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Embryonic Stem Cells

Differentiation and Pluripotent Alternatives

Edited by Michael S. Kallos

EMBRYONIC STEM CELLS – DIFFERENTIATION AND PLURIPOTENT ALTERNATIVES

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Meet the editor

Dr. Michael S. Kallos, PhD, PEng is an Associate Professor of Chemical Engineering in the Schulich School of Engineering, and Associate Director of the Pharmaceutical Production Research Facility (PPRF), both at the University of Calgary. He is also the Director of the Biomedical Engineering Graduate Program and the Associate Director of the Center for Bioengineering Research and Education (CBRE), both at the University of Calgary. His research interests include stem cell bioprocess design, bioreactors, tissue engineering and mass transfer. He was awarded the Alberta Science and Technology (ASTech) "Leader of Tomorrow" Award in 2002, a Faculty of Engineering Service Award in 2005, and was named one of Calgary's Top 40 Under 40 in 2009. He has also won numerous Teaching Awards from his peers and students, including "Professor of the Year" and "Outstanding Excellence in Teaching".

Contents

Preface

Embryonic stem cells have immense therapeutic potential, but for cell therapy these pluripotent cells will have to be differentiated towards cells of interest before transplantation. Controlled, robust differentiation processes will require knowledge of the signalling pathways and mechanisms which will obviously be unique for each differentiated cell type. There are a tremendous variety of approaches and techniques, and this book, *Embryonic Stem Cells - Differentiation and Pluripotent Alternatives* and its companion, *Embryonic Stem Cells - Basic Biology to Bioengineering*, serve as a snapshot of many of the activities currently underway on a number of different fronts.

This book is divided into eight parts and provides examples of the many tissue types that embryonic stem cells are being pushed towards, as well as alternative sources for pluripotent stem cells.

Part 1: General Differentiation
Chapters 1-3 present a number of different aspects of embryonic stem cell differentiation including signalling pathways, epigenetic factors, bioactive lipids and retinoid signalling.

Part 2: Neural and Retinal Differentiation
Chapters 4-7 examine how neural cell types are generated from embryonic stem cells, including looking at some interesting imaging techniques and a review of stem cell therapy for retinal diseases.

Part 3: Cardiac and Other Myogenic Differentiation
Chapters 8-12 present a number of aspects of cardiomyocytes differentiation including detailed characterization of the differentiated cells. In addition the use of ESC-derived myogenic cells for neuromuscular diseases is discussed.

Part 4: Endothelial Differentiation
Chapters 13-14 describe endothelial differentiation with a focus on the signalling transduction pathways involved.

Part 5: Hepatic Differentiation
Chapters 15-16 examine another interesting application of embryonic stem cells for hepatic tissue engineering.

Part 6: Osteogenic Differentiation
Chapter 17 looks at osteogenesis and the developmental path from pluripotent cells to osteoblasts.

Part 7: Pluripotent Alternatives Induced Pluripotent Stem Cells (iPSCs)
Chapters 18-21 discuss induced pluripotent stem cells (iPSCs) starting with a great review of the iPSC story to date. The next chapters present new techniques to generate iPSCs including from cord blood cells. An interesting application of iPSCs in the modelling of neurological diseases is described as an example of one of the many uses of these cells.

Part 8: Pluripotent Alternatives Other Cell Sources
Chapters 22-25 describe alternative sources of pluripotent stem cells. These include very small embryonic/epiblast like stem cells, multipotent dental stem cells, pluripotent stem cells from testis and amniotic fluid stem cells.

In the book *Embryonic Stem Cells - Differentiation and Pluripotent Alternatives*, the story begins with a foundation upon which future therapies and uses of embryonic stem cells can be built.

I would like to thank all of the authors for their valuable contributions. I would also like to thank Megan Hunt who provided me with much needed assistance and acted as a sounding board for early chapter selection, and the staff at InTech, particularly Romina Krebel who answered all of my questions and kept me on track during the entire process.

Calgary, Alberta, Canada, September 2011

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Part 1

General Differentiation

1

Role of Signaling Pathways and Epigenetic Factors in Lineage Determination During Human Embryonic Stem Cell Differentiation

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1. Introduction

Human embryonic stem cells (hESCs) are culture-adapted cells that were originally derived from the inner cell mass (ICM) of the blastocyst-stage embryo [1]. HESCs are pluripotent cells that can be propagated indefinitely in culture, while retaining the *in vivo* properties of ICM cells; they can give rise to all tissues of the three germ layers (ectoderm, mesoderm and endoderm). Due to their pluripotency, hESCs have been the subject of intense research since they were initially isolated in 1998. HESCs can serve as model systems to study early human development, in addition to providing a potentially unlimited source of functional tissues for use in drug evaluation and regenerative medicine. Nevertheless, despite major advances, the exact molecular mechanisms that govern the self-renewal and differentiation of hESCs remain unclear. Indeed, a mechanistic understanding of the molecular processes regulating hESC fate can elucidate early events in human development and enable the development of protocols for efficient generation of functional tissues. Here we review the molecular mechanisms that regulate hESC fate; specifically, we focus on the role of signaling pathways and factors regulating epigenetic changes, in hESC self-renewal and lineage-specific differentiation.

In hESCs, as in embryos, differentiation is triggered by developmental cues such as morphogens or cytokines that are present in the extracellular space. These morphogens or cytokines bind to their cognate plasma membrane-bound receptors and activate specific signaling pathways inside the cell. Activation of signaling pathways involves a sequence of phosphorylation events that eventually result in the regulation of specific transcription factors. These transcription factors, in turn, can recruit other co-factors and directly cause transcription of downstream genes. Furthermore, transcription factors can recruit histone modifying and chromatin remodeling enzymes to reshuffle the epigenetic structure, such that pluripotency genes become inaccessible for transcription and are repressed, whereas lineage-specific genes become accessible and are activated. This sequence of events finally leads to expression of lineage-specific proteins such as transcription factors and structural proteins, causing a morphological change in the cell. Also, pluripotency associated transcription factors and other pluripotency-associated genes are permanently repressed, thereby completing the process of differentiation. Thus, the process of differentiation is a

rather complex cascade of events, controlled by signaling pathways, transcription factors, epigenetic factors and lineage-specific proteins. While significant understanding of each of these functional groups (i.e., signaling pathways, transcription factors, epigenetic factors and lineage-specific proteins) has been gathered in isolation, very little is known about the interactions amongst these groups, particularly in the context of hESC differentiation. In part, interactions amongst these groups confer lineage specificity to the process of differentiation and mediate the development of specific tissues upon exposure of hESCs to certain morphogens. In this review, we focus on the role of signaling pathways, transcription factors and epigenetic factors in the context of lineage-specific differentiation of hESCs and summarize the various links between these groups. Our goal is to present a mechanistic overview of the sequence of molecular events that regulate the differentiation of hESCs along various lineages.

2. The signaling pathways

As briefly described earlier, the self-renewal and differentiation of hESCs is governed by several developmental cues. The most well known among these are cytokines that trigger specific signaling pathways. These extracellular ligands initiate signaling through interactions with ligand-specific cell surface receptors. Receptor-binding typically results in association of multiple receptor subunits and activation of the kinase domains of receptors or other receptor-bound effector proteins. This triggers a sequence of phosphorylation events involving various other proteins, finally resulting in the activation or inhibition of transcription factors. These transcription factors in turn are directly responsible for activating or repressing their target genes. Thus, a group of signaling pathways is usually responsible for modulating gene expression in hESCs, leading to control of the transcriptome, the proteome and ultimately cellular physiology. In this section, we summarize key signaling pathways that have been implicated in the maintenance of undifferentiated hESCs and their lineage-specific differentiation.

2.1.1 The transforming growth factor-β pathway

The transforming growth factor β (TGF-β) pathway is well known for its involvement in embryonic development and patterning, as well as in epithelial-to-mesenchymal transformations and carcinogenesis [2-3]. This pathway (extensively reviewed elsewhere [2, 4-5]) is divided into two branches: the Activin/Nodal branch and the Bone Morphogenetic Protein (BMP) branch. The Activin/Nodal pathway is activated by ligands such as Activin, Nodal and TGFβ1. These ligands bind to their Type II receptor, which then recruits the Type I receptor. The Type II receptor phosphorylates the intracellular domain of the Type I receptor, creating a binding site for SMAD2 and SMAD3 transcription factors. Upon binding, SMAD2/3 is phosphorylated by the Type I receptor, leading to subsequent dissociation of SMAD2/3. Phosphorylated SMAD2/3 then associates with SMAD4 and can enter the nucleus to modulate gene expression. The BMP branch is activated by the BMP ligands; binding of BMP ligands results in phosphorylation of the type I receptor by the type II receptor, and subsequent intracellular binding and phosphorylation of SMAD1/5/8. Phosphorylated SMAD1/5/8 forms a complex with SMAD4 and subsequently enters the nucleus. Unlike ligands in the Activin/Nodal branch, the BMPs have high affinities for the type I receptor and bind weakly to the type II receptor; Activin/Nodal bind with high

affinity to their type II receptors and do not directly interact with their type I receptors. Numerous other proteins can modulate the localization and activity of SMADs (reviewed in [6-8]). Additionally, the activated TGFβ pathway receptors can also activate the Mitogen Activated Protein Kinase (MAPK) Pathway [9].

2.1.2 Role in lineage determination

The TGFβ pathway plays a significant role during embryogenesis across many species including flies, fishes, amphibians and mammals (reviewed in [10]). Specifically among vertebrates, Nodal is expressed throughout the epiblast [11] and is required for specification of mesoderm and endoderm from the epiblast [12]. Low levels of Nodal lead to mesoderm formation whereas high levels lead to endoderm formation [13]. Absence of Nodal signaling through active inhibition leads to ectoderm formation [12, 14]. Within the ectoderm, high levels of BMP cause formation of epidermis while low levels cause neural plate formation [15]. Intermediate levels of BMP lead to neural crest formation at the borders of the neural plate, although this a necessary but not a sufficient condition [16].

Not surprisingly, *in vitro* protocols for differentiating hESCs resemble *in vivo* conditions present during embryogenesis. Specifically, Activin causes endoderm differentiation from hESC cultures, while Activin and BMP simultaneously lead to mesoderm formation [17-21]. Inhibition of both Activin/Nodal and BMP causes neural differentiation [22] and this differentiation proceeds through an epiblast-like intermediate. Intriguingly, some amount of Activin/Nodal signaling is essential for hESC pluripotency; the pluripotency factor Nanog is a direct target of Smad2/3 [23-25] and inhibition of Activin/Nodal causes upregulation of BMP and subsequent trophectoderm differentiation [26]. Short term BMP treatment causes mesoderm formation [27] whereas long term BMP treatment leads to trophectoderm formation [28]. The disparity of BMP treatment leading to trophectoderm differentiation and not epidermal differentiation, as during embryogenesis, can in part be attributed to the fact that hESCs are derived from ICM cells and not epiblast cells. Thus, even though *in vivo* embryogenesis serves to provide guidelines for carrying out *in vitro* differentiation of hESCs, major challenges still remain. The biggest of these challenges is perhaps the heterogeneity in lineages of differentiated cells obtained through most *in vitro* protocols. This is primarily because most differentiation protocols rely on embryoid body (EB) formation which results in a heterogeneous environment for cells within the EB, and leads to heterogeneity in the lineages obtained after differentiation. Another challenge is the inability to form an absolute mechanistic link between the culture conditions used to differentiate hESCs, and the differentiation behavior seen in hESCs. This is mostly because the composition of serum, B27, serum replacer, conditioned medium and other components of media used for differentiation studies, are unknown [29].

2.2 The Fibroblast Growth Factor pathway

The Fibroblast Growth Factor (FGF) pathway is also known for its involvement in embryonic development and patterning, as well as in regulation of cell growth, proliferation and motility (extensively reviewed in [30-34]). The FGF pathway is activated when the FGF ligands, which have a strong affinity for Heparan Sulfate Proteoglycans (HSPGs), bind to FGF Receptors (FGFRs) forming a 2:2:2 combination of FGF: FGFR: HSPG on the cell surface. The ensuing receptor dimerization causes transphosphorylation of tyrosine residues in the intracellular domains of FGFRs through their tyrosine kinase domains. The

phosphorylated tyrosines of FGFRs cause recruitment of the GRB2/SOS complex and its subsequent activation. SOS then activates RAS, which triggers the MAPK cascade, finally leading to the activation of extracellular-signal related kinases (ERKs). Activated Erk1/2 can phosphorylate and control the activity of a wide range of proteins [35]. Notably, Erk1/2 can phosphorylate the linker region of SMAD1 and inhibit BMP signaling [36-37]. Additionally, activated FGF receptors can also recruit FRS2/GRB2 and activate them, leading to recruitment of GAB1. GAB1 activates the Phosphatidylinositol 3-kinase (PI3K) pathway. Thus, FGF can also activate the PI3K pathway in a cell-specific context.

2.2.1 Role in lineage determination

The FGF pathway is needed for proper embryonic development in vertebrates. Experiments in mice have shown that FGF4 is required for ICM proliferation and maintenance [38-39], and for trophectoderm and primitive endoderm development [40]. FGF4 is secreted by the ICM and supports trophectoderm maintenance [41]. Interestingly, activation of HRas1 which is a component of the MAPK pathway, in mouse embryonic stem cells (mESCs) caused upregulation of Cdx2, a trophectoderm marker, and trophectoderm stem cells were derived from these mutants [42]. FGF4 secreted by ICM also causes primitive endoderm differentiation [43]. Further along the developmental timeline, FGF signaling is required for the induction of paraxial mesoderm and for maintenance (but not induction) of axial mesoderm [44-46]. FGF is required for primitive streak formation and cell migration during gastrulation [45, 47]. Inhibition of FGF is required for blood development [48-50], whereas activation of FGF signaling is required for neural differentiation [51]. However the mechanism by which FGF aids in embryonic neural differentiation is not fully understood [52-53]. *In vitro* studies have mimicked the role of FGF signaling in hESC maintenance and differentiation. FGF2 is required for maintaining hESCs in a pluripotent state [24, 54-55]. FGF signaling is also required for maintenance of mouse trophectoderm stem cells which are derived from the trophectoderm tissue of mouse embryos [56]. While there are no specific studies which delineate the inductive and maintenance/proliferative roles of FGF during hESC differentiation, work with mESCs have given ambiguous results, some showing that autocrine FGF2 is essential for neural differentiation [57] while others showing that FGF2 has a role in maintenance rather than induction of neural differentiation [58-59]. However, FGF signaling does seem to be necessary for inducing the posterior nervous system in vertebrate embryos [53, 60].

2.3 The Wnt pathway

The Wnt pathway is widely implicated during various stages of embryonic development, homeostasis as well as in cancer. This pathway (reviewed in [61-62]) has canonical and non-canonical branches. The canonical Wnt pathway is activated by binding of Wnt ligands to the Frizzled receptors and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptors leading to the recruitment of Dishevelled to the Frizzled receptor. This causes recruitment of Axin to the receptor complex, causing the subsequent deactivation of Axin. In the absence of Wnt signaling, Axin associates with GSK3β, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and β-catenin. CK1 and GSK3β phosphorylate β-catenin causing it to be degraded. Upon Wnt activation, Axin is inhibited and β-catenin becomes de-repressed, and subsequently enters the nucleus to function as a transcription factor. Various co-factors associate with β-catenin and control its promoter specificity, thus dictating the

target genes activated or repressed by β-catenin [63-64]. The non-canonical Wnt pathway acts independently of β-catenin and is also required during embryogenesis. The details of this Ca^{2+}-dependent pathway are reviewed in [62].

2.3.1 Role in lineage determination

The canonical Wnt pathway is activated during gastrulation [65] and mutation of Wnt3 blocks primitive streak formation resulting in lack of mesoderm and endoderm [66] (the primitive streak-specific transcription factor Brachyury is a direct target of Wnt3a signaling [67]). Similar defects are seen in Lrp5/6 double mutants and β-catenin loss-of-function mutants [68-69]. Interestingly, expression of Cripto, a co-receptor for Nodal signaling, is missing in β-catenin loss-of-function mutants [70]. Also, β-catenin is indispensable for endoderm formation and loss of β-catenin causes definitive endoderm to change into pre-cardiac mesoderm [71]. Although loss of Wnt signaling leads to loss of mesoderm formation, inhibition of Wnt signaling is required for a cardiac fate [65, 72], once pre-cardiac mesoderm has been induced. Remarkably, similar reports for the role of Wnt signaling have been obtained through *in vitro* differentiation studies in hESCs. Over-expression of β-catenin in hESC cultures lead to primitive streak formation [73]. Inputs form Activin/Nodal and BMP pathways are necessary for further lineage specification into mesoderm/endoderm. Blocking BMP signaling abolishes mesoderm and leads to endoderm formation, whereas Activin/Nodal is required for endoderm formation [73]. Wnt is required for mesoderm differentiation but must be inhibited thereafter for cardiac mesoderm formation [74].

2.4 The Phosphatidylinositol-3Kinase pathway

The Phosphatidylinositol-3Kinase (PI3K) pathway regulates cell survival, apoptosis and has been implicated in cancer. The pathway as well as its role in cancer is reviewed in [75-78]. It also has been, in select cases, implicated in lineage-specific hESC differentiation [79]. The pathway is activated when PI3K is phosphorylated; this can happen through binding of Insulin to the Insulin receptor or of Insulin-like growth Factor (IGF) to Insulin-like Growth Factor Receptor (IGFR), or as previously discussed, by recruitment and activation of GAB1 by FGFR. Activated PI3K phosphorylates Phosphatidylinositol (4, 5)-biphosphate to Phosphatidylinositol (3, 4, 5)-triphosphate and creates a docking site for proteins with a pleckstrin homology (PH) domain, such as Akt. Once Akt is properly docked, it is phosphorylated and activated by protein-dependent kinase 1 (PDK1). Akt can then dissociate and activate/repress numerous proteins by phosphorylating them [80]. The PI3K pathway has not received much attention during vertebrate embryogenesis, though some recent studies have emerged to show that it is necessary for normal embryo development [81-82]. Homozygous null mutations in the p110β subunit of PI3K cause embryonic lethality before formation of the blastocyst [83]. Thus, *in vivo* studies do not implicate PI3K signaling in differentiation of cells, but rather in maintenance of cell viability [82]. It has been hypothesized that growth factors maintain PI3K signaling during embryogenesis to guard against ectopic or metastatic growth of cells, since such ectopic/metastatic cells do not receive enough growth factors and enter the default apoptotic pathway [84]. In contrast, *in vitro* studies with hESCs have shown some supportive role for the PI3K pathway in definitive endoderm differentiation. Inhibition of PI3K signaling enhances definitive endoderm differentiation by Activin [85]. Other conflicting reports show that PI3K signaling stabilizes β-catenin during definitive endoderm formation [73]. A major challenge in

elucidating the possible role of this pathway during differentiation is the inability to decouple its role in cell viability. Therefore, careful studies need to be designed to assess the extent of its role in causing differentiation.

2.5 The Hippo pathway

So far we have focused on developmental cues in the form of morphogens, i.e. protein ligands that physically diffuse through the embryonic tissue and pattern the embryo. Another developmental cue that has recently emerged to be of significant importance during embryogenesis is cell-cell contact. An increase in cell-cell contact is sensed by the cell through the activation of the Hippo pathway (reviewed in [86-87]). The Hippo pathway is important for organ size control, tumorigenesis, epithelial-to-mesenchymal transformation and cell-cell contact inhibition. Although the molecular mechanisms of cell-cell contact sensing are not fully understood, an increase in cell-cell contact is known to ultimately lead to phosphorylation of MST1/2 kinases. They then associate with SAV1 and phosphorylate the LATS1/2 kinases. Upon phosphorylation, LATS1/2 kinases recruit MOB1 and phosphorylate YAP and TAZ, both of which are homologues with non-redundant functions [86, 88]. Phosphorylation of TAZ and YAP leads to their association with 14-3-3 proteins and subsequent cytoplasmic retention [89-90]. YAP and TAZ act as co-factors for various transcription factors such as TEADs, RUNX, PAX3 and SMAD1/2/3/7 and modulate their nuclear localization and/or activity [91-96]. Additionally, TAZ can associate with DVL2 and inhibit its phosphorylation by CK1, thus possibly inhibiting β-catenin activation by Wnt factors [97]. Therefore, the nucleocytoplasmic shuttling of YAP and/or TAZ can lead to changes in the activity levels of associating transcription factors, leading to differentiation [93]. Indeed, low cell-cell contact at the periphery of the embryo leads to Yap activation, which in concert with Tead4, leads to trophoblast differentiation in mouse blastocysts [98]. The inner cell mass and epiblast tissues show predominantly cytoplasmic and weakly nuclear localization for Yap, Taz and phospho-Smad2 [98-99], in agreement with the fact that Taz controls Smad2 localization [93]. While cells of the mouse ICM continue to differentiate, *in vitro* cultures derived from ICM (i.e. mESCs) maintain high levels of Yap. Yap is downregulated during *in vitro* mESC differentiation and upregulated in mouse and human induced pluripotent stem (iPS) cells [100]. Ectopic Yap expression maintains mESC phenotype even under differentiation conditions. Though it remains to be seen whether downregulation of Yap/Taz is the cause of differentiation of ICM cells *in vivo*, experiments with hESCs show that downregulation of TAZ initiates neural differentiation [93]. Tead and Yap suppress terminal neuronal differentiation and maintain neural progenitor populations in the vertebrate neural tube [94]. Tead2 and Yap activate Pax3 expression during neural crest formation [101]. Tead1/2 and Yap also maintain the notochord which is formed from the axial mesoderm [102]. Thus the co-activators YAP and TAZ are important for the activity of many transcription factors during embryogenesis. However, further studies are needed to uncover the specific inductive/maintenance roles of these co-factors and their responsiveness to Hippo signaling in these tissues.

2.6 Crosstalk between signaling pathways

There is a vast amount of crosstalk between the various pathways described here, thus adding additional complexity in the regulation of downstream transcription factors [103]. As described earlier, the TGF-β pathway can activate the MAPK pathway directly

downstream of receptor activation [9]. Both TGF-β and FGF pathways can activate the PI3K pathways directly at the receptor level [9, 30]. However, the crosstalk between pathways is cell-specific, since the available pool of interacting proteins depends on the cell-type. Also, the promoter accessibility of downstream genes is dependent on cell-type. Therefore, here we restrict our discussion to crosstalk events identified specifically in hESCs. Activin/BMP signaling induces Wnt ligand expression in hESCs [74]. Also, Activin regulates FGF, Wnt and BMP pathways in hESCs [104]. Inhibition of Activin/Nodal signaling causes downregulation of Wnt3, FGF2, FGF4 and FGF8 expression [26] and upregulation of BMP signaling [26] while activation of Activin signaling causes upregulation of Wnt3 and FGF8 expression [104]. Interestingly, upregulation of Activin signaling also causes upregulation of Nodal and Lefty expression. Cerberus1, an inhibitor of Nodal signaling, is a downstream target of both Wnt and Nodal pathways in hESCs [105]. Expression of Cripto, a co-activator of Nodal signaling, is upregulated by FGF signaling in hESCs [24]. As described earlier, YAP, which is regulated by the Hippo pathway, controls the nuclear localization of Smad2 in hESCs [93]. Thus, it can be seen that hESCs exhibit considerable endogenous signaling wherein, signaling pathways not only control their own ligand expression but also the expression of ligands of other pathways.

3. Other regulators of differentiation

While morphogens and other developmental cues act as the environmental input to hESCs and trigger the process of differentiation, the molecular mechanisms responsible for carrying out differentiation inside the cell are complex and require many key factors. These factors are required for the following: 1) to bring about a change in gene expression which causes the cell to transition into the new lineage-specific physiology, 2) to reshuffle the epigenetic structure of the genome, and finally 3) to make the new epigenetic structure permanent, lending stability to the newly formed cellular physiology. We will now discuss these intracellular factors that mediate various aspects of the differentiation process.

3.1 MicroRNAs

MicroRNAs (miRNAs) have emerged as a new paradigm for regulating gene expression at the post-transcriptional level. The role of miRNAs in embryogenesis, stem cell fate and cancer is reviewed in [106-108]. Transcription factors regulate promoter regions of miRNAs, which upon synthesis can target many mRNAs and lead to downregulation of protein synthesis. MiRNAs, which upon transcription are called pri-miRNAs, fold into secondary structures with characteristic hairpin-loops. These are recognized by Drosha, which cleaves the hairpin-loop structures to generate pre-miRNAs. Pre-miRNAs are then exported to the cytoplasm and recognized by Dicer, which cleaves one of the strands and incorporates the other into the RNA-induced silencing complex (RISC). Once incorporated into RISC, the single-stranded miRNA recognizes target mRNAs (usually many different mRNA targets) through partial sequence complimentarily, and causes down-regulation of protein synthesis. It is now known that miRNAs play an important role in embryogenesis [109] and lineage-determination, and that many lineages have their characteristic miRNA expression patterns, akin to characteristic mRNA expression patterns [110]. The role of miRNAs in embryogenesis is evident from the fact that Dicer mutant mouse embryos die during gastrulation [111], while Dicer deficient zebrafish embryos do not develop beyond day8 [109]. The role of miRNAs in embryonic stem cell pluripotency and differentiation has also

been demonstrated recently [107]. Dicer deficient mESCs fail to differentiate *in vitro* as well as *in vivo* [112]. Over-expression of miR-302 leads to reprogramming of human hair follicle cells and human skin cancer cells to form iPS cells [113-114]. During mESC differentiation, miR-134, miR-296 and miR-470 target and downregulate the transcription factors Nanog, Sox2 and Oct4 [115]. MiR-200c, miR-203 and miR-183 target and repress Sox2 and Klf4, both of which are involved in maintaining pluripotency in mESCs [116]. Similarly, during hESC differentiation, miR-145 targets and represses OCT4, SOX2 and KLF4 [117]. Sall4, another pluripotency-related transcription factor, is positively regulated by the ESC cell cycle regulating (ESCC) family of miRNAs and negatively regulated by the let7 family [118]. Additionally, miRNAs are also implicated during later stages of differentiation. The muscle-specific miR-1 controls cardiomyocyte differentiation and proliferation in mice by targeting the Hand2 transcription factor [119]. miR-181 controls hematopoietic differentiation in mice [120] and miR-143 regulates adipocyte differentiation [121]. MiR-196 is involved in HOX gene regulation [122-124] and the miR-200 family regulates olfactory neurogenesis [125]. MiRNAs have also been implicated in skin morphogenesis [126]. Most intriguingly, transfection of muscle-specific miR-1 or brain-specific miR-124 into human HeLa cells shifts the mRNA expression profile towards that of muscle or brain cells, respectively [127].

The expression of miRNAs is regulated by transcription factors which bind promoter regions of genes harboring miRNAs; more than half of known mammalian miRNA genes are within host gene introns and are spliced after transcription [128]. For example, Activin A signaling regulates the expression of ~12 miRNAs in hESC cultures [129]. OCT4, NANOG and SOX2 occupy the promoter regions of ~14 miRNAs in hESCs [130]. Additionally, miRNAs can be regulated directly by signaling pathways. In smooth muscle cells, BMP4 or TGF-β signaling causes increased processing of pri-miR-21 and pri-miR-199a [131] and regulates the processing of numerous other miRNAs [132]. The MAPK/ERK pathway can regulate miRNA maturation in the cytosol by controlling phosphorylation of TRBP, which functions with Dicer [133]. However, it is largely accepted that miRNAs do not trigger differentiation but rather, are required for carrying out the process of differentiation [107]. It is hypothesized that miRNAs are required to dampen the stochastic noise in mRNA transcription levels of genes during the process of differentiation. Thus, miRNAs add another layer of complexity to gene regulation during the process of differentiation, by fine-tuning active mRNA levels of a gene.

3.2 Epigenetic factors

Our discussion on signaling pathways focused on how a change in gene expression during differentiation is initiated; while miRNAs are most probably required to stabilize the mRNA levels against stochastic perturbations during differentiation. However, to provide long-term stability to the new gene expression pattern, the epigenetic structure of the genome needs to be changed. Epigenetic factors are responsible for modulating the epigenetic structure of hESCs, while it is pluripotent (reviewed in [134]) as well as while it goes through differentiation. The epigenetic structure of the genome dictates the promoter regions that would be accessible to transcription factors for initiating transcription; the heterochromatin, being densely packed, is inaccessible whereas the euchromatin is loosely packed and readily accessible. The epigenetic structure of hESCs is different from that of differentiated cells. The epigenetic structure also differs across various lineages of differentiation. Thus, epigenetic factors are involved in changing the epigenetic structure of the genome in a lineage-dependent fashion.

Epigenetic factors comprise broadly of histone modifying enzymes and chromatin remodeling complexes. The concerted action of both is needed to bring a stable change in the epigenetic landscape. Histone modifying enzymes are enzymes that modify histones post-translationally and create an epigenetic code of various acetylations, ubiquitinations and methylations throughout the genome (the histone code hypothesis [135-136]). This code is then recognized by chromatin remodeling enzymes, which alter the higher order structure of nucleosomes by creating heterochromatin and euchromatin. By controlling the formation of heterochromatin and euchromatin, these epigenetic factors control promoter accessibility and gene expression during differentiation. Differentiation is thought to proceed through activation of lineage-specific genes and repression of pluripotency genes [137]. This requires epigenetic factors to create repressive histone modifications on pluripotency genes (which were hitherto active) and reciprocally, to create activating histone modifications on lineage-specific genes (which were hitherto repressed). Permanent modification of histones also allows for epigenetic stability of the differentiated cell, which now becomes locked in this lineage. There is also some feedback from chromatin remodeling enzymes back to histone modifying enzymes. This means that certain chromatin remodeling enzymes can recruit back specific histone modifying enzymes for changing the histone code further. This is thought to provide more robustness to this system of epigenetic modification, thus lending further stability to the differentiated phenotype.

3.2.1 Histone Acetyltransferases

Of the various histone modifications, acetylation and methylation are critical for regulating the chromatin structure and gene expression [138]. These histone modifications, which create the genome-wide histone code, are regulated by Histone Acetyltransferases (HATs), Histone Deacetylases (HDACs), Histone Methyltransferases (HMTs) and Histone Demethylases. Histone Acetyltransferases are further classified into five families [139-140]: the Gcn5-related HATs (GNATs); the MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60)-related HATs; p300/CBP HATs; general transcription factor HATs; and nuclear hormone-related HATs. In humans, the identified GNAT-related HAT complexes are PCAF, STAGA and TFTC. All three complexes have the chromatin-binding bromodomain which targets these complexes to chromatin. The bromodomain specifically recognizes and binds acetylated histones [141-142]. In mammals, the identified MYST-related HATs are Moz, Qkf, Mof, Tip60 (homologue of yeast NuA4 [143]) and Hbo1. The TIP60 complex contains the chromatin-binding chromodomain. The chromodomain of yeast SAGA HAT complex has been shown to recognize methylated histones [144] raising the possibility that TIP60 may also be recruited to methylated histones in humans. Together with the case of the bromodomain containing complexes, this implies that HATs may be recruited to specifically tagged histones and may function in a signaling cascade to modify the epigenetic map of the genome [145]. *Mof* homozygous null mice lack H4K16 acetylation and arrest at blastocyst stage [146]. Homozygous null *Tip60* mutant mice also die during blastocyst stage[147]. Tip60 has also been implicated in pluripotency of ESCs [148]. Qkf is required for normal development of neurons of the cerebral cortex [149], whereas Moz is required for normal hematopoietic stem cell development [150-152].

3.2.2 Histone Deacetylases

The family of Histone Deacetylases is classified into four groups [153-155]: the Class I HDACs (yeast Rpd3-like) comprising of HDAC1/2, HDAC3 and HDAC8; the Class II

HDACs (yeast Hda1-like) comprising of HDACs4-7, HDAC9 and HDAC10; the Class III HDACs (Sir2-like) comprising of SIRT1-7; and the Class IV HDACs (HDAC11-like) comprising of HDAC11. Of these, HDAC1 and HDAC2 have been identified in numerous complexes [156], namely: the SIN3 co-repressor complex, the nucleosome remodeling and deacetylase (NuRD) complex, the CoREST complex, the Nanog and Oct4 associated deacetylase (NODE) complex and the SHIP1 containing complex. HDACs complexes become associated with transcription factors through mediator proteins such as Sin3, NCoR, SMRT, CtBP and TLE [157]. Hdac1 and Hdac2 are important for embryonic development, especially during myogenesis, neurogenesis, haematopoiesis and epithelial cell differentiation [156]. The HDAC complexes NuRD and SIN3 are critical during different stages of embryonic development [158]. Mice embryos lacking Mbd3 or p66α, components of the NuRD complex, die during embryonic development [159-160]. *Mbd3* null mice show normal segregation of trophoblast and primitive endoderm, but fail to develop embryonic ectoderm and extraembryonic ectoderm [161]. ICM cells of these embryos continue expressing Oct4 and the primitive endoderm marker Gata4 and fail to expand in number. Further, even though the primitive endoderm is present, the visceral endoderm fails to form. Analogously, the ICM cells derived from *Mbd3* null mice did not expand ex-vivo and mESCs could not be formed [161]. *Mbd3* null mESCs could initiate differentiation but could not commit to the differentiated lineages [162]. Mbd3 was also shown to suppress trophoblast commitment of mESCs [163]. P66α, however, was not required for proper blastocyst formation and implantation, and *p66a* null mice died later during embryogenesis [160]. Mi-2β, another component of the NuRD complex, is important for haematopoiesis, lymphopoiesis and skin development [164-167]. Similar to *Mbd3* and *p66a*, *Sin3a* null mice embryos also die after implantation [168-169]. The ICM derived from these embryos shows severely retarded proliferation ex vivo [168]. *Sin3b* null embryos show defects in erythrocyte and granulocyte maturation and in skeletal development [170]. The Class III HDACs, known as Sirtuins (SirTs), are also implicated during differentiation and mammalian development [171]. SirT1 is highly expressed in ESCs and decreases during differentiation [172]. During late development, SirT2 modulates skeletal muscle and SirT1 modulates white adipose tissue differentiation [173-174]. Under oxidative stress, SirT1 causes astroglial differentiation in mouse neural progenitor cells [175]. SirtT2 controls gametogenesis in mice embryos [176].

3.2.3 Histone Methyltransferases

Various Histone Methyltransferases (HMTs) exist in the mammalian genome and many putative HMTs are yet to be discovered [177]. The major mammalian HMTs include Ash1l, Dot1l, Ezh1-2, G9a, GLP, Mll1-5, Nsd1, Prdm1-6, Prdm8-16, PrSet7, Setd1-7, Setdb1-2, Setmar, Smyd1-5, Suv39h1-2, Suv4-20h1-2 and Whsc1/l1. Their requirement during specific stages of mammalian development is comprehensively reviewed in [177]. These HMTs are associated with specific histone methylation activities on H3 and H4 histones. Although most of identified methylation marks are promiscuous and need further study, some histone methylation marks correlate well with gene activity. Transcriptionally active genes display H3K4me3 on their promoter region and H3K36me3 across the gene body, while repressed genes are enriched in H3K27me3 over the gene body, with some amount of H3K9me3 and H4K20me3 [178-180]. H3K4 methylation, which is associated with gene activation, is induced by Mll1-5, Setd1a/b and Ash1l. Therefore, these HMTs are critical during mammalian development. Mutations in *Mll1* lead to embryonic lethality in mice [181-182]

and cause aberrant regulation of Hox genes. Generation and/or expansion of hematopoietic stem cells, is abrogated in these embryos [183]. *Mll2* null mice are capable of blastocyst formation and normal implantation without any lineage-specific growth abnormalities, but die later during embryonic development [184]. Very few genes are misregulated in *Mll2* null mESCs, though *Mll2* is needed for spermatogenesis [185]. *Mll3* mutant mice show impaired differentiation towards the adipocyte lineage [186], while *Mll5* mutant mice show impaired hematopoietic development [187]. H3K27 methylation, which is associated with gene repression and is important for embryonic development, is caused by the Ezh1-2 HTMs. Again, *Ezh2* knockout causes early embryonic lethality in mouse embryos [188]. These embryos fail to complete gastrulation. Ezh2 is also shown to regulate epidermal and hematopoietic differentiation during embryogenesis [189-191]. H3K9 methylation is also associated with gene repression, and is induced by G9a, GLP, Prdm2, Setdb1 and Suv39h1-2. No gene targets for the Suv39h enzymes have been discovered though *Suv39h* double mutant mice display impaired viability as well as sterility [192]. In contrast, *Setdb1* knockout causes early embryonic lethality in mouse embryos due to aberrant blastocyst formation, and mESCs cannot be derived from these mutant blastocysts [193]. Setdb1 also controls the switch between osteoblastogenesis and adipogenesis from bone marrow mesenchymal progenitor cells [194]. Similar to *Setdb1*, both *G9a* and *GLP* null mice also show embryonic lethality, including aberrant somitogenesis and aberrant neural tube formation [195-196]. G9a inactivates ~120 genes during mESC differentiation including Oct4 and Nanog, in concert with DNA Methyltransferases Dnmt3a/b [197-198]. G9a is also implicated for genomic imprinting in the mouse placenta [199].

3.2.4 Histone Demethylases

In humans, the identified histone demethylases include the KDM (Lysine (K) Demethylase) families of demethylases (KDM1-6), the PHF family and the JMJD6 family (reviewed in [200-201]). As with histone methyltransferases, histone demethylases are critical for embryonic development. The KDM1 family comprises of KDM1A and KDM1B. Homozygous deletion mutants of *Kdm1a* are early embryonic lethal and do not gastrulate [202]. *Kdm1a* null ES cells are pluripotent but do not form embryoid bodies and do not differentiate [202]. *Kdm1b* mutant mice embryos are maternal embryonic lethal and defective in imprinting [203]. The KDM2 family comprises of KDM2A and KDM2B, of which, KDM2B is implicated in osteogenesis from mesenchymal stem cells [204]. The KDM3 family consists of KDM3A, KDM3B and JMJD1C. KDM3A is required for spermatogenesis [205-206]. KDM3A is positively regulated by Oct4 and depletion of KDM3A from ES cells leads to differentiation [207]. The KDM4 family consists of KDM4A, KDM4B, KDM4C and KDM4D, of which, KDM4C is also positively regulated by Oct4 [207]. Depletion of KDM4C causes differentiation in ES cells and KDM4C also positively regulates Nanog expression [207]. The KDM5 family comprises of KDM5A, KDM5B, KDM5C and KDM5D, of which, KDM5A has been implicated in differentiation [208]. The KDM6 family consists of KDM6A, UTY and KDM6B. KDM6A and KDM6B are shown to regulate HOX gene expression during development [209-210]. KDM6B also controls neuronal differentiation and epidermal differentiation [211-213]. The PHF family includes JHDM1D, PHF2 and PHF8, while the JMJD6 family includes only JMJD6. JHDM1D is required for neural differentiation in mESCs and knockdown of *Jhdm1d* blocks neural differentiation [214].

3.2.5 DNA Methyltransferases

The DNA Methyltransferases (DNMTs) in humans include DNMT1, DNMT2 and DNMT3 (reviewed in [215-216]). The identified isoforms of DNMT1 are DNMT1s, DNMT1o, DNMT1b and DNMT$^{\Delta E3\text{-}6}$. Members of DNMT3 are DNMT3a, DNMT3b and DNMT3L, of which, DNMT3a has isoforms DNMT3a1-4 and DNMT3b has isoforms DNMT3b1-8. *Dnmt1* null mice embryos die after gastrulation, before the 8-somite stage [217]. Double homozygous null mutations in *Dnmt3a* and *Dnmt3b* in mice embryos also caused similar phenotypes, with defects in neural tube closure and embryonic lethality at presomite stage [218-219]. During normal development, Dnmt3b is expressed in ICM, epiblast, embryonic ectoderm and spermatogonia of mouse embryo, whereas Dnmt3a is expressed throughout the embryo after E10.5 [220-221]. Dnmt3b is also expressed in progenitor population during hematopoiesis, spermatogenesis and neurogenesis [222]. During terminal neuronal differentiation, expression shifts to Dnmt3a [222]. Dnmt3l is required for genomic imprinting and female homozygous *Dnmt3l* null mice die during embryogenesis due to imprinting defects [223]. These mice show reduced spongiotrophoblast differentiation and excess trophoblast giant cell formation. Male homozygous *Dnmt3l* null mouse embryos show impaired spermatogenesis, but are viable [224]. Dnmt3a is also required for imprinting and spermatogenesis [225-226]. Curiously, triple homozygous knockout of *Dnmt1*, *Dnmt3a* and *Dnmt3b* causes no change in ESC self-renewal [227]. However, *Dnmt1* null ESCs with extremely low CpG methylation levels do not differentiate [228]. Similar blockage of differentiation is also observed in dual *Dnmt3a/Dnmt3b* homozygous null mutants [228].

3.2.6 Chromatin remodeling enzymes

Chromatin remodeling enzymes are involved in controlling the higher order structure of chromatin by creating heterochromatin and euchromatin, and utilize the energy of ATP to do so. Chromatin remodeling enzymes thereby control cell fate during differentiation of ESCs (reviewed in [229]). During *in vivo* development, chromatin remodeling enzymes have been shown to be important for myeloid differentiation, erythropoiesis, T-cell development, adipogenesis, neurogenesis and myogenesis (reviewed in [230]). Mammalian chromatin modeling enzymes are categorized into three families: SWI/SNF, ISWI and CHD. The SWI/SNF family is characterized by the presence of either Brg1 or Brm as the catalytic subunit, and is further categorized into two subfamilies: Baf and Pbaf [231]. *Brg1* homozygous null mice die at peri-implantation stage and their ICM as well as trophoblast tissues die [232]. Similar phenotypes are observed homozygous mice null for *Snf5*, another subunit of SWI/SNF complexes [233]. However, downregulation of Baf60c causes late embryonic lethality due to defects in cardiac and skeletal muscle development [234]. Similarly, ablation of *Baf180* leads to defects in heart development and placental trophectoderm development, and subsequent embryonic lethality [235] However, homozygous null mutations in *Baf155* caused early embryonic lethality due to failure of ICM cells, though trophoblast giant cells were found to be normal [236]. Heterozygotes null for *Baf155* showed defective brain development. In mESCs, knockdown of Brg1 leads to loss of self-renewal and impaired ability to differentiate to ectoderm and mesoderm [237]. Inactivation of *Baf250b* has been associated with reduced self-renewal and increased differentiation [238]. Interestingly, ablation of *Baf250a* caused failure of mesoderm formation in mouse embryos as well as in ESC-based embryoid body cultures [239]. However, primitive endoderm differentiation and neuronal differentiation could be established in these cells. Brg1, Baf47, Baf155 and Baf57 are

required to suppress Nanog expression during differentiation and knockdown of Baf155 or Baf57 lead to de-repression of Nanog levels during differentiation [240].
The ISWI family of chromatin remodelers is characterized by the presence of the Snf2h or Snf2l ATPase subunits that interact with unmodified histones. Snf2h is a part of the RSH, WICH, NoRC, CHRAC and ACF chromatin remodeling complexes. Snf2l is a part of the NURF and CERF complexes. Similar to *Brg1*, *Snf2h* homozygous null mice die at peri-implantation stage and both ICM and trophectoderm tissues degenerate [241]. Cecr2, a component of CERF, has been implicated in neural tube formation in mice [242]. Human NURF has also been implicated in neuronal development [243]. Interestingly, homozygous mice null for *Bptf*, a component of NURF, fail to form the visceral endoderm [244]. Bptf is required for ectoderm, mesoderm and both definitive and visceral endoderm development from ESCs in embryoid body cultures. Association between NURF complex and Smad transcription factors are necessary for endoderm formation [244]. The CHD family of chromatin remodelers is characterized by the presence of two chromodomains, with affinity for methylated histones. The CHD family is categorized into three subfamilies: family I with CHD1-2, family II with CHD3-4 and family III with CHD5-9. Downregulation of Chd1 in ESCs leads to impaired pluripotency, such that the cells are incapable of primitive endoderm and cardiac mesoderm differentiation and become prone to neural differentiation [245]. CHD3 and CHD4 are also found in NuRD histone deacetylase complexes described previously. Mutations in *Chd7* are embryonic lethal, showing that Chd7 is also important for embryonic development [246].

3.2.7 Regulation of epigenetic factors by transcription factors

Significant literature has been accumulated concerning the regulation of epigenetic factors by transcription factors. Transcription factors lie downstream of signaling pathways, raising the possibility that signaling pathways can control the recruitment of epigenetic factors to specific promoter regions. The vast number of interactions between the Smad transcription factors and epigenetic factors is extensively reviewed in [157]. Smads 1-4 can directly interact with HATs p300 and CBP [247] while Smads 2-3 can also interact with PCAF [248]. Smads 1, 2, 3 and 5 can interact with the HAT GCN5 [249] while Smad5 can also interact with the histone methyltransferases Suv39h2 [250]. Smad7 is acetylated and protected from degradation by p300 [251]. Smads 6 and 7 can also bind and recruit HDACs [252]. As discussed before, "bridging" proteins such as NCoR, Sin3, SMRT, CtBP and TLE can also help epigenetic factors to associate with transcription factors. Smad6 can directly bind CtBP [253]. Smads 3 and 4 can interact with NCoR and Sin3 through Dach1 [254]. Smad1 can also associate with Sin3a through Dach1 [255]. Smads 2, 3 and 4 can recruit NCoR as well as Sin3/HDAC through Ski [256-257]. Smad3 can associate with Hdac1 through TGIF2 [258]. Similarly, the transcription factor β-catenin can recruit epigenetic factors and influence the epigenetic state of cells (reviewed in [64]). β-catenin can associate with the HATs p300 and CBP [259-260] as well as TIP60 [261]. β-catenin can also associate with the chromatin remodeling factors Brg1 and ISWI [64]. Additionally, β-catenin can interact with the histone methyltransferases Mll1/Mll2 [261]. The Erk kinase, which is downstream of the MAPK pathway, can phosphorylate CBP [262], Smads 1-4 [263-265] as well as Brg1 and Brm [266]. Similarly, the Akt kinase, which is downstream of PI3K signaling, can associate with SWI/SNF components Ini1, Baf155 and Baf170, and can phosphorylate Baf155 [267]. Akt can interact with the histone methyltransferases Setdb1 [268] and Ezh2 and can phosphorylate and inhibit Ezh2 [269]. Akt can also phosphorylate and activate p300 [270]. Interestingly, the pluripotency factor Oct4 has also been shown to associate with numerous epigenetic factors

including components of the NuRD HDAC complex (Chd4, p66α, p66β, Mbd3, Mta1-3 and Hdac1), chromatin remodeling proteins (Brg1, Baf155 and ISWI) and DNA methyltransferases (Dnmt3a and Dnmt3l) [271].

3.3 Cell polarity

Cell polarity is a feature of cellular physiology exhibited by epithelial cells. It refers to uneven spatial distribution of proteins across the cell, causing different parts of the cell to have different morphology and functions. Cell polarity can be classified as apical-basal polarity (epithelial cells), anterior-posterior polarity (neurons) and planar polarity (cochlea). In mammals, the apical-basal polarity of epithelial cells is regulated by three distinct protein complexes: the Crumbs/PALS1/PATJ complex, the Par3/Par6/aPKC complex and the Scribble/Dlg/Lgl complex. A detailed review of these complexes and their function in controlling epithelial architecture, cell migration and tumorigenesis can be found in [272]. The apical-basal polarity is lost during epithelial-to-mesenchymal transformation (EMT), a process that changes epithelial cells to mesenchymal cells with no apical-basal polarity and occurs during embryogenesis, fibrosis and cancer metastasis. Importantly, recent findings have indicated that epithelial cell polarity is required for early mouse embryogenesis and may be the driving factor for differentiation of certain early lineages. For example, the cell polarity regulator Par6 is required for proper trophectoderm formation in mouse embryos [273]. Downregulation of Par3 or aPKC drives blastomeres towards ICM instead of trophectoderm [274]. Interestingly, the Crumbs polarity complex has been found to interact with components of the Hippo signaling pathway [99], and the Hippo has been implicated in trophectoderm formation as discussed in the review. It will therefore be interesting to study whether cell polarity proteins indeed control trophectoderm differentiation during early stages of embryogenesis.

4. Conclusions

The development and morphogenesis of the embryo is under strict control by a rather small set of signaling pathways. However, the presence of multiple ligands and multiple receptors, numerous transcription factor-binding partners and significant crosstalk between pathways gives rise to vast complexity within this small set of signaling pathways. Further, lineage-specific differentiation is also controlled by complex regulation of various histone acetyltransferases, histone deacetylases, histone methyltransferases, histone demethylases, DNA methyltransferases and chromatin-remodeling enzymes. A detailed and molecular-level understanding of the determinants of lineage specificity of differentiation has only recently begun to emerge through studies of the signaling pathways and their downstream factors. We have summarized the signaling pathways and miRNAs associated with differentiation to various lineages in **Table 1**. Along similar lines, various histone modifying proteins and chromatin remodeling proteins associated with various lineages are summarized in **Table 2**. Even though these tables present a concise mechanistic linkage between various regulators of ESC differentiation, questions regarding how lineage-specific transcription factors are regulated and how a balance between opposing factors, such as HATs and HDACs, or HMTs and histone demethylases, is achieved in the cell remain largely unanswered. Further, the possible role of lineage-specific transcription factors in the recruitment of epigenetic factors also remains largely unknown. Thus, our understanding of the lineage-specificity of differentiation is still rudimentary and requires significant additional research.

Lineage	Signaling Pathways	Link with Lineage-specific Transcription Factors	Micro-RNAs
HESC	Activin/Nodal [23-24] FGF [24, 38-39, 54-55]	Smad2/3 activates Nanog promoter [25] Nanog binds and inhibits Smad1[275]	miR-302 [113-114] miR-145 [117]
Ectoderm	Block Activin/Nodal [12, 14]		
Mesoderm	Low Activin/Nodal [13, 18-19] Short-term BMP [18-19, 27] FGF [44-47], Wnt [66, 68-69, 73]	Smad1/5/8 binds Nanog promoter [25] Wnt3a activates Brachyury [67] β-catenin regulates Cripto promoter [70]	
Definitive Endoderm	High Activin/Nodal [13, 17, 20-21] Wnt [66, 68-69, 71, 73] Low PI3K [21, 85]	Wnt3a activates Brachyury [67]	
Epidermis	Block Activin/Nodal [12, 14] High BMP [15]	Smad1/5/8 binds Nanog promoter [25]	
Neural Plate / Neurogenesis	Block Activin/Nodal [12, 14, 22] Block BMP [15, 22] FGF [51, 53, 57, 60]		
Neural Crest	Block Activin/Nodal [12, 14] Low BMP [16], FGF [51, 57] Hippo [101] (?)	Smad1/5/8 binds Nanog promoter [25]d2 activates Pax3 promoter [101]	
Trophectoderm	Block Activin/Nodal [26] Long-term BMP[27-28] FGF [40-42, 56], Hippo [98]	Smad1/5/8 binds Nanog promoter [25] Ras/MAPK upregulates Cdx2 [42] Yap and Tead4 coactivate Cdx2 [98]	
Primitive Endoderm	FGF [40, 43]		
Hematopoietic Mesoderm	FGF [48-49]	FGF controls Runx1, Lmo2, Scl [50]	miR-181 [120]
Cardiac Mesoderm	Block Wnt [74]		miR-1 [119]
Adipocytic Mesoderm			miR-143 [121]

Table 1. Summary of signaling pathways, their link with lineage-specific transcription factors and micro-RNAs during embryogenesis and during hESC differentiation

Lineage	Epigenetic Factors					Link with Lineage-specific Transcription factors
	HAT	HDAC	HMT	HDM	DNMT	Chromatin Remodeling	
HESC	Tip60 [148]	NuRD [162]	Setdb1 [193]	KDM3A, KDM4C [207]		Brg1 [232] Baf155 [236] Baf250b [238] Snf2h [241]	Oct4 upregulates KDM3A and KDM4C. KDM4C upregulates Nanog [207], Oct4 associates with NuRD [271]
Ectoderm		NuRD [161]				Brg1 [237] NURF [244]	
Mesoderm						Brg1 [237] Baf250a [239] NURF [244]	Smad2 interacts with NURF [244], β-catenin can interact with Brg1 [64]
Definitive Endoderm						NURF [244]	Smad2 interacts with NURF [244]
Gametes		SirT2 [176]	Mll2 [185]	KDM3A [205-206]	Dnmt3l [224]		
Neural Plate/ Neurogenesis	Qkf [149]	HDAC1-2 [156], SirT1 [175]	G9a, GLP [195-196]	KDM6B [211-212] JHDM1D[214]	Dnmt3a, Dnmt3b [218-219]	Baf155 [236] Snf2l [242-243]	JHDM1D upregulates FGF4 [214]
Trophecto-derm		NuRD [163]			Dnmt3l [223]	Brg1 [232] Baf180 [235] Snf2h [241]	
Hematopoietic Mesoderm	Moz [150-152]	HDAC1-2 [156], NuRD [165-167]	Mll1 [183] Mll5 [187]				Smad2-4, 6-7 can interactwith HDAC1-2 [252, 256-258], β-catenin can interact with Mll1 [261]
Cardiac Mesoderm		HDAC1-2 [156]				Baf60c [234] Baf180 [235] CHD1 [245]	Smad2-4, 6-7 can interact with HDAC1-2 [252, 256-258]
Adipocytic Mesoderm		SirT1 [174]	Mll3 [186]				
Myogenic Mesoderm		HDAC1-2 [156] SirT2 [173]				Baf60c [234]	Smad2-4, 6-7 can interact with HDAC1-2 [252, 256-258]
Primitive Endoderm		NuRD [161]				CHD1 [245]	

Table 2. Summary of epigenetic factors and their link with lineage-specific transcription factors during embryogenesis and during hESC differentiation. Abbreviations: HAT: Histone Acetyltransferase, HDAC: Histone Deacetylase, HDM: Histone Demethylase, DNMT: DNA Methyltransferase

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6. References

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2

Bioactive Lipids in Stem Cell Differentiation

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1. Introduction

Bioactive lipids are lipids with cell signaling functions. In the last two decades, they have become increasingly important in many fields of biology. They are the main diffusible mediators of inflammatory responses in tissues and regulate the polarity of cellular membranes. They are also critical for cell fate decisions during stem cell differentiation by inducing apoptosis or sustaining cell survival and polarity. The bioactive lipids discussed here belong to the classes of phospho- and sphingolipids. Mainly three different types of lipids and their function in stem cell differentiation will be reviewed in detail: phophatidylinositols (PIPs), lysophospholipids and eicosanoids, and the sphingolipid ceramide and its derivative sphingosine-1-phosphate (S1P).

2. Biological Function of bioactive lipids in stem cell differentiation

2.1 Phosphatidylinositols

The phosphatidylinositols PI(3,4)P2 and PI(3,4,5)P3 generated by class I phosphatidyinositol-3-kinase (PI3K) upon induction of tyrosine receptor kinases or G-protein coupled receptors (GPCRs) are known to be the major activators of the Akt/PKB cell signaling pathway for cell survival and differentiation (Callihan et al., 2011; Frebel &Wiese, 2006; Layden et al., 2010; Paling et al., 2004; Storm et al., 2007; Umehara et al., 2007). The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid phosphatase that catalyzes the hydrolysis of PIP3 to PIP2, which leads to inactivation of the Akt/PKB cell signaling pathway and loss of pluripotency in stem cells (Groszer et al., 2001; Korkaya et al., 2009; Otaegi et al., 2006). PTEN is a tumor suppressor mutated in many types of cancer and it is critical for the controlled growth of embryonic tissue and ES cells.

PTEN converts PIP3 into PIP2 (Fig. 1). Since PIP3 activates the Akt/PKB cell signaling pathway, thus PTEN catalyzing PIP3 hydrolysis is a negative regulator of Akt/PKB. Consistent with this function, deletion of PTEN activates Akt/PKB-dependent cell signaling pathways (Groszer et al., 2001). PTEN mutations are often found in human cancers such as glioblastoma, prostate cancer, and breast cancer. Loss of function of this tumor suppressor gene results in the up-regulation of the Akt/PKB-to-β-catenin pathway (Fig. 2A) (Korkaya et al., 2009). Akt/PKB phosphorylates and therefore, inactivates glycogen synthase-3β (GSK-3β), a protein kinase in the Wnt signaling pathway that phosphorylates β-catenin (Doble &Woodgett, 2003; Ikeda et al., 2000; van Noort et al., 2002). The oncogene β-catenin is an important adhesion protein and transcription factor for genes involved in proliferation. When phosphorylated by GSK-3β, β-catenin (in a protein complex with adenomatous

polyposis coli or APC) is proteolytically degraded and thus, adhesion lost and proliferation reduced. Consistent with this function, deletion of β-catenin results in loss of pluripotency and early embryonic death of the respective knockout mouse (Haegel et al., 1995). Likewise, deletion of PTEN results in increased β-catenin levels and increased pluripotency or malignancy (Groszer et al., 2001). Therefore, the PTEN vs. PI3K-to-Akt/PKB antagonism is interesting in two biological contexts with respect to stem cell differentiation: maintenance of pluripotent stem cells and tumorigenesis of cancer stem cells. In the first context, inhibition of PTEN, activation of PI3K and Akt/PKB, or inhibition of GSK-3β will be useful to maintain pluripotent ES cells. In the second context, activation of PTEN, inhibition of PI3K and Akt/PKB, or activation of GSK-3β may be a useful strategy to eliminate cancer stem cells.

In the cultivation process of ES cells, elevated expression of the transcription factors Oct-4 and Nanog is essential for maintenance of pluripotency (Bhattacharya et al., 2003; Sato et al., 2004). It has been shown that two cell signaling pathways are critical for this regulation: the janus kinase/signal transducer and activator of transcription 3 (Jak/Stat3) and the Akt/PKB signaling pathways (Fig. 2A) (Kelly et al., 2011; Paling et al., 2004). In the cultivation of mouse ES cells, the most important growth factor activating Stat3 and Akt/PKB is LIF (leukemia inhibitory factor), an interleukin 6 class cytokine binding to LIF receptor α (LIFRα) (Cartwright et al., 2005; Niwa et al., 1998; Okita &Yamanaka, 2006; Schuringa et al., 2002; Takao et al., 2007). In vitro, LIF is added to the medium when cultivating undifferentiated mouse ES cells on feeder fibroblasts and in feeder-free culture. In vivo, LIF is generated by the trophoectoderm from where it penetrates the inner cell mass, the source of pluripotent ES cells in the pre-implantation embryo. In human ES cells, the role of LIF as "guardian" of pluripotency is taken over by fibroblast growth factor (FGF) (Lanner &Rossant, 2010; Li et al., 2007) (Fig. 2A). Binding of FGF-2 to the FGF receptor 2 (FGFR2) activates similar cell signaling pathways in human ES cells as stimulated by LIF in mouse ES cells: Jak/Stat3, mitogen-activated protein kinase (MAPK), and Akt/PKB (Lanner &Rossant, 2010; Li et al., 2007). However, FGFR-dependent signaling is very diverse and it depends on individual receptor protein complexes which specific response is elicited by FGF. For example, in mouse ES cells, FGF-2 is used to maintain the multipotent neuroprogenitor stage and to prevent further neuronal differentiation. In human ES cells, supplementation of the serum-free cell culture medium with FGF-2 is critical to prevent apoptosis and to maintain pluripotency.

The role of lipids as the key factors in the PI3K-to-Akt/PKB-to-β-catenin cell signaling pathway is obvious since phosphatidylinositols (PIPs) are lipids by provenance. Unfortunately, PIPs are not applicable as exogenous factors that can be simply added to stem cell media since these lipids are part of an intracellular cell signaling cascade not easily accessible to the outside of the cell. However, there are other lipid-regulated pathways that are dependent on the activation of cell surface receptors, which is of tremendous advantage if one attempts to use lipids as exogenously added growth or differentiation factors (see section 2.2). The two receptors involved in maintenance of pluripotency, LIFRα and FGFR2 are both tyrosine receptor kinases which are not directly activated by lipids, although indirect regulation by so-called "lipid rafts" has been discussed (see section 2.4) (Lee et al., 2010b; Yanagisawa et al., 2004b, 2005).

In addition to using natural lipids as ligands, stem cell differentiation can also be modulated by pharmacologic reagents that are either lipid analogs, inhibitors of enzymes in lipid

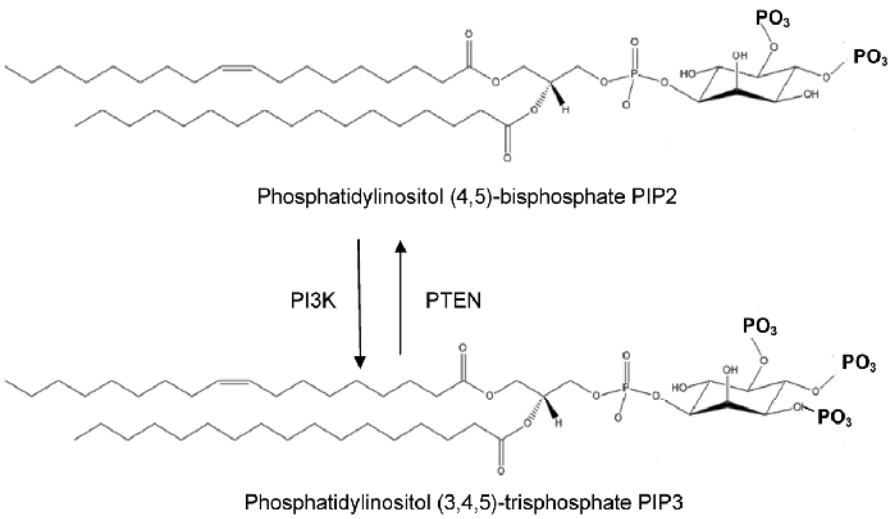

Fig. 1. Metabolism of phosphatidylinositols in the PI3K-to-Akt/PKB cells signaling pathway for ES cell pluripotency. PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10

metabolism, or drugs targeting downstream effectors of lipid-regulated cell signaling pathways. Two drugs that are inhibitors of protein kinases in the LIFRα and FGFR2 pathways have been tested on their effect on pluripotency: LY294002 and indirubin-3-monoxime, two inhibitors specific for PI3K and GSK-3β, respectively (Chen et al., 2006; Chen et al., 2000; Ding &Schultz, 2004; Ding et al., 2003; Lyssiotis et al., 2011; Otaegi et al., 2006; Paling et al., 2004; Sato et al., 2004). The PI3K inhibitor LY294002 has been shown to reduce the capacity of mouse and human ES cells to self-renew and to undergo subsequent steps of lineage specification and differentiation (Paling et al., 2004). These effects are likely to involve differentiation stage-specific (contextual) other cell signaling pathways downstream (or parallel) to the PI3K-to-Akt/PKB signaling axis. While it may not be desired to interfere with ES cell pluripotency, LY294002 and other PI3K and Akt/PKB inhibitors are currently tested for cancer treatment, in particular for targeting cancer stem cells (Bleau et al., 2009; Plo et al., 1999). If one desires to sustain self-renewal of ES cells, GSK-3β inhibitors such as indirubin-3-monoxime or BIO are attractive candidates. BIO has been successfully used to maintain pluripotency in human ES cells (Sato et al., 2004). Additional effectors targeting GSK-3β are synthetic agonists of the Wnt receptor Frizzled, however, their use in stem cell differentiation is not yet sufficiently investigated (Lyssiotis et al., 2011).

Interestingly, inhibitors of the mitogen activated protein kinase (MAPK) pathway such as the MAPK kinase (MEK) inhibitor PD98059 have been used with mouse ES cells to promote self-renewal or pluripotency (Buehr &Smith, 2003; Li et al., 2007). This appears paradoxical since LIFRα as well as FGFR2 are known to activate MAPK, which suggests that activation of MAPK is involved in pluripotency. However, only transient MAPK activation to promote G1 re-entry is useful for self-renewal while prolonged activation will promote differentiation (Fig. 2B). Therefore, a combination of LIF with the MAPK-kinase (MEK) inhibitor PD95059 activating PI3K-to-Akt/PKB while inhibiting MAPK signaling has been successfully used to promote pluripotency in mouse ES cells, but also to enhance the

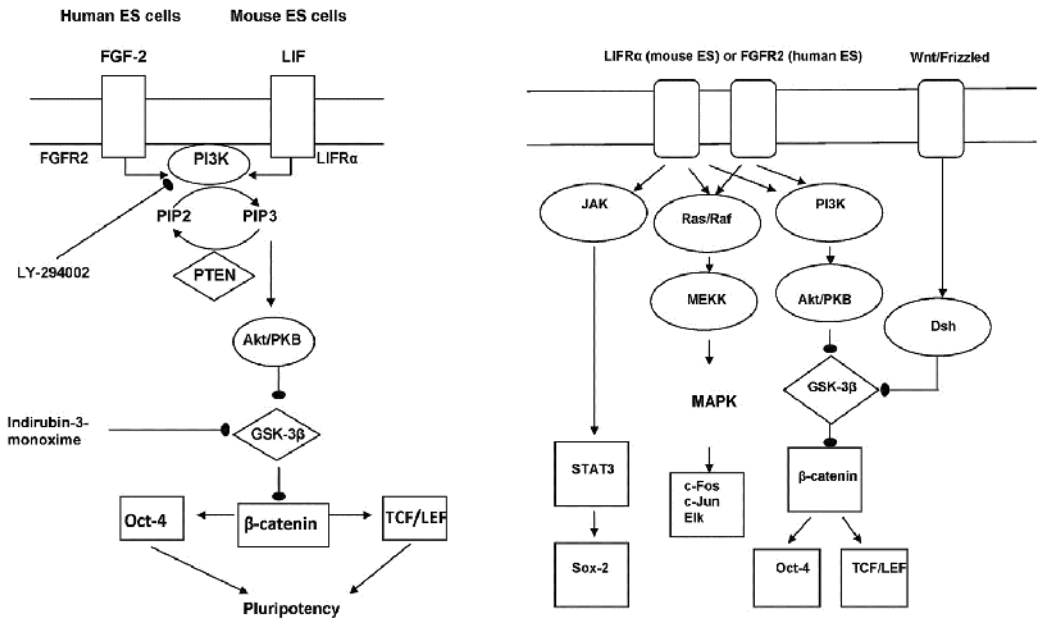

Fig. 2. Cell signaling pathways for ES cell pluripotency. Elliptic circles label enzymes that promote pluripotency, while diamonds label enzymes that reduce pluripotency and promote differentiation. MAPK shows both, pro-pluripotency or pro-differentiation activity in human or mouse ES cells, respectively.

generation of induced pluripotent stem (iPS) cells (Li et al., 2007; Lyssiotis et al., 2011). The situation in human ES cells, however, is different. In contrast to mouse ES cells, inhibition of the MAPK cell signaling pathway reduces the potential of undifferentiated human ES cells to self-renew, indicating that FGFR2-mediated activation of Ras/Raf-to-MEK-to MAPK is critical for human ES cell pluripotency (Ding et al., 2010). A similar role has been found for Bmp4, which promotes pluripotency in mouse and differentiation in human ES cells (Bouhon et al., 2005; Zeng et al., 2004; Zhang et al., 2010). It is quite possible that this difference depends on which other pathways for pluripotency are co-activated such as Jak/Stat3 in mouse or Activin in human ES cells. Bioactive lipids are important in that they co-regulate several cell signaling pathways critical for pluripotency and differentiation of ES cells, in particular MAPK and PI3K downstream of ClassA/Rhodopsin-like GPCRs, which will be discussed in the next section.

2.2 Lysophospholipids and eicosanoids

Lysophospholipids (LPLs) are lipids generated by hydrolytic cleavage of fatty acid from glycerophospholipids, which is catalyzed by phospholipases. Distinct phospholipases cleave off either one of the two (PLA1 and PLA2) or both (PLB) fatty acid residues, or they cleave off the phosphate-containing head group (PLC) or the alcohol (PLD) (Gardell et al., 2006; Hla et al., 2001; Hla et al., 2000; Lin et al., 2010; Meyer zu Heringdorf &Jakobs, 2007; Okudaira et al., 2010; Radeff-Huang et al., 2004; Tigyi &Parrill, 2003; Ye et al., 2002). PLA2 generates arachidonic acid, the precursor for the generation of eicosanoids, a group of inflammatory mediators including prostaglandins and leukotrienes (Funk, 2001; Jenkins et

al., 2009; Khanapure et al., 2007; Lambeau &Gelb, 2008; Szefel et al., 2011; Wymann &Schneiter, 2008). Similar to the PLD reaction, lysophospholipase D or autotaxin generates lysophosphatidic acid (LPA) from lysophosphatidylcholine (Nakanaga et al., 2010; Okudaira et al., 2010; Samadi et al., 2011). LPA receptors are critical in cell proliferation and tumorigenesis and have recently been shown to promote proliferation of human neural precursor cells (Callihan et al., 2011; Hurst et al., 2008; Lin et al., 2010; Pebay et al., 2007; Pebay et al., 2005; Pitson &Pebay, 2009).

Arachidonic acid, generated by PLA2 from phospholipids such as phosphatidylcholine (Fig. 3A) is converted to a variety of pro-inflammotory eicosanoids among which prostaglandins, thromboxanes, and leukotrienes are the most important signaling lipids (Fig. 3B). The effect of eicosanoids on ES cells is not well understood and research is mostly limited to results with mouse ES cells. Interestingly, lysophospholipids such as LPA and eicosanoids such as prostaglandin E2 (PGE2) appear to activate similar downstream cell signaling pathways, mainly the PI3K-to-Akt/PKB, MAPK, and Wnt/GSK-3β pathways (Callihan et al., 2011; Goessling et al., 2009; Logan et al., 2007; North et al., 2007; Pebay et al., 2007; Pitson &Pebay, 2009; Yun et al., 2009). In contrast to LIFRα or FGFR2, however, stimulation of Akt/PKB by PGE2 has not been reported to sustain pluripotency, but is rather anti-apoptotic/cell protective and promotes stem cell proliferation. This may not be surprising since generation and conversion of arachidonic acid is often a response to hypoxic insults, which can damage mitochondria and induce apoptosis. Notably, inhibition of eicosanoid biosynthesis reduces the potential of mouse and human ES cells to self-renew, indicating a role of eicosanoids in stem cell maintenance or pluripotency (Yanes et al., 2010). Thromboxane has not been described to play a role in stem cell differentiation, maybe because its main function is rather confined to platelet aggregation. In contrast, prostacyclin, a similar eicosanoid in platelet aggregation has been shown to promote cardiogenic differentiation from human ES

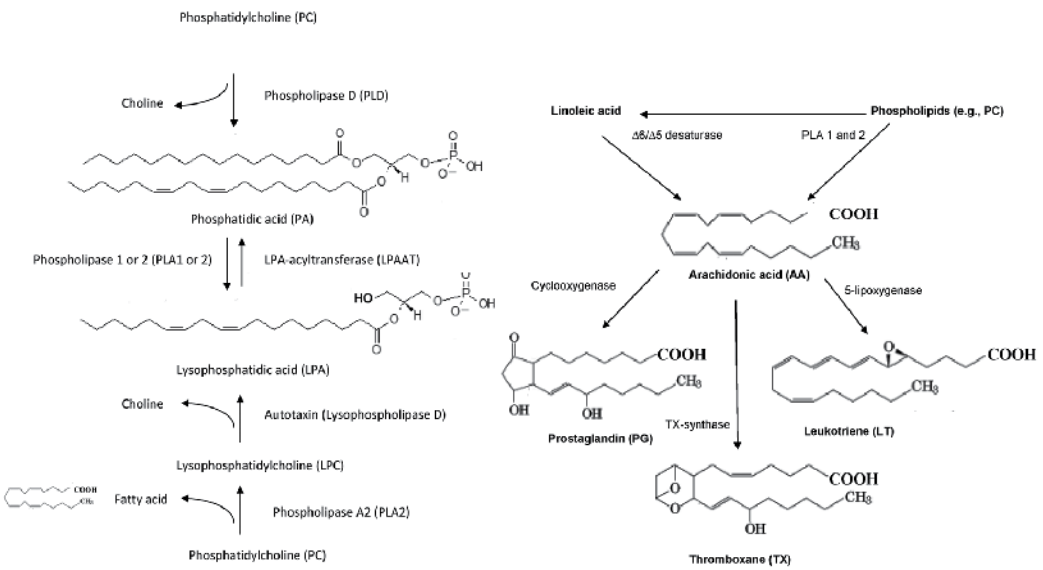

Fig. 3. Biosynthesis pathways in lysoposphatidic acid (LPA) and eicosanoid metabolism

cells (Chillar et al., 2010; Xu et al., 2008). In addition to prostacyclin, leukotriene of the LTD4 type has been used in several studies to promote proliferation and cardiovascular differentiation of mouse ES cells (Finkensieper et al., 2010; Funk, 2001; Kim et al., 2010).
The effect of prostaglandins and other eicosanoids on ES cells is worth discussing in an important aspect of human health care. Inhibitors of cyclooxygenase 2 (Cox-2), the enzyme critical for PGE2 production, are taken by nearly everyone to ease up head ache, back pain, and inflammation. The Cox-2 inhibitor aspirin is one of the most successfully administered drugs world-wide. A recent study on the negative effect of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin on the differentiation of human ES cells suggests that one has to be careful with the use of NSAIDs when human ES cells are to be transplanted for heart tissue repair (Chillar et al., 2010). These observations suggest that eicosanoids are important in cardiogenic/cardiovascular differentiation of ES cells.
The eicosanoid as well as lysophospholipid receptors belong to the family of Class A Rhodopsin-like GPCRs (Callihan et al., 2011; Hla et al., 2001; Kostenis, 2004; Lin et al., 2010; Pitson &Pebay, 2009; Radeff-Huang et al., 2004). They mediate the activation of downstream cell signaling pathways through different types of GTPases, mainly Gi, Gq, and G12/13,

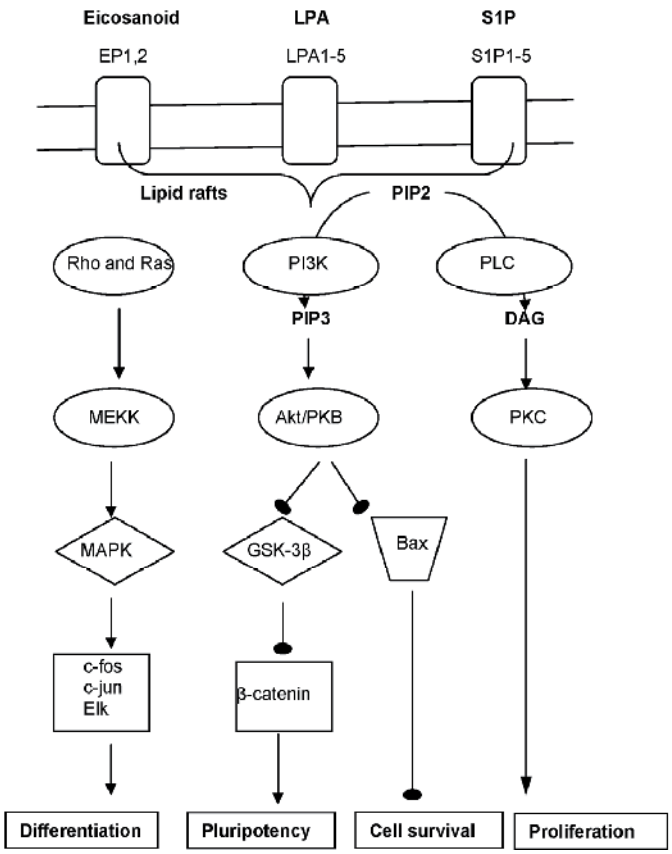

Fig. 4. GPCR-dependent cell signaling pathways with similar function for ES cell pluripotency and differentiation. DAG, diacylglycerol; PLC, phospholipase C; EP, eicosanoid receptor.

acting upon PI3K-to-Akt/PKB (Gi), Ras-to-ERK (Gi, Gq) Rho (G12/13), and PLC-to-PKC (Gq) cell signaling pathways for pluripotency and cell survival (Akt/PKB), proliferation (Rho and PKC), and differentiation/specification (MAPK) pathways (Fig. 4). Hence, combinations of particular cell signaling lipids with cytokines or growth factors such as LIF or FGF-2 activating similar effectors have been found to be useful in directing stem cell fate toward pluripotency, proliferation, or differentiation, respectively (Hurst et al., 2008; Kilkenny et al., 2003; Layden et al., 2010; Pebay et al., 2007; Radeff-Huang et al., 2004). There are five GPCRs for each LPA and sphingosine-1-phosphate (S1P) expressed in mouse and human ES cells.

2.3 Ceramide and sphingosine-1-phosphate

Sphingolipids are acyl (fatty acid) derivatives of the amino alcohol sphingosine. They encompass sphingosine, ceramide, and ceramide derivatives such as sphingomyelin, ceramide-1-phosphate, S1P, and glycosphingolipids (Fig. 5A for structures) (Bartke &Hannun, 2009; Chalfant &Spiegel, 2005; Chen et al., 2010; Futerman &Hannun, 2004; Hannun et al., 2001; Hannun &Obeid, 2002, 2008; Lebman &Spiegel, 2008; Merrill et al., 1997; Spiegel &Milstien, 2003; Strub et al., 2010; Takabe et al., 2008). Important biological functions of sphingolipids are cell signaling for inflammation, apoptosis, cell cycle regulation, and autophagy (Bartke &Hannun, 2009; Basu &Kolesnick, 1998; Bieberich, 2004, 2008a; Futerman &Hannun, 2004; Gulbins &Kolesnick, 2003; Haimovitz-Friedman et al., 1997; Hannun &Obeid, 2008; Morales et al., 2007). Most recently, particular sphingolipids have also been implicated in ES cell differentiation and cell polarity (Bieberich, 2004, 2008a, b, 2010; Bieberich et al., 2003; Bieberich et al., 2001; Bieberich et al., 2004; Gardell et al., 2006; Goldman et al., 1984; Harada et al., 2004; Hurst et al., 2008; Jung et al., 2009; Pebay et al., 2007; Pebay et al., 2005; Pitson &Pebay, 2009; Salli et al., 2009; Walter et al., 2007; Wang et al., 2008a; Wong et al., 2007; Yanagisawa et al., 2004a). Ceramide has been shown to induce apoptosis specifically in residual pluripotent stem (rPS) cells that cause teratomas (stem cell-derived tumors) after stem cell transplantation. S1P has been found to promote oligodendrocyte differentiation (see section 3.2. for discussion).

Ceramide is the precursor of all bioactive sphingolipids. It is synthesized in three different metabolic pathways. Figure 5B shows that sphingolipid metabolism is integrated into phospholipid (i.e., PC), one carbon unit (i.e., choline), fatty acid (i.e., palmitoyl CoA for de novo biosynthesis and other fatty acids in the salvage pathway), and amino acid (i.e., serine in de novo biosynthesis) metabolism (Bartke &Hannun, 2009; Bieberich, 2004, 2008a; Chen et al., 2010; Futerman &Hannun, 2004; Futerman &Riezman, 2005; Gault et al., 2010; Hannun et al., 2001; Luberto &Hannun, 1999). In cell cultures, plenty of these precursors are provided in the medium, which may not necessarily reproduce the metabolic situation of stem cells or other cell types in vivo. Recently, our group has found that neural crest-derived stem or progenitor cells are sensitive to alcohol due to ethanol-induced elevation of ceramide and induction of apoptosis (Wang &Bieberich, 2010). Apoptosis can be prevented by supplementing the medium with CDP-choline, This effect can be explained by providing excess of substrate required to drive conversion of ceramide to SM using the interconnection of the Kennedy pathway for phospholipid biosynthesis and the SM cycle (Fig. 5B). Choline can also be replenished from the one carbon unit metabolism, which establishes the interconnection of sphingolipid metabolism with this metabolic pathway.

The fatty acid metabolism interconnects with sphingolipid biosynthesis twice, in the de novo and salvage pathways. The de novo pathway uses palmitoyl CoA and serine for a

condensation reaction that is the first step in ceramide biosynthesis. Since serine is used as the second substrate, de novo biosynthesis ties into the amino acid metabolism as well. The salvage pathway uses a variety of activated fatty acids for re-attachment to sphingosine (Fig. 5B). While supply with precursors for lipid metabolism may not be critical in vitro, specialized tissues or cells such as astrocytes providing nutrients and metabolic precursors to neurons or neural stem cells in vivo maybe more sensitive toward lipid imbalances as observed in fetal alcohol syndrome and Alzheimer's disease (Adibhatla &Hatcher, 2008; Cutler et al., 2004; De Vito et al., 2000; Hirabayashi &Furuya, 2008; Jana et al., 2009; Jana &Pahan, 2010; Muscoli et al., 2010; Riboni et al., 2002; Satoi et al., 2005; Wang et al., 2008b). In particular, neural stem cells are confined to distinct morphological cell complexes which tightly control the interaction with other cells and therefore, comprise "metabolic niches" that may control supply with metabolic precursors and lipid cell signaling factors.

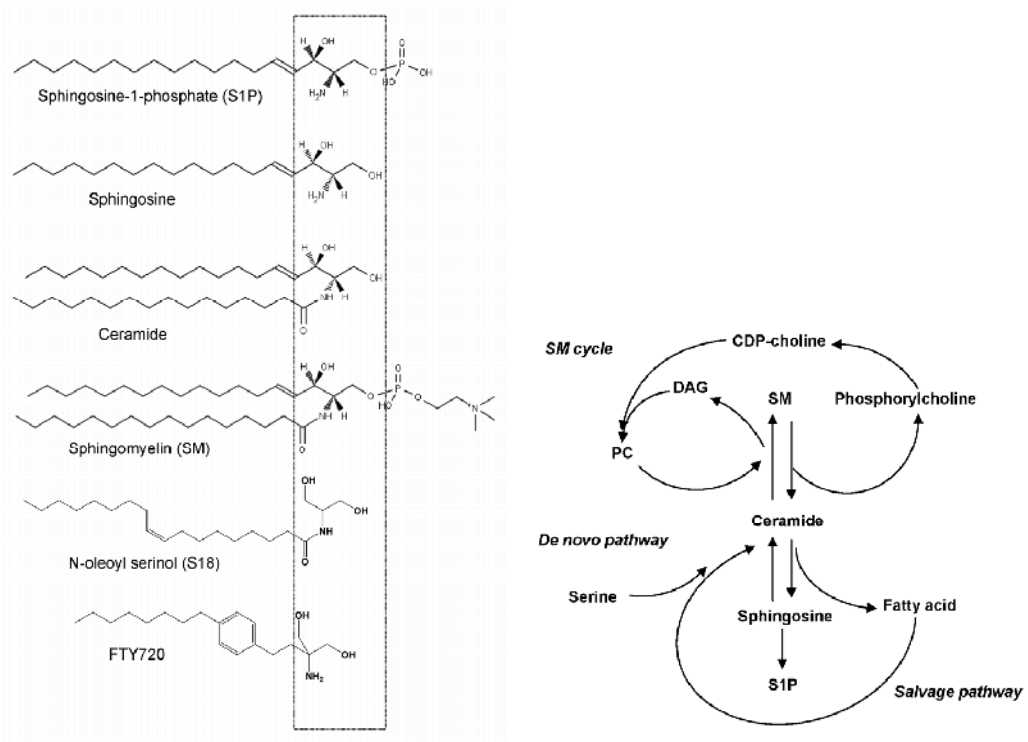

Fig. 5. Structures of ceramide precursors and derivatives with cell signaling function and interconnection of ceramide metabolism with other lipid and amino acid metabolism. N-oleoyl serinol (S18) or FTY720 are analogues of ceramide or S1P, respectively. Box shows common structural motif.

Regulation of sphingolipid metabolism by its interconnection with other lipid metabolic pathways has a direct impact on lipid-dependent cell signaling. Ceramide is the precursor of S1P, which is a ligand for five distinct S1P receptors (S1P1-5) on the cell surface and also binding partner/co-factor for at least three intracellular proteins, histone deacetylase 1 and 2

(HDAC 1 and 2) in the nucleus, the E3 ubiquitin ligase TRAF2, and prohibitin in the mitochondria (Alvarez et al., 2010; Callihan et al., 2011; Hait et al., 2009; Hait et al., 2006; Hurst et al., 2008; Pitson &Pebay, 2009; Radeff-Huang et al., 2004; Sanchez &Hla, 2004; Spiegel &Kolesnick, 2002; Strub et al., 2011) . The effect of intracellular S1P on stem cell differentiation is not known. However, there is solid experimental evidence that S1P has profound effects on ES cells and ES cell-derived neural progenitors via S1P receptors, which will be discussed in section 3.2.

2.4 Terpenoids, sterols, glycosphingolipids, and lipid rafts

The previous sections discussed bioactive lipids that are known to act through lipid receptors or binding proteins. There are many more lipids that regulate cell signaling pathways through a mechanism known as "lipid rafts" or "lipid microdomains" (Bieberich, 2008a; Lee et al., 2010b; Lingwood &Simons, 2010; Miljan &Bremer, 2002; Ohanian &Ohanian, 2001; Yanagisawa et al., 2005). Lipid rafts are areas in the cell membrane (or intracellular membranes) that emerge from the self-assembly of lipids in an ordered structure. They are believed to show high affinity to specific cell signaling proteins such as growth factor or cytokine receptors, which leads to clustering and activation of these receptors. Therefore, bioactive lipids can affect stem cell differentiation in two different ways: direct interaction with lipid receptors such as GPCRs and lipid raft-dependent activation of growth factor or cytokine receptors such as LIFRα or FGFR2 (Bieberich, 2008a; Bryant et al., 2009; Gutierrez &Brandan, 2010; Lee et al., 2010b; Yanagisawa et al., 2005). Lipids that form rafts are sphingomyelin, cholesterol, and glycosphingolipids. In addition, signal transduction proteins such as Ras can be modified with fatty acids (palmitoylation) or terpenoids (farnesylation, geranylation) and glycophosphatidylinositol (GPI anchor), which tremendously increases membrane binding and raft association (Levental et al., 2010; Lingwood &Simons, 2010; Resh, 2004; Roy et al., 2005). It has been shown that particular glycosphingolipids termed gangliosides can regulate ES cell differentiation by the activation of FGFR2 and other receptors in lipid rafts (Bieberich, 2004; Yanagisawa et al., 2005). An example for this mechanism is the corrective activity of the ganglioside GM1 on the effect of the fungus toxin fumonisin B1, which causes neural tube defects by inhibiting sphingolipid biosynthesis (Gelineau-van Waes et al., 2005; Marasas et al., 2004). It has also been demonstrated that the activity of sonic hedgehog, a morphogen critical for germ layer formation is functionally dependent on palmitoylation and modification with cholesterol (Gofflot et al., 2003; Guy, 2000; Incardona &Roelink, 2000; Karpen et al., 2001; Kelley et al., 1996; Lewis et al., 2001; Li et al., 2006). Inhibition of cholesterol biosynthesis with statins leads to aberrant embryo development. Although these are impressive examples of the effect of lipid modification and lipid raft formation on stem cell differentiation and embryo development, it is presently not known how to specifically utilize this mechanism in controlling the differentiation of ES cells. It is also not clear, which differentiation potential cholesterol has besides being critical for lipid raft formation. There is a plethora of steroid hormones such as estrogen or progesterone that are bioactive lipids activating nuclear receptors critical for embryo development. Progesterone is a particularly curious case since it is added to most of the supplements (e.g., N2, B27) found in defined media used for the in vitro maintenance and differentiation of ES cells. The use of this and other bioactive and synthetic lipids for the in vitro differentiation of ES cells will be discussed in the following section.

3. Bioactive lipids and their use for in vitro differentiation of embryonic stem cells

3.1 Induction of apoptosis in teratoma-forming stem cells by ceramide analogs

The reliability and safety of current stem cell differentiation protocols is still a matter of controversy. Many studies have shown that even when using similar protocols for the in vitro differentiation of ES cells, transplantation can lead to the formation of teratomas (Baker, 2009; Bieberich, 2008b; Blum &Benvenisty, 2008; Fong et al., 2010; Fujikawa et al., 2005; Lee et al., 2009; Vogel, 2005; Wang et al., 2010). Teratomas are stem cell-derived tumors that are fatal if they grow in the brain or heart. Teratomas may arise from any type of pluripotent cells, including induced pluripotent stem (iPS) cells. Therefore, they are a major safety concern, in particular when using larger numbers of ES or iPS cell-derived cells. Our studies have shown that teratomas arise from a particular type of residual pluripotent stem (rPS) cells that maintain the expression of the pluripotency transcription factor Oct-4 and fail to differentiate or undergo apoptosis (Bieberich, 2008a, b, 2010; Bieberich et al., 2003; Bieberich et al., 2004). However, we have also found that they co-express prostate apoptosis response 4 (PAR-4), a protein that sensitizes cells toward ceramide-induced apoptosis. Using a water-soluble ceramide analog termed N-oleoyl serinol or S18, which was for the first time synthesized in our laboratory, we were able to rid stem cells grafts of teratoma-forming rPS cells (Bieberich et al., 2002; Bieberich et al., 2000). We have shown that S18 promotes binding of atypical PKC (aPKC) to PAR-4, which inhibits the aPKC-activated NF-κB cell survival pathway and induces apoptosis in rPS cells (Bieberich, 2008a; Krishnamurthy et al., 2007; Wang et al., 2009; Wang et al., 2005). These cells are eliminated because they are sensitive to S18. Neural progenitor cells will survive and undergo further differentiation because they show no or only low level expression of PAR-4.

3.2 Induction of oligodendrocyte differentiation by S1P and S1P analogs

It has been shown that S1P and the S1P prodrug analog FTY720 promote cell survival and differentiation of primary cultures of oligodendroglial precursor cells (OPCs) (Bieberich, 2010; Coelho et al., 2010; Jung et al., 2007; Miron et al., 2008a; Saini et al., 2005). We have found that teratoma-forming rPS cells do not express the S1P receptor S1P1, which makes them vulnerable to ceramide or S18-induced apoptosis (Bieberich, 2008b, 2010; Bieberich et al., 2004). In contrast, ES cell-derived neural progenitor cells express S1P1. Our studies have shown that in the presence of S18 and FTY720, neural progenitor cells will survive and undergo oligodendroglial differentiation because they are insensitive to S18 (PAR-4 is not expressed). At the same time, OPC differentiation is promoted by FTY720 or S1P (S1P1 is expressed). Implantation of S18 and FTY720-treated neural progenitors does not result in teratoma formation and leads to integration of the grafted cells into highly myelinated areas of the brain such as the corpus callosum (Bieberich, 2010). Therefore, a combined treatment with ceramide analogs and S1P analogs or S1P receptor agonists is a promising strategy to control ES cell differentiation toward OPCs that are useful for treatment of de- or dysmelination diseases such as multiple sclerosis. Interestingly, the addition of S1P analogs to the ceramide analog S18 resulted in a shift of predominantly neuronal differentiation (as promoted by S18 alone) of ES cells toward oligodendroglial lineage, which is an impressive example for the impact of bioactive lipids on stem cell differentiation.

3.3 Synthetic lipids as small molecular effectors for ES cell differentiation

The use of defined media supplemented with small molecule effectors that control the in vitro differentiation of stem cells is a promising strategy to generate transplantable progenitor cells that have not been in contact with animal-derived products such as serum. Currently, there are more than twenty compounds available that specifically induce differentiation of ES cells toward progenitors of bone, heart, muscle, or brain tissue. Although most of these compounds are not considered bioactive lipids because they are synthetic drugs not found in biological organisms, almost all of them are lipids with respect to their chemical structure. One of the first synthetic lipids used a small molecule effector for ES cell differentiation is a bioactive lipid with critical function in brain development: retinoic acid (Dinsmore et al., 1996; Guan et al., 2001; Hu et al., 2009; Jiang et al., 2010; Lee et al., 2010a; Liour et al., 2006; Mayer-Proschel et al., 1997; Mummery et al., 1990; Murashov et al., 2005; Osakada &Takahashi, 2011; Plachta et al., 2004). Mouse and human ES cells respond to a brief exposure to retinoic acid by accelerating differentiation into motoneurons, interneurons, and even oligendendrocytes when combined with specific growth factors such as FGF-2 or platelet-derived growth factor (PDGF). Another bioactive lipid used for in vitro differentiation of mouse and human ES cells, in particular toward oligodendroglial lineage is thyroid hormone (T3) (Glaser et al., 2007; Kang et al., 2007).

In addition to these naturally occurring lipids, synthetic lipids have been isolated from chemical libraries using various bioassays for ES cell differentiation. Indirubin-3-oxime type compounds for maintenance of pluripotency have already been discussed in section 1. A more detailed discussion of these small molecule effectors can be found in the following articles (Ding et al., 2003; Lyssiotis et al., 2011; Zhu et al., 2010). The advantage of these compounds emerges from their lipid-like structure, which allows for penetration of the blood brain barrier. Provided that toxicity issues do not prevent the use of these compounds in vivo, bioactive and synthetic lipids are likely to develop into powerful pharmacologic drugs that can be used for in vitro differentiation of ES or iPS cells and then after transplantation, for further treatment of the patient to enhance the in vivo differentiation of the grafted cells. One of the first drugs with this dual potential of in vitro and in vivo use is FTY720 (Bieberich, 2010; Coelho et al., 2007; Lee et al., 2010a; Miron et al., 2008a; Miron et al., 2008b; Napoli, 2000). It is quite expectable that many of these "dual use" drugs will play an important role in the clinical application of ES and iPS cells.

4. Conclusions and perspectives

The goal of this chapter was to review current knowledge on bioactive lipids in embryonic stem cell differentiation. One of the important results of this analysis is the insight into the interconnection between lipid metabolism and signaling function. Unlike most proteins, lipids can be converted into derivatives that either complement or antagonize each other's cell signaling function. For example, conversion of PC to LPA and eisosanoids has similar effects on enhancing pluripotency. On the other hand, conversion of ceramide to S1P can have opposite functions, in particular with respect to apoptosis and survival of pluripotent stem cells. Another important insight is that most bioactive lipids cooperate with cytokine and growth factor receptors providing the possibility to combine these factors with the respective lipids in defined media for controlled stem cell differentiation. For example, FGF-2 can be combined with the ceramide analog S18 to promote neuronal differentiation, or with S18 and FTY720 to enhance specification to oligodendroglial lineage. This provides the

opportunity to generate bioactive lipids or lipid analogs that can be applied for in vitro differentiation of stem cells and then for further treatment of the patient who has received the stem cell graft. These "dual use" bioactive lipids will be of tremendous value for the therapeutic application of stem cells.

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3

Retinoid Signaling is a Context-Dependent Regulator of Embryonic Stem Cells

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1. Introduction

Although the beneficial effect of certain foods, such as liver, egg or carrot is known from ancient remedies, one of the common active substances, called Vitamin A, was not identified until 1913, when it has been independently discovered by Elmer McCollum at the University of Wisconsin–Madison, and Lafayette Mendel and Thomas Burr Osborne at Yale University. Since then numerous studies have come to light documenting the effect of vitamin A on the health of the individual from birth to adult age. Hale has demonstrated among the first that deprivation of vitamin A during pregnancy induces congenital ocular malformation (Hale, 1933). Wilson and Warkany later described several other congenital malformations that occurred in fetuses from vitamin A-deficient (VAD) rats affecting the genito-urinary tract, heart and great vessels, ocular and respiratory system (Wilson and Warkany 1947; Wilson and Warkany 1950; Warkany 1954).

