a major ECM molecule produced by alginate cultured chondrocytes. Sensitive assays have been developed for the quantification of glycosaminoglycans (GAGs) and nitric oxide (NO) produced by alginate cultures [121, 122]. Decorin is also synthesized in small amounts but it is rapidly lost from the agarose or alginate gel [117]. Alginate culture can be used to induce the re-expression of cartilage-specific genes (aggrecan and collagen II) by dedifferentiated human articular chondrocytes cultured in

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alginate beads. However, alginate is unable to restore the chondrocyte phenotype in SV-40 transformed cells [123]. Thus, articular chondrocytes embedded in alginate gel can produce *de novo* a matrix rich in collagens and proteoglycans [124]. The alginate culture system appears to represent a relevant model for maintaining primary populations of chondrocytes and inducing the redifferentiation of dedifferentiated human chondrocytes, especially when they have been expanded several times by passaging in monolayer culture. This approach is particularly useful when small cartilage biopsies are available. New approaches to cartilage tissue-engineering have combined isolated cells with polymer scaffolds (polyglycolic acid fibre meshes and alginate gels) for the purpose of generating new cartilage (neo-cartilage) [125].

Figure 5. Chondrocyte culture in alginate beads.

6.3. 3–Dimensional cartilage culture systems — Agarose gels

Agar is a gelatinous substance derived from algae. It is a mixture of two components: the linear polysaccharide agarose, and a heterogeneous mixture of smaller molecules called agaropectin. Agar and agarose have been used extensively for cell culture. Agar gels are have been used throughout the world to provide a solid surface containing medium for the growth of bacteria

and fungi. As a gel, an agarose medium is porous and therefore can be used to re-create the 3-dimensional environment that chondrocytes are accustomed to in articular cartilage. Culturing chondrocytes in agarose gels is actually very similar to alginate beads. Agarose gels proved to be particularly useful for studies on proteoglycans produced by avian [126], porcine [127] and bovine [128] chondrocytes. Agarose gels re-create a biomimetic 3-dimensional environment and stimulate ECM production by chondrocytes [129]. One of the most important studies on chondrocyte differentiation and redifferentiation was done using agarose gels by Benya and Shaffer [130]. As described earlier serial monolayer culture results in chondrocyte dedifferentiation and loss of phenotype. When chondrocytes dedifferentiate in monolayer culture they stop producing proteoglycans and type II collagen and instead secrete a different ECM consisting predominately of type I collagen and a low level of proteoglycan synthesis. Benya and Shaffer used agarose gels to demonstrate that dedifferentiated chondrocytes re-express the differentiated phenotype, producing proteoglycans and cartilage specific collagens [130]. The same outcome was achieved years later using alginate beads [131]. The original work of Benya and Shaffer demonstrated that a complete return to the differentiated collagen and proteoglycan producing chondrocyte phenotype is possible in agarose gels. Their results also emphasized the essential role of the spherical cell shape in the modulation of the chondrocyte phenotype and demonstrate a reversible system for the study of gene expression [130].

It is important to bear in mind that articular cartilage is subjected to dynamic compressive loading during normal activity and this influences chondrocyte metabolism. Mechanical forces are key determinants of connective tissue differentiation. Agarose gels, alginate beads and other 3-D gel systems are well established for studying the effects of dynamic compression on chondrocytes [132, 133]. These techniques have gained significant popularity over the last two decades [134] and are still in use today to study patterns of gene expression in response to dynamic compression [135] and chondrocyte mechanotransduction pathways [136]. The preservation of the chondrocyte phenotype and the gradually increasing proteoglycan synthesis in agarose and alginate gels are promising methods for creating and engineering tissue implants for cartilage repair. These techniques can also be used to create new cartilage tissue from the joints of food producing animals (i.e. cattle, sheep, pigs) without having to sacrifice many smaller laboratory animals. This is an important area of cartilage tissue engineering with important consequences for animal research.

6.4. 3-Dimensional cartilage explant culture

Explant culture is a technique used for the isolation of cells from a piece of tissue. Tissue harvested in this manner is called an explant. The tissue is harvested under sterile conditions and explants are placed in a cell culture dish containing growth media. In some explant cultures (i.e. skeletal muscle) progenitor cells migrate out of the tissue and grow on the surface of the dish. These primary cells can then be further expanded and exploited. In cartilage explant culture cells remain in their surrounding extracellular matrix and this accurately mimics the *in vivo* environment (see Figure 6). The method became established in the mid 1970's [137, 138] and was used to study the effects of prostaglandins [139-141] and rheumatoid synovium [142] on cartilage degeneration. The establishment of cartilage explant culture later became an important prelude to the development of monolayer culture systems of chondrocytes [143].

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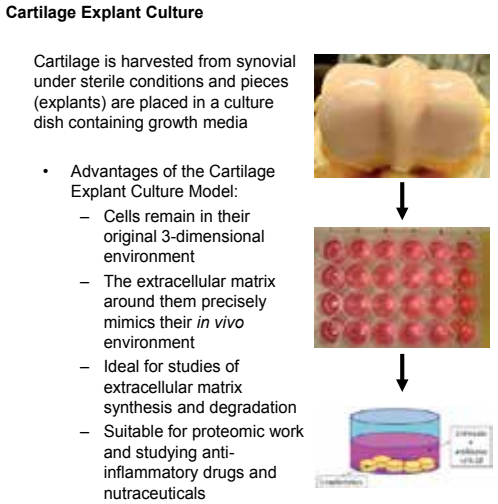

Figure 6. Overview of cartilage explant culture and its unique advantages.

6.4.1. 3-Dimensional High-Density and Pellet Cultures of Chondrocytes

High-density culture is a 3-dimensional system that has been in the literature since the early 1970's. High-density suspension cultures of chondrocytes were initially used to study cartilage matrix protein synthesis by mammalian chondrocytes [144, 145], specifically mucoprotein [146] and proteoglycan [147] biosynthesis. In an important study published in 1977 the method was refined by von der Mark and von der Mark who used tissue culture plastic dishes on an agar base to monitor chondrogenesis of stage-24 chick limb mesodermal cells *in vitro* by analyzing the onset of type II collagen synthesis [148]. This work demonstrated that high-density cultures may be used for the analysis of cell specific types of collagen and provide a useful model for detailing the specific events in the differentiation of mesenchymal cells *in vitro*. Several subsequent studies used chick limb bud mesenchymal cells and chick sternal chondrocytes cultured in high-density to study chondrogenesis and proteoglycan synthesis [149] and fibronectin expression [150]. We have successfully used this model as a model for chondrogenic, osteogenic and tenogenic differentiation of mesenchymal stem cells and redifferentiation of dedifferentiated chondrocytes [151-154].

The high-density model exhibits a number of characteristics that make it particularly suitable for studies on chondrogenesis. In the first 24 hours of the high-density chondrocytes culture, cells form prechondrogenic areas composed of densely packed cells with intercellular interactions (gap junctions); these are surrounded by a perichondrium of flat fibroblast-like cells [155] resembling the situation during the early stage of chondrogenesis *in vivo*. The extensive cell-cell interactions during the first step of chondrogenesis are crucial for cell aggregation [156, 157]. The high-density culture system allows investigations during chondrogenesis starting from early blastema/mesenchymal stem cell condensations until cartilage maturation. This chondrogenic development is accompanied by enlargement of cartilage nodules, which

is not due to cell proliferation but rather is due to appositional growth, which includes the transition of perichondral cells into chondrocytes. These conclusions are based on the almost complete absence of mitotic figures in cartilage and perichondrium and on the continuous increase in nodule size during cultivation [156].

6.4.2. Co–cultures of chondrocytes and synoviocytes

The synovial joint contains several important tissue components. These are articular cartilage, synovium, subchondral bone and fat pads (adipose tissue). Developing *in vitro* models that include all of these tissues is a major challenge. Co-culture models involve growing distinct cell types in a combined culture environment. The mixing of different cell types in culture is normally avoided, although it is becoming increasingly apparent that this approach can be used to model the cellular interactions that occur *in vivo*. Some studies of cellular interactions may actually require direct cell-cell contact in a 2-dimensional model where two different cell types are grown in mixture. Other studies may require proximity between the two cell types without any actual cell-cell contact. In this scenario keeping the two types apart can allow investigators to study the effect of one cell type on another. In a synoviocyte-chondrocyte co-culture model synoviocytes grown on a filter insert may be stimulated with pro-inflammatory cytokines or other inflammatory mediators (i.e. phorbol esters such as phorbol myristate acetate (PMA), reactive oxygen species such as hydrogen peroxide or a combination of iron and ascorbic acid to simulate inflammation-like radical attacks) before bringing the "activated" cells in contact with chondrocytes. The use of cell culture inserts controls the physical contact and also the duration of that contact. It is also possible to grow different cell types on either side of the membrane, allowing a simulation of tissue behaviour to be built and studied. A co-culture model of rat chondrocytes and a rabbit synoviocyte-like cell line (HIG-82, [158]), has been used to show that chondrocytes establish protective mechanisms against reactive oxygen species by interacting with synoviocytes [159]. This co-culture system presents a model to study mechanisms of inflammation in articular joints under well-defined conditions [159]. With the financial support of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) we have established a novel co-culture model of primary equine synoviocytes and primary equine chondrocytes for using equine joints derived from the abattoir[9]. We have not fully exploited this equine model system. However, since chondrocytes and synoviocytes are the main cell types present in articular joints, such co-culture models have been used for metabolic studies [160] and have the capacity to recapitulate the inflammatory and catabolic events that occur in an arthritic joint.

7. Conclusions

Cell culture has a bright future. It is a fundamental and core component of tissue engineering and regenerative medicine. In this context the majority of studies carried out to date have used the well-established method of growing cells on 2-D plastic or glass substrates. There is

9 http://www.nc3rs.org.uk/researchportfolio/showcatportfolio.asp?id=254

increasing use of 3-D cell cultures in research areas as diverse as drug discovery, cancer biology, regenerative medicine and basic life science research. There are many methods to facilitate the growth of 3-D cellular structures including nanoparticle facilitated magnetic levitation, gels, beads and solid matrices, self-assembling scaffolds and hanging drop plates. The culture of mammalian cells for toxicity testing or drug screening is likely to increase in the future. The biotechnology, pharmaceutical and cosmetic industries urgently need 3-D cell culture models that more accurately mimic living tissues and organs. This kind of technology will gradually increase in importance because of the vast array of natural and combinatorial products that require screening and the increased pressure from regulatory authorities to reduce animal testing. Most importantly, the pressure to reduce animal testing will stimulate scientists to create more robust biomimetic culture models and culture systems that may eventually eliminate the need for using animals for antibody production and vaccine development. There are many exciting and emerging areas that have not been reviewed in this chapter. This chapter has focused on biomimetic models of joint and muscle. Since joint tissues contain a diverse number of cell types, we have focused our efforts on *in vitro* models of chondrocytes and skeletal muscle cells.

It is becoming clear that OA is a disease of the entire joint rather than any single component and deterioration of associated skeletal muscle masses around an affected joint is a well-reported phenomena. Changes in the highly adaptive skeletal muscle may precede changes seen in other tissue types so analysis of the transcriptome/metabolome could have great value in prognosis and diagnosis. It is possible to conduct such studies in humans although such experiments are logistically complicated, requiring appropriate subject numbers that adhere to complicated inclusion and exclusion criteria and expensive consumables. It could however be argued that a more beneficial approach would be to utilise a "pre-clinical model" to further refine and develop hypotheses before introduction in the human being. This approach would clearly also decrease the need for animal work.

A number of animal models of arthritis have been developed to study arthritic disease pathogenesis and evaluate the efficacy of candidate anti-inflammatory and anti-arthritic drugs for clinical development [161-167]. These animal models may involve injection of inflammatory agents into the joint, surgical creation of joint instability or surgical replication of joint trauma. Such models not only cause a considerable amount of pain and suffering but also none of them have a proven track record of predictability in human disease. Consequently, there is an acute need for developing novel and alternative *in vitro* models that mimic the biology of cartilage [168-172]. Many studies have used articular cartilage and synovial tissues to establish *in vitro* models of the joint. In our laboratories we have developed explant models of articular cartilage inflammation, co-cultures of primary synoviocytes and chondrocytes as well as 3-dimensional high-density cultures of chondrocytes and MSCs [152, 153, 173-175]. We have also determined the potential of phytochemicals as stimulators of chondrogenic differentiation. These culture models of MSCs, fully differentiated cells and co-cultures have potential as ''proof of principle'' ethically acceptable ''3Rs'' models for engineering biomimetic models of articular cartilage *in vitro* for the purpose of replacing and reducing the use of animals in arthritis research. This approach could help reduce the number of laboratory animals used in arthritis research and may provide a realistic alternative to experimental animals.

Articular cartilage damage is a persistent and increasing problem as the ageing population expands and treatments to achieve biological repair have been challenging [176]. Cartilage tissue engineering has been around for over 20 years. However, none of the approaches available so far have been able to achieve the consistency, effectiveness and reliability that are required for clinical applications. Tissue engineering of a mechanically resilient cartilage construct that meets the structural and functional criteria for effective functional integration into a defect site in the host is a difficult endeavour [177]. One of the fundamental weaknesses of all the models available to date is that none of them possess the normal zonal organization of chondrocytes that is seen *in vivo* (i.e. superficial, middle, deep and calcified zones) and the local composition of extracellular matrix in each zone. This structural organisation is a pre-requisite for normal cartilage function and the success of any future clinical applications. The currently available 3-D models produce fairly homogeneous populations of cells without the ability to achieve any zonal organization *in vitro* [176]. The ability to produce a construct that recapitulates the zonal and structural architecture of the original tissue is currently lacking. Even the mechanically stable scaffolds that have been created so far do not allow regeneration of a sufficiently large mass of structurally and functionally competent cartilage construct especially if they were constructed and seeded with 2-D passaged (monolayer) chondrocytes in combination with a biomimetic carrier or scaffold [177]. This is one reason why future studies must begin with 3-D cultured chondrocytes maintained in a physiologically relevant microenvironment that replicates the ionic, osmotic and biomechanical milieu of cartilage. The 3-D and microenvironmental impact on cell phenotype is a significant factor creating cartilage constructs within biomimetic scaffold constructs [177].

In summary, it may be difficult to imagine research being done without animal models but it is worth pointing out that *in vitro* models of joint and musculoskeletal tissues have been around for several decades and new researchers are increasingly adopting them for work on musculoskeletal diseases. Therefore, it is our optimistic view that in future many animal models could potentially be replaced with biomimetic and animal sparing alternatives in a variety of research disciplines and applications.

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Chapter 22

Importance of Extracellular Environment for Regenerative Medicine and Tissue Engineering of Cartilagious Tissue

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Additional information is available at the end of the chapter

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1. Introduction

Cartilage degeneration caused by osteoarthritis (OA) and trauma is of great clinical consequence, given the limited intrinsic healing potential of the cartilaginous tissue. OA is the most common joint disease in world populations. Pain during activities of daily living is a common presenting complaint of individuals with OA and is also associated with a decrease in quality of life for people with OA. Its incidence increases with age, and thus this degenerative disease is a major problem in ageing populations. OA is a multifactorial disease of the joints characterized by gradual loss of articular cartilage. In the recent years, the mechanism of chondrocyte differentiation has come to be well understood owing to the advancement of molecular biology, and researches have rapidly progressed for bioengineering or tissue engineering technique, where it is aimed to regenerate/reconstruct tissues by simulating the process of cell or tissue differentiation during development. Articular cartilage is composed mainly of collagen/proteoglycan (PG) and water. PG accounts for about 7 - 10% of cartilage tissues, and aggrecan, which is a member of PG representing macromolecules, plays a key role for mitigation of mechanical stress imposed on the cartilage tissues (Maroudas, 1979). A fall in PG concentration is one of the first changes in OA with consequent deleterious effects on the mechanical behaviour of cartilaginous tissues (McDevitt & Muir, 1976, Venn & Maroudas 1977). Among the components of aggrecan, negatively-charged Glycosaminoglycan (GAG) produces a high osmotic pressure in the cartilage tissue, and water is therefore absorbed in the cartilage tissues. As a result, the collagen networks are inflated, and the cartilage tissues acquire elastic resistance characteristic to cartilage tissues to protect from compression force. Thus, the

strength of cartilage tissues strongly depends on the density of aggrecan (Kempson et al., 1970, Maroudas, 1979, Maroudas & Bannon 1981). Therefore, in order to produce cartilage tissues that can tolerate mechanical force of about 10-20 Mpa using the tissue engineering technology, it is necessary to generate sufficient PG (Hodge et al., 1986). PG/GAG generation depends on the amount of GAG production, the capacity of GAG retention in the tissues, and the concentration of cells (Kobayashi et al., 2008). There is now an increasing interest in developing biological methods of cartilage repair for these disorders with attainment of the correct biomechanical properties critical for success (Brittberg et al. 1994, Minas, 2001, Risbud & Sittinger 2002, Schaefer et al. 2002 Ochi et al. 2002, Robert et al. 2003). The stiffness of cartilaginous tissues is thus strongly dependent on aggrecan content. Therefore, one of the targets of successful repair is thus that GAG concentration of the tissue-engineered construct should approach that of the native cartilage. First of all, it is important to establish the optimum culture conditions for the generation of cartilaginous tissues. In this study, we examined how physiological levels of extracellular osmolality and cell density influence PG accumulation in chondrocytes in a three-dimensional culture system. And also, we evaluated the influence of transforming growth factor-β (TGF-β) and fibroblast growth factor-2 (FGF-2), which are involved on the metabolism of PGs by cartilage cells cultured under low-osmotic conditions.

2. Changes of the extracellular environment in the articular cartilage with age

Unlike other connective tissues, articular cartilage represents avascular tissue. The chondrocytes may receive nutrients via the vascular system under subchondral bone as well as via the synovial fluid and are supplied by diffusion, helped by the pumping action generated by compression of the articular cartilage or flexion of the elastic cartilage (Brower, et al. 1962, Mankin 1963, Maroudas, et al. 1968, Hodge & McKibbin 1969, McKibbin & Holdsworth 1966, O'Hara et al. 1990). Nutritional supply to the cartilaginous tissue is affected by the architecture of the vascular system and the porosity in the subchondral bone (Fig.1A). Nutrients move from the vascular systems under subchondral bone that supply the cartilaginous tissue, through the subchondral bone and the dense matrix of the cartilaginous tissue, to the chondrocytes. Its limits transport of large molecules into and out of the cartilaginous tissue. For small solutes such as glucose, lactate acid, and oxygen, both experimental and modeling studies have shown that solute transport is accomplished mainly by diffusion (Mauck et al. 2003a), hence, the movement of fluid in and out of the cartilaginous tissue as a result of the diurnal loading pattern has little direct influence on transport. Gradients in the concentration arise depending on the balance between the rate of supply of glucose or oxygen from the blood supply to the cells and the rate of cellular consumption (Stockwell, 1991, Hall, et al. 1993, Lee & Urban, 1997, 2002).

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The cartilaginous tissue is avascular, and the metabolic activity of its cells is regulated by various factors in the extracellular matrix, such as oxygen concentration (Lane et al, 1977, Lee & Urban, 1997, 2002), extracellular osmolality (Urban & Bayliss, 1989, Urban et al., 1993, Bush & Hall, 2001, Palmer et al., 2001, Erickson et al., 2001, Bush et al., 2005, Negoro, et al., 2008), pH (Wilkins & Hall, 1995, Gray et al.,1988), mechanical stress (Gray et al. 1989, Urban 1994, 2000), and various growth factors (Morales & Roberts, 1988, Luyten et al, 1988, Morales, 1994, Van Osch, et al. 1998, Huck, 2001). The cell density of the normal human cartilaginous tissue is 2– 4 x 10^6 cells/mL, and the extracellular environment differs markedly from that of other tissues, with an oxygen saturation of 1–6%, pH of 6.8–7.1, and extracellular osmolality of 350–450mOsm. So, the cartilaginous extracellular environment is relatively hypoxic, unlike the case of other tissues. And also, the articular chondrocytes are exposed to a unique osmotic environment, which varies throughout the depth of cartilage, and in response to mechanical loading or pathological conditions. It is said that such a harsh environment suppresses chondrocyte differentiation and maintains the nature characteristic to chondrocytes. Thus, compared to other connective tissues, cartilage grows and repairs more slowly.

The articular cartilage is essential for absorbing shock and maintaining normal joint environment, and, regardless of the cause, degeneration of articular cartilage can result in irreversible osteoarthritis. Osteoarthritis is commonly referred to as wear-and-tear arthritis. There are other parts of joint anatomy, like subchondral bone, that play a significant role in osteoarthritis. Subchondral bone is the layer of bone just below the cartilage. The subchondral bone does not normally provide a barrier to diffusion. In aging and degeneration, however, the subchondral bone tends to calcify by unknown mechanisms and the apparent permeability of this plate decreases with age, as the subchondral bone becomes more sclerotic (Lane, et al., 1977, Lane, & Villacin, 1980, Botter et al., 2011). The consequent fall in supply of nutrients to the chondrocytes inhibits their ability to synthesize and maintain the matrix and even leads to cell death (Kühn, 2004). In osteoarthritis, subchondral bone becomes thicker than usual (Fig1B). Adult hyaline articular cartilage is progressively mineralized at the junction between cartilage and bone. It is then termed articular calcified cartilage. A mineralization front advances through the base of the hyaline articular cartilage at a rate dependent on cartilage load and shear stress. Intermittent variations in the rate of advance and mineral deposition density of the mineralizing front, lead to multiple "tidemarks" in the articular calcified cartilage. Adult articular calcified cartilage is penetrated by vascular buds, and new bone produced in the vascular space in a process similar to endochondral ossification at the physis. A cement line demarcates articular calcified cartilage from subchondral bone. Vascular channels connect the marrow spaces of trabecular bone with the calcified cartilage layer, thus nourishing the deeper cartilage layers that cannot be nourished by synovial fluid (Duncan, 1987, Milz & Putz, 1994). These vascular channels also nourish osteocytes in the subchondral bone plate, unlike osteocytes in trabecular bone, which receive nourishment from marrow tissue. In aging, however, the subchondral bone tends to calcify by unknown mechanisms (Fig.9B). This tide mark (calcification) acts as a barrier to nutrients transport and is thought to be a major factor in the development of osteoarthritis. Cellular parameters are very important in regulating nutrient

levels, with levels of oxygen or pH falling with increases in rates of cell metabolism or cell density. For the chondrocytes to remain viable, the levels of extracellular nutrients and pH must remain above critical values. Because disc cells obtain ATP primarily by glycolysis, glucose is a critical nutrient. The cells start to die within twenty-four hours if glucose concentration falls below 0.2 mM and the efficiency of glucose transport into the cell is likely reduced at this glucose concentration (Windhaber et al., 2003). The rate of cell death increases when pH levels are acidic. The cell viability is reduced even with adequate glucose at pH 6.0. The osmotic environment of chondrocytes in the articular cartilage changes with loading and pathologic states. The osmolality of the extracellular matrix is regulated by negatively charging the GAG chains of PGs which adjust ionic composition. Particularly, extracellular osmolality is controlled by negatively charged PGs. It is now evident that an increase in the concentration of PGs which control ionic composition causes an increase in the osmolality, and conversely, a decrease in PGs reduces osmolality (Maroudas, 1981). Maroundas et al. (1975) investigated the osmotic pressures in articular sections extending to the sagittal sections and reported that the osmotic pressure in the articular cartilage is about 370-400 mOsm and were decreased in the degenerated articular cartilage. Thus, it may be said that osmotic pressure gradient disturbance associated with reduced PGs is an important factor contributing to the development of disc degeneration. The results also suggest that standard culture mediums do not provide an appropriate ionic and osmotic environment for chondrocytes.

The physico-chemical environment created and maintained by chondrocytes in turn has a powerful effect on cartilaginous metabolism. However, the supply of nutrients from vascular systems at the subchondral bone to the cartilaginous tissue of osteoarthritis is likely to be affected, causing the extracellular environment to deteriorate and some cells in degenerate cartilage are senescent (Kühn, 2004). This environment is often neglected by it can strongly influence matrix turnover or the responses of chondrocytes to growth factors or other external stimuli. Such limitations apply to all avascular tissues including tissue engineered constructs.

3. Effect of extracellular osmolality on glycosaminoglycan production and cell metabolism with time in culture

The effect of change in extracellular osmotic pressure on the amount of GAG production has not been well understood. It is important to initially know what culture condition provides the optimal environment for generation of articular cartilage. Therefore, we conducted an in vitro study to investigate the effects of change in extracellular osmotic pressure on cartilage cell morphology, GAG production, and cartilage cell metabolism, using the alginate beads system for three-dimensional culture of articular chondrocytes (Negoro, et al., 2008). Cells were obtained from metacarpal phalangeal joints of 18-24 month bovine. They were cultured for 6 days in alginate beads at 4 million cells/ml in DMEM containing 6% FBS under 21% O_2, Medium osmolality was altered by NaCl addition over the range 270-570 mOsm and monitored using a freezing point osmometer.

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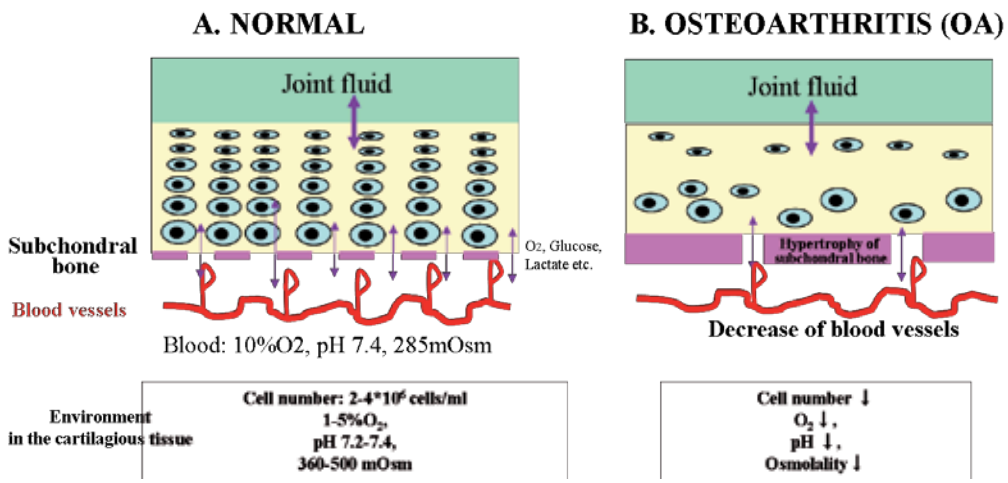

Figure 1. Changes of nutrient supply rote and extracellular environment of cartilaginous tissue with aging. (A) Normal cartilaginous tissue, (B) Osteoarthritis (OA).(A) This schema shows a representation of healthy articular cartilage overlying the subchondral trabecular bone. Although chondrocytes occupy less than 1% of articular cartilage, they are responsible for maintaining the integrity of the extracellular matrix by balancing macromolecular synthesis with breakdown. The matrix that surrounds them confers a mechanically resilient surface to the articulating bones within joints, and comprises collagens (principally collagen II), other noncollagenous proteins, and proteoglycans. In addition to structural support and absorption of shock offered by the subchondral bone, its small vessels and probably the interstitial bone fluid in osteocyte canaliculi, provide important nutrition to the cartilage. (B) The right schema shows some cartilage erosion, as seen in OA. The number of chondrocytes decreases with aging, but it is unknown whether this decline is caused by apoptosis, or insufficient supply of nutrients from the end plate. Ossification of the subchondral bone (tide mark) occurs with aging and is one of the major causes of cartilaginous degeneration. This leads to deterioration of the extracellular environment in the cartilaginous tissue and causes cellular impairment that is followed by a decline of matrix metabolism, resulting in progression to osteoarthritis. The nutrient supply for cells and the extracellular environment of the cartilaginous tissue have a considerable influence on the outcome of treating osteoarthritis by bioengineering techniques.

3.1. Cell viability

After 2, 4 and 6 days of culture, the chondrocyte viability rate was 90% or higher in all of the 4 osmolality groups (Fig.2). So, chondrocyte viability was not modified by the difference in extracellular osmolality. However, confocal microscopy showed that the cells were the largest under 270 mOsm and became smaller with increasing osmotic pressure (Fig.3). Under transmission electron micrographs of chondrocytes, at 370 and 470 mOsm all cells appeared viable, with large nuclei, dotted with chromatin and abundant rough endoplasmic reticulum (Fig.4B, C). The cells appeared active throughout the beads. In the beads cultured at 270mOsm, however, all cells were swelling with numerous cytoplasmic vacuoles and lipid droplets (Fig. 4A). Many cells had blebbing and these cells undergoing oncosis were seen. Under 570mOsm, many cells were reduced in size and blebbing was visible in the nuclei (Fig 4D,5A,5B).

3.2. GAG production and cell metabolism

Osmotic environment of cells in cartilage tissues is altered significantly by loading and morbid conditions. The cartilage tissues sustain static load and prolonged cyclical loading all the time

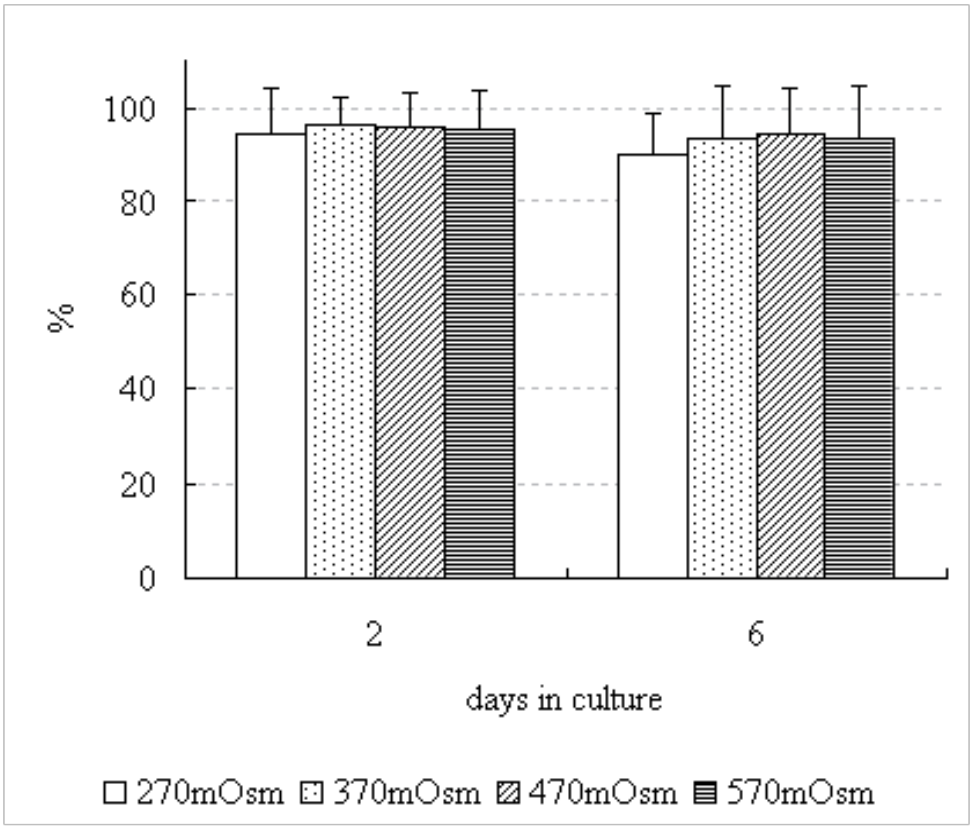

Figure 2. The cell viability by manual counting using a Live/Dead assay kit containing fluorescent probes. After 6 days of culture, the survival rate of cells was 90.3 ± 8.7, 93.3 ± 11.5, 94.4 ± 9.6, and 93.3 ± 11.5% (mean ± SEM) respectively in the 270, 370, 470, and 570 mOsm groups. The percentage of live and dead cells in sections was similar for the high and low osmolality cultures.

and osmolality imbalance occurs in the articular cartilage. To overcome osmotic imbalance and acquire new equilibrium, fluid is exuded from the tissue, and the PG level, cation level, and osmotic pressure are increased as a result. The chondrocytes always sustains high osmotic pressure. When loading is removed the tissue, fluid is slowly absorbed in turn, and the normal osmotic status is recovered. Urban, et al. (1993) incubated chondrocytes isolated from the articular cartilage in commercially- available DMEM solutions set at 250-270 mOsm of osmolality for 2 hours. Their experiment showed that the chondrocytes swelled by about 30-40% in the above osmolality condition and chondrocytes incubated in a medium set at 350-400 mOsm for osmolarity were most close to the size of chondrocytes in the intact tissues and synthesized the highest amount of PG. Hopewell & Urban (2003) investigated the effect of extracellular osmolality on chondrocytes cultured in alginate beads. Their study showed decreased sulphate incorporation rate for the cells incubated at high osmolality for 4 hours, recovery of sulphate incorporation rate for the cells incubated at high osmolality for 24 and 48 hours, and a higher sulphate incorporation rate than the original level for the cells incubated

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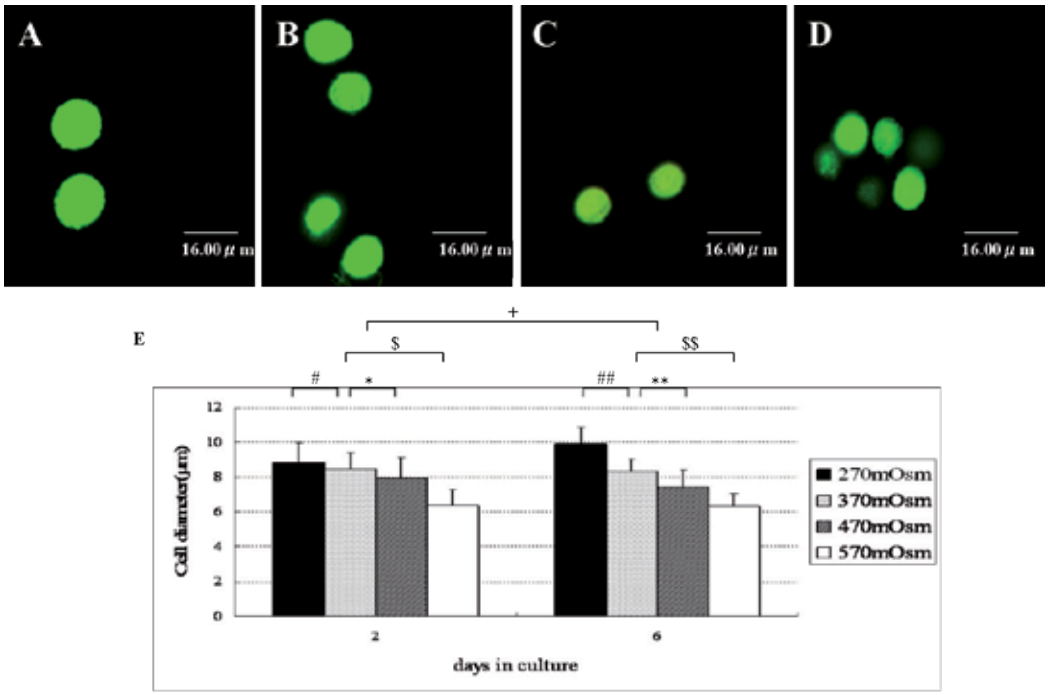

Figure 3. Confocal microscopy showed that the chondrocytes were greatest in the 270 mOsm group and diminished gradually along with the increases in osmolality. The diameters of cells measured obviously decreased in the higher osmolality groups ($P<0.05$). After 6 days of culture, the chondrocyte diameter was 9.9 ± 0.9, 8.4 ± 0.6, 7.4 ± 0.9, and 6.3 ± 0.8 μm (mean ± SEM) respectively in the 270, 370, 470, and 570 mOsm groups. The cell diameter was already established by 2 days of culture. The cell diameter increase with time in culture ($P<0.05$). (Reproduced with permission from Negoro K, Kobayashi S, et al. Effect of osmolality on glycosaminoglycan production and cell metabolism of articular chondrocyte under three dimensional culture system. Clin Exp Rheumatol 26: 527-533,2008.)

further. Based on these results, they indicated that chondrocytes are sensitive to osmolality and are able to adjust for high osmolality during short time. Bush, et al (2005). reported a single impact caused temporal and spatial changes to in situ chondrocyte viability with cell shrinkage occurring in the majority of cells. However, chondrocyte shrinkage by raising medium osmolality at the time of impact protected the cells from injury, whereas swollen chondrocytes were markedly more sensitive. These data showed that chondrocyte volume could be an important determinant of the sensitivity and response of in situ chondrocytes to mechanical stress. And also, Erickson, et al (2001). indicated that osmotic stress causes significant volume change in chondrocytes and may activate an intracellular second messenger signal by inducing transient increases in intracellular calcium ion. Palmer, et al (2001). measured the aggrecan promoter activity and mRNA levels using bovine monolayer chondrocytes subjected to hyper-osmotic loading for different time periods from 1 minute to 24 hours. They concluded the hyper-osmotic loading regulates aggrecan gene expression and cell size in isolated. Thus, mechanical compression of cartilage is associated with a rise in the interstitial osmotic pressure, which can alter cell volume and activate volume recovery pathways.

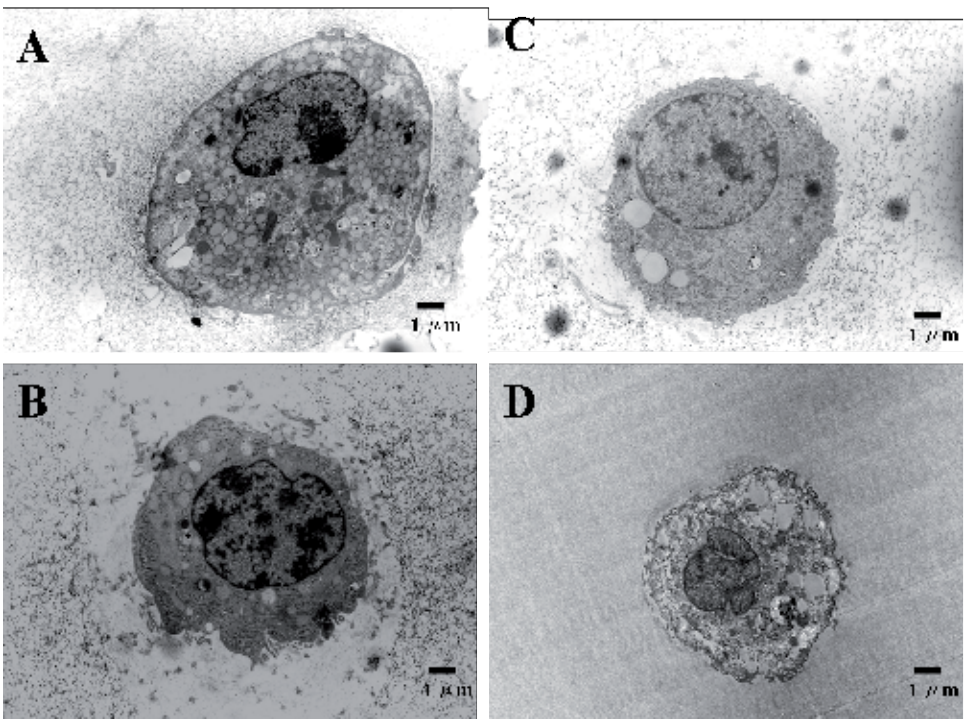

Figure 4. Electron micrograms of nucleus pulposus cells in the centre of the beads.A. under 270mOsm, B. under 370mOsm, C. under 470mOsm, D. under 570mOsm.At 270mOsm, the cell was swelling with numerous vacuoles and cytoplasmic organelles destroyed were visible. This cell undergoing oncosis were seen. At 370 and 470mOsm, all cells appeared viable. At 570mOsm, the cell and nuclei was reduced in size and chromatin condensation was visible in the nuclei. (Reproduced with permission from Negoro K, Kobayashi S, et al. Effect of osmolality on glycosaminoglycan production and cell metabolism of articular chondrocyte under three dimensional culture system. Clin Exp Rheumatol 26: 527-533,2008.)

In our study, GAG accumulation was measured using a modified dimethylmethylene blue (DMB) assay. GAG production was largest in the 370mOsm, and the capacity for GAG production and cell metabolism (lactate production) was low under hypo-osmolality and hyper-osmolality, and cell deaths were often observed on electron microscopy. While total GAG in beads/ml of beads volume increased with the duration of culture, it was greatest in the 370 mOsm group and lowest in the 270 mOsm group during culture (Fig.6A). The total GAG in beads/ml of beads volume was greatest in the 370 mOsm group, being 0.298±0.035 (mean ± SEM) mg/ml at day 6. It was lowest in the 270 mOsm group than in the other osmolality groups, being 0.122±0.019 mg/ml. In the high osmolality group at 570 mOsm, the total GAG in beads/ml of beads volume was greater than that in the 370 and 470 mOsm group after 2 days of culture, while the percentage of increased diminished subsequently until day 6. Similarly the rate of GAG in beads per live cell was the highest in the 370 mOsm group during culture (Fig.6B). While the total GAG in beads/million cells increased with the duration of culture, it was greatest in the 370 mOsm group and lowest in the 270 mOsm group during up to 6 days of culture. In the high osmolarity group at 570 mOsm, the total GAG in beads/million cells was

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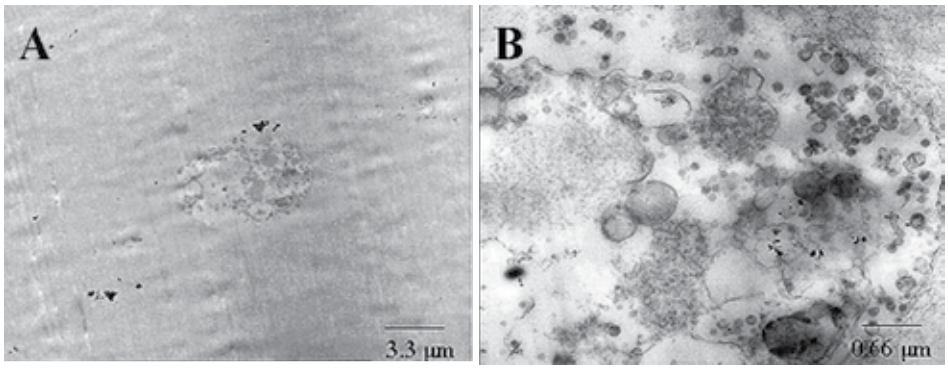

Figure 5. Under 570mOsm, Some cells undergoing apoptosis were seen. Cells with condensed and fragmented nuclei and condensed chromatin (apoptotic bodies) and with cytoplasmic organelles destroyed were visible. (Reproduced with permission from Negoro K, Kobayashi S, et al. Effect of osmolarity on glycosaminoglycan production and cell metabolism of articular chondrocyte under three dimensional culture system. Clin Exp Rheumatol 26: 527-533, 2008).

greater than that in the 270 mOsm group after 2 days of culture, while the percentage of increased diminished subsequently until day 6. The cell cultured at 370 and 470 mOsm were thus more active and accumulated significantly more GAG than cells cultured at 270 and 570 mOsm with time. In this study, the cells incubated at 370 mOsm produced the greater amount of GAG, and the cells incubated at high osmolality for 2 days showed a similar trend for GAG production to the results of Hopewell's experiment (2003) using isolated articular cartilage. However, the cells incubated further for 6 days produced a lower amount of GAG in the condition of high osmolarity and showed the profile of cell death (apoptosis) under electron microscope. Thus, this study indicates that chondrocytes is unable to adjust for such non-physiological conditions lasting for a long time and this phenomenon plays a critical role in the development of cartilage degeneration and resultant OA.

The lactate production was measured enzymatically and the rate of sulphate GAG synthesis was measured using a standard ^{35}S-sulphate radioactive method. Fig.7A shows the effect of extracellular osmolalities on lactate production by chondrocytes, a marker for total energy production. The rate of lactate production per live cell significantly decreased with time in culture. Lactate production was significantly decreased in hypo-osmolality (270 mOsm) group compared with the other groups. Thus, cell metabolism was decreased with the duration of culture, but metabolic hypofunction persisted under hypo-osmolality. Similarly the rate of sulphate incorporation per live cell was the highest in the 370 mOsm group during culture, and was decreased with an increase in extracellular osmolality (Fig. 7B). It was the lowest in the hypo-osmolality (270 mOsm) group during culture. The cells cultured at 370 and 470 mOsm were more active significantly more sulphate incorporation per live cell than cells cultured at 270 and 570 mOsm. The rate of sulphate incorporation fell more steeply than lactate rates with time in culture.

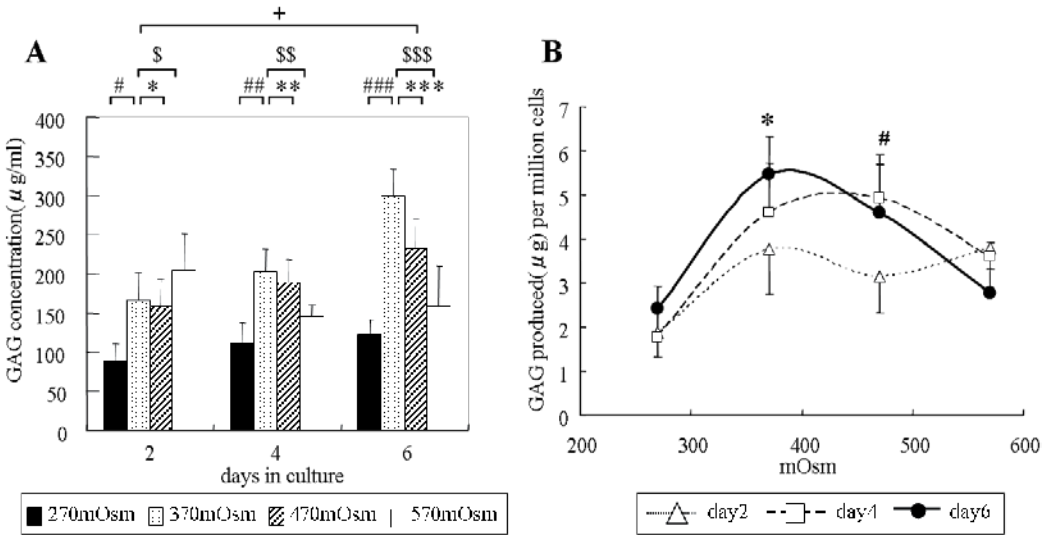

Figure 6. Effect of extracelluler osmotic change on GAG accumulation/tissue volume (A) and GAG produced per million cells (B). This figure gives pooled data for 4 representative osmolality from 3 separate experiments for cells cultured by articular chondrocytes. (A) GAG accumulation/tissue volume was significantly increased at 370 and 470 mOsm with time in culture. This was the highest in the 370 mOsm group and the lowest in the hypo-osmolality group (270 mOsm) decreased after 6 days of culture. In the hyper-osmolality group (570 mOsm), the rate of GAG accumulation/tissue volume wasn't decreased after 2 days of culture when compared with the 370 mOsm group. Values are mean ± standard error. (Scheffe, *: P<0.05). (B) GAG produced per million cells was significantly increased at 370 and 470 mOsm with time in culture (*,#: P<0.05, Scheffe between between 2 and 6 days)] This was the highest in the 370 mOsm group and the lowest in the hypo-osmolality group (270 mOsm) decreased after 6 days of culture. In the hyper-osmolality group (470 and 570 mOsm), the rate of total GAG produced per million cells wasn't decreased after 2 days of culture when compared with the 370 mOsm group. (Reproduced with permission from Negoro K, Kobayashi S, et al. Effect of osmolarity on glycosaminoglycan production and cell metabolism of articular chondrocyte under three dimensional culture system. Clin Exp Rheumatol 26: 527-533,2008).

In this study, the chondrocytes produced the highest amount of GAG in the osmolality condition of 370 mOsm after 2 and 6 days of culture. The amount of GAG production was obviously lower in the low osmolality cultures than in the culture at the optimal osmolarity close to that in the normal cartilage. On electron microscopy of chondrocytes cultured under varying levels of osmolality, cells under 370 mOsm generally showed normal nuclei and cytoplasm, while cells under hypo-osmolality presented oncotic changes, with cellular swelling and destructed organelles of the cytoplasm. On the other hand, cells cultured under hyper-osmolality were reduced in size and some cells underwent apoptosis. Manjo & Joris (1995) reported oncosis is a form of cell death accompanied by cellular swelling, organelle swelling, blebbing, and increase membrane permeability. They also showed that necrosis can occur after both forms (oncosis and apoptosis) of cell death. Therefore, its mechanism is based on failure of the ionic pumps of the plasma membrane induced by the changes of extracellular osmotic environment. Thus, our physiological and morphological study showed the articular chondrocytes is unable to adjust for such non-physiological conditions lasting for a long time and this phenomenon plays a critical role in the development of cartilage degeneration and resultant OA.

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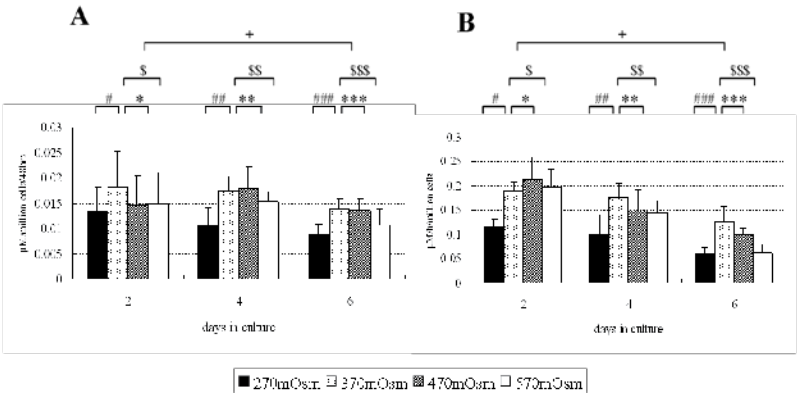

Figure 7. Effect of extracellular osmotic change on lactate production rate (A) and ^{35}S-sulphate incorporation rate (B). (A) The rate of lactate production per live cell decreased with time in culture (+: P<0.05, 2 way ANOVA with repeated measures among 2,4 and 6 days). After 4 and 6 days of culture, lactate production was clearly decreased under hypo-osmolality (270 mOsm) compared with other levels of osmolality. (Scheffe between 270 and 370 mOsm [#: P=0.405, ##,###: P<0.05], 370 and 470 mOsm [*: P=0.707, **: P=0.988, ***: P=0.997], or 370 and 570 mOsm [$: P=0.738, $$: P=0.559, $$$: P=0.083]). (B) Sulphate incorporation rates fall with time in culture (+: P<0.05, 2 way ANOVA with repeated measures among 2,4 and 6 days). Values are mean ± standard error. It was clearly decreased in the hypo-osmolality (270 mOsm) groups compared with the 370 mOsm groups during culture. After 6 days of culture, lactate production was clearly decreased under the 570 mOsm groups compared with the 370 mOsm groups. (Scheffe between 270 and 370 mOsm [#,##,###: P<0.05], 370 and 470 mOsm [*: P=0.537,**:P=0.446,***:P=0.109], 370 and 570 mOsm [$: P=0.98, $$:P=0.366, $$$:P<0.05]). (Reproduced with permission from Negoro K, Kobayashi S, et al. Effect of osmolarity on glycosaminoglycan production and cell metabolism of articular chondrocyte under three dimensional culture system. Clin Exp Rheumatol 26: 527-533,2008).

The osmotic pressure in the cartilage tissues is obviously higher than the plasma osmolarity (about 280 mOsm), and chondrocytes exist in the extracellular environment different from that of other tissues. This study indicated that adjustment of osmolality is very important for the culture of chondrocytes. At cell densities found *in vivo* (standard conditions) in the cartilage tissue viz, 4 million cells/ml and GAG concentration in beads cultures was 0.298 mg/ml at 370 mOsm in 6 days. Assuming that the initial production rate is maintained and that there is no loss of GAG, it is calculated that > 1000 days of culture is necessary to produce a GAG concentration equal to the in vivo GAG concentration of 7% per wet weight (viz. 70 mg/ml). That is, it is suggested that chondrocytes need to be cultivated at the cell density of 4 x 106 cells/ml for more than 1 year in order to construct cartilage tissue in the GAG concentration of about 70 - 100 mgs/ml, which is equal to the GAG concentration in the normal cartilage tissues, using cell culture technology.

4. Effect of growth factor on glycosaminoglycan production in articular chondrocytes

Growth factors, such as transforming growth factor-β (TGF-β) and fibroblast growth factor-2 (FGF-2), have demonstrated a great potential as cartilage anabolic factors because of their

ability to induce matrix synthesis and promote repair in cartilage. TGF-β is one of the anabolic factors involved in cartilage maintenance and appears to be a good candidate for cartilage repair. TGF-β is a stimulator of extracellular matrix production, like collagen type II and proteoglycan (PG), in chondrocytes and it downregulates matrix-degrading enzymes (Edwards, et al. 1987). High amounts of TGF-β are stored in healthy cartilage (Pedrozo, et al. 1998, Redini F, et al. 1997, Morales, et al. 1991a,b, Burton-Wurster & Lust,1990), whereas in OA cartilage the expression of TGF-β is reduced (Verdier et al. 2003). FGF-2 belongs to the family of heparin-binding growth factors (Gospodarowicz, et al. 1987).This proteins are known to induce chemotactic, angiogenic, and mitogenic activity, and play an important role in early differentiation and development (Powers et al., 2000, Burgess & Maciag, 1989). Cell expansion in the presence of FGF-2 was shown to promote differentiation of stromal cells into cartilaginous tissues (Martin, et al. 1998). Similary, FGF-2 was shown to retain the chondrogenic differentiation potential of extensively expanded stromal cells (Tsutsumi, et al. 2001). Like wise, pellet cultures established from FGF-2 treated human mesenchymal stem cells were larger in size and contained a higher level of proteoglycans than untreated cells (Solchaga, et al. 2005). Martin et al. (1999a, 2001) showed that the FGF-2 treatment increased cell number during monolayer expansion and the differentiation capacity of expanded chondrocytes in subsequent 3-dimensional culture.

The most important problems in cartilage regeneration medicine are to supply nutrients to cells activated by grafting or growth factors and to maintain a healthy extracellular environment. However, it has been shown that the extracellular osmotic pressure decreases with cartilage degeneration in candidates for treatment. In this study, cells were isolated from the cartilage from metacarpal phalangeal joints of 18-24 month bovine. Alginate beads containing cartilage cells collected from adult bovine (4 million cells/ml) were prepared. Three-dimensional culture was done for 5 days under a low osmotic condition (270 mOsm) as seen osteoarthritis or a normal osmotic condition like that of healthy cartilage (370 mOsm). Transforming growth factor-β (TGF-β) and fibroblast growth factor-2 (FGF-2) were added every day. GAG production was assessed by the dimethylmethylene blue (DMB) assay after 5 days. Fig. 8 shows the effect of osmorality and growth factor on the amount of GAG accumulated with time in culture. Significantly more GAG was accumulated by cells cultured at 370 Osm than low osmoralities (270 mOsm). The cells cultured with the growth factors (TGF-β or FGF-2) were more active and accumulated significantly more GAG accumulated than cells cultured without the growth factors. However, GAG accumulated was significantly lower for cells cultured at 270 mOsm than those at 370 mOsm. Thus, the clinical application of cartilage regeneration medicine needs to be advanced by providing appropriate physiological conditions with consideration of age-related cartilaginous changes.

The interrelationships between cell density, cell viability and activity, and diffusion distance resulting from nutrient supply constraints, limit the rate at which GAG can be accumulated in three-dimensional constructs. GAG accumulation depends on GAG production per cell and on cell density. GAG accumulation thus appears necessarily slow, and the general finding that cultures of >7 months are required to achieve concentrations of GAG similar to those seen in vivo may not be easily overcome (Kellner et al. 2002, Roughley, 2004). An increase in GAG

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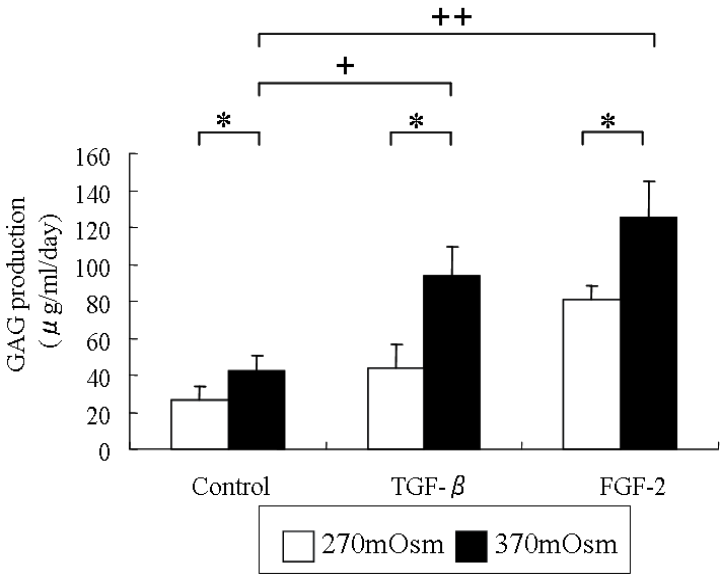

Figure 8. Effect of extracelluler osmotic change and growth factors on GAG accumulation/tissue volume. This figure gives pooled data for 2 representative osmolality from 6 separate experiments for cells cultured by articular chondrocytes. GAG production at 370 mOsm was about 42.1±9.1 μg/ml/day, while it was only about 26.3 ± 4.3 μg/ml/day at 270 mOsm. During culture at 370 mOsm, GAG production was increased about 2-3 times by addition of growth factors, while there was a clear decrease in the response of GAG production to growth factors at 270 mOsm compared with that seen at 370 mOsm. Incubation with growth factors, enhances GAG production during culture at a normal osmotic pressure, but cell function is decreased in degenerated cartilage. Values are mean ± standard error. (*: Scheffe between 270 mOsm and 370 mOsm medium, #: P<0.05, Scheffe between control and every growth factors).

production rate per cell can be induced by addition of growth factors, but the relative increase which can be achieved is limited (usually two–threefold under optimal conditions) and the consequent increase in metabolic demand can lead to a fall in pH in the construct center and thus severely limit growth factor efficacy. In this study, addition of TGF-β, and FGF-2 to constructs was found to have big effect on the concentration of accumulated GAG under low osmoralities. Thus, increasing cell metabolism potentially should increase GAG deposition, but leads to a more nutrients demands.

5. Effect of cell density on the rate of glycosaminoglycan accumulation by chondrocytes

Glycosaminoglycan (GAG) accumulation in constructs is dependent on the rate of GAG production per cell and on the cell density. It seems intuitive, therefore, that increasing cell density should increase rate of GAG deposition, as indeed has been shown in several studies (Almarza & Athanasiou, 2005, Mauck et al., 2002, Mauck et al., 2003b, Saini & Wick, 2003, Williams et al., 2005, Kobayashi et al., 2008). However, it is apparent from these studies that

GAG accumulation in the construct does not increase in proportion to cell density and, indeed, GAG production per cell appears to fall at high cell densities.

We used alginate gels in the form of beads as a model system (Fig.9). Cells were isolated from bovine metacarpal phalangeal joints. They were cultured in alginate beads in DMEM containing 6% FBS under 21% O_2 at cell densities from 1-33 million cells/ml. The amount of GAG accumulated in a typical culture of bovine articular chondrocytes increased with time in culture. After 7 days a bimodal response was evident with the concentration of GAG accumulated rising as cell density was increased from 1-10.4 million cells/ml and then falling gradually as cell density was further increased (Fig.10A). However the GAG production per million cells fell as cell density was increased (Fig.10B).

Next, we mainly examined two initial seeding cell densities viz. 4 million cells/ml (Fig.9A) and 25 million cells/ml (Fig.9B); these represent cell densities often used in alginate beads and found in vivo in the cell density in adult cartilage from the bovine metacarpal-phalangeal joint.

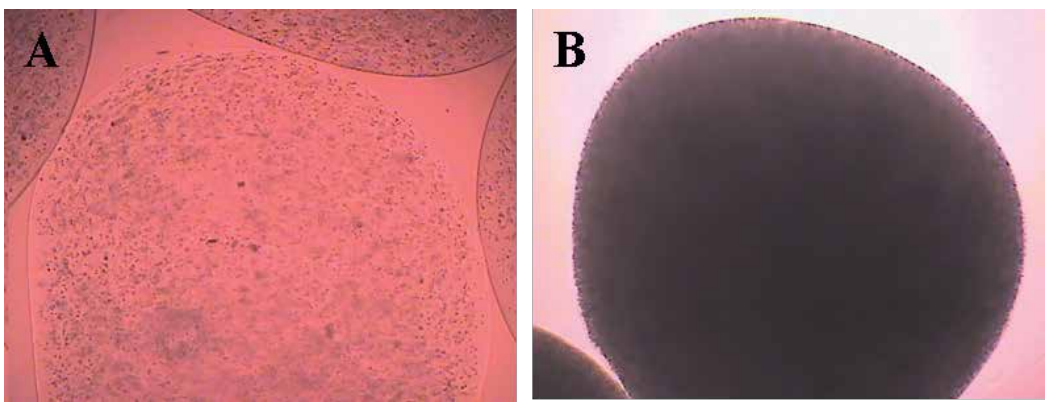

Figure 9. Three-dimensional cell culture system of alginate beads. (A) 4 x 10^6 cell/ml, (B) 25 x 10^6 cell/ml

Significantly more GAG was accumulated by cells cultured at high (25 million cells/ml) than low (4 million cells/ml) densities and in agreement with results shown in Fig.11, GAG accumulated also increased with time in culture. At 4 million cells/ml, the concentration of GAG in the bead reached 520.9 ± 62.4μg/ml in 5 days. These concentrations could be increased to 1297.2 ± 115.2 μg/ml by raising cell density to 25 million cells/ml (Fig.11A). The increase in amount of GAG accumulated was not directly proportional to increase in cell density; although the beads at high cell density contained more than 6 times as many cells as those at low cell density, they only produced only 2-3 times as much total GAG. After 5 days culture at 4 million cells/ml, GAG accumulation per cell was 166.3 ± 38.9μg GAG/million cells (Fig.11B). These amounts fell to 70.9 ± 23.9 μg/million cells when cell density was increased to 25 million cells/ml. Thus, cells cultured at low density were more active and accumulated significantly more GAG per cell than cells cultured at high density. Evidence of greater cellular activity for cells cultured at low cell density was also seen from measurements of lactate production per live cell; lactate production was significantly higher for cells cultured at low density than for those

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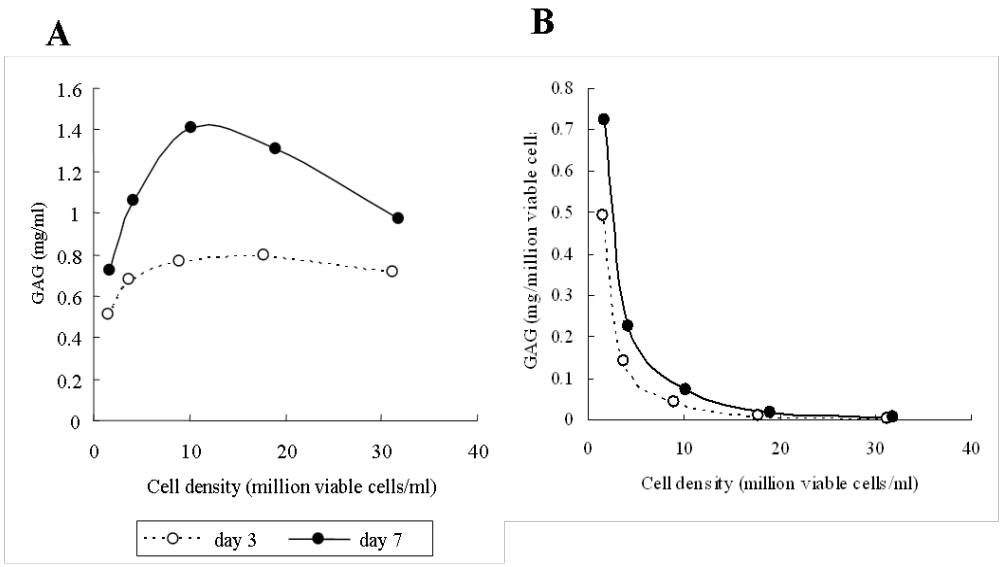

Figure 10. Typical results showing effect of cell density on GAG deposition (A) and on GAG accumulation per million cells (B) by articular chondrocytes. Cells were isolated, encapsulated in alginate beads at cell densities ranging from 1 to 33 million cells/ml. Beads were cultured for 7 days at 5 wells/bead in 2 ml medium, 2 wells for each cell density and cultured for 7 days in DMEM containing 6% serum. Beads were then dissociated for cell counting and assay of total GAGs.

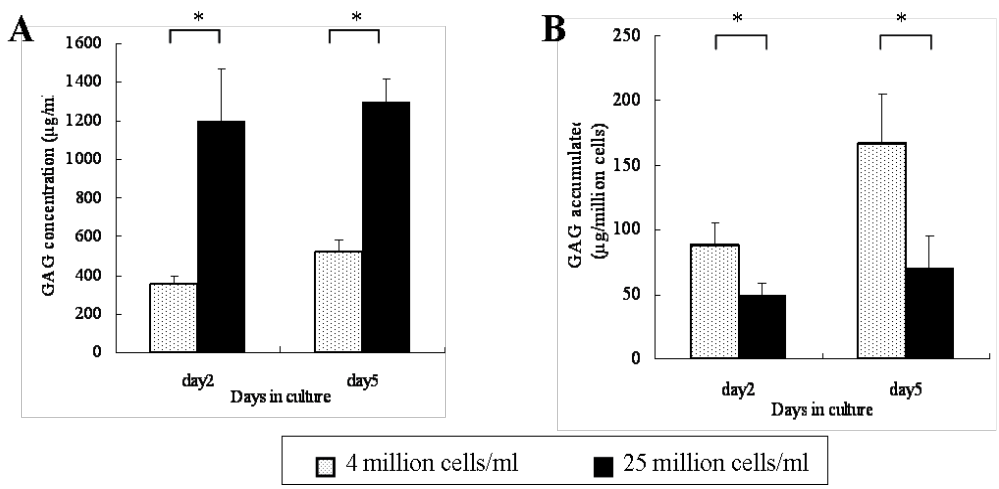

Figure 11. Effect of cell density on GAG concentration (A) and GAG accumulation per million cells (B) by articular chondrocytes after 2 days and 5 days in culture. Cells were encapsulated in alginate beads, cultured in DMEM with 6% serum under air and GAG concentration and cell density measured after 2 and 5 days culture. These figures give pooled data for the two representative cell densities from 3 separate experiments. Values are mean ± standard error. *, : Significant difference ($P<0.05$) between the high cell density (25 million cells/ml) and the low cell density (4 million cells/ml) using non-paired t test.

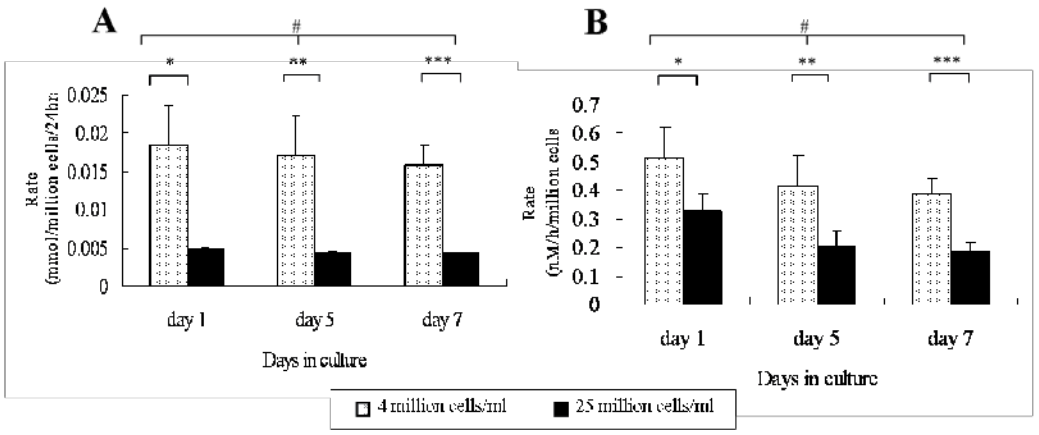

Figure 12. Effect of cell density on lactate production rate (A) and 35S-sulphate incorporation rate (B). (A) Cells were cultured under standard conditions in beads containing 4 and 25 million cells/ ml (1.0ml medium, 5 beads/well) for up to 7 days, with complete medium change daily. Representative beads were dissociated for cell counting and viable cell density/bead recorded. Lactate in the medium was measured at days 1,5 and 7, after 24 hours culture and rates per million cells/24 hrs reported. High cell density lead to a fall in cellular metabolism (*,**,***: P<0.05, Paired t test between high [25 million cells/ml] and low cell density [4 million cells/ml]). Lactate production rate fall with time in culture (#: P<0.05, 2 way ANOVA with repeated measures among 1, 5 and 7 days).(B) At days 1,5 and 7, tracer sulphate was added to the fresh medium of 3 wells, the beads were cultured in the radioactive solution for 4 hours, the beads dissociated and cell density and sulphate incorporation measured (Fig 3B). Results are given as means ± s.e.m of 3 independent experiments. Sulphate incorporation rates fall with increase in cell density (*,**,***: P<0.05, Paired t test between high [25 million cells/ml] and low cell density [4 million cells/ml]) and with time in culture (#: P<0.05, 2 way ANOVA with repeated measures among 1, 5 and 7 days).

cultured at high density (Fig 12A). Lactate production also decreased with time in culture, more rapidly at high than at low cell densities. The rate of sulphate incorporation per live cell was also greater at low than at high cell densities (Fig.12B), though the difference was less marked than that seen in Fig.12A; sulphate incorporation fell more steeply than lactate production with time in culture.

The change in percentage of live and dead cells with time in culture at the periphery and centre of beads is shown in Fig.13 for cells cultured at low (4 million cells/ml) and high cell densities (25 million cells/ml), respectively. For cells cultured at 4 million cells/ml, 100% of the cells were viable at the both the periphery (Fig 13A) and in the centre (Fig 13B). It can be seen that by day 2 of culture at high cell densities, while almost all the cells at the periphery were alive (Fig. 13C,E), 30 percent of the cells in the bead centre were dead (Fig.13D,E). Similar percentages were dead at day 5 of culture, suggesting the profile of viable cells across the bead was established early in culture.

From sections of beads cultured for 5 days at 4 and 25 million cells/ml and then stained with safranine O to visualise the sulphated GAGs accumulated (Fig.14A-D), there was a noticeable difference between staining at the bead periphery (Fig.14A,C) and the bead centre (Fig. 14B,D). From densitometric measurements, GAG accumulated at centre of beads cultured at 25 million cells per ml was only 60-70% of that accumulated at the periphery (Fig.14E). Less staining was seen in beads cultured at 4 million cells per ml as seen also from chemical

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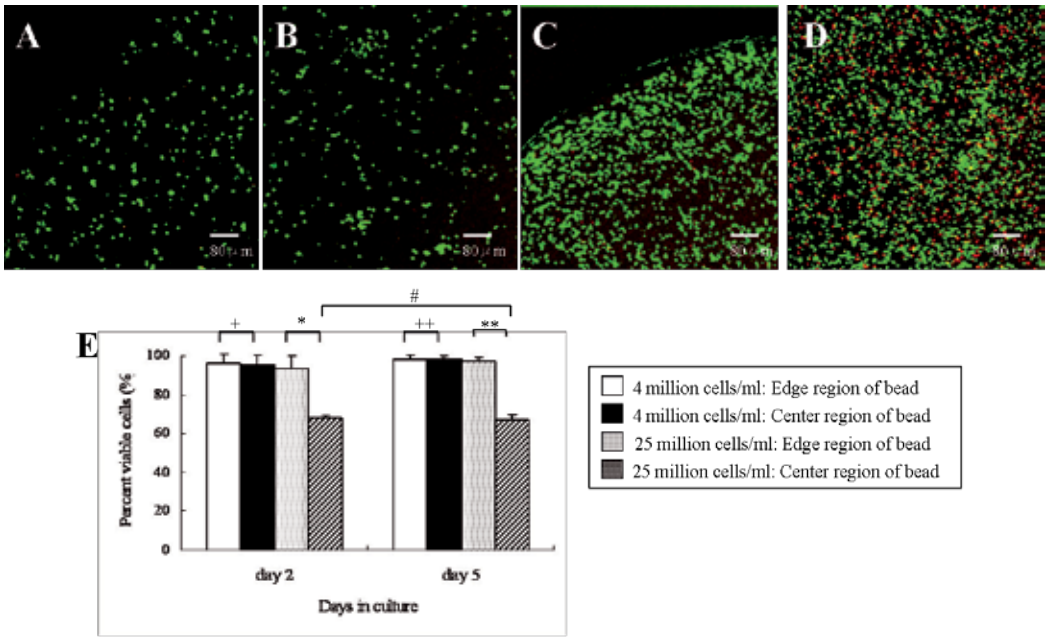

Figure 13. Effect of cell density on cell viability under conforcal microscope.This shows the variation of cell viability at the edge and centre of beads with time and cell density. Cell viability was determined using a live/dead assay kit; live cells (green) and dead cells (red) were counted manually. Results are means and s.e.ms of percentage of viable cell from 4 representative beads. Figs 13A and 13B shows the periphery and central region respectively of a typical bead cultured at 4 million cells/ml after 5 days. Figs 13C and 13D shows the periphery and central region of a bead cultured at 25 million cells/ml after 5 days. Fig 13E shows the variation of cell viability with region (edge versus centre). At high cell density (25 million cells/ml), cell viability is lower in the centre than at the edge (+: P=0.977, ++: P=0.893, *, **: P<0.05, 2 way ANOVA with repeated measures between edge and centre).

analysis. At this low density however there was no significant profile of GAG accumulation across the bead, with the amount accumulated in the centre similar to that accumulated at the periphery.

At low cell density, transmission electron micrographs indicated that all cells appeared viable and active (Fig.15A). Chondrocytes cultured at high cell density appeared viable at the bead periphery (Fig.15B). However cells undergoing apoptosis were seen in the centre; the cells and nuclei were reduced in size and chromatin condensation was visible in the nuclei (Fig. 15C,D). These results are in agreement with those of others who have found regions of cell death in the center of constructs or even of microsphere aggregates (Martin et al., 1999b, Mercier et al., 2004, Obradovic et al., 1999), and that glycosaminoglycan accumulation may highest at the construct peripheries. In addition, others have also found that increasing cell density or cell number does not necessarily increase matrix accumulation (Mercier et al., 2004).

These avascular constructs, unless experimentally perfused, rely on diffusion for supply of nutrients to the cells (Obradovic et al., 2000) simulating the condition seen in cartilaginous tissue. In avascular tissues and in constructs, there are steep gradients of oxygen and other nutrients between the surface and center of the tissue or constructs (Kellner et al., 2002, Malda

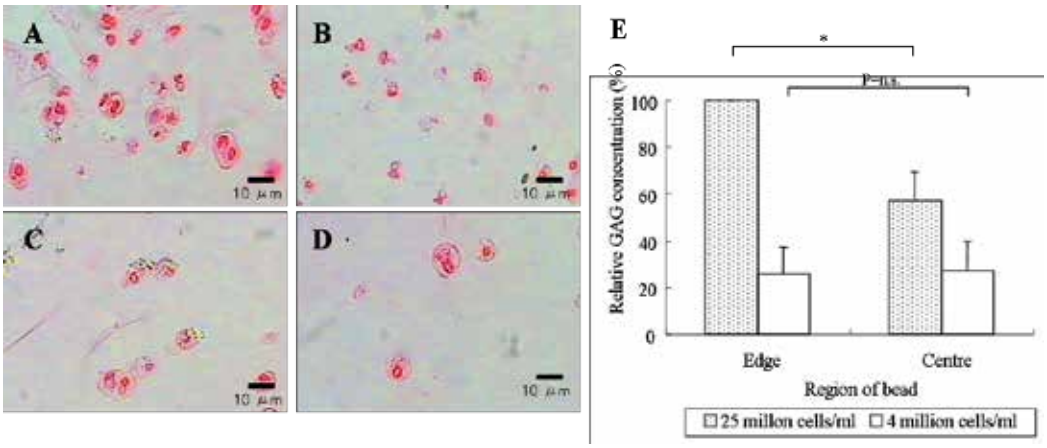

Figure 14. Effect of cell density on GAG deposition by Safranine O staining after 5 days culture.Fig 4A shows a 20micron section through a typical bead of chondrocytes cultured at 25 million cells/ml (A,B) and at 4 million cells/ml (C,D) cultured for 5 days. Images at the periphery (A,C) and at the centre (B,D) were captured digitally and the GAG around cells was quantified using image-analysis. Results are reported as the fraction of stained area in the peripheral and central regions of the beads and data normalized to results at 25 million cells/ml (E). At high cell density, the area of the staining was higher at the edge of the bead (*: P<0.05, Paired t test between edge and centre). At low cell density, however, there was no significant profile of GAG accumulation between edge and centre (P=0.712, Paired t test between edge and centre).

et al., 2004). The steepness of these gradients, and hence the nutrient concentrations in the center of the construct, depend not only on the geometry and properties of the tissue or construct but also on the cell density and the cellular activity (Haselgrove et al., 1993, Zhou et al., 2004, Soukane et al., 2005). Thus, in any particular construct or tissue, an increase in cell density will lead to a corresponding fall in the concentration of nutrients such as oxygen and glucose, and an increase of metabolic by-products such as lactic acid (Zhou et al., 2004), leading, once cell density has risen sufficiently, to a fall in rates of cell metabolism and glycosaminoglycan synthesis (Gray et al., 1988, Ysart & Mason, 1994). If cell density is sufficiently great, oxygen and glucose concentrations and pH levels can fall to levels which can no longer sustain viable cells (Horner & Urban, 2001) leading to the necrotic region in the construct center. Diffusional nutrient transport is thus a limitation on the number of viable and active cells which can be maintained in any construct or tissue; indeed, viable cell density is inversely related to diffusion distance both in disc and in constructs (Horner & Urban, 2001, Stockwell, 1971).

6. Physical limitations to biological repair and tissue engineering

The interrelationships between cell density, cell viability and activity, and diffusion distance resulting from nutrient supply constraints, limit the rate at which GAG can be accumulated in three-dimensional constructs. GAG accumulation depends on GAG production per cell and on cell density. At low cell densities, cells may be functioning optimally but the low cell density

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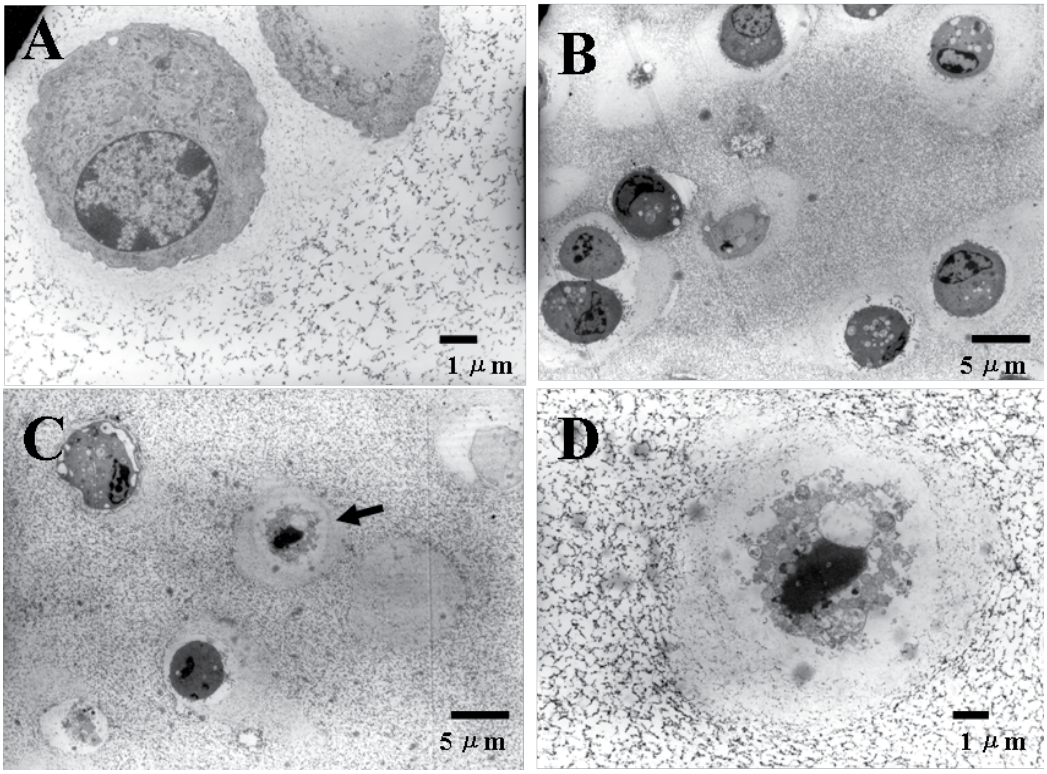

Figure 15. Electron micrographs of central and peripheral articular chondrocytes cultured at low (A) and high cell density (B-D). These pictures show representative cells from the central and peripheral regions of beads cultured at low (4 million cells/ml) and high (25 million cells/ml) for 5 days. (A) Central region, low cell density, cells appear normal. (B-D) High cell density (25 million cells/ml). (B) Bead periphery, some cells appear normal. (C) Central region showing cells undergoing apoptosis (arrow). The cells and nuclei were reduced in size and chromatin condensation was seen in the nuclei in comparison of the cells in the periphery. (D) High power magnification of apototic nucleus pulposus cell.

limits the rate of GAG accumulation. At high cell densities, more GAG is deposited at least initially, but nutrient gradients particularly in the center of constructs, reduce the rate of GAG deposition per cell and may even lead to a fall in cell number if cells die. GAG accumulation thus appears necessarily slow, and the general finding that cultures of >7 months are required to achieve concentrations of GAG similar to those seen in vivo may not be easily overcome (Kellner et al., 2002, Roughley, 2004). The different maneuvers which have been tried to increase GAG production all have limitations. An increase in GAG production rate per cell can be induced by addition of growth factors, by providing mechanical or ultrasound stimulation or through alterations to scaffold properties (Blunk et al., 2002, Richmon et al., 2005, van der Kraan et al., 2002, Kuo & Lin, 2006), but the relative increase which can be achieved is limited (usually two–three fold under optimal conditions) and the consequent increase in metabolic demand can lead to a fall in pH in the construct center (Wu, M.H. et al., 2008) and thus severely limit growth factor efficacy. Indeed, addition of anabolic growth factors, such as

Transforming growth factor-β (TGF-β) (Morales, et al. 1988, 1991a,b, Luyten, et al. 1988, van Osch, et al. 1998, Diao, H. et al. 2009,), bone morphogenetic protein-2 (BMP-2) (van Beuningen, et al. 1998, Blaney Davidson, et al. 2007), bone morphogenetic protein-7 (BMP-7) (Chubinskaya, et al. 2007, Hayashi , et al. 2008), insulin growth factors-1 (IGF-1) (Luyten, et al. 1988, van Osch, et al. 1998, Fortier, et al. 2002, Goodrich, et al. 2007) and fibroblastic growth factor (FGF) (Martin, et al. 1999a, Maehara, et al. 2010), to constructs was found to have little effect on the concentration of accumulated GAG although it increased construct size. In addition, GAG production rates appear to fall with time in culture in many different systems also limiting GAG accumulation (Mercier et al., 2004). Increasing cell density potentially should increase GAG deposition, but leads to a lower activity per cell, and also, in general, has not been found to increase GAG deposition rates (Panossian et al., 2001). It should also be noted that tissue in vivo cannot support too high a cell density, so in vitro culture of constructs at high cell density could lead to cell death after implantation.

Culture conditions such as stirring or perfusion (Freyria et al., 2000, Seidel et al., 2004) appear able to overcome diffusive transport initially, but as GAG concentrations rise and the hydraulic permeability of the construct falls, convective transport also is reduced and rates of GAG deposition slow. GAG concentrations were reported to reach 5% by wet weight within 2 months but took a further 5 months to increase to 7% GAG. In view of the long culture times which appear necessary to achieve the required GAG composition in vitro, achievement of in vivo concentration before implantation of a construct may be an unrealistic and possibly unnecessary goal for tissue engineered disc. It has been suggested that about 2-3 fold amount of GAG can be produced using anabolic growth factors. Even if such growth factors are used, more than 100 days of culture is thought to be necessary. Furthermore, it has been reported that turnover of GAG in the cartilage tissue takes about 2 - 3 years (Maroudas, 1975). So, GAG is slowly synthesized in the biological condition, and a long time is necessary to construct articular cartilage with adequate mechanical strength even if cells are maintained in active status by three-dimensional culture. Anabolic growth factors were obvious tools to enhance cartilage repair. Recently, Carragee et al (2011). reported that the bone morphogenic protein-2 (BMP-2) has cancer risk associated with the use of BMP-2 in spinal fusion surgery. In the future, orthopaedic surgeons must exercise caution to use the anabolic growth factors in clinics.

At present, the target diseases of treatment utilizing bioengineering (tissue engineering) such as chondrocytes implantation are local lesions such as traumatic cartilage defect and osteochondritis dissecans (Bittberg, 1999, Minas, 2001, Ochi, et al. 2002, Robert, et al. 2003) (Fig. 16A). For regeneration of extensive degenerated cartilage in OA, it is necessary to secure a large amount of chondrocytes for implantation. If a large graft is implanted, a nutritional problem may occur as explained above. In addition, the subchondral bone needs to be healthy to obtain the normal function of articular cartilage such as dispersion of load. But, lesion of OA is not localized in the cartilage layer but involves the subchondral bone (e.g., osteosclerosis) (Fig.16B). The osteosclerosis which is the subchondral bone, covers the bone-cartilage junction with age and looks to close the nutritional route through these vascular system (Trueta, 1963, Havelka, et al. 1984). Therefore, even if regeneration of cartilage is achieved by means of hyaline cartilage, the regenerated cartilage may sustain overload and may be degenerated

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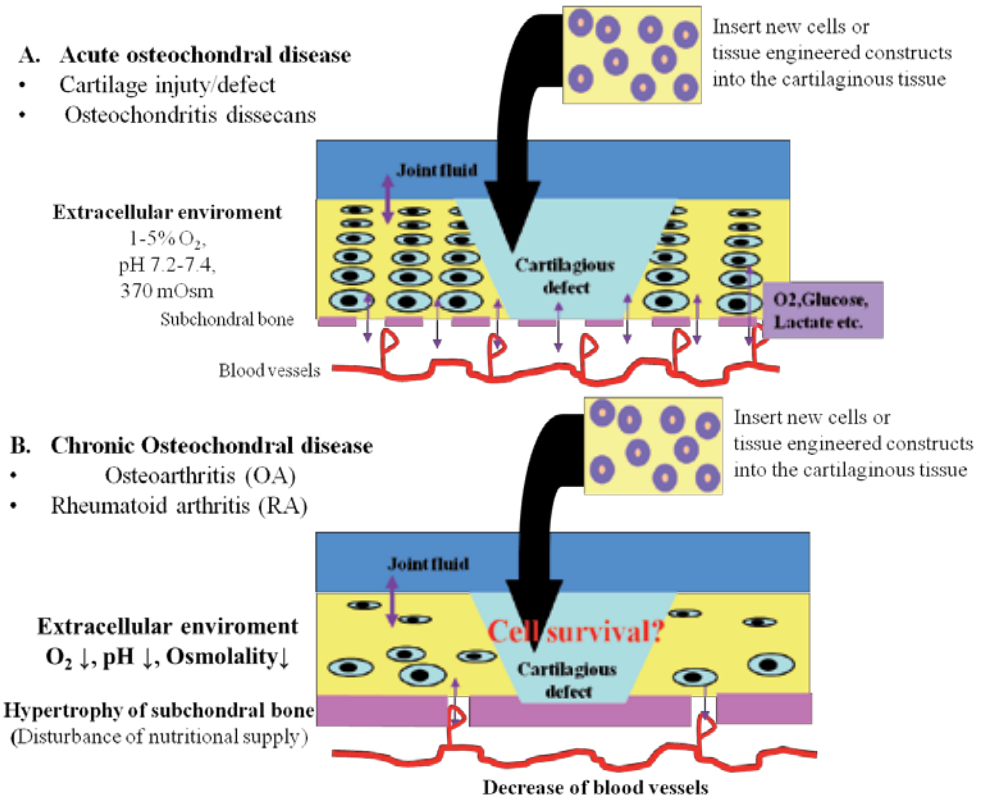

Figure 16. Target diseases of treatment utilizing bioengineering or tissue engineering technique.(A) Chondrocytes implantation is local lesions such as traumatic cartilage defect and osteochondritis dissecans at present. However, GAG is slowly synthesized in the biological condition, and a long time is necessary to construct articular cartilage with adequate mechanical strength even if cells are maintained in active status by culture.(B) Biological repair depends on the cartilaginous tissue maintaining a population of viable and active cells. If, in some degenerate cartilaginous tissues, nutrient supply is impeded (as it seems to be) hence resident cells are inactive or die. Therefore, we have to consider chemical and ionic environments in the degenerative cartilage before performing the tissue engineering of cartilaginous tissue.

again unless the mechanical environment is modified together. Cellular repair using autologous chondrocyte transplantation appears successful even though chondrocytes are implanted with no matrix at all. Under these conditions, remodeling in vivo appears to produce a cartilage-type matrix under some conditions. Tissue engineered composites implanted with low GAG appeared to accumulate GAG in vivo, withstand physiological loading, and remodel towards a hyaline-type matrix. Perhaps optimization of such processes is a more useful goal.

7. Conclusions

There is increasing interest in the using biological methods to repair osteoarthritis. Biological repair depends on the articular cartilage maintaining a population of viable and active cells.

Adequate nutrition of the cartilaginous tissue influences the outcome of such therapies and, hence, must be considered to be a crucial parameter. Therefore, it is very important to maintain an appropriate physicochemical environment to achieve successful cartilage repair by biological methods and tissue engineering procedures.

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Chapter 23

Potential of Different Tissue Engineering Strategies in the Bladder Reconstruction

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Additional information is available at the end of the chapter

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1. Introduction

Organism's functions are provided by different biological apparatus and one of them is essential for maintaining the integrity of this system. Indeed activities of each organs lead to the cellular production of metabolites. These metabolic products are discharged into the blood system to be supported by the urinary apparatus. The purification of the blood is essential to preserve the homeostasis of the organism and the blood pressure. The upper urinary tract is composed of kidneys which filter the blood to evacuate the excessive water, ions and toxic metabolic products. Then, this mixture called terminal urine is transported in the lower urinary tract by the ureters. The lower urinary tract consists of the bladder which stocks the urine until its evacuation by the urethra. The terminal urine is cytotoxic because of its composition of nitrogenous and potential carcinogenic elements, also its pH which varies between 4.5 and 8.3 [1, 2]. Therefore, the storage of urine need to be safe, this is why the bladder possesses two specific characteristics.

Firstly, the bladder wall is watertight in order to prevent the urine from seeping through the different tissue layers and damage their structure. Secondly, the bladder is compliant in order to keep a low pressure during the urine filling, and prevent reflux towards the upper urinary tract which could lead to kidneys failure. Thereby, the bladder is able to adapt its capacity to the volume of the accumulated urine. These properties are entirely related to the nature and the structure of different tissues which compose the bladder wall (Figure 1). As shown in the figure 1, the tissue directly in contact with urine is the urothelium. This is an epithelium highly specialized regarding the watertight function and adaptation to large changes in urine volume.

This distensible property is assumed by the pseudo-stratified character of the urothelium, while the water tightness is provided by the most differentiated urothelial cells known as umbrella cells. These cells are endowed with very tight junctions in the apicolateral side, while a well-organized protein barrier, called uroplakin plaque, covers the luminal surface [3, 4]. The urothelium rest upon the lamina propria: an extracellular matrix which serves of nutritive and informative support for cells. The lamina propria is mostly made of collagen of type I and III [5, 6]. Another type of extracellular matrix is found at the lower level. It contains three organized smooth muscle cells bundles responsible for urine emptying because of their contractile property. This extracellular matrix is characterized by the presence of an elastic fibres network which allows the distension of the bladder and a low pressure during filing.

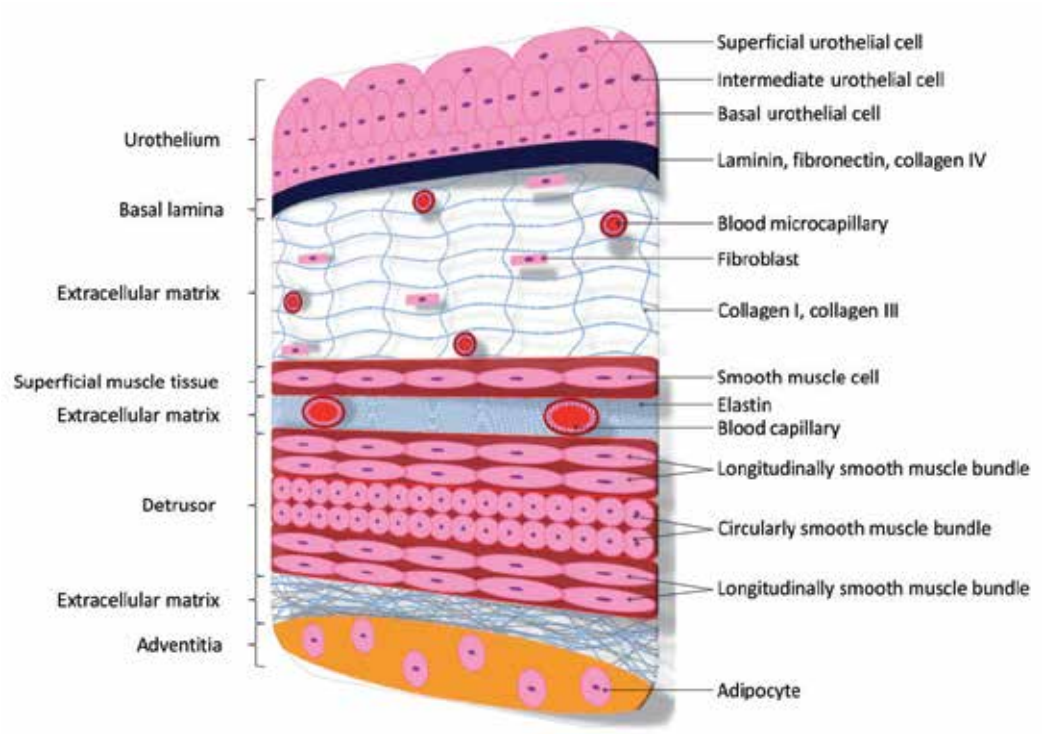

Figure 1. Scheme of bladder wall.

Every year 400 million person of all ages suffers from urinary disorders. Several congenital disorders such as bladder exstrophy or neurogenic bladder can affect the function of this organ. But there are also acquired bladder problems like traumatisms, inflammations, chronic interstitial cystitis and cancer which is the sixth most detected. All of these pathologies may require surgical augmentation or reconstruction of bladder wall to restore the storage capacity. The first application of a free tissue graft for bladder replacement was reported by Neuhof in 1917 [7], when fascia was used to augment bladders in dogs. Since that first report, diverse

methods have been proposed for this type of surgical intervention, but actually the gold standard is the bladder replacement/repair with autologous segment of the gastrointestinal tract, also named Enterocystoplasty [8, 9]. It has the advantage of being highly vascularized, promoting the survival of the graft. Unfortunately, this technique is associated with multiple short and long-term complications well documented (Table 1). The most frequent is metabolic disturbance, but mucus secretion, stone formation, bladder perforation and malignancy have also been found many years after enterocystoplasty [10-13]. These complications predominantly result from the difference between the absorption property of intestinal mucosa and the watertight function of the bladder epithelium, concerning the contact with urine.

	BLADDER
Pathologies	Congenital malformations Neurogenic bladders Bladder cancers Interstitial cystitis
Common treatment	Surgical bladder repair or reconstruction with intestinal segment. The choice of the segment depend mainly on age and medical history.
Associated complications	Intestinal dysfunctions Hypocontractility Hematuria, dysuria, urolithiasis Neoplasia Mucus production Metabolic imbalances [1]: delay of growth, and reduction of bone density in pediatric patients.

Table 1. Complications associated with current bladder treatment.

The lack of autologous tissue with similar properties to the native bladder is a limitation which led numerous research groups to develop alternative approaches. These last years, many fundamental knowledge concerning bladder cells and matrix have emerged, and have constituted an essential aid to the in vitro elaboration of various bladder models. This chapter will explain the different sources of cells used, the different type of engineered matrices, and the advanced concerning the techniques of in vitro culture. The emphasis will be placed on qualities and inconveniences of each approach, as well as the clinical potential of the engineered models.

2. Cellular source

Although the bladder is composed of many type of cells, the most harvested for vesical tissue-engineering is urothelial and smooth muscle cells. Urothelial cells are organized into three

layers which are anchored to the basal lamina. Basal cells, reside in the lower layer [14]. These progenitor cells develop themselves into intermediate cells, and differentiate into umbrella cells which are the most mature urothelial cells. The degree of urothelial differentiation is defined by the expression of specific proteins, such as keratins, claudins and uroplakins (Table 2) [15]. Smooth muscle cells have a fusiform shape and are assembled into bundles also organised into three layers. In the outer and inner layers, the smooth muscle bundles are oriented longitudinally, while those of the middle layer circularly. In each bundle, a single smooth muscle cell is innervated and action potential can propagated to neighboring cells in order to causes a coordinated contraction. The proliferation and differentiation of urothelial and smooth muscle cells are interdependent, because of factors released from these cells [16].

	UROTHELIAL CELLS			
Markers	basal	lower intermediate	upper intermediate	superficial
K5 - 10 - 17				
K13				
K14				
K7 - 8 - 18 - 19				
K20				
ZO1				Intercellular junctions
Cldn3				Intercellular junctions
Cldn4				Intercellular junctions
Cldn5				Intercellular junctions
Cldn7			Intercellular junctions	
Occldn			Intercellular junctions	
UPIa/Ib/II/IIIa				

Table 2. Markers of urothelial cells (white = no presence, light-blue = low presence, dark-blue = strong presence).

The importance to associate cells to a urological substitute was described by numerous studies [17-19]. These works showed that bladder substitute seeded with cells led to better in vivo regeneration, than the use of scaffold only. Cells help the graft integration; this is why the latest bladder substitutes are generally constituted by a combination of scaffold and cells.

2.1. Urological cells

Urothelial cells culture dates back to more than 30 years [20], and two major methods are developed for their extraction. The most former method is the explant technique. It consists to put a fragment of biopsy in nutritive medium, and let cells to migrate. Then, sequential action

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of trypsin will permit to harvest different cells with distinct adhesion properties. The other way is the enzymatic technique which consists to detach the urothelium from basal lamina, undergoing matrix extracellular protease action (thermolysin, dispase, collagenase IV) [21, 22]. This last method is faster, led to a suitable yield and a good purity. Finally to obtain enough cells, an amplification stage must be engaged. Generally, epithelial medium is used and supplemented with serum or/and specific growth factor (e.g. epithelial growth factor).

Smooth muscle cells were first described in 1913. In the same manner of urothelial cells, the two approaches could be used for extraction [23]. In the case of enzymatic treatment, the collagenase will be used to digest the extracellular matrix, made of collagen I principally, to liberate these cells. In the amplification stage, it is important to know that the serum percentage could modify the phenotype (contractile or secretory) and the functional property (electrophysiological) of these cells [24-26].

Endothelial cells are more and more frequently extracted and used for bladder reconstruction. Indeed, after transplantation the graft need to be rapidly vascularized to survive in vivo. Endothelial cells could organize themselves into capillary or secrete angiogenic factors which could improve a certain inosculation between the substitute and the host vasculature [27]. Some teams harvest endothelial cells from human umbilical vein (HUVEC), what asks ethical question, and other achieve these cells extraction from bladder microcapillaries which appeared more physiological for the elaboration of the vesical substitute. In this last case, enzymatic treatment could be used and the harvested cells must be purified with beads coupled with a specific endothelial marker (e.g. PECAM-1) [21].

2.2. Stem cells

In some situation the bladder is too affected and no healthy cells could be harvested to elaborate a tissue-engineering substitute. So, stem cells could represent a serious alternative and major avenue in the regenerative medicine. These cells are characterised by their capacity to maintain themselves by symmetrical division. But in a second phase, asymmetrical division occurs and leads to a daughter stem cell and a daughter differentiated cell. This last event makes difficult the in vitro preservation of stem cells and the constitution of an usable stock.

Embryonic stem cells are pluripotent and therefore can evolve into cells of all three embryonic layers (ectoderm, mesoderm and endoderm). The method of culture to enable specific differentiation pathways are not yet discovered, but the in vivo benefits have already been shown [28-29]. Unfortunately, their aptitudes to initiate teratoma, the need of genetic donor-host match to avoid immune-rejection and their potential illegal and non-ethical character compromise their clinical use.

Autologous stem cells could resolve these limitations. They present a low possibility of tumor malignancy and an exact histocompatibility. These cells niche within many adult tissues, in order to maintain homeostasis during tissue turnover and repair. However, they present lower proliferation capacity and plasticity which restricted their use.

Induced pluripotent stem cells appeared in 2006 and consist of reversion of differentiated cells into stem cells [30]. The reprogramming of adult cells requires the introduction of specific

transcription factors (Oct4, Sox2, Klf4 and Myc), which allow to acquire differentiation potential comparable to the embryonic stem cells [31, 32]. This ethical approach provides an easy and important cell sources for clinical application. However, even if a major risk of cancer has been corrected [33], epigenetic changes not yet entirely documented.

3. Nature of scaffolds

Once the cells have been obtained, they must be seeded onto a support (Table 3). The scaffolds are not only a physical support, but also supplier of biochemical information. It is difficult to encourage an appropriate in vitro cellular behaviour because spatial and temporal evolutions occurred during organogenesis. The complexity of extracellular matrix sequentially increases from morula to blastocyst stage [34, 35], and the signalisation between cells and extracellular matrix proteins change in a dynamic way [36]. In the present state of our knowledge, laminin is firstly synthetized to allow cellular adherence, while collagen IV and fibronectin appear more latter to initiate the cellular migration [37]. In a second phase, these extracellular matrix proteins form an organized matrix, the basal lamina, which is essential for the development of specialized tissue like epithelium [38]. Thus, tissue engineering must tend to create a controlled cellular microenvironment, taking into account the physiological process, to reach the experimental organogenesis goal. In another hand, scaffolds must offer comparable physical properties and have the capacity to confront the same mechanical strain. In the case of vesical repair, the substitute must combine resistance with elasticity to adapt itself to cyclic pressure caused by bladder filling and emptying. The scaffold biocompatibility is the starting point of material choice, and many products have been experimented with a clinical application point of view.

STEPS	DESCRIPTION
Harvesting bladder cells	Very small biopsy is taken. The different tissues are separated. Cells from each tissue are collected.
Cells amplification	Protocols exempt of biological risks. Using serum must be from the patient. Using growth factors must be recombinant.
Assessing cells into a support	Seeded cells must be functional and located at their physiological area.
Surgical implantation	The substitute must be functional before the bladder repair.

Table 3. General procedure in bladder reconstruction field.

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3.1. Natural matrices

Autologous tissues have been tested for bladder repair before the standard use of intestinal segment in the beginning of the eighties [1]. The skin, the omentum, the stomach, the pericardium have been used with very limited success [39-42]. Indeed, the epithelium of these tissues is not specialized in watertight function. So, the direct contact between the non-urological tissues and the toxic urine leads mainly to the formation of fibrosis and the contraction of the graft [43, 44].

Acellular tissue matrices represent a growing interest in the urological regenerative medicine. They are prepared from native tissue with a decellularisation and sterilisation process [45]. The purpose is to abolish immunological potential by discarding cells with physical, enzymatic or chemical protocols [46]. This materials offer the advantages of mechanical and biochemical environment ideal for the cellular recognition. Their matrix architecture is relatively preserved and the physiological organization of extracellular proteins can generate appropriate signalling to induce a suitable cellular behaviour [47]. Unfortunately, the decellularisation and sterilisation protocols include offensive biophysical and biochemical elements (temperature, pH, ionic detergents) which can denature extracellular matrix proteins and damage the physiological environment [48]. Compared to other scaffolds, the ability to provide a nutritive supply by neovascularization after the graft, and therefore to promote the graft survival constitutes an attractive advantage which justifies the choice of acellular matrices [49, 50]. Small intestinal submucosa (SIS) [51] and bladder acellular matrix graft (BAMG) [52, 53] are the most tested for bladder reconstruction in animal models. Microscopic analyses generally show a relative cellular organization, probably due to the presence of growth factors [54]. But functional tests such as watertightness or compliance are very seldom evaluated.

Concerning SIS, studies are conflicting. Some experiments describe the benefit to use SIS with contractility testing and immunostaining analysis [18], while other report fibrotic scarring observations and contraction during in vivo bladder augmentation in canine model [55]. Histologically, the urothelial and smooth muscle cells seeding led to a suitable adherence but without cellular maturation. This lack of regenerative potential could be caused by an absence of the appropriate extracellular proteins-cells communications, due to the fact that intestinal and bladder matrices are not similar [56]. It is known that extracellular matrix composition and structure presented by the bladder basal lamina have an impact on urothelial cells behaviour [57]. This is why bladder acellular matrix graft (BAMG) has been also tested. However, porcine bladder augmentation with a seeded BAMG demonstrated a local cells infiltration which remained limited in the periphery [58]. The insufficient cellular organization resulted in calcification process within the graft and its shrinkage. This incomplete cellular migration within the BAMG could be attributed to the decellularisation treatment, because it was reported that laminin and fibronectin are not preserved [59]. Thus, even if urological cells are placed into a familiar environment, the alteration of matrix proteins and the removal of intrinsic growth factors do not permit an optimal cellular signalisation [60]. Moreover, the disruption of the extracellular matrix compromises the specific architecture and leads to the loss of mechanicals properties, such as elasticity or resistance [61]. Optimization of these

protocols is in progress and the damages caused by the preparation of decellularised tissue tend to be corrected [62, 63].

Intestinal segment and bladder wall are richly vascularized tissues, and this specificity is not negligible for the survival of the graft. However some parameters limit the clinical use of BAMG and SIS such as their xenogeneic origin and the associated risks of contamination by pathogens. Generally, the matrix proteins are highly conserved between the different species and seem to be non-immunogenic but an epitope, which is present in many species except human and a category of monkeys, was found in marketed SIS extracellular matrix [64]. Numerous postoperative inflammations have been reported [65] and in vitro DNA residues have been detected [66]. Because of their biological nature, the acellular tissues constitute a great potential for bladder reconstruction but the protocols of preparation must be improve to better preserve the extracellular matrix composition/architecture and to remove effectively any cellular fragments [67].

3.2. Synthetic scaffolds

Synthetic polymers are made of chemically assembled macromolecules and could have different physical properties (thermoplastics, thermo-hardenings and elastomers). The use of synthetic scaffolds, supported by the capacity to deliver any three-dimensional shape at low-price, would allow to overcome the lack of native tissue available for transplantation. Each characteristic of this type of material can be controlled. The hydrolysis degradation of synthetic substitutes is important in the context of organ repair. The material used for bladder reconstruction must remain stable until the organization of seeded cells and the migration of surrounding cells. But during the terminal tissue remodelling, the substitute degradation must follow the tissue regeneration. The degradation rate of synthetic materials can be determinate by the molecular weight and the nature of biopolymer used for copolymerisation. Then the degraded fragments can be treated by the metabolic pathways [68-70]. The size of pores can also be modulated in order to influence the cellular migration, vascular invasion, and diffusion of nutrients, waste and oxygen [71, 72]. The cell adherence and growth can be enhanced by synthetic support, as shown for the reconstruction of different damaged tissues [73]. But in the case of bladder substitute, the in vitro evolution of seeded urologic cells into a mature cellular tissue has not yet demonstrated. There are fundamental differences between synthetic and biological molecules. The size range of fibers, their biophysical and biochemical properties are not comparable, and the challenge for these supports is to enhance a suitable interaction between cells and synthetic microenvironment, in spite of the dissimilarities existing with the physiological context.

Silicone, polyvinyl sponge and teflon, have been firstly tested for bladder reconstruction with the help of synthetic materials [74-78]. The advantage was to construct neobladders in a reproducible way, but the poor cytocompatibility, the lack of vascularization and their immunological potential led to significant complications. Because of the absence of cellular development, and urothelial cells particularly, the direct contact with urine caused the formation of fibrosis and contraction of the alloplastic graft.

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The more recent synthetic polymers including poly(ethylene glycol) (PEG), poly l-lactic acid (PLLA), poly(lactic-co- glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), and polyurethane (PU), are used to pursue the bladder tissue-engineering research [79]. Few successes have been reported, but like in the case of acellular tissue matrices, it has been shown that the cell seeding play an important role concerning the graft integration [19]. This experience of canine bladder reconstruction have been led at mid-term with PGA scaffold coated with PLGA, and contrarily to the seeded synthetic substitute, contracture and inflammatory response have been observed with free scaffold one month post-transplantation. In spite of association of urothelial cells with a high proliferative capacity when urothelium is damaged [80], the surrounding cells of the host do not migrate in the whole surface of tissue-engineered bladder. The only one microscopic result demonstrated a well development of an urothelial tissue onto the seeded scaffold, with the presence of muscle bundles, but no analysis was led to assess the degree of urothelial maturation or the contractility function of smooth muscle cells. The potential of synthetic materials to support urothelial differentiation have not been proven yet. The same team have combined collagen with their PGA scaffold to lead bladder augmentation in patients with end-stage bladder disease [81]. The strategy is to supply a three-dimensional shape and mechanical resistance with the help of PGA, and to induce a cellular signal with the help of the collagen. It was shown that the biological activity provided by collagen improve the cellular propagation and development of seeded urothelial and smooth muscle cells. Additionally, omentum was used as a vascularization bed to support the graft survival [82]. Even if significant improvement of vesical capacity has been shown for only one of the seven patients, great strategic progresses have been made to initiate the elaboration of a composite scaffold able to communicate with cells and enhance their appropriate and terminal development. The scale of cellular environment and signalisation induced by specific protein sequences have been taking into account with the advent of biotechnologies.

3.3. Nanobiotechnologies

The nanotechnologies, which emerged in the last decade of the twentieth century, are defined by specific functions induced by the nanoscale dimension or the nanoscale organization of a material [83]. This characteristic could permit to overcome the problems encountered with the classic synthetic substitutes made of micrometer scale elements. The interest to include nanometer elements in the composition of synthetic scaffolds is to reproduce the scale of the size of native extracellular matrix proteins, and better manage cellular behaviour [84]. Recent researches demonstrated the effect of roughness surface, comparable with the roughness of the native bladder, on smooth muscle cells adhesion and urothelial cells development [85-87]. On another hand, the surface of synthetic materials could be modified with different chemistry, topography or roughness parameters, to improve the interaction with proteins. Thus, extracellular matrix proteins could be added to the scaffold in order to enhance the cellular interaction and to tend toward natural signalisation. Recent publication reported that vitronectin adsorption is improved by 20% if the roughness of synthetic surface is augmented with nanotechnology process, compared to nanosmooth surface [88]. A concrete example is illustrated by the increase of fibronectin adsorption onto synthetic material, because of the

augmentation of roughness surface by adding carbon nanotubes and without changing the chemical properties.

Smooth muscle cells phenotype is affected by the nanometer topography of synthetic surfaces. This phenomenon was illustrated by recent works. One of them reported the elaboration of PLGA/PU materials with different feature dimensions. It is appeared that more the features size is comparable to the nanometer scale, like the extracellular matrix proteins, more the adhesion of bladder smooth muscle cells is improved [85]. A new chemically etching technique, which breaks ester and ether bonds via NaOH and HNO3 treatments, was tested by the same team to convert synthetic surface from micrometer to nanometer scale [89]. It was shown that this chemical treatment can generate nanoscale features on various synthetic surfaces (PLGA, PCL, PU), and then enhances the functions of bladder smooth muscle cells, compared to the conventional nanosmooth polymers. It is noted that micron, submicron and nanostructured polymers was generated with this same chemical protocol, and the development of bladder smooth muscle cells have been improved on the nanostructured surface, independently of a chemical action. So, any type of synthetic scaffold could be nano-engineered and promote bladder smooth muscle cell functions, such as elastin and collagen secretion [86]. Further studies demonstrated that the direct action of nanometer and submicron scale surface features is to promote the adsorption of proteins from serum present in culture media. Therefore it seems that the bladder smooth muscle cells behaviour is more directly improved by this serum proteins coating rather than the synthetic nanostructured environment [90, 91]. These observations lead us to believe that the next generation of synthetic substitute could be grafted with soluble proteins in order to ameliorate the cellular signalisation.

Urothelial cells growth is also affected by the roughness of synthetic surfaces [87]. With nanometer rough synthetic surface, results show the improvement of adhesion and proliferation of urothelial cells, and also the reduction of calcium stones, often noticed with conventional synthetic materials. In vivo studies were performed in rodent model and confirmed the benefits showed with bladder smooth muscle cells. But nanostructured synthetic scaffold did not resist the contact with urine, leading to post-graft bladder leaks [92]. Further optimizations must be proposed to induce cellular differentiation. Fibronectin is recognized to induce cell migration, laminin for its adherence potential, therefore synthetic scaffolds will be completed with bioactive nano-elements. Protein sequences implicated in cellular signalisation will be used such as RGD peptide, a cell adhesive integrin-binding peptide found in most of extracellular matrix protein [93]. The development and maturation of seeded cells must be evaluated at longer term, while in vivo testing will occurred throughout animal models more comparable to human (the porcine urologic system is the nearest of human [94]).

4. The self-assembly method

The natural and synthetic materials have their own characteristics (Table 4), specific bioactive elements for the first and processing reproducibility for the second. The advances deserve a particular attention even if these experimental scaffolds could not be recommended at that

time for clinical application. Acellular matrices present the risk of incomplete decellularisation and variability of biological activity. While synthetic materials are too far from the composition and architecture of physiological extracellular matrix. But one of the common disadvantages of these two models is the possibility of immunological response in vivo and therefore a graft rejection.

BIOMATERIALS	CHARACTERISTICS
Non autologous natural matrices	+ Biologically organized tissue. + Specific growth factors reservoir. + Augmentation of tissue availability. - Damaged architecture of matrix. - Presence of cellular fragments. - Risk of xenogeneic contamination.
Synthetic matrices	+ Control of physical properties. + Biodegradable. + Evolution towards nanoscale. - The deficiency of biological signalization. - Cicatrization with the host's tissue. - The cost.
Autologous matrix	+ Limitation of immunoreaction. + Self-assembled tissue. + Evolution towards physiological stimuli. - Lack of *in vivo* investigation.

Table 4. Types of engineered bladder substitute.

Autologous and functional substitute represents the ideal alternative to avoid immunosuppressive therapy and to enhance the in vivo regeneration. The tissue-engineering field proposes a new approach to attain these objectives: the self-assembly method [95]. This technique is based on the use of the own cells of patient and their capacity to differentiate in vitro in order to form a mature tissue. At the Laboratoire d'organogénèse expérimentale (LOEX) many autologous substitutes are elaborated with controlled culture conditions and without the help of exogenous biomaterials. Human cornea [96], psoriatic skin model [97], microvascularized tissue-engineered skin substitute [98] has been developed for clinical repair or pharmacological investigations. Based on this method, an autologous vesical tissue was elaborated with a view to future clinical bladder repair/augmentation.

4.1. Autologous vesical substitute

A minimally invasive preoperative technique was developed to extract the bladder cells from the same small biopsy. Enzymatic method allows the harvesting of bladder cells with high purity [21]. Fibroblast, urothelial, endothelial and smooth muscle cells can be grown in the

laboratory setting. Appropriate growth factors (e.g. epidermal growth factor for urothelial proliferation or vascular endothelial growth factor for endothelial cells) permit a good cellular expansion and the constitution of sufficient cellular bank.

The preparation of engineered vesical tissue begins with the elaboration of matrix support [99]. In presence of ascorbic acid [100, 101], fibroblasts have the ability to synthesize and assemble their own collagen fibers, and form an extracellular matrix layer which could be manipulated. The autologous matrix layers are superimposed to provide a sufficient mechanical resistance and to create a three-dimensional biological environment. It is known that mesenchymal-epithelial interactions play critical role in tissue development [102-105]. In the urological tract, mesenchyme regulates epithelial maturation and functional activities, while epithelium also contributes to the mesenchymal cells differentiation [106]. This is why the self-assembled scaffold is made of a specifically organized extracellular matrix where cells can develop themselves with appropriate biological signalling. Urothelial cells are seeded on the top of fused matrix layers and cultivated until they have proliferated on the whole surface, then we induced epithelial maturation with the use of the culture at air/liquid interface (Figure 2).

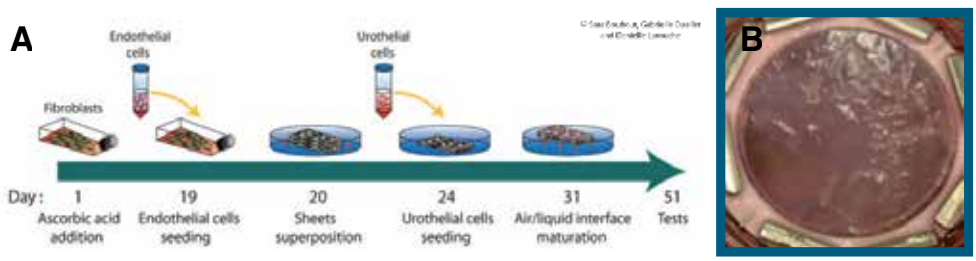

Figure 2. The self-assembly method (A) used to elaborate the engineered bladder tissue (B).

Masson's trichrome staining displayed a homogenous distribution of collagen I and the covering of stratified urothelium was roughly similar to a native vesical mucosa. Cytokeratin 8/18 staining confirmed the well widespread of a stratified urothelium, and claudine-4 staining demonstrated the presence of tight junctions which are important to avoid urine infiltration. More interestingly, the permeability evaluation at urea was performed on the reconstructed vesical tissues and their watertightness profile is comparable to the native bladder. Conversely, the permeability test realized on self-assembled matrix, devoid of reconstructed urothelium, showed a fast diffusion of urea. This result supports the necessity to have a mature urothelium, in order to avoid urine extravasation, followed by in vivo necrosis and fibrosis of tissues. An attention was paid to the endothelialization of the substitute in order to encourage the post-graft survival. LOEX laboratory obtained a rapid inosculation between a capillary-like network of reconstructed skin and the host vasculature [6]. Thus endothelial cells, harvested from bladder microcapillaries, can be added before the seeding of urothelial cells, and cultured in order to form microcapillary-like network within the self-assembled substitute. The endothelialized vesical substitute displayed an also good watertightness profile than the native bladder, and mechanical properties were acceptable to allow suturing. The potential limit of

this method is the incertitude to dispose of healthy bladder cells, especially in a case of end-stage disease. This is why the question concerning the getting of an appropriate source of cells remains in reflection, but in some case, urological cells could be harvested from upper segment of urethra because of its sharing of the same embryologic origin with the bladder [107]. The self-assembled vesical model still must to be completed and tested in vivo, but its autologous character and its efficiency as a barrier to urea are essential properties to tend toward an ideal substitute for bladder augmentation.

4.2. Culture conditions

In regenerative medicine domain, the goal of tissue engineering is to deliver a functional substitute. It consists in obtaining specific cells with sufficient purity and quantity, and inducing their differentiation in order to ensure their biological role. Self-assembled extracellular matrix provides both a physical and informative support for urological cells, but the control of cellular proliferation/differentiation balance deserve to be better taking into account. Several studies reported an altered maturation of urothelial cells in vitro, particularly a defect of uroplakins synthesis [108] and their incomplete assembly at the apical surface of urothelial cells [109, 110]. The pathway of differentiation is induced by defined intracellular signals. Transcription factors such as Fox-A1, PPARγ and RXR, have been identified as being involved in the expression of proteins implicated in the urothelial functions [111]. The bladder is subjected to different pressure during the emptying/filling cycle, and the cells are constantly exposed to mechanical stresses (e.g. hydrodynamic strength) [112]. It was shown that mechanical stress acts on survival, migration and proliferation of bladder cells [113-115]. More accurately, cyclic hydrostatic pressure seems to promote uroplakin trafficking/maturation [116-118], and to influence the growth of bladder smooth muscle cells by activating Rac1 signaling pathway [119], or their proliferation via the PI3K activation [120]. Even if the molecular mechanism acting under cyclic pressure remains not elucidated, the conviction that physiological stimulations would be required for the differentiation of bladder cells cultured in vitro is more and more evocated in the literature [121-123].

In the beginning, mechanical stimuli were included in cellular culture protocols and interactions between cells or between cells and extracellular matrix proteins were analyzed [124, 125], but physiological conditions were not yet investigated. In 2008, the mimic of bladder filling and emptying was tested through a bioreactor which applies hydrostatic pressure waves in a cyclic way [126]. Porcine BAMG was used and the expression of extracellular matrix proteins was more elevated under dynamic condition. This result is encouraging for acellular matrix application. Indeed, acellularisation treatment deteriorates the matrix architecture and the obtained tissue after treatment had a too high porosity [127]. Because of its property to support cell proliferation and migration, hyaluronic acid could be used in static regime to decrease the porosity observed after the acellularisation process [128], but this step could be replaced by placing the reconstructed substitute under physical stress. The urothelial cells were also influenced since mechanical stimulations induced the increasing of uroplakin II expression. The effects of physical strain must be studied in more details because gene expression does not constitute sufficient information, since the protein functionality depends on its

complete synthesis, its good folding and its appropriate localization. But these preliminary results encourage the integration of mechanical phase in the process of engineered bladder. This is why we designed a bioreactor which is scheduled to reproduce the physiological intravesical pressures at the fetal stage, and replace the air/liquid phase used for our self-assembled model. Briefly, our self-assembled vesical tissue is placed between two chambers, with urothelial side face to the pressure chamber. To mimic the low pressure maintained during filling phase, 5 cm H2O is applied during few hours. In the last hour the pressure slowly increase until 15 cm H2O, and then decline quickly to zero in a few seconds in order to simulate the voiding and complete the urination cycle. Compared to static condition, short-term dynamic culture significantly improves the urothelial development and the watertightness profile of the self-assembled substitute [129]. These results are in conformity with outcomes related in our dynamical engineered urethra which displayed an increase of uroplakins immunostaining at urothelial cells surface [130]. Whatever the substitute model, the mechanical stimulations must take more importance within protocols of in vitro bladder reconstruction. When must the dynamic phase intervene? How long time must it intervene? Should the pressure cycle follow a constant scheme or must it evolved during the process? Better understanding of bladder cells mechanotransduction may ameliorate the setting up of a dynamical environment appropriate to the reconstruction of a mature and functional vesical tissue.

5. Conclusion

Bladder exposition to diverse pathologies could jeopardize its function of elastic and watertight reservoir. To date, the clinical technique for bladder repair is associated to a high level of morbidity. Based on the well documented post-operative complications, it is appear that the ideal bladder substitute must combine the compliance conferred by the nature and architecture of the matrix support, with a urinary barrier provided by the differentiation degree of urothelium. Natural and synthetic scaffolds were investigated to reproduce the bladder abilities and some successes were furthered the urological tissue-engineering domain. But due to their poor mechanical stability, immune responses, and incomplete cellular maturation, these models remain insufficiently developed to be used in clinical application. At present, teams which support the acellularised or polymeric substitute are working on the next generation of engineered bladder model. For example, nanodimensional surface features would be included in order to imitate the nanometer topography of native tissue, and therefore, to enhance interactions between bladder cells and the proposed environment. Among all biotechnologies, the self-assembly method proves to be a promising approach to elaborate a vesical substitute comparable to the structure and function of native tissue. The good watertightness of reconstructed mucosa and its autologous character will permit a suitable integration in vivo, and promote the cellular expansion. The application of appropriate culture method, such as dynamical regime, will lead to the maturation of the reconstructed connective tissue and its urothelium. The capacity of the self-assembled tissue to be pre-endothelialized might avoid the necrosis of the graft attributed to the lack of synchronized neovascularization. Another aspect which is rarely taking into account is the capacity for a graft to growth with

pediatric patient. The self-assembled tissue is made of autologous cells only, and constitutes a serious alternative in the urological tissue-engineering field. Research continues its efforts to optimize the different reconstructed substitutes, and agree the necessity to evaluate them at long-term through bladder with specific dysfunctions.

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Chapter 24

Advances in Bone Tissue Engineering

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Additional information is available at the end of the chapter

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1. Introduction

1.1. The need for engineered bone

While bone is inherently capable of regeneration, complications such as excessive bone loss impede healing, necessitating the use of bone grafts. In the United States alone, an estimated 15 million fractures occurs annually, including 1.6 million hospital admissions for traumatic fractures and 2 million osteoporotic fractures, costing over 60 billion dollars and calling for 1.6 million bone grafts each year [1]; a growing demand for bone grafts is similarly observed worldwide. In such applications, autologous bone grafts continue to be regarded as the "gold standard" for bone repair. However, this may not be practicable in cases involving large bone loss. Additionally, patients suffer from significant donor site morbidity, as well as poor outcomes in older patients [2, 3]. Allogeneic bone grafts may alternatively be used, but pose potential risks of immune rejection and pathogen transmission [4]. Additionally, the limited number of donors is unable to cope with the clinical demand. Consequently, alternative approaches to provide efficacious and reliable bone grafts are being actively pursued.

The advent of tissue engineering, where the aim is to generate functional tissue, has raised the possibility of engineering bone in vitro [5]. Over the past few decades, a wealth of progress in bone tissue engineering has been achieved, particularly in cell sources, developing biocompatible and biodegradable scaffolds, designing bioreactors to enhance in vitro osteogenic priming, and identifying growth factors that can induce or promote endogenous bone and vascular formation [6]. Numerous pre-clinical trials with various animal models have produced optimistic results [7].Despite the initial optimism, the lack of translation into a clinical setting suggests significant issues remain, including optimisation of cell sources, choice of biomaterial, in vitro preparation and the mode of delivery.

1.2. Normal bone anatomy

As seen in figure 1, bone tissue is organised into cancellous or cortical bone. Cancellous bone (also referred to as trabecular bone or spongy bone), is porous, providing structural support and organisation for bone marrow interspersed inside. In contrast, cortical bone is the compact bone surrounding the marrow space, and confers mechanical strength to bone. The outer layer that covers the surface of cortical bones is the periosteum, containing mainly blood vessels and osteoblasts, which are activated during appositional growth and bone repair [8].

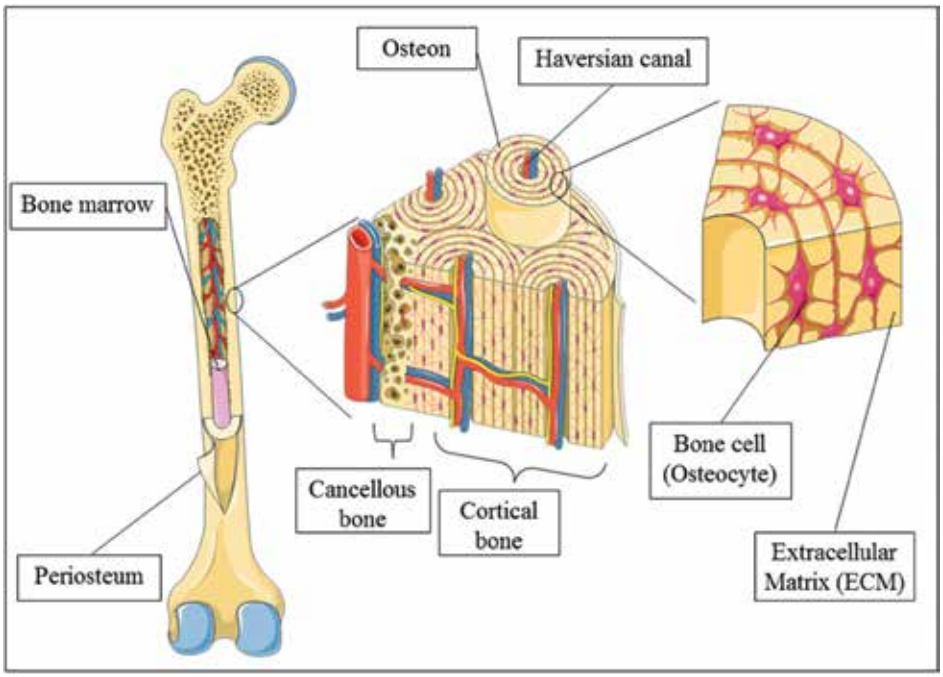

Figure 1. Schematic overview of bone, depicting gross overview, and cellular distribution. (Figures were produced using Servier Medical Art) In particular, osteoprogenitors may be found abundantly in the periosteum and bone marrow, where they perform critical roles in bone repair. Additionally, bone is observed to be highly vascularized, in both the intramedullary canal and periosteal region.

The basic functional unit of the cortical bone is the osteon (or haversian system) which contains cells and extracellular matrix organised in a lamellar pattern, surrounding the haversian canal, in which nerves and blood vessels reside [9]. Within the osteon, osteoblasts and the mature osteocytes exist, contributing to the generation and maintenance of ECM that gives bone it's structural strength [10]. These are derived from osteoprogenitors that reside in the bone marrow and periosteum. Also present are the myeloid-derived osteoclasts that mediate bone resorption; further elaboration on the roles and functions of these cells is provided below. Compared to other tissue, the ECM found in bone is highly mineralized to confer mechanical strength. Calcium and phosphate are found in bone ECM in the form of hydroxyapatite crystal ($Ca_{10}(PO_4)_6(OH)_2$), interspersed with Type I collagen [11], while the exact composition remains

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to be elucidated [12]. This unique composite of biological molecules (collagen) and inorganic minerals (calcium phosphate) provides bone with high mechanical strength, as well as toughness to resist impact.

1.3. Bone physiology

Bone is a highly dynamic tissue, which undergoes constant remodelling through one's lifetime. Homeostasis is achieved through the combined actions of osteoprogenitors, osteoblasts, osteocytes, as well as osteoclasts. In general, bone formation is effected through the proliferation of osteoprogenitor cells and differentiation into osteoblasts, which are responsible for the regulation of mineralization and collagen production through expression of functional proteins such as osteocalcin and alkaline phosphatase [13, 14]. Osteoblasts which get trapped in the ECM eventually differentiate to mature osteocytes forming syncytial networks that function to support bone structure and metabolism. Bone actively undergoes remodelling in response to environmental stimuli, such as physiologic influences or mechanical forces, in accordance with Wolff's law. This process involves a balance between bone formation as described above, as well as bone resorption by osteoclasts [15], and it is crucial for renewing bone, to maintain bone strength and mineral homeostasis [16].

The bone is able to undergo significant regeneration in response to trauma and fractures. Regeneration progresses through the three phases of inflammation, repair and remodelling [17]. The immune system can be seen to be intricately involved in the process of healing and inflammation is required for effective healing [18]. Subsequently, the repair and remodelling processes are initiated in the periosteum, which contain a rich population of osteoprogenitors and osteocytes that proliferate and migrate to the defect site, forming a bony bridge to effect fracture healing. When this bridging does not happen, hypertrophic non-union occurs, necessitating the use of bone grafts.

2. Stem cell sources

Over the last few decades, stem cells have emerged as a key player in tissue engineering, both for in vitro generation of bones, and in vivo bone regeneration. Two hypotheses have been proposed to describe the mechanism of healing affected by stem cells. In the conventional approach, osteogenic stem/progenitor cells are proposed to participate in new bone through direct differentiation into functional tissue. More recently, it has emerged that trophic factors secreted by administered stem/progenitor cells can promote functional tissue regeneration [19]. Much research is currently focused on understanding the influence of stem cell origin and culture conditions on clinical outcome.

2.1. Adult stem cell sources

The human body retains a small amount of osteoprogenitor cell population with differentiation potential even in adulthood. The main osteogenic progenitor cells – mesenchymal stem cells (MSCs) are considered to consist of a variety of cell populations, with no unique marker to

delineate a MSC to-date [20]. Therefore, in some studies, the term "mesenchymal stem cells" is used to represent osteoprogenitor cells [21]. The current consensus on the characteristics of a bona-fide MSC should include: 1) expression of cell-surface markers which are non-haemopoietic, non-endothelial and incorporates a number of other cell-surface markers, including CD73, CD105 and CD90, and 2) the capacity for tri-lineage differentiation into osteocytes, chondrocytes and adipocytes [22].Common sources of MSCs include bone marrow, fat tissue and the periosteum [23].

MSCs are most abundantly found in the bone marrow (BM), and BM-MSCs are widely utilised for both autologous or allogeneic transplantations [20]. BM-MSCs can be easily expanded in culture, and induced to differentiate into osteoblasts through culture in a high phosphate environment [24]. In tissue engineering applications, BMMSCs have been used to generate bone graft by loading into appropriate scaffolds, followed by osteogenic culture [25],. BM-MSCs may offer favourable characteristics, including availability as an autologous cell source, and thus, non-immunogenic cell source [26]. In contrast to suggestions that BM-MSC are immunoprivilleged and thus suitable for allogeneic – unmatched transplantation [27], a few studies have emerged that reported rejection of murine BM-MSCs following infusion into immune competent recipients [28]. Besides, adult BM-MSCs though advantageous in autologous source availability, have shown relatively low efficiency in osteogenic differentiation in vivo in several comparative studies [29, 30].

The periosteum is involved in extraosseous fracture repair, and is thus also thought to contain osteoprogenitors. Indeed, stem cells derived from periosteal tissue showed better mineralization and neovascularization ability than adult bone marrow derived cells and demonstrating good efficacy in healing a calvarial defect model [31]. However, its potential as a cell source is limited by the low volume of periosteum which can be harvested, as well as the complexity of the harvesting process [32]. Further optimization in terms of isolation and culture conditions will be required before it can be a practical source.

Adipose tissue represents another potential source of osteogenic stem cells [33]. Adipose derived stem cells (ADSCs) can be isolated from fat tissues harvested by liposuction or surgical fat section, which are typically considered medical waste [34]. Similar to BM-MSCs, ADSCs respond to osteogenic induction through upregulation of alkaline phosphatase and other key osteogenic proteins, and differentiate into osteoblasts capable of depositing minerals [35]. Additionally, ADSCs have been shown to ne efficacious in bone repair, facilitating the repair of critical size defects in both calvarial and femoral segmental defects [36, 37]. However, the efficacy of bone repair by using ADSCs alone is relatively low compared with marrow-origin MSCs and more primitive MSCs [38, 39].

The adult stem cells represent a rich autologous source for bone tissue engineering, and the long-history study accumulate mature methodology to facilitate their application in pre-clinical trials or even clinical trial [40, 41]. Yet the low efficiency of in vitro bone generation and in vivo bone regenerative capacity may call for optimized induction strategy or alternative sources.

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2.2. Embryonic Stem Cell (ESC) and induced Pluripotent Stem Cell (iPSC)

In the last decade, the osteogenic induction of embryonic stem cells (ESCs) and the creation of induced pluripotent stem cells (iPSCs) presented new cell sources for bone tissue engineering [42, 43]. However, expansion of ESCs and iPSCs to clinically useful numbers is logistically challenging, and autologous use is not possible in the case of embryonic stem cells. Moreover, precise control of differentiation is necessary before applying to clinical use. This is especially so since the presence of undifferentiated "rogue cells" may result in tumour formation after transplantation [23].

ESCs are defined by their pluripotency, and can be induced into osteogenic cells through different methods in vitro [44, 45]. It is showed that human ESCs can be induced into osteogenic cells and form mineralized bone in vivo without presence of teratoma [46]. However, more investigations will be required to address the potential of teratoma in embryonic stem cells transplantation. Mature methods for specific osteogenic differentiation are also needed to overcome non-specific differentiation before clinical implementation can be contemplated.

As an alternative, iPSCs have recently emerged as a potential cell source for regenerative medicine [43]. Compared to ESCs, iPSCs face fewer ethical challenges, and are able to serve as an autologous cell source. iPSCs have been successfully differentiated into osteoblasts like cells [47], or through induction into a MSC phenotype [48, 49], allowing ready expansion of the iPSC-derived MSC before their direct differentiation into osteoblasts. However, similar issues to that of ESC remain, and concerns remain over the potency of the cells. Additionally, the concept of reprogramming remains poorly understood, and leaving questions on the fate of cells in vivo [49]. Therefore, although the differentiation capacity qualifies the iPSC as a potential source for bone engineering, yet a lot more efforts have to be done before it can be competent.

2.3. Fetal stem cell sources

Compared with adult stem cells, fetal stem cells are relatively more primitive, and have higher proliferative and differentiation capacity. MSCs have been derived from various fetal tissues in early to mid-gestation, including the blood, bone marrow, liver, pancreas, kidney thymus and bone [50]. Of these, fetal BM-MSC has shown particular utility for bone tissue engineering as reviewed extensively by Zhang et al [19]. However, its availability may be limited by ethical issues especially where fetal tissues are used to derive MSC [51].

As an alternative source, MSC has also been derived from umbilical cord blood at term gestation, albeit at very low frequencies [51], with optimised protocols achieving up to 60% success in its derivation [52]. Aside from the cord blood, MSC has also been derived from the umbilical cord vessels and matrix, the placenta and fetal membranes [53]. Although these sources of MSC are plentiful and readily harvested, their utility for bone tissue engineering is somewhat limited compared to fetal bone marrow derived MSC [19, 29].

2.4. Conclusion

Stem cell sources for bone tissue engineering have been widely explored recently, and several studies have been conducted to compare the different cell sources [21, 29, 30, 44, 54]. Table 1 below summarizes some of these studies and compares the main properties of different stem cell sources.

Cell origin	Proliferation rate	Osteogenic capacity	Mineral deposition	Main Limitations	Key Advantages
Adult bone marrow	Low	Medium	Medium	Limited efficiency	Autograft accessibility
Adipose	Low	Low	Low	Low efficiency	Autograft accessibility
Periosteum	High	High	High	Less resource	Autograft accessibility
Embryonic stem cell	High	Medium	High	Non-specific differentiation	Least mature, highest potential
Cord blood	Medium	High	Low	Autograft non-accessibility	High potential, good efficiency
Fetal bone marrow	High	Very high	Very high	Autograft non-accessibility	Highest efficiency

Table 1. A comparison of stem cell sources based on comparative studies

3. Biomaterials

The development of scaffolds is a major aspect in bone tissue engineering research. On one hand, these scaffolds should be rigid and resilient since they function as the main supporting frame work of bone graft. On the other hand, they should also be porous, biocompatible, osteoinductive and osteoconductive so that bone tissue can regenerate within the scaffolds [55]. In addition, a relatively slow degradation rate is crucial to provide mechanical support prior to complete native bone regeneration. The scaffolds can be made of natural, synthetic materials or suitable composites.

3.1. Natural materials

Natural materials applied to bone tissue engineering include biological polymers (such as collagen and hyaluronic acid), as well as inorganic materials (such as hydroxyapatite and tri-calcium phosphate). Intuitively, naturally occurring materials in native bone, such as collagen, are favoured as the possess the innate biological cues that favour cell attachment and promote chemotactic response when being implanted in vivo [56]. When used as grafts implanted in vivo, those polymers are readily remodelled by the resident cells to the internal environment. Besides, the fibrous property of polymers allows manipulation during scaffold fabrication, so

that the scaffold's structure and porosity can be easily controlled [57, 58]. However, the telopeptide within these polymers may be immunogenic, and some of the polymer's nature (poor inherent rigidity and high degradation rate) limit their application in bone repair.

The main minerals in bone matrix, hydroxyapatite (HA) and tri-calcium phosphate (TCP), are other candidates for bone scaffolds. Their mechanical properties are able to provide the mechanical support at the defect area after transplantation. However, these minerals are inherently brittle, and may perform poorly in response to impact. Currently, they are usually combined with polymer materials with higher fracture toughness to achieve optimized performance in bone tissue engineering application [59].

3.2. Synthetic materials

As compared to natural materials, synthetic materials may be designed and customised for highly specified chemical and physical properties. These properties contribute to controllable mechanical properties of the scaffolds, including tensile strength, resiliency and degradation rate and to tailor desirable biological outcomes, such as reducing risks of toxicity, immunogenicity and infectionscan [60].Synthetic materials, however, lack bioactive properties such as biocompatibility, osteoinductivity and osteoconductivity, necessitating further modification prior to use.

The most often used synthetic materials for three dimensional scaffolds are saturated poly-α-hydroxy esters, including poly lactic acid (PLA), poly glycolic acid (PGA), poly lactic-co-glycolic acid (PLGA), and poly caprolactone (PCL) [60]. They can be processed by techniques such as gas forming, phase separation, fused deposition, and 3D printing [61-64]. The choice of polymers and fabrication techniques for three dimensional scaffolds used in tissue engineering are a major aspect in material science, and much progress in this field has been made in the last few decades [65].

As most materials alone showed some form of limitations, now researchers mostly design and fabricate composite materials that combine polymers and inorganic minerals, to let the different nature of materials complement each other, and attain optimal and controllable degradation rate and mechanical properties. The combination can be varies and the fabrication methods are diverse [66].

4. Emerging research theme: Addressing the need for vascularisation

4.1. Vascularisation as limiting bottleneck

Much effort has been focused on generating tissue engineered bone grafts in vitro, and several attempts have been made to heal bone defects with engineered grafts in vivo. The achievements in bone tissue engineering led considerable progress in finding potent osteogenic cell sources and suitable biomaterials, as well as the development of scaffolds and the use of bioactive factors. Currently, the attention has gradually shifted to strategies to improve vascular

formation in tissue engineered bones as it emerges as the most crucial factor in ensuring graft survival and hence bone repair.

Having a network of blood vessels within a tissue-engineered graft is important for maintaining cellular survival particularly within the core of large bone grafts [67]. It has been shown that after implantation, neo-bone tissues were found only at where a vascular network was present [68]. Poor angiogenesis has been identified as the main reason for implant failure and is currently acknowledged as a major challenge in tissue engineering [69-71].

Currently, there are various strategies that are under investigation for improving vascularization in tissue engineered grafts. These include the induction of vascularization in vivo, the design of scaffolds specific to improving vascularization, and prevascularization techniques using coculture systems [72]. The prospect of functional vascularized bone graft for defect healing brings a bright future for clinical application.

4.2. Induction of angiogenesis and vasculogenesis

Angiogenesis and vasculogenesis are natural vascularization process that occurs in tissue development and wound healing. The endothelial cells function as the main mediator of neovascularization through forming new blood vessels by angiogenesis and they can be expanded by vasculogenesis. In some studies, endothelial cells were used to generate capillary-like structure and connect vessels in vitro [73]. However, it is unclear as to whether these vascular generation approaches are effective in inducing vascularization in engineered bone graft in vivo.

In addition to directly using endothelial cells to form vessel network, some growth factors related to angiogenesis are used as another method to improve vascularization in engineered tissue. These factors include vascular endothelium growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) [74]. A major advantage of utilizing growth factors instead of living cells is that the risk of host rejection can be excluded. However, it seems inefficient using growth factors alone, because of the random formation of new vessels within implanted bone [68]. More importantly, the inappropriate delivery of growth factors may induce excessive angiogenesis which could cause severe pathogenic process such as tumour development, atheroscleosis, and proliferative retinopathies [68, 71].

4.3. Scaffold design to promote vascular formation

Aside from the selection of molecular and cellular mediator for effective vascularization, the choice of material used is also closely related to the vascular formation ability of the engineered bone graft. The scaffolds have been designed to allow vascular in-growth through a macroporous structure and incorporating vascular cues such as with the use of growth factors and/or cells, rather than just serving as osteoconductive surfaces [75].

Over the past years, the selection of material candidates for bone tissue engineering scaffolds has been focused on the compatibility of bone cell attachment and growth. But now, much attention has also been switched to homing vascular formation [76]. The materials can impact

the vascularization outcome of the bone graft from two aspects: 1) supporting the endothelial cells growth and forming vessels; 2) incorporating active molecules that help to introduce vascular formation. For example, the usage of silk fibroin and polycaprolactone (PCL) as components for 3D porous scaffolds exhibited a good support to endothelial cell growth and consequent vascularization [69, 77]; while poly lactic-co-glycolic acid (PLGA) and poly lactide-co-glycolide (PLG) scaffolds showed the ability to incorporate VEGFs and release them locally so that angiogenesis was improved [78, 79].

Additionally, the structural properties, such as geometry and porosity can also affect the angiogenic ability of the scaffolds. Narayan and Venkatraman reported that the pore size of scaffolds have a profound influence on the growth of endothelial cells, with enhanced cell growth with smaller pore sizes and lower interpose distances [80]. Currently, the influence of scaffold design on its osteoconductivity and vascular conductivity is still unclear and deserves more investigations [81].

4.4. The use of stem cells for neo-vascularisation

Compared to mature endothelial cells, stem/progenitor cell candidates has been shown to exhibit higher proliferative capacity and differentiation potential [82]. Endothelial progenitor cells undergo vasculogenesis and have been shown to improve vascularization through the release of a milieu of angiogenic factors [83, 84].

In addition to using endothelial progenitor cells to induce vascularization, stem cell candidates are also needed for bone formation. Therefore, a co-culture system of the cells from different lineages has been proposed by various groups which reviewed comprehensively by Liu et all [72]. Several attempts have been made in generating vascularized bone graft for defect healing through co-culture systems, combine endothelial lineages with osteogenic cell types on different types of scaffolds [85-89].We have recently shown that co-cultured human fetal mesenchymal stem cells (hfMSC) and umbilical cord blood derived endothelial progenitor cells (UCB-EPC) seeded on PCL-TCP macroporous scaffolds induced more neo-vascularization and better bone formation, compared with the use of hfMSC alone [88].

5. Summary

A large number of bone grafts are required annually for clinical treatment of severe bone fracture. The limitations in autograft and allograft restricted their clinical application. Alternatively, tissue engineering approach may offer a new solution to produce bone grafts for clinical use. Over the last twenty years, tissue engineering of the bone has made remarkable progress, although the problems of translating into clinical application still remain.

Various types of stem cells have been used to form mineralized bone in vitro. In contrast, there were much fewer studies focused on the healing efficacy and its potential side effects. One main barrier is the complicated in vivo environment, which has profound interactions with the implanted cell types. This is especially so for allogeneic cells, where the host immune

reaction is likely to play a very important role, with the macrophage system currently being under intense investigations [90, 91].

The use of biomaterials and development of scaffolds are especially important for engineering bone grafts, because they need to provide mechanical support during bone repair and bioactive aspect for bone formation. In order to obtain optimal mechanical properties, and high biocompatibility, numerous composite materials were designed to acquire integrated properties from the individual components. Recently, more stringent requirements brought forth in the scaffold design for complete bone healing efficacy, such as inducing neo-vascularization during the formation of bone.