In 1968 Saunders and Gasseling have shown that grafting a posterior margin zone (called zone of polarizing activity, ZPA) of a chick embryo limb bud to the anterior side is able to induce an extra set of limb structures (Saunders 1968). It suggested that the ZPA region contains a diffusible morphogen. Surprisingly, retinoic acid (RA), a derivative of vitamin A, has been found to have the same effect on the anterior side of the bud (Tickle, Alberts et al. 1982). There was a doubt that retinoic acid is responsible *in vivo* for the phenomenon, but in 1987 Thaller and Eichele demonstrated the graded distribution of endogenous retinoic acid from posterior to anterior in the limb bud (Thaller and Eichele 1987). This was the time when retinoic acid became known as the first morphogen. Part of the truth that later RA was found to act indirectly, via induction of sonic hedgehog (Shh), and actually Shh is the true morphogen signal peptide produced by the ZPA (Riddle, Johnson et al. 1993).

Our understanding on how vitamin A and its derivatives (called retinoids) are able to have such morpho-regulatory effect on the body has dramatically increased in parallel with the evolution of molecular biology methodologies. Development of cloning strategy, establishment of cDNA library made possible the identification of a receptor for the active derivative of vitamin A by the Evans and Chambon laboratories (Giguere, Ong et al. 1987; Petkovich, Brand et al. 1987). This opened the way to clarify the role of retinoids in embryonic development. Several aspects of the retinoid signaling have been described in the last two deacades using genetically modified mice. Excellent reviews are available summarizing these *in vivo* results (Duester 2008; Dolle 2009; Mark, Ghyselinck et al. 2009).

At the same time, *in vivo* system did not make it possible to understand the molecular mechanism in details. Thus, different cell lines were used, including embryonic carcinoma (EC) and embryonic stem (ES) cells. ES cells have the ability to resemble normal embryonic development much closer than any other cell lines and allow one to study the molecular mechanisms in the context of differentiation.
In this chapter we will focus on recent studies using embryonic stem cells as a model system to deconvolute the retinoid signaling in depth. The first section 'Retinoid signaling pathway' will introduce briefly the present accepted molecular mechanism of retinoid action. We will then go through the retinoid field in the context of ES cell pluripotency and in context of early differentiation. Unconventionally, we will discuss a set of genes that were identified in high-throughput methods as potentially retinoic acid regulated genes in ES cells, including many that were previously not implicated in RA regulation. The chapter intends to serve as a starting point to the better orientation in retinoid field and embryonic stem cells.

2. Retinoid signaling pathway

There are two sources of dietary vitamin A. Retinoids are immediately available to the body from intracellular stores. In contrast, precursors (such as carotenoids, mainly found in plants) first must be converted to active forms. The predominant retinoid present in the mammalian fasting circulation is the retinol. Retinol is bound to a 21 kDa retinol-binding protein (RBP4) since it is not soluble in aqueous environments (Noy 1992). Two active derivatives, all-*trans* retinoic acid (atRA) and 13-*cis*-retinoic acid can be also found in the fasting plasma (Horst, Reinhardt et al. 1995), however at much lower concentration (for instance, atRA is 7.3-9 nmol/l in rats) (Cullum and Zile 1985). Retinol is transported into the cytoplasm of the target cell through RBP receptor Stra6 (Kawaguchi, Yu et al. 2007). Cellular retinol binding proteins (CRBPI and II) are able to bind this cytosolic retinol in many cell types, functioning as storage of retinol. For biological effects retinol must be converted into its active forms, and that process involves two steps. Alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs) are catalyzing the first step, the oxidation of retinol to retinaldehyde (retinal). The second step is the oxidation of retinaldehyde to retinoic acid (RA) that is catalyzed by retinaldehyde dehydrogenases (Aldh1a1, Aldh1a2 and Aldh1a3). Retinoic acid is not produced by all cell of the body. Cells without active RA synthesis may have access to it, since it can be also taken up from the environment. RA can chemically exist as several different isomers including atRA, 9-cis-RA, 11-cis-RA, 13-cis-RA, etc, showing some tissue specific distribution. In the cytoplasm RA binds to intracellular retinoic acid binding protein 2 (Crabp2) and delivers to the nucleus where it serves as a ligand for the retinoic acid receptor – retinoid X receptor heterodimer (RAR:RXR) (reviews in (Blomhoff and Blomhoff 2006; Duester 2008; D'Ambrosio, Clugston et al. 2011).
RA acts via the activation of RAR:RXR heterodimer. RAR (RARα, RARβ and RARγ) and RXR (RXRα, RXRβ and RXRγ) are two families of nuclear receptors that bind DNA and directly regulate transcription (review in (Chambon 2005)). RARs and RXRs exhibit the conserved structure of nuclear receptors, including DNA-binding domain (DBD) and C-terminal ligand binding domain (LBD) (review in (Bain, Heneghan et al. 2007)). RARs bind atRA, and the RXR partner bind 9-*cis*-RA. However, 9-*cis*-RA is normally undetectable except when retinol is present in excess (Mic, Molotkov et al. 2003). When RA binds to the RAR partner of RAR:RXR heterodimers it binds to a regulatory DNA element, called RA

response element (RARE) (Umesono, Giguere et al. 1988; Glass 1994). The excess of RA is further oxidized and degraded by Cyp26 enzymes (Cyp26a1, b1 and c1) (Figure 1).

During embryonic development RA acts as a morpho-regulator, forming an anterior-posterior (A–P) concentration gradient (Casci 2008) and cross-talks with other key embryonic signals, especially fibroblast growth factors (FGFs) and sonic hedgehog (Shh) (Niederreither and Dolle 2008). Production by Aldh1a1-a3 and elimination together determine the local concentration of RA. Cells can read their position in the RA gradient (considering the gradient of other morphogenes) and differentiate accordingly to these signals. Knockout of both Aldh1a2 and Cyp26a1, the two key enzymes responsible to determine the local RA concentration, lead to early morphogenetic defects and embryonic lethality, indicating the importance of the precise regulation of RA concentration along the body (Niederreither, Subbarayan et al. 1999). Interestingly, Cyp26a1 expression itself is inducible by RA (Ray, Bain et al. 1997), and has two identified RARE (Loudig, Maclean et al. 2005), suggesting a negative feedback mechanism to control the RA concentration. After all, Cyp26a1 is not expressed posteriorly, where RA is the highest, raising the possibility that Cyp26a1 is controlled by another signals as well. Fibroblast growth factor (FGF) is one of the candidates that can be responsible for RA independent regulation. Accordingly, the elimination of FGF signaling was found to shift the expression of Cyp26a1 posteriorly (Casci 2008).

Present model proposes that unliganded RAR heterodimerizes with RXR and binds to DNA. In the absence of ligand the heterodimer is often complexed with corepressor proteins (such as SMRT, NCoR, etc.) (Nagy, Kao et al. 1997) and inhibits gene expression. Indeed, RA receptors are present not only where RA signaling is active, but also in anterior regions in which RA is fully absent. This is, as we will show later, true for undifferentiated embryonic stem cells, as well. Unliganded RAR:RXR thought to have a repressor function. Such active repression of RAR signaling has important regulatory role, for instance it is required for the proper head formation (Koide, Downes et al. 2001). Ligand binding stimulates a cascade of events resulting the release of corepressor complexes, recruitment of transcriptional coactivators (NCoA, CBP/p300, etc.) and thus initiation of transcription (Figure 1) (Glass and Rosenfeld 2000; Rochette-Egly 2005). Alternatively, ligand dependent repression of target genes is also known (Horlein, Naar et al. 1995).

Strikingly, while in embryonic carcinoma (EC) or HL60 cells RA induces cell cycle arrests and differentiation (Flynn, Miller et al. 1983; Mummery, van Rooijen et al. 1987) in hepatocytes RA promotes cell proliferation (Ledda-Columbano, Pibiri et al. 2004). Cell-type specific expression of coregulators may resolve this contradiction and provide an additional context-dependent level of RA-signaling control. Little is known about the expression of RA coregulators in ES cells and early differentiation. Gudas recently showed an example for the complexity of mechanism that control RA-mediated transcription in fibroblasts versus stem cells. It was found that while RARβ_2 is induced in both cell types by RA; Hoxa1 and Cyp26a1 are induced only in F9 stem cells, but not in fibroblasts. Coactivators and RNA Pol II recruitment are following this pattern. Strikingly, recruitment of Suz12 (a polycomb protein), H3K27me3 (repressive epigenetic modification), H3K9 and K14 acetylation (activation) and methylated CpG islands are just partly able to explain this phenomenon (Kashyap and Gudas 2010). Thus, simple gene expressional studies are not enough to clarify the context-dependent role of coregulators in cell fate commitment. Combination of single cell analysis and genome-wide screen will be required to unexplore this level of regulation. Potential applications and an outlook will be discussed briefly at the end of this chapter, in the “Future directions”.

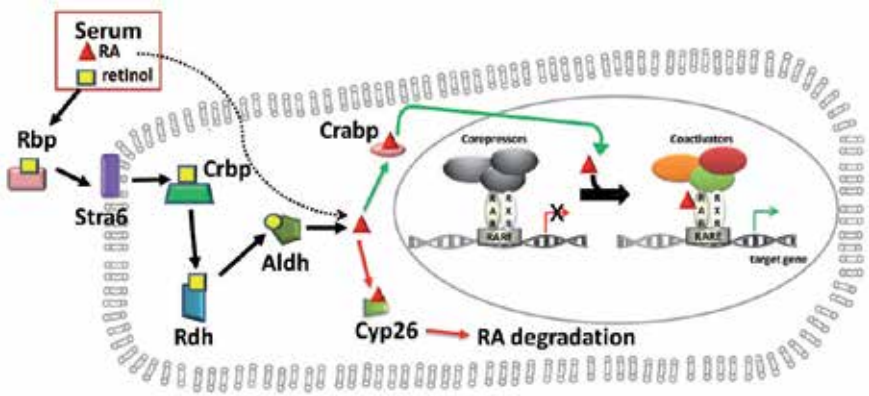

Fig. 1. Overview of retinoid signaling. Retinol is taken up from the serum by the cells via retinol binding protein (Rbp) receptor Stra6. Intracellular retinol is reversibly oxidized to retinal by retinol dehydrogenases (Rdh) and further oxidized to biologically active RA by retinal dehydrogenases (Aldh). Alternatively, cells can take up RA from the environment. Depending on the cell necessities the unwanted RA is eliminated by Cyp26 enzymes or transported into the nucleus and activate the transcription via RAR:RXR. According to the present model, unliganded RAR:RXR bind corepressor complexes while activating ligands replace them to coactivators and promote transcriptional activation.

3. Retinol in ES pluripotency

In standard mammalian cell cultures blood derived fetal bovine serum (FBS) or fetal calf serum (FCS) are widely used in 1-20% to supplement the basal media providing essential factors for the cells. Media for embryonic stem cell culturing contains notable, typically 15% FBS and other factors, provided by the feeder cells. Due to the requirement of these components, ES-cell self-renewal was thought to be dependent on multifactorial stimulations. However, serum-free condition and feeder-independent cultures have been developed in recent years with success. These cultures require addition of Bmp4 and LIF (Williams, Hilton et al. 1988; Qi, Li et al. 2004). LIF can act via activation of Jak-Stat3 signaling pathway, however a complex interplay of different signaling pathways and transcription factors, such as Nanog, Oct3/4, Fgf4, Sox2, etc. are all together responsible for the pluripotency. Recently, it was shown that inhibition of FGF receptor tyrosine kinases and the inhibition of ERK cascade in combination with LIF addition are able to maintain cellular growth capacity and self-renewal of ES cells, providing a ground state to ES cells (Ying, Wray et al. 2008). These studies excluded the requirement of other extrinsic signals, such as FBS content. However, developing embryo is in direct contact with the serum, thus further studies of FBS will help us to better understand its role in embryogenesis.

Certification of defined sera usually does not include the information of its retinol content. According to our data, an ES-qualified serum contains notable retinol (Simandi, Balint et al. 2010). It is questionable whether this high retinol content of serum is playing any role in maintenance of normal phenotype of undifferentiated ES cells. Jaspal S. Khillan and his research group have focused on whether retinol affects any key signaling pathways in ES pluripotency. Interestingly, they found that it can suppress the differentiation of ESCs by up-regulating the expression of Nanog (Chen, Yang et al. 2007). The activation of Nanog by

retinol appears to be independent of previously described LIF/Stat3, Oct3/4-Sox2, bone morphogenic proteins (BMPs), and Wnt/beta-catenin pathways. Importantly, forced constitutive expression of Nanog was shown to sufficiently prevent ES cell differentiation and render self-renewal constitutive even in the presence of active FGF/Erk signaling (references in (Silva and Smith 2008)).

The ability of retinol to influence gene expression and cell fate commitment is thought to make possible by enzymes controlling the conversion steps of the biologically active forms. In other words, only that cell is able to respond to the retinol which is expressing the necessary enzymes and receptors, listed above in section 'Retinoid signaling pathway'. Using global gene expression analyses we found, that only RDHs, Crabp1, RARs (Rarγ, Rarβ) and RXRs (Rxrα, Rxrβ) are expressed unambiguously detectable levels in ES cells. RBPs, ALDHs and Cyp26s are present at very low mRNA levels in undifferentiated embryonic stem cells (Simandi, Balint et al. 2010). These results suggest that endogenous production of RA from the retinol content of serum is unlikely to take place in undifferentiated ES cells. Indeed, it has been demonstrated by HPLC analysis that retinol is not metabolized to RA in ES cells in the presence of LIF (Lane, Chen et al. 1999). Thus, presence of retinol in the serum is not in contrast with the importance of RA in differentiation, since endogenous RA synthesis is not taking place to induce retinoid signaling and differentiation. The fact, that RBP neutralizing antibody fail to prevent retinol-mediated up-regulation of Nanog, suggests a new and independent retinol mediated pathway. Indeed, retinol can activate PI3kinase directly via insulin-like growth factor-1 (IGF-1) receptor/IRS-1 (Chen and Khillan 2010). The exact mechanism by which retinol interacts with IGF-1 receptor is not clear at this stage. As the authors hypothesize, retinol may be required to preserve the integrity of stem cells in early-stage embryos before the lineage restricted differentiation is determined. Verifying this striking mechanism, IGF-1/IGFBP-1 has been already shown to facilitate the establishment of a stem-cell line (Lin, Yen et al. 2003). Further studies confirming the positive effect of retinol on stem cell culture may raise the adaptation of purified retinol in routine stem cell culturing and it may further revise the composition of serum-free media.

However active ligand of RAR is not produced in undifferentiated ES cells, important to note, that as the circulating serum, the FBS may also contain active derivatives of retinol, such as atRA or 13-cis RA in low but detectable concentration. AtRA, although fully ionized in free solution at pH 7.4, is uncharged when within a lipid environment (Noy 1992). Uncharged atRA is able to traverse the membrane and enter the cell (Figure 1) (Chen and Gudas 1996). It was discussed above that even ES cells express RARs and RXRs and Crabp, minimally required to respond for a stimuli. In our study we could detect atRA content of the cell culture serum and we could detect atRA by LC-MS in the cells, suggesting that atRA is taken up by the cells. Furthermore, we found that it has impact on basal gene expression level which could be inhibited by BMS493, an inverse RAR agonist (Simandi, Balint et al. 2010). In conclusion these data suggest that the artificial combination of retinol and RAR antagonist may enhance the quality of ES cultures.

4. Retinoid receptors in undifferentiated ES cells

Even in undifferentiated ES cells Rarγ, Rarβ, Rxrα and Rxrβ are expressed, however their function is not clear at this moment. According to the "unliganded receptor-repression" model their DNA binding influences the expression of adjacent genes. The binding site of

the RAR:RXR heterodimer on the DNA is the so called RA response element (RARE). RARE is consisting two consensus half-sites separated by a variable length of DNA. The sequence of this consensus half-site is most typically 5′-A**GGTCA**-3′ (Balmer and Blomhoff 2005). According to the relative direction of the half-sites, direct repeat, inverted repeat and everted repeat can be distinguished (Honkakoski and Negishi 2000). Type II nuclear receptors, such as RARs and RXRs typically bind to a direct repeat (DR), separated by 1, 2 or 5 nucleotides (called DR1, DR2, DR5, respectively). The RAREs tipically located in target genes, however large number of DR2-type elements are present within Alu repeats (Vansant and Reynolds 1995) (Figure 2 show some of the well known RA target genes and their RAREs).

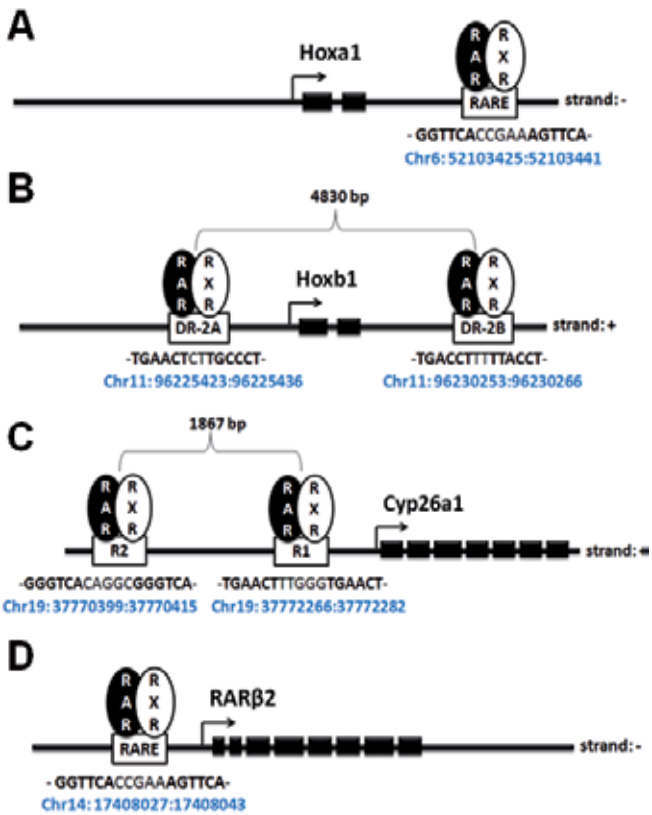

Fig. 2. RARE elements of well-known RA target genes. RAR binding occurs most typically on DR5 (Hoxa1, Cyp26a1, RARβ2) and DR2 (Hoxb1) elements.

Chromatin immunoprecipitation (ChIP) is a powerful and widely applied technique for detecting the association of individual proteins with specific genomic regions. ChIP can be combined with microarray technology to identify the location of specific proteins on a genome-wide basis (called ChIP-chip) (Aparicio, Geisberg et al. 2004). A recent study has performed ChIP-chip on mouse embryonic fibroblasts and ES cells overexpressing tagged Rarγ (Delacroix, Moutier et al. 2010). Rarγ is the dominantly expressed subtype of RAR in ES cells, thus it confirms the biological relevance of this analysis. 354 target loci bound by the RAR were identified in MEFs and 462 loci in undifferentiated ES cells. These sites

obviously do not cover the full repertoire, but as this was the first large-scale study, it served many striking results. First, it was found that only a minority of the 354 MEF loci were present in ES cells, suggesting cell-specific occupancy of RAR binding. This can be an important mechanism contributing to the distinct of retinoid signaling seen in different cell types. Not unexpectedly, in ES cells RAR was found to occupy genes with a wide variety of functions: pluripotency (Oct3/4, Lin28, or Utf1), differentiation (Lefty1, Lefty2), important signaling pathways (Nodal, Notch4, sonic hedgehog pathway components, several FGF), a more systematic discussion of regulated genes will be provided in the next section. In MEF cells RAR occupancy (ChIP-chip data) and RA regulation (microarray) has been analyzed together, showing that only small subset of RAR bound genes were regulated by RA. In undifferentiated ES cells such dataset is still not available. It cannot be completely excluded that formaldehyde cross-linking or overexpression of Rarγ gives its artificial detection by ChIP. However, many of those RAR bound, but not regulated genes were found to be regulated by RA in other cell types, suggesting the good source of data and a highly cell type and context dependent regulation. Moreover, part of the genes, bound by unliganded RAR, showed H3K4me3 (associated with active chromatin) and expression. Data is lacking to show whether there is a partial repression in case of these genes or unliganded RAR binding do not influence their expression at all. Latter would suggest that unliganded RAR silences only small subsets of bounded target genes. This is at least partly in contrast with the previously mentioned "unliganded RAR – corepressors recruitment" model (shown on Figure 1) and confute one at this moment to unambiguously predict which gene and which cell type will respond to the stimuli. In the same study the sequence of RAR-bound loci has been compared. Interestingly, majority of the loci did not contain consensus DR1, -2 or -5 elements, rather compromised one or several anomalously spaced consensus half sites. Strikingly, there was no correlation between the presence/absence of recognizable DR elements and RA regulation. Bioinformatic analyses are considering the sequence diversity of RAR binding sites and the varying distance between the direct repeats only in a limited level. Genome-wide RAR binding studies in different cell types combined with advanced bioinformatic analysis of target loci may help to build a better prediction model and further investigate the biological aspects of different binding sites and the potential role of epigenetic state of these regions. One of such pioneer study (Mahony, Mazzoni et al. 2011) will be discussed in the next section.

5. Retinoic acid induced downregulation of stem cell pluripotency and differentiation induction

The moment that we revoke LIF from the media of undifferentiated ES cells results in the spontaneous differentiation of cells. This can be further induced by allowing them to form aggregates, called embryoid bodies (EB). We have looked for the early changes during the four day embryoid body formation using genome-wide expressional analysis and found ~1000 genes to be regulated. Important genes involved in retinoid signaling, such as Stra6, Aldh1a2, Rarβ, etc. are not among them, only the expression of the RA induced-RA eliminator Cyp26a1 is elevated, suggesting that RA signaling is not activated yet in this stage and the incidental RA level is actively cleared away. This is in correlation with the findings that presence of RA decreases the viability of blastocyst (Huang, Shen et al. 2003) and explain the importance of uterine Cyp26a1 activity in the maintenance of pregnancy, especially during the process of blastocyst implantation (Han, Xia et al. 2010). Potentially, an

RA-independent, FGF signaling controlled Cyp26a1 expression may be responsible for this for RA clearance in day 4 EBs. This explains why Cyp26a1 could upregulate in the presence of RAR inverse agonist BMS493. In contrast, following the post-implantation RA is sorely needed. Absence of RA in this stage cause serious malformations, including axial rotation, shortens along the anterioposterior axis, disturbed limb bud development, finally resulting early embryo death (Niederreither, Subbarayan et al. 1999).

Currently, the effector molecules and cascade signaling of the early retinoid signaling in the vertebrate embryo are barely known. Mechanistic studies showed that RA is not just initiating the differentiation, but also actively decrease the pluripotency via binding to RARE in Oct3/4 promoter (Pikarsky, Sharir et al. 1994) and regulation of LIF-signaling (Tighe and Gudas 2004). Indeed, Oct3/4 is not changing significantly between day 0 and day 4 in our data, but it is actively down-regulated following RA-treatment. In the same time, neural marker DCX becomes upregulated (Simandi, Balint et al. 2010). This suggests clearly the importance of retinoic acid-free (but not retinol-free) environment to the undifferentiated embryonic stem cells. The importance of RA in pluripotency can be studied from a different viewpoint, namely, whether retinoid signaling is able to influence the cell reprogramming. Yamanaka and contributors have shown that introduction of four factors (Oct3/4, C-Myc, Klf4 and Sox-2) into mouse embryonic fibroblast (MEF) cells induce the reprogramming of the differentiated cells and driving back them to embryonic stem cell-like cells, called induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka 2006). A recent study has investigated how different nuclear receptors influence the iPS generation from MEFs. This screen included 19 nuclear receptors, among them Rarα, Rarγ and Rxrα. Surprisingly, none of the overexpressed retinoid receptors influenced significantly the obtained iPS colony numbers (Heng, Feng et al. 2010). Importantly, this study did not use ligands or antagonists to activate (or repress) the overexpressed receptors.

More is known about the ES differentiation promoting effect of RA. Over the years, many RA-regulated differentiation pathways have been discovered first in embryonic carcinoma (EC) and then adopted to ES cells as well. The most studied mouse EC cell lines include F9 cells, which can be induced by RA to differentiate into primitive, parietal, and visceral endodermal cells; and P19 cells, which can differentiate to endodermal and neuronal cells upon RA treatment. ES cells can be induced to differentiate into a number of different cell types; many of which require RA treatment (Soprano, Teets et al. 2007).

To understand in depth how retinoic acid is able to drive a given differentiation pathway, one should identify a set of regulated genes by high-throughput methods. Here, a very important issue is the applicable concentration of retinoic acid. Typically, a regulated gene shows dose dependent effect. From this dose dependent curve one can determine the half maximal effective concentration (EC_{50}) of the given gene. This value may vary in case of other target genes. Thus, it can happen that with the application of lower retinoic acid concentration one will induce only the most responsiveness genes (lower EC_{50}). A solution for this problem can be a high RA concentration that will give the maximal response (saturation). However, this posing is just partly correct. First, in the embryonic development RA grade has very important effect in the anterior-posterior patterning and deep impact on cell fate commitment. This can be true for stem cell differentiation as well. Strikingly, there is no systematic study available using different concentration of RA comparing sets of regulated genes genome-wide. Theoretically, a low concentration of RA results in the expression of “gene A”, a higher concentration will also express “gene B”, which may negatively affect the expression of “gene A” by a negative feedback mechanism.

Limited number of studies demonstrated the dose-dependent effect of RA on a given pathway. For instance, it was shown that higher concentrations of RA induce a dorsal phenotype, while lower concentrations of RA induced a more ventral phenotype during RA induced neural differentiation (Okada, Shimazaki et al. 2004). Not only the concentration, but also the timing of RA signal might be very important. According to the established protocols, different cell types (such as adipocyte, pancreatic cell, neural cells, primordial germ cells, etc.) requires RA signal in various time-points during their differentiation (Bost, Caron et al. 2002; Bibel, Richter et al. 2007; Eguizabal, Shovlin et al. 2009).
The exact molecular events that lead from a pluripotent stem cell to a fully differentiated cell following RA treatment are yet to be determined. Considering the above discussed time- and dose-dependent effect, gene expressional studies can determine the early signaling pathways induced by retinoic acid. We have performed such a study on 4 day-old embryoid bodies treated with 5 μM ATRA for 12 hours and identified 70 annotated genes that were regulated at least 2-fold. At first glance it seems a low number of genes, but another independent study could detect similarly only 96 significantly differently expressed genes (Mahony, Mazzoni et al. 2011). Gene expressional studies cannot clearly classify sets of genes that are directly regulated by RAR. A new method, namely ChIP-Seq, combines chromatin immunoprecipitation (ChIP) with parallel DNA sequencing to identify global binding sites for any protein of interest (Johnson, Mortazavi et al. 2007). A recently published paper investigated the ligand-dependent dynamics of RAR binding during early differentiation using ChIP-Seq. In the absence of RA, ChIP-Seq enrichment could be detected at 1822 sites in embryoid bodies. After 8 hours of exposure to RA this number did not really changed (enrichment at 1924 sites). The most frequent motifs were DR2 and DR5. Confirming the previously discussed ChIP-chip dataset, only 507 sites bound constitutively RAR. This is further query the accepted model of constitutively binding RARs. More strikingly, in the same study they could detect a ligand-dependent shift in RAR's preference from DR0 and DR1 (absence of ligand) to DR5 (presence of ligand).

6. Retinoic acid driven signaling pathways in embryonic stem cells

Vitamin A-deficient and genetic animal models revealed the importance of retinoic acid signaling in embryonic development and organogenesis. In early development, RA participates in the organization of the trunk by providing an instructive signal for posterior neuroectoderm, foregut endoderm and a permissive signal for trunk mesoderm differentiation. At later stages, RA contributes to the development of the eye and other organs (reviewed in (Duester 2008). Presumably, *in vitro* experiments in stem cell cultures are able to reveal the *in vivo* molecular targets of retinoid action during the development. Indeed, we will show hereinafter that genes identified in cell culture experiments match *in vivo* findings. However, one should assume that these *in vitro* cultures are not able to perfectly mimic the spatio-temporal events.
Binding of RA to its receptor induces differentiation mainly via one of the following mechanisms: 1) Initiate changes in interaction of RAR:RXR and co-regulators (SMRT, NCoR, NCoA, CBP/p300, etc.), resulting transcription of primary target genes; 2) Induces expression of transcription factors (Hox, Cdx, etc) and other signaling molecules, resulting transcription of secondary target genes. Regulation of non-coding RNAs may serve as additional level of retinoid action (Rossi, D'Urso et al. 2010); 3) Alters interactions with proteins involved in epigenetic regulation (Kashyap, Gudas et al. 2011). Furthermore, there

are proteins that bind at or near RAREs resulting a more complex mechanism of retinoid action (for review, see (Gudas and Wagner 2011).
In the followings we will discuss the potential role of genes identified in ChIP-chip study of ES cells (Delacroix, Moutier et al. 2010), in day 2 EBs by ChIP-Seq study (Mahony, Mazzoni et al. 2011) or in our microarray study (Simandi, Balint et al. 2010), focusing especially on those which were found in more than one of these studies. In fact, the overlap between the results is remarkable. The results of these experiments provide a list about genes that are primary (ChIP-chip, ChIP-Seq) or secondary response to RA (microarray) considering the technical limitation of such experiments. Based on the published biological functions, we divided arbitrarily some of the genes into functional subgroups. An asterisk (*) mark genes where previously identified or predicted RARE could be identified. Following the gene name, an arrow marks the atRA effect for the expression of the given gene (↑, ↓), up and down, respectively.

6.1 Regulators of RA metabolism

The following genes are well known players in the retinoid metabolism (see Figure 1 and 3). At the same time many of them are involved in the proper eye function, and very likely important in its development. For more details about retinoid signaling in embryonic eye development look for recent reviews in (Duester 2009), (Cvekl and Wang 2009).

Cyp26a1* (cytochrome P450, family 26, subfamily a, polypeptide 1) (↑)

Cyp26 enzymes have been shown to restrict the availability of RA to the transcriptional machinery (review in (Pennimpede, Cameron et al. 2010). HPLC and LC-MS-MS identified 4-OH-RA, 4-oxo-RA and 18-OH-RA and other oxidized products formed as metabolites by Cyp26a1 (Chithalen, Luu et al. 2002). Cyp26a1 expression is directly regulated by RA, and presumably independently regulated by FGF (Casci 2008). Microarray analyses revealed that RA-treated Cyp26a1 $^{-/-}$ ES cells exhibited lower mRNA levels than wild-type ES cells for genes involved in differentiation, particularly in neural and smooth muscle differentiation (Langton and Gudas 2008). One intriguing possible role of Cyp26a1 is to regulate the formation of waves or pulses of RA during the dynamic development of some structures, most notably the hindbrain (Sirbu, Gresh et al. 2005).

Dhrs3 (dehydrogenase/reductase (SDR family) member 3), also known as retinal short-chain dehydrogenase/reductase (retSDR1) (↑)

It has been first described in cone photoreceptors suggesting its possible responsibility for reduction of all-trans-retinal in photoreceptor outer segments that serve as the first step in the regeneration of bleached visual pigments (Haeseleer, Huang et al. 1998). Furthermore, it was found to be expressed also in placenta, lung, liver, kidney, pancreas, and retina; many of these tissues known to actively metabolize retinol. Thus, it suggests that Dhrs3 acts as a generic all-trans-retinol dehydrogenase in many tissues, ensuring that deleterious levels of all-trans-retinal do not accumulate. It was later demonstrated that Dhrs3 is induced by RA in a wide array of cell lines derived from different human tissues and a recent study has shown that Dhrs3 respond oppositely to RA antagonists. Using morpholino knockdown and mRNA over-expression, this study demonstrated that Dhrs3 is required to limit RA levels in the embryo, primarily in the central nervous system (CNS) (Feng, Hernandez et al. 2010). These together suggest that Dhrs3 is an RA-induced feedback inhibitor of RA biosynthesis, and it acts very similarly than Cyp26a1. Whether the RA function is direct or indirect in

Dhrs3 regulation remained to be undetermined. Both ChIP-chip and ChIP-Seq analysis could identify Dhrs3; however typical binding site was not identifiable.

Rarb* (retinoic acid receptor, beta) (↑)

The various RAR subtypes (α, β, γ) are widely but differentially expressed during murine embryonic development, and most tissues express one or more of the subtypes and isoforms during development in different combinations (Dolle 2009). The expression of Rarβ gene is spatially and temporally restricted in certain structures in the developing embryo (segmented brain (r7), hypothalamus, ventricular neuroepithelium, pigmented epithelium, proximal bronchi, myocardium, etc.), suggesting that Rarβ could play specific roles during morphogenesis. In contrast, mice lacking all isoforms of Rarβ develop normally and there is no obvious alteration in the spatial pattern of expression of Hox genes (Luo, Pasceri et al. 1995). These experiments demonstrate that Rarβ is not absolutely required for embryonic development, maybe due to the redundancy in their role among the RAR isoforms.

Rbp1* (retinol binding protein 1, cellular), also known as CRBP1 (↑)

As it was discussed previously, retinol and retinaldehyde in the cytoplasm are associated with cellular retinol-binding proteins (CRBPs). CRBP1 is the most widespread, but it displays some tissue specificity (Dolle, Ruberte et al. 1990). Functional analysis of CRBP1 suggested its role in retinol storage and release where high levels of RA are required for specific morphogenetic processes (Ghyselinck, Bavik et al. 1999). However, CRBP1-deficient mice did not show any morphological defects, did not result altering organogenesis and CRBP1 ablation did not change RA-dependent gene expression, indicating that absence of CRBP1 is not life-threatening, at least under conditions of maternal vitamin A dietary sufficiency (Ghyselinck, Bavik et al. 1999).

Stra6 (stimulated by retinoic acid gene 6) (↑)

Existence of a cell-surface receptor for RBP has been predicted since the mid-1970s. In 2007, Kawaguchi and coworkers could identify Stra6 that acts as a high affinity cell surface receptor for RBP (Kawaguchi, Yu et al. 2007), however Stra6 is not expressed in all retinoid metabolizing tissues. Interestingly, cell culture experiments have shown that Stra6-expressing cells, preloaded with retinol, are able to release more retinol into the culture medium than cells without expresson of Stra6 (Isken, Golczak et al. 2008), suggesting that Stra6 acts as a bidirectional transporter of retinol. Potentially, Stra6 depending on intracellular retinol/retinoic acid concentration, coordinate retinol uptake /removal, thus avoiding cells to take up toxic amount of retinol. Stra6 mutation was found to lead severe congenital abnormalities in humans, including congenital heart defects, mental retardation and lung hypoplasia (Pasutto, Sticht et al. 2007).

6.2 Regulators of body axis patterning

Retinoic acid has been described as a first morphogen (Slack 1987). Indeed, it is responsible for the regulation of several genes implicated in body axis determination. However, the embryonic axis formation is a very complex process, regulated by additional factors, such as Wnt, Shh, Bmp and FGF signaling (Meyers and Martin 1999; Diez del Corral and Storey 2004). Final outcome of the differentiation is the resultant of these signaling pathways. Most of the following genes are well known RA signaling targets, the reason for listing them here is to demonstrate the role of RA in body axis determination.

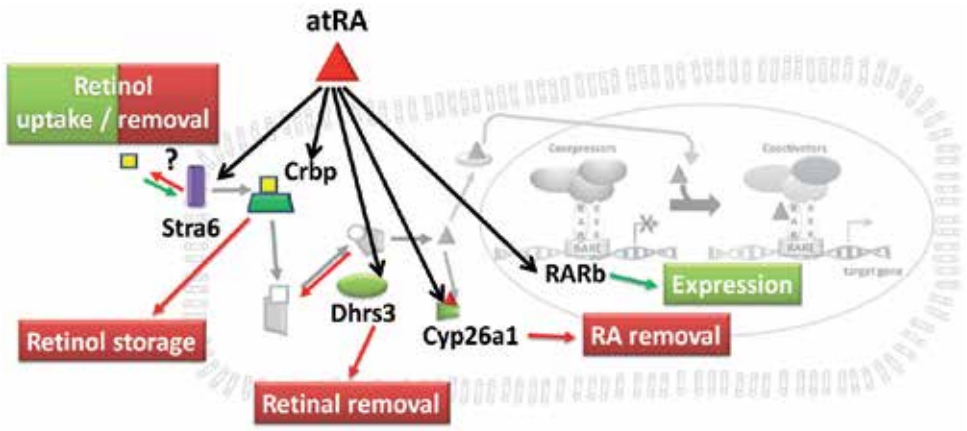

Fig. 3. Effect of all-trans retinoic acid treatment on the retinoid pathway in EBs. Addition of atRA induce its removal via multiple pathway (in red). Stra6 may responsible to eliminate the intracellular retinol, Crbp1 may hold back the retinol from further steps, Dhrs3 participates in retinal removal, while Cyp26a1 can degrade the produced RA. These mechanisms may have a role to protect the blastocyst from the toxic effect of retinoids.

Cdx1* (caudal type homeobox 1) (↑)

The Cdx genes (Cdx1, 2 and 4) are relatives of Hox genes (see below) and believed to derive from a common ProtoHox ancestral cluster (Chourrout, Delsuc et al. 2006). Cdx regulate anterior-posterior (AP) vertebral patterning, at least in part, through direct regulation of Hox gene expression via Cdx-binding sites, which are often found in clusters throughout the Hox cluster (Gaunt, Drage et al. 2008). Prior work has shown that Cdx1 is target of Wnt, RA and FGF signaling, suggesting that Cdx1 is responsible to convey the activity of these signaling molecules to Hox genes. Indeed, RARE and Lef/Tcf-response elements (LRE) has been identified in the proximal promoter of Cdx1. Interestingly, mutation of LRE greatly reduce the induction of Cdx1 by RA, demonstrating a requirement for Wnt signaling in the regulation of this gene by retinoids (Beland, Pilon et al. 2004). Retinoic acid plays a key role in early stages of Cdx1 expression at embryonic day 7.5. Surprisingly, Cdx1 -/- mice are viable and fertile, however showing anterior homeotic transformations of vertebrae (Subramanian, Meyer et al. 1995). These abnormalities are concomitant with posterior shifts of Hox gene expression domains in the somitic mesoderm.
In ES cell derived EBs Cdx1 expression is increasing, peaking between day 2 and day 3 which is the reported window of hemangioblast and blood fate specification (day 3 to day 4 of EB differentiation). Ectopic expression of Cdx1 in differentiating EBs up-regulated Hoxa and Hoxb posterior cluster genes (Hoxa6, Hoxb8, Hoxb9) *(McKinney-Freeman, Lengerke et al. 2008)*.

Hox (homeobox) **genes** (* in the text) (↑)

Hox genes are organized in gene clusters (Hoxa, Hoxb, Hoxc, and Hoxd) and show a strict coordinated expression, reglated by among others Cdx genes. Retinoic acid is not only through the regulation of Cdx, but also directly a well-established regulator of Hox genes along the anterior-posterior axis in higher animals. Genes in the 3′ ends of Hox clusters are induced by RA, while 5' Hox genes are not induced by RA (Langston and Gudas 1992;

Langston, Thompson et al. 1997). Indeed, microarray and ChIP-Seq studies identified several members of the Hox family (Hoxa1*, a2*, a3*, a4, a5*, a10 and Hoxb1*, b2*, b3*, b4*, b5*, b6*, Hoxc4 etc.). Hoxc and Hoxd clusters are not induced upon RA treatment in ES cells and day 2 or day 4 old EBs. Several RARE has been identified in the Hox cluster by ChIP-Seq, many of them have been described in previous publications (Popperl and Featherstone 1993; Dupe, Davenne et al. 1997; Packer, Crotty et al. 1998; Huang, Chen et al. 2002).

Lefty1 (left right determination factor 1) (↑)

A distinctive and essential feature of the vertebrate body is a pronounced left-right asymmetry (review in (Mercola and Levin 2001). Left-right asymmetric signaling molecules in mammals include three transforming growth factor beta (TGFbeta)-related factors, Nodal, Lefty1 and Lefty2 that are responsible for the mirror-image anatomy. They are all expressed on the left half of developing mouse embryos. Nodal acts as a left-side determinant by transducing signals through Smad and FAST and by inducing Pitx2 expression on the left side. Lefty proteins are antagonists that inhibit Nodal signaling (Hamada, Meno et al. 2001). Nodal and Lefty are expressed in the pancreas during embryogenesis and islet regeneration. *In vitro* studies demonstrated that Nodal inhibits, whereas Lefty enhances, the proliferation of a pancreatic cell line (Zhang, Sterling et al. 2008).

Meis1 and **Meis2*** (myeloid ecotropic viral integration site) (↑)

Homeodomain proteins of the Meis subfamily are expressed dynamically in several organs during embryogenesis and exert potent regulatory activity through their interaction with Hox proteins and other transcription factors. Meis1-deficient embryos have partially duplicated retinas and smaller lenses than normal. They also fail to produce megakaryocytes, display extensive hemorrhaging, and die by embryonic day 14.5 (Hisa, Spence et al. 2004). In addition, Meis1-deficient embryos lack well-formed capillaries, although larger blood vessels are normal. Definitive myeloerythroid lineages are present in the mutant embryos, but the total numbers of colony-forming cells are dramatically reduced (Azcoitia, Aracil et al. 2005).

Pbx2* (pre B-cell leukemia transcription factor 2) ((-), see in the text)

It has been found that Pbx2 -/- embryos are born at the expected Mendelian frequencies and exhibit no detectable abnormalities in development and organogenesis (Selleri, DiMartino et al. 2004), however they show limb abnormalities (Capellini, Di Giacomo et al. 2006). Supporting its role in this phenomenon, its elevated expression in limb buds after RA treatment has been demonstrated. Retinoic acid response element in the promoter of Pbx2 has been identified in both ChIP-chip and ChIP-Seq study, even without ligand activation. However it seems to be not regulated by atRA in ES cells or EBs according to the microarray data, suggesting a more context dependent regulation of this gene by RA. As Pbx proteins have been shown to dimerize with Hox proteins and act as a cofactor, we included here (Moens and Selleri 2006). Pbx2 is believed to be involved in the control of proximodistal axis formation in mouse (Capellini, Zewdu et al. 2008).

Tshz1 (teashirt zinc finger family member 1) (↑)

Tshz1 is detected from E9.5 in the somites, and also present in the spinal cord, limb buds and branchial arches. Tshz1 -/- mice exhibit Hox-like vertebral malformations and homeotic transformations in the cervical and thoracic regions, suggesting that Tshz1 and Hox genes are involved in common pathways to control skeletal morphogenesis (Core, Caubit et al.

2007). Previous studies suggested Tshz1 as an RA regulated gene, however direct evidence was not shown yet.

6.3 Testis development, spermatogenesis, female fertility

Generation of putative primordial germ cells (PGCs) has been reported during the differentiation of mouse and human ESCs in *in vitro* systems using RA (reviewed in (Zhou, Meng et al. 2010)). *In vivo* PGCs come from the proximal epiblast and migrate subsequently into the mesoderm, the endoderm and the posterior of the yolk sac (Bendel-Stenzel, Anderson et al. 1998). The mechanisms responsible for the pluripotent epiblast cells to become PGCs involve bone morphogenetic protein 2 (Bmp2), Bmp4, Bmp8b, Prdm1 and Prdm14 (Ying and Zhao 2001; Edson, Nagaraja et al. 2009). Migration of PGCs requires interferon-induced transmembrane proteins 1 and 3, Kit ligand and Cxcl12 (Molyneaux, Zinszner et al. 2003). Germ cells continue to proliferate by mitosis upon arriving in the genital ridge. Spermatogonial lineage does not enter meiosis before puberty, and the proliferation is inhibited by androgens. In contrast, germ cells in the female gonad continue to proliferate by mitosis and enter meiosis. These are raising the evidence of testicular meiosis-preventing and ovarian meiosis-inducing factors. Initiation of meiosis in the fetal ovary has been suggested to require retinoic acid (Bowles, Knight et al. 2006). Germ cells fail to enter meiosis and remain undifferentiated in embryonic vitamin A-deficient ovaries, suggesting that retinol regulates the initiation of meiosis I (Li and Clagett-Dame 2009). Another study revealed, that RA-degrading enzyme Cyp26b1 in fetal testis is responsible to delay meiosis until postnatal development (Koubova, Menke et al. 2006). However, RA acts too widely in mammalian development to account, by itself, for the cell-type and temporal specificity of meiotic initiation. The following RA regulated genes have been found related to germ cell development; many of them only partly characterized yet.

Agpat3* (1-acylglycerol-3-phosphate O-acyltransferase 3) also known LPAAT3 (↑)

Agpat3 was shown to have both lysophosphatidic acid acyltransferase (LPAAT) and lysophosphatidylinositol acyltransferase (LPIAT) activities. It is mainly expressed in the liver, kidney, and testis. Present studies suggest its role in PI (phosphatidylinositol) production of the testis. Interestingly, expression of Agpat3 in the testis is enhanced significantly in an age-dependent manner (Yuki, Shindou et al. 2009).

Nrip1 (Nuclear receptor interacting protein 1) also known as RIP140 (↑)

A nuclear protein that specifically interacts with the hormone-dependent activation domain AF2 of nuclear receptors. Mice null for Nrip1 gene are viable, but females are infertile because of complete failure of mature follicles to release oocytes at ovulation stage. Heterozygous females are only partially affected (White, Leonardsson et al. 2000).

Rec8* (REC8 homolog (yeast)) (↑)

Gene expression of Rec8 is strictly confined to spermatocytes and spermatids in male mouse and oocytes in female mouse. Restricted expression pattern of Rec8 mRNA implies its essential role in meiosis in both sexes of mammals (Lee, Yokota et al. 2002). It was found that Rec8 is a key component of the meiotic cohesin complex. During meiosis, cohesin is required for the establishment and maintenance of sister-chromatid cohesion, for the formation of the synaptonemal complex, and for recombination between homologous chromosomes. Importantly, Rec8 -/- mice are born in sub-Mendelian frequencies and both sexes have germ cell failure causing sterility (Xu, Beasley et al. 2005).

Stra8* (stimulated by retinoic acid 8) (↑)

Encodes a protein that is crucial for mammalian germ cells to enter into pre-meiotic stages. Microarray analysis of whole murine embryonic ovary and postnatal testis time course data revealed a single peak of Stra8 expression in each organ at the onset of meiosis; at E14.5 in the ovary and 10 days postpartum in the testis (Hogarth, Mitchell et al. 2011). Stra8 is specifically expressed in mammalian germ cells before their transition from mitosis to meiosis and its expression is observed only in the postnatal testis. Stra8 associates with DNA and possesses transcriptional activity (Tedesco, La Sala et al. 2009). Stra8 mRNA and protein were induced in cells treated by all-trans and 9-cis retinoic acids in P19 embryonal carcinoma cells (Oulad-Abdelghani, Bouillet et al. 1996), however using Aldh1a2 -/- mice (lacking RA synthesis) it was found that Stra8 expression is detectable even in the absence of RA (Kumar, Chatzi et al. 2011).
The following genes are also regulated by atRA and could be detected with ChIP-chip or ChIP-Seq. They are related to fertility, testis or germ cells, but due to the limited available data, they are not discussed here in details: **Wdr40b** (↑), **Nr0b1** (↑), **Cxcl12** (↑), **Kit** (↑), **Tcp11** (↑).

6.4 Neuroectodermal cell fate commitment

Other chapters of the book prominently construe with the neural differentiation of stem cells. Present protocols are widely using RA to induce the neural differentiation of stem cells; however the exact mechanisms remained largely unknown yet. The fact that endogenous RA is starting to synthetize in mouse embryo after forebrain and midbrain neuroectoderm induction is raising the possibility that RA is actually not required for neural induction (Duester 2008). However, RA induced Hoxa1 activity is clearly essential for the neuronal differentiation (Martinez-Ceballos and Gudas 2008).
The following genes were found to be related to the neural differentiation or functioning. They may have role in the neural specification only. Some of them are putatively involved in the eye development as well.

Ankrd43* (ankyrin repeat domain 43) (↑)

Very limited information is available for this gene. It is among those seven genes which were found to be differentially expressed in progenitors in the lateral and medial ganglionic eminences (LGE and MGE). LGE progenitors produce striatal projection neurons and olfactory bulb interneurons, whereas MGE as well as caudal ganglionic eminence (CGE) progenitors produce cortical and hippocampal interneurons. Thus, its RA regulation suggests the role of RA in neural specialization (Tucker, Segall et al. 2008).

Glra2 (glycine receptor chloride channel, alpha 2 subunit) (↑)

This gene mediates inhibitory neurotransmission in the spinal cord, brainstem and retina. During development, Glra2 is expressed in the retina, in the spinal cord, and throughout the brain. Mice with a targeted deletion of Glra2 show no gross morphological or molecular alterations in the nervous system. (Young-Pearse, Ivic et al. 2006).

Gpr124 (G protein-coupled receptor 124) (↑)

GPR124 is highly expressed in central nervous system (CNS) endothelium. Its deletion resulted embryonic lethality through CNS-specific angiogenesis arrest in forebrain and neural tube. Conversely, GPR124 overexpression throughout all adult vascular beds produced CNS-specific hyperproliferative vascular malformations (Kuhnert, Mancuso et al. 2010).

6.5 Adipocyte differentiation

Adipogenesis is a complex process by a multifaceted transcriptional regulatory cascade. In recent years data have emerged indicating a role of retinoids in adipose tissue. Retinoids has been shown to inhibit preadipocyte to adipocyte differentiation by repressing Pparγ and Rxrα activities (Ziouzenkova, Orasanu et al. 2007), however we should note that the embryonic stem cell derived adipogenesis requires RA in the very beginning (Bost, Caron et al. 2002). The role of such early RA signal in adipocyte differentiation remained largely unknown.

Cidea (cell death-inducing DNA fragmentation factor, alpha subunit-like effector A) (↑)

Cidea-null mice are lean and resistant to diet-induced obesity and diabetes, indicating a role for Cidea in energy balance and adiposity (Zhou, Yon Toh et al. 2003). Thus, Cidea can be a potential RA regulated gene in ES cell that influence the adipocyte differentiation.

Nrip1 (discussed also in "Regulators of RA metabolism" section) (↑)

Knockout male and female mice are smaller than wild-type littermates. Nrip1-null cells show elevated energy expenditure and express high levels of the uncoupling protein 1 gene (Ucp1), carnitine palmitoyltransferase 1b, and the cell-death-inducing DFF45-like effector A (Cidea) (Christian, Kiskinis et al. 2005).

Rbp1* (retinol binding protein 1, cellular), also known as CRBP1 (discussed also in "Regulators of RA metabolism" section) (↑)

Interestingly, a recent study showed that CRBP1-deficient mice fed with high-fat diet (HFD) lead to increased adiposity. Similarly, suppression of CRBP-I expression *in vitro* enhanced adipocyte differentiation (Zizola, Frey et al. 2010).

Rarb* (discussed also in "Regulators of RA metabolism" section) (↑)

Rarβ activation has been shown that both sufficient and necessary to trigger commitment of mES cells to adipocytes (Monteiro, Wdziekonski et al. 2009).

7. Future directions

Microarray completed with ChIP-chip or ChIP-Seq is useful to determine sets of RA regulated genes and map the binding sites of RAR:RXR heterodimer. We have shown that RA treatment of ES cells or EBs induces expression of genes involved in retinoid metabolism, body-axis regulation, gonad development and neural differentiation. Other recently introduced methods, such as RNA-Seq (Wang, Gerstein et al. 2009) and Gro-Seq (Core, Waterfall et al. 2008), still have not been used in retinoid research, and may serve more detailed picture about the direct effect of RA (detailed description of genome-wide approaches in aspect of stem cell research is reviewed in (Zhang and Huang 2010). Future experiments should be complemented with studies investigating the co-occupancy of RAR, RXR, RNA Pol II and coregulators (SMRT, NCoR, NCoA, CBP/p300, etc.) genome-wide to get information about the direct gene regulation and its temporal dynamics. Development of data processing methods and integration of system biology will be also required for analyzing the complexity underlying the regulatory networks in stem cells and during cell fate commitment (Macarthur, Ma'ayan et al. 2009).

There are other technical challenges limiting our recent possibilities. Several cell-type specific differentiation protocol has been published, many of them resulting heterogeneous cell population. This is mainly due to our poor understanding on interactions of different signal cascades. Uncontrolled content of FBS, heterogeneity during EB formation, not adequate

ligand treatment may also affect the quality of cell culture. For instance, embryonic stem cell derived embryoid bodies often show heterogeneity in their size that may have impact on cell fate commitment (Mansergh, Daly et al. 2009). Adherent monolayer cultures have been used to get more homogenous differentiation. It has been successfully used for neural differentiation (Pachernik, Esner et al. 2002), however many of the lineage differentiation is still require the EB formation, implying the importance of a three-dimensional structure. Replacement of EBs by scaffolds can provide such three-dimensional environment in which cells have access to nutrients and space to grow. Recent studies are using scaffolds to induce high organization of stem cells during differentiation. For instance, one of the most recent study reported the dynamic, autonomous formation of the optic cup structure from a three-dimensional culture of mouse embryonic stem cell aggregates (Eiraku, Takata et al. 2011).

During development, morphogens are secreted locally and presented to embryonic cells in a spatially and temporally controlled manner to direct appropriate differentiation and tissue formation. This seems to be true for RA production as well. *In vitro* addition of RA to the media of differentiating EBs does not accurately replicate this process. In addition, the diffusion of RA into EBs may be restricted by the formation of an exterior shell composed of collagenous matrix and tight E-cadherin mediated cell–cell adhesions at the EB surface. Biodegradable microspheres to deliver morphogens directly within EBs may enable production of more homogeneous populations of differentiated cells (Bratt-Leal, Carpenedo et al. 2010). A study examined ESC differentiation in response to microsphere-mediated delivery of RA (Li, Davidovich et al. 2011). The authors found that after 10 days of differentiation, the RA microsphere-containing EBs formed large cystic structures that comprised the majority of the EBs. Genome-wide analysis revealed more pronounced up-regulation in visceral endoderm, epiblast, and early primitive streak markers and down-regulation in mesoderm and definitive endoderm differentiation. Thus, in the existing stem cell differentiation protocols we need to revisit the usefulness of cytokine/ligand delivering microspheres.

Finally, important to note that RT-PCR, western blot or even microarray analysis are able to reveal only the average response of the cell population to a given signal (Figure 4). This is clearly giving only limited information. Single cell analyzing methods will bring us closer to understand the dynamics of cell fate commitment. This field is expected to accelerate soon and will change recently used methodologies.

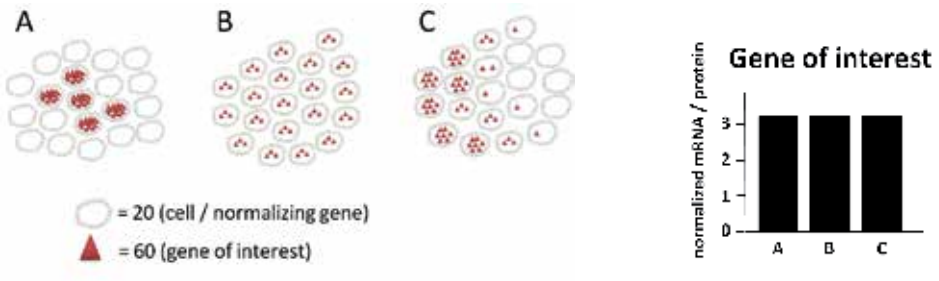

Fig. 4. Population measurements can obscure the heterogeneity of the cells within the embryoid body. For instance, western blot or RT-PCR are not able to determine whether only a few cell are expressing the gene of interest on high level (A), every cell on middle level (B) or there is a polarity (C).

8. Conclusions

Embryonic cells are widely used model system to understand the early steps of embryonic development. Over the last years, many RA-regulated pathways have been discovered in EC and ES cells using a diverse set of techniques. In this chapter we have summarized the results of recent studies using high-throughput approaches (microarray, ChIP-chip, ChIP-Seq) to understand the mechanistic of retinoid signaling in early embryonic development. However, many questions remained to be unanswered. We should face the new technical challenges to be able to work out these issues.

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Part 2

Neural and Retinal Differentiation

4

Pluripotent Stem Cells as an *In Vitro* Model of Neuronal Differentiation

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1. Introduction

Embryonic stem (ES) cells are derived directly from inner cell mass (ICM) of mouse or human preimplantation embryos (Evans & Kaufman 1981, Martin 1981, Thomson et al., 1998). They are pluripotent, once they are able to differentiate *in vitro* and *in vivo* into derivatives of the three embryonic germ cell lines: mesoderm, endoderm and ectoderm (Fig. 1). The establishment of protocols for direct *in vitro* differentiation of pluripotent stem cell (PSC) into desirable cell type is extremely important for their use in therapies, for the studies of human diseases, and also for biochemical, toxicological and pharmacological studies (Pederson 1999, Sukoyan et al., 2002, Wobus & Löser 2011). Therefore, focusing on the PSCs use in these studies, many efforts have been devoted to the establishment of stem cells models with a particular emphasis to their *in vitro* differentiation into mature and functional neurons.

The nervous system is the most complex system in the organism and its formation usually involves four stages: specification of the neural cells identity (neural or glia), neural migration and axon formation, synapse formation (with target neurons, muscle or gland cells) and synaptic connection refinement (elimination of axons branches and cells death) (Müller 2006). It is well-known that many genes are involved in the process of neuronal stem cells fate specification (Aiba et al., 2006). This process depends on the specific environment during organogenesis, after birth and during adult life. The temporal and spatial factors are essential for neuronal differentiation, due to the multilayer nature of cortex (Müller 2006). Although numerous publications have reported PSCs differentiation toward neurons, many important questions are not answered yet, especially in respect to the equivalency of the *in vitro* PSCs model and *in vivo* central nervous system (CNS) development. Accomplishments in these directions would represent a crucial starting point for the stem cell therapies and drug discovery. A number of important protocols have been set up for the differentiation of PSCs into neurons, which mainly lead to the coexistence in the culture of differentiated neurons and non-neural cells, together with neural precursors

and undifferentiated PSCs (Okabe et al., 1996, Li et al., 1998, Mujtaba et al., 1999, Baharvand et al., 2007). Most of these protocols are short-lasting, which therefore does not allow a careful analysis of the neurons maturation, aging, and death processes.
In this chapter, we describe principal methods of PSCs differentiation into neurons *in vitro*. Next, we present a method developed by our group, which established a long-term culture of committed neuronal precursors and functional neurons from mouse embryonic stem (mES) cells. In addition, using this long-term protocol we demonstrated the temporal and spatial localization of microtubule-associated proteins, such as, Lis1 (Lissencephaly-1) and Ndel1 (nuclear distribution element-like) in neuronal precursors and differentiated neurons. These both proteins have been shown to be essential for neuronal differentiation during the CNS development. Regardless of the relevance of these proteins for neuronal differentiation, their expression during PSCs differentiation was marginally explored.

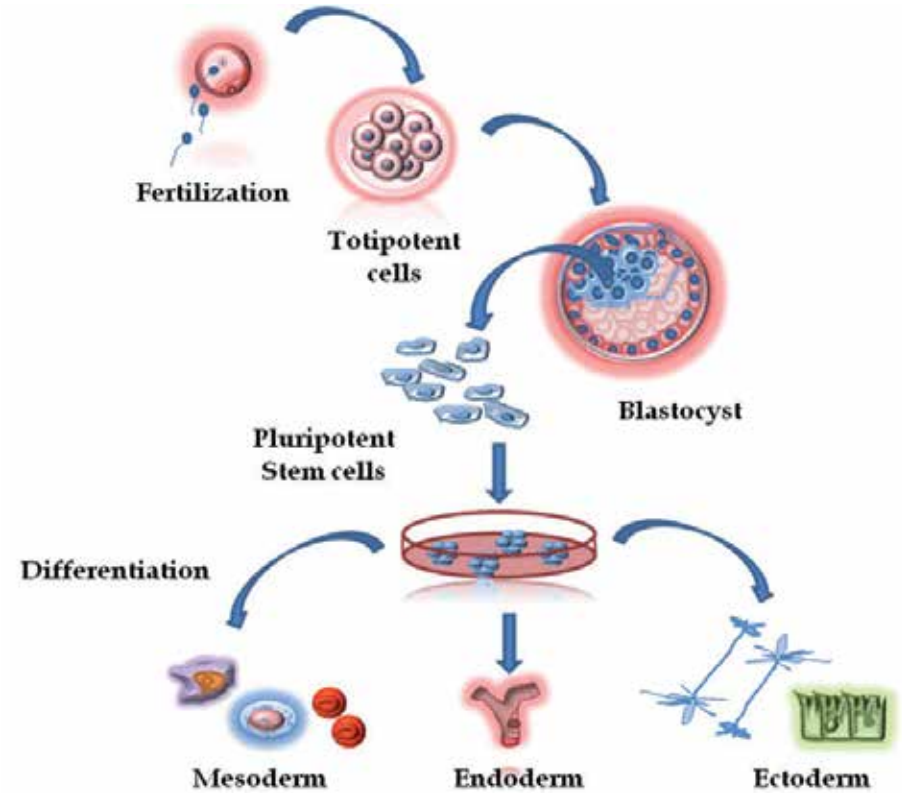

Fig. 1. ES cell isolation and differentiation.

2. Pluripotent stem cells as a model of *in vitro* differentiation

ES cells are powerful biological model, which can provide important information for our knowledge regarding the cell commitment and differentiation during development process (O'Shea 1999, Wobus & Boheler 2005). Multiple methods have been developed in order to induce *in vitro* PSCs differentiation and to obtain the desirable cell phenotype (Baharvand et al., 2004, Keller 2005). It has been found that ES cells are able to differentiate spontaneously

within cell aggregates, when feeder layers and required factors to maintain pluripotency are removed. These aggregates, denominated embryoid bodies (EBs), resemble early post implantation embryos, although chaotically organized inside. It is assumed that EBs formation initiates spontaneous differentiation of ES cells to the three embryonic germ layers (Evans & Kaufman 1981). Innumerous studies have addressed the issue of cell specific differentiation of ES cells. In Figure 2 we summarize the main cell phenotypes, which can be induced to differentiate from ES cells *in vitro* under specific culture conditions. Those accomplishments are the result of a dynamic interaction between knowledge of embryonic development and empirical testing, targeted at reproducing *in vitro* cell specification conditions found in the developing embryo.

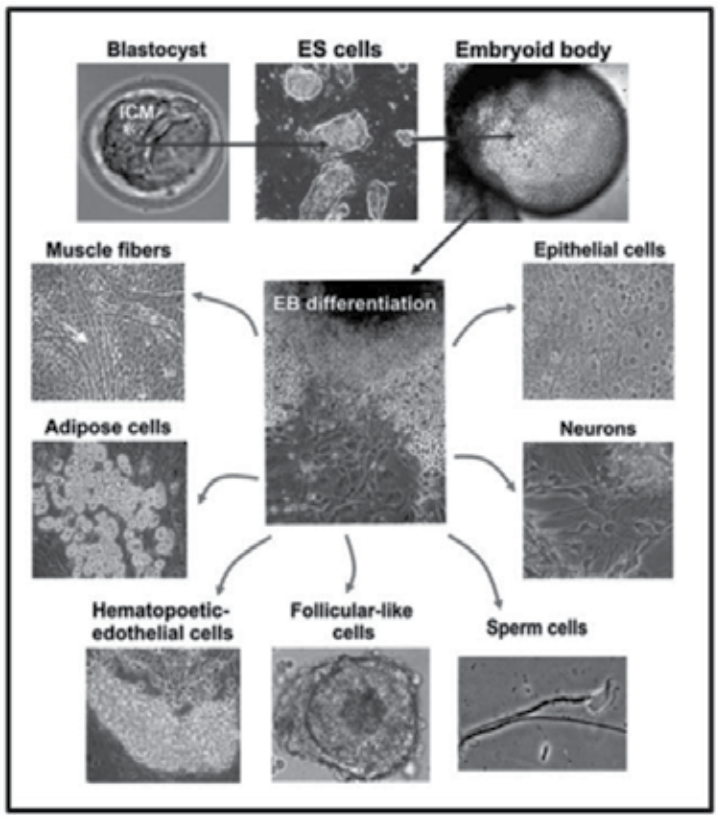

Fig. 2. ES cell differentiation *in vitro*. ES cells are isolated from the ICM of the blastocyst. These cells can be induced to form EBs, which are structures that contain representatives of the three embryonic germ layers. Under the appropriate culture condition, the EBs can be induced to differentiate into several types of cells *in vitro*.

2.1 Mesoderm specification

Mesoderm is the germ layer responsible for the development of muscle, bone, cartilage, blood, and connective tissue. Blood and endothelial cells are the first cell types to form in the developing vertebrate embryo at around six days of gestation. This event leads to the formation of the yolk sac, an extraembryonic membrane composed of adjacent mesodermal

and primitive (visceral) endodermal cell layers, which give rise to blood and endothelial cells (Baron 2001). *In vitro* differentiation of ES cells in EBs allows the generation of blood islands containing erythrocytes and macrophages (Doetschman et al., 1985), whereas differentiation in semisolid medium is efficient for the formation of neutrophilis, macrophages, and erythroid lineages (Wiles & Keller 1991). In an attempt to identify potential inducers of the hematopoietic lineage, researchers indicated Wnt3 (Proto-oncogene protein) as an important signaling molecule that plays a significant role in enhancing hematopoietic commitment during *in vitro* differentiation of ES cells (Lako et al., 2001). The hematopoietic cells derived from ES cells have been characterized by specific gene expression patterns and by cell surface antigens (Wiles & Keller 1991, Wang et al., 1992). However, the most important aspect was to characterize these cells in the functional capacity, by demonstrating long-term multilineage hematopoietic repopulating properties in an animal model (Palacios et al., 1995).

Cardiomyocytes readily differentiate from aggregates composed of mES cells in the presence of high concentration of serum (around 20%), and display properties comparable to those observed *in vivo*: they express similar cardiac gene expression patterns, present sarcomeric structures, and demonstrate contractility triggered by cardiac-specific ion currents, as well as the expression of membrane-bound ion channels. This type of differentiation can develop spontaneously or be induced by differentiation factors including dimethyl sulfoxide (DMSO) and retinoic acid (RA), or small molecules, such as Dynorphin B and cardiogenol derivatives (Fassler et al., 1996). Human ES cells also hold the ability to differentiate into cardiomyocytes, which show similar properties to those derived from mES cells (Kehat et al., 2001). Furthermore, it is well established that ES cells can efficiently differentiate into several other mesodermal cells types, including mesenchymal cell-derived adipogenic (Dani et al., 1997), chondrogenic (Kramer et al., 2000), osteoblast (Buttery et al., 2001), and myogenic cells (Rohwedel et al., 1994). In all of these experiments, the cell type derivation was induced by specific differentiation factors.

2.2 Endoderm specification

Endoderm is responsible for deriving the pancreas and liver. Regarding the therapeutic interest for the treatment of hepatic failure and diabetes mellitus, hepatic and pancreatic cells are of special interest. Thus, since these cells could be derived from ES cells new hope has emerged (Soria 2001). These *in vitro* derived cells showed hepatic-restricted transcripts and proteins, and were able to integrate and to function in a host liver following transplantation (Chinzei et al., 2002). Recently, researchers demonstrated that hepatocyte-like endodermal markers were also detected in ES cell derivatives (Yamada et al., 2002).

The potential use of ES cells for treatment of diabetes was enhanced by the perspective of deriving insulin-producing pancreatic endocrine cells. Researchers at NovoCell, Inc., a biotechnology company in the USA, have developed an *in vitro* differentiation process that mimics pancreatic organogenesis. By directing cells through stages resembling definitive endoderm, gut-tube endoderm, pancreatic endoderm and endocrine precursor, they were able to convert human ES cells to endocrine cells capable of synthesizing the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin (D'Amour et al., 2006). Moreover, in pre-clinical trials, the same group showed that those ES-derived pancreatic cells efficiently generated glucose-responsive endocrine cells after implantation into mice, and those insulin producing cells, in turn protected animals from streptozotocin-induced hyperglycemia (Kroon et al., 2008).

2.3 Ectoderm specification

The embryonic ectoderm is an embryonic germ layer, which can produce various cell lineages during development. Of particular interest, the differentiation of ES cells into neuronal cells was published independently by three groups in 1995 (Bain et al., 1995, Fraichard et al., 1995, Strubing et al., 1995). Gene expression and electrophysiological studies of cell derivatives from PSCs indicated the presence of the all three major cell types of the brain: astrocytes, oligodendrocytes, and neurons (dopaminergic, GABAergic (gamma-aminobutyric-acid-releasing), serotonergic, glutamatergic and cholinergic neurons) (Lee et al., 2000, Rolletschek at al., 2001, Aubert et al., 2002) (Fig. 3). Thus, these studies opened first perspectives regarding ES cell models for the study of neurodegenerative disorders. Human ES cells are also able to generate the neural epithelium (Thomson et al., 1998, Reubinoff et al., 2000, Zhang et al., 2001). However, although neural progenitors derived from ES cells could be enriched and directed to differentiate into mature neurons, astrocytes, and oligodendrocytes (Carpenter et al., 2001), experimental data obtained until recently could not demonstrate the formation of a given neuron subtype (Lee et al., 2000, Rolletschek at al., 2001, Aubert et al., 2002). The possibility of generating neurons *in vitro* signals for a first step towards exploring the therapeutic potential of ES cells for Parkinson's disease (Svendsen, 2008).

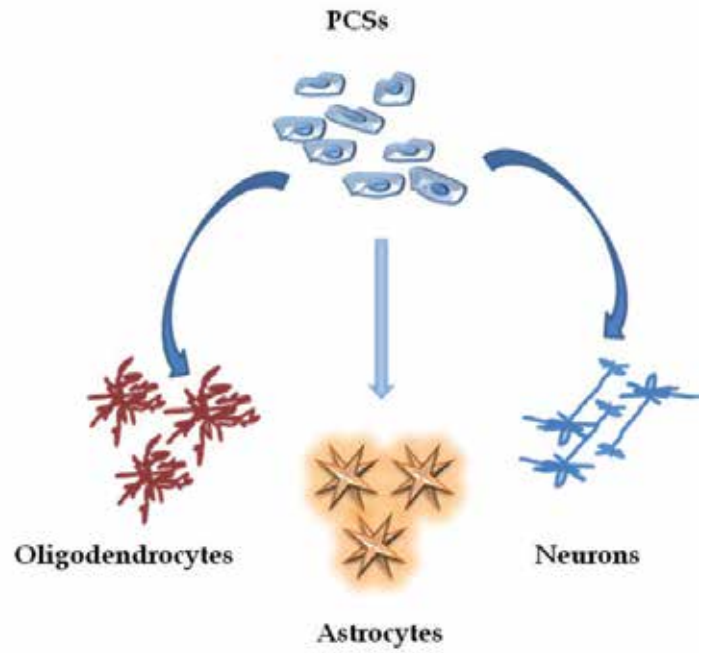

Fig. 3. All three major cell types of CNS derivative from PSCs: oligodendrocytes, astrocytes, and neurons.

2.3.1 Three dimensional (3-D) model of PSCs differentiation

Currently, common protocol comprises three steps: EBs (3-D model) formation, derivation of primitive neuroepithelial cells from EBs and generation of differentiated neural cell types. The most widely used method to induce neuronal differentiation is to enzymatically or mechanically lift the PSCs colonies and place them into low-adherence culture dishes or flasks

without substrate, feeder cells, or mitogens, where they form EBs (Carpenter et al., 2001, Colombo et al., 2006, Baharvand et al., 2007). The culture media formulations for EBs vary significantly between different works (Ng et al., 2005, Yoon et al., 2006). Next, EBs are transferred to serum-free culture media and are plated onto laminin (poly-lysine)-coated dishes in order to generate an adherent culture and to differentiate into neuroepithelial cells. The EBs undergo spontaneous differentiation and the formation of clusters of small elongated cells surrounding a central zone, free of cells, so-called neural rosettes was showed (Pankratz et al., 2007, Pankratz & Zhang 2007). These rosettes resemble the morphology of the primitive neural tube and express early neural marker antigens such as Nestin (type VI intermediate filament (IF) protein) and Musashi-1 (RNA-binding proteins expressed in the CNS), but not markers of more mature neural cells. These rosettes were observed in majority of the studies of induction of neural differentiation of PSCs. PSCs neuroepithelial differentiation method are widely used to generate neural progenitors and mature neural cell types. The neuroepithelial cells obtained by this approach express the neuroepithelial transcription factors, such as PAX6 (Paired box gene 6), Sox1 (Sex determining region Y-box 1), and Sox2 (sex determining region Y-box 2), in about 90% of the total differentiated progenies (Li et al., 2007, Pankratz & Zhang 2007). Neuronal differentiation can also occur without EBs formation. In this case, specific growth factors or the co-culture of PSCs with cells of a particular origin that have been found to produce factors of neuronal cell specification are used to accelerate the differentiation towards one cell type or lineage of interest.

RA is an important regulator of the nervous system development, regeneration and maintenance (Zhang 2006, Maden 2007). Although, rosette formation occurrs during spontaneous *in vitro* differentiation of PSC-derived EBs, addition of RA enhances significantly the yielding of rosettes and mature neurons. Therefore, the predominant number of studies uses RA alone or in combination with other factors, e.g. bFGF (*basic fibroblast growth factor*). Additionally, neural differentiation can also be induced by the withdrawal of bFGF/EGF (*epithelial growth factor*) and exposure to BDNF (*brain-derived neurotrophic factor*), NGF (*neuronal growth factor*) or other factors into the culture medium.

The above described protocol and its modifications commonly produce mixed population of neuronal cells, which contain precursors, neurons and glial cells. This mixed population needs the application of further protocols for selection and enrichment, in order to obtain almost pure population of precursors or neurons, suitable for pharmacological screening or therapeutic applications.

2.3.2 Bi-dimensional (2-D) model of PSCs differentiation

Primary neural stem cells (NSC) can proliferate *in vitro,* forming multicellular floating spherical clusters, commonly referred as neurospheres, which are mainly composed by committed progenitor cells. When adhered on substrate, these neurospheres differentiate into functional neurons (Reynolds & Weiss, 1992; Chojnacki & Weiss, 2008). Our group aimed at developing a protocol for PSCs differentiation into neurons, which resemble the differentiation pattern of NSC-derived adherent neurosphere (AN). This protocol comprises five steps: EBs formation, culturing of floating EBs in the presence of RA, EBs adherence, formation of AN (2-D model), composed by commuted neuronal precursors and generation of neurons from AN. We further referred adherent EBs as ANs. It is worth mentioning that this protocol avoids the formation of rosettes.

The details of 2-D protocol are presented in Figure 4. An enzymatic digestion with trypsin of mES cells were used in order to obtain a feeder-free cell suspension. The mES cells were

plated in culture flask, which allows rapid adherence of feeder cells. The EBs were obtained in low serum (5%) basal culture medium, following routine protocol of hanging drop method. Next, EBs were transferred into low-adherence culture dishes without substrate that allows adherence, and the neuronal differentiation was induced by the addition of RA (at final concentration of 0.1 μM). The EBs were maintained under non-adherent serum-free culture conditions (neurobasal (NB) medium supplemented with B27), for additional 4 days. Next, RA was removed and the EBs was transferred to poly-lysine treated plastic dishes in order to form ANs. The ANs were maintained in serum-free conditions for additional 7 days. At this moment of neuronal differentiation, outgrowth of neuron-like cells on the periphery of ANs was clearly observed (Fig. 5). These ANs were caught in small pieces and mechanically transferred into another Petri dish. After 3-4 days, these small ANs start to produce outgrowth of neurons. This process of ANs mechanical splitting and transfer can be repeated several times continuously producing ANs and neurons.

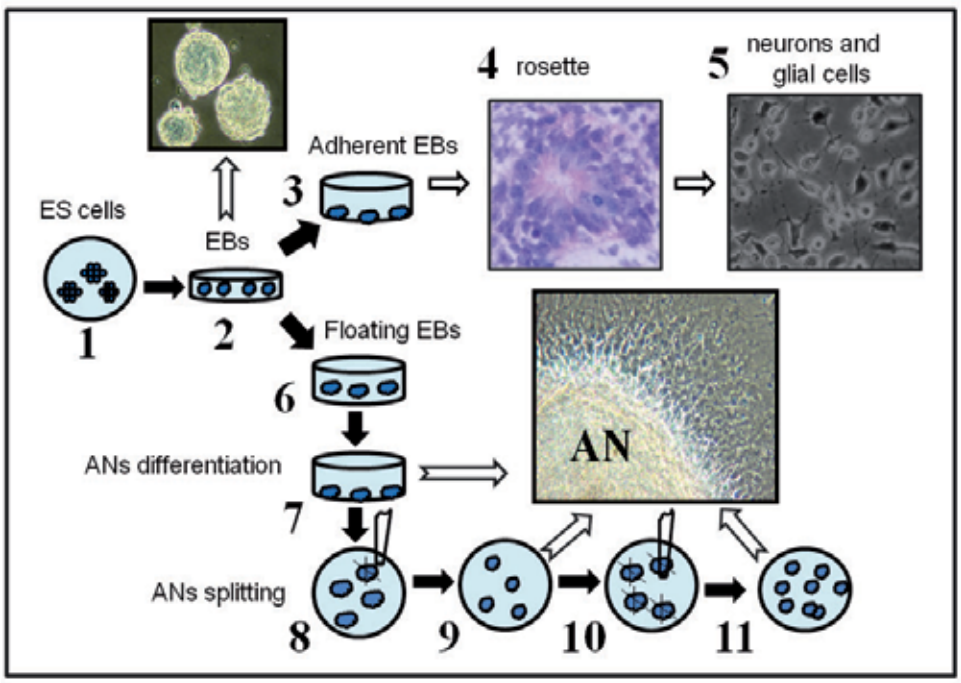

Fig. 4. Differentiation of ES cells towards neurons. 1-2: EBs formation; 3-6: 3-D model; 7-11:2-D model. (1) Pluripotent ES cells in basal culture medium. (2) EBs formation using suspension cell culture or hanging drop protocol. (3-5) Adherence of EBs, in serum-free medium with or without RA, production of neuroepithelial cells, (4) rosette formation, (5) neurons (white-brilliant) and glial cells (black) production. (6-11) culture of floating EBs in serum-free medium in the presence of RA, (7, 9, 10) RA removal, EBs adherence and ANs formation, production of neuronal precursor and mature neurons, (8, 10) ANs mechanical splitting and transfer using glass pipette. White arrows showed in (2) EBs (phase contrast), in (3) rosette (Hematoxylin & Eosin staining) in (5) neurons (phase contrast), in (7, 9, 11) AN with outgrowing neurons (phase contrast).

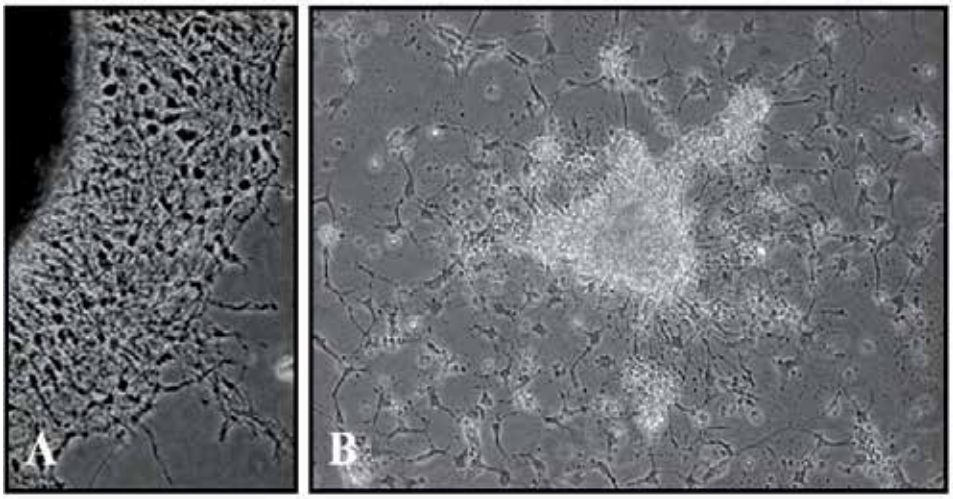

Fig. 5. Differentiation of PSC-derived adherent neurospheres. A) AN (black) producing a network of connected neurons. B) AN (white) after mechanical splitting and transfer. Migration of neurons also can be observed. Light microscopy (phase contrast).

These ANs present expression of the neural progenitor cell markers, such as Sox1 and Nestin just after plating. Following differentiation, the inner part of the AN, continuously expressed Sox 1 and Nestin proteins, while outgrowing neurons, which form an extensive neurite net around the AN expressed beta III-tubulin (neuron-specific marker) (Fig. 6).

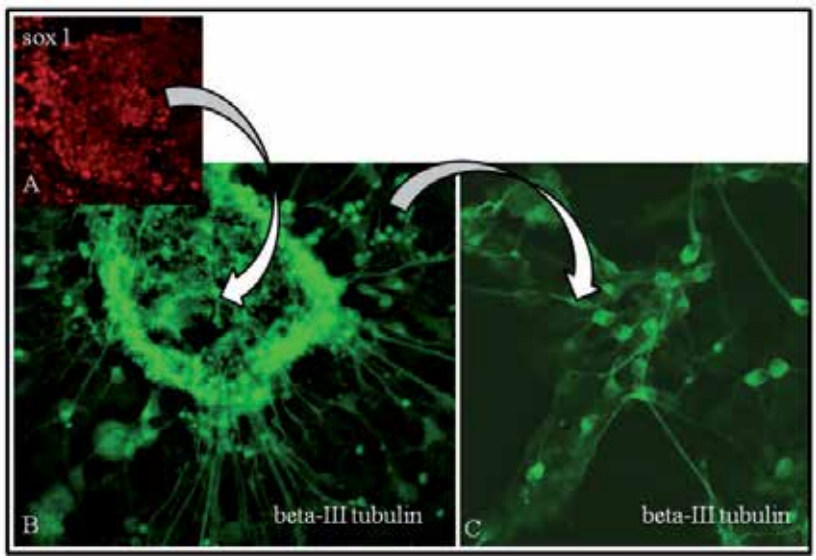

Fig. 6. Expression of neuroepithelial and neuronal markers in AN. A) Expression of Sox 1 protein (red), which express in the nucleus of progenitor cells localized in the inner part of AN. B) Expression of beta-III tubulin protein (green) at the periphery of AN and in outgrowing neurons. C) Higher magnification of extensive neurite net in (B) showing interconnected neurons.

The expression of other neuron-specific proteins, such as MAP2 (microtubule-associated protein 2), NF-M (neurofilament medium protein), Tau (a microtubule-associated protein), NeuN (neuronal nuclei marker), GABA and 5-HT (5-hydroxytryptamin), was observed in neurons derived from ANs each time after splitting and mechanical transfer, which was maintained during three months. Electrophysiological analysis, by using the patch-clamp technique, in long-lasting culture of AN-derived mature neurons, showed the presence of ionic channels and membrane electrical potentials typical of electrically excitable cells, which is a characteristic feature of functional CNS neurons.
This method of mechanical splitting and transfer of ANs is advantageous because it avoids the trauma associated to the trypsin treatments and mechanical dissociation and, so forth, may improve the survival of committed precursors able to differentiate into neurons. It is well-known that CNS precursors are localized in stem cell niche of organisms, which guarantees their continuous growth and renewing, and also the production of differentiated cells. In our model, ANs provide a constant microenvironment (*in vitro* niche) for the neuronal progenitor cells, which can be maintained for at least twelve weeks in culture, following repetitive mechanical splitting and transfer. Since expression of GFAP (Glial fibrillary acidic protein) gene has not been detected, it seems that AN direct the fate of non-committed precursors toward the neurons generation.

2.3.3 Importance of lineage selection for transplantation studies in regenerative medicine

A majority of available protocols for neural differentiation result in the generation of multiple cell types of committed neural precursor to a fully differentiated, post-mitotic neural cell. The selection and expansion of ES-derived neural precursors is a material for transplantation studies focusing on diseases as Parkinson's or Alzheimer's disease, or neural damage following stroke or injury. Such protocol is important due to the elimination of PSCs from the transplanted cell population, which can generate teratocarcinomas (Zhang et al., 1996, Deacon et al., 1998, Bjorklund et al., 2002). Commonly genetic engineering methods are used for lineage selection on differentiating ES cells to purify neural precursors. These techniques rely on the introduction of a reporter/selection cassette into a locus with restricted expression in the desired cell type by homologous recombination. Thus, to address lineage selection of neuronal precursor one copy of the pan-neural gene Sox1 has been replaced by the dual selection/reporter cassette egfpIRESpac in ES cell line, which confers cell-autonomous green fluorescence and puromycin resistance to cells that express Sox1. This gene is not expressed in ES cells. Its expression is limited to neuroectodermal cell, and undifferentiated ES cells are not fluorescent. Upon neural differentiation, Sox1 is activated and the cells produce green fluorescence enabling further purification of both neural and non-neural cells generated during differentiation. Fluorescence-activated cell sorting (FACS) is used for the isolation of both Sox1–GFP-positive and -negative cells allowing further analysis (Li et al., 1998, Pevny et al., 1998, Wood et al., 1999, Ying et al., 2003).
The comparison of AN and the above described techniques demonstrated that we succeeded to establish very simple and long-term protocol for generation of Sox1 positive cells. It is useful to note that 3 months of several mechanical splitting, Sox1-positive cells maintain the expression and continuously produce outgrowing beta III-tubulin-positive cells, while expression of GFAP gene has not been detected (Hayashi et al., 2010). Quantification of precursors and mature neurons demonstrated stable production of both Sox1 and beta III-tubulin proteins during the first 2 months. At the end of the third month, Sox1-nestin-

positive cells were maintained at a similar level as before (~83%), whereas the number of immature neurons (~45%) decreased 1.5-fold, suggesting delay of the maturation process (~32%). Moreover, we showed that under the described conditions, dopaminergic, GABAergic, and serotonergic neurons can be produced. Therefore, generation of 2-D model is of great importance because allows expansion of neural progenitor without genetic modification from primary ES cell culture. Our AN protocol is especially advantageous for the future of regenerative medicine and treatment of neurodegenerative diseases, which will provide more tools for a safety clinical protocol with the advantage of lacking the intermediate effects from non-neural cells.

2.3.4 Importance of microtubule associated proteins for neuronal differentiation

Neuronal migration has been studied extensively in diverse mammalian species and the sequence of events that occurs during cortical development is shared by all mammals. (Gleeson & Walsh 2000, Walsh & Goffinet 2000). During neurogenesis, neural precursors are generated, which proliferate and differentiate into immature postmitotic neurons. These immature cells migrate from the ventricular zone (VZ) to preplate, a layer at the surface of the developing cerebral cortex, splitting the preplate and forming the cortical plate, which further develops into the cortex. Following immature neurons migration from the VZ, cortical lamination is established in an inside-out fashion. In the deep of the cortex, the earliest-born neurons end up, while later-born neurons localize at more superficial layers of the cortex residing near the pial surface. Synaptogenesis and apoptosis of neurons occur at the final stages of cortical development. Indeed, the migration of neurons requires the same steps, which is necessary for migration of any cell type. The signals of environment for attraction and repulsion; the nucleus dislocation from central position to the periphery, a process called nucleokinesis; and a mechanism for migration end up. Microtubule associated proteins (MAPs), for instance, Lis1 and Ndel1, have been shown to be essential for neuronal differentiation and cell migration during the CNS development and also in the adult nervous system.

2.3.4.1 Lis1 and Ndel1

Haploinsufficiency of Lis1 results in lissencephaly, a human neuronal migration disorder (Reiner et al., 1993, Saillour et al., 2009). Patients with type 1 Lissencephaly disorder, have a reduction in brain folding, and aberrant distribution and orientation of neurons in several brain regions. Lis1 binds with high affinity to a protein called Ndel1. Both proteins can complex with cytoplasmic dynein, the retrograde microtubule motor. Lis1 and Ndel1 are proposed to be important for the regulation of dynein-related events in mitosis and migration (Shu et al., 2004, Yamada et al., 2008, Youn et al., 2009, Hippenmeyer et al., 2010, Zyłkiewicz et al., 2011). Thus, PSCs can provide an important model to study migration defects related to MAPs.

Lis1 is a central component of a protein complex, evolutionarily conserved from fungus to human that regulates nuclear migration (Morris, 2000). Lis1 is able to regulate neuronal migration efficiency in a dose-dependent manner (Gambello et al., 2003). Reduced Lis1 activity results in severe defects in the radial migration of multiple types of neurons, including neocortical projection neurons (Tsai et al., 2005).

Ndel1 is important for normal cortical development and it is involved in microtubule organization, nuclear translocation, and neuronal positioning, in concert with various other proteins, including Lis1 (Shu et al., 2004, Youn et al., 2009). Mutations in the mammals Lis1

gene result in neuronal migration defects (Reiner et al., 1993, Youn et al., 2009, Saillour et al., 2009), while knockdown or ablation of cortical Ndel1 function also results in impaired migration of neocortical projection neurons (Sasaki et al., 2005, Youn et al., 2009). Lis1 and Ndel1 co-localize predominantly in the centrosome in early neuroblasts, and later, redistributes to axons during neuronal development (Shu et al., 2004, Guo et al., 2006, Bradshaw et al., 2008, Hayashi et al., 2010). Thus, Lis1 and Ndel1 are essential for normal cortical neuronal migration and neurite outgrowth.

Currently, Lis1 and Ndel1 were shown to have additional, important functions in the cytoplasmic dynein pathway. They participate in nuclear and centrosomal transport in migrating neurons (Shu et al., 2004, Tsai et al., 2005). Additionally, they influence a centrosome positioning in migrating non-neuronal cells (Dujardin et al., 2003, Stehman et al., 2007, Shen et al., 2008) as well as chromosome alignment, and mitotic spindle orientation (Faulkner et al., 2000, Siller et al., 2005, Liang et al., 2007, Stehman et al., 2007, Vergnolle & Taylor 2007). McKenney and co-authors (2010), using biochemical and biophysical approaches, investigated whether and how Lis1 and NudE (Ndel1) affect dynein motor activity. Results obtained in this work apparently explain the requirement for Lis1 and NudE in the transport of nuclei, centrosomes, chromosomes, and the microtubule cytoskeleton. Additionally, they provide new insight into the molecular basis for lissencephaly, and the mechanism of action of these proteins in a broad range of biological functions.

2.3.4.2 Expression of Lis1 and Ndel1 in ANs

Regardless of the relevance of these proteins for neuronal differentiation, their expression during PSCs differentiation is not well explored yet. Our group was the first to analyze intracellular localization of both proteins in mES cells, undifferentiated and during *in vitro* neural differentiation (Hayashi et al., 2010). The expression of both Lis1 and Ndel1 proteins was observed in undifferentiated cells, which presented co-localization within the perinuclear region (Fig. 7). At early stages of differentiation, just after formation of ANs, Lis1 expression was observed in the cytoplasm, while Ndel1 was in the perinuclear region of committed cells. Following differentiation, when ANs grow in size, the expression of both proteins was no more observed in the area of committed cells. Both Lis1 and Ndel1 proteins were visualized in outgrowing neuritis. Additionally, they co-localized with Tau, which is a marker of MAPs, involved in the microtubule assembly and stabilization. In the same way, Ndel1 and MAP2 were also co-localized. In non-rosette MAP2 positive neurons, Lis1 and Ndel1 proteins co-localized in neuronal cell body and growing axons (Fig. 7).

In attempt to mimic the development of cortical layers *in vitro* and to study the cell migration during the differentiation process, which can be assessed by the analysis of the expression pattern of these proteins, the ANs were allowed to grow for 15 days without splitting. Significant variation in spatial distribution of Lis1 and Ndel1 proteins were observed within 2-D ANs. The expression of Lis1 was observed in the inner part of ANs, in the cells presenting rosette morphology. Unexpectedly, Ndel1 was not expressed in rosette forming cells. Both proteins were co-localized in the cytoplasm of the cells showing neuroblast-like morphology, which were found close to the periphery of AN. Lis1 protein was expressing in the cells very closely localized to Ndel1 expressing cells, which, in turn, were close to the region of outgrowing neurons. Co-localization of Lis1 and Ndel1 expression was detected in cells from upper layer of ANs. Ndel1 was found to interact with centrosomes, suggesting that these cells are early neuroblasts (Fig. 8).

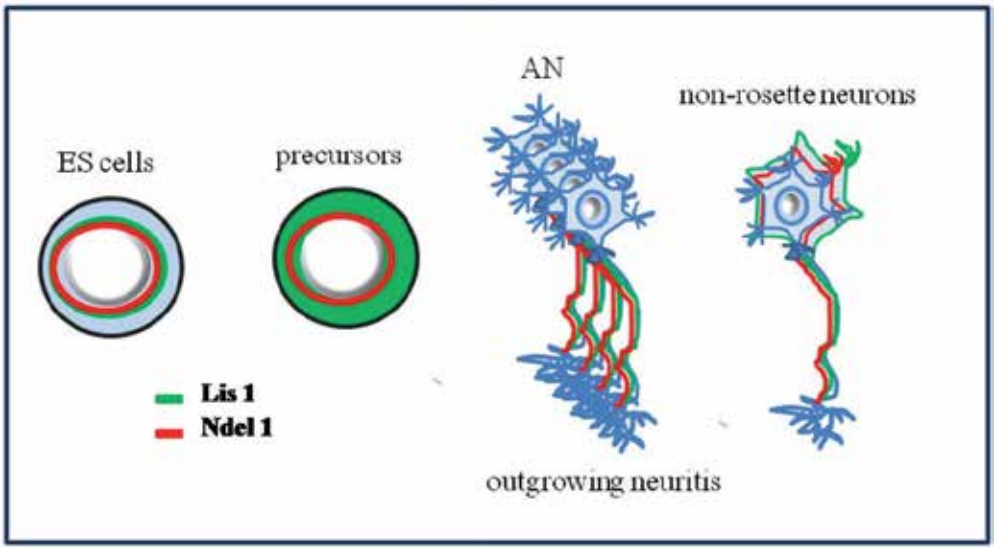

Fig. 7. Schematic presentation of expression pattern of Lis1 and Ndel1 during neuronal differentiation of PSCs. In undifferentiated ES cells, both Lis1 and Ndel1 show a perinuclear co-localization. In neuronal precursors, Lis1 presents a cytoplasmatic and Ndel1 a perinuclear localization. In neurons, at the periphery of ANs, both Lis1 and Ndel1 co-localize in the outgrowing neurites. In non-rosette neurons, these proteins co-localize in neuronal body and neurites.