The achievements in engineering bone tissue so far are encouraging, while new challenges and opportunities are bringing the perspective of bone tissue engineering to a new height in clinical application. In the near future, tissue engineering approaches will achieve full tissue transplantation and engineered bone graft will be mature enough for bone fracture repair treatment.

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Chapter 25

Bone Engineering: A Matter of Cells, Growth Factors and Biomaterials

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Additional information is available at the end of the chapter

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1. Introduction

1.1. A connective tissue

Bone is a highly specialized form of connective tissue that is nature's provision for an internal support system in all higher vertebrates. It is a complex living tissue in which the extracellular matrix (ECM) is mineralized, conferring marked rigidity and strength to the skeleton while still maintaining some degree of elasticity. In addition to its supportive and protective organic ions, it actively participates in maintaining calcium homeostasis in the body.

Bone is composed of an organic matrix that is strengthened by deposits of calcium salts. Type I collagen constitutes approximately 95% of the organic matrix; the remaining 5% is composed of proteoglycans and numerous noncollagenous proteins. The crystalline salts deposited in the organic matrix of bone under cellular control are primarily calcium and phosphate in the form of hydroxyapatite (HA).

Morphologically there are two forms of bone: cortical (compact bone) and cancellous (spongy bone). In cortical bone, densely packed collagen fibrils form concentric lamellae, and the fibrils in adjacent lamellae run in perpendicular planes as in plywood. Cancellous bone has a loosely organized, porous matrix. The differences between cortical and cancellous bone are both structural and functional. Differences in the structural arrangements of the two types are related to their primary functions: cortical bone provides the mechanical and protective functions and cancellous bone provides the metabolic functions.

1.2. Bone cell structure and function

Bone is composed of four different cell types (Fig. 1). Osteoblasts, osteoclasts, and bone lining cells are present on bone surfaces, whereas osteocytes permeate the mineralized interior. Osteoblasts, osteocytes, and bone lining cells originate from local osteoprogenitor cells, whereas osteoclasts arise from the fusion of mononuclear precursors, which originate in the various hemopoietic tissues.

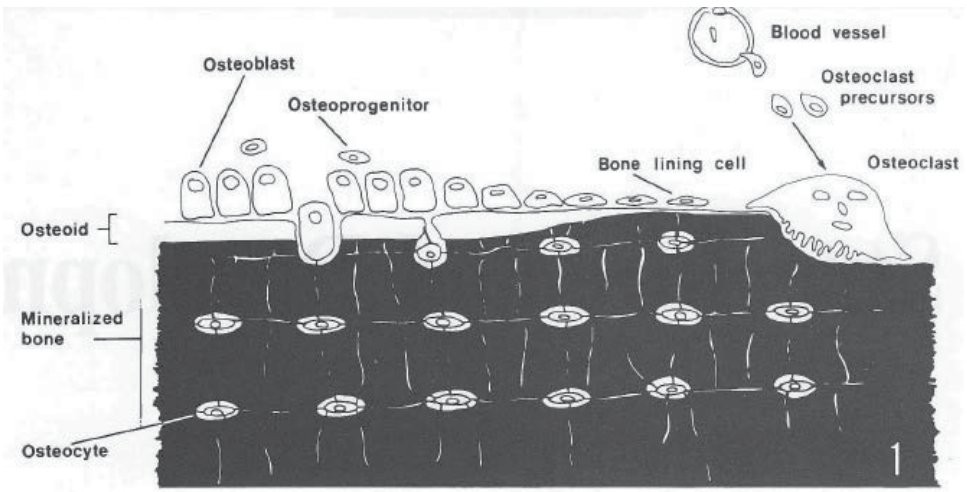

Figure 1. The origins and locations of bone cells. *Taken from Academic Press Inc., with permission*

Osteoblasts are the fully differentiated cells responsible for the production of the bone matrix. Portions of four osteoblasts are shown in Figure 2. An osteoblast is a typical protein-producing cell with a prominent Golgi apparatus and well-developed rough endoplasmic reticulum. It secretes the type I collagen and the noncollagenous proteins of the bone matrix.

The staggered overlap of the individual collagen molecules provides the characteristic periodicity of type I collagen in bone matrix. Numerous noncollagenous proteins have been isolated from bone matrix (Sandberg, 1991) but to date there is no consensus for a definitive function of any of them. Osteoblasts regulate the mineralization of bone matrix, although the mechanism(s) is not completely understood. In woven bone, mineralization is initiated away from the cell surface in matrix vesicles that bud from the plasma membrane of osteoblasts. This is similar to the well-documented role of matrix vesicles in cartilage mineralization (Hohling et al., 1978). In lamellar bone, the mechanism of mineralization appears to be different. Mineralization begins in the hole region between overlapped collagen molecules where there are few, in any, matrix vesicles (Landis et al., 1993) and appears to be initiated by components of the collagen molecule itself or noncollagenous proteins at this site. Whatever the mechanisms of mineralization, collagen is at least a template for its initiation and propagation and there is always a layer of unmineralized bone matrix (osteoid) of the surface under osteoblasts. Matrix deposition is usually

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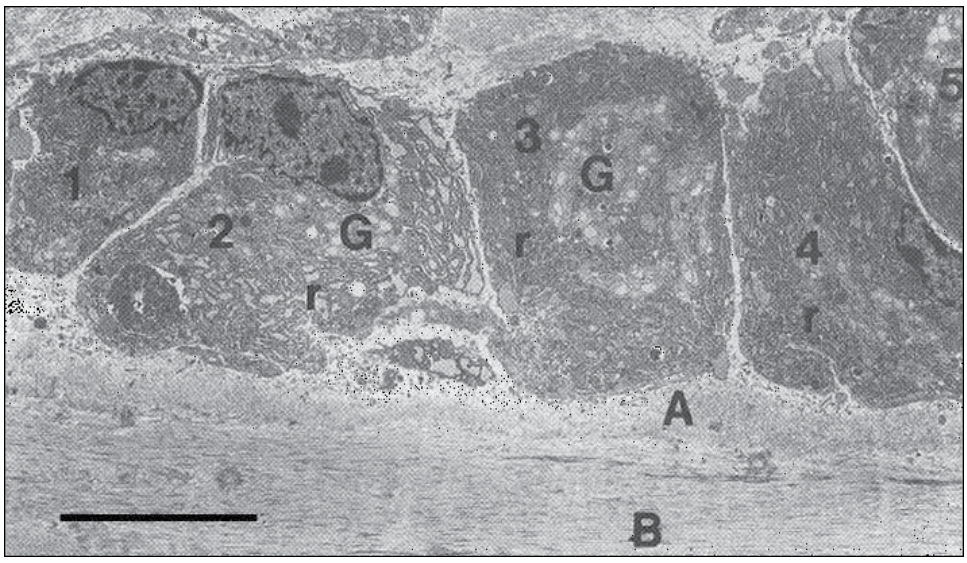

Figure 2. Transmision electron micrograph of osteoblasts (numbered) on a bone surface in which the collagenous matrix has been deposited in two layers (A and B) at right angles to each other. The Golgi apparatus (G) and rough endoplasmic reticulum (r) are prominent cytoplasmic organelles in osteoblasts. (Original magnification x2800, bar 0.1 μm). *Taken from Academic Press Inc., with permission*

polarized toward the bone surface, but periodically becomes generalized, surrounding the osteoblast and producing the next layer of osteocytes. Deposition of mineral makes the matrix impermeable and to ensure a metabolic lifeline, osteocytes establish numerous cytoplasmic connections with adjacent cells before mineralization.

The osteocyte is a mature osteoblast within the bone matrix and is responsible for its maintenance (Buckwalter et al., 1995). These cells have the capacity not only to synthesize, but also to resorb matrix to a limited extent. Each osteocyte occupies a space, or lacunae, within the matrix and extends filopodial processes through canaliculi in the matrix to contact processes of adjacent cells by means of gap junctions. Because diffusion of nutrients and metabolites through the mineralized matrix is limited, filopodial connections permit communication between neighbouring osteocytes, internal and external surfaces of bone and with the blood vessels traversing the matrix. The functional capacities of osteocytes can be easily ascertained from their structure.

Bone lining cells are flat, elongated, inactive cells that cover bone surfaces that are undergoing neither bone formation nor resorption. Because these cells are inactive, they have few cytoplasmic organelles. Little is known regarding the function of these cells; however, it has been speculated that bone lining cells can be precursors for osteoblasts.

Osteoclasts are large, multinucleated cells which resorb bone. When active, osteoclasts rest directly on the bone surface and have two plasma membrane specializations: a ruffled border and a clear zone. The ruffled border is the central, highly infolded area of the plasma membrane where bone resorption takes place. The clear zone is a microfilament-rich, organelle-free area

of the plasma membrane that surrounds the ruffled border and serves as the point of attachment of the osteoclast to the underlying bone matrix. Active osteoclasts exhibit a characteristic polarity. Nuclei are typically located in the part of the cell most removed from the bone surface and are interconnected by cytoskeletal proteins (Watanabe et al., 1995). Osteoclasts contain multiple circumnuclear Golgi stacks, a high density of mitochondria, and abundant lysosomal vesicles that arise from the Golgi and cluster near the ruffled border.

When a fracture occurs, a set of signals is triggered. These are both local signals and systemic ones; some of these signals are mediated by neuronal impulses (Nordsletten et al., 1994), by the haematoma at the site of the fracture and by the trauma caused to the tissues surrounding the fracture (Einhorn, 1998). These signals can be divided into two interactive and interchangeable categories: inflammatory signals and bone building signals. These factors mitigate the migration of phagocytotic cells to the area of the fracture, removing the necrotic tissue and propagating the in-growth of new blood vessels to the site of the fracture, thus providing nutrients and cells to the fracture site and starting the healing cascade. If at the end of the healing process osteo-integration (of the new bone together with the native bone) is not achieved, even with the best type of scaffolds, the chances of long-term success are dismal (Avila et al., 2009).

2. Bone tissue engineering

2.1. The basic concepts

Today great hope is set on Regenerative Medicine in all medical fields and, of course, it has developed to be of interest in orthopedics, being bone defects one of the main focus. In the last two decades, Regenerative Medicine approaches have been extensively studied to improve bone healing, or even generate functional bone tissue to substitute lost bone in orthopedics, neurosurgery, and dentistry. These types of studies include two different strategies of cell-based therapy: in the first approach, called Cell Therapy, cells are applied to substitute damaged cells within a tissue to reconstitute its integrity and function. The second approach, called Tissue Engineering, is more complex and encompasses three approaches: bioactive molecules (growth factors (GFs), cytokines, ECM compounds and hormones) that encourage tissue induction; cells and cell substitutes that will respond to the signals; the seeding of cells into three dimensional matrices, with specific adhesion properties and degradation rates, to compose a tissue-like construct to substitute lost parts of the tissue, or even organs; and a good nutritious support (angiogenesis) (Fig. 3).

Stem cells (SCs) are of particular interest in Regenerative Medicine, since they inhere several unique characteristics that distinguish them from other cell types. SCs represent unspecialized cells, which have the ability to differentiate into cells of all three germ layers, different adult cell types, and represent the only cell type which has the ability to renew itself indefinitely. It is important to distinguish embryonic stem cells (ESCs), which are truly pluripotent from multipotent adult stem cells and only found in early developmental stages of the organism. The successful dedifferentiation of somatic cells into a pluripotent ESC-like status by trans-

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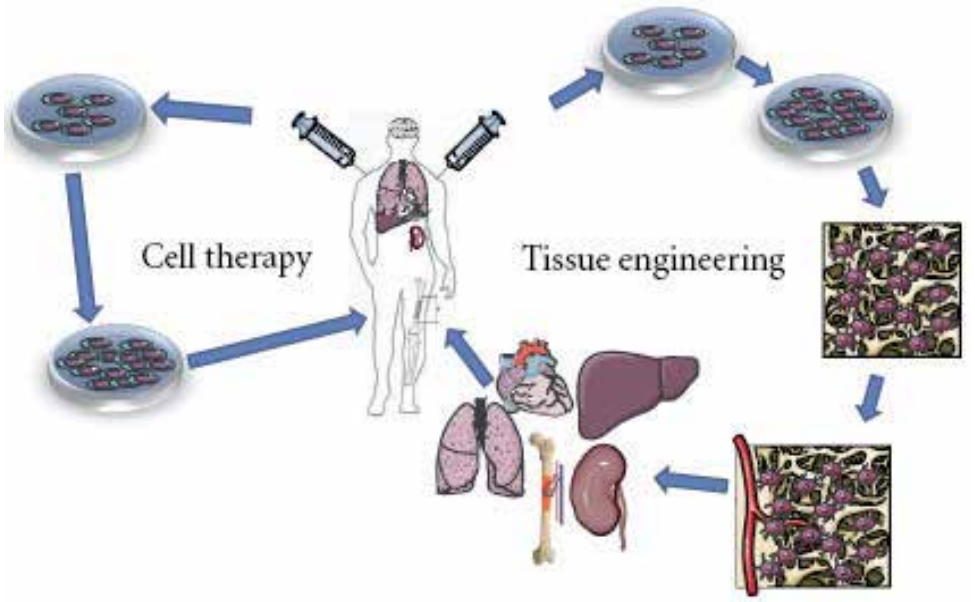

Figure 3. The two strategies of stem cell application in Regenerative Medicine. Stem cells are either isolated from the patient (autologous transplantation) or from other donors (allogenous transplantation). The cells are expanden *in vitro* and either applied directly to the patient to substitute lost cells (Cell Therapy), or seeded into 3 dimensional scaffolds (Tissue Engineering) and differentiated into the demanded cell type. The composed artificial tissue construct is subsequently implanted into patients' tissue defect. *Taken from A. Schmitt et al. 2012, with permission*

fection with four embryonic transcription factors, the so-called induced pluripotent stem cells (iPS cells), provide the possibility of autologous therapy with pluripotent and easily accessible cells in the future. Beside the great potential this technique undoubtedly represents, it bears some essential safety problems which are currently far from being solved. In contrast, a variety of multipotent adult SCs exists in assumedly all tissues of the organism. They are responsible for maintaining the integrity of the tissue they reside in. Usually, these adult SCs show limited differentiation potential to tissues of one germ layer.

Bone regeneration is a physiological process which can be observed in healing fracture and continuous remodeling along by adult life. Bone holds the most regenerative ability of human tissues contained on the mayor source of osteogenic cells capable for forming bones, the bone marrow (BM). However, a loss considerable amount of bone due to any anomalies such as severe trauma, skeletal deformations, bone tumor resection or periprosthetic osteolysis can obstruct this capacity. Nevertheless, the capacity of proliferation and differentiation of BMSCs, as well as cells concentration, are reduced with the increase of the age of the patient. Many *in vitro* studies were performed to investigate applicability of different SC types for bone regeneration. Here, promising capacity for differentiating towards bone cells, enhancing bone healing and vascularization could be proven for ESCs and different adult mesenchymal stem cells (MSCs). However, due to the ethical and safety concerns, which currently forbid applications of ESCs or iPS cells in patients, we will focus on adult stem cells for therapeutic

applications. Therefore, MSCs presently seem to be the most promising candidates for bone regeneration, due to their excellent osteogenic differentiation capacity. They can be isolated from a number of adult mesenchymal tissues, among others, umbilical cord blood, peripheral blood, placenta, synovial fluid, adipose tissue, skeletal muscle or BM, as mentioned, where they contribute to normal tissue turnover and repair. Recently, the multitude of cell surface markers used in various studies has been limited to a marker panel representing, in addition to plastic adherence and differentiation capacity, the minimal criteria for the identification of MSCs. The molecular mechanisms of human MSCs regulation and the importance of specific GFs during the different stages of osteogenic differentiation, as well as the secreted signaling proteins known as *Wnts,* implicated in various differentiation programs including osteogenesis, are subjects of intensive research right now. Several studies have demonstrated improved results of MSCs therapy with genetically modified cells which produce osteogenic and angiogenic GFs in a local delivery of therapy strategy for bone healing. Also, there is recent information about the use of endothelial progenitor cells (EPCs) that improves the treatment of fracture and bone regeneration.

Besides their unique ability to differentiate into different cell types, MSCs secrete a variety of cytokines, showing anti-inflammatory activity and create an anabolic microenvironment. Furthermore, direct cell-cell contact immunomodulation has also been shown. On one hand, they indirectly influence tissue regeneration by secretion of soluble factors. On the other hand, they are able to modulate the inflammatory response. The differentiation potential of MSCs in bone engineering has been extensively studied *in vitro* and *in vivo.* By first time, Urist (1965), and Reddi and Huggins (1972) showed the capacity of a molecule, called bone morphogenetic protein (BMP), with potent osteoinductive properties in healing fractures and bone regeneration. Their experiments demonstrated the presence of osteoinductive cytokines in bone matrix that have abilities to induce MSCs differentiation into osteoblasts. The GFs, also including transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) or insulin-like growth factor (IGF), among others, are delivered of paracrine or autocrine manner, generating a chemotaxis process toward MSCs by recruitment, which induce their differentiation.

Extracellular matrix (ECM) is the native scaffold in most tissues. Besides the direct injection in the surrounding tissue, biomaterials are frequently used as carriers for cells, bioactive molecules and drugs. These materials have to be immune-compatible and nontoxic, whereas the bio-degradation process must neither release toxic substances nor tissue-toxic concentrations of degradation products. Scaffolds must be of three-dimensional structures, with great influence on cell growth and differentiation, and must be highly porous with interconnected pores of a diameter of at least 100 μm to allow ingrowth of cells and vessels. Despite the tissue engineering of bone, for which various inorganic materials, such as HA, calcium phosphate, calcium carbonate (due to their similarity to bone mineral, as well as their inherent biocompatibility and osteoconductivity), or glasses was tested, mainly organic biomaterials have been investigated for scaffold production. These are either naturally derived, for example, collagen, fibrin, agarose, alginate, gelatin, silk or hyaluronic acid; or produced synthetically, such as polyhydroxyacids. Since natural bone consists of an ECM with nanosized apatitic minerals

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and collagen fibers that support bone cell functions, it is of interest to manufacture a synthetic biomimetic scaffold to i) contain nano-apatite crystals, together with fibers to form a matrix that supports cell attachment; ii) have mechanical properties similar to those of bone; and iii) encapsulate and support cells for osteogenic differentiation. Different methods have been employed in the fabrication of nanomaterials for bone engineering, such as the principle of electrospinning, that produce a variety of synthetic biomaterials, or the novel thermally induced phase separation (TIPS) technique to fabricate nanofibers to mimic natural collagen fibers. Rapid developments in this field of nanotechnology will be a key for many clinical benefits in the field of bone tissue engineering. The main advantage is that several novel biomaterials can be fabricated into nanostructures that closely mimic the bone in structure and composition. The optimization in the surface features of scaffolds has strongly improved cell behavior in terms of adhesion, proliferation, differentiation and tissue formation in three dimensions.

3. Stem cells as source

The popularity of SCs in the clinical arena has significantly increases, given the rapid improvement in our understanding of their biology. Classically, SCs are defined by their capacity to retain an undifferentiated state for a prolonged period while retaining the potential to differentiate along one lineage (unipotent), multiple lineages (multipotent), or into all three germ layers (pluripotent) (Young, 2003). These cells can be broadly categorized into two major classes: embryonic and adult SCs.

Embrionic stem cells (ESCs), isolated from the inner cell mass of the blastocyst, are pluripotent cells with the potential of differentiating into tissues from all three germ layers (Fig. 4) (Buehr et al., 2008).

While ESCs have significant regeneration capacity, their clinical application has been limited as a result of multiple factors including: 1) a propensity to form teratomas, 2) ethical concerns with isolation, 3) rejection by the host immune system after transplantation, and 4) the use of a feeder layer to retain an undifferentiated state *in vitro* (Cho et al., 2010). Recently discovered, another source of pluripotent SCs are induced pluripotent stem (iPS) cells, derived from somatic cells treated with few defined factors (Hamilton et al., 2009). While iPS cell-based therapy has the potential to revolutionize the field of Regenerative Medicine, many obstacles must be overcome before their clinical application can be realized (Lengner et al., 2010).

3.1. Mesenchymal stem cells as candidates

Furthermore, naturally occurring adult SCs have also been identified and categorized into their hematopoietic stem cells (HSCs), a source of various hematopoietic cell lineages, and nonhematopietic SCs, which can give rise to cells of mesenchymal origin (de Barros et al., 2010). Many reports have suggested that these nonhematopoietic SCs, also known as mesenchymal stem cells (MSCs), can be isolated from a wide variety of adult tissues such as blood, adipose, skin, mandibule, trabecular bone, fetal blood, liver, lung and even the umbilical cord and placenta

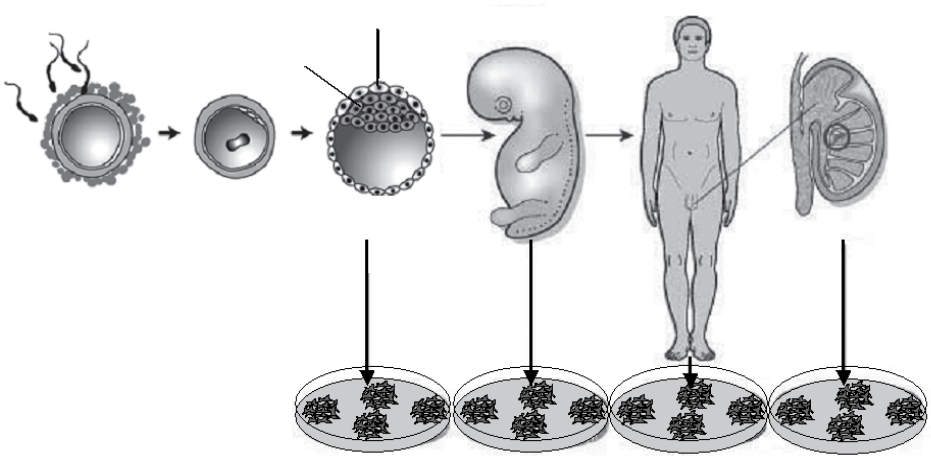

Figure 4. Origin of the different types of stem cells available. Derivation of embryonic stem cells (ESCs), embryonic germ cells (EGCs) and adult stem cells (SCs). *Taken from M.E. Arias and R. Felmer 2009, with permission*

(Steinhardt et al., 2008). The wide range of sources, methods, and acronyms are standardized by the International Society for Cellular Therapy in 2005.

Upon harvest, these cells can be expanded *in vitro* with high efficiency without sacrificing differentiation capacity (Kassem, et al., 2004). While these multipotent progenitor cells share many similar characteristics, they can be differentiated based on their expression profile and differentiation propensity along various lineages (Wagner et al., 2005). Amongst the various sources, MSCs isolated from the BM are considered to have the greatest potential for multilineage differentiation and have been the most characterized (Kuznetsov et al., 2009).

MSCs were initially described by Friedenstein and colleagues more than 40 years ago as adherent cells, with a fibroblast-like appearance capable of differentiating *in vitro* into osteoblasts, chondroblasts, adipocytes, and tenocytes (Friedenstein et al., 1968; Alonso et al., 2008; Prockop et al., 2009; Andrades et al., in press (a). Unlike ESCs, MSCs provide the flexibility of autologous transplantation, circumventing ethical concerns or immunological rejection (Igura et al., 2004). These cells also play a sentinel role in proliferation and differentiation of hematopoietic cells (Briquet et al., 2010). Mankani et al. illustrated that the formation of both hematopoiesis and mature bone organ is correlated with the high local density of MSCs (Mankani et al., 2007). Additionally, MSCs are considered to be immune privileged and have the capacity for allogenic transplantation a property has been used in the clinical setting for the treatment of various autoimmune diseases (Le Blanc et al., 2008). While many studies have suggested that MSCs are immunoprivileged and do not undergo rejection, others have cast doubt on this notion, showing that in certain scenarios, MSCs induce immune rejection (Nauta

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et al., 2006) (Fig. 5). More investigations should be conducted to provide further insight into the specific interaction between these progenitor cells and the host immune system.

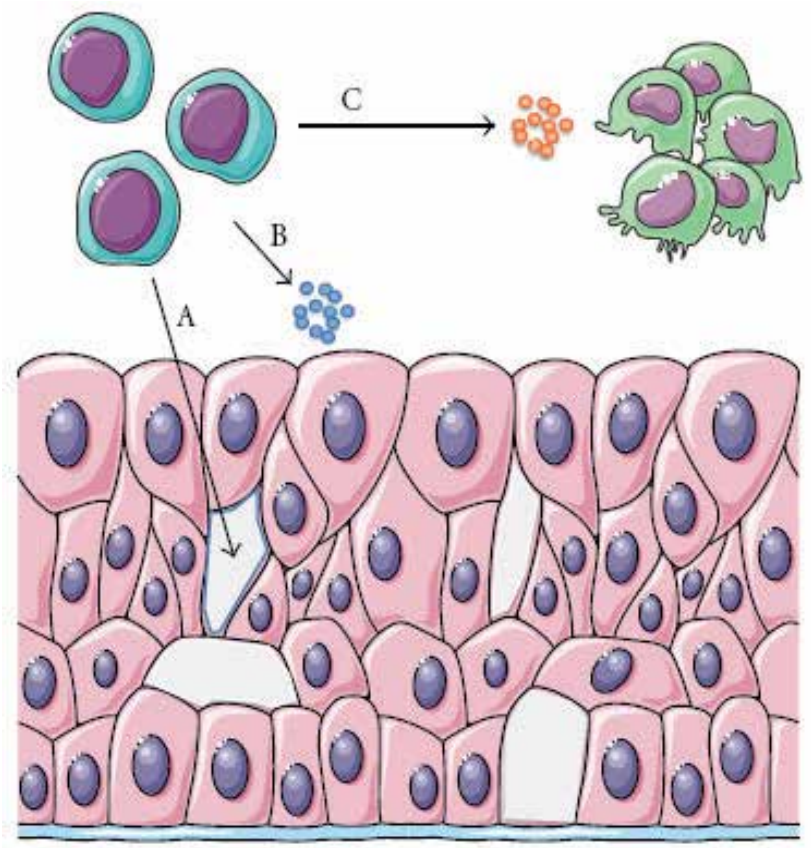

Figure 5. Stem cells participate in tissue regeneration in different ways. They directly differentiate into tissue-specific cells and thus substitute damaged or lost cells (A). They indirectly influence tissue regeneration by secretion of soluble factors. Here they promote vascularization, cell proliferation, differentiation within the tissue (B) and modulate inflammatory processes (C). *Taken from A. Schmitt et al. 2012, with permission*

Considerable effort has been put forth to identify specific surface markers that characterize MSCs, yet disagreement within the literature has prevented the creation of definitive standards. The minimal criteria identified by the International Society for Cellular Therapy for identifying MSCs requires the cells: 1) to be plastic adherent while maintained in cell culture; 2) to express CD73, CD90, and CD105, and lack expression of CD11b, CD14, CD19, CD34, CD45, CD79-alpha, and HLA-DR; and 3) to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* (Dominici et al., 2006; Claros et al., 2008; 2012). Additional studies have also suggested that CD146 is considered an important marker of BM progenitor cells (Sorrentino et al., 2008). These guidelines were set in place to enable a unified approach for comparison amongst different studies.

BM is generally considered milieu plentiful for various cell types, collectively referred to as stromal cells. Amongst these, the multipotent subset of MSCs comprises a small fraction (<0.01) (Dazzi et al., 2006), yet despite their small numbers, the relative ease with which MSCs can be harvested has propelled their experimental use. Researchers have pioneered the creation of stable animal models aimed at mimicking human conditions to study the therapeutic capacity of these BM-derived cells (Kadiyala et al., 1997). Because of their ubiquity, tolerance of expansion, paracrine capabilities, and multipotency, the potential for clinical applications of MSCs in the orthopaedic realm is countless (Becerra et al., 2011).

The first problem that arises when Cell Therapy methods are used to rebuild bone tissue is how to obtain a sufficiently large number of osteocompetent cells for the intervention to be successful. Hence, the idea of using SCs, which are self-renewing and differentiate into various tissues, surfaced.

4. Direction by growth factors

Growth factors (GFs) serve a critical role in Regenerative Medicine, facilitating tissue growth *in vitro* and repair *in vivo.* In the case of skeletal tissues, they are being using to regulate chemotaxis, proliferation, and differentiation of MSCs. Also, selected hormones, cytokines, and nutrients are potentially useful in controlling MSCs growth.

A GF is a signaling biomolecule, commonly polypeptide, that is not a nutrient. Typically they act as signaling molecules via binding to specific receptors on the same cells that secrete the factors (autocrine signaling) or on neighboring cells (paracrine signaling). The binding of the receptor initiates a cascade of cellular reactions, often involving the activation of specific gene transcription. These cellular activities lead to alterations in cell proliferation, differentiation, maturation, and production of other GFs and ECM, all of which result in the formation of specific tissues. Unlike hormones, which act on cells distant from the source (endocrine signaling), GFs have a local (nonsystemic) effect and are often secreted at low concentrations (Fig. 6).

4.1. Transforming growth factor-beta (TGF-β) superfamily

Members of this superfamily, as bone BMPs, growth differentiation factors (GDFs) and TGF-βs, are involved in the different stages of repair bone (intramembranous and endochondral bone ossification) during bone repair (Gerstenfeld et al., 2003).

4.1.1. Transforming growth factor-beta (TGF-β)

The term transforming growth factor beta is applied to the superfamily of length well-known growth factors involved generally with connective tissue repair and bone regeneration present in many types of tissue (Lieberman et al., 2002). TGF-β exists as five isoforms, three of them have received the most attention regarding fracture repair and proliferation of MSCs (TGF-β1, TGF-β2, TGF-β3), although TGF-β3 has the most pronounced effect on increases proliferation

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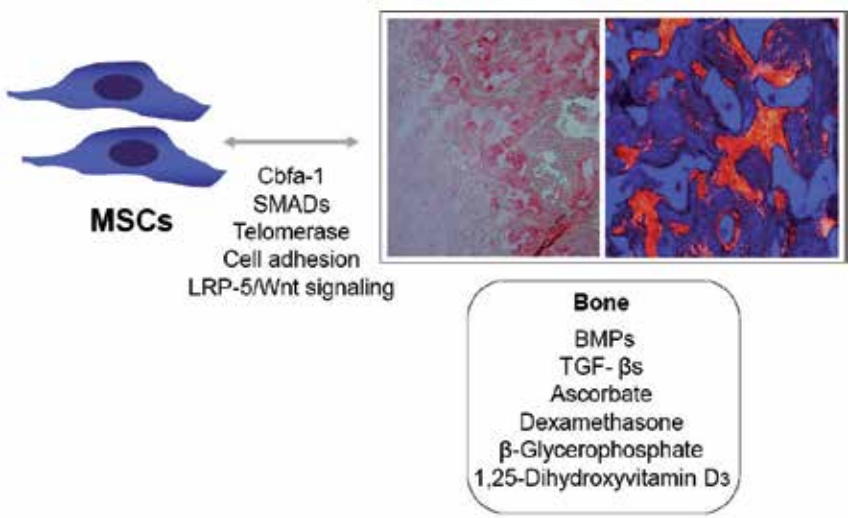

Figure 6. Growth factor regulation of BM-derived MSCs differentiation. Among the classes of bioactive factors, such as matrix ligand, mechanical stimulation, and cell shape, GFs exert strong effects on the regulation of the lineage differentiation of MSCs. Boxed GFs and hormones are used to control bone differentiation *in vitro.* Factors under the arrow have been implicated in promoting regulation differentiation. Pictures represent two histological sections, stained with Sirius red and observed under light (left, femur segmental resection) and polarized (right, profile of a hydroxyapatite implant) microscopes. New bone tissue appears in red. *Results obtained in LABRET-UMA*

of MSCs and chondrogenesis (Weiss et al., 2010). All TGF-β members superfamily are synthesized as large precursors which are proteolytically cleaved to yield mature protein dimers (Massague et al., 1994). TGF-β signaling that involves two receptor types, TGF-β receptor type I and type II, occurs when factors from the family bind a type II serine/threonine kinase receptor, recruiting another similar transmembrane protein (receptor I). Receptor I phosphorylates the primary intracellular superfamily signal effector molecules, SMADs, causing their translocation into the nucleus and specific gene transcription (Valcourt et al., 2002). TGF-β and members of this growth factor family can also signal via the mitogen activated protein tyrosine kinase (MAPK), Rho GTPase and phosphoinositide 3kinase (PI3K) pathways (Zhang, 2009).

Like PDGF, they are synthesized and found in platelets and macrophages, as well as MSCs and some other cell types (Barnes et al., 1999), acting as paracrine and autocrine fashion (Fig. 7). TGF-βs inhibit osteoclast formation and bone resorption, thus favoring bone formation over resorption by two different mechanisms (Mohan & Baylink, 1991). The TGF-β activates fibroblasts and preosteoblasts to increase their numbers, as well as promoting their differentiation toward mature functioning osteoblasts. It influences the osteoblasts to lay down bone matrix and the fibroblast to lay down collagen matrix to support capillary growth (Marx et al., 1996). They also play a role in osteogenesis, its actions are diverse and it is thought to influence the activity of BMPs (Salgado et al., 2004). TGF-β1 plays a pivotal role in the process and site of fracture healing where appears elevated levels in humans, as well as in other mammals, as it enhances the proliferation and differentiation of MSCs and is chemotaxis on bone cells (Sarahrudi et al., 2011).

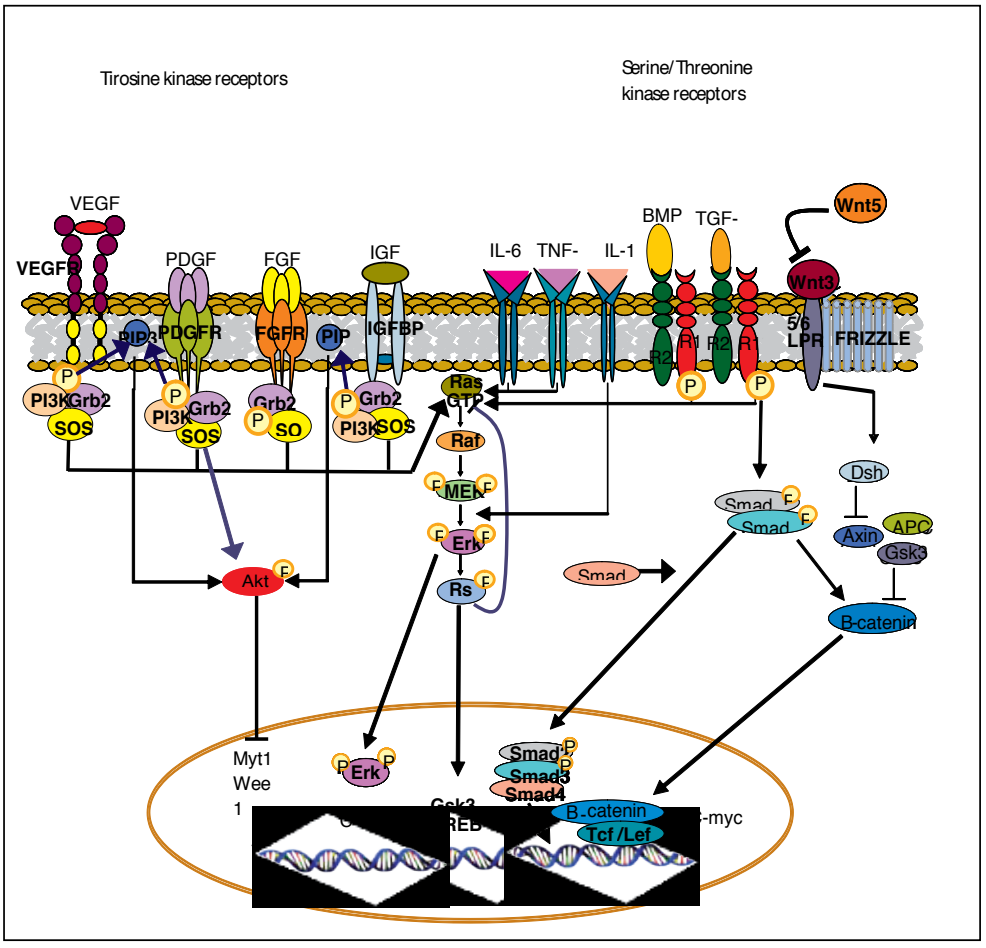

Figure 7. The signal cascade inside the cell after the receptor binding of GFs involves in bone repair. *Taken and modified from L. Barnes et al. 1999, and Rodrigues et al. 2010, with permission*

Also, our group have demonstrated that osteogenic precursor cells can be selected from a mixed population of BM MSCs by virtue of their distinctive survival responses in the presence of a recombinant human TGF-β1 fusion protein (Andrades et al., 1999a; 1999b; Andrades and Becerra, 2002a; Andrades et al., 2003; Becerra et al., 2006; Claros et al., in press), engineered to contain an auxiliary collagen binding domain (rhTGF-β1-F2) (Fig. 8), and further, that these selected cells exhibit unique properties in the chodroosteogenic lineage that can ultimately be utilized to therapeutic advantage

4.1.2. Bone morphogenetic proteins (BMPs)

The first BMP was identified by Urist (1965). He observed the ability from demineralized bone matrix (DBM), to induce ectopic bone formation when implanted under the skin of rodents, and showed that there was a recapitulation of all the events that taking place during skeletal

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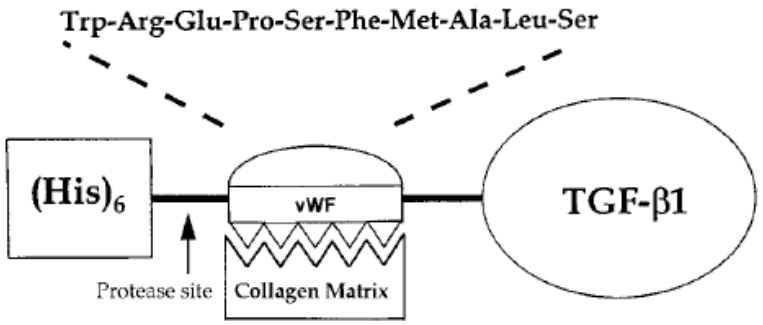

Figure 8. Schematic representation of the genetically engineered fusion construct, containing a histidine purification tag, a protease site, an auxiliary von Willebrand Factor collagen binding domain. Recombinant hTGF-β1-F2 applied to a bovine collagen matrix as vehicle and delivery system could be of advantage in promoting the survival, proliferation, differentiation, and colony mineralization of the osteogenic precursor cell population. It plays a crucial role in early stages of osteogenic commitment and differentiation. *Results obtained in LABRET-UMA*

development. In 1971, it was named as the responsible factors BMPs. More lately, others searchers, as Reddi and Huggins (1972) demonstrated that these molecules are important during development. Even present, at least many than 30 BMPs have been identified and BMP ´s functions have been studied by means of analysis of mutant genes and knockout experiments in mice. Different BMPs, among others member of the TGF-βs superfamily, trigger a serine/ threonine kinasa cascade of events that induce the formation of cartilage and bone (Fig.7).

During fracture repair, BMPs are produced by MSCs, osteoblasts, and chondrocytes, and bind to cells by direct interaction or are accumulated and subsequently delivered of ECM to promote the bone generation. These proteins induce a cascade of cellular pathways that promote cell growth, migration and differentiation of MSCs to repair the injury, stimulates angiogenesis, as well as synthesis of ECM and play a regulatory role in tissue homeostasis (Reddi, 2001). The different BMPs act in different temporal scale during bone repair. In studies of fracture healing, BMP-2 mRNA expression showed maximal levels within 24 hrs of injury, suggesting that this BMP plays a role in initiating the repair cascade. Other *in vitro* studies examining marrow MSCs differentiation have shown that BMP-2 controls the expression of several other BMPs, and when its activity is blocked, marrow MSCs fail to differentiate into osteoblasts (Edgar et al., 2007).

BMP-3, BMP-4, BMP-7, and BMP-8 are expressed during bone repair, from days 14 to 21, when the resorption of calcified cartilage and osteoblastic recruitment are most active, and bone formation takes place. Our group has demonstrated that BMP-7 is capable of selecting a cell population from BM which, in a three dimensional collagen type I gel, achieves skeletogenic potential under *in vitro* and *in vivo* environments (Andrades et al., 2001; Andrades and Becerra, 2002b; Andrades et al., 2003). BMP-5 and BMP-6 and other members of the TGF-βs superfamily are constitutively expressed from days 3-21 during fracture in mice, suggesting that they have a regulatory effect on both intramembranous and endochondral ossification. BMP-2 to BMP-8 show high osteogenic potencial, however BMP-2, BMP-6, and BMP-9 may be the most potent

inducers of MSCs differentiation to osteoblasts, while the others, stimulate the maturation of osteoblasts (Cheng et al., 2003).

The first BMP extracted in a highly purified recombinant form was BMP-2. In preclinical models, BMP-2 has the ability to induce bone formation and heal bone defects and promote the maturation and consolidation of regenerated bone. Recombinant human BMP-7 and BMP-2 are among the first growth factor based products available for clinical use to treat patients afflicted with bone diseases. A large number of studies have been performed to determine appropriate carriers for BMPs (Cheng et al., 2003).

In vitro cultures, MSCs and osteoblasts exhibit a high number of BMP receptors and synthesize the BMP antagonist's noggin, which are capable of blocking osteogenesis as MSCs differentiate into osteoblasts. BMP antagonists are important in normal bone turnover and regulation. The expression of the BMP antagonists, as noggin, which blocks BMP-2, BMP-4, and BMP-7 interaction with its receptor, also is modulated during bone repair (Balemans et al. 2002).

4.1.3. Wnt proteins

The Wnt pathway was initially identified as a proto-oncogene in mammary tumors that was activated by integration of the mouse mammary virus (Nusse & Varmus, 1982). Since then, it has been the subject of many studies. It knows Wnt proteins are secreted cysteine-rich glycosylated family proteins to share a highly conserved pattern of 23–24 cysteine residues and several asparagines-linked glycosylation sites (Li et al., 2006). In mature tissues, Wnt pathway play a regulator role of osteogenesis and stem/progenitor cells self-renewal, it is involved in bone formation, and also cellular adhesion and migration through their indirect interactions with the cadherin pathway (Arnsdorf et al., 2009).

Wnt proteins are divided towars to activate one of two main signaling pathways that consist of the Wnt1 class, also called Wnt/β-catenin or canonical Wnt pathway and Wnt5a class, Wnt/Ca2+ or non-canonical pathway. Several lines of evidence have demonstrated the importance of canonical Wnt signaling in promoting osteogenesis *in vitro* and *in vivo* (Chung et al., 2009). Wnt signaling is a prime target for bone active drugs and the approaches include inhibition of Wnt antagonist like Dkk1, sclerostin, and Sfrp1 with neutralizing antibodies and inhibition of glycogen synthase kinase 3β (GSK-3β), which promotes phosphorylation and degradation of β-catenin. Enhancement of Wnt signaling either by Wnt overexpression or deficiency of Wnt antagonists (ten Dijke et al., 2008) is associated with increased bone formation in mice and humans. Gain of function mutations of *LRP5/6* that lead to impaired binding of Dkk-1 (Dickkopf-1 is a secreted Wnt antagonist that binds LRP5/6) to this Wnt coreceptor are associated with increased bone mass (Boyden et al., 2002).

In spite of osteogenic inhibitory function of canonical Wnts, this pathway plays a positive role in bone homeostasis *in vivo* (Liu et al., 2009). Canonical Wnt signaling in osteoblast differentiation is modulated by Runx2 and osterix transcription factors (Hill et al., 2005). Quarto et al. (2010) have shown canonical Wnt signaling can either inhibit or promote osteogenic differentiation depending on the status of cell (cellular differentiation degree undifferentiated vs. differentiated), the threshold levels of its activation (existence of a differential activation of

canonical Wnt signaling between an undifferentiated MSC and osteoblast), and Wnt ligands concentration showing *in vitro* and *in vivo* data correlated results for Wnt3a treatment of calvarial defects created in juvenile mice where rise activation of canonical Wnt signaling inhibited osteogenic differentiation of undifferentiated MSCs, whereas increased the mineralization of differentiated osteoblasts.

4.2. Growth hormone and insulin-like growth factors (GH and IGF)

In clinic, the patients that present short stature are treated with the Growth Hormone (GH); for this reason, many researcher study the effects of GH in the treatment for osteoporosis and repair bone fracture. It is released by pituitary gland and travels through the circulation to the liver, where target cells are stimulated to release IGF. There are two IGFs identified: IGF-I and IGF-II. Various studies have shown that both IGF-I and IGF-II (Swolin et al., 1996;) are delivered by osteoblasts, chondrocytes, endotelial cells, and bone matrix, and they are detected by recruitment MSCs and bone cells in a paracrine/autocrine manner thanks to the presence of six insulin growth factor-binding proteins (IGFBPs), which modulate their action by intracellular tisone kinase cascade.

IGF-II is the most abundant GF in bone matrix. However, IGF-I is 4 to 7 times more potent in synthesis of bone matrix (type I collagen and non-collagen matrix proteins) (Lind, 1996). IGF-II acts on phase of endochondral bone formation and induces type I collagen production, stimulates cartilage matrix synthesis, and cellular proliferation. Both factors have been localized in bone studies of animals and humans with GH-deficient levels. The expression and secretion of IGFBPs, IGF-I and IGF-II (Birnbaum et al., 1995) changes during *in vitro* MSCs cultures. Prisell et al. (1993) showed that IGF-I mRNA was expressed during the MSCs recruitment and proliferation; however IGF-II mRNA expression happened later, during endochondral bone formation by osteoblasts and chondrocytes. IGF production is not only under the control of GH, is also regulated by estrogen, PTH, cortisol (inhibits IGF-I synthesis), local GFs and cytokines (Ohlsson et al., 1998). This abundant supply of IGFs is necessary to promote bone formation, bone repair, and MSCs cell proliferation and differentiation.

4.3. Fibroblast growth factor (FGF)

FGF is a secreted glycoproteins family whose signals are implicated in wound healing and angiogenesis, which influence in cellular proliferation, differentiation, migration, survival and polarity transduced through their receptors (FGFR1, FGFR2, FGFR3 and FGFR4). These receptors are constituted of extracellular immunoglobulin-like (Ig-like) domains and cytoplasmic tyrosine kinase activity domain. FGF proliferation signals occur through the tyrosine kinase cascade in various target cell types (Ng et al., 2008).

The various FGF receptors display varying affinities for each of the members of the FGF family and are expressed in a wide variety of tissues including indeed, bone. As with many of the tyrosine kinase receptors, activation of the intrinsic tyrosine kinase activity occurs through receptor dimerization in response to ligand binding. An additional complexity may be added to the receptor-ligand association through the binding of FGF li-

gand by either secreted or membrane-bound proteoglycans, heparin-like proteoglycans in particular because their high affinity (Givol & Yayon, 1992). Nine members of the FGF family have been identified of which, the most abundant in human tissue are FGF-1 (acid character) and FGF-2 (basic character) (Lieberman et al., 2002). FGFs are important regulators of fracture repair expressed by MSCs, maturing chondrocytes and osteoblasts and have been demonstrated to enhance TGF-β expression in osteoblastic cells (Bolander, 1998). They play a role in maintaining the balance between bone-forming cells and bone-resorbing cells and promote angiogenesis. Specifically, FGF-2 not only maintains MSCs proliferation potential, it also retains a slight osteogenic, adipogenic and chondrogenic differentiation potentials through the early mitogenic cycles; eventually, however, all of the MSCs differentiate into the chondrogenic line (Yanada et al., 2006).

4.4. Platelet derived growth factor (PDGF)

PDGFs are potent mitogens of MSCs (Ng et al., 2008) which express all forms of the GF: PDGF-A and PDGF-C at higher levels, and PDGF-B and PDGF-D at lower levels, such as both receptors type PDGFRα and PDGFRβ through which PDGF signaling is transduced (Tokunaga et al., 2008). PDGF is a dimeric molecule can exist either as a homodimeric (PDGF-AA, PDGF-BB, etc) or a heterodimeric form (PDGF-AB) according to the relative levels of each subunit generating a level of ligand complexity in cells in which both polypeptides are expressed. The different PDGF isoforms exert their effect on target cells by binding with different specificity to two structurally related protein tyrosine kinase receptors, denoted as the α-receptors and β-receptors, which are autophosphorylate ligand bound (Tokunaga et al., 2008). Several groups have found PDGF-BB to induce both proliferation and migration in MSCs (Fierro et al., 2007). While PDGFRβ inhibits osteogenesis, however, PDGFRα has been observed to induce osteogenesis. Akt signaling has been proposed to mediate both the suppression and induction of osteogenesis by PDGFR signaling (Tokunaga et al., 2008).

These molecules acts as paracrine manner stimulating mitogenesis of the marrow SCs and endosteal osteoblasts transferred in grafts to increase their numbers by several orders of magnitude. It also begins an angiogenesis of capillary budding into the graft by inducing endothelial cell mitosis and macrophage activator effect. It is known to emerge from degranulating platelets at the time of injury. PDGF also increased hMSC proliferation like Wnt (Liu et al., 2009). PDGF recruits MSCs and promotes chemotaxis and angiogenesis (Salgado et al., 2004).

5. Biomaterials as support

Natural bone consists of an ECM with nanosized apatitic minerals and collagen fibers that support bone cell functions. It is advantageous for a synthetic biomimetic scaffold to: (1) contain nano-apatite crystals similar to those in bone, together with fibers to form a matrix that supports cell attachment; (2) have mechanical properties similar to those of bone; and (3) encapsulate and support cells for osteogenic differentiation and bone regeneration. The success

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in regenerating a damaged tissue using the tissue engineering approach depends on the various types of interactions between the cells, scaffolds, and GFs. Besides, an understanding of the phenomena of cell adhesion and, beyond, the function of the proteins involved in cell adhesion on contact with the materials and the purpose depends of supramolecular assembly (scaffolding) of biomimetic biomaterials such as collagens, proteoglycans, and cell adhesion glycoproteins such as fibronectins and laminin.

Osteogenesis is highly dependent on the substrate carrier used, which has to provide a favorable environment into which bone cells can migrate before proliferating, differentiating, and depositing bone matrix (i.e., osteoconduction) (Ono et al., 1999). At the cell level, substrates of this kind must have specific biochemical (molecular) properties, physicochemical characteristics (surface free energy, charge, hydrophobicity, and so on), and a specific geometric conformation (they must be three dimensional and show interconnected porosity) (Jin, 2000). From the biomaterial point of view, the scaffolds used for bone engineering purposes have to meet a number of criteria, including (1) biocompatibility (nonimmunogenicity and nontoxicity); (2) resorbability (showing resorption rates commensurate with the bone formation rates); (3) preferably radiolucency (to allow the new bone to be distinguished radiographycally from the implant); (4) osteoconductivity; (5) mechanical properties to match those of the tissues at the site of implantation; (6) easy to manufacture and sterilize; and they must be (7) easy to handle in the operating theater, preferably without requiring any preparatory procedures (in order to limit the risk of infection).

The bone substitute materials intended to replace the need for autologous or allogeneic bone, consist of bioactive ceramics, bioactive glasses, biological or synthetic polymers, and composites of these. Biological polymers, such as collagen and hyaluronic acid provide guidance to cells that favors cell attachment and promotes chemotactic responses, but, a disadvantage is immunogenicity for the potential risk of disease transmission. On the other hand, other alternative is synthetic polymers such as polyfumarates, polylactic acid (PLA), polyglycolic acid (PGA), copolymers of PLA and PGA (PLGA), and polycaprolactone. Nevertheless, there are a wide range of bioactive inorganic materials similar in composition to the mineral phase of bone, for example, tricalcium phosphate, HA, bioactive glasses, and their combinations; and all of these can be tailored to deliver ions such as Si at levels capable of activating complex gene transduction pathways, leading to enhanced cell differentiation and osteogenesis. Hydrogels, such as polyethylene glycol or alginate-based, are to provide a three-dimensional cellular microenvironment with high water content, this is suitable for cartilage regeneration. Polyethylene glycol (PEG) hydrogels were investigated as encapsulation matrices for osteoblasts to assess their applicability in promoting bone tissue engineering. Non-adhesive hydrogels were modified with adhesive Arg-Gly-Asp (RGD) peptide sequences to facilitate the adhesion, spreading, and, consequently, cytoskeletal organization of osteoblasts. Finally, mineral deposits were seen in all hydrogels after 4 weeks of *in vitro* culture, but a significant increase in mineralization was observed upon introduction of adhesive peptides throughout the network. Potentially, the cell suspension could be injected into the body through a needle and photopolymerized through the skin to provide a non-invasive technique to enhance bone regeneration.

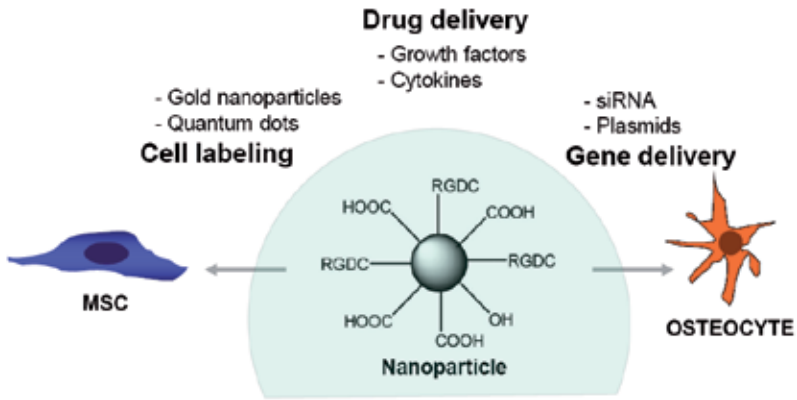

Figure 9. Overview of nanoparticle applications in bone regeneration

Biomaterials such as polymers, ceramics, and metals are widely used in bone for regenerative therapies, including in bone grafts and in Tissue Engineering as well as for temporary or permanent implants to stabilize fractures (Navarro et al., 2008). In recent years, biomaterials in general and bone-related implant materials in particular have been considerably refined, with the objective of developing functionalized materials, so-called smart materials, containing bioactive molecules to directly influence cell behaviour (Mieszawska and Kaplan, 2010). Rapid developments in nanotechnology have yielded many clinical benefits, in particular in the field of bone tissue engineering. The main advantage in that several novel biomaterials can be fabricated into nanostructures that closely mimic the bone in structure and composition. The optimization in the surface features of biomaterials has strongly improved cell behaviour in terms of adhesion, proliferation, differentiation and tissue formation in three dimensions. In this context, nanoparticles that are in the same size range as integral parts of natural bone, such as HA crystals or cellular compartments, are promising candidates for local applications. In bone, locally applied nanoparticles may be suitable for numerous potential uses with respect to the improvement of tissue regeneration, the enhanced osseointegration of implants, and the prevention of infections.

Increasingly refined nanoparticles are being developed for a wide range of applications (Fig. 9). These include cell labelling to broaden research possibilities as well as to improve and noninvasively monitor cell therapy approaches (Bhirde et al., 2011; Andrades et al., in press (b). Moreover, drug delivery systems with improved pharmacologic characteristics are being developed. They promote enhanced therapeutic outcome by providing controlled release of bioactive molecules, such as growth factors or anticancer drugs (Allen and Cullis, 2004). In addition, gene therapy concepts with good prospects are required for future treatment options based on intracellular manipulation (Evans, 2011).

The heterogeneous picture of research on the interactions of nanoparticles with MSCs makes it difficult to draw general conclusions. However, it becomes clear that parameters such as chemistry, size, and shape in some cases greatly affect the particle uptake behaviour of MSCs as well as their natural differentiation potential. Different strategies for nanoparticle applica-

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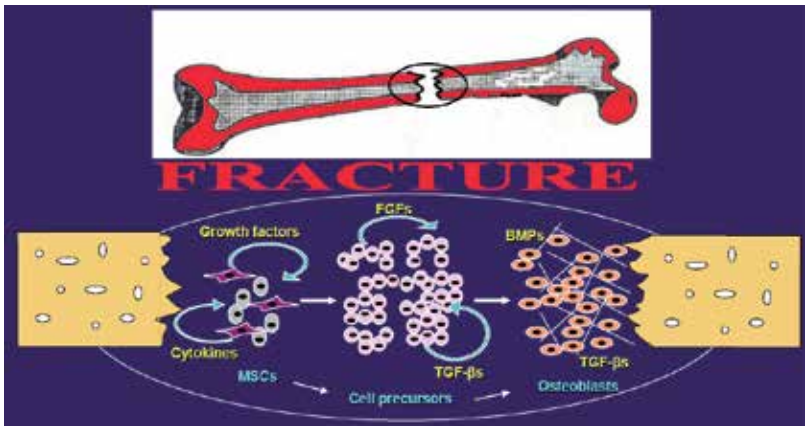

Figure 10. Bone fracture repair and regeneration is a question of balance among cells, growth factors and biomaterials.

tion in bone (i.e., as cell-labeling agents and for drug or gene delivery) have great potential for monitoring and supporting tissue regeneration.

6. Conclusion

Over the last decades we have advanced in many aspects of bone defects treatment. We have good understanding of the components involved in the healing of bone. Osteoprogenitor cells are necessary to replace the inserted scaffold and to create new bone tissue. These cells, MSCS, can come from the periosteum, the BM, or from chemotaxis and blood vessels entering the haematoma at the fracture site. Specific mechanical and biological stimulants cause the cells to differentiate into osteoblasts, which are the bone forming cells (Fig. 10). However, in critical size bone defects the natural migration of osteoprogenitor cells does not suffice for fracture healing. In normal conditions MSCs are rare (one in 10 million cells) (Pittenger et al., 1999). However, when a bone is broken, these cells, using special probing signals, roam in the blood and settle in the fracture site, differentiate into bone cells and start to construct the callus. The number of stem cells differs from person to person and is affected by age, sex and environmental factors.

Also, we have strived forward in defining different components of bone regeneration and have achieved a good combination of biology and technology leading to solid and reproducible answers to the *in vitro* and animal *in vivo* problem of bone defects. However, there is still one more step to take (the human *in vivo* step). There are scant data with respect to this part of the question, and in the next few years this field must undergo a transition, giving clinicians tools to deal with these critical everyday problems. The solution will come from a collaborative work of biologist, surgeons, engineers and chemists who possess the social understanding that there has to be a limit to the cost that the patient (and the society) can bear for healing a fracture.

Consequently, the search for the new bone regeneration strategies is therefore a key international priority fuelled by the debilitating pain associated with bone damage, and the increasing medical and socioeconomic challenge of our aging population.

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Chapter 26

Adaptation and Evolution in a Gravitational Environment — A Theoretical Framework for the Limited Re-Generative Post-Natal Time Window of the Heart in Higher Vertebrates

Michele Mario Ciulla, Gianluca Lorenzo Perrucci and Fabio Magrini

Additional information is available at the end of the chapter

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[Who are you who live in these many forms?]

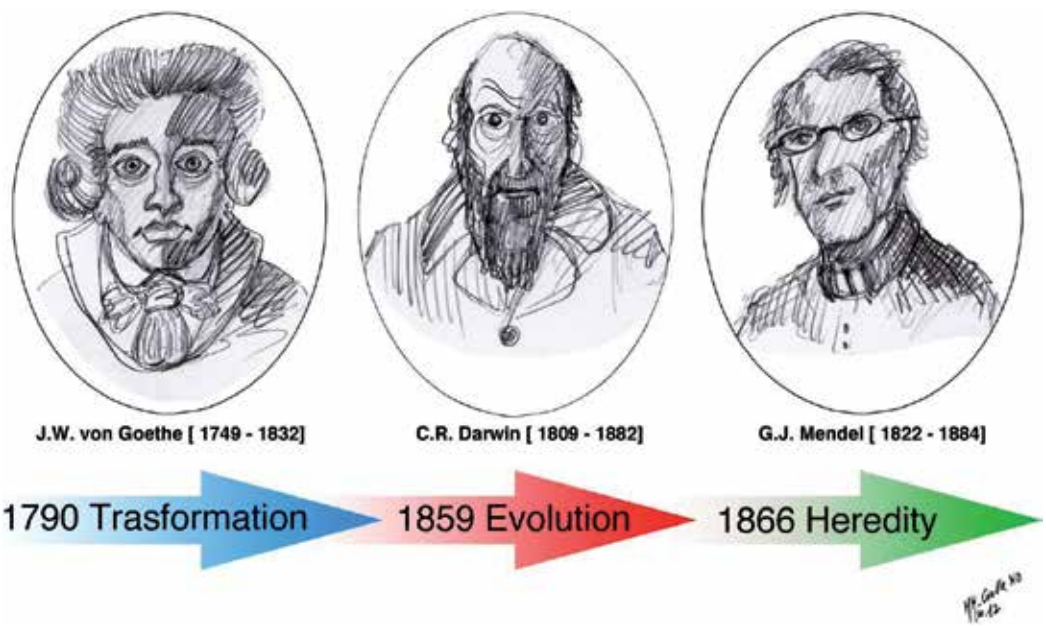
The Thin Red Line, 1998. Movie. Directed by Terrence MALICK. USA

1. Introduction

The origin and the meaning of life on the earth was traditionally attributed to an intelligent creator as an independent act until 1859 when Charles Darwin compiled the book The Origin of Species (Darwin, 1859). In this book Darwin introduced the *theory of evolution by natural selection* (Figure 1, central panel) opening a new perspective to read what looks like the *largest experiment on earth* called biological life. This new perspective has proposed a reversal of the traditional view where the intelligence is placed above the experiment by introducing the concept of *transformation or evolution* (Dennett, 2009). In this upside down view intelligence is not excluded but, rather, is within the experiment and drives the so called "struggle for life" in a dynamic planet where habitats are continuously destroyed and created.

Interestingly some years before, the *idea of transformation* was anticipated by the poet Johann Wolfgang von Goethe. In his book "The Metamorphosis of Plants" originally published in 1790, he wrote: *"Everyone who observes the growth of plants, even superficially will notice that certain*

external parts of them become transformed at times and go over into the forms of the contiguous parts" (von Goethe, 1790). What then seemed merely a poetic yearning on the wonder of nature, indeed, proposed the *transformation of the parties* (Figure 1, left panel) itself as a creative principle instead of a single external creative act. This *imaginative vision* provided by a poet shows how intuition and imagination can be a source of inspiration in the search for new knowledge and a place of convergence between literature and science (Pelaprat and Cole, 2011). The extension of the concept of transformation from the plant to the animal kingdom and the theory of evolution are further insights undertaken by non-poets with great imagination which certainly meets the definition of *creative scientists* (Boxenbaum, 1991). Thus, in the progress of human knowledge, the claim that science is superior to the literature, simply because it has to do with *facts* and the literature with *imagination*, has no basis because the idea behind every (great) scientific discovery is inspired by intuition, a kind of ability that does not use inference or reason (Beveridge, 1957).



Small cameos representing the faces of visionary scientists who introduced the concepts of transformation, evolution and inheritance.

Figure 1. The visionary scientists

Looking now at biological life with imagination, it is clear how it may seem the largest experiment, at least, on earth since, till now, we do not know whether there are other ongoing experiments like this in the *universe*. We know that many attempts to search for other forms of life in the universe have been made since the Sixties but, to date, without significant results (Wilson, 2001). Thus we cannot exclude that life on earth is, in fact, the *only* result of a larger experiment that we might call *life in the universe* (Aldiss, 2001).

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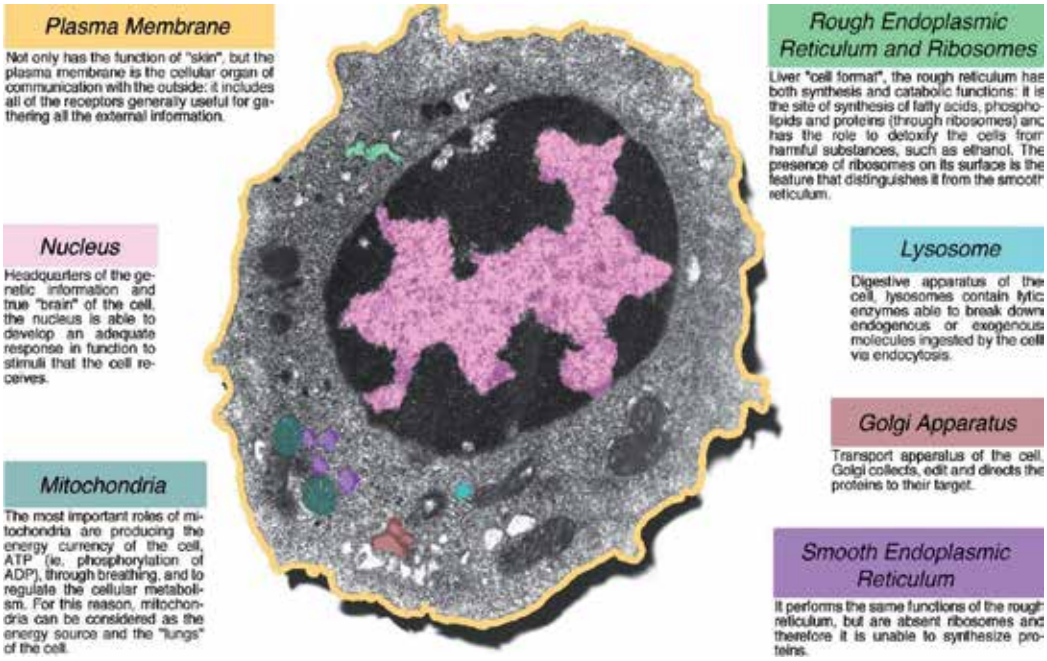
Returning back on earth, since here the experimental conditions vary continuously because of the regular and irregular environmental changes, there is no certainty about the results. If we try to define the experiment, this could be in summary to *assess life on earth*, and its process, through *trial and error*. Assuming that the experiment is started, to ensure its continuity in the presence of *errors* that can result from an *unfavorable interaction with the environment*, the method to explain how it works proposed by evolutionists implies a kind of continuous refinement of life through the *adaptation process* in order to get the possibility of a new trial with the environment. And it works since this challenge goes on from the evolution of the first *living organism,* represented by *ancestral bacteria,* dated about 3,500 millions of years ago till now (Kutschera, 2009).

Especially under adverse environmental condition, consisting mainly in climatic changes, a better adaptation to the environment obviously increases the chances of survival and gives continuity to the experiment. Therefore is the fittest organism that survives, which is to be considered a *prototype* that has passed the compatibility test with the environment, a process described by evolutionists as *natural selection*. In this framework it is logical to assume that allocating resources for adaptive processes in order to buffer environmental changes is an essential condition for life. The survival obtained through adaptation is temporary because it depends on each organism's life cycle and ends with death. To date the death still represents an essential phase in the experiment of organic life as it is the natural end of each life cycle; in addition, since the dead organism undergoes, under appropriate environmental conditions, to decomposition into basic elements, it can therefore be claimed that death, and after-death processes, also promote the accessibility of the basic elements for the vital functions of the other still living organisms that represent the ecosystem of the earth (Marschner and Kalbitz, 2003).

In order to overcome the time constraints imposed by the life cycle of individual organisms and ensure continuity to the experiment is required to introduce *the possibility of an offspring* and thus to apply the adaptation processes on a population consisting of copies (n>1) of the same organism.

This result can be achieved by duplication, a simple biosynthetic way to keep track of itself by division in use in *prokariotic* cells. Thus duplication ensures continuity to a single prototype, but this is not enough to respond to *major climate changes* of the earth, whose major effects can be deducted from the rate of extinction of living organisms (Jablonsky, 1994; Raup and Sepkoski, 1982).

In such dramatic context it is obvious that having more prototypes of living organisms to be tested provides more guarantees of continuity to the experiment. Therefore the *evolution of species* from a common ancestor, placed at the origin of what is commonly called the *tree of life*, is a way to adapt the biological life to randomly environmental changes (Kussell and Leibler, 2005) and must be seen as a *necessity* in evolution. Emerging of *diversity* is linked to the evolution of the *eukaryotic cell*, representing a real breakthrough in cell organization and function, which occurred about 2,500 million years ago. In this cell has its origin the *endosymbiosis*, an advanced form of *phagocytosis* that consists in *using the competence* of other organisms, instead of the energy, to re-organize cellular functions more efficiently, a starting point for the evolution of the cellular organization and *multicellularity* (Figura 2).



Electron microscopy image of a lymphocyte (courtesy of Paola Braidotti, BSc). The subcellular organelles with clear analogies with organs and/or apparati of complex multicellular organisms, such as humans, have been highlighted with pseudo-colors.

Figure 2. The cell: a body in miniature

Diversity is at the origin of the eukaryotic cell that adopts very early the *meiosis*, a new biosynthetic procedure evolved from the *mitosis* (Wilkins and Holliday, 2009) that allows *reproduction* instead of simply *duplication*. Intimately related to the development of *sexual reproductive cycles* (Antonovics et al., 2011) the *mitosis* generates more variability thus contributing to the overall *diversity*.

All these *adaptive responses* are coordinated by an *expert program* that enables living organisms to interact with the environment in an *active manner* ensuring at the same time *continuity* and *change* in the subsequent generations. This program, while remaining substantially the same over time, has ferried the biological life on earth through five *mass extinctions*, defined as times when the Earth loses more than three-quarters of its species, namely Ordovician, Devonian, Permian, Triassic and Cretaceous Periods. The *size of the experiment* has changed over time, from the first life forms to *complex organisms*, through the same basic mechanisms, showing the *major cellular evolution* at the origins followed by a very rapid expansion of life forms and a substantial stasis (Cavalier-Smith, 2006) but to understand the general principle by which the program reproduces itself, we will have to wait for some experiments on peas. A few years after the publication of The Origin of Species, Gregor Mendel, trying to understand with his experiments what any good farmer knows, which consists in the fact that through the selection of varieties of plants and their interbreeding is possible to obtain a better product, introduced

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the general principles of heredity (1866). His remarks were neglected until the early twentieth century when they provided the inspiration for the birth of *genetics*, the study of *heredity in biology* (Figure 1, right panel).

The term genetics was first used by William Bateson in 1905, but to identify the program code that at this point can be called *genetic program*, and how it is transmitted we should wait until 1953 in which the structure of DNA is discovered by other scientists with great imagination James D. Watson and Francis Crick. The rest is a more recent history. Having cracked the DNA code of humans and other living organisms, all scientists facing the incredibly high level of homology between different species have turned their interest towards the *proteome*, opening the so-called *post-genomic era* (Gromov and Celis, 2000). This new branch aimed at the analysis of the *functional state of the genome* has begun to develop. Unlike the genome, the proteome is much more complex and dynamic, and undergoes radical changes both in ontogeny and in different states. The proteome of any cell is unique and provides qualitative and quantitative information on proteins, thus giving a dynamic picture of genome expression under varying conditions (Gromov and Celis, 2000). The post-genomic era, in fact, strengthens the bonds of evolutionary biology with Darwin's theory of natural selection recovering the role of the environment in the experiment. But natural selection is still a mechanism at work? Yes it is, more in some geographical areas than in others (Stajich and Hahn, 2005), but is also working in a quite novel ways through the effects of changes in the environment caused by the so-called modernization (Hunter, 2007).

Although the question of the *origins of the life* after Darwin has been removed or limited in certain areas of science simply confining the *teleological question* to a purely human level (Ayala, 1999), we believe that the mission of modern science is still to find teleological explanations to everything that is humanly intelligible.

Even if none of us can be considered an expert on the origin of life on earth because of the lack of *fossil evidence*, the new synthetic view including *evolution* and *genetics* has contributed to define the main lines along which the *life experiment* on earth has evolved in a continuously *changing environment* (Graham, 2011). Trying to *conceptualize* the main steps in the evolution of *complex organisms*, we can identify three main lines, the first is the *organization* the second is the *diversity* and the third is the *adaptation*.

2. The organization

The *organization* or, better, the ability to organize itself, is the main feature of *living organisms*, but to date no one can state with certainty whether this property is intrinsic or acquired during evolution. Geological research has shown that the age of planet earth is about 4,560 million years (Dalrymple, 1991). Thus, as we saw earlier, we will have to wait about 1,000 million years from the origin of the earth for the evolution of the first living organism consisting in *ancestral bacteria* or *archaea*. In this somewhat *obscure* lapse of time, that have lasted at least 3,500 million years, much space is left to the imagination, but it is reasonable to assume that living organisms

are endowed with the ability of *self-organization* and that this capability has evolved from elementary properties of chemical compounds essential to kick off life on earth.

This obscure stage has possibly involved *abiotic molecules* slowly evolving towards self-replicating forms (Paragraph 1) in a kind of *chemical evolution* (Martin and Russell, 2003) that goes beyond this discussion. Self-replicating means keep track of itself and preserving memory of the experiment, but the question is, obviously, about the nature of the first self-replicating molecule; however, since replication is accomplished in modern cells through the cooperative action of *proteins* and *nucleic acids*, there is a general agreement on their essential contribution to the development and maintenance of any living organism. Hence at the origin of the cellular organization are elementary properties that, through self-replication, are transmitted to the offspring.

The appearance of the *prokaryotes* dating back approximately 3,500 million years ago represents the first remarkable result of this process, but is just a step towards the refinement of life. A real breakthrough in cellular organization is the advent of the *eukaryotes* where elementary properties are re-arranged to a higher level of complexity. But, how was it possible? Nobody knows, however this seems to be related to the endosymbiosis, an evolved form of phagocytosis that consists in using the competence of other organisms, instead of the energy. From this peculiar type of *symbiosis* (Mereschkowsky, 1926) derive certain essential properties of the cellular organization, such as *cooperation*, development of specific skills or *competence*, *complexity increase*, development of *interaction patterns* up to *multicellularity*.

The appearance of multicellular organisms as occurred fairly rapidly in the experiment of life must be seen as an evolutionary stage that does not necessarily involve a significant increase in the complexity of the genetic program (Prochnik et al., 2010); this process has been reproduced recently in vitro by using an eukariotic model (Ratcliff et al., 2012).