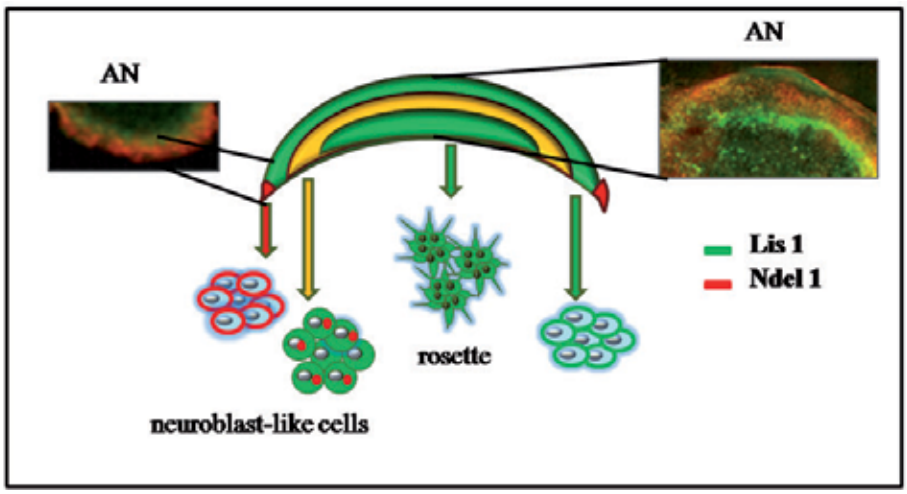

Fig. 8. Schematic presentation of Lis1 and Ndel1 expression in different layers of AN. Lis1 (green) expression was observed in the inner part of AN in the cells organized in rosettes. Intermediate layer (yellow) is composed by the neuroblast-like cells, which express both proteins, Lis1 (green) in the cytoplasm and Ndel1 in centriolos (red) and less in cytoplasm. Upper layer is composed mainly by Lis1 and at the periphery of AN by Ndel1 expressing cells. Proteins expression is also demonstrated by immunofluorescence within AN located on both sides of schematic presentation (Confocal microscopy + Fluorescence).

Interaction of Lis1 and Ndel1 with other cytosolic proteins had been well studied using cultured non-neuronal and/or neuronal cells and the expression of both Ndel1 and Lis1 genes in early neuroblasts derived from embryonic and adult tissues was observed (Sasaki et al., 2000, Shu et al., 2004). Our data demonstrated an expression of only Ndel1 without Lis1 expression in the centrosome region in neuroblast-like cells within differentiated AN. Variation of spatial distribution of Lis1 and Ndel proteins expression was also observed. The ES cells isolated from, for instance, Lis1 or Ndel1 knockout mice, followed by their differentiation into neuronal cells using the present protocol, will permit the elucidation of the real role of each protein during the neuronal differentiation process. Our data suggest that further analysis involving other important MAPs are necessary to allow a better comprehension of the migration mechanism(s) and of the specification fate of neuronal cells during differentiation.

3. Conclusions

PSCs have the capacity to differentiate *in vitro* into neuronal cells spontaneously through EBs formation or in monolayer culture. EBs 3-D model is shown to be more efficient model, which can be improved using serum-free culture conditions and inductors of differentiation (e.g. RA). Following this protocol, neuroepithelial cells could be obtained, which formed rosettes. Further selection and enrichment protocols are needed to isolate culture of committed neuronal precursor, neurons and/or glial cells. This 3-D model provides short-term culture of neuronal cells, which did not allow analysis of neurons migration and survival. It is of note that these AN can be maintained even in the absence of growth factors, without lacking the capacity to produce functional neurons.

Our study demonstrated that ANs is a long-term protocol, which can be used to analyze the process of neuronal differentiation in dynamics. Plating of intact ANs also provides a window of time to the precursor cells for establishing their fate in a 2-D environment. ANs model avoid a stage of rosettes formation directly producing committed progenitors and non-rosette neurons, mimicking process of differentiation of neurospheres form CNS. Mature neurons, obtained from ANs, display ionic channels and membrane electrical potential, which are typical of electrically excitable cells and are also a characteristic feature of functional CNS neurons.

Following mechanical splitting and transfer, these ANs grow continuously, confirming their auto-renewing properties similarly to progenitors of CNS. When maintained untouched during prolonged period (at least 15 days), progenitors inside growing ANs undergo further cell specification. As we demonstrated by the analyses of expression of Lis1 and Ndel1 proteins, both presented differential spatial distribution within the ANs. The discrepancy between patterns of expression of these proteins in neuroblasts isolated from embryonic or adult mouse neuronal tissues, and in those AN-derived cells was observed. AN-derived neuroblasts demonstrated only Ndel1 location in centrosome region, instead of showing the location of both proteins in this region. This indicates that miss expression of proteins, which are responsible for neuronal cells division and migration, can occur during *in vitro* differentiation.

Thus, our protocol provides an efficient experimental model for studying neuronal *in vitro* differentiation mimicking early development, as well as it represents a novel source of functional cells that can be used as tools for testing the effects of drugs on functional neuronal cells.

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5

Characterization of Embryonic Stem (ES) Neuronal Differentiation Combining Atomic Force, Confocal and DIC Microscopy Imaging

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1. Introduction

The nervous system development is of tremendous fundamental importance, but it is immensely challenging because of the complexity of both its architecture and function (Staii et al., 2011). Understanding the formation of this ordered complexity is one of the greatest challenges of modern science. Mechanisms by which progenitor cells differentiate and acquire their cell identity are just beginning to be fully understood (Gilbert, 2000; Lai & Johnson, 2008).

A potent tool for studying differentiation and development *in vitro* has been offered to researchers by the isolation of mouse embryonic stem (ES) cells derived from the inner cell mass of blastocyst-stage embryos more than 20 years ago (Evans & Kaufman, 1981; Martin, 1981). Two unique characteristics distinguish them from all other organ-specific stem cells identified to date. First, they can be maintained and expanded as pure populations of undifferentiated cells for extended periods of time retaining normal karyotypes. Second, they are pluripotent, capable to generate every cell type in the body. The pluripotent nature of mouse ES cells was formally demonstrated by their ability to contribute to all tissues of adult mice, including the germline, following their injection into host blastocysts (Bradley et al., 1984). In addition to their developmental potential *in vivo*, ES cells display a remarkable capacity to form all differentiated cell types in culture (Keller, 2005).

In vitro differentiation of embryonic stem (ES) cells recapitulates early events in the development of the mammalian nervous system: ES cells can both generate and respond *in vitro* to signals that normally regulate embryonic development (Fraichard et al., 1995; Keller, 2005). This knowledge has been transported *in vitro* designing several different protocols that have evolved to promote neuroectoderm differentiation (Cai & Grabel, 2007; Keller, 2005). Each of the three major neural cell types of the central nervous system—neurons, astrocytes, and oligodendrocytes—can be generated, and relatively pure populations of each can be isolated when cultured under appropriate conditions (Okabe et al., 1996; Barberi et al., 2003). In addition to the generation of these different neural populations, conditions have been established for the development of different subtypes of neurons. Thus neurons

of different parts of the neural tube were successfully generated including spinal motoneurons, midbrain dopaminergic neurons, spinal cord interneurons, Purkinje and granule cells of cerebellum, hypothalamus and finally cortical pyramidal cells (Gaspard & Vanderhaeghen, 2010).

These neurons do not only have the correct morphology and express specific markers but they are also functional: electrophysiological data from ES-derived neurons with different protocols validate their functional differentiation *in vitro* and there are evidence of the formation of synapse between ES-derived neurons or between an ES-derived neuron and a mature neuron in organotypic slices as well as integration of ES-derived neurons *in vivo*. Moreover, networks formed by ES-derived neurons display functional properties remarkably similar to those of hippocampal neurons (Ban et al., 2007 and references herein).

The isolation of human ES cells in 1998 (Thomson et al., 1998) dramatically elevated the interest in the therapeutic promises of ES cells differentiation, in particular for brain repair. Therefore ES have emerged as a powerful tool in neurobiology, helping address longstanding questions in an entirely novel way (Gaspard & Vanderhaeghen, 2011).

If the cell is to become a neuron, the next decision is what type of neuron it will be. After fate is determined, still another decision gives the neuron a specific target (Gilbert, 2000). Neurons find their targets by protruding axons driven by the motile apparatus of growth cones. The surface of growth cones contains receptors for extracellular guidance cues that integrate this information into directional movement towards the target cell (Grzywa et al., 2006). Filamentous (F)-actin is the primary cytoskeletal element that maintains the growth cone shape and is essential for proper axon guidance, whereas microtubules are essential for giving the axon structure and serve an important function in axon elongation (Dent & Gertler, 2003). Thus growth cones act as sensors, signal transducers and motility devices and understanding their fate is a key question.

While the role of guidance molecules (Dickson, 2002) for growth cone movements, underlying signalling of both the F-actin and microtubules is well established (Dent & Gertler, 2003; Schaefer et al., 2002), relatively little is known about the three-dimensional structure of growth cones, which is difficult to determine *in vivo* in the mouse embryo and impossible in the human embryo (Keller, 2005).

Cellular three-dimensional structures can be analyzed with different imaging techniques. Despite the enormous advancements brought about by electron and scanning probe microscopy, about 80% of all microscopy investigations in the life sciences are still carried out with conventional lenses and visible light. Taking advantage of the optical transparency of cells, light microscopy uniquely provides noninvasive imaging of the interior of cells (Hell, 2007).

Discovered and first patented in 1957 by Marvin Minsky, confocal microscope, a predecessor to today's widely used **confocal laser scanning microscopy (CLSM)** has allowed a major advance in biological imaging of cell structure and physiology in thick specimens, in three dimensions and in time. CLSM offers high-quality three-dimensional fluorescent images over conventional wide-field optical microscopy, due to its ability to control the depth of a focal plane to eliminate out-of-focus light or glares in specimens and its capability to collect serial (i.e., z-stack) optical images for thick specimens without the need for physical sectioning of the tissue. In this way, thin, optical sections with greater resolution and contrast and with greater sensitivity than conventional, wide-field microscopes are produced (Dailey et al., 1999; Park et al., 2010; Pawley, 2006).

Confocal laser scanning microscopy (CLSM) utilizes the optical pathway of a regular optical microscope and in combination with immunofluorescence histochemistry has been widely used to simultaneously map the distribution and localization of different cellular components (extracellular and intracellular macro-molecules, including proteins, nucleic acids and lipids, as well as intracellular ions such as calcium) helping to understand intracellular mechanisms (Dailey et al., 1999; Rajwa et al., 2004). Moreover, it is possible to monitor the process and dynamics of living cells with high temporal resolution (Hell, 2007; Park et al., 2010).

The 200 nm resolution limit of the CLSM is restricted by the diffraction limits of the microscope objective and does not permit accurate evaluation of the sample height. The z-resolution of CLSM for any given wavelength is always at least 2 times less than the corresponding xy-resolution thus restricting a detailed structural image that can be achieved when exploring biological samples (Doak et al., 2008; Rajwa et al., 2004).

Great advances in optical microscopy such as near field scanning optical microscopy (NSOM), stimulated emission depletion microscopy (STED), photoactivated localization microscopy (PALM), and stochastic optical reconstruction microscopy (STORM) have been achieved to overcome the optical diffraction limit (Hell, 2007 and 2009; Schermelleh et al., 2010), but there are still constraints for realizing time-resolved dynamics or three dimensional imaging (Park et al., 2010). These techniques provide an excellent spatial resolution in the xy plane also in live specimens but relying on the presence of fluorophores cannot provide information on the overall morphology of cells.

State-of-the-art transmission and scanning electron microscopy techniques are technically demanding, relatively costly, and time-consuming. Moreover, the possibility to specifically label and visualize multiple cellular structures or components in one specimen is still limited. Furthermore, chemical fixation, contrasting procedures and/or physical sectioning can cause artifacts and exclude the option to observe living cells (Schermelleh et al., 2010).

With the discovery of **Atomic force microscopy (AFM)**, amazing progress has been made in the imaging of biomolecules with sub-nanometer resolution (Binnig et al., 1986; Engel et al. 1997; Fotiadis et al., 2002; Kasas et al, 1993; Lal & John, 1994; Müller et al., 1999), comparable to scanning electron microscopy. To date, AFM has been used increasingly and is progressively becoming a usual benchtop technique. The volume of scientific publications citing AFM increases continuously and papers with a biological emphasis reached more than 21% of total publications (as in 2006) (Parot et al., 2007).

AFM is a scanning probe device, which generates an image by systematically scanning a sharp tip mounted on the end of a flexible cantilever over the samples' surface. The tip interacts with the surface causing the cantilever to bend. A laser beam acting as an optical lever is deflected from the end of the cantilever to a position sensitive photo-diode that measures the cantilever deflection. In this way a topographic map of the surface is generated (Doak et al., 2008).

AFM can be used in ambient air or under liquid and provides an unprecedented way to image the morphological structure of the surfaces of cells and other biological samples, i.e. it images the surfaces where most of the regulatory biochemical and other signals are directed (Lal & John, 1994). By functionalizing the cantilever tip with appropriate molecules, (Reddy et al., 2004; Li et al., 2006), it is possible to localize specific molecules on the surface of the cell and to obtain a high resolution map of their localization. Furthermore, AFM, by measuring forces within and between biological molecules, can provide additional

biophysical information on molecular characteristics by acting as a sensor that quantifies the interaction forces between the tip and sample as they are brought into and out of contact (Butt et al., 2005, as cited in Doak et al., 2008).
In the case of neurons, the majority of AFM imaging has focused on the cell body, axon, and synaptic vesicles, whereas less attention has been paid to the growth cone and its underlying cytoskeletal structures (Grzywa et al., 2006; Laishram et al., 2009; McNally et al., 2005; Parpura et al., 1993; Xiong et al., 2009; Yunxu et al., 2006). In particular the morphological characterization at nanometer scale of ES-derived growth cones with AFM is largely unexplored.
As a stand-alone technique, AFM can provide unique biophysical and ultra-structural information on a sample, but a limitation is the difficulty in correlating structural or mechanical features with functionality (Kellermayer et al., 2006; Doak et al., 2008). Therefore it is necessary to combine AFM imaging with other techniques to obtain functional information. In particular, with fluorescent confocal microscopy specific subcellular components can be stained allowing functional studies.
AFM microscopy is a scanning method and therefore cannot be used to investigate the motion of large biological samples at a high temporal resolution, which are better viewed with conventional video imaging with CCD cameras (Cojoc et al., 2007; Niell and Smith, 2004; Schaefer et al., 2002) or time-lapse confocal microscope (Dailey et al., 1999; Fishell et al., 1995). It is possible to follow moving biological structures with AFM only when small details such as filopodia are imaged (McNally et al., 2005), or the temporal resolution is substantially decreased (Yunxu et al., 2006).
Here we describe how combining three different imaging techniques (time-lapse DIC imaging, AFM on both fixed and living neurons and fluorescence confocal microscopy) used separately, at different times, on different instruments - but on the same samples it was possible to provide a morphological characterization of ES-derived growth cones related to their movement (Ban et al., 2011) and hypothesize a possible functional role of the fragmented structures observed only in a fraction of growth cones.

2. Combined AFM, confocal and DIC imaging for characterization of ES-derived neuronal growth cones

2.1 Morphological characterization of ES-derived growth cones

AFM imaging on more that one hundred fixed cells (n=119) revealed different morphologies of ES-derived growth cones, as shown in Figure 1.
ES-derived neuronal precursors were obtained using the protocol described previously (Ban et al., 2007). Cells were plated on coverslips and induced to differentiate for 24 hours. During this period of culture, ES-derived neurons extended neurites with growth cones moving forward, retracting and exploring the environment with their filopodia. The structure of the differentiating growth cones was analyzed by AFM in contact mode in liquid. Cells were plated at a density of $3x10^4$ cells/cm^2 in order to obtain isolated growth cones to avoid overlapping structures. This low density, however, is sufficient for the neuronal survival in culture.
At this stage of differentiation, very different structures were observed with the diameter of growth cones that varied from 1.5 to 28 μm and height that varied from 65 to 593 nm. They could be classified in three different morphological groups: in the first one (Figure 1D-I) they appeared swollen and smooth, with several filopodia spreading from the central domain. The

height differed substantially from one growth cone to another but in this group (68% of growth cones analyzed) it always exceeded 200nm. In the second group (21% of cases) growth cones were flat with few or no filopodia (Figure 1A-C) and their height was consistently below 200nm. In the third group (11% of cases) growth cones showed a ruffled and fragmented structure with several holes (Figure 1J-L). The height of these growth cones was also below 200nm, and their thickness almost vanished in some regions. All these structural details were not recognizable with optical microscope due to their small dimensions.

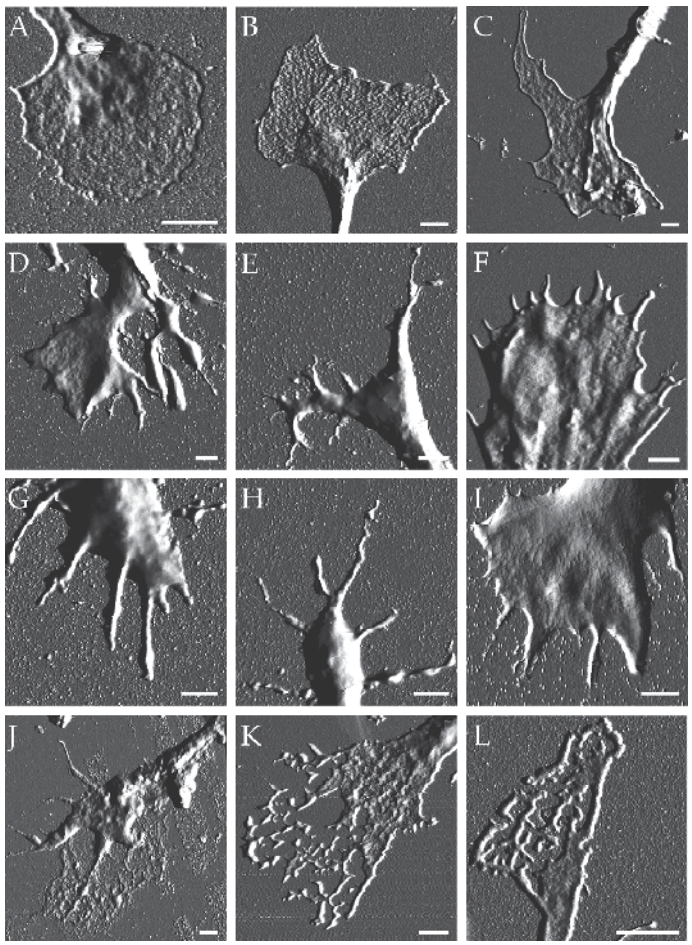

Fig. 1. High resolution AFM images of ES-derived growth cones fixed after 24 h of differentiation. Growth cones could have flat and rough (A-C), swollen and smooth (D-I) or fragmented (J-L) structures. Scale bar, 2μm.

Although during image acquisition the AFM tip interacts with the cell surface and could modify or damage the cell membrane, we demonstrated that the fragmented structures were not an artifact of AFM scanning by acquiring a series of images of a compact growth cone at increasing tapping forces of 200, 1000 and 3000 pN respectively. In 4 independent experiments performed, the height profile of the growth cones at varying forces showed a decrease in height caused by a compression of the cell structures, probably due to the

residual cell elasticity, after the fixation procedure. However, when the imaging force was restored to the initial value of 200pN, the cell morphology was exactly the same as in the first scan and no damage was introduced (Ban et al., 2011).
Holes and fragments of growth cones were not fixation artefacts. In fact, using three different fixation methods both fragmented and compact growth cones were present on the same coverslip and sometimes on neighbouring cells. Fragmented structures were also observed in growth cones of living neurons analyzed by AFM: in 6 out of 23 imaged cells the growth cones retracted leaving behind fragments with variable dimensions. In 3 cases holes in the growth cones were observed and in one case the holes were already present at the first scan. The physiological dynamics was poorly affected by AFM scans and the cantilever tip did not damage the growth cones since in majority of cases (14 out of 23 imaged cells) their compact structure was preserved during the scanning period even though cycles of protrusion and retraction were observed. Moreover, fragmentation observed under living conditions (13% of cases) had comparable proportion in respect to what observed with fixed cells (11% of cases). Altogether, these observations suggested that the fragments observed on the fixed cells were originated before fixation and that fragmentation is a physiological phenomenon (Ban et al., 2011).
We hypothesized that the different morphologies observed in the growth cones might correlate with different types of movement. For doing so we took advantage of the 3 techniques described to study the motion of living growth cones and trying to correlate it with structure and expression of specific subcellular components.

2.2 Preparation of coverslips

To be able to collect images with all these techniques (each of them with different performaces, resolution and collection modes) but from the same cell it was necessary to have a position marker on the sample.
In our case we prepared a home-made coverslips on which ES-derived neuronal precursors were plated and induced to differentiate. But commercial coverslips with numerical grid could serve the same purpose.
Coverslips with printed markers were prepared by optical lithography and metal evaporation on 24mm diameter glass coverslips. A polymeric photoresist was deposited on one side of the coverslips by spin coating. The indexed pattern was produced by UV exposure through a patterned optical mask. A 20nm titanium layer was finally deposited and stripped by lift-off techniques (Figure 2A).
In the free space between printed markers the movement of neurons and their growth cones could be clearly monitored (Figure 2B). Time-lapse DIC and AFM imaging can be performed on living cells while immunofluorescence assays (Figure 2C) and high resolution AFM (Figure 2D) can be done at very different time intervals after fixation (from few hours up to many months if samples are kept at +4°C in paraformaldehyde).

2.3 Correlation between movement and structure/function

Theoretically movement and structure could be analyzed imaging living cells with AFM. When performing AFM on living cells images could be obtained in tapping mode at 0.6 scan line per second and with a maximum of 256 scan lines. In this way the acquisition of AFM images required several minutes and, due to the movement of growth cones and to the fluidity of the membrane, a high resolution AFM image could not be achieved. Giving higher temporal resolution, time-lapse differential interference contrast (DIC) imaging was

used. Images with resolution between 512x512 and 1024x1024 pixels were acquired every 10 seconds, for a total of 80 frames (total duration 13 minutes and 20 seconds) and the motion of more than 100 growth cones was collected.

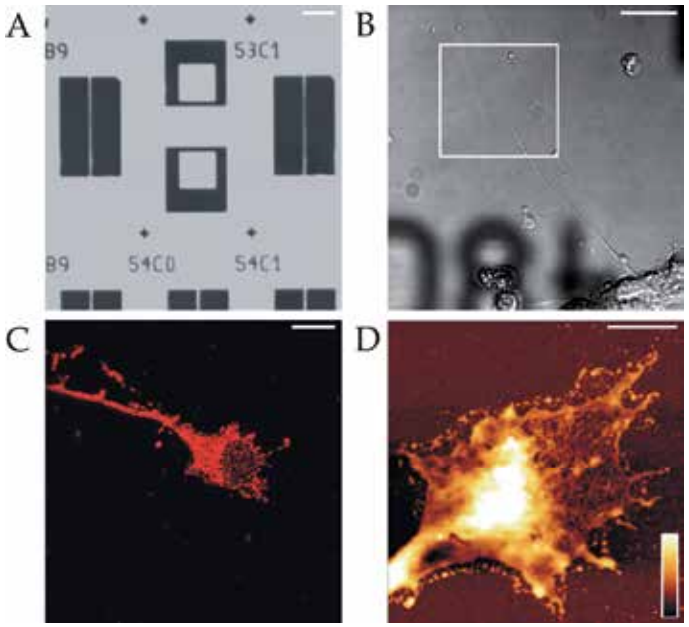

Fig. 2. (A) An example of a coverslip with printed markers and numbers. Scale bar, 100 μm. (B) ES-derived growth cone (inset) imaged with DIC using a confocal microscope. Scale bar, 25 μm. (C) Confocal image (1024x1024 pixels) of a growth cone stained for with an antibody against neural cell adhesion molecule (NCAM). Scale bar, 10 μm. (D) AFM image (1024x1024 pixels) of the same growth cone. Scale bar, 5 μm. Color bar from 0 to 350nm (From Kondra et al., 2009, Journal of Neuroscience Methods, ELSEVIER).

From the time-lapse analysis 4 different types of movements were observed: exploring (43/104), growing (16/104), retracting (17/104) and stasis (28/104). 29 of these cells were imaged with AFM. Cells were fixed immediately after video imaging, AFM with a resolution between 512x512 and 1024x1024 pixels was performed followed by staining with appropriate antibodies.

Filopodia of exploring growth cones (9 out of 29 cells) moved with a velocity up to 80 nm s^{-1} with an average value of 25±7 nm s^{-1} exploring very efficiently the surrounding free space independently in all directions. Some filopodia were continuously extending and retracting, changing rapidly in their length, while some others were freely exploring maintaining their shape. AFM imaging revealed a smooth and compact surface. The growth cones had average height of 852 ± 235 nm and their filopodia had a height varying between 67 and 219 nm (n=40; average height 98.8 ± 48.3nm).

Neurites of growing growth cones (5 out of 29 cells) could grow by 1-5 μm in 2-10 minutes and in two cases a neurite grew up to 14 μm in less than 2 minutes. As for exploring growth cones, in all cases AFM showed a compact and smooth surface. Filopodia height reached almost 2 μm and their average length was 3.5 μm (n=20).

Retracting growth cones (7 out of 29 cells) moved with a velocity ranging from 80 to 135 nm/sec, retracting their filopodia and the whole neurites from the original position. After fixation, AFM scanning revealed the presence of fragments near the tip of fast retracting growth cones. The confirm that these fragments were actual parts of the growth cone left behind by the neuron came from AFM imaging of living neurons. In all 6 retracting growth cones observed, fragments with an average diameter of 85 ± 27 nm and an average height of 75 ± 28 nm were detected.

Growth cones were classified as static (8 out of 29 cells) when the external contour of the growth cone remained in the same position and the length of their neurite did not change during DIC observation. These growth cones had an apparently intact shape when viewed with time-lapsed DIC images (Figure 2B), but their 3D shape was highly fragmented when viewed with AFM (Figure 2D). Static growth cones were thinner and rarely reached 200nm height.

When the same growth cone was analyzed with immunofluorescence - with actin and tubulin to follow cytoskeletal component underlying growth cone movement or neural cell adhesion molecule (NCAM) to visualize cell membrane contours - the two images were difficult to compare due to the different resolution (Figure 2C and D). Moreover, AFM and CLSM images contain different information: every pixel at location (x,y) in the AFM images provides a direct measurement in nm of the sample height while fluorescence images acquired with a confocal laser scanning microscope characterize the emitted fluorescence at the same location.

In order to integrate the information derived from AFM and immunofluorescence analyses it is important to properly align or superimpose these different images. This problem has been extensively studied in Computer Vision, where it is referred as "Registration" of different images (Kondra et al., 2009).

2.4 Image registration

Image registration is the process of overlaying two or more images of the same scene taken at different times, different viewpoints, and/or by different imaging modalities. Differences between images are introduced because of different imaging conditions, such as different viewing points or light conditions or because of the use of two different microscopes, as in the case under consideration. Registration transforms one image – usually the sensed image – so that it becomes aligned to the reference image. Image registration is a crucial step in image analysis and can be solved using methods used in computer vision (Trucco & Verri, 1998), in which the final information is obtained by integrating various data sources like in image fusion (Zitova & Flusser, 2003). Images taken from different modalities may undergo a linear deformation in scale, translation, rotation and sometimes shearing i.e. affine deformation.

The word registration is used with two different meanings (Hill et al., 2001). The first meaning is to determine a transformation of one image so that features in the sensed image can be put in a one-to-one correspondence to features in the reference image. The symbol T is used to represent this type of transformation. The second meaning of registration enables also the comparison of the intensity at corresponding positions. The symbol T_i is used to describe this second meaning of registration, which incorporates the concepts of re-sampling and interpolation.

T is a spatial mapping. The more complete mapping T_i maps both position and associated intensity value from image A to image B. Therefore T_i maps an image to an image, whereas T maps between coordinates of image A into coordinates of image B. To overlay two images that have been registered, or to subtract one from another, is necessary to know T_i, not just

T. T_i is only defined in the region of overlap of the image fields of view, and has to take into account of image sampling and spatial resolution.

Before doing the registration, it is necessary to select the correct type of transformation required for the images under consideration. The most widely used transformations are Linear, Affine and Projective. The linear transformation is used when shapes in the sensed image are unchanged, but are distorted by some combination of translation, rotation, and scaling. Straight lines remain straight, and parallel lines are still parallel. This is the case of the same biological sample viewed by two microscopes using different objectives and imaging systems, such as an AFM (Figure 2D) and a CLSM (Figure 2C). Registration using affine transformation is necessary when shapes in the sensed image are distorted also by shearing or a linear deformation. In this case, straight lines remain straight, and parallel lines remain parallel, but rectangles become parallelograms. Projective transformation is used when the scene appears tilted. Straight lines remain straight, but parallel lines converge toward vanishing points that might or might not fall within the image.

If we consider two images of the same biological sample, the first one acquired using AFM and the second using a CLSM, the two imaging systems differ because of different scale factors, by a rigid translation of their origin and a possible rotation of their axis. It is assumed that the two imaging systems do not introduce any deformation. Registration of the two images consists in the determination of the transformation parameter by aligning properly the two images.

The unknown parameters in the transformation matrix can be estimated either by matching a selected number of points or landmarks in the two images or by matching entire contours in the two images. The first method is used when it is possible to identify in the two images enough points corresponding to the same physical structure, such as the tip of a dendrite, or a small vesicle. The second method is used in presence of rounded biological structures, with no obvious marks and it is more convenient to put in correspondence two contours than isolated points.

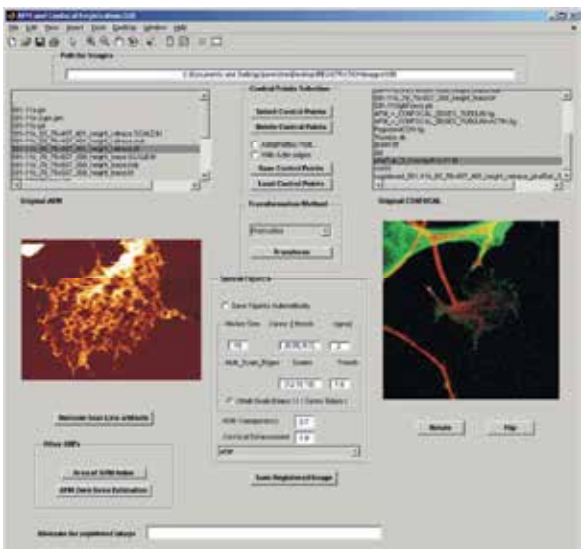

Fig. 3. Snapshot of the graphical user interface (From Kondra et al., 2009, Journal of Neuroscience Methods, ELSEVIER).

To apply these methods to the images derived from different microscopes we have implemented our Registration program using Matlab7.1 (The MathWorks Inc. http://www.mathworks.com) a standard tool common in the computer vision and biomedical community for statistical analysis and production of figures and images of analyzed data and results.
For a more detailed mathematical description of the different transformations and description of the program see Kondra et al., 2009, Appendix 1, 2 and 3).
Corresponding points or contours in AFM image and confocal image were marked by hand using a friendly graphical user interface developed for this particular purpose (Figure 3).
Registration by points selection method is illustrated in Figure 4. The operator identifies N (minimum 3) points in the AFM image (Figure 4A) that are put in correspondence with N points in the confocal image (Figure 4B). Corresponding points in Figure 4A and B are indicated by the same number. By using these correspondences the parameters determining the transformation T are obtained by solving a system of linear equation and the confocal image can be registered. In this way same physical points in the original AFM and in the new registered confocal image, shown in Figure 4C, have the same location.
To make more user friendly the selection of points, confocal image can be rotated in a sequence of 90 degrees and/or flipped to make a approximate alignment with AFM image (compare Figure 2C with Figure 4B). Control Point Selection Tool in Matlab was used to mark the control point pairs in the image to be registered, the input image (Figure 4B), and the image to which you are comparing it, the base image (Figure 4A) corresponding points were initially specified by pointing and clicking in the input and base images so that each point specified in the input image had a match in the base image.

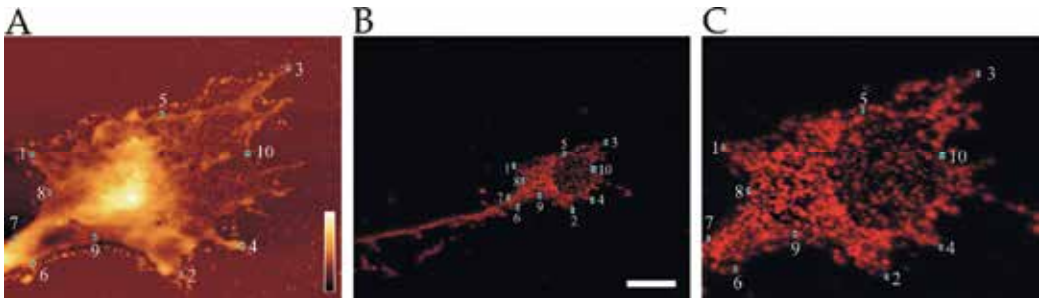

Fig. 4. Example of registering images using points selection method. (A) AFM image of ES-derived growth cone. Color bar from 0 to 350nm. (B) Confocal image of the same ES-derived growth cone. Scale bar, 10 μm. (C) Registered confocal image (From Kondra et al., 2009, Journal of Neuroscience Methods, ELSEVIER).

The advantage of this tool is that, after the first three pairs are selected, it is sufficient to select a point in the input image and the Control Point Selection Tool estimates its match point in the base image or *vice versa* automatically, based on the geometric relationship of the previously selected control points. Another advantage of this tool is that if the image is not registered properly, it is possible to change the position of control points to get the exact superimposition.
The alternative method is registration by aligning contours with Procrustes Analysis. Procrustes was a robber in Greek mythology. He would offer travellers hospitality in his

road-side house, and the opportunity to stay the night in his bed that would perfectly fit each visitor. As the visitors discovered to their cost, however, it was the guest who was altered to fit the bed, rather than the bed that was altered to fit the guest (Hill et al., 2001). Let us consider that the AFM image is like the bed and is the guest. So using the Procrustes' method means that confocal image is altered by scaling and/or rotating it to approximately fit AFM image.
The number of landmarks available depends on the structure of the image, and there may be differences in opinion between scientists as to which landmarks are consistent and locatable. Marking of the corresponding points thus becomes difficult when there are few corners in the structure, and thus exact location of the points is impossible and can differ from observer to observer. This problem can be solved by marking many points along the contour of the structure in both images. The contours are then interpolated so that both of them contain the same number of equally spaced points (for mathematical description of transformation see Kondra et al., 2009).
An example of this method is illustrated in Figure 5. A contour was marked by continuously clicking on points following the borderline of the structure of interest in AFM image (Figure 5A) and in the confocal image as well (Figure 5B). As a consequence of Procrustes analysis, confocal image was properly transformed and put in correspondence with AFM image (Figure 5C).

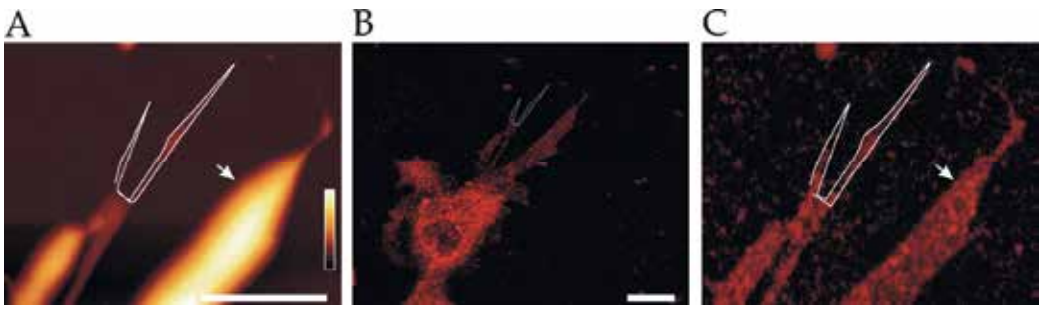

Fig. 5. Example of registering images using Procrustes Analysis. (A) AFM image of ES-derived growth cone. Scale bar, 10μm. Color bar from 0 to 2200 nm. (B) Confocal image of the same ES-derived growth cone stained for neural cell adhesion molecule (NCAM). Scale bar, 10μm. (C) Registered confocal image of membrane protein NCAM (From Kondra et al., 2009, Journal of Neuroscience Methods, ELSEVIER).

2.5 Identification of nanometric structures and subcellular component in static fragmented growth cones

With registration of confocal and AFM images it was possible to further analyze the composition of fragments characteristic of static and retracting growth cones. Their structure seemed compact when viewed with time-lapsed DIC images (Figure 6A), instead AFM (Figure 6B) revealed highly fragmented 3D shape. Profiles of AFM images of static growth cones showed isolated fragments with height that varied from 50 to 150 nm (Figure 6E). Registered confocal images demonstrated that they contain actin filaments but not tubulin and they are positive for neural cell adhesion molecule (NCAM) indicating that fragments left behind by growth cones are formed by chunks of actin filaments enveloped by the cell membrane (Figure 6C and D).

Height of fragments and of filopodia had a similar distribution varying from less than 30 nm up to 300 nm (Ban et al., 2011). Immobile growth cones not only were surrounded by detached fragments, but they also had holes. Growth cone regions surrounding these holes had a height varying from 20 to 90 nm and holes had an area varying between 0.03 to 0.650 μm^2. Fragments had a height varying from 40 to 400 nm with an area varying between 0.4 to 6μm^2.
During the time-lapse DIC imaging none of the neurons with static growth cones showed signs of membrane blebbing or cell shrinking typical of apoptotic cell indicating that the observed fragmentation was not associated with apoptosis. Moreover, nuclear staining by Hoechst revealed that none of the eight cells that were also analyzed by AFM and immunofluorescence after fixation had an apoptotic nucleus. We can therefore conclude that growth cone fragmentation is not part of the apoptotic process.

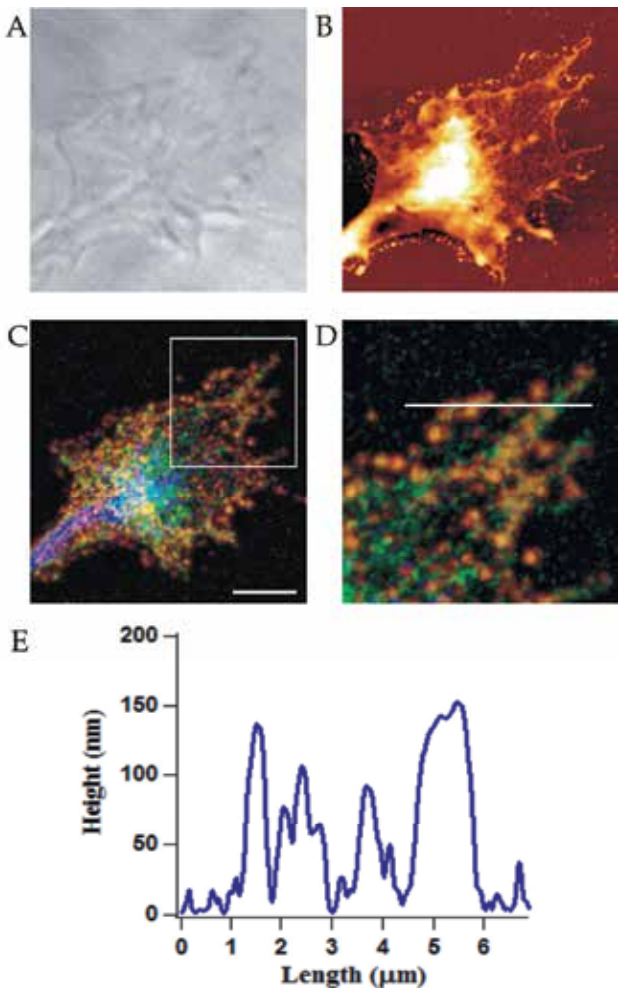

Fig. 6. Static growth cones. (A) DIC, (B) AFM topography and (C) registered confocal image of the same growth cone stained for F-actin (green), NCAM (red) and β-tubulin III (blue). Scale bar, 5 μm. (D) Confocal image of the inset shown in C. (E) Height profile along the white line shown in D.

3. Discussion

Three different imaging techniques used separately, at different times, on different instruments - but on the same samples and the possibility to compare AFM and confocal images due to the development of the registration method (Kondra et al., 2009) allowed us to obtain a morphological characterization of ES-derived growth cones.

Integrated AFM and CLSM instrument has been developed and it offers the advantage of parallel analysis of the same sample with nanometer-scale spatial resolution, frame/second temporal resolution, and chemical identification through fluorescence detection can be done simultaneously for live cells (Doak et al., 2008; Park et al., 2010).

In addition to optical microscopy, AFM can be combined with other instruments and techniques, such as microfluidic liquid cell (Schoenwald et al., 2010), patch-clamp (Pamir et al., 2008) or ultramicrotome (Efimov et al., 2007) providing a novel insights to further understanding of cellular structure-function relationships getting down to the scale of single molecule.

Although there are several studies where AFM is combined with confocal microscope (Doak et al, 2008; Moreno Flores & Toca-Herrera., 2009; Kassies et al., 2005; Owen et al., 2006; Park et al., 2010), our method is useful in cases where combined fluorescence (confocal) and atomic force microscope is not available. In addition, performing different assays separately permitted optimization of sample preparation for each experiment. For example, AFM on fixed cells was performed in contact mode, in liquid (phosphate buffer saline (PBS)) while for immunofluorescence assays, coverslips were mounted with commercial mounting medium, in order to reduce photobleaching during the repetitive number of confocal scans required to zoom in the region corresponding to the growth cone. Our AFM microscope was provided with fluorescence setup and we collected some fluorescent images while performing AFM. However, in PBS the samples bleached rapidly and the resulting images had poor definition.

The combination of AFM and fluorescence confocal microscopy on fixed cells, and of time-lapse DIC and AFM imaging on living cells allowed us to correlate morphology of ES-derived growth cones to their movement prior fixation providing therefore both structural and functional insights.

Growth cones actively exploring the environment before fixation had a smooth external surface. In contrast, growth cones which were immobile before fixation revealed a fragmented shape, composed of several nanoscale structures either partly attached or completely isolated from the rest of the growth cone. In addition, by using AFM on live specimen, fragmentation was observed in some of the retracting growth cones. Therefore the morphology of ES-derived growth cones depends on their overall motility.

Growth cones with micrometric size holes of retinal ganglion cell axons were previously observed *in vivo* (Godement et al., 1994; Mason & Erskin, 2000). They appear in the spread growth cones and are predicted to form from the fusion or contact of lamellar extensions of the growth cone as they enfold radial glial processes. However, although morphologically similar, these two findings reflect a different biological phenomenon: first because our observations were the result of a technical approach measuring at nanometric level and second, because we never found fragmented structures in growing growth cones.

The fragmentation can be related to growth cones pruning that occurs during the early development of the central nervous system, where an excessive outgrowth of projections need to be refined to achieve precise connectivity (Faulkner et al., 2007). The selective

elimination of neuronal cell processes, or neurites, is an essential step during normal development and occurs through retraction, degeneration, or a combination of both (Franze et al., 2009).

This phenomenon resembles the growth cone collapse induced by several factors like mercury (Leong et al., 2001), X-ray (Al-Jahdari et al., 2008) or semaphorin in the absence of growth factors (Tamagnone & Comoglio, 2004). The fragmentation of the growth cone we observed is a local phenomenon, similar to what observed for Lysophosphatidic acid (LPA) induction of collapse *in vitro* which, in contrast with other collapsing treatments, is reversible and not toxic (Saito, 1997). However, to our knowledge, this is the first time that this collapse-like phenomenon was observed to occur spontaneously.

Previous investigations have shown the formation of migration tracks resulting from the release of cellular material onto glass surfaces and artificial matrices for a number of cell types (Fuhr et al., 1998; Kirfel et al., 2004; Richter et al., 2000; Zimmermann et al., 2001). Macroaggregates left behind migrating keratinocytes contain high amounts of β1 integrin and parts of the fibronectin and laminin receptors. They lack, however, of any cytosolic proteins including actin and of the adhesion complex constituents talin and vinculin. In our experimental conditions, fragments left behind by growth cones are composed of cell membrane but also of cytoplasmic proteins such as F-actin, as it was reported for other migrating cells (Fuhr et al., 1998). Fragments could correspond to filopodia originally present on the growth cone in that position also because the height distribution of filopodia and fragments was similar, suggesting that they may be composed of similar building/dismantling blocks. The existence of building blocks could agree with a previous study (Parpura et al., 1993) where it was found that the height of an hippocampal growth cones corresponds to a multiple of the heights of individual filopodia possibly due to overlying actin bundles arising from different filopodia.

The release of a fragment might be energetically advantageous for faster retraction and/or change in growing direction compared to recycling of distal elements. However it cannot be excluded that fragments might act as guidance signals for neighboring neurons.

Vertebrate semaphorins are either secreted or associated with the cell surface. *In vitro* and *in vivo* experiments have implicated semaphorins in the guidance of elongating axons and dendrites, as well as in axon branching, pruning and degeneration (Tamagnone & Comoglio, 2004). The primary role of Sema 3A in the nervous system is to repel growth cones from inappropriate areas and to help steer both axons and migrating cells along the correct trajectory (Brown & Bridgman, 2009). When added in bath, they cause rapid collapse of growth cones, followed by axon retraction (Kolpak et al., 2009).

The fragmentation of growth cones that have not established contact or by pruned contact here observed could therefore serve as a migrating track for other neurons, by exposing semaphorins or other membrane proteins that act as receptors and/or ligands for axon guidance.

4. Conclusion

The combination of AFM and fluorescence confocal microscopy on fixed cells, and of time-lapse DIC and AFM imaging on living cells allowed us to obtain a morphological characterization of ES-derived growth cones related to their movement providing both structural and functional insights.

In particular the registration method allowed to superimpose images taken from different modalities. Registration, however, is not restricted to confocal and AFM images but is a versatile tool for combined studies. The main advantage is that there is no need for sophisticated and combined microscopes but with conventional instruments and AFM, structural information at nanometer scale is combined with functional studies.

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6

Oligodendrocyte Fate Determination in Human Embryonic Stem Cells

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1. Introduction

Oligodendrocytes (OL) are cells of the glial lineage which play a critical role in the central nervous system (CNS) by producing the multilamellar protein, myelin. Oligodendrocytes extend processes which wrap around the axons of several neurons. This myelin sheath increases neuronal conduction by decreasing ion leakage and capacitance of the axonal membrane and increasing saltatory conduction. Damage to oligodendrocytes results in loss of myelin and consequent functional impairment due to loss or decay of neuronal conduction. Demyelination in the CNS is the hallmark of conditions such as spinal cord injury (**SCI**), transverse myelitis and multiple sclerosis – resulting in functional impairment across the sensory, motor and cognitive domains. Current research on treatment of SCI focuses on the implantation of oligodendrocyte progenitor cells (OPC) into the injured region to achieve remyelination of the spared axons and potentially repair the sensory and motor pathways. However, this approach requires a large and relatively pure population of OPCs, which are differentiated from other multipotent cell lineages. Human embryonic stem cells have been used in several studies for this purpose and hence, it is important to understand the molecular mechanisms regulating the differentiation of pluripotent hESCs to oligodendrocytes. Elucidation of such mechanisms may lead to the development of more efficient differentiation protocols and the derivation of cells with improved myelination potential for implantation in injured spinal cords.

While the molecular basis for hESC differentiation into OL lineage cells is not completely understood, several recent studies have filled some of the gaps in our knowledge of the field. The journey from a pluripotent state to commitment to the OL fate is characterized by a complex interplay between genetic regulation and epigenetic modifiers such as histone modification, DNA methylation and microRNAs (miRNAs). Global miRNA expression profiling of OL differentiation from hESCs has revealed that a significant number of the differentially expressed miRNAs have targets hypothesized to be involved in myelination and OL development. Such studies are complemented by histone modification analyses in ESCs which show that specific histone acetyltransferases (HATs) and histone deacetylases (HDACs) are up- or down-regulated during different time points in OL differentiation to

guide myelin production. This review focuses on the role of miRNAs in oligodendrocyte development from human embryonic stem cells and elucidates a new protocol developed to obtain oligodendrocytes from human embryonic stem cells with high yield and purity.

2. MicroRNA regulation of oligodendrocyte development

To date, many studies have attempted to unravel mechanisms of stem cell fate determination by studying gene expression by measuring messenger RNA (**mRNA**) expression. However, in the early-mid 90's a novel class of RNA molecules was discovered that regulated gene expression at the level of translation in *C. elegans*. These RNAs were shown to regulate the translation of a target mRNA, by directly base-pairing to its 3'-untranslated region (3' UTR) (Lee, Feinbaum & Ambros 1993, Wightman, Ha & Ruvkun 1993). Nevertheless, the broad significance of these small RNAs was not fully appreciated until only a few years ago when many similar molecules were further identified in *C. elegans*, Drosophila, and in some mammals (Lee, Ambros 2001, Lagos-Quintana et al. 2001), and these newly recognized regulatory RNAs were termed microRNAs (**miRNAs or miRs**). To date, sequencing libraries of cloned small RNAs and bioinformatic analyses of genomic sequences have led to the identification of ~500 verified mammalian miRNAs while studies exploiting the high degree of conservation of miRNAs across mammalian genomes have suggested that as many as 1000 human miRNAs are likely to exist (Berezikov et al. 2005, Bentwich et al. 2005). Despite these great advances in the identification of miRNAs, our understanding of their roles in cellular processes remains at a very early stage although there is evidence suggesting that miRNA dysfunction contributes significantly to oncogenesis and stem cell self-renewal.

MiRNAs are found in plants, viruses and animals and are generated via a multi-stage process. In mammals they are first transcribed as long precursor transcripts (~60-80 nucleotides) known as primary (pri)-miRNAs containing a hairpin loop (Lee et al. 2002). Pri-miRNAs are then processed into shorter hairpin shaped precursors (pre)-miRNAs in the nucleus and transported to the cytoplasm. There, the RNase-III enzyme Dicer performs a second cleavage to generate a double-stranded 21-23 nucleotide RNA molecule (Bernstein et al. 2001, Grishok et al. 2001, Hutvágner et al. 2001, Ketting et al. 2001, Knight, and Bass 2001). A large protein complex known as the RNA-induced silencing complex, or RISC, associates with this RNA duplex and unwinds it. Generally, only one strand is stably incorporated into RISC while the other is discarded and rapidly degraded (Meister et al. 2004). After the RISC is formed the miRNAs guide it to the target mRNA that is subsequently cleaved or translationally silenced. The degree of complementarity between a miRNA and its target dictates the mechanism of silencing (Meister et al. 2004, Hutvágner et al. 2004, Brennecke et al. 2003)

While degradation occurs predominantly in plants, in mammals the predominant mechanism appears to be through imperfect base-pairing between a miRNA and its target, resulting in translational silencing through a complex mechanism that appears to involve inhibition of both translation initiation and elongation (Pillai, Bhattacharyya & Filipowicz 2007).

MiRNAs are attractive candidates for regulating stem cell identity, which includes self-renewal and cell fate decisions, as their ability to simultaneously regulate many targets provides a means for coordinated control of gene action (Chen et al. 2007). Although direct functional roles in stem cell biology are just emerging, corroborating evidence from

expression patterns, predicted targets, and from overexpression studies suggests miRNAs are key regulators (Cheng et al. 2005). For example, the loss of Dicer1 in mice has been demonstrated to cause embryonic lethality as well as loss of stem cell populations implicating a role for miRNAs in stem cell self-renewal (Bernstein et al. 2003, Murchison et al. 2005). Based on several reports from mouse and human studies, distinct sets of miRNAs are expressed specifically in pluripotent stem cells and not in adult tissues further implicating their role in stem cell self-renewal. In addition, Argonaute family proteins, which are key components of the RISC complex, have also been shown to be required in the maintenance of germline stem cells in different organisms (Carmell et al. 2002).

The role of miRNAs in oligodendrocyte fate determination has been a recent field of research and a few studies have reviewed the state of the current knowledge on the subject (He et al. 2011, Emery 2010, Dugas, Notterpek 2011).. This chapter aims to review the advances made in understanding oligodendrocyte fate determination by miRNAs and elucidate a novel protocol to derive OLs from embryonic stem cells in vitro to study their development.

2.1 MicroRNAs in glial fate determination

Zheng et al first reported that miRNAs were essential for the "developmental switch from neurogenesis to gliogenesis" (Zheng et al. 2010). The authors deleted Dicer, a protein necessary for functional miRNA synthesis, in the mouse ventral spinal neuroepithelium, where embryonic gliogenesis occurs. Neural patterning and motor neuron development occurred even in the absence of miRNA formation, showing that they are probably not involved in neuron maturation. However, oligodendrogenesis in the ventral spinal cord was blocked in the absence of Dicer, as confirmed by the complete absence of PDGFRα+ cells and a huge reduction in Sox10+ cells in the spinal cord parenchyma of Dicer mutants. On similar lines, astrogliogenesis was also found to be miRNA-dependent, as the study found that Dicer deletion led to the complete block of the development of a subset of astrocytes from the ventral neuroepithelium. This was confirmed by the lack of GFAP immunostaining of a small region flanking the floor plate when compared to the control. However, this study did not implicate any specific miRNA species as being involved in the regulation of gliogenesis in the developing spinal cord.

2.2 MicroRNAs in oligodendrocyte development

A 2009 study by Shin et al definitively established that miRNAs were involved in epigenetic regulation of oligodendrocyte function. This was performed by the OL-specific knockout of *Dicer* in postnatal mice. The Dicer protein is essential for synthesis of functional miRNAs and it was found that its elimination in postnatal OLs led to demyelination, oxidative damage, inflammatory astrocytosis and microgliosis in the brain. This phenotype resulted primarily from the disruption of miR-217 and consequent upregulation of its target ELOVL7 (elongation of very long chain fatty acids protein 7). Overexpression of ELOVL7 leads to lipid accumulation in the myelin rich regions of the brain and significant decrease in peroxisomal β-oxidation activity (Shin et al. 2009).

In the first study to analyze the global miRNA expression pattern in oligodendrocytes, Lau et al identified 98 miRNAs expressed by developing oligodendrocytes in the postnatal rat brain and found that 37 of these have an mRNA bias (Lau et al. 2008). In addition, the predicted protein targets of 13 miRNAs were dynamically regulated during OL

development. In particular, the study identified miR-9 as a candidate miRNA with an important regulatory role during the transition from OPC (A2B5+/GalC−) to premyelinating OL (A2B5+/GalC+) and it was found that miR-9 downregulated the expression of the peripheral myelin protein (**PMP**) 22. PMP22 is expressed by Schwann cells and is a component of the compact myelin of the peripheral nervous system. Interestingly, miR-9 is not expressed by Schwann cells.

Another study identified miR-23 and its target, Lamin B1 (**LMNB1**) as a regulatory component of OL differentiation (Lin, Fu 2009). Genomic duplication of *LMNB1* has been implicated in autosomal dominant leukodystrophy (Padiath et al. 2006), a disease characterized by severe myelin loss in the CNS. Nuclear lamins such as LMNB1 interact with heterochromatin to regulate DNA synthesis and transcription and LMNB1 specifically regulates OL function; this was confirmed by the repression of transcription of OL-specific genes such as myelin basic protein (**MBP**), proteolipid protein (**PLP**) and myelin oligodendrocyte glycoprotein (**MOG**) by overexpression of LMNB1. The Lin et al study showed that miR-23 downregulates LMNB1 in normal OLs and that downregulation of LMNB1 by miR-23 was important for the development of OLs from glial cell cultures (Lin, Fu 2009). The effect of LMNB1 on differentiation was confirmed by the observation that increased *LMNB1* gene dosage caused an arrest in differentiation and led to the development of an MBP and PLP negative phenotype. This was hypothesized to be due to either faulty transcription of these genes or defective nuclear export.

A 2010 study by Zhao et al identified miR-219 and miR-338 as OL-specific miRNAs. This was confirmed by the complete absence of these two miRNAs in the spinal cord of Olig1 mutant mice. Fluorescent in situ hybridization revealed that miR-219 and miR-338 positive cells were found only in the white matter of the spinal cord, which is where OLs reside. Further, it was found that these two miRNAs promoted OPC maturation in vitro and in vivo. Transfection of OPC-enriched cultures with these miRNAs led to a significant increase in the number of MBP+ cells (a marker of mature OLs) and a slight decrease in the number of PDGFRα+ OPCs. MiR-219 and miR-338 were also found to be necessary for OL maturation. The study also identified Sox6 and Hes5 as potential protein targets of these miRNAs. These two transcription factors are inhibitors of OL maturation and have been shown to downregulate myelin gene expression and oligodendrocyte maturation (Kondo, Raff 2000, Liu et al. 2006). Sox6 and Hes5 are downregulated by miR-219 and miR-338. The authors suggest that commitment to the oligodendroglial lineage would require downregulation of transcription factors which induce differentiation to other neural cell fates and this was confirmed by the observation that miR-219 and miR-338 also downregulated other proneurogenic transcription factors such as NeuroD1, Isl1 and Otx2. In summary, these two miRs promote OL maturation by simultaneously inhibiting the inhibitors of OL differentiation and transcription factors which promote differentiation to non-OL neural cell fates.

Another recent study confirmed the importance of miR-219 and miR-338 in OL differentiation and maturation (Dugas et al. 2010). In addition, it also found miR-138 to be strongly upregulated during OL differentiation. Among the three, miR-219 was found to be the strongest inducer of OL differentiation and its transfection in a purified OPC population increased the number of differentiated OLs expressing both early (CNP, MBP) and late (MOG) OL markers. OLs induced by transfection with miR-138 only expressed early OL markers. As expected, OL-specific miRNAs were found to target genes that are repressed during OL differentiation. In addition, this study also found that miR-219 downregulates inhibitors of OL differentiation. The validated targets of miR-219 were found to include

PDGFRα, Sox6, FoxJ3 and ZFP238. PDGF has been known to be an OPC mitogen for multiple decades (Besnard et al. 1987). ZFP238 and FoxJ3 had previously not been functionally characterized in OPCs and this study confirmed that FoxJ3 and ZFP238 are inhibitors of OL differentiation by constitutively expressing these genes in OPCs and observing significantly reduced differentiation to the mature OL stage.

A study by Letzen et al was the first to perform a global miRNA analysis of cells isolated from 8 developmental stages from the pluripotent embryonic stem cell state to a mature, myelinating OL state (Letzen et al. 2010). Significantly, this study analyzed the expression of miRNAs in human cells as opposed to rodent models used in prior investigations. The study detected 183 miRNAs over the eight stages of oligodendrocyte maturation and also identified the highest differentially expressed miRNAs at each transition in the pluripotent-to-mature OL differentiation scheme. These differentially-expressed miRNAs were then matched to potential protein targets which revealed proteins such as chromosome 11 open reading frame 9 (**C11orf9**), claudin-11 (**Cldn11**), myelin transcription factor 1-like (**Mytl1**), myelin-associated oligodendrocyte basic protein (**Mobp**), myelin protein zero-like 2 (**Mpzl2**), and discoidin domain receptor tyrosine kinase 1 (**Ddr1**). The study focused on two miRNAs – miR-199a-5p and miR145, both of which show a decreasing expression level during the early to mid OP transition. C11orf9, which appears to be the human analog of the mouse myelin regulatory factor (MRF) was the target protein predicted for these 2 miRNAs. MRF has been shown to be necessary for myelination and OL maturation (Emery et al. 2009). It is thought that the decrease in miR199a-5p levels during OPC maturation may be parallel to the observed increase in MRF expression during the same time window, implying that miR-199a-5p may be one of the regulators of myelination and OPC maturation. MiR-214 was also observed to be strongly downregulated during the early-to-mid OPC transition and its predicted target is myelin-associated oligodendrocyte basic protein (MOBP), an important structural component of myelin.

2.3 Oligodendrocytes, miRNA and disease

Dysfunctional oligodendrocytes due to misregulation by miRNAs have recently been implicated in diseases of the central nervous system. For instance, mutations in several miRNAs have been discovered in the pathogenesis of multiple sclerosis (**MS**) (Junker, Hohlfeld & Meinl 2010). Specifically, miR-219 and miR-338 showed the maximum relative reduction in expression when the entire microRNAome of healthy, mature OLs was compared with chronic, inactivated MS tissue lesions (Junker et al. 2009). Other reports have also shown that miRNAs provide a mechanism for resistance against viral infection, and that miR-122 levels were significantly reduced in OLs infected with the Borna disease virus. This is significant as the Borna disease virus causes neurological disease in animals, and recent findings have implicated that it may also play a role in some human neurological and psychiatric conditions including bipolar disorder and depression (VandeWoude et al. 1990). In this case, overexpression of miR-122 inhibited viral protein synthesis and viral gene transcription and translation (Qian et al. 2010).

In one study to examine the possible role of miRNAs in the development of schizophrenia, the expression levels of 435 miRNAs in postmortem brain samples of schizophrenic patients were compared with those of psychiatric healthy controls (Moreau et al. 2011). In this study, 19% of the miRNAs analyzed showed misexpression in the schizophrenic samples, with these miRNAs mostly being downregulated compared to the healthy controls. On the other hand, a previous study identified differential expression of 28 miRNAs in the postmortem

dorsolateral prefrontal cortex (Brodmanns Area) of schizophrenic individuals (Santarelli et al. 2011). 89% of these 28 miRNAs were upregulated and quantitative PCR was used to validate this finding for miR-328, miR-17-5p, miR-134, miR-652, miR-382, and miR-107. This elevation was linked to the observed upregulation of Dicer activity in these samples. The role of miRNAs in other neurological disorders has been reviewed (Xu, Karayiorgou & Gogos 2010).

3. Methods of oligodendrocyte differentiation

Unlike animal models, the study of oligodendrocyte development in humans has been generally limited in scope by the lack of models both in vivo and vitro that can be utilized to study this process. However, human embryonic stem cells provide an exciting model to help identify mechanisms that regulate the differentiation of oligodendrocytes. This includes elucidating the role of miRNAs in regulating the oligodendrocyte fate. This section describes a novel protocol for generating oligodendrocyte fated cells from human embryonic stem cells which have been developed in part from several published protocols (Nistor et al. 2005, Kerr et al. 2010).

3.1 Materials

3.1.1 Buffers and solutions

PBS: Phosphate buffered saline, without Ca^{+2} and Mg^{+2}, pH 7.0 (Invitrogen).
Trypsin/EDTA: HBSS containing 0.05% trypsin and 0.53 mM EDTA (Sigma).
Collagenase: 1mg/ ml collagenase IV (Invitrogen) in PBS, filtered.

3.1.2 Culture media

Basic culture medium for **MEF**: (Mouse embryonic fibroblast line PMEF-CF1 from Millipore): DMEM high glucose, supplemented with 10% Fetal Bovine Serum (Invitrogen), 2 mM glutamax (Invitrogen), 5 U/ml penicillin–streptomycin (Invitrogen), and 0.1 mM non-essential amino acids (**NEAA**).
Basic culture medium for undifferentiated human embryonic stem cells: DMEM/F12 (Invitrogen), supplemented with 20% Knockout Serum Replacement (Invitrogen), 2 mM glutamax (Invitrogen), 5 U/ml penicillin–streptomycin (Invitrogen), 0.1 mM non-essential amino acids and 3.5 µl β-mercaptoethanol supplemented with basic fibroblast growth factor (**FGF2**).
Basic culture medium for neural and oligodendrocyte progenitors: Neural Basal Media, supplemented with 10% Bovine Serum Albumin in PBS, filtered (Gibco), 0.5% N2 supplement (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma) and 25 ug/ml gentamicin (Invitrogen).

3.1.3 Fixatives

4% Paraformaldehyde: 4g paraformaldehyde in 100 ml PBS. To dissolve the paraformaldehyde, the PBS was preheated at 90°C and sodium hydroxide (NaOH) added drop-wise slowly until the solution turns clear. The solution was cooled down before use.

3.2 Cell culture

Human ESCs were maintained on irradiated mouse embryonic fibroblasts (**MEFs**) in ESC growth media. Prior to differentiation, ESCs were separated from the feeder layer using a

solution of 1 mg/ml collagenase and plated on a 4% matrigel (BD Biosciences) substrate for 5 to 7 days and fed feeder-conditioned media daily. Cells were subsequently differentiated into embryoid bodies (**EBs**), neural progenitors (**NPC**), glial restricted precursors (**GPC**), and oligodendrocyte precursors (**OPC**) and finally mature oligodendrocytes (**OL**).

3.2.1 Preparation of MEF feeder layers

MEFs were maintained in basic MEF culture medium. The feeder cells were prepared the day before ESCs are expanded. The MEFs were plated at a density of 10 x 10^4 cells/cm^2 onto 0.1% w/v gelatin (Stem Cell Technologies) -coated culture dishes. Next morning, the feeder plates were γ-irradiated with a dose of 50 Gy (5000 rads) to induce cell cycle arrest. After irradiation, the culture medium was replaced. MEFs can also be mitotically-inactivated by treatment with mitomycin C (Sigma) at a concentration of 10 μg/ml for 4 hours at 37°C. The ESCs were plated on top of the MEF feeder layer using collagenase as described below. The medium was replaced daily with fresh medium supplemented with growth factors.

3.2.2 Human embryonic stem cells

The derivation and maintenance of human embryonic stem cells has been covered at length and details can be found in the following reviews (Park et al. 2003, Draper et al. 2004, Lu et al. 2006, Suemori et al. 2006, Ludwig et al. 2006, Hoffman, and Carpenter 2005). In brief, a culture plate with mitotically inactivated MEF feeder cells was prepared 24 h before use with ESCs with MEF basic culture media. The medium was removed from a culture dish of ESCs and the dish was washed with PBS. ESC can be expanded using either collagenase or trypsin. In both cases, however, over trypsinization of the colonies should be avoided because it will reduce cell viability. This could occur by incubating cells too long with enzyme and/or over triturating. For one 10 cm plate of ESCs, 1 ml of collagenase or trypsin solution was added and placed in incubator at 37°C for 5 to 10 min. The plate was removed from the incubator, the bottom of the plate scraped with a 5 ml pipette, making sure that all colonies had been lifted off, and then triturated slowly until only very small clumps were obtained. The colonies were not broken up into single cells as unlike their mouse counterparts, human ESCs do not survive well as single cells. The cell suspension was transferred into a 15 ml conical tube, 10 ml complete medium added and centrifuged for 5 to 10 min at 200 g at room temperature (**RT**). The pellet was resuspended in complete medium and placed onto mitotically-inactivated MEF feeder cell plate containing ESC complete medium with FGF2. The medium was replaced daily until ESC colonies reached optimal size for further passage. ESCs usually expanded by 5 fold after 5-7 days.

3.2.3 Embryoid body formation

Neural differentiation of ESCs was initiated via embryoid body (EB) suspensions. Embryonic stem cells were plated and dissociated onto 10 cm matrigel-coated tissue culture dishes as described above for passaging ESCs onto 10 cm plates with MEF. When ESCs were transferred onto matrigel they were grown in basic ESC culture media that had been conditioned on MEFs (live or irradiated) for 24 hours (12 ml of media per 10cm of confluent feeders), filtered and supplemented with 4 ng/ml FGF2. MEF-conditioned media was replaced on a daily basis until colonies were large and tight, almost near confluency in the wells.

To generate embryoid bodies, the medium was removed from a culture dish of ESCs and the dish washed with PBS. For one six well plate of ESCs, the cells were incubated in 0.5 ml

of 1 mg/ml collagenase in PBS (avoid trypsinization as it can affect cell viability) and placed into the incubator at 37ºC for 5 to 10 min. After removing the tube from the incubator, the cells were scraped with a cell scraper or lifter. The cell suspension was transferred to a centrifuge tube very gently, 10 ml of neural basal media supplemented with 200 ng/ml noggin, 20 ng/ml FGF2, and 20 ng/ml FGF4 (all growth factors from R&D Systems) was added and centrifuged for 5 min at 200 g, (RT). The supernatant was removed and the pellet gently resuspended into complete medium and placed onto a non-adherent or low adhesive 10 cm culture plate (Corning).

Neural EBs were fed daily by sedimentation. This was performed by tilting the dish at a 45 degree angle, waiting 5 minutes, and carefully aspirating with a 5ml pipette only half of the media so as to not discard the EBs floating in suspensions. EBs were grown for 10-25 days until they produced a transparent morphology. EBs were characterized by their loss of pluripotent markers, AP, OCT4, and NANOG and initiation of early markers of neuroectodermal fate including SSEA1, SOX1, PAX6 and Islet1 (**Figure 1**). The time may vary for different cell lines to reach this stage. Thus, the time at which is takes EBs to express these markers is dependent on the ESC line employed and so the cells should always be characterized at this point before proceeding forward. This can be performed by RT-PCR and by immunohistochemistry by freezing EBs in cryoprotectant mount media such as O.C.T. (Sakura Finetek TissueTek) and antibody staining (**Table 1**). The timeline provided here is based on our experiences using ESC lines H1 and H9 generated by Thomson et al (Thomson et al. 1998).

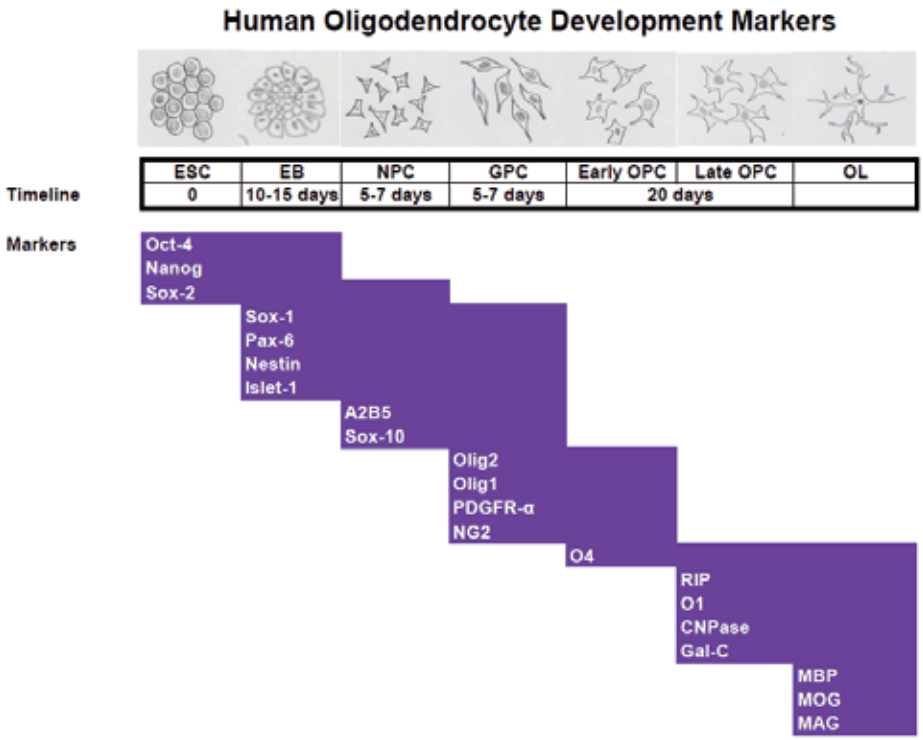

Fig. 1. Timeline of oligodendrocyte differentiation from pluripotent human embryonic stem cells, showing the expression of relevant cellular markers at each stage.

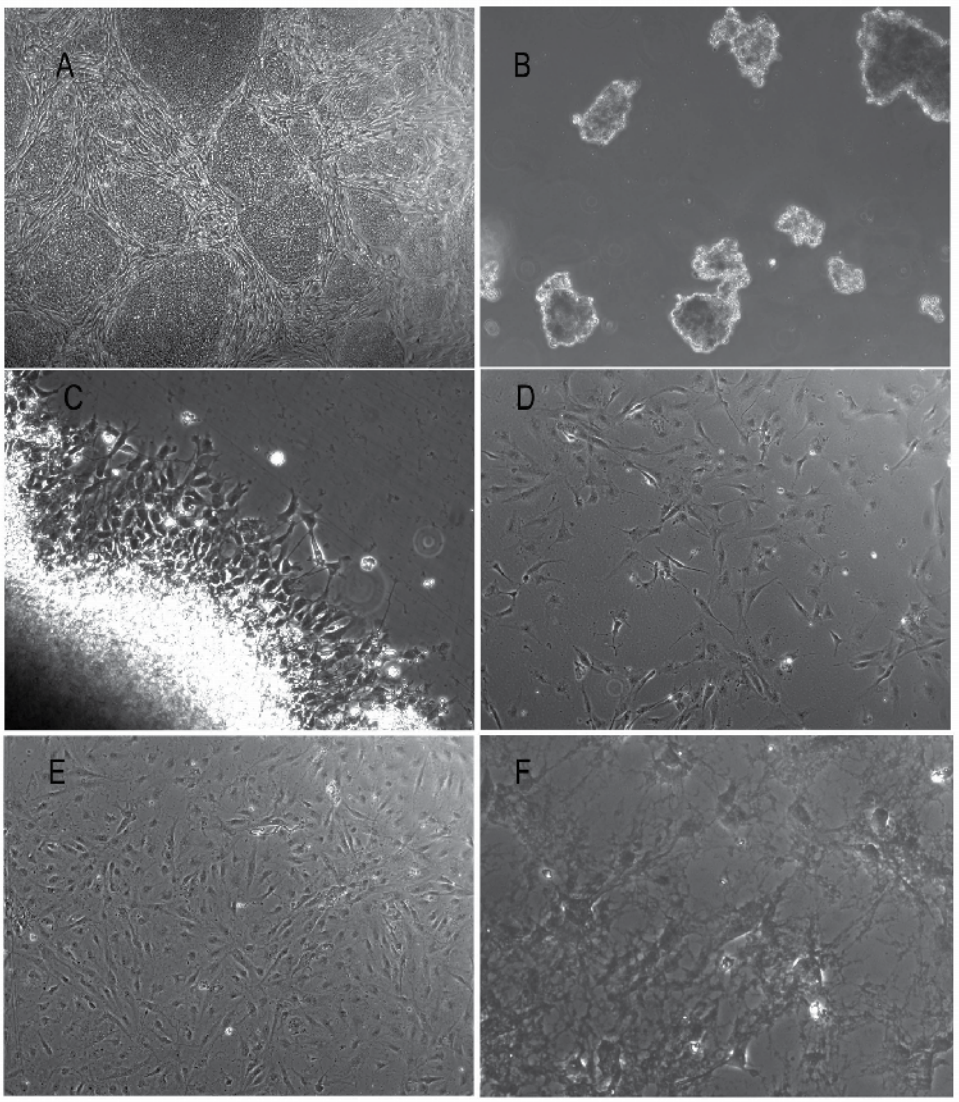

Fig. 2. **Phase contrast images of various stages of oligodendrocyte differentiation.**
A. Pluripotent human embryonic stem cells on MEFs, B. EBs one day after being generated by the scraping method, C. NPCs sprouting from the periphery of a plated EB on matrigel after 24 hrs, D. OPC 9 days after 2 expansions in OPC inducing medium, and OLs one week after being exposed to OL inducing media.

3.2.4 Neural progenitor cells

10 cm tissue culture plates were coated with 3 ml of 4% matrigel in neural basal media for one hour prior to plating EBs. Human ESC Qualified Matrigel Matrix was thawed overnight at 4°C then diluted 1:24 (approximately 4% Matrigel) in cold neural basal medium. Afterwards, the matrigel was removed and EBs plated in 10 ml of neural basal media supplemented with 20 ng/ml FGF2 for 5 to 10 days. In this time, EBs spread over the plate

and became confluent. Most cells expressed neural fated markers including nestin and neural surface marker (A2B5) (**Figure 1**). NPCs at this stage were capable of deriving cells with both neuronal and glial fates. NPCs were fed daily.

3.2.5 Glial progenitor cells

NPCs were passaged in 1 to 3 splits for expansion using collagenase as described below (*Passaging Cells*) and replated on matrigel coated plates for differentiation into glial progenitor fate. Twenty-four hours after replating, media was changed to neural basal media supplemented with 20ng/ml EGF. Cells were fed daily for 10-14 days. GPCs began to express NG2, PDGFRα, and Olig1 (**Figure 1**). These cells can differentiate into either astrocytes or oligodendrocytes at this stage depending on the growth factors employed.

3.2.6 Oligodendrocyte progenitor cells

To promote differentiation into OPC, the media was supplemented with 20 ng/ml PDGF-AA. Cells were fed daily and begin to express OPC markers O1, O4, CNP, RIP, and GalC after one week (**Figure 1**). After 7–10 days the OPC morphology could be detected and was visible on the culture dish (**Figure 2**). By two weeks greater than 95% pure OPC could be obtained. Pure populations of OPCs can be obtained by FACs or MACs using O1 and O4 antibody selection using standard procedures. Once an OPC line is derived, cells can be expanded using 1 mg/ml collagenase in PBS onto gelatin-coated plates for several months before they reach senescence.

3.2.7 Oligodendrocytes

Terminal differentiation to mature oligodendrocytes was initiated by the addition of 50 ng/ml T3 (triiodothyronine, Sigma) and removal of PDGF-AA from the neural basal medium. Mature OLs demonstrate complex morphology after 3-5 days and express myelin-associated proteins such as MAG, MOG, MOBP, and MBP (**Figure 1**). These cells do not survive as long as the progenitor cells and begin to die off after the first week. In total, their survival under these culture conditions is between 1-3 weeks after maturity.

3.2.8 Passaging cells

3 ml of 0.1% matrigel is placed on a 10cm tissue culture plate. The medium is removed from a culture dish of OL-fated cells and the dish washed with PBS. For one six well plate, the cells were incubated in 0.5 ml of 1 mg/ml collagenase in PBS (avoid trypsinization as it can affect cell viability) and cells placed into the incubator at 37ºC for 5 to 10 min. After removing the tube from the incubator, the cells were scraped with cell scraper or lifter. The suspension was mixed by gently pipetting up and down the cell clumps until a single-cell suspension was obtained. Importantly, to reduce cell mortality, the extent of cell disruption was controlled by pipetting only 5 to 10 times and with a 5ml pipette. The cell suspension was transferred to a centrifuge tube and centrifuge for 5 min at 200g at RT. The supernatant was removed and pellet resuspended in complete medium and placed onto the matrigel-coated plates. Medium was replaced daily until the cells were confluent before passaging onto a larger culture dish plate.

3.2.9 Freezing cells

From one confluent 6-well ESCs, NPCs, GPCs or OPCs were dissociated using collagenase as described above. These were rinsed in neural basal media for neural cells and ESC media

for ESCs at 200g for 5 min. The supernatant was aspirated and the pellet resuspended in freshly prepared 1X Freezing Media (for neural cells: 50% Neural Basal Media, 40% FCS, 10% DMSO; ESC freezing media**:** 50% Fetal Bovine Serum (Defined, Hyclone), 40% Neural Basal Media (Invitrogen) and 10% DMSO (Sigma), filtered). 1 ml of freezing media was added dropwise, mixing well after each addition. The suspension was transferred into sterile freezing vials, and placed into cryovessels. The cells were frozen overnight at -80^{o}C, and then transferred to the liquid nitrogen freezer after 24 hours.

3.2.10 Thawing out cells

The vial of frozen cells was removed from the nitrogen freezer and transferred to a 37^{o}C heat water bath to thaw by gentle shaking (thawing generally takes only 1-2 minutes). The suspension was transferred into 15 cc conical tube and 10 ml of culture media added and centrifuged at 200g for 5 min. The supernatant was removed, culture medium added, and plated directly onto a fresh matrigel coated plate for NPs, GPCs and OPCs or feeder plate for ESCs.

Name	**Company**	**Product Code**
Oct-4	BD Biosciences	611203
Nanog	EBioscience	14-5768-82
Sox-2	Abcam	ab15830-100
Sox-1	Abcam	ab22572
Pax-6	Abcam	ab5790
Nestin	Millipore	mab5326
Islet-1	DSHB	394D5
A2B5	Millipore	mab312r
Sox-10	Santa Cruz Bio.	sc17343
Olig1	Millipore	mab5540
PDGFR-a	Abcam	ab61219
NG2	Millipore	ab5320
O4	Millipore	mab345
O1	Millipore	mab344
RIP	Abcam	ab2035
CNPase	Millipore	mab326r
MBP	Abcam	ab7349-2

Table 1. Specific antibodies for oligodendrocyte characterization

3.3 Cell characterization

The identity of a cell in the differentiation pathway from pluripotent embryonic stem cells to mature oligodendrocytes is most commonly studied by its morphology, its gene expression and by analyzing the expression of protein markers by immunodetection.

Morphologically, undifferentiated ESCs generate colonies on top of feeders, NPC are more columnar or cuboidal in shape while GPCs and OPCs generate bipolar cells. Mature OLs demonstrate a complex network of dendritic processes (**Figure 2**).

Immunofluorescence (**IF**) staining of plated cells is more accurate morphology and the simplest method of analyzing protein expression to define the different stages of OL potential (**Table 1** lists sources of reliable antibodies for OL characterization). Here we present a standard protocol for IF staining for OL fated cells. **Figure 3** demonstrates staining at various stages of OL differentiation.

Cells were plated onto 24-well culture plates. Before cells became confluent, cells were washed twice in PBS and the excess PBS was removed. Cells were fixed in 4% PFA in PBS for 10 minutes at RT. The fixative solution was removed and wells washed twice with PBS for 5 min each. Cells were permeabilized and blocked with PBS solution containing 0.2% (w/v) Triton X-100 and bovine serum albumin (BSA; Sigma) at a concentration 1% (w/v) in PBS and incubated for 5 minutes at RT (for cells surface markers this step was skipped). Primary antibody (1:50 dilution) was added after dilution in antibody dilution buffer (PBS with 0.1% (w/v) BSA) and incubated for 1 hr at RT. Sample was washed three times with PBS for 5 minutes each. Appropriate secondary antibody was added (Alexa488 or Alexa546 conjugated antibodies, 1:200; Molecular Probes) in antibody dilution buffer and incubated in a humidified dark chamber for 1 hr at RT. Washed three times with PBS for 5 min each. Nuclei were stained with DAPI solution (Sigma) for 10 min at RT in a humidified chamber in the dark. DAPI solution was aspirated and 0.5 ml PBS was added to each well. Samples were examined under a fluorescence microscope with appropriate filters within 24 hrs as signal diminishes very rapidly. After 2 to 3 days most of the fluorescence signal is bleached. Sealant tape and aluminum foil was applied around the plates and stored in the dark at 4^{o}C (short term storage only).

4. Results

4.1 Morphological analysis of differentiated cells

As shown in **Figure 2**, oligodendrocytes were successfully differentiated from human embryonic stem cells by our protocol described above. Embryoid bodies (Fig. 2b) were generated from pluripotent embryonic stem cells colonies (Fig. 2a). Plating these embryoid bodies on matrigel led to their adhesion and appearance of neural progenitor cells (Fig. 2c), which were then differentiated to glial progenitors (Fig. 2d) and subsequently oligodendrocyte progenitor cells (Fig 2e). Removal of all growth factors from the media led to the appearance of mature oligodendrocytes (Fig. 2e).

4.2 Immunocytochemistry of differentiated cells

EBs were characterized by their loss of pluripotent markers, AP, OCT4, and NANOG and initiation of early markers of neuroectodermal fate including SSEA1, SOX1, PAX6 and Islet1 (**Figure 1**). Upon contact with matrigel, EBs spread over the plate and became confluent. Most cells expressed neural fated markers including nestin and neural surface marker (A2B5)(**Figure 1**). After 5-7 days in NP media and addition of EGF, GPCs appeared and began to express NG2, PDGFRα, and Olig1 (**Figure 1**). Subsequent addition of PDGF-AA after 7 days led to the appearance of OPC markers O1, O4, CNP, RIP, and GalC (**Figure 1**). Finally, mature oligodendrocytes express myelin-associated proteins such as MAG, MOG, MOBP, and MBP (**Figure 1**).

Immunofluorescent images for a few of these markers are shown in **Figure 3.**

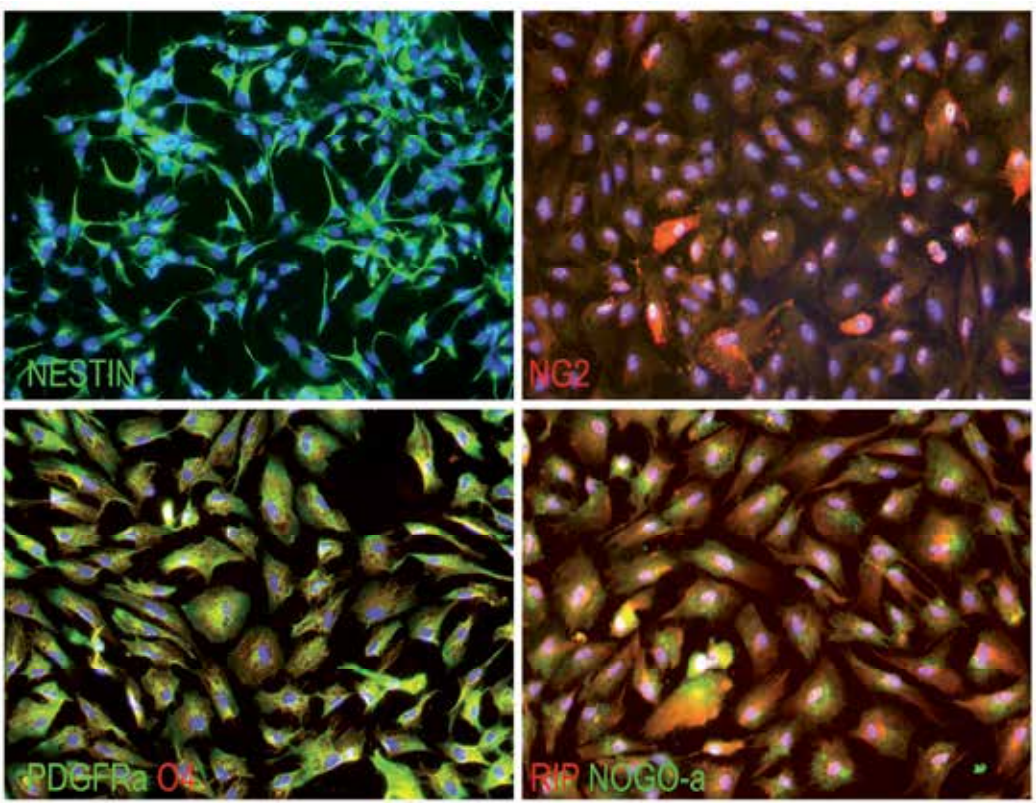

Fig. 3. Indirect immunofluorescence microscopy to examine differentiation of human ESCs into the oligodendrocyte lineage. The nuclei are stained blue by DAPI. Nestin (green) is shown for neural progenitors while NG2 (red), PDGFRa (green) O4 (red), RIP (red) and NOGO-a (green) are expressed by 5-day old oligodendrocyte progenitor cells.

5. Conclusion

In conclusion, a novel protocol adapted from previously published ones for oligodendrocyte development from pluripotent human embryonic stem cells has been developed. The cells express the established neural, glial and oligodendroglial markers as they advance along the differentiation timeline and we have confirmed this using indirect immunofluorescent microscopy.

Also, we have reviewed the current state of knowledge about microRNA regulation of oligodendrocyte differentiation from pluripotent cells and myelination of CNS neurons by those cells. It is hoped that the protocol for obtaining OLs in high purity will aid in the unraveling of more mechanisms of miRNA regulation of myelination so that this knowledge can eventually be used for the development of a more efficient approach to the stem cell based therapy of spinal cord injuries and other demyelinating disorders.

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7

Stem-Cell Therapy for Retinal Diseases

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1. Introduction

1.1

Stem cell (SC) therapy is not a new concept. In the aftermath of the bombings of Hiroshima and Nagasaki in 1945, researchers discovered that bone marrow transplanted into irradiated mice produced hematopoiesis (Lorenz, 1951). Hematopoietic stem cells (HSCs) were first identified in 1961 and their ability to migrate and differentiate into multiple cell types was documented (Till, 1961).

Distinct SC types have been established from embryos and identified in the fetal tissues and umbilical cord blood (UCB) as well as in specific niches in many adult mammalian tissues and organs such as bone marrow (BM), brain, skin, eyes, heart, kidneys, lungs, gastrointestinal tract, pancreas, liver, breast, ovaries, prostate and testis (Siqueira, 2010). All SCs are undifferentiated cells that exhibit unlimited self renewal and can generate multiple cell lineages or more restricted progenitor populations which can contribute to tissue homeostasis by replenishing the cells or to tissue regeneration after injury (Lanza, 2004; Mimeault, 2006).

Several investigations (Mimeault, 2006; Ortiz-Gonzalez, 2004; Trounson, 2006) have been carried out with isolated embryonic, fetal and adult SCs in a well-defined culture microenvironment to define the sequential steps and intracellular pathways that are involved in their differentiation into the specific cell lineages. More particularly, different methods have been developed for the *in vitro* culture of SCs, including the use of cell feeder layers, cell-free conditions, extracellular matrix molecules such as collagen, gelatin and laminin and diverse growth factors and cytokines (Mimeault, 2004; Siqueira, 2010).

1.2 Overview of the retinal anatomy

The retina is approximately 0.5 mm thick and lines the back of the eye. The **optic nerve** contains ganglion cell axons running to the brain and incoming blood vessels that open into the retina to vascularize the retinal layers and neurons. A radial section of a portion of the retina reveals that the **ganglion cells** (the output neurons of the retina) lie innermost in the retina closest to the lens and front of the eye, and the photosensors (the **rods** and **cones**) lie outermost in the retina against the retinal-pigment epithelium (RPE) and choroid. Light must, therefore, travel through the thickness of the retina before striking and activating the rods and cones. Subsequently, the absorption of photons by the visual pigment of the photoreceptors is translated first into a biochemical message and then into an electrical

message that stimulates all of the succeeding neurons of the retina. The retinal message concerning the photic input and some preliminary organization of the visual image into several forms of sensation are transmitted to the brain from the spiking discharge pattern of the ganglion cells (Kolb, 2005).
RPE cells support photoreceptor survival and are involved in, for example, ion and nutrient transport, formation of the blood-retina barrier and light absorption.
They are also responsible for phagocytosis of the photoreceptor outer segments, which is important for the renewal of photoreceptor membranes. Interestingly, it has been demonstrated in a chicken model that, RPE in the postnatal stage of life is similar to that found in the embryonic retina with regard to specific gene expression.
Furthermore, the generation and *ex vivo* expansion of RPE from human embryonic stem cells (hESCs) has been extensively studied and characterized. Moreover, hESC-derived RPE cells have been demonstrated to be functional in *ex vivo* conditions. More recently, the *in vitro* differentiation of RPE and photoreceptors from human induced pluripotent stem (iPS) cell cultures provid another potential tool for transplantation purposes and additionally enables avoidance of host immune reactions (Machalinska, 2009).

1.3 Retinal diseases

Age-related macular degeneration (AMD), glaucoma and diabetic retinopathy are the three most common causes of visual impairment and legal blindness in developed countries (Bunce, 2006). One common denominator of these conditions is progressive loss of the neural cells of the eye [photoreceptors, interneurons and retinal ganglion cells (RGC)] and essential supporting cells such as the RPE. Retinal dystrophies [retinitis pigmentosa (RP), Stargardt's disease, Best disease, Leber congenital amaurosis, etc.] all evolve with early loss of photoreceptors and subsequent loss of RGC. Recent years have seen enormous progress in the treatment options that stop the progression of AMD from a neovascular state to fibrosis, that slow down the progression of glaucoma by reducing intraocular pressure, and that prevent progression of diabetic retinopathy by optimizing glycemic control and treat retinal neovascularization early (Chakravarthy, 2010; Maier, 2005; O'Doherty, 2008; Mohamed, 2007). However, irreversible visual loss still occurs in a significant proportion of cases. Research is aimed at developing novel treatments using neuroprotective and regenerative strategies.
SCs can potentially be used for both neuroprotection and cell replacement. Intravitreal delivery of neurotrophic factors slows down photoreceptor degeneration in rodent models of RP, RGC loss in glaucoma models and optic nerve and optic tract trauma, but the effect may be temporary. Slow-release preparations and gene therapy approaches used to induce retinal cells to secrete neurotrophic factors are two ways to induce longer-term effects. A third option is to use SC as long-term delivery agents, possibly encapsulated in a device, because many SC either secrete neurotrophins naturally or can be genetically engineered to do so (Otani, 2004; Dahlmann-Noor, 2010).
Progress has also been made in the field of photoreceptor, RPE and RGC replacement by SC and progenitor cells, although long-term restoration of visual function has been confirmed. The recent discoveries that human fibroblasts can be ''reprogrammed'' to behave like embryonic SC and that adult eyes harbor retinal progenitor cells, also increase the potential availability of SC for transplantation, including autologous transplantation and stimulate intrinsic ''self-regeneration, '' which could potentially overcome a lot of the problems associated with non-autologous transplantation in humans (Dahlmann-Noor, 2010).

2. Potential sources of stem cells for cell therapy in retinal diseases

2.1 Bone marrow-derived stem cells

Bone marrow-derived SCs have been proposed as a potential source of cells for regenerative medicine (Machalinska, 2009; Enzmann, 2009). This is based on the assumption that HSCs isolated from BM are plastic and are able to "transdifferentiate" into tissue-committed SCs for other organs (e. g., heart, liver or brain). Unfortunately, the concept of SC plasticity was not confirmed in recent studies and previously encouraging data demonstrating this phenomenon *in vitro* could be explained by a phenomenon of cell fusion or, as believed by our group, by the presence, of heterogeneous populations of SCs in BM (Müller-Sieburg, 2002; Spangrude 1988). The identification of very small, embryonic-like SCs in BM supports the notion that this tissue contains a population of primitive SCs, which, if transplanted together with HSCs, would be able to regenerate damaged tissues in certain experimental settings. Cells from BM are easily and safely aspirated. After administering local anesthesia, about 10 mL of the BM is aspirated from the iliac crest using a sterile BM aspiration needle; subsequently mononuclear bone marrow SCs are separated using the Ficoll density separation method (Siqueira, 2010) (Figure 1).

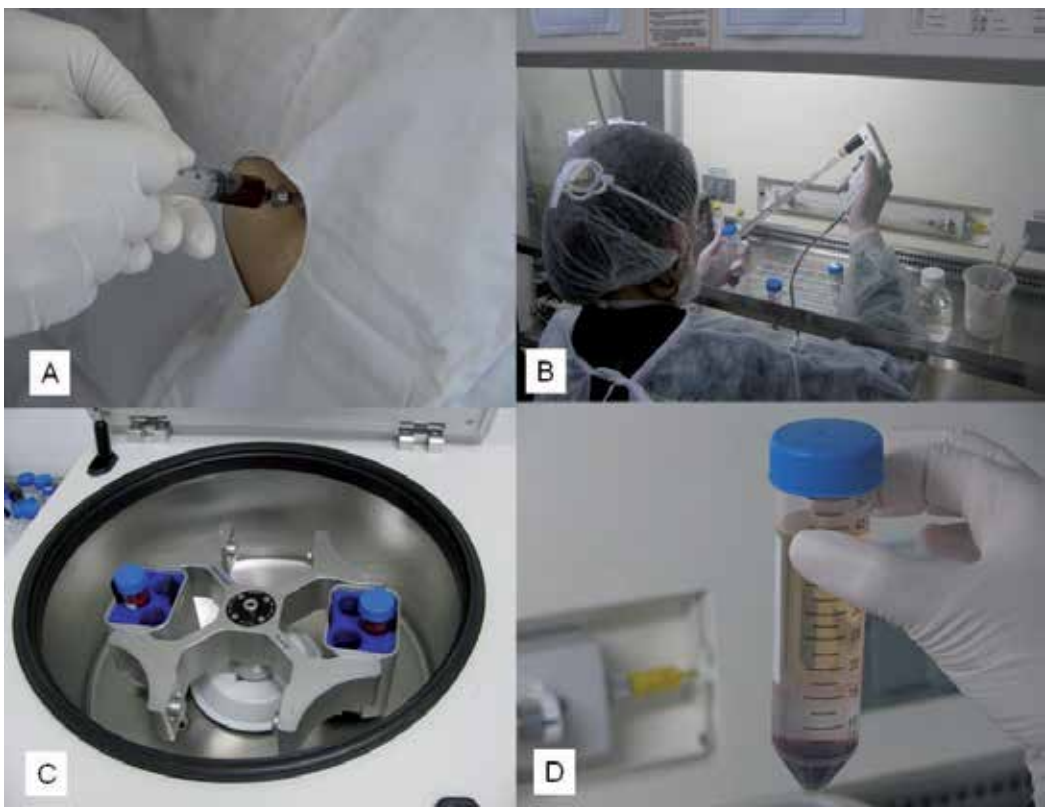

Fig. 1. Sequence of photos showing the collection of bone marrow (A) and the initial separation of the mononuclear cells using Ficoll-Hypaque gradient centrifugation (B) (C) (D) (Siqueira RC 2010)

SC-based therapy has been tested in animal models for several diseases including neurodegenerative disorders, such as Parkinson disease, spinal cord injury, and multiple sclerosis. The replacement of lost neurons that are not physiologically replaced is pivotal for therapeutic success. In the eye, degeneration of neural cells in the retina is a hallmark of such widespread ocular diseases as AMD and RP. In these cases the loss of photoreceptors that occurs as a primary event as in RP or secondary to loss of RPE, as in AMD, leads to blindness (Machalinska 2009; Siqueira 2010).

BM is an ideal tissue for studying SCs because of its accessibility and because proliferative dose-responses of bone marrow-derived SCs can be readily investigated. Furthermore, there are a number of well-defined mouse models and cell surface markers that allow effective studies of hematopoiesis in healthy and injured mice. Because of these characteristics and the experience of BM transplantation in the treatment of hematological cancers, bone marrow-derived SCs have also become an important tool in regenerative medicine. The BM harbors at least two distinct SC populations: HSCs and multipotent marrow stromal cells (MSC).

2.1.1 Hematopoietic stem cells

HSCs are multipotent SCs that give rise to all the blood cell types including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cells).

HSCs are found in the BM of adults, which includes in femurs, hips, ribs, the sternum and other bones. Cells can be obtained directly from the hip using a needle and syringe (Figure 1), or from the blood following pretreatment with cytokines, such as G-CSF (granulocyte colony stimulating factors), that induce cells to be released from the BM compartment. Other sources for clinical and scientific use include UCB and placenta (Ratajczak, 2004; Müller-Sieburg 2002).

In reference to phenotype, HSCs are identified by their small size, lack of lineage markers, low staining (side population) by vital dyes such as rhodamine 123 (rhodamine-dull, also called rholo) or Hoechst 33342 and presence of various surface antigenic markers, many of which belong to the cluster of differentiation series: CD34, CD38, CD90, CD133, CD105, CD45 and also c-kit and SC factor receptor (Müller-Sieburg, 2002; Nielsen, 2009; Kuçi, 2009; Challen 2009 ; Voltarelli 2000; Voltarelli 2003). Otani (2004) demonstrated that whenever a fraction of mouse or human adult bone marrow–derived SCs [lineage-negative hematopoietic stem cells (Lin-HSCs)] containing endothelial precursors stabilizes and rescues retinal blood vessels that would ordinarily completely degenerate, a dramatic neurotrophic rescue effect is also observed. Retinal nuclear layers are preserved in two mouse models of retinal degeneration, *rd1* and *rd10*, and detectable, albeit severely abnormal, electroretinogram recordings are observed in rescued mice at times when they are never observed in control-treated or untreated eyes. The normal mouse retina consists predominantly of rods, but the rescued cells after treatment with Lin-HSCs are nearly all cones. Microarray analysis of rescued retinas demonstrates significant upregulation of many antiapoptotic genes, including small heat shock proteins and transcription factors.

Some reports have demonstrated the clinical feasibility of the intravitreal administration of autologous bone marrow-derived mononuclear cells (ABMC) in patients with advanced degenerative retinopathies (Jonas, 2008 and 2010). More recently, our group conducted a prospective phase I trial to investigate the safety of intravitreal ABMC in patients with retinitis pigmentosa or cone-rod dystrophy, with promising results (Siqueira, 2011).

2.1.2 Multipotent Mesenchymal Stromal Cells (Mesenchymal Stem Cells)

Mesenchymal stem cells (MSCs) are progenitors of all connective tissue cells. In adults of multiple vertebrate species, MSCs have been isolated from BM and other tissues, expanded in culture and differentiated into several tissue-forming cells such as bone, cartilage, fat, muscle, tendon, liver, kidney, heart, and even brain cells.

According to the International Society for Cellular Therapy (Horwitz, 2005), there are three minimum requirements for a population of cells to be classified as MSCs. The first is that MSCs are isolated from a population of mononuclear cells on the basis of their selective adherence to the surface of the plastic of culture dishes, differing in this respect to bone marrow hematopoietic cells, a disadvantage of this method of identification is the possible contamination by hematopoietic cells and cellular heterogeneity with respect to the potential for differentiation. The second criteria is that CD105, CD73 and CD90 are present and that CD34, CD45, CD14 or CD11b, CD79, or CD19 and HLA-DR are not expressed in more than 95% of the cells in culture. Finally, the cells can be differentiated into bone, fat and cartilage (Phinney, 2007).

A number of studies have shown that bone-marrow-derived MSCs can differentiate into cells expressing photoreceptor proteins when injected into the subretinal space (Gong, 2008; Castanheira, 2008). Interestingly, it has been suggested that rat MSCs can be made to express photopigment (rhodopsin) *in vitro* simply by adding epidermal growth factor to the culture media (Zhang, 2008). Additionally, though other retina-relevant cell types have been engineered, a number of studies have shown that BM or adipose tissue MSCs are converted to RPE (Gong, 2008; Arnhold, 2006; Vossmerbaeumer 2009). As with work on other neuronal phenotypes, however, there has now been a reassessment of the ability of MSCs to differentiate into functionally useful retinal cells. Some studies have shown that transplanted bone marrow MSCs do not differentiate into neural retinal cells (YU, 2006). In an *in vitro* rat retina-explant model, untreated MSCs seemed to transdifferentiate into microglia109 in a way reminiscent of earlier work on MSC transplants in other neurological tissue (Azizi 1998). Some limited improvement was seen with pre-treatment with BDNF, NGF, and bFGF in terms of morphological differentiation into retinal neurons and expression of NF200, GFAP, PKC-alpha, and recoverin, but these cells did not express Rhodopsin (Erices, 2000).

In an ischemic retina rodent model, MSCs injected into the vitreous cavity have been shown to mature (with expression of neuron-specific enolase and neurofilament) and secrete CNTF, bFGF, and BDNF for at least 4 weeks (Li, 2009). Animal studies have also demonstrated that subretinal transplantation of MSCs delays retinal degeneration and preserves retinal function through a trophic response (Inoue, 2007). UCB-derived MSCs have also been shown to be neuroprotective of rat ganglion cells (Zwart, 2009). Very recently, the intravenous administration of bone marrow-derived MSCs was shown to prevent photoreceptor loss and preserve visual function in the RCS rat model of RP.

A role for genetically-modified MSCs may emerge in the treatment of subretinal neovascularization. It has been shown that bone-marrow-derived MSCs accumulate around subretinal membranes induced by retinal laser burns.

Intravenous injection of mouse bone-marrow MSCs genetically engineered to secrete pigment epithelium derived factor resulted in smaller neovascular complexes (Hou, 2010).

2.2 Induced pluripotent stem cells

Current methods of producing SCs from adult somatic cells offer an alternative cell source for transplantation. Induced pluripotent stem (iPS) cells are morphologically identical to

embryonic SCs, display similar gene expression profiles and epigenetic status and have the potential to form any cell in the body (Takahashi, 2006 and 2007; Yu, 2007). These cells have been employed to generate cells for the treatment of various diseases including diabetes, cardiovascular disease, sickle cell anemia, Parkinson's disease and hemophilia (Zhang, 2009; Hanna, 2007; Xu, 2009; Wernig, 2008). Meyer et al. 2009 recently showed that iPS cells can differentiate into retinal cell types whilst a paper by Buchholz et al. 2009 showed that human iPS cells can be differentiated into retinal pigment epithelial cells which display functionality in vitro.

Carr (2009) demonstrated that iPS cells can be differentiated into functional iPS-RPE and that transplantation of these cells can facilitate the short-term maintenance of photoreceptors through phagocytosis of photoreceptor outer segments. Long-term visual function is maintained in this model of retinal disease even though the xenografted cells are eventually lost, suggesting a secondary protective host cellular response.

While this particular line of iPS-RPE cells cannot be used as a direct therapy due to viral insertions of pluripotency genes, recent advances in iPS cell reprogramming technology, including the use of small molecules (Huangfu, 2008; Shi, 2008; Li, 2009), piggyBac transposition (Woltjen, 2009; Kaji, 2009), non-integrating episomal vectors (Yu, 2009) and manipulation of endogenous transcription factors (Balasubramanian, 2009) should eliminate the risks associated with the integration of SC genes into the genome. Furthermore, the finding that blood cells can be used to derive iPS cells (Loh, 2009) may remove the need for the invasive biopsies required to collect somatic cells and accelerate the ethical production of SC-derived tissue for therapeutic use.

2.3 Human Embryonic Stem Cells

The human embryonic stem cell (hESC) is defined as a cell that can both renew itself by repeated division and differentiate into any one of the 200 or more adult cell types in the human body. An hESC cell arises from the eight-cell stage morula. Outside of normal development, hESCs have been differentiated *in vitro* into neural cell types and even pigmented epithelium, although controlling their differentiation has proven challenging. Several hESC lines exist and are supported by public research funds. The use of hESCs has significant limitations, including ethical issues, and a risk of teratoma formation, but the chief problem is that we are still struggling to understand the developmental cues that differentiate hESCs into the specific adult cell types required to repair damaged tissues (MacLaren, 2007).

Nistor et al. (2010) showed for the first time that three-dimensional early retinal progenitor tissue constructs can be derived from hESCs. Three-dimensional tissue constructs were developed by culturing hESC-derived neural retinal progenitors in a matrix on top of hESC-derived RPE cells in a cell culture insert. An osmolarity gradient maintained the nutrition of the three-dimensional cell constructs. Cross-sections through hESC-derived tissue constructs were characterized by immunohistochemistry for various transcription factors and cell markers. Tissue constructs derived from hESC expressed transcription factors characteristic of retinal development, such as pax6, Otx2, Chx10, retinal RAX; Brn3b (necessary for differentiation of retinal ganglion cells) and crx and nrl (role in photoreceptor development). Many cells expressed neuronal markers including nestin, beta-tubulin and microtubule-associated protein.

Assessments of safety and efficacy are crucial before hESC therapies can move into the clinic. Two important early potential hESC applications are the use of retinal pigment

epithelium (RPE) for the treatment of age-related macular degeneration and Stargardt's disease, an untreatable form of macular dystrophy that leads to early-onset blindness. Long-term safety and function of RPE from hESCs in preclinical models of macular degeneration was demonstrated by Lu et al. (2009).

They showed long-term functional rescue using hESC-derived RPE in both RCS rats and Elov14 mice, which are animal models of retinal degeneration and Stargardt's disease, respectively. Good manufacturing practice-compliant hESC-RPE survived subretinal transplantation in RCS rats for prolonged periods (> 220 days). The cells sustained visual function and photoreceptor integrity in a dose-dependent fashion without teratoma formation or untoward pathological reactions.

Near-normal functional measurements were recorded at > 60 days survival in RCS rats. To further address safety concerns, a Good laboratory practice-compliant study was carried out in the NIH III immune-deficient mouse model. Long-term data (spanning the life of the animals) showed no gross or microscopic evidence of teratoma/tumor formation after subretinal hESC-RPE transplantation.

These results suggest that hESCs could serve as a potentially safe and inexhaustible source of RPE for the efficacious treatment of a range of retinal degenerative diseases.

In 2010, the US Food and Drug Administration (FDA) granted Orphan drug designation for RPE cells of Advanced Cell Technology, Inc. (ACT) to initiate its Phase 1/2 clinical trials

Fig. 1. Intravitreal injection of autologous bone marrow–derived stem cells in a patient with retinitis pigmentosa (Siqueira RC, 2010)

using retinal pigment epithelial (RPE) cells derived from hESCs to treat patients with Stargardt's Macular Dystrophy (SMD). Moreover, in 2011 the company received a positive opinion from the Committee for Orphan Medicinal Products (COMP) of the European Medicines Agency (EMA) towards designation of this product as an orphan medicinal product for the treatment of Stargardt's disease.

	Type of study	**Type of injury or illness**	**Route used**	**Type and source of cells**
Atsushi Otani et al.	Experimental study in animals	Mice with retinal degenerative disease	Intravitreous transplantation	Adult bone marrow–derived lineage-negative hematopoietic stem cells
Wang S et al.	Experimental study in animals	Retinitis pigmentosa	Tail vein	Pluripotent bone marrow-derived mesenchymal stem cells
Li Na & Li Xiao-rong & Yuan Jia-qin	Experimental study in animals	Rat injured by ischemia/reperfusion	Intravitreous transplantation	Bone marrow mesenchymal stem cells
Uteza Y, Rouillot JS, Kobetz A, et al.	Experimental study in animals	Photoreceptor cell degeneration in Royal College of Surgeon rats	Intravitreous transplantation	Encapsulated fibroblasts
Zhang Y, Wang W	Experimental study in animals	Light-damaged retinal structure	Subretinal space	Bone marrow mesenchymal stem cells
Tomita M	Experimental study in animals	Retinas mechanically injured using a hooked needle	Intravitreous transplantation	Bone marrow-derived stem cells
Meyer JS et al.	Experimental study in animals	Retinal degeneration	Intravitreous transplantation	Embryonic stem cells
Siqueira RC et al.	Experimental study in animals	Chorioretinal injuries caused by laser red diode 670N-M	Intravitreous transplantation	Bone marrow-derived stem cells
Wang HC et al.	Experimental study in animals	Mice with laser-induced retinal injury	Intravitreous transplantation	bone marrow-derived stem cells
Johnson TV et al.	Experimental study in animals	Glaucoma	Intravitreous transplantation	Bone marrow-derived mesenchymal stem cell
Castanheira P et al.	Experimental study in animals	Rat retinas submitted to laser damage	Intravitreous transplantation	Bone marrow-derived mesenchymal stem cell
Jonas JB et al.	Case report	Patient with atrophy of the retina and optic nerve	Intravitreous transplantation	bone marrow-derived mononuclear cell transplantation

	Type of study	Type of injury or illness	Route used	Type and source of cells
Jonas JB et al.	Case report	Three patients with diabetic retinopathy, age related macular degeneration and optic nerve atrophy (glaucoma)	Intravitreous transplantation	bone marrow-derived mononuclear cell transplantation
Siqueira RC et al. gov clinical trial. NCT01068561	Clinical Trial Phase I	Five patients with retinitis pigmentosa	Intravitreous transplantation	bone marrow-derived mononuclear cell transplantation
Siqueira RC et al. Ethics committee of Brazil. Register: 16018	Clinical trial Phase II	50 patients with retinitis pigmentosa	Intravitreous transplantation	bone marrow-derived mononuclear cell transplantation
Siqueira RC et al. Ethics committee of Brazil. Register 15978	Clinical trial Phase I/II	Ten patients with macular degeneration	Intravitreous transplantation	bone marrow-derived mononuclear cell transplantation
Advanced Cell Technology http://www. advancedcell. com/	Clinical trial Phase I/II	12 patients with Stargardt's Macular Dystrophy	Subretinal transplantation	retinal pigment epithelial (RPE) cells derived from human embryonic stem cells (hESCs)

Table 1. Clinical and experimental studies using cell therapy for retinal diseases

3. Conclusion

Stem cells maintain the balance between somatic cell populations in various tissues and are responsible for organ regeneration. The remarkable progress of regenerative medicine in the last few years indicates promise for the use of stem cells in the treatment of ophthalmic disorders. Based on the above mentioned mechanisms, experimental and human studies with intravitreal bone marrow-derived stem cells have begun (Table 1). The history starts to be written in this very promising therapeutic field.

4. Acknowledgment

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Part 3

Cardiac and Other Myogenic Differentiation

8

Transcriptional Networks of Embryonic Stem Cell-Derived Cardiomyogenesis

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1. Introduction

Embryonic stem cells are pluripotent cells that, if cultured under specific conditions, give rise to clusters of beating cardiomyocytes. Beating cardiomyocytes, also dubbed beating areas, display gene expression profiles and functional properties similar to adult cardiomyocytes. In line with this, a wide heterogeneity has been observed in embryonic stem cell-derived cardiomyocytes, resembling thus the distinct characteristics of atrial, ventricular and conductive cardiomyocytes.

It has been recently demonstrated that two distinct cardiogenic precursor cells contribute to the developing heart. The first heart field contributes to the cardiac linear straight tube while a second population of cells adds cells to both arterial and venous pole of the cardiac tube, delimiting thus the second heart field. As cardiogenesis advances the first heart field mainly gives rise to the left ventricle, whereas the second heart field contributes to the right ventricle, outflow tract and the atrial chambers. A third population of cells, with distinct gene expression fingerprint has been demonstrated to form the inflow tract, suggesting the possible existence of a third heart field.

Over the last years we have gained insights about the transcriptional mechanisms that govern the distinct heart fields, however, our understanding about if such endogenous program is recapitulated in embryonic stem cell-derived cardiomyogenesis remains elusive. Within this review we elaborate about the current state-of-the-art of the transcriptional networks that operate during embryonic stem cell-derived cardiomyogenesis, with special emphasis on the development of heart field transcriptional networks.

2. Cardiogenic potential of embryonic stem cells

Embryonic stem cells are pluripotent stem cells derived from the inner cell mass of the developing blastocyst. *In vitro* culture of embryonic stem cells, if nurtured under specific conditions, can give rise to distinct cell types of endodermal, mesodermal and ectodermal lineages, including thus beating cardiomyocytes (Miller-Hance et al. 1993). Such capabilities have raised the possibility of using embryonic stem cells as a source to heal the damaged heart (Chinchilla & Franco, 2006; Franco et al., 2007). However, several constrains has obstructed this purpose. Leaving apart ethical concerns, mainly applicable to human

embryonic stem cells, technical and scientific obstacles also have contributed to slow down this quest. We shall update in this book chapter the state-of-the-art progress made to conquer the challenging aim of converting embryonic stem cells into beating cardiomyocytes as suitable therapeutical tools.

It is incredible to observe that a subset of cells hunted from the inner mass cells of the developing blastocyst, set in appropriate cell culture conditions, are able to progressively proceed *in vitro*, differentiating into neurons, fibroblasts, cardiomyocytes as well as in several other cells types (Lanza et al., 2004). The real question is then; does this occur mimicking the early step of embryogenesis? Several studies have demonstrated that upon the initial phases of *in vitro* embryonic stem cells culture, the aggregating cells that form the embryonic bodies in the hanging drops acquire a rather well organized three-dimensional structure, by which externally located cells express ectodermal markers, while internally located cells express mesodermal and endodermal makers (Doetschman et al., 1985, Wobus & Boheler, 1999; Boheler et al., 2002). Thus, these findings support the notion that cell specification and determination of embryonic stem cells into a discrete cell type would mainly follow the endogenous signal pathways. Therefore, in order to understand how stem cells can lead to cardiomyocytes, efforts should be made to learn the natural routing of a mesodermal cell that will contribute to the heart. In essence we need to learn how cardiac development is achieved.

3. Transcriptional control of cardiac muscle development

Over the last two decades, our understanding of the cellular and molecular mechanisms that govern cardiac development has greatly advanced. Initial steps of mesoderm commitment from the lateral plate mesoderm to the forming heart are mainly directed by interplay between Bmp, Fgf and Wnt signaling (Barron et al., 2000; Lopez-Sanchez et al., 2002; Marques et al., 2008). As soon as the cardiogenic mesoderm is committed, several transcription factors, such as Nkx2.5, Gata4, Srf, Hand2 and Mef2c are activated, which play crucial roles during cardiogenesis as revealed by loss-of-function experiments in mice (Lyons et al., 1995; Srivastava et al., 1995; Kuo et al., 1997; Lin et al., 1997). Cardiogenesis proceeds by the formation of two concentrical tissue layers, an external myocardial and internal endocardial layer. To date it remains elusive how each cardiogenic lineage is distinctly established and whether they share a common progenitor (Linask & Lash, 1993; Eisenberg & Bader, 1995) or, on the contrary, they are distinctly derived from separate precursors (Cohen-Gould & Mikawa, 1996; Wei & Mikawa, 2000). As the myocardium is configured, it has been recently demonstrated that two distinct cell populations contribute to the developing heart; the first heart field (FHF) will contribute to the linear heart tube and subsequently will give rise mainly to the left ventricle (Kelly & Buckingham, 2002), whereas a second population of cells is subsequently recruited, namely the second heart field (SHF), contributing to the rest of the developing heart (Kelly et al., 2001; Cai et al., 2001; Waldo et al., 2001; Kelly & Buckingham, 2002). FHF derivatives express Nkx2.5 but are negative from islet-1, while SHF cells express both Nkx2.5 and islet-1. In addition, SHF derivates can be subdivided in two distinct regions, according to their entry site to the developing heart; a) anterior SHF leads to the formation of the right ventricle and outflow tract, and its contribution is governed by signaling emanating from Fgf8- and Fgf10-expressing cells (Watanabe et al. 2010), as well as contribution from Tbx1 signaling at the arterial/pharyngeal pole of the heart (Huynh et al., 2007; Liao et al., 2008), b) posterior SHF

will contribute to the atrioventricular canal as well as right and left atria, and its contribution is directed by Fgf10 signaling at the venous pole of the heart (Kelly et al., 2001). In addition, complex regulatory networks are operative in the embryonic heart providing differentiation cues to the developing myocardium, as illustrated by the complex expression pattern of T-box genes, including therein Tbx2, Tbx3, Tbx5, Tbx18 and Tbx20 (Singh & Kispert, 2010; Greulich et al., 2011), Hand1 and Hand2 providing systemic *vs* pulmonary cues (Srivastava et al., 1995; Thomas et al., 1995) as well as left/right positional cues as illustrated by Pitx2 (Franco & Campione, 2003; Chinchilla et al., 2011). Thus, shared and distinct transcriptional pathways are governing first and second heart field deployment, leading in both cases to activation of a core cardiac transcriptional regulatory network as illustrated in **Figure 1**.

In addition, a third population of cells, with distinct gene expression hallmark as compared to first and second heart field cardiac precursors, has been demonstrated to form the inflow tract of the heart, suggesting the possible existence of a third heart field (Mommersteeg et al., 2010). Nkx2.5 /islet-1 negative cells, but positive for Tbx18, contribute to the formation of the caval veins (Mommersteeg et al., 2010). However, recent Cre-based lineage tracing experiments have challenged this notion, since all myocardial component of the venous pole, including the atrial appendages and the caval and pulmonary veins, have been reported to be derived from islet-1 and Nkx2-5 positive cells (Ma et al., 2008).

Concomitant with the deployment of the first and second heart field precursor cells, differentiation into distinct cardiomyocyte cell types is occurring, providing thus distinct working chamber and conduction system myocardium. Tbx2 and Tbx3 have been reported to play a fundamental role controlling gene expression pattern within the atrioventricular node (Bakker et al., 2008; Aanhaanen et al., 2009, 2011). Shox2, Tbx3 and Tbx18 have been demonstrated to play a crucial role on the sinoatrial node formation (Hoogaars et al., 2007; Wiese et al., 2009; Espinoza-Lewis et al. 2009). Furthermore, cardiomyocyte subtypes progressively emerge during cardiogenesis, such as distinct atrial and ventricular chamber myocardium, although the transcriptional regulation of such cell identities remains rather elusive. In chicken, Irx4 plays a crucial role in this step (Bao et al., 1999; Bruneau et al. 2000), however such function is not conserved in mice (Bruneau et al., 2001), which might be partially taken by Coup-tfII (Pereira et al., 1999; Wu et al., 2011). Similar events also are applicable for the cardiac conduction system, in which nodal and bundle branch fascicles are developed, each of them with distinct functional capabilities, yet their transcriptional regulation remains rather unexplored (Franco & Icardo, 2001; Miquerol et al. 2011).

In the adult heart, some of the developmental differences are progressively smoothened, in such a way that we can consider the adult heart being composed of two types of working myocardium (atrial and ventricular) and three distinct types of conductive cells (SAN and AVN node, His and bundle branches, and Purkinje fibers). Curiously, novel transmural differences emerge in the adult ventricular myocardium (Yan & Antzelevitch et al., 1996, 1999; de Castro et al., 2005), which are crucial for correct function of the heart (Constantini et al. 2005).

In its important to highlight that during cardiogenesis, the heart is progressively acquiring novel functional capabilities, which are reflected on the progressive onset of expression of contractile, conductive and cytoskeleton proteins. At the contractile level, sarcomeric genes such as myosins, actins and troponins, and cytoskeleton proteins as tropomyosin and actinins are differently expressed already at early stages of development (Lyons et al., 1990; Franco et al., 1998) providing functional heterogeneity to the developing myocardium, as

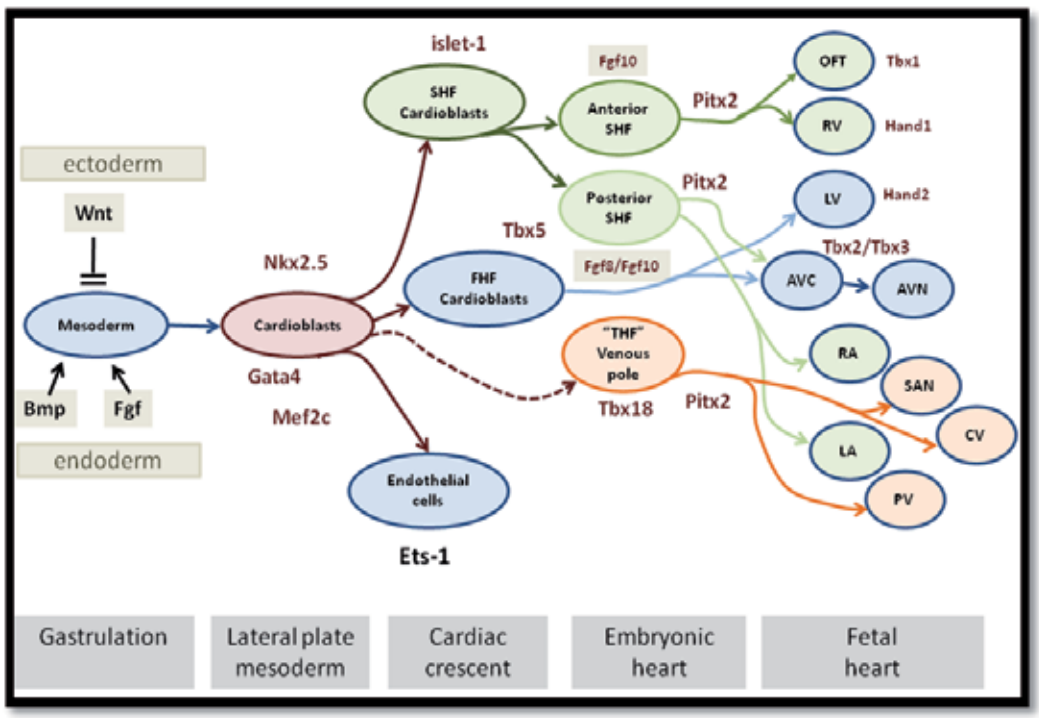
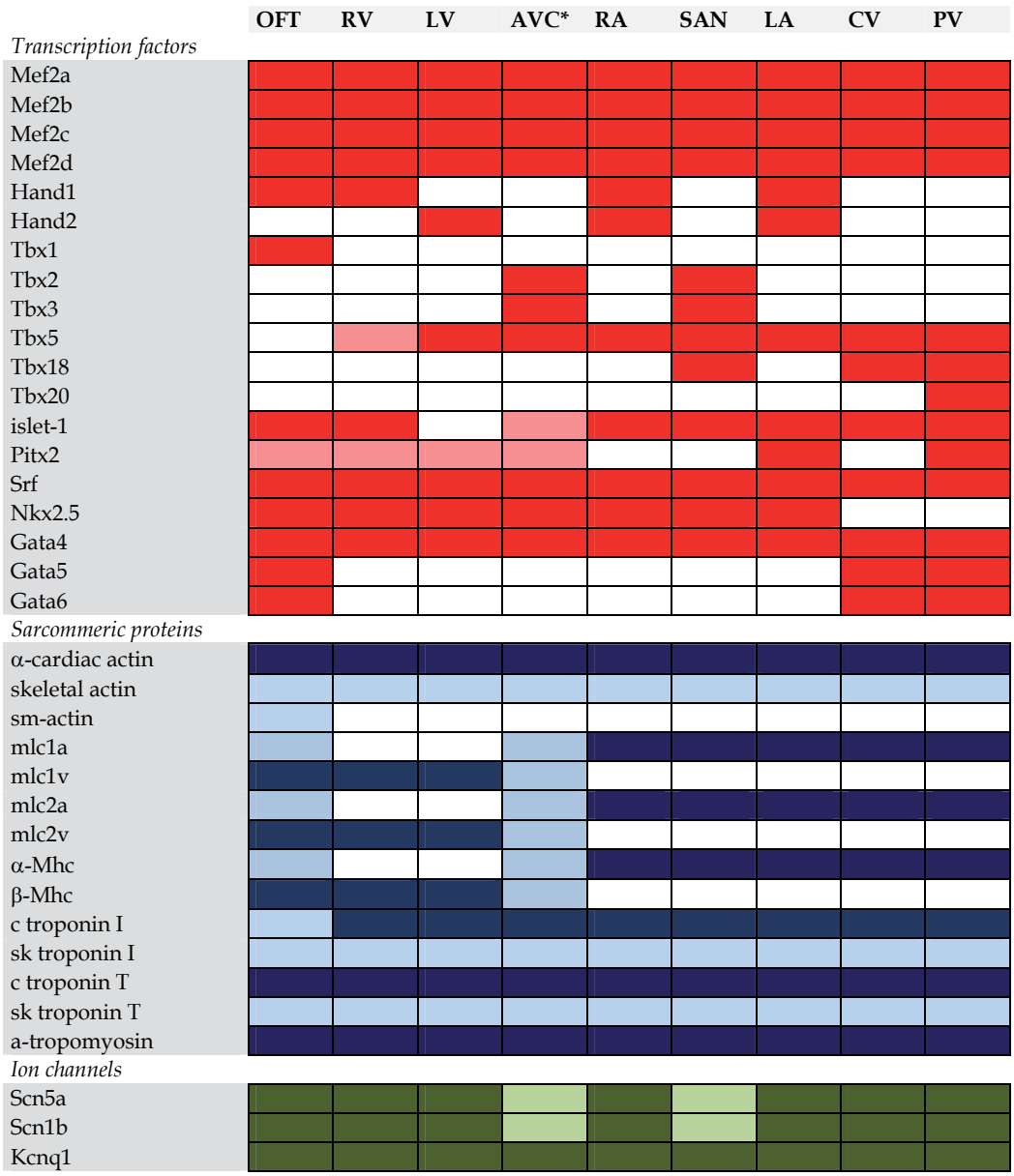


Fig. 1. **Schematic representation of embryonic cardiac development.** At gastrulation, nascent precardiac mesoderm listens to signal emanating from the surrounding ectodermal and endodermal tissues, committing the cells into the cardiogenic lineage, i.e. cardioblasts. Cardioblasts are characterized by the expression of core cardiac transcription factors, including Nkx2.5, Gata4 and Mef2c. Soon thereafter, cardiomyocyte and endothelial cell lineages emerged and within the cardiomyocyte lineage, two distinct populations of cells can be recognized, first heart field (FHF) and second heart field (SHF) cells. A third population of cells is originating soon thereafter, which lacks Nkx2.5 or islet-1 expression, yet, lineage tracing evidences using Cre/LoxP system suggested they have had a common ancestry (Cai et al., 2003; Zhou et al., 2008). FHF contributes mainly to the left ventricle and the atrioventricular canal (AVC). AVC will eventually be remodeled as septation proceeds, contributing therein to the formation of the atrioventricular node, which is characterized by Tbx2 and Tbx3 expression. Interestingly, SHF have two distinct components, an anterior SHF contributing to the outflow tract and right ventricle, listing to signal emanating from the pharyngeal arches domain, such as Fgf8 and Fgf10, and a posterior SHF leading to the right and left atrial chambers, listing to signals such as Fgf10. The "third" heart field contributes to the sinous venosus, forming therefore the caval veins and the sinoatrial node, and to the pulmonary myocardium which constitutes the atrial septa and the pulmonary veins (Franco et al., 2000). Importantly, almost all previously mentioned cardiac lineages listen to left/right signaling clues provided by the homeobox transcription factor Pitx2 (Campione et al., 1999; Franco & Campione, 2003).

detailed in **Table 1**. Similarly, ion channels and gap junctional proteins are progressively expressed in distinct regions of the developing heart (Gros & Jongsma, 1996; Franco et al. 2001; de Castro et al., 2005)(**Table 1**), providing the bases for the onset of a persitaltoid contraction in first instance and subsequently of synchronous contraction at late embryonic stage, displaying thus an apex-to-base pattern of activation. In essence, if we take thus as a reference our knowledge about cardiac development, it sounds reasonable that such information could be applicable to embryonic stem cells in order to obtain beating cardiomyocytes.



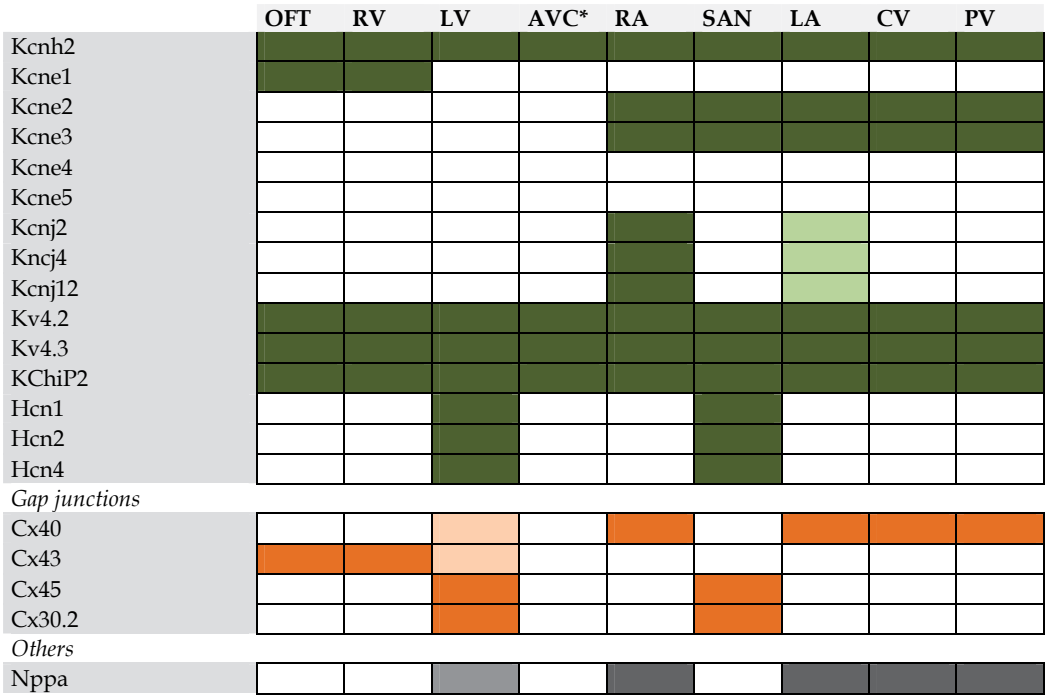


Table 1. Graphical respresentation of the expression profiles of cardiac-enriched transcription factors, sarcomeric proteins, ion channels and gap junctional proteins during embryonic heart development, in distinct regions of the embryonic/fetal heart. OFT, outflow tract; RV, right ventricle, LV, left ventricle, AVC, atrioventricular canal, RA, right atrium, LA, left atrium, SAN, sinoatrial node, CV, caval veins, PV, pulmonary veins. *AVC will lead in the adult heart to the atrioventricular node.

4. Understanding transcriptional control of *in vitro* cardiogenesis

The formation of beating cardiomyocytes from undifferentiated embryonic stem cell has been an important focus of scientific research, as it can be witnessed by the number of publication in this front. Several of the signaling pathways involved in *in vivo* cardiogenesis are also recapitulated *in vitro*, as depicted in **Figure 2**. Importantly, initial reports provided evidences that embryonic stem cell-derived cardiomyocytes display morphological, molecular and functional characteristics similar to adult cardiomyocytes, displaying therefore expression of sarcomeric and gap junctional proteins (Sachinidis et al., 2003; van Kempen et al., 2003; Fijnvandraaf et al., 2003ab). Additional experiments, demonstrated that most ion channels that are natively configure the cardiac action potential are also expressed during embryonic stem cell-derived cardiomyocytes (van Kempen et al., 2003). Furthermore, elegant spatio-temporal studies also nicely illustrate the progressive onset of the core transcriptional cardiac and ion channel expression during embryonic stem cell-derived cardiomyogenesis (Fijnvandraaf et al., 2003ab, van Kempen et al., 2003). However, several caveats were soon arising. Firstly, the fact that embryonic stem cell cultures, although capable of providing a source of cardiomyocytes, yield on average to a low percentage (<20%). Secondly, beating areas display distinct contraction rates, suggesting that large

heterogeneity was observed from beating area to beating area, which might have gone unappreciated since most studies were done using RT-PCR methods. Thirdly, it was unclear if all cells within a beating area were equally differentiated. Sorting out these key questions is compulsory before any therapeutical strategy could be envisioned since a large number of cardiomyocytes is required, which will need to be morphologically homogeneous in order to avoid the chance of generating arrhythmias, and sufficiently and adequately differentiated in order to limit the oncogenic propagation of undifferentiated or poorly differentiated embryonic stem cells.

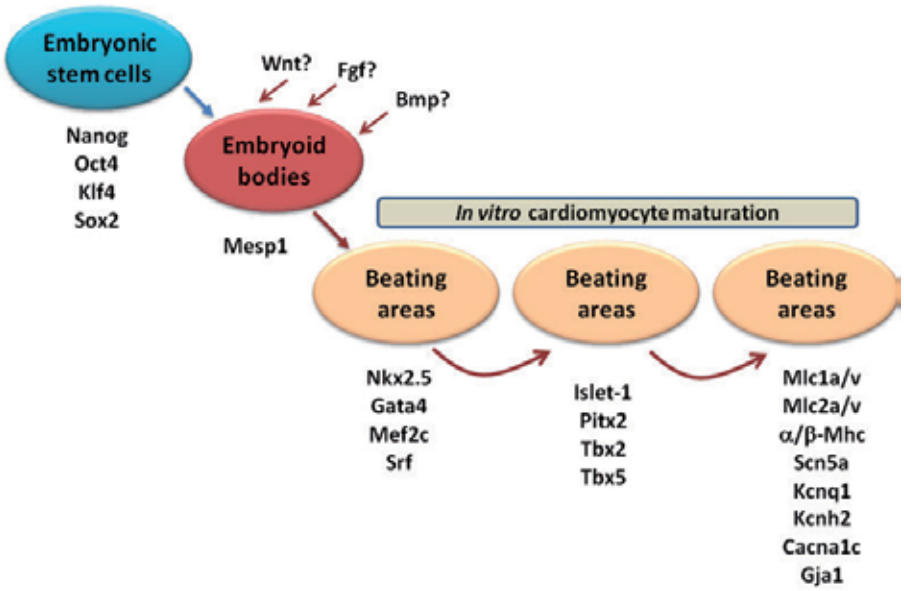
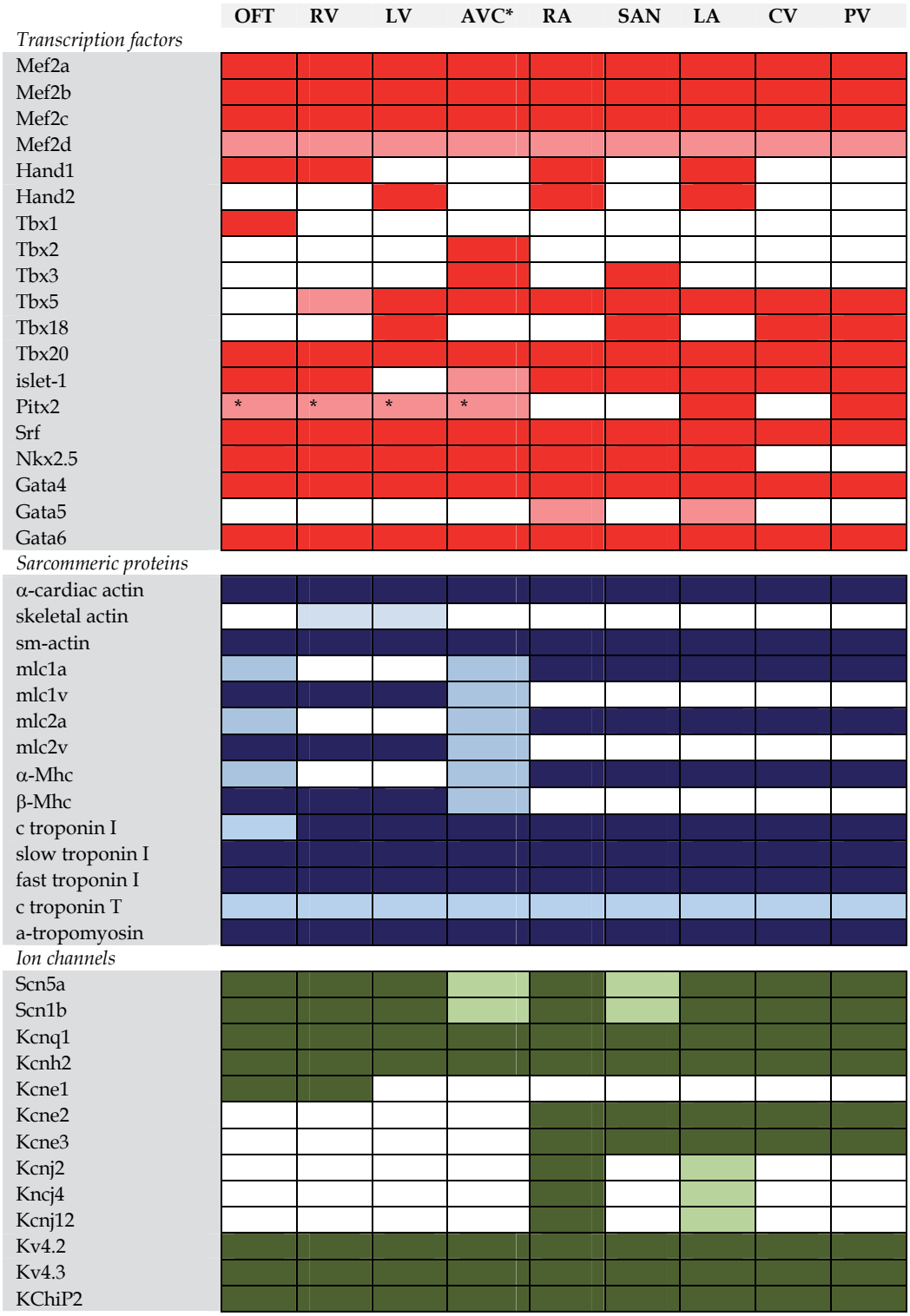


Fig. 2. **Schematic representation of the signaling pathways involved in embryonic stem cell–derived cardiogenesis.** Pluripotency makers such as Nanog, Oct4, Klf4 and Sox2 are expressed in undifferentiated embryonic stem cells. As soon as differentiation is initiated, these makers are down-regulated and mesoderm specification genes such as Mesp1 are expressed. Soon thereafter, as the beating areas are conformed, core cardiac transcriptional regulatory genes are up-regulated, and progressively the nascent cardiomyocytes acquire sacormeric and ion channel proteins, as depicted in **Table 2**.

First approaches to increase the efficiency of converting embryonic stem cells into cardiomycytes has been obtained by mimicking the inductive signals that naturally convert precardiogenic mesodermal cells into nascent cardioblasts (Hidai et al., 2003; Holtzinger et al., 2010). Bin et al. (2006) demonstrates that Bmp2 treatment of mouse embryonic stem cells increased the efficiency of obtaining beating areas, providing evidences that core transcriptional factor Nkx2.5 and structural proteins such as troponin-T and alpha-MHC were increased. Further evidences on the pivotal role of Bmp signaling in embryonic stem cell-derived cardiomyogenesis were reported by Rajasingh et al. (2007). More recently, Kim et al. (2008) provides similar evidences using human embryonic stem cells. However, other authors have reported that Bmp2 overexpression can also lead to induction of other mesodermal lineages such as smooth muscle cells (Blin et al., 2010) or condroblasts,


OFT
RV
LV
AVC*
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SAN
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Transcription factors
Mef2a
Mef2b
Mef2c
Mef2d
Hand1
Hand2
Tbx1
Tbx2
Tbx3
Tbx5
Tbx18
Tbx20
islet-1
Pitx2
Srf
Nkx2.5
Gata4
Gata5
Gata6
Sarcommeric proteins
α-cardiac actin
skeletal actin
sm-actin
mlc1a
mlc1v
mlc2a
mlc2v
α-Mhc
β-Mhc
c troponin I
slow troponin I
fast troponin I
c troponin T
a-tropomyosin
Ion channels
Scn5a
Scn1b
Kcnq1
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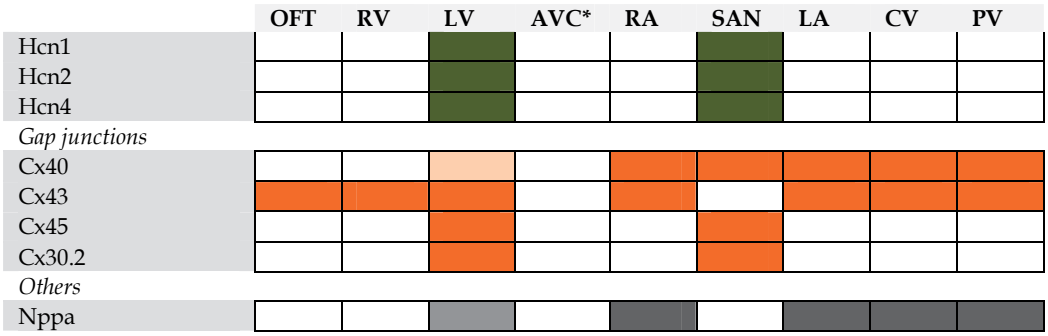

Table 2. Graphical respresentation of the expression profiles of cardiac-enriched transcription factors, sarcomeric proteins, ion channels and gap junctional proteins during embryonic stem cell differentiation. ESC, embryonic stm cells, EB, embryoid bodies, BA2, beating areas at 2 days of culture, BA7, beating areas at 7 days of culture, EB14, beating areas at 14 days of cultures.

osteoblasts and adipoblasts, if cultures with supplementary co-factors (zur Nieden et al., 2005). Thus, these data suggest that combinatorial treatments might even further enhance embryonic stem cell-derived cardiomyogenesis. In this context, Evseenko et al. (2010) has elegantly evaluated the initial stages of mesoderm commitment during human embryonic stem cell differentiation, demonstrating the presence of endogenous cardiogenic morphogens such as activin A, Bmp4, Vegf and Fgf2. Laflamme et al. (2007) demonstrate that Bmp4 treatment increased *in vitro* cardiomyogenesis using human embryonic stem cells and Paige et al. (2010) has elegantly shown a balanced interplay between activin A/Bmp4 and Wnt/β-catenin is needed to efficiently induce mesodermal lineage formation and subsequent cardiomyocyte development in human embryonic stem cells. Similar findings are also observed using mouse embryonic stem cells (Taha et al., 2006, 2007; Taha & Valojerdi, 2008; Takei et al., 2009; Verma & Lenka, 2010). In addition to the role of Bmp signaling, several studies have reported the pivotal role of Fgf signaling controlling mouse embryonic stem cell-derived cardiomyogenesis (Dell´Era et al., 2003; Ronca et al. 2009). Importantly, Fgf signaling, in conjunction with Bmp signaling enhances cardiomyocyte formation of other stem cell sources, such as bone marrow stem cells (Degeorge et al., 2008) or P19 carcinoma cell line (Hidai et al., 2003), reinforcing the notion that Fgf signaling is necessary (embryonic stem cells) and sufficient (other sources) to induce cardiomyocyte development. Curiously, novel signaling pathways, such as Sdf-1/Cxcr4 (Chiriac et al., 2010) and Vegf (Chen et al., 2006) also play a determinant role in cardiogenesis, although their links to Bmp, Fgf and Wnt signaling remains unexplored.

Understanding of the embryonic stem cell-derived cardiomyogenesis has also been largely unraveled by manipulation of several cardiac enriched transcription factors. Gata4 and Gata6 deficient mice suggested a pivotal role of this transcription factor in early cardiogenesis (Kuo et al., 1997; Xin et al., 2006). Gata4 deficient embryonic stem cells have been reported to disrupt visceral endoderm formation and thus hematopoiesis (Soudais et al., 1995; Bielinska et al., 1996, 1997; Morrisey et al., 2000; Pierre et al., 2009). Similar findings are also observed in Gata6 deficient embryonic stem cells (Pierre et al., 2009). Importantly, cardiomyogenesis is not affected in Gata4 deficient embryonic stem cells (Narita et al., 1997) but over-expression of Gata4 in embryonic stem cells enhances cardiogenesis (Grepin et al.,

1997) and visceral endoderm (Holtzinger et al. 2009) suggesting that Gata4 is not necessary but is sufficient to induce cardiomyocyte differentiation. Nkx2.5 deficient mice display cardiac embryonic lethality (Lyons et al., 1995), supporting a pivotal role for Nkx2.5 in cardiomyogenesis. In this context, over-expression of Nkx2.5 in embryonic stem cells increases the expression of cardiogenic markers at the expenses of hematopoietic markers such as Gata-1 (Caprioli et al., 2011). Similarly, enhanced expression of Mef2c in embryonic stem cells increases cardiomyogenic differentiation (Puceat et al., 2003), in line with its determinant role during heart development as demonstrated by genetic deletion in mice (Lin et al., 1997). Comparable over-expression approaches in embryonic stem cells have been described for the homeobox transcription factor Pitx2, a left-right signaling pathway determinant (Campione et al., 1999, 2002). Enhanced expression of Pitx2 leads to increased expression of cardiac markers (Lozano-Velasco et al., 2011) supporting a role of this transcription factor in cardiogenesis. In addition, overexpression of Tbx5, a pivotal transcription factor associated with Holt-Oram syndrome (Li et al., 1997), in P19 embryonic carcinoma cells (Fijnvandraat et al., 2003abc) display similar findings. Overall, these studies illustrate the pivotal role of distinct transcription factors with reported enhanced expression during cardiogenesis, as well as it also provides the entry to previously unknown transcription factors as key elements for cardiomyogenic lineage differentiation, as it is the case for the transcription factors hhLIM (Zheng et al., 2006) and Rb (Papadimou et al., 2005) or the GTPase Rac1 (Puceat et al., 2003).

Importantly, although these reports provided evidence of enhancing cardiomyocte formation by the usage of discrete growth factors and/or transcription factors, and subsequently identifying cardiac specific molecular markers such as Nkx2.5, Gata4 and sarcomeric proteins (i.e troponin T and alpha-actinin), it remains elusive if cardiomyocyte heterogeneity in terms of lineage origin (FHF, SHF), gene expression (atrial/ventricular/ nodal) or function (working/conductive) is observed. We have recently reported that mouse embryonic stem cell-derived cardiomyocytes display a dynamic temporal expression of FHF and SHF makers which are reminiscent of the *in vivo* cell lineage deployment (Lozano-Velasco et al. 2011). In addition, large heterogeneity in gene expression, displaying distinct atrial-, ventricular- and nodal-like patterns (Fijnvandraat et al. 2002, 2003abc) and functional heterogeneity (van Kempen et al., 2003) displaying distinct cardiac action potential configurations, have been extensively reported. In this context, is it important to highlight that overexpression of Pitx2 mainly directs the expression of SHF cardiomyocytes, since both islet-1 and Mef2c were up-regulated, whereas FHF marker Nkx2.5 was unaltered (Lozano-Velasco et al., 2011). Furthermore overexpression of Pitx2 enhances Tbx5 expression and thus Nppa (Anf) and Gja5 (Cx40)(Lozano-Velasco et al., 2011), in line with previous Tbx5 over-expression findings (Fijnvandraaf et al., 2003). Thus, it is plausible that enhance cardiomyocyte commitment by distinct inductive signals might generate cellular heterogeneity in a similar fashion as in the developing heart. If so, these observations might hindrance their therapeutical usage since, for example, engrafting nodal-like cells in the ventricular chambers might lead to ectopic electrical foci and thus to arrhythmias. In this context, searching for transcriptional factor cocktail which might homogenize the cardiomyocyte outcome is a plausible strategy, as recently reported to convert induced pluripotent fibroblasts into cardiomyocytes (Ieda et al. 2010) or hepatocytes (Huang et al., 2011).

Differentiation of embryonic stem cells into beating cardiomyocytes can therefore be naively observed by simply developing embryoid bodies or enhanced by supplementing these

embryonic stem cells and/or embryoid bodies with a subset of growth factors and/or transcription factors. **Table 2** summarizes the current state of the art knowledge about the dynamic expression of distinct transcription factors, sarcommeric proteins, ion channels and gap junctions during embryonic stem cell-derived cardiomyogenesis. However, the question arising is: are all beating areas differentiated into cardiomyocytes or are there remaining non-differentiated embryonic stem cells or contaminating cells that might differentiate into other embryonic lineages? This is a crucial question if we aim to use them *in vivo*. We have recently reported that fine-dissection of beating areas have minor contamination of endodermal- or ectodermal cells (Lozano-Velasco et al., 2011) at distinct developmental stages. Thus, it seems that a rather homogeneously differentiated cluster of cells is normally achieved. It remains to be elucidated if undifferentiated embryonic stem cells remain in those areas. *In vivo* approaches suggest that indeed this might be the case, yet titering the number of engrafted cells results in absence of teratomas at the long run (Behfar et al., 2005, 2007; Yamada et al. 2009).

5. Conclusions and perspectives

Over the last decade we have started to understand the molecular mechanisms that govern cardiac formation and we are translating these findings to the manipulation of embryonic stem cells opening promising avenues to enhance cardiomyocyte differentiation. We have learnt how to increase the number of cardiomyocyte produced, and we have learnt that embryonic development is faithfully recapitulated *in vitro*, including the onset of first and second heart field transcriptional programs. Manipulation of these transcriptional machinaries will be therefore the upcoming challenges for the next years to come in order to facilitate the generation of fully differentiated, structurally similar and functionally homogeneous cardiomyocytes. Searching for transcription factor cocktails or opening new strategies such as those emerging from the microRNA world (Ivey et al. 2008; Chinchilla et al., 2011) constitutes the next goals, as recently illustrated for miR-499 (Wilson et al., 2010). Thus, in summary, an important part of the route has been walked, and the way ahead seems promising with the reward of achieving therapeutically usable embryonic stem cell-derived cardiomyocytes.

6. Acknowledgments

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9

Human Pluripotent Stem Cells in Cardiovascular Research and Regenerative Medicine

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1. Introduction

Heart disease is one of the leading causes of mortality worldwide. Because adult cardiomyocytes (CMs) lack the ability to regenerate, malfunctions or significant loss of CMs due to disease or aging can lead to cardiac arrhythmias, heart failure, and subsequently death. Heart transplantation for patients with end stage heat failure is limited by the number of donor organs available. Cell-based therapies offer a promising alternative for myocardial repair, but there are significant challenges involved. The transplantation of human CMs, eg fetal CMs, is difficult for practical and ethical reasons, thus cells of non-cardiac lineage, such as skeletal myoblasts (Murry et al., 1996; Menasche et al., 2003) and mesenchymal stem cells (Shake et al., 2002; Toma et al., 2002), have been considered as alternatives. Animal studies and clinical trials involving these cells have yielded conflicting results. Transplanted non-cardiac cells such as bone marrow-derived hematopoietic cells do not transdifferentiate into the cardiac lineage (Balsam et al., 2004; Murry et al., 2004). They also do not integrate into the host myocardium. For instance, the lack of electrical integration of skeletal myoblasts after their autologous transplantation into the myocardium resulted in the generation of malignant ventricular arrhythmias, which led to the premature termination of clinical trials involving skeletal myoblasts (Menasche et al., 2003; Smits et al., 2003). Therefore, an alternative cell source is needed.

2. Human embryonic stem cells and induced pluripotent stem cells

Human embryonic stem cells (hESCs), isolated from the inner cell mass of blastocysts, can self-renew while maintaining their pluripotency to differentiate into all cell types (Thomson et al., 1998), including CMs (Kehat et al., 2001; Mummery, C. et al., 2002; Xu, C. et al., 2002; Xue et al., 2004; Mummery, C. et al., 2003). Therefore, hESCs may provide an unlimited *ex vivo* source of CMs for cell-based heart therapies. The laboratories of Yamanaka (Takahashi et al., 2007) and Thomson (Yu et al., 2007) showed that adult somatic cells can be

reprogrammed to become pluripotent hES-like cells (a.k.a. induced pluripotent stem cells or iPSCs) via the forced expression of four pluripotency genes (Oct4, Sox2, c-Myc, and Klf4 *or* Oct4, Sox2, Nanog, and Lin28) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Aasen et al., 2008). More recent studies have further demonstrated the successful use of less pluripotency factors (Huangfu et al., 2008; Kim, J. B. et al., 2008; Nakagawa et al., 2008) and non-viral methods (e.g., with synthetic modified RNA (Warren et al., 2010)) to reprogram somatic cells into iPSCs. Although concerns such as induced somatic coding mutations (Gore et al., 2011) have yet to be fully addressed, hiPSCs have morphology, gene expression profile, epigenetic status, *in vitro* and *in vivo* differentiation capacities similar to hESCs (Takahashi et al., 2007; Yu et al., 2007).

3. Differentiation of hESC into CMs

Previous studies studies have demonstrated that hESCs and hiPSCs can spontaneously differentiate into CMs when they aggregate in suspension to form embryoid bodies (Xu, C. et al., 2002; Zwi et al., 2009). Recent studies have focused on improving the yield and purity of CM differentiation. For instance, hESC differentiation into the CM lineage can be enhanced by coculture with visceral endoderm-like cells (Mummery, C. et al., 2003; Mummery, C. L. et al., 2007). Recently, an effective protocol for cardiac differentiation has been successfully developed by the Keller laboratory involving the stage-specific addition of growth factors, including BMP4, Activin-A, DKK, bFGF etc, to drive sequential differentiation into the epiblast, mesoderm and CMs, resulting in greatly increased yield of up to 50%, as gauged by the proportion of cells that express cardiac troponin T (Yang et al., 2008). Other approaches have also been pursued, utilizing different extracellular matrices, serum (Passier et al., 2005) and insulin elimination (Xu, X. Q. et al., 2008). Besides improving the yield of CM differentiation, the isolation of a pure population of CMs is also important in order to prevent malignancy and arrhythmias. Various purification methods have been developed including Percoll gradient centrifugation (Xu, C. et al., 2002), optical signatures (Chan, J. W. et al., 2009) and genetic selection based on the expression of a reporter protein under the transcriptional control of a cardiac-restricted promoter (e.g., α-myosin heavy chain (α-MHC) (Anderson et al., 2007), ventricular myosin light chain (MLC-2v) (Huber et al., 2007; Fu et al., 2010)).

4. Properties of hESC-derived CMs (hESC-CMs)

4.1 Human ESC-CMs have molecular and structural properties of CMs

Gene expression profiles have been examined for hESC-CMs cultured in different laboratories using different differentiation protocols and from different hESC lines (Kehat et al., 2001; Xu, C. et al., 2002; Snir et al., 2003; Norstrom et al., 2006). There is now consensus that hESC-CMs express transcription factors and structural proteins specific to human cardiomyocytes (Kehat et al., 2001; Xu, C. et al., 2002; Snir et al., 2003; Norstrom et al., 2006). Human ESC-CMs express cardiac-specific transcription factors such as NKx2.5, GATA4 and Mef-2 (Kehat et al., 2001; Xu, C. et al., 2002), which are expressed in the precardiac mesoderm but persist in the heart during development. Structural components of the myofibers can also be detected in hESC-CMs. These include α–, β- and sarcomeric-myosin heavy chain (MHC), atrial and ventricular forms of myosin light chain (MLC-2a and –2v), tropomyosin, α-actinin and desmin (Kehat et al., 2001; Xu, C. et al., 2002; Norstrom et al.,

2006). Two members of the troponin complex, cardiac tropinin T, which binds to tropomyosin, and cardiac tropinin I, which regulates Ca^{2+}-sensitive muscle contraction, are also present in hESC-CMs (Kehat et al., 2001; Xu, C. et al., 2002; Norstrom et al., 2006). At the ultrastructural level, hESC-CMs show clearly identifiable sarcomeres and intercalated discs (Kehat et al., 2001; Snir et al., 2003). Morphologically, single hESC-CMs show spindle, round, and tri- or multiangular morphologies, rather than the more defined rod shape of mature cells (Xu, C. et al., 2002). Sarcomeric striations are organized in separated bundles, reminiscent of the pattern seen in human fetal CMs, and rather than the highly organized parallel bundles seen in human adult CMs (Mummery, C. et al., 2003). These data suggest that hESC-CMs display molecular and structural properties consistent with immature human CMs.

4.2 Human ESC-CMs have immature Ca^{2+} handling properties

4.2.1 Mechanism of Ca^{2+}-induced Ca^{2+}-release and Ca^{2+} transient

The contractile apparatus of CMs is dependent on the rise and decay of intracellular Ca^{2+}, known as the Ca^{2+} transient. During an action potential (AP) of adult CMs, Ca^{2+} entry into the cytosol through sarcolemmal L-type Ca^{2+} channels triggers the release of Ca^{2+} from the intracellular Ca^{2+} stores (sarcoplasmic reticulum or SR) via the ryanodine receptors (RyR). This process, the so-called Ca^{2+}-induced Ca^{2+}-release (Bers, 2002), escalates the cytosolic Ca^{2+} ($[Ca^{2+}]i$) to activate the contractile apparatus for contraction. For relaxation, elevated $[Ca^{2+}]i$ gets pumped back into the SR by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and extruded by the Na^{+}-Ca^{2+} exchanger (NCX) to return to the resting $[Ca^{2+}]i$ level. Such a rise and subsequent decay of $[Ca^{2+}]i$ is known as Ca^{2+} transient. Given the central importance of Ca^{2+}-induced Ca^{2+}-release in cardiac excitation-contraction coupling, proper Ca^{2+} handling properties of hESC-CMs are crucial for their successful functional integration with the recipient heart after transplantation. Indeed, abnormal Ca^{2+} handling, as in the case of heart failure, can even be arrhythmogenic.

4.2.2 Human ESC-CMs have functional SRs

Dolnikov et al (2006) were the first to study the Ca^{2+}-handling properties of hESC-CMs in detail (Dolnikov et al., 2006). They reported that Ca^{2+} transients recorded from spontaneously beating or electrically stimulated hESC-CMs respond to neither caffeine nor ryanodine; hESC-CMs recorded as beating clusters also displayed a negative force-frequency relationship that is different from adult CMs. Based on these observations, the authors concluded that hESC-CMs are immature, do not express functional SRs, and that their contractions result from trans-sarcolemmal Ca^{2+} influx (rather than Ca^{2+} release from the SR). Given the paucity of related data, our laboratory performed a comprehensive analysis to better define the Ca^{2+} handling properties of hESC-CMs by comparing Ca^{2+} transients from hESC-CMs and human fetal left ventricular (LV) CMs (16–18 weeks) (Figure 1, adopted with permission from Liu et al 2007 *Stem Cells* Vol. 25, No. 12: pp.3038-3044). Upon electrical stimulation, all of hESC-CMs and fetal LV-CMs generated similar Ca^{2+} transients. However, caffeine induced Ca^{2+} release in 65% of fetal LVCMs and 38% of hESC-CMs. Ryanodine significantly reduced the electrically evoked Ca^{2+} transient amplitudes of caffeine-responsive but not -insensitive hESC-CMs and slowed their upstroke; thapsigargin, which inhibits SERCA, reduced the amplitude of only caffeine-responsive hESC-CMs and slowed the decay (Liu et al., 2007). The discrepancy between our findings and those of Dolnikov et al can be largely attributed to the newly identified caffeine-responsive population.

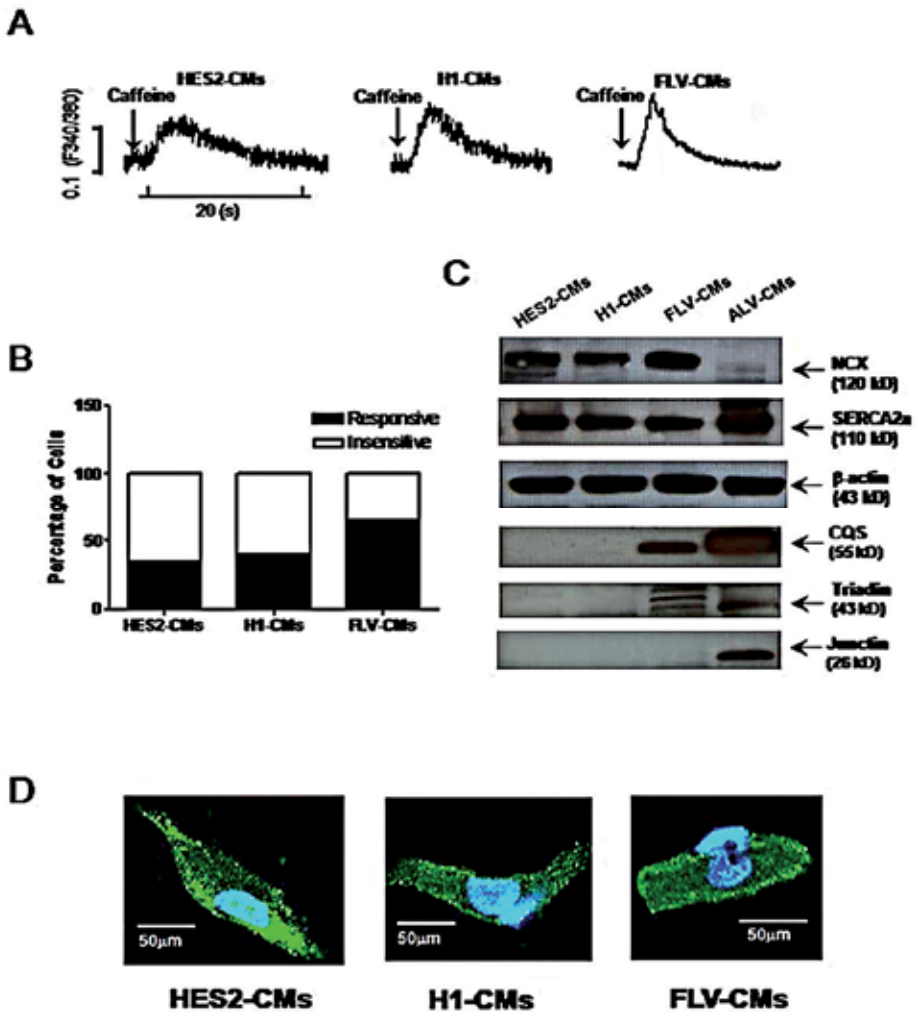

Fig. 1. Ca^{2+} handling properties of hESC-CMs
A) Representative tracings caffeine-induced Ca^{2+} transients of hESC-CMs (HES2-, H1-CMs) and fetal (F) LV-CMs. B) % of caffeine-responsive and -insensitive cells. C) Expression of various Ca^{2+} handling proteins. β-actin was used as the loading control. D) Immunostaining of RyRs. Adopted with permission from Liu et al 2007 *Stem Cells* Vol. 25, No. 12: pp.3038-3044.

4.2.3 Differential expression of Ca^{2+} handling proteins

While hESC-CMs express functional SRs, their Ca^{2+} handling properties are immature and are similar to those of fetal CMs. The functional immaturity of hESC-CMs may be attributed to the expression pattern of Ca^{2+} handling proteins (Figure 1C) (summarized in Table 1, adopted with permission from Kong et al 2010 *Thromb Haemost* Vol. 104, No. 1: pp.30-38). Compared to adult CMs, hESC-CMs express significantly lower levels of RyR, SERCA, phospholamban, calsequestrin and higher levels of calreticulin and NCX (Liu et al., 2007). The regulatory proteins junctin, triadin, and calsequestrin (CSQ) are expressed in adult LV-CMs but are completely absent in hESC-CMs (Liu et al., 2007).

		hESC-CMs	Fetal LVCMs	Adult LVCMs
Expression levels of Ca^{2+}-handling proteins.	RyR	++	++	++++
	SERCA	+++	+++	++++
	Phospholamban	-	++	++++
	CSQ/Triadin/Junctin	-	+	++++
	Calreticulin	++++	++++	+
	NCX	+++	++++	+
Ca^{2+} transient properties	Basal $[Ca^{2+}]_i$	++	+++	++++
	Amplitude	++	++	++++
	Decay	++	++	++++
	Upstroke	++	++	++++

Table 1. Ca^{2+} handling properties of hESC-CMs, fetal and adult LVCMs.
Adopted with permission from Kong et al 2010 *Thromb Haemost* Vol. 104, No. 1: pp.30-38.

4.2.4 T-Tubules are absent in hESC-CMs

Transverse (t) tubules are invaginations in the sarcolemmal membrane that concentrate dihydropyridine receptors and bring them spatially close to RyRs residing on the SR membrane located deeper in the cytoplasm (Brette and Orchard, 2003; Brette and Orchard, 2007). By physically minimizing the diffusion distance, RyRs in CMs can participate in Ca^{2+}-induced Ca^{2+}-release without a lag. The result is a synchronized, faster, and greater transient $[Ca^{2+}]$i increase from the peripheries to the center, creating a uniform Ca^{2+} wavefront across the transverse section with simultaneous recruitment of all SR. Fast and synchronized activation of RyRs translates into a greater Ca^{2+} transient amplitude, recruitment of more actin–myosin cross-bridge cycling, and generation of greater contractile force. The presence of t-tubules is therefore crucial to the mature Ca^{2+} handling of CMs. Lieu et al used fluorescent staining (Figure 2A and C, adopted with permission from Lieu et al 2009 *Stem Cells Dev* Vol. 18, No. 10: pp.1493-1500) and atomic force microscopy (Figure 2B and D) to detect the presence of t-tubules and showed that the latter is absent in hESC-CMs. Consistent with CMs deficient of t-tubules, hESC-CMs also exhibit a U-shaped Ca^{2+} wavefront that is caused by a delayed Ca^{2+} increase in the central region of the cell relative to the peripheral region (Lieu et al., 2009) (Figure 2E).

4.2.5 Attempts to improve Ca^{2+}-handling properties by genetic modification

We hypothesize that the differential expression of key CM proteins underpins the immaturity of hESC-CMs relative to adult CMs. We tested this idea by overexpressing CSQ in hESC-CMs. CSQ is the most abundant, high-capacity but low-affinity, Ca^{2+}-binding protein in the SR that is anchored to the RyR. The cardiac isoform CSQ2 can store up to 20 mM Ca^{2+} while buffering the free SR $[Ca^{2+}]$ at ~1 mM. This allows repetitive muscle contractions without rundown. While CSQ is robustly expressed in adult CMs, it is completely absent in hESC-CMs (Liu et al., 2007). We hypothesized that forced expression of CSQ in hESC-CMs would induce functional improvement of SR. We tested this hypothesis by transduction of hESC-CMs with the recombinant adenovirus Ad-CMV-CSQ-IRES-GFP (Ad-CSQ) and demonstrated that Ad-CSQ significantly increased the transient amplitude, upstroke velocity, and transient decay compared with the control and a truncated mutant (Liu et al., 2009) (Figure 3, adopted with permission from Liu et al 2009 *Am J Physiol Cell*

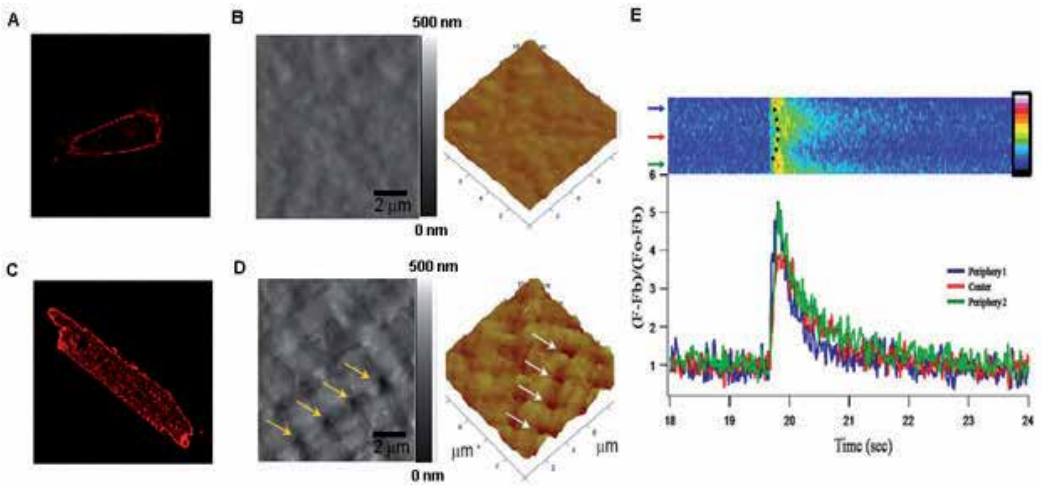

Fig. 2. T-tubule imaging of a hESC-CM and a mature ventricular CM.
Confocal microscopic images of a hESC-CM (A) did not show intracellular fluorescent spots like those in an adult guinea pig ventricular CM (C) suggesting the absence of t-tubules. The absence of t-tubules in ESC-CMs was further confirmed by atomic force microscopy imaging of an adult ventricular cardiomyocyte (D) showing regularly spaced pores in the sarcolemma that coincide with the Z-lines, while hESC-CM (B surface showed comparatively smoother topology with no presence of invaginations that are indicative of t-tubules. E) Electrically induced Ca^{2+} transient in hESC-CMs. Top: Time progression linescans of pseudo-colored transient increase in intracellular Ca^{2+} across the mid-plane of a hESC-CM showed a U-shaped wavefront. Bottom: Quantified Ca^{2+} transient of linescans of the top panel. Adopted with permission from Lieu et al 2009 *Stem Cells Dev* Vol. 18, No. 10: pp.1493-1500.

Physiol Vol. 297, No. 1: pp.C152-159). These results showed that immature Ca^{2+}-handling properties of hESC-CMs can be rescued by genetic modification and improved our understanding of CM maturation.

4.3 Human ESC-CMs demonstrate immature electrophysiological properties similar to 'embryonic' CMs

He et al (2003) were the first to study the electrophysiological properties of hESC-CMs (He et al., 2003). They characterized the contractions and action potentials (APs) from beating EB outgrowths cultured for 40 to 95 days and showed that hESC can differentiate into a heterogeneous mixture of CMs, with APs classified as 'nodal-like', 'embryonic ventricular-like' and 'embryonic atrial-like', analogous to CM specification into pacemaker, ventricular and atrial CMs. The latter two classes are considered 'embryonic' based on their Maximum diastolic potential and more depolarized resting membrane potential, and "slow" type APs based on low dV/dt_{max}. Unlike adult CMs, which are normally electrically silent yet are excitable upon stimulation, the majority of hESC-CMs fire spontaneously, exhibiting a high degree of automaticity. Our laboratory examined triggered activity and found that 'embryonic ventricular-like' CMs exhibit delayed after depolarization, suggesting that hESC-CMs can be arrhythmogenic.

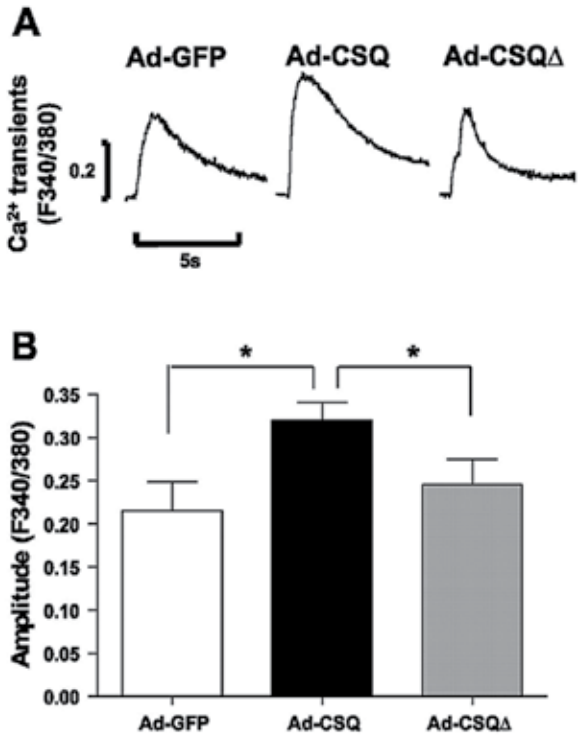

Fig. 3. Effect of CSQ overexpression on hESC-CMs.
A) Representative electrically-induced Ca^{2+} transient tracings for Ad-GFP (n=12) and Ad-CSQ (n=29) and Ad-CSQΔ (truncated mutant) (n=14) transduced hESC-CMs. B) Bar graphs of amplitude.* $P < 0.05$, ** $P < 0.01$. Adopted with permission from Liu et al 2009 *Am J Physiol Cell Physiol Vol. 297, No. 1*: pp.C152-159.

Subsequent studies were done to assess if hESC-CMs mature over time to acquire electrophysiological properties that are comparable to those of adult CMs and results are conflicting. Sartiani et al (2007) examined the AP of CMs over 3 months of culture and concluded that the molecular and functional expression of ion channels in hESC-CMs change over time, although they still do not reach the phenotype typical of adult VCMs (Sartiani et al., 2007). This is in contrast to findings by Pekkanen-Mattila et al (2010), which show that although one third of hESC-CMs exhibit a more mature phenotype, these changes are not correlated with time in culture (Pekkanen-Mattila et al., 2010). Taken together, these data suggest that hESC-CMs are functionally immature and present an arrhythmogenic risk. Therefore, facilitated *in vitro* maturation is important for the translation of hESC-CMs to the clinic and other applications (such as disease modeling, drug discovery and cardiotoxicity screening).

Our group sought to define the immature proarrhythmic electrophysiological properties observed in hESC-CMs by examining the role of different currents in automaticity (Azene et al., 2005; Siu et al., 2006; Xue et al., 2007; Lieu et al., 2008; Chan, Y. C. et al., 2009). I_{K1} (the inward-rectifier K^+ current encoded by Kir2.1), which stabilises a negative resting membrane potential, is important for suppressing automaticity and we hypothesize that its

absence in hESC-CMs may underlie their immature phenotype. Consistent with this, forced Kir2.1 expression alone sufficed to render the electrical phenotype indistinguishable from that of primary adult ventricular cells (Lieu DK, Fu JD and Li RA, unpublished data). These proof-of-concept experiments show that developmentally arrested Ca^{2+} and electrophysiological phenotypes of hESC-CMs can be rescued. We are currently developing a non-genetic, non-pharmacologic method to drive global maturation, by targeting the microenvironmental niches and other non-cell autonomous means.

5. The use of hESC-CMs for myocardial repair and bioartificial pacemakers

Myocardial infarction is the major worldwide cardiovascular disorder in humans and is the leading cause of death in many parts of the world. Immediately after a heart attack, oxygen starvation of myocardial tissues leads to cell death, often resulting in irreversible and permanent damage to the heart. Despite some improvements in short term management of acute myocardial infarction, long term prognosis remains poor. Sudden cardiac death due to ventricular arrhythmias remains a leading cause of morbidity and mortality in the industrialised world, claiming well over 300,000 lives annually in the United States alone. After myocardial infarction, the heart undergoes hypertrophy in an attempt to compensate for loss of CMs, and cardiac fibroblasts secrete collagen and other extracellular matrix proteins during scar formation, leading to impaired cardiac function. Since terminally differentiated CMs have very limited potential for regeneration, transplantation is the only treatment for end-stage heart failure. However, this is hampered by the lack of suitable donor organs and tissues. Cell-based therapy is thus a promising option for myocardial repair. A range of cell sources have been considered, including bone marrow cells, skeletal myoblasts and smooth muscle cells, but their non-cardiac identity has presented major problems. They either do not differentiate into the cardiac lineage or they do not integrate well into the host myocardium (Menasche et al., 2003; Smits et al., 2003; Balsam et al., 2004; Murry et al., 2004). As discussed in previous sections, hESC-CMs have functional and structural properties very similar to human embryonic/fetal CMs and is therefore a very promising cell source. Transplantation of hESC-CMs to mouse/rat hearts showed that the cells survived, formed myocardial tissue and promoted functional improvement in rat models of myocardial infarction (Laflamme et al., 2007; Mignone et al., 2010). More detailed studies into the maturation of hESC-CMs, as well as the electropysiological consequences of hESC-CM transplantation are required before these early successes can be translated into clinical therapies.

Normal rhythms originate in the SA node (SAN), a specialized cardiac tissue consisting of only a few thousands pacemaker cells. Malfunction of cardiac pacemaker cells due to disease or aging can cause rhythm generation disorders. Current treatments include pharmacological intervention and/or implantation of electronic pacemakers, but they are associated with significant shortcomings (e.g. increased susceptibility to infection, haemorrhage, lung collapse and death infection, finite battery life, patient discomfort related to the permanent implantation of a foreign device, and lack of intrinsic responsiveness to neural and hormonal regulation). Therefore 'bioartificial-pacemakers', made up of transplanted cells with pacemaker properties, may be a desirable alternative.

There are two major strategies for creating bioartificial-pacemakers. The first is to confer pacemaker ability on cells that are normally silent. For instance, adult atrial and ventricular

CMs are electrically silent unless they are stimulated by signals transmitted from the SAN. This is due to the absence of I_f, encoded by the HCN channel family, and the presence of I_{K1}. Several gene-based approaches have been pursued to induce pacemaker activity in these normally silent cells. Our group took a protein engineering approach to define criteria important for pacing and created a bioengineered construct of HCN (Lesso and Li, 2003; Tsang et al., 2004; Tsang et al., 2004; Tse et al., 2006; Xue et al., 2007). This engineered-construct was shown to produce pacing *in vitro* and *in vivo* (Tse et al., 2006; Xue et al., 2007). In a sick sinus syndrome porcine model, pacing of the heart was restored and originated from the site of focal transduction in the left atrium with HCN-construct injection (Tse et al., 2006). Somatic gene transfer to create such a gene-based bioartificial pacemaker significantly reduces the dependence on device-supported pacing by electronic pacemaker from 85% to 15%. Alternatively, hESCs can be differentiated into pacemaker-like derivatives for transplantation to recreate a cell-based bioartificial pacemaker (Kehat et al., 2004; Xue et al., 2005). Of note, the construction of cell-based bioartificial-pacemakers requires much fewer cells (several thousands) than myocardial transplantation (hundreds of millions). Furthermore, the spherical SAN is structurally less complex than the left ventricular myocardium. Our group is currently exploring the possibility of using nodal progenitors. We are also testing non-invasive catheter-based delivery techniques for implantation as well as long-term safety and efficacy.

6. Creation of engineered cardiac tissue constructs

The ventricular myocardium is a highly complex structure consisting of aligned, connected CMs, stromal cells and a vascular network systematically embedded in a mesh of extracellular matrix. Indeed, hESC-CMs differentiated *in vitro* lack the sub-cellular organization and higher order structural 2- or 3-dimensionality seen in adult heart. To more closely recapitulate the *in vivo* environment of the heart, various groups have used different approaches to manipulate the surface and geometry of the culture platform, cell and matrix composition. For instance, Luna et al used a tunable culture platform comprised of biomimetic wrinkles to simulate the heart's complex anisotropic and multiscale architecture and showed that the hESC-CMs cultured on these 'microgrooved' substrates display the typical tropomyosin banding pattern consistent with organized sarcomeric structure patterns (Luna et al., 2011) (Figure 4, adopted with permission from Luna et al 2011 *Tissue Eng Part C Methods* Vol. 17, No. 5: pp.579-588). Quantitative assessment based on nuclei shape and actin organization show that the hESC-CMs exhibit increased alignment on microgrooved substrates compared with controls. Functionally, aligned monolayers of hESC-CMs display anisotropic conduction properties with distinct longitudinal and transverse velocities, a signature characteristic of the native heart, not seen in control randomly organized monolayers (Lieu, Wang, Khine and Li, unpublished data). In another approach to mimic the structure of the heart, the Costa lab was among the first to construct 3-D engineered cardiac tissue constructs including cardiac papillary-like muscle strips as well as ventricle-like "organoid" chambers that exhibit key characteristics of cardiac physiology by ejecting fluid and displaying force-frequency and pressure-volume relationships (Kim, Do Eun et al., 2006; Lee et al., 2008). These studies were originally performed using rat cardiac myocytes but are now being applied to hESC/iPSC-CMs. Further optimization of hESC/iPSC-based cardiac tissue constructs will not only provide powerful tools for disease modeling, drug/cardiotoxicity screening and clinical translations,

but physiologic 3D environment also promises to reveal novel insights not possible with conventional rigid 2D culture systems.

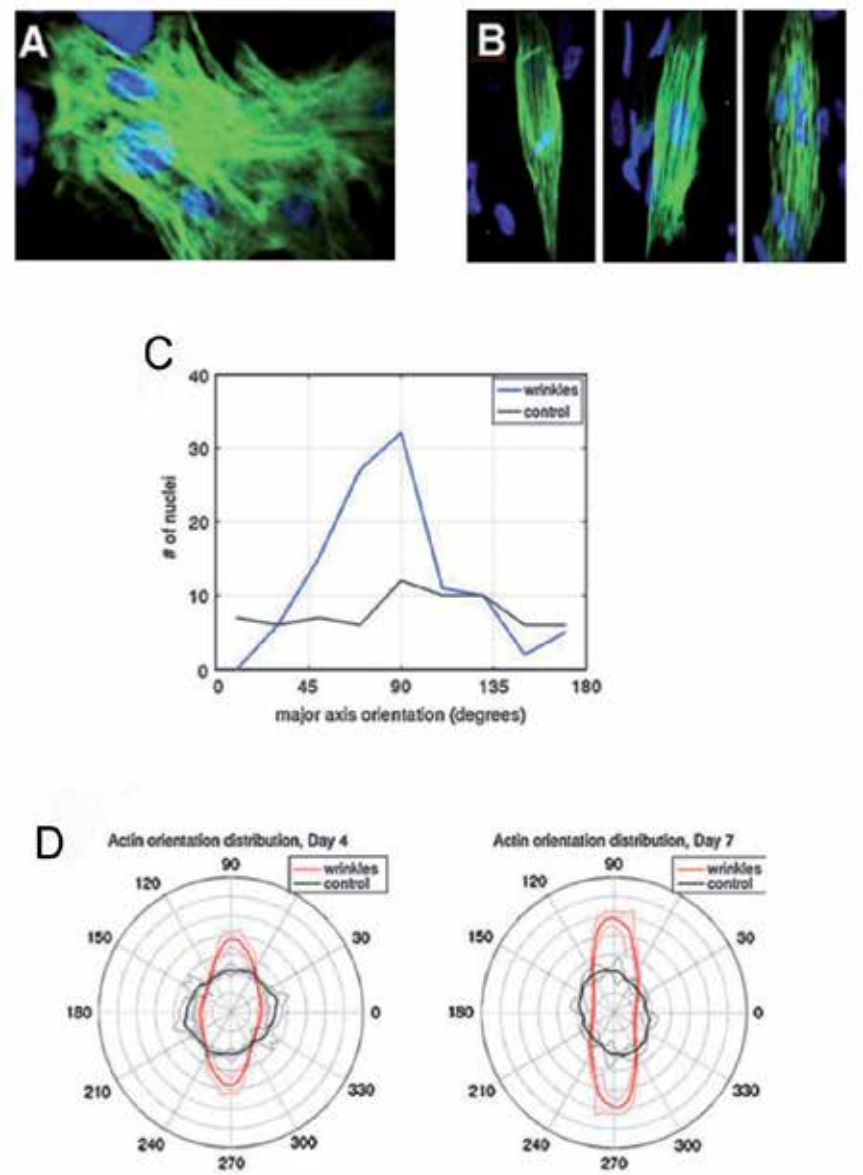

Fig. 4. Confocal micrographs of hESC-derived CMs alignment on wrinkles.
Human ESC-CMs were isolated and cultured on flat substrate (A) and wrinkle substrates (B) for 8 days. Green indicates tropomyosin staining, blue nuclear staining DAPI.
C) Image processing was used to detect the orientation of the DAPI-labeled nuclei. D) Anisotropy analysis of control (black) versus green (on wrinkles) showing that 90° is direction of wrinkles. The thinner lines indicate the standard deviations. Adopted with permission from Luna et al 2011 *Tissue Eng Part C Methods* Vol. 17, No. 5: pp.579-588.

7. Pharmacological testing using hESC-CMs

Adverse cardiac side effects is one of the most frequent reasons that cause drugs to be removed from the market. For instance, Vioxx, a once widely used COX-2 inhibitor prescribed to patients with arthritis and other conditions causing acute and chronic pain, was withdrawn from the market due to unexpected cardiotoxicity. Therefore, cardiotoxicity screening is necessary to test the efficacy and safety of new drug treatments. Cardiotoxicity arises from various mechanisms, including the modulation of signaling pathways and/or interference with the I_{Kr} current. The hERG channel, which produces the I_{Kr} current, is robustly blocked by a large class of drugs. This current has a major role in cardiac repolarization, so it affects the length of the action potential and the QT interval (the duration of ventricular depolarization and subsequent repolarization). QT prolongation may lead to arrhythmias, e.g. torsade de pointes, with potentially lethal consequences. Since adult CMs do not proliferate, animal CMs, isolated hearts or non-cardiac cells, which express cardiac ion channels are used although their non-human origin and instability in culture greatly limit their usefulness. Human ESC and hiPSC may provide an unlimited cell source for cardiotoxicity screening. Preliminary studies have already established that hESC-CMs/hiPSC-CMs do express the hERG mRNA (Sartiani et al., 2007; Tanaka et al., 2009), and display an outward 'I_{Kr}-like' current that is sensitive to selective blockers of I_{Kr} and canonical long QT-inducing inhibitors including E4031, sotalol, and cisapride (Sartiani et al., 2007; Caspi et al., 2009). When treated with a range of cardiac and non-cardiac drugs, hESC-CM also exhibit dose responses predictive of clinical effects (Braam et al., 2010). These suggest that hESC-CMs may be a suitable model for cardiotoxicity testing, although issues of CM purity and maturation should be considered in the design of future experiments, as already discussed elsewhere. It is hoped that high-throughput pharmacological systems involving hESC/hiPSC-CMs will soon be developed.

8. Human ESC-CMs/hiPSC-CMs as models of cardiovascular diseases

The use of hESC-/hiPSC-CMs as models of cardiac disorders is an exciting area of research. Previously, transgenic mouse models were used to study human cardiac diseases, but these mouse models do not always fully recapitulate the same phenotypes as those seen in humans. For instance, electrophysiologically, mice also have shorter action potential duration and higher heart rate compared to humans (Danik et al., 2002), limiting their usefulness as models of some disorders such as arrhythmias. Human pluripotent stem cell derived CMs are therefore logical suitable alternative. Reprogramming technology pioneered by Yamanaka and Thomson has led to the creation of disease- or patient-specific iPSCs. The derivation of hiPSC from patients with a range of diseases including adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker muscular dystrophy, Parkinson disease, Huntington disease, juvenile-onset, type 1 diabetes mellitus, Down Syndrome/trisomy 21, and the carrier state of Lesch-Nyhan syndrome has been reported (Park et al., 2008). More recently, Ebert et al (2009) reported the generation of hiPSC line from a patient of spinal muscular atrophy and these cells maintained the disease genotype and generated motor neurons that showed selective deficits compared to those derived from the patient's unaffected mother (Ebert et al., 2009). Some advances have also been made in the area of cardiovascular research. For instance, hiPSC models of long-QT syndrome type 1

and type 2 were generated (Moretti et al., 2010; Itzhaki et al., 2011). CMs differentiated from these hiPSC recapitulated the electrophysiological features of the disorders such as prolongation of AP and arrhythmogenicity and enabled the groups to study the pathogenesis of the diseases. Itzhaki et al (2011) also used the hiPSC-CMs to evaluate the potency of existing and novel pharmacological agents that may either aggravate or ameliorate the long-QT syndrome type 2 disease phenotype. These studies illustrate the potential of human iPSC technology to model the abnormal functional phenotype of inherited cardiac disorders and to identify potential new therapeutic agents.

9. Conclusion

Adult CMs lack the potential to regenerate. Human ESC and hiPSCs, with their potential for unlimited self-renewal and differentiation, offer an exciting means of generating human CMs for research and regenerative medicine. Concentrated effort by research groups worldwide has resulted in higher efficiency of cardiogenic differentiation and better characterization of hESC-/hiPSC-CMs. Experiments using animal models have demonstrated functional improvement after hESC-CM transplantation. However, substantial hurdles have to be overcome before hESC-/hiPSC-CMs can be translated into clinical applications. For instance, hESC-CMs are functionally immature, limiting their use for transplantation and as disease models. More studies are required to evaluate the long term effect of hESC-/hiPSC-CMs transplantation. Nonetheless, there is much reason to believe that hESC and hiPSC technology will bring significant benefit to cardiac research and treatment.

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10

Human Pluripotent Stem Cell-Derived Cardiomyocytes: Maturity and Electrophysiology

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1. Introduction

Human pluripotent stem cells comprise embryonic and induced pluripotent stem cells. Both of these are able to give rise to all cell types of an individual, including heart muscle cells or cardiomyocytes. First stable human embryonic stem cell (hESC) lines were derived in 1998 by James A. Thomson and his co-workers (Thomson et al., 1998). First cardiomyocytes derived from hESCs were made in 2001 by Kehat and co-workers (Kehat et al., 2001) and after that many research groups have derived and studied human pluripotent stem cell derived cardiac cells and their properties. In 2007 the first human induced pluripotent stem cells (hiPSCs) were produced by Shinya Yamanaka's (Takahashi et al., 2007) and James A. Thomson's (Yu et al., 2007) groups from dermal fibroblasts, and hiPSCs have subsequently also been shown to be able to give rise to cardiomyocytes (Zhang et al., 2009, Zwi et al., 2009). Pluripotent stem cell derived cardiac cells have a great potential for cardiotoxicity testing, for preclinical testing of new chemical entities for the pharmaceutical industry and hopefully in the future for cell therapy in myocardial infarction and heart failure. However, before these goals are achieved thorough characterization of the cardiomyocytes is needed to ensure their feasibility for these applications.