Furthermore, since the evolution of multicellularity has not resulted in the replacement of the *prokaryotic prototype* that is still alive, for example, in *modern bacteria*, it seems logical to assume that this stage represents a necessity in evolution (Furusawa and Kaneko, 2000) for *some* living organisms expecially when exposed to highly selective environmental conditions. These extreme conditions on earth are possibly responsible for the *evolutionary peaks* recorded after long periods of stasis (Eldredge et al., 2005) and thus for the evolution of multicellularity. At this regard it is noteworthy that archaea and bacteria, in spite of their early evolution, exibit a very small number of species (about 5,000) if compared with multicellular organisms, and are associated with a high level of resistance in all ecosystems (Staley, 2006). Therefore, it is plausible to assume that bacteria are a *source of backup* capable of restarting the experiment of life on earth even after catastrophic climate change or, possibly, in other places in the universe (Wickramasinghe, 2004).

Returning to the metaphor of the *tree of life*, having a common trunk or origin means to share, at least, some features and/or functions, and in multicellularity some properties of unicellular organisms are reallocated on a larger scale with the evolution of *cellular differentiation* and *specialization*. This new kind of cooperation establishes *functional hierarchies* and leads from the development of *finely detailed pattern* up to the evolution of fully developed *complex organisms*(Furusawa and Kaneko, 2000).

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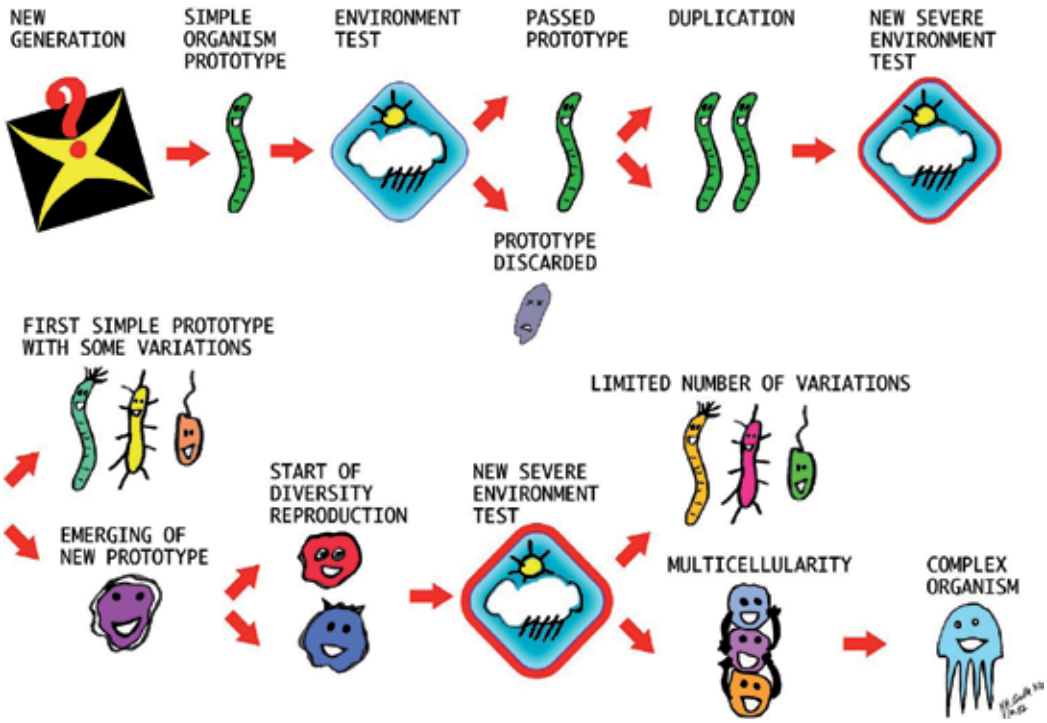
The evolutionary stages, from the development of the first prototype to multicellular organisms, are conceptualized in the block diagram in Figure 3.



The black box [*new generation* machine] represents the obscure stage of the experiment of life, originating the first prototype [*single organism prototype*]. The environmental tests are possibly responsible for the evolutionary peaks, generating others prototypes [*variations, diversity*] up to multicellular and complex organisms.

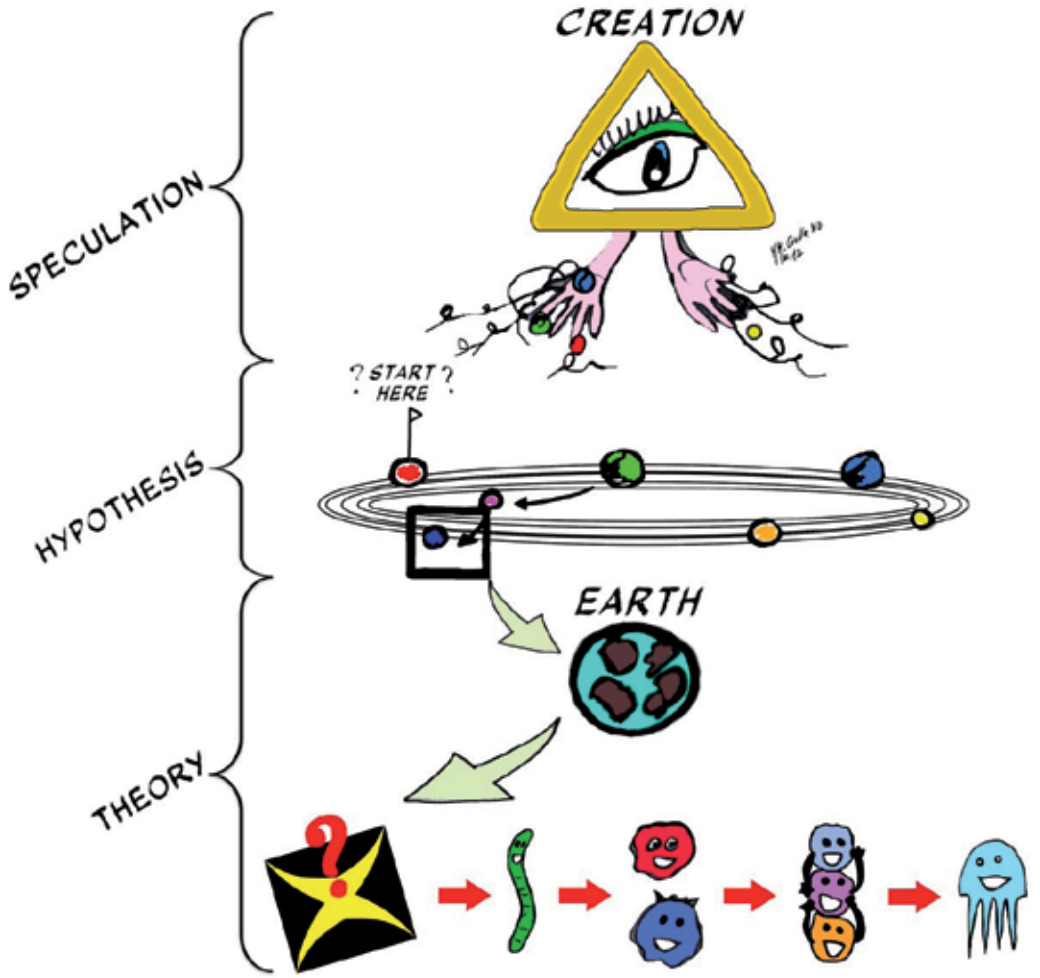
Figure 3. Conceptualization of evolutionary stages

3. The diversity

The *diversity* in biology is "...the variety and variability among living organisms and the ecological complexes in which they occur." (Assessment., 1987). The diversity can also be calculated as the *number* of different items and their *relative frequency*; these items are organized at many levels, ranging from entire ecosystems to the chemical structures that are the molecular basis of heredity (Paragraph 2).

As we have seen before, the diversity originated very early in the tree of life with a very rapid expansion characterized by the evolution of an extraordinary variety of living organisms in a relatively short time followed by a long lasting substantial stasis (Eldredge et al., 2005). This expansion phase is possibly the second *obscure stage* in the experiment of life, after the initial

one (Figure 3), since it is really hard to imagine a cause-effect relationship during those phases beyond the theories of transformation-evolution (Figura 4).



Schematic representation of the origin of life and diversity. The upper part is a speculation and represent an intelligent creator above the experiment of life in the universe. The intermediate part is a representation of the hypothesis that life on Earth has an extraterrestrial origin thanks to space vectors such as meteorites or asteroids. The lower part recalls the second obscure stage [generation machine] and the evolutionary theory explaining the origin of diversity.

Figure 4. On the origin of life

The road to the diversity is marked by milestones including the transition from *duplication* to *reproduction* and this innovation is believed to coincide with the evolution of the *eukaryotic* cell that adopts the *meiosis*. This peculiar form of cell division produces greater variability contributing to increase the diversity of living organisms.

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But writing of diversity in biology can be complicated, precisely because of different points of view covering the whole scenario; indeed, from a purely *descriptive* point of view, there are both visible and invisible differences. Focusing on a *population* represented by all the living organisms that belong to the same species and live in the same geographical area, since individuals are not identical, some visible differences are expected and these differences are even more noticeable when taking into account *different populations* or species.

Nonetheless the visible differences give way to *basic similarities* when are taken into consideration the internal features, invisible at naked eyes. These common features include the general *molecular structures* and principles that are the basis of *biochemical functions* in all living organisms and clearly demonstrate the concept of *branching of the tree of life* starting from a *common ancestor*.

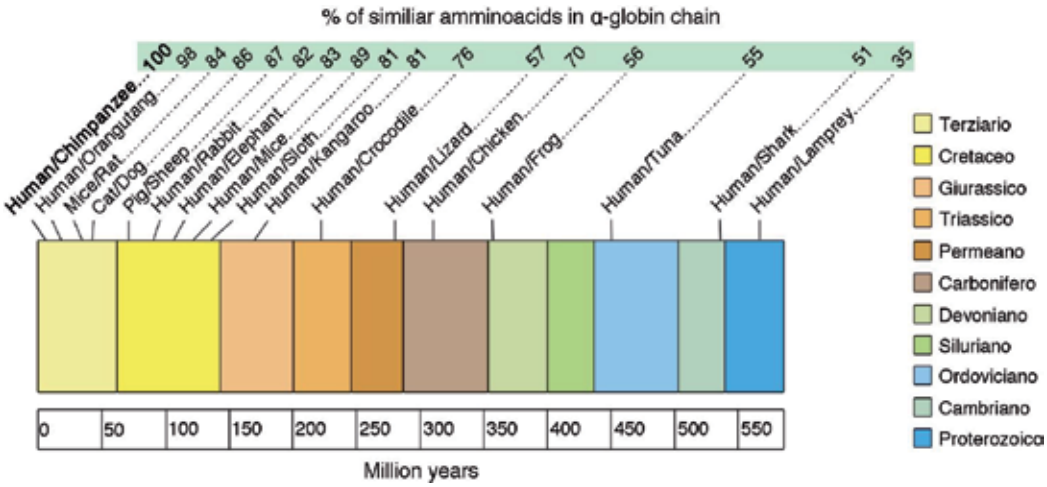
Thus the biological life is characterized by a *partial and transient independence* as the result of the dynamic interaction between the environment and the biochemical function of some *common macromolecules* such as lipids, nucleic acids, namely DNA and ribonucleic acid (RNA), proteins and carbohydrates reallocated on a larger scale with the evolution of *cellular differentiation* and *specialization*.

The *nucleic acids* of all organisms are constituted by the same set of nucleotides and the proteins blocks are almost made by the same amminoacids. Moreover, several *cellular events* share the same mechanisms and machinery; events like mitosis, DNA duplication, protein syntesis are indeed based on the same molecular steps and the molecular structures involved are the same both in prokayotes and eukaryotes, such as histones for chromatin packing, polymerase for DNA duplication, ribosomes for protein syntesis and so on.

From a molecular point of view, only the analysis of structures, such as the analysis of how many and which amino acids compose a protein with a similar function in two different species, can state the diversity, assess the *phylogenetic proximity* between species and outline the branches of the tree of life (Figure 5).

Looking now at the *genetic program* that determines the shape and the function of living organisms, it is represented by different *gene sequences* that, because of *diploidy*, in humans consists of two or more copies of genes called *alleles*. Thus the *gene pool* of a population is made by the totally of alleles, their *combination* or *aplotype* and their *relative frequencies* among the individuals. Thus the *genetic diversity* describes the existence of many different versions of the same individual, a *different phenotype* is the result of a genetic variation in a specific environment and is called *variant*. As stated before, the *genetic diversity* among individuals in a given population and even more between species, increases the chances of survival in case of highly selective environmental conditions; thus *evolution* takes place when there are *any* changes in the genetic pool that increase the adaptation process (Paragraph 1). There are few processes that can lead to *new genetic combinations* and the most relevant for this discussion are *mutation*, *recombination* and *natural selection*, therefore we do not take into account the effects of *migration* into a population from another one, with different gene frequencies, as a *source of variation*.

Essentially these processes can be *random*, meaning that they take place independently of the needs of the organism, *probabilistic* and *directional*; mutations are random, recombination is



The picture shows the estimated geological age of the last common ancestor of each pair of specified animals. Each time estimate is based on comparisons of amino acid sequences of orthologous proteins; more time had a pair of animals to evolve independently, smaller is the percentage of amino acids remaining identical. The final estimates and the time scale has been calibrated to match the fossil evidence that the last common ancestor of mammals and birds lived 310 million years ago. Numbers in the top bar gives data on sequence divergence for a particular protein chosen arbitrarily, i.e the α-chain of hemoglobin. The clear irregularities in growing divergence with increasing time reflect the randomness of the evolutionary process and, probably, the action of natural selection, which drives particularly rapid changes in some organisms subjected to special physiological requirements.

Figure 5. Phylogenetic proximity

probabilistic, depending on the distance between genes (Russell, 1998), and natural selection is directional since it follows a direction which favors the survival of the fittest organism.

Both mutation and recombination define the *allelic variability*, which determines a difference between the genotypes of individuals; this variability will be further shaped by the environment, that ultimately determine the presence of different *phenotypes*, based on instructions dictated by the *genotype* (Russell, 1998). Mutations, as source of variation, are quite limited since their rates are very low. On the other side, recombination is a primary source of variation; most of the attention of evolutionary geneticists has focused on the extensive *genetic recombination* that takes place during meiosis and, in particular, during pairing of sisters chromatides and its significance as a generator of genetic diversity in organisms with sexual reproduction (Charlesworth, 1988; Edwards, 2000).

In organisms with asexual reproduction, such as bacteria, recombination is very limited since mutations are the only source of gene combinations, thus, asexual organisms may evolve more slowly, under natural selection, than sexual ones (Griffiths et al., 1999). Although the mutations play a more limited role as a source of variability in multicellular organisms with sexual reproduction, together with the effects of gene recombination, they can still be transmitted to offspring only by the *germ cells*.

In multicellular eukaryotes these cells are the connection between the different generations since they are the only ones that undergo *meiosis*, as well as *mitosis*, in contrast to *somatic cells*,

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practically all the others, that divide only by mitosis. In organisms that have evolved forms of *sexual reproduction*, the life cycles start through the union of two germ cells, male and female, and their nuclear fusion originates the *zygote*. Several reasons support the evolutionary necessity and the benefits of sexual reproduction in multicellular organisms (Roze, 2012; Wong and Wessel, 2006) and their prevalence in animals (Engelstadter, 2008).

Thus the zygote is a *diploid cell* resulting from the mating of two *haploid cells*, that gives rise, after three cell divisions, to the first *stem cells*. These cells are the only ones capable of differentiating into all cell types that make up a multicellular organism. This extreme versatility is known as *potency*, it is maximal during the pre-natal life in the *embryonic stem cells*, and progressively decreases in post-natal life remaining confined within the *adult stem cells*.

With regard to the definition given at the beginning of the paragraph, it should be remarked that the *biodiversity* implies the overall biological variability, including both genetic and environmental features. Environmental changes, both cyclic and irregular ones, may be a serious challenge for the experiment of life causing usually a *switch* in the phenotype. There are two opposite types of switching of the phenotype: the *reactive type*, which occurs as a direct response to an external cause detected by a *sensory mechanism,* such as a receptor, and the *stochastic type*, which occurs without the mediation of a sensory mechanism (Kussell and Leibler, 2005).

The *phenotypic diversity* is generated by *phenotype switching* caused mainly by stochastic mechanisms; thus the *population diversity* and the coexistence of subjects differently adapted to a certain environment, can be a way to respond to *irregular environmental changes* (Soll and Kraft, 1988; Perez-Martin et al., 1999; Bayliss et al., 2001; Lachke et al., 2002; Bonifield and Hughes, 2003; Kearns et al., 2004; Balaban et al., 2004).

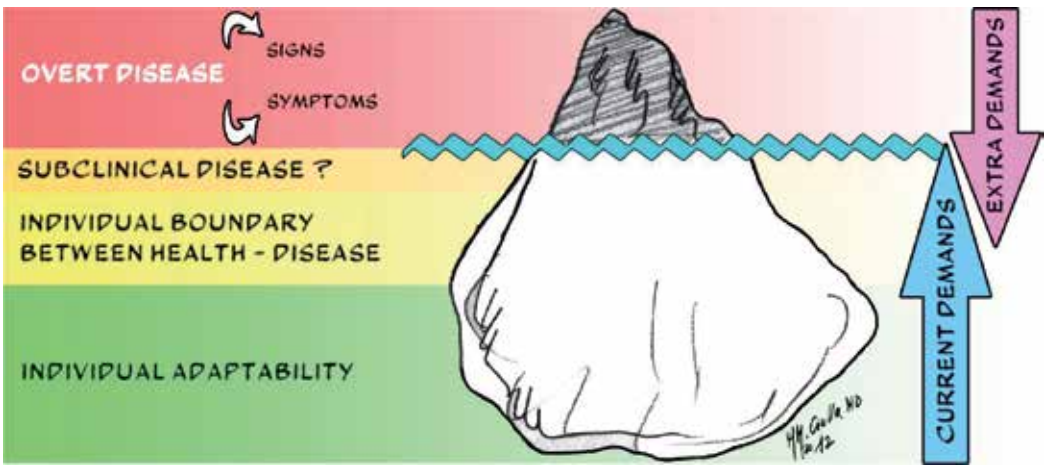
4. The adaptation

It is impossible to speak of life in a biological sense without postulating a *certain degree of independence* from the environment where life itself takes place. Whatever its level of complexity, a living organism is a *biological system* delimited by a kind of boundary to face the *external environment* and capable to maintain a stable, constant condition by regulating himself and the *internal environment*.

The demarcation of self with a *membrane* and the establishment of an *internal environment*, opposed to the external one, is a formal requirement to any living organism and the condition of *relative equilibrium* called *homeostasis* (Cannon, 1929) provides the biological system an appropriate level of independence from the environment that allows to acquire other essential properties such as response to stimuli, development, growth and reproduction.

Any change, either decremental or incremental, in the internal/external environment of the cell/organism such that it requires an active response from the cell/organism can be termed demand (Ciulla et al., 2011). By using the simplified approach *demand-response*, it is possible to classify the demand as a function of its *type*, *intensity* and *duration*. Since the possible responses

of the cell/organism are limited and fall within the *adaptive processes*, the demands can be further classified in current or extra according to the efficiency with which they can be handled. Current demands can be adequately dealt with in a physiological context, even though some degree of cell/organism injury can still occur; extra demands cannot be sufficiently buffered, and lead to functional impairment and, eventually, to disease or, as the last resort, to death. Anyhow the final outcome of the changes imposed on living organisms depends on the *ratio demand-response*; an excess in demand and/or a defect in the response for the lack or depletion of adaptive resources, can represent a serious problem for the survival of the organism. In such view, *disease* is the result of an *unfavorable interaction* between the cell/organism and the environment or, by simplifying, between the genetic resources and the environment. The succession of cyclical and stochastic environmental changes suggests that, during the life cycle, the current demands represent the bulk of all demands while extra demands are more often occasional. The relation between current and extra demand can be depicted by using the iceberg metaphor (Figure 6). Thus, the *possibility of damages* and *disease* caused by *unfavorable interactions* with the environment are foreseen and their occurrence is managed by coping biological resources involved in tissue maintenance and repair of damages.



Representation of the iceberg metaphor, illustrating the boundary between health as a result of *individual adaptability* and disease. The tip of the iceberg corresponds to overt disease; the huge part below the water line is where individual adaptability successfully buffers environmental demands; just below the surface is the grey zone of subclinical disease. Demands are defined as current or extra according to how efficiently they can be handled by the single organism.
Modified from an Open Access source: http://www.intechopen.com/books/advances-in-regenerative-medicine/inflammation-angiogenesis-cross-talk-and-endothelial-progenitor-cells-a-crucial-axis-in-regenerating.

Figure 6. The iceberg metaphor to understand diseases

Therefore, as changes in the internal/external environment occur continuously, it is evident that *allocating biological resources* in order to buffer changes is an essential requirement for life and living organisms have, indeed, evolved specific *adaptive processes* to meet the demands imposed by changes in the environment. In this context, it is therefore not superfluous to

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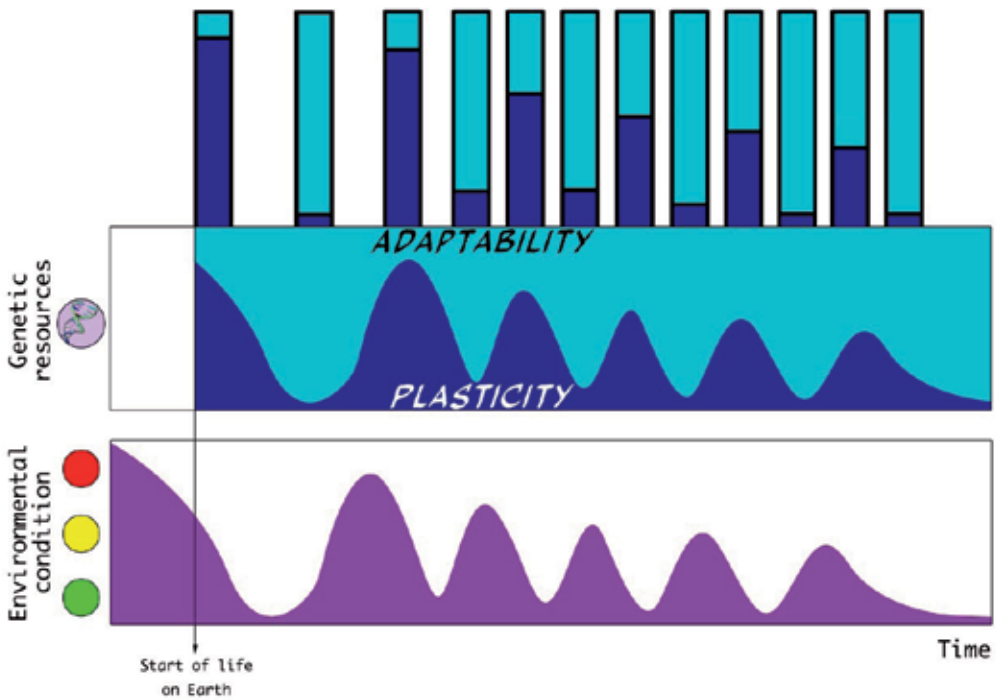
remind that the *availability of resources* is only possible if there is a corresponding *genetic resource*. The *genetic program* that governs *genetic resources* is a *finite sequence of instructions* whose possible combinations are somehow limited by the same program. Furthermore, especially in complex organisms that require *stages of development* to reach a final *adult form* and a *full functionality*, genetic resources are not only quantitatively limited but also *temporally regulated* by the constraints of the life cycle. Thus in the same manner will also be limited the *adaptive processes*, including *organism adaptation* or *plasticity* and *adaptability* (Kutschera, 2009). The *plasticity* is the ability to express a broad variety of phenotypes in response to environmental changes and, in complex organisms with sexual reproduction, is at a maximum during *embryogenesis* and *early extrauterine life*. The *adaptability* could instead be applied to the more limited process of adaptation which occurs in adult life and varies between organs and species (Figura 7).



The lower panel shows the environmental fluctuations as result of temperature changes (street light colors) during the history of Earth. The upper panel shows how genetic resources respond accordingly to these fluctuations by tuning adaptability and plasticity. The bars depict which resource is mainly involved in each time points.

Figure 7. Relationship between plasticity and adaptability in a changing environment

But why are these processes limited in adult life? In this regard, it should be remembered that in complex organism the evolution towards multicellularity is a form of adaptation to the environment that involves a high cost in terms of biological resources; in other words,

differentiation, specialization, establishment of functional hierarchies and development of a *fully developed complex organisms* implies a considerable expense of biological resources, thus reducing the availability of resources for other forms of adaptation in adult life such as the *re-generation* process.

Therefore in the presence of an *extra demand* causing a damage the response of complex organisms in adult life mainly consists in *functional* and *structural adaptation* since *re-generation* processes after *injury* are limited. Indeed in such organisms, *generation* and *re-generation* are capabilities shared by the same deputy cells named *stem cells*, the only ones capable of differentiating into all cell types that make up a multicellular organism (Paragraph 2). The number of stem cells is limited and, as we saw earlier, their *potency* is maximal during the pre-natal life in the *embryonic stem cells*, and progressively decreases in post-natal life remaining confined within the *adult stem cells*.

These cells are therefore to be understood as a kind of *functional reserve*, restricted to a *specific tissue*, that could be recruited to support adaptation processes that allow the organism to better fit in with the changed environment and, thus, attain a new equilibrium. At this regard the *functional hierarchy* of complex organisms allocates *functional reserve* where it is needed and establishes the *distribution of the available resources*. In complex organisms such as mammals, many adult tissues contain populations of adult stem cells that have the capacity for *renewal after disease* or *aging*; these tissues include brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis. Thus, the primary role of adult stem cells in a living organism is to *maintain* and *repair* the tissue in which they are found. Unfortunately there is a very small number of adult stem cells in each tissue, with large numerical differences between a tissue and another, and, therefore, the *re-generative potential* is unevenly distributed and, in any case, is very limited. The reason why some *highly specialized tissues* have limited regenerative capacity is not yet known, but we can not exclude that the extreme structural and functional specialization reached by some tissues is an inherent limit to regeneration (Table 1).

Recently a number of experiments have reported that certain adult stem cell types can differentiate into cell types seen in organs or tissues other than those expected from the cells' predicted origin or *lineage*; for example, brain stem cells that differentiate into blood cells or blood-forming cells that differentiate into cardiac muscle cells, suggesting the idea that cells might have an *alternative fate* after maturation according to their specific identity. The two known processes by which cells are able to turn into other cell types are: *trans-differentiation*, consisting in the direct conversion from one cell type to another, and *de-differentiation*, or the reversion to a less-differentiated cell type and the subsequent maturation to a different lineage. Thus the *cell identity*, which had so far been considered a rigid and durable characteristic involving a one-way process from precursor to mature cell, was shown to exhibit not only intrinsic plasticity (Scadden, 2007) but also a certain degree of adaptation depending on the interplay between genome and microenvironment. In fact it was demonstrated that mature cells are able to switch not only their functional phenotype but also their gene expression profile into that of stem cells, thereby acquiring pluripotent plasticity (Ciulla et al., 2011). The

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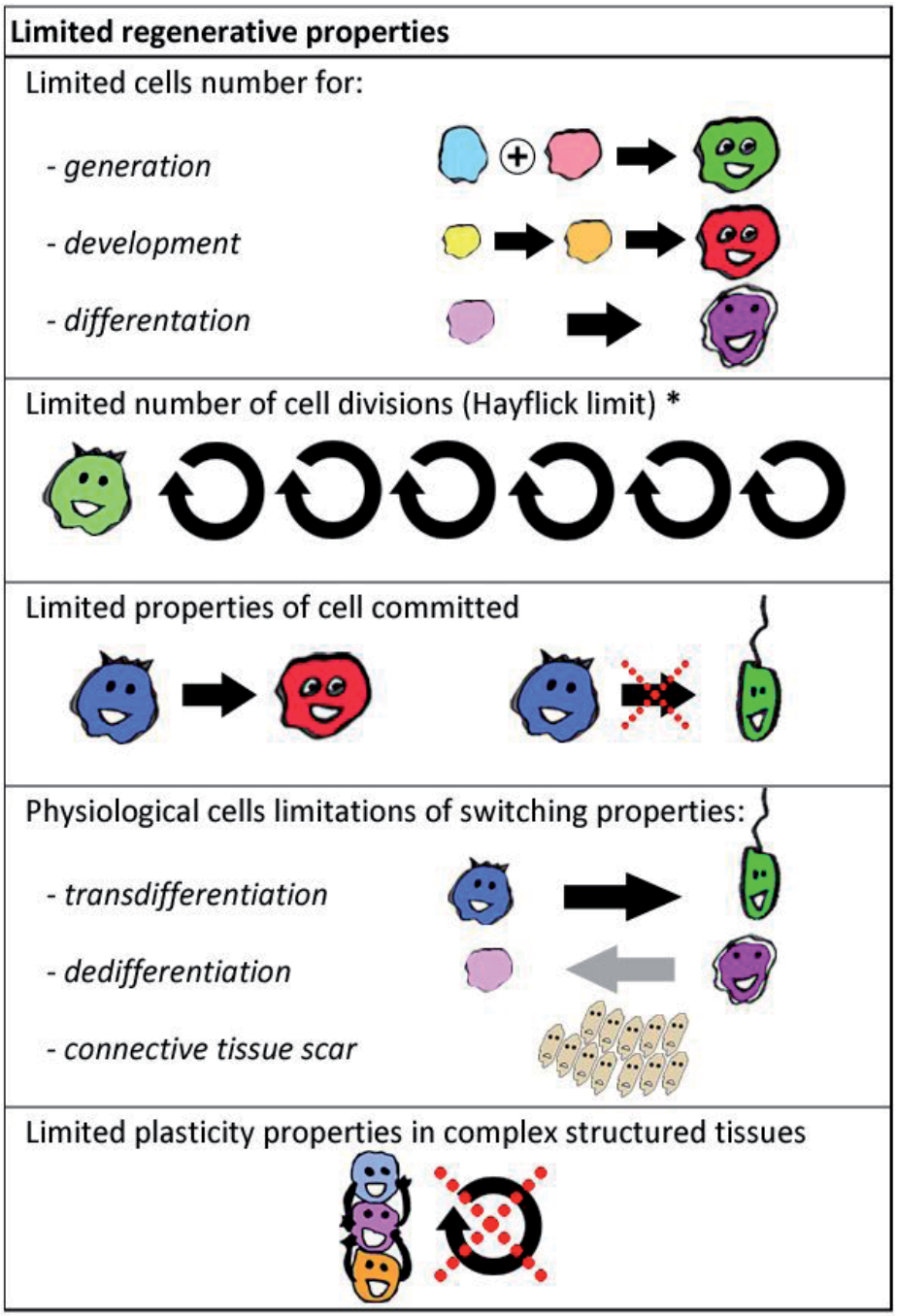
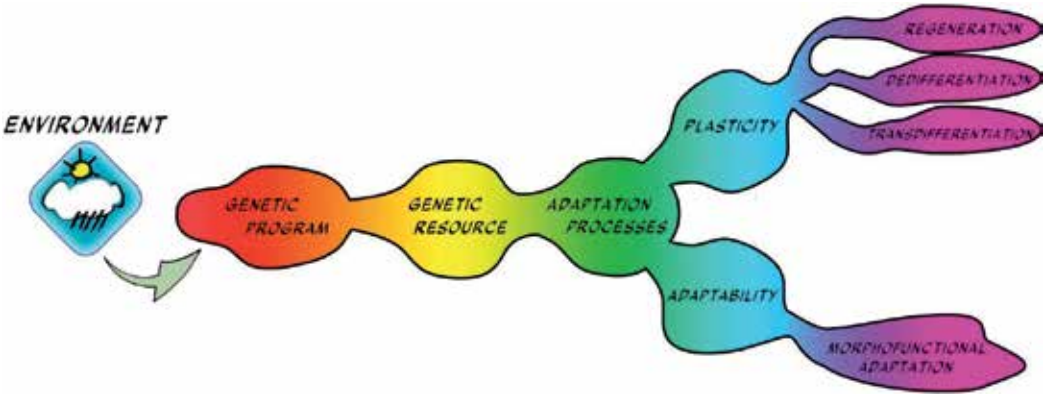


* Hayflick demonstrated in 1965 a limited number of cell division (Hayflick, 1965)

Table 1. Limited regenerative properties of cells in complex organisms

alternative fate has indeed opened a new window of opportunity revealing the significant opportunities that may arise from *engineering of adaptive processes* (Figure 8).



Representation of biological resources allocation by genetic program in order to buffer changes in the internal/external environment. All living organisms have evolved specific *adaptive processes* to meet the demands imposed by environment.

Figure 8. Allocation of biological resources for adaptation processes

In the last decade the topic of *adult stem cell repair* of the infarcted myocardium was among the most popular in the scientific community and has gained growing popularity among top scientific journals. The objective of these studies is, therefore, to optimize the adaptive processes by probing the possibility of *manipulating the cellular identity*. By suggesting possible *alternative fates* for the cells, this research model clashes with the *dogma of the cell cycle* and is faced with the problem of how to ascertain the identity of the cells in a context in which the identity itself is no longer a certainty but, rather, a dynamic concept (Ciulla et al., 2011).

Thus the issue of cell identity or phenotype led to develop alternative techniques to direct visualization of histological structures; among them the refined, however complex, techniques of *fluorescence microscopy* based on the confocal representation of *fluorochromes* visible at different wavelengths. The employment of these laboratory techniques in the belief that *methodological complexity* equates to biological soundness has produced a paradox culminated in a scientific nonsense: the same experiment, carried out by different investigators, produced discrepant findings, as illustrated by an article on Nature (Orlic et al., 2001). After this setback the isolated instances of *trans-differentiation* observed in some vertebrate species following transplantation of adult stem cells have been debated by the scientific community and the observations so far made have been explained alternatively as a result of the fusion of a donor cell with a recipient one; in addition, even when trans-differentiation has been detected, only a small percentage of cells undergo to this process. This episode, seen as a drama by the scientific community, points out that as the complexity of a study increases its informative content paradoxically deteriorates. The scientific basis for this type of reasoning can easily be found in the field of mathematics, according to Gödel's Incompleteness Theorem: *a great complexity is a source of incompleteness because it increases the likelihood that true sentences cannot*

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be proved (Calude and Jurgensen, 2005). In this regard it should be emphasized that the direct visualization of histological structures across a large number of fields coupled with immuno-histochemistry assay on contiguous slices in clear guarantees, in any case, a high spatial resolution (Kwok et al., 2010; Ciulla et al., 2013).

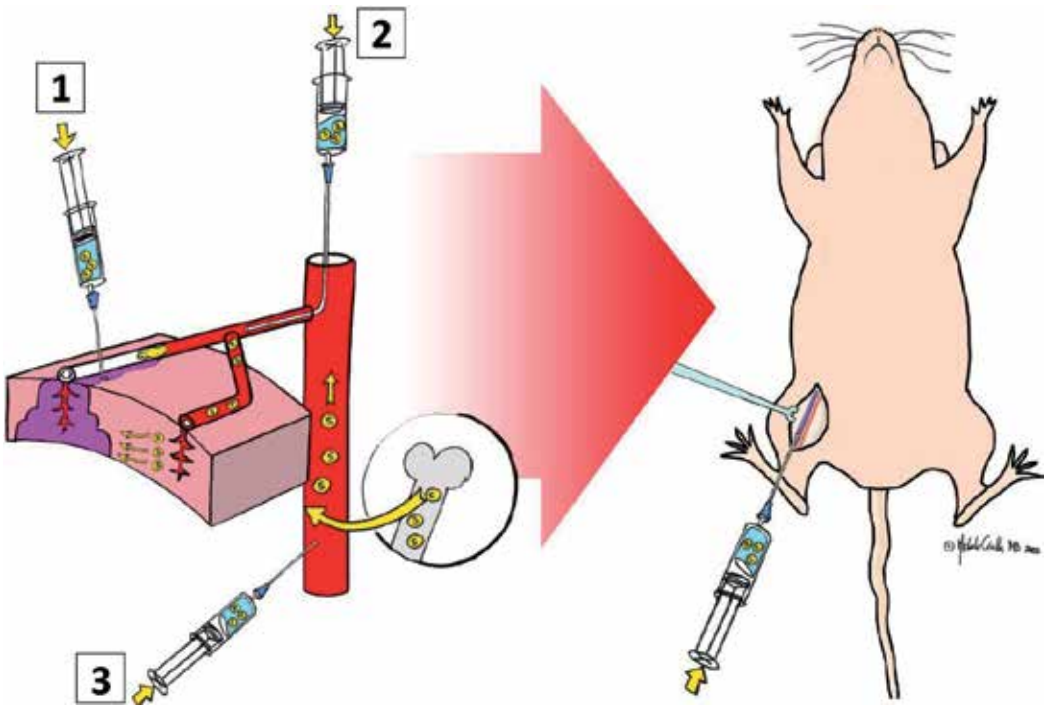
Nonetheless research on adult stem cells continues to generate great enthusiasm and has led researchers and clinicians to ask whether adult stem cells could be used for transplants. In the era of biomedicine, transplantations and tissue engineering, an emerging practical issue, however, is what kind of adult stem cells should be used to optimize the adaptive processes after tissue damage. Pioneering studies have focused on the most versatile adult stem cell such as the *hematopoietic* one that, starting from a common precursor, is able to give rise to very different cell lines. It must be remembered that adult hematopoietic stem cells transplantation has been used as a medical procedure in the field of hematology and oncology since the fifties (Rebulla and Giordano, 2004).

Once collected from a donor and administered peripherally to a recipient, the ability of these hematopoietic precursors, identified as *bone marrow mononuclear cells*, to travel through the circulation and to selectively targeting an area of *experimental myocardial damage* produced by means of *cryoinjury* (Ciulla et al., 2004b) has been demonstrated in rats (Ciulla et al., 2003) (Figure 9).

Furthermore, the study of the *mechanisms of homing* of these cells, also showed that this phenomenon is proportional to the extent of the damage (Ciulla et al., 2004a) and, finally, their contribution consists mainly in giving rise to new, actually working, vessels (Ciulla et al., 2006; Ciulla et al., 2007). Another instance is that transplanted cells might have also a *paracrine effect* such as to modulate the response to injury (Ciulla et al., 2008). In the perspective of the autologous infusion, it has been shown that adult stem cells, once removed from the body, have very limited ability to divide, making generation of large quantities of stem cells difficult (Ciulla et al., 2006). Despite these limitations, with a view to improve the healing process in humans, the advantages of using adult stem cells should be remarked as they allow to avoid the ethical and political issues associated with the use of embryonic stem cells.

5. Evolution in a gravitational environment or gravity as an evolutionary force

The gravity of the Earth refers to the *acceleration* that the Earth imparts to any given body on its surface by virtue of its *mass*; this acceleration is directed toward the center of the Earth and is approximately 9,81 m/sec^2. More precisely, according to the law of gravitation formulated by Isaac Newton to 1665, the attractive force between two bodies, in this case the earth and any object, is directly proportional to the product of the masses and inversely proportional to the square of their distance. The *mass* is an intrinsic property of any body and is a constant in classical physics, on the contrary the *weight* varies and *on Earth* is the result of *mass* for *Earth's gravitational acceleration*. Gravity or, better, the Earth's *gravitational field* hasn't significantly changed since the origins and is considered a *constant* although there are some differences in



The main routes used for the administration of hematopoietic precursors. Left drawing, 1) direct injection in the infarcted area; 2) direct coronary injection and 3) peripheral injection. Right drawing, peripheral injection in vivo in the femoral vein in rat.

Figure 9. Administration of adult stem cells in experimental model of myocardial injury

its distribution since the Earth is not a perfect sphere with a constant density and, due to its rotation, is subject to the *inertial force*.

By virtue of the gravity of the Earth, also living organisms have a weight that affects every aspect of life including biological ones. The life originated from the sea, landing on the ground of certain organisms and the experience of gravity has, therefore, required the development of specific mechanisms to counteract the effects of gravitational acceleration. Subsequently for some species the transition from the horizontal position to the vertical one has required the adoption of *sensors for the gravity* and of systems to allow the movement and the fine adjustment of the displacement of body fluids. (Table 2).

These postural changes from the horizontal position to the vertical one are cyclical and mainly related to the rhythms of activity and rest that are typical of each species. Furthermore, especially in placental mammals such as humans, for *n* months, first the embryo and then the fetus, are immersed in a fluid and compressed by a capsule that exerts a counter-pressure that diminishes the direction and the force of gravity creating a *microgravity environment*. This exposure to microgravity cause changes in gene expression in a variety of developing organ systems in live embryos (Shimada et al., 2005) thus the meaning of *prenatal life* goes beyond

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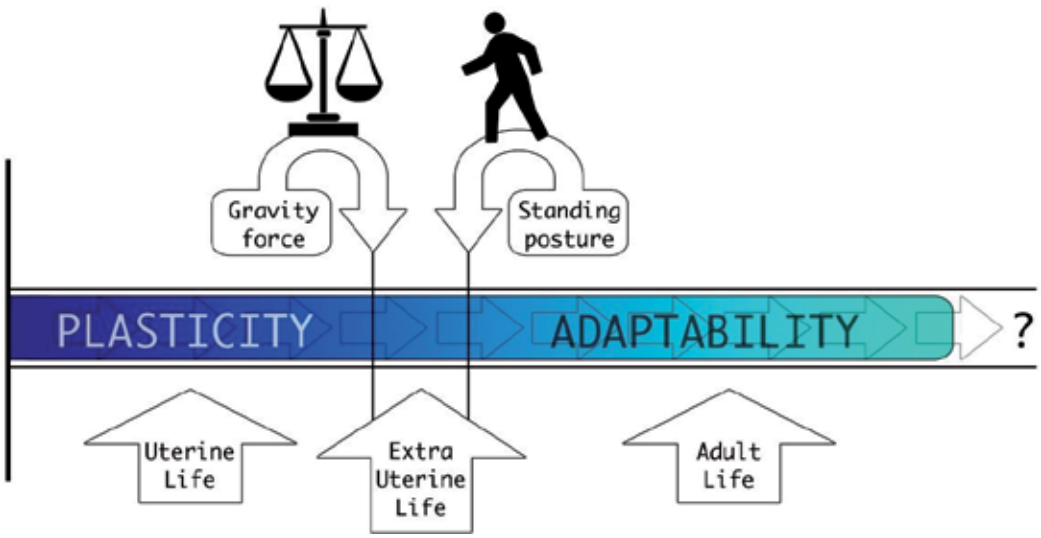
Evolution in a gravitational environment
Sensors *Sensors for the gravity*
Standing and locomotion *Structural support* *Postural stability*
Fluid and Flow regulation *Fine adjustment of the displacement of body fluids*

Table 2. Requirements for the evolution of a complex organism in a gravitational environment

the simple effect of *mechanical protection* played by amniotic fluid as it covers an essential role in initiating the processes of long-term expression of the genetic program of the embryo. These different developmental pathways are ultimately triggered by the *external environment* through the maternal mediation (Fligny et al., 2009); the shift from uterine life to extra uterine life is concurrent with the progressive decrease of plasticity in favor of adaptability (Figure 10).



The transition from a microgravity environment (uterus) to a gravitational one and the assumption of standing posture in humans are potential stimuli shifting adaptive resources from plasticity to adaptability.

Figure 10. Gravity challenges and adaptive resources allocation during human life

The effects of gravity on organisms can be studied easily by varying the *gravity level* by simulating microgravity on the earth or in flight. The *simulated microgravity* is commonly

obtained on Earth by *head down tilt*, *prolonged bed rest* or *dry water immersion* (Magrini et al., 1992), in flight, during the fall phase of *parabolic flight in jet aircraft* or in orbit around the Earth with *spacecraft* (Table 3).

Simulated microgravity
On Earth - *Head down tilt* - *Prolonged bed rest*
In flight or in orbit around the Earth - *Parabolic flight in jet aircraft* - *Orbit flight in spacecraft*

Table 3. Experimental approaches to simulate microgravity

Since these techniques counteract some of the effects of gravity, it is believed they can provide important information relevant to biology in a gravitational environment.

Unfortunately the precise role of gravity on evolution is difficult to determine in complex organisms because, until now, the stay in a *microgravity environment* has been too limited and no higher vertebrate has passed at least one life cycle in microgravity to see the effects on subsequent generations. These simulations, even if limited in time, may nevertheless provide useful information on *adaptive processes* to counteract the force of gravity in different organisms and, in such sense, the responses of the cardiovascular system are paradigmatic. Previous observations suggests that prolonged dry water immersion after birth in rats is able to dissociate the effects of body growth and aging on systolic blood pressure since the microgravity reduces the needing for load-bearing structures and then the body weight, temporarily blunting systolic blood pressure rise (Magrini et al., 1992).

It should be remarked that the ability to survive in a gravitational environment is inversely proportional to the size, in other words unicellular organisms such as bacteria are much less sensitive to gravity when compared to complex organisms with blood circulation and subject to cyclical postural changes. These observations are derived from the study of extremophiles, i.e organisms capable of surviving in extreme environments and similar to those that are supposed to exist on other planets (Rothschild and Mancinelli, 2001; Brack and Pillinger, 1998).

Due to this lower sensitivity to changes in gravity, as demonstrated also by the growth at hyper-accelerations (Deguchi et al., 2011) and also to the high level of resistance in all ecosystems, bacteria have indeed the potential to travel in space and colonize planets with different gravity levels. This theoretical possibility, and some studies on meteorites, have led to the suggestive hypothesis that life on Earth has an extraterrestrial origin thanks to space vectors such as meteorites or asteroids (Mautner, 2002). This kind of exogenesis, challenged at the

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theoretical level (Di Giulio, 2010), moves indeed elsewhere the problem of the origin of life but, at the same time, is in line with the concept of a solid common trunk for the tree of life which possibly consists in extremophile bacteria.

Since the bacteria are *single-celled organisms*, in assessing the effects of gravity a point that deserves emphasis is the impact of the microenvironment in which the cells are suspended which affects nutrient supply and disposal of waste with potential cumulative effects (Klaus et al., 1997).

6. The heart of the matter: Circulation of blood in a gravitational environment

Despite little changes over time in the Earth's gravitational field, the effects of gravity on living organisms cannot be properly appreciated without taking into account some factors related to the development of multicellularity that have introduced significant changes in the experiment of life on earth. Among these factors, the development of blood circulation and the propensity to postural change have certainly contributed to substantially increase the role of gravitational factors.

In complex organisms the development of a cardiovascular system fulfills the need for transport of nourishment, oxygen and metabolic waste as well as the need for communication between distant districts (Ciulla et al., 2011). The impacts of the *assumption of standing position* in mammals during evolution may have represented a real challenge for the cardiovascular system and, certainly a step forward in the freedom to move in space (Gisolf, 2005). This challenge presents itself in every individual after birth when the assumption of the upright position translocates the intravascular volume from the cardiopulmonary area to the periphery eliciting appropriate *neuro-humoral responses* whose objective is to control the dynamics of body fluids and blood pressure in a gravitational environment (Magrini et al., 1989). It should be recalled at this point that the heart of higher vertebrates is a quite complex multi-chambered pump that contracts synchronously; for its function, a number of components have to be generated; thus not surprisingly, several factors are involved in regulating the target genes responsible for both morphogenesis and function (Hoogaars et al., 2007).

Indeed the cardiovascular system begins to adapt to the gravitational loading before birth and, in particular, when the fetus, growing, begins to come into contact with the amniotic sac (Sekulic et al., 2005). This takes place towards the end of the pregnancy and is essential for the proper development of *sensory receptors* (Bradley and Mistretta, 1975) thus the microgravity resulting from immersion in the amniotic fluid does not mask the effects of gravity on the fetus. After birth, the newborn is immediately exposed to the gravitational load that, as we have seen, also varies depending on the posture. Exposure to gravitational stimuli may also have played a key role in the evolution of the position of the heart in relation to the circulation; taking, for example, three types of snakes that live in different environments, such as trees, land and sea, it was noticed that the position of the heart is closer or more distant from the head as a function of the gravitational load, being more distant in water snakes (Lillywhite,

1988). Therefore, even if gravity is a rather constant parameter on earth, it conditions the development of living organisms, before and after birth, with an increasing impact depending on having a blood circulation and, at the same time, on the propensity to postural change, and both of these characteristics are typical of higher vertebrates.

Finally, several findings suggest that gravity continues to play a decisive role *during aging*, since the reduction of loading conditions that characterizes the more sedentary life typical of the elderly has important effects on the organism that resemble, in many respects, to what happens in microgravity environment during prolonged space flight (Vernikos and Schneider, 2010). In particular, the reduction of motion and acceleration typical of *aging* and of the *prolonged space flight*, can decondition the cardiovascular reflexes, altering the control of blood pressure with *orthostatic hypotension*, and lead to a reduction of muscle mass and the loss of calcium from the bones.

7. Generative and re-generative time windows: Definition and meaning

As we saw earlier, *generation* and *re-generation* are capabilities shared by the same deputy cells named *stem cells*, the only ones capable of differentiating into all cell types that make up a complex organism. The number of stem cells is limited as well as the number of generations, and their *potency* is maximal during the pre-natal life in the *embryonic stem cells*, and progressively decreases in post-natal life remaining confined within the *adult stem cells*. These processes have very tight *time constraints* to allow the *harmonious development* and *maintenance* of a very complicated structure such as a higher vertebrate; thus generative and re-generative processes are only possible at the opening of a *specific time-window* that coordinates them through the activation of specific gene sequences. In this respect, the evolution towards multicellularity is a form of adaptation to the environment that involves a high cost in terms of biological resources to handle *differentiation, specialization, establishment of functional hierarchies* and development of a *fully developed complex organisms* (Figure 11).

It is therefore evident that the availability of resources for other forms of adaptation in *adult life* such as the *re-generation* process for *maintenance* or *repair* is limited, especially if we take into account the complex organisms. These *time-windows* have very different characteristics, the *generative time-window* close formally when the organism reaches its full development, although with large time differences between the various tissues. On the other hand, the *re-generative time-window* differ depending on the tissue and on the functional hierarchy that it covers; more precisely, in *permanent tissues*, the re-generative time window is *formally closed* and the cells that compose them cannot divide after generation; in *stable tissues* the window *can be re-opened* in the event of a *limited damage* to allow the *repair*; finally, in *labile tissues*, the window *remains open* almost throughout the life cycle for maintenance and repair (Figure 12).

It should be recalled, therefore, that the experiment of life on earth includes the *possibility of damages* caused by *unfavorable interactions* with the environment and, above all, that there are also some solutions to fix them (Ciulla et al., 2011). These solutions range from the optimal one which consists, obviously, in the re-generation, to that of lower efficacy such as the replacement

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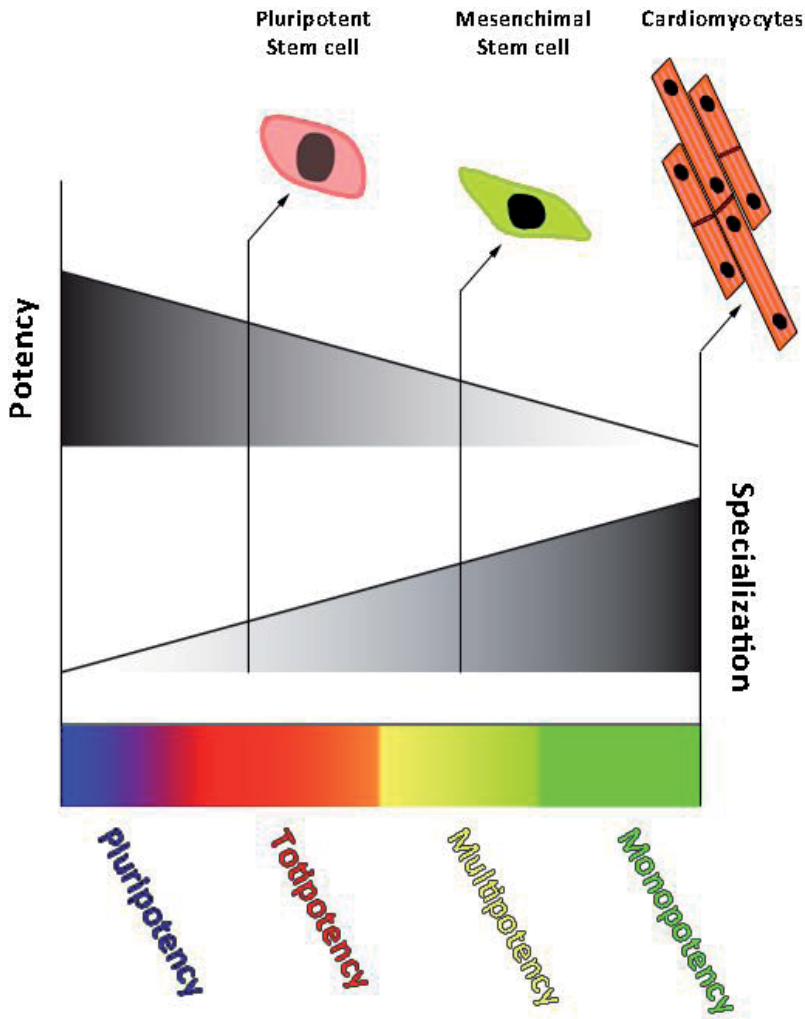
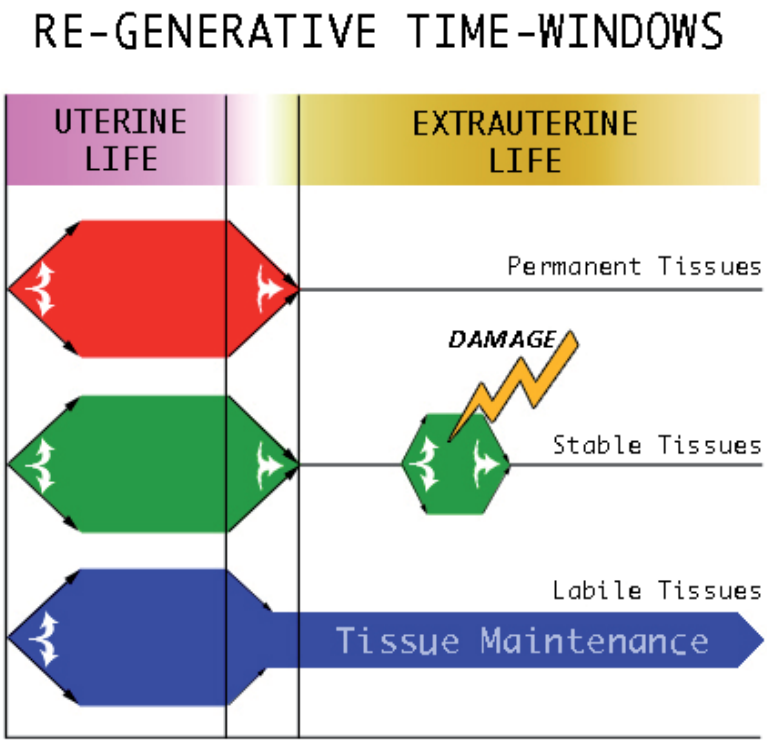


The number of stem cells and their replication cycles are limited; their potency is maximal during pre-natal life (embryonic stem cells) and progressively reduces in post-natal life (adult stem cells). Generative and re-generative processes are possible only at the opening of a specific time-window (Figure 12). The potency of the cells is inversely proportional the degree of cellular specialization, that is thus maximal at monopotency.

Figure 11. Relationship between potency and specialization of cells

of damaged tissue with *scar tissue*, a process known as *scarring*. Thus, the primary roles of *adult stem cells* in a living organism is to support the *functional reserve* by *maintaining* and, eventually, *repairing* the tissue in which they are found, according to a specific re-generative time window. Unfortunately there is a very small number of adult stem cells in each tissue, with large numerical differences between a tissue and another, and, therefore, the *re-generative potential* is unevenly distributed and, in any case, is limited. It should be remarked that in the *vascular system* a vast reservoir of adult stem cells is maintained for *renewal processes* including



Master plan of re-generative time-windows in the three main tissues, according to the cell replication capabilities during human life. The white arrows indicate the opening and the closing of each window.

Figure 12. Re-generative time-windows

the continuous production of the endothelial cells lining the vessel wall and of the circulating blood elements.

In complex organisms the functional hierarchy is paramount in allocating this functional reserve where it is needed establishing the priorities in the *distribution of the available resources*. In mammals many adult tissues contain populations of adult stem cells that have the capacity for *renewal after disease* or *aging* but some *highly specialized tissues* -such as heart and brain- have a very limited re-generative potential. At this regard, we cannot exclude that the extreme structural and functional specialization reached by some tissues is a further and, possibly, inherent limit to re-generation.

Thus, returning to a broader view, it is a matter of fact that in the complex interplay between organization of the living matter, natural selection and adaptation, mammals have evolved with limited or no re-generative capabilities of the heart after birth and the reasons for this apparent flaw have to be sought in organization and allocation of resources in a hierarchically structured multi-cellular organism with an evolved system of transport and communication, such as the circulation of blood.

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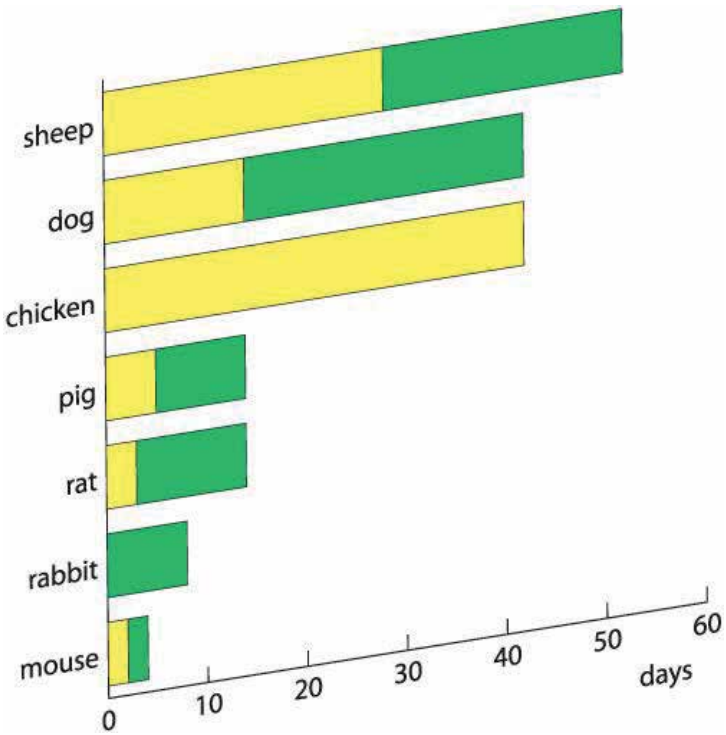
Focusing on *adaptive resource allocation*, it is widely acknowledged that during the post-natal development of the heart in higher vertebrates there exists a limited generative time-window. In figure 13 we show the upper limit of such time-window for different animal species.



During the post-natal cardiac development exists a limited time-window of potential tissue regeneration; the graph shows the lower (yellow) and the upper (green) length of this period. The data were taken from the following references: (Li et al., 1997; Brodsky et al., 1988; Hoerter et al., 1981; Clubb and Bishop, 1984; Burrell et al., 2003; Morgan and Beinlich, 1997; Bishop and Hine, 1975). Data collected by courtesy of Paola Nicolini, MD.

Figure 13. Limited generative time-window of the heart in higher vertebrates

Since generation and re-generation of the heart rely on the same physiological reserve consisting in proliferating cardiomyocytes, is logical to assume that the generative and re-generative time-windows coincide, but the questions on re-generative potential still open are:

1. *Why the limited re-generative capacity of the heart is a current problem?*

2. *What is the physiological significance of this post-natal time-window?*

3. *What are the clinical implications of this last re-generative chance for the heart?*

1. *Why the limited re-generative capacity of the heart is a current problem?*

In humans what appears to be a flaw may not have been a problem for thousands of years until, in the twentieth century, the increase in *life expectancy* (Burger et al., 2012) has given rise

to diseases generally less frequent in the first three or four decades of life. Therefore, the significant increase in *morbidity* and *mortality* related to cardiovascular disease, seen mainly in Western countries in the last years (Smil, 1989; Ramsden et al., 2009), has brought to the foreground the problem of *cardiac damage* and of its repair. According to this broader view of the problem, the limited regenerative capacity should be read in the context of the processes associated with aging (Frangogiannis, 2012). At this point it should be emphasized that in Western societies cardiovascular disease have a cost and this cost is fully or partly covered by the community; therefore, as economic resources are finite and health care costs are programmed at the political level on the basis of emerging issues, the new opportunities arising from research on adult stem cells have represented in recent years an attractive location for public and private funds. As in the overall budget military spending represents an item in competition with those of health, research on adult stem cells has the great merit to redirect spending towards scientific research in the biomedical field.

2. *What is the physiological significance of this post-natal re-generative time-window?*

Some studies have tried to characterize the post-natal re-generative window by using its potential to repair a myocardial damage. Among them, one of the most impressive for the scientific community was conducted a few years ago on the zebrafish with the amputation of a limited portion of the cardiac apex (Poss et al., 2002). The results were in line with the expectations since this kind of injury is coherent with the pathophysiology of teleost fish and urodele amphibians which are known to regenerate body parts, and the resected ventricle was regenerated (Poss et al., 2002; Neff et al., 1996). More recently, a similar experiment was conducted on mice achieving a similar regenerative result (Porrello et al., 2011) even if the resection of about 15% of the ventricular myocardium has a poor clinical applicability to humans. Unfortunately the goal of both these studies, was not to elucidate the physiological significance of this re-generative time-window but, rather, to evaluate its potential under *extreme experimental conditions* far from a clinical setting, even if hypothetical.

In this regard, the limited duration of the re-generative cardiac time-window and its placement between fetal and postnatal life clearly argues for its physiological function linked to the development of the cardiovascular system in a gravitational environment whose impact grows, indeed, after the birth. The plasticity of the heart is therefore required in mammals to support the new load conditions: birth is an event that carries great challenges for the heart, not only because there is a change from the fetal to the neonatal circulation which imposes haemodynamic stresses on the ventricles but also because it is marked by a transition from a microgravitational to a gravitational environment, with gravity playing a key role in the maturation of the cardiovascular system (Magrini et al., 1989).

Thus it cannot be excluded that the use of this post-natal potential for *re-generative purposes* that go beyond the physiology, such as the repair of a myocardial damage, would interfere with normal cardiac development.

About the mechanism by which myocytes proliferate, the proliferation of cardiomyocytes implies their dedifferentiation and the previous cited study (Porrello et al., 2011) provided evidence of sarcomeric disassembly, but this is somewhat far from a biological point of view. Indeed, with

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the sole exception of cancer stem cells it is generally accepted that cell division does not require the cell to move backwards along its differentiation pathway. Other authors (Bersell et al., 2009) suggest that sarcomeric disassembly occurs in *differentiated* cardiomyocytes and is the process by which the cell reorganises its contractile apparatus to avoid interference with karyo- and cytokinesis. But the most likely hypothesis is that the replicating cells belong to an earlier stage of differentiation since possibly are adult cardiac stem cells committed to the myogenic lineage. There is convincing data that in vitro *c-kit*+ stem cells show an immature morphological and functional phenotype, including sarcomeric disassembly (Beltrami et al., 2003).

Finally, as it is known that the physiological processes taking place during postnatal cardiac growth lie along a continuum in time (Ahuja et al., 2007) it is reasonable to suppose that the proliferative response to amputation, in terms of intensity and duration, would also exhibit a temporal gradient. Unfortunately it is not possible to track its dynamics since none of these studies investigate multiple time-points and neglect the intermediate time-window. In future studies aimed to the characterization of this re-generative time-window valuable information might be obtained by exploring earlier time-points around the time of birth, possibly including the late prenatal period. Moreover, considering that physiological reserves are finite, to exclude that the use of these resources does not interfere with cardiac development, both the contractility and the residual proliferative potential of the heart should be evaluated carefully.

3. *What are the clinical implications of this last re-generative chance for the heart?*

As we have seen, this time-window that appears to be the last post-natal re-generative "chance" of the heart argues for a function that seems to be closely linked to the development of the cardiovascular system in a gravitational environment and, in particular, to support the new load conditions at birth. Thus it is difficult hypothesize its role in a specific clinical scenario. Assuming a clinical role for this time-window restricted to the neonatal period, it is possible to take into consideration neonatal acute myocardial infarction (AMI) as clinical scenario, but AMI is rare, usually due to congenital heart disease, paradoxical coronary artery thromboembolism or perinatal asphyxia in premature newborns with severe respiratory distress (Poonai et al., 2009), and is often extensive (Fesslova et al., 2010; Abdurrahman et al., 1999; Iannone et al., 1975) and with an extremely high mortality rate, approaching 90% (Poonai et al., 2009). This, of course, raises questions about the possibility to use the re-generative resources for reparative purposes in this context. On the other hand, if applied to the clinical setting of adult AMI, it would be more appropriate since, according to recent epidemiological data, the last few decades have witnessed a marked decrease in infarction severity, with a growing predominance of non-ST elevation AMI (NSTEMI) over transmural AMI (Roger et al., 2010). Unfortunately this time-window is almost completely closed soon after birth in mammals, therefore his only possible clinical use in adults requires the exact knowledge of the mechanisms that control its opening and closing in order to re-open it when necessary as to repair a myocardial damage. Finally, it is possible to speculate that further experiments in microgravity environment, such as prolonged space flight, can provide further information on the functioning of the post-natal re-generative time-window of the heart.

8. Concluding remarks

This text traces its origin in the lectures given to students, in the discussions held with PhD students and in some years spent in cardiovascular research, and more precisely, in hematopoietic adult stem cells. All it has been then placed in an evolutionary perspective that has helped to give the text a teleological perspective; however, being the territory of the origins and evolution of life on earth almost without scientific evidences, the text leaves wide space for the imagination of the authors.

The text was then carefully reviewed to avoid repetitive forms that relate to citations; unfortunately, in some cases, this may have led to borderline syntactic forms, so we apologize to our readers. Finally, the images, with some exceptions, are an attempt to conceptualize the evolution and the adaptive processes and, therefore, do not have a data base to which to refer, however, we believe that they can illustrate complex phenomena not yet completely known.

9. Summary

In the complex interplay between organization of the living matter, natural selection and adaptation, mammals have evolved with limited or no re-generative capabilities of the heart after birth.

The reasons for this apparent flaw is far from being understood, however, they are closely related to the concept of organization and allocation of resources in a hierarchically structured multi-cellular organism with an evolved system of transport and communication, such as the circulation of blood. In humans this flaw may not have been a problem for thousands of years until, in the twentieth century, the increase in life expectancy has given rise to diseases generally less frequent in the first three or four decades of life. Therefore, the significant increase in morbidity and mortality related to cardiovascular disease, seen mainly in Western countries in the last years, has brought to the foreground the problem of cardiac damage and of its repair. In order to develop new therapies for cardiovascular damage aimed at reawakening and, possibly, expanding the limited re-generative capabilities of the heart is necessary to reconsider the basic concept on adaptation and functional reserve allocation in complex organisms.

In this regard, the demonstration of a temporary re-generative window in the post-natal heart of higher vertebrates may provide an opportunity to investigate when, why and how the re-generative capabilities are self-limited in some organs by the genetic program.

Therefore in this chapter we will consider this window that appears to be the last post-natal re-generative "chance" of the heart and try to place it in the general context of the adaptation by assuming that its meaning is, at least in part, related to the cardiovascular adjustment in a gravitational environment.

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Chapter 27

Skeletal Muscle Regeneration for Clinical Application

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Additional information is available at the end of the chapter

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1. Introduction

Comprising nearly 50% of the human body [1] skeletal muscles compose the machinery that sets the body in movement. When well-trained, muscles have the capability to protect joints and bones from daily waste and trauma [2]. They hold an intrinsic protective mechanism against cancer formation and metastasis settling [3] and are at the same time the main energy reservoir of the body storing more than 80% of our glycogen reserve [4]. Hence, muscle tissue is associated to several functions and networks with different parts of the body. It is composed of muscle fibers, the contractile units, which are bound together by connective tissue. Most importantly, skeletal muscles display an astonishing regenerative capacity [5]. Due to resident stem cells, one week after severe trauma new myotubes are already being formed, and within 28 days after trauma muscle regeneration is almost complete [6]. These intrinsic features turn the skeletal muscle into a very interesting topic of study in regenerative medicine. Taking advantage of the regenerative potential of stem and precursor cells, skeletal muscle is constantly renewed in response to injury, damage or aging. It is this natural process that researchers are about to harness in order to help patients with many muscle diseases and diseases that causes weakness or destruction of the muscle - for instance stress urinary incontinence (SUI), muscular dystrophy. In this chapter, the focus will be on the regeneration of the skeletal muscle and especially in the case of incontinence. Urinary incontinence is the involuntary loss of urine and is a major medical problem affecting millions of people worldwide. It impairs the quality of life of patients and involves high healthcare costs. The main reason provoking SUI is the damage of the sphincter muscle due to childbirth, surgical treatments (as prostatectomy) or as an effect of aging. Current treatment encompasses behavioral training, pelvic floor exercising, drugs, medical devices and surgery. Unfortunately, all these options permit only limited recovery: short-term relief and are often accompanied with complications. The ultimate goal will be to prevent disease progression and to restore the tissue and its functions.

Stem cell therapy as a treatment for skeletal muscle diseases is becoming a reality and it represents a promising alternative for muscle regeneration and for treating SUI in a more complete and definitive manner.

In this chapter, the homeostasis and maintenance of skeletal muscle is explained in order to understand the basis behind muscle regeneration. As different types of stem cells have been demonstrated to form fibers and to develop into skeletal muscle, cell sources for a muscle cell therapy is discussed. Some of them have also been applied successfully in preclinical and clinical studies that are going to be described. Finally, we are going to highlight the parts important for the translational effort into clinics including biomaterials, cell delivery, imaging, regulatory affairs, and manufacturing.

2. Muscle homeostasis

2.1. Satellite cells are the secret of skeletal muscle regeneration

The secret of skeletal muscle staggering regenerative capacity is found in the specific components of its cell niche. The muscle tissue is composed of long and slender cells that form muscle fibers grouped in bundles (Figure 1). Adjacent to these myofibers, a heterogeneous pool of subsarcolemmal progenitor and stem cells known as muscle satellite cells (SC), respectively committed to myogenic differentiation or to self-renewal, guarantee a fast and efficient regenerative process after trauma [7]. These cells, activated by injury [8], work hierarchically to maintain the *in situ* pool of cells (Figure 1) and to reconstruct damaged tissue in less than one month by differentiating into new myotubes.

2.2. Injury and inflammation — The role of inflammation

After trauma an inflammatory infiltrate can be observed when neutrophils, macrophages, satellite cells and later myoblasts work chronologically together cleaning up damaged fibers and reconstructing new functional myotubes. Neutrophils are the first cells to arrive at the site of injury, followed by macrophages three hours after damage [6]. Through the combined action of free radicals, growth factors and chemotactic factors these inflammatory cells contribute both to injury and repair [9]. Without the neutrophil-related oxidative and proteolytic modifications of damaged tissue, phagocytosis of debris would not be possible [10]. Macrophages are the major housecleaners that remove remaining debris of fibers. Furthermore, macrophages produce proteases to lyse the sarcolemma membrane, which allows activation and proliferation of SC [11]. Dismantling of the extracellular matrix is key to SC activation, and the up-regulation of metalloproteinase is required for muscle regeneration [12]. Macrophage infiltration is also important for SC activation and proliferation by activating NF-κB via TWEAK ligand [13]. Quiescent SCs are still found between the basal membrane and sarcolemma until the third day after injury. Subsequently, they are slowly replaced by cells with large nuclei, nucleoli, and cytoplasmatic processes filled with ribonucleoprotein granules. These myoblasts display an initial exponential growth phase and after the seventh day they start to form myotubes with centrally placed nuclei and peripheral my-

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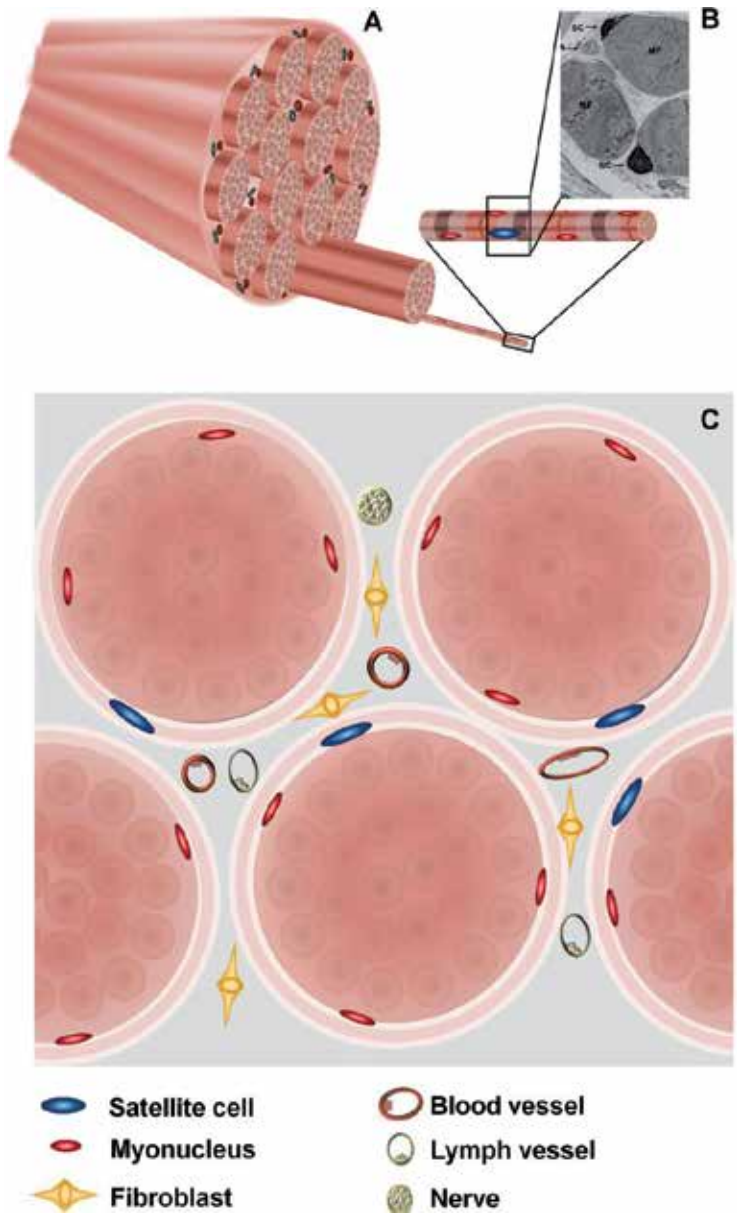

Figure 1. The muscle niche is the secret of skeletal muscle astounding regenerative capacity. Attached to bones, skeletal muscle are organs composed of skeletal muscle tissue, connective tissue, nerves and blood vessels. Each individual skeletal muscle is composed by hundreds or thousands bundles of muscle fibers that are single cylindrical muscle cells. (A) The connective tissue surrounding each muscle is called epimysium, and its projections that separate muscle bundles are called perimysium. (B) The connective tissue between single muscle fibers is called endomysium and servers as the muscle satellite cells (SCs) niche. SCs are subsarcolemmal cells that can be activated to regenerate new muscle fibers. (C) Skeletal Muscle tissue is not only formed by muscle fiber, but also by acellular matrix, cellular components, blood and lymphatic vessels and nerves. Altogether, these muscle niche components play a distinct role on muscle regeneration and on muscle progenitor cell regulation.

ofibrils. On the periphery of these newly formed myotubes a new population of subsarcolemmal quiescent cells replenishes the SC pool [6]. Finally, mature myofiber nuclei do not display mitotic figures throughout the regeneration process, demonstrating that the damaged fiber cannot heal itself without the activation of satellite cells.