2. Differentiation of cardiomyocytes from pluripotent stem cells

Cardiomyocytes can be differentiated from pluripotent stem cells by multiple methods and the differentiation event is quite rapid, 10-20 days. However, all the differentiation methods have common problems, which include uncontrolled differentiation, low differentiation rate and heterogeneous differentiated cell population. In addition, the cardiomyocyte differentiation efficiency has been shown to vary markedly between different hESC lines (Pekkanen-Mattila et al., 2009). The differentiation methods are described in more detailed manner below and summarized in Figure 1.

2.1 Spontaneous differentiation in embryoid bodies

Cardiomyocyte differentiation from hESCs and hiPSCs in EBs has been described in many reports (Figure 1 A) (Itskovitz-Eldor et al., 2000, Kehat et al., 2001, Burridge et al., 2007, Zhang et al., 2009). In addition to cardiomyocyte generation, EB differentiation is widely

used also in production of other cell types such as neuronal cells, hematopoietic cells, adipocytes and chondrocytes (Pera & Trounson, 2004). For the whole existence of hESCs, EB differentiation has been widely used differentiation method for its relatively simple and inexpensive nature regardless the low differentiation rate. For example, if aiming at cardiac differentiation, under 10% of the EBs formed contain beating areas (Kehat et al., 2001).

2.2 Differentiation with mouse visceral endoderm –like cells

A little more directed way to differentiate cardiomyocytes from hESCs is in co-culture with mouse endodermal-like (END-2) cells (Figure 1B) (Mummery et al., 2003, Passier et al., 2005). The differentiation inducing factors are secreted from END-2 cells and therefore END-2 conditioned medium can also be used in cardiomyocyte differentiation (Graichen et al., 2008). With END-2 methods, cardiogenic differentiation potential can be enhanced with serum-free medium supplemented with ascorbic acid (Passier et al., 2005) or adding cyclosporine A to the culture medium (Fujiwara et al., 2011).

END-2 cells support the differentiation towards endodermal and mesodermal derivatives (Mummery et al., 2003, Passier et al., 2005, Beqqali et al., 2006). This is in accordance with embryonal development studies, which have shown that anterior visceral endoderm is essential in normal heart development (Lough & Sugi, 2000). The specific mechanism or the specific factors inducing cardiac differentiation by END-2 cells are, however, not clearly known. It has been suggested that removal of insulin by END-2 cells could have a role in this differentiation method. Insulin inhibits cardiac differentiation by suppressing endoderm and mesoderm formation and favouring ectoderm differentiation (Freund et al., 2008) therefore the elimination of insulin from the medium by END-2 cells could favour the cardiac differentiation. Additionally, another more promising mechanism inducing cardiac differentiation by END-2 cells has been suggested to be prostaglandin I2 (PGI2) (Xu et al., 2008).

2.3 Cardiac differentiation with defined growth factors

The combination of activin A and BMP-4 with matrigel has been used in differentiation protocols for cardiomyocytes (Figure 1C) (Laflamme et al., 2007). These factors enhance mesoendoderm formation, an early precursor cell lineage which gives rise to mesoderm and endoderm (Laflamme et al., 2007). Mesoderm is the origin of cardiac cells , but cardiac differentiation inducing signals are in large extent arising from endoderm (Lough & Sugi, 2000).

A stepwise differentiation protocol has also been developed by Gordon Keller's group (Yang et al., 2008, Kattman et al., 2011). This protocol involves induction of primitive streak-like population, in addition to formation of cardiac mesoderm and expansion of cardiac lineages. The protocol is based on EB differentiation and is comprised of three stages. Growth factors BMP-4, FGF, activin A, vascular endothelial growth factor (VEGF) and dickkoptf homolog 1 (DKK1) were used in varying combinations. Mesoendoderm formation has also been induced by Wnt3A, an activator of the canonical Wnt/β-catenin signalling pathway (Tran et al., 2009).

3. Characteristics of differentiated cardiomyocytes

Cardiac differentiation can be followed by multiple markers at gene and protein expression levels. During early stages of differentiation mesoderm formation is detectable by the elevated mRNA level of Brachyury T. Brachyury T expression peak is detected at day 3 in

END-2 co-cultures (Beqqali et al., 2006, Pekkanen-Mattila et al., 2009) and a day later in EBs (Bettiol et al., 2007, Pekkanen-Mattila et al., 2010). The cardiac differentiation cascade can be

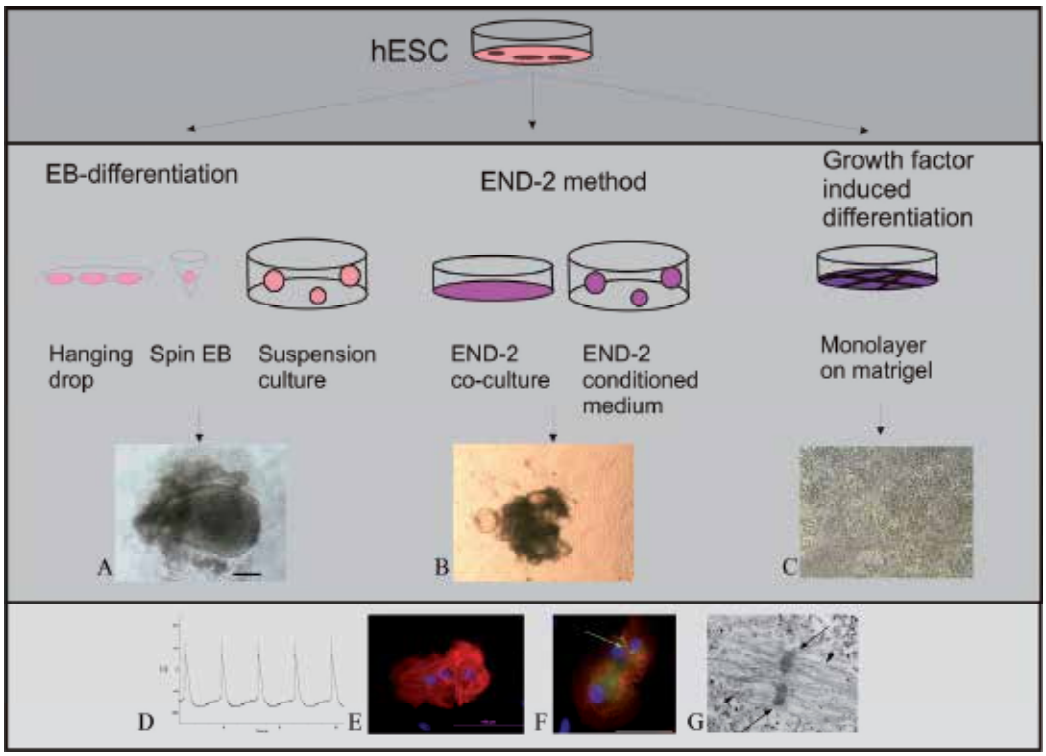

Fig. 1. Differentiation methods for hESC derived cardiomyocytes (middle column) and characteristics of the differentiated cells (undermost column). *EB-differentiation* can be performed in three ways, EBs can be formed either in hanging drops: enzymatically dissociated stem cells are pipetted in suspension into small drops on petri dish which is then inverted. EBs form in these drops and they can be plated down afterwards. EBs can be formed also in 96-well-plate wells, where single cell suspension is added and EB formation is forced by sentrifuging. Traditional was to EB formation is however the suspension method, where EBs form spontaneously in suspension from enzymatically dissociated hESCs. The picture A represents a 12-day old EB is attached to the bottom of cell culture dish. *Differentiation with END-2-cells* can be performed in two ways; hESC are plated onto END-2 cell layer and differentiated as co-culture or hESC are differentiated as EBs in END-2 conditioned medium. The picture B is taken from END-2 co-culture after one week of differentiation. Third way presented here is to culture hESCs on matrigel and initiate differentiation towrads cardiac lineage with *growth factors*, such as Activin-A and BMP-4. Differentiation is performed in a monolayer of cells (C).
The first sign for differentiated cardiomyocytes is the formation of beating areas. hESC-CM have the ability to contract spontaneously and in addition to fire spontaneously action potentials (D). Differentiated cells are stained positively with troponin T (E) and connexin 45 can be seen in the border areas of two troponin T positive cells (F). Electronmicroscopy (G) reveals sarcomere structures with Z-bands (marked with arrows) in the differentiated hESC-CMs.

followed further by the expression of the cardiac regulatory transcription factors such as Islet-1 (ISL-1), Mesp 1, GATA-4, NKX2.5 and Tbx6 (Graichen et al., 2008, Yang et al., 2008).
The first clear indication for emergence of cardiomyocytes is the formation of spontaneously beating aggregates or areas in the cell culture dish (Kehat et al., 2001, Mummery et al., 2003). The number of beating areas has been used in quantifying differentiation efficiency even though the beating areas contain varying numbers of cardiomyocytes (Passier et al., 2005, Pekkanen-Mattila et al., 2009, Pekkanen-Mattila et al., 2010). The amount of cardiomyocytes has also been quantitated using flow cytometry (Kattman et al., 2011). Electron microscopy studies reveal that the differentiated cardiomyocytes contain myofibrils which are first organized randomly throughout the cytoplasm. However, organized sarcomeric structures occur at later stages of differentiation with A, I, and Z bands (Figure 1G). In the vicinity of the sarcomeres, mitochondria are also present. In addition, cells have intercalated disks with gap junctions and desmosomes (Kehat et al., 2001, Snir et al., 2003, Pekkanen-Mattila et al., 2009). Cardiac structural proteins such as troponin I, T or C (Figure 1 E), myosins and α-actinin are also present in differentiated beating cells (Kehat et al., 2001, Mummery et al., 2003).
The beating function of heart muscle is the result of chain reactions between many ionic currents through cell membranes and sarcomeric proteins in the cytoplasm. Cardiomyocytes express ion channels and gap junction proteins on the cell membrane and these proteins are needed for transmission of electrical stimuli from cell to another (Figure 1 F) (He et al., 2003, Sartiani et al., 2007). Furthermore, there are intracellular channels such as ryanodine receptor 2 (RyR2) which are responsible for the calcium-induced calcium release (CICR) from the sarcoplasmic reticulum (Fabiato, 1983, Dolnikov et al., 2006, Satin et al., 2008). All these channels function in a cascade which results in synchronous contraction of the heart mediated by sarcoplasmic proteins.

4. Cardiomyocytes during embryonic development and stem cell differentiation

The development of the heart is composed of series of complicated differentiation events and morphogenetic changes (Buckingham et al., 2005). Additionally, electrical activities of cardiac cell change during development. For example, the average beating rate of human neonatal cardiomyocytes is ~140 beats per minute and in adult cardiomyocytes ~80 beats per minute (Huang et al., 2007). The data of human cardiac embryology is, however, very restricted and, thus, most information is based on animal models. Microelectrode recordings from chick embryo revealed that the first contracting cardiomyocytes have pacemaker like action potentials. According to these measurements, the membrane diastolic potential in the first pacemaker like cells is in the range of -35 mV, action potential amplitude is relatively small and Ca^{2+} dependent action potential upstroke is slow (Sperelakis & Shigenobu, 1972).
Similar postnatal development changes are seen in rabbit models. The beating rate decreases, action potential duration increases and the maximal diastolic potential (MDP) reaches more negative values during development (Toda, 1980). Allah and co-workers investigated in rabbits mRNA levels of several ion channels and Ca^{2+} handling proteins such as hyperpolarization activated cyclic nucleotide-gated potassium channel 4 (HCN4), $Na_{V1.5}$, $Ca_{V1.3}$, NCX 1, $K_{V1.5}$, ERG, K_VLQT1 (also known as KCNQ1) and minK and they observed decreased mRNA levels of all these factors and suggested that this could explain the postnatal decrease in the beating rate and increase of action potential duration (Allah et al., 2011).

Ca-handling in cardiomyocytes also changes during embryonic development. According to ultrastructural studies, the sarcoplasmic reticulum is not completely developed at the early developmental stages and therefore neonatal cardiomyocytes are suggested to be more dependent on transsarcolemmal Ca^{2+} influx than sarcoplasmic Ca^{2+} release (Brook et al., 1983, Nakanishi et al., 1987, Nassar et al., 1987, Klitzner & Friedman, 1989). Therefore Na^{+}/Ca^{2+} exchanger (NCX) have been suggested to have a important role in excitation-contraction coupling during the early developmental stages (Klitzner et al., 1991, Wetzel et al., 1991, Wetzel et al., 1991, Huynh et al., 1992) . Indeed, NCX gene and protein expression, in addition to NCX current have been shown to be enhanced at the early developmental stages (Artman, 1992, Artman et al., 1995, Chin et al., 1997, Haddock et al., 1997, Gershome et al., 2011).

Asp and colleagues compared the cardiac marker and ion channel expression of human embryonic stem cell derived cardiomyocyte (hESC-CM) clusters to human fetal, neonatal and adult atrial and ventricular origin heart tissue samples (Asp et al., 2010). They found the beating frequencies between hESC-CM clusters to vary substantially. They could identify two groups, one having slow (< 50 beats per minute[bpm]) and the other high (> 50 bmp) beating rate and they suggested these could represent ventricular and atrial type of cardiac cells, respectively. They also demonstrated that hESC-CMs had higher *NKX2.5* and cardiac muscle actin mRNA expression levels than the heart samples. The *NKX2.5* expression level, however, decreased over time in culture finally approaching that of the heart samples. Cardiac troponin T was more strongly expressed in ventricular samples and in hESC-CMs compared to atrial samples. The levels of cardiac troponin T mRNA also decreased over time in hESC-CMs. Phospholamban was expressed less in the atrial samples and hESC-CMs compared to ventricle samples. α-myosin heavy chain, which is normally mostly expressed in atrial tissue, was more strongly expressed in hESC-CMs with beat rates over 50 bpm and in adult atrial tissue samples whereas β-myosin heavy chain was more expressed in the ventricular heart samples and less in all the other heart preparations of hESC-CMs. The α-myosin heavy chain mRNA levels increased with increasing age of the hESC-CMs. Across all the heart tissue and hESC-CM samples cardiac RyR2, L-type calcium channel, and $Na_{V1.5}$ sodium channel mRNA were similarly expressed. HERG mRNA expression in hESC-CMs was similar to neonatal and adult atrial samples. *HCN2* was expressed in a more comparable way to the ventricular samples. Only *HCN4* expression differed between the hESC-CMs, with those having the beating rate of less than 50 bpm having lower expression. Overall, it was concluded that the difference between slow beating and fast beating hESC-CMs paralleled the human atrial and ventricular tissues (Asp et al., 2010). Despite small differences in expression levels, the conclusion from this study was that stem cell -derived cardiac cells share many similarities with human heart tissue and thus stem cell -derived cardiac cells are a good cellular model for human heart.

5. Electrophysiology of human pluripotent stem cell derived cardiomyocytes

The electrical properties of pluripotent stem cell derived cardiac cells have been studied principally either with patch clamp analysis of single cells or with microelectrode array (MEA) platform using beating cell aggregates.

5.1 Patch clamp analysis

Patch clamp method has been developed to study ion channels in excitable membranes. (Sakmann & Neher, 1984). In this technique micropipette is attached to the cell membrane

by a giga seal and this can be exploited to measure current changes and voltage across the membrane. This technique has been widely used in detailed electrical analysis of pluripotent stem cell derived cardiomyocytes.

Key cardiac ion channel types (and respective currents in brackets) involved in the human ventricular action potential include $Na_{V1.5}$ (I_{Na}), $K_{V4.3}$ (I_{to}), $Ca_{V1.2}$ ($I_{Ca,L}$) $K_{V11.1}$ (I_{Kr}), $K_{V7.1}$ (I_{Ks}), and $K_{ir2.X}$ (I_{K1}) (Pollard et al., 2010). These ion channels mediate the complex interaction between the currents and result in the characteristic action potential shape which can be divided into five different phases (Figure 2). Phase 0 of the action potential is the depolarization of the cardiomyocytes from the negative membrane potential to positive, called the upstroke. This is followed by phase 1, the short transient repolarization that is followed by phase 2, the plateau at slightly less positive membrane potential than the

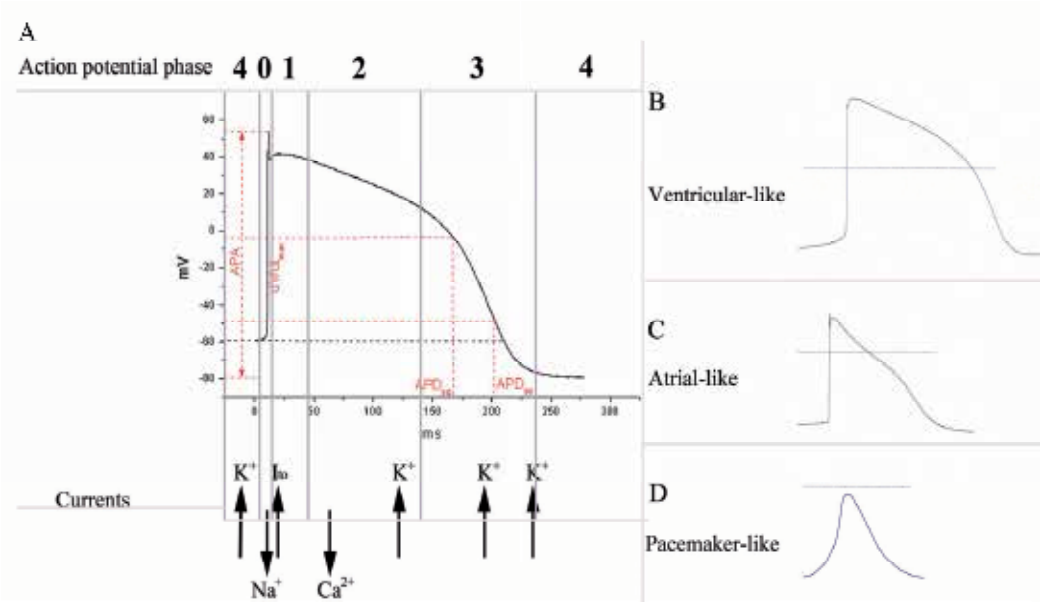

Fig. 2. Action potential phases and specification of cardiomyocyte subtypes. (A) Action potential (AP) parameters: Action potential amplitude (APA), maximum rate of rise of the action potential (dV/dtmax), action potential delay (ADP) and membrane diastolic potential (MDP). AP phase 0 is a rapid depolarization phase when the sodium channels are activated and membrane permeability is increased to Na+. Rapid depolarisation is followed by rapid repolarization phase 1 and plateau phase 2, where Ca2+ ions are entered to the cell throught L-type calcium channels. At phase 3, calcium channels are inactivated and repolarization is caused by outward potassium currents. Repolarization is due to the currents carried mainly by the slow Iks and rapid Ikr components of the delayed rectifier potassium channels. The Ikr current is produced by hERG channel (encoded by the human ether-à-go-go-related gene). By contrast, inward potassium current contributes to the maintenance of the resting membrane potential, phase 4. B-D. Classification of ventricular (B), atrial (C) an pacemaker-like (D) action potentials. Ventricular action potential has a prominent plateau phase whereas atrial action potential is more triangularly shaped. Pacemaker-like cells are characterized by slower upstroke velocity and amplitude if compared to ventricular and atrial type of cells.

maximal upstroke value. Phase 2 is followed by phase 3, which is the repolarization back to the resting membrane potential. The resting state of the membrane potential is phase 4 (Nerbonne & Kass, 2005).

Sartiani and co-workers have investigated the expression of different ion channel proteins and respective ion currents with patch clamp technique in undifferentiated hESCs and in their early- (days 15 to 40) and late-stage (days 50-110) cardiomyocyte derivates using the EB differentiation method (Sartiani et al., 2007). Some ion currents were present readily in the undifferentiated cells (I_{Kr}, I_f, $I_{ca,L}$). The properties of these currents were modified during hESC-CM development and some new currents (I_{to} and I_{K1}) were introduced, so the cardiomyocytes achieved maturation over time. With regard to the I_{to}, transient outward potassium current, the two isoforms $K_{v1.4}$ and $K_{v4.3}$ were differentially expressed in the developing cardiomyocytes at mRNA level. The shorter $K_{v4.3}$ splice variant was expressed in over 57-days-old cardiomyocytes whereas the longer one was expressed in the earlier cardiomyocytes. The $K_{v1.4}$ isoform is expressed at least from day 25 onward. Despite $K_{v4.3}$ mRNA expression in hESCs I_{to} current was not present in them. I_{to} could be detected using patch clamp on day 12 in the developing hESC-CMs and it was higher in later-stage (57 days old) cardiomyocytes compared to earlier ones (Sartiani et al., 2007).

Another repolarizing current (I_{Kr}), encoded by the human ether-a-go-go related gene (*HERG*) channel is also expressed in hESCs as well as the developing cardiomyocytes. However, the shorter splice variant *HERG1b* mRNA was only expressed in the developing hESC-CMs. Using patch clamp an outward K^+-current sensitive to E-4031, a selective blocker of the I_{Kr} current, could also be recorded in hESCs. *HCN* isoforms encode the I_f depolarization current. *HCN1* and *HCN4* were expressed more strongly in the undifferentiated hESCs and early cardiomyocytes than in late cardiomyocytes. *HCN4* was also expressed in the adult heart, whereas *HCN1* was not. *HCN2* was expressed in the adult heart as well as strongly in hESCs and early and late hESC-CMs. Voltage clamp experiments revealed I_f currents in hESCs and early and late developing cardiomyocytes. However, during cardiomyocyte maturation, the I_f activation rate decreased. With regard to another depolarization current $I_{K1,}$ the $K_{ir2.1}$ mRNA was already present in hESCs but the current could only be measured from the developing cardiomyocytes (Sartiani et al., 2007).

Voltage-dependent Ca^{2+} current ($I_{Ca,L}$) is mediated by α1C subunit of the calcium channel in many tissues and this subunit is encoded by *CACNA1C* gene. Sartiani and co-workers demonstrated mRNA expression of *CACNA1C* to be present both in undifferentiated hESCs and in the cardiomyocytes. Also, the $I_{Ca,L}$ current could be recorded from hESCs as well as the hESC-CMs. During hESC-CM development the action potential upstroke velocity and action potential duration increased significantly. The beating rate on the other hand decreased during cardiomyocyte maturation and the diastolic depolarization rate flattened in the late cardiomyocytes. Pharmacological interventions also demonstrated intact ion channel function and expected responses were obtained with E4031 and $BsCl_2$ (I_{K1} blockers), zatebradine (I_f blocker), and lacidipine ($I_{Ca,L}$ blocker). Finally, stimulation with isoprenaline proved intact β-adrenergic signalling in the stem cell derived cardiomyocyte (Sartiani et al., 2007).

Taken together, the cardiomyocytes seem to achieve a more mature cardiac phenotype over time in cell culture, even though this has not been confirmed in all studies (Pekkanen-Mattila et al., 2010). The I_{to} and I_{K1} currents could serve as markers for hESC cardiac differentiation (Sartiani et al., 2007) since they appear only later in cardiac differentiation. I_{to} current has also been shown to increase in postnatal rat cardiomyocytes (Guo et al., 1996,

Shimoni et al., 1997) and I_{K1} current has been shown to stabilize the diastolic potential in myocytes (Silva & Rudy, 2003).

With regard to the EB and END-2 co-culture differentiation methods our own experiments demonstrated that the EB method produces slightly more cardiomyocytes with consistent beating rate and more cardiomyocytes with ventricular type action potentials. The EB method also produced cardiomyocytes with significantly more hyperpolarized MDP (Pekkanen-Mattila et al., 2010). Low expression of I_{K1} current in developing hESC-CMs seems to be responsible, at least in part, for their low MDP (Sartiani et al., 2007). Cardiomyocytes produced with both methods did not differ in their upstroke velocity (Pekkanen-Mattila et al., 2010a).

Dolnikov and colleagues studied mechanical functions of hESC derived cardiac cells. They found that hESC-CMs have a negative force-frequency relation, whereas mature human myocardium the relationship is positive (Dolnikov et al., 2005). They also found that blocking the ryanodine receptor or the sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPase did not affect the hESC-CM contraction as it usually does in mature cardiomyocytes. Furthermore, caffeine did not result in increase of intracellular calcium concentration. However, in subsequent studies caffeine-indused release of intracellular Ca^{2+} has been documented both in hESC and hiPSC -derived cardiac cells (Satin et al., 2008, Itzhaki et al., 2011). Both RyR mediated release of intracellular Ca^{2+} stores as well as the reuptake of Ca^{2+} by SERCA into endoplasmic reticulum were reported to occur the same way as in cardiac tissue. These results indicate that Ca^{2+} handling in both hESC and hiPSC-derived cardiac cells are functional and further indicate the potential of these cardiac cells in the future applications in basic research as well as in translational cardiac research.

5.2 Microelectrode array platform

In addition to the more traditional patch clamp (Hamill et al., 1981) studies the MEA platform (Reppel et al., 2004) offers an easy and convenient medium-throughput technique to assess the electrical properties of the differentiated cardiomyocytes (Reppel et al., 2005). Action and field potential curves achieved by patch clamp and by MEA are represented in Figure 3. The MEA platform presents an advantageous additional tool for cardiac safety studies in addition to the more traditional Langendorff heart organ model, conventional patch clamp electrophysiology studies and heterologous expression systems of ion channels, especially the hERG potassium channel (Meyer et al., 2004)..

The MEA system allows examination of multicellular cardiac syncythia, thus enabling electrocardiogram-like mapping of their field potential properties. Cardiac repolarization in hESC-CMs can be therefore investigated with MEAs and it has been demonstrated that drug effects can be investigated using this platform (Reppel et al., 2005). With MEA system, Caspi and co-workers were able to investigate drug effects on hESC-CMs. E-4031 is a compound that blocks I_{Kr} repolarizing current and this effect can be seen as prolongation of the cardiac field potential (FP) cycle in the electrocardiogram. The authors were able to demonstrate a dose-dependent effect where the field potential duration (FPD) prolonged by escalating concentrations of E-4031. Sotalol, a class III antiarrhythmic agent, also increased FPD as did quinidine and procainamide, both class IA antiarrhythmic agents. Cisapride, a gastrointestinal prokinetic drug that was withdrawn for the market due to adverse cardiac side effects, prolonged the FPD as well, as seen on the MEA recordings (Caspi et al., 2009, Liang et al., 2010).

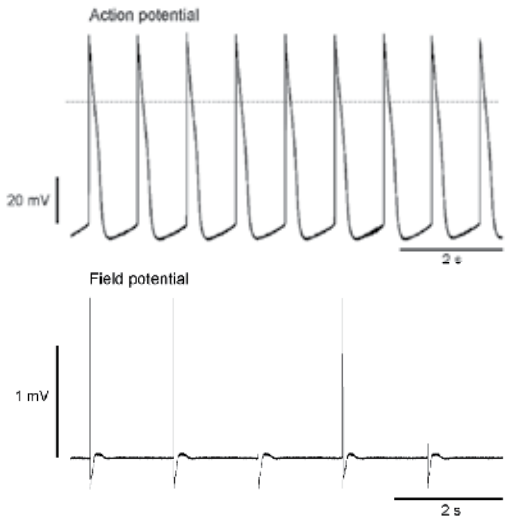

Fig. 3. Action and field potentials recorded from human embryonic stem cell –derived cardiomyocytes. Action potentials can be measured from single cardiomyocytes in current clamp mode using the patch clamp technique. Field potentials can be measured from a multicellular cardiac syncytium with microelectrode arrays.

To assess the practicality of using hESC-CMs in conjunction with the MEA platform in pharmacotoxicological testing, Braam and colleagues investigated the effects of various drugs on the FPD of cardiomyocytes derived from human ES cells (Braam et al., 2010). They tested 12 compounds for their potential in prolonging repolarization (FPD) in hESC-CMs. Despite the relatively lower maturation of hESC-CMs compared to mature cardiomyocytes, they could be used in predicting the clinically observed cardiotoxic effects. Blocking of sodium, calcium and *HERG* potassium channels by lidocaine, nifidipine, and E-4031, respectively, had expected effects on hESC-CM field potential properties. Quinidine and sotalol, both used clinically to prolong repolarization, increased hESC-CM FPD at concentrations near the unbound effective therapeutic plasma concentration (ETCP unbound). They also tested drugs which have been known to prolong the QT interval in patients and noticed that the FPD prolongations too place at concentrations that were not as near the ECTP unbound range. The varying FP shapes in hESC-CM recordings did not affect the results, meaning that FPD changes in these cardiomyocytes can be reliably detected despite the large variety of FP shapes (Braam et al., 2010). The study provided, for the first time, data on the effects of large number of tested compound over a high concentration range on the FP properties of hESC-CM.

6. Applications for pluripotent stem cell-derived cardiomyocytes

6.1 Human model for development of cardiomyocytes and cardiac electrophysiology

The amount of data about electrophysiological changes during human cardiac differentiation is limited. Data is mostly based on animal models, but due to the physiological differences between species this data cannot be always applied directly to humans. Human pluripotent stem cells differentiate into functional cardiomyocytes and these cells also mature in culture (Sartiani et al., 2007). Therefore they provide a good model

to study development of complex network of cardiac ion channels involved in signal transduction and cardiomyocyte contraction.

6.2 Drug screening and safety pharmacology

Many cardiac and also non-cardiac drugs have been withdrawn from the market because of toxic effects on heart and its function. Even though all new chemical entities (NCE) are tested according to requirements during drug development process, unforeseen effects such as syncope, arrhythmia, sudden death, polymorphic ventricular tachycardia (Torsade de pointes [TdP]) are occasionally seen only in clinical trials or when the drug is already on the market (Redfern et al., 2003, Roden, 2004, Lexchin, 2005). Pharmaceutical regulatory authorities have specified and expanded the requirements for safety testing and recommend that tests are done with two mammalian species, one rodent and other nonrodent species. Tests include electocardiographic recordings and also histological studies of the heart (ICH, 2005, ICH, 2005, EMEA, 2008).

With regard to proarrhythmic potential, the QT interval is the cornerstone of the guidelines for the assessment of new chemical compounds (ICH, 2005, ICH, 2005). A number of drugs can potentially prolong the QT interval (Fenichel et al., 2004, Roden, 2004), and it is also a leading cause for use restriction and market withdrawal (Roden, 2004), the International Conference of Harmonization has defined the evaluation of this risk for new chemical entities as standard preclinical process (Bode & Olejniczak, 2002, Cavero & Crumb, 2005).

Delayed rectifier potassium current (I_{Kr}) is responsible in part for the repolarization of the action potential (Vandenberg et al., 2001, Pollard et al., 2008). Inhibition of this hERG channel ($K_{V11.1}$) and the subsequent inhibition of the I_{Kr}, is the predominant basis of drug-induced QT prolongation and TdP (Hancox et al., 2008; Redfern et al., 2003). Currently a number of preclinical models and assays have been employed by pharmaceutical companies (Carlsson, 2006, Pollard et al., 2008). These assays include in vivo QT assays, such as ECG telemetry of conscious dogs (Miyazaki et al., 2005), and in vitro assays, such as repolarization assay, which detects changes in the action potential delay (APD) of cardiac tissues (isolated animal Purkinje fibres, papillary muscles or cardiomyocytes) or the hERG channel assay where hERG current expressed in heterologous cell system (such as CHO or HEK293 cells) or native I_{Kr} is characterized (Finlayson et al., 2004, Martin et al., 2004). However, current methods are not fully adequate (Redfern et al., 2003, Lu et al., 2008). They are costly and the in vivo assays are ethically questionable because of the large number of animals used. Therefore there is a need for an in vitro method based on human cardiac cells that would bring additional value and reliability for testing novel pharmaceutical agents. Cardiomyocytes derived both from hESC and iPS cells have many potential applications in the pharmaceutical industry including target validation, screening and safety pharmacology. These cells would serve as an inexhaustible and reproducible human model system and preliminary reports of the validation of hESC-CM system already exist (Braam et al., 2010, Mandenius et al., 2011).

6.3 Disease modelling with induced pluripotent stem cell –derived cardiomyocytes

The hiPSC technology (Takahashi et al., 2007, Yu et al., 2007) presents a great opportunity to investigate diseases in cell culture that would otherwise be challenging to study. Although the full potential of this method is still to be realized in cardiac research, some preliminary results provide encouragement to investigate this path further.

One condition that is challenging to study in patients in terms of underlying molecular mechanisms is the long QT syndrome (LQTS). This condition can be either genetic or acquired as a side effect of using certain therapeutic drugs. The first LQTS modelling using hiPSCs was reported in 2010 (Moretti et al., 2010). Pluripotent stem cells were reprogrammed from fibroblasts of two family members having LQTS type 1, and cardiac cells derived from these hiPSCs with LQT1 genotype had prolonged action potential. Additionally increased arrhythmogenicity could be demonstrated with isoproterenol. Similar findings have been reported about LQTS type 2 (Itzhaki et al., 2011, Matsa et al., 2011).

Cardiomyocytes have also been derived from hiPSCs from a patient with Timothy syndrome (Yazawa et al., 2011). These patients have mutation in the *CACNA1C* gene that encodes the $Ca_V1.2$ calcium ion channel in humans. The cardiomyocytes having Timothy syndrome genotype exhibited irregular contractions and excessive calcium influx as well as action potential prolongation, irregularities in the electrical activation and abnormal calcium transients in the ventricular-type cardiomyocytes (Yazawa et al., 2011).

Taken together these results provide optimism for modelling different cardiac disease phenotypes in cell culture conditions. This allows for more detailed dissection of the pathological pathways and molecular interactions within and between the cells. An additional benefit is also the fact that these studies can be now carried out in patient-specific cells which provide suitable genomic backgrounds for more optimal comparison between the clinical disease phenotype and results obtained *in vitro*.

7. Challenges in pluripotent stem cell research

While human pluripotent stem cells represent a promising new tool for pharmacological and toxicological testing and hopefully also for regenerative therapies in the future some hurdles remain to be cleared before we can achieve those goals efficiently. With regards to hiPSC a question remains how close these cells are to hESCs in their properties. While they fulfil the criteria required for pluripotent stem cells, some recent studies suggest that they retain some aberrant epigenetic reprogramming compared to ES cells (Lister et al., 2011). Both cell types have similar global methylomes, but the reprogrammed iPS cell seem to retain some memory of the somatic cell DNA methylation patterns in addition to the methylation patterns that are specific for iPSCs (Lister et al., 2011). However, the cardiomyocytes differentiated from hiPSCs and hESCs seem to have very similar global transcriptomes (Gupta et al., 2010).

Another issue with both, hESCs and hiPSCs, are the currently suboptimal differentiation protocols for desired differentiated cell types. Cardiomyocytes are no exception in this case and several protocols for more efficient differentiation have been experimented with. Recently, increased yields of cardiomyocytes have been obtained by stage-specific optimization of the activin/nodal and bone morphogenetic protein (BMP) signalling (Kattman et al., 2011). The directed differentiation protocol of hESC-CMs in a monolayer, with activin A and BMP4 supplementation, represents another step forward in creating more efficient differentiation methods (Laflamme et al., 2007). In hiPS reprogramming omission of c-Myc from the four factor Yamanaka cocktail has been shown to enhance their cardiogenic potential (Martinez-Fernandez et al., 2010).

To achieve better cardiomyocyte differentiation efficiencies with pluripotent stem cells we need to gain more insight into the lineage-specification steps that govern the transformation

of pluripotent stem cells to committed progenitors and finally to mature differentiated cardiomyocytes. For example, $ISL1^+$ cardiac progenitors are able to give rise to cardiomyocytes, smooth muscle, and endothelial cell lineages and these progenitor populations can be expanded in cell culture (Bu et al., 2009). KDR^+ cells derived from human embryonic stem cells have also been shown to give rise to cardiac progenitor cells (Yang et al., 2008).

8. Conclusion

In conclusion, human pluripotent stem cell derived cardiomyocytes have very similar electrophysiological properties as human heart tissue and, thus, they have a great potential in the future to benefit pharmaceutical and toxicological industry. Additionally, with these cells we are closer than ever before to individualized, patient-specific treatments. However, a lot of basic research is still required before we can utilize the full advantage of human pluripotent stem cell –derived cardiac cells.

9. References

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Maintenance Of Calcium Homeostasis in Embryonic Stem Cell-Derived Cardiomyocytes

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1. Introduction

Myocardial infarction is one of the major causes of morbidity and mortality in many developed countries. A potential method for treatment of such disease is the cell replacement therapy which involves the transplantation of cardiomyocytes (CMs). However, CMs are of very limited supply. Embryonic stem cells (ESCs) isolated from the inner cell mass of blastocysts are capable of self-renewal and can differentiate into all cell lineages, including CMs (He et al., 2003; Kehat et al., 2002; Kehat et al., 2001; Moore et al., 2008; Mummery et al., 2003; Ng et al., 2010; Thomson et al., 1998; Xu et al., 2002; Xue et al., 2005). Therefore, ESCs can be an excellent source of CMs for regenerative medicine.

Calcium (Ca^{2+}) is a universal signaling molecule that regulates a wide variety of cellular functions. In CMs, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) plays an important role in the contraction and relaxation of CMs. The $[Ca^{2+}]_i$ is tightly regulated by many proteins, including ion channels, receptors, pumps, and exchangers that are located on the cell surface plasma membrane and on the sarcoplasmic reticulum (SR). The aim of this book chapter is to provide a thorough review on Ca^{2+} handling in ESC-derived CMs.

For each protein of interest, some basic information on the protein was firstly presented. Then, changes in the expression of the protein and their contribution to the Ca^{2+} homeostasis and Ca^{2+} transient as described in human (h) and/or mouse (m) ESC studies were presented. *In vivo* data in mouse embryo studies were also presented for comparison purposes.

The information reviewed in this chapter would be important not only for understanding the basic biology of early differentiating CMs, it would also be important for providing insights into the future uses of ESC-derived CMs for cell replacement therapies.

2. Regulation of intracellular calcium level by voltage-operated calcium channels

Voltage-operated Ca^{2+} channels are typically composed of five subunits, namely α_1, α_2, β, δ and γ (Catterall, 1995; Catterall, 2000; De Waard et al., 1996; Dolphin, 2006; Moreno Davila,

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1999). The α_1 subunit forms the ion conduction pore of the channels while the other subunits modulate the functions of the channels. The α_1 subunit is itself a tetramer of proteins, each of which consists of six transmembrane segments (S1-S6). This subunit confers all of the major properties to voltage-operated Ca^{2+} channels in that it contains a voltage sensor at segment S4 and forms a Ca^{2+}-selective pore at segments S5-S6. The other subunits are regarded as to providing ancillary functions to the channels. The α_2 and δ subunits are two products of the same gene; the protein is cleaved into two peptides following translation. Subsequent disulfide bond formation anchors the extracellular α_2 subunit to the membrane via the transmembrane δ subunit. The β subunit is a cytoplasmic protein; it contains an α_1-binding pocket, where specific amino acids in the linker region between segments S1 and S2 of the α_1 subunit bind (Richards et al., 2004). The γ subunit is a monomer, which consists of four transmembrane segments (S1-S4). A diverse range of functionally distinct voltage-operated Ca^{2+} channels are formed by the combinations of different isoforms that have been identified for each of these subunits. The α_1 subunit is encoded by a family of ten distinct genes (the Ca_V gene family), which can be divided into three sub-families; Ca_V1 consists of $Ca_V1.1$-1.4, Ca_V2 consists of $Ca_V2.1$-2.3 and Ca_V3 consists of $Ca_V3.1$-3.3 (Catterall et al., 2005). The α_2/δ and β subunits are each encoded by four genes; $\alpha_2/\delta_{1\text{-}4}$ and $\beta_{1\text{-}4}$ respectively, while the γ subunit is encoded by eight genes; $\gamma_{1\text{-}8}$ (Arikkath and Campbell, 2003; Yang et al., 2011). Voltage-operated Ca^{2+} channels function by regulating the entry of extracellular Ca^{2+} into cells. Two types of voltage-operated Ca^{2+} channels exist in adult CMs (Catterall et al., 2005; Ono and Iijima, 2005). These are T-type Ca^{2+} channels, which open in response to more negative membrane potentials at about >-70mV for very short durations of 0.5 to 2 milliseconds (transient activation), and L-type Ca^{2+} channels, which open in response to less negative membrane potentials at about >-30mV for relatively longer periods of 0.5 to 10 milliseconds (long-lasting activation) (De Waard et al., 1996).

2.1 T-type calcium channels

T-type Ca^{2+} channels could be the simplest type of voltage-operated Ca^{2+} channels known thus far. Its structure was initially predicted *in silico* by homology to other α_1 subunits known at the time (Perez-Reyes, 2006). Since expression of the synthetic products was able to reproduce most of the electrophysiological properties observed in its native form, T-type Ca^{2+} channels were thought to consist of only one α_1 subunit; the other ancillary subunits (α_2, β, δ and γ) were considered absent. Although later co-expression studies could describe a role for the other ancillary subunits in the regulating functions of T-type Ca^{2+} channels *in vitro*, presence of these subunits have not been validated *in vivo* (Dolphin et al., 1999; Dubel et al., 2004; Green et al., 2001; Hobom et al., 2000; Lacinova and Klugbauer, 2004). Therefore, further investigations will be required to ascertain the genuine structure of this type of voltage-operated Ca^{2+} channel.

The α_1 subunit of T-type Ca^{2+} channels is encoded by either one of the three Ca_V3 genes, among which $Ca_V3.1$ and $Ca_V3.2$ are expressed in sinoatrial (SA) nodal cells of the heart (Bohn et al., 2000; Perez-Reyes, 2003). The SA node is the primary site, where spontaneous rhythmic action potentials are initiated. At the beginning of each action potential in an SA nodal cell, an influx of extracellular Na^+ into the cytosol, known as the funny current (I_f), first depolarizes the cell to about -50mV, at which T-type Ca^{2+} channels on the membrane open to allow an influx of extracellular Ca^{2+} into the cytosol. This produces a T-type Ca^{2+} current ($I_{Ca,\ T}$), which further depolarizes the cell to about -40mV. L-type Ca^{2+} channels on the membrane then open at this membrane potential to allow a greater influx of extracellular

Ca^{2+} into the cytosol and produce an L-type Ca^{2+} current ($I_{Ca,\ L}$) to a membrane potential until the threshold to produce an action potential is reached. Action potentials generated in the SA nodal cells are conducted to CMs within the working myocardium via gap junctions, ultimately resulting in regular contractions of the adult heart.

$Ca_V3.1$ mRNA and protein were simultaneously detected as early as embryonic day 14 (E14) after the heart has fully matured *in vivo* within mouse embryos (Cribbs et al., 2001). In a separate experiment, the mRNA level of $Ca_V3.1$ was moderately reduced from E18 when compared to its expression at adult stage (Yasui et al., 2005). Ni^{2+} at a high concentration of 100μM could be used to selectively block 50% of $Ca_V3.1$ T-type channels. The application of Ni^{2+} at this concentration was found to reduce $I_{Ca,\ T}$ by about 40% in CMs derived from mouse embryos at E12.5, indicating that the $Ca_V3.1$ T-type channels were functional by this time-point (Cribbs et al., 2001). In addition, homozygous null $Ca_V3.1^{-/-}$ mice displayed bradycardia, i.e. a reduced rate of cardiac contraction (Mangoni et al., 2006). Expression of $Ca_V3.1$ has also been studied *in vitro*. Its mRNA could be detected as early as day 5 and generally increased up until its last measurement at day 15 post-differntiation in ht7 mouse ESC- (mESC-) derived CMs (Mizuta et al., 2005). In R1 mESC-derived CMs, however, $Ca_V3.1$ mRNA could only be detected at two copies per cell by day 12, with its expression reaching the highest level at day 23 and declining by day 34 post-differentiation; the expression pattern reflected the amplitudes of $I_{Ca,\ T}$ measured at these time-points. (Zhang et al., 2003). A reduction of about 46% in $Ca_V3.1$ expression from day 9.5 to day 23.5 post-differentiation was also reported in EMG7 mESC-derived CMs; this down-regulation was found to be associated with a decrease in the contraction rates of the CMs in that $I_{Ca,\ T}$ was smaller in non-contracting myocytes at day 23.5 when compared to contracting CMs at day 9.5 and that the contraction rate at day 9.5 could be reduced via the application of 10μM efonidipine, an L- and T-type blocker, to the CMs (Yanagi et al., 2007). These data suggested a role for $Ca_V3.1$ in regulating the early contractions observed in the developing embryonic heart; by maintaining these contractions exclusively in developing pacemaker cells, other cells could then develop into non-contracting atrial or ventricular cells.

$Ca_V3.2$ mRNA was detected as early as eight-week gestation in humans (Qu and Boutjdir, 2001). In mouse, the expression of $Ca_V3.2$ was also measured at three time-points; its mRNA was detected as early as E9.5 and then its level was decreased by E18 until it was no longer detected at adult stage (Yasui et al., 2005). Again, $Ca_V3.2$ has been studied *in vitro*. Its mRNA was detected as early as day 5, but peaked at day 6 and gradually declined and was still detectable up until the last measurement at day 15 post-differentiation in ht7 mESC-derived CMs (Mizuta et al., 2005). In line with this finding, $Ca_V3.2$ mRNA level was also found to be down-regulated by about 24% from day 9.5 to day 23.5 post-differentiation in EMG7 mESC-derived CMs (Yanagi et al., 2007). Ni^{2+} at a low concentration of <50μM could be used to selectively block 50% of $Ca_V3.2$ T-type channels. When Ni^{2+} was applied at 40μM to ht7 mESC-derived CMs at day 8 post-differentiation, $I_{Ca,\ T}$ was found to be evidently decreased by about 60%, which signified the presence of functional $Ca_V3.2$ T-type channels by this time-point. Homozygous null $Ca_V3.2^{-/-}$ mice showed no sign of cardiac arrhythmia, but cardiac fibrosis with age-dependent severity (Chen et al., 2003). These data suggested a role for $Ca_V3.2$ in regulating the growth and maturation of the developing embryonic heart.

2.1.1 L-type calcium channels

L-type Ca^{2+} channels are distinguished from other voltage-operated Ca^{2+} channels that open at high membrane potentials for long periods on the basis of their sensitivity to 1, 4-

dihydropyridines (DHPs) (Hess et al., 1984). DHP-binding sites are contained within the α_1 subunit of L-type Ca^{2+} channels, which are encoded by either one of the four genes of the Ca_V1 sub-family. Among these, $Ca_V1.1$ is expressed predominantly in skeletal myocytes, $Ca_V1.2$ in adult CMs and $Ca_V1.3$ exclusively in SA nodal and atrial cells of the heart (Catterall, 2000; Qu et al., 2005). Unlike T-type, L-type Ca^{2+} channels are typical voltage-operated Ca^{2+} channels, which are made up of five subunits. Together with one of each of the $\alpha_2/\delta_{1\text{-}4}$, $\beta_{1\text{-}3}$ and $\gamma_{4,\ 6\text{-}8}$ ancillary subunits, $Ca_V1.2$- and $Ca_V1.3$-encoded α_1 proteins form the functional L-type Ca^{2+} channels that are found within the adult heart (Freise et al., 1999; Hosey et al., 1996; Yang et al., 2011).

$Ca_V1.2$ L-type Ca^{2+} channels are responsible for cardiac excitation-contraction coupling (E-C coupling), a mechanism by which an action potential is transformed into contraction in adult CMs. Upon the arrival of an action potential from SA nodal cells, L-type Ca^{2+} channels, which are clustered at transverse tubules (T-tubules), briefly open at the sarcolemma to allow an influx of extracellular Ca^{2+} into the cytosol of the CMs. This only accounts for about 30% of the increase in cytosolic Ca^{2+}, which is maintained and magnified through a process known as calcium-induced calcium release (CICR) (Bers, 2002). CICR is mediated via ryanodine receptor (RyR) Ca^{2+} release channels, which detect the $I_{Ca,\ L}$ and open to release Ca^{2+} from its intracellular stores within the SR into the cytosol of the CMs. This rise enhances binding of Ca^{2+} to troponin within the CMs, ultimately bringing about contractions of the adult heart. Conversely, $Ca_V1.3$ L-type Ca^{2+} channels are responsible for producing $I_{Ca,\ L}$, which constitutes one of the ionic currents to initiate action potentials at the SA node. Hence, these L-type Ca^{2+} channels contribute in maintaining the pacemaker activity of the adult heart (see 'T-type calcium channels' for details).

Consistent with its role in E-C coupling in the adult heart, $Ca_V1.2$ mRNA was detected as early as eight-week gestation in humans, with its level culminating to its maximum at adult stage (Qu and Boutjdir, 2001). $Ca_V1.2$ mRNA was also present *in vitro* at <40 days post-beating in H9.2 human ESC- (hESC-) derived CMs (Satin et al., 2008). In H7 hESC-derived CMs, Ca^{2+} transients that were detected at day 17 post-differentiation could be eliminated by the application of 10μM diltiazem, an L-type specific antagonist; this signified the functionality of L-type channels by this time-point (Zhu et al., 2009). Similar to humans, the expression of $Ca_V1.2$ was also up-regulated from its initial detection at E9.5 when the earliest contractions were observed by about three-fold at E15.5 when the heart has fully matured in mouse embryos; its protein was detected by E12.5 in mouse embryos (Acosta et al., 2004; Xu et al., 2003). In mESCs, patch clamp and Ca^{2+} imaging experiments indicated the functional expression of the $I_{Ca,\ L}$ starting from differentiation day 7, even before the appearance of spontaneous contractions (Kolossov et al., 1998). The current density of I_{Ca} continued to increase to day 10, the day of beginning of spontaneous contractions in most differentiating embryoid bodies (EBs). Thereafter, similar current density was recorded on day 17, the last day of measurement (Kolossov et al., 1998). Homozygous null $Ca_V1.2^{-/-}$ mice died *in utero* by E14.5, probably due to an absence of CICR-regulated contractions, resulting in reduced oxygen supply to the embryos (Seisenberger et al., 2000). Despite the lethality observed, normal rhythmic contractions were sustained in the $Ca_V1.2^{-/-}$ mice until E12.5. For this reason, CICR was initially thought to be non-essential for regulating the early cardiac contractions observed before E12.5 until later time-points. This conclusion was further supported by the fact that both the contractions and Ca^{2+} oscillations of D3 mESC-derived CMs were insensitive to the application of 50nM nisoldipine, a specific L-type channel blocker, at days 8-11 post-differentiation (Viatchenko-Karpinski et al., 1999). In addition,

these cells were insensitive to the application of high K^+ when compared to D3 mESC-derived CMs at day 16 post-differentiation; exposure to high K^+ at this stage would normally lead to immediate hyper-contraction and death of these cells. Albeit these findings, the notion that $I_{Ca,\ L}$ was not required at earlier stages of development was overthrown when evidence for the existence of a compensatory mechanism emerged; the expression of $Ca_V1.3$ was eminently up-regulated at E9.5 and E12.5 by four fold to produce a DHPR-insensitive $I_{Ca,\ L}$ in $Ca_V1.2^{-/-}$ mouse embryos when compared to wild-type (Xu et al., 2003). This has not only reinstated the importance of $I_{Ca,\ L}$ in maintaining cardiac contractions, even at the embryonic stage of the heart, but also appointed a new role for $Ca_V1.3$ to substitute for $Ca_V1.2$ and preserve its role in CICR for E-C coupling in its absence. In contrast with $Ca_V1.2$, $Ca_V1.3$ mRNA was detected by E9.5, with its level elevated only by a modest amount at E15.5 in mouse embryos. Its protein was also detected by E12.5 in mouse embryos (Xu et al., 2003). Analogous to $Ca_V3.1^{-/-}$ mice, homozygous null $Ca_V1.3^{-/-}$ mice developed bradycardia, consistent with its primary role in regulating the pacemaker activity of the heart (Platzer et al., 2000; Zhang et al., 2002). In R1 mESC-derived CMs, the expression of L-type Ca^{2+} channels could be detected as early as day 7 post-differentiation; given that the application of 1μM nifedipine, an L-type channel-specific antagonist, could eliminate Ca^{2+} transients that were normally observed at early (days 9-11) and intermediate (days 13-15), but only partially inhibit those at late (18-21 days post-differentiation) stages, this indicated that L-type channels were, in fact, playing a more dominant role at earlier developmental stages (Fu et al., 2006b).

2.2 Regulation of intracellular calcium level by ligand-operated calcium release channels

2.2.1 Ryanodine receptor (RyR) calcium release channels

RyR-Ca^{2+} release channels of a molecular mass of greater than two Mega-Daltons probably form the largest ion channels known thus far (Lanner et al., 2010). RyR-Ca^{2+} release channels are composed of homo-tetramers of RyR proteins. Owing to its enormous size, structural elucidation of these channels has been a major challenge. Nonetheless, RyR-Ca^{2+} release channels have been predicted to be largely cytoplasmic, with an ion-conducting pore consisting of around four to twelve transmembrane segments.

Three mammalian isoforms of RyRs exist; RyR1 is predominantly expressed in skeletal myocytes, RyR2 in CMs and RyR3 in astrocytes. The isoforms differ in three particular regions, which have been named domains D1-3 (Ma et al., 2004). As mentioned earlier in this chapter, RyR-Ca^{2+} release channels are induced to open in response to Ca^{2+} influx via L-type Ca^{2+} channels to facilitate E-C coupling in muscle cells. In skeletal myocytes, E-C coupling takes place through direct physical interaction between RyR1 and the D2 region, which is located between segments S2 and S3 of the $Ca_V1.1$-encoded α_1 subunit of L-type Ca^{2+} channels. In CMs, however, sequence divergence in the D2 domain between RyR1 and RyR2 means that RyR2 cannot physically interact with the $Ca_V1.2$-encoded α_1 subunit of L-type Ca^{2+} channels; E-C coupling can, therefore, only occur via CICR. RyR-Ca^{2+} release channels are, thus, organized into large arrays at junctions between the SR and the sarcolemma, in close proximity to the $Ca_V1.2$ L-type Ca^{2+} channels in adult CMs (Bers, 2004). Localized Ca^{2+} release events are referred to as Ca^{2+} sparks, which are collectively synchronized by $I_{Ca,\ L}$ to produce large, whole cell Ca^{2+} transients in the CMs.

Release of Ca^{2+} from the SR stores greatly increases the amount of cytosolic Ca^{2+} available to bind troponins in adult CMs. Adult CMs are mostly occupied by bouquets of thick and thin

filaments. Thick filaments are composed of myosin II molecules, while thin filaments are made up of troponin, tropomyosin and actin molecules. Troponin is itself a globular complex, which consists of three subunits, namely troponin T (TnT), troponin C (TnC) and troponin I (TnI). The TnT subunit binds tropomyosin, which coils around strands of actin molecules in the thin filament. Binding of Ca^{2+} to the TnC subunit induces a conformational change in the TnI subunit, thereby removing the steric hindrance on actin from tropomyosin. Binding of myosin II to actin can then occur, inducing a conformational change in the complex. This pulls the actin-associated thin filament pass the myosin II-associated thick filament, resulting in a contracting phenomenon in the CMs.

RyR2 protein was present as early as day 17 post-differentiation in H7 hESC-derived CMs (Zhu et al., 2009). In H9.2 hESC-derived CMs, the application of a puff of 10mM caffeine was able to induce Ca^{2+} release from the SR as early as day 2 post-beating; this signified the functionality of RyR-channels in these cells at this early time-point (Satin et al., 2008). In mouse embryos, RyR2 mRNA was detected as early as E8.5, the level of which continued to increase up until its last measurement at E16.5 (Rosemblit et al., 1999). Ca^{2+} release from the SR was inducible by the application of 10mM caffeine, indicating the functionality of RyR2 channels by E8.5 in mouse embryos. In a different study, RyR2 protein was also detected at E18 and the detection persisted until adult stage (Liu et al., 2002). Homozygous null $RyR2^{-/-}$ mice died at E10, displaying morphological abnormalities in the heart tube (Takeshima et al., 1998). RyR2 expression has also been studied *in vitro* in mESC-derived CMs. RyR2 mRNA and protein were detected as early as day 5 and day 9 post-differentiation in mESC-derived CMs respectively (Boheler et al., 2002; Fu et al., 2006a). Immunohistochemistry revealed a continuous increase in RyR immunofluorescence intensity in differentiation day 15-25 CMs compared with differentiation day 8-11 CMs, suggesting an increasing density of RyRs during cardiac differentiation (Sauer et al., 2001). The application of 10mM caffeine elicited Ca^{2+} transients, the amplitudes of which increased from day 8 to day 17 post-differentiation in mESC-derived CMs (Kapur and Banach, 2007). Hence, RyR2 channels were functional by day 8 post-differentiation in mESC-derived CMs. $RyR2^{-/-}$ knockout R1 mESC-derived CMs exhibited no difference in the amplitudes of Ca^{2+} transients but contractions at a reduced rate from early (days 9-11) to intermediate (days 13- 15) to late (days 18-21 post-differentiation) differentiation stages when compared with wild-type (Fu et al., 2006a; Yang et al., 2002). The applications of 10μM ryanodine and 10mM caffeine were able to inhibit and induce Ca^{2+} release from the SR respectively. Cardiac differentiation was not affected, as indicated by the number of contracting colonies present in the differentiating cultures. Both effects of ryanodine and caffeine were seen to increase with time post-differentiation, indicating that SR Ca^{2+} loads increased during differentiation (Fu et al., 2006a; Sauer et al., 2001). Altogether, these findings suggested a role for RyR to mediate SR Ca^{2+} release, thereby regulating the rate of the earliest contractions observed in the developing embryonic heart, and that this regulation increases with differentiation.

2.2.2 Inositol 1, 4, 5-trisphosphate Receptor (IP_3R) calcium release channels

IP_3R Ca^{2+} release channels represent a type of enigmatic intracellular Ca^{2+} channels in that both its structure and function(s) are not fully understood (Foskett et al., 2007; Taylor et al., 2004; Taylor and Tovey, 2010; Yule et al., 2010). IP_3R-Ca^{2+} release channels can be formed from either homo- or hetero-tetramers of IP_3R proteins. Each subunit of IP_3R proteins contains an IP_3-binding domain at its N-terminus and six transmembrane segments (S1-6) at its C-terminus; the Ca^{2+}-selective pore is formed at segments S5-6. Three mammalian

isoforms of IP_3R exist, namely IP_3R1, IP_3R2 and IP_3R3. These isoforms differ in their binding affinities for IP_3, with IP_3R2 being the most and IP_3R3 being the least sensitive to the ligand (Iwai et al., 2007; Tu et al., 2005). All three IP_3Rs are expressed in human adult CMs, but their subcellular localizations have not been studied (Nakazawa et al., 2011; Uchida et al., 2010). In rat adult CMs, these are expressed at about 50-fold lower than those of RyRs; IP_3R1s are localized around the nuclear envelope and SR, both of which are connected, while IP_3R2s are dispersed throughout the cytosol in a punctate pattern (Bare et al., 2005; Li et al., 2005; Moschella and Marks, 1993). Thus far, the role of IP_3Rs in the adult heart has not been defined, but an up-regulation in their expression has been associated with conditions of cardiac failure in that the mRNA levels of IP_3R1 and IP_3R2 were increased by 123% and 93% respectively in failing compared with normal heart tissues (Ai et al., 2005; Go et al., 1995).

Activation of IP_3R-Ca^{2+} release channels is regulated by both the concentration of cytosolic Ca^{2+} ions and the binding of IP_3 to one or more of the IP_3-binding domains (Iino, 1990; Taylor and Laude, 2002). Low concentrations of cytosolic Ca^{2+} ions are known to activate pore opening, while high concentrations inhibit it. IP_3 is a second messenger that is generated via a G protein-coupled receptor-mediated signal transduction pathway. G protein-coupled receptors are transmembrane receptors that are able to sense external stimuli and relay these signals into the cell via their interactions with cytosolic G proteins. G proteins are heterotrimeric complexes, which consist of a GDP-bound G_α subunit and a $G_{\beta\gamma}$ dimer. G protein-coupled receptors are classified into different types according to the isoform of the G_α subunits contained within their G protein-interacting partners. Generation of IP_3 involves the stimulation of a G_q type of G protein-coupled receptors. Upon stimulation with their agonists, a conformational change is induced in the G_q protein-coupled receptors. This causes an exchange of GDP for GTP in the G_α subunits, which then dissociate from their G_q protein complexes. GTP-bound G_α subunits of G_q protein complexes are responsible for activating phospholipase C. It is this enzyme, which hydrolyses phosphatidylinositol 4, 5-bisphosphate (PIP_2), a phospholipid component of the plasma membrane, to ultimately yield IP_3 and another second messenger, diacylglycerol (DAG). IP_3 then binds to and activates IP_3R-Ca^{2+} release channels.

IP_3R mRNA was present at detectable levels in D3 mESCs at the undifferentiated state (Yanagida et al., 2004). Application of 5μM ATP generated a Ca^{2+} transient in the undifferentiated D3 mESCs, which was inhibited by pre-treatment with 75μM 2-APB, an IP_3R blocker. This verified the functionality of IP_3R-channels in these cells. In H9.2 hESCs, immunostaining indicated that both IP_3R1 and IP_3R2 were expressed in hESC-dervied CMs (Sedan et al., 2010; Sedan et al., 2008); and the expression of IP_3R2 was shown to gradually decline with maturation as revealed by quantitative RT-PCR (Satin et al., 2008). The expression of IP_3R was also tested during development *in vivo*; its mRNA was first measured and detected as early as E5.5 in mouse embryos; it was present at high levels by E8.5 and continued to increase until E16.5 when its level started to drop (Rosemblit et al., 1999). These were demonstrated to be functional at two time-points; application of 5μM of IP_3 was able to induce Ca^{2+} release from the SR in CMs derived from mouse embryos at E5.5 and E8.5. Application of 5μM xestospongin C, an IP_3R antagonist, to mouse embryos at E10 diminished its Ca^{2+} spiking; washing the drug out allowed slow recovery of this spiking activity (Mery et al., 2005). Likewise, application of 5μM xestospongin C also abrogated the Ca^{2+} spiking activity observed in CGR8 mESC-derived CMs at day 8-10 post-differentiation. In addition, during the whole course of cardiac differentiation of R1 mESCs, 2-APB

decreased both the amplitude and upstroke velocity of Ca^{2+} transients, with the inhibitory effect decreased as differentiation proceeded, suggesting that IP_3R contributes to the Ca^{2+} transient and its effect decreases with differentiation (Fu et al., 2006b). In an attempt to discriminate between the roles of RyR- and IP_3R2- channels, 50μM ryanodine was first used to block RyR-channels before the application of 5-20μM IP_3-AM to CMs derived from mouse embryos at E10 (Rapila et al., 2008). This was able to induce a Ca^{2+} leak from the SR of the CMs in a concentration-dependent manner, whereby the slopes of the Ca^{2+} transients were elevated, despite its frequency stayed unchanged. In the absence of ryanodine, however, application of 10μM IP_3-AM led to an increase in the frequency of Ca^{2+} transients and, hence, increased contractions in the CMs at E10. These data suggested a role for IP_3R in regulating the rate of the earliest contractions observed in the developing embryonic heart, perhaps by providing a source of Ca^{2+} to bind RyRs so as to increase its open probability for greater Ca^{2+} release from the SR, as suggested by Rapila *et al*. (Rapila et al., 2008) In support of this finding, genetic knockdown of IP_3R in CGR8 mESC-derived pacemaker cells resulted in weak and infrequent contractions, although differentiation was not affected, whilst mice over-expressing the IP_3R2 gene developed mild cardiac hypertrophy by three months of age (Mery et al., 2005; Nakayama et al., 2010).

2.3 Regulation of intracellular calcium level by exchanger and pump

As previously mentioned, upon arrival of cardiac action potential, activation of L-type Ca^{2+} channel followed by CICR increases $[Ca^{2+}]_i$ for the contraction process. Subsequently, the excess Ca^{2+} has to be removed in order to initiate relaxation. In CMs, this Ca^{2+} removal process is mediated by different Ca^{2+} extrusion mechanisms via the action of sodium-calcium exchanger (NCX) and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA).

2.3.1 Sodium-calcium exchanger (NCX)

NCX contains 9 transmembrane segments with a large loop at the cytoplasmic side (Philipson and Nicoll, 2000). Mutation experiments demonstrated the importance of α-helices in the transmembrane segments for the transport activity of NCX (Nicoll et al., 1996). The large intracellular loop is found to be not essential for the transport; however, it is important for catalyzing the ion translocation reaction and has important regulatory functions (Philipson and Nicoll, 2000). This loop consists of multiple regulatory sites, including the regulatory Ca^{2+} binding site and the exchanger inhibitory peptide (XIP) region. The activity of NCX is known to be regulated by at least 4 factors, including Ca^{2+} (DiPolo, 1979), Na^+ (Hilgemann et al., 1992), PIP_2 (Hilgemann, 1990), and phosphorylation (Iwamoto et al., 1996). Binding of Ca^{2+} to the Ca^{2+} binding site is required for activating the Na^+-Ca^{2+} exchange activity (Giladi et al., 2010; Ottolia et al., 2004; Wu et al., 2010). On the other hand, the XIP region is responsible for the Na^+-dependent inactivation and is involved in the elimination of the Na^+-dependent inactivation process by PIP_2 (Matsuoka et al., 1997). The activity of NCX is also regulated by phosphorylation, with stronger phosphorylation leading to higher NCX activity (Reppel et al., 2007a). Requirements of direct phosphorylation for up-regulation of NCX function by PKA and PKC are still in debate. A recent study by Wanichawan *et al*. demonstrated that the PKA phosphorylation site in full-length NCX1 is inaccessible, suggesting that NCX1 is not a direct substrate of PKA (Wanichawan et al., 2011). On the other hand, another study showed that the activity of NCX1 is dependent on PKC, although direct phosphorylation by PKC is not required (Iwamoto et al., 1998). NCX exists in 3 different isoforms, namely NCX1, NCX2 and NCX3.

NCX1 is referred as the cardiac NCX isoform as it is highly expressed in CMs but only in a lesser extent in other tissues such as brain and kidney (Lee et al., 1994).

NCX is classified as a secondary active transporter, which uses the energy stored in the electrochemical gradient in Na^+ to extrude Ca^{2+} out of the cells, while the electrochemical gradient of Na^+ is maintained by the Na^+/K^+-ATPase. Under normal condition, NCX operates in the forward mode in which it constitutively brings 3 Na^+ into the cells and extrudes 1 Ca^{2+} in each translocation cycle. The forward mode is stimulated in response to a rise in $[Ca^{2+}]_i$, and it serves to bring $[Ca^{2+}]_i$ back to normal level. In CMs, the primary role of NCX is to extrude Ca^{2+} after excitation under normal physiological conditions (Philipson and Nicoll, 2000). Some studies also showed that NCX functions in shaping the cardiac action potential. Application of NCX blocker KB-R7943 leads to shortening of plateau phase of cardiac potential (Spencer and Sham, 2003); similarly, CMs from NCX knockout mice also has a shorter AP when compare to wild-type mice (Pott et al., 2005), while induced over-expression of NCX leads to a longer plateau (Wang et al., 2009). On the other hand, NCX also operates in the reverse mode in response to membrane depolarization in CMs. During the depolarization phase of action potential when the $[Ca^{2+}]_i$ has not reached the peak of Ca^{2+} transients, the reverse mode is predominant (Sah et al., 2003). Ca^{2+} influx via the reverse mode of NCX has been suggested to act synergistically with Ca^{2+} influx via L-type Ca^{2+} channels to trigger Ca^{2+} release from the SR as $I_{Ca,\,L}$ are small at depolarized membrane potential (Sah et al., 2003). In addition, NCX can positively regulate SR Ca^{2+} load via the reverse mode action (Hirota et al., 2007). Interestingly, under pathophysiological conditions such as cardiac failure, NCX also operates in the reverse mode to allow additional Ca^{2+} influx for contraction in order to compensate for the reduction in Ca^{2+} release from SR (Gaughan et al., 1999).

NCX is found to be essential for embryo development. Several studies showed that NCX1 is expressed restrictedly in the embryonic heart during early development. NCX knockout are embryonic lethal at ~9-11 days post coitum with immature heart development (Cho et al., 2000; Koushik et al., 2001; Reuter et al., 2003; Wakimoto et al., 2000). Molecular studies demonstrated that NCX mRNA expresses before the appearance of spontaneously beating mESC-CMs; the expression persists thereafter in the CMs (Fu et al., 2006b). It is suggested that expression of NCX at that early stage is essential for early EC-coupling as SR is not well-developed at that stage, NCX is hence essential for maintaining the proper Ca^{2+} homeostasis even in the very early stage cardiac development (Reppel et al., 2007a; Reppel et al., 2007b).

Two approaches were used to demonstrate the functional expression of NCX in ESC-CM. In Otsu *et al*. (Otsu et al., 2005), function of NCX was indirectly assessed by using high concentration of NCX blocker KB-R7943. Application of KB-R7943 induced sustained elevation of $[Ca^{2+}]_i$, and this elevation increased as differentiation of mESC-CMs proceeded (Otsu et al., 2005). Apart from using pharmacological blocker, direct measurement of NCX activity was performed by using patch-clamping. I_{NCX} was found to be increased as hESC-CMs developed from day 7+40 to day 7+90 (Fu et al., 2010). However, CMs derived from murine embryonic heart at late stage (E16.5) showed a significantly lower I_{NCX} density when compared to CMs at early stage (E10.5) (Reppel et al., 2007a), probably due to the high phosphorylation status of NCX in early stage. Consistently, upon differentiation, the proportion of Ca^{2+} extrusion by NCX declined from day 9 to day 17 in mESC-CMs (Kapur and Banach, 2007). The discrepancy between different studies on the absolute functional expression of NCX as development proceeds is unknown. Nonetheless, it is clear that NCX is important for Ca^{2+} extrusion in differentiating CMs and the decreased contribution by

NCX to Ca^{2+} extrusion as development proceeds may be explained by the gradual development of SERCA on the SR.
In the study by Fu *et al*., the basal $[Ca^{2+}]_i$ of mESC-CMs in both early and late developmental stages was increased after applying Na^+-free solution, suggesting that NCX is functional in maintaining Ca^{2+} homeostasis (Fu et al., 2006b). Interestingly, Na^+-free solution completely blocked the Ca^{2+} transients in CMs from late developmental stage but not the CMs from early developmental stage, suggesting that NCX starts to regulate Ca^{2+} transients only in the late developmental stage (Fu et al., 2006b). Similar results were obtained from hESC-CMs (Fu et al., 2010). Basal $[Ca^{2+}]_i$ was marginally increased in CMs at day 7+90 after applying Na^+-free solution, but the same was not observed in CMs at day 7+40. In addition, irregular Ca^{2+} transient pattern was observed in day 7+90 CMs treated with Na^+-free solution (Fu et al., 2010). These suggested that the contribution of NCX to Ca^{2+} transients becomes more important as development proceeds in ESC-CMs.

2.3.2 Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)

Details of the structure and function relationship of SERCA have been extensively reviewed (Periasamy et al., 2008; Periasamy and Huke, 2001; Toyoshima, 2008; Toyoshima and Inesi, 2004; Wuytack et al., 2002). SERCA is a single polypeptide with ~1000 amino acid residues located on the ER/SR membrane. It consists of 10 transmembrane (M) domains and 3 cytosolic domains, including actuator (A) domain, phosphorylation (P) domain, and nucleotide-binding (N) domain. A domain regulates the Ca^{2+} binding and release. N domain is connected to the P domain; it contains the adenosine binding site and forms the catalytic site. On the other hand, the γ-phosphate reacts with an amino acid residue in the P domain. SERCA utilizes the energy derived from ATP hydrolysis to pump Ca^{2+} against concentration gradient from the cytosol to the lumen of ER/SR. Two Ca^{2+} are transported by hydrolysis of one ATP in each catalytic cycle.
Regulation of SERCA is mainly achieved by the action of SR membrane proteins phospholamban and sarcolipin (Asahi et al., 2003a; Edes and Kranias, 1987; MacLennan et al., 2002; MacLennan and Kranias, 2003; Simmerman and Jones, 1998; Traaseth et al., 2008). De-phosphorylated form of phospholamban interacts with SERCA and inhibits the pumping activities by decreasing the Ca^{2+} affinity of SERCA. Phospholamban exists in monomeric or pentameric form, while the monomeric form is inhibitory. Phosphorylation of phospholamban favors the formation of pentameric form, which in turns relieves the inhibitory effect on SERCA. Phosphorylation of phospholamban is regulated by cAMP-dependent protein kinase (Schwinger et al., 1998) and Ca^{2+}/calmodulin-dependent kinase (Ji et al., 2003). Sarcolipin is a shorter homolog of phospholamban (Hellstern et al., 2001). Unlike phospholamban, sarcolipin has no obvious phosphorylation site (Odermatt et al., 1997); therefore, the effect of sarcolipin on SERCA inhibition is mainly controlled by altering the expression level of sarcolipin (Odermatt et al., 1998). Sarcolipin interacts directly with SERCA and inhibits its function by decreasing the Ca^{2+} affinity of SERCA. Sarcolipin can also exert its superinhibitory effect on SERCA by forming the tertiary complex phospholamban-sarcolipin-SERCA (Asahi et al., 2002; Asahi et al., 2003b). In addition, sarcolipin stabilizes the SERCA-phospholamban complex in the absence of phospholamban phosphorylation and also inhibits phospholamban phosphorylation (Asahi et al., 2004).
SERCA plays a vital role in Ca^{2+} cycling between SR and cytosol, and this is important for EC-coupling. Over-expression of SERCA2a improved cardiac contractility by increasing SR Ca^{2+} loading and frequency of Ca^{2+} transients (Baker et al., 1998; He et al., 1997; Maier et al.,

2005; Prasad et al., 2004). On the other hand, homozygous SERCA2a knockout mice are embryonic lethal (Periasamy et al., 1999), while heterozygous knockout mice are alive and able to reproduce (Shull et al., 2003). Ji *et al*. has reported that the content of SR Ca^{2+} stores and the amplitude of Ca^{2+} transients were decreased by 40-60% and ~30-40%, respectively, in heterozygous CMs (Ji et al., 2000). Interestingly, heterozygous CMs showed a reduced phospholamban expression, an enhanced phospholamban phosphorylation, and an upregulated NCX expression. However, these changes in Ca^{2+} handling proteins were not sufficient to compensate the effects on contractility by the loss of SERCA2a, indicating that SERCA2a is a critical regulator in controlling the E-C coupling of CMs (Ji et al., 2000). Apart from genetic manipulation of the expression level of SERCA, function of SERCA can also be assessed by using the pharmacological blocker thapsigargin. Acute application of thapsigargin caused the decrease in Ca^{2+} transient amplitude, rate of decay of Ca^{2+} transients, and duration of action potential in isolated ventricular myocytes (Kirby et al., 1992), again suggesting the involvement of SERCA in the E-C coupling of CMs.

In vertebrates, SERCA is encoded by three genes, including the SERCA1, SERCA2, and SERCA3. Alternative splicing of the transcripts from these genes produces more than 10 SERCA isoforms. In CMs, SERCA2a and SERCA2b are expressed, with SERCA2a being the predominant form (Periasamy and Kalyanasundaram, 2007).

SERCA2a mRNA is present before initial contraction of mESC-CMs, but has no obvious change in expression level during further differentiation (Fu et al., 2006b). From embryo studies, SERCA2 protein increased from E9.5 to E18 in mouse heart (Liu et al., 2002). In human, it was reported that SERCA2a protein level remained steady between 8 to 15th week gestation, and started to increase afterwards (Qu and Boutjdir, 2001). Role of SERCA in regulating Ca^{2+} transients in ESC-CMs has been studied by several groups. Zhu *et al*. demonstrated that SERCA inhibitors, including thapsigargin and cyclopiazonic acid, reduced ~70% amplitude of Ca^{2+} transients in hESC-CMs, but had no effect on the time of decay (Zhu et al., 2009). In case of mESC-CMs, thapsigargin reduced both the amplitude and decay of Ca^{2+} transients, but exerted similar inhibitory effect on CMs from the 3 developmental stages (Fu et al., 2006a; Fu et al., 2006b). These findings are therefore consistent with the mRNA expression level of SERCA during mESC-CM differentiation. However, the contribution of Ca^{2+} removal by SERCA is estimated to be more important as differentiation proceeds based on Ca^{2+} imaging experiments (Kapur and Banach, 2007). Therefore, the role of other SR Ca^{2+} handling proteins cannot be neglected. For example, expression level of calsequestrin increases as SR matures (Fu et al., 2006a). This increases the capacity of SR Ca^{2+} load and may account for the requirement of greater contribution of Ca^{2+} removal by SERCA in later developmental stage of mESC-CMs.

To summarize for the whole chapter, Figure 1 represents a summary of the relative contributions of different proteins responsible for regulating Ca^{2+} transients in early differentiating ESC-CMs as development proceeds.

3. Conclusion

In summary, early differentiating ESC-CMs have already developed a scheme for regulating their $[Ca^{2+}]_i$ for E-C coupling. The relative contributions of the proteins that regulate Ca^{2+} transients alter upon the maturation of CMs. By comparing the regulation of $[Ca^{2+}]_i$ in ESC-CMs and that in adult CMs, we can obtain important insights into the potential strategies for 'fine-tuning' ESC-CMs to better-suit different therapeutic and research purposes.

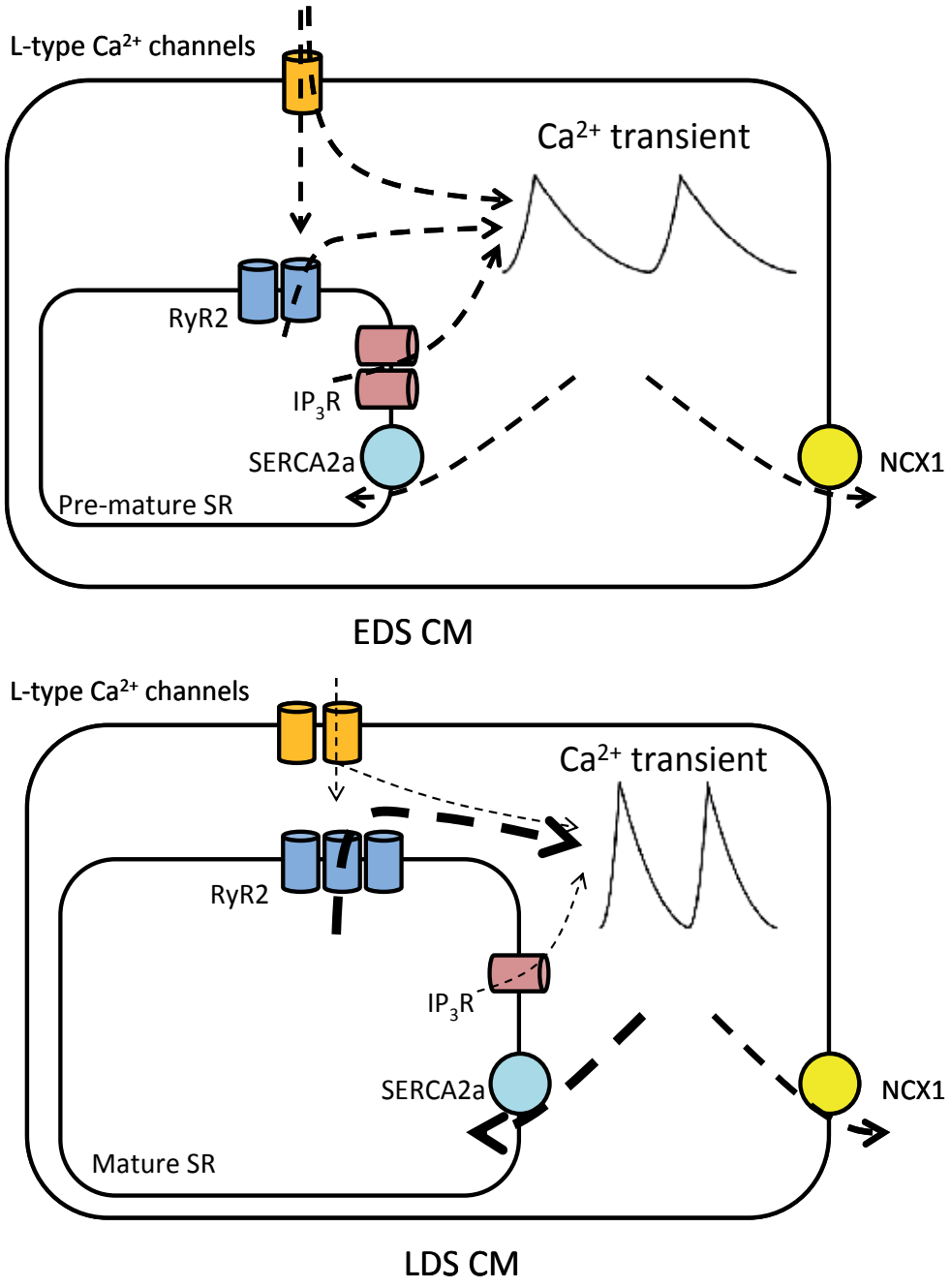

Fig. 1. Summary of the regulation of Ca^{2+} transients in CMs at early differentiation stage (EDS) and late differentiation stage (LDS). Number of a particular protein represents the relative changes in the expression level of that particular protein as differentiation proceeds. Thickness of the arrows represents the relative contribution of a particular path to the Ca^{2+} transients as differentiation proceeds.

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12

Myogenic Differentiation of ES Cells for Therapies in Neuromuscular Diseases: Progress to Date

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1. Introduction

The neuromuscular disorders are a heterogeneous group of genetic diseases characterized by progressive degeneration and impaired regeneration of skeletal muscle, resulting in weakness. The mobility of patients is very reduced, leading, depending on the disease severity, to wheelchair dependency and reduced life expectancy and quality.
Currently there are no proven treatments for these diseases, except for palliative measures to improve a patient's quality of life. Nevertheless, cell therapy using embryonic or somatic stem cells is considered to offer the best potential for success, and many projects are now being undertaken to evaluate the therapeutic possibilities of this approach.
Theoretically, due to their pluripotency, embryonic stem cells can give rise to any type of tissue, and raises the possibility of successfully treating many diseases. However, a simple injection of ES cells into various body locations in model organisms often leads to formation of undesirable teratomas and not to healthy new tissues. Accordingly, ES cells must be partially differentiated and selected prior to injection to increase the likelihood of implantation and growth of tissue exhibiting differentiation of the desired type.
Although some modest advances have been achieved, to date the use of ES cells for therapy of neuromuscular disorders still remains a distant goal. In this review we mainly consider the role of embryonic stem cells in neuromuscular therapeutic approaches.

2. Muscular dystrophies

The neuromuscular disorders form a heterogeneous group of genetic diseases characterized by progressive loss of muscular strength caused by defects in or absence of muscle proteins and also to imbalance between rates of tissue degeneration and regeneration. There is great clinical variability, ranging from extremely mild to severe forms (Emery, 2002).
More than 30 genetically defined forms are recognized, and in the last decade, mutations in several genes coding for the sarcolemmal, sarcomeric, cytosolic or nuclear muscle proteins have been reported. Deficiencies or loss of function of these proteins leads to variable degrees of progressive muscle degeneration, which in turn results in progressive loss of motor ability (Vainzof et al., 2003). The principle proteins involved occupy specific niches in muscle cells: dystrophin (*Dmd*), sarcoglycan (*Sgca*) and dysferlin (Dysf) are sarcolemmal or peri-sarcolemmal proteins; laminin alpha 2 (*Lama2*) and collagen type VI (*Col6*) are

extracellular matrix proteins; telethonin (Tcap) and actin alpha 1 (*Acta1*) are sarcomeric proteins; calpain 3 (*Capn3*) and FKRP (Fkrp) are cytosolic enzymes; and emerin (Emd) and lamin A/C (*Lmna/c*)are nuclear proteins (Figure 1).

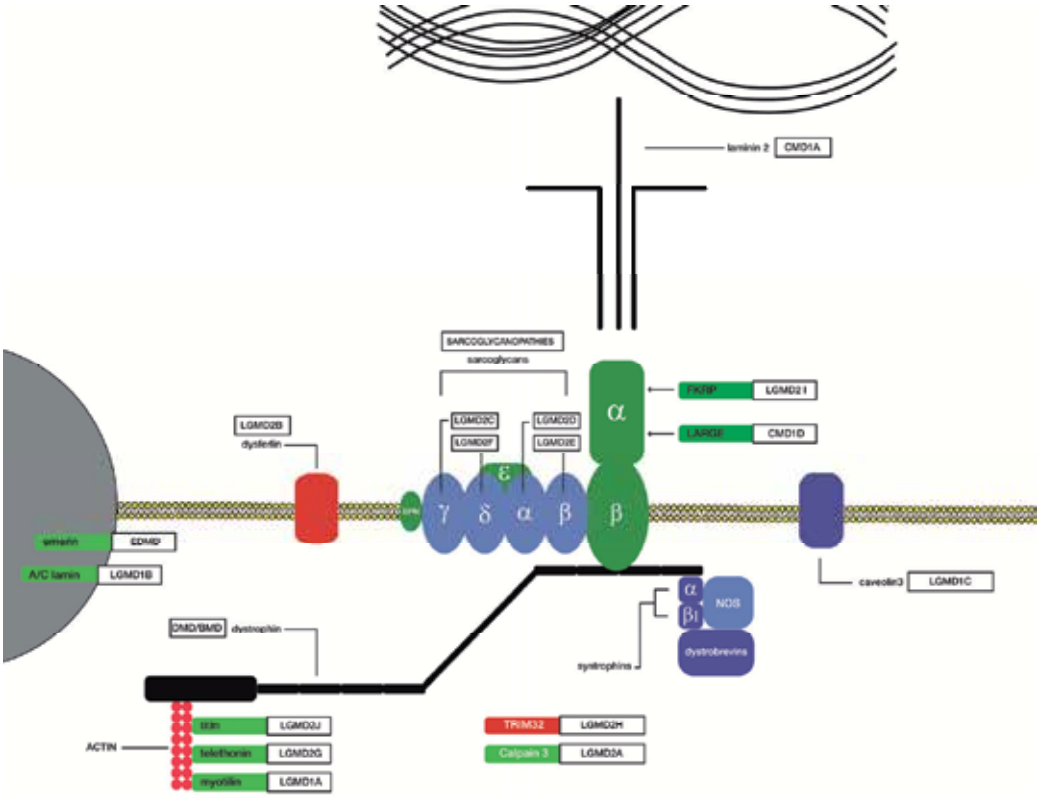

Fig. 1. Schematic representation of proteins involved in the process of muscle degeneration in neuromuscular disorders, localized at the sarcolemma, the sarcomere, the cytosol and the nucleus. The boxes contain the disease name associated with the protein. LGMD – limb-girdle muscular dystrophy; EDMD- Emery-Dreifuss muscular dystrophy; FKRP – fukutin-related protein; NOS – nitric oxide synthase.

Defects in components of the dystrophin-glycoprotein complex (DGC) are known to be an important cause of different forms of muscular dystrophies (Ervasti & Campbell, 1993; Yoshida & Ozawa, 1990). The DGC is an oligomeric complex connecting the subsarcolemmal cytoskeleton to the extracellular matrix. The DGC consists of dystroglycan (α- and β-DG), sarcoglycan (α, β-, γ-, δ- and ε-SG) and syntrophin/dystrobrevin sub-complexes. The intracellular link of the DGC is the protein dystrophin that plays an important structural role in muscle fibers. Mutations in the dystrophin gene cause the most common form of neuromuscular disorder, namely X-linked Duchenne muscular dystrophy (DMD) (Hoffman et al., 1987) with a frequency of 1 in 3000 males in most populations studied. Further, this disease epitomises the severe need for efficient treatment modalities, since 1/3rd of all patients arise from new mutations and the disease frequency can never be modulated efficiently by genetic counselling approaches.

Mutations in the genes coding the four SG proteins cause severe forms of limb-girdle muscular dystrophies type LGMD2D, 2E, 2C and 2F. The peripheral membrane glycoprotein α-DG, a receptor for the heterotrimeric basement membrane protein laminin-2, binds to β-DG and so completes the connection from the inside to the outside of the cell (Straub and Campbell, 1997). Mutations in the *Lama2* gene, encoding the α2 chain of laminin-2, cause α2-laminin deficiency, and a severe form of congenital muscular dystrophy (CMD1A) linked to human chromosome 6q (Tomé et al., 1994). In addition, some forms of muscular dystrophy have recently been associated with genes encoding putative or known glycosyltransferases. Muscle protein analysis in these patients shows a hypoglycosilation of α-dystroglycan and a consequent reduction of numerous ligands components of the extracellular matrix, such as laminin 2 (Muntoni et al., 2004). Other milder forms of muscular dystrophy are caused by mutations in genes coding the enzyme calpain 3 (*Capn3*), the sarcolemmal protein dysferlin (*Dysf*), and the sarcomeric protein telethonin (*Tcap*) (Vainzof & Zatz, 2003).

2.1 Animal models for neuromuscular diseases

Several animal models, manifesting phenotypes observed in neuromuscular diseases have been identified in nature or generated in laboratory. These models generally present physiological alterations observed in human patients, and can be used as important tools for genetic, clinical and histopathological studies (Vainzof et al., 2008).

The *mdx* mouse is the most widely used animal model for Duchenne muscular dystrophy (DMD) (Bulfield et al., 1984). Although it is a good genetic and biochemical model, presenting total deficiency of dystrophin in muscle, this mouse is not useful for clinical comparisons, because of its very mild phenotype. The canine golden retriever MD model presents a more clinically relevant model for DMD in humans due to the much larger size of the animals, significant muscle weakness progression and premature lethality.

Models for autosomal recessive limb-girdle MD include the *SJL/J* mice that develop spontaneous myopathy resulting from a mutation in the Dysferlin gene, which is a specific model for LGMD2B (Bittner et al., 1999). For the human sarcoglycanopathies (SG), the BIO14.6 hamster is the spontaneous animal model for δ-SG deficiency, while some canine models with deficiency of SG proteins have also been identified (Straub et al., 1998). More recently, using homologous recombination in embryonic stem cells, several mouse models have been developed with null mutations in each one of the four SG genes. All sarcoglycan-null animals display a progressive muscular dystrophy of variable severity, and share the property of a significant secondary reduction in the expression of the other members of the sarcoglycan subcomplex, and other components of the Dystrophin-glycoprotein complex.

Mouse models for congenital MD include the *dy/dy* (dystrophia-muscularis) mouse, and the allelic mutant *dy2J/dy2J* mouse, both presenting a significant reduction of α2-laminin in the muscle and a severe phenotype. The myodystrophy mouse (*Largemyd*), harbors a mutation in the glycosyltransferase Large, which leads to altered glycosylation of α-DG, and a severe phenotype (Grewal et al., 2001).

Other informative models for muscle proteins include the knockout mouse for myostatin, demonstrating that this protein is a negative regulator of muscle growth (Patel & Amthor, 2005). Additionally, the stress syndrome in pigs, caused by mutations in the porcine *Ryr1* gene, helped to localize the gene causing malignant hyperthermia and Central Core myopathy in humans (Yang et al., 2006).

The study of animal models for genetic neuromuscular diseases, in spite of some differences with their equivalent human disease phenotypes, can provide important clues to understanding the pathogenesis of these disorders in humans and are also very valuable for testing strategies for cellular therapeutic approaches.

3. Muscle development

Activating key genes in a sequence similar to that occurring in the normal organism is a reasonable approach to obtain differentiated muscle cells in vitro. This depends on understanding the gene pathways leading to myogenic differentiation.
Skeletal muscle development can be divided into a number of principal stages: determination of the cell fate (myoblast formation); myoblasts proliferation; alignment and fusion of myoblasts; formation of myotubes; maturation of myotubes and muscle fibre formation (Figure 2). Different molecular factors regulate each step in a particular and very ordered manner.

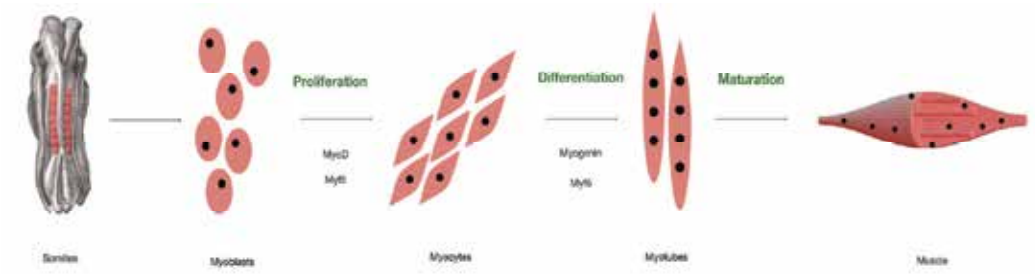

Fig. 2. Schematic of the myogenic cascade

3.1 Anatomy of embryonic myogenesis – Myotome formation

The majority of skeletal myogenic progenitors arise from somites, which are transitory condensations of paraxial mesoderm on either side of the neural tube and notochord. As the maturation of somites progresses, myogenic progenitor cells are confined to the epithelium of the dermomyotome, which give rise to the dermis and the skeletal muscle of the trunk and limbs (Buckingham, 2006).
The dermomyotome is subdivided into the hypaxial dermomyotome – the source of the lateral trunk muscles and limb muscles – and the epaxial dermomyotome – the source of the deep back musculature (Parker et al., 2003). In this structure, it is possible to distinguish two lips: the hypaxial and epaxial lips, from which cells delaminate and migrate under the dermomyotome, forming the myotome, an intermediate structure (Buckingham, 2001) (Figure 3).

3.2 Molecular markers and regulatory factors

Pax3 and *Pax7* are markers for cells derived from the dermomyotome and recently formed muscle masses. *Pax3* expression is involved in progenitor muscle cell formation and is essential for the definition and migration of these cells to their proper location in the body. *Pax3* acts mainly during embryogenesis, while *Pax7* is more important in adult myogenesis.

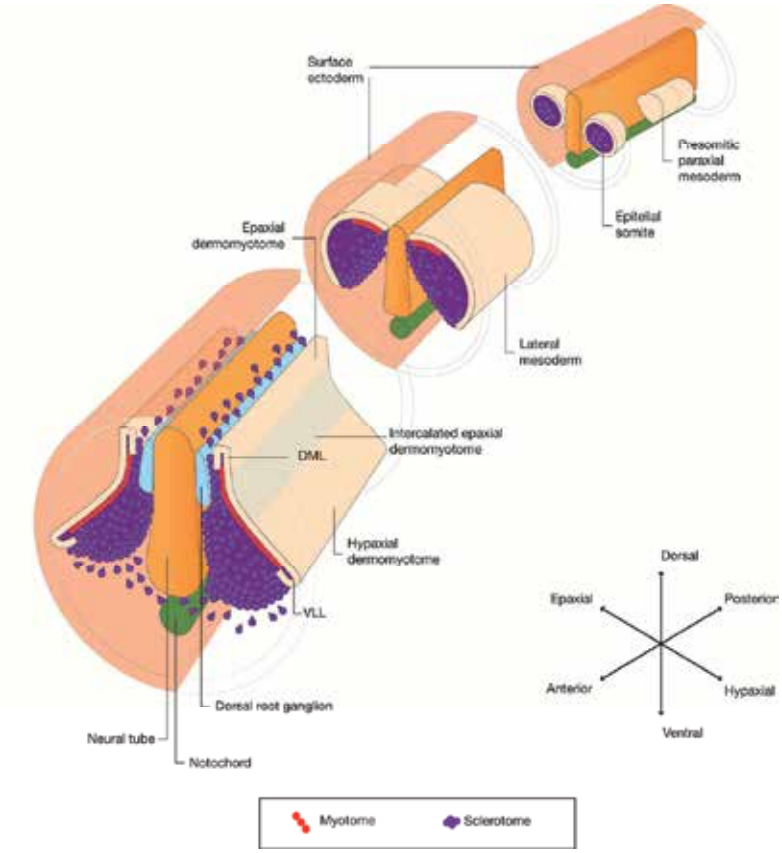

Fig. 3. The origin of different muscles in the embryo. VLL – ventral lateral lip. DML – dorsal medial lip. (Adapted from Buckingham, 2001).