2.3. The role of the muscle niche in muscle regeneration

Components of the muscle niche are important for skeletal muscle regeneration and satellite cell activation. The basal lamina is the common anatomic site of satellite cells and also contributes to cell fate. The basal lamina is rich in $\alpha 7\beta 1$ integrin which acts directly in the anchorage, adhesion and quiescence of satellite cells [14]. These integrin functions also comprise the migration and proliferation of developing myoblasts [15], the formation and integrity of neuromuscular junctions [16], as well as the binding of muscle fibers. Another integrin, VLA-4, is expressed as myotubes form and influences the alignment and fusion of myoblasts [17]. Finally, the calcium-dependent cell adhesion protein M-cadherin is a morphoregulatory molecule facilitating myoblast fusion and cell adhesion to its adjacent myofibers [18, 19].

The surrounding acellular matrix (ACM) contains a number of components that can influence the behavior and regulate the growth of muscle progenitor cells. The ACM is a source of hepatocyte [20] and fibroblast [21] growth factors, which act on the activation of satellite cells, proliferation and inhibition of differentiation. Another factor produced by the ACM is the endothelial growth factor, which promotes satellite cell activation and cell survival after injury [22]. Finally, the aged ACM is capable of impairing the regenerative potential of satellite cells and inducing fibrosis by activating the Wnt signaling pathway [23].

Fibroblasts are the main source of collagen in the muscular interstitial space [24]. They continuously promote the formation of the basal lamina during myogenesis [25] and after muscle injury proliferate hand in hand with Pax7 positive satellite cells, orchestrating the fine balance between muscle reconstruction and fibrosis formation [26]. These fibroblasts prevent premature activation and differentiation of muscle progenitor cells, thereby avoiding depletion of the pool of satellite cells. Accordingly, satellite cells are sufficient to regulate the ingrowth of fibroblasts and fibrosis formation [26]. Fibroblasts are also involved in myosin switch from fetal to adult muscle, specially promoting Myosin Heavy Chain type 1 expression (slow twitch) in several limb muscles in the fetal mouse and in the soleus in the adult muscle [27].

Circulating and locally produced soluble factors participate in the signaling pathway that regulates satellite cell activity. During exercise and stretching muscle fibers liberate hepatocyte growth factor (HGF) through nitric oxide stimulation and induce activation of satellite cells [28]. HGF can also activate satellite cells by activating the sphingolipid signaling cascade upon disruption of the laminin-integrin adhesion in the event of trauma [29]. Furthermore, the insulin-like growth factor 1 (IGF-1), a potent mitogen produced locally during muscle hypertrophy and injury, can induce activation, proliferation and differentiation of satellite cells [8, 30]. In contrast, mysotatin, a growth differentiation factor and member of the TGF-beta protein family secreted by adult skeletal muscle, is capable of inhibiting activation and self-

renewal of quiescent cells [31]. Finally, a hormone produced by the thyroid gland and responsible for inducing hypercalcemia named Calcitonin [32], has been associated with delay of satellite cell activation [33]. Together all these components and products of the muscle niche are key regulators of all the development and regeneration processes of skeletal muscle.

2.4. Satellite cells are also required for exercise related muscle turn–over

Exercise is capable of activating muscle gene transcription within seconds and these molecular responses can last for hours even after exercise cessation [34]. During endurance exercise, muscle consume large amounts of oxygen to generate energy by breaking down carbohydrates and posteriorly fat [35]. Muscle fibers are not in a smooth continuous muscle contraction during exercise, but rather act as a series of small groups of fibers contracting at the same time [36]. This occurs due to stimulation of neuromuscular junctions of terminal branches of axons whose cell body is in the anterior horn of the spinal cord. Altogether, these nerve and muscle components comprise the motor unit [37] and conduce impulses that enable sharp muscle contraction within milliseconds [38]. A signaling pathway is then activated by rapamycin kinase (mTOR) leading to hypertrophic changes in muscle mass [39]. The opposing effect is found during starvation when the AMP-activated protein kinase (AMPK) is switched on to up-regulate energy-conserving processes and ultimately induce muscle atrophy [39]. However, exercise is sufficient to increase the pool of stem cells reversing the effects of atrophy after prolonged limb immobilization [40].

After a trauma or during exercise nitric oxide is liberated and modulates the activation of satellite cells [41, 42]. Another evidence of this cell addition during exercise is the decrease of telomeres length detected in marathon runners, which correlates to their running hours [43]. Endurance exercise has been reported to stimulate the production of free radicals like nitric oxide [44], which has been shown to again induce activation of satellite cells thereby increasing muscle turn-over [28]. On the other hand, during muscle atrophy caused by limb immobilization an apoptotic decrease of myonuclei occurs [45] associated with a decrease in mitotic activity of satellite cells [46]. These findings underline the involvement of satellite cells in the regulation of muscle mass during exercise.

2.5. Markers for satellite cells

A transcriptional network controls progression of both embryonic and adult muscle stem cells [47]. Quiescent muscle embryonic progenitor cells can be identified by the co-expression of the paired-domain transcription factors Pax3 and Pax7 (Figure 2) and are maintained as a self-renewing proliferative population [48]. During embryogenesis Pax3 is required to maintain muscle progenitor cells in the somite and further induce cell migration to the required site of skeletal myogenesis [49]. Indeed the normal expression of Pax3 seems to be decisive for the development of normal muscle, and its mutation promotes malignant growth and induces tumorigenesis in alveolar rhabdomyosarcoma tumor cells [50]. However, its down-regulation is necessary for final cell commitment to myogenesis and leads to rapid and robust entry into the myogenic differentiation program [49]. The expression of transcription factor Pax7 is detectable in cells starting from the embryonic

muscle progenitor to the quiescent and activated satellite cells (Figure2). Its induction in muscle-derived stem cells induces satellite cell specification by restricting alternate developmental programs [51].

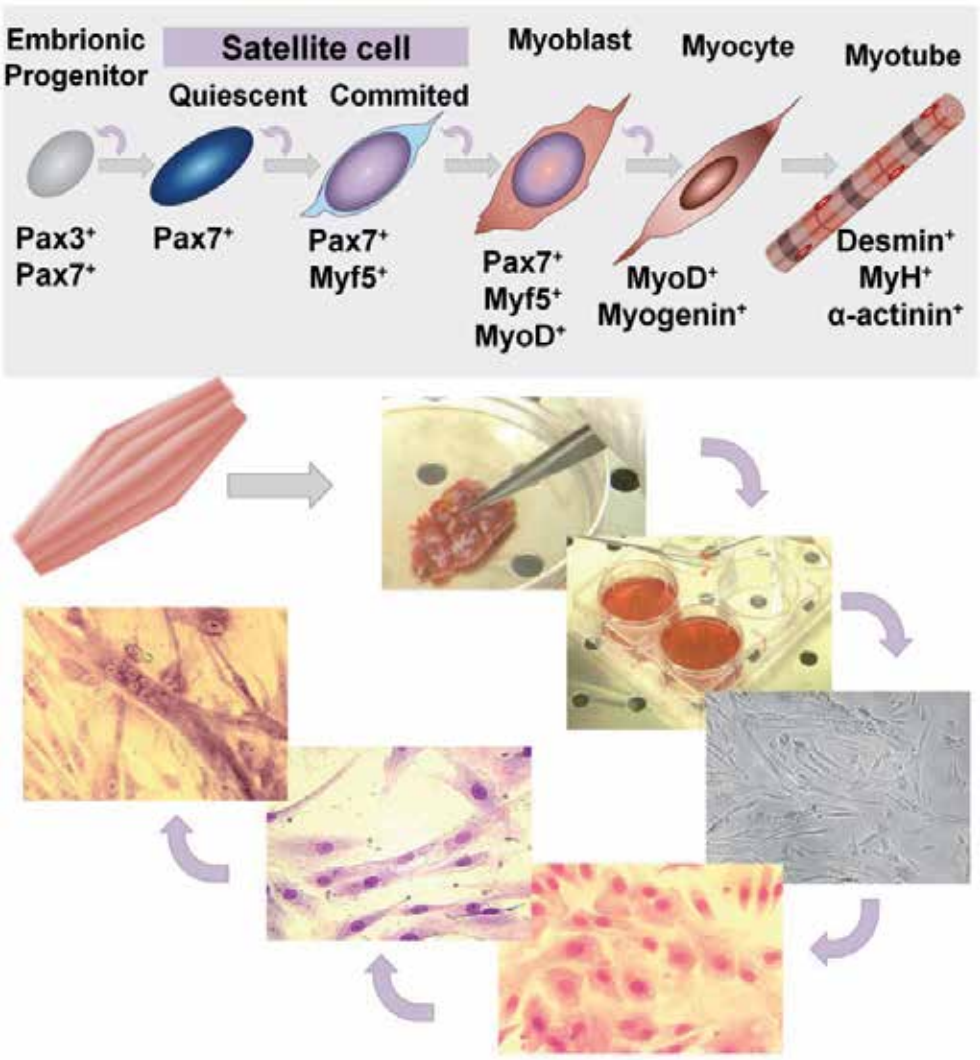

Figure 2. Myogenic cell characterization and culture. Myogenic cell lineage can be identified in each differentiation state and pursue tightly regulated proliferation and differentiation cycles. From the embryonic state until the terminal differentiation into muscle fibers an intricate network of transcription factors regulates the fate of muscle progenitor cells. These cells can be isolated from any skeletal muscle tissue, grown in culture and reimplanted into a damaged muscle to promote muscle regeneration.

Specific molecular markers have been demonstrated to distinguish between activated and quiescent SC. Quiescent satellite cells express the transcription factor Pax7, after activation in

co-expression with MyoD [52]. This dual expression is followed by a proliferative phase, down-regulation of Pax7 and terminal differentiation. If Pax3 and Pax7 down-regulation do not occur *in vitro* differentiation is blocked [53, 54]. In this context microRNAs (miRNAs) play a regulatory role conferring robustness to developmental timing by posttranscriptional repression of genetic programs of progenitor and satellite cells [55]. They allow rapid gene program transitions from proliferation to differentiation, blocking PAX3 [56] and Pax7 [57] activity in progenitor and satellite cells.

This interplay during development is required to ignite the commitment of satellite cells to the myogenic program, to activate the myogenic regulatory factors Myf-5 and MyoD and to promote terminal muscle differentiation [55] [58] [59], which are decisive to subsequent myoblast cell cycle progression or exit into differentiation. Through the action of the myogenic regulatory factors (MRFs), Myf5 and MyoD, the muscle progenitor cells ($Pax3^+$) and quiescent satellite cells ($Pax3^+/Pax7^+$) become muscle lineage committed and activated myoblasts [60]. They express *Myf5* and *Mrf4* and rapidly give rise to $Desmin^+$ cells, whose differentiation is regulated by myogenin, MyoD and MRF4 [61]. Completing these regulatory features, MyoD is also a main player in the intricate epigenetic cascade that controls skeletal myogenesis [62].

3. Muscle regeneration

3.1. Cell sources for skeletal cell therapy

Several types of cell populations have been identified as potentially efficient in muscle regeneration, especially in cell therapy. They are able to self-renew, proliferate and form muscle fibers. Among these cells some are muscle derived and some are from other origin.

3.2. Muscle–derived cells

Muscle satellite cells, which are squeezed between the plasma membrane and basement membrane of muscle fibers, are the natural source of muscle regeneration during homeostasis or after injury in postnatal stages [63]. They are specifically expressing the paired box transcription factor pax7 [51] and have been shown to be efficient in the muscle regeneration process. One study illustrated that as little as seven satellite cells were able to generate more than 100 muscle fibers in irradiated muscle [64]. Though, satellite cells isolated from different muscles are not equivalent: they produce muscle fibers with variable contractile abilities depending on the muscle of origin [65]. This can be explained by the fact that a satellite cell pool does not seem to consist of a homogeneous population of cells [66-70]. Once activated, satellite cells are triggered toward proliferation and differentiation by giving rise to muscle precursor cells that fuse and form skeletal muscle fibers [71]. The two techniques used to isolate muscle precursor cells are selection of single fibers that are cultured or mechanical processing of muscle biopsies and enzymatic treatment with a mixture of collagenase and dispase [64, 72-74]. The first method is claimed to be less aggressive and to better preserve the cells.

Another type of cells is isolated from muscle biopsies through a series of preplating stages. These cells are also recognized to have a myogenic profile and are capable to fuse and form skeletal muscles fibers. They are known as muscle-derived stem cells (MDSC) with characteristics of non-committed progenitor cells [75, 76] and are most probably originating from blood vessel walls [77]. Similarly, other cells types isolated from the muscle compartment such as mesoangioblasts and pericytes are involved in the muscle regeneration but are of non-myogenic origin. These are vessel-associated progenitors, not expressing myogenic markers such as Myf5 and MyoD even though they can differentiate to myotubes and fuse to form fibers [78-80]. More cell types with non-myogenic profile are found in the skeletal muscle and have recently been demonstrated to form fibers. Hence, skeletal myogenic precursors or muscle stem cells sorted by FACS are capable to reconstitute fibers in rodent models [72, 81]. The first type of cells is characterized by expression of β1-integrin (adhesion protein) and CXCR4 (SDF-1 receptor), the second type by α7-integrin (adhesion protein) and CD34 markers. Side populations are also isolated from muscle tissues and are expressing specific surface markers [82]. They are distinct from satellite cells and have been used successfully in muscle regeneration in rodent models [83-87]. Surprisingly, more types of cells of the skeletal muscle tissue can contribute to muscle regeneration. In fact, recently, a new type of myogenic cells, localized in the area of the interstitium between muscle fibers, has been characterized and is known as PW1-interstitial cells (PICs). They are characterized as positive for cell stress mediator PW1 but negative for Pax7; though they possess myogenic profile *in vitro* and lead to muscle regeneration *in vivo*, which includes the generation of satellite cells [88]. Hence, various types of cells isolated from skeletal tissue either mechanically or by flow cytometry are capable to regenerate muscle. In addition to the muscle there are more sources of stem/precursor cells isolated from other compartments.

3.3. Sources outside of the skeletal muscle compartment

Mesenchymal stem cells (MSC) are procured from bone marrow biopsies and are multipotent stem cells that give rise also to skeletal muscle fibers and participate to restore the satellite cell niche [89]. These cells are well characterized and involved in many different applications due to their multipotency as it is the case for adipose-derived stem cells (ADSC). The latter are easily harvested by liposuction, cultured *in vitro* and injected to restore muscle in the case of SUI [90, 91]. Embryonic stem cells, induced pluripotent stem cells and umbilical cord blood have been demonstrated to be good alternatives for skeletal muscle regeneration [74, 92, 93]. However, precaution should be taken when these types of cells are considered for further development in clinics, as different types of viruses are used during the process of myogenic induction. In addition, there are still potential tumorigenicity issues with this sort of cells that need to be solved before further clinical application.

Hence, the sources of stem/progenitor cells for skeletal muscle regeneration are large. Though, several important factors need to be considered when choosing the optimal source for treating patients. Autologous cell therapy avoids immunogenic reaction and therefore complications after the implantation procedure. Therefore, autologous satellite/muscle precursor cells are advantageous for muscle regeneration. They are committed to muscle restoration and

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therefore the most convenient cells for applications in cell therapy. Their dedication to one lineage offers an advantage over other, previously discussed sources, which are multipotent and hence differentiate also into non-muscular tissue cells. Furthermore, satellite / muscle precursor cells can be isolated in a simple procedure and are easily expanded in a GMP facility. They produce enough cells to be injected after 2-3 weeks, which is much faster than the 5 - 6 weeks required for muscle derived stem cells. For allogenic application, mesenchymal and adipose derived stem cells represent valid alternatives when satellite cells cannot be extracted from the skeletal muscle.

4. Preclinical and clinical studies

Several animal studies have been addressing the problem of stress urinary incontinence (SUI) and different strategies have been tested to restore continence, either by applying pharmacological therapies, bulking agents, sling surgical procedures or cell-based therapies [94]. Until now, the first three strategies mentioned above are commonly applied in clinics. However, the outcomes are associated with adverse events and limited effectiveness in middle and long terms [95-97]. Therefore, cell-based therapies are aiming to bring new solutions to the treatment of SUI. Numerous preclinical studies have been implementing stem/progenitor cell injections for restoration of muscle contraction in SUI. Animal models that mimic SUI are crucial for the understanding of effects and benefits of the different therapies options.

4.1. Animal model

To stimulate SUI in animals, various methods were applied. The goal is to injure one or several aspects of the urinary continence mechanisms to provoke incontinence as found in patients. The methods comprise the compression of the muscular and neurological system involved in continence by vaginal distension [98, 99], crush of the pudental nerve [100], damaging of anatomic supports such as fascia and pubourethral ligament [101-104] or destruction of intrinsic urethra by periurethral cauterization, urethral sphincterectomy, pudendal nerve transection and botulinum toxin periurethral injection [105-112]. One has to note that vaginal distension or pudendal nerve injury are relatively limited models due to the fact that the injury is naturally recovered after 2 weeks and thereby does not mimic an irreversible SUI. Eberli et al. have been describing a large animal model for SUI that was followed for 6 months. In this study, the sphincter muscle of dogs has been irreversibly damaged by surgically removing part of it. During the follow-up, the dogs were permanently affected by this procedure with long term decrease in sphincter pressures [107].

4.2. Preclinical studies

Rats are the preferred animal models for studying safety and efficacy of several cell types for treatment of SUI (table.1).

Cell type	Animal model	SUI model	Injection Target organ	Time point Weeks	Reference	Year
MDSC	SCID mice / Rats	Cryoinjury bladder	Bladder	1 to 4	Huard et al.	2002
MPC	Mice	Noxetin	Urethral	2 to 4	Yiou et al.	2002
MDSC	Rats	Sciatic nerve section	Urethral	4	Lee et al.	2002
MDPC	Rats	Sciatic nerve section	Urethral	2	Cannon et al.	2003
MPC	Rats	Electrocoagulation	Urethral	0.7 to 4	Yiou et al.	2003
MDSC	Rats	Pudendal nerve section	Urethral	12	Lee et al.	2004
MDC	Rats	Electrocauterization	Urethral	2 to 6	Chermansky et al.	2004
MDSC	Rats	Sciatic nerve section	Urethral sling	2	Cannon et al.	2005
MDC fibroblasts	Rats	Sciatic nerve section	Urethral	4	Kwon et al.	2006
Myoblasts	Rats	Cryoinjury / noxecin	Urethral	1 to 6	Praud et al.	2007
Myofibers	Pigs	Urethral injury	Myofiber implantation	4	Lecoeur et al.	2007
MDSC_FACS sorted	Rats	Nerve transection / sphincter injury	Urethral	4 to 12	Hoshi et al.	2008
ADSC	Rats	Vaginal dilatation	Urethral	4	Lin et al.	2010
BMSC	Rats	Sciatic nerve section	Urethral sling	4 to 12	Zou et al.	2010
BMSC	Rats	urethrolysis / cardiotoxin	Urethral	13	Kinebuchi et al.	2010
MDSC	Rats	Pudendal nerve section	Urethral	1 to 4	Xu et al.	2010
Myoblasts / ADSC	Rats	Vaginal dilatation	Urethral	4	Fu et al.	2010
UCBSC	Rats	Electrocauterization	Urethral	2 to 4	Lim et al.	2010
BMSC	Rats	Pudendal nerve section	Urethral	4 to 8	Corcos et al.	2011
BMSC	Rats	Pudendal nerve section	Urethral	4	Kim et al.	2011
BMSC	Rabbits	Cryoinjury	Urethral	1 to 2	Imamura et al.	2011
ADSC	Rats	Pudendal nerve section	Periurethral	3 to 4	Wu et al.	2011
ADSC	Rats	Pelvic nerve section	Periurethral	2 to 4	Watanabe et al.	2011
ADSC	Rats	Pudendal nerve section	Periurethral	8	Zhao et al.	2011
MPC	Dogs	Urethral sphincterecomy	Periurethral	24	Eberli et al.	2012
BMSC	Rats	Pudendal, other nerves section	Urethra and baldder neck	1 to 8	Du et al.	2012
MSC	Rats	Vaginal dilatation	intravenously	0.6 to 1.4	Cruz et al.	2012

Abbreviations: ADSC, adipose-derived stem cells; BMSC, bone-marrow mesenchymal stem cells; MDC, muscle-derived cells; MDSC, muscle-derived stem cells; MPC, muscle precursor cells; MSC, mesenchymal stem cells; UCBSC, umbilical cord blood stem cells;

Table 1. Animal studies for treating stress urinary incontinence based on cell therapy.

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Muscle derived cells were the first cells to be used for urethral regeneration and to demonstrate that cell therapy might represent an option for the treatment of SUI. Hence, MDSC, myoblasts, MPCs or muscle fibers injected around the injured area were surviving, participating in fiber formation and re-establishing muscle contractility [113-118]. In rat models, it was shown that the injected MDSC – isolated by preplating procedures or FACS-sorted - were participating actively in muscle regeneration for up to 3 months [119, 120]. Interestingly, in a dog study, a rare large animal model for SUI, transplanted MPCs were efficiently restoring the sphincter pressure to 80% of normal values during a half year follow-up period [121]. Concerning the speed of regeneration, Cannon et al. noticed 87% recovery after only 2 weeks post-injection and Chermansky et al. a full recovery after 4 weeks with myoblast and 6 weeks with MDSC [113, 116, 122]. Hence, muscle derived cells are able to incorporate the urethral structure and help recovering continence by reconstructing new fibers and connections with the surrounding cells – nerves, Schwan cells, vessels etc. [119, 121]. However, they are not the only kind of cells facilitating this cell therapy. Bone marrow derived mesenchymal stem cells, adipose-derived stem cells, umbilical cord blood stem cells (UCBSC) have been proven to also restore continence in animal SUI models [123-135]. In fact, different studied showed that these cells are contributing to the formation of fibers and contractile muscles which permit to control urinary leakage. When compared to common procedures for treatment of SUI such as the injection of collagen bulking agent, ADSC cell therapy provided better results [132]. Moreover, the association of cells with biomaterials seems to enable further improvements as observed with BMSC and sling systems or MDSC with fibrin glue [135, 136]. To improve this cell therapy model, Zhao et al. took account of the fact that muscle regeneration is an interaction process involving paracrine factors produced by surroundings cells and combined with ADSC the nerve growth factors. This method stimulated muscle regeneration and demonstrated that combining different cell types could be beneficial for muscle restoration in SUI.

4.3. Clinical trials

Several clinical trials applying cell therapy in SUI have been conducted in the last decade (table.2).

Safety and efficacy of this strategy have been proven with several types of cells in women and men. As the procedures differ from one trial to another straight comparisons between them are difficult. Additionally, the recruited patients suffered from different levels of SUI - from moderate to severe- and some studies even included complementary therapies such as electrical stimulation or exercises to improve the results. Nevertheless, the results were highly promising and have demonstrated that a solution for patients suffering of SUI is within reach. Surprisingly, the first cells to be used in a clinical trial for SUI was not muscle derived cells but chondrocytes isolated from auricular cartilage that were expanded in culture before injection in female patients. Out of 32 treated patients 26 had an improved situation and 50% were continent after one year [137]. This was the only clinical study using chondrocytes for voiding dysfunction. Muscle derived cells (myoblasts and MDSC) are the most frequently used cells in muscle regeneration for SUI in both genders. Myoblasts have been used in many trials and were injected in or around the external urethral sphincter. The efficiency was stated to be

Cell type	Source	Patients / n	Injection	Target organ	Delivery biomaterial	Time point Months	Outcomes Measurements	Reference	Year
Chrondrocytes	autologous	Women / 32	Trans/peri-urethral	Bladder neck	calcium alginate	12	81% improved 50% continent	Bent et al.	2001
Myoblasts and fibroblasts	autologous	Woman / 123	Transurethral	Urethra	autologous serum collagen	12	79% continent 13% improved	Mitterberger et al.	2007
Myoblasts and fibroblasts	autologous	men / 63	Transurethral	Urethra	autologous serum collagen	12	65% continent 17% improved	Mitterberger et al.	2008
MDSC	autologous	Women / 8	Trans/peri-urethral	External sphincter	none	3 to 24	63% improved 13% continent	Carr et al.	2008
Myoblasts	autologous	Boys / 7 Girls / 1	Transurethral	External sphincter	none autologous serum	12 to 18	88% improved 38% continent	Kajbafzadeh et al.	2008
UCBSC	allogenic	Women / 39	Transurethral	Submucosa	none	12	72% improved 9% continent	Lee et al.	2010
Myoblasts	autologous	Women / 12	Transurethral	External sphincter	none	12	50% improved 25% continent	Sèbe et al.	2011
MDC	autologous	Men / 222	Transurethral	External sphincter	none	at least 12	12% continent 42% improved 46% no efficacy	Gerullis et al.	2012
ADSC	autologous	Men / 3	Transurethral	External Urethra sphincter Submucosa space	none	6	improvement	Yamamoto et al.	2012
Myoblasts	autologous	Women / 38	Intrasphincteric	External Urethra sphincter	none	1.5	78.4% improved 13.5% cured 8.1% unchanged	Blagange et al.	2012

Abbreviations: MDC, muscle-derived cells; MDSC, muscle-derived stem cells; UCBSC, umbilical cord blood stem cells.

Table 2. Clinical trials for treating stress urinary incontinence based on cell therapy.

between 50% and 88% in a follow-up of 12 months [138-141]. Even if the designs between studies differ, the combination of cell therapy with electrical stimulation or/and pelvic floor exercises may explain the variation between the values. In fact, a cell therapy with the application of myoblasts alone seems to provide a 50% improvement [139, 141], improving to 78.4% if electrical stimulation is added [138] and reaching 88% with pelvic floor exercises [140]. This approximate comparison can encourage future clinical studies to combine other therapies and exercises with cell therapies in order to optimize the outcome. Myoblasts have also been combined with fibroblasts mixed in a collagen solution. The results were impressive: 79% of treated women and 65% of the men reached continence [142, 143]. As a Lancet publication of this group was retracted, these results should be handled with precaution and should be confirmed by other groups [144]. Other muscle-derived cells have been injected in patients with SUI. Since 2008, MDSC have been applied in several clinical trials [145, 146] with improvement rates of 53% after 1-year follow-up with 10 million cells injected, 63% with 20 million and 67% with 50 million. The efficiency of the cell therapies seems to be dose-dependent. This was confirmed by Kaufman et al. in a 6-month dose escalating study, where improvements increased with the dose of injected cells. The best results were obtained with 200 million MDSC injected [146]. Interestingly, no serious adverse effects were observed even when numbers of UCBSC as high as 400 million were applied [147]. In this latter case, 72% of

39 patients were more than 50% satisfied 12 months post-injection. This represents another type of cells that is suitable for SUI treatment. Although the cell therapy with UCBSC is allogenic, no immunosuppressive effects were observed during this cure. As a source of multipotent stem cells, ADSC were trusted in recovering the contractility of the sphincter muscle in patients [148]. Certainly, the encouraging preclinical studies enabled transplantation of ADSC in patients suffering from SUI. However, only 3 patients were treated so far. Peri-urethral injection of ADSC seems to be safe and showed improvement of the sphincter contraction after 6 months follow-up. The use of total nucleated cells associates with lysates seems to be another good option for treating SUI. This type of cells significantly helped all treated patients in the study: 100% noticed improvement in their situation and 88% reached complete continence after 6 months. Hence, these clinical trials show that different sources of cells were able to improve the continence level of patients suffering from SUI.

5. Non–biological translational work

The ability to regenerate muscle tissue from patient derived cells would have profound impact on many human diseases. Cell therapy is within reach as a novel treatment option for incontinence, reflux, vocal cord dysfunction and other muscle-related pathologies. However, the carrier used for cell delivery and the techniques used to inject the cells are still being optimized.

5.1. Cell delivery

It has been demonstrated for more than a decade that cells injected in a saline solution carrier are able to ectopically form contractile muscle [149]. However, further studies have reported very poor cell survival rates (5-20%) associated with myogenic cell implantation without embedding into protein based carriers that support cell settling into their new niche [150, 151].

Species-specific cues play an important role in cell affinity to carriers. A previous study demonstrated advantages using collagen rather than matrigel coated dishes, boosting cell growth and differentiation potential [73]. In contrast, another study with porcine satellite cells demonstrated cell preference to matrigel coated dishes and growth decrease on collagen layers[152]. Moreover, three-dimensional (3D) matrigel coated PLGA (poly lactic-co-glycolic acid) scaffolds were capable of improving cell survival when compared to direct cell injection [153]. However, the same study failed to demonstrate a comparative improvement of matrigel coated PLGA with other cell carriers. Furthermore, matrigel has not presented advantage *in vivo a*s a carrier for myogenic cells when compared to hyaluronic acid-photoinitiator (HA-PI) complex. It rather downgraded the quality of muscle structure and decreased the total number of new myofibers after cell injection [154].

Collagen is a main component of the natural extracellular matrix of skeletal muscle, it is therefore expected that satellite cells would have their functionality up-scaled in a collagen rich environment [155]. Combined with electrical stimulation collagen induces three-dimensional expansion of muscle precursor cells *in vitro* and in syngeneic recipient muscle [156]. Cell cycle analyses of engrafts implanted into a 3D collagen sponge highlighted the increment of

cell fractions in proliferating phases, with 80% of cell survival [157]. In addition, the use of parallel aligned collagen nanofibers yielded good proliferation and enabled the generation of aligned cell layers [158]. Finally, grafts of myoblasts seeded into three-dimensional collagen scaffolds and implanted into injured sites in mice demonstrated improvement in muscle healing, innervation and vascularization [159]. Altogether these recent studies confirm that collagen is a very promising matrix for satellite cell ingrowth and an ideal carrier for the transplantation of myogenic cells.

5.2. Imaging techniques for guided cell implantation in vivo

The success of cell transplantation into a specific site *in vivo* is directly dependent of 3 key points: cell source, cell carrier and injection technique. The first two were previously discussed in this chapter. We dedicate this section to the discussion of injection techniques used so far to inject myogenic cells into a specific injury site. The application of myogenic cells was already used for the treatment of male and female patients suffering from urinary incontinence, the involuntary loss of urine that represents a hygienic and social problem [160]. Transurethral ultrasound guided injections of autologous cells isolated from limb skeletal muscle biopsies were so far the method of choice [161, 162]. This method is also standard for the injection of bulking agents like collagen in the clinical practice [163]. Finally, ultrasound guidance was also used to monitor percutaneous trans-coronary-venous transplantation of autologous myoblasts in infarcted myocardium [164, 165].

Recently magnetic resonance imaging (MRI) has gained attention as a useful tool for guidance during injection of drugs and potentially of cells [166]. Pulsed focused ultrasound is a new ultrasound technique that associated with magnetic resonance guidance was recently suggested as a new imaging modality that may be utilized to target cellular therapies by increasing homing to areas of pathology [167]. It has also been demonstrated to increase drug uptake into a specific target in the prostate [168] and brain [169]. This same technique has been shown to facilitate the delivery of neural stem cells into a specific site in the brain [170]. Overall, the most successful deliveries of myogenic cells have been done either operatively in 3D scaffolds or in collagen carrier that facilitates cell settling into the new cell niche. Ultrasonography is still the most adaptable and widely used imaging technique allowing visualization of the injury zone and real time needle guidance. However, new approaches combining MRI and ultrasonographic pulses are very promising methods that need to be further studied and adapted for cell injection in different anatomic sites. Moreover, MRI is used in tracking stem cells after injection [171, 172]. In fact, it is important not only to inject the cells at the right place but also to ensure that cells are not migrating to other parts and pursuing their role in regenerating the tissue of interest. Additionally, developments in MRI technology, especially in scanning technics, offer the possibility to follow the differentiation process of injected MPCs and their fate in making fibers [173].

5.3. Regulation and guidelines

The application of cell-based therapies is not only advancing scientifically but also regulations are adapting and including the new scientific discoveries for clinical use. The relevant health

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agencies all around the world are creating committees that are modifying the regulations in order to take account of these new categories of products that are cell-based. Stem cell based therapies are part of advanced therapies, which are therapies based on genes, or cells, or tissues [174]. Concerning this emerging branch of medicinal products, the regulations are new and still in development. They have their own classification, distinct from chemical and biologic drugs, transplantation organs and medical devices. Though, they can be sometimes included in these categories. In Europe, the European Medicines Agency (EMA) is in charge of improving the standards and reviewing the applications for stem cell based therapies, which are part of Advanced Therapy Medicinal Products (ATMP), and they are found in regulation (EC) N° 1394/2007 [175]. The Committee for Advances Therapies (CAT) is the body responsible within EMA of this new field of science and its approval for marketing. The goals are to protect the patient from contaminated tissues/cells, to avoid the inappropriate handling of tissues/cells and to guaranty safety and efficacy of therapies. The documents are providing a regulatory framework that is coherent with existing ones, specific to biological and chemical entities for instance. Hence, before starting any clinical trial on human, several requirements are to be fulfilled. The cell-based product needs to be grounded on a sound and solid scientific work that is confirmed in pre-clinical studies, which show its quality, safety and efficacy. During this preparation phase, CAT is available for giving advice in preparing all the relevant files for obtaining clinical trials authorization or latter for marketing authorization. Guidelines are specifying aspects of pharmacovigilance, risk management planning, monitoring, labeling, safety, efficacy follow-up and traceability. The submission process should comply with these requirements in order to receive the green light for starting clinical trials or entering the market. During product development and clinical investigations guidelines have also been adapted by CAT for stem-cell based therapies for specifications on Good Manufacturing Practice (GMP) and Good Clinical Practice (GLP) [176]. In the US, the Office of Cellular, Tissue, and Gene Therapies (OCTGT) - part of the Center for Biologics Evaluation and Research (CBER) in FDA- is responsible of the cellular therapies products [177]. They are regulated by human cells, tissues, and cellular and tissue-based products (HCT/Ps) under the authority of Section 361 of the Public Health Services (PHS) Act as well as Title 21 of the Code of Federal Regulations (CFR) part 1271 [178]. The OCTGT are making sure that the cell-based products meet safety, purity, potency and effectiveness qualifications. EMA and FDA are collaborating closely together in the Advanced Therapies Medicinal Product cluster. The development of regulatory frameworks is not equal in all countries and is independent from a state to another state. However, at the international level, regulatory agencies are working together in sharing and harmonizing the regulatory frameworks for cellular therapy products through the International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use (ICH), the Pan-American health Organization (PAHO), WHO and Asia-Pacific Economic Cooperation (APEC). This global interaction facilitates the development of the cellular therapy field and prepares in bringing the products to the markets. As the experience is right now limited in this field, this discussion panels permits to cover the different applications and cases among the countries and therefore increase the knowledge levels among the participants and the regulatory boards. In addition, it creates convergence in the develop-

ment of the regulations and guidelines concerning different aspects: manufacturing, quality assurance, quality control and pre-clinical studies [179].

Therefore, the regulations and guidelines have been reviewed and adapted for some of them in order to be applied in the field of cell therapy. This paves the road for regenerating the sphincter muscle by using stem cells.

5.4. Production of cell–therapies

Besides, chemical drugs, medical devices and biotechnology drugs, advanced therapies are developed and offer tailored solutions for patients. These therapies are based on genes, cells or tissues.

Cell therapy for skeletal muscle is one of many therapies that are in translational phase and can be applied in near future on treating patients. As it is involving individuals' health and the cell product is delivered to human, safety concerns are raised. In fact, cell therapy product – as an investigational or marketed one- needs to meet requirements as any medicinal product or medical device. The goal is to deliver a consistent, safe, good quality and well-defined product. Therefore, Good Manufacturing Practice (GMP) is requested for the development of cell-based product, or its production for the market, and it consists on guidelines and regulations that advertise quality principles for manufacturing biological products. These rules are covering all the processes from the biopsy up to the final product. It involves several aspects:

Quality management, buildings and facilities, the equipment, the personnel, the documentation, the materials management, the processes in production, the monitoring, the packaging and labeling, the storage and distribution, the laboratory controls.

Advanced therapies are new technology. Hence, protocols, guidelines and regulations that are used for existing medicinal product cannot be transposed literally for cell therapies and need adaptations. However, the goals stay the same: safety, quality and efficacy.

5.5. Manufacturing process

In cell therapy, the starting material represents a critical part that takes account of donor eligibility criteria including age, tissue quality, source accessibility and viral testing. For skeletal muscle cell therapy, as described above, the sources are multiple and the efficiency of most of them is good in regenerating muscle in the case of SUI.

As soon as the biopsy is received in the manufacturing site, the GMP requirements have to be followed. Hence, quality management should be applied at all production steps: processing, testing, release, storage and transport.

Manufacturing cell product necessities safe and certified raw materials and components for cell culture and preparation. In addition, upon reception to the GMP facility, the materials need to be tested in-house regarding quality and safety. Only then, the products can be released and accepted into the production area by the responsible for quality in the facility. It is highly recommended by the regulations to use supplements – as cytokines and growth factors- from human origin and therefore some adaptations are needed in the production protocols coming

from the research laboratories. One of the major problems in the cell culture is to replace the fetal bovine serum (FBS). Most of the protocols are still based on this animal derived product. Recently, some efforts have been made to work with xeno-free medium by replacing FBS with human serum and platelet lysate [180]. In the case of MPCs, one of the major sources of cells for muscle cell therapy as described above, pooled human platelet lysate was demonstrated to be a good alternative to FBS [181]. Other factors are important and must be controlled as cell seeding, growth rate, differentiation process, markers expression, potency of the cells in making contractile fibers. The protocols for each step - from receiving the biopsy up to the final product -must be standardized and approved by local authorities before starting clinical trials. Standardization means that clear and details protocols should be written and followed without deviation or modifications. Quality controls are done not only for starting materials but also at critical steps in production. Quality is a key parameter that applies to all levels of the cell therapy production: building and facilities environments, equipment, production, labeling, storage and distribution. The quality unit performs all the controls to show the purity of the products, the cleanness of the environment, the maintenance of the equipment and the respect of the specifications set for obtaining a safe, effective and potent cell product. In muscle cell therapy, the cell population should have a pure or a very high percentage of cells expressing markers of skeletal cell as described above.

All the stages and elements related to the GMP facility or the production process should be documents to insure traceability of every single action. The documents should be prepared, reviewed, approved and distributed as specified in established and written procedures. All these demanding steps require qualified personnel, well-trained in working in GMP facilities. It includes good sanitation and health habits and the right skills to accomplish the work with products for cell therapy. Finally, internal and external audits are conducted regularly to verify the respect of the GMP regulations and guidelines as validated by the GMP facility and the authorities.

6. Outlook: Concerns and improvements

6.1. Effects of age

A decline of approximate 30% in muscle strength and 40% in muscle volume occurs between the second and seventh decades of life [182]. Also the total number of MPCs and their proliferation potential in culture gradually decrease in an age-dependent manner [183] due to apoptosis [184]. Additionally cell fate is tightly defined by the interactions with the microenvironment and the host age is of key importance, as the stem cell regenerative capacity reduces in aged niches [185]. We have reported that although human MPCs can be successfully isolated and grown from patients of all ages and genders (figure 3), both elderly and male donors provide unstable and slower growing cells *in vitro* with decreased contractile output *in vivo* [186]. Hence, a combination of stem cell and gene therapy might be needed in older patients [187, 188].

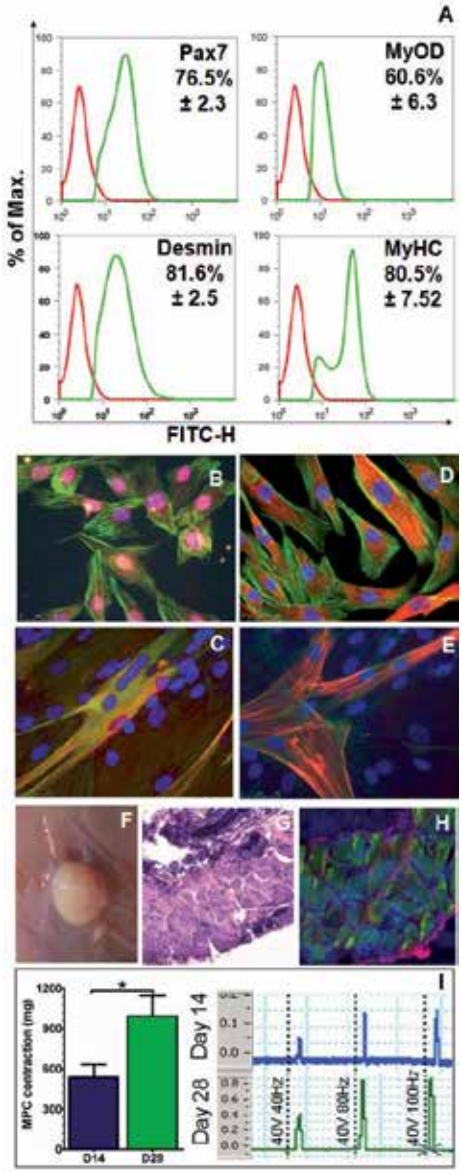

Figure 3. Muscle progenitor cells identification *in vitro* and muscle formation after transplantation *in vivo*. Myogenic cells isolated from the *Rectus abdominis* of patients undergoing abdominal surgery, grown in culture and characterized by FACS, immunohistochemistry *in vitro*. Tissue formation was evaluated *in vivo* by Hematoxilin and Eosin staining and immunohistochemistry. Function was assessed by electromyography. A: FACS analyses of cells in P2 expressing Pax 7, MyOD, desmin and upon differentiation induction Myosin Heavy Chain (MyHC). An IgG Isotype control (red curve) was used to determine the background, whereas positive cells are plotted as a green curve. Immunocytochemistry of cells in culture expressing, MyOD (B), MyHC (C), desmin (D), sarcomeric α-actinin (E) (green -Phalloidin 488, blue – DAPI, red - mM anti-IgG Cy3). Muscle cells injected subcutaneously in nude-mice revealed muscle formation *in vivo* (F, G, H) and contraction upon eletrical stimulation (I). HE stained (G) and labelled with sarcomeric α-actinin-Cy3 and PKH67 (H). Muscle function significantly improved over time (I), with contraction strength still increasing after 4 weeks.*p=0.015

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6.2. Overcoming pitfalls by reactivating muscle metabolism, tissue vascularization and innervation

In the context of muscle reconstruction, gene therapy is not aimed at rectifying a genetic mutation, but at boosting the myogenic potential and ultimately the muscle functionality of the injected autologous muscle cells. Two key factors have been demonstrated to improve the quality of satellite cells for transplantation: a better vascularization [189] and endurance exercise [190]. We have previously described that an angiogenic modification of muscle precursors can overcame some of the limitations of aged muscle cells [189]. For future application expanding the knowledge produced on this study and therapeutically combining it with the intrinsic adaptation effects of endurance exercise would be of major interest. In this context, studies using muscle-specific PGC-1α transgenic animals demonstrated that ectopic expression of PGC-1α in muscle seems sufficient to evoke a trained phenotype avoiding muscle atrophy [191]. Upon activation, PGC-1α in turn controls many, if not all of the adaptations of skeletal muscle to endurance exercise [192]. Hereafter, PGC-1α muscle-specific transgenic animals exhibit high endurance, oxidative muscle fibers, an increase in mitochondrial biogenesis and oxidative metabolism, augmented muscle capillarization and a remodeling of the neuromuscular junction [193, 194].

Although innervation of the newly implanted tissue is also essential to engineer a functional muscle tissue there is few approaches that could effectively promote nerve ingrowth after transplantation. Some studies described a spontaneous nerve ingrowth from the neighbor tissues into the newly transplanted sites [195, 196], but non-invasive methods to induce nerve ingrowth after newly formed muscle engrafts are still to be investigated. We have recently proposed that magnetic stimulation supports regeneration of injured muscle with activating resident stem cells or supporting integration of newly implanted myoblasts [197, 198]. Exposition of injured limb and co-cultures of muscle cells and neurons to magnetic fields was sufficient to trigger synapses, induce acetylcholine receptors clustering and cause typical muscular metabolic adaptations verified during endurance exercise [197]. Notwithstanding, magnetic stimulation mimicked the effects of exercise inducing PGC-1α up-regulation, induces myogenic cells differentiation and increases nerve fibers and acetylcholine receptor clustering after cell transplantation [198]. New efforts in establishing functional innervation, proper vascular network and the development of a high endurance resistance muscle are going to be the three main pillars supporting future translational studies and bringing myogenic cell transplantation from bench to bedside.

7. Conclusion

Regeneration of skeletal muscle for SUI is becoming a reality and the cell therapy may soon be available to patients. Tremendous progresses have been made to understand the science behind the natural process of skeletal muscle regeneration that involves primarily satellite cells and their progenitors, MPCs. In addition, these cells are now well-characterized with several markers at different stages of proliferation and differentiation. They are also interacting

actively with their environment, which is composed of different types of cells. These neighboring cells have a significant influence on the environment and on stimulating the factors that trigger satellite cells renewal, proliferation and differentiation into myofibers. The process is complicated and involves cocktails of factors and cells. However, the interaction between these parameters is better-understood and applied in research and preclinical studies to ameliorate lack of early vascularization and innervation. In clinical trials, the first results are promising and many patients with SUI were treated successfully. The cell sources are important for a successful skeletal muscle cell therapy but they must be accompanied by a set of tools to ensure the safety and the quality of the process: culture medium, biomaterials, imaging for injection and follow-up. The advances have been made and the solutions are ready, even at the regulatory level. Although, there is not yet a standardized cell therapy for SUI, the solutions and the first results are encouraging. The cell therapy for SUI treatment will be certainly part of the choices that urologists will adopt very soon in hospitals.

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Chapter 28

Delivery Systems and Role of Growth Factors for Alveolar Bone Regeneration in Dentistry

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Additional information is available at the end of the chapter

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1. Introduction

Growth factors (GFs) have been investigated for the purpose of alveolar bone regeneration in periodontal, reconstructive and pre-prosthetic surgery, often with a view to rehabilitation with dental implants. Results are promising, and research is currently focusing on developing an effective delivery system capable of ensuring a controlled and localized GF release and activity. In fact, one of the main issues relating to GFs concerns how to control their effects over time so as to guarantee their effective action in the various phases of bone healing. This chapter provides a review of the literature on GFs used for bone regeneration in dentistry, emphasizing the most recent developments relating to local delivery systems.

2. Mechanism of action of growth factors (GFs)

Growth factors (GFs) are protein molecules that have a role in controlling biological processes, such as cell growth, proliferation, differentiation and repair. GFs cannot pass through a cell's membrane; they must bind to high-affinity cell receptors in order to take effect. Many GFs stimulate several cell populations, while others are less versatile and specific to a particular cell line.

In dentistry, numerous GFs have been investigated in terms of their effect on hard and soft tissue healing and regeneration.

Whatever the tissue involved, the healing process always involves a series of molecular, biochemical and cellular events that can be grouped into three overlapping phases: inflammation, proliferation, and remodeling.

Inflammation begins spontaneously straight an after injury has occurred and lasts for 1 to 4 days. It is characterized by clotting in the wound, the release of signal molecules to recruit immune cells, and the release of specific enzymes (matrix metalloproteinases, MMPs) that clean the wound. The proliferative phase takes place between 4 and 21 days after wounding, when fibroblasts are stimulated to invade the site of the wound and produce extracellular matrix components. Highly-vascularized granulation tissue is formed and the gap is closed. The final remodeling phase can take up to a year, during which time the immature scar is converted into a stable, less vascularized tissue that exhibits good mechanical proprieties, followed by the growth of regenerated tissue.

GFs have been used in dentistry in all these phases. The most often studied GFs are probably the bone morphogenetic proteins (BMPs), discovered by Urist, who found that protein mixtures obtained from demineralized, lyophilized segments of bone were responsible for bone formation after implanting in rabbit muscle tissue [1].

BMPs are multifunctional cytokines that belong to the transforming growth factor-β (TGF-β) superfamily. They are not only involved in direct ectopic bone formation (hence their name of *bone morphogenetic proteins*), they also modulate several developmental processes, prompting numerous authors to suggest other names: for instance, Reddi suggested that they be should be called *body morphogenetic proteins*, given their extensive roles in various tissues [2].

Over 20 BMPs with various functions have been identified in humans. They have a major role in embryogenesis and in the maintenance and repair of many skeletal and non-skeletal tissues in adults [3]. BMP-1 is actually not considered a member of the BMP family, but a misnamed protein with chordinase and procollagen proteinase activities, implicated in pattern formation during the development of a number of organisms [4]. BMPs are mainly related to bone and cartilage formation, though BMPs 8b, 10 and 15 have no role in these processes, and BMPs 12, 13 and 14 are called cartilage-derived morphogenetic proteins (CDMPs) because they induce chondrogenic phenotypes rather than osteogenesis [2,5], whereas a definite bone-inducing role during bone formation has been observed for BMPs 2, 4, 6, 7 and 9 [6].

BMPs play a pivotal part in skeletal morphogenesis and repair, promoting the differentiation of mesenchymal cells into osteoblasts and inducing new bone formation. BMPs are involved in regulating mesenchymal cell differentiation and proliferation by stimulating intracellular signaling pathways. BMP signals are transmitted by the plasma membrane receptors to the nucleus through multiple signaling pathways that can be divided into two groups, the Smad and non-Smad pathways [3,7]. At the cell surface, BMP ligands bind with BMP receptors, triggering specific intracellular pathways that activate and influence gene transcription. Of the three types of receptor for the TGF-β superfamily, only types I and II appear to have significant roles in BMP binding and signaling. Five type I receptors (ALK1 [Acvrl1], ALK2 [ActRI], ALK3 [BRIa], ALK4 [ActRIb] and ALK6 [(BRIb]), and three type II receptors (BRII, ActRIIa, and ActRIIb) have been identified [8], plus a short form of BRII [9]. Type III TGF-β receptors have

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also been shown to have a role in BMP signaling, by mediating epithelial to mesenchymal cell conversion [10].

Canonical Smad-dependent TGF-β first binds to receptors type I and type II, and then signals are transduced to their Smads. Activated Smads form a complex with Smad4 and cross the nuclear membrane into the nucleus, where they regulate the expression of transcriptional factors and transcriptional coactivators that are important in osteoblasts (Dlx5, Runx2 and Osx). It has recently been demonstrated that, following TGF-β induction, the Smad and the p38 MAPK pathways converge on the Runx2 gene to control mesenchymal precursor cell differentiation [11].

As for the isolation of BMPs, after Urist's experiments, BMPs were obtained from the bones of various species, including rabbit, cow and human. Nowadays, BMPs are produced and purified using DNA recombinant technology and essentially two expression systems, in mammalian cells or bacteria [6]. Recombinant human BMP-2 (rhBMP-2) and recombinant human BMP-7 (rhBMP-7) are currently the only proteins in the group to been approved by the US Food and Drug Administration (FDA) for clinical use in humans, which explains why they are clearly the most extensively evaluated BMPs [12].

Another GF of interest in dentistry is the growth and differentiation factor (GDF), the structure of which closely resembles some BMPs, so it could be included in the BMP family. GDF-5 is also known as BMP-14, or cartilage-derived morphogenetic protein 1, because it induces chondrogenic phenotypes rather than osteogenesis [6]. GDF-5 gene mutations give rise to different types of dysplasia and can result in the autosomal recessive syndromes of brachypod in mice and Hunter-Thompson or Grebe-type chondrodysplasia in humans, involving a loss of joints in both humans and mice [13-15]. Francis-West and colleagues [14] showed that GDF-5 can modulate the initial stages of chondrogenesis by increasing cell adhesion, and can increase chondrocyte proliferation in the later stages of skeletogenesis.

The osteoinductive potential of GDF-5 has been found smaller than that of other members of the BMP family, though numerous studies have confirmed its crucial role in skeletal morphogenesis. Several *in vitro* experiments have demonstrated that rhGDF-5 stimulates osteogenic differentiation and promotes angiogenic activity by increasing vascular endothelial growth factor gene expression in fat- or bone-marrow-derived stromal cells. The osteoinductive activity of rhGDF-5 has also been examined in numerous *in vivo* model systems [13].

Another GF extensively investigated for clinical applications is the platelet-derived growth factor (PDGF), which is synthesized by platelets, monocytes, macrophages, endothelial cells and osteoblasts. This is a dimeric molecule consisting of disulfide-bonded, structurally similar A- and B-polypeptide chains that combine to form homo- and heterodimers. The biologically most potent of these PDGFs is PDGF-BB, which has been thoroughly investigated. The PDGF isoforms exert their cellular effects by binding to and activating two structurally related protein tyrosine kinase receptors, called the alpha-receptor and the beta-receptor [16,17].

PDGF is stored in the alpha granules of circulating platelets and is released during blood clotting in the event of soft or hard tissue injury. Once it has been released from the platelets, PDGF binds to specific cell surface receptors and promotes rapid cell migration (chemotaxis)

and proliferation (mitogenesis) at the site of injury. In particular, *in vitro* and *in vivo* studies have demonstrated that PDGF is a potent chemotactic and mitogenic factor for gingival and periodontal ligament fibroblasts, cementoblasts and osteoblasts [18].

Since the first animal study conducted by Lynch and co-workers [19], extensive *in vitro,* preclinical and clinical studies have been performed using PDGF, alone or in combination with other GFs, for incrementing bone vertically and horizontally, and for treating periodontal and peri-implant defects. The positive outcomes of these studies provide strong evidence of the safety and predictably of rhPDGF combined with specific scaffolds in periodontal and peri-implant regeneration, suggesting promising clinical applications [18,20,21].

Although a large body of preclinical and clinical data has been obtained for only a few GFs, others have nonetheless been assessed for possible applications in clinical practice.

The activity and osteoinductive potential of fibroblast growth factor (FGF) have been the object of various studies [22-24]. FGF signaling reportedly interacts with BMP signaling in bone formation, showing a synergic action on osteogenesis [11].

Few studies have considered the use of parathyroid hormone (PTH) as a factor for modulating bone augmentation and healing [25]. PTH binding activates PTH1R to stimulate several downstream effectors and also drives the internalization of the PTH1R(PTH type I receptor)-TGFβRII (TGF-β type II receptor) complex, which attenuates both TGF-β and PTH signaling on bone development. The transcriptional factor/cAMP response element binding protein (CREB) mediates PTH signaling in osteoblasts, and the PTH-CREB signaling pathway serves as an effective activator of BMP-2 expression [11].

Transforming growth factor-β (TGF-β) [26-27], vascular endothelial growth factor (VEGF) [24], and insulin-like growth factor (IGF) [28] are also the object of studies regarding the biological properties of these bioactive molecules.

3. Clinical application of GFs in dentistry

Given the biological properties of GFs, a major focus of research has concerned the clinical application of the osteoinductive proteins, such as some BMPs, for enhancing new bone formation. Bone loss involving the teeth may be secondary to diseases such as periodontitis, cystic diseases or tumors, or the consequence of trauma. Alveolar bone augmentation procedures are often needed for the purpose of inserting dental implants for prosthetic rehabilitation.

Missing teeth can be replaced with prostheses supported on dental implants, which can only be inserted in patients with an adequate alveolar ridge height and/or thickness, so bone augmentation procedures enable implant treatments in cases in which it would otherwise not be an option. Bone augmentation procedures can be performed prior to implant placement (in a two-stage procedure), or during the same surgical procedure (one-stage procedure), using numerous materials and techniques.

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Various options have been described [29], including: autogenous bone grafts, allografts, xenografts, alloplastic grafts, barrier membranes for guided bone regeneration (GBR), growth factors (and BMPs in particular), platelet-rich plasma (PRP), inlay grafting, onlay grafting, ridge expansion, and distraction osteogenesis.

Tonetti et al. [30] described various techniques that have been developed to correct inadequate vertical and horizontal bone volumes, such as guided bone regeneration (GBR), sinus lift and onlay bone grafting.

Bone augmentation techniques have also been promoted as a means for treating periodontal and peri-implant diseases in an effort to regenerate lost periodontal or peri-implant soft and hard tissues [31-32].

Autogenous bone grafts are still considered the gold standard for bone repair in most cases, though there are some restrictions in their use in clinical practice because of the morbidity of the harvesting procedures and the limited amount of bone available. Many authors have consequently been studying the biocompatibility and effectiveness of other materials as potential substitutes for autogenous bone grafts.

The most recent and promising approach consists in applying osteoinductive growth factors to promote new bone formation (protein therapy) [33], providing a new alternative to autogenous grafts and other bone substitutes.

Combining growth factors with osteoinductive scaffolds may facilitate a faster and more significant enhancement of new bone formation thanks to the delivery of the growth factors at the site of the graft, and because their three-dimensional stability provides protection during the gradual replacement of the graft with newly-formed bone. Numerous materials have been used in combination with GFs, including inorganic bovine bone, porous hydroxyapatite and demineralized human bone matrix.

Numerous pre-clinical and clinical studies have looked into how GF implantation influences bone augmentation and implant osteointegration, focusing particularly on recombinant human BMP-2 (rhBMP-2), rhBMP-7 and recombinant human growth and differentiation factor-5 (rhGDF-5), combined with a variety of biomaterials used as scaffolds and delivery systems.

Although the potential value of GFs in alveolar bone regeneration and augmentation has been highlighted by numerous authors [6,31,34-35], it is still difficult to assess the different biological potential of each growth factor, because few analyses have compared different growth factors under identical *in vivo* conditions [24].

There is still much to learn about osteogenic growth factors: only a handful of growth and differentiation factors have been the object of clinical evaluation [6,18,25] and further studies are needed to identify predictable clinical outcomes.

3.1. Pre-prosthetic surgery for the purpose of dental rehabilitation with implants

Several surgical techniques and materials - including the use of GFs - have been introduced with a view to increasing bone volume in order to enable the placement of dental implants.

The systematic literature review conducted by Jung and coworkers [25] assessed the clinical, histological and radiographic outcomes after BMP-2, BMP-7, GDF-5, PDGF, and PTH had been used for localized alveolar ridge augmentation. Altogether, 74 studies met the authors' inclusion criteria, including 6 on the outcome of BMP-2 for localized alveolar ridge augmentation in humans; the remainder were pre-clinical studies involving BMP-2, BMP-7, GDF-5, PDGF, and PTH. For all the GFs other than BMP-2, no human studies met the inclusion criteria. Concerning the animal studies, most of those on BMP-2 (43 out of 45) showed a positive effect of this growth factor. Six of 8 studies reported a positive effect of BMP-7. The one animal study on GDF-5 spoke of a statistically significant increase in bone volume. Five of 10 studies involving the use of PDGF also reported a statistically significant increase in bone volume. Four animal studies identified a significantly greater bone regeneration in cases treated with PTH than in controls. In the six human studies, BMP-2 influenced local bone augmentation, with a dose-dependent increase in bone volume. The dose of BMP-2 delivered seemed to have an impact on treatment outcome, local bone regeneration being greater for higher BMP-2 doses [36-38], with a smaller decrease in bone height at extraction socket sites [39]. Four of these six human studies were designed as randomized-controlled clinical trials (RCT) [37-40], the other two as prospective cohort studies [36,41]. The locally-applied dose of BMP-2 ranged from 0.5 to 1.75 mg/ml, or 0.12 to 3.4 mg/patient, respectively. An absorbable collagen sponge (ACS) was used in five studies, while Jung et al. [40] used a demineralized bovine bone matrix (DBBM) as a carrier. The treatments included sinus floor augmentation [38,41], extraction socket preservation [36-37,39], augmentation of localized ridge defects [36], and lateral ridge augmentation combined with simultaneous implant placement [40].

The 16-week open-label study conducted by Boyne and coworkers [41] assessed the safety and efficacy of implanting BMP-2 delivered on an absorbable collagen sponge (rhBMP-2/ACS) for two-stage maxillary floor sinus augmentation. The dose of rhBMP-2 ranged from 1.77 to 3.40 mg per patient. Significant bone growth was documented by computed tomographic (CT) scans in all evaluable patients (11/12), with an overall mean response of 8.51 mm in height (±4.13 mm). Histology on core bone biopsies obtained when the dental implant was inserted confirmed the good quality of the bone induced by rhBMP-2/ACS.

In a more recent RCT, Boyne and colleagues [38] found no statistically significant differences in terms of the increase in ridge height, as measured using CT scans, between their treatment and control (bone graft) groups, and even a narrower ridge width in the former after using BMP-2/ACS in two-stage maxillary floor sinus augmentations.

Bianchi et al. [37] investigated the efficacy of different concentrations of rhBMP-2 in regenerating bone in alveolar defects in the anterior maxilla, reporting a positive outcome in terms of bone volume augmentation.

Another RCT [39] compared the efficacy of rhBMP-2 in two different concentrations, delivered on ACS, with placebo ACS alone in 80 patients requiring local alveolar ridge augmentation for buccal wall defects (> or =50% buccal bone loss around the extraction socket) immediately after tooth extraction of the maxillary bicuspids. They found no statistically significant effects of BMP-2 on the treatment outcome when a lower dose was used, but a statistically significant

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positive effect of a higher dose (1.50 mg/ml rhBMP-2/ACS). In addition, bone density and histology revealed no differences between newly-induced and native bone.

Finally, Jung et al. [40] tested whether adding rhBMP-2 to a xenogenic bone substitute mineral could improve guided bone regeneration in the case of bone defects requiring lateral bone augmentation procedures and simultaneous implant placement. Following implant insertion (baseline), the peri-implant bone defect height was measured from the implant shoulder to the first implant-bone contact. The authors reported a positive, but statistically insignificant effect of BMP-2 on the amount of newly-formed bone (37±11.2%) compared with the control group (30± 8.9%). On the other hand, they found more mature lamellar bone (76±14.4% versus 56±18.3%) and a greater area of bone-to-graft contact (57±16.2% versus 30±22.6%) at the BMP-2-treated sites.

Various methods have been described for increasing bone volume before or at the time of positioning implants [25], one of the best-documented of these methods being GBR for intra-oral bone augmentation. To overcome some of the drawbacks of this technique, e.g. a long treatment time, the difficulty of predicting any vertical bone augmentation, the risk of infection after membrane exposure, research has concentrated on the use of bioactive molecules that induce local bone formation. Using the GBR technique, the width and height of the alveolar ridge is increased in areas of insufficient bone volume by applying barrier membranes, alone or in combination with bone grafts or substitutes.

Misch [42] published a human case series of atrophic posterior mandible augmentation prior to implant insertion, using recombinant human BMP-2 2/absorbable collagen sponge (rhBMP-2/ACS) and titanium mesh. All the 10 implants involved in the study, inserted after a 6-month healing period, became integrated and were restored with single crowns.

Many *in vivo* studies used critical-size supra-alveolar peri-implant defect models and other bone augmentation methods simultaneously with implant insertion. In an animal study, Sigurdsson et al. [43] found that defect sites implanted with rhBMP-2⁄ACS showed signs of a statistically significant and clinically relevant vertical alveolar bone augmentation by comparison with controls (ACS). Although the titanium implant was osseointegrated after a 16-week healing interval, the BIC (bone-to-implant contact) was lower than in resident bone, as was to be expected; the newly-induced bone was often in a thin layer on the implant surface, probably due to the unpredictability of ACS in providing adequate space for new bone formation.

Wikesjö and colleagues [44] subsequently used a critical-size supra-alveolar peri-implant defect model to study the efficacy of an ePTFE GBR device in supporting rhBMP-2⁄-induced bone formation in dogs. The space-providing macro-porous membrane was characterized by the ability to prevent the compression of the rhBMP-2/ACS construct, while allowing for vascularization via the gingival connective tissue. The authors compared GBR alone with rhBMP-2(0.4 mg)⁄ACS and rhBMP-2(0.4 mg)⁄ACS combined with GBR. Histometric analysis on block biopsies after an 8-week healing interval revealed the best results in the third sample, i.e. the GBR-rhBMP-2⁄ACS combination, which revealed bone formation filling the dome-shaped GBR device, with a vertical bone gain at the turned implants averaging 4.7 ± 0.2 mm,

and an induced bone area of 9.6 ± 0.7 mm^2, generating a highly-significant correlation between the induced bone area and the space provided by the GBR device. This study highlighted the crucial importance of providing space in order to obtain clinically significant benefits from a BMP construct.

Jung et al. [45] ran a randomized-controlled clinical trial with a split-mouth design, in which implants were placed in sites exhibiting lateral bone defects and patients were randomly selected for treatment with demineralized bovine bone mineral and bioresorbable collagen membrane, with (test) or without (control) the addition of rhBMP-2. After an average healing period of 6 months, a reentry operation was performed for abutment connection and prosthetic reconstruction. At the 3-year follow-up, all 34 implants in all 11 patients were clinically stable and radiologically osseointegrated. At the 5-year follow-up, 32 implants were stable and functioning, while 2 were not re-examined because the patient had moved away. The survival rate of the implants examined at 3 and 5 years was therefore 100% for both the test and the control sites. The periapical radiographs of the test and control sites also showed no peri-implant radiolucency at the 3- and 5-year follow-up examination, demonstrating healthy peri-implant tissues with minimal marginal bone loss, and only minor prosthetic complications were recorded. In short, both the test and the control sites revealed excellent clinical and radiological outcomes after 3 and 5 years, with no statistically significant differences in any of the parameters examined (though the authors emphasized the need for a larger group of patients in future studies).

In a micro-CT study in dogs, Al-Hazmi and co-workers [20] assessed the efficacy of using PDGF-BB and xenografts, with or without collagen membranes, for GBR around immediate implants with buccal dehiscence defects. They concluded that using PDGF and xenografts resulted in greater BBT (buccal bone thickness), BBV (buccal bone volume), VBH (vertical bone height) and BIC (bone-to-implant contact) when used alone rather than in combination with a collagen membrane. Their results are consistent with the report from Simion et al. [46], who said that barrier membranes may interfere with the chemotactic effect of GFs on periosteal pluripotential mesenchymal cells.

Further studies are nonetheless warranted to investigate the influence of barrier membranes on the periosteal pluripotential mesenchymal cells [20].

Most of the clinical studies on rhPDGF have focused on periodontal and peri-implant regeneration, and only a few human studies have investigated ridge preservation for implant placement in extraction socket defects [47], or three-dimensional ridge augmentation [48].

In a pilot study, Nevins et al. [47] tested whether mineralized collagen bone substitute (MCBS) combined with recombinant human platelet-derived growth factor-BB (0.3 mg/mL) could generate enough viable bone in buccal wall extraction defects to enable implant placement.

In a more recent clinical study, Nevins and colleagues [49] focused on human buccal plate extraction socket regeneration with recombinant human platelet-derived growth factor BB or enamel matrix derivative. Buccal plate resorption is a critical issue when it comes to implant placement. They compared four groups: A (mineral collagen bone substitute [MCBS] scaffold alone), B (MCBS with recombinant human platelet-derived growth factor BB [rhPDGF-BB; 0.3

mg/mL]), C (MCBS with enamel matrix derivative [EMD]), and D (a combination of EMD with bone ceramic). Grafting was done at the time of extraction, advancing the buccal flap for primary closure. Histology on trephine core biopsies of the implant site performed 5 months later, at the time of implant placement, identified new bone healing around the biomaterial scaffolds with no statistically significant differences between the four treatment groups. There was a histomorphometric trend towards a greater quantity of new bone in the rhPDGF-BB-treated group, with the most favorable ridge morphology for the purposes of an optimal implant placement at reentry surgery.

Simion et al. [48] reported on two human cases of patients who underwent three-dimensional ridge augmentation using a xenograft combined with rhPDGF-BB. In the first patient, a deproteinized bovine block infused with rh-PDGF was attached to the alveolar crest with two screws to obtain a horizontal ridge augmentation. The second patient underwent a vertical ridge augmentation procedure involving deproteinized bovine bone particles embedded in a collagen matrix soaked in rhPDGF-BB. Three titanium dental implants were placed in each patient 5 months later with excellent clinical and histological outcomes, mean that rhPDGF-BB in combination with a deproteinized bovine graft has promise in applications for regenerating large three-dimensional alveolar defects in humans.

3.2. Dental implant surface coatings with GFs

Another interesting approach to enhancing alveolar ridge augmentation with a view to dental implant placement involves using implants coated with GFs.

Wikesjo and colleagues [35] reviewed the literature on implants coated with a bone-inductive factor capable of stimulating local bone formation and osseointegration. They concluded that rhBMP-2 can be delivered successfully for the purposes of inducing local bone formation and osseointegration by using screw-type endosseous oral implants with titanium oxide surfaces with open pores as a carrier. They also found that purpose-designed implant surfaces coated with rhBMP-2 resulted in the formation of Type II bone and significant osseointegration without any need for biomaterials or devices for GBR.

In an *in vivo* animal model, Susin et al. [50] used the critical-size supra-alveolar peri-implant defect model to assess the potential of a purpose-designed porous titanium oxide implant surface coated with rhBMP-7 for inducing alveolar bone formation and enhancing osseointegration. The animals received implants coated with rhBMP-7 at 1.5 or 3.0 mg/ml randomized to the contralateral jaw quadrants. The authors found clinically relevant bone formation and osseointegration with no statistically significant differences in terms of bone formation between the sites treated with rhBMP-7 at 1.5 or 3.0 mg/ml. Histology showed an increase in the height and area of the bone, and the newly-formed bone exhibited the same characteristics as the contiguous resident bone. Their observations support the significant clinical value of rhBMP-7 in inducing bone regeneration, but the authors made the point that higher concentrations were associated with some local side effects.

Other authors [e.g. 51-52] have investigated *in vivo* the potential of an rhGDF-5 coating on an oral implant with a porous titanium oxide surface for stimulating local bone formation, including osseointegration and vertical augmentation of the alveolar ridge.

Polimeni and co-workers [51] examined a bilateral critical-size, 5 mm, supra-alveolar peri-implant defect model in dogs. Six animals received implants coated with 30 or 60 μg rhGDF-5, and another six animals received implants coated with 120 μg rhGDF-5 or left uncoated (controls). The implants coated with rhGDF-5 displayed only limited peri-implant bone remodeling in the resident bone, as measured using fluorescent bone markers, with the 120 μg dose coinciding with a more advanced remodeling than the 60 and 30 μg doses. These results suggest a dose-dependent osteoinductive and/or osteoconductive effect of rhGDF-5-coated oral implants. Leknes et al. [52] performed an *in vivo* study in dogs that consisted in placing different kinds of implant in the alveolar ridge of the posterior mandible following the surgical extraction of the premolars and reduction of the alveolar ridge. Six animals were treated with implants coated with rhGDF-5 in doses of 30 or 60 μg/implant in contralateral jaw quadrants, while six received implants coated with rhGDF-5 at 120 μg/implant or uncoated implants (for control purposes), using a split-mouth design. The radiographs showed a dose-dependent formation of mineralized tissue significantly greater than around the uncoated implants, the greatest increase corresponding to the implants coated with 60 μg and 120 μg of rhGDF-5, and amounting to approximately 2.2 mm in both cases at 8 weeks. The authors also reported no adverse events, such as peri-implant bone remodeling, implant displacement, or seroma formation.

The above-mentioned studies indicate that these GFs have great potential for stimulating clinically relevant local bone formation, though it should be emphasized that further studies are essential to address their most appropriate dosage, carriers, and applications, as well as the long-term prognosis of GF-coated titanium implants.

3.3. Maxillary sinus lift procedure

Sinus floor elevation with immediate or delayed dental implant placement is a well-known technique for dental rehabilitation in cases of severe atrophy of the posterior maxilla due to the extension and pneumatization of the maxillary sinus. Many materials, such as autografts, xenografts, and synthetic bone substitutes, have been shown to achieve acceptable clinical results when used in maxillary sinus floor augmentations [53]. The use of GFs with various carriers and dosages has recently been investigated in combination with sinus augmentation procedures too.