The induction of myogenesis in the cells of somites is conducted by factors secreted by the notochord and neural tube: various members of the *Wnt* family and *Shh*, are responsible for the activation of MRFs (myogenic regulatory factors). Expression of *Ctnnb1* (β-catenin) dependent *Wnt6* signalling is important for the maintenance of the epithelial structure of the dermomyotome that is essential for the ordered progression of myogenesis. In epaxial muscle, *Wnt* family members are involved in *Myf5* and *MyoD* regulation through a complex cascade of gene regulation that includes the action of *Shh* as a positive regulator of *Myf5* (Parker et al., 2003) (Figure 4 A).

The MRFs concerned are *MyoD*, *Myf5*, myogenin and *Myf6* (*Mrf4*) and each one has a defined role in regulating skeletal muscle development and differentiation, directing the expression of genes responsible for the formation of the contractile machinery of the muscle (Bryson-Richardson & Currie, 2008). All have a homologous bHLH domain, required for DNA binding and dimerization with transcription factors of the E-protein family. The complexes of MRF-E proteins bind to a specific consensus sequence found in the promoters of many muscle-specific genes.

In hypaxial muscle, the MRFs are up-regulated by *Pax3*, which in turn, is regulated by family members of the sine oculis homeobox (*Six*) and eyes absent (*Eya*) genes (Bryson-Richardson & Currie, 2008) (Figure 4 B).

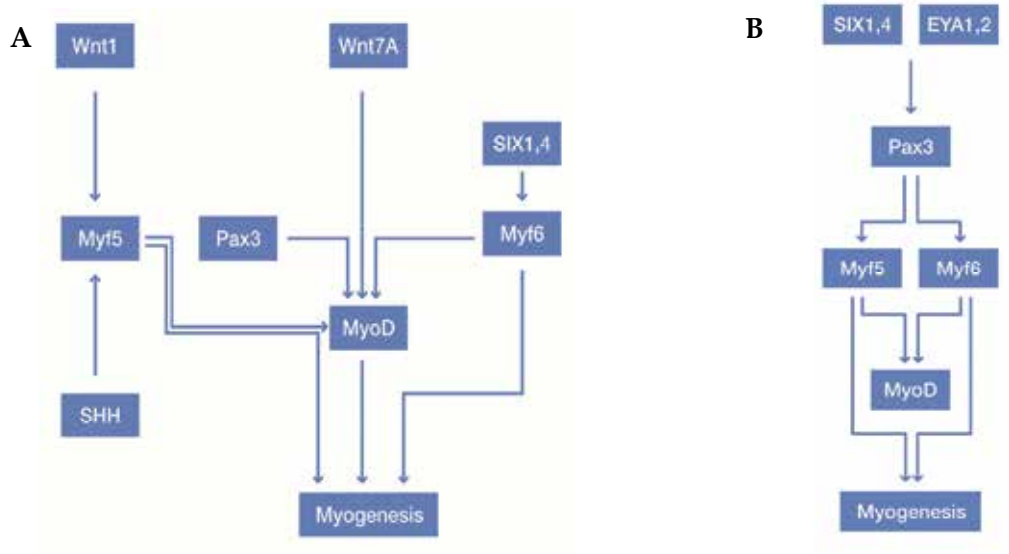

Fig. 4. Gene regulatory networks in A- epaxial muscle (left) and in B- hypaxial muscle (right).

Another important family of transcription factors is the myocyte enhancer factor-2 (*Mef2*), also involved in the expression of many muscle-protein genes in mouse (Naya & Olson, 1999). The *Mef2* family acts in conjunction with MRFs, especially *Myf6* and *Myog* (myogenin), to coordinate myoblast terminal differentiation.

3.3 Myogenesis in the adult – muscle regeneration

The cells responsible for regeneration in adult muscle are the satellite cells, localized under the basal lamina of muscle fibers. The satellite cells are partially undifferentiated myogenic precursor cells capable of both self-renew and differentiation into new myogenic cells (Relaix & Marcelle, 2009).

In response to injury, and under the stimulus of several myogenic factors, these cells are activated, start to proliferate and differentiate, fuse to pre-existing fibers (hypertrophy), or generate new fibers (hyperplasia) in a process recapitulating muscle development (Hawke & Garry, 2001). Myogenic determinants involve the components of the family of transcription factors called muscle regulatory factors (MRFs), include: a) *Myf5* and *Myod1*, responsible for muscle-cell type determination and satellite-cells activation; b) *Myf6* (also called *Mrf4*) and *Myog*, responsible for muscle differentiation (Brand-Saberi & Christ, 1999).

In addition to satellite cells, there are other cell types that contribute to muscle regeneration: bone-marrow derived cells (Ferrari et al., 1998), muscle side-population cells (Gussoni et al., 1999), $CD34^+/Sca1^+$ cells (Torrente et al., 2001; Lee et al., 2000) and cells of vascular origin (Figure 5).

The regenerative capacity of the satellite cells, however, is finite, and the exhaustion of the pool of precursor cells is an important factor contributing to the progressive muscle deterioration observed in human and murine muscular dystrophy. In fact, the exhaustion of satellite cells is the primary cause of onset of symptoms in Duchenne muscular dystrophy.

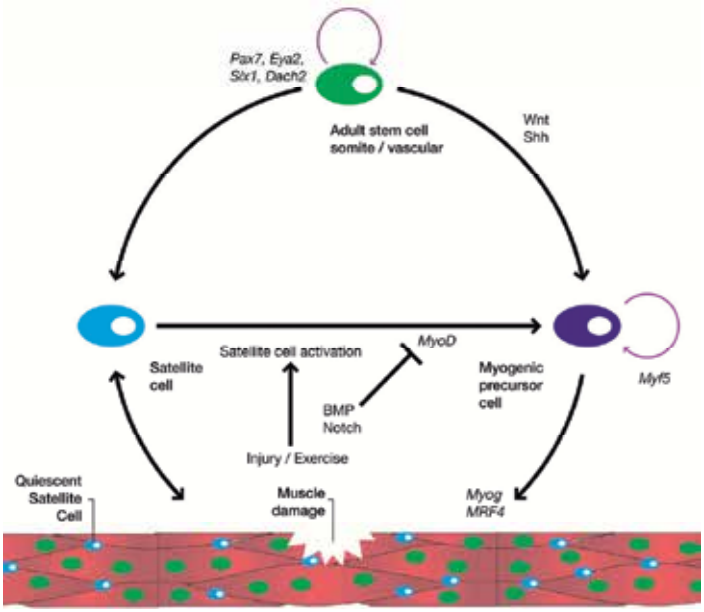

Fig. 5. Adult myogenesis and precursors cell types. Adapted from Parker et al., 2003.

Thus, cell therapies for muscular dystrophies can also focus on the re-establishment of the muscle's satellite cell pool, which reduces rapidly when there are excessive cycles of degeneration and regeneration.

4. ES cells in myogenic differentiation

Any attempt to direct the differentiation of ES cells must be designed so that the majority of cells will start to differentiate, as far as possibly, simultaneously into the desired cell type with avoidance of teratoma formation caused by non-committed pluripotent stem cells. Induction of appropriate myogenesis is a relatively difficult task given the unique architecture of muscle tissue.

There are three main potential approaches to directed differentiation of ES cells into muscle: the use of muscle specific growth and differentiation factors, genetic modifications and use of genetically modified feeder cells (Grivennikov, 2008). The first two have already been tested for myogenic induction, but to date, there are no reports on the use of modified feeder cells for this purpose.

For the differentiation of ES cells into different cell lineages, the cells must be cultivated in aggregates called embryoid bodies (EBs) by the hanging drop method (Figure 6), in which the 3-dimensional structure of the embryoid bodies, in combination with application of growth factors favouring myogenesis, encourages the stem cells to differentiate into myoblasts. The ES cells are first cultivated in drops of medium containing an exact number of cells leading to formation of EBs within 2 days, following which, the EBs are transferred into suspension cultures for some additional days resulting in adhesion of the EBs onto the bottom of tissue culture plates. The medium for EB cultivation is changed for one supporting myogenic differentiation at the time of EB adhesion. Studies suggest that the EBs need to be cultivated for five days in the suspension phase to obtain maximal differentiation into skeletal muscle cells.

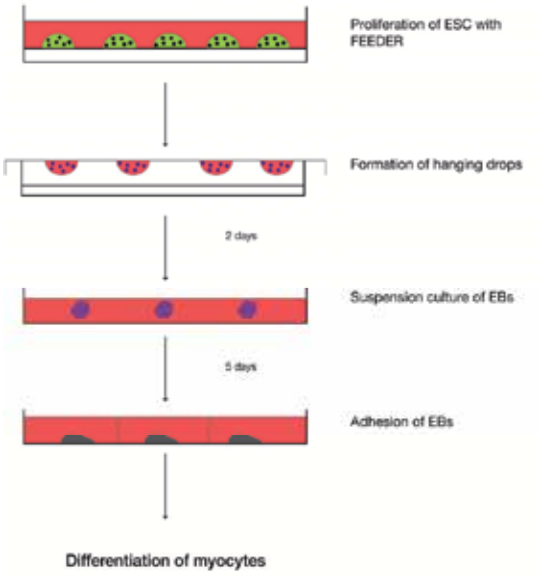

Fig. 6. Hanging drop method

4.1 *In vitro* induction of myogenesis

The use of chemical compounds to induce differentiation is a quite simple approach based on media supplementation. In some cases, different cell types can be obtained from the same compound according to the substance's concentration. For instance, 1μM retinoic acid causes neuronal differentiation, while 25nM retinoic acid enhances skeletal myogenesis (Kennedy et al., 2009).

One of the first reports of myogenic differentiation of ES cells was reported by Rohwedel et al. (1994), based on protocols developed by Wobus et al. in 1988 (2002), which depend on a prior differentiation in embryoid bodies followed by a posterior treatment with 1% DMSO (dimethyldisulfoxide) or 10^{-8}M retinoic acid. The myogenic cells obtained in this work were identified as myoblasts and myotubes by hematoxylin-eosin staining. The first myosin-positive and desmin-positive skeletal muscle cells appeared four days after EB adhesion. Myogenin-positive myocytes were identified after six days and fusion into myotubes on the seventh day. Neuronal cells also appeared mostly before the differentiation of skeletal muscle cells, which reached a maximum in nine days. However, some EB outgrowths containing ES cells, which decreased with prolonged time of differentiation. Using RT-PCR the authors detected expression of the myogenic regulatory factors myogenin, *Myf5*, *Myf6* and *Myod1*, indicating that ES myogenic differentiation *in vitro* resembles myogenesis *in vivo* (Rohwedel et al., 1994).

Retinoic acid (RA) is a derivate of vitamin A and has different roles in various processes in embryonic development and regulates the expression of several hundreds of genes (Blomhoff & Blomhoff, 2006), including MRFs. In stem cells and myoblast cells lines, RA enhances skeletal myogenesis at low concentrations (Edwards & McBurney, 1983; Halevy & Lerman, 1993; Albagli-Curiel et al., 1993, as cited in Kennedy et al., 2009). In the work of Kennedy et al. (2009), mouse ES cells and P19 cells (pluripotent embryonal carcinoma cells) were differentiated with various RA concentrations, ranging from zero to 50 nM and 1%DMSO, that promotes skeletal myogenesis, but not cardiogenesis. They detected

increasing transcript levels of *Meox1*, *Pax3* and *Myod1* (all skeletal muscle markers) in the presence of various concentrations of RA by RT-PCR and showed that transcription peaked at a concentration of 25nM.

Prelle et al. (2000) overexpressed the *Igf2* gene (*insulin-like growth factor 2)* in mouse embryonic stem cells to evaluate this protein as a stimulator of myogenesis. *Igf2* was identified as an autocrine differentiation factor in myoblasts (Stewart et al., 1996, as cited in Prelle et al., 2000). It is also a survival factor during the transition from proliferation to differentiation in myoblasts and overexpression of *Igf2* in myoblasts results in enhanced differentiation characterized by accelerated expression of myogenin mRNA and extensive myotube formation (Stewart & Rotwein, 1996, as cited in Prelle et al., 2000). Embryoid bodies were formed by overexpression of *Igf2* in ES cells and myocytes were observed three days after adhesion, with myotube formation four days later and commencement of myotube contraction after ten days. They also detected the expression of the myogenic proteins titin and sarcomeric *Myhc* (myosin heavy chain). On the last day of differentiation (day 23), the large contracting myotubes showed a regular sarcomeric organization of both titin and myosin proteins.

By semi-quantitative RT-PCR, the expression onset and intensity of the skeletal muscle-specific genes *Myf5*, *Myod1* and *Myog* showed an increase in EB outgrowths overexpressing *Igf2*, with a similar pattern of expression to that occurring in vivo. Compared with non-transformed cells, the cells overexpressing *Igf2* showed an accelerated myogenic differentiation, associated with enhanced expression of MRFs, without effects in the sarcomeric structural organization.

We recently tested different factors for differentiating murine embryonic stem cells into muscle. In terms of morphology, the cells obtained are very similar to myoblasts in primary culture. The cells obtained also express mRNA from some proteins typical of the process of myogenesis, but proteins of mature muscle were not detected. To induce myogenesis in mES cells, EBs were cultured in media with 1%DMSO or 5µL of 10^{-7}M *Igf2*. After 13 days, cells were harvested for mRNA and protein analysis. Cells treated with *Igf2* visually seemed to differentiate more rapidly and with a larger proportion of cells morphologically similar to myoblasts than the cells treated with DMSO (data not published) (Figure 7).

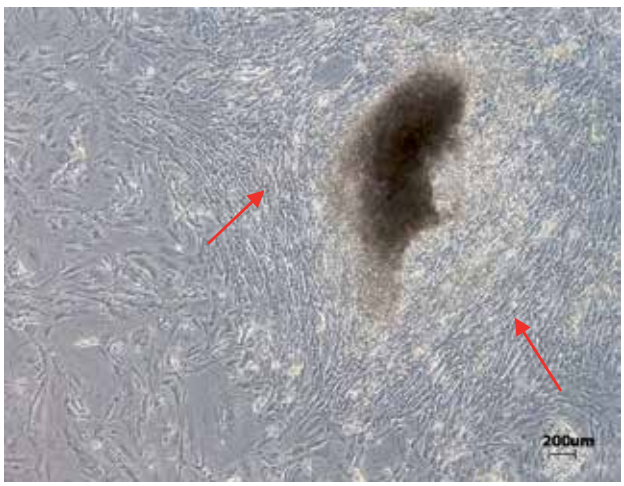

Fig. 7. Embryoid body from mES cells treated with Igf2 twelve days after adhesion. Cells indicated by the arrows are similar in morphology to myoblasts.

4.2 *In vivo* experiments and potential of therapies for NMD

Due to the lack of any available therapy for NMDs, cell therapy has been suggested as a promising alternative. Several attempts to use adult stem cells have been carried out, but with limited results. However, due to their greater pluripotency, ES cells show a greater potential for cell therapy, but can also form teratomas, when simply injected into the organism without predifferentiation, which must be avoided. Methods must be developed for inducing differentiation of the ES cells into the desired cell type before therapy, in such a way as to obtain a uniform population of differentiated cells, and, ideally, without the presence of undifferentiated ES cells.

Although there are a reasonable number of articles describing derivation of cell lines with some skeletal muscle features, the functional results are far from ideal and more basic research into *in vitro* culturing procedures is required. The skeletal muscle tissue has a unique architecture, and it is very complicated to reproduce this *in vitro*, rendering it impossible to obtain totally differentiated cells prior to transplantation. Accordingly, transplants are best made with cells that are already committed to differentiating into the myogenic lineage but have not yet completed the process, which will occur *in vivo* following transplantation.

Co-culturing of muscle stem/precursor cells from skeletal muscle with EB outgrowths can induce myogenic differentiation in vivo. This was achieved by Bhagavati & Xu, (2005) by obtaining muscle from normal mice and deriving ES cells by pre-plating muscle fragments and culturing EBs over them. The myogenic differentiation obtained in this manner is probably due to the myogenic stem/precursor cells being provided an optimal developmental environment by inductive signals for the EBs. Alternatively, it is possible that the occasional cell fusion between ES cells and myogenic precursor cells, results in the reprogramming of a limited number of ES cells. To test the potential of these cells to form skeletal muscle *in vivo*, the ES cells derived from co-culturing were injected into *mdx* mice via intramuscular injection. The muscles were analyzed after two weeks by immunohistochemical and *in situ* hybridization analyses. Dystrophin positive fibers that were lying on the surface of recipient muscle fibres were observed in 2 out of 8 injected mice. However, no functional evaluation was performed to test whether this newly derived muscle tissue was functionally normal (Bhagavati & Xu, 2005).

Zheng et al. (2006) used three different media, and treatment with 10mM 5-azacytidine in some experiments, to induce myogenic differentiation in EBs derived from hES cells. The 5-azacytidine treatment reduced cell proliferation and caused the cells to elongate. The expression analysis showed that the drug decreased the expression of *Met* and *Pax3*, but increased the expression of *Pax7* and *Myod1*. The expression of *Myf5*, *Des* (desmin), *Myhc*, *Tnni1* (troponin I), *Ncam1* was observed under all culture conditions, but occurred in the absence of myotube and myofiber formation. This indicates that, although the treated cells had the potential to initiate myogenic gene expression, they were not yet committed to complete the process and form muscle cells. The transplantation of the human ES-derived precursors to NOD-SCID mice injured with cardiotoxin and irradiation resulted in the incorporation of approximately 28% of cells into host myofibers. In the adult environment, hES cells derived precursors followed the same sequence of muscle development as during embryogenesis: myoblasts expressed muscle-specific structural proteins, fused together to form myotubes and to mature into myofibers. Hybrid regenerated myofibers displayed striated myofibrils and expressed desmin, actinin, troponin I, dystrophin (human) and myosin heavy chain. The transplanted hES cells also gave rise to satellite cells, which can provide a semi-permanent source of donor cells (Zheng et al., 2006).

A mouse ES cell line (ZHTc6-MyoD) was established by Ozasa et al. (2007) by introducing a *Myod1* transgene controlled by a Tet-Off system. This cell line is feeder-free, proliferates indefinitely and has the potential to differentiate almost exclusively into the myogenic lineage in the absence of doxycycline, and without pre-differentiation into embryoid bodies. To start the differentiation process, doxycycline is removed and a differentiation medium containing 4% fetal bovine serum (FBS) is used. Although other FBS concentrations were tested, the best one for differentiation was found to be 4%. The morphology of ZHTc6-MyoD cells in an undifferentiated state is round, but after changing to differentiation medium, they became elongated. After seven days, they started to fuse into myotubes, and occasional light muscle contractions were observed. Besides *Myod1* expression, the expression of desmin (day 4), myogenin (day 4) and dystrophin (day 8) was detected by Western blotting. *Pax7*, *Myod1*, desmin, myosin heavy chain and dystrophin expression was assayed by immuno-histochemical analysis. The cell's potential to differentiate into myofibers *in vivo* was also investigated by intramuscular injections into mdx mice and clusters of dystrophin-positive myofibers were detected in the injected area (Ozasa et al., 2007).

The use of hES cells in regenerative medicine requires pure cell populations, so that specific precursors are isolated; this is important to control cell differentiation into the desired lineage and avoid problems, such as teratomas. Barberi et al. (2007) developed a feeder-free induction system in monolayer culture to derive mesenchymal precursors from hES cells. The isolation of mesenchymal precursors was made using FACS (fluorescent activated cell sorting) of CD73+ cells, from which skeletal myoblasts were isolated by selecting for the expression of *Ncam1* (neural cell adhesion molecule 1) in another round of cell sorting. When induced with N2 medium, these skeletal myoblasts underwent terminal differentiation and formed contractile myotubes. The majority of *Ncam1*+ cells expressed *Myod1* and *Myog* in both undifferentiated and differentiated condition; however, the expression of mature muscle protein markers, such as myosin heavy chain 2a, desmin, skeletal muscle actin and sarcomeric myosin were present only in cells in terminal differentiation. After transplantation into a muscle injury model, long-term engraftment of hES cell-derived skeletal myoblasts was observed (Barberi et al., 2007).

One of the reasons for the difficulty in obtaining skeletal muscle progenitors from ES cells is the scarcity of paraxial mesoderm formation in the embryoid body, due to the lack of signals from the neural tube and notochord that are only present in the embryo. Enhancement of paraxial mesoderm formation was achieved by Darabi et al. (2008) by inducing *Pax3* by doxycyclin and sorting EBs for the presence of the PDGF-α receptor (*Pdgfr1)* a paraxial mesoderm marker, and absence of *Flk1* (synonymous with *VEGFR* in humans), which is a lateral plate mesoderm marker. The PDGFαR^{+}Flk1^{-} (*Pdgfr1*$^{+}$/*Flk1*$^{-}$) cell population is enriched under *Pax3* induction for doxycycline, once paraxial mesoderm expansion is *Pax3* dependent in the embryo; this population shows proliferative capacity and myogenic potential. However, terminal muscle differentiation occurs only when *Pax3* expression is removed and the cells exposed to a differentiation medium containing 2% horse serum. When transplanted into the tibialis anterior muscle of *Rag2*$^{-/-}\gamma c^{-/-}$ mice injured with cardiotoxin, the *Pdgfr1*$^{+}$/*Flk1*$^{-}$ cells do not form teratomas and show muscle regeneration without the necessity of continuous *Pax3* induction. In *mdx* mice, these cells exhibited significant engraftment and better functional properties (Darabi et al., 2008).

The majority of transplantation experiments have been carried out in recessive models of muscular dystrophy, so it is unknown whether the same cellular therapies would produce equivalent results in dominant models. To address this question, Darabi et al. (2009) tested the therapeutic potential of the *Pax3* induced *Pdgfr1*+/*Flk1*- cells in *Frg1* transgenic mice, a

model for the dominant facioscapulohumeral muscular dystrophy (FSHD). Consistent with the results of the previous work, these cells showed appropriate engraftment and brought amelioration to muscle contractile properties, especially in males, perhaps due to gender differences. This work confirms the therapeutic potential of stem cells to treat both recessive and dominant forms of muscular dystrophy.

According to Sakurai et al. (2008) the *Pdgfr1*+ population, when either positive or negative for *Flk1*, clearly consisted of paraxial mesoderm precursors, confirmed by the expression *Msgn1* (mesogenin), *Mesp2* and *Tbx6*. However, myotome and myogenic markers are not expressed by *Pdgfr1*+ cells, indicating that these cells have progressed to an early somatic stage, but are not yet completely committed to a specific lineage. When transplanted into the injured quadriceps femoris muscle of *KSN* nude mice, the *Pdgfr1*+ mesodermal progenitors are localized in the interstitial zone of muscles, adjacent to the myofibers. This localization suggests that these progenitor cells differentiated into satellite cells, which is confirmed by the expression of *Pax7* and *Cd34*. Some progenitor nuclei are found in the center of myofibers, indicating the contribution of *Pdgfr1*+ cells to muscle regeneration (Sakurai et al., 2008).

The culture of EBs in a differentiation medium composed by DMEM, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 5% horse serum and 10% fetal bovine serum, gives rise to cells expressing *Pax7*, but not *Myod1*, indicating the presence of quiescent satellite cells (Chang et al., 2009). These *Pax7* positive cells were enriched by fluorescense activated sorting using the surface marker SM/C-2.6 antibody, typical of quiescent adult and neonatal mouse satellite cells. The SM/C-2.6 positive cells represented 15.7% of the EB derived cells. The SM/C-2.6 positive population showed myogenic potential both *in vitro* and *in vivo* experiments. *In vivo*, GFP^+SM/C-2.6-positive cells were injected directly into cardiotoxin damaged muscles of mdx mice and were localized between the basal lamina and the muscle cell plasma membrane, which is the same location as satellite cells. After a second injury, a contribution of the injected cells to regeneration was observed, evidenced by the presence of GFP positive muscle fibers with central nuclei. In addition, the GFP^+SM/C-2.6-positive cells were able to proliferate, and to replace recipient satellite cells that were still present 24 weeks after transplantation, which is important for a continuous regeneration of the surrounding tissue (Chang et al., 2009).

A summary of all published experiments to date are in table 1.

ES cell type	Strategy	Animal models	Markers	Delivery	Reference
mESC	media supplementation with DMSO	only in vitro experiments	none	only in vitro experiments	Rohwedel et al., 1994
mESC	IGF-II overexpression	only in vitro experiments	none	only in vitro experiments	Prelle et al., 2000
mESC	co-culture with muscle cells culture	*mdx* mice/ Rag2-/-γc-/- mice	none	intramuscular	Bhagavati & Xu, 2005
hESC	media supplementation 5-azacytidine	*NOD-SCID* mice	none	intramuscular	Zheng et al., 2006
ZHTc6 mESC	MyoD gene-inducible system	*mdx* nude mice	Sca+/c-kit+/CD34-	intramuscular	Ozasa et al., 2007
hESC	FACS of mesenchymal precursors	*SCID/Beige* mice	CD73+/CD56+	intramuscular	Barberi et al., 2007
mESC	Pax3 induction/ FACS	*mdx* mice/ *Rag2-/-γc-/-* mice	$PDGFαR^+/Flk1^-$	intramuscular/systemic	Darabi et al., 2008
mESC	FACS of paraxial mesoderm precursors	*KSN* nude mice	PDGFαR+/Flk1- or Flk+	intramuscular	Sakurai et al., 2008
mESC	media supplementation DMSO and RA	only in vitro experiments	none	only in vitro experiments	Kennedy et al., 2009
mESC	Pax3 induction/ FACS	*Fyg1* mice	$PDGFαR^+/Flk1^-$	intramuscular/systemic	Darabi et al., 2009
mESC	media composed of 5% HS and 10% FBS	*mdx* mice	SM/C-2.6+	intramuscular	Chang et al., 2009

Table 1. Summary of experiments testing the myogenic potential of ES cells.

5. Conclusion

Several attempts to achieve appropriate differentiation of ES cells and implantation into model organisms have been tested with mixed results. The differentiation into the muscular lineage could be successfully obtained *in vitro*, as evidenced by the expression of myogenic markers. However, despite attempts to direct *in vivo* differentiation, to date few reports have documented successful long term therapeutic results using these procedures. Strategies, such as selecting subpopulations of undifferentiated or partially differentiated embryonic stem cells before any kind of implantation treatment is undertaken, are emerging and should result in more homogenous cell populations and, diminish the frequency of maturation of stem cells into undesired cell types. In fact, the combination of several approaches, including cell sorting, genetic modification, and prior *in vitro* induction will probably be necessary to obtain successful therapeutic outcomes with ES cells.

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Part 4

Endothelial Differentiation

13

Dissecting the Signal Transduction Pathway that Directs Endothelial Differentiation Using Embryonic Stem Cell-Derived Vascular Progenitor Cells

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1. Introduction

Blood vessels are essential for embryonic development and tissue homeostasis in adults (Coultas et al., 2005). They supply oxygen and nutrients and remove metabolic waste from tissues. Blood vessels also participate in intercellular communication. Secretion of growth/differentiation factors by blood vessels is essential for liver and pancreas organogenesis (Matsumoto et al., 2001; Lammert et al., 2001). Additionally, developing sympathetic neurons are guided by endothelins secreted by blood vessels (Makita et al., 2008), and liver regeneration in adults is triggered by factors secreted by liver sinusoidal endothelial cells (Ding et al., 2010).

In mice, vascular progenitor cells first develop in the posterior primitive streak in response to fibroblast growth factor-2 (FGF-2) and bone morphogenetic protein 4 (BMP4), and they are marked as vascular endothelial growth factor receptor 2 (VEGFR2)–positive mesodermal cells (Fig. 1; Park et al., 2004; Flamme et al., 1995). These precursor cells are committed for development into the hematopoietic and/or vascular lineage (hemangioblasts or angioblasts), and they migrate into extra-embryonic sites including the yolk sac and allantois, as well as into intra-embryonic sites (Huber et al., 2004; Hiratsuka et al., 2005). In the yolk sac, these progenitors aggregate and form clusters known as blood islands. The outer cells of the blood islands differentiate into endothelial cells, whereas the inner cells give rise to hematopoietic progenitor cells. These cells subsequently form primary capillary plexuses (vasculogenesis). In contrast, intra-embryonic angioblasts do not form a plexus intermediately and directly assembles into the dorsal aorta or cardinal vein. The extra-embryonic primary capillary plexuses then fuse with the intra-embryonic vessels to form a complete vascular network (angiogenesis). Angiopoietins and Tie receptors, along with vascular endothelial growth factor-A (VEGF-A) and Notch, are involved in this process (Thurston et al., 2003). Finally, mural cells that have differentiated in response to

transforming growth factor-β (TGF-β) are recruited to nascent vessels by platelet-derived growth factor (PDGF) secreted by endothelial cells (Betsholtz et al., 2005; Lebrin et al., 2005). This entire process ultimately leads to mature blood vessel formation.

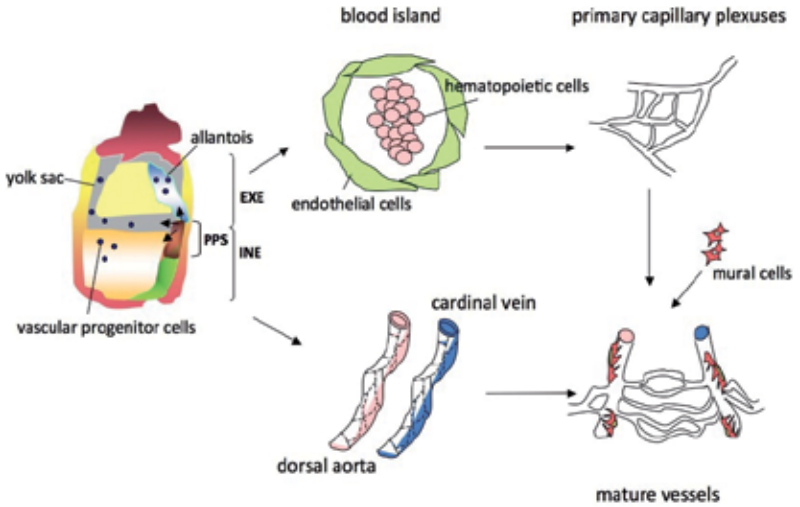

Fig. 1. Blood vessel formation during mouse development. EXE, extra-embryonic ectoderm; INE, intra-embryonic ectoderm; PPS, posterior primitive streak

A number of extracellular signaling molecules are involved in vascular development, some of which, including FGF-2, angiopoietins, PDGF, and VEGF-A, transmit their signals through receptor tyrosine kinases (RTKs). Signal transduction pathways downstream of RTKs have been extensively studied, and most share downstream effector components such as the Ras-mitogen–activated kinase (Ras-MAPK) and the phosphatidylinositol-3′ kinase (PI3K) pathways. Intriguingly, these RTKs still transmit distinct signals and play unique roles that drive vascular development. The molecular basis for the observed signaling specificity remains unclear. To better understand this important process, we focused on signaling downstream of VEGFR2, a functional receptor for VEGF-A, using embryonic stem cell–derived VEGFR2$^+$ vascular progenitor cells (Suzuki et al., 2005; Kawasaki et al., 2008; Sase et al., 2009). VEGFR2-null mice demonstrate severe defects in vasculogenesis, and VEGFR2 signaling is thought to play crucial roles in embryonic blood vessel formation (Shalaby et al., 1995; Shalaby et al., 1997; see below for details).

2. Use of differentiating embryonic stem cells for the study of lineage specification

To elucidate the signaling pathways important for the endothelial differentiation of vascular progenitor cells, we employed an *in vitro* vascular differentiation system using mouse embryonic stem cells (ESC) (Hirashima et al., 1999; Yamashita et al., 2000). *In vivo*, vascular progenitor cells must migrate to the correct microenvironment where they receive a cue for endothelial specification and further proliferate. It is challenging to identify the contributions of distinct signaling pathways to each *in vivo* event that occurs during vascular development, but the use of the *in vitro* system has allowed us to focus on the signaling

events required for endothelial specification by providing well-defined supplements to the culture medium.

We used ESC-derived vascular progenitor cells because it is practically impossible to prepare a sufficient amount of vascular progenitor cells from mouse embryos for biochemical study. In this *in vitro* system, ESCs are differentiated by culture on type IV-collagen–coated dishes in the absence of leukemia inhibitory factor (LIF) for four days, and during this time, mesodermal cells expressing VEGFR2 are induced. VEGFR2$^+$ cells (comprising 5–10% of the total cell population) are then sorted by magnetic-activated cell sorting (MACS) and used as vascular progenitors. These cells differentiate into α-smooth muscle actin-positive (αSMA$^+$) mural cells resembling vascular smooth muscle cells in the presence of PDGF-BB or serum, whereas they differentiate into platelet-endothelial adhesion molecule 1-positive (PECAM1$^+$) endothelial cells in response to VEGF-A (Fig. 2; Yamashita et al., 2000; Ema et al., 2003; Watabe et al., 2003). Alteration of the differentiation fate of vascular progenitor cells was also examined by limiting dilution assay. VEGFR2$^+$ cells were seeded at low density (90–120 cells/cm^2) and allowed to form single-cell–derived colonies for four days. After immunostaining, the numbers of PECAM1$^+$ or αSMA$^+$ colonies, which reflect the fate of differentiation, were counted. In the presence of VEGF-A, endothelial differentiation occurs at the expense of mural differentiation: stimulation with VEGF-A increases PECAM1$^+$ colonies and decreases αSMA$^+$ colonies, while the total number of colonies remains constant. Thus, VEGF-A stimulation alters the differentiation fate of vascular progenitor cells.

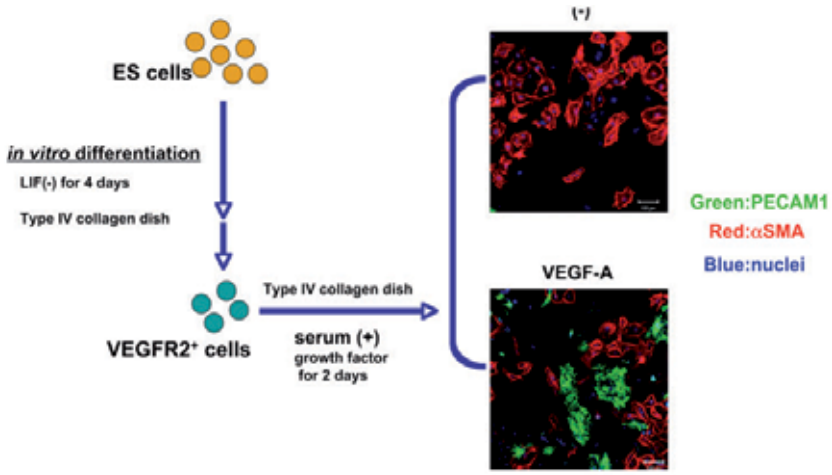

Fig. 2. *In vitro* vascular differentiation system (Yamashita et al., 2000)

This *in vitro* system was originally established using CCE embryonic stem cells. However, we used MGZ-5 cells and MGZRTcH cells because they are more amenable to transgene expression. Accordingly, we first studied MGZ-5 cells expressing genes contained in the pCAGIP vector (Fujikura et al., 2002). MGZ-5 cells express polyoma large T antigen, and this allows the efficient episomal propagation of plasmids with a polyoma origin of replication such as pCAGIP. Thus, transgenes contained within the pCAGIP vector are stably expressed in MGZ-5 cells (supertransfection method, Gassmann et al., 1996). In later experiments, we used MGZRTcH cells derived from MGZ-5 cells (Masui et al., 2005) in which a target gene

can be knocked into the *ROSA26* locus by homologous recombination using the Cre-*lox*P system, and gene expression is under the control of tetracycline (Tc) (tet-off system, Masui et al., 2005). Importantly, the *ROSA26* locus is minimally affected by epigenetic silencing during cell differentiation (Zamobrowicz et al., 1997). Therefore, expression of a transgene knocked into this locus can be regulated by Tc during nearly any stage of differentiation.
Using this *in vitro* system, we have successfully pursued two different lines of investigation of vasculogenesis: examination of downstream effectors of VEGFR2 signaling (Section 3) and screening of pharmacological inhibitors (Section 4).

3. VEGFR2 transmits unique signals to induce endothelial differentiation

VEGFR2, also known as Flk1 in mice and KDR in human, is a member of the VEGFR family of RTKs, which contains three members, VEGFGR1-3 (Fig. 3; Shibuya & Claesson–Welsh, 2006). VEGFRs share characteristic structural features: they are composed of seven extracellular immunoglobulin-like domains, a transmembrane region, and an intracellular region with intrinsic tyrosine kinase activity. They have distinct expression profiles and different ligand specificities among VEGF family members.
VEGFR1, also called Flt-1 (fms-like tyrosine kinase 1) is expressed in vascular endothelial cells, hematopoietic stem cells, macrophages, and monocytes. VEGFR2 is expressed in both vascular and lymphatic endothelial cells. VEGFR3, also called Flt-4 (fms-like tyrosine kinase 4), is principally expressed in lymphatic endothelial cells.

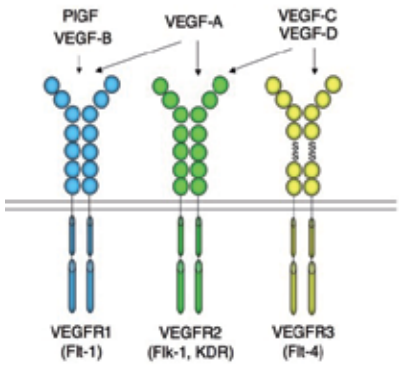

Fig. 3. VEGFR family members and their cognate ligands. The tyrosine kinase domains of VEGFRs are separated by the "kinase insert" of ~60 amino acid residues, a structural feature common to PDGF receptors and FGF receptors. The fifth immunoglobulin-like domain of VEGFR3 has an internal cleavage. The resultant N-terminal and C-terminal polypeptides are linked together by a disulfide bond.

VEGF family members, including VEGF-A, -B, -C, -D and placenta growth factor (PlGF), regulate the development, growth and function of vascular as well as lymphatic endothelial cells (Ferrara et al., 2003). VEGF-A is bound by VEGFR2 and VEGFR1, which transmit signals essential for vasculogenesis and angiogenesis. VEGF-C and VEGF-D are bound by VEGFR2 and VEGFR3 and are both involved in lymphangiogenesis (Karkkainen et al., 2004; Baldwin et al., 2005). PlGF and VEGF-B signal through VEGFR1, and they contribute to pathological angiogenesis (Carmeliet et al., 2001; Mould et al., 2003) and endothelial fatty acid uptake (Hagberg et al., 2010).

3.1 Essential role of VEGFR2 during vasculogenesis

Individual knock-out mice lacking VEGFR1, VEGFR2, or VEGFR3 all demonstrate vascular defects. VEGFR1-deficient mice exhibit disorganized vasculature secondary to endothelial cell overgrowth (Fong et al., 1995). VEGFR3 knockout mice have defects in vascular remodeling and develop fluid accumulations in the pericardial sac (Dumont et al., 1998). Blood vessel formation occurs in VEGFR1$^{-/-}$ and VEGFR3$^{-/-}$ mice. VEGFR1 and VEGFR3 are thus indispensable for angiogenesis but not vasculogenesis. In contrast, VEGFR2 plays essential roles during vasculogenesis. It is first expressed in the vascular progenitor cells at the posterior primitive streak. VEGFR2-deficient mice die *in utero* between 8.5 and 9.5 d.p.c. due to lack of organized blood vessels and hematopoietic cells. VEGFR2 signaling is required for the formation of blood islands at extraembryonic sites (Shalaby et al., 1995), where vascular endothelial cells and hematopoietic cells differentiate to form primary plexuses. In the absence of VEGFR2 signaling, vascular progenitor cells fail to migrate to extraembryonic sites from the posterior primitive streak (Shalaby et al., 1997). In the embryo proper, VEGFR2 signaling is required for endothelial specification of the vascular progenitor cells (Shalaby et al., 1997). Potential endothelial precursor cells are located at the correct anatomical niche, but they fail to complete the differentiation pathway. The identified roles of VEGFR2 signaling in vascular development *in vivo* include promoting the proliferation, migration, and differentiation of progenitor cells. Additionally, VEGF-A$^{+/-}$ mice have defects in vascular development (Carmeliet te a., 1996; Ferrara et al., 1996), but VEGF-C$^{-/-}$ mice and VEGF-D$^{-/-}$ mice exhibit lymphatic defects (Karkkainen et al., 2004; Baldwin et al., 2005). Therefore, the VEGF-A/VEGFR2 axis appears to be essential for vasculogenesis.

Because VEGFR2^{+} mesodermal cells can give rise to lineages other than endothelial and hematopoietic cells, including vascular mural cells, skeletal muscle cells, and cardiomyocytes (Motoike et al., 2003; Ema et al., 2006), differentiation of VEGFR2^{+} cells should be appropriately specified. We examined the nature of this unique and specific signaling.

3.2 VEGFR2, but not VEGFR3, directs endothelial differentiation

Although VEGFRs differ in their ligand-binding properties, their intracellular domains share structural similarities. However, the signaling properties of VEGFRs remain incompletely understood.

VEGF-A, which initiates signaling from both VEGFR1 and VEGFR2, promotes the differentiation of endothelial cells from ESC-derived VEGFR2^{+} cells, whereas PlGF, a specific ligand for VEGFR1, fails to induce endothelial differentiation despite VEGFR1 expression by precursor cells (Yamashita et al., 2000). Thus, VEGFR1 signaling does not direct endothelial differentiation. We then examined the ability of VEGFR3 to induce endothelial differentiation (Suzuki et al., 2005). While VEGFR3 is not expressed by VEGFR2^{+} vascular progenitor cells, we ectopically expressed VEGFR3 to examine whether VEGFR3 signaling induces endothelial cell differentiation.

3.2.1 VEGFR3 fails to induce endothelial differentiation

VEGFR3 cDNA was introduced into MGZ-5 ES cells using the supertransfection technique followed by drug selection (Gassmann et al., 1996). VEGFR2^{+} vascular progenitor cells were then collected from the transfected cells after *in vitro* differentiation (Suzuki et al., 2005).

These cells differentiated into PECAM1$^+$ endothelial cells in response to VEGF-C stimulation. Although VEGF-C is bound by both VEGFR2 and VEGFR3, it does not promote endothelial differentiation through endogenous VEGFR2 signaling, because it failed to induce endothelial differentiation of mock-transfected cells. We next examined the effect of VEGF-C(C152S), a mutant that selectively stimulates VEGFR3 (Kirkin et al., 2001). This mutant did not induce endothelial differentiation, but it caused vascular progenitor cell differentiation into mural cells (Suzuki et al., 2005). Thus, VEGFR3 signaling alone is not sufficient to induce endothelial differentiation. It remains possible that VEGF-C induces endothelial differentiation through heterodimer formation between ectopic VEGFR3 and endogenous VEGFR2 in these experimental conditions. Collectively, these findings indicate that VEGFR2 has unique properties among VEGFR family members even in this *in vitro* system.

3.2.2 The intracellular domain of VEGFR2 can transmit signals for endothelial differentiation

We next examined the intracellular events downstream of VEGFR2 that are required for endothelial differentiation. For this purpose, we constructed a chimeric receptor (denoted R32) that is composed of the extracellular and the transmembrane domains of VEGFR3 fused with the intracellular domain of VEGFR2 (Fig. 4; Sase et al., 2009). We then established ES cell lines expressing either R32 or VEGFR3 under the control of the Tc-regulated promoter in MGZRTcH cells (denoted Tc-R32 and Tc-VEGFR3, respectively).

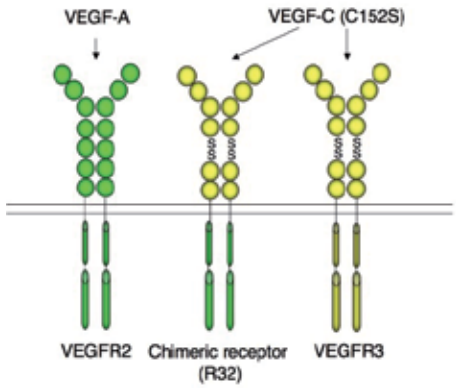

Fig. 4. Schematic structure of the chimeric receptor R32

VEGFR2$^+$ vascular progenitor cells prepared from these cell lines differentiated into PECAM1$^+$ cells following VEGF-A stimulation, indicating that these cell lines retain their ability to differentiate into endothelial cells. However, VEGFR2$^+$ cells derived from Tc-R32, but not Tc-VEGFR3, differentiated into PECAM1$^+$ cells upon stimulation with VEGF-C(C152S). The PECAM1$^+$ cells were also positive for other endothelial markers (VE-cadherin, CD34, and endoglin) and appeared to be endothelial cells. Therefore, the differences in phenotypes between VEGFR2$^+$ cells derived from Tc-R32 and Tc-VEGFR3 cells are due to intrinsic properties of the intracellular domains of VEGFR2 and VEGFR3. The intracellular domain of VEGFR2 is sufficient to direct endothelial differentiation of ESC-derived vascular progenitor cells.

3.2.3 Tyrosine 1175 of human VEGFR2 is required for endothelial specification of vascular progenitor cells and endothelial cell survival

The role of signal transduction pathways downstream of VEGFR2 for cell proliferation, migration and survival have been well explored in mature endothelial cells. Five tyrosine residues (Y951, Y1054, Y1059, Y1175, and Y1214) have been identified in the intracellular domain of VEGFR2 as major phosphorylation sites (Matsumoto et al., 2005). Y1054 and Y1059 are located in the activation loop of the kinase domain and are required for the activation of intrinsic kinase activity. The remaining tyrosine residues are located outside of the kinase domain and are required for recruitment of downstream effectors. Phosphorylation of Y1175 leads to phospholipase Cγ-activation, followed by protein kinase C (PKC) β-mediated Raf activation to induce cell proliferation (Takahashi et al., 2000). Y1175 is also involved in the activation of the PI3 kinase pathway through the adaptor protein Shb (Welch et al., 1994). In contrast, phosphorylation of Y951 leads to cell migration and actin stress fiber organization through interactions with T cell specific adaptor (TSAd) (Matsumoto et al., 2005). Phosphorylation of Y1214 is also implicated in actin stress fiber remodeling through the p38 mitogen activated kinase pathway (Lamalice et al., 2004). Y951 and Y1175 are unique tyrosine residues in VEGFR2, whereas Y1214 is conserved in VEGFR2 and VEGFR3.

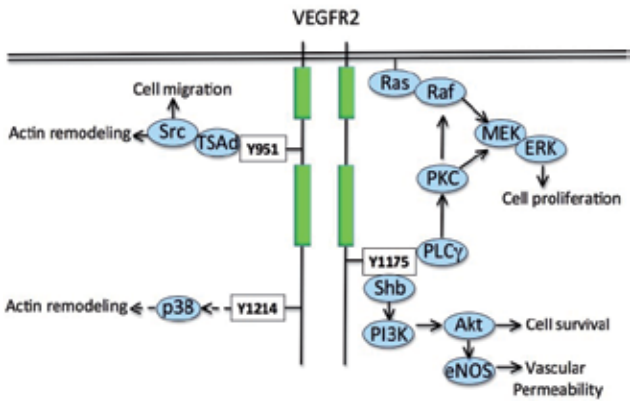

Fig. 5. Signal transduction downstream of VEGFR2

Y1173 in mouse VEGFR2 (corresponding to Y1175 in humans) is indispensable for blood vessel formation *in vivo* (Sakurai et al., 2005). VEGFR2 Y1173F knock-in mice died between E8.5 and E9.5, similar to VEGFR2-null mice. In Y1173F knock-in mice, VEGFR2$^+$ vascular progenitor cells failed to migrate into the yolk sac to form blood islands, and, because vasculogenesis was aborted and progenitor cells did not receive specification signals, it remained unclear whether Y1173 is also important for endothelial specification of vascular progenitor cells. Therefore, we addressed this question using the *in vitro* differentiation system.

3.2.3.1 Tyrosine 1175 of human VEGFR2 is required for endothelial differentiation

To examine the roles of individual tyrosine residues in the induction of endothelial differentiation, we constructed three mutants of the R32 chimeric receptor (R32Y951F, R32Y1175F, and R32Y1214F) in which the indicated tyrosine residues were mutated to phenylalanine. After confirming the ability of these mutants to activate effective signals, we

established MGZRTcH ES cell lines expressing these mutant receptors (Tc-R32Y951F, Tc-R32Y1175F, and Tc-R32Y1214F). VEGFR2+ vascular progenitor cells were prepared from these cell lines and examined for endothelial differentiation in the presence of VEGF-C(C152S) (Sase et al., 2009).

VEGFR2+ cells derived from Tc-R32Y951F and Tc-R32Y1214F differentiated into endothelial cells following treatment with VEGF-C(C152S) whereas those from Tc-R32Y1175F failed to do so. To exclude the possibility that signals associated with residues Y951 and Y1214 could compensate for one another to promote endothelial differentiation, we also established a cell line expressing a double mutant, Tc-R32Y951/1214F. VEGFR2+ cells derived from this cell line retained their ability to undergo endothelial differentiation. These findings indicate that signaling from Y1175 plays a central role in the endothelial differentiation of vascular progenitor cells, but residues Y951 and Y1214 are dispensable for this function.

In this *in vitro* differentiation system, endothelial cells appear only when vascular progenitor cells are successfully specified into endothelial cells and when the differentiated endothelial cells are able to survive and proliferate (Fig. 6, top panel). Endothelial cells are unable to survive in the presence of serum alone, and they typically require growth factors such as VEGF-A or FGFs for survival and/or proliferation. We investigated whether signaling from Y1175 plays a role in specification, survival, or both processes.

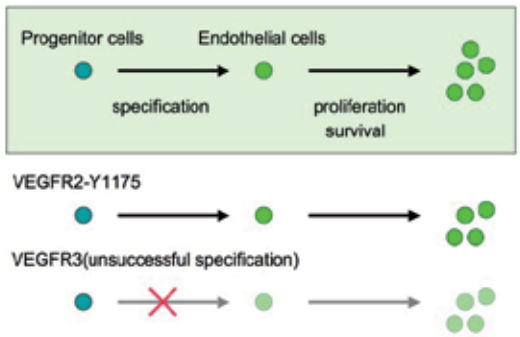

Fig. 6. VEGFR signaling and endothelial differentiation

3.2.3.2 Tyrosine 1175 of human VEGFR2 is required for endothelial survival

We first performed an endothelial survival assay (Fig. 7) with Tc-R32, Tc-R32Y1175F, and Tc-VEGFR3. ESC-derived VEGFR2+ cells were cultured with VEGF-A in serum-free medium for two days to induce mature endothelial cells, and the endothelial cells were then cultured in serum-free medium with or without VEGF-C(C152S) to activate the chimeric receptors.

The number of endothelial cells cultured in the absence of VEGF-C(C152S) was considerably decreased within 12 h. VEGF-C(C152S)–induced signaling from R32 led to a significant recovery in the number of cells, but VEGF-C(C152S) treatment of cells expressing R32Y1175 failed to do so. Thus, Y1175 is involved in the transmission of survival signals to endothelial cells. As described in 3.2.1, VEGFR3 signaling is not sufficient for inducing endothelial differentiation from VEGFR2+ vascular progenitor cells. However, VEGFR3 signaling increased the survival of ESC-derived endothelial cells. We also found that LY294002, an inhibitor of PI3K, abrogated the effects of VEGFR3 on endothelial survival. These findings suggest that VEGFR3 signaling promotes endothelial survival despite its inability to direct endothelial specification (Fig. 6).

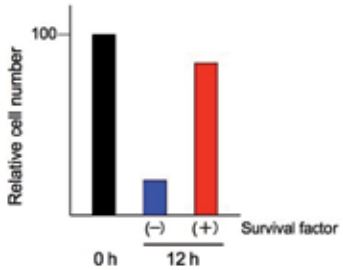

Fig. 7. Endothelial survival assay. When ESC-derived endothelial cells are cultured in serum-free medium, the cell number largely decreases within 12 h, while in the presence of an appropriate survival factor, the cell number is maintained.

3.2.3.3 Tyrosine 1175 of human VEGFR2 is required for endothelial specification

We next examined the involvement of signaling from Y1175 in the endothelial specification of vascular progenitor cells. Because R32Y1175F failed to transmit appropriate survival signals, we supplemented the cell cultures with a low dose of FGF-2 (0.5 ng/ml) to promote endothelial cell survival. VEGFR2$^+$ cells derived from Tc-R32Y1175F failed to differentiate into endothelial cells upon stimulation with VEGF-C(C152S) although those derived from Tc-R32 successfully underwent differentiation. Thus, human VEGFR2 Y1175 is essential for the induction of endothelial cells from vascular progenitor cells through both specification of VEGFR2$^+$ cells and their subsequent survival (Fig. 6).

3.2.4 The VEGFR2-Y1175-PLCγ1 pathway is indispensable for endothelial specification

In mature endothelial cells, Y1175 of human VEGFR2 primarily transmits cell proliferation signals through the recruitment of PLCγ and cell survival signals through the recruitment of Shb (Fig. 5). Intriguingly, PLCγ1 deficient mice are embryonically lethal secondary to a lack of vasculogenesis and erythrogenesis despite the presence of hemangioblasts (Liao et al., 2002). Thus, PLCγ1 may be involved in the specification of vascular progenitor cells and/or subsequent survival of endothelial cells. We hypothesized that R32Y1175F failed to induce endothelial differentiation because it does not activate PLCγ1.

3.2.4.1 Knockdown of PLCγ1 attenuated endothelial differentiation

To elucidate the function of PLCγ1 in endothelial differentiation, we established a stable ES cell line in which expression of PLCγ1 can be knocked down by expression of pre-miRNA under the control of Tc (Tc-miRNA-PLCγ1) (Sase et al., 2009). We used this system to perform gene silencing studies because we had difficulty introducing siRNA oligonucleotides to VEGFR2$^+$ vascular progenitor cells; we were able to easily introduce them into ES cells or differentiated endothelial cells, however (Kawasaki et al., 2008). It is likely that differentiating ES cells may be resistant to transfection at certain stages of development.
Expression of the miRNA targeting PLCγ1 resulted in a modest decrease in PLCγ1 protein expression and decreased appearance of endothelial cells, indicating that PLCγ1 signaling is required for endothelial differentiation.

3.2.4.2 Constitutively active PLCγ1 induces endothelial specification

We next constructed a constitutively active form of PLCγ1, PalmPLCγ1, in which a sequence for myristoylation and palmitoylation was added to its N-terminus. This mutant protein is

constitutively localized at the plasma membrane. We then established a stable ES cell line harboring Tc-regulatable PalmPLCγ1 (Tc-PalmPLCγ1) or a negative control cell line (Tc-empty) and examined the differentiation of VEGFR2$^+$ cells derived from these cell lines.
The induced expression of PalmPLCγ1 did not lead to the appearance of PECAM1$^+$ cells from VEGFR2$^+$ progenitor cells, and, after performing an endothelial survival assay, we found that PalmPLCγ1 did not transmit signals for cell survival in ESC-derived endothelial cells. We next examined endothelial differentiation of VEGFR2$^+$ cells in the presence of a low dose of FGF-2 to support survival of endothelial cells. Stimulation with PalmPLCγ1 plus FGF-2 reconstituted signaling for endothelial differentiation although stimulation with only FGF-2 did not affect the number of differentiated endothelial cells. PalmPLCγ1-expressing progenitor cells treated with FGF-2 became positive for PECAM1, VE-cadherin, CD34, and endoglin, and these findings indicate that PLCγ1 signaling is involved in the endothelial specification of vascular progenitor cells but not in the survival of differentiated endothelial cells (Fig. 8). Consistent with this, VEGFR3, which transmits signals for endothelial survival but not endothelial specification, failed to activate PLCγ1 (Sase et al., 2009).
At present, the signaling pathways downstream of Y1175 that mediate survival of endothelial cells remain to be elucidated. However, Shb may be a good candidate for a signaling effector because it activates the PI3K pathway.

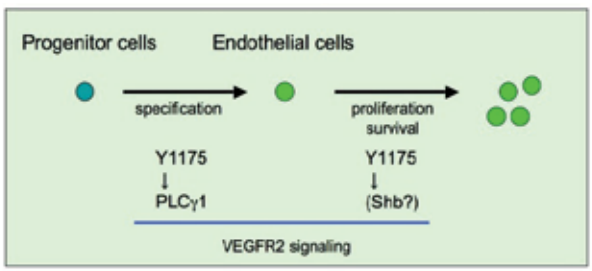

Fig. 8. Signaling from VEGFR2-Y1175 for induction of endothelial cells *in vitro*

4. Ras signaling specifies endothelial lineage

In addition to the chimeric receptor approach described above, we also used various pharmacological inhibitors to study endothelial differentiation (Kawasaki et al., 2008), and we found that temporally regulated Ras signaling plays a crucial role in endothelial specification downstream of PLCγ1.

4.1 A farnesyltransferase inhibitor, FTI-277, inhibits VEGF-A–induced endothelial differentiation of ESC-derived VEGFR2$^+$ cells

To determine the signaling components involved in VEGF-A–induced endothelial differentiation from vascular progenitor cells, we screened various low molecular weight compounds targeting signaling molecules for their ability to inhibit endothelial differentiation *in vitro*. Among the compounds tested, we found that the farnesyltransferase inhibitor FTI-277 (Lerner et al., 1995) selectively affects endothelial differentiation. FTI-277 interferes with the farnesylation of small G proteins and their subsequent association with the plasma membrane, thus abrogating their signaling functions. FTI-277 inhibited the appearance of VEGF-A–induced PECAM1$^+$ cells, but that of αSMA$^+$ was not. The effect of

FTI-277 was also confirmed in a limiting dilution assay. In the presence of FTI-277, the number of PECAM1$^+$ colonies was decreased while αSMA$^+$ colonies were increased. Importantly, the total number of derived colonies was not significantly changed. These findings indicate that FTI-277 specifically inhibits the endothelial differentiation of ESC-derived VEGFR2$^+$ cells.

We next performed an *ex vivo* whole-embryo culture assay to confirm the effects of FTI-277 on vascular development in a mouse embryo (Fig. 9; Kawasaki et al., 2008). Concepti at embryonic day 6.75 (E 6.75) were picked out from the uteri of pregnant mice and cultured *in vitro* for three days, and, in the absence of further manipulation, PECAM1$^+$ blood vessels formed in the yolk sac during this time. However, FTI-277 treatment led to a reduced number of PECAM1$^+$ vessels, but the overall development of the yolk sac was not affected. Quantitative RT-PCR analysis also indicated that PECAM1 expression was decreased in the presence of FTI-277, while the expression of αSMA was not affected. These findings suggest that FTI-277 selectively suppresses vascular development. Importantly, endothelial differentiation induced by constitutively active PalmPLCγ1 plus FGF-2 was inhibited by FTI-277, indicating that FTI-277 targets signaling pathway(s) downstream of PLCγ1.

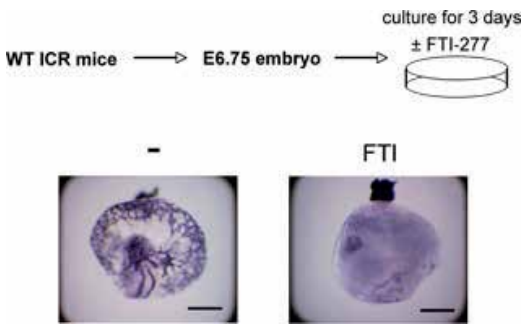

Fig. 9. FTI-277 inhibited *ex vivo* yolk sac vascularization (Kawasaki et al., 2008. Originally published *in Journal of Cell Biology*. doi: 10.1083/jcb.200709127). E6.75 concepti were picked out and cultured with or without FTI-277 (10 μM) for three days and whole-mount stained for PECAM1.

4.2 Loss of H-Ras attenuates endothelial differentiation of VEGFR2$^+$ cells *in vitro*

Because the principal targets of FTI-277 include H-Ras, we hypothesized that H-Ras signaling could be involved in the VEGF-A–induced endothelial differentiation of vascular progenitor cells. To examine this possibility, we established an ES cell line based on MGZRTcH cells in which a pre-miRNA sequence targeting H-Ras is expressed under the control of Tc (Tc-miR-H-Ras). In Tc-miR-H-Ras cells, expression of endogenous H-Ras was efficiently knocked down in the absence of Tc (Tet-off system). A limiting dilution assay was performed using Tc-miR-H-Ras–derived VEGFR2$^+$ cells in the presence or absence of Tc. In the absence of Tc (H-Ras knocked-down condition), the number of VEGF-A–induced PECAM1$^+$ colonies was decreased while the number of αSMA$^+$ colonies was increased compared to cells grown in the presence of Tc (PECAM1:αSMA=51.6%:48.4% to 36.8%:63.2%). These findings suggest that H-Ras plays a role in the endothelial differentiation of VEGFR2$^+$ progenitor cells.

4.3 Loss of H-Ras attenuates endothelial differentiation in mouse embryos

To examine the importance of H-Ras signaling during vascular development *in vivo*, we investigated the vascular phenotype of H-*ras* knockout mice. Because heterozygous H-*ras*$^{+/-}$ mice produced homozygous H-*ras*$^{-/-}$ offspring in the expected Mendelian ratio as previously reported (Ise et al., 2000), we focused on the vascular phenotype during early development. There were no clear differences in the vascular phenotypes of wild-type (WT) and H-*ras*$^{+/-}$ embryos. Vascular anomalies were found in the brain of 73% of H-*ras*$^{-/-}$ embryos examined at E9.5 although they contained similar numbers of somites as their WT littermates (Fig. 10; Kawasaki et al., 2008).

We next double-stained the cephalic region of the embryos for PECAM1 and VEGFR2, the earliest marker of endothelial cell differentiation. In H-*ras*$^{+/-}$ embryos, numerous complex vascular networks were stained for both PECAM1 and VEGFR2, but vascular structures positive for either PECAM1 or VEGFR2 were strikingly reduced in H-*ras*$^{-/-}$ embryos. However, these defects were transient because no abnormalities were apparent in E10.5 H-*ras*$^{-/-}$ embryos. Collectively, these data suggest that H-Ras signaling is involved in the *in vivo* differentiation of endothelial cells although it is not indispensable.

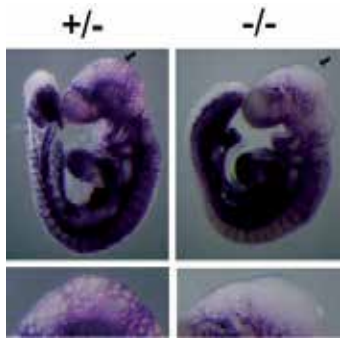

Fig. 10. Whole-mount PECAM1 staining of E9.5 H-*ras*$^{+/-}$ and H-*ras*$^{-/-}$ mice (Kawasaki et al., 2008. Originally published *in Journal of Cell Biology*. doi: 10.1083/jcb.200709127).
Magnifications of the area marked with arrows in the top are shown in the bottom.

4.4 A constitutively active G12V mutant of H-Ras induces PECAM1$^+$ cells from VEGFR2$^+$ progenitor cells

We next established an ES cell line carrying a constitutively active form of H-Ras (Tc-H-Ras[G12V]) under the control of Tc, and we examined the differentiation of VEGFR2$^+$ cells derived from this cell line. When H-Ras[G12V] expression was suppressed by Tc treatment, the appearance of PECAM1$^+$ cells was VEGF-A–dependent. However, expression of H-Ras[G12V] led to the VEGF-A–independent induction of PECAM1$^+$ cells. These PECAM1$^+$ cells were also positive for other endothelial markers including CD34 and endoglin, and they incorporated AcLDL. Additionally, the aggregated VEGFR2$^+$ cells derived from Tc-H-Ras[G12V] cells formed tube-like structures even in the absence of VEGF-A when cultured in type I collagen gel for seven days, indicating that vascular formation by VEGFR2$^+$ cells is dependent on H-Ras signaling. Furthermore, Tc-H-Ras[G12V] cells formed vascular structures when subcutaneously injected with Matrigel into the abdominal region of mice, dependent upon the expression of H-Ras[G12V]. These findings suggest that active Ras induces the differentiation of cells with characteristics of endothelial cells from VEGFR2$^+$

progenitor cells (Kawasaki et al., 2008). Additionally, data from a limiting dilution assay showed that expression of H-Ras[G12V] led to endothelial differentiation at the expense of mural differentiation. Thus, the fate of cell differentiation was altered by Ras signaling.
Although Ras signaling induces the expression of VEGF-A (Rak et al., 1995; Gruger et al., 1995), Ras-induced endothelial differentiation was not dependent on autocrine stimuation by induced VEGF-A. Endothelial differentiation by H-Ras[G12V] proceeded normally in the presence of SU5614, an inhibitor of VEGFR2 kinase (Spiekermann et al., 2002), or VEGFR1 (Flt1)-Fc chimeric protein which competes with VEGFR2 for binding to VEGF-A. Collectively, these findings suggest that differentiation depends on intracellular Ras signaling.

4.5 Signaling for endothelial differentiation is mediated through the Ras-Erk pathway

Ras proteins activate both the Raf/MEK/Erk and the PI3K/Akt pathways. To determine the signaling pathway(s) that mediate Ras-induced endothelial differentiation, we established ES cell lines in which H-Ras effector mutants H-Ras[G12V, T35S] or H-Ras[G12V, Y40C] can be inducibly expressed. The effector mutants H-Ras[G12V, T35S] and H-Ras[G12V, Y40C] preferentially activate the Raf/MEK/Erk and PI3K/Akt pathways, respectively (Joneson et al., 1996). When H-Ras[G12V, T35S] was expressed, PECAM1$^+$ colonies increased in number, while αSMA$^+$ colonies were decreased as determined by limiting dilution assay. In contrast, when H-Ras[G12V, Y40C] was expressed, the numbers of PECAM1$^+$ colonies and αSMA$^+$ colonies were unchanged. These findings suggest that the Ras-PI3K pathway does not affect the determination of cell fate, and we concluded that the Ras-Erk pathway specifies the endothelial fate of VEGFR2$^+$ progenitor cells.

4.6 Kinetics of Ras activation by VEGF-A

Ras proteins are activated by extracellular stimuli including hormones, cytokines and growth factors. ESC-derived VEGFR2$^+$ cells differentiate into endothelial cells upon stimulation with VEGF-A, but not upon stimulation with PDGF-BB, a known activator of Ras signaling. Thus, the activation statuses of Ras are different between that downsteam of VEGFR2 and that downstream of PDGF receptors.
We examined the effects of FTI-277 treatment at different time points after VEGF-A stimulation. FTI-277 inhibited endothelial differentiation even when added 3 h after VEGF-A stimulation, indicating that Ras activation later than 3 h after stimulation plays a role in endothelial differentiation. We hypothesized that the specificity of Ras signaling induced by VEGFR2 may be attributed to the timing of Ras activation, and we investigated the window within which Ras is specifically activated by VEGF-A, focusing on the period later than 3 h after VEGF-A stimulation.
We examined the levels of phosphorylation of Erk, a downstream effector of Ras, 3–12 h after stimulation with VEGF-A. Erk phosphorylation peaked at 6 h and 9 h after stimulation, suggesting that Ras could be activated with a similar time course. We next examined the activation of Ras in cells stimulated with VEGF-A or PDGF-BB for 6 h, and the patterns of Ras activation in response to VEGF-A or PDGF-BB were markedly different. At 6 h after stimulation, VEGF-A caused intense activation of Ras and Erk, whereas PDGF-BB failed to activate both Ras and Erk. At 5 min after stimulation, both VEGF-A and PDGF-BB induced activation of Ras and Erk although to different extents. Activation of Ras and Erk by VEGF-A was also observed at 9 h, but not at 3 h after stimulation (Fig. 11). Importantly,

phosphorylation of Erk around 6 h after VEGF-A stimulation was sensitive to FTI-277. Activation of the Ras-Erk pathway 6–9 h after stimulation with VEGF-A thus appears to direct endothelial differentiation of VEGFR2$^+$ progenitor cells. These findings provide mechanistic insights into signaling events required for cell specification through widely-shared effector molecules.

4.7 VEGF-A–specific Ras activation precedes the expression of endothelial markers

We finally examined whether the expression of vascular markers is induced after the delayed activation of the Ras-Erk pathway during *in vitro* differentiation of vascular progenitor cells. The mRNA expression of the endothelial markers PECAM1 and VE-cadherin increased from 12 h after VEGF-A stimulation. Intriguingly, the expression of VEGFR2 in VEGF-A–stimulated cells was similar to that seen in non-stimulated cells up to 6 h after treatment. However, at time points later than 12 h after stimulation, VEGFR2 expression was up-regulated in VEGF-A–stimulated cells, but it was down-regulated in non-stimulated cells. These observations suggest that specification to endothelial lineage occurs between 6–12 h after stimulation with VEGF-A, and this is preceded by the delayed activation of Ras (Fig. 11). We conclude that VEGF-A stimulation of VEGFR2$^+$ vascular progenitor cells specifically induces Ras-Erk activation around 6–9 h after stimulation, and this event in turn specifies endothelial differentiation.

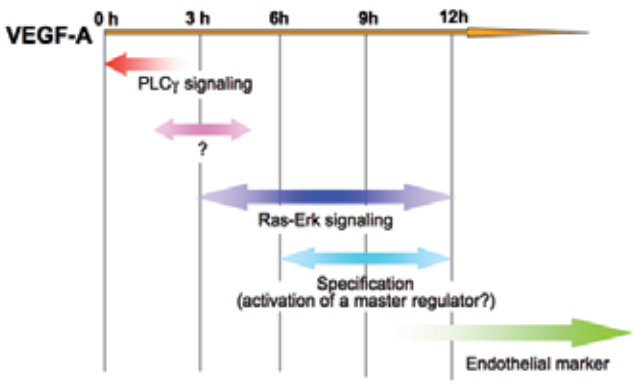

Fig. 11. Kinetics of endothelial differentiation of vascular progenitor cells after VEGF-A stimulation. The link between PLCγ and Ras-Erk (the second double-headed arrow) remains to be elucidated.

5. Unsolved questions in signaling for endothelial differentiation

The development of multicellular organisms requires the orchestrated interactions of a wide variety of cells, including growth, migration, and differentiation. Extracellular factors as well as intracellular signaling molecules are involved in the regulation of cellular processes during development. Each signaling molecule appears to have defined functions that are dependent upon the appropriate cellular context.

VEGFR2 signaling plays a central role in *de novo* blood vessel formation. In an *in vitro* embryoid body culture system, VEGFR2$^{-/-}$ ES cells still give rise to endothelial cells with low efficiency (Schuh et al., 1999). This may be due to an effects of FGF-2 included in the culture

medium (Schuh et al., 1999), because FGF-2 induces endothelial differentiation of ESC-derived VEGFR2$^+$ cells to a modest extent (Kano et al., 2005). VEGFR2 signaling appears to be the primary pathway for controlling endothelial specification with high efficiency, although it is not the sole pathway capable of mediating these effects. We found that activation of the Ras-Erk pathway 6–9 h after stimulation with VEGF-A plays a critical role in endothelial specification of vascular progenitor cells. The mechanism(s) regulating the delayed activation of Ras and the downstream signaling of the Ras-Erk pathway are the next questions to be solved.

5.1 Upstream and downstream of the delayed Ras activation induced by VEGF-A

In ESC-derived VEGFR2$^+$ progenitor cells, the PKC-dependent pathway (Fig. 5) appears to be activated early, since phosphorylation of Erk was notably increased while activation of Ras was modest 5 min after VEGF-A stimulation (Kawasaki et al., 2008). In contrast, the Ras pathway strongly induced Erk phosphorylation 6–9 h after stimulation, a finding supported by the inhibition of Erk phosphorylation by FTI-277. The mechanism(s) controlling the delayed activation of Ras remain unclear. At present, we only know that PLCγ1 lies upstream of the delayed Ras activation. It is possible that Ras activation is mediated through the transcriptional induction of some signaling molecules and/or activation of other signaling receptor(s). It is also important to identify transcription factors that are phosphorylated by Erk to better understand the signaling specificity of early and late Erk activation. VEGFR2$^+$ cells develop into mural cells in the absence of growth factor, and mural differentiation may be the default fate of VEGFR2$^+$ cells. During the differentiation of vascular progenitor cells, induction of mural markers is delayed compared to the induction of endothelial markers by VEGF-A: expression of αSMA was increased at 24 h while PECAM1 became expressed from 12 h after stimulation. It appears likely that VEGFR2$^+$ cells in which the delayed Ras-Erk signaling is activated before the determination of cell fate to mural cells are differentiated into endothelial cells. However, it remains possible that VEGF-A signaling merely suppresses mural differentiation, thus altering the default differentiation fate. Alternatively, it is possible that VEGF-A signaling not only induces endothelial differentiation but also suppresses mural differentiation.

Although Ras appears to be activated downstream of PLCγ1 in vascular progenitor cells after stimulation with VEGF-A, constitutively active Ras transmits sufficient signals for full endothelial differentiation while constitutively active PLCγ1 transmits signals only for endothelial specification. This discrepancy remains unexplored. It is likely that constitutively active effector molecules may transmit artificial signals. In particular, G12V mutants of Ras proteins are resistant to intrinsic negative regulation through hydrolysis of GTP. H-Ras[G12V] may constitutively send strong signals that are sufficient to fully induce endothelial differentiation.

5.2 Phenotypes of H-Ras knockout mice

We examined vascular formation in H-*ras*$^{-/-}$ mice, and vascular anomalies were found in the brain of 73% of E9.5 H-*ras*$^{-/-}$ embryos. However, there were no obvious abnormalities in E10.5 H-*ras*$^{-/-}$ embryos, consistent with the previous report that H-*ras* knockout mice are born and grow normally (Ise et al., 2000). These findings suggest that H-*ras*$^{-/-}$ embryos catch up for the delay in vascular formation in the cephalic region until E10.5. It is possible that expression of other Ras family members, N-Ras and K-Ras, is upregulated, and these

proteins may compensate for the loss of H-Ras (Ise et al., 2000). Alternatively, a reduction of endothelial differentiation in the absence of H-Ras may be permissive for embryonic development. Compensatory growth of differentiated endothelial cells may offset the reduction in endothelial differentiation. It appears likely that N-Ras and K-Ras are also involved in endothelial specification because treatment with FTI-277 or knockdown of H-Ras failed to completely inhibit endothelial specification induced by VEGF-A in the *in vitro* vascular differentiation system.

5.3 Towards more efficient differentiation of endothelial cells

In recent years, revascularization therapy using endothelial progenitor cells (EPCs) has been studied for the treatment of ischemic disorders and arteriosclerosis. In this therapy, EPCs implanted at sites of disease form new vascular structures. Identification of the signaling pathways required for endothelial differentiation could provide valuable information for establishing a highly efficient endothelial differentiation system. This would be a boon to the field of regenerative medicine.

The efficiency of endothelial differentiation of vascular progenitor cells *in vitro* remains ~50%. Differentiating ES cells actively proliferate, indicating that they are going through cell cycles. Recent work revealed that Ras can efficiently transmit signals during G1 phase but not during G1/S or S/G2 (Sakaue–Sawano et al., 2008). Signals for endothelial specification are likely successfully transmitted in vascular progenitor cells at G1 phase (Fig. 12). Manipulations that control the cell cycle status may improve the efficiency of specification of vascular progenitor cells to endothelial cells, which is transmitted principally through Ras proteins.

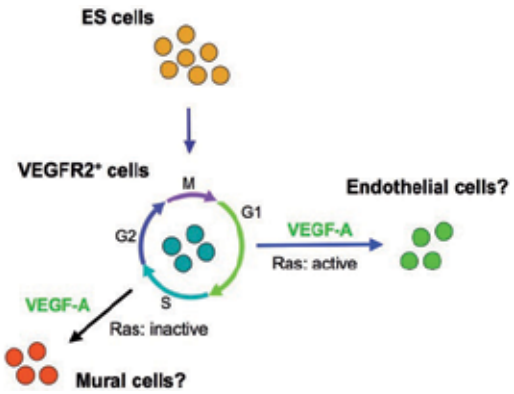

Fig. 12. Possible cell cycle–dependent signaling for endothelial differentiation through Ras activation

Modulation of the VEGFR2 signaling pathway at the level of receptor–ligand interaction may also improve the efficiency of endothelial differentiation *in vitro*. Recently, protein kinase A was found to induce expression of VEGFR2 and its co-receptor neuropilin-1, thus enhancing vascular progenitor potential (Yamamizu et al., 2009).

6. Conclusions

Vascular endothelial growth factor 2 (VEGFR2) is a critical signaling component controlling many aspects of vasculogenesis during embryonic development, including proliferation,

migration, and endothelial differentiation of vascular progenitor cells. However, the signaling pathways specifying endothelial differentiation downstream of VEGFR2 are poorly understood. We investigated these signaling pathways using an *in vitro* endothelial differentiation system (Kawasaki et al., 2008; Sase et al., 2009). Using a variety of different approaches including chimeric receptors, gene silencing, gain-of-function mutants, and pharmacological inhibitors, we dissected the signal transduction pathway that directs endothelial differentiation (Fig. 13). In response to VEGF-A stimulation, VEGFR2 transmits signals for specification of vascular progenitor cells and survival of endothelial cells through Y1175. PLCγ1, a downstream effector that is recruited to phosphorylated Y1175, relays endothelial specification signals. Activation of H-Ras that occurs at a delayed phase after VEGF-A stimulation triggers endothelial specification. The unique activation of signaling molecules downstream of VEGFR2/PLCγ/Ras is essential for transmitting signals controlling endothelial specification. At the present time, the link between PLCγ1 and H-Ras remains to be identified. Shb, another downstream effector recruited to phosphorylated Y1175, may be involved in the survival of endothelial cells through activation of the PI3K pathway. VEGFR3 lacks the ability to induce endothelial specification, and this may be due to its inability to activate PLCγ1, although it activates the PI3K pathway to support endothelial cell survival. It has previously been shown that Y1175 signaling is indispensable for migration of vascular progenitor cells to the site of their differentiation (Sakurai et al., 2005). Therefore, Y1175 of VEGFR2 appears to be a central node transmitting signals required for endothelial differentiation including migration, specification, and subsequent cell survival.

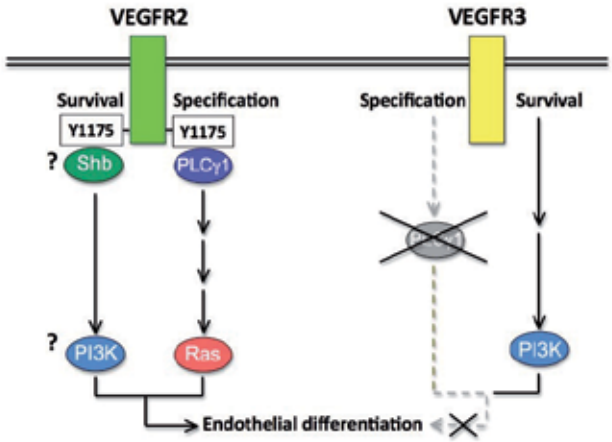

Fig. 13. Signal transduction pathways for endothelial differentiation from VEGFR2

7. Acknowledgement

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14

Endothelial Differentiation of Embryonic Stem Cells

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1. Introduction

Embryonic stem (ES) cells are a rich source of multiple different cell types of diverse lineages. Significant advances have recently been made in our understanding of the molecular mechanisms by which ES cells differentiate into endothelial cells. The differentiation of ES cells into endothelial cells can be enhanced by certain growth factors, environmental cues, cell-cell interactions, and extracellular matrix. A wide variety of signal transduction pathways and transcription factors have been shown to participate in this process. The use of ES cells for endothelial differentiation are not only of interest with respect to the molecular mechanisms but also for identifying sources of endothelial cells that can be used for a number of therapeutic purposes. The purpose of this chapter is to review recent advances in the molecular mechanisms underlying ES differentiation into endothelial cells and how endothelial cells derived from ES cells are being used therapeutically.

2. Culture conditions

ES cells are derived from the inner cell mass of a growing blastocyst [1]. As such, they have the capacity to differentiate into all the cell types of an organism [2]. To maintain their undifferentiated state ES cells are generally grown in the presence of feeder cells or leukemia inhibitory factor (LIF). In the absence of inhibitory factors ES cells can spontaneously differentiate into cells with characteristics of one of three germ layers; mesoderm, endoderm, or ectoderm. A variety of approaches have been used to differentiate human and mouse ES cells that include: (1) aggregating ES cells into three-dimensional cell masses called embryoid bodies (EBs); (2) by co-culturing the ES cells with other cell types; and (3) by culturing the ES cells on specific matrix surfaces together with defined media.
Murine ES cells have been used for several years to study differentiation along multiple lineages. Culturing ES cells into EBs has been the most frequently used model to study EC differentiation. The appearance of structures consisting of immature hematopoietic cells surrounded by endothelial cells and the formation of vascular-like channels suggests that the EBs produce factors necessary not only for endothelial differentiation but also for

vasculogenesis. These structures closely resemble the so-called blood islands of the yolk sac. One of the major advantages of this culture method is the highly reproducible timing of molecular events that occurs during the process of differentiation. The induction of both hematopoietic and endothelial markers at precise time points during the differentiation has greatly facilitated investigation of factors that promote or inhibit EC differentiation or hematopoiesis. Their close association has also led to commonly held belief that the hematopoietic and endothelial lineages stem from a common precursor known as the hemangioblast. The markers of hematopoiesis and endothelial differentiation also permit the isolation of specific populations of cells at different stages of differentiation. An example of one of the earliest markers of the endothelial lineage is VE-cadherin, and of the hematopoietic lineage is CD41. One of the potential disadvantages of this model is that only a fraction of the cells, on the order of 5-10%, ultimately become endothelial cells.

Human ES cells have also been used to generate EBs as a model of EC differentiation or hematopoiesis. endothelial cells can be isolated and propagated from human EBs at vary time points during differentiation. The EBs can also be used as a model of angiogenesis in addition to EC differentiation and vasculogenesis. 11-day old EBs are cultured within three dimensional type 1 collagen matrix gels that is supplemented with angiogenic growth factors including VEGF and FGF2 [3]. The sprouting of new vessels can be observed radiating out from the EBs into the surrounding matrix.

A second mouse model of ES cell differentiation is a two dimensional system that takes advantage of the calvaria stromal cell line OP9. ES cells are initially grown as EBs and then a subset of cells expressing VEGF-R2 are separated by flow cytometry. These VEGF-R2 cells are grown on the OP9 cells they gradually differentiate into endothelial cells expressing VE-cadherin and CD31 (PECAM-1). Interestingly, the endothelial cells exhibited two morphological characteristics. On the one hand endothelial cells grew as sheets of cells and on the other hand the cells coalesced into cord-like structures. It was determined that the sheet-like Endothelial cells were of lymphatic origin, whereas the other endothelial cells were more like blood endothelial cells. Two soluble factors made by the OP9 cells that contribute to the differentiation of these cells into lymphatic endothelial cells include Angiopoietin-1 and VEGF-C.

A third method of culturing the murine ES cells into endothelial cells consists of isolating a subpopulation of ES cells that express the VEGF receptor 2 (VEGF-R2) and allowing them to differentiate on an acellular matrix such as type IV collagen [4]. By adding growth factors to a serum free media it was determined that VEGF was a critical factor for differentiating VEGF-R2 expressing ES cells into endothelial cells.

3. Markers of the endothelial lineage

A critical determinant of endothelial differentiation is the identification of selective markers that separate the endothelial lineage from other lineages. Because of the close association between the hematopoietic and the endothelial lineage, the existence of a bipotent cell called the hemangioblast capable of differentiating into either the endothelial or hematopoietic lineages was suggested and identified [5,6]. A commonly used marker to define this cell type is the VEGF receptor 2 (VEGF-R2). Using an embryoid body (EB) model of ES the temporal expression pattern of several endothelial markers was evaluated by PCR and immunohistochemistry [7]. The expression of VEGF-R2 on day three of differentiation

preceded the expression of all of the endothelial markers. The expression of PECAM-1, Tie-1, Tie2, and VE-cadherin, followed shortly thereafter on days 4 and 5 of differentiation. Furthermore, the number of cells expressing these EC-specific markers represented a small fraction of the overall number of ES cells that appeared to coalesce into discreet regions of the EBs and by day 12 of differentiation when they formed primitive vascular structures. The number of cells expressing the endothelial marker PECAM-1 was enhanced by adding the growth factor VEGF on day 6 of ES cell differentiation [7]. We have similarly used the embryoid body model to identify a population of VEGF-R2 expressing mouse embryonic stem cells that also express the endothelial marker VE-cadherin and that are distinct from those that express the hematopoietic marker CD41 [8].

The close association between the hematopoietic and endothelial lineages that is seen during ES cell differentiation has also been observed in a variety of different organisms including the mouse, chicken, zebrafish and humans during embryonic development. In the mouse for example, cells of the hematopoietic and endothelial lineages are first observed in the yolk sac in regions called blood islands. In addition to the yolk sac, the generation of blood is first observed in the developing embryo in a region called the aorta-gonad-mesonephros (AGM), in the vitelline and umbilical arteries, and in the allantois/placenta region [9 10,11]. In the AGM region hematopoietic cells are found in close association with the endothelial wall of the aorta suggesting that these early hematopoietic cells are generated from an early intermediate called the hemogenic endothelium [9,12]. Additional support for the hemogenic endothelium has come from in vitro studies of mouse embryonic stem cell differentiation. Blast colony-forming cells (BL-CFCs) were identified that can give rise to cells of both the endothelial and hematopoietic lineages [5,6]. More recently, studies using time-lapse photography and FACS analysis of differentiating mouse embryonic stem cells, have demonstrated that the hemangioblast generates hematopoietic cells through the formation of a hemogenic endothelium intermediate [13]. In particular, $Tie2^{hi}c\text{-}Kit^{+}CD41^{-}$ expressing cells were identified as a population of cells that constitute the hemogenic endothelium.

Further differentiation of endothelial cells into cells of arterial, venous, and lymphatic endothelium has also been shown to occur in ES cells and do so in the developing embryo prior to the development of blood flow. The differentiation of embryonic stem cells into arterial endothelial cells is dependent on the presence and concentration of VEGF. When mouse ES cells are cultured in the presence of lower molecular weight isoforms of VEGF (120 and 164) they differentiate into endothelial cells expressing arterial markers [14]. The differentiation of ES cells into arterial versus venous endothelial cells is also dependent on the dose of VEGF. Whereas at low concentration of VEGF (2ng/ml) cultured VEGF-R2 expressing mouse ES cells differentiate into endothelial cells expressing venous EC markers such as neuropilin-2 (NRP2), at higher concentrations of VEGF (50 ng/ml) the same cells will differentiate into EC expressing the arterial markers Ephrin B2 and neuropiin-1 (NRP1) [15]. ES cells have also been used study their differentiation into lymphatic endothelial cells. For example, murine ES cells were aggregated to form EBs and then were cultured in a 3-dimensional collagen matrix for up to 18 days [16]. Treatment with a combination of growth factors including VEGF-C and VEGF-A enhanced the formation of lymphatic endothelial cells in the EBs [17]. An alternative approach of promoting the differentiating ES cell along the lymphatic lineage is to use VEGF-R2 expressing ES cells and to co-culture them with OP9 stromal cells [18]. The differentiation of the VEGF-R2 cells into lymphatic endothelial cells is dependent upon VEGF-C and angiopoietin-1.

4. Signal transduction pathways

A variety of signal transduction pathways have been implicated during the process of endothelial differentiation. At the top of the hierarchy of signal transduction molecules are the hedgehog (HH) family of signaling proteins, which play a number of different roles in determination of cell fate, embryonic patterning, and morphogenesis [19]. HH signaling activates the transcription factor GLI. Inhibition of Indian hedgehog (IHH) during the differentiation of mouse or human ES cells blocks their ability to differentiate along the endothelial and hematopoietic lineages [20] [21]. In addition to IHH, several studies have suggested a role for sonic hedgehog (SHH) in endothelial differentiation. In the absence of SHH, angioblasts fail to form into EC tubes or vascular networks [22]. Administration of SHH was able to promote the differentiation of human multipotent adult progenitor cells into arterial endothelial cells both in vivo and in vitro [23].

Bone morphogenic proteins (BMPs) belong to the TGF-beta family of proteins that are involved in regulating cell proliferation, survival, and differentiation during embryogenesis [24]. In particular, BMP-2 and BMP-4 are known mediators of endothelial function and differentiation during embryogenesis and ES cell differentiation [25,26]. BMP appears to function downstream of the HH proteins during the in vitro differentiation of ES cells along the EC lineage. Inhibition of EC differentiation by blocking the HH pathway can be rescued with BMP-4 [20]. Furthermore, when human ES cells are cultured in the presence of BMP-4, this augments their differentiation along the endothelial lineage [25].

BMPs can also activate a number of downstream signal transduction pathways. For example, the induction of angiogenesis by BMP-2 is dependent upon activation of the canonical and non-canonical WNT pathways [26]. BMPs can also activate the MAP kinase signaling pathways. For example, BMP-4 activation of EC sprouting is dependent upon p38 MAP kinase [27]. Inhibition of EC migration by BMPs is dependent upon the JNK and ERK pathways [28]. In contrast to BMP-2 and BMP-4, two BMPs, BMP-9 and BMP-10 inhibit the growth of endothelial cells and promote endothelial quiescence [29,30]. The role of BMP-9 and BMP-10 in endothelial differentiation has not been studied, however in contrast to other endothelial cells, BMP-9 promotes the proliferation of mouse embryonic-stem cell derived endothelial cells [31].

WNT signaling is involved in processes that determine cell fate, self-renewal of stem cells, polarity, and organogenesis. There are three classical WNT pathways: (1) the canonical or WNT/β-catenin pathway; (2) the planar cell polarity pathway; and (3) the WNT/Ca^{2+} pathway [32]. WNT5A is a mediator of EC proliferation, survival, and differentiation. WNT5A is expressed in the developing vasculature of several organs including the skin, retina, stomach, and liver [33]. WNT5A functions predominantly through the non-canonical WNT pathways. Signaling cascades activated by WNT5A include the protein kinase C (PKC) and c-Jun n-terminal kinase (JNK) pathways [34]. WNT5A can also inhibit the activity of the canonical WNT/β-catenin pathway [35]. WNT5A contributes to the regulation of the differentiation of ES cells along the endothelial lineage [36]. WNT5A deficient ES cells cannot differentiate into endothelial cells. In endothelial cells WNT5A can activate both the canonical and non-canonical WNT pathways. The WNT/Ca^{2+} pathway is the predominant WNT pathway activated in WNT5A deficient ES cells exposed to exogenous WNT5A. A role for WNT signaling has also been evaluated during the differentiation of the hemangioblast in human ES cells [37]. Administration of the WNT inhibitor dickkopf1 markedly inhibited the differentiation of ES cells towards the endothelial or hematopoietic

lineages. Likewise, when ES cells were cultured in the presence of WNT1, there was a marked increase in the number of hemangioblast like cells. In contrast, when ES cells were exposed to WNT5A, they did not have the same effect of increasing the number of hemangioblasts, suggesting that WNT5A principally acts at later stages of EC differentiation [38].

5. Transcription factors

A variety of transcription factors are known to play a critical role in cellular differentiation during embryonic development. In particular, selected transcription factors are known to regulate the differentiation of embryonic stem along the endothelial or hematopoietic lineages. As mentioned above, the hemangioblast is a bipotent cell capable of differentiating into either endothelial or hematopoietic cells. The basic helix-loop-helix (HLH) transcription factor SCL (Tal1) has been shown to be critical for blood and endothelial cell development [39]. SCL is expressed early during embryogenesis in hematopoietic and endothelial cells and its disruption in either mouse or zebrafish leads to severe defects in vasculogenesis [40-42]. SCL is also an early marker of the hemangioblast in embryonic stem cells during their differentiation towards the hematopoietic and endothelial lineages [43]. In this model of ES cells were cultured in serum free conditions and sequentially exposed to BMP4, activin A, bFGF, and VEGF. As the mesodermal marker brachyury gradually decreases there is an increase in the expression of SCL together with two other transcription factors Runx1 and Hhex. SCL deficient ES cells are unable to generate either primitive or definitive hematopoietic cells [44]. SCL also appears to be critical for the development of the hemogenic endothelium [13]. SCL deficient ES cells failed to differentiate into $Tie2^{hi}$c-$Kit^{+}CD41^{-}$ cells that constitute cells with the capacity to differentiate into hematopoietic and endothelial cells.

Members of the ETS family of transcription factors have also been shown to play a role in the regulation of EC-specific gene expression and EC differentiation. For example, ER71 has been shown to be critical for endothelial differentiation and vascular development in mice and zebrafish [45,46]. In ES cells ER71 is critical for the expression of VEGF-R2 [47]. ER71 appears to induce VEFGF-R2 downstream of BMP, Notch, and Wnt signaling. Another ETS factor that is expressed in the vasculature and regulates hematopoiesis is Fli-1. Fli-1 deficient mice die at embryonic day E12.5 of defective vasculogenesis leading to cerebral hemorrhage [48]. Fli-1 deficient ES cells also exhibit defective hematopoiesis with a marked reduction in the number of blast colony forming cells. In contrast to most other ETS factors we and other groups have shown that the ETS factor ERG exhibits an EC-restricted expression pattern [49-52]. Furthermore, it has also been shown that several EC-restricted genes including VE-cadherin, endoglin, and vWF, are regulated by ERG [53-55]. In addition to its role in regulating EC-restricted genes we have recently shown that ERG is critical effector of EC differentiation of ES cells that appears to be independent of hematopoiesis [56]. ERG was selectively expressed in $VEGF\text{-}R2^{+}VE\text{-}caherin^{+}$ cells and not in $VEGF\text{-}R2^{+}CD41^{+}$ cells. Suppression of ERG in ES cells by lentivirally delivery of shRNA resulted in a significant reduction in EC differentiation but not hematopoietic cells.

There are several transcription factors that facilitate the differentiation of ES cells into arterial, venous, and lymphatic endothelial cells. The critical transcription factor that promotes the differentiation of the hemangioblast or VEGF-R2 expressing cells into venous endothelial cells is COUP-TFII [15]. The differentiation of these cells was dependent upon the dose of VEGF. Whereas low doses of VEGF (2-10 ng/ml) induced the differentiation of VEGF-R2 cells into venous endothelial cells expressing high levels of COUP-TFII, high doses

of VEGF (50 ng/ml) repressed COUP-TFII levels and induced the differentiation of VEGF-R2 cells into endothelial cells expressing markers of arterial endothelial cells such as Ephrin B2. Transcription factors that are preferentially upregulated in arterial endothelial cells include the HLH factors Hey1 and Hey2. Notch signaling appears to be critical for promoting differentiation of ES cells into arterial endothelial cells. Inhibition of Notch signaling with a gamma secretase inhibitor preferentially leads to the expression of venous EC markers and a repression of arterial markers [15]. An environmental stimulus that promotes the differentiation of ES cells into arterial endothelial cells is hypoxia. Exposure of ES cells to hypoxia was associated with an increase in the expression of the Notch ligand Dll4 and the transcription factors Hey1 and Hey2 [57].

The prototypic transcription factor that regulates the differentiation of ES cells into lymphatic endothelial cells is Prox1. Prox1 expression can be induced by three-dimensional culture of murine ES cells into EBs in collagen matrices in the presence of VEGF-C and VEGF-A [16]. Similarly when VEGF-R2 cells were co-cultured with OP9 cells expression of Prox1 was observed in lymphatic endothelial cells on day 3 [18]. Expression of Prox1 in blood endothelial cell leads expression of lymphatic markers and transdifferentiation into lymphatic endothelial cells [58]. Another transcription factor that regulates the expression of the VEGF-R3, which binds to VEGF-C, is the T box transcription factor Tbx1 [59]. Although Tbx1 is not required for LEC differentiation it is required for the growth and maintenance of lymphatic vessels. A transcription factor that is involved in regulating later stages of lymphatic EC maturation is the forkhead transcription factor Foxc2 [60,61]. Foxc2 was shown to be important for the development of lymphatic valves and controlling interactions between lymphatic endothelial cells and mural cells.

6. Therapeutic implications

One of the ultimate goals of developing culture methods that promote the differentiation of stem cells into endothelial cell is to provide a source of cells for a variety of different therapeutic applications. One obvious application would be to generate new blood vessels or repair existing blood vessels in clinical settings where blood flow is compromised. As an initial proof of concept a population of VEGF-R2 expressing mouse ES cells were injected into chicken embryo hearts. These cells were shown to be able to integrate into the host vasculature and differentiate into two cellular components of the vasculature, endothelial cell and smooth muscle cells [62]. Based on these promising studies embryonic stem cell derived endothelial cell have been used in animal models of human disease to promote angiogenesis and ultimately improve blood flow. For example, murine ES cell derived endothelial cell were injected either intramuscularly or intra-arterially in a hindlimb model of ischemia, and were shown to engraft at the site of ischemia and improve tissue perfusion [63]. One of the concerns of using ES cells for therapeutic purposes is the potential formation of teratomas from undifferentiated ES cells. In the hindlimb ischemia study no teratomas formed when ES cell derived endothelial cell were used compared to their uniform development within two weeks when undifferentiated ES cells were used. A similarly promising study was done using human ES cell (hESC) derived endothelial cells [64]. The hESC derived endothelial cells were shown to engraft into blood vessels at sites of myocardial ischemia using a mouse myocardial infarction model. Together these exciting proof-of-concept studies provide evidence that ES cell derived endothelial cells can be used in a variety of settings to promote blood vessel growth at sites of ischemia. One of the major

challenges in using the currently available stem cells as a source of endothelial cells for therapeutic applications is the potential of significant immune responses to the engrafted cells. One potential mechanism of overcoming this hurdle more recently is the development of induced pluripotent stem (iPS) cells in which skin fibroblasts from any individual can be transformed into ES cells by introducing four transcription factors [65]. These iPS cells have subsequently been cultured to promote their differentiation into vascular cells that are very similar to those obtained from human ES cells [66].

7. Acknowledgements

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Part 5

Hepatic Differentiation

15

Stem Cells for HUMAN Hepatic Tissue Engineering

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1. Introduction

1.1 Clinical implications

End-stage liver disease is a life-threatening condition for which the only effective medical treatment available to date is orthotropic liver transplantation. Other approaches are needed because of the severe shortage of donors. These alternatives include cell transplantation and extracorporeal bioartificial livers. Since adult hepatocytes do not readily proliferate in culture, and healthy human livers are used to meet transplantation needs and are therefore not available for other purposes, a major challenge for these cell-based therapies is to identify a reliable hepatocyte source[1]. In the case of extracorporeal devices, many have suggested the use of animal sources (e.g. rat and pig), where immunoisolation may be possible. This is not likely to be a viable option for cell implantable modalities since these implants must be vascularized to function properly, and as a result would be exposed to xenogeneic immune rejection[2]. An increasingly plausible approach is the use of hepatocytes derived from embryonic stem cells (ESCs), as recent studies – reviewed in greater detail below – show that it is possible to differentiate ESCs into hepatocyte-like cells with high yields. Furthermore, recent developments with induced pluripotent stem cells (iPSCs) suggest that many of the procedures used to differentiate ESCs could be used on iPSCs, thus making it possible to derive patient-specific syngeneic hepatocytes. Besides therapeutic applications, hepatocytes derived from human ESCs can be used for a variety of other applications, such as toxicity drug screening, where the use of human cells is much preferred compared to animal cells that often vary in sensitivity and metabolism of xenobiotics and drugs.

1.2 Industrial implications

One of the fundamental challenges facing the development of new chemical entities within the pharmaceutical industry is the extrapolation of key in vivo parameters from in vitro cell culture assays and animal studies. Development of microscale devices and screening assays incorporating primary human cells can potentially provide better, faster and more efficient prediction of in vivo toxicity and clinical drug performance[3]. With this goal in mind, large strides have been made in the area of microfluidics to provide in vitro surrogates that are

designed to mimic the physiological architecture and dynamics. Current embodiments of this synergy cover various microelectromechanical (MEMs) devices that contain hepatocytes, for use in drug metabolism screening and toxicology assessment.

1.3 Liver function and structure

The liver, a key metabolic and detoxification center, contains parenchymal cells called hepatocytes (70%) and various nonparenchymal cell types such as sinusoidal endothelial cells, stellate (fat-storing, Ito) cells, Kupffer cells, and cholangiocytes (bile duct cells). Hepatocytes are responsible for most liver-specific functions, including albumin synthesis, detoxification of ammonia into urea and glutamine, bile and cholesterol production [4]. The liver is also critical for maintaining circulating glucose levels via gluconeogenesis during fasting. A large array of enzymes is responsible for the detoxification of organic compounds, either endogenous (such as many hormones) or exogenous (drugs and toxins) via two sequential mechanisms described as Phase I and Phase II biotransformations. There is an extensive body of literature that has shown that controlling environmental parameters, in other words the culture conditions, which consist of the type of substrate used, spatial orientation of the cultured cells, addition of growth factors, and the combinatorial effects of these parameters, is critically important to induce and maintain a high level of hepatocellular viability and function[5]. The functional and structural complexity of the liver organ has been very difficult to reproduce ex vivo in hepatocyte culture systems. Some of these challenges have been partially met using novel cell culture approaches as well as microfabrication techniques that can emulate the size scales of the liver sinusoid. While all of these studies describe various techniques to boost in vitro hepatocyte function, they do not resolve the limited access to primary hepatocytes, which do not proliferate to any significant degree outside of the liver[1]. This is crucial because despite modifications in the culture environment, large numbers of mature hepatocytes are needed, and yet, are not available, for clinical applicability. Herein, we focus primarily on the challenge of securing a sufficient supply of high functioning hepatocytes for clinical and industrial applications using a stem cell differentiation platform.

1.4 Adult stem cells

To address cell source issues more effectively, research into alternate hepatocyte precursor populations has been conducted. Unlike differentiated cells, hepatoblasts are not only capable of expressing differentiated function, but are also able to self-renew. A few hepatoblasts have been identified that have the capacity to differentiate into mature hepatocytes and include bipotential precursors for hepatocytes and biliary cells, and hematopoietic stem cells [6].

In scenarios following severe hepatic injury, liver regeneration is attributed to a potential stem cell compartment located within the smallest branches of the intrahepatic biliary tree, which gives rise to the bipotential cells known as oval cells [7]; [8]. Oval cells are characterized as small cells with a high nucleus-to-cytoplasm ratio, oval shaped nucleus, and the ability to express markers of both fetal hepatocytes and biliary cells [9]; [10]. Oval cells have been shown to require growth factors such as transforming growth factor alpha (TGFα), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) for progression through the cell cycle as well and subsequent differentiation toward mature hepatocytes [11]. Despite the large number of observations describing liver growth

processes driven by oval cell proliferation and differentiation into hepatocytes, oval cells are difficult to isolate and the molecular mechanisms behind these processes must still be elucidated.
Hematopoietic stem cells (HSCs) have also been induced to differentiate along hepatocyte specific pathways. For example, one experimental system utilized HSC transplantation to alleviate liver disease in fumarylacetoacetate hydrolase (FAH) deficient mice [12]. FAH deficiency leads to liver dysfunction and eventual lethality. Following HSC transplantation, liver function was reconstituted. However, it is unclear whether the HSCs or HSC progeny that repopulated the liver. In addition, the mechanism that induces differentiation toward mature hepatocytes is unclear.
Despite the fact that hepatoblasts exhibit the potential to provide a renewable hepatocyte cell source, these cells are hard to isolate and exist in very low numbers [13]. In addition, the full efficacy of utilizing these precursor cells is questionable, since the long-term functional stability of hepatocytes obtained from these systems has yet to be assessed.

2. Embryonic stem cells and induced pluripotent stem cells

There are multiple stem cell starting paths for hepatocytes: 1. ESCs, 2. iPSCs, 3. Endoderm precursors and 4. Hepatic stem cells. However, due to their robust nature and large body of literature, we will focus on the first two. Furthermore these two cell types are readily abundant and have a higher proliferative capacity, thereby providing a strong potential starting point for the aforementioned applications.

2.0.1 Embryonic stem cells (ES cells)

Embryonic stem cells, derived from the inner cell mass of the blastocyst [14], have been proposed as another potential source for the generation of mature hepatocytes. ES cells are pluripotent and can be induced to differentiate into any cell type. When cultured in the presence of an anti-differentiation agent such as leukemia inhibitory factor (LIF) and with or without a feeder layer, these cells can proliferate while maintaining pluripotency [15]. Upon removal of the anti-differentiation agent, ES cells begin to spontaneously differentiate.

2.0.2 Induced pluripotent stem cells (iPS cells)

A new area of research has developed in recent years, majorly in part due to the legislative restrictions certain countries have placed on ES cell research. One area that has shown strong advancement is the area of induced pluripotent stem cells (iPS cells). iPS cells are the result of reprogramming somatic cells to a pluripotent state which resemble ES cells with respect to morphology, proliferation (self- renewal), surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, epigenomics and telomerase activity [16, 17]. Human iPS cells' autologous nature offers several advantages over human ES cells in regards to potential patient specific therapeutics, the study of disease state, study of developmental processes, drug discovery as well as drug toxicity on differentiated hepatocytes while avoiding the ethical issues associated with isolation and the usage of human ES cells as illustrated in Figure 1.
Yamanaka coined the term induced pluripotent stem cells in 2006 while inducing a pluripotent state in mouse somatic cells by direct reprogramming [16]. The same year, his lab demonstrated the generation of induced pluripotent stem cells from adult human

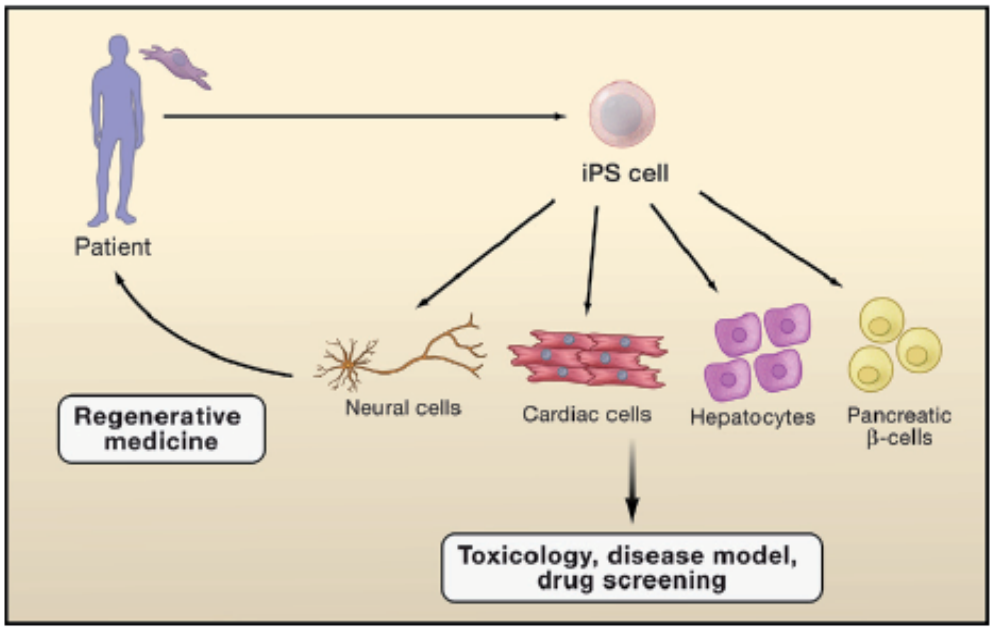


Fig. 1. **Applications of IPS Cell Technology**
Patient-derived iPS cells can produce various somatic cells with the same genetic information as the patient. These cells could be used to construct disease models and to screen for effective and safe drugs, as well as to treat patients through cell transplantation therapy. (Figure taken from [20], figure 1)

fibroblasts by retroviral transfection of four factors: Oct3/4, Sox2, c-Myc and Klf4 [16, 18]. Since then, others have generated human iPS cells using different combination of factors while preserving Oct3/4 and Sox2 as the core factors needed for pluripotency reprogramming while Oct3/4 is the most important[19, 20]. The main risk associated with the use of human iPS derived cells for transplantation is directly influenced by the iPS generation methods which may involve risk of DNA modification (gene deletion, viral gene incorporating into the human genome and gene expression alterations) which may lead to insertional mutations that will affect iPS function, differentiation potential and tumorigenesis [20, 21]. The human iPS cells generated by the Yamanaka group, for example, had more than 20 retroviral integration sites in total which may increase tumor formation [16, 18]. That risk has accelerated the search for new methods for generating clinically safer iPS cells such as the use of non-integrating episomal vectors or the use of molecules that promote or enhance reprogramming of somatic cells to iPS cells to improve reprogramming efficiency and reduce genomic alterations due to viral integration [21, 22].

2.0.3 Induced pluripotent stem cells are similar, but not identical to embryonic stem cells

Although iPS cells share similar features with ES cells as mentioned above, they are not identical. The reprogramming process does not involve genetic transformation but rather an epigenomic one. Lister et al. have shown that iPSCs are not identical to ESCs with respect to epigenomic profile by profiling whole-genome DNA methylation at single-base resolution

in five human iPSC lines, along with methylomes of ES cells, somatic cells, and differentiated iPSCs and ES cells [17]. In addition, iPSCs maintain some epigenomic features which resemble the somatic cell they have been generated from [17]. Cell memory based on epigenomics may suggest that, for example, iPSCs which were generated from primary hepatocytes as a somatic cell source will yield more or better functioning iPSC derived hepatocytes compared to those generated from iPSCs which were generated from fibroblasts as a somatic cell source. These discrepancies between human ES and iPS cells led to the question of what is the difference between the two stem cell types in regards to pluripotency and differentiation potential? And what is the best method to assess it? The ultimate test for pluripotency in the mouse system is the generation of a chimeric mouse which was successfully generated by blastocyst microinjection of mouse ES and iPS cells. Obviously, this test is not applicable to human ES and iPS cells and, therefore, other methods are being used to determine the pluripotency of the cells such as teratoma formation. As in the case of human ES cells, human iPS cells have the potential to differentiate to any of the three germ layers and form teratomas when transplanted subcutaneously into a severe combined immunodefficient (SCID) mouse [16, 18]. Unfortunately, teratoma formation does not guarantee full reprogramming as many mouse ES cell-like cell lines form teratomas but fail to produce germline chimeras [20]. The ultimate method to assess the differentiation potential of human ES and iPS cells to mature and functional hepatocytes is to compare the two with respect to mature hepatocytes gene and protein expression profiles, as well as metabolic activity, drug clearance, glycogen storage, urea and albumin synthesis and secretion while using the same differentiation method.

In addition to the functionality of the differentiated hepatocytes, the efficiency of the process is of high importance due to the potential of human ESCs and iPSCs to differentiate spontaneously into cells from the three germ layers. Complete differentiation of stem cells in vitro is especially important when used for cell based therapies. Heterogeneous populations at the end of the differentiation process composed of differentiated cells as well as stem cells may lead to teratoma formation at the site of transplantation as well at other locations to which the cells migrated.

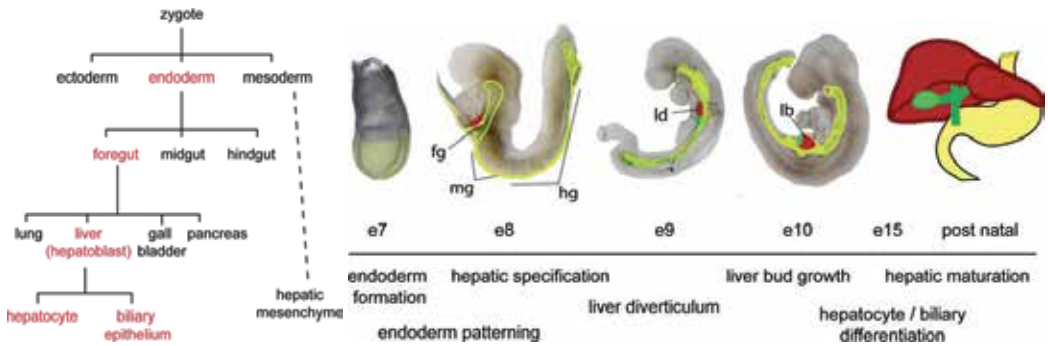
2.1 Traditional embryonic stem cell and induced pluripotent stem cell differentiation paradigms

Many paradigms currently exist to specifically direct the differentiation of embryonic stem cells toward a hepatocyte lineage in vitro, while utilizing the knowledge of embryological pathways occurring in vivo during normal liver development. This process involves numerous stages and is influenced by cytokines as well as cell-matrix interactions in a temporal and spatial manner. When developing new paradigms for direct differentiation of human ES and iPS cells into mature and functional hepatocytes in vitro with high efficiency, one must refer to the developmental process of the liver during embryonic development.

2.1.1 Hepatocyte differentiation during embryogenesis

The main stages are illustrated in Figure 2A. In the first phase, ES cells differentiate to endodermal cells. ES and iPS cells are pluripotent and therefore can give rise to any of the three germ layers: ectoderm, mesoderm and endoderm where the latter, more specifically the anterior- ventral definitive endoderm, give rise to the cells of the liver. The expression of

Wnt signaling inhibitors in the anterior endoderm represses the Wnt/β catenin pathway and was shown to be required for liver specification in the endoderm [23]. Signaling by the transforming growth factor beta (TGF*β)* growth factor Nodal at relatively high concentrations initiates endoderm formation. Nodal signaling stimulates the expression of a core group of endoderm transcription factors including the HMG domain DNA-binding factor Sox17 and the fork head domain proteins Foxa1–3 (HNF3*α/β/γ*) which in turn regulate a cascade of genes committing cells to the endoderm lineage [24]. FoxA2 and GATA4 serve as transcription factors for the alb1 gene which encodes for serum albumin and appear early in the pre-liver hepatic domain of ventral foregut endoderm and later in liver [25, 26].



(A) (B)

Fig. 2. **Embryonic development of the liver.**
(A) Development of the liver is illustrated from the standpoint of cellular differentiation (red) from uncommitted endoderm to functional adult hepatocytes and biliary epithelium. (B) The schematic shows mouse embryos at different stages of development with the endoderm tissue highlighted in yellow, the liver in red, and the gall bladder in green. The major developmental events are listed below. The endoderm germ layer is formed during gastrulation (e6.5-e7.5). Throughout gastrulation and early somite stages of development (e7-e8.5) the endoderm is patterned along the A-P axis into foregut (fg) midgut (mg) and hindgut (hg) progenitor domains. Morphogenesis forms foregut and hindgut pockets as the endodermal cup is transformed into a gut tube. By e8.5 hepatic fate specified in a portion of the ventral foregut endoderm adjacent to the heart. As the embryo grows the endoderm forms a gut tube and the liver domain moves to the midgut. The liver diverticulum (ld) forms by e9 and expands into an obvious liver bud (lb) by e10. The liver grows, and by e15 hepatoblasts are differentiating into hepatocyte and biliary cells. Final maturation of the liver is gradual and continues into the postnatal period.
(Figure taken from[74], figures 1 and 2)

In the second phase, we see the differentiation from definitive to hepatic endoderm. The onset of liver development is characterized by the commitment of midgut endoderm to become liver through interactions with cardiac mesoderm which secrete fibroblast growth factors (FGFs) [27]. FGFs signaling in the foregut endoderm activated the MAPK pathway is necessary for initiation and stabilization of hepatic differentiation [28]. Bone morphogenetic proteins (BMPs) (BMP2, BMP4, BMP5, and BMP7) signaling from the septum transversum mesenchyme also contributes to hepatic gene induction in the endoderm [29-32]. Wnt

signaling along this stage is suppressed but is required in the following stage where the hepatic endoderm outgrows into the liver bud[23]. In addition, HNF3β and activin A signaling are involved in the process of the specification from endodermal stem cells toward the hepatic epithelial lineages as indicated in Figures 2 and 3 [31, 32]. Under the influence of these signals, endodermal cells (the liver bud) migrate from the ventral foregut into the extracellular matrix (ECM) rich septum traversum, forming the primordial hepatoblasts. This migration is accompanied by major remodeling of the extracellular matrix surrounding the hepatic cells. Some investigators have tried to induce this process in vitro by differentiating ES and iPS cells using various types and configurations of ECM [33].

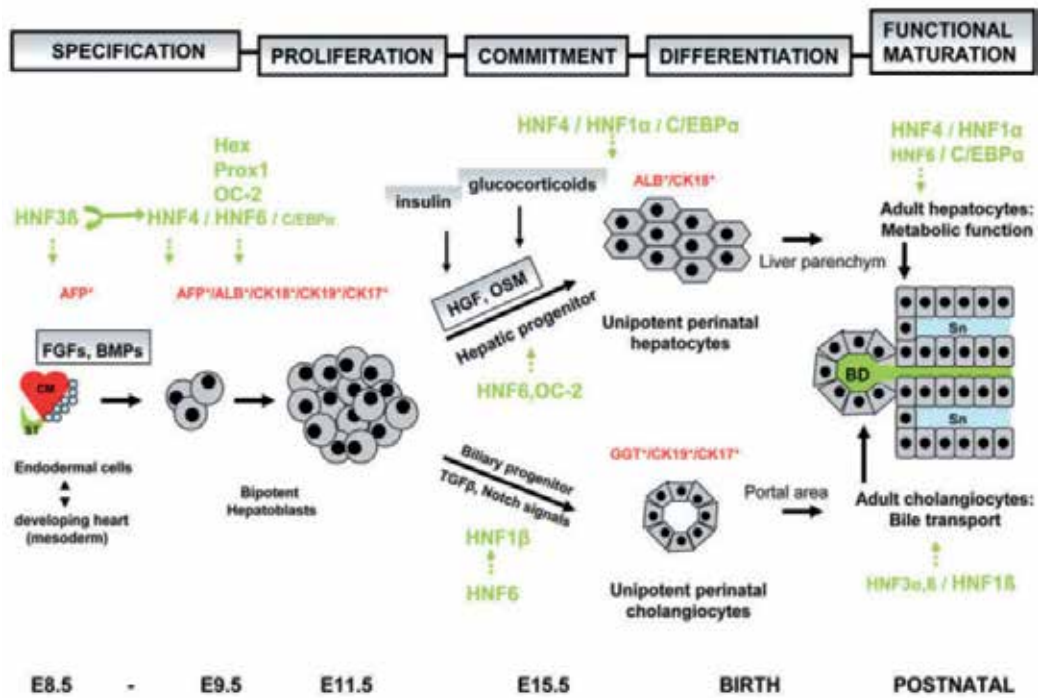


Fig. 3. **Schematic presentation of fetal liver development.** The establishment of a fully functional liver architecture is not accomplished before postnatal stages and follows upon a sequential array of tightly regulated intra- and extracellular signaling pathways, including liver-enriched transcription factors (LETFs) and growth factors, cytokines, glucocorticoids and hormones, respectively. To distinguish the level of expression and/or regulating role among diverse LETFs, different letter sizes are used. Abbreviations: ALB, albumin; AFP, alpha-fetoprotein, BMP, bone morphogenic proteins; C/EBP, CCAAT enhancer binding protein; CK, cytokeratin; CM, cardiogenic mesoderm; E, embryonic day in rodent liver development; FGF, fibroblast growth factors; GGT, c-glutamyltransferase; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; OC-2, Onecut transcription factor; ST, septum transversum; TGF, transforming growth factor. (Figure taken from[31], figure 2)

In the third phase, the liver primordium is induced to invade the septum transversum, giving rise to fetal hepatocytes (primordial hepatoblasts) which are bi-potent and may

proliferate of later differentiate into hepatocytes and cholangiocytes (biliary cells). Markers of hepatoblasts inherent in this phase are GATA4, HNF4alpha, HNF6, hepatic alpha-fetoprotein [AFP], albumin [ALB], and biliary cytokeratin (CK 17/CK19) [29, 30, 32].
Hematopoietic stem cells (HSCs) colonize the liver bud, thereby emitting a growth signal for the liver. Hematopoietic tissue and hepatoplasts subsequently release additional growth factors to further develop both tissues. Hepatoblasts continue to proliferate and start expressing placental alkaline phosphatase, intermediate filament proteins (cytokeratins CK14, CK8, and CK18), c-glutamyltransferase, and later also alpha1-antitrypsin, glutathione S-transferase P, C/EBPα, lactate dehydrogenase, and muscle pyruvate kinase [29-32].
At this stage three cell populations exist: (a) hepatocyte-committed cells that exclusively express hepatocyte markers, such as AFP and ALB, (b) cholangiocyte-committed progenitor cells, expressing biliary cell markers such as CK19, and (c) a bipotential hepatoblast population, expressing both hepatic and biliary markers. Bipotential hepatoblasts then proceed through a series of maturation steps which entail proliferation, cellular growth, and functional maturation. These final in vivo steps are induced by various extracellular signals such as: (1) dexamethasone, which induces albumin production and downregulates alpha-fetal protein production; (2) transforming growth factor beta, which inhibits hepatocyte proliferation and increases albumin production; (3) oncostatin M, mostly produced by HSCs, which induces tight cell-cell contacts, necessary for maximum differentiated hepatocyte function, maintenance of albumin production, and upregulation of other various hepatocyte functions; (4) HGF, excreted by mesenchymal cells or nonparenchymal liver cells, antagonizes the latter process, resulting in support of growth and differentiation of the fetal hepatocytes. The hormone insulin synergistically promotes this effect [27, 29, 31].
In parallel, the percentage of bipotent cells is markedly reduced. At this point, although cells continue to proliferate, most of them are unipotent and irreversibly committed to either the hepatic or cholangic lineage [29-32]. Complete functional hepatic maturation ultimately takes place after birth upon coassistance of HGF, produced by the surrounding nonparenchymal liver cells (sinusoidal, stellate, and endothelial cells) [27, 31]. About half of the active genes tested in the liver are bound by the transcription factor HNF4 alpha and it is suggested that it directly regulates hepatic genes and is an important transcription factor during the differentiation process of ES and iPS cells into mature hepatocytes [34, 35].
It is important to mention that mouse iPS cells exhibit full potential for fetal liver development in embryos derived solely from mouse iPS cells by tetraploid complementation compared to wild-type embryos as indicated by similar levels of hepatocyte marker mRNA and liver specific cell type protein expression levels. In addition, liver Hematoxylin and Eosin (H&E) stained sections presented a similarity in liver morphology between the two [36].

2.1.2 Induction of ES and iPS cell differentiation into hepatocyte-like cells in vitro

The resultant paradigms, taking these cues into account, can thus be broadly grouped in terms of temporal regulation through cytokine addition or spatial regulation using various extracellular matrix configurations. Various configurations for generating hepatocyte-like cells in vitro involve culturing human ES and iPS cells on extra cellular matrix and inducing differentiation with hepatocyte development stage specific factors in order to maximize the yield of cells at each stage which will determine the overall differentiation yield of the process.

As mentioned above, the first step involves the differentiation of pluripotent human ES into endodermal cells and most differentiation paradigms utilize Activin A which mimics Nodal signaling. At the end of this stage, most of the cells lost the expression of the pluripotent marker Oct4 while concomitantly gaining strong expression of definitive endoderm (DE) transcription factors Sox17, FoxA2 as well as GATA4 [36-38]. The later steps of differentiation induce endodermal like cells to differentiate into mature and fully functional hepatocytes while utilizing different ECMs and various factors for about 15 additional days [36-38].

The human ES cells exhibited a similar differentiation efficiency as human iPS cells while using the same differentiation procedure (about 81% albumin positive cells) as well as similar hepatocyte mRNA fingerprints [36] . In addition, cell properties of primary human hepatocytes such as albumin and urea secretion, as well as cytochrome CYP450 activity were similar in vitro between hepatocyte-like cells generated from human ES as well as those generated from human iPS cells, although lower compared to primary isolated hepatocytes (10 fold difference for urea and albumin secretion and 30 fold difference for CYP450 isozyme activities after phenobarbital induction) [38]. While comparing the expression of a series of genes encoding phase I and phase II hepatic enzymes between cadaveric liver samples and hepatocyte-like cells, it was indicated that the level of mRNA was similar between the ES and iPS derived hepatocyte-like cells while lower compared with adult liver samples in most cases [36]. This may indicate that differentiated cells are not mature enough and this step of the differentiation procedure demands further optimization [36, 38].

Human ES and iPS cell derived hepatocytes were shown to contribute to the liver parenchyma in vivo and were stained positive for albumin 7 days after their injection to the right lateral liver lobe of newborn mice, which exemplifies the potential of human iPS cells in regenerative medicine therapeutics [36].

2.2 Differentiation paradigms utilizing tissue engineering

A key requirement for effective tissue engineering is the cellular environment that allows the cells to maintain the functional capacity observed in the in vivo environment. Often the environment mimics some critical aspects of the in vivo setting through proper control of the materials and mechanical properties as well as the chemical milieu.

A consideration with tissue-engineered constructs is the presence of exogenous chemical and mechanical stimuli such as soluble growth and differentiation factors as well as mechanical forces (e.g., cyclic mechanical loading, fluid shear).

2.2.1 Matrix configuration

Culturing cells in various controlled three-dimensional (3D) environments has proven to be a successful differentiation technique. This is thought to be a result of mimicking the *in vivo* histoarchitecture of liver, incorporating cell-matrix interactions as well as soluble cues. Several ECMs are present in liver development. One study compared the effect of using type I collagen, fibronectin, laminin, and Matrigel™ on directing hepatic differentiation. Cells were cultured for 7 days in embyoid bodies (EBs) before being transferred to one of the substrates. This mimics to some extent the liver development stage where endodermal cells migrate into the ECM rich septum transversum. Type I collagen was shown to cause the greatest increase in liver specific genes, with Matrigel™ also showing a beneficial effect[39].

Therefore, one research group utilized a collagen scaffold as a 3D network for hepatic differentiation. Mouse ES cells were cultured in an EB configuration and implanted into a collagen scaffold and cultured in media containing aFGF, HGF, oncostatin M, dexamethasone, and insulin transferrin sodium selenite (ITS). Cells cultured in the 3D configuration showed gene expression of AFP, ALB, glucose 6-phosphate (G6P) and tyrosine aminotransferase (TAT) after 6 days in cultures as opposed to 12 days in an EB configuration alone. However, these cells stained positive for albumin protein, but not for the CK-18 protein. Following implantation into the median lobe of the liver of a nude mouse for 14 days, the cells stained positive for both CK-18 and albumin proteins. This result signifies that cells were not completely matured by the in vitro protocol [40]. While this study showed the advantages of 3D culture, most studies fail to demonstrate a genetic or functional advantage relative to standard monolayer differentiation protocols. Other groups have used 3D cultures to provide a configuration suitable for direct implantation, eliminating the need to remove cells from culture by using biodegradable and/or biocompatible materials. For example, one group used a polyurethane foam spheroid culture to direct differentiation of mouse ES cells. Embryoid bodies were inoculated into a block of polyurethane foam (PUF) to induce the formation of spheroids in the pores of the scaffold. Induction of hepatic differentiation was accomplished by supplementing the media with aFGF, HGF, oncostatin D, dexamethasone, and ITS. Analysis of gene expression showed the expression of endoderm markers transthyretin (TTR) and AFP as well as hepatocyte specific albumin, arginase, and tryptophan 2,3-dioxygenase (TDO) expression. Notably, ES cell derived hepatocyte like cells demonstrated ammonia clearance and albumin secretion rates within the range, albeit on the low end, of those seen for primary rat hepatocytes seeded in the PUF scaffold. However, this method of differentiation is lengthy and immature endoderm markers were still present at the end of the 30 day protocol[41]. Biodegradable polymer scaffolds have also shown to be an effective three-dimensional environment for hepatic differentiation. One study allowed ES cells to form EBs for 5 days before being mixed them with Matrigel™. The cell suspension was then seeded into a rigid polymer network comprised of poly-L-lactic acid (PLLA) and polyglycolic acid (PGA). The cells formed spheroids along the polymer fibers and were cultured for 20 days with dexamethasone, dimethyl sulfoxide (DMSO), FGF4, HGF, oncostatin M, and ITS. After the culture period, the cells showed expression of AFP, ALB, G6P, TTR, and CK-18 and the ability to secrete albumin, uptake low-density lipoprotein (LDL) and store glycogen [42].

Encapsulation of ES cells in alginate has been explored as a method for the control of hepatic differentiation. The capsules allow the diffusion of nutrient, oxygen, and growth factors into the capsules while sequestering the cells. This technique has been widely used in the past to induce stem cell differentiation and maintain hepatocyte function, making it an ideal candidate for controlled hepatic differentiation. For instance, one group encapsulated 5-day-old embryoid bodies derived from mouse ES cells in a 2% alginate solution. The media was supplemented with aFGF, HGF, oncostatin M, dexamethasone, and ITS, similar to those factors previously used by other research groups. RT-PCR analysis determined that this methods results in the expression of endoderm (AFP) and hepato-specific (ALB, Cyp7A1, TAT, TTR, and CK18) genes. The cells produced albumin and urea, but only with growth factor supplementation. If one takes a closer look at the temporal addition of growth factors in these systems, several similarities exist. FGF is usually added at early time points in order to promote differentiation of definitive endodermal cells into hepatic endoderm cells. This is almost always followed by HGF supplementation, mimicking signaling from the

mesenchyme which promotes growth and differentiation of fetal hepatocytes. Lastly, oncostatin M, dexamethasone, and ITS are added, as these factors are known to promote hepatocyte maturation. Sequential supplementation therefore mimics in vivo development. In addition, ES cells are allowed to form embryoid bodies prior to being cultured in a 3D environment. This allows the formation cell-cell interactions known to increase hepatocyte function during development. Although these aforementioned methods yield hepatocyte-like cells from a functional perspective, growth factors are expensive and therefore these approaches are not amendable to scale up for clinical application.

A different approach is the use of cellular encapsulation to control lineage commitment and final maturation of murine ES cells. The novelty of this method lies in the fact that no growth factor supplementation is required to direct hepatic differentiation. This was accomplished through the manipulation cell seeding density and alginate concentration. These two variables dictate the size of cellular aggregates that form within the capsule which in turn direct differentiation. It was found that a 2.0% w/v alginate concentration and a $5x10^6$ cells/mL seeding density were the optimal parameters for hepatic differentiation [43, 44]. Genetic analysis showed the expression of a variety of Cyp450s as well as CK-18. In addition, albumin and urea secretion as well as glycogen storage were shown and reached a maximum around day 20 in culture. This method demonstrates a way to obtain a high yield of hepatocyte-like cells through inducing cell-cell interactions known to upregulate hepatocyte function during development, thus eliminating the need for growth factor supplementation. In addition, the cells can be recovered from the capsule through depolymerization without effecting cell viability. Scalability is also significant, as generating large numbers of cells would simply involve producing large batches of capsules. With all these advantages taken into consideration, this technique is amenable to the mass production of hepatocyte like cells and thus has the potential for clinical utility.

2.2.2 Coculture

A co-culture of ES cells with another cell type present during hepatic development or in the adult liver cell has been shown to induce hepatic differentiation. The supporting cell type directs the ES cells towards the hepatic lineage by introducing cues resulting from soluble factors, cell-cell interactions, or a combination of the two. Thus, this approach is performed by separating the cells with a porous membrane or with the cells in direct contact with one another. Both methods have been shown to induce hepatic differentiation of embryonic stem cells with careful choice of feeder layers. In fact, investigators often develop their own cell lines optimized to drive hepatic differentiation. For example, one group first developed a protocol to differentiate endoderm cells by culturing the cells on Matrigel™ and supplementing the media with activin A and HGF [45]. The endoderm cells were separated by fluorescence-activated cell sorting (FACS), made possible by the transfection of enhanced GFP (EGFP) under the control of an AFP promoter. The purified cells were then further exposed to a co-culture with MLSgt20 cells, a cell line cloned from fetal murine stromal cells experimentally shown to promote hepatic differentiation in ES cells [46]. Combining the results of the two studies, the AFP positive endoderm cells were contact co-cultured with the MLSgt20 cells. The co-cultured cells expressed markers for both immature (GATA4, AFP) and mature (Alb, TAT, TO, CYP3a4/7) hepatocytes at the end of the culture period. The cells also showed the ability to clear ammonia and store glycogen. However, gene expression was not identical to that of adult human liver hepatocytes [47]. A drawback of contact co-culture is that the hepatocyte like cells must be separated from the supporting cell

type after the culture period. To eliminate the need for purification of differentiated cells, one group co-cultured three human liver non-parenchymal cell lines with ES cells using a transwell membrane. Mouse ES cells containing a GFP gene with and albumin promoter were cultured in suspension for 2 days to facilitate embryoid body formation. The EBs were then cultured on a poly-amino-urethane (PAU) coated non-woven polytetrafluoroethylene (PTFE) fabric that allowed the cells to adhere. The media was supplemented with basic FGF and activin A for 3 days. The ES cells were then co-cultured in Matrigel layered transwells with the human cells lines growth-arrested with mitomycin C. Cholangiocytes, liver endothelial, and hepatic stellate cell lines were chosen due to their secretion of soluble factors that have shown to be important for liver regeneration. Cholangiocytes generate IL-6 and TNF-α, liver endothelial cells produce FGF4 and vascular endothelial growth factor (VEGF) and hepatic stellate cells produce HGF. The cells were co-cultured with media supplementation of dHGF and DMSO for 8 days and dexamethasone for the final 3 days of culture. The differentiated GFP positive cells were separated by cell sorting and showed a yield of 70%. These cells expressed both hepatocyte markers as well as endoderm markers, demonstrating that the cells were not fully mature. They also stained positive for albumin and GFP, secreted albumin, and metabolized ammonia, lidocaine, and diazepam. However, albumin secretion and metabolic activity occurred at lower levels than primary mouse hepatocytes[48]. Another example of a non-contact co-culture was demonstrated using cynomolgus monkey ES cells with mouse fetal liver-derived cells to simulate the environment of the developing liver. The ES cells lost pluripotent markers and expressed AFP, ALB, CYP7A1, and HNF4α, an important transcription factor for mature hepatocytes. The cells also stained positively for AFP, albumin, alpha1AT, and HNF4α, as well as for Hep Par 1, an anti-hepatocyte antibody. Functional analysis also showed glycogen storage through Periodic acid-Schiff (PAS) staining as well as ammonia clearance[49].

While the aforementioned co-culture systems utilize non-parenchymal cells or fetal liver cells as a support cell type, the use of hepatocytes as the feeder cell type in co-culture differentiation schemes has also been attempted. Moore et al. examined the effects of co-cultivated hepatocytes on the hepatospecific differentiation of murine ES cells[50]. Hepatocytes co-cultured with cadherin-expressing ES cells markedly enhanced ES cell differentiation toward the hepatic lineage, as demonstrated by hepatic-like cuboidal morphology, heightened gene expression of the late maturation marker G6P in relation to the early marker AFP, and the intracellular localization of albumin. The effect was mediated by cadherin, since it was reversed through E-cadherin blockage and inhibited in control ES cells with reduced cadherin expression. Direct contact co-cultures of hepatocytes and ES cells maximally promoted ES cell commitment towards hepato-differentiation. This study showed that both soluble signaling and cell-cell interaction creates a synergistic effect that drives hepatic differentiation.

Cho et al. developed another co-culture method with hepatocytes[51]. A collagen gel was formed on tissue culture dishes and primary rat hepatocytes were plated after gelation. A thick collagen layer was then deposited on top of the hepatocytes. We have previously shown that this collagen sandwich hepatocyte culture maintains hepatocyte function in vitro. Murine ES cells were then seeded on the thick collagen layer and cultured in this configuration for 10 days. At this stage, the ES cells were removed from the collagen layer by dispase treatment while leaving the collagen sandwiched hepatocytes intact. RT-PCR demonstrated the presence of endoderm markers Foxa2, Sox17, and AFP and the absence of

mesoderm and ectoderm markers. The presence of Foxa2 and AFP were confirmed by immunostaining, and flow cytometry showed that they were expressed in 95% of cells. In addition, the cells proliferated and stopped expressing Oct4, a marker for pluripotency. These results showed that mouse ES cells cultured on top of collagen-sandwiched hepatocytes differentiated and proliferated into a uniform and homogeneous cell population of endoderm-like cells. However, the endoderm cells did not express albumin, signifying that they had not yet committed to the hepatic lineage. To further mature the cells, they were co-cultured for 20 days with fibroblasts due to the in vivo interactions of the endoderm with the mesenchyme during development. The media was supplemented with oncostatin M, ITS, and dexamethasone. The resulting hepatocyte like cells expressed hepatospecific genes albumin, alpha-1-antitrypsin (AAT), CK-8, CK-18, TTR, and CYP2A13 and displayed morphology similar to that of primary rat hepatocytes. Immunostaining demonstrated the presence of albumin and CK-18 while functional analysis showed the cells could synthesize urea. This study showed the generation of a homogeneous population of hepatocyte-like cells from ES cells[51]. However, the drawback of this method is that two separate co-cultures are required to direct differentiation, with one feeder cell being the scarce cell type that we are trying to generate.

2.2.3 Metabolic engineering

A distinguishing feature of adult hepatocytes is the high content of mitochondria and high level of oxidative metabolism. On the contrary, ES cells contain a much smaller amount of mitochondria and produce energy mainly through the glycolytic pathway. Recent studies show that metabolic additives that promote carbon backbone oxidation can be used to help direct differentiation towards the hepatocyte lineage. One such method utilizes sodium butyrate treatment to generate an enriched population of hepatocyte-like cells from embryonic stem cell [52]. ES cells were plated on gelatin and the media was supplemented with DMSO for the first 5 days of culture. Sodium butyrate replaced DMSO for the next 6 days, and cells were replated onto collagen coated or non-coated polystyrene at various time points in order to perform metabolic analysis. Significantly higher levels of urea secretion and albumin positive cells were observed in a 2.5 mM sodium butyrate condition on both substrates. It was also shown that mitochondrial mass increased from days 5-8 in culture, which is characteristic of hepatic differentiation. However, the percentage of albumin positive and high mitochondrial activity cells was still less than mouse hepatoma cells. These results imply that these cells represent an immature hepatocyte phenotype. A subsequent study was conducted in order to further differentiate the cells into mature hepatocyte-like cells [53]. The immature cells were treated with S-NitrosoAcetylPenicillamine (SNAP), a nitric oxide donor. Nitric oxide is known to induce the synthesis of mitochondria, thus possibly facilitating a further increase of mitochondrial mass to levels seen in mature liver cells. After the 11 days of culture conditions that were found to induce partial differentiation using sodium butyrate, cells were replated on day 12 and supplemented with various dilutions of SNAP in DMSO for the next 3 days. A 500 μM concentration of SNAP significantly increased glucose consumption, lactate production, and the percentage of albumin positive cells. From a functional perspective, SNAP treatment also increased urea and albumin secretion as well as cytochrome P4507a1 activity relative to the other culture conditions. These studies demonstrate that simply altering the ES cell's metabolic activity to more closely resemble those of mature hepatocytes is an alternative

strategy to developing hepatocyte-like cells. This novel method circumvents the need for media supplementation with expensive cocktails of growth factors or co-culture with another cell type.

3. Utilizing hepatocyte-like cells

Techniques aimed at obtaining a homogeneous population of hepatocyte-like cells with functional characteristics similar to native hepatocytes from ES cells are rapidly improving. As these methods progress, applications taking advantage of a constant supply of hepatocytes are being developed in parallel. One application is the use of hepatocytes for the treatment of liver failure. With the gap between those on the waiting list for a liver transplant and the organs available for transplantation growing every year, a renewable source of hepatocyte-like cells could potentially alleviate this problem. Bioartificial livers containing functional hepatocyte-like cells could be used to keep patients alive while waiting for a transplant. In addition, decellularizing livers unsuitable for transplant and reseeding them with functional cells could increase the donor pool. In conjunction, these two cutting-edge methods could reduce the number of patients who die on the waiting list. Finally, the important role the liver plays in the metabolic clearance of drugs makes hepatocytes an important research tool for the pharmaceutical industry. By including microfluidic technology, the number heaptocytes needed per assay can be greatly reduced, facilitating high-throughput screening of new chemical entities. This next section will discuss the current state of these applications of hepatocyte-like cells derived from ES cells.

3.1 Decellularized liver scaffolds for implantation

Using hepatocyte-like cells for transplantation is a potentially very exciting application. As mentioned earlier, a large gap exists between the number of patients waiting for a liver transplant and the number of available liver grafts. With this gap growing each year, an alternative source of transplantable livers is greatly needed. Although the differentiation of hepatocyte-like cells from embryonic stem cells has been accomplished through a variety of methods, creating a three-dimensional organ structure is challenging due to the nutrient and oxygen limitations that occur in the engineered tissue. The liver consists of a complex network of extracellular matrix proteins as well as microvasculature critical to organ function. One approach to provide the complex 3D environment of the liver is not to try to recreate it from scratch, but rather to use natural decellularized liver scaffolds. Data show that the native 3D matrix can promote cell engraftment, survival, and sustained hepatic function over time. In addition, by preserving the native microvasculature structure, the risk of ischemic damage or lack of perfusion is minimized. This approach has the potential of utilizing livers unsuitable for transplantation as a source of scaffolds to seed hepatocyte-like cells derived from ES cells, thereby increasing the number of livers available for transplantation.

Using the liver as a scaffold requires that the organ be decellularized while leaving the 3D architecture intact. Following decellularization, the scaffold must be reseeded with the proper cell types engrafting to their appropriate locations within the tissue to promote function. This has been accomplished by perfusing sodium dodecyl sulfate (SDS) solutions through the portal vein of a rat liver for 72 hours [54]. The result was a translucent structure free of cells which preserved the 3D architecture of the liver, Figure 4a. Most notably, the

microvascular tree was preserved, as shown in Figure 4b. Using the scaffold, cells were seeded by injecting 12.5 million cells in 4 doses at 10-minute intervals. This approach resulted in a grafting efficiency greater than 90%, and perfusion of the recellularized scaffold showed 80% viability over 2 days. Since a liver consists of non-parenchymal cells as well as hepatocytes, epithelial cells were also seeded and engrafted in the lining of the vessel. Moreover, epithelial cell seeding did not affect hepatocyte viability, providing proof of concept that other cell types could also be successfully seeded. The scalability of the system was also tested, injecting a total of 200 million cells using the 4-dose approach. This cell total represents 20% of the adult rat liver mass, double the amount required to provide therapeutic benefit.

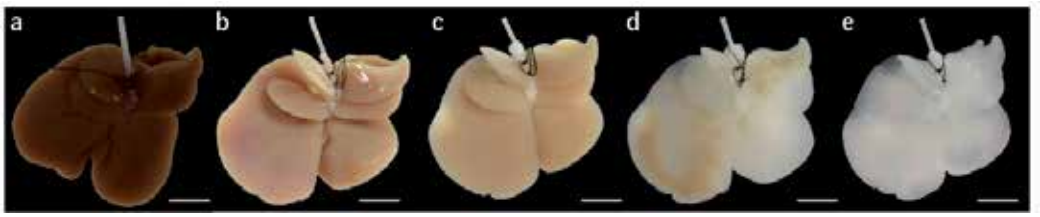


Fig. 4a. Rat liver during the decellularization process. SDS was perfused through the portal vein, leaving a translucent, cell free structure which maintained native liver architecture. From left to right, the images show the liver at 0, 18, 48, 52, and 72 hours of the SDS perfusion, respectively. (Figure taken from [54], Figure 1)

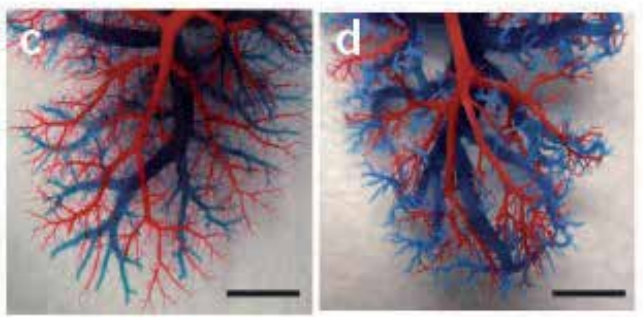


Fig. 4b. Portal (red) and venous (blue) corrosion cast of normal (left) and decellularized liver (right). Comparison of the two shows that the decellularization process preserves the native liver microvasculature. (Figure taken from [54], Figure 2)

Analysis of the reseeded hepatocytes showed gene expression similar to that of long-term stable cultures (e.g. collagen sandwich) of hepatocytes. Immunohistochemical analysis of UGT1a, G6pc, and albumin showed staining at levels comparable to adult livers. Functional analysis showed urea synthesis significantly higher that hepatocyte sandwich and similar albumin secretion rates. However, albumin production was much lower than that of adult rat livers. The recellularized liver was then transplanted into a rat for 8 hours to investigate the effect of shear stress due to blood flow. Transplantation did not affect hepatic function, viability, or morphology in any significant way. Other investigators successfully decellularized a liver and seeded the matrix with both hepatocytes and other epithelial cells

[55]. These studies demonstrate the feasibility of using a decellularized liver matrix as a scaffold for hepatocytes. The inclusion of other non-parenchymal cells and evaluation of the transplant after long periods in vivo still needs to be performed prior to bringing this approach to the bedside. This may further require the development of techniques to derive hepatic nonparenchymal cells from ES cells.

3.2 Bioartificial liver devices

The shortage of donor livers has also prompted researchers to develop bioartificial liver (BAL) devices, **Figure 5**. These devices aim to provide temporary assistance to patients with liver failure while waiting for a donor organ. Early BAL concepts were modified dialysis systems which did not incorporate living cells, [56] and were found to be limited in efficacy. Since the liver provides a host of biochemical processing and detoxification functions that are essential to life, it was thought that an effective device should contain liver parenchymal cells (e.g. hepatocytes). Cells were added to the dialysis systems, and again poor results were obtained, in this case because the bioreactor design itself did not allow for sufficient metabolite transport (especially oxygen), and as a result the cells did not function properly or even survive. Next, a support structure was built that allows for the convective and diffusive transport of plasma metabolites to and from the cells in the device [57]. Many creative operational strategies and designs exist [58]; [59]; [60], ranging from packed bed bioreactors [61, 62], to flat plate bioreactors [63]. It was found that hepatocytes can only

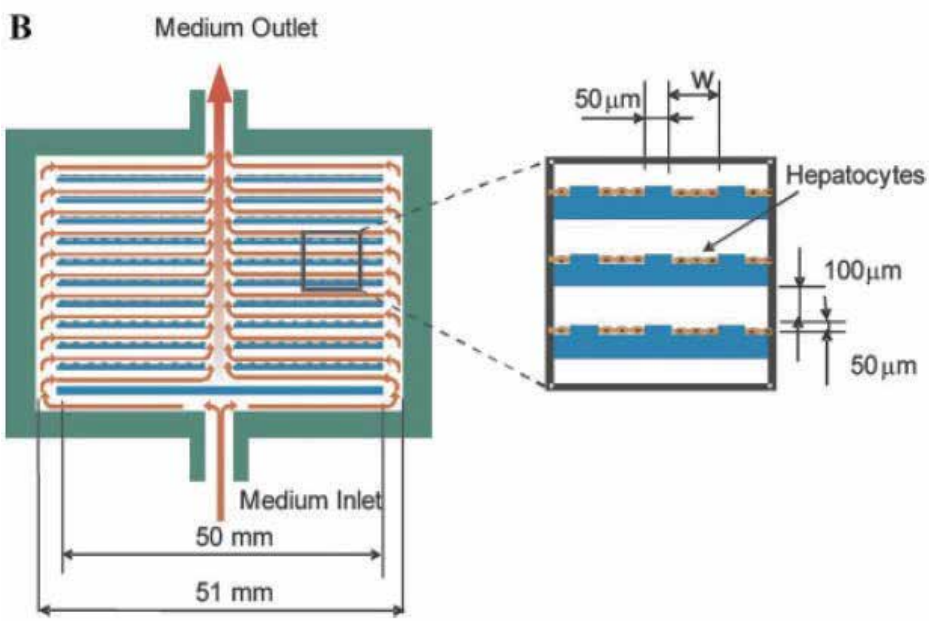


Fig. 5. **Schematic of a radial flow Bioartifical Liver Device.** Red arrows show the direction of medium flow and in blue is the location of the seeded hepatocytes. The design includes patterned microgrooves to decrease shear stress on the seeded hepatocytes. (Figure taken from [67], Figure 1)

withstand low levels of fluid shear stress, therefore in a recent modification of the flat plate geometry, cells were seeded at the bottom of grooves to allow for higher flow rates, thus increasing mass transport within the device, while keeping shear stress at the cell surface to a low level [64, 65].

The high metabolic activity of hepatocytes makes the transport of oxygen a significant technical concern in BAL devices. In fact, transport limitations have led to the loss of hepatic function over time and the failure of many BAL devices based on hollow fiber technology that have undergone clinical trials. To alleviate this issue, semi-permeable membranes have been introduced into devices to increase the oxygen tension within the device. However, a device would require a surface area of approximately 10 m^2 to provide therapeutic benefit [66]. Therefore, the incorporation of an internal membrane oxygenator would create scale up issues. To address these problems, a radial stacked plate bioreactor was developed to increase the surface area to volume ratio of the device. The design was devoid of a semi-permeable membrane and therefore required perfusion at high volumetric flow rates in order to create the necessary oxygen tension to maintain hepatocyte function. Nevertheless, hepatocytes will also lose function above a critical shear stress. Therefore, photolithographically patterned microgrooves were incorporated to shield the cells from the resulting high shear rates required for sufficient convective oxygen transport.

The requirement of high-flow rates and low shear stress is a non-trivial engineering problem. In order to examine the effect of microgrooves, computation fluid dynamics (CFD) was employed to characterize the device. CFD analysis showed that varying width of the microgrooves with the distance from the center of the bioreactor results shear stresses well below detrimental levels throughout the device. Incorporating an oxygenator within the perfusion circuit, design and operating parameters were optimized that provided sufficient convective oxygen delivery and acceptable shear stresses. These critical parameters resulted in 95% viability of hepatocytes under perfusion after 36 hours within the device. In addition, urea and albumin secretion levels were significantly higher than a flat plate bioreactor and comparable to hepatocytes co-cultures with fibroblasts. The device produced 2,100 µg of albumin per day, as compared to $19x10^5$ µg/day required for a therapeutic effect. To be clinical useful, this device would need to be scaled by a factor of 905, which would allow the device to be operated without exceeding the recommended clinical priming volume[67].

As opposed to *ex vivo* bioartificial liver devices, an implantable liver assist device (LAD) has been developed by engineering a hepatic organoid. The implantable construct comprised of a PAU-coated PTFE non-woven fabric covered with a polyethylene-vinyl alcohol membrane layered with a polyester supporting fabric. Based on the method described in Section 2.2.2, human cholangiocytes, liver endothelial cells, and hepatic stellate cells were injected into an LAD coated with FGF-2 to facilitate angiogenesis. The LAD performance was evaluated through implantation into mice after a 50% hepatectomy for 7 days. While LAD with only hepatocytes did not survive, devices with non-parenchymal cells exhibited the formation of organotypic structures in the LAD similar to the liver acinus. Additionally, in vitro analysis showed that the organoid significantly improved ammonia and lidociane clearance as well as albumin secretion relative to device with seeded with hepatocytes alone. This study showed that the inclusion of non-parenchymal cells in liver assist devices has the potential of improving *in vivo* performance.

3.3 In vitro drug screening systems

According to the Pharmaceutical Research and Manufacturers of America (PhRMA) U.S. drug companies spent $62.4 billion on research and development in 2009. Studies indicate that it can cost more than $800 million, of which 80% is spent on clinical trials and development, and will take between 8 and 10 years of development to bring a new drug to market. Among candidate drugs that make it past Phase I clinical trials, 50% fail due to human toxicity and bioavailability issues. Moreover, of all candidate drugs, 90% do not make it through final stages of development. This tremendous attrition rate has not improved in recent years. To curb such costly failures, a significant amount of research has been dedicated to identifying in vitro screening systems; i.e., approaches that can be utilized in preclinical phases of discovery and development that offer greater utility in predicting in vivo subcellular and cellular physiological responses.

Currently, a majority of hepatic in vitro screening assays employed within the field of drug metabolism and pharmacokinetics (DPMK) utilize hepatocytes cultured under fully static conditions. In such assays, hepatocytes are either adhered to the bottom of a microtiter well-plate to which culture medium containing candidate drug(s) is subsequently added; or else the hepatocytes are maintained inside the microtiter well in suspension in the media. The microtiter plate is shaken to facilitate mixing and transport, but there is no means for providing a continuous flow of culture media over the cells.

The lack of continuous flow produces functional limitations in these conventional static systems when compared to microfluidic systems. The presence of flow helps regulate the concentrations of both metabolites and cellular by-products in the immediate vicinity of the hepatocytes; whereas in a static system these concentrations are ever-changing until a saturation/depletion condition is attained through accumulation, uptake, and reaction. In a flow system it is possible to maintain a pseudo steady state (equilibrium) which results in optimal working characteristics. Furthermore, in a static well the system is usually mass transport limited; flow can remedy this by the addition of convective mode of mass transport.

A variety of devices have been developed for this purpose [68-70], and in particular to determine parameters for multi-compartmental physiologically-based pharmacokinetic (PBPK) models, Figure 6. For example, a multi-tissue compartmental device has been developed that incorporates a large liver compartment for the assessment of drug absorption both in the liver as well as in other metabolizing tissue types [71-73]. More specifically, the Hμrel® Corporation has developed a liver-specific microfluidic chip that focuses on liver metabolism, with the possible application to liver toxicity assessment, Figure 7. The Hμrel® device allows for the seeding of various cell types to emulate the *in vivo* components affecting pharmacokinetics. The device consists of separate chambers, each seeded with a specific cell type. Device testing demonstrated that the flow co-culture format in the device resulted in better predictions of *in vivo* clearance rates compared to static cultures and flow-based monoculture models, across a wide range of cytochrome P450s. The reason for increased hepatocyte function when flow is incorporated is not yet known for certain. One possibility is that increased mass transport in the system leads to a thinner boundary layer in addition to faster removal of unwanted by-products, together resulting in an observed increased clearance. Regardless of the mechanism, the Hμrel® device yields an in vitro analogue to PBPK models utilizing minimal amounts of cells and drug, thus facilitating high-throughput screening of new chemical entities.

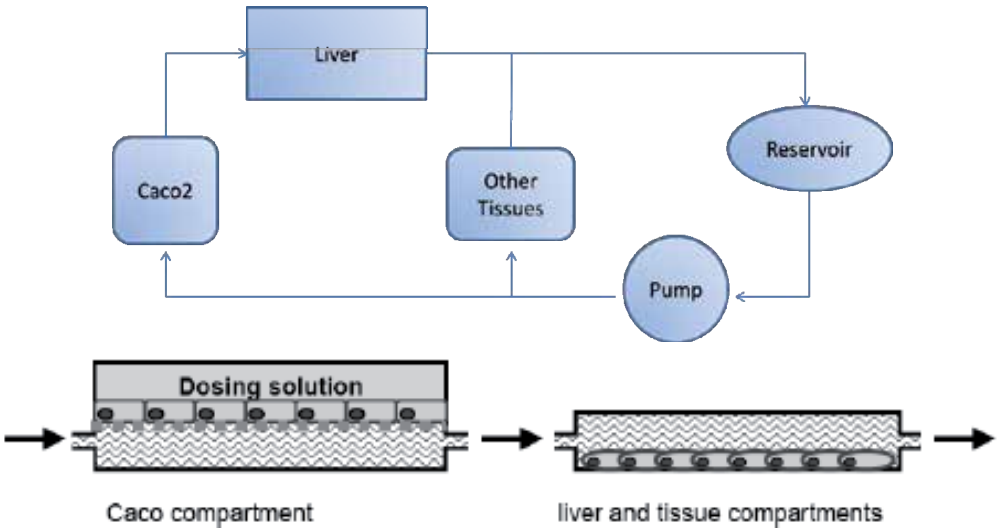


Fig. 6. **Example of multi-compartmental MEMs model utilized to determine PBPK values for use in in silico simulations.** The new chemical entity is dosed on one side of the Caco-2 cells, where it must be absorbed prior to reaching the microchannels. The absorbed drug then passes into the liver for metabolic clearance prior to being re-circulated and reaching the target tissue.

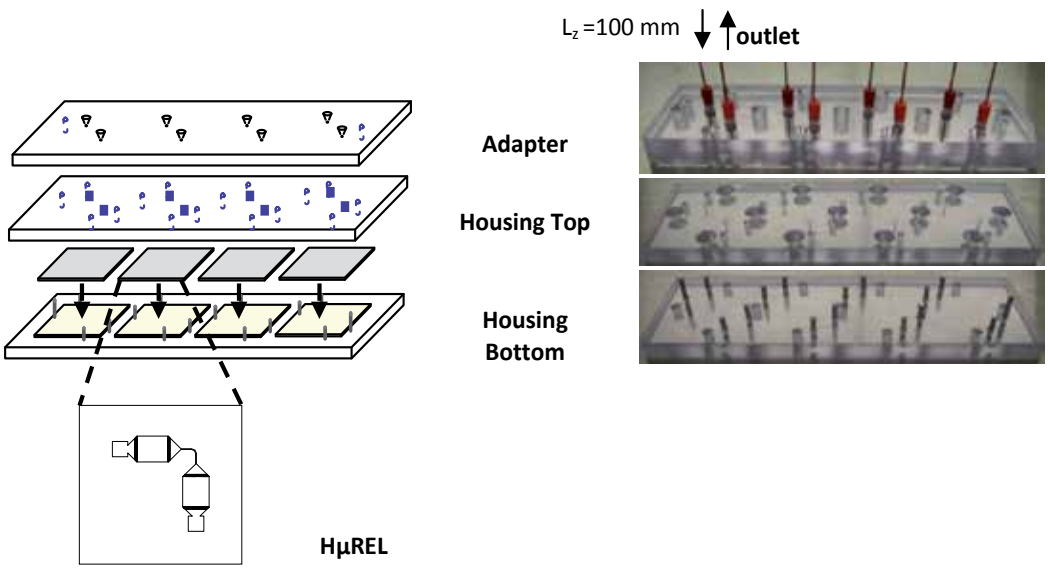


Fig. 7. **Geometry of the static and flow configurations in the HμREL® system.** The assembly of a HμREL® housing set with four biochips. The components of the chip housing and interface are, from the top – adapter, housing top, HμREL® biochips, housing bottom. The complete setup of the HμREL® prototype instrument then involves connecting inlet and outlet tubing to the adapter. The inlet tubing is then fed through a peristaltic pump, and connected to a reservoir that contains the media with the test compound of interest. The outlet tubing is also inserted into the reservoir to complete the recirculation loop. (Figure taken from [75], Figure 1)

4. Future directions

Considerable progress has been made in differentiating ESCs into liver cells; however, current protocols have not yet produced cells that express a completely adult-like mature hepatocyte. In fact, criteria that define what is an acceptable functional human stem cell-derived hepatocyte will need to be established and standardized. ESC differentiation protocols typically do not yield a pure hepatocyte population, and often times sorting protocols are needed. Methods to scale-up such protocols to the therapeutic scale of a human patient will need to be developed. There remains safety concerns (e.g. tumoregenicity) when using ESCs and iPSC for cell transplantation which cannot easily investigated in rodent models and will require further analysis in more "human-like" systems, such as nonhuman primates. On a short-term basis, human hepatocytes derived from ESCs or iPSCs may be effectively used for toxicology studies on xenobiotics as well as drug safety screening. The development of devices that contain such cells for high throughput testing is an important avenue for the future in this area, and special consideration should be taken to make such systems easy to use at the point of care or in the field. The ability to derive cells that are patient-specific provides a unique opportunity to better understand patient variability in their sensitivity to drugs as well as potentially develop individualized patient-specific drug regimens.

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16

Hepatic Differentiation of Human Embryonic and Induced Pluripotent Stem Cells for Regenerative Medicine

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1. Introduction

A hepatocyte is one of the most desired cells for regenerative medicine as well as for pharmaco-toxicological testing. Hepatocytes, the parenchymal cells of the liver, possess a wide range of functions, including protein synthesis/storage, detoxification, transformation of carbohydrates, synthesis of cholesterol, bile salts and phospholipids, as well as the excretion of exogenous and endogenous substances (Blouin et al 1977).

The various functions of the liver are essential for the maintenance of the human body and liver failure is often only treatable by organ liver transplantation (OLT). The shortage of donated organs, however, limits this treatment option available to patients. Since the Model for End-Stage Liver Disease and Pediatric End-Stage Liver Disease (MELD/PELD) liver allocation system was introduced in February 2002, the usage of borderline-quality donated livers for OLT has increased (Freeman et al 2002). As a result, the number of patients on waiting lists for OLT has stabilized; however, more than 15,000 patients await OLT annually in the United of States alone (Thuluvath et al 2010).

Hepatocyte transplantation (HT), a cell-replacement therapy for liver disease, could decrease the number of the patients on the waiting list. HT is particularly useful for metabolic liver diseases that do not require whole organ replacement but rather a partial restoration of specific liver metabolic functions (Fox et al 1998, Strom et al 1997). The feasibility and the therapeutic efficacy of HT have been demonstrated in several clinical trials (Strom et al 2006). However, the limited availability of human hepatocytes due to organ shortage prohibits the provision of this promising therapy to patients as a standard option. Human hepatocytes can be isolated from cadaveric livers that are donated but not suitable for OLT (Strom et al 1982). The number of unused donated livers has decreased since the MELD liver allocation system was initiated. This dilemma has led to an urgent demand for developing stem cell-derived functional human hepatocytes as an alternative. Stem cell-derived hepatocytes could also be utilized in artificial liver bio-devices for patients with acute liver failure who may not need the OLT (Gerlach et al 2010, Gerlach et al 2008, Soto-Gutierrez et al 2006a). An unlimited supply of stem cell-derived hepatocytes could facilitate the development of these cell-based therapies for treatment of life-threatening liver diseases.

Moreover, these stem cell-derived hepatocytes could be utilized not only for therapeutic applications but also for pharmaco-toxicological testing (Davila et al 2004). It is anticipated that stem cell-derived hepatocytes will be important as *in vitro* tools for testing drug safety, thereby significantly facilitating drug development. Toxicity assessment for drug development could be conducted with phenotypically and genotypically standardized human hepatocytes. In this regard, genetically homogeneous stem cell-derived hepatocytes would be superior to primary human hepatocytes. Human stem cell-derived hepatocytes would be particularly useful for conducting some infectious disease research as well. As a case in point, hepatocytes derived from animals have been demonstrated to not be useful for the study of human hepatitis virus biology such as HCV (Cai et al 2007).

A number of different stem/progenitor cells have been proposed as an alternative cell source for generation of functional hepatocytes – these include fetal hepatocytes (Suzuki et al 2000), adult hepatic progenitor/stem (epithelial) cells (Alison & Sarraf 1998), bone-marrow derived-stem cells (Gilchrist & Plevris 2010), and adipose-derived stem cells (Schaffler & Buchler 2007). Although the biological potential of fetal hepatocytes and adult hepatic progenitor cells is promising, ethical issues and limited availability hampers development for use in the clinic. Relatively small numbers of stem/progenitor cells would be insufficient for use to generate stem cell-derived hepatocytes in the clinic, particularly for HT, as HT recipients require transplantations of more than a billion cells at a time (Fox et al 1998). Some reports claimed that there was hepatic differentiation of mesenchymal stem cells from adipose tissue or bone marrow. However, the differentiation to functional hepatocytes, remains controversial (Goodell 2003, Wagers & Weissman 2004). Further research efforts are required to gain consensus for utilizing these adult stem cell-derived hepatocytes in the clinic.

To date, pluripotent stem cells are the most realistic candidate for the generation of stem cell-derived hepatocytes. Pluripotent stem cells possess nearly unlimited self-renewal capability *in vitro* and are able to differentiate into all three germ layers. Human embryonic stem cells (hESCs) are derived from blastocysts (Thomson et al 1998), and several protocols have been published for the differentiation of hESCs to functional hepatocytes. Additionally, recent breakthroughs in the field of induced pluripotent stem (iPS) cell technology (Takahashi & Yamanaka 2006) have provided yet another promising source to generate stem cell-derived hepatocytes. The human iPS cells may overcome the ethical concerns of utilizing blastocyst-derived pluripotent stem cell and may provide patient specific immunotype cells so as to avoid the lifelong use of immunosuppressants (Takahashi et al 2007). Disease-specific iPS cells, derived from patients who suffer from various congenital liver diseases, such as alpha1-antitrypsin deficiency, familial hypercholesterolemia, and glycogen storage disease, are anticipated to become important tools for studying the mechanisms underlying the pathogenesis of these diseases and investigations of new treatments (Ghodsizadeh et al 2010). Moreover, human iPS-derived hepatocytes would be an unlimited source for materials that are genetically homogeneous for conducting consistent pharmaco-toxicological evaluations. For example, an iPS-derived hepatocyte library could be established based upon single-nucleotide polymorphisms (SNPs) in drug metabolizing enzymes (eg. cytochrome P450s), and this could enable evaluation of drug metabolism with specific SNP-type hepatocytes. These data could be utilized to design personalized therapies for future "made-to-order" medicine (Medine et al 2010).

In this chapter, we describe the developmental progression from a pluripotent stem cell state to hepatocytes and provide an overview of protocols for hepatic differentiation of

pluripotent stem cells. Enrichment strategies of functional hepatocytes and functional evaluation methodologies are also discussed.

2. Lessons from liver development

A reasonable approach to induce hepatic differentiation from pluripotent stem cells is to recapitulate actual embryonic developmental events *in vitro*. Liver development occurs through a series of cell-to-cell interactions between the embryonic endoderm and its nearby mesoderm (Duncan 2003, Zaret 1996). A number of genes and signaling pathways that are involved in this process have been identified (Lemaigre & Zaret 2004). Understanding the molecular pathways controlling hepatogenesis is essential in designing protocols for inducing hepatic differentiation *in vitro*. Although our knowledge of embryonic liver development is based on studies from non-human embryos, it is anticipated that the fundamental processes are similar if not identical to those in human liver development. Studies from mouse, chicken, zebrafish and Xenopus have shown that much of hepatogenesis is evolutionarily conserved (Roberts 2000, Tian & Meng 2006). Numerous signals are required at each step of hepatogenic development (Lemaigre & Zaret 2004, Zhao & Duncan 2005). Here, we review these sequential events – we have divided them into four stages to highlight the crucial cues at each stage for a better understanding of hepatic differentiation protocols that will be described in subsequent sections.

2.1 Stage 1. Toward definitive endoderm

The earliest event of organogenesis during mouse embryonic development is gastrulation. At this phase (ED6.5-7.5), the definitive endoderm layer is formed from mesendoderm lineage cells (Tada et al 2005). The process begins with the migration of Brachyury (T) positive cells through the anterior region of the primitive streak and through the Node, located at the anterior-most position of the streak (Vincent et al 2003). It has been shown that signaling by a member of the transforming growth factor (TGF)-β superfamily, specifically Nodal, initiates both endoderm and mesoderm formation (Vincent et al 2003). The epidermal growth factor family molecule, Cripto-1/FRL1/cryptic, plays a central role as a co-receptor for Nodal. Nodal/ALK signaling in conjunction with Cripto contributes to generate endoderm precursor cells and for the subsequent specification of definitive endoderm (DE) (Strizzi et al 2005). The duration and magnitude of Nodal signaling influence the specification of mesoderm or endoderm. The physical position of cells relative to the Node also seems to control this specification. Exposure to longer and higher levels of Nodal signaling induce endoderm specification, whereas lower stimulation of Nodal signaling promotes differentiation into the mesoderm (Schier 2003). Nodal signaling targets a core group of endoderm transcription factors genes expression including Sox17, Foxa2 (HNF3β) (Shen 2007).

2.2 Stage 2. Influence from cardiogenic mesodermal cells and septum transversum mesenchyme

Following gastrulation, the definitive endoderm is patterned along the A-P axis into foregut, midgut and hindgut progenitor domains (Dessimoz et al 2006). Morphogenesis forms foregut and hindgut pockets as the endodermal cup is transformed into a gut tube. At ED8 the definitive endoderm cells occupy a portion of the ventral foregut adjacent to the heart. Signaling cues from cardiogenic mesodermal cells such as fibroblast growth factors (FGFs) (Jung et al 1999) and cues from the septum transversum mesenchyme (STM), such as bone

morphogenic proteins 2 and 4 (BMP2, BMP4) are essential to direct cell fate to a hepatic lineage (Rossi et al 2001). It has been demonstrated that mouse embryonic foregut explants express albumin when co-cultured with cardiac mesoderm (Calmont et al 2006, Rossi et al 2001). Studies using explant systems have shown that the cardiac mesoderm can be replaced by exogenous FGF1 or FGF2 supplementation (Jung et al 1999). BMP signaling is required, but not sufficient, for hepatic induction in explants (Rossi et al 2001).

2.3 Stage 3. Hepatoblast proliferation

The domain that eventually becomes the liver moves to the midgut, and the liver diverticulum forms by ED9 (Rifkind et al 1969). By ED9.5, the basal lamina breaks down and hepatoblasts delaminate and migrate into the STM. The liver diverticulum expands into a pronounced liver bud by ED10. The liver bud then undergoes tremendous growth and becomes the major site of fetal hematopoiesis. The massive proliferation and protection from apoptosis are regulated by paracrine signals from adjacent mesenchyme. These signals are including the FGFs, BMPs, Wnts, and the TGFβ signaling pathways. Hepatocyte Growth Factor (HGF) signaling is also required for hepatoblast migration and proliferation (Birchmeier et al 2003).

Although Wnt/β-catenin signaling appears to repress liver fate during earlier endoderm patterning stages of development, β-catenin has the opposite effect and promotes hepatic growth in the liver bud at this stage (ED10) (McLin et al 2007, Micsenyi et al 2004, Monga et al 2003). Immunohistochemical studies showed TGF- β3 expression in the liver bud mesenchyme at ED13.5 (Pelton et al 1991). Similarly, Stenvers *et al* showed a predominant expression of the TGF-β receptor III (TbetaRIII) mRNA in liver at midgestation. They also demonstrated that TbetaRIII gene disruption induced apoptosis in the liver at ED13.5 (Stenvers et al 2003).

2.4 Stage 4. A bifurcation point to hepatocyte or cholangiocyte

The differentiation of bi-potential hepatoblasts into hepatocytes or cholangiocytes begins around ED13 of mouse development. Hepatoblasts, adjacent to the portal vein, transform into cuboidal cells, which eventually forms the intrahepatic bile ducts during the prenatal period through the ductal plate remodeling process (Lemaigre 2003, Raynaud et al 2011), while other hepatoblasts gradually differentiate into mature hepatocytes. It has been shown that oncostatin M (OSM) promotes hepatic maturation (Kamiya et al 2001) During this phase, the liver bud transitions from a hematopoietic organ to a metabolic organ. Coexisting hematopoietic cells also play a significant role in the hepatic maturation process. For example, OSM is released from the hematopoietic cells and activates a JAK/Stat3 signaling pathway via the gp130 receptor (Ito et al 2000, Kamiya et al 1999), and intrahepatic structures are organized by ED15. The final maturation of the liver is a gradual process and continues into the postnatal period. Many hepatic enzymes such as cytochrome P450s shift from a fetal form to a mature form during the postnatal period.

3. Hepatic differentiation of pluripotent stem cells

Currently hepatic differentiation strategies from pluripotent stem cells can be classified into three approaches; 1) via formation of spontaneous embryoid body (EB), 2) co-culture with supporter cells, and 3) directed hepatic differentiation via stepwise stimulation by defined growth factors.

3.1 Embryoid body formation and spontaneous hepatic differentiation

Mouse ESCs aggregate in suspension to form spheroid clumps of cells called embryoid bodies (EBs). Removal of leukemia inhibitory factor (LIF) induces spontaneous differentiation into three germ layers (Itskovitz-Eldor et al 2000). Chinzei *et al* reported that AFP and albumin gene expressions were detected in EBs after 9 days and 12 days, respectively, without additional exogenous growth factors (Chinzei et al 2002). However, the spontaneous differentiation of hepatocytes in EBs is inefficient (usually under 10%) and is highly cell line dependent (Lavon & Benvenisty 2005, Schwartz et al 2005, Shirahashi et al 2004). To increase the ratio of hepatic cells in the EBs, differentiation can be directed by exposure to exogenous growth factors. Hamazaki and colleagues demonstrated that the combination and consecutive supplementation of FGF, HGF, OSM, and dexamethasone efficiently induced hepatic differentiation (Hamazaki et al 2001). In regards to human ESCs, hepatic induction using the EB formation approach was reported by Schuldiner and colleagues (Schuldiner et al 2000). These studies with EB formation provided evidence that mouse and human pluripotent stem cells were able to differentiate to the hepatic lineage *in vitro*. The 3D structure of EBs has an advantage over the 2D culture system in further inducing functional maturation. However, the uncontrolled heterogeneous cell populations in EBs and the variation in size and morphology prohibit further clinical applications.

3.2 Co-culture of pluripotent stem cells with supporter cells

Another approach for hepatic differentiation is providing *in vivo*-like supplements including cytokines, growth factors, and extracellular matrixes. These elements can be provided by co-culturing appropriate supporter cells, such as hepatic mesenchymal cells. A co-culture strategy for hepatic differentiation was first applied using Thy1-positive mesenchymal cells derived from mouse fetal liver (Ishii et al 2005). An enhanced green fluorescent protein (EGFP)-AFP labeling system was used to isolate hepatic lineage committed ES-derived cells, which were subsequently co-cultured with the Thy1 positive mesenchymal cells. Several late-phase hepatic markers such as tyrosine amino transferase, tryptophan 2,3-dioxygenase, and glucose-6-phosphatase were expressed in the co-cultured ES-derived hepatocytes. Fair et al reported that co-cultured mESCs with chick embryonic cardiac mesoderm cells expressed mesendoderm genes, Sox17α, HNF3β, GATA4, after 24 hours (Fair et al 2003). They also demonstrated that the xenogeneic cell-to-cell contact between mESCs and the chick cells, apart from providing cytokines/growth factors, may also provide signals via direct cell-to-cell interaction. In contrast, Saito et al demonstrated that diffusible factors from mouse fetal liver-derived cells were sufficient to stimulate hepatic differentiation of cynomolgus monkey ES cells (Saito et al 2006). They also demonstrated that induction of hepatic differentiation utilizing the co-culture system was faster than chemical/growth factor induction. Soto-Gutierrez et al combined a co-culture system of human liver nonparenchymal cell lines with growth factors. This protocol resulted in the successful derivation of 70% albumin positive cells from mESCs (Soto-Gutierrez et al 2007). These data indicate the feasibility of the co-culture system for inducing hepatic differentiation from pluripotent stem cells. However, it might be difficult to reproduce hepatic differentiation by co-culturing with primary cells as the quality and the secreted factors are uncertain. Moreover, the contamination of the supporter cells and the difficulty in the scale-up of hepatocyte production are issues that hamper clinical applications.

3.3 Guided hepatic differentiation with specific factors

The *in vitro* developmental capability of ES cells has been shown with spontaneous differentiation with EB formation. The co-culture studies indicate that exogenous signals

could induce hepatic differentiation. As described in section 2, extensive developmental biology studies have uncovered the pathways controlling hepatogenesis. To mimic the sequential stimulation of *in vivo* hepatogenesis, various combinations of stepwise growth factor stimulation strategies have been tested.

The critical first step is to efficiently induce definitive endoderm (DE) commitment of ESCs. Based on researches of mesendoderm and definitive endoderm differentiation in rodent and Xenopus, it was reasonable to expect that a high concentration of Nodal is key to the induction of definitive endoderm differentiation (Schier 2003). In most studies, a high dose of Activin A was used as a substitute for Nodal. Activin shares the same receptors (ActRII and ALK4) with Nodal but does not require the association of Cripto to initiate the Nodal/Smad2 signaling pathway (Strizzi et al 2005). After a few days of Activin treatment, most of the ES cells express definitive endoderm genes (Sox17 and Foxa2), while mesoderm, pluripotency and the extraembryonic endoderm genes are down regulated (D'Amour et al 2005, Kubo et al 2004). Further studies indicate that generation of hESC-DE requires two conditions: signaling by Activin/Nodal family members and the release from inhibitory signals generated by PI3K through insulin/IGF (McLean et al 2007). In this approach, using a high-density monolayer culture, pluripotent stem cells are cultured in a feeder cell-free system and mesendoderm differentiation is induced by a defined serum-free medium, supplemented with high-dose of Activin A (e.g. 100ng/ml) (Yoshie et al 2010).

The signaling pathways regulating foregut formation can be recapitulated in cell culture by the addition of specific growth factors such as FGFs and BMPs. A number of groups have used this combination to generate hepatic cells (Dan & Yeoh 2008, Ochiya et al 2009). The endoderm specification stage is followed by HGF stimulation to expand the hepatoblast population (LaBrecque 1994). Hepatic maturation is normally regulated and promoted by some combination of OSM and Dexamethasone. (Agarwal et al 2008, Baharvand et al 2008, Cai et al 2007, Soto-Gutierrez et al 2006b, Teratani et al 2005).

Hay and colleagues introduced a similar stepwise induction concept, except using chemicals to induce hepatic differentiation in ES and iPS cells (Hay et al 2008a, Hay et al 2008b, Sullivan et al 2010). A combination of Activin A and the histone deacetylase inhibitors (HDI), sodium butyrate, were utilized for the definitive endoderm induction and 1% DMSO for further hepatic specification (Hay et al 2008b). Although the effect of butyrate for inducing differentiation has been known for a long time (Leder & Leder 1975), the role of HDI in the hepatic differentiation process was not totally elucidated. McLean showed that a combination of Activin and a PI3K inhibitor is more efficient to induce DE differentiation (McLean et al 2007). Recent progress on studies in signal transduction pathways has led researchers to control not only the initiation of signal transduction by adding exogenous growth factors, but also the regulation of intracellular signaling to induce hepatic differentiation in a more precise and efficient manner. Touboul et al used the PI3K inhibitor, LY294002, for DE induction and activin receptor-like kinase (ALK) 5 inhibitor, SB431542, for hepatic specification. Currently, these protocols still require combinations with recombinant growth factors. Since mass production of these recombinant proteins are cost prohibitive, a totally defined chemical protocol would be ideal for a large-scale hepatocyte production.

Although hepatic differentiation protocols are now more sophisticated, fully differentiated stem cell-derived hepatocytes that possess identical hepatic functions to primary adult hepatocytes have yet to be produced. Using a unique primary hepatocyte culture system, Michalopoulos's group demonstrated that a combination of dexamethasone, HGF, and EGF was required for formation of liver-like tissue. Dexamethasone induces expression of both

HNF4α and C/EBP-α, essential transcription factors for hepatocyte differentiation, while HGF and EGF induce members of the TGF-β family and HNF6β, which are essential for maintenance of hepatic functions (Michalopoulos et al 2003). Although this unique culture system has not been tested with stem cell-derived hepatic cells, this combination and culture system may be essential to achieve further hepatic maturation.

4. Enrichment strategies of stem cell derived hepatocytes

Despite utilizing these well-designed differentiation protocols on mouse and human ES cells, hepatic induction from pluripotent stem cells results in mixtures with non-hepatic cells. Generally speaking, in the areas of stem cell-derived cell studies, there has not been a differentiation protocol that is able to induce 100% of this desired target cells based upon current technologies. This is one of the issues that is hindering further clinical development. Particularly in the use of pluripotent stem cells, contamination with undifferentiated cells is one of the biggest concerns due to their tumorigenic potential (Ben-David & Benvenisty 2011, Dressel et al 2008). Therefore, further investigations will be necessary to obtain clinically applicable stem cell-derived hepatocytes. One of the approaches is to develop techniques that allow for isolation and purification of specific hepatic subtypes.

For basic research, it is common to use genetic selection techniques. Under this strategy, undifferentiated pluripotent stem cells are genetically modified to carry either a reporter gene, usually a green fluorescence protein (EGFP) gene or an antibiotic resistance gene under the transcriptional control of a hepatic-promoter such as alpha-fetoprotein (Yin et al 2002). The transgenic cells are then induced to differentiate and subsequently selected based on the activation of the hepatocyte-specific promoter. The major disadvantage of this approach is the risks associated with the genetic modification which may lead to insertional oncogenesis. In addition, these transcriptional markers are also common to immature hepatocytes. It is, therefore difficult to designate one gene as a definitive single marker of hepatic differentiation.

An endogenously expressed surface marker, asialoglycoprotein receptor (ASGPR), has been proposed as a cell surface marker for functional hepatocytes (Li et al 2008, Treichel et al 1995). Recent studies have utilized ASGPR to identify mature hepatic cells from a mixed population of fetal hepatocytes (Ring et al 2010). ASGPR is a transmembrane hepatocellular surface carbohydrate binding glycoprotein that lacks terminal sialic acid residues (=asialoglycoproteins). Characterization of the ASGPR has revealed its functional role in the binding, internalization and transport of a wide range of glycoproteins in a selective manner via the process of receptor-mediated endocytosis. The expression of the ASGPR has been clinically correlated to loss of hepatic functions in liver diseases associated with cancer, viral hepatitis, and cirrhosis. Basma et al enriched hESC-derived hepatic cells using the ASGPR marker (Basma et al 2009). Although the G6Pase, albumin, and TAT mRNA expressions were dramatically improved by this enrichment step, mRNA expression of both Oct4 and AFP were still detectable. This data indicates that a single enrichment step could not completely eliminate the undifferentiated stem cell or the progenitor cell contamination.

Kumashiro et al used a silica-based colloidal medium (Percoll) and PECAM-1 antibodies to separate mESCs-derived hepatocytes from Oct4 positive undifferentiated cells. The ES-derived hepatocytes were transplanted into the CCl4-injured mouse liver and this led to improved liver function. Importantly, unlike unselected ES-derived hepatocytes, the enriched ES-derived hepatocytes did not develop teratomas in the recipients' liver (Kumashiro et al 2005).

Conclusively, the hepatic enrichment studies demonstrate the advantages of selecting hepatic cells from mixed ES-derived cell population. However, there is to date, no single standard technology to achieve sufficient hepatocyte enrichment or non-hepatic cell exclusion. The influence of other cell types on hepatic differentiation is one aspect that requires further studies. Non-hepatic cells such as cholangiocyte contamination may have a positive influence for further maturation in stem cell-derived hepatic cells. For future clinical applications, genetic modification of stem cell will not be acceptable. Although the ASGPR enrichment protocol is promising, the need for a large quantity of antibody remains a substantial financial burden for large-scale production of stem cell-derived hepatocytes. Thus, additional investigations are needed to find novel strategies to enrich clinically viable stem cell-derived hepatocytes.

5. Characteristics of pluripotent stem cell derived hepatocytes

One of the difficulties for stem cell research is the interpretation of *in vitro* data on the extent of terminal differentiation. Since human primary hepatocytes lose their hepatic functions *in vitro* within a week after their isolation, it is clearly a challenge to induce full maturation status equivalent to that of adult hepatocytes in stem cell-derived hepatocytes *in vitro*. With this in mind, the *in vitro* evaluation for hepatic differentiation has to be undertaken very carefully using multi-pronged approaches. The evaluation should be conducted at 1) the transcription level, 2) the translation level, 3) performing biochemical (*in vitro*) functional tests, as well as 4) *in vivo* functional assays.

5.1 Transcriptional evaluation. Hepatic marker genes expression

Many genes have been proposed as hepatocyte-specific genes. The most commonly cited genes are; albumin, alpha-fetoprotein (AFP), cytokeratin-18, glucose-6-phosphatase (G6P), phosphoenolpyruvate carboxykinase (PEPCK), alpha-1-antitrypsin (A1AT), bilirubin, uridine diphosphate-glucuronosyltransferase, coagulation factor VII. However, some of these are also expressed in other tissues such as extra-embryonic yolk sac. AFP, for example, is expressed in both the liver and the yolk sac. Therefore, AFP expression needs to be demonstrated along with definitive endoderm marker genes, Sox17 and CXCR4. Transcriptome studies on mouse liver development have revealed that the expression patterns of these hepatic markers are dramatically altered during hepatogenesis. For instance, Jochheim et al. demonstrated that HNF4α gene expression peaked on ED11.5 and decreased until ED13.5, after which, it gradually increased but its levels were not as high as at ED11.5 (Jochheim et al 2003). Therefore, quantitative RT-PCR data must be carefully interpreted, as a downregulation of HNF4α mRNA expression could still be an indication of increased hepatocyte maturation. Genes encoding cytochrome P-450 (CYP)-7A1, bilirubin, uridine diphosphate-glucuronosyltransferase, coagulation factor VII, and asialoglycoprotein receptors are considered late phase hepatic marker genes and are not expressed in the yolk sac. Therefore, these genes could be useful as markers for mature hepatocytes. In contrast, G6P, CYP3A4 and PEPCK are also deemed late-phase marker genes, however, their expression is not liver specific. CYP7A1 is a member of the cytochrome P450 superfamily of enzymes. This liver specific endoplasmic reticulum membrane protein is involved in the cholesterol catabolic pathway, which converts cholesterol to bile acids. Other CYP enzymes are responsible for oxidative metabolism of most therapeutic drugs. Therefore, it is important to demonstrate the expression of these genes in the stem cell-derived hepatic cells

for drug testing. Among the CYP enzymes, 3A4, 1A2, 2C19, 2D6, are dominant players for drug metabolism in human liver.
Ideally the gene expression levels in stem cell-derived hepatic cell at the end of the differentiation protocol should be comparable to that of an adult hepatocyte. Concurrently, evaluations for non-hepatocyte specific genes to determine the extent of differentiation to specific hepatic lineage should also be performed.
With regards to the development of drug metabolizing enzymes such as CYP genes, both the pattern and the level of expressions are important. Many metabolic and detoxifying enzymes are expressed at significant levels only after birth. Therefore, the gene expression ratio of the fetal form to the adult form could be used as a determinant of the status of hepatic maturation. For example, fetal liver cells express CYP3A7, a fetal form of CYP3A4. Higher 3A4/3A7 ratio indicates increased hepatic maturation of stem cell-derived hepatic cells (Ek et al 2007, Miki et al 2011).

5.2 Translational evaluation. Hepatic marker proteins expression

Gene expression does not necessarily translate to protein production and activity. Using a hepatic differentiation protocol, Hay et al demonstrated that Oct4 protein was undetectable after the second day, while Oct4 mRNA remains detectable until day 11 of the protocol. Along the same vein, albumin mRNA expression was observed at day 11 but albumin protein was detected only starting at day 15 (Hay et al 2008b). Therefore, immunohistochemistry and/or Western blot analyses must be performed to verify the expression of key proteins at the end of any differentiation protocol. AFP, Albumin, A1AT, and cytokeratins (8, 18, 19) are common proteins that were used in many reports. As mentioned in section 4, some cell surface marker proteins (e.g. ASGPR) are also important.

5.3 Biochemical functional assay

Demonstration of albumin production and secretion from stem cell-derived hepatic cells is the gold standard assay to demonstrate functional protein synthesis. In the human body, albumin is the most abundant protein in serum and is produced specifically by the liver. Therefore, serum albumin has been used as an indicator to evaluate liver function in a clinical setting. Although albumin production and secretion are essential, it is however insufficient to use this as a marker for stem cell-derived hepatic cells. It has been shown the albumin gene is expressed at a relatively early stage of hepatogenesis, and furthermore, visceral endoderm cells such as yolk sac cells also produce albumin (Meehan et al 1984). Therefore, albumin production from stem cell-derived hepatic cells must be further supported through a combination of other assays.
The ability to metabolize ammonia is also a standard assay to evaluate hepatic detoxification function. *In vivo*, ammonia is a metabolic product generated from dietary amino acids, and 80-90% of ammonia is converted into urea by hepatocytes through the urea cycle. To test this function *in vitro*, excess amount of ammonium chloride is added to the culture medium (ammonia challenge). Ammonia clearance or urea production can then be measured as functional readout of the assay. Since precise parameter adjustment for measuring ammonia level in culture media is difficult, the standard protocol is measuring urea concentration in the supernatant via the enzymatic urease method. A pitfall in this assay is the possibility of arginase leakage from damaged cells due to the excess ammonium chloride, which could produce urea independently from the urea cycle activity. Therefore, the acquisition of urea metabolism function should be further confirmed by the expression of key enzymes in the

urea cycle, such as arginase I (ARG1), argininosuccinate lyase (ASL), argininosuccinate synthetase 1 (ASS1), carbamoyl-phosphate synthetase 1 (CPS1), and ornithine carbamoyltransferase (OTC) (Mavri-Damelin et al 2008, Miki et al 2011).
To meet the demand for pharmaco-toxicological applications, demonstration of activities of xenobiotic-metabolizing phase I and phase II enzymes and phase III transporters must be satisfied (Xu et al 2005). To evaluate phase I activities, CYP-dependent monooxygenase activity assays; 7-ethoxycoumarin-O-deethylase (ECOD assay) (Lubet et al 1985), 7-ethoxyresorufin-O-deethylase (EROD assay), and specific oxidation of testosterone are commonly used. In measuring phase II activities, glucuronosyltransferase activity and sulfotransferase activity toward p-nitrophenol, and glutathione S-transferase activity are commonly used. Functional assays for phase III transporters have yet to be standardized. Measurement of fluorescent protein conjugated bile acid could be useful to evaluate one of the phase III transporters, i.e. the bile salt export pump (BSEP/ABCB11), for activity in stem cell-derived hepatocytes (Yamaguchi et al 2010).