Ho and colleagues [54] assessed the efficacy of various bioimplants used in maxillary sinus lift procedures with the lateral window approach in a rabbit model. They compared particulated autogenous bone, demineralized bone matrix (DBM), DBM combined with purified BMP-7 (BMP-7/DBM bioimplants), and bioimplants consisting of a poloxamer gel with BMP-7 in two different doses. In their animal model, BMP-containing bioimplants had produced more new bone and a greater new bone surface area at 2 weeks than autografts, but the advantage of these bioimplants subsequently seemed to be lost, since the differences between the bioimplants and the autografts had disappeared by 8 weeks. The authors concluded that BMP-

containing bioimplants prompt a more rapid bone formation, possibly offering a greater implant stability earlier in the healing period, and therefore enabling clinicians to place osseointegrated implants in augmented maxillae sooner after grafting.

In a clinical study, Boyne and colleagues [38] compared different concentrations of rhBMP-2 (0.75 and 1.5 mg/mL), delivered on an absorbable collagen sponge (ACS) carrier, with bone grafts to identify a safe and effective concentration of rhBMP-2 for use in maxillary sinus floor augmentation procedures. Judging from density measurements on CT scans obtained before and 4 months after treatment, and 6 months after functional loading of the dental implants, and from core biopsies obtained at the time of placing the dental implant, they established that the 1.5 mg/mL dose of rhBMP-2/ACS was more appropriate in a pivotal, randomized, multicenter study to compare rhBMP-2/ACS with conventional bone graft for staged maxillary sinus floor augmentation to support dental implants for long-term functional loading.

These data prompted a randomized, parallel evaluation of rhBMP-2/ACS and autogenous bone grafts for two-stage maxillary sinus floor procedures [55]: 160 individuals with less than 6 mm of native bone height in the posterior maxilla were randomized for treatment with 1.5 mg/mL rhBMP-2/ACS or an autograft. Height and density measurements were obtained on CT scans, and core biopsies obtained at the time of dental implant placement underwent histological examination. A significant amount of new bone had formed by 6 months postoperatively in both treatment groups, but there was a significant difference in the density of the newly-induced bone at the 6-month follow-up, which was denser in the bone graft group than in the group treated with rhBMP-2/ACS. Six months after dental restoration (functional loading), however, the bone induced in the rhBMP-2/ACS group was significantly denser than in the bone graft group. No major differences emerged between the two groups in terms of the histological parameters. 17% of the patients in the autograft group experienced long-term parasthesia, pain, or gait disturbance relating to the bone graft harvest. Adverse reactions frequently recorded in the rhBMP-2/ACS group related to excessive facial swelling, and this edema was attributed to the chemotactic cellular recruitment to the site of rhBMP-2 implantation and neovascularization of the grafted area; although it was severe, this edema did not adversely affect the outcome. This study confirmed the efficacy and safety of rhBMP-2/ACS by comparison with bone grafting for sinus floor augmentation, given the morbidity, cost, and increased surgical time associated with the harvesting of autogenous bone.

Kao and coworkers [56] measured the bone formation after a lateral window sinus augmentation with recombinant human BMP-2/ absorbable collagen sponge (rhBMP-2/ACS) in combination with Bio-Oss by comparison with the results achieved with a Bio-Oss graft alone. Histology demonstrated that less new bone formed in patients treated with rhBMP-2/ACS + Bio-Oss than in those treated with Bio-Oss alone, pointing to a negative effect on bone formation of combining rhBMP-2 with Bio-Oss for maxillary sinus augmentation.

Gruber and coworkers [57] studied a GF closely related to the BMP family - the recombinant human growth and differentiation factor-5 (rhGDF-5) - in an *in vivo* study involving the use of different materials in sinus floor augmentation procedures in Goettingen miniature pigs. They demonstrated that associating rhGDF-5 with β-tricalcium phosphate

(β-TCP) enhanced bone formation by comparison with the results obtained using the β-TCP carrier material alone.

In a further study using a split-mouth study design, the same authors [13] compared rhGDF-5-coated β-TCP with particulated autogenous bone grafts combined with the scaffold material (β-TCP). In each minipig, the sinus floors were augmented (simultaneously inserting the dental implants) with β-TCP mixed with autogenous cortical bone chips on one side, and using β-TCP coated with two different concentrations of rhGDF-5 on the contralateral side. Histology and histomorphometric analyses demonstrated that rhGDF-5-coated β-TCP not only enhanced new bone formation, but also - by comparison with a combination of β-TCP and autogenous bone chips - induced a significant increase in VD (volume density) and BIC (bone-to-implant contact) in the augmentation material.

Stavropoulos et al. [58] ran a prospective, multicenter, randomized clinical trial to examine the histological outcome of maxillary sinus lifting with rhGDF-5/β-TCP or β-TCP and autogenous bone (β-TCP/AB) composite. Thirty-one patients requiring unilateral maxillary sinus floor augmentation with a residual alveolar bone height <5 mm were treated using a lateral window approach. Cylindrical biopsies were harvested with a trephine bur during implant site preparation 3 or 4 months after sinus floor augmentation (three groups (a) rhGDF-5/b-TCP and a 3-month healing period, (b) rhGDF-5/b-TCP and a 4- month healing period, and (c) b-TCP/AB and a 4-month healing period). Histological and histometric analyses showed that sinus augmentation with rhGDF-5/β-TCP resulted in new bone in comparable amounts and of similar quality to the bone obtained with a β-TCP/AB composite graft, suggesting that rhGDF-5/β-TCP could eliminate the need for AB grafting in sinus lift procedures.

Though these favorable regenerative findings are encouraging, further studies are needed to ascertain the influence of GFs on the amount and quality of new bone formation, and on the implant survival rate after sinus lift procedures.

3.4. Periodontal regeneration

Periodontitis is a widely prevalent inflammatory disease of the tissues supporting the teeth, characterized by a progressive loss of bone and attachment.

The ultimate goal of periodontal therapy is the regeneration of periodontal tissues, which consists in stimulating new cementum formation, new alveolar bone apposition, and a functionally-oriented periodontal ligament reconstruction. Various techniques have been suggested for promoting periodontal tissue regeneration, using different bone graft materials that have gained clinical acceptance in the treatment of periodontal defects.

To overcome the weaknesses of conventional regenerative procedures, the predictability of which may be limited to selected case types, using GFs with biocompatible scaffolds to promote tissue regeneration may represent a new and promising periodontological approach.

After preliminary *in vitro* experiments, extensive *in vivo* preclinical studies have been performed to assess the potential and safety of using various GFs, alone or in combination, to treat periodontal defects.

A recent animal study by Oortgiesen et al. [23] investigated the regenerative potential of an injectable macroporous calcium phosphate cement (CaP) combined with BMP-2 or fibroblast growth factor-2 (FGF-2) in intrabony defects. After 12 weeks, only the CaP revealed limited effects on both periodontal ligament (PDL) and bone healing, while a good response in terms of bone healing was also seen with CaP/BMP-2 and CaP/FGF-2. The best PDL healing scores coincided with the combined CaP/FGF-2 treatment, suggesting that associating a topical application of FGF-2 with an injectable CaP might be a promising treatment for the purposes of periodontal regeneration.

Ishii and colleagues [22] investigated the effect of the combined use of basic FGF-2 and beta tricalcium phosphate (β-TCP) on root coverage in a dog model, finding that FGF-2/β-TCP enhanced the formation of new bone and cementum without any significant root resorption.

Kitamura et al. [59] undertook a multi-center, randomized, double-blind, placebo-controlled, dose-finding study on the potential of local applications of FGF-2 in periodontal regeneration. Modified Widman periodontal surgery was performed, during which 200 μL of the investigational formulation containing 0% (vehicle alone), 0.2%, 0.3%, or 0.4% FGF-2 was administered to 2- or 3-walled vertical bone defects in 253 adult patients with periodontitis. The primary outcome was the percentage of bone fill visible on radiographs 36 weeks after administering the treatment. All the doses of FGF-2 were significantly superior to the vehicle alone ($p < 0.01$) in terms of the percentage of bone fill, and this percentage peaked in the 0.3% FGF-2 group. No significant differences were observed between the four groups in terms of the regained clinical attachment (CAL), with all patients scoring around 2 mm (this was judged to be due to the different healing patterns between the FGF-2 groups and the 'vehicle alone' group). Conventional periodontal surgery (which corresponds to the 'vehicle alone' group) usually gives rise to long junctional epithelial attachments, but manual probing cannot precisely distinguish fibrous from epithelial attachments, so the difference in healing pattern cannot be reflected in the CAL regained by the different treatment groups. This limitation could have been overcome by histology, but this was not done for ethical reasons. No clinical safety issues emerged in this study. These results support the efficacy and safety of topical FGF-2 applications for periodontal regeneration in humans.

When implanted in furcation defects exposed surgically or by inflammatory processes in *Papio ursinus*, recombinant human osteogenic protein-1 (hOP-1) or BMP-7 tends to induce cementogenesis with the insertion of *de novo* generated Sharpey's fibers. Long-term studies on *P. ursinus* after hOP-1 implantation show a highly-organized periodontal ligament space with periodontal ligament fibers cursing from the newly-formed and mineralized cementum to the regenerated alveolar bone, with a multitude of supporting capillaries throughout the periodontal ligament space [60].

In an experimental study by Teare et al. [27], binary applications of hOP-1 and hTGF-β(3) were implanted in Class II furcation defects of the mandibular molars of Chacma baboons (*P. ursinus*) to induce periodontal tissue regeneration. Sixty days after implantation, the animals were killed and histological and histomorphometric studies led the authors to conclude that

hOP-1 and hTGF-β(3) in Matrigel(®) matrix induced substantial periodontal tissue regeneration and cementogenesis.

In their review, Ripamonti et al. [61] emphasized the induction of bone formation by the osteogenic proteins of the TGF-beta superfamily in the nonhuman primate, *P. ursinus*.

In a recent study in beagle dogs, Kim and co-workers [62] compared a candidate β-tricalcium phosphate (β-TCP) carrier technology with the absorbable collagen sponge (ACS) benchmark for supporting rhGDF-5-stimulated periodontal wound healing/regeneration in intrabony periodontal defects. Both solutions stimulated the formation of functionally-oriented periodontal ligament, cellular mixed-fiber cementum, and woven/lamellar bone, but bone regeneration (height and area) was significantly greater for the rhGDF-5/β-TCP construct. The structural integrity of the β-TCP carrier preventing compression while providing a framework for bone ingrowth may account for these results.

A phase IIa randomized controlled clinical and histological pilot study was conducted to assess rhGDF-5/β-TCP for periodontal regeneration [63]. Twenty chronic periodontitis patients participated in the study, each with at least one tooth scheduled for extraction with a probing depth (PD) ≥6 mm and an associated intrabony defect ≥4 mm following basic periodontal therapy. Participants (one defect/patient) were randomized to receive open flap debridement (OFD) + rhGDF-5/β-TCP (n = 10) or OFD alone (control; n = 10). Both protocols resulted in statistically significant clinical improvements. Descriptive statistics showed a greater reduction in PD after OFD with rhGDF-5/β-TCP than after OFD alone (3.7 ± 1.2 versus 3.1 ± 1.8 mm; $p = 0.26$), as well as less gingival recession (0.5 ± 0.8 versus 1.4 ± 1.0 mm; $p < 0.05$) and a greater CAL gain (3.2 ± 1.7 versus 1.7 ± 2.2 mm; $p = 0.14$) at the deepest aspect of the defect. Block biopsies of the defect sites were collected 6 months after surgery and prepared for histology. Five biopsies (1 rhGDF-5/β-TCP; 4 OFD) were deemed unsuitable for histological or histometric evaluation. Bone regeneration height (2.19 ± 1.59 versus 0.81 ± 1.02 mm; $p = 0.08$) and PDL (2.16 ± 1.43 versus 1.23 ± 1.07 mm; $p = 0.26$), cementum (2.16 ± 1.43 versus 1.23 ± 1.07 mm; $p = 0.26$) and bone regeneration area (0.74 ± 0.69 versus 0.32 ± 0.47 mm^2; $p = 0.14$) were greater at sites treated with rhGDF-5/β-TCP compared to controls. These differences failed to reach statistical significance, however, and the authors said that further studies on larger samples will be needed to verify these findings.

The potential of PDGFs for promoting new bone formation and/or periodontal wound healing/regeneration has been examined in a variety of pre-clinical animal models. *In vivo* experimental studies have been performed using PDGF-BB alone or in combination with other GFs, such as insulin-like growth factor (IGF), and shown that these growth factors promoted new bone, cementum and periodontal ligament formation *in vivo*.

The first human clinical trial testing the effect of rhPDGF/rhIGF-I in periodontal defects was reported by Howell and colleagues [64] with promising results.

Early human clinical studies used rhPDGF-BB combined with bone allografts. An alternative is to use a synthetic system, such as β-tricalcium phosphate (β-TCP). Since rhPDGF applications have proved clinically effective in the treatment of intrabony defects, this growth factor has also been considered for the treatment of soft tissue recession defects [18].

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Jayakumar and coworkers [65] ran a double-blind, prospective, parallel, active-controlled, randomized, multi-center clinical trial on the efficacy and safety of rhPDGF-BB with β-TCP in human intraosseous periodontal defects. Fifty-four patients with periodontal osseous defects were randomly grouped for treatment with rhPDGF-BB/β-TCP or β-TCP alone. A total number of 50 defects in 25 patients in the rhPDGF-BB/β-TCP group and 25 in the β-TCP group were ultimately available for statistical analysis. The radiographic parameters considered were linear bone growth (LBG) 6 months after surgery and percent bone fill (% BF), both of which were found significantly higher in the rhPDGF-BB/β-TCP group than in the β-TCP group. There also emerged a significantly higher area under the curve for clinical attachment level gain from 0 to 6 months, and a greater reduction in PD at the third and sixth month than after β-TCP treatment alone. The implantation of rhPDGF-BB/β-TCP for the treatment of intraosseous periodontal defects was safe and well tolerated, and resulted in clinically and statistically significant improvements in bone formation parameters and soft tissue outcomes.

Preliminary investigations thus indicate that GFs have great potential for improving periodontal regeneration, but randomized clinical trials must be conducted to gain a better understanding of the role of GFs in periodontal treatments, focusing particularly on establishing the safety and efficacy of their application.

4. Growth factor delivery systems

The great potential of GFs in bone regeneration has been discussed by numerous authors [6,31,34-35]. BMP-2 and BMP-7 have a marked effect on bone and cartilage growth and the maintenance of homeostasis during bone remodeling [66]. One of their limitations, on the other hand, seems to be the unpredictable nature of the resulting tissue regeneration *in vivo*. It has been suggested that the clinical efficacy of recombinant human forms of BMPs (rh-BMPs) depends on the carrier system used to ensure an effective delivery of adequate protein concentrations to the site being treated [67]. BMPs are soluble proteins and, delivered in a buffer solution, they undergo rapid degradation, leading to an insufficient bioavailability. Other factors, such as protein competition, enzymatic activity, temperature, pH and salt concentration, may also influence the total amount of active protein available immediately after its administration [68].

In 2007 Giannoudis et al. [69] came up with the "Diamond Concept" to describe the conditions needed for osteogeneration, i.e. mechanical stability at the site of the defect, and osteogenic cells combined with osteoinductive growth factors and a suitable carrier or delivery system.

The main purpose of the delivery system is to ensure adequate protein concentrations at the defect site for as long as it takes to enable the regenerative cells to migrate, proliferate and differentiate [33].

A localized, controlled release is also necessary to prevent any unwanted and uncontrolled ectopic bone formation in non-bony body tissues [70]. Supra-physiological concentrations resulting from imperfect GF release kinetics have been correlated with severe clinical

complications, including generalized hematomas in soft tissues and peri-implant bone resorption. Other potential concerns theoretically include carcinogenicity and teratogenic effects [70].

Few authors have investigated the influence of GF release kinetics on bone regeneration. In physiological bone repair, some growth factors (such as BMP-2) are expressed mainly during the early inflammatory phase. Others are up-regulated during the chondrogenic and osteogenic phases, and have a biphasic expression pattern or are constitutively expressed [33]

In vivo studies demonstrated that higher BMP-2 retention times were more osteoconductive [71], and that prolonged BMP-2 delivery enhanced the protein's osteogenic efficacy by comparison with a shorter-term delivery of an equivalent dose in a rat model [72]. Release should preferably be sustained over time, either in large single doses or in multiple smaller-dose applications. In evaluating the timing of the protein release, it is important to consider the dynamic nature of the healing zone, which depends on the type, location and appearance of the defect, the patients' age and gender, their hormone and nutritional state, and any diseases, as well as other parameters influencing release rate, including the protein's size and conformational changes, solubility, polymer/scaffold composition/geometry, and molecular weight [33].

Dose and concentration parameters are available for orthopedic clinical applications, where different anatomical sites require different therapeutic doses depending on the degree of vascularization, defect size and the number of resident responding cells. Supraphysiological dosages range from 0.01 mg/ml in small animal models (e.g. rats) to 0.4 mg/ml in rabbits, to more than 1.5 mg/ml in non-human primates [33].

Growth factor release from a delivery system may be diffusion-controlled, chemical or enzymatic reaction-controlled, solvent-controlled, or controlled by a combination of these mechanisms. Diffusion-controlled release is governed by the protein's solubility and diffusion coefficient in the aqueous medium, protein partitioning between the aqueous medium and the material of the delivery system, protein loading and the diffusional distance. Chemical or enzymatic reaction-controlled systems include erodible systems, in which the protein is physically immobilized in the carrier matrix and released as the carrier undergoes degradation and dissolves. In solvent-controlled systems, the protein is embedded in a carrier matrix and a diffusional release occurs as a consequence of the rate-controlled penetration of the solvent (water) in the system [33].

Several GF delivery systems and carriers have been suggested for use in bone regeneration applications in an effort to find the optimal strategy for optimizing their clinical effectiveness and minimizing complications.

Delivery systems and carriers used for bone GFs should meet general requirements (Table 1) such as biocompatibility, predictable biodegradability, and the ability to provoke appropriate inflammatory responses. They must also have the following features: easy and cost-effective to manufacture; stability; easy handling and storage [33].

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Biocompatibility
Predictable biodegradability
Low immunogenicity and antigenicity
Enhancement of cellular vascularization and attachment
Affinity with BMPs and bone
Maintenance and enhancement of BMP bioactivity
Malleability and ease of manufacture
Safety, stability, sterility, availability and cost-effectiveness
Regulatory agency approval for the clinical application of interest
Controlled protein release at an effective dose for the appropriate period of time

Table 1. General requirements for BMP delivery systems

Carrier materials have been generally divided into four classes (Table 2): natural-origin polymers (collagen, hyaluronic acid, gelatin hydrogel complex, alginates and chitosan); inorganic materials (synthetic bone grafts, hydroxyapatite, calcium phosphates and bioactive glasses); synthetic biodegradable polymers (polylactic acid PLA, polyglycolide PLG, and their polymers PLGA, cholesterol-bearing pullulan nanogel CHPA), and composites (combinations of materials from the above different classes) [33].

To date, only BMP-2 and BMP-7 have been approved by the US Food and Drug Administration for human use in specific orthopedic applications, delivered using absorbable collagen sponges [33].

4.1. Collagen

Collagen is the protein most abundant in the connective tissue of mammals and the main non-mineral component of bone. It has been prepared in powders, membranes, films and implantable absorbable sponges, as well as in aqueous forms. Although it is versatile and easy to manipulate, the manufacture of collagen carriers is highly sensitive to several factors (including mass, soaking time, protein concentration, sterilization, buffer composition, pH and ionic strength) that directly affect rhBMPs binding [73]. Absorbable collagen sponges (ACS) have been evaluated in numerous in vivo models and clinical trials [6, 38,74-76]. In patients requiring staged maxillary sinus floor augmentation, rhBMP-2/ACS safely induced adequate bone formation for the purpose of placing and functionally loading endosseous dental implants [38]. The use of rhBMP-2/ACS without any concomitant bone grafting materials in critical-size mandibular defects prompted an excellent regeneration in a case review of 14 patients [75]. On the other hand, a recent study by Kao et al. demonstrated a more limited bone formation after a lateral-window sinus augmentation procedure involving rhBMP-2/ACS combined with Bio-Oss than when Bio-Oss was used alone [56].

Although they do away with the need to harvest autologous bone (with the associated pain), the use of animal-derived collagens is limited by their xenogenic nature: anti-type I collagen antibodies reportedly developed in almost 20% of patients treated with rhBMP-2/ACS [6]. In addition, collagen sponges are usually sterilized with ethylene oxide prior to soaking the

Class	Types	Advantages	Disadvantages
Natural polymers	Collagen (gels, nano fibers, scaffolds and films) Fibrin glue Alginate and chitosan	Biocompatible, biodegradable, soluble in physiological fluids, natural affinity with BMPs	Immunogenicity (xenogenic), pathogen transmission, sensitivity to sterilization process
Inorganic materials	Synthetic bone grafts CPC (calcium phosphate cement) Bioactive glasses, hydroxyapatite, hyaluronic acid, tricalcium phosphates, metal, ceramics and calcium sulfate	Osteoconductive, affinity with BMPs	Brittle, difficult mold, some formulations are exothermic
Synthetic polymers	PLLA and PGLA and their copolymers CHPA	Easy to process and sterilize, flexible to tailor and reproducible, excellent chemical and mechanical properties	Inflammatory response, localized pH drop and limited biological function
Composites	Collagen-HA and titanium PLLA	Depending on the combination of the different materials' characteristics	Complex to manufacture

Table 2. Major classes of carrier materials

sponge in the BMP solution, and this can affect the GF release kinetics or the protein's bioactivity [73].

4.2. Alginate and chitosan

Alginate is a non-immunogenic polysaccharide used in a wide range of tissue engineering applications for its gel-forming properties. Alginate hydrogels allowing for a controlled, prolonged release of BMPs have only been studied in the preclinical phase, with promising results in vitro [72,77]

Chitosan is a cationic glucopolymer well known for its biological, chelating and adsorbing properties, and has been used as a BMP-2 carrier in a rat critical-size mandibular defect model, with positive results on histological and histomorphometric analysis [78].

4.3. Hyaluronic acid

Hyaluronic acid is a naturally-occurring biopolymer that plays a significant part in wound healing. It has been associated with an improved bone formation in mandibular defects by comparison with collagen sponges, when both were used to carry rhBMP-2 [79].

4.4. Hydroxyapatite

Hydroxyapatite (HAP) is well known for its osteoconductivity and has been widely used as a bone substitute material in clinical practice since the 1970s because of its ability to bond directly with bone [80]. Synthetic HAP comes in ceramic or non-ceramic, cementable forms, and has been evaluated as a scaffold and a controlled-release carrier, demonstrating lack of resorption and limited bone induction [6]. It has been combined with tri-calcium phosphates, collagen and other materials to form rigid, resorbable, porous carriers, in which case delivery and bone formation were generally found better than when HAP was used alone [81,82].

4.5. Synthetic biodegradable polymers

Unlike natural polymers and collagen, synthetic polymers pose no problem of immunogenicity or risk of disease transmission.

The most commonly-used polymers are polylactic acid (PLLA) and polyglycolic acid (PLGA). Bioresorbable PLLA/PLGA copolymers have been found superior to collagen when used to deliver rh BMP-2 to mandibular defects in the rat [83].

4.6. Bone grafts and derived composite materials

Bone grafts act as scaffolds for the ingrowth of vessels and bone-forming cells. During this osteoconductive bone regeneration process, the scaffold allows for bone to grow on its surface and inside the pores in the material. Given the biological limitations of other osteoconductive materials and the donor site morbidity after bone harvesting, the combination of osteoconductive scaffolds with osteoinductive proteins, such as BMPs, has been a major focus of research. [13,84]

Bone substitutes for use in dental and maxillofacial surgery are classified in three groups according to their origin. Allogenic bone grafts are derived from human donors, xenogenic bone grafts from other species (mostly bovine, but also equine, porcine and coralline), and the last group comprises the synthetically-produced materials. Synthetic bone grafts aim to imitate the natural bone's structure. The most widely used are the calcium phosphates, including hydroxyapatite, tri-calcium phosphates (TCP) and composites of the two. By means of a thermal treatment (sintering) and subsequent cooling they can be transferred into ceramics with a very solid but porous structure and a rough surface closely resembling human bone.

Recent studies have reported successful bone regeneration after grafting on periodontal defects, using sinus floor elevation techniques, and in post-extraction socket defects using TCP carriers [58,65, 85].

Clinical studies reporting results of GFs delivery systems in oral surgery are revised in Table 3.

Some authors have also investigated the application of GFs to dental implant surfaces to stimulate local bone formation and osteointegration. In preclinical studies, functionalized titanium implant surfaces coated with rhBMP-2 have been shown to be able to stimulate bone formation around implants [35, 86]

References	Study design	Total number of patients	Protein	Carrier	Application	Main findings
Jung et al. 2003 [40]	RCT	11	rhBMP-2	Xenogenic bone (Bio-Oss)	Maxillary implant placement	rhBMP-2 has the potential to predictably improve and accelerate guided bone regeneration therapy
Boyne et al. 2005 [38]	RCT	48	rhBMP-2	ACS	Maxillary sinus floor elevation	rhBMP-2/ACS safely induced adequate bone for the placement and functional loading of dental implants
Herford and Boyne 2008 [75]	Case review	14	rhBMP-2	ACS	Mandibular defect	Bone formation could be identified radiographically after 5 to 6 months
Van den Bergh et al. 2000 [76]		3	rhBMP-2	Type I collagen	Maxillary sinus floor elevation	Potential for initiating bone formation in the human maxillary sinus within 6 months after a sinus floor elevation, but its behavior is currently not sufficiently predictable in this application
Kao et al., 2012 [56]	Clinical trial		rhBMP-2	ACS and xenogenic bone	Sinus floor elevation	Less bone formed in patients treated with the rhBMP-2/ACS/xenogenic bone device
Alonso et al. 2010 [89]	RCT	16	rhBMP-2	Collagen	Alveolar defect closure in cleft lip and palate patients	Satisfactory bone healing at 6 months and reduced morbidity
Stavropoulos et al. 2011 [63]	RCT	20	rhGDF-5	β-TCP	Regeneration of periodontal defects	Greater alveolar regeneration, differences not statistically significant
Nevins et al. 2011 [49]	Cohort study		rhPDGF	Mineral collagen scaffold	Socket preservation	No statistically significant differences were observed
Stavropoulos et al. 2011 [58]	RCT	31	rhGDF-5	TCP	Sinus floor elevation	Comparable amount and similar quality of bone formation as in controls
Jayakumar et al. 2011 [65]	RCT	54	rhPDGF	TCP	Regeneration of periodontal defects	Increased bone formation and soft tissue healing

References	Study design	Total number of patients	Protein	Carrier	Application	Main findings
McAllister et al. 2010 [85]	RCT	12	rhPDGF	β-TCP	Socket preservation	Similar histological findings at 3 months
Triplett et al. 2009 [55]	RCT	160	rhBMP-2	ACS	Sinus floor elevation	Induced bone was significantly denser, no marked differences in histological parameters.

Table 3. Clinical studies on GF delivery systems applicable in oral surgery

4.7. Gene delivery methods

The potential applications of gene therapy have recently expanded to include the local treatment of bone defects. Gene transfer methods may circumvent many of the weaknesses of protein delivery to soft tissue wounds. The application of growth factors or soluble forms of cytokine receptors by means of gene transfer offers a greater sustainability than the use of a single protein application. Gene therapy may make growth factors more readily bio-available.

Gene transfer is accomplished by using viral and non-viral vectors. Examples of viral vectors are retroviruses, adenoviruses (Ads), and adeno-associated viruses (AAV), and non-viral vectors include plasmids and DNA polymer complexes.

Some authors have studied gene delivery via adenoviral or liposomal vectors carrying information for encoding recombinant human GFs combined with a collagen matrix in animal models [87,88].

5. Conclusion

The role of growth factors for alveolar bone regeneration in dentistry is a recent field of research, with a relative paucity of clinical studies. Findings seem to demonstrate a positive effect of GFs on intraoral hard and soft tissues healing, and the bone regeneration associated with implant therapy represents one of the main scenarios of interest. For the time being, however, the application of GFs in this field is limited by the dubious results, complications and side effects encountered so far. In particular, one of the main problems seems to be the relationship between the GF delivery and the timing of the healing process. Among the delivery systems tested to date, only collagen matrices have correlated with successful clinical results, albeit with some limitations. Other potential delivery systems have been studied only in a few animal models, and the currently available data are not enough for any final conclusions to be drawn. The development of dedicated and more "sophisticated" GF delivery systems is probably the most interesting area of research for the future.

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Section 4

Clinical Perspective of Tissue Engineering and Cell-Based Therapies

Chapter 29

Regenerative Medicine for Neurological Diseases with the Use of Electrical Stimulation

Masahiro Kameda

Additional information is available at the end of the chapter

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1. Introduction

In current clinical practice, tissue plasminogen activator (t-PA) is the gold standard drug to be administred within three hours after the onset of cerebral infarction. In the near future, it will become acceptable to administer it within 4.5 hours of onset. Even if t-PA administration cannot be utilized for cerebral infaction patients because of time window limitations, the intravascular technique using the Merci(R) retreiver and the Penumbra(R) system can be applied to some patients with cerebral infarction. Not all cerebral infarction patients can be saved by these clinical therapeutic methods. Despite surviving the acute phase of cerebral infarction as a result of these clinical therapeutic methods, some patients suffer from permanent hemiparesis in the chronic phase. It is for this reason that regenerative medicine must play more important role in solving this problem.

We have two types of regenerative medicine approaches to patients with neurological diseases such as cerebral infarction and Parkinson's disease. The first approach involves exogenous stem cells for stem cell transplantation [1-3], and the second involves the enhancement of endogenous stem cells. In terms of neural stem cell transplantation (allogenic transplantation), we demonstrated the neuroprotective effect of adult neural stem and progenitor cells that were modified to secrete Glial cell line–derived neurotropic factor (GDNF) in a transient ischemia model of rats [4]. However, we have not demonstrated the same effect by autologous transplantaion. Stem cells were transplanted in the acute stage of an ischemia animal model in many studies, which showed the neuroprotective effect of stem cell transplantation. It is difficult to show the same effect of stem cell transplantation in the chronic phase of ischemia. As a result, if we want to show the same effect by autologous stem cell transplantation, we must transplant autologous stem cells in an acute-stage ischea model of rats. Unfortunately, we do not yet have a technique to immediately expand autologous adult neural stem cells. Our previous experi-

ment showed that it took several weeks to obtain a sufficient number of autologous adult neural stem cells [5]. Many researchers have pointed out that the effect of these stem cell transplantations is derived not from cell replacement, which is the original purpose of stem cell transplantation, but mainly from the trophic effect brought about by transplanted stem cell secretions. Generally speaking, compared to exogenous stem cell transplantation, the enhancement of endogenous stem cells is expected to be less invasive. Previous reports showed that an enriched environment and running enhanced endogenous stem cell generation [6] and that trophic factors produced by these enhanced endogenous stem cells had a neuroprotective effect on these neurological disorders. Deep brain stimulation (DBS) is one of the gold standards of treatment for Parkinson's disease (PD) in current clinical pratice. Recent reports have showed that DBS can enhance endogenous stem cells in a PD model of rats [7]. Although phase 3 or 4 clinical trials with DBS for depression and obesity and epidural electrical stimulation for pain are currently ongoing, they are not being performed for regenerative medicine (http://clinicaltrials.gov). Based on this report, we examined whether electrical stimulation has therapeutic potential for CNS diseases by activating endogenous stem cells.

2. Electrical stimulation for cerebral infarction

After the onset of cerebral infarction, more endgenous stem cells are produced in the SVZ and these endogenous stem cells migrate and differentiate into neurons in order to replace the area of infarction. Most of these newly produced endogenous stem cells cannot survive, however, and die within a few weeks [8]. That is why we expected that enhancing the survival of these newly produced endogenous stem cells or enhancing the production of endogenous stem cells by electrical stimulation could result in an improved neurorescue effect.

The invasiveness of the electrical stimulation of the brain varies with the position of the electrode, e.g., brain parenchyma, brain surface (subdural), epidural space, or skin. In the DBS for Parkinson's disease, electrodes are inserted in deep brain areas such as the subthalamic nucleus. Although DBS is considered to be one of the gold standards of therapy for Parkinson's disease, less invasive and more effective stimulation techniques are urgently required. Compared to the use of exogenous stem cells such as stem cell transplantation, the enhancement of endogenous stem cells is expected to be less invasive. We believe that continuous epidural stimulation is the best choice as it is less invasive and can be expected to produce a similar neuroprotective effect as DBS.

To examine the neurorescue effect of electrical stimulation for cerebral infarction, we applied epidural electrical stimulation to an acute-stage cerebral infarction model of rats. To identify the best stimulation parameter, we stimulated the rats with different frequencies and intensities (see Table). We found that continous electrical stimulation with low frequency produced a better neurorescue effect compared to that with high frequency. Moreover, this low-frequency continous epidural electrical stimulation had a better neurorescue effect through an increase in trophic factors such as BDNF, GDNF, and VEGF (Fig.1), suppressed inflamatory response (Fig. 2), enhanced angiogenesis (Fig.2) and played anti-apoptotic effect with the

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upregulation of phosphorylated Akt (Fig.3). LY294002 administration into the lateral ventricle suppressed the neurorescue effect of electrical stimulation (Fig.4), which showed that this anti-apoptotic effect was exerted through the PI3K-Akt signaling pathway [9].

	0	2	10	50	(Hz)
100	3.9±1.5	7.6±3.2	8.2±2.2	4.4±1.7	(LPT at 3 days)
	6.3±2.7	11.8±2.2	11.1±3.2	6.2±0.9	(LPT at 1 week)
	28.0±1.5	21.3±1.4	21.8±6.9	30.5±8.7	(infarct volume)
200		8.0±2.7			(LPT at 3 days)
		10.3±3.8			(LPT at 1 week)
(μA)		23.9±8.2			(infarct volume)

Table 1. The table presents Limb Placement Test (LTP) scores (3 days and 1 week after MCAO) and the percentages of infarct volumes measured 3 days post-MCAO relative to the intact side of rats receiving electrical stimulation with several parameters. Initially, 0, 2, 10, and 50 Hz were used with 100 μA current. Next, exploration using 200 μA was performed. Based on this experiment, we decided that 2 Hz 100 μA stimulaton was the optimal therapeutic condition. This table was reproduced and/or modified from the original article [9].

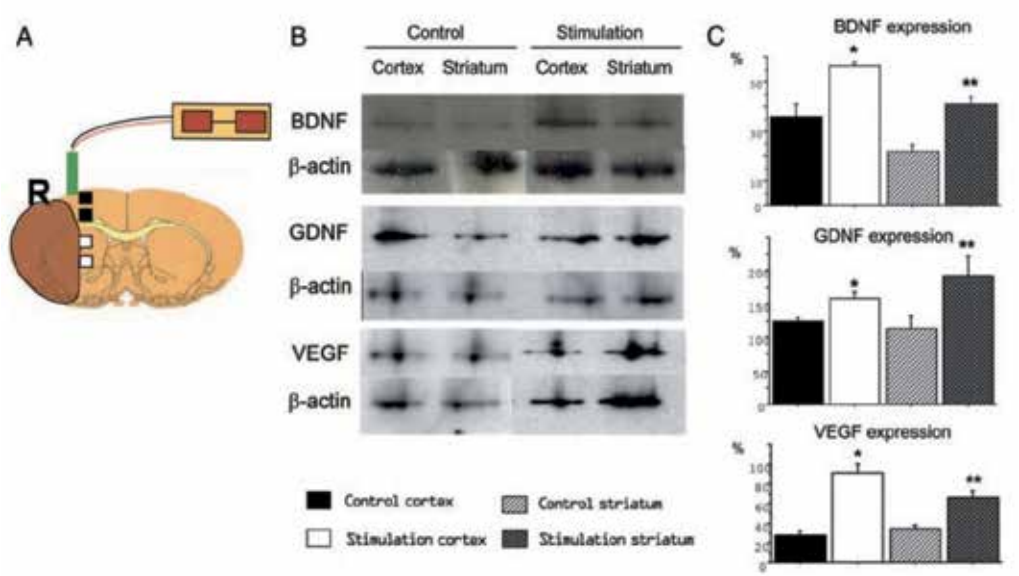

Figure 1. (A) The schematic diagram shows the regions in the cortex and striatum of rats with electric stimulation. Black box, cortex; white box, striatum. Each pair of brain tissues in the cortex and striatum per hemisphere was punched. (B) Upregulation of neurotrophic factors such as BDNF, GDNF, and VEGF was observed in the stroke rats receiving electric stimulation. (C) Quantification. *$p < 0.05$ versus cortex of the control rats. **$p < 0.05$ versus striatum of the control rats. This figure was reproduced and/or modified from the original article [9].

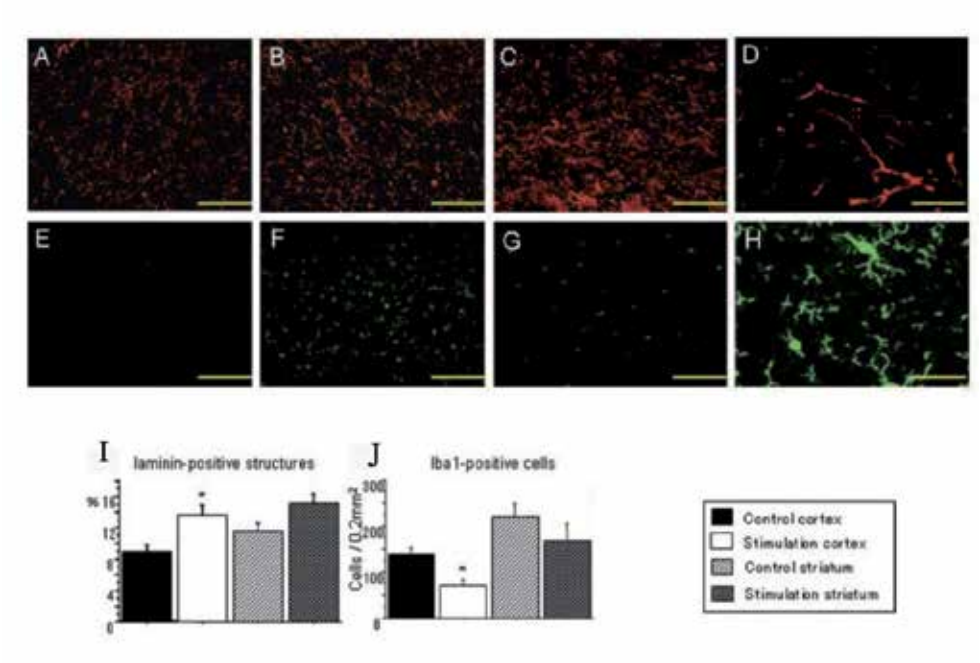

Figure 2. Laminin staining (A–D and I) and Iba-1 staining (E–H and J) show angiogenic and anti-inflammatory effects in the stimulation cortex were exerted by electric stimulation. A and E are the intact cortex, B and F are nonstimulated cortex, and C, D, G, and H are the ischemic cortex (D and H are with high magnification). *$p < 0.05$ versus cortex of the control rats (I and J). Scale bar: 100 μm (A–C, E–G), 25 μm (D, H). This figure was reproduced and/or modified from the original article [9].

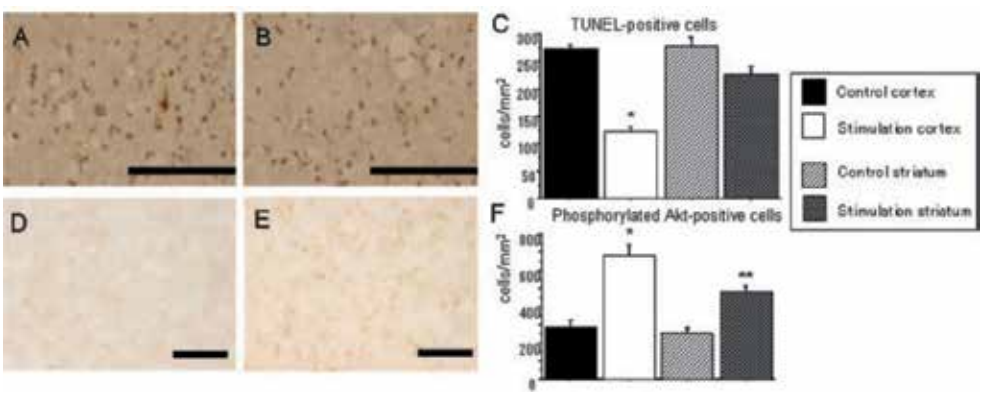

Figure 3. Anti-apoptotic effects of electric stimulation were exerted through phosphorylated Akt. (A-C): TUNEL staining revealed the anti-apoptotic effect of electrical stimulation (B) compared to those in the control group (A). Scale bar: 100 μm. (C): Quantitative analyses. *$p < 0.05$ versus cortex of the control rats. (D–F): Phosphorylated Akt staining revealed a surge of stained cells in the ischemic cortex with electric stimulation (E) compared with the control group (D). (F) Quantitative analyses. *$p < 0.05$ versus cortex of the control rats. **$p < 0.05$ versus striatum of the control rats. This figure was reproduced and/or modified from the original article [9].

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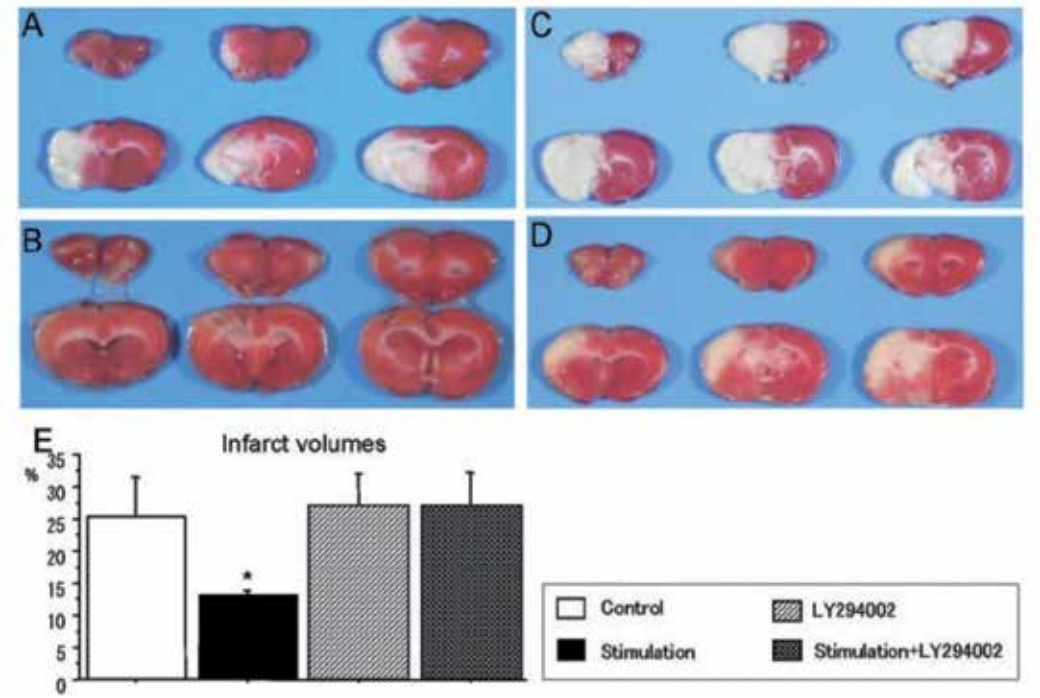

Figure 4. LY294002 blocked the neuroprotective effects of electric stimulation. The infarct volumes of rats receiving electric stimulation (B) were significantly decreased compared with those of control rats (A) and ischemic rats that received LY294002 alone (C). In contrast, the neuroprotective effects of electric stimulation were blocked by intraventricular administration of LY294002 (D). Quantitative analysies of infarct volumes is shown in E. *$p < 0.05$ versus all other groups. This figure was reproduced and/or modified from the original article [9].

As the next step, we applied epidural electrical stimulation to a chronic-phase cerebral infarction model of rats. However, we could not obtain the same neuroprotective effect. This is why we needed to change the method. According to the clinical application of DBS for Parkinson's disease, we decided to insert an electrode in the infarct striatum although this method is more invasive than epidural stimulation. Before giving striatum electrical stimulation to the chronic-phase ischemia model of rats, we applied electrical stimulation to normal rats and found that low-frequency continuous striatal electrical stimulation upregulates GDNF and vascular endothelial growth factor (VEGF) and enhances the neuronal differnetiation of endogenous stem cells and angiogenesis. We then applied striatal stimulation to the chronic-phase ischemia model of rats. We conducted magnetic resonance imaging 28 days after ischemic induction and inserted electrodes into the striatum based on these MR images followed by electrical stimualation for a week; rats were sacrificed on day 60. In this experiment, we found that striatum stimulation exerted behavioral improvement and MR images taken after striatal stimulation showed a reduced infarct volume compared with those taken before stimulation. Moreover, striatal stimulation during the chronic-phase ischemia model of rats resulted in enhanced migration of neural progenitor cells from SVZ to ischemic

penumbra, enhanced neuronal differentiation, and suppressed microglial activation in the ischemic penumbra [10].

3. Long-Term Potentiation (LTP) and neurogenesis

It is well established that new neurons are produced continuously in the SVZ and the subgranular zone (SGZ) of the dentate gyrus (DG) during adulthood [11-13]. Given this fact, many researchers have focused their attention on discovering how to stimulate these resident precursors to enhance neurogenesis. Environmental enrichment and voluntary running [14] are now widely accepted ways to enhance neurogenesis.

Long-term potentiation (LTP) is induced by brief high-frequency tetanization of excitatory afferents and is observed as a long-lasting enhancement in the efficacy of synaptic transmission [15]. LTP is considered to be a cellular model of learning [16] and memory [17]. Moreover, LTP is speculated to be one possible mechanism of enhanced motor function recovery resulting from electrical stimulation to the cortex after stroke [18]. Based on the accumulated evidence of hippocampal neurogenesis, the relationship among hippocampus-related function, LTP, and neurogenesis has been examined [19-22]. For example, environmental enrichment and running enhanced hippocampus-related memory function as well as LTP. More importantly, electrophysiological reports have demonstrated that recently created neurons contribute to synaptic plasticity [23] and that LTP correlates with neural plasticity, compensating for ischemia-induced damage [24]. These findings support the hypothesis that enhancing neurogenesis may be useful in the restoration and repair of a damaged brain and emphasizes the importance of finding a more effective way to stimulate neurogenesis. As mentioned above, a number of reports are available that describe the relationship between neurogenesis and LTP but these mainly focus on neurogenesis-dependent changes in LTP [19, 21, 22]. Only a few reports have shown that LTP per se can enhance neurogenesis [25-27].

Neural activity was recently demonstrated to stimulate a large number of hippocampal latent precursors, including a self-renewing stem cell population that only becomes activated following depolarization [28]. Given a number of recent reports linking LTP and hippocampal neurogenesis, we examined whether LTP is able to enhance proliferation of the neural precursors and neurogenesis in the adult mouse dentate gyrus. In this study, we stimulated the perforant pathway unilaterally with several stimulation protocols and analyzed hippocampal precursor numbers using both the neurosphere assay and BrdU immunostaining. In the neuropshere assay, we found that induction of late-LTP, but not early-LTP, was required to activate latent precursors in the mouse dentate gyrus and that neurosphere number was positively correlated with LTP magnitude (Fig.5 & Fig.6). In the BrdU immnostaining, proliferation and differentiation in the dentate gyrus was significantly enhanced in the only mice in which LTP was successfully induced. In contrast, protocols that failed to induce LTP, induced only early LTP; used low-frequency stimulation (LFS) or included a pharmacological blocker of LTP (CPP: (3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid), all failed to activate neurogenesis (Fig.7). These findings demonstrate that LTP can activate latent neural precursor cells in the adult mouse dentate gyrus, leading to increased neurogenesis [29].

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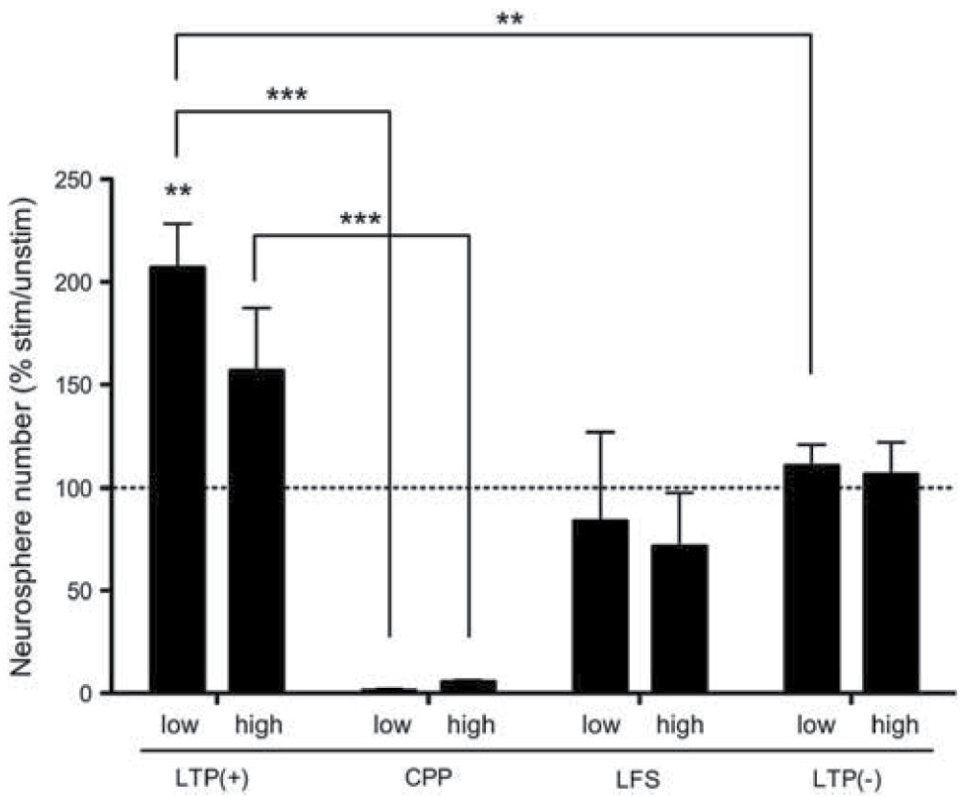

Figure 5. LTP enhances proliferation of neural precursors. A neurosphere formation assay was performed 2 days after stimulation. Under normal K^+ conditions, only the LTP(+) group revealed a significant increase in neurosphere number from the stimulated hemisphere relative to the control hemisphere. Furthermore, a significantly greater number of neurospheres was generated from the stimulated hippocampi of the LTP(+) group compared with the simulated hippocampi of the CPP group and LTP(-) group. When CPP was administered to block the NMDA receptor, neurosphere formation was significantly reduced compared with the LTP(+) group. low: low K^+, high: high K^+. $^{**}p < 0.01$, $^{***}p < 0.001$. This figure was modified and/or reproduced from the original article [29].

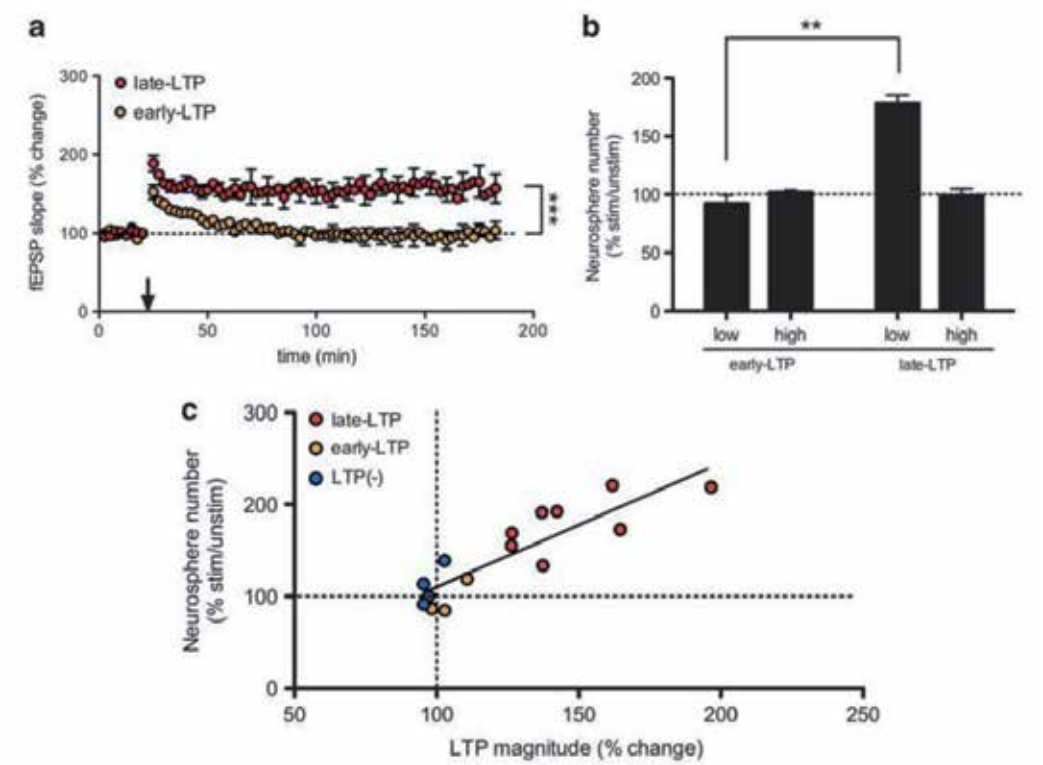

Figure 6. Late-LTP but not early-LTP can activate hippocampal precursors in vitro. (a) At the end of recording, the magnitude of potentiation in the late-LTP group was significantly greater than that in the early-LTP group. (b) Following the induction of late-LTP, a significant increase in the number of neurospheres was observed in the low K^+ condition compared with the early-LTP group. (c) The magnitude of LTP, calculated 60 min after HFS, showed a significant positive correlation ($r = 0.879$, $n = 15$, $p < 0.0001$, Pearson's correlation) with the number of neurospheres grown in vitro, after hippocampi were dissected 2 days after HFS. The arrow indicates HFS, error bars indicate SEM, low: low K^+, high: high K^+. $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: fEPSP, field extracellular postsynaptic potential; HFS, high-frequency stimulation. This figure was modified and/or reproduced from the original article [29].

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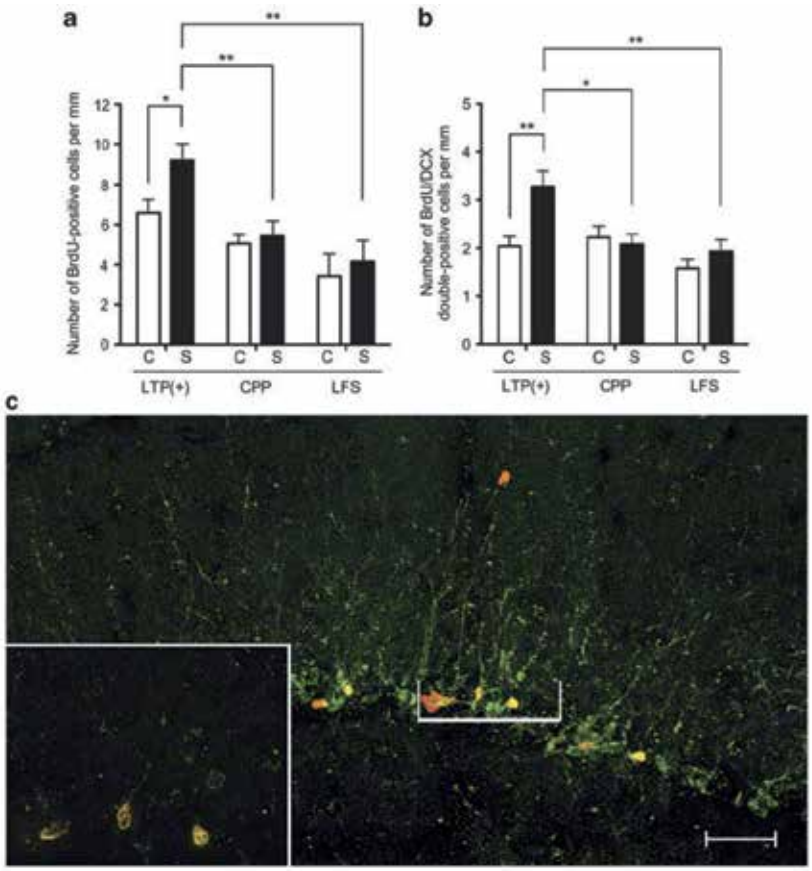

Figure 7. Proliferation and differentiation were significantly enhanced in the LTP(+)-stimulated hippocampus. (a) The number of BrdU-positive cells per length of the dentate gyrus (mm) was significantly enhanced in stimulated hippocampi of the LTP(+) group compared to the unstimulated hemisphere. Furthermore, there was a greater number of BrdU-positive cells following LTP induction compared with the CPP group and LFS group. (b) Following LTP induction, the number of BrdU/DCX double-positive cells per perimeter length of the dentate gyrus (mm) was significantly enhanced in the stimulated hippocampus compared with the control hippocampus. BrdU/DCX-double labeling also revealed a significantly greater number of neurons in the stimulated hippocampi of the LTP(+) group compared with the stimulated hippocampi of the CPP and the LFS groups. (c) Representative maximum intensity projection image of BrdU (red) and DCX (green) immunostaining reconstructed from 40 optical sections of the LTP-stimulated hippocampus of the LTP(+) group. Inset shows a higher magnification. Scale bar: 50 mm in main image, 20 mm for inset. *$p < 0.05$, **$p < 0.01$. Abbreviations: C, control side; DCX, doublecortin; S, stimulated side. This figure was modified and/or reproduced from the original article [29].

In the future, these findings can be clinically applied to individuals suffering from depression, Alzheimer's disease, and age-related cognitive decline explained by neuronal loss. In fact, electrical convulsive seizure is clinically performed for severe depression and this therapy showed increase of the proliferation and neuronal differnetiation of hippocampal precursors in a depression model of animals [30-32]. For Alzheimer's disease, a previous report showed that transcranial direct current stimulation enhanced aspects of memory performance in both healthy controls and individuals with Alzheimer's disease [33]. However, an operation for

electrode insertion is needed, and this is invasive for patients. To reduce invasiveness, transcranial magnetic stimulation (TMS), which is currently clinically applied to patients with severe depression, would solve this problem. For age-related cognitive decline, TMS is currently the main therapeutic option; however, more evidence needs to be collected. Continuous learning and exercise is the best method to overcome age-related cognitive decline.

In the future, we need to clarify the mechanism of electrical stimulation and neurogenesis in more detail. In the electrical stimulation conducted for cerebral infarction, we found that the optimal stimulation parameter was low frequency; however, in this LTP experiment, neurogenesis was enhanced by HFS that can induce LTP. Disease model (cerebral infarction) or healthy model is a crucial difference between these two experiments. This difference in animal condition might be one possible explanation for this discrepancy in optimal stimulation parameters. As written above, we found the therapeutic potentials of electrical stimulation; however, further studies and ethical consensus are required for its application in a clinical setting because electrical stimulation has the potential to modulate the human mind and cognition.

4. Conclusion

The therapeutic benefit of electrical stimulation to neurological diseases was reviewed in terms of the enhancement of endogenous stem cells. Electrical stimulation was demonstrated to play a neuroprotective and/or neurorescue effect in the ischemia model of animals. For intact animals, LTP is most effective at activating hippocampal latent stem cells. Electrical stimulation should be considered a possible therapeutic method in the future, although further studies and ethical consensus are required before use in the clinical setting.

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Chapter 30

Pigmented Skin Models: Understand the Mechanisms of Melanocytes

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Skin and melanocytes

Skin is composed of three layers: epidermis, dermis and hypodermis. The epidermis is organized into five layers in which there are different cell types. The most important cell type in the epidermis is the keratinocytes which constitute approximately 95 % of the total epidermal cells. Melanocytes, Langerhans cells, Merkel cells and inflammatory cells form the remaining 5 % [1]. Among these cells, the melanocytes, which are dendritic cells, are the second most important cell type in the epidermis. They have the capacity to synthesize melanin, a skin pigment. Melanocytes are not only found in the skin, but can also be observed in hair, eyes, ears and central nervous system (Table 1) [2-3]. Their different localization gives them different functions in the organism, but they all keep a common function: melanogenesis [1].

1.1.1. Skin melanocytes

Skin melanocytes are localized in the basal layer of the epidermis, at the junction of the dermis, and their dendrites expand between keratinocytes of the next layer. These dendrites allow melanocytes to make contact with the keratinocytes for the melanin transfer. This cell-to-cell contact stimulates proliferation and differentiation of melanocytes due to growth factors produced by the keratinocytes [4]. Thus, the melanosome, organelle containing melanin pigments, can be transferred to the adjacent keratinocytes, which store the pigments, and degrade them when they move to the skin surface [5]. When the pigments are in keratinocytes, they give a color to the skin with a mix of other pigments such as carotenoids and hemoglobin derivatives [6-7]. However, melanin is the principal pigment present in the skin [7] and can be

Body localization	Functions
Skin	Skin pigmentation
	Photoprotection
Hair	Hair pigmentation
Eyes	Vision
	Photoprotection
	Eye pigmentation
Ears	Hearing
	Protection against high intensity noise
Central nervous system	Scavenge toxic cations

Table 1. Melanocyte functions dependent on the body localization

found in two different colors: yellow/red (pheomelanin) and brown/black (eumelanin) [8]. These two types of pigment are one of the explanations for different ethnic skin color in the world. The other causes will be covered in the next section. However, ultraviolet radiations (UVR) are the main factor causing pigmentation variation in the skin. They can produce photodamage, erythema, mutations, vitamin D synthesis and tanning of the skin. Current research on the mechanism of UV-induced pigmentation (tanning) suggests that UVR induced DNA damage, and the mechanism for repair of these damages by a specialized endonuclease increases the production of melanin [6, 9]. This quantity of produced melanin acts as a skin protective barrier against the UVR by absorbing the UV photons [10]. Some research demonstrates that people who have more pigmented skin have less risk of developing skin cancer or sunburn. Moreover, eumelanins, which are presented in high quantity in dark skin, are more photoprotective than pheomelanins. They absorb free radicals generated in the cells by UVR, thus preventing DNA damage [11].

1.1.1.1. Ethnic skin types

The different ethnic skin color worldwide depends on various factors such as UV exposition, genetics, environmental factors and skin pigments [12]. Melanin is the principal pigment which can affect skin color in several ways. The number of melanocytes, the melanogenic activity, the melanin type, the size and the number of melanosomes and the distribution in the epidermis can also affect the skin pigmentation [6]. In 2002, Alaluf *et al*. demonstrated that Causasian skin was characterized by a low number of melanocytes (1 for 36 keratinocytes), small melanosomes and light pigments such as pheomelanins while black skin was characterized by the presence of a higher number of melanocytes, larger melanosomes, a higher quantity of melanin and more eumelanins [13]. It seems that the size of melanosomes is important in the skin pigmentation because large melanosomes are found in the epidermis as single particles, while small melanosomes tend to aggregate them. It could have the effect that the large melanosomes are transferred to keratinocytes individually and thus, can absorb light better than melanosomes transferred in complex [14].Their distribution in the epidermis is equally very important and research demonstrated that in white skin, melanosomes are

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completely degraded before the *stratum corneum* while in dark skin, melanosomes are found in this layer [15]. Therefore, all these characteristics contribute to ethnic groups their specific skin color.

1.1.2. Other melanocytes

1.1.2.1. Hair and eye melanocytes

Melanocytes have a predominant role in skin color, but they have equally the same function in hair and in eyes. In hair, they are localized in the bulb, at the bottom of the hair follicles, to give the hair's color. The same pigments found in skin, such as pheomelanin and eumelanin, are also responsible for the different colors. Counter to skin melanocytes that rarely proliferate, hair melanocytes can proliferate and differentiate in each hair cycle. This cycle includes the growing phase (anagen) in which there is melanogenic activity and hair is pigmented. At the end of this phase, the hair follicle regresses, the melanogenic activity decreases and the melanocytes retract their dendrites to finally stop the pigmentation. These non-active melanocytes are replaced by new melanocytes, which are recruited from the pigment cell reservoir, to restart a new hair cycle [16]. In eyes, there are two types of melanocytes: conjunctival and uveal. The first type is localized in the conjunctiva and transfers melanin to conjuctival epithelium while the second is localized in the iris and produces as well as stores melanin, but it does not transfer melanin to any other cells [17]. The quantity or the type of melanin in uveal melanocytes characterizes different eye colors. Such as in the skin, melanin contains in uveal melanocytes may have a protective role for the eyes against UVR. However, few studies have been conducted on this subject and uveal melanocytes remain unknown.

1.1.2.2. Ear and central nervous system melanocytes

Melanocytes have a well-known pigmentation function, but they also have an important role in ears and in the central nervous system. Melanocytes in ears are located in the stria vascularis of the cochlea, which is formed of three cell types: marginal cells, basal cells and intermediate cells [18]. Intermediate cells are composed of two types of melanocytes, such as light cells that are able to synthesize melanin, and dark cells that are incapable. They are necessary to the normal function of ears, and damages or loss of these cells can cause the hearing loss [19]. Some studies demonstrated that albino guinea pigs are more sensitive to high intensity of noise than pigmented guinea pigs because of their low quantity of melanocytes [20]. In the nervous central system, melanocytes are distributed on the meninges, particularly on the leptomeninges that cover the brain. Their role in the organism is not yet well-determined, but it is known that leptomeninge melanocytes have the capacity to capture toxic cations and free radical species from the blood circulation [19].

1.1.3. Melanogenesis

Melanocytes originate in neural crest precursor cells: the melanoblasts. Melanoblasts migrate, proliferate and differentiate into melanocytes to reach their destination, such as skin, eyes, hair, meninges and ears [3]. Melanin is synthesized in these melanocytes in specialized lysosome-

like organelles named melanosomes. These organelles have four stages of maturation in which the melanosome begins unpigmented and ends pigmented [5]. The process of melanin synthesis is called melanogenesis and it needs three enzymes to assure its good working: tyroninase, tyrosinase-related protein 1 (TRP1) and tyrosinase-related protein 2 (TRP2). These three enzymes are necessary for the regulation of the melanogenesis, but tyrosinase is the limiting factor of this pathway. It catalyzes the first two reactions of the biosynthesis of melanin that are necessary for producing eumelanin and pheomelanin [21] while TRP1 and TRP2 are only involved in the pathway of eumelanin [1]. Tyrosinase uses tyrosine, DOPA and 5,6-dihydroxyindole (DHI) as substrate to produce respectively DOPA, DOPA-quinone and DHI-melanins (Figure 1). Tyrosinase activity is regulated by some factors such as the pH, which needs to be optimal at 6.8 in melanosomes [22], and the melanocyte-stimulating hormone (α-MSH) secreted by melanocytes. The α-MSH binds melanocortin receptor-1 (MC1R), which is expressed by melanocytes, and triggers eumelanin pigments production whereas when α-MSH does not recognize MC1R, pheomelanin pigments are generated [9]. Following this production of melanin, the melanosomes which have pigments are transported towards the end of the melanocyte dendrite by actin and tubulin filaments. Then, melanosomes are transferred to keratinocytes (when it is skin melanocytes). The mechanism of transfer is still unknown, but there exist some hypothesis about this mechanism such as cytophagocytose [23], discharge melanin in the intercellular space [24], or transfer by filopodia [25]. Once in keratinocyte, melanosomes are distributed around the nuclei and, in response to UVR, they form a supranuclear melanin cap on the sun-exposed side of the nuclei to protect DNA against the UVR damages [26]. Melanin pigments are degraded with the keratinocytes when they move to the epidermal surface for their differentiation [11].

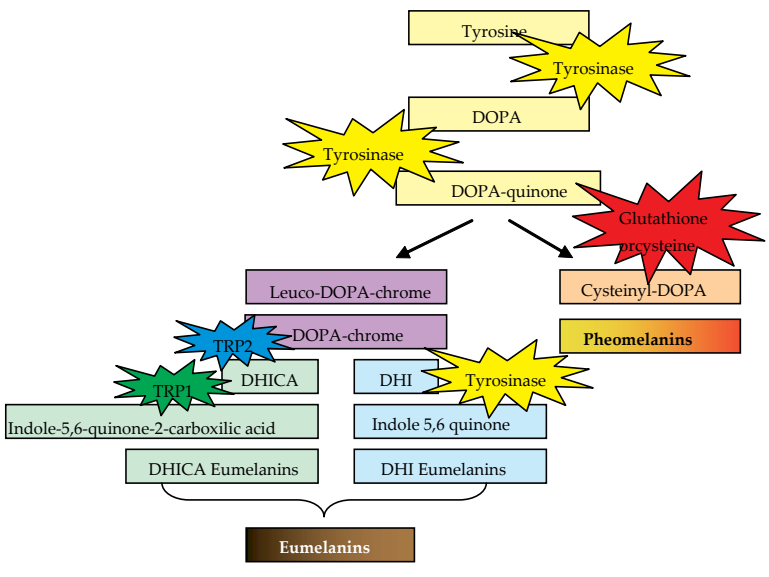

Figure 1. Melanogenesis mechanism. TRP1: Tyrosinase-related protein 1; TRP2: Tyrosinase-related protein 2; DHI: 5,6-dihydroxyindole; DHICA: 5,6-dihydroxyindole-2-carboxylic acid.

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1.2. Pigmentation diseases

Disorders in melanogenesis or in melanocytes can lead to different skin pigmentation pathologies. Some disorders are characterized by a loss of skin pigmentation (hypopigmentation) while others are recognized by the presence of dark plaques on the skin formed by an increase of pigmentation (hyperpigmentation).

1.2.1. Hypopigmentation disorders

Hypopigmentation disorders affect skin pigmentation by the destruction of melanocytes, by preventing development of melanocytes and by inhibiting or retarding melanin production. Vitiligo is characterized by the first mechanism, piebaldism by the second, while oculocutaneous albinism and tinea versicolor are characterized by the third mechanism [27]. These three mechanisms lead to white macules/plaques on the skin because of the lack of melanin pigments.

1.2.1.1. Vitiligo

Vitiligo is an autoimmune hypopigmentation disorder that affects approximately 1-2 % of the population worldwide. This disease is characterized by the presence of white macules or patches on the skin caused by the loss of functioning epidermal melanocytes [28]. Studies suggest three principal hypotheses on the mechanisms of the melanocyte destruction: autoimmunity, neural and toxic hypothesis [29-30]. Autoimmune diseases such as thyroid diseases [31] and diabetes mellitus [32] are often associated with vitiligo. These diseases cause defects in the immune system, which can cause destruction of melanocytes and the loss of pigmentation [29]. In addition, antibodies against melanocytes were found in serum of patient, and these can engage the apoptosis of melanocytes when they are present [33]. T cells were also found in perilesional vitiligo plaque biopsies and they are enriched with cytotoxicity against melanocyte antigens [34]. The neural hypothesis is based on the contact of the melanocytes with nerve endings in depigmented skin [35]. Neuropeptides and nerve growth factors such as tumor necrosis factor-α, intercellular adhesion molecule-1 and interferon-γ were found in perilesional skin, which suggest that nerves can have a role in destruction of melanocytes [30]. The toxic hypothesis suggests that the mechanism of natural protection of melanocytes is defective. The melanocytes are unable to eliminate toxic molecules, and these are accumulated in the cells [36]. More than these three mechanisms, the loss of melanocytes can be induced by environmental factors, genetic predispositions [37], apoptosis or metabolic dysfunctions [38]. All these hypotheses are a good way to understand the pathology, but the one single mechanism of the melanocyte destruction in vitiligo is still unknown.

1.2.1.2. Piebaldism

Piebaldism is an uncommon hypopigmented disorder characterized by the presence of a congenital white forelock and white macules on the extremities, forehead, frontal scalp and ventral trunk [39]. This pathology is caused by mutations of loss-of-function in the *KIT* gene that encodes for the stem cell growth factor receptor expressed in mastocytes and in melano-

cytes. When activated, *KIT* is essential to the development of melanocytes and stimulates their proliferation [40]. These mutations prevent the development and the proliferation of melanocytes, thereby causing white macules without melanocytes in skin and in hair.

1.2.1.3. Oculocutaneous albinism

Oculocutaneous albinism (OCA) is characterized by a disorder in the melanin synthesis in the melanosome due to mutations in specific genes. Tyrosinase or other enzymes essential to the melanogenesis are absent or dysfunctional, resulting in an incapacity in the melanosome to synthesize melanin [41]. People affected by this disease have a complete or a partial loss of pigmentation of the skin, hair and eyes, with the reduced pigmentation in eyes causing a lowering of visual acuity. Four types of albinism exist, all characterized by mutation in a different gene (Table 2). The most severe type is OCA1A, which is characterized by a complete loss of hair and skin pigmentation, while eyes are light blue almost pink. People affected by OCA1B can develop pigmentation on skin, hair and eyes, but they have a characteristic temperature-sensitive pigmentation. Hairs on hands and feet can be pigmented, while body hairs stay depigmented. The most common type worldwide is the OCA2 characterized by various amount of cutaneous pigment and better vision than OCA1. OCA2 and OCA4 have the same clinical characteristics, but differ in the responsible gene. People affected by OCA3 have characteristic red hair and reddish brown skin [42].

1.2.1.4. Tinea versicolor

Tinea versicolor is a common pigmentation disorder that can be characterized by round shaped hypopigmented and hyperpigmented macules on the face, trunk and arms. These macules are caused by the fungal infection, *Malassezia*, which is a genus normally found in the skin flora [43]. Several factors play a role in the transformation of the benign form of the fungi to the parasitic form. Fatty skin, exposure to sunlight, genetic predispositions, malnutrition and corticosteroids can lead to development of lesions [44]. This lipophilic fungus metabolizes various fatty acids and releases, as one of the metabolites, azelaic acid. This acid acts as an inhibitor of tyrosinase, blocking the transformation of tyrosine in melanin pigment, resulting in hypopigmented macules on the skin. Tinea versicolor is often found in young adults because their sebaceous glands are very active due to the action for sex hormones [45]. The mechanism of hyperpigmented macules is not as well-known as the previous mechanism. Some studies demonstrate that melanosomes are larger in hyperpigmented macules than melanosomes in normal skin and in white macules, but the cause of these enlarged melanosomes is still unknown [14, 45-46].

1.2.2. Hyperpigmentation disorders

Hyperpigmentation disorders are characterized by darker skin that can be caused by an increase of melanin synthesis or an increase of the melanocytes in the epidermis. "Café-au-lait" macules, Addison's disease and postinflammatory hyperpigmentation are characterized by an increased production of melanin, while melanoma is the result of the two causes.

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OCA type	Responsible gene
OCA1	Tyrosinase
OCA2	*P* gene
OCA3	*TRP1*
OCA4	*SLC45A2*

Table 2. Gene responsible of oculocutaneous albinism (OCA) different types. TRP1: Tyrosinase-related protein 1

1.2.2.1. "Café-au-lait" macules

"Café-au-lait" macules are characterized by light to dark brown spots of 1 to 20 cm on the skin that can be of a congenital origin, or appear during life [47]. These spots are often the first sign for the diagnosis of type 1 neurofribromatosis, a disorder caused by mutations in the *NF1* gene, and which can lead to benign and malignant tumors of the peripheral nerve sheath [48]. "Café-au-lait" macules are caused by an increase of melanin in melanocytes and the presence of larger melanosomes in keratinocytes, but the mechanism is still not well-known [49].

1.2.2.2. Malignant melanoma

Malignant melanoma is one of the most severe skin cancers affecting both men and women. It begins in melanocytes which have been mutated and proliferates to form an irregular naevus, a dark pigmented spot on the skin. In several cases, melanoma begins by forming a normal mole, which is grows increasingly and becomes a metastatic melanoma [50]. Some factors can affect the proliferation of the melanocytes such as genetic factors [51], environmental factors [50], spontaneous mutations [52] and endocrine factors [53]. The most important factor playing a role in this cancer is ultraviolet radiation exposure. UVR cause damages to DNA, the cell usually being able to repair this by the transcription of one of the tumor-suppressor genes, for instance, *p53*. The protein produced by *p53* genes stops the cycle cell, preventing the reproduction of DNA mutations. When there are mutations in the *p53* gene, the cell cycle can not be stopped and mutations in DNA are reproduced and accumulated in the cell that will lead to a hyperproliferation of melanocytes and a melanoma [54]. Other genes such *GADD45*, *PTCH*, *p16*, and oncogenes such as *Bcl-2*, *ras*, *c-fos* can be involved in the pathway of UV-induced melanoma [55].

1.2.2.3. Postinflammatory hyperpigmentation

Postinflammatory hyperpigmentation occurs after cutaneous inflammation or injury and causes dark plaques on skin. It can affect all types of skin, but dark-skinned people are mostly affected, especially after acne [56]. Hyperpigmented plaques are caused by an overproduction of melanin or an irregular distribution of melanin in the epidermis [57]. The exact mechanism is not well-known, but it has been shown that cytokines, chemokines, inflammatory mediators such as leukotrienes, prostaglandins E2 and D2, thromboxane-2, interleukin IL-1 and IL-6, TNF-α and some others can stimulate melanocyte activity and promote the production of melanin [58].

1.2.2.4. Addison's disease

Addison's disease, also called adrenal cortical insufficiency, is a rare endocrinal disorder that in more than 50 % of cases is caused by an autoimmune disease. The adrenal glands can not produce enough steroid hormones, resulting in a deficiency in corticosteroids [59]. One of the first indicator symptoms of this disorder is the development of a diffuse hyperpigmentation of the skin (DHP). DHP is caused by the increase of adrenal corticotrophic hormone (ACTH) in the circulation, which has the same precursor molecule to α-MSH, the melanocytes-stimulate hormone. Considering that ACTH has approximately the same composition as α-MSH, they will have the same function in the organism: stimulate melanin production [60]. This production of melanin gives people affected by Addison's disease a brown skin, intensified at the sites that are exposed to light and pressure, in the skin folds, lines of the hands, nipples and areas of scarring [59].

1.3. Available treatments

1.3.1. Cosmetic treatments

Make up, topical dyes and tanning cream are frequently used to cover up undesirable plaques caused by different pigmentation diseases. Cosmetics treatments are practiced to improve the quality of live for young people that can not undergo surgeries because it is not recommended before adulthood. In vitiligo and in piebaldism, dihydroxyacetone (DHA), the browning ingredient in tanning formulations, can be used to camouflage depigmented lesions. DHA polymerizes amino acid of the skin creating pigmentation similar to UV tanning and covering the depigmented plaques of vitiligo and piebaldism patients [61]. However, no melanin pigment is produced and DHA is much less protective against UV than melanin [62]. Some other tanning products can be useful for people affected by a hypopigmentation disorder, while for the hyperpigmentation disorders, patients may use cosmetics such as make-up to cover up their lesions. In postinflammatory hyperpigmentation, scars of acne are frequently concealed with makeup and allow affected people to have a better quality of life [56].

1.3.2. Therapeutic treatment

1.3.2.1. Hypopigmentation disorders

Therapeutic treatments, unlike cosmetic treatments, are durable and usually treat the disease. For hypopigmentation disorders, treatment objectives are to increase the production of melanin and the quantity of melanocytes. In vitiligo, the most popular treatment is psoralen-UVA phototherapy (PUVA). This treatment uses the extract of plants such as 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and a synthetic compound, trisoralen (TMP). These compounds can be used orally or in topical agents by patients, after which, they have to be exposed to sunlight or UVA radiation [36]. PUVA affects skin by increasing the number and the activity of melanocytes in the epidermis resulting in an augmentation of the pigmentation [63]. Another attractive treatment for vitiligo is immunomodulators. It is suggested that, in this disease, T cells play a role in destruction of melanocytes, and researchers are trying to

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find immunomodulators that will inhibit them. Cyclosporine is one of them, and prevents the activation of T cells by inactivation of the calcineurin, which is a regulate transcription factor of T cells [64]. Levamisole is another immunomodulators that has been studied for vitiligo treatment, and its sound effectiveness has been demonstrated [65]. The mechanism of action of this compound is not well-known, but no side effect was reported. Vitiligo and piebaldism can be treated with surgeries such as suction blister grafting [36, 66], noncultured melanocytes transplantation [67], cultured epidermis grafting [68] and autologous minigrafting [30]. In tinea versicolor, considering that the disease is caused by a fungus, the principal treatments are topical or oral antifungal agents. Nonspecific topical antifungal agents exist that do not directly affect the fungus, and specific topical antifungal agents that specifically affect the fungus. The most popular nonspecific agents are selenium sulfide and benzol peroxide which chemically remove the infected tissue and prevent a recurrence. Azoles, terbinafine and ciclopiroxolamine are some groups of drugs that are frequently used as specific topical agents to directly attack the fungus [69]. Most of these agents can be taken orally as specific topical agents, and are more effective and simpler for the patient [43]. For oculocutaneous albinism, unfortunately, no treatments are reported to be effective for repigmentation of affected people.

1.3.2.2. Hyperpigmentation disorders

In hyperpigmentation disorders, therapeutic treatments have to diminish the melanin production and the quantity of melanocytes. "Café-au-lait" macules are mainly treated with lasers. These lasers must emit a specific wavelength that will be well-absorbed by the chromophore being treated. In this disease, the referred chromophore is the melanin that has a well-absorbance at 694 nm [70]. Melanin pigment absorption decreases when the wavelength of the laser increases, and thus in this treatment, it is important to choose the appropriate wavelength [71]. For malignant melanoma, several treatments are available, but research for more effective treatments still continue. Currently, the main treatment is the excision of the malignant melanoma, with excision of a large region around the site to make sure that no cancer cell remains. If there are metastases in many other organs, surgery can rarely treat it. In these cases, patients resort to chemotherapy, using drugs which will kill cancer cells by passing into the bloodstream. If the melanoma is recurrent, patient will undergo radiation therapy, high energy rays that cause damage to cancer cells and inhibit their growth, preventing the spread of other malignant melanomas. Immunotherapy is also used for the treatment of this cancer. This therapy consists of strengthening the immune system of the patient so that he can fight against cancer cells. Cytokines such as interpheron-α, interleukin-2 and TNF can be used to stimulate the patient's immunity [72-73]. Unlike the previous two diseases, postinflammatory hyperpigmentation is principally treated with the utilisation of medications such as hydroquinone, mequinol, retinoids, azelaic acid and ascorbic acid [56]. Hydroquinone, azelaic acid and mequinol all affect tyrosinase to inhibit the melanin production, but not in the same way. Hydroquinone and azelaic acid will interfere with tyrosinase, while mequinol acts like a competitive substrate of tyrosinase [74]. Retinoids such as tretinoin and tazatorene help the penetration of other medications through the skin barrier by causing skin irritation and inducing the apoptosis of mature melanocytes [75]. Ascorbic acid suppresses the melanin production by reducing the formation of quinones, creating a lack in the melanogenesis process

[76]. However, postinflammatory hyperpigmentation can be treated by a peeling surgery, a technique that uses chemical products for destruction of a part of the dermis and/or the epidermis [77]. Finally, diffuse hyperpigmentation in Addison's disease is usually treated with mineralocorticoid and glucocorticoid [78]. These two corticosteroids will compensate for the lack of corticosteroids, reduce the production of ACTH, and thus, reduce the production of melanin pigments. Considering that it is a rare disease, treatments are not very abundant. The development of pigmented skin models could be useful for studying unknown mechanisms involved in these disorders, and for developing more relevant treatments with few side effects.

2. Challenges for the development of pigmented skin models

2.1. Tissue engineering

It is known that there is a real need for human organs available for transplantations [79]. Each year, people die while they are waiting for a compatible organ. Just in Canada, in 2011, a person needing an organ had a 30 to 40% probability of not receiving it [80]. Moreover, there are some problems with allogeneic grafts, such as problems of incompatibility and reject preoccupations. To get rid of these problems, a new approach emerged at the end of the 1980s [81]. This approach, called tissue engineering, is a science that combines both biology and engineering expertise. Its goal is to develop biological substitutes for maintaining, repairing or regenerating human organs or tissue, such as skin [82-83].

2.2. Skin substitutes

Skin substitutes are useful in different fields. They can be grafted onto patients suffering from severe burns or chronic wounds such as skin ulcer [84-85]. They can also be used for fundamental research to analyze skin functional mechanisms. Moreover, skin substitutes can be used for cosmetic testing to replace animal testing [86]. While skin is the interface tissue between human body and exterior environment, it is an organ particularly exposed to chemical and mechanical wounds and to pathological agents. Furthermore, it is an important organ in area and complexity both in structural and functional ways [87]. Consequently, to regenerate a human skin with all its functionality is a big challenge.

2.2.1. Skin substitute characteristics

In recent years, many skin substitutes have been reported, becoming more and more similar to natural human skin, but different from each others, and still not perfectly simulating skin functionality. Those substitutes can be characterized by different factors. Provenance of cells used to produce the substitute can be either allogeneic, xenogeneic or autologous. Presence of autologous cells allows reduction of incompatibility and graft reject problems [88]. The purpose of substitutes can also vary between a permanent, semi-permanent or temporary use [83]. Depending on the use wanted, complexity of substitutes will change and can be either monolayered or bilayered. As there is no perfect substitute that has been developed to this

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day, research and development on skin substitutes are still very current. Many aspects must be considered in the elaboration of a skin substitute so that it will be as close as possible to human skin. First, histological properties must be evaluated. Indeed, a perfect skin substitute must have both dermis and epidermis, with an epidermis well-differentiated and the presence of all its four principal layers: *stratum basale, stratum spinosum, stratum granulosum* and *stratum corneum*. Moreover, skin structure is important and can be evaluated by the expression or not of different markers and their localization. Furthermore, skin plays an important role as a protective barrier against chemical, biological and mechanical aggression. Consequently, the presence of this barrier functionality is important. A method to evaluate the barrier efficacy is to perform permeability analysis. Finally, *stratum corneum* lipid organization is also important in skin barrier functionality. This organization must be well-structured and features can be analyzed by ATR-FTIR [88]. One of the lacks in the currently available skin substitutes is the absence of melanocytes that lead to a hypopigmentation of the skin where the substitute is grafted. While melanocytes are part of the skin protection against UV irradiations, the lack of this molecule in skin substitutes can become problematic for the patient, beyond the cosmetic preoccupations [89]. Therefore, there is a need for developing skin substitutes with melanocyte cells. Moreover, incorporation of ill melanocytes in skin models allows the study of melanocyte skin diseases such as melanoma. While tests on animal skin are not always representative of human skin and can be ethically problematic, the development of pigmented skin models becomes a real need.

3. *In vivo* and *in vitro* pigmented skin models

Human melanocytes have been cultured selectively for two decades when, in 1982, Eisinger and Marko published their work on selective proliferation of human melanocytes. Their selective culture was based on the proprieties of phorbol ester. Indeed, at a certain concentration, this compound is toxic for keratinocytes, but not for melanocytes. By adding it to an epidermal cell solution, it allows the selective proliferation of melanocytes only [90]. In 1986, Topol *et al.* went further and reported the first pigmented human skin equivalent. This equivalent consisted of human neonatal melanocytes plating onto a dermal skin substitute with keratinocytes. They were added before those cells overgrew the dermal equivalent [91]. Since that year, many other models have been developed.

3.1. *In vivo* models

3.1.1. Spontaneous and induced mutations

Spontaneous mutation models can be use to study some diseases when the mutations have similarity with human diseases. In melanocyte skin diseases, there are not many spontaneous mutation models available. One of them was developed in 1981 by Smyth which proposed a chicken model to study human vitiligo [92]. Indeed, he developed a mutant line of chickens characterized by a higher than normal spontaneous postnatal cutaneous amelanosis. This affection has similarity to human vitiligo. They are both a consequence of melanocyte de-

struction. Those chickens are called "chickens of the autoimmune delayed-amelanotic" (DAM) or "Smyth chickens".

In melanomas studies, spontaneous mutations on mice are rare because, even if some chemical agents can induce them, it takes a long time, and the melanomas are not very representative of human ones, with the frequent absence of metastases that are frequent in human [93]. Most of the models found in the literature use other animals. An example is Millikan *et al.* which proposed in 1974 a Sinclair swine model to study pigment tumors [94]. Lesions developed by those swine are similar histologically and clinically to some different human tumors. Indeed, they show flat lesions that can be compared to human junctional neavus and elevated lesions similar to human compound neavus. Some other lesions found in swine are raided blue tumors, the counterpart of human blue neavus, peripheral depigmentation, the counterpart of vitiligo, and ulcerative tumors, the counterpart of melanoma. Previously, in 1966, Chernozemski has proposed a Syrian hamster model which presented spontaneous or induced by DMBA melanomas [95]. DMBA was also used to induce melanomas on Guinea pigs by Clark *et al.* ten years later [96]. Clark *et al.* have reproduced Edgcomb's and Mitchelich's work of 1963 and had shown that tumors in guinea pigs have some similarities with those in humans, but are not histogenetically the same. In 1989, Setlow *et al.* reported a platyfish-swordtail hybrid model susceptible to melanoma when exposed to UV radiations [97]. This fish had already been used by other authors in the past such as Anders *et al.* who used it in 1984 for melanoma research [98]. Setlow, in his work, studied different strains of this fish and their response to some UV wavelengths to find two that were susceptible to melanomas under UV irradiations. Fish of those two strains developed melanomas that were quite similar to the human ones. The principal difference was the presence of melanophores in fish melanomas. In vertebrates, melanophores represented the last stage of pigmented cells differentiation. The same year, Ley *et al.* also reported an animal model of melanomas induced by UV radiations using South American opossum [99]. In their study, they also used the concept of photoreactivation repair pathway for DNA damages to investigate pyrimidine dimer's implication in melanoma induction. Their research allowed them to make two principal conclusions: first, they concluded that UV radiations can be used to induce malignant melanoma; second, they came to the conclusion that pyrimidine dimer is involved in melanoma formation, and that the radiations induced DNA damages. Reported spontaneous models of melanoma have been less and less frequent in the recent years probably as a result of the improvement of science in different fields such as gene modifications and *in vitro* models.

3.1.2. Transgenic models

Transgenic animals can be useful for mimicking some human diseases such as melanoma. In 1991, Bradl *et al.* reported a transgenic mouse with the simian virus 40 (SV-40) controlled by a tyrosinase promoter [93] that promotes ocular and cutaneous melanomas. The melanomas reported were histopathologically the same as their human counterparts. One year later, Iwamoto *et al.* also reported a transgenic mouse model for studying melanocyte tumors. Their model consisted of a mouse metallothionein promoter enhancer coupled to a *ret* oncogene inductor [100]. They developed four independent mouse lines:

three of those lines were well-predisposed to developing melanocytic tumors, and the other one reported an acceleration of melanogenesis with no clear proliferative disorders. In 1994, Klein-Szanto *et al.* also used the SV40 driven by tyrosinase promoter to develop a transgenic mouse model. Their model allowed them to study the induction of malignant skin melanomas by short ultraviolet radiation exposure and without chemical carcinogen application [101]. Inbred line choice and other factors such as the age of the mice and the intensity of the UV treatment can be modified in the protocol to improve melanoma induction. In 1997, Takayama *et al.* proposed a new transgenic mouse model, also using metallathionein promoter driving, this time an hepatocyte growth factor/scatter factor (HGF/SF) [102]. They wanted to study the oncogenic role of those factors. Their transgenic mice developed a large variety of tumors, including melanoma. Some tumors, including this one, overexpressed HGF/SF. The same year, Chin *et al.* reported another transgenic mouse model using a different gene. Indeed, their model consisted of H-ras driven by tyrosinase promoter with INK4a knockout mice [103]. Their studies allowed them to conclude that development of melanoma can be accelerated by both the loss of INK4a allele and the activation of Ras. In 2009, Goel *et al.* reported a BRAFV600E transgenic mouse [104]. Indeed, in more than half melanoma cases, there is presence of a mutation that affects BRAF, a protein activated by Ras. Transgenic mice presented benign melanocytic hyperplasia of which progression to the melanoma stage depended on BRAF expression. In 2011, Meyer *et al.* used *ret* transgenic mice to study melanoma evolution. They also studied inflammatory tumor microenvironment when there is enrichment of myeloid-derived suppressor cells (MDSCs). They concluded from their studies that, before starting an immunological treatment for melanoma, the immune status should be controlled and the immunosuppressive microenvironment should be neutralized [105]. However, transgenic models are not the best for mimicking some cancers such as melanoma because, such as spontaneous mutations, they present a lack of metastase production. Indeed, it is known that the importance or not of metastases in large amount is crucial for the patient's survival. By consequence, it is often a target in treatment development and so their presence is important to have a valuable model.

3.1.3. Xenotransplantation models

Another type of model that can be used to study human melanoma consists of xenotransplantation of human skin onto an animal. For models on mice, three different types of this animal are mainly used: athymic nude mice, severe combined immunodeficient mice (SCID) and spontaneous AGR129 mice models. The immune systems of those mice differ from those of normal mice so that their immunological potential is decreased. Athymic nude mice do not have T cells because of the absence of a thymus. SCID mice do not have either T cells, and are also deficient of B cells. AGR129 mice do not have either T or B cells as SCID mice, and also have immature natural killer cells [106]. In 1993, Juhasz *et al.* proposed a xenotransplantation model of human skin grafted onto either nude or SCID mice. Beforehand, human melanomas were injected onto the human skin graft [107]. This

model can allow reproduction of human melanomas and their metastases because tumour cells invaded human vessels, and in more than half case, metastases were found in lungs. In 1998 Atillasoy *et al.* proposed another model for studying UV irradiations and chemical carcinogen implication in skin melanoma development. Their model consisted of human newborn foreskin grafted onto RAG-1 mice. Mice were separated into four groups and received different treatments. While the first group was the control group and received no treatment, the second received a treatment of DMBA, a chemical carcinogen. The third group received UVB irradiations and the last one, both DMBA and UVB irradiations [108]. Only DMBA treatment was not conclusive, as the only impact was the development of melanocytic hyperplasia in 16%. UVB only treatment was a little better as it caused solar lentigo in 23% and melanocytic hyperplasia in 68%. Combined treatment caused solar lentigo in 38% and melanocytic hyperplasia in 77% and was the only one that generated melanoma in 2.1% after 15 months. This is representative of melanoma incidence in Caucasian Americans that is 1.4%. In the last years, xenotransplantation models were also used to test different treatments. For example, in 2010, Schicher *et al.* used a xenotransplantation model of a SCID mouse with a human melanoma graft to test treatment of Erlotinib combined with some chemotherapeutics agents [109]. Erlotinib is a treatment already used for non small cell lung cancer. The melanomas treated with a combined treatment showed a higher reduction than those only treated with chemotherapeutic agents.

3.1.4. Other models

Other testing systems that can be used are injection of mouse melanoma into mice. For that, some mouse melanoma cell lines have been produced. One of these lines originated from one of the rare spontaneous melanomas in mice, an event that occurred on the ear of a C57BL/6 mouse in 1954. It was Fidler *et al.*, who at the beginning of the 1970s really set up the line known as B16 melanoma cell line [110]. Those murine melanoma cells can be used for studying melanoma treatments as reported by some authors. For example, in 2002, Lucas *et al.* injected B16 melanoma in C57BL/6 mice in order to test injection by electroporation of interleukin-12 [111]. They tested an intratumoral and an intramuscular treatment. While the first one was useful for treating tumour in 47% of the cases, the second one was not conclusive. They also tried those two treatments in a nude mouse model, but neither the intratumoral or the intramuscular treatment worked. Those results allowed them to conclude that T cells probably have a role in the melanoma regression, at least in this model. The same year, Garcia-Hernandez *et al.* investigated on the implication of interleukin-10 in the promotion of B-16 melanoma growth [112]. This study allowed them to conclude that IL-10 seems to have a role in this promotion in three fields: first, they simulate the proliferation of the tumour-cells; second, they have implications for the angiogenesis process and third, they are implicated in the immunosuppression. B16 melanoma cells can therefore be useful to many types of studies.

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3.2. *In vitro* models

3.2.1. Monolayers

Monolayer models are characterized by the culture of one cell type, which has previously been extracted from a skin biopsy. Cells can be extracted from normal or lesional skin such as cells of skin affected by hypo- and hyperpigmentation disorders. Monolayer models are useful for studying melanocyte properties and testing different conditions or drugs. In 2008, in a comparative study of melanocytes culture and melanocytes-keratinocytes co-culture, Liu *et al*. demonstrated the effect of melanogenic stimulators (α-MSH and L-tyrosine) and inhibitors (arbutin and hydroxybenzyl alcohols (HBA)) in the two conditions [113]. Results showed that α-MSH and L-tyrosine increased the melanin content of melanocytes, and that the increase was better in the co-culture with keratinocytes. 4HBA and arbutin inhibit the melanogenesis in the two conditions, but, in co-culture, the inhibition was much better than in melanocytes alone. These results suggest that cytokines released by keratinocytes can have an effect on the regulation of melanin synthesis, and that the co-culture model has interesting properties for testing drugs related to the treatment of pigmentation disorders. In the same vein as Liu *et al*.,Criton *et al*. tested 22 N-hydroxy-N-phenylthiourea and N-hydroxy-N-phenylurea analogues, which could inhibit tyrosinase activity and reduce melanin synthesis on melanocyte culture [114]. Results showed that compound 1 inhibits tyrosinase and reduces 78 % of melanin synthesis. It is a promising candidate for the treatment of hyperpigmentation disorders to replace whitening agents that have undesirable effects. These studies demonstrate that monolayer models allow testing of several conditions, and the possibility of observing melanocytes behavior in some pigmentation disorders.

3.2.2. Collagen gels

Unlike monolayer models, which are composed of only one cell type, collagen gel models allow formation of a dermis on which more than one cell types can be seeded. Globally, for the construction of a pigmented equivalent with collagen gel, fibroblasts, keratinocytes and melanocytes are extracted from a skin biopsy and are cultured separately. Fibroblasts are seeded onto the collagen gel, then, after few weeks of culture, keratinocytes and melanocytes can be seeded onto fibroblasts and collagen matrixes [115]. These types of equivalent that contain various cell types are useful for studying the interactions between keratinocytes and melanocytes and understanding different mechanisms of pigmentation disorders. Recently, Duval *et al*. developed a pigmented skin model using collagen, such as a dermal matrix that is very representative of normal human skin with melanocytes [116]. They demonstrate that their model has a functional pigmentary system by the presence of melanocytes well-developed with melanosomes, the expression of tyrosinase, TRP1 and TRP2, the transfer of melanosomes containing melanin to keratinocytes and the stimulation of melanin synthesis by α-MSH. This model has most of the normal human skin melanocyte characteristics [9] and seems to be an interesting pigmented skin model for studying cell interactions of the pigmentary system. A study with this type of skin model has observed the response of melanocytes after UV radiation [117]. Archambault *et al*. demonstrated by a comparative study of monolayer

melanocyte culture and pigmented skin equivalent that melanocytes have a better capacity for surviving UVR than melanocytes in culture. These results suggest that keratinocytes and fibroblasts secrete factors that enhance melanocytes survival and migration, which could explain UVR-induced pigmentation by melanocytes. Unlike other teams, Freeman *et al*. elaborated another technique of culture with collagen gels [118]. They put a complete skin biopsy, which was affected by a melanoma, onto a collagen gel that contained fibroblasts. This technique allowed conservation of the *in vivo* properties of the melanoma and observation of the proliferation and other characteristics *in vitro*. All of these models composed with a collagen matrix are representative of human skin, and are effective for the study of pigmentation disorders. However, collagen models may not be useful for testing drugs, because they can be absorbed by the collagen and a higher quantity of drugs must be used [119].

3.2.3. De-Epidermized Dermis (DED)

Such as collagen gels, DED allowed the construction of pigmented skin equivalent that reproduced human skin for studying the pigmentary system. Unlike collagen gels, this method has a native extracellular matrix and a basal membrane that facilitates melanocyte adhesion. Principally, DED preparation is very similar. From a skin biopsy, the epidermis is removed and the dermis is incubated in saline solution or undergoes freezing-thawing cycles to kill cells [120]. After this treatment, the dermis remaining is called a dead de-epidermized dermis, and is ready to be seeded by keratinocytes and melanocytes. In 1993, Todd *et al*. used this model to demonstrate the effect of UV radiation on a pigmented equivalent [121]. They demonstrated that, after UV radiation, there is an increase of TRP1 activity, an increase of pigmentation and an increase of DOPA-positive melanocytes such as observed *in vivo*. In 2000, for a better understanding of melanoma invasion, Dekker *et al*. developed and characterized a DED skin equivalent with four types of melanoma cells [122]. They observed the expression of different integrins that play an important role in the behavior of melanoma cells. Their model is a useful tool for studying melanoma and other mechanisms involved in this cancer. In 2007, Cario-André *et al*. developed a DED skin model with normal and non-lesional vitiligo cells to understand if the loss of melanocytes in vitiligo is caused by a detachment of melanocytes due to stress factors [123]. The model with non-lesional vitiligo cells contains less melanocytes than the model with normal cells, and hydrogen peroxide and epinephrine could be the cause of the detachment of melanocytes. Todd, Dekker and Cario-André models demonstrated that de-epidermized dermis is an interesting model for studying different pigmentation disorders and, in comparison with collagen gels, DED produced an epidermis, which is closer to the native epidermis than collagen gels [124].

3.2.4. Commercial models

Commercial *in vitro* skin models have been developed to reduce tests on animals, replace animal models and refine methodologies. For the cosmetic industry, it allows testing of the toxicity of their products and their pharmaceutical effects on a complete human epidermis and dermis. Several companies produce skin models and some of them produce equally pigmented skin models that are useful for testing photoprotection, whitening agents and repigmentation

products. The list of the main commercial pigmented skin models and their principal features is presented in Table 3.

Models	Type of models	Epidermis	Epidermis + dermis	Different degree of pigmentation	References
SkinEthic™	Cells seeded on a polycarbonate filter	x		x	[125]
MelanoDerm™	Cells seeded on a collagen gels	x		x	[126]
StratiCell™	Cells seeded on a polycarbonate filter	x		x (only 2)	
Melanoma skin model™	Cells seeded on a collagen gels		x		[127]

Table 3. Different commercial pigmented skin models

4. Conclusion

Melanocytes are underestimated cells that are more than pigmentation cells. They protect the skin against environmental factors and they are necessary for sight and hearing. However, melanocytes can also be the cause of some skin disorders and cancer. Even more mechanisms involved in pigmentation disorders remain unknown and need to be elucidated upon in order to give affected people a better quality of life. *In vitro* pigmented skin substitutes produced by tissue engineering and *in vivo* models are useful tools for understanding these mechanisms and developing appropriated treatments or drugs.

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Chapter 31

Cell Therapy and Muscle Regeneration: Skeletal Myogenic Differentiation of Urine-Derived Stem Cells for Potential Use in Treatment of Urinary Incontinence

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Additional information is available at the end of the chapter

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1. Introduction

Stress urinary incontinence (SUI) is the prevalent form of urinary tract infection [1], affecting nearly 34 million women in the US [2,3]. In men undergoing prostatectomy, postoperative removal of the catheter leads to urine leakage due to SUI in 50% of cases [4]. The costs of medicine and health care related to SUI are estimated to be $26 billion US dollars annually [5]. Damage to the sphincter muscle around the middle segment of urethra and its connective tissue and nerves is a major cause of SUI [6]. The urethra consists of proximal, middle, and distal segments. The midurethra is a critical component in the urethral sphincter complex, which mainly consists of the inner smooth muscle and outer striated muscles. The striated muscle contributes the most to the complex. Urethral striated muscle is significantly reduced with increasing age. Thus, in patients with SUI, when the lost striated muscle can be restored, amelioration of SUI can be expected. Otherwise, the smooth muscle plays a lesser role, but it is still a desirable treatment target to recover sphincter function [7]. Strategies to replace skeletal muscle cells with stem cell therapy could be used in treatment of SUI.

2. Cell therapy for USI

Pharmaceutical treatment of SUI has not been successful [8]; in addition, periurethral injection of bulking agents has poor long-term efficacy and is associated with complications such as voiding dysfunction, abscess formation, and pulmonary embolism [9]. Several surgical procedures such as urethral sling surgery or installation of an artificial sphincter have been used to treat patients with SUI over last three decades [10]. Although these procedures can

reinforce weak muscle tissue at the pelvic floor or around the urethra, deficient urethral sphincter function remains. Furthermore, surgery for SUI sometimes causes complications such as infection and postoperative voiding difficulty [11]. Autologous adult stem cell injection therapy for SUI has recently provided a promising alternative for sphincter tissue regeneration for repair of SUI. Stem cells obtained from skeletal muscle [12], bone marrow [13-15], umbilical cord [16], adipose tissue [17], and more recently, induced pluripotent cells [18] are regarded as possible candidates for use in this therapy. However, harvesting these types of cells is invasive and may cause complications. Furthermore, the amount of tissue that can be safely harvested in some patients, such as skeletal muscle-derived progenitor cells or bone marrow stem cells (BMSCs) in older individuals, limits clinical applications [19]. Thus, an autologous stem cell source that can be obtained using non invasive techniques would be desirable.

Mesechymal stem cells (MSCs) are often used as a cell source for cell therapy in two ways. First, stem cells are implanted directly into the tissues where repair is needed. By secreting paracrine factors, MSCs promote angiogenesis, decrease fibrosis, and recruit stem cells from native tissues to complete the repair, replacement, and regeneration processes at the injured sites. In addition, the surrounding normal cell- and tissue-based signals from the host environment guide the undifferentiated stem cells to give rise to the specific target cells required for tissue regeneration [20]. Second, stem cells are induced to differentiate into the target cells or tissue–like cells *in vitro*. The induced cells are then implanted into defective sites where normal cells and tissues are not available.

In clinical settings, most patients with SUI acquire chronic injuries related to urethral dysfunction spanning years or even decades. In cases with extensive injuries and fibroblast formation, it would be better to induce the stem cells to differentiate into a myogenic lineage before injection, since the unhealthy or diseased environment (e.g. muscular dystrophy) may not be able to provide efficient differentiation cues required for efficient stem cells differentiation. Therefore, our strategy is to guide USCs to give rise to myogenic differentiation and then lead to repairing the deficiency, and also determine whether USCs can secrete paracrine factors to recruit the resident cells from the host to participate in tissue repair.

3. Stem cell properties of urine-derived cells

Tissue-specific stem cells are rare in each tissue or organ. These cells play an important role in replacing aged, injured, and diseased cells and promoting tissue regeneration. However, it is a challenge to isolate these cells for therapeutic purposes. Recently, we successfully established a primary culture system to isolate and repopulate stem cells from regular voided human urine [21]. These cells possess stem cell properties, such as robust proliferation potential and multipotential differentiation capabilities, and are termed urine-derived stem cells (USCs) [22] (Fig.1). USCs expressed a whole set of MSC/pericyte markers and some key cell surface markers, such as CD 105, CD 90, CD 73, and CD 146, but not hematopoietic stem cell makers such as CD 31 and CD34 [21, 23](Fig.2). These cells maintain high telomerase activity and a normal karyotype in culture medium, even after several passages. The cells can differentiate

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into mesodermal cell lineages, such as osteocytes, chondrocytes, adipocytes, and myocytes, including smooth muscle cell differentiation and endodermal lineages (e.g. endothelial and urothelial cells) [23,24]. Additionally, USCs can be isolated and cultured from almost every urine sample. Our recent data demonstrate that 100 ml of urine contains about 3-10 USC clones [23]. On average, 12 USC clones can be isolated from one urine sample (about 200 ml). To induce stem cells to differentiate into skeletal muscle cells more efficiently, cells at the early passage are recommended. Usually, it takes 4 weeks for one cell clone to generate $3.2X10^7$cells at an early passage (p4) [24]. Therefore, at least $4x10^8$ USCs ($12x3.2 \times 10^7$cells) can be generated from one urine sample, which are enough cells for use in the treatment of any grade of SUI.

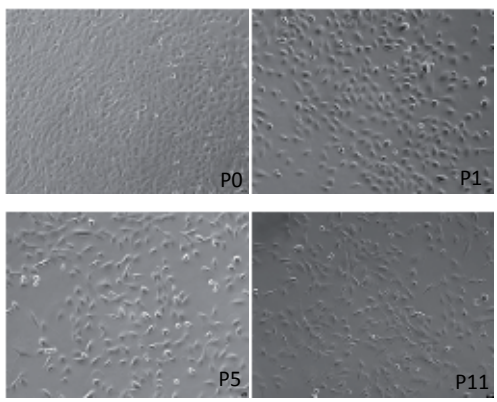

Figure 1. Morphology of USC: Phase contrast microscopic appearances of urine derived stem cell clones showing "rice-grain" like morphology.

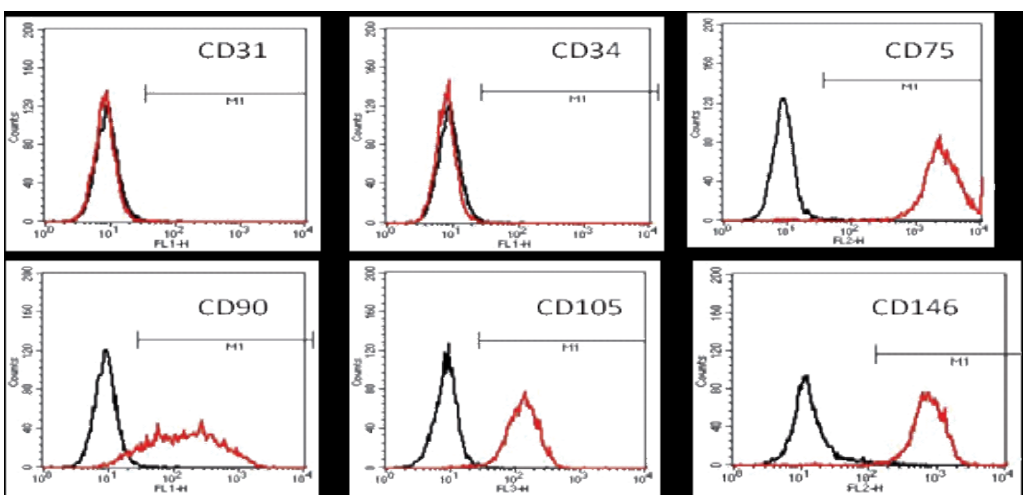

Figure 2. Stem cell surface marker of USC cell clones.

4. Myogenic differentiation of USCs

Our previous studies demonstrated that USCs can be efficiently induced to differentiate into smooth muscle cells [23, 24]. After myogenic differentiation with TGF-1 (2.5 ng/ml) and PDGF-BB (5.0 ng/ml), induced USCs displayed spindle-shape morphology, and expressed smooth muscle-specific gene and protein markers (such as smoothelin, myosin, and desmin). In addition, these cells exhibited a contractile pattern similar to bladder smooth muscle cells. In our recent study, we demonstrated that USCs can give rise to skeletal muscle-like cells with myotube formation in standard myogenic differentiation media (i.e. SkGM2/Differentiation Medium. USCs are culture in Sk-MC growth Medium (SkGM-2, Lonza) for 2 weeks followed by Differentiation medium (DMEM:F-12 [1:1], 2% Horse serum and 1% Penecillin/streptomycin). Differentiated USCs expressed specific skeletal muscle cell transcripts and protein markers (myoD, myogenin, myosin, and Myf5). *In vitro* calcium labeling studies showed that skeletal myogenic differentiated USCs resulted in increased calcium efflux. After the induced cells ($1x10^6$) mixed with 2% collagen-I gel (100 μl) were implanted into the tibialis anterior muscle of nude mice for one month, the grafted cells maintained expression of skeletal muscle cell markers *in vivo*. The USCs differentiated into a skeletal muscle cell lineage, which could be used as a potential source for cell injection therapy in the treatment of urethral sphincter dysfunction.

5. Paracrine factors of USCs

We have recently demonstrated that USCs are able to secrete several angiogenic growth factors, such as VGEF, PDGF, FGF-1, IGFBP-1 and 3, MMP-9, and angiogenin when UCSs in culture media were assessed by ELISA. *In vivo* studies showed that USCs can stimulate the resident cells to migrate from the host into the graft tissue. More cells with no human nuclear protein expression were observed in the sites where USCs were implanted, compared to a cell-free injection sites.

There are several potential advantages to using USCs as a cell source for cell therapy, including the following: 1) USCs can be easily harvested by a non invasive method and grown in culture; 2) they do not require enzyme digestion or culture on a layer of feeder cells to support cell growth; 3) they start as one single-cell colony in the initial culture, which creates more homogeneous cells after differentiation; 4) since they possess telomerase activity, they can generate more cells and provide a longer life span for tissue regeneration; 5) they possess higher plasticity and more efficiency when induced to differentiate into other functional cells, including endothelial and muscle cells; and 6) as autologous cells, USCs do not raise ethical issues or cause immune reactions to engineered implants. Therefore, obtaining and using stem cells from urine could be an attractive alternative to the standard urological tissue biopsies currently used in cell therapy and tissue engineering. Additionally, myogenic differentiated USCs might be used in other diseases involved in sphincter dysfunction via an endoscopic procedure, including muscle sphincter-deficient diseases such as vesicoureteral reflux, gastroesophageal reflux disease [25,26], and anal incontinence [27].

6. Conclusions

USCs as pericytes/mesenchymal stem cells could be easily obtained by a non invasive approach. These cells are able to extensively expand and can be induced to differentiate efficiently into a skeletal myogenic cell lineage. In addition, myogenically-differentiated USCs expressed skeletal muscle cell gene and protein markers. Moreover, USCs are able to recruit resident cells from the host to participate in tissue regeneration by their secretion of paracrine factors. Use of autologous USCs may serve as a more convenient and low-cost cell source comparable in quality to other types of adult stem cells, and could generate a large number of cells in vitro for potential use in treatment of patients with SUI or other diseases due to sphincter deficiency.

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Chapter 32

Autologous Muscular Treatment Options for Endstage Heart Failure — A Critical Appraisal of the Dynamic Cardiomyoplasty (DCMP) vs. a New Concept of a Closed-Loop Controlled DCMP (CLC-DCMP)

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Hans-H. Sievers

Additional information is available at the end of the chapter

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1. Introduction

Dynamic cardiomyoplasty (DCMP) aims at improving cardiac function in cases of severe heart failure by wrapping the latissimus dorsi (LD) muscle, (usually left LD) around the ventricles and stimulating it electrically, synchronously to the ventricular function (Figure 1, Figure 2). It is a surgical treatment option mostly for pharmacologically untreatable heart failure. The first successful clinical application was performed in 1985 (Broussais Hospital, Paris, [1]). Since then, more than 1.000 surgeries have been performed worldwide [2]. The clinical results of the DCMP however did not live up to the expectations due to the loss of muscle strength [3][4] and muscle damage [5][6].

In DCMP, a special kind of tissue engineering is applied using an electric stimulation on autologous skeletal muscles (electrical muscular tissue engineering). The fiber type changes from the fatiguing type IIa to the fatigue resistant type I [7], [8]. Type IIx fibers disappear. The gene expression for myosin heavy chains IIa (MHCII) is changed into heavy chains I (MHCI) [3]. Intramuscular collaterals are opened [9], [10] and enhanced and capillary density is increased [11].

A critical analysis of more than 20 years and more than 1.000 clinical cases should demonstrate it's clinical impact [1], [2], [4–6], [12–145]. Clinical efficacy concerning survival, clinical outcome indicated by NYHA- class, ejection fraction of the left heart ventricle (EF) was evaluated by the use of more than 100 relevant reports. A comparison of the DCMP therapy

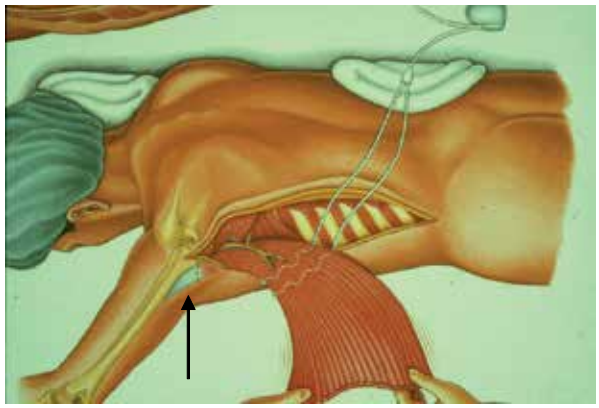

Figure 1. Latissimus dorsi muscle (LDM) is dissected free from the left thoracic wall before placing it into the thoracic cavity via a window of the thoracic wall after a partial resection of the second rib. Artery, vein and nervus thoracodorsalis remain untouched. LDM's tendon is cut (arrow) before it's re-fixation to the thoracic wall. Two stimulation electrodes are placed wavelike around the branches of nervus thoracodorsalis at a distance of 6 cm.

to other treatment options for end-stage heart failure is performed in this report. And last but not least new experimental insights concerning the DCMP procedure should be demonstrated. This experience from clinical application over more than 20 years and new experimental data about dynamic cardiomyoplasty procedures should become compared and discussed. From these insights, conclusions should be drawn to improve clinically results from DCMP which are needed urgently for a more effective treatment of pharmacologically non-treatable heart failure.

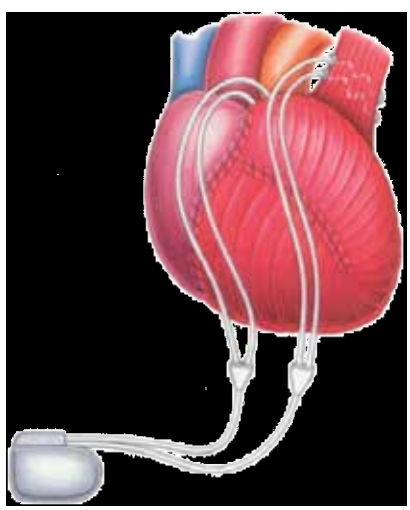

Figure 2. When latissimus dorsi muscle (LDM) has been transferred intra-thoracally it is wrapped around the heart and stimulated electrically by a myostimulator via two muscular electrodes near the branches of the thoraco-dorsal nerve. Stimulation bursts are synchronized with the heart action via additional epicardial sensing electrodes.

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2. Clinical efficacy of DCMP

2.1. Survival

An analysis of survival curves of different investigators for DCMP-procedures shown in figure 3 may give insights in factors determining the best survival rate.

2.1.1. Survival rate and NYHA — Class

Observing and comparing the survival curves in figure 3 demonstrates, that a pre-operative NYHA class III has a better long term survival than NYHA class IV. In the upper half of the diagram with the higher survival, NYHA III is predominantly present while in the lower half mostly appears NYHA IV.

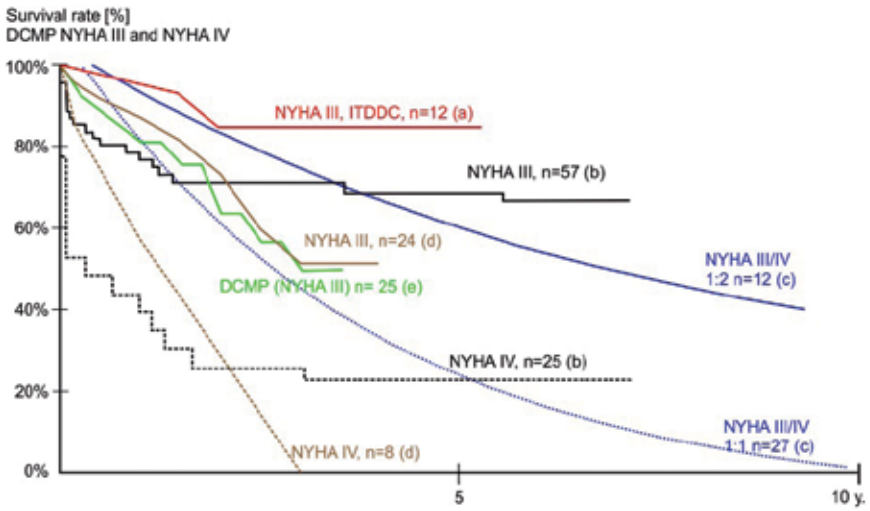

Figure 3. Survival rates of patients with a DCMP published by different authors (a) Rigatelle et al, 2002 (ITDDC = Italien Demand Dynamic Cardiomyoplasty) *[118]* (b) Chachques et al. 1997 [37] (c) Benicio et al. 2003, [20] (d) Bocchi et al. 1994, [25] (e) Bocchi et al. 1996 [23]

To demonstrate the influence of the pre-operative NYHA class, results of the "French DCMP Experience" from the year 1997 are selected from figure 3 and documented in figure 4 separately. The survival rate of class NYHA III patients at 5 years after DCMP was 68% compared to NYHA IV with 23 % (Chachques JC, 1997) [37]. Another group reports (figure 3 (d)) about no survivor of pre-operative NYHA IV patients 3 years after the DCMP procedure [25].

2.1.2. Survival rate and stimulation pattern

A re-evaluation of long-term outcomes of dynamic cardiomyoplasties from the Sao Paulo Group Brazil [20] in 43 patients with dilated cardiomyopathies showed impressive results

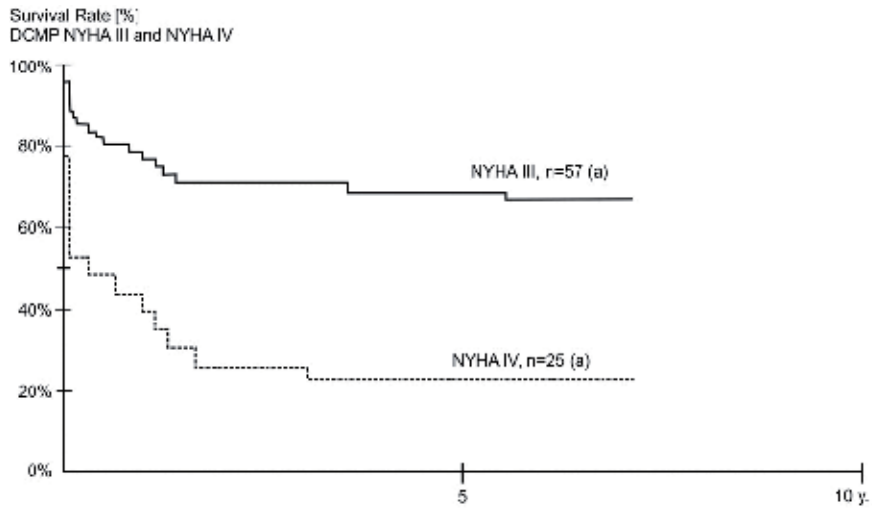

Figure 4. Kaplan-Meier statistics of the event-free survival after dynamic cardiomyoplasty according to pre-operative New York Heart Association functional class (upper curve NYHA III, lower curve NYHA IV) (a) Chachques, 1997, [37]

using different stimulation modes by synchronizing the electrical stimulation of the muscle wrap to the heart in 1:1 and 1:2 mode (stimulations bursts per heart beat). In 1:2 mode after 10 years of DCMP, there is a survival of about 40 % while in 1:1 there is no survivor. A muscle damage as shown in the histology (figure 11, chapter 2.5, [5]) using also a 1:1 stimulation pattern may be the reason of this insufficient result.

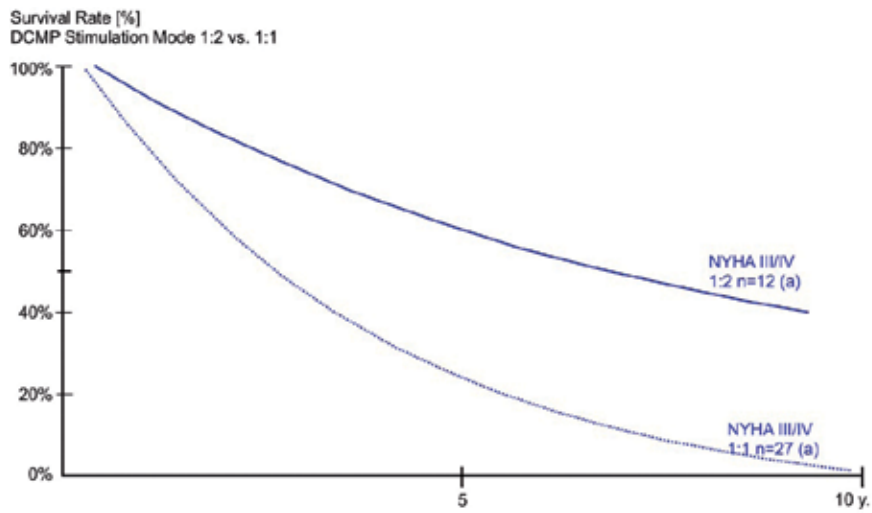

Figure 5. Survival rate of DCMP procedures 10 years after different synchronization modes with the heart contractions. 1:2 synchronization results in a 40 % survival, while in a 1:1 mode there was no survivor. (a) Benicio et al. 2003, [20]

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The highest survival rate, upper curve in figure 3 (a), has been reported by Rigatelli et. al 2003 [118]. They applied the lowest mean pulse frequency of all investigators and used a day and night regime with a myostimulator on at day and off at night.

2.2. NYHA — Class

In order to determine the best course of therapy, physicians often assess the stage of heart failure according to the New York Heart Association (NYHA) functional classification system. This system relates symptoms to everyday activities and the patient's quality of life.

- Class I (Mild): No limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, or dyspnea (shortness of breath).
- Class II (Mild): Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in fatigue, palpitation or dyspnea
- Class III (Moderate): Marked limitation of physical activity. Comfortable at rest, but less than ordinary activity causes fatigue, palpitation, or dyspnea
- Class IV (Severe): Unable to carry out any physical activity without discomfort. Symptoms of cardiac insufficiency at rest, if any physical activity is undertaken.

In 101 from 139 publications about clinical DCMP authors report about the efficacy of DCMP on the clinical severity in heart failure using the classification of the New York Heart Association (NYHA). The DCMP outcome is reported between 1989 and 2009.

In figure 6 the higher bars in all cases represent the pre-operative NYHA-class and the lower bars show the post-operative results. In summary there is a significant improvement from NYHA 3.26 ± 0.63 to 1.69 ± 0.34 ($p<0.05$, figure 7).

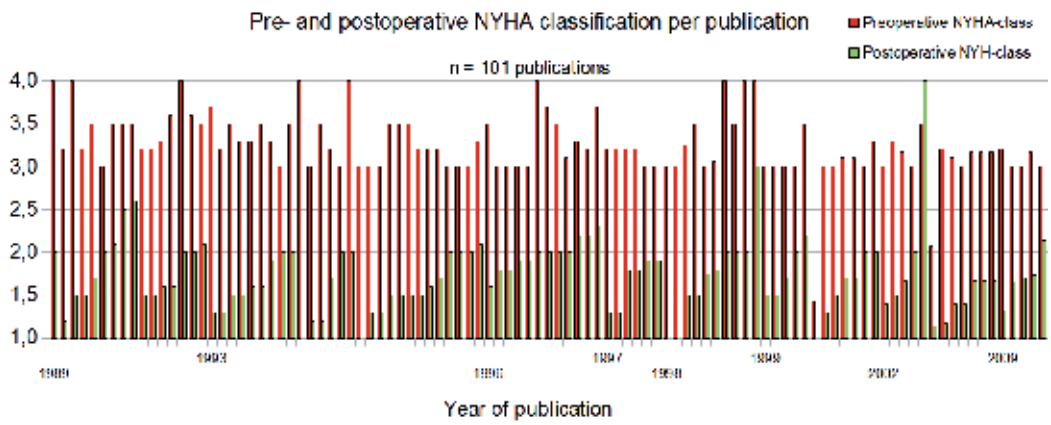

Figure 6. Clinical outcome between 1989 and 2009 evaluating 101 reports from literature shows an improvement of NYHA from pre-operatively 3.26 to post-operatively 1.69 ($p< 0.05$).

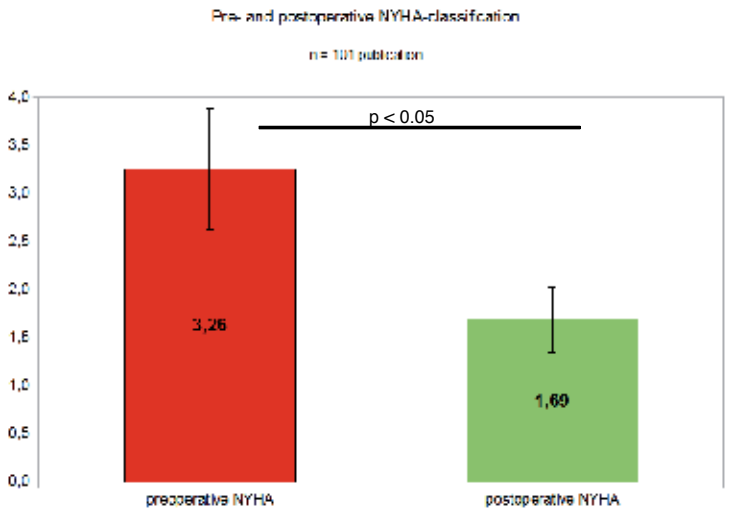

Figure 7. Mean values of the pre-operative (left) and post-operative (right) NYHA-classes (p< 0.05).

2.3. Hospitalization rate

Evaluating data from 264 patients of 7 publications [31], [35], [40], [96], [118], [136], [138], the pre-operative hospitalization rate was 2.79 ± 0.83 days/year. The post-operative hospitalizationrate of DCMP patients was significantly decreased with 0.48 ± 0.23 days/year (Figure 8).

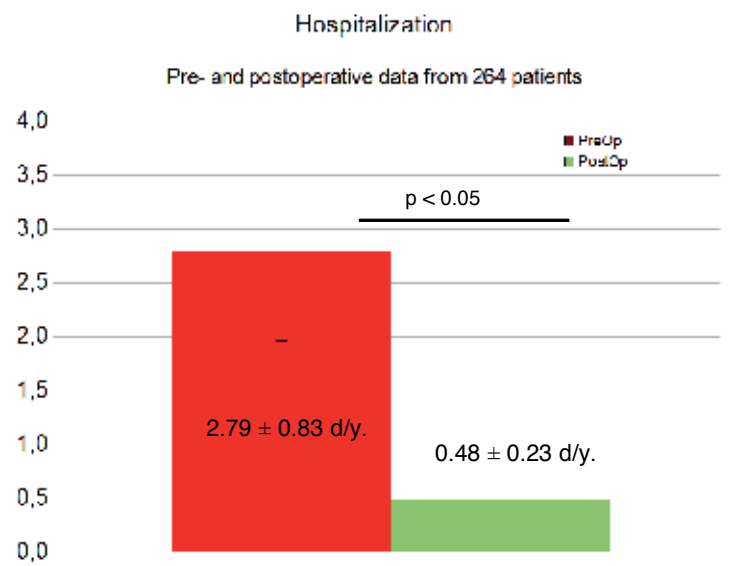

Figure 8. Mean values of the pre-operative (left) and post-operative (right) hospitalization (p< 0.05).

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2.4. Ejection Fraction (EF)

According to the clinical improvement documented by the NYHA-decline in figure 6, there is an increase of the ejection fraction (EF) of the left heart ventricle from 21.8 ± 4.5 to 28.9 ± 5.9 ($p < 0.05$). Figure 9 demonstrates an overview of the ejection fraction (EF) of 89 DCMP relevant reports.

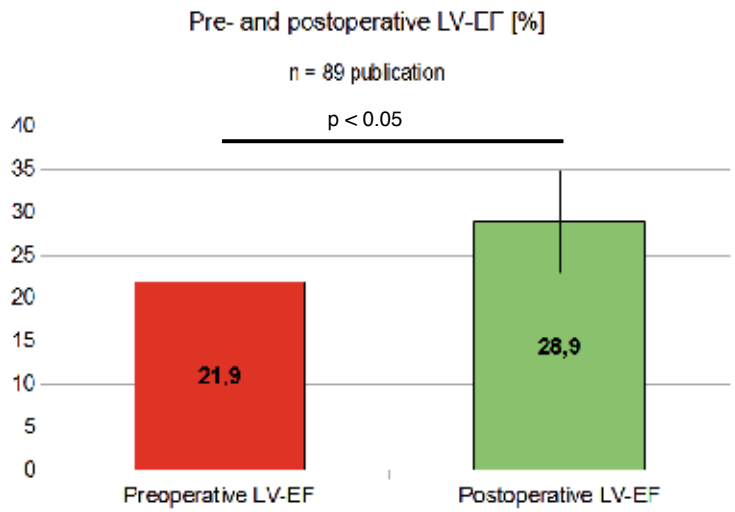

Figure 9. Ejection fraction (EF) after DCMP improved from 21.9 to 28.9%

2.5. PV-loop evaluation

The conductance catheter method visualizes an impressive documentation of DCMP' s clinical efficacy. It is performed by heart catheterization where a conductance catheter is placed along the longitudinal axis of the left heart ventricle (LV). It enables a real time volumetry of the stroke volume of the LV by recording the changes of intra-ventricular blood conductivity caused by a volume reduction (stroke volume). A simultaneous measurement of the LV's pressure allows generating pressure-volume loops in real time. The area surrounded by the loop represents the stroke work of the left heart ventricle. Thus the influence of an activated DCMP on the hemodynamic of the LV can be demonstrated as shown in figure 10(n=9 patients). A stimulated DCMP enhances the stroke work of the left ventricle.

In three patients A, B and C (Figure 11, [74]), left ventricular pressure-volume loops and relations performed by the conductance method are recorded before the cardiomyoplasty procedure and 1 year after surgery. As demonstrated by example in the baseline panel for patient A, multiple pressure- volume loops are used to measure end-systolic pressure-volume

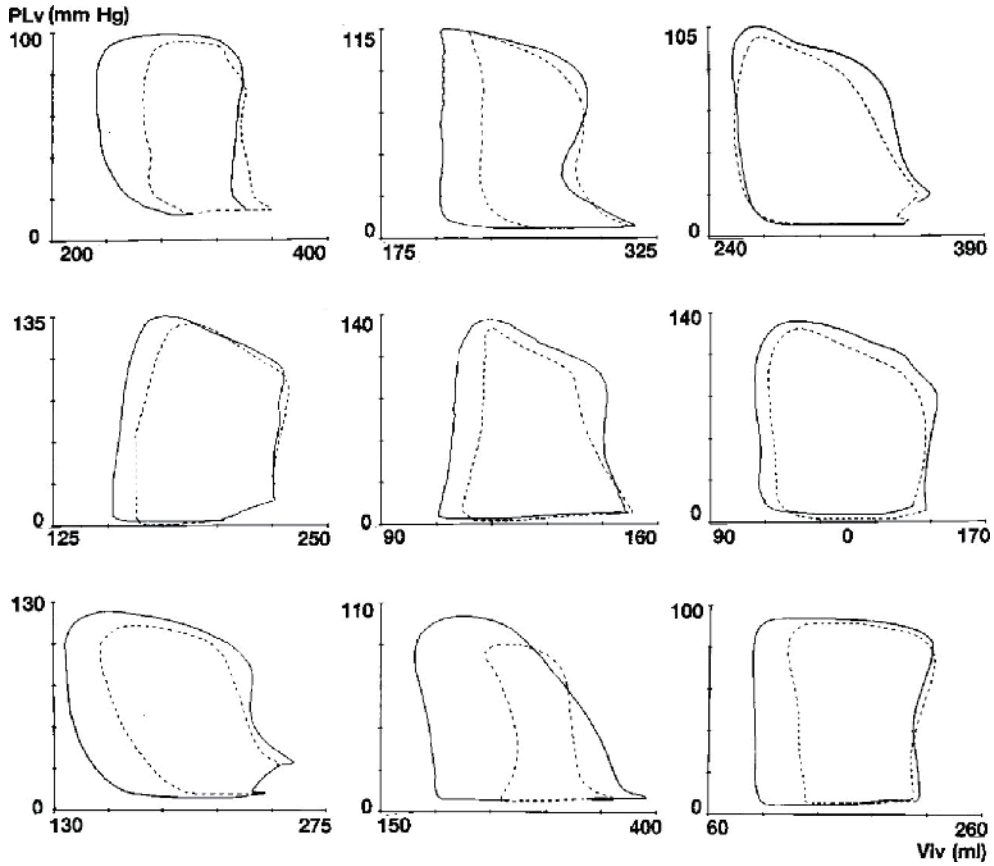

Figure 10. Pressure-volume-loops of 9 patients. The dotted lines represent non-supported and the solid lines show DCMP-supported heart cycles. Vlv expresses the volume of the left ventricle and Plv represents left ventricular pressure. (Schreuder et al.1995 [130])

relation (ESPVR) and diastolic pressure-volume relation (DPVR), defining the active and passive limits of cardiac performance, respectively.

Summarizing the results of Kass et al., the most important finding is a leftward shift of the PV-loop, indicating a volume reduction of the dilated, failing heart. This reverse remodelling improves left ventricular performance.

2.6. Histology

Dynamic cardiomyoplasty patients had an initial clinical improvement followed by a decrease in cardiac failure indices mostly in a 1:1 stimulation mode [20]. A histopathological study of the skeletal muscle was undertaken by Gutierrezz et al. 2001 [5] to explain this. Latissimus dorsi fragments from 15 patients submitted to dynamic cardiomyoplasty in a 1:1 conditioning were analysed by light microscopy. The interval between surgery and obtaining the specimens (13 from necropsies, two from heart transplants) ranged from 37 days to 6 years. Nuclear

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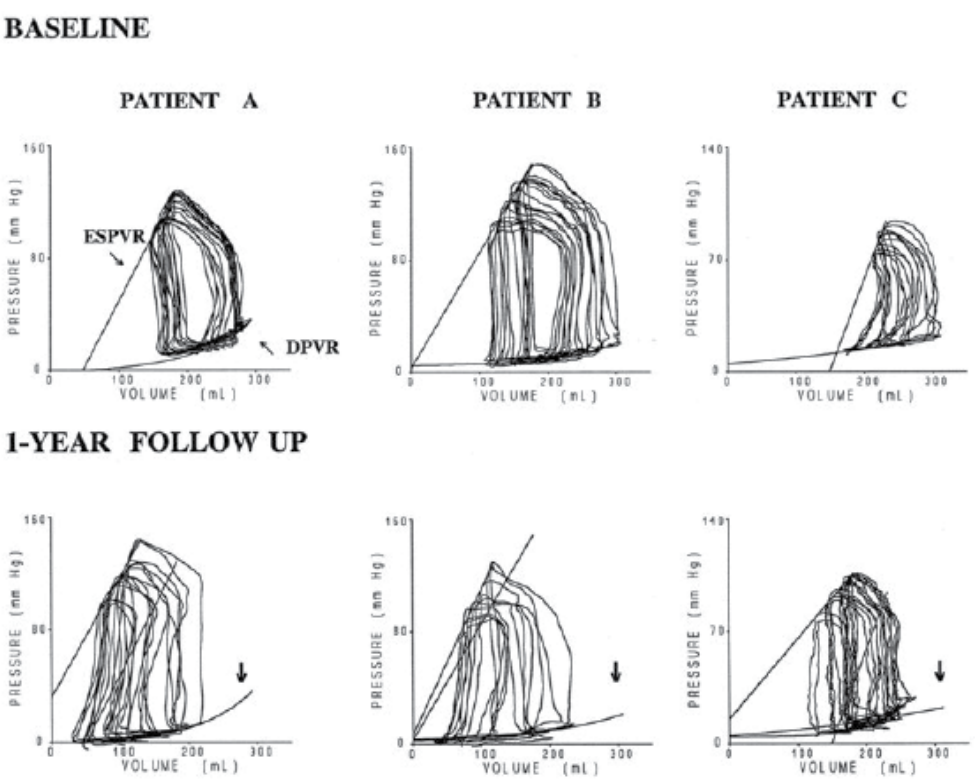

Figure 11. Effect of cardiomyoplasty on cardiac function by pressure volume relations one year after the operative DCMP procedure [74].

clumps and internalization, the presence of round fibres, inflammation, and fibrosis were analysed semi-quantitatively; the thickness of muscle fibres and the percentage of tissue fat were measured by image analysis. The quantitative data were also compared, in 12 cases, with gender- and age-matched necropsy controls. The mean thickness of muscle fibres in cases and controls was 27.21 ± 5.33 and 40.84 ± 9.42 μ, respectively (p=0.001). The percentage of tissue fat in cases and controls was 12.04 ± 12.66% and 0.93 ± 0.91%, respectively (p=0.008). Accordingly, a negative correlation was found between the duration of graft usage and the mean diameter of fibers, characterizing muscle atrophy (R=-0.66, p=0.01). The longer the post-surgical period, the more intense the degenerative lesions. This study shows that skeletal muscle used in human dynamic cardiomyoplasty may atrophy and be replaced by fat when stimulation is synchronized to every cardiac beat [20]. These findings could play a role in explaining the long-term results in DCMP procedures as shown in Fig.5 chapter 1.2.1 with no survivors after 10 years of an electrical stimulation in a 1:1 mode.

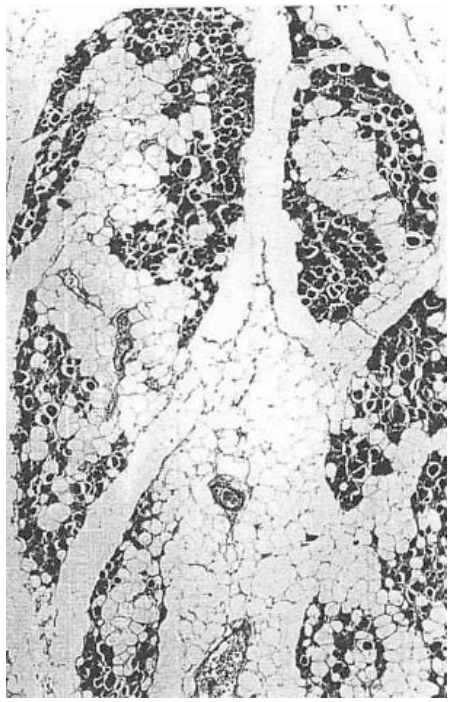

Figure 12. Histology of severe muscle damage is demonstrated using an 1:1 over stimulation. (Gutierrez et al. J Pathol 2001; 194: 116-121) [5]

3. DCMP in comparison to other clinical treatment options for patients with end-stage heart failure

3.1. Medical treatment

The only prospective randomized study comparing DCMP with the medical treatment is C-SMART (Cardiomyoplasty Skeletal Muscle Assist Randomized Trial, [145]). All 103 patients suffered from cardiac failure of NYHA III. C-SMART showed that after 6 months quality of life and sub-maximal physical performance was increased significantly. The main limitation of this study was the short observation time of 6 months. This American study was finished due to the inability to recruit further patients. After these 6 months survival rate did not differ significantly. Due to the knowledge that the DCMP is effective after a longer post-operative period interval we have to rely on retrospective studies only including DCMP patients [20], [118] and medical treated over more than 4 years ([23], [146], [147], [148]). The most favourable medical therapy in NYHA III was comparable to the best results of DCMP patients in the same NYHA class. Summarizing the results of these studies comparing DCMP with medical treatment, following statements can be made:

1. As long as medical treatment is effective, it should be continued.

2. If medical therapy in NYHA III gets insufficient and patients are going to move towards NYHA IV, a DCMP is indicated. The poor results of a medical treatment applied in NYHA IV is visible in the lowermost curve of figure 13 (f).

3. Patients having been in NYHA IV and recovered to NYHA III are candidates for a DCMP as well.

4. When a DCMP is stimulated by a stimulation pattern with less stimulation pulses, the surgical treatment might become superior to a medical treatment in future (compare topmost curve of figure 13 (a) stimulated with less pulses and using a day and night regime, having no stimulation at night.)

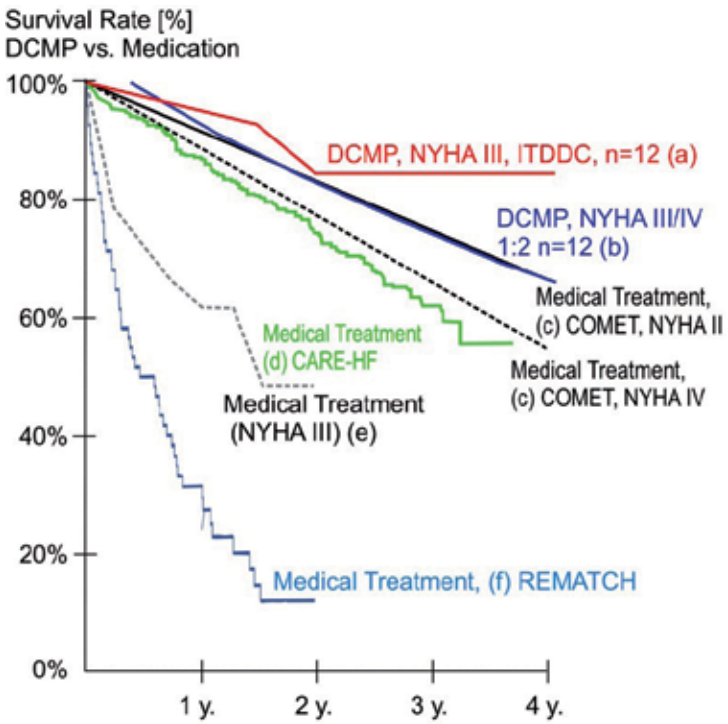

Figure 13. Clinical efficacy of the DCMP (NYHA III/IV, 1:2) in comparison to medical treatment options. (a) Rigatelli, 2002, [118] (b) Benicio, 2003, [20] (c) COMET, Poole-Wilson, 2003 [146] (d) CARE-HF, Cleland, 2005 [148] (e) Bocchi, 1996, [23] (f) REMATCH, Rose, 2001, [147]

3.2. Cardiac resynchronization therapy (CRT)

Comparing survival rate of DCMP and cardiac resynchronization therapy (CARE-HF, CRT-group, [148]), some authors found superior and others equivalent results. The DCMP procedure is more invasive and expensive than a CRT. But indication for a CRT is a heart failure in combination with a desynchronization of the left and right ventricle (left or right bundle branch

block). Additionally CRT is not effective in one third of these patients. Those are candidates for a DCMP.

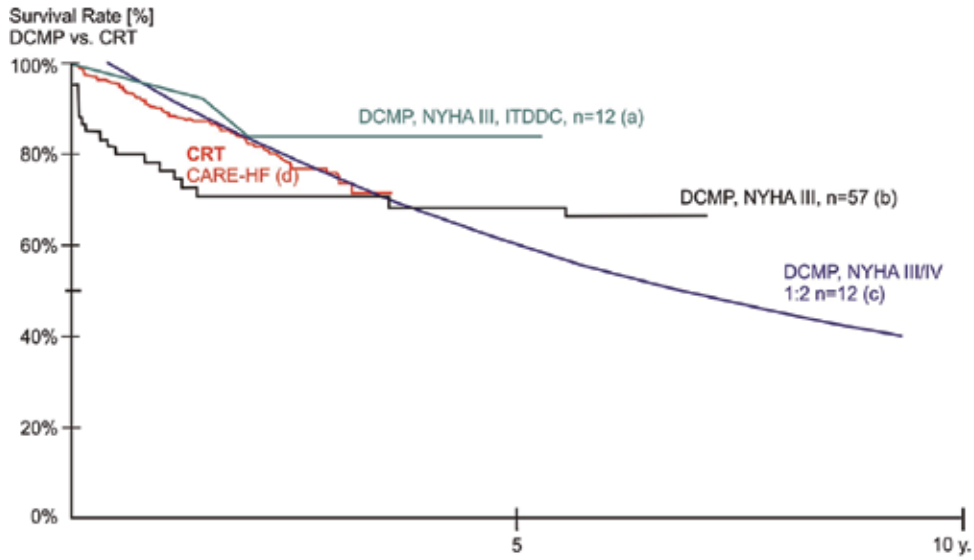

Figure 14. Efficacy of the DCMP in comparison to the cardiac resynchronisation therapy (CRT) in patients with bundle branch blocks. (a) Rigatelli, 2002 [118], (b) Chachques 1997, [37], (c) Benicio, 2003 [20], (d) CARE-HF Cleland, 2005 [148]

3.3. Heart transplantation

Comparing clinical results from the DCMP to those from the heart transplantation (HTX), their survival rates are comparable (figure 15, (a,b,c) vs. (d) [ISHLT, Heart Transplantation, Annual Report 2011 [149])). Objection however is, HTX is mainly suitable to NYHA class IV and DCMP to NYHA III. Taking in account the tremendous lack of donor hearts, it is to consider to apply the DCMP as early as possible in the non-effective medical treatment of NYHA III patients, to avoid a manifest stadium NYHA IV. Furthermore patients with a DCMP have no contraindication for HTX procedure. That is why DCMP can be regarded as a bridge to transplant [2] over years. In conclusion DCMP can contribute to a therapy of end-stage heart failure postponing HTX for years.

3.4. Left heart assist devices

Figure 16 indicates that a therapy by left heart assist devices (LVADs) is inferior to HTX, but due to the lack of donor hearts it is often the only treatment option in severe heart failure. A DCMP in NYHA III however may postpone a LVAD or HTX procedure. A comparison of DCMP and LVADs [150] count similar limitations like mentioned in HTX above, concerning the comparison of groups with different NYHA classes.

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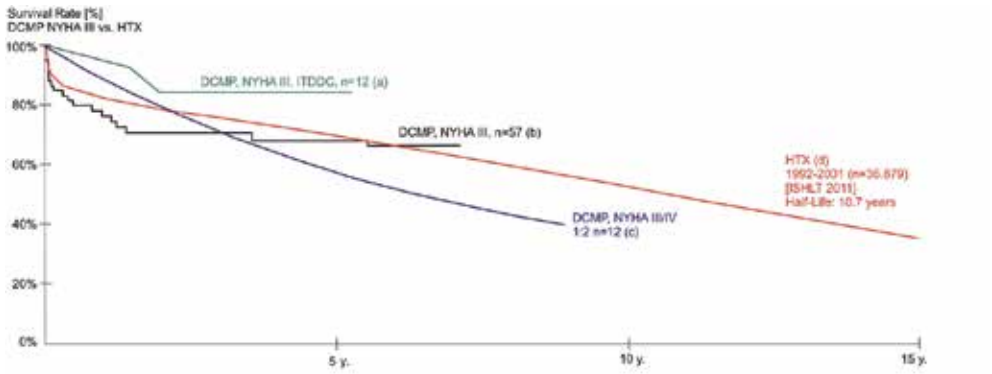

Figure 15. Clinical efficacy of a DCMP treatment in comparison to heart transplantation (HTX). (a) Rigatelli,2002, [118] (b) Chachques, 1997, [37] (c) Benicio, 2003 [20] (d) ISHLT, 2011, [149]

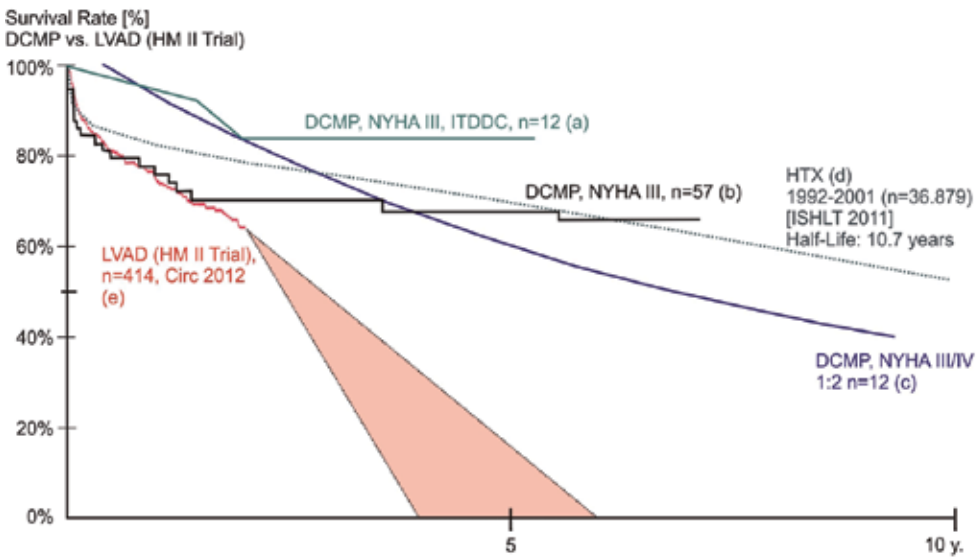

Figure 16. Efficacy of DCMP in comparison to mechanical assist devices (HeartMate II) and heart transplantation (HTX). (a) Rigatelli, 2002, [118], (b) Chachques, 1997 [37], (c) Benicio, 2003 [20], (d) ISHLT, 2011, [149] (e) Park, 2012, [150]

3.5. Stem cell therapy

The stem cell therapy of the human myocardium with mesenchymal stem cells (MSCs) has been evaluated in several clinical studies [151], [152]. The MSCs were applied intracoronarilly after myocardial infarction, and intra-myocardially solely and in combination with aorto- coronary bypass-surgery [153]. Thereby the ejection fraction (EF) of the left heart ventricle was seldom enhanced by more than 5% in the long time. The therapeutic effect never lasted longer than 6 to 9 months which was due to the disappearance of the MSCs

from the myocardium within several months. Thereafter MSC's were found predominantly in liver and spleen. Thus DCMP treatment might be superior to a MSC' s therapy, however there are not enough comparative clinical data yet. A combination of both seems favourable. An additive therapeutic effect specially with a stem cell transforming into cardiomyocytes and a permanent homing within the myocardium might enhance this therapy (Guldner et al. 2006, [154], 2009, [155]).

4. Summarizing appraisal of the clinical efficacy from DCMP

Evaluating 139 papers from literature about clinical dynamic cardiomyoplasty, DCMP showed to be effective accordingly to survival rate, NYHA class, hospitalization rate and ejection fraction of the hemodynamic from the left heart ventricle. Survival rate was clinically relevant higher in NYHA class III than in NYHA IV (Figure 4, [37]) and additionally more patients survived synchronized in 1:2 mode than in 1:1 pacing (Fig.5, [20]). Quality of life showed to be enhanced by a decreased NYHA of 1.57 (pre-operatively NYHA 3.26 ± 0.63 to post-operatively NYHA 1.69 ± 0.34 [$p<0.05$]). The ejection fraction of the left heart ventricle was increased from 21.9 ± 4.5 % to 28.9 ± 5.9% ($p<0.05$). A better clinical outcome is documented in cases with a reduced electrical stimulation [4], [33], [34], [117–125] accompanied with a day and night regime. A higher stimulation frequency might result in a muscle damage [5]. Dynamic cardiomyoplasty in comparison to other treatment options for end-stage heart failure like heart transplantation, resynchronization therapy, mechanical left ventricular assist devices and stem cell therapy has a clearly defined therapeutic indication. These are patients with a non-pharmacologically treatable heart failure of the NYHA class III and the non-responders of the resynchronization therapy (CRT). Due to poor therapeutic results, NYHA IV class patients are not to be considered for a DCMP.

5. Rational of pre-stimulation

Enhanced capillary density in skeletal muscle, induced by different stimulation patterns, has been well investigated in small animals [156], [157] and only a few investigations are in big animals [11]. But these experiments seldomly correlate capillary density with blood flow. Thus, an evaluation of blood flow, and capillary density of a clinically feasible two weeks electrically in-situ-conditioning in latissimus dorsi muscle (LDM) was investigated at rest and exercise in human sized animals. In five adult female Boer goats (52±9 kg), the left LDM (group I) was stimulated in-situ electrically over 14 days (10 Hz, 2-5V, 12h/day, 1h on-1h off). The right LDM served as control (group II). After conditioning, the LDMs blood flow was measured at rest and exercise (electric muscle stimulation over 10 minutes) by an ultrasonic flow probe around the A. thoracodorsalis. Muscle samples of different regions of LDM were harvested to evaluate the capillary to fiber ratio, mean diameter of the muscle fibers and their myosin heavy chain composition (type I /II).

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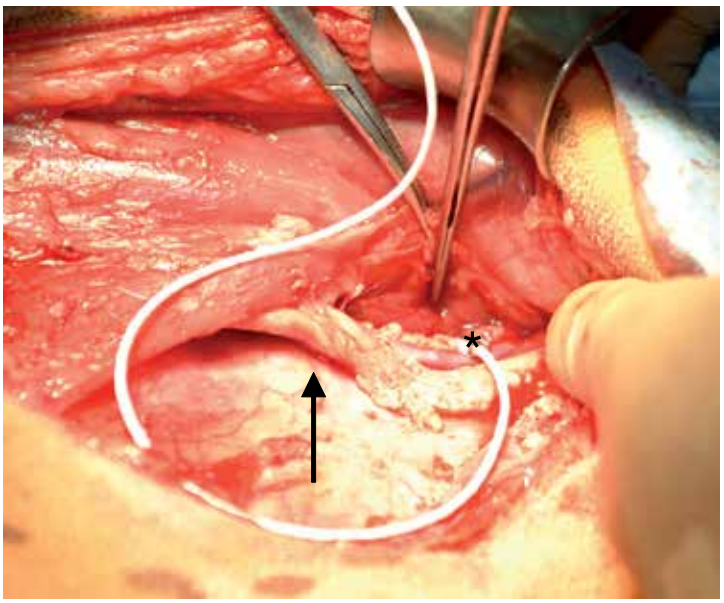

Figure 17. Operative situs in a goat during flow evaluation.The neuro-vascular bundle including the arteria thoraco-dorsalis leading to the latissimus dorsi muscle is demonstrated (arrow). The flow probe (*) is situated around the arteria thoraco-dorsalis.

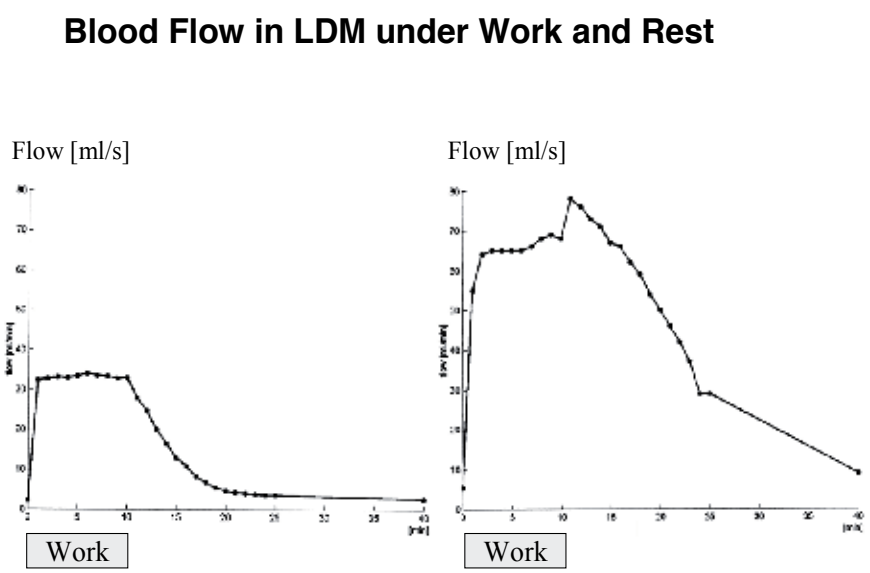

Figure 18. Blood flow within the Arteria thoraco-dorsalis of a non pre-stimulated LDM in a goat in-situ (left) in comparison to the same setting 14 days after electrically pre-stimulation of LDM in-situ (right) The pre-stimulated LDM (right) showed a higher blood flow under work conditions during a 10 minutes burst stimulation (work).

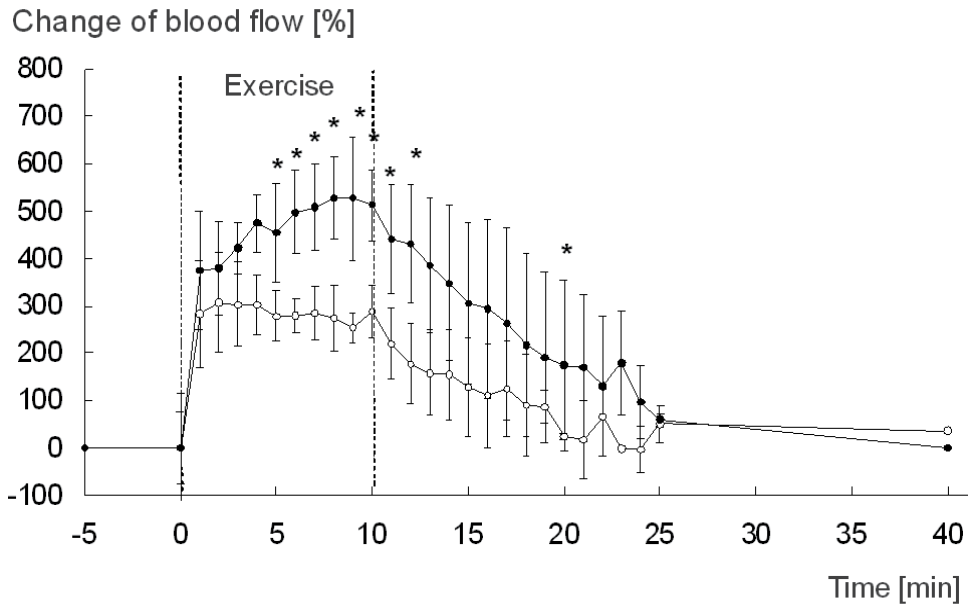

Figure 19. Change of blood flow [%] in arteria thoracodorsalis of non- (white spots)- and pre-stimulated (black spots) LDM ($p \leq 0.05$*) under rest, exercise (stimulation) and recovery. The two weeks stimulated muscle (n=5) shows a significant higher blood flow under exercise and recovery (*).

In group I, the pre-stimulated muscle (black spotted curve) shows an increased mean blood flow from 5.0±2.4 ml/min at rest to 30.2±2.7 ml/min under exercise. In group II, blood flow was 5.1±2.8 ml/min at rest and 19.9±0.9 ml/min under exercise. Therefore flow under exercise increased to 51.8% ($p \leq 0.01$).

5.1. Collaterals

Within latissimus dorsi muscle there exist two arterial territories. A proximal one supported by the thoracodorsal artery and a distal territory supplied by perforating arteries deriving from intercostal arteries. Mobilization of the latissimus dorsi muscle as a functional graft necessarily involves division of perforating arteries that enter the distal portion of the muscle causing an ischemic tissue with a loss in function.

Between the two arterial territories anastomotic connections are well described by Salmons et.al 1998 [9]. In clinical applications, the existence of anastomotic connections offers the prospect of maintaining flow to the distal part of the grafted muscle. Electrical stimulation enhances blood flow through these anastomoses (Tang et al, 1998 [10]) demonstrated in a sheep model that a pre-stimulation of LDM in situ over two weeks increases blood flow in the distal part of LDM to a normal range while a reduction of flow by 50% was found in the untreated muscle. Own experience with pre-stimulated LDM in situ in goats confirm these findings. Pre-stimulated LDM's distal part looked well perfused after dissection but ischemic without pre-stimulation. We conclude, electrical pre-stimulation of the latissimus dorsi muscle in situ

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reduces the acute distal ischemia of LDM caused by surgical manipulations, and promotes subsequent recovery of blood flow.

In conclusion, applying pre-stimulation in-situ before a DCMP procedure might result in a relevant functional advantage and might prevent muscle damage in the distal muscle tissue of the LDM.

5.2. Capillary density

Experimental own data from big animal experiments in Fig.20 and Fig.21 and experimental papers of others dealing with muscle powered cardiac assist show that a pre-stimulation of the LDM in-situ enhances LDM's capillarization.

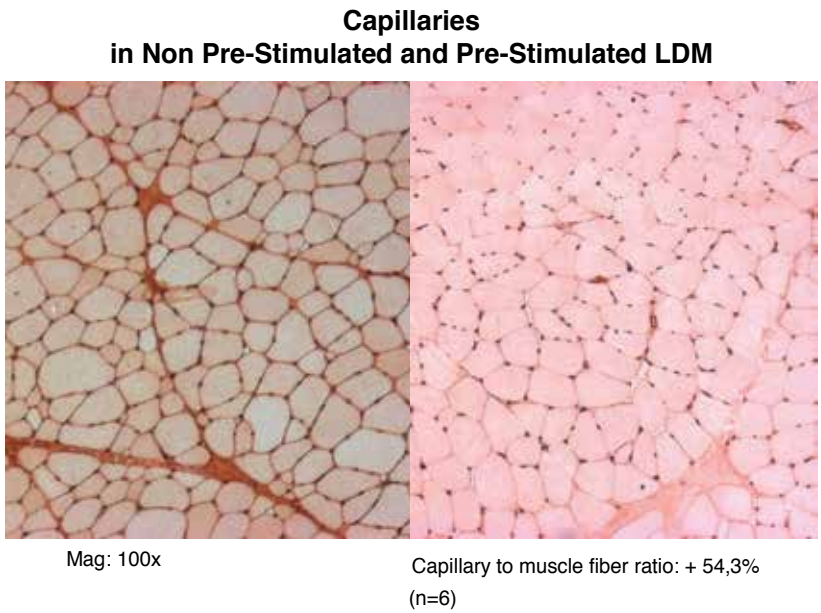
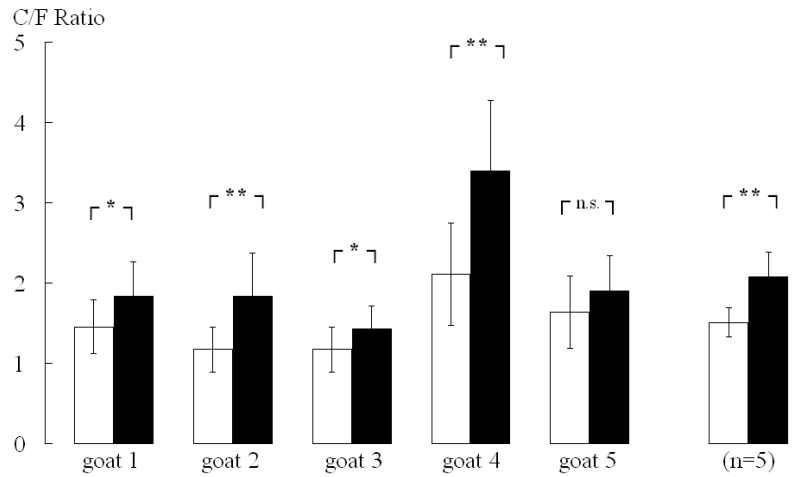


Figure 20. Histologic visualization of cross-section areas from LDMs muscle fibers surrounded by capillaries (black dots), in the stimulated muscle (right) exists a higher number of capillaries than in the non-stimulated LDM (left).



C/F ratio in muscular tissue in five boore goats of non (white block)- and pre-stimulated (black block) LDM (p≤0.05*; p≤0.01**,n.s. = not significant;).

Figure 21. 14 days of electrical stimulation increased the capillary to fiber ratio in goat's LDM (n=5) to 38% (black columns) in comparison to the non-stimulated contra- lateral LDM control (white columns). (* $p < 0.05$; ** $p < 0.01$; n.s. not significant)

Ischemic damage in the latissimus dorsi muscle may limit the success of cardiomyoplasty. Electrical pre-stimulation of the muscle in situ is known to enhance capillarization and thoracodorsal perfusion to the distal latissimus dorsi muscle immediately after grafting. Use of a pre-stimulated graft may therefore improve the outcome of skeletal muscle cardiac assistance (Tang et. al,1998, [10])

6. Closed-Loop Controlled Stimulation (CLC-Stimulation)

Since the basic experimental investigations of Salmons [7] and Pette [3], [8] it was evident, that the number of electrical pulses to a muscle fibre determines its fibre type. They showed that it is possible to transform a fast twitching fatigable IIa muscle into a fatigue resistant type I fibre muscle by increasing the number of pulses within a defined stimulation pattern. At the first glance it seemed to be ideal to substitute a damaged non-fatigable heart muscle by a slow twitching non-fatigable skeletal muscle. However, this dream was not to fulfil the expectations of muscle powered cardiac assistance clinically due to a tremendous loss of power in type I fibre muscles combined with a reduced contraction velocity. Nevertheless the DCMP showed to be effective clinically in certain limits as shown before.

Experimental investigations of Lopez-Guajardo et al. 2001 [158] demonstrated that a mean pulse reduction within certain limit results in a strong and non-fatigable muscle. But there was

a limitation: the time of usage of this non-fatigable type II a muscle was restricted. Otherwise the muscle transformation would continue into type I fibres with all the negative consequences of power loss and a decreased contraction velocity. Therefore a controlled pulse management is mandatory to keep non-fatigable type IIa muscle fibres. This restriction in pulses however, goes along with a time-wise limited usage for a cardiac support. This fact has major implications for a restricted usage in muscle powered cardiac assist with no blood contact like the DCMP and even more in muscular blood pumps [159–161].

To fulfil these demands in electrically stimulated muscular cardiac support to maintain a non-fatigable strong muscle with a preserved contraction velocity, myostimulators are mandatory to enable a closed-loop controlled stimulation.

6.1. Technical basics for a closed-loop controlled muscle stimulation

In order to protect the assisting muscle, the new Microstim myostimulator will prevent chronic overstimulation which results in an undesired muscle fiber transformation and degeneration. The fiber transformation to type I would lead to a subsequently reduced contraction velocity and muscle force accompanied by a reduced cardiac support with structural and functional muscle damage.

A closed-loop is designed to limit the amount of applied stimulation pulses below a certain maximum value of mean stimulation frequency over a given period, e.g. 0.7 Hz mean stimulation frequency within 24h. This closed-loop is used to maintain the fast and powerful twitching type IIa muscle fibres needed for a sufficient muscle powered cardiac assistance.

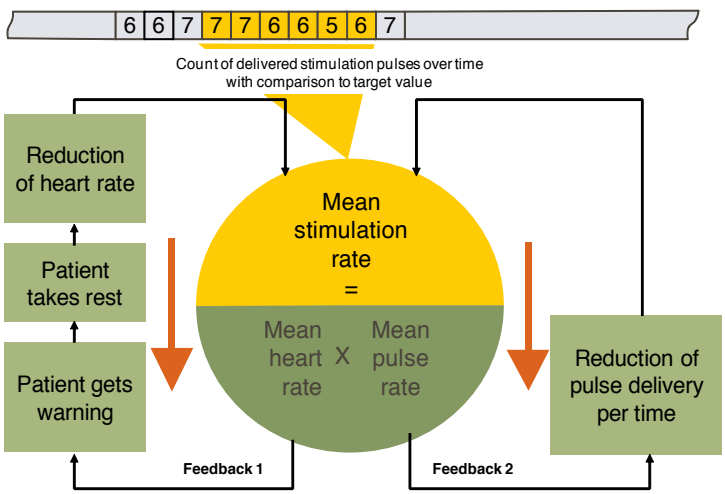


Figure 22. Closed-loop control to prevent muscular overstimulation. In feedback path 1, the patient receives a warning message via RF from the implanted myostimulator to a wearable patient monitor. Feedback path 2 intrinsically reduces the pulse delivery in accordance to programmed parameters.

In two groups of goats, skeletal muscle ventricles (SMVs) were shaped intra-thoracically [159], [160]. In group A (n=6) goat's LDM was not pre-stimulated and SMVs contracted by bursts of a mean pulse frequency of 5 Hz. This mean frequency of approximately 5 Hz has been used clinically in more than 1000 cases up to now, resulting in severe muscle damage as shown above in chapter 2.2. Group B, LDM with pre-stimulation over 14 days as shown above and with a controlled stimulation with a mean pulse frequency not exceeding 1Hz mean pulse frequency showed a preserved muscle tissue (figure 23, right). This new stimulation regime in group B became feasible by the newly available myostimulator with an integrated feature for muscle protection (MyoSen ® Myostim GmbH, Wismar, Germany).

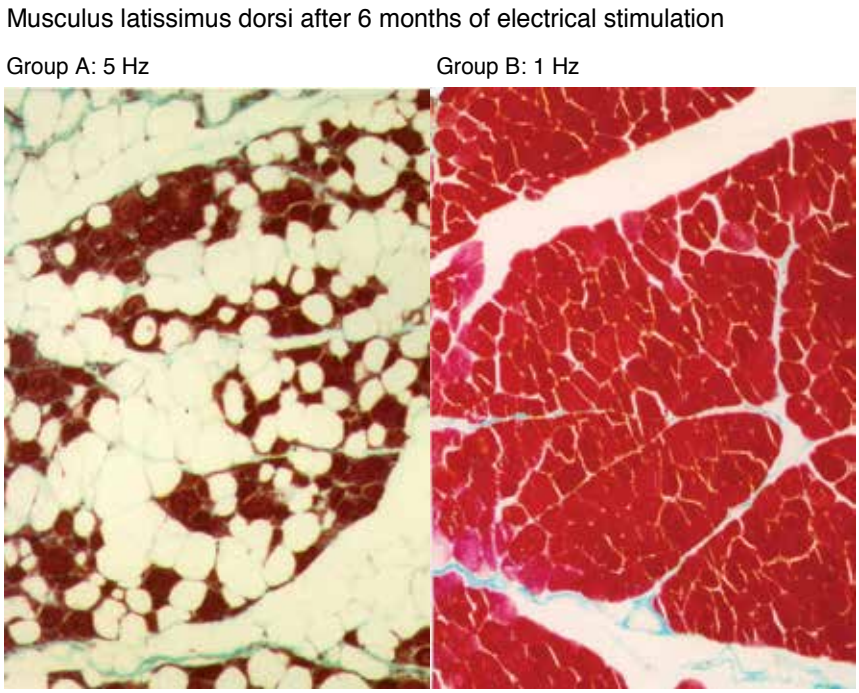


Figure 23. Histology of LDM 6 months after electrical stimulation with 5Hz and without pre-stimulation resulting in severe muscle damage (left) and LDM with pre-stimulation over 14 days and closed-loop controlled stimulation with a mean pulse frequency of 1Hz and preserved muscle tissue.

7. DCMP vs. CLC- DCMP — Clinical implications

This special kind of "electrical tissue engineering" of the LDM in situ 14 days before wrapping the LDM around the heart and a controlled electrical stimulation pattern with a reduced mean pulse frequency minor 1Hz should significantly increase the clinical efficacy of DCMP which has been described above. The new pre-stimulated, controlled cardiomyoplasty (CLC-CMP)

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or its combination with intra-myocardial injections of adult stem cells (Stem Cell Cardiomyopexy) might open new fields in the therapy of pharmacologically untreatable heart failure.

Comparing DCMP vs. CLC- DCMP, the expected efficacy of CLC- DCMP, well based on facts as shown above, should become an effective and reliable treatment option for end-stage heart failure with much better therapeutic results as shown in clinical DCMP up to now. A combination with an intramyocardial stem cell therapy is desirable [154], [155].

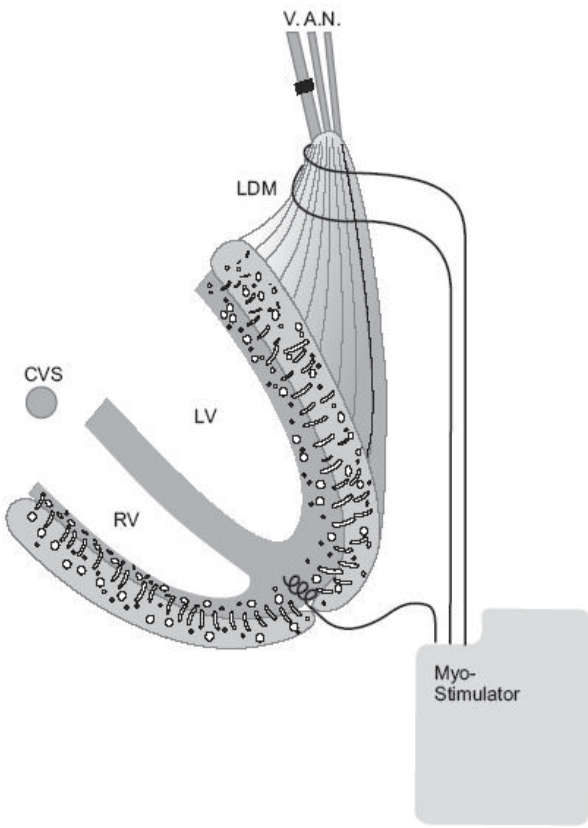

Figure 24. Pre-stimulated and such hypercapillarized muscle, wrapped around the heart for indirect revascularization and girdling using a stimulation with a reduced number of pulses as a controlled cardiomyoplasty (CLC-DCMP).

8. Conclusion

DCMP has been effective clinically but did not fulfil the therapeutic expectations. Additional treatment processes like pre-stimulation in situ and CLC-DCMP-pacing may increase clinical efficacy for patients with a refractory medical treatment in NYHA III in future.

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Chapter 33

Complications of Post-Transplant Immunosuppression

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Additional information is available at the end of the chapter

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1. Introduction

Transplantation is one of the revolutionary fields in modern medicine that has saved thousands of lives. The continuous refinement of surgical techniques and the availability of potent immunosuppressive drugs have made transplantation the most effective treatment option for patients with end stage organ failure. Over 25 000 organ transplants are performed in the USA each year and survival rates following transplantation are now approaching 90% at 1 year and 75% at 5 years, depending on the organ transplant (kidney, liver, pancreas, heart, lung, intestine). Central to this success was the introduction of drugs that suppress the immune system and prevent rejection. Indeed, across organs, the use of current immunosuppression *regimens* effectively prevents acute rejection in the majority of patients. As a result, the incidence of graft loss due to acute rejection has decreased dramatically compared to the early era of transplantation.

This success of organ transplantation has led to a growing population of immunosuppressed transplant recipients with prolonged survival with a functioning graft, but also with prolonged exposure to the side effects and complications of chronic immunosuppression. Indeed, the burden of chronic immunosuppression post-transplant has become a growing concern among transplant physicians, although its impact is currently smaller compared to two decades ago, following the introduction of new and less-toxic immunosuppression *regimens* (see below). However, chronic immunosuppression remains associated with significant morbidity: as an example, the majority of patients treated with calcineurin inhibitors develop some degree of renal function impairment and up to 10% progress to kidney failure requiring dialysis or kidney transplant (see below).

The consequences of chronic immunosuppression on multiple organ systems are becoming increasingly evident and often new symptoms or disorders develop post-transplant as a consequence of the side-effects of immunosuppressive drugs (ie opportunistic infections,

malignancies, diabetes mellitus, hypertension and others, see below). The onset of new post-transplant conditions requiring treatment has multiple implications in terms of function of the graft, patient compliance and cost. Therefore, strategies to limit and prevent the complications of prolonged immunosuppression post-transplant are needed.

Here we present briefly the most common immunosuppression *regimens* currently used in abdominal organ transplantation and we review current major complications and challenges of prolonged immunosuppression after transplant. We will discuss issues common to abdominal organ transplants including liver, intestine, pancreas, kidney, without entering into organ-specific issues. The discussion is limited to adult transplant recipients, since pediatric transplantation raises a number of issues specific to this age group in terms of immunosuppression *regimens* and its complications including growth-related issues and compliance. In addition, a pediatric transplant recipient has potentially an expected more prolonged exposure to chronic immunosuppression than an adult and, as a consequence, more time to develop complications.

We will also present current strategies in the management of complications of immunosuppression and ways to limit the burden of immunosuppression. Finally, we report on current research and indicate future directions to improve post-transplant immunosuppression.

2. Current immunosuppression *regimens* in abdominal organ transplantation

Compared to the early era of transplantation, a substantial progress has been made since the early 1990s in developing effective immunosuppressive agents to prevent allograft rejection. As a result, graft and patient survival rates have dramatically improved. In addition, highly immunogenic organs such as heart, lungs and intestine, previously characterized by a high incidence of failure due to rejection, are now being successfully transplanted since more potent immunosuppressive drugs have become available.

Most immunosuppressive agents target T lymphocytes, which are primary mediators of the alloimmune response and effectors of the rejection process. Current immunosuppression protocols usually include two or more agents to target different steps or mechanisms of the alloreactive immune response. The combination of multiple drugs not only increases the efficacy of the immunosuppression *regimen* but also often allows dose reduction of one or more of the drugs in an attempt to limit the associated toxicity (see below). Recently, other agents have been introduced that target B lymphocytes and other mechanisms involved in the alloimmune response including complement and others mechanism of the innate immune system (see below). As a result, an increasing number of immunosuppressive agents are now available (Table 1).

There are also new drugs being evaluated in clinical trials that target novel mechanisms and pathways of the immune response in attempt to reduce the burden of side effects and complications of agents currently available [1,2].

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Antibodies	Alemtuzumab, Atgam, Basiliximab, Daclizumab, OKT3, Thymoglobulin
Antimetabolites	Azathioprine, Mycophenolate mofetil, Mycophenolate sodium
Calcineurin-inhibitors	Cyclosporine, Tacrolimus, Voclosporine
Corticosteroids	Methylprednisolone, Prednisone
Co-stimulation blockers	Belatacept
Proliferation- inhibitors	Everolimus, Sirolimus
Others	Bortezomib, Infliximab, Rituximab

Table 1. Immunosuppressive agents currently available

Immunosuppression is usually heavier in the peri-operative period and early post-transplant (induction) when the risk of rejection is higher due to a number of factors including preservation injury of the graft and sudden exposure of the recipient immune system to a load of foreign antigen. Later, depending on graft function and tolerability, immunosuppressive doses are gradually reduced (maintenance) to levels adequate to prevent rejection and avoid toxicity. Although there are reports of "tolerant" patients, who maintain a functioning graft after discontinuation of immunosuppression) (see below), these are rare and exceptional cases and immunosuppression needs to be continued lifelong, inevitably exposing the recipient to the long term effects of chronic immunosuppression. Since the is no single optimal immunosuppression *regimen*, post-transplant care strives to achieve the delicate balance between effective prevention of rejection and avoidance of toxicity. The doses of immunosuppressive drugs are usually adjusted according to target trough levels, which vary among organs and among transplant programs.

2.1. Antibodies

Polyclonal antithymocyte globulins (Atgam, Thymoglobulin) are prepared from the serum of rabbits immunized with human thymocytes. Antithymocyte globulins contain cytotoxic antibodies that bind to CD2, CD3, CD4, CD8, CD11a, CD18, CD25, CD44, CD45 and HLA class I and II molecules on the surface of human T lymphocytes. The mechanism of action of depleting antibodies is to reduce the number of circulating lymphocytes by direct cytotoxicity, both complement and cell-mediated. Anti-CD3 monoclonal antibodies (OKT3) is a mouse monoclonal antibody against CD3. It binds to T-cell receptor-associated CD3 complex and depletes and alters T-cells. Its use has declined since newer immunosuppressive drugs have reduced the incidence of rejection episodes.

Non-depleting antibodies (Basiliximab, Daclizumab) block lymphocyte function by binding to cell surface molecules involved in the regulation of cell function. The main uses of antibodies in post-transplant immunosuppression are during induction and for the treatment of severe or steroid-resistant rejection (review in [3]). The risk of opportunistic infections (viral, fungal) is higher after profound T cell depletion, especially if prolonged, compared to the use of non-

depleting agents. Adverse effects include fever, chills, thrombocytopenia, leukopenia, hemolysis, respiratory distress, serum sickness, and anaphylaxis.

2.2. Antimetabolites

Azathioprine, a derivative of 6-mercaptopurine functioning as an antimetabolite to decrease DNA and RNA synthesis, has been used for many years since the early era of organ transplantation in combination with corticosteroids. The mechanism of action of azathioprine is to incorporate into and to halt DNA replication by blocking the *de-novo* purine synthesis in lymphocytes. Adverse effects include myelosuppression (leukopenia, thrombocytopenia), nausea, vomiting, diarrhea, hepatitis, cholestasis and alopecia. In the last 10 years azathioprine has been largely replaced by mycophenolate mofetil and mycophenolate sodium (two preparations of mycophenolic acid) in many transplant programs.

Mycophenolic acid. Unlike other cell types that can "recycle" purines from the process of cell turnover, lymphocyte proliferation and responses are dependent on the *de novo* purine synthesis; mycophenolic acid blocks the action of the key enzyme inosine monophosphate dehydrogenase (IMPDH), a rate limiting step in the biosynthesis of purines crucial to cell cycling in T and B lymphocytes. Consequently, the proliferation and clonal expansion of T and B lymphocytes is prevented, with the effect of reducing the alloreactive immune response, including antibody production and the generation of cytotoxic T cells and other effector cells. In addition, mycophenolic acid suppresses the glycosylation and the expression of adhesion molecules, thereby decreasing recruitment of lymphocytes and monocytes into sites of inflammation and graft rejection.

Two formulations of mycophenolic acid are now available, mycophenolate mofetil and mycophenolate sodium. Both formulations are non-nephrotoxic and are being used in calcineurin-inhibitors sparing regimens in attempt to reduce the risk of renal failure (see below). The main side effects of mycophenolate mofetil are gastro-intestinal intolerance (diarrhea), reported in up to 45% of patients and often requiring dose reduction or discontinuation. The enteric-coated mycophenolate sodium was designed to reduce the mycophenolic acid-related gastro-intestinal adverse effects: the enteric coating dissolves at pH levels ≥5, thus delaying the delivery of the active compound mycophenolate acid until the small intestine.

2.3. Calcineurin-inhibitors

The most commonly used class of immunosuppressive drugs currently used in organ transplantation are calcineurin inhibitors (CNIs). Indeed, calcineurin inhibitors (cyclosporine and tacrolimus) are main immunosuppressive agents in use today in virtually every transplant program. Their immunosuppressive effect results from the blockage of the production of pro-inflammatory cytokines including IL-2, INF-γ, TNF-α and from inhibition of T cell activation and proliferation. Their mechanism of action is to inactivate calcineurin, an essential enzyme for the function of T cell lymphocytes. Calcineurin, an intracellular calcium/calmodulin phosphatase triggered by the engagement of T cell receptor by donor MHC, dephosphorilates

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nuclear factor for activated T cells (NF-AT) which in turn promotes the transcription of cytokine genes. The main adverse effect associated with the use of CNI is renal function impairement: virtually all people who take a CNI will develop some degree of kidney toxicity and up to 10% will progress to kidney failure. With more people taking CNIs for longer and longer periods of time the consequences of calcineurin inhibition on other organ systems - particularly kidney function - have become a growing concern. In addition to nephrotoxicity, other adverse effects of CNI include hyperkalemia, hypomagnesemia, nausea, vomiting, diarrhea, hypertrichosis, hirsutism and gingival hyperplasia. Tacrolimus, a more potent CNI compared to cyclosporine, shares the same mechanism of action and the same risk of nephrotoxicity. Tacrolimus binds to a cytoplasmic protein FK506-binding protein 12 (FKBP12) to create a complex that inhibits phosphatase activity of calcineurin. Tacrolimus, like CsA, inhibits signal transduction pathways linked to the T-cell receptor for antigen at the level of JNK and p38 kinase. While the abnormal cosmetic side effects (hypertrichosis and hirsutism) are less frequent with tacrolimus compared to cyclosporine, glucose intolerance and neurotoxicity (headache, seizures) are more common. Voclosporin, a cyclosporine analog with reduced nephrotoxicity, is a novel calcineurin inhibitor being developed for organ transplantation and currently in clinical trials: preliminary results showed a reduced risk of post-transplant diabetes compared to tacrolimus while maintaining the same efficacy in preventing rejection in kidney transplantation [4].

2.4. Corticosteroids

Corticosteroids (methylprednisolone, prednisone) were the first immunosuppressive drugs to be used in transplantation and remain today first line treatment across organs for both prevention and treatment of rejection. The multiple anti-inflammatory and immunomodulatory effects on a wide variety of cells including lymphocytes, granulocytes, macrophages, monocytes and endothelial cells are well known and the molecular mechanisms of action of steroids have been described extensively [5]. Briefly, corticosteroids down regulate cytokine gene expression in lymphocytes, antagonize macrophage differentiation, inhibit neutrophil adhesion to endothelial cells thereby decreasing their extravasation to the site of inflammation, decrease circulating eosinophil and basophil counts, inhibit IgE-dependent release of histamine and leukotriene from basophils and inhibit degranulation of mast cells. Additionally, glucocorticoids downregulate endothelial cell function including expression of class II MHC antigen and expression of adhesion molecules. Based on these multiple effects on different cellular components of the immune response corticosteroids are very effective in preventing and treating acute allograft rejection, although there are instances of steroid-resistant rejection episodes. The multiple side effects of steroids are also well known and include impaired wound healing, increased risk of infection, hypertension, weight gain, hyperglycemia, osteoporosis, fluid retention, hirsutism, acne and cataracts. Side effects may have an important impact especially in the long term and in children (ie growth pattern), therefore multiple trials of steroid withdrawal and steroid-free regimens have been designed in an attempt to limit the side effects of corticosteroids.

2.5. Costimulation blockers

Costimulation blockers represent a new class of immunosuppressants with a different mechanism of action compared to calcineurin inhibitors [6]. Costimulation (or signal 2) refers to the amplifying signal received by the T lymphocyte after interaction with ligands presented by antigen presenting cells. This costimulation amplifies the initial T cell activation event (or signal 1) resulting from the engagement of T cell receptors with donor antigens. Indeed, T cells undergoing signal 1 without signal 2 become unresponsive and undergo apoptosis. Several costimulatory pathways mediate the interactions between the surface of T cells and antigen presenting cells. One of the most studied pathways involves the surface molecule CD28 on lymphocytes and the B7 family of molecules on antigen presenting cells. This signaling pathway has become an attractive target for the development of novel immunosuppressive drugs. Two humanized fusion proteins have been developed to inhibit costimulatory signaling, abatacept and belatacept. The latter has been used in clinical kidney transplantation [7].

2.6. Proliferation inhibitors (mTOR-inhibitors)

This group includes everolimus and sirolimus, two of the most recently introduced immunosuppressive agents in clinical transplantation, acting with a mechanism of action different from calcineurin-inhibitors and from antimetabolites. Sirolimus will be discussed first, being the first mTOR inhibitor to be used in clinical transplantation. Sirolimus (also known as rapamycin) is a bacterial macrolide antibiotic produced by a strain of Streptomyces hygroscopicus isolated from a soil sample collected from the island Rapi Nui, commonly known as Easter Island. Although originally an antifungal agent with potent anti-candida activity, side effects precluded its use as an antifungal, and it has since been used primarily as an immunosuppressant. Sirolimus and everolimus are members of a newer class of immunosuppressive agents called inhibitors of the mammalian target of rapamycin. Sirolimus binds the intracellular immunophilin FKB12, the same intracellular binding protein of tacrolimus, but with different mechanism of action. After binding the immunophilin, the complex sirolimus-immunophilin inhibits a protein called mammalian target of rapamycin (mTOR). Inhibition of mTOR results in selective inhibition of synthesis of new ribosomal proteins which are essential for progression of the cells from the G1 to the S phase. This results in blockage of T cell activation. In addition, sirolimus has been associated with inhibition of fibroblast growth factors required for tissue repair. The half life of sirolimus is 60 hours which allows single daily dose unlike other agents given twice daily and this has an important impact on patient compliance to immunosuppression *regimens*. Everolimus is a modified form of sirolimus to improve its absoprion. Its half life is shorter and is administred twice daily. Everolimus is currently undergoing clinical trials in transplantation in attempt to reduce the nephrotoxicity of calcineurin inhibitors [8,9]. The adverse effects of mTOR inhibitors include thrombocytopenia, leukopenia, anemia, arthralgias, hyperlipidemia, pneumonitis, and diarrhea. There have also been reports of wound complications (delayed wound healing, incisional hernia) in the post-transplant period, an affect probably secondary to its antiproliferative effects on fibroblasts. Oral ulcers were seen with the liquid preparation; however, this seems to be less frequent with the use of the pill preparation.

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2.7. Other novel immunosuppressive agents

In this group we include antibodies that act on different targets than T cells. Bortezomib is an antineoplastic agent originally developed for the treatment of multiple myeloma. It is a proteasome inhibitor that induces apoptosis in rapidly dividing cells with active protein synthesis like plasma cells. In kidney transplantation it has been reported to revert antibody-mediated rejection [10]. Anti–tumor necrosis factor (TNF) reagents (Infliximab) are monoclonal antibodies that bind with high affinity to TNF-alpha and prevent the cytokine from binding to its receptors. It is approved for treating the symptoms of rheumatoid arthritis. In transplantation it has been investigated in the treatment of severe rejection after intestinal transplantation [11]. Rituximab is a monoclonal antibody directed against the CD20 antigen on B cells. It is approved for the treatment of certain types of non-Hodgkin lymphoma and to reduce the signs and symptoms of moderate to severe rheumatoid arthritis. In transplantation its use is currently being studied in treating some forms of antibody-mediated rejection [12] and as part of desensitization protocols in highly sensitized transplant recipients [13].

3. Complications of prolonged immunosuppression post-transplant

As a result of the success of effective immunosuppression, many more transplant recipients live now longer after transplant compared to decades ago and have time to manifest the long term effects of chronic immunosuppression. This has become increasingly more evident with the longer follow-up of successful transplant recipients. Indeed, after achieving excellent survival rates across organs, a constant focus of research and current clinical trials are now concentrating on how to reduce or prevent or antagonize the burden of chronic immunosuppression. It is becoming increasingly clear that if an effective control of rejection on the one hand protects the graft function and prolongs patient survival, at the same time the patient is exposed to the risk of complications of prolonged immunosuppression and also to new post-transplant disease, even in presence of excellent graft function. These complications result from either persistently low immune defenses as a result of immunosuppressive therapy (infections and malignancies) or as a result of side effects of immunosuppressive drugs, which affect virtually every organ system (renal function impairment, diabetes, cardiovascular disease and others, see below).

3.1. Infections

The most obvious consequence of a decreased immune defense is the increased risk of infection. Indeed, infectious complications are among the most common causes of morbidity and mortality after transplantation. Improved immunosuppressive *regimens*, while reducing the incidence of allograft rejection, have increased the susceptibility to opportunistic infections. In addition, other factors including malnutrition, co-morbidities associated with end stage renal or liver disease and alterations of the muco-cutaneous barriers following the transplant procedure contribute to increase the risk of infections post-transplant. Post-transplant infections have been classified in 1.peri-operative infections (during the first month post-

transplant, usually nosocomial infections or donor-derived), 2. early post-transplant infections (within the first 6 months, usually due to reactivation of latent infections, mostly viral) and 3. late infections (occurring usually after 6 months from transplant, mainly community acquired infections) [14]. Strategies to prevent infections post-transplant are based on either universal prophylaxis (administration of antimicrobial therapy to all patients at risk of infection for a limited period, usually 3 to 6 months post-transplant) or pre-emptive therapy (monitoring patients at established intervals for early detection and treatment of infection). A large number of viruses, bacteria and fungi can cause significant infections post-transplant. Here we discuss the most common viral, bacterial and fungal infections post-transplant.

3.2. Viral infections

The most common viral infections post-transplant are caused by viruses listed in Table 2.

Adenovirus
Cytomegalovirus (CMV)
Epstein-Barr virus (EBV
Herpes simplex (HSV)
Influenza-Parainfluenza
Polyoma (BK)
Rotavirus
Varicella-zoster virus

Table 2. Viral infections post-transplant

Cytomegalovirus and Epstein-Barr virus, among others, are causing significant morbidity post-transplant and will be discussed here.

3.2.1. Cytomegalovirus [15,16]

The incidence of CMV infection post-transplant (ie detection of active viral replication in the recipient) ranges from 25 to 50%, depending on the organ [17] The incidence of CMV disease (ie organ damage by CMV infection) is lower, reported between 3 and 14% [18]. The main risk factors for CMV infection and disease include serology mismatch (donor CMV IgG positive, recipient CMV IgG negative), degree of imunosuppression, use of antilymphocyte antibodies for the treatment of rejection and the type of graft (more common in lung and intestinal transplant, likely related to the heavy immunosuppression *regimens* used in these recipients. Manifestations of CMV disease vary from flu-like symptoms to invasive organ disease. Most commonly affected are the gastrointestinal tract (ulcers), the lungs (pneumonitis) and the liver (hepatitis). The morbidity associated with CMV post-transplant is not only related to its direct effects (see above) but also to its indirect effects, including increased risk of rejection, of other infections and of EBV-related lymphoproliferative disorders [19] (see below). Prophylaxis of

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CMV is usually with either intravenous ganciclovir or with oral valgancyclovir, an oral prodrug of ganciclovir with equivalent drug exposure [18]. Standard treatment of invasive disease usually requires intravenous ganciclovir for 2-3 weeks, often extended for a longer period to treat severe disease. Foscarnet and cidofovir are alternative agents active on CMV but are rarely used because of their toxicity

3.2.2. Epstein-Barr virus [20, 21]

EBV is a DNAvirus associated with the common, usually self-limited infectious mononucleosis affecting young immunocompetent subjects. In the transplant recipient EBV infection may cause significant morbidity and mortality related to the development of post-transplant lymphoproliferative disorders (PTLD). EBV transforms and immortalizes B cell, which proliferate uncontrolled when the surveillance of EBV immunocompetent T cells is lacking secondary to immunosuppression. EBV infection post-transplant occurs either as primary infection, especially in children, or as reactivation. Risk factors for PTLD include primary EBV infection in a seronegative transplant recipient, the net state of immunosuppression (especially the use of antilymphocyte antibodies) and prior CMV infection. Quantitative EBV viral load assays are used for surveillance, diagnosis and disease monitoring. Non-PTLD manifestations of EBV disease post-transplant vary from mononucleosis-like viral syndrome to organ involvement (lungs, liver, gastrointestinal tract, bone marrow). PTLD presents a wide spectrum of histology and clinical presentations, from benign self-limited lymphoproliferation to aggressive disseminated lymphoma [22]. These lymphoproliferation are commonly extranodal and the transplanted organ may be involved as well. Outside the allograft, typical sites of involvement include the liver, gastrointestinal tract, skin and central nervous system.

The incidence of PTLD varies across organs from 1-5 % in kidney and liver transplant to as high as 15-20% in intestinal transplant recipients [23]. Based on morphologic, immunophenotypic, and molecular criteria, PLTD are classified into 4 pathologic categories: early lesions, polymorphic, monomorphic, and classical Hodgkin lymphoma.They present with a wide spectrum of pathologic and clinical manifestations ranging from benign lymphoid hyperplasia to aggressive lymphomas. Given the pathologic and clinical heterogeneity of PTLD, treatment is often individualized. Although there is no generally accepted protocol, treatment includes reduction or discontinuation of immunosuppression and a combination of rituximab (a chimeric anti-CD20 monoclonal antibody), chemotherapy, antiviral therapy and surgical resection depending on the aggressivity (review in [20] and [24]). New strategies are being tried such as adoptive immunotherapy [25].

3.3. Bacterial infections

The most common bacterial infections post-transplant are listed in Table 3.

The majority of bacterial infections early post-transplant (first month) are hospital acquired and are usually characterized by a high incidence of multidrug-resistance (review in [26]). Opportunistic bacterial infections, usually occurring between 2 and 6 months post-trasnplant, are caused by Listeria monocytogenes and Nocardia spp. Six months after transplant or later,

when immunosuppression is generally lowered, community-acquired bacterial infections are the most common, especially urinary tract infections by *E. coli* and *S. pneumonia* pneumonia.

Gram negative
Bacteroides and other anaerobes
Enteric bacteria
Pseudomonas
Gram positive
Staphylococcus spp
Streptococcus spp
Enterococcus spp (incl VRE)

Table 3. Common bacteria of post-transplant infections

Common bacterial infections post-transplant affect the urinary tract, the respiratory tract, the surgical wound and the bloodstream. The incidence of bacterial urinary tract infections ranges between 4.4% in non renal transplant recipients and 7% in renal transplant recipients, most commonly secondary to *E. coli* [27]. In one study the need for immediate post-op dialysis was risk factor for bacterial urinary tract infection in kidney transplant recipients, whereas age and diabetes were main risk factors in non renal transplant recipients [27].

Skin and wound infections, although not life-threatening, are common after solid organ transplantation. One study reported an incidence up to 45 % in kidney-pancreas recipients [28]. Most common isolates are *S. aureus*, but also enteric gram negative bacteria in abdominal organ recipients. The incidence of pneumonia also varies between organs from 7.3% within the first year after kidney transplant [29] to 22% after liver transplant [30] to 36% in lung transplant recipients [31] and is associated with prolonged intensive care stay and hospital stay. The source of bacterial bloodstream infections after transplant, in addition to intravenous catheters, include the respiratory tract, the urinary tract and the abdomen. However, often the source of bacteremia is not identified. Both Gram negative and Gram positive bacteria are isolated but in recent years methicillin-resistant staphylococci and vancomycin-resistant enterococci have become more common [32]. The presence of polymicrobial infection, the early onset of bacteremia after transplantation, the association with pneumonia, liver failure or kidney failure increase the mortality risk associated with bacteremia, reported up to 25% in lung transplant recipients [33].

3.4. Fungal infections

Fungal infections post-transplant cause significant morbidity and increase the mortality risk (review in [34]). Candida *spp*. and Aspergillus *spp.* are the most common causes of invasive fungal infections after transplant.

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Aspergillus spp.
Candida spp
Cryptococcus spp
Hystoplasma capsulatum

Table 4. Most common fungal infections post transplant

The incidence of invasive fungal infections varies across organs between 7 and 14% in pancreas transplant, 5-42% in liver transplant, 15 to 35% in lung transplant and 40-59% in intestinal transplant [35]. There are multiple risk factors related to the net state of immunosuppression (high doses of corticosteroids, use of antibody induction) and peri-operative factors such as prolonged complex operations including re-transplantation and renal dysfunction [36]. In addition, concomitant viral infections (ie CMV) exert immunomodulatory and immunosuppressive effects that increase the risk of fungal infections [37]. The clinical manifestations of invasive Candida infection vary across organs and include wound, intra-abdominal (peritonitis), thoracic (tracheobronchitis, pneumonitis) and bloodstream infection. The majority of cases of invasive Aspergillosis involve the lungs with single or multiple nodular infiltrate that may become cavitary lesions (Figure 1)

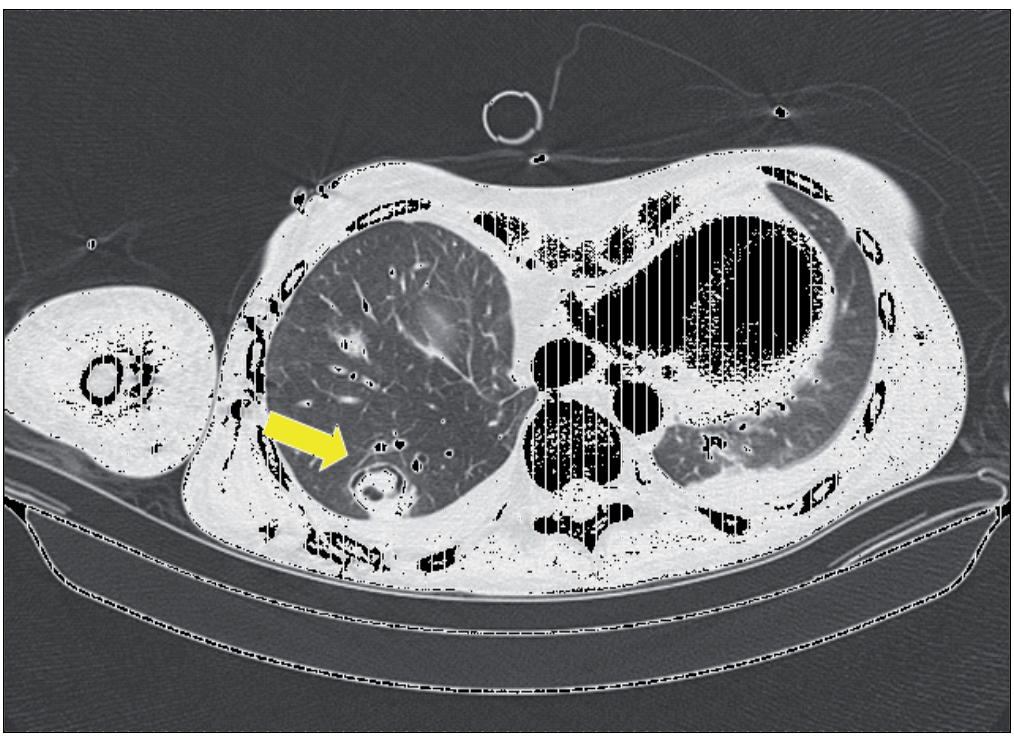

Figure 1. Pulmonary aspergillosis

The second and most invalidating site of invasive aspergillosis is intracranial, causing mental status alterations, seizures and focal neurologic deficits secondary to brain abscesses that most commonly involve the fronto-parietal lobes. The angiotropic character of Aspergillus infection tends to cause vascular invasion resulting in intracranial infarcts or hemorrhagic lesions (Figure 2)

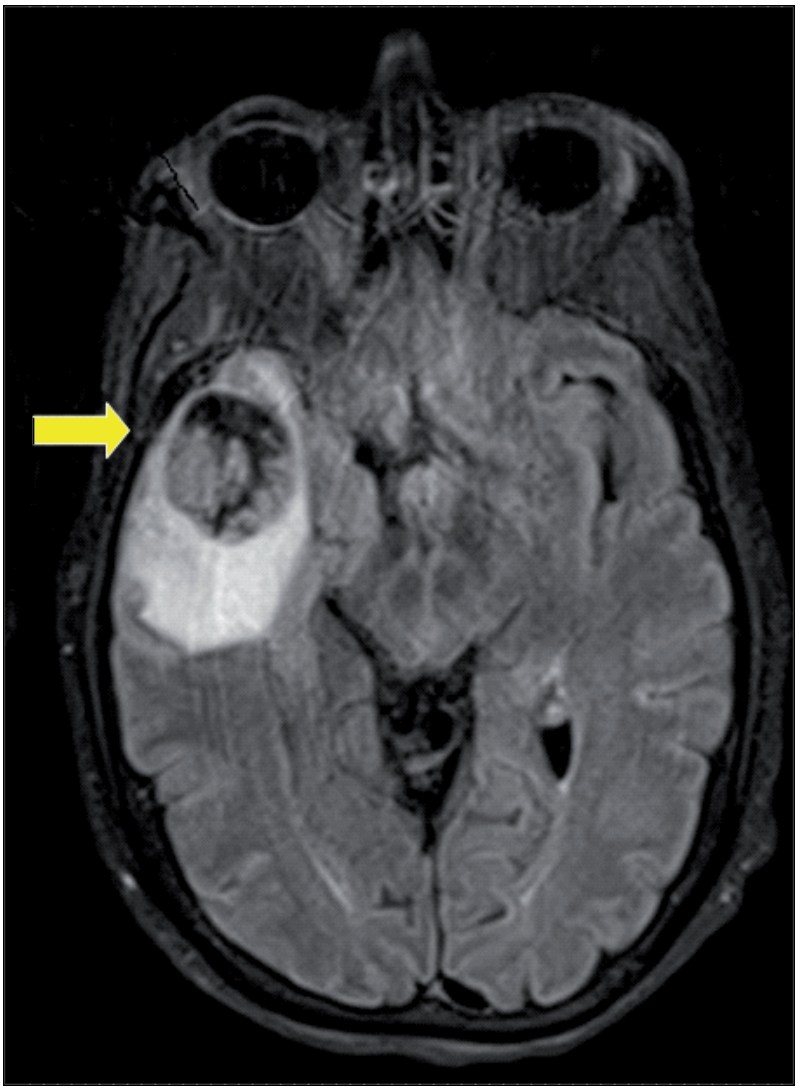

Figure 2. Intracranial aspergilloma

The overall 3 month mortality risk from invasive fungal infections after transplant across organs has been reported up to of 29% [34] and key strategies remain prophylaxis of high risk recipients and early diagnosis and prompt treatment.

3.5. Malignancies

In addition to increased risk of infections, chronic suppression of the immune defences is associated with increased risk of malignancies. The incidence has been reported 3- to 5-fold higher in transplant recipients than in the general population and increases with the length of follow up. It has also been reported that after 25 years of immunosuppression, about half of the recipients are at risk of developing some kind of tumor [38]. Indeed, in renal transplant recipients, cancer is the third most common cause of death after cardiovascular accidents and infections [39].

Squamous and basal cell carcinomas of the skin are the most common *de-novo* malignancies, accounting for almost half of cancers post-transplant, although recently melanoma has been re-emphasized as also having an increased frequency following transplantation. The ratio of squamos and basal cell carcinoma is 4:1, the opposite of what is found in the immunocompetent population. Squamous cell carcinoma is more aggressive in transplant recipients compared to the general population, tends to recur and occasionally to metastasize [40]. Risk factors for skin cancer post-transplant, in addition to prolonged immunosuppression, are age, skin type and exposure to ultraviolet radiation. Therefore, attempts are made to reduce the dose of maintenance immunosuppression after developing skin cancer and to reduce skin exposure and to seek dermatology yearly survey in order to reduce the risk of recurrence. In addition, clinical data have shown beneficial effects of the use of mTORinhibition (sirolimus, see above) in preventing cancer development in transplant recipients. It is likely that these effects are the result of sirolimus'antitumor and antiangiogenic properties [41]. The second most common group of malignancies after transplant are lymphoproliferative disorders (PTLD), usually Epstein-Barr Virus-induced (see above, review in [42]). Excluding skin cancer and lymphoproliferative disorders, the incidence of *de-novo* malignancies (gastrointestinal, pulmonary and others) has been reported between 0.7% and 5.6% at 5 years [43]. A more recent study reported that the risk of *de-novo* malignancies after liver transplantation is 2-3 times higher than the general population [44].

3.6. Renal dysfunction

The effects of immunosuppressive drugs extend far beyond lowering the immune defense of transplant recipients and have an impact on virtually every organ system. Among them, renal dysfunction is a common and significant complication of solid organ transplantation. Long-term use of calcineurin inhibitors as part of immunosuppressive *regimens* is considered to be a major contributing factor in the development of chronic kidney disease (CKD). Renal failure post-transplant is associated with a 4-fold increase in mortality risk [45]. The incidence of renal dysfunction varies across organs depending on the length and level of calcineurin-inhibitors based immunosuppression. The mechanism of nephrotoxicity, like in hypertension, is thought to be related to alterations of the vascular tone of the endothelium at the level of the afferent arteriole [46]. However, CNI cause both acute and chronic nephrotoxicity. Acute nephrotoxicity involves afferent arteriolar vasoconstriction and reduced renal plasma flow, and is predictably associated with high trough levels. In contrast, chronic CNI-induced nephrotoxicity is not predicted by individual trough levels, and is characterized by potentially irrever-

sible structural changes including arteriolopathy, tubulointerstitial fibrosis and, eventually, glomerulosclerosis. Among other factors implicated in renal dysfunction post-transplant are hypertension, diabetes, pre-transplant renal function impairment and post-transplant acute kidney injury [47]. Several strategies have been proposed in attempt to reduce the risk of nephrotoxicity post-transplant, including CNI reduction or avoidance (review in [48]) and conversion to mycophenolate mofetil-based [49] or sirolimus- based [50] immunosuppression *regimens*. Although there is no general consensus on the optimal combination of immunosuppressive agents for maintenance of graft function while minimizing nephrotoxicity, it has become increasingly evident that immunosuppression *regimens* may need to be individualized based on patient- and organ-specific factors (see below).

3.7. Cardiovascular disease

Post-transplant, several preexisting risk factors like hypertension, dyslipidemia and hyperglycemia usually get exacerbated resulting in accelerated atherosclerosis causing significant cardiovascular disease post-transplant, including ischemic heart attack, congestive heart failure, cerebrovascular accidents and peripheral vascular disease. Indeed, cardiovascular disease is the most common cause of death in transplant patients, with a 2.5-fold greater risk of cardiovascular mortality and threefold greater risk of ischemic events compared to the general population [51]. Prevention strategies to limit the impact of cardiovascular disease after transplant include lifestyle modifications, correction of modifiable risk factors (hypertension, diabetes mellitus and dyslipidemia, see below) and tailoring of immunosuppression [52].

3.8. *Hypertension* (review in [53])

De- novo hyprtension post-transplant or the acceleration of hypertension (>140/90 mmHg) is common after solid organ transplantation, affecting up to 50%- 75% of patients within the first weeks to months [54] and can pose a significant hazard both early and late after transplant. Both calcineurin inhibitors cyclosporine and tacrolimus have been associated with development or worsening hypertension post-transplant [55]. Since CNI based immunosuppression *regimens* are very common in virtually every transplant program, is it no surprise that hypertension remains a major cardiovascular risk factor in organ transplant recipients. CNIs are known to increase sympathetic tone, vasoconstriction and to cause sodium dependent volume expansion [56]. Studies have demonstrated the beneficial effect of lowering blood pressure post-transplant and the association of controlled blood pressure with prolonged patient and graft survival [57].

3.9. Diabetes mellitus

New-onset diabetes after transplantation (NODAT) refers to the occurrence of diabetes in previously non-diabetic persons after organ transplantation. The incidence of NODAT vary by organ transplanted and post-transplant interval. The estimated rates at 12 months post-transplant are 20-50% for kidney transplants, 9-21% for liver transplants, and approximately 20% for lung transplants [58]. However, a meta-analysis of 56 studies across all organs reported a 13.5% incidence of NODAT when the diagnosis was made using current guidelines. In

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previous studies using different criteria for the diagnosis of diabetes post-transplant, including transient peri-operative hyperglycemia, the reported incidence was higher up to 21% in renal transplant recipients [59]. The risk factors for NODAT are the same as in the general population with the added effect of immunosuppressive medications, namely corticosteroids, calcineurin inhibitors and sirolimus. Among calcineurin inhibitors, tacrolimus was found to be more diabetogenic than cyclosporine [60]. However, both calcineurin inhibitors and steroids play a major role. Both CNIs have been associated with decreased insulin sensitivity and reduced insulin release. The reduced insulin release might result from CNI induced damage to pancreatic beta cells. Comparing the CNIs, most studies show higher rates of post-transplant diabetes mellitus with tacrolimus use compared to cyclosporine [61]. Other risk factors are pretx diabetes and obesity. Both pre-existing diabetes and NODAT are important cardiovascular risk factors, with a 2–5 times increased risk of cardiovascular disease, compared with transplant recipients without diabetes. In addition, new-onset diabetes is also an independent risk factor of graft failure and graft loss in kidney transplantation [62]. Monitoring of HbA1C is not recommended before three months following transplantation because the test may not be valid until new hemoglobin has been synthesized and glycated for the appropriate period in the diabetogenic post-transplant setting [63]. The management of post transplant diabetes follows the principles of treatment in non transplant populations but in addition it often requires adjustments in the immunosuppression *regimens*. Although these adjustments should be weighed against the risk of allograft rejection, options include reduction or weaning of corticosteroids and switching maintenance immunosuppressive drugs to less diabetogenic agents.

3.10. Dyslipidemia

The prevalence of dyslipidemia after transplantation has been reported up to 60-70% [64].

The mTOR inhibitors (sirolimus and everolimus, see above) have been associated with increased risk of dyslipidemia [65]. As hypercholesterolemia has been associated with increased prevalence of cardiovascular diseases, blood cholesterol levels should be maintained in the range recommended by practice guidelines, especially in transplant recipients receiving mTOR inhibitors.

4. Strategies to reduce the burden of immunosuppression and future directions

Strategies to limit the impact of chronic immunosuppression include protocols of drug minimization towards individualization of organ-specific immunosuppression *regimens*, development of new non-nephrotoxic agents and trials of tolerance induction. Drug minimization *regimens* are being explored in select patient populations to improve the safety of current immunosuppression protocols while preserving their efficacy. This strategy is based on the concept that, over time, the risk of rejection decreases and, at the same time, the cumulative risk for toxicity increases. Studies have concentrated on corticosteroids minimization and

calcineurin-inhibitors minimization (review in [66]). Careful patient selection and close monitoring of graft function are mandatory steps for a successful conduct of a drug minimization attempt in order to avoid rejection and graft loss.

At present we still lack reliable methods to identify transplant recipients who can be weaned of immunosuppression, although a number of candidate assays have been proposed to identify operationally tolerant patients. Among them, transcriptional profiling with either microarray or real-time PCR is currently a promising approach [67]. Peripheral and intra-graft expression markers of immune activation are used as tools to guide patient selection and monitor the progress of drug minimization trials [68]. In renal transplantation, non-invasive urine biomarkers have been described by measuring mRNA of inflammatory cytokines [69]. In addition, studies on urine proteomics allowed to identify different causes of graft dysfunction [70]. These non-invasive tools with or without protocol allograft biopsy offer the opportunity to monitor patients enrolled in trials of drug minimization.

In recent years, advances in immunosuppression that target specific pathways of the alloimmune response have been developed (review in [71]). In particular, new medications targeting the processes related to ischemia-reperfusion injury are currently under evaluation [72]. The ischemic insult to the allograft associated with the procurement and implantation processes contributes to trigger the immune activation of the recipient via the release of immunologically active substances known as damage-associated molecular patterns (DAMPs) [73]. In addition, new agents are being developed acting on the cellular and humoral mechanisms of the adaptive immune response. These include antibodies and fusion proteins interfering with T-cell-mediated activation via LFA-1/ICAM-1, CD2/LFA-3, CD40/CD154, and CD28/B7.1 and B7.2 interactions [74]. Furthermore, intracellular targets involved in T- and B-cell activation pathways are being evaluated, including protein kinase C inhibitors, Janus-associated kinase (JAK) inhibitors, and proteasome inhibitors. Several new medications demonstrate promise in inhibiting donor-directed humoral immunity by targeting B-cell-activating factor (BAFF) and complement activation pathways. Finally, other strategies are targeting the "memory" component of the T-cell repertoire [75] or the regulatory component [76].

Currently, transplant recipients are bound to lifelong immunosuppression. However, there have been reports of rare instances of "tolerance", defined as the maintenance of allograft function without immunosuppression. Although several definitions of tolerance have been proposed ("complete tolerance", "prope" tolerance, "operational" tolerance and others) and consensus is still lacking on the underlying mechanisms involved in tolerance, indeed there are patients who either intentionally or accidentally fail to reject the allograft and maintain allograft function while under minimal or no immunosuppression. As an example, in 1993 a series was reported of 11 liver transplant recipients maintaining normal liver function following the discontinuation of all immunosuppressive drugs as a consequence of either noncompliance or lymphoproliferative disorders [77]. Unfortunately, due to the heterogeneity of the human immune response, it has been so far prohibitively difficult to replicate these results on a larger number of patients and to establish tolerance in the clinical setting. The individualization of immunosuppression, identification of biomarkers of tolerance and of rejection and real-time monitoring of post-transplant immune responses may facilitate

induction of lasting tolerance in humans [78,79] The advancement of many high-throughput 'omic techniques such as genomics, proteomics and metabolomics has allowed to identify potential mechanisms of specific graft injury and to develop novel biomarkers for acute rejection, chronic rejection and operational tolerance [80,81]. Finally, the pharmacogenomics of organ transplantation has emerged recently as a complement to the immunogenetic information that has accumulated over the past decade [82]. Polymorphism studies focus on genes that interact across the group of immunosuppressive drugs (cyclosporin, tacrolimus, sirolimus and corticosteroids) such as CYP3A5, ABCB1, IMPDH1 and IMPDH2, and cytokines and growth factors. Although not routinely used in the clinic, it is expected that in the near future clinical pharmacogenomics techniques will become additional tools in the management of organ transplant patients.

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Few events in science have captured the same level of sustained interest and imagination of the nonscientific community as Stem Cells, Tissue Engineering, and Regenerative Medicine. The fundamental concept of Tissue Engineering and Regenerative Medicine is appealing to scientists, physicians, and lay people alike: to heal tissue or organ defects that the current medical practice deems difficult or impossible to cure. Tissue engineering combines cells, engineering, and materials methods with suitable biochemical and physiochemical factors to improve or replace biologic functions. Regenerative medicine is a new branch of medicine that attempts to change the course of chronic disease, in many instances regenerating failing organ systems lost due to age, disease, damage, or congenital defects. The area is rapidly becoming one of the most promising treatment options for patients suffering from tissue failure. This book of Regenerative Medicine and Tissue Engineering fairly reflects the state of the art of these two disciplines at this time as well as their therapeutic application. It covers numerous topics, such as stem cells, cell culture, polymer synthesis, novel biomaterials, drug delivery, therapeutics, and the creation of tissues and organs. The goal is to have this book serve as a reference for graduate students, post-docs, teachers, scientists and physicians, and as an explanatory analysis for executives in biotech and pharmaceutical companies.

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