5.4 *In vivo* analysis

These *in vitro* hepatic functional assays are essential and useful. However, the most definitive proof for the functionality of stem cell-derived hepatic cells would be the demonstration of hepatic engraftment *in vivo* using animal models (Soto-Gutierrez et al 2006a, Yamamoto et al 2003). It would be ideal if stem cell-derived hepatic cells could improve survival rates in lethal disease models. Transgenic mouse models of congenital liver diseases would be candidates for such assays. The fumarylacetoacetate hydrolase (FAH) deficiency mouse model (Lagasse et al 2000) and urokinase-type plasminogen activator-transgenic SCID mice (Tateno et al 2004) have been previously and provide a means to test stem cell-derived hepatocytes in a liver transplantation setting. Unfortunately such animal models are not widely available and also require significant technical expertise to perform hepatocyte transplantation in neonatal animals.
Nevertheless, whether human or rodent, primary hepatocytes tend to lose their differentiated characteristics in culture. One question that arises is whether the stem cell-derived hepatic cells have to be fully differentiated *in vitro*. It is anticipated that stem cell-derived hepatic progenitor cells spontaneously differentiate to fully mature hepatocytes in their correct endogenous environment. Once hepatic lineage commitment is confirmed, stem cell-derived hepatic progenitor cells could be an acceptable source for cell replacement therapy. The endogenous mouse liver environment is suitable to test this hypothesis. It is therefore extremely important to establish standard animal models that are easy to experiment with, and also widely available for stem cell-derived hepatic cell transplantation.

6. Conclusion and future perspectives

Hepatic differentiation is a sequential dynamic event that involves various types of cells and complex signaling networks. Although the signaling pathways and growth factors that are involved in definitive endoderm specification have been well documented, only portions of the mechanisms involved in the human hepatogenic development have been defined. Therefore, the goal of inducing directed differentiation of pluripotent stem cells toward hepatocytes remains a big challenge. A number of differentiation protocols have been described to generate hepatocytes from pluripotent stem cells. Collectively, various hepatic

differentiation studies demonstrate how the exposure of various growth factors to pluripotent stem cells, with the appropriate timing and doses, is essential for directing the differentiation process from early mesendoderm via definitive endoderm towards more functionally mature hepatocytes. Based on the recent advances in the field of developmental biology, hepatic differentiation protocols have been modified to provide more than 70% albumin positive cells from mESCs (Soto-Gutierrez et al 2007) and 35% of ASGPR positive cells from hESCs (Touboul et al 2010). It appears that iPS cells possess a similar capacity to hES cells to generate hepatocytes using similar differentiation protocols (Sullivan et al 2010).

It must be noted that the stem cell-derived hepatocytes described to date generally exhibit fetal hepatocyte-like gene expression profiles and immature functional characteristics compared to authentic adult primary hepatocytes. Recent finding suggested that a 3D dynamic bioreactor culture system which provides an *in vivo*-like culture environment induced teratoma-like multi-directional differentiation of human ESCs (Gerlach et al 2010). When a hepatic differentiation protocol was applied in this system, the 3D dynamic culture system induced further hepatic maturation, including tissue-like structure formation (Miki et al 2011). Although the superiority of the culture conditions in the 3D dynamic culture system has been clearly demonstrated, it is difficult to determine which parameters are critical for this advantage in hepatic maturation. Using matrigel or collagen gel, it has been shown that static 3D culture conditions prolong hepatic function of primary hepatocytes (Kono et al 1997). It has been proposed that the 3D structure allows more physiological cell-to-cell interactions, and induces polarity in hepatocytes (Haouzi et al 2005). Baharvand et al demonstrated that hESCs are more efficiently differentiated to hepatic cells under static 3D culture conditions than conventional 2D culture conditions (Baharvand et al 2008).

Dynamic culture conditions are, not surprisingly, more advantageous than static culture conditions (Vinci et al 2011). Unlike static cultures, the dynamic culture system provides continuous and gradual medium change under controlled oxygen tension and temperature. Taken together, the dynamic 3D culture system can provide a more physiological and homeostatic environment that could be favorable for stem cell-derived hepatocyte maturation. The continuous supply of fresh medium via nano-size porous hollow fibers also provides micro steady flow around the cells (Gerlach et al 2010) and low shear stress that could further stimulate maturation signals through sensory systems (e.g. cilia) on the polarized hepatic cells (Decaens et al 2008). Furthermore, it is noteworthy that in the human body, the liver passively but dynamically moves with the movements of diaphragm. In addition to the oxygen concentration in the blood flow, such movement could be a factor to induce rapid postnatal hepatic maturation events such as cytochrome isotype switching from CYP3A7 to 3A4.

As reviewed, the current differentiation protocols demonstrated the feasibility of hepatic cell production from pluripotent stem cells. Further optimization will be required to better define hepatic differentiation protocols and improved culture conditions to obtain fully differentiated functional hepatocytes *in vitro*. On the other hand, for cell replacement therapy applications, such *in vitro* full maturation may not be essential. Both fetal and adult hepatocytes demonstrated bilirubin conjugating activity in the Gunn rat after cell transplantation (Borel-Rinkes et al 1992). Hepatic progenitor cells could mature in the endogenous hepatic environment and thereby provide therapeutic effects to patients. The direction of research for this goal will be generating hepatic lineage committed cells *in vitro*, establishing an appropriate enrichment protocol, and critically evaluating the efficacy and safety of these cells in animal models.

In conclusion, the promise of pluripotent stem cells for the generation of hepatocytes and with further investigations will eventually lead us to a more in-depth understanding of mechanisms of hepatogenesis and hepatic maturation. The widespread utility of pluripotent stem cell-derived functional hepatocytes for basic research and pharmaceutical applications, could also become a reality. In the future, stem cell-derived hepatocytes are anticipated to have an enormous impact on the treatment of liver disease.

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Part 6

Osteogenic Differentiation

17

Osteogenesis from Pluripotent Stem Cells: Neural Crest or Mesodermal Origin?

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1. Introduction

Research in stem cell biology has the potential to dramatically alter the way we understand the vast complexity and coordination that is required for an organism to develop and function. The creation of therapeutic tools that will inevitably accompany these discoveries in this field of research may completely revolutionize our approach to medicine in the 21st century.

In this chapter we will examine one facet of stem cell research that holds great potential to improve the quality of life for millions of individuals; the study of osteogenesis from pluripotent stem cells. Despite its overt rigid structure, which provides mechanical support and protective functions, bone is a highly dynamic tissue that is tightly regulated to serve multiple roles in the body. Bone tissue is constantly being remodeled by the actions of the osteoblasts, the bone forming cells, and the osteoclasts, the bone resorbing cells. The improper balance of these cells can result in a number of bone-related and osteodegenerative diseases. Osteoporosis, for example, is estimated to effect 75 million individuals in Europe, Japan and the US alone, and thus the potential benefits of understanding the processes regulating osteogenesis may be quite far reaching.

Despite the similarity of the bone tissues found in the adult mammalian skeleton, there are three different sources from which bone is derived in the developing embryo (Fig. 1). Two of these bone origins are from mesodermal progenitors, where cells from either the lateral plate or paraxial mesoderm contribute to the appendicular or axial skeleton, respectively. The third origin of bone tissue can be traced back to ectodermal cells where neural crest progenitors differentiate into many of the bones within the craniofacial region. Differences in the origin in bone are also paralleled in differences seen in the bone formation process. Most bones of mesodermal origin develop via the process of endochondral bone formation, whereas the bones of ectodermal origin form by a process called intramembranous bone formation. These processes differ most generally in the series of cell differentiations that lead to the mature tissue. In endochondral bone formation the mesenchymal progenitors differentiate into chondrocytes, which lay down the cartilaginous framework that is eventually replaced by the mineralized matrix of invading osteoblasts, while the chondrocytes undergo apoptosis. In intramembranous bone formation, the progenitors differentiate directly into osteoblasts. In addition, mature bone tissues house adult stem cell niches, such as those composed of mesenchymal or hematopoietic stem cells. These cells are

the source for diverse cell types throughout the life of the organism and are critical for normal maintenance and overall physiology.

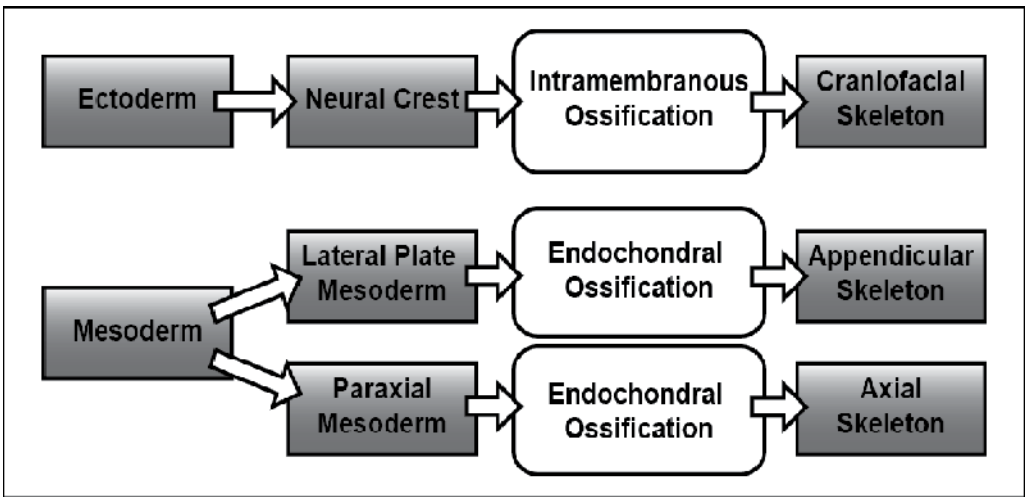

Fig. 1. Embryonic origins of bone tissue

While it is widely accepted that pluripotent stem cells have the capability to give rise to osteoblasts, it has only recently been examined whether they do so through a mesodermal route or through progenitors with neural crest characteristics. This chapter will provide a review of the current understanding of the different progenitors that contribute to the aforementioned bone formation processes and regulatory networks known to play critical roles in these cells. It will further examine the experimental manipulations in stem cell culture systems that have allowed us to derive neural crest and mesodermal type osteoprogenitors *in vitro*. However, it remains elusive whether a neural crest type progenitor and a mesodermal progenitor will have the same capacity to repair bone when transplanted or whether one will be superior to the other in a certain transplantation site. In order to systematically assess the influence of the type of progenitor and the transplantation site as well as the process of bone formation that is typically used as repair mechanism in a particular transplantation site, this chapter therefore also summarizes bone tissue engineering studies that have been undertaken using these diverse progenitors and that will bring us closer to eventual clinical applications that this exciting field of research will provide.

2. Pluripotent stem cells to bone

Both *in vitro* and *in vivo* studies continue to elucidate the developmental program that pluripotent stem cells take to their eventual differentiated states. One such program is the development of bone tissue; and research in this field has already made a positive impact on the lives of individuals in various clinical trials (Giordano et al., 2007). However, before these applications become commonplace in the medical field, further study is required to improve both our understanding and methodologies. This chapter seeks to give a broad overview of a diverse range of topics, from differentiation of pluripotent stem cells along osteogenic lineages, some current approaches in applying stem cell based bone engineering

for potential clinical applications, and concluding with a discussion of different bone origins and their respective developmental pathways.

2.1 Embryonic stem cells

Pluripotent stem cells can be distinguished from adult stem cells based on their nature of origin, but first and foremost based on their more versatile differentiation capability. This unsurpassed differentiation capability is known as pluripotency, the potential to generate cell types from the three embryonic germ layers: the mesoderm, the ectoderm and the endoderm. One class of pluripotent stem cells, the embryonic stem cells (ESCs) have been under fervent ethical debate since their initial derivation. The crux of this debate can be attributed to their source being a cluster of cells found in the blastocyst, an early pre-implantation embryonic stage. This cluster of cells, the inner cell mass (ICM), is established directly after the developing embryo has gone through the first fate decision, in which the trophoectoderm secedes from the ICM. While this outer trophoectodermal layer of the blastocyst eventually gives rise to the placenta, the *in vivo* fate of the ICM is to develop into the embryo proper, which contains cell types of the three germ layers. Mirroring this capability of the ICM, isolated ESCs also have the capacity to give rise to cell types of all three germ layers when differentiated *in vitro* (Itskovitz-Eldor et al., 2000).

ESCs were first derived from mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981) and since then their derivation has been reported from a number of mammals including: hamster, rabbit and rat (Doetschman et al., 1988; Giles et al., 1993; Iannaccone et al., 1994). Although these alternative rodent ESC lines have never gained recognition as model systems, their utilization continues to provide insights into stem cell biology. As for primate ESC derivation, the initial challenges that plagued the field for years were finally overcome by Thomson et al. (1995), and this study laid the ground for the establishment of human ESCs by the same team just shortly before the turn of the century (Thomson et al., 1998).

In addition to the pluripotent nature, it is their second characteristic of being capable of unlimited proliferation that ESCs first became an attractive cell source for regenerative therapies. This propagation in the undifferentiated state can be supported in culture with the addition of leukemia inhibitory factor (LIF) (Williams et al., 1988). Since LIF is inefficient in maintaining the undifferentiated state in human ESCs, the molecular cues needed maybe released by murine embryonic fibroblast feeder layers, which both human ESCs and murine ESCs can be grown on (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). In feeder-independent conditions, basic fibroblast growth factor (bFGF) is able to maintain the pluripotent state of human ESCs (Amit et al., 2003).

2.2 Differentiation of ESCs into bone cells

Historically the developmental program that pluripotent stem cells take to form bone tissue was first elucidated using murine ESCs. Buttery and coworkers were the first to show that mESCs maintained in medium supplemented with beta-glycerophosphate and ascorbic acid had mineralized in culture, a hallmark feature of bone tissue formation (Buttery et al., 2001). In the past decade, numerous protocols have then been established that allow ESC differentiation into bone and cartilage and their characteristic cell types, the osteoblasts and osteoclasts as well as the chondocytes. The studies that describe formation of osteoblasts typically all assess the ability of the cells to secrete an organic matrix composed of collagen type I (COL I) and proteoglycans, the deposition of inorganic hydroxyapatite and the expression of osteoblast-specific genes (Davis et al., 2011; Handschel et al., 2008; Shimko et al., 2004).

One difference between these protocols however, is the choice of additional osteogenic inducers. While beta-glycerophosphate and ascorbic acid are absolutely necessary for the cells to calcify, the additional supplementation of either dexamethasone, retinoic acid or 1,25alpha $(OH)_2$ vitamin D_3 (VD_3) each can significantly increase the amount of bone nodules and expression of osteogenic markers in both mouse and human ESC cultures (Buttery et al., 2001; Phillips et al., 2001; Sottile et al., 2003; zur Nieden et al., 2003).

Similar to endochondral bone formation in the embryo, osteogenesis from ESCs *in vitro* can be direct or the future bone can at first undergo a chondrocyte phase. Both processes have been described for ESCs. For example, during ESC *in vitro* intramembranous ossification, osteoblasts would be specified through a mesenchymal precursor and then directly into the osteoblastic fate. In this case, markers for hypertrophic chondrocytes should be absent or should only be minimally expressed. In turn, ESC differentiation would model embryonal endochondral ossification when ESCs would first differentiate into chondrocytes, then undergo hypertrophy and give way to osteoprogenitors that calcify. Hegert and colleagues, supported by data from our group, have shown that chondrogenic ESC cultures indeed can be manipulated to calcify, whereby such ossification results in a lower calcium content of the matrix then the direct (non chondrocyte-mediated) differentiation (Hegert et al., 2002; zur Nieden et al., 2005). This direct chondrocytic differentiation is mediated by growth factors of the transforming growth factor family, including bone morphogenic proteins and TGFβ1 (Kramer et al., 2000; Hegert et al., 2002; zur Nieden et al., 2005; Toh et al., 2007). Under such treatment the cartilage-specific transcription factors Sox9 and scleraxis are up-regulated at early stages of differentiation (Kramer et al., 2002, 2005; zur Nieden et al., 2005). The addition of BMP also increased the formation of cartilaginous matrix, comprised of collagen, proteoglycans and ECM proteins and expression of collagen mRNAs found in cartilage, such as collagen type II and collagen type X, the latter being indicative of chondrocytes undergoing hypertrophy (Kielty et al., 1985). ESC cultures containing such hypertrophic chondrocytes also initiate expression of osteoblast-specific mRNAs. This overlap of the chondrocyte-specific and the osteoblast- specific differentiation program suggest that ESCs may be undergoing the endochondral bone formation process.

In addition to growth factors and chemicals that direct differentiation through the endochondral or intramembranous route, different physical means have also been utilized to induce ESC differentiation into bone. While murine ESCs are typically grown into small (i.e. approximately 300-400 μm) agglomerates of differentiating cells called embryoid bodies (EBs) (Trettner et al., 2011), as the first stage of differentiation, human ESCs can alternatively be induced to differentiate by overgrowing colonies on a plate (Karp et al., 2006). Further osteogenic differentiation can be observed when intact EBs or dissociated EB cells are cultured in the presence of osteogenic supplements (Buttery et al., 2001; Cao et al., 2005; Chaudhry et al., 2004; zur Nieden et al., 2003). Woll and coworkers trypsinized mouse EBs into single cell suspensions and plated those at very low clonal density (Woll et al., 2006). They reported that approximately 60-80% of single-cell derived colony formation exhibited matrix mineralization as determined by von Kossa staining. Further qPCR analysis of osteoblast markers supported the potential of these cells to undergo osteogenesis, although there was heterogeneity between colonies in expression of these specific markers. Despite this heterogeneity between these individual colonies, the clonal expansion from a single cell offers an easy approach to dissect the differentiation pathway leading to bone cell formation.

This seems to be of particular importance as ESCs can be lead to differentiate from pluripotency into mesenchyme and subsequently bone, whereby mesenchyme may be

specified either from a mesodermal or neural crest derived origin. More recent studies have indeed reported the generation of mesenchymal stem cell like cells from ESCs as well as the isolation of progenitors with osteogenic properties that were mesoderm or neural crest derived (Aihara et al., 2010; Olivier et al., 2006; Sakurai et al., 2006; Trivedi and Hematti, 2007).

A few days into the differentiation, ESCs will express T-Brachyury, a gene that is typically transcribed in the primitive streak when the early embryo undergoes gastrulation to establish the three germ layers (Beddington et al., 1992). The primitive streak contains cells with mesendodermal character, a subpopulation of cells that can later become osteoblasts. T-Brachyury expression is often used to characterize the output of differentiating mesendoderm (Gadue et al., 2006; Nakanishi et al., 2009) and is thus also informative to the very early differentiation events of osteogenesis. Similarly, modeled after the early lineage decisions *in vivo*, activin and nodal induction may be used to enhance the percentage of mesendodermal cells positive for Goosecoid (Gsc), E-cadherin, and platelet-derived growth factor receptor alpha (PDGFRα) (Tada et al., 2005), which are a combination of markers expressed by organizer cells in the primitive streak region. During subsequent development, this triple-positive cell population diverges to Gsc+ and either E-cadherin or PDGFRα positive intermediates that later differentiate into definitive endoderm and mesodermal lineages, including calcified osteopontin expressing osteoblasts (Tada et al., 2005).

While the mesendodermal progenitors are being established in the process of gastrulation *in vivo*, neurulation has already initiated in the anterior part of the embryo. Therefore, specification of neural crest populations may occur *in vitro* during ESC differentiation at around the same time or slightly after T-Brachyury+ or Gsc+ populations are found. In the embryo, neural crest cells emerge from the dorsal epithelium of the neural tube after it has formed, undergo epithelial-to-mesenchymal transitions and become highly migratory. These cells later disperse to and incorporate within skin tissue (i.e. melanocytes) as well as neurons and glia in the peripheral nervous system (Chung et al., 2009; Dupin et al., 2007; Weston, 1991). Due to the multitude of cell types that arise from the highly specific population of neural crest cells, it is sometimes regarded as the fourth germ layer. In addition, the neural crest is generally considered to be the source of a population of cells deemed the ectomesenchyme, which produces a variety of mesenchymal tissues including craniofacial cartilage and bone (Morrisskay et al., 1993; Smith and Hall, 1990). More recently however, the view that mesenchymal cell types are established from progenitor populations of neural crest origin was challenged by Weston and colleagues, who suggest that neural crest and ectomesenchyme are developmentally distinct progenitor populations, possibly distinguishable by the expression of E-cadherin and PDGFRα (Weston et al., 2004).

While it seems widely established that ESCs have the capacity to differentiate into osteoblasts from these various origins, other questions related to the feasibility of their clinical use are still under investigation. As pluripotent cells, ESCs are particularly attractive for the treatment of critical size bone defects that require large numbers of cells as an illimitable source of progenitors, be it mesoderm or neural crest derived MSCs or even more committed osteoprogenitors. More recently, a new less ethically controversial source of pluripotent cells has been discovered in the artificial creation of induced pluripotent stem cells (iPSCs). In this method mature, fully differentiated cells are reprogrammed to a pluripotent state. Explicitly, pluripotency-associated genes are shuttled into somatic cells, e.g. fibroblasts or keratinocytes (Aasen et al., 2008; Okita et al., 2007; Takahashi and Yamanaka, 2006), and brought to expression before they are silenced, which is just enough

to turn the differentiated cells into ESC-like cells with a pluripotent pheno- and genotype. Only five years after their discovery, iPSCs have been recently exploited to study osteogenesis and have already been shown to possess comparable differentiation capacity (Bilousova et al., 2011).

3. Bone tissue engineering

The current gold standard for bone tissue replacement is the autologous graft, which utilizes bone tissue that has been extracted from another site within the patient's own body. However, there is only a limited amount of bone tissue that can feasibly be harvested without inducing considerable donor site morbidity (Rose and Oreffo, 2002). On the other hand, surgical procedures using an allograft, where the bone is harvested from a cadaver, can provide enough material to correct large-scale bone defects. However, this approach carries its own disadvantages including potential immunorejection and pathogen transmission. Techniques involving synthetic materials such as metals and ceramics are continually being used and explored as alternatives to these approaches, but these substitutes continually fall short of bone grafts in areas such as host site integration and tensile strength (Rezwan et al., 2006; Yaszemski et al., 1996). Thus, the attractive features and potential versatility of stem cells offers the investigator an exciting source to improve and develop new technologies that may significantly enhance the efficacy of these procedures.
Currently a popular approach in applying stem cells to *de novo* bone synthesis is the *in vitro* culturing or 'seeding' of cells onto scaffolding materials that can be used for subsequent implantation. In order for this approach to be successful there are a number of essential properties that a researcher must keep in mind when designing the appropriate scaffolding material. These properties will have a direct effect on both the colonization of the scaffold and its successful incorporation into host bone tissue. To achieve an optimal scaffold design a number of considerations such as biocompatibility, porosity, pore size, osteoinductivity and conductivity (including biomolecule incorporation), biodegradability, and mechanical properties must be accounted for (Salgado et al., 2004). Thus, reaching this goal will be a challenge that requires the coordinated efforts of researches across the diverse disciplines of material and biological sciences.

3.1 Mesenchymal stem cells in bone tissue engineering

Beyond the type of scaffold used in a particular study, the choice of seeded cell type will also play a critical role in the creation of *de novo* bone tissue. Starting with the most differentiated cell type, seeding a scaffold with harvested autologous osteoblasts superficially seems attractive because of their inherent cellular program to develop new bone. However, using this cell type is problematic because of low initial concentrations following harvest and relatively poor proliferation capacity *in vitro*. Also, if these treatments are designed to not only amend bone defects, but also to alleviate bone disorders, it is unlikely that harvested osteoblasts will have the suitable characteristics to be effective. Another possible cell type is the multipotent adult mesenchymal stem cell.
Mesenchymal stem cells (MSCs) are unspecialized adult stem cells that reside in mature somatic tissues, predominantly the bone marrow in the long bones. There they share the niche with hematopoietic stem cells, but differ from them in the array of specialized daughter cells that they can generate. MSCs were first described forty five years ago by

Friedenstein and colleagues, when they first found this heterogeneity in differentiation capacity between cells isolated from bone marrow. While they described the cells as ossific progenitor cells of stromal origin in rats in this first study, subsequent studies proved the multilineage differentiation potential of these cells into fibroblasts, chondrocytes and other cells of connective tissue coining the term mesenchymal stem cell (Friedenstein et al., 1966, 1976, 1987; Tondreau et al., 2004a, b; Johnstone et al., 1998; Young et al., 1998; Niemeyer et al., 2004).

Despite the fact that the scientific community has long exploited MSCs to understand the processes of osteogenic and chondrogenic differentiation as well as for the study of adult stem cell maintenance (Bruder et al., 1990; Gazit et al., 1993; Grayson et al., 2006; Hong and Yaffe, 2006), the isolation of the non-hematopoietic mesenchymal stem cell from bone marrow or other tissue sources remains complex. Initially, Friedenstein isolated the MSCs by their tight adherence to plastic (Friedenstein et al. 1976). Yet, newer studies suggest that by isolating MSCs based on their plastic adherence, a portion of mesenchymal stem cells are lost (Zhang et al. 2009). Unfortunately, the fibroblast-like MSCs show a variable profile of surface marker expression (Simmons und Torok-Storb, 1991; Jiang et al. 2002; Vogel et al. 2003), which makes it difficult to isolate them based on a specific marker set. A few years ago, a group of investigators with extensive track records in MSC research has agreed on specific characteristics that need to be met by a cell in order to be called an MSC (Dominici et al., 2006, the International Society for Cellular Therapy position statement). For example, CD14, CD34 or CD45 mark hematopoietic cells and are therefore considered negative markers for MSCs. The most commonly used markers for the detection and purification of MSCs are CD90 (Thy-1 cell surface antigen), CD105 (endoglin) and CD73 (ecto-5'-nucleotidase) (Pittenger et al., 1999; Dominici et al., 2006). Both CD105 and CD73 are constitutively expressed by MSCs, however are also expressed by endothelial cells (Gougos und Letarte, 1988; Airas et al., 1995). Therefore, a combinatorial approach using CD106 (vascular cell adhesion molecule 1) is also recommended in the literature to identify MSCs, as CD106 is only expressed on the MSC surface, but not on endothelial cells (Pittenger et al., 1999; Osborn et al., 1989). Stro1 (Stenderup et al., 2001), glycophorin A (Pittenger et al., 1999; Reyes et al., 2001; Jones et al., 2006), D7-fib (Jones et al., 2002) and p75 (Quirici et al., 2002) have also been associated with MSCs recently, but are not contained in the International Society for Cellular Therapy position statement.

Currently, the use of bone marrow derived mesenchymal stem cells (BDMSCs) to study bone tissue generation is popular because these can be harvested from the patient's own body, thereby removing concerns of immunorejection and disease transmission. Because the transition of BDMSC studies to clinical applications is currently more direct, and not enveloped in ethical considerations, there have been many studies looking at the differentiation capacity of BDMSCs *in vivo* (Arinzeh et al., 2003; Bruder et al., 1998; Gao et al., 2001; Kotobuki et al., 2008). BDMSCs are already used in preclinical trials for treatment of osteogenesis imperfecta and non-union bone fractures (Le Blanc et al., 2005; Tuch, 2006).

However, this does not exclude the necessity to examine ESCs as a potential source of bone engineered cells. In fact, improvements in the techniques of somatic nuclear transfer (Byrne et al., 2007) and creating iPSCs (Nakagawa et al., 2008; Yu et al., 2007), make it quite plausible that the protocols derived from the study of ESCs may someday become more applicable to the future of regenerative medicine than their adult stem cell counterparts. In addition, there are drawbacks from using BDMSCs, including the limited number that can be obtained, more restricted proliferation and differentiation capacities when compared to

ESCs, and they may also harbor undesirable characteristics when harvested from unhealthy bone. So although the use of MSCs has progressed further in clinical applications of bone tissue engineering, the examination of ESCs as a potential source for repairing bone defects and disorders still merits a great deal of attention.

3.2 Embryonic stem cells for bone tissue engineering

Since Levenberg and colleagues (2003) demonstrated the potential to create complex tissue structures on 3D scaffolds using differentiating human ESCs, a number of investigations sought to refine and optimize the conditions required to engineer specific tissue types within 3D scaffolds. In 2004, Chaudhry and colleagues (2004) were the first to demonstrate the feasibility of inducing mineralization of murine ESC derived cells within 3D poly L-lactic acid (PLLA) scaffolds. To accomplish this goal the team initially differentiated murine ESCs into osteoblast progenitor cells in 2D culture. EBs were initially formed, which were then subsequently transferred into suspension dishes for 3 days in the presence of retinoic acid, and then were grown in the presence of β-glycerophosphate and ascorbic acid. EBs were trypsinized and seeded onto PLLA scaffolds. After four weeks of subsequent culture in osteogenic media, the scaffolds showed extensive bone nodule formation on the surface of the scaffold and evidence of cell invasion/mineralization with the interior, as demonstrated by electron microscopy and von Kossa staining. Molecular characterization of the cells that had colonized the scaffold also revealed expression of the osteoblast specific markers osteocalcin, osteopontin and alkaline phosphatase (Alk Phos).

When discussing synthetic scaffolds for tissue engineering it is important to realize that not only the composition of the material itself is important, but that the nano-scale architecture can also play a critical role in the successful colonization of the material. Smith and colleagues (2009) developed a fabrication method of producing a nanofibrous PLLA scaffold in an attempt to mimic a collagen matrix. These were compared to traditional 'solid-walled' PLLA scaffolds in both 2D and 3D osteogenic culture systems. It was found that the 3D nanofibrous matrices expedited differentiation of mouse ESCs as revealed by markers runx2, an osteoblast-specific transcription factor (5 times greater), bone sialoprotein (8.5 times greater) and osteocalcin (2.9 times greater). These scaffolds were also found to contain greater amounts of COL I (5.5 times) and calcium (3 times) when cultured for 28 days. Another point of interest from this study showed that the nanofibrous scaffold, unlike all the other materials tested, was also able to support osteogenesis without the addition of osteogenic supplements. Although, the osteogenic output was not as robust as when cultured with media supplemented with ascorbic acid, β-glycerophosphate, and dexamethasone. Thus, it appears that the nano-scaled architecture of these scaffolds mimics the endogenous ECM.

These differences in geometry presumably create a more appropriate spatial context to facilitate cell-cell interactions and communication for bone tissue development. In addition, it was previously found that this nanofibrous scaffold absorbed four times the amount of serum proteins than their traditional solid walled counterparts (Woo et al., 2003). Thus, the ability of this nano-scaled architecture to both improve the spatial arrangement of cells and to absorb more growth factors, demonstrates how attention to microscopic manufacturing of materials can greatly enhance the potential and success of these scaffolds.

The availability of a blood supply, especially to large bone grafts, is critical for engineered tissue transplant efficacy. The creation of a flap for transplantation is one surgical approach to address this issue. A flap is tissue that already has a vasculature system in place to support nutrient and gas exchange. Although not explored in ESC-derived grafts, studies

performed with BDMSCs demonstrate the feasibility of this approach. Warnke and colleagues performed an interesting clinical demonstration of this technique in 2004. Here, a seeded scaffold intended to repair a large resection of the patient's mandible was first implanted within the patient's latissimus dorsi muscle. This *in vivo* incubation period allowed time for the graft, a titanium mesh cage filled with hydroxyapatite blocks coated with recombinant human BMP7, to develop vasculature. The graft was initially seeded with solution containing autologous bone and natural bovine bone-mineral extract. After seven weeks the implant was removed along with the muscle tissue containing the thoracodorsal artery and vein, which had provided the circulation to the implant, and was transplanted into the patient's jaw. Bone mineral density was measured using non-invasive 3D chromatography and revealed continuous improvement for the duration of 38 weeks (Warnke et al., 2004). Due to ethical considerations, a biopsy of the implant was not undertaken. However, mineralized scar tissue in areas of implant overgrowth was histologically examined and showed young cancellous bone formation containing viable osteoblasts and osteocytes. The patient's continual smoking and alcohol abuse compromised the initial favorable prognosis of the treatment, and unfortunately the patient had passed away 15 months following the operation (Warnke et al., 2006). Also due to the nature of the procedure, which precluded the use of control implants, statistical analyses were not performed. However this study provides at least an initial demonstration of principle within a human subject, and may eventually serve as a model to vascularize engineered bone tissue *in vivo*.

To examine the differences in the *in vivo* osteogenic capacity of between BDMSCs and ESCs, Tremoleda and colleagues (2008) implanted chambers that were cell-impermeable. These chambers contained either BDMSCs or ESCs that had been cultured *in vitro* for 4 days in standard osteogenic media. Since the pore size of these chambers precluded the passage of cells but allowed the diffusion of growth factors and other macromolecules, comparison of the intrinsic capacity for differentiation of these cell types became more straightforward. After 79 days post-implantation within nude mice, the authors reported no qualitative differences between the bone tissue formation between the BDMSCs, H7, and H9 embryonic stem cells. Although an interesting finding revealed that the ESC lines used did not require the *in vitro* osteogenic culture prior to implantation to form de novo bone tissue, which was unlike the BDMSCs, which required this pretreatment. Thus, although a significant difference between these cell types with the same osteogenic treatment was not uncovered in this study, the fact that ESCs required less coaxing and were more primed to respond to the bone tissue environment may be capitalized upon in future studies.

However, one of the major concerns when using ESCs for reparative medicine is the potential for residual undifferentiated cells to form teratomas following *in vivo* introduction. As such, undifferentiated murine ESCs form teratomas when injected into a healthy knee joint (Wakitani et al., 2003). The rate at which the teratomas grew in the knee joint however was slower than upon subcutaneous injection, suggesting that the microenvironment in the knee joint is not as favorable for ESC proliferation as for example a subcutaneous injection site. Surprisingly, if cells were injected into an inflammatory environment caused by a full-thickness osteochondral defect, the cells integrated and repaired the defect even in an allogenic setting (Wakitani et al., 2004).

Also, our group was recently able to show that ESCs lose their teratoma formation capacity with progressing osteogenic differentiation and maturation *in vitro*, whereby the *in vitro* microenvironment used to steer differentiation influences their teratoma formation capacity

in vivo. Whereas spontaneously differentiated cells formed teratomas in 16% of the cases when taken from day 10 old cultures, 30-day osteogenic cultures did not show any sign of teratoma formation upon subcutaneous injection (Taiani et al., 2009).
Highlighting the concern of teratoma formation further, Nakajima and colleagues (2008) seeded mouse ESCs embedded in a collagen matrix into osteochondral defects within the knee joints of mice. Their investigation focused on the differentiation potential of these cells when the joint was either free to move or physically immobilized. They revealed that the mechanical environment appears to have a dramatic effect on the differentiation outcome of these implanted cells. Three weeks post operation, the defects were examined and the free-moving joints were shown to contain cartilaginous tissue formation with favorable histological characteristics. Surprisingly, when the joint was immobilized a teratoma formed in every instance of study. Thus, considering the close link between chondrogenesis and osteogenesis (to be discussed further in next section), it is important to note the results here and recognize that the mechanical environment into which undifferentiated stem cells are placed can have important consequences.

3.3 ESC-derived MSCs

Another cell type that has been recently gained attention as a possible therapeutic source is the embryonic stem cell-derived mesenchymal stem cell (ESC-MSC) in which ESCs are induced along the mesenchymal stem cell lineage. For a more detailed overview of the markers and techniques used to isolate such mesenchymal stem cell like cells from ESCs, the reader is referred to two recent reviews by Hematti (2011) and zur Nieden (2011). In one study of this cell type Barberi and colleagues (2005) demonstrated that cells initially differentiated along a paraxial mesoderm lineage were able to undergo osteogenesis *in vitro*. They found that this induced and sorted cell type (i.e. using the mesenchymal stem cell marker CD73) was able to undergo osteogenesis, by various staining assays and expression of bone specific markers. Similarly, Hu and colleagues derived human ESC-MSCs, and examined their capacity to differentiate into bone forming cells (Hu et al., 2010). When these cells were cultured in the presence of dexamethasone and BMP-7, they found that both Alk Phos levels and calcium deposition was statistically higher in dishes containing both supplements. This improvement found with both supplements was a synergistic one, as revealed through the modest effect when BMP-7 was used independently. When these cells were grown on 3D PLLA nanofibrous scaffolds, similar to that of Smith and colleagues (2009) discussed earlier, they exhibited growth throughout the scaffold and demonstrated extensive mineralization.
The *in vitro* osteogenic capacity between isolated human MSCs and derived human ESC-MSCs, was directly compared by de Peppo and others (2010). In this study they designated human ESC-MSCs as human embryonic stem cell-derived mesodermal progenitors hES-MPs and used a similar approach to that of Hu et al. (2010) to derive this cell type (Karlsson et al., 2009). Here they demonstrated that *in vitro* culture of hES-MPs resulted in faster ECM mineralization as compared to human MSCs. These results were contrary to their Alk Phos assays, which showed significantly greater activity of Alk Phos in human MSCs at every point during the first five weeks of differentiation. This apparent discrepancy may reflect a differential dependence of Alk Phos to mineralize the ECM between these cell types. In addition, this study examined the osteogenic capacity of cells in relation to their passage number. In every assay performed the osteogenic capacity decreased as passage number increased for all cell types examined. Although they reported that the hES-MPs were more buffered against this diminishing capacity, it brings attention to the problem with serial

passages, which are inexorably tied to the requirements of tissue engineering, and their resulting potential to undergo osteogenesis.

The apparent discrepancy in relative Alk Phos activity was also found by Bigdeli and others (2010) when they compared the osteogenic capacity of human MSCs and a derived human ESC line (Bigdeli et al., 2008), which could be expanded on culture plastic without the support of feeder layers or other dish coatings such as Matrigel. Utilizing this cell line allowed the investigators to perform more direct comparison of the two cell types, since the typical differences between culture conditions were eliminated. Like the aforementioned study (de Peppo et al., 2010), they found that although Alk Phos expression was significantly lower at each time point examined, the derived human ESC line was better able to mineralize the extracellular matrix when compared to human MSCs. These results were further supported by ion mass spectrometry of the mineralized ECM, which demonstrated the signature of natural hydroxyapatite.

A study comparing osteogenesis of murine MSCs and murine ESCs derived from the same mouse strain (Shimko et al., 2004) also revealed this pattern where the mineral content was not directly correlated to Alk Phos activity. Thus, although Alk Phos activity is used frequently in studies of osteogenic differentiation, the level of enzyme activity may not directly correspond to the potential of the cells to mineralize the extracellular matrix. In addition, diverse Alk Phos levels may not necessarily suggest that more or less osteoblasts were formed, but may simply reflect different maturation kinetics of the different cell types.

Shimko et. al, (2004) went further in characterizing the mineralized matrix between murine MSCs and murine ESCs derived from the same mouse strain and cultured in the same conditions. As compared to natural hydroxyapatite found in bone, where the ratio of calcium to phosphorous is: 1.67:1; murine ESCs exhibited a ratio far closer (1.26:1) than murine MSCs (0.29:1). Mouse ESC cultures also contained, on average, a mineral content 50 times greater than mouse MSCs. However, once again reflecting distinct differentiation kinetics, pathways, or inherent differences in mineralization capacity, Alk Phos activity was significantly higher in MSCs throughout the course of the experiment. In addition, expression of osteocalcin and COL I in mouse ESCs was delayed relative to mouse MSCs. Thus, murine MSC differentiation appeared to be more reflective of natural osteogenesis, when examining organic matrix components and gene expression. On the other hand, the quantity and quality of the mineralization found in murine ESCs significantly surpassed what was exhibited by murine MSCs.

Although transferring the techniques of osteogenic induction of ESCs from flat culture dishes towards 3D scaffolds have demonstrated initial success, there continues to be the need for method refinement in order for these approaches to bone engineering become widely accepted. One such area of study where current knowledge is lacking is an understanding of the possible differentiation pathways that are normally found in vertebrate development these cells take in attempts at bone tissue engineering.

4. Different embryonic bone origins

Both *in vitro* and *in vivo* studies continue to elucidate the developmental program that pluripotent stem cells take to their eventual differentiated states, among them the osteoblast. Because of their capacity to differentiate into any cell type of the body, pluripotent stem cells may differentiate through the neural crest route or the mesodermal route, followed by mesenchymal specification. Similarly, ossification from pluripotent stem cells may occur

through intramembranous bone formation or endochondral bone formation. In regard to the *in vivo* source of mesenchymal cells, which differentiate into bone in the appropriate developmental context, there also appears to be multiple developmental origins. The earliest MSCs appear to arise from Sox1+ neuroepithelium through a neural crest intermediate stage (Takashima et al., 2007) and not from mesoderm progenitors as previously believed.
The process of fracture healing also occurs through both intramembranous and endochondral means, which is dependent on the mechanical conditions at the fractured site (Claes et al., 1998). When dissecting the steps of bone development, far more is known about the endochondral pathway than the intramembranous process. The most overt difference between these two pathways is that either chondrocytes will arise from mesenchymal condensations, which subsequently apoptose and are replaced by invading osteoblasts, or there is a direct differentiation into osteoblasts themselves. Thus the differential influence and the necessity of chondrocytes highlight the most apparent differences between these bone-forming pathways. Thus, in order to optimize bone tissue-engineering procedures there is a need to understand the molecular basis underlying different bone formation processes. However, a current review of the literature demonstrates large holes in our understanding of these multiple routes in which bone naturally forms and how they are recapitulated in experimental systems. The remaining part of this chapter will be devoted towards our preliminary understanding of these processes with particular emphasis to their roles in bone tissue engineering.

4.1 Endochondral ossification

In the endochondral process mesenchymal cells condense and differentiate into proliferating chondrocytes, which take on the general shape of the future bone. These chondrocytes eventually fall out of the cell cycle and these post-mitotic chondrocytes undergo hypertrophy. In this stage of development the mature hypertrophic chondrocytes lay down cartilage-specific proteins into the surrounding matrix. This cartilaginous framework provides molecular cues, which attracts invading vasculature along with osteoblasts, which will replace the cartilage intermediate. Osteogenesis occurs directly adjacent to hypertrophic chondrocytes. It appears that both the parathyroid hormone (PTH)-related peptide (PTHrP) and its receptor PPR are critical in the process osteogenesis via the endochondral pathway. In mice, upon disruption of the either PTHrP or PPR, the formation of ectopic hypertrophic chondrocytes is accompanied by ectopic bone collar formation (Karaplis et al., 1994). To determine if the hypertrophic chondrocytes induce osteogenesis in adjacent cells and is not a spatial/temporal coincidence, Chung and colleagues (2001) studied transgenic mice that express constitutively active PPR under the control of a chondrocyte specific promoter. This constitutive action resulted in suppression of hypertrophic chondrocyte formation and concurrent suppression of bone collar and primary spongiosa development. In addition, when these transgenic mice were mated to PTHrP-/- mice the resulting rescue of the ectopic bone formation, supported the conclusion that hypertrophic chondrocytes are responsible for the induction of osteogenesis in adjacent tissue.
Regulation of the PTHrP/PPR signal appears to be controlled by one of the members of the hedgehog family of paracrine factors, Indian hedgehog (Ihh). Members of this signaling family are found throughout the animal kingdom and take on a number of critical roles in the developing organism. Here, Ihh is expressed by both prehypertrophic and hypertrophic chondrocytes. This signal mediates the expression of PTHrP by cells of the perichondrium, which in turn binds to PPR on chondrocytes. Ihh and PTHrP signaling thereby creates a

negative feedback loop which suppresses differentiation of the proliferating chondrocytes into hypertrophic ones (Lanske et al., 1996; Vortkamp et al., 1996). Thus, this balance of signals dictates the spatial positioning of the hypertrophic chondrocytes. However, the role of Ihh appears to have a broader impact on osteogenesis than its PTHrP-dependent regulation of chondrocyte maturation.

St-Jacques and colleagues (1999) demonstrated that Ihh also plays a role in chondrocyte proliferation and the direct development of osteoblasts in endochondral bones. Previous studies have demonstrated a critical role of Wnt signaling and β-catenin localization as well (Gong et al., 2001; Kato et al., 2002). Hu and others (2005) found nuclear β-catenin localization within the cells of perichondrium indicating an upstream role of Ihh signaling to facilitate proper Wnt signaling. Furthermore, Ihh null mice do not exhibit osteocalcin expression within endochondral bones, whereas this expression is readily detected within the intramembranous bones of the skull and clavicle. This differential dependence of Ihh signaling underscores one of the differences between endochondral and intramembranous bone formation.

When assessing the role of local synthesis of VD_3 in transgenic mice that exhibited a chondrocyte-specific loss-of-function Cyp27b1, the enzyme that converts 25-hydroxyvitamin D_3 into the active form VD_3, it was found that the hypertrophic zone was expanded (Naja et al., 2009), thereby increasing both bone mass and trabecular size and number. The classical view that VD_3 synthesis (active form) was restricted to the kidneys and that this hormone's influence on bone tissue regulation was an indirect consequence of altering calcium and phosphate homeostasis had to be reevaluated. The authors suggest their results can be explained by a reduced osteoclast recruitment, which follows from a reported delay of vascularization that may be attributed to a reduction of VEGF found. Conversely, overexpression of Cyp27b1 under a chondrocyte-specific promoter resulted in the opposite expression profile and phenotype. These results are in accordance with chondrocyte specific VD_3 receptor ablation experiments, which showed impaired vascularization and osteoclast number in endochondral bone (Masuyama et al., 2006). As opposed to the traditional view of the role of VD_3 in bone biology as an indirect mediator of mineral uptake, these experiments demonstrate a functional role of this metabolite in regulating endochondral bone formation.

Some investigators have explored an approach to bone tissue-engineering by mimicking the development of mammalian long bones, where the creation of cartilage scaffolds *in vitro* are implanted *in vivo*. This approach hinges on the idea that the body will recognize this cartilage scaffold as an intermediate step in the endochondral bone formation process and will then proceed to ossify this construct. In the formation of endochondral bone, chondrocytes are exposed to very low oxygen levels and their survival is dependent on the expression of the transcription factor hypoxia-inducible factor-1 (For review see: Pfander and Gelse, 2007). Thus, the natural ability of chondrocytes to withstand the low oxygen supply can provide the time needed for new vasculature to develop and reach the core of the implant before widespread cell necrosis.

Jukes et al. (2008) tested whether *in vitro* differentiation of ESCs along chondrocyte lineages on scaffolds could improve *in vivo* osteogenesis following implantation. ESCs were initially induced along a chondrogenic pathway for 21 days on ceramic scaffolds. These chondrogenically-primed scaffolds were then subsequently implanted in immunodeficient mice and were found to exhibit nascent bone tissue formation when examined 21 days post-operatively. For comparison, primary chondrocytes and adult MSCs of human, goat, and bovine origin were used in lieu of the chondrocyte-induced ESCs. It was found that each cell

type demonstrated differential abilities to form bone tissue *in vivo*. Interestingly, the goat MSCs resulted in the highest degree of bone tissue formation, and it appeared that this formation occurred via an intramembranous pathway.

Farrell et al. (2009) further examined the *in vitro* chondrogenic-priming of scaffolds using human MSCs and reported limited success. After the cells were cultured on collagen–GAG scaffolds for three weeks in chondrogenic media, the scaffolds were implanted subcutaneously in nude mice. Although cell survival and angiogenesis was found higher in the chondrogenically-primed scaffolds, as opposed to scaffolds that were osteogenically-primed, there appeared to be no *de novo* osteogenesis. The authors reported the chondrogenically-primed scaffolds showed evidence of the initial progression of endochondral ossification, yet were unable to proceed through the later stages of osteoblast-induced mineralization. When mineralization was induced *in vitro* prior to implantation, the nascent angiogenesis that was previously obtained was compromised. Thus, it appears that for this approach to be successful, the timed release of additional factors *in vivo* is needed to promote the osteogenic replacement of the cartilaginous scaffold.

When examining the developmental pathway of bone-tissue engineered constructs, by either endochondral or intramembranous routes, it makes sense that different cell types will mature along different pathways even when presented to the same conditions. Tortelli and others (2010) revealed how the differentiated state of implanted cells affects subsequent ossification and host cell recruitment to the graft site. They seeded hydroxyapatite scaffolds with either human MSCs or osteoblasts. When differentiated osteoblasts were used to seed the scaffolds, ossification occurred through an intramembranous pathway, as revealed by the lack of cartilage markers by immunohistological examination. This intramembranous ossification appeared to be more rapid and thus accounted for more bone deposition within the same time period when compared to the MSC-seeded scaffolds. However, MSC scaffolds, which ossified in an endochondral fashion, were able to facilitate nascent vascularization of the graft. This highlights the fact that engineered bone grafts may one day be tailored to a patient's need depending on factors such as speed of graft ossification and site incorporation. In addition this study shows that implanted MSCs can progress through the endochondral pathway, but as the aforementioned study by Farrell et al. (2009) demonstrates this process currently cannot be split into an early *in vitro* stage that can be 'picked up' later *in vivo*. However, if the process of endochondral bone formation is elucidated further and applied to tissue engineering, then it is feasible that this approach may one day become a viable avenue to repair large bone defects.

Although mimicking the development of long bone through endochondral ossification of scaffolds maybe appropriate in some contexts, intramembranous ossification may be suitable for other applications in regenerative medicine. The body utilizes both of these systems in different contexts depending on certain conditions whose reasons remain to be fully characterized. Nonetheless, it appears quite probable that bone tissue engineering need not only be tailored to the individual but also the specific bone defect or disease in order to be completely effective.

4.2 Intramembranous ossification

As for intramembranous bone formation, not only is little known about the process itself, but the developmental pathway of the cells leading to the formation of the tissues within the cranial skeleton is still not well understood.

4.2.1 Neural crest cells

As incipiently indicated, the migrating cranial neural crest cells form bone mostly through intramembranous ossification. Initially neural crest cells become committed to either an ectomesenchymal (i.e. producing tissues such as cartilage, bone and connective tissue) or a non-ectomesenchymal (i.e. producing neurons, glia and pigment cells) lineage. The ectomesenchymal tissue is also referred to in the literature as mesectoderm. Blentic and others (2008) describe how migrating neural crest cells in chick and zebrafish embryos commit to either fate. Cells that migrate into the pharyngeal arches are induced to respond to FGF signaling within these embryonic structures, resulting in the expression of the homeobox gene Dlx2. Concurrently, early neural crest markers Sox10 and FoxD2 are downregulated, which are still expressed in the neural crest cells that have not invaded the pharyngeal arches and thus are fated to become non-ectomesenchyme. Whether or not neural cells migrate into the pharyngeal arches appears to be determined by the timing of their emergence from the neural tube. Although not fully understood, it appears that early migrating cells 'fill up' the pharyngeal arches and the cells that migrate later are thus more likely to find residence outside of the arches and become non-ectomesenchyme (Blentic et al., 2008).

4.2.2 Neural crest and mesodermal progenitors in intramembranous bone formation

The parietal bone, which is of paraxial mesoderm origin and the frontal bone, which is of neural crest origin, both form via intramembranous ossification, thus making the study of calvarial bones an attractive platform to study the possible differences in bones of different embryonic origins. Quarto et al. (2010) examined the osteogenic capacity of first passage osteoblasts that were obtained from these respective bones in mice. Frontal bone-derived osteoblasts from post-natal day 7 and day 60 mice were found to exhibit greater mineralization capacity, as revealed by Alk Phos activity, von Kossa and Alizarin Red S staining. This was also supported by expression data of the bone-specific markers osteocalcin and runx2. These *in vitro* observations were reinforced by the relative healing capacity of these two bones. The successful healing of 2mm defects was found within the frontal bone in the majority of mice at 8 weeks post-injury, whereas complete healing was not typically found in same sized injuries of the parietal bones within the same time period.

The investigators uncovered a higher level of endogenous canonical Wnt signaling in frontal bone osteoblasts as compared to parietal bone osteoblasts that may be responsible for this differential regenerative propensity. By modulating Wnt signaling through exogenous addition of Wnt3a or transfecting osteoblasts with constructs that increase β-catenin signaling in parietal bones to frontal bone levels, and vice versa, the authors showed a reversal of osteogenic potential of these cells. Thus, providing strong evidence that that the enhanced osteogenic potential of frontal bone osteoblasts can be at least be partially attributed to these differences in endogenous Wnt signaling.

Xu and colleagues (2007) found that osteoblasts derived from the frontal bone proliferated faster and attached to culture dishes better than osteoblasts that were harvested from parietal bone. This may be linked to the fourfold greater expression of osteoblast-specific cadherin that they found within frontal bone osteoblasts. The parietal bone osteoblasts did however show double the Alk Phos activity at the time points examined. When cultured in the presence of osteogenic inducing factors, such as VD_3, the frontal bone osteoblasts showed a much more robust bone nodule formation. However, expression of osteogenic differentiation markers, such as osteopontin, Col1, and Wnt5a was significantly greater in

the parietal bone derived cells. Members of the FGF signaling cascade were also differentially expressed between these two cell types. Thus, the frontal and parietal bones appear superficially similar yet exhibit an number of different characteristics such as growth kinetics, regulation by signaling cascades and varying marker expression, all of which demonstrate that these bones are not as similar as they initially appear to be.

To further examine the regenerative osteogenic capacity of cells from different embryonic origins Leucht and others (2008) engineered mice in which developing cells of neural crest origin would irreversibly express GFP. Tissues from mesodermal origin were also induced to express β-galactosidase. Following skeletal injury in either the mandible or the tibia resulted in natural bone regeneration where the progenitor pool which became new bone tissue was derived from the same embryonic origin of the injured bone itself (i.e. cells from neural crest origin repaired mandible defects, and cells of mesodermal origin repaired tibia defects). The investigators then performed a number of transplant experiments where skeletal progenitor cells were implanted into bone of different embryonic origin. Interestingly neural crest derived progenitors were able to form more new bone when implanted ectopically into tibia injury sites, than if they were implanted back into their endogenous environment within mandible injuries. Conversely, when mesoderm derived progenitors were implanted into mandible injuries, an abundance of cartilage formed, which over time ossified via an endochondral pathway.

These results suggest a difference in the underlying reparative plasticity of cells from different origins. *In vitro* analysis demonstrated that mesoderm osteoprogenitors proliferated faster than the corresponding neural crest osteoprogenitors. However, the cells of neural crest origin were able to differentiate faster based on Alizarin Red S staining and qPCR of osteogenic markers. The authors went further to try to understand the possible molecular mechanisms underlying this difference (Leucht et al., 2008). They found that in the adult mice Hoxa11 expression was maintained in the tibia and was absent in the mandible. For neural crest osteoprogenitors, which originally lack Hoxa11 expression, they began to express Hoxa11 when ectopically placed in the tibia. This switch in expression was not found in the mesodermal osteoprogenitors, which continued to express Hoxa11 even when placed in the Hoxa11-negative environment of the mandible. This study once again reiterates how the molecular identity of cells used for transplantations can be a crucial factor in determining the success of a stem cell based bone graft. In addition, it may be true that osteoprogenitors of neural crest origin may be best suited as the stem cell source of bone grafts because of their greater plasticity to adapt to local environments.

4.2.3 ESC-derived neural crest stem cells

When ESC cultures are osteogenically induced following standard differentiation procedures, it is seldom examined which developmental progenitors are responsible for the terminally differentiated osteoblasts. Although some studies have differentiated ESCs along defined lineages and then determined their osteogenic capacity, Lee et al. (2008) reported the isolation and propagation of human neural crest cells from human ESCs. Initially they cultured human ESCs in neural induction media and then mechanically removed and replated the resulting neural rosettes. Cells that were doubly positive for the neural crest markers p75 and HNK-1 were further cultured and revealed a CD73 positive population. This marker expression indicates the presence of neural crest-derived mesenchymal stem cells. This CD73+ population could be osteogenically induced as revealed by Alizarin Red S, and Alk Phos staining, and bone sialoprotein expression.

In a similar study Jiang and colleagues (2008) also used a FACS enrichment strategy for p75 and HNK-1 positive neural crest cells after co-culture of human ESCs with PA6 stromal cells, although the osteogenic differentiation potential of such isolated neural crest cells was not determined. In another study cranial neural crest-like cells were derived from human ESCs, not by co-culture but instead though EB formation (Zhou and Snead, 2008). Here, FACS purification of neural crest cells was performed based on the expression of Frizzled3, a Wnt receptor, and cadherin11, a cadherin specifically expressed in the gastrulating embryo and migrating neural crest cells (Kimura et al., 1995). Only about 1% of cells were double positive for these selected markers and were able to self-renew and maintain multipotent differentiation potential, including runx2 positive osteoblasts with the capability to calcify. Although not definitive demonstrations of the isolation of osteoprogenitor stem cells from different germ layer-derived populations, these studies offer compelling evidence that cells existing in *in vitro* culture conditions can recapitulate the neural crest osteogenic pathways found in the developing embryo.

5. Conclusion

In summation, pluripotent stem cells are a particularly attractive source to develop new technologies and techniques to address many debilitating bone disorders and defects, and we have come far in the understanding and characterization of osteogenesis. Although more investigations and innovations are needed before regenerative bone biology becomes commonplace, the future holds great promise in this field of research.

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Part 7

Pluripotent Alternatives – Induced Pluripotent Stem Cells (iPSCs)

18

The Past, Present and Future of Induced Pluripotent Stem Cells

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1. Introduction

Our body is derived from only one cell, a fertilized egg. At the birth, the body consists of 220 kinds of somatic cells. The fertilized egg divides many times during development. The resulting cells differentiate into many kinds of somatic cells, and a fertilized egg can differentiate into all of the different types of cells, including intraembryonic and extraembryonic tissues. This ability is called totipotency. Fertilized eggs differentiate into various kinds of somatic cells. However, somatic cells do not divide and differentiate into other types of somatic cells after differentiation in a disorderly manner. There are two possibilities to explain this. First, somatic cells can completely lose their potential to differentiate into other kinds of cells during development, or second, they may retain their potential, but such potential may be suppressed after development. The studies to elucidate how these processes occur were the origin of reprogramming science and regenerative medicine.

In 1938, Spemann was the first person to carry out nuclear transplantation, but the experiment failed (Spemann, 1938). In 1952, Briggs and King transplanted the nucleus of a frog blastula into enucleated unfertilized eggs. The eggs developed into tadpoles. This was the first cloned animal with nuclear transplantation, and the origin of the cloning technique. An interesting discovery was that the later the nucleuses were taken during the developmental stage, the lower the efficiency of generating clone frogs. It was impossible to produce a cloned frog using the nucleus from a stage later than the development of a tailbud. At that time, they thought the information in the nucleus changed during development (Brigge & King, 1952). However, Gurdon arrived at a different conclusion from Briggs and King. He transplanted the nuclei of small intestinal epithelial cells into enucleated unfertilized eggs and obtained tadpoles (Gurdon, 1962). His data suggested that the nucleus of a somatic cell could be reprogrammed, and thereby regain the ability to differentiate into many kinds of cells. In 1997, the cloning of a sheep demonstrated that mammalian somatic cells could also be reprogrammed (Wilmut, 1997). These data suggested that the information in the nucleus did not change irreversibly during development, and indicated that somatic cells have the potentially ability to differentiate into other kinds of cells after development.

2. What are ES cells?

It was necessary for the growth of developmental engineering and reprogramming science to make cells that can easily expand and maintain the ability to differentiate into many kinds

of cells in a cell culture system. Embryonic stem (ES) cells fulfilled these characteristics. ES cells have two important abilities, self-renewal and pluripotency. The ability for self-renewal allows these cells to grow semipermanently. Pluripotency, as described above, is the potential for differentiation into many kinds of cells which make up the body, such as muscle cells, neural cells and so on. Mouse ES cells injected into mouse blastocysts contribute to the formation of all tissues in the body. The mice generated from embryos injected with ES cells are called chimeric mice. These abilities of the ES cells made it possible to generate a large number of any type of cells that is desired.

ES cells are established from fertilized eggs. The inner cell mass of blastocyst-stage embryos are transformed into ES cells. The fertilized egg is first cultured on feeder cells, which provide several necessary factors to the egg and ES cells. A few days later, the cells of the inner cell mass start to grow under culture conditions. ES cells are established from these growing cells. In 1981, mouse ES cells were established (Evans & Kaufman, 1981), and human ES cells were established in 1998 (Thomson et al 1998). Interestingly, the characteristics of human ES cells are different from those of mouse ES cells. The morphology of human ES cells was more like that of cynomolgus ES cells, which had been established several years before the mouse ES cells. Moreover, the optimal culture conditions differ between human ES cells and mouse ES cells. For example, leukemia inhibitory factor (LIF) and bone morphogenic protein (BMP) are important for maintaining the abilities of mouse ES cells in vitro. On the other hand, basic fibroblast growth factor (bFGF) and Activin A are required for the maintenance of human ES cells (Boiani & Schöler 2005).

Mouse ES cells are commonly used as a tool to generate transgenic and gene-targeted animals. These animal models have controbuted to the progress made in basic and medical sciences. Human pluripotent stem cells, including ES cells, are expected to be a good source of regenerative medicine, because of their outstanding capacities such as self-renewal and pluripotency.

3. Why generate artificial pluripotent stem cells?

There are several outstanding issues surrounding the use of ES cells for clinical applications. One of them is immunological rejection. ES cells are generated from fertilized eggs, which can have different immunizing antigens from the recipient who received the regenerative medicine developed using these cells. If the somatic cells from ES cells are transplanted into recipients, then the cells are rejected by the patient's own immune system. To overcome this problem, a new technique was developed. In this technique, the nuclei of an individual's somatic cells are transplanted into enucleated unfertilized eggs. The eggs can then be used to make ES cells expressing the individual's own immunizing antigens. These ES cells were called nuclear transfer ES cells (ntES cell), and the somatic cells derived from ntES cells are not rejected by the recipient after transplantation. These ntES cells have been established from not only mouse, but also from monkey cells (Rideout et al., 2000; Byrne et al., 2007). However, there have been no reports of human ntES cells. A likely reason for this is that the efficiency of generating ntES cells is very low, thus requiring a lot of embryos. This is not only a technical challange but also poses ethical problems. Generating human embryos for research is questionable and in case human nuclear transplanted embryos are implanted in a uterus, a cloned human would be generated.

Another ethical problem is the use of ES cells for clinical applications. To generate ES cells, it is necessary to either injure or break up embryos, which are the origin of human life. To

avoid these problems, ES cells were generated from embryos which arrested their own development or from poor-quality embryos generated for in vitro fertilization treatments. The embryos which would be discarded as of low quality for fertilization treatment are used to make ES cells. Moreover, ES cells can be generated from single blastomers of embryos, and the biopsied embryos still can grow normally (Chung et al., 2006; Klimanskaya et al., 2006; Chung et al., 2008). That is, ES cells can be generated without embryonic destruction. However, there are still discussions ongoing about how these "origins of human life" are handled by humans, and many countries have legislation preventing the development and use of ES cells. Therefore, it is necessary to be able to make pluripotent stem cells artificially for clinical applications. One of the major goals of nuclear reprogramming research is to generate ES-like cells by the conversion of somatic cells.

4. The search for reprogramming factors

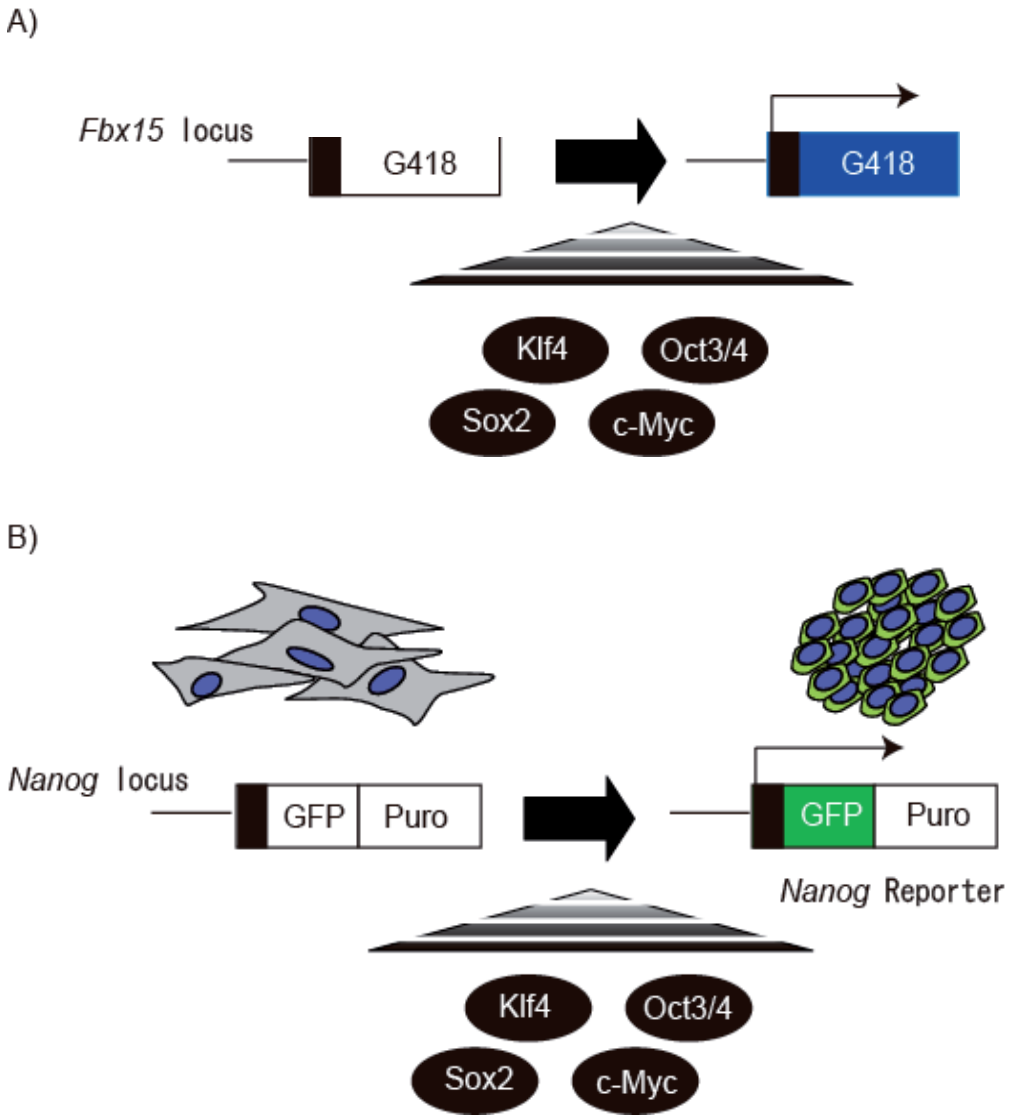
One of the most difficult points for finding ways to convert somatic cells to pluripotent stem cell was to identify reprogramming factors. There had been hints based on the previous research on ES cells. For example, it was known that ES cells could induce pluripotency in somatic cells. When mouse somatic cells were hybridized with mouse ES cells, the nuclei of the somatic cells were reprogrammed. The genes which were normally expressed specifically in ES cells started to be expressed from the genome of somatic cells after hybridization. These hybridized somatic cells could differentiate into all three germ layers. When human ES cells were used for hybridization, the nuclei of human somatic cells were also reprogrammed. These data demonstrated that there were factors (reprogramming factors) which could induce pluripotency in somatic cells that were present not only in oocytes, but also in ES cells.

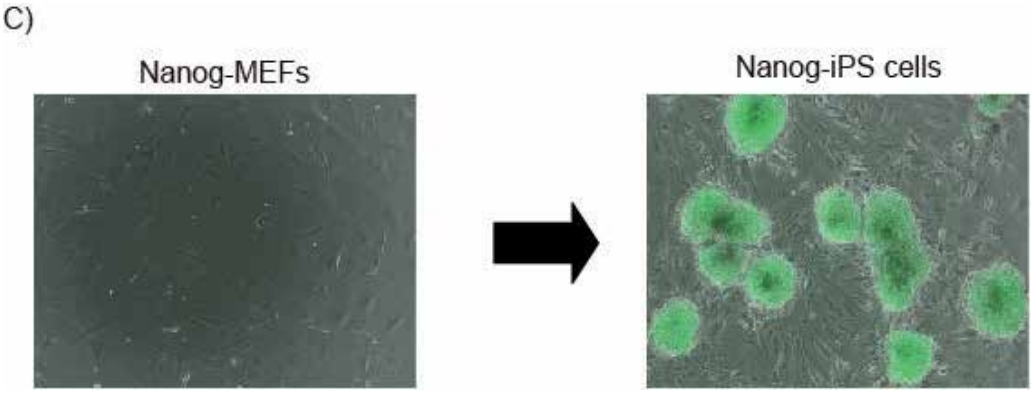
It was hypothesized that tsuch reprogramming factors would be important factors for maintaining pluripotency in ES cells, and that the identification of the factors required by ES cells would also indicate the factors required for the reprogramming of somatic cells. These factors would be expressed highly and specifically in ES cells. Next, Then gene expression pattern was compared between ES cells and somatic cells to narrow down the candidates of reprogramming factors using a computer database. The selected genes were named ES cell associated transcripts (ECATs). These ECATs were expressed highly and specifically in ES cells. They also were shown to play important roles in maintaining the properties of ES cells. For example, Nanog is one of the ECATs. In the absence of Nanog, mouse ES cells differentiate into visceral or parietal endoderm, and do not maintain the properties of ES cells. An overexpression of Nanog also maintains the self-renewal of ES cells, independent of LIF (Mitsui, et al., 2003; Chambers, et al., 2003).

In the 1990s, the transcription network involved in maintaining the pluripotency and self-renewal of ES cells gradually started to become clearer. Oct3/4 was discovered to be one of key factors that make ES cells unique. Oct3/4 is expressed in ES cells, germ cells and also differentiated cells. However, the expression level of Oct3/4 is strictly regulated strictly by the transcription network in ES cells. A mere 1.5-fold increase in the expression of Oct3/4 induces the differentiation of ES cells to primitive endoderm. A reduction in the expression of Oct3/4 by half leads ES cells to generate trophectoderm (Niwa et al, 2000).

Moreover, several oncogenes were also shown to be important for maintaining ES cells. The Myc family of genes plays an important role in maintaining ES cells. Max is an important partner required for the functions of Myc. If max is knocked out, Myc family genes such as

c-Myc, L-Myc and N-Myc cannot exert their effects. Max knockout ES cells cannot survive. Several genes were selected from the ECATs, important transcripts for ES cells and oncogenes as candidates of reprogramming factors. It was necessary to create an assay system which evaluated candidates reprogramming factors for their ability to reprogram somatic cells. Fbx15 is another of the ECATs. That is, Fbx15 is expressed specifically in ES cells, and not in somatic cells. Fibroblasts with a G418 antibiotic resistance gene in the Fbx15 locus were used for the assay system. Normal cells cannot survive in the presence of G418. If the fibroblasts are reprogrammed by the candidates, their Fbx15 locus is activated, the G418 resistance genes are expressed, and the fibroblasts are resistant to G418. The cells that were reprogrammed cells by the candidate could then be selected with G418 (Fig1.A).





A - An illustration of the Fbx15 reporter system. Fbx15 is a marker gene of ES cells which is specifically expressed in ES cells. Fbx15 is not expressed in fibroblasts. If fibroblasts are reprogrammed by reprogramming factors, then the Fbx15 locus is activated in the cells. These reprogrammed cells thereafter demonstrate resistance for G418; a tocix antibiotic to mammalian cells.
B - An illustration of the Nanog reporter system. The Nanog locus is inactivated in somatic cells. On the other hand, the Nanog locus is activated in reprogrammed cells. The reprogrammed cells are positive for GFP (green fluorescent protein) and also show resistance for Puromycin ,
C- MEFs and iPS cells carrying the Nanog reporter system. The iPS cells are positive for GFP driven by the Nanog reporter system.

Fig. 1. Reporter system

5. Creating the world's first iPS cells

Candidate reprogramming genes were introduced into mouse embryonic fibroblasts (MEF) carrying a Fbx15 reporter system. When 24 candidates were introduced into MEF at the same time, G418 resistant mouse ES-like colonies appeared about 2 weeks later. The 24 candidates were narrowed down to just 4 factors: Oct3/4, Sox2, klf4 and c-Myc. The cells reprogrammed from somatic cells by these four factors were named "induced pluripotent stem cells" (iPS cells). Their global gene expression patterns were similar to those of mouse ES cells. The proliferation of iPS cells was also similar to ES cells. The iPS cells can differentiate into all 3 germ layers in vitro and in vivo. The iPS cells generated using the Fbx15 reporter system could also contribute to mouse embryos, but the chimeric embryos did not survive until birth (Takahashi & Yamanaka, 2006). These data indicated that these first iPS cells had many features like ES cells, but were not completely ES cell like. These iPS cells were considered to be only partially reprogrammed, so the reporter system was improved to facilitate the development of completely reprogrammed iPS cells. Nanog and Oct3/4 are more tightly associated with pluripotency in ES cells than Fbx15. The iPS cells established using a Nanog or Oct3/4 reporter system (Fig1.B,C) contributed to chimeric mouse embryos which survived beyond birth, and these improved iPS cells contribute to the germline of chimeric mice (Okita et al.,2007; Wernig et al.,2007; Maherali et al., 2007) (Fig,2). Moreover, it was reported that cloned live pups could be generated using iPS cells by tetraploid complementation (Kang et al., 2009). These studies strongly suggest that mouse iPS cells are substantially comparable to mouse ES cells, at least in terms of their differentiation potential.

A - iPS cells are injected into blastocysts to make chimera mice. iPS cells are injected into mouse blastocysts (middle) using a micro manipulation system consisting of a holding- (left) and a transfer pipette (right)
B, C - Mouse iPS cells expressing red fluorescent proteins are injected into mouse blastocysts. The mouse iPS cells contribute to all tissues in the mice bodies. The right mouse pup is the chimera.

Fig. 2. Contribution of iPS cell to all tissues in chimera mice

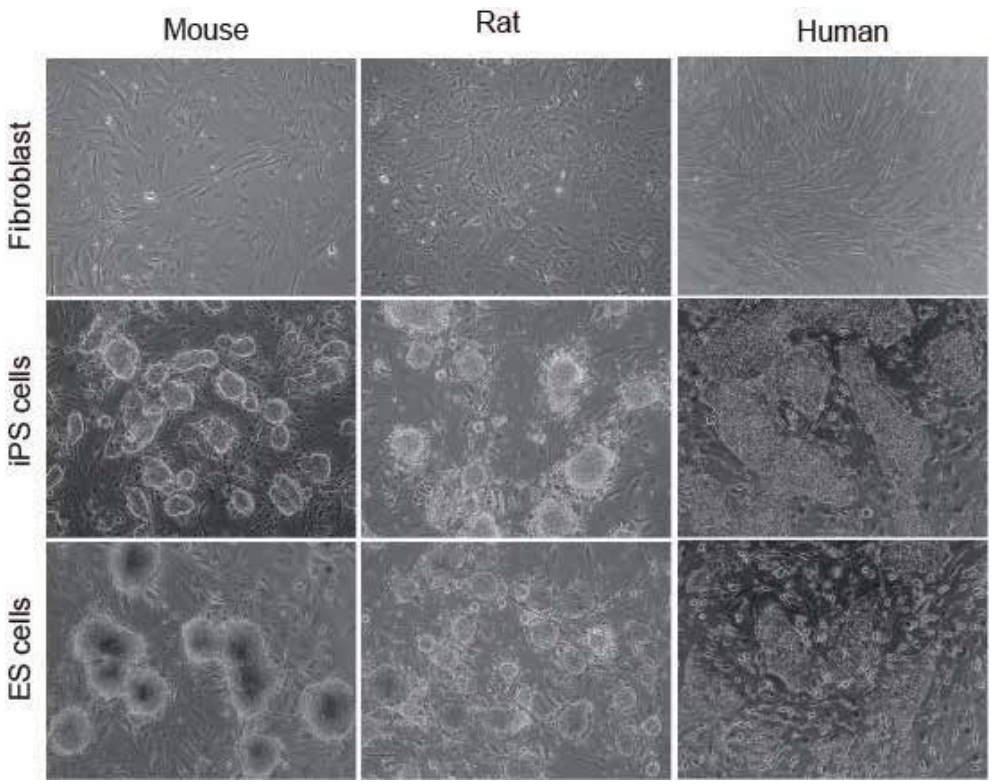
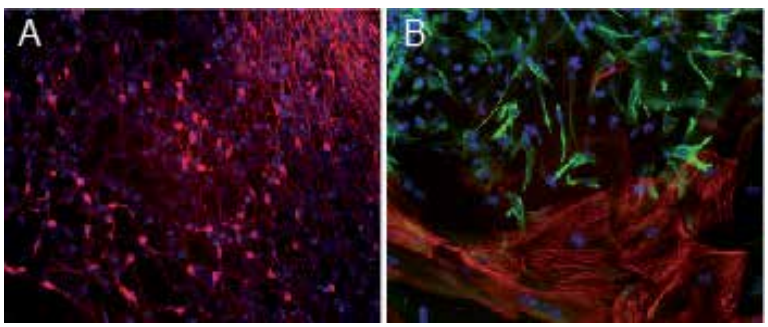


Fig. 3. Comparing the morphologies among the mouse, rat and human.
The mouse, rat and human morphologies of fibroblasts, iPS cells and ES cells. Mouse, rat and human iPS cell colonies are morphologically very similar to ES cell colonies. Mouse and at iPS and ES cell colonies are round shaped. On the other hand, human iPS and ES cell colonies are flat shaped and different for many properties compared to mouse and rat.

In 2007, Human iPS cells were established (Takahashi et al., 2007; Yu et al., 2007). This was only one year after the establishment of mouse iPS cells, which is remarkable considering that it took about 15 years to establish human ES cells after establishing mouse ES cells. The establishment of the human iPS cells was the result of the accumulation of knowledge regarding human ES cells and mouse iPS cell induction. Human iPS cells were established using two different combinations of reprogramming factors. Our group used Oct3/4, Sox2, and Klf4 with or without c-Myc. Another group used Oct3/4, Sox2, and Nanog, with or without Lin28 (Takahashi et al., 2007; Yu et al., 2007). Therefore, Oct3/4 and Sox2 were common between our combinations and the other group's combinations. Human iPS cells can differentiate into all 3 germ layers in vitro (Fig.4) and in vivo (Fig.5). Up to now, rat, monkey, pig, dog and rabbit iPS cells have been established, however, the germline transmission of these iPS cells has not yet been reported (Liu et al., 2008; Jing et al., 2008; Wenlin etal., 2008; Esteban et al.,2009; Zhao et al.,2009; Shimada et al,. 2009; Wu et al., 2010; Honda et al., 2010).



A - In vitro differentiation of human iPS cells into neurons (Red).
B - In vitro differentiation of human iPS cells into smooth (Red) and striated muscles (Green).
Fig. 4. Human iPS cells were differentiated in vitro.

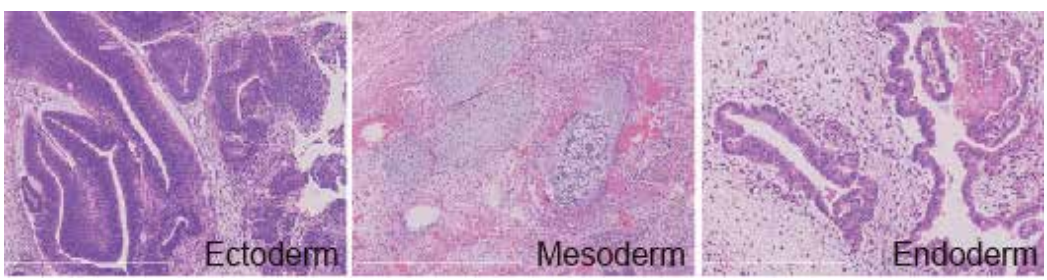


Fig. 5. In vivo differentiation of human iPS cells.

Human iPS cells are transplanted into testes of immunocompromised mice. After about 10 weeks, human iPS cells form teratomas. The teratomas are analysed histologically with haematoxylin and eosin staining. iPS cells are differentiated into all three germ layers.

6. Increasing the efficiency of generating iPS cells

In the beginning of iPS cell research, the generation efficiency was very low, when Oct3/4, Sox2 and klf4 were used. It was found that the addition of c-Myc increased the efficiency more than 100-fold. Although c-Myc is not essential for iPS cell induction, it is a very effective factor for increasing the efficiency. The stability of c-Myc is regulated by the glycogen synthase kinase 3 (GSK3), which negatively regulates the Wnt pathway. Phosphorylated c-Myc is rapidly degraded by the ubiquitin-proteosome pathway. Therefore, Wnt may enhance the generation efficiency of iPS cells with Oct3/4, Sox2 and klf4 (Marson et al., 2008). However, precisely role of c-Myc during iPS cell induction is still unclear. Even when iPS cells were induced with c-Myc, the overall efficiency calculated from the number of potentially reprogrammable cells was less than 1%. Therefore, further improvements in the reprogramming efficiency were needed. There are three main ways to increase this efficiency: inducing iPS cells with the help of chemicals, adding more reprogramming factors, and changing the combination of reprogramming factors.

6.1 Increasing the efficiency with chemical compounds

6.1.1 Chemicals affecting DNA and histone modifications

DNA and histone modifications regulate the gene expression patterns in cells. These modifications stably maintain the gene expression pattern to ensure the proper characteristics of the cells. During iPS cell generation, these modifications are changed dramatically (Deng et al., 2009). One of DNA methyltransferase inhibitors, 5-azacytidine, improved the efficiency of reprogramming by nuclear transfer. Several inhibitors of DNA methyltransferase, such as 5-azacytidine, BIX-01294, RG108, etc. improved the efficiency of iPS cell generation. Inhibitors of histone deacetylases, for example valproic acid (VPA), butyrate, and trichostatin A, also increased the iPS cell generation efficiency. iPS cells could be induced using just Oct3/4 and Sox2 with VPA (Xu et al., 2008; Shi et al., 2008; Huangfu et al., 2008; Danwei et al., 2008; Mali et al., 2010; Zhou et al., 2010). It was thought that the effects of these chemicals which change DNA and histone modifications was due to inhibition of genes expressed in somatic cells and the induction of those expressed in ES cells. However, these chemicals have low specificity. They change the global DNA and histone modifications. Therefore, they inhibit not only the expression of genes which define somatic cells, but also those which are important for ES and iPS cells. As a result, if the concentration of these chemicals, the length of the treatment, or the original somatic cells are different, these chemicals may either have no effect or may even decrease the efficiency of iPS cell generation.

6.1.2 Chemicals affecting molecular signaling pathways

The inhibition of the Tgf-β (transforming growth factor- β) pathway increases the efficiency of mouse iPS cell generation. This inhibition is effective during the early stage of iPS cell induction. It is thought that the mechanism of Tgf- β inhibition is as follows: Fibroblasts are mesenchymal cells, while iPS cells are epithelial cells. Fibroblasts need to be converted to epithelial cells during iPS cell induction (Payman et al., 2010). The Tgf- β pathway accelerates the epithelial-to-mesenchymal transition. Therefore, the inhibition of the Tgf- β pathway improves the iPS cell generation efficiency by accelerating the mesenchymal-to-epithelial transition (Maherali et al., 2009). The combined inhibition of the MAPK pathway and the Tgf- β pathway has a synergistic effect (Tongxiang et al., 2009) to generate human iPS cells. Moreover, using just an Oct3/4 transgene, mouse iPS cells can be generated from

neonatal human epithelial keratinocytes with a combination of compounds including sodium butyrate (a histone deacetylase inhibitor), PS48 (an activator of 3'-phosphoinositide-dependent kinase-1), A-83-01 (a TGF-β inhibitor) and PD0325901 (a MAPK inhibitor) (Zhou et al., 2011). The use of 8-bromoadenosine 3', 5'-cyclic monophosphate (8-Br-cAMP), a cyclic AMP analog, also improves human iPS cell induction efficiency (Wang & Adjaye, 2010). It is thought that 8-Br-cAMP exerts its pro-induction effect by decreasing the expression of p53 and increasing the expression of Cyclins.

6.2 Promoting the efficiency by adding more reprogramming factors

Suppressing p53 gene (TP53) expression enhances the efficiency of generating both mouse and human iPS cells (Kawamura et al., 2009; Rowland et al., 2009; Hong et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal Banito et al., 2009). The p21 gene is one of the p53 downstream targets. The p21 protein binds and inactivates the G1/S-cyclin dependent kinase (cdk) and S-Cdk complexes to stop the cell cycle. Overexpression of p21 negated the amplifying effect of p53 suppression during iPS cell transduction. Inhibition of the retinoblastoma protein (RB) also improves the efficiency of iPS cell generation. RB inhibits E2F, which accelerates the transcription of S-phase genes such as Cyclin E and Cyclin A. The complex of G1-cdk and Cyclin D1 phosphorylate RB. Phosphorylated RB cannot bind and inhibit E2F, allowing the cell cycle to progress from the G1 phase to the S phase. Cyclin D1 also increases the iPS cell generation efficiency. Rem2 GTPase is one of the suppressors of the p53 pathway. Rem2 is an important player to maintain human ES cells. Rem2 enhances the reprogramming by regulating p53 and cyclinD1 (Edel et al., 2010). These data suggest that accelerating cell proliferation enhances the iPS cell generation efficiency. Promoting cell proliferation accelerates the stochastic process of reprogramming. However it is thought that the amplifying effect of p53 inhibition does not only result from the acceleration of cell cycle. It is known that p53 directly binds to the promoter region of the Nanog gene and suppresses its expression in mouse ES cells (Sabapathy et al., 1997; Qin et al., 2007). There is a possibility that p53 directly regulates the gene expression pattern during iPS cell generation.

Lin28 is also effective for increasing the efficiency of reprogramming. Lin28 was used to generate some of the first human iPS cells. Like c-Myc, Lin28 is effective, but not essential, for the generation of human iPS cells. Lin28 is also effective in combination with Oct3/4, Sox2, Klf4 and c-Myc. Lin28 can interfere with the maturation of miRNA and promote their degradation by uridylation of miRNA (Heo et all., 2008 2009). Let-7 is one of the Lin28-associated miRNAs, and regulates the translation of several genes including c-Myc, K-Ras, Cyclin D1 and Hmga2. However, the mechanism(s) underlying the effects of Lin28 are still unclear (Kim et al., 2009; Viswanathan et al., 2009).

Tbx3 also improves the efficiency of mouse iPS cell generation. The association between Tbx3, Nanog and Tcf3 is important for pluripotency and self-renew of ES cells. Moreover, the efficiency of germline transmission of mouse iPS cells with Tbx-3 is higher than that with just Oct3/4, Sox2 and Klf4 (Jianyong et al ., 2010).

E-Cadherin also enhanced the mouse reprogramming efficiency in combination with Oct3/4, Sox2 and Klf4 or Oct3/4, Sox2, Klf4 and c-Myc. E-Cadherin is a molecule that mediates cell-cell interactions, and is upregulated during iPS cell induction. An antibody against the extracellular domain of E-cadherin reduced the efficiency of iPS cell generation. These data indicated that the cell-cell contact mediated by E-cadherin plays an important role in reprogramming (Chen et al., 2010).

In addition, some micro RNAs also enhance the efficiency of iPS cell generation. The mir-290 cluster is highly expressed in mouse ES cells. The efficiency of mouse iPS cell generation with Oct3/4, Sox2 and klf4 was improved by miR-291-3p, miR-294, miR-295, which are included in the cluster of mir-290. However, they are not effective with c-Myc. While c-Myc binds to the promoter of the mir-290 cluster, introducing c-Myc could not induce the expression of the mir-290 cluster in fibroblasts. The promoter of the mir-290 cluster is regulated negatively by histone modifications in fibroblasts. These data suggest that the mir-290 cluster is one of targets which are regulated by histone modification (Robert et al., 2009).

6.3 Promoting the efficiency by using different combinations of reprogramming factors

L-Myc is one of Myc family members. L-Myc is more effective for iPS cell induction than c-Myc. Moreover, mouse iPS cells established with L-Myc contribute to the germline more efficiently than iPS cells with c-Myc (Nakagawa et al., 2010).

Utf1 also improves the efficiency of mouse iPS cell generation. The number of mouse iPS colonies generated using a combination of Oct3/4, Sox2, klf4 and Utf1 was 10 times higher than that with Oct3/4, Sox2, Klf4 and c-Myc (Zhao et al., 2008).

Recently, both human and mouse iPS cells were established using just the miR-302/367 cluster in the absence of any other reprogramming factors. The miR-302/367 cluster is highly expressed in ES and iPS cells, and is one of the target of Oct3/4 and Sox2. The use of just the miR302/367 cluster reprogrammed both human and mouse cells more efficiently and rapidly than the combination of Oct3/4, Sox2, Klf4 and c-Myc (Anokye-Danso et al., 2011).

7. The methods for generating iPS cells

There are two main methods to generate iPS cells. These are the genomic integration method and the genomic integration-free method.

7.1 The Genomic integration method

7.1.1 Retrovirus systems

Retrovirus systems were used to generate the world's first mouse and human iPS cells (Takahashi et al., 2006, 2007). The reprogramming factors introduced by a retrovirus system are strongly and stably expressed in somatic cells. The retrovirus system can efficiently introduce several reprogramming factors into cells at the same time. For these reasons, a retrovirus system efficiently generates iPS cells. Therefore, retrovirus systems are suitable for investigating the mechanism of iPS cell induction. Moreover, reprogramming factors introduced into somatic cells by the retrovirus system are gradually silenced during the reprogramming progress (Okita et al., 2007). This is good for iPS cell generation, because the expression of reprogramming factors in reprogrammed cells sometimes induces differentiation and cell death. Moreover, expressing transgenic reprogramming factors into reprogrammed cells can induce tumorigenicity.

7.1.2 Lentivirus systems

Lentiviral vectors were also used to generate some of the first human iPS cells (Yu et al., 2007). Lentivirus can infect not only the dividing cells, but also non-dividing cells. Lentivirus infection therefore occurs independent of cell division. The reprogramming factors introduced by lentiviruses are stably expressed and less silenced than those

introduced by retroviruses. Thus, drug-inducible transgene expression systems were made because of these characteristics of lentiviral vectors to investigate the mechanism of iPS cell induction (Hockemeyer et al., 2008; Maherali et al., 2008).

7.2 Genomic integration-free method

The genes introduced by either retroviral or lentiviral vectors permanently integrate into the genome. Such integration increases the risk of tumorigenicity for two reasons: the first reason is that the integration can interrupt genes and gene promoters; the second reason is that the integrated factors could be reactivated unexpectedly by nearby promoters. For clinical applications, these issues will need to be overcome. Recently, two principal ways to generate iPS cells without genomic integration were developed. One of them is removing the genomic integration after establishing iPS cells. The other is establishing iPS cells without integration vectors.

7.2.1 Removing genomic integration after establishing iPS cells

Cre-mediated recombination can be used to remove transgenes from the iPS cell genome. Human iPS cells have been established using lentiviral constructs including loxP sequences in their long terminal repeat (LTR). Established iPS cells can be treated with Cre recombinase in order to excise the lentiviral cassettes. However, the LTR sequence still remains in the genome (Soldner et al., 2009).

A "piggybac" transposon vector system can also solve this problem. Using this system, the integrated reprogramming factor can be removed seamlessly. Transposase has activities for both the insertion and excision of transposon vectors by recognizing the TTAA tetra-nucleotide sequence in the host genome (Kaji et al., 2009 Woltjen et al., 2009 Yusa et al., 2009).

7.2.2 Generating iPS cells without genomic integration of reprogramming factors

The first integration-free iPS cells were generated from mouse somatic cells with adenoviral vectors or conventional expression vectors (Okita et al, 2008; Stadtfeld et al, 2008). Recently, episomal vectors were used to generate human iPS cells (Yu et al., 2009). Episomal vectors consist of the replication origin and an Epstein-Barr nuclear antigen (EBNA). The EBNA vector can self-replicate and maintain the expression of transgenic reprogramming factors without genomic integration. However, the efficiency of reprogramming with episomal vectors was 10 times less than that with integration vectors. However, the efficiency was recently improved using episomal vectors encoding Oct3/4, Sox2, Klf4, L-Myc, Lin28 and a short hairpin RNA against p53 (Okita et al., 2011). This method is very promising for clinical application because the possibility of episomal vector genomic integration is very low, although it is still not zero. There is a little possibility that this vector may accidentally integrate into the genome accidentally. This possibility should be kept in mind when planning trial for clinical application.

The Sendai RNA virus is also a promising vector that can be used to generate clinical-grade iPS cells. This virus does not enter into the nucleus for replication, transcription or translation. Therefore, there are no risks of insertion of reprogramming factors introduced by this virus. The transduction efficiency using this virus is comparable to that using retrovirus system. iPS cells were also established from less than 1 ml of peripheral blood using this system(Seki et al. 2010). If deficient sendai viral vectors are used for iPS induction, the vectors can be removed by siRNA (Nishimura et al., 2011).

There are also other ways to establish iPS cells virus-free. In one study, the Oct3/4, Sox2, Klf4 and c-Myc proteins were modified so that they could easily pass through the cell membrane. Both human and mouse iPS cells were established using these proteins (Hongyan et al., 2009; Dohoon et al., 2009). Another recent method used synthetic modified mRNA to generate iPS cells. RNA usually is unstable, and cells with foreign RNA are usually destroyed by the interferon response. The authors modified the medium and RNA to reduce the interferon response and improve RNA stability. The reprogramming factor from the introduced RNA was expressed stably and highly in the cells. Using this method, the possibility of genomic integration is very low, because of the nature of RNA. However, the possibility of cell damage in iPS cells generated using this method is slightly increased through the stressful induction method that requires consecutive introduction of RNA into cells for 2 weeks and artificial inhibition of the cell interferon response. A newer method to establish both human and mouse iPS cells used just miRNA, miR-200c, 302 and 369. These iPS cells were named mi-iPS cells (Miyoshi et al., 2011).
Regardless of the method used to generate iPS cells, the quality of the cells should be examined from various points of view and in depth before using the iPS cells for clinical applications.

8. Applications of iPS cells

The major benefit of iPS cells is that they make it possible to obtain differentiated cells in the required quantities. It is expected that iPS cells can be used for regenerative medicine and drug discovery (Fig. 6).

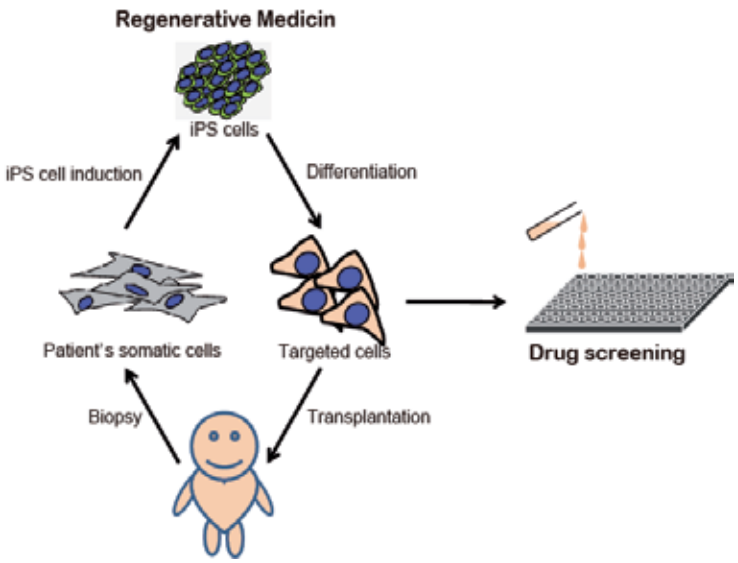


Fig. 6. A Schematic illustration for the application of iPS cells

iPS cells derived from patients are useful for regenerative medicine and drug discovery. Somatic cells are taken by biopsy from patients. Patient specific iPS cells are then established from the somatic cells and differentiated into targeted cells. If the targeted cells are transplanted into a disease site, then this would bethat is very promising for regenerative

medicine. Moreover, if disease phenotypes are reproduced using patient specific iPS cells in vitro, patient specific responses to drugs could be evaluated for individual therapies.

8.1 Using iPS cells for regenerative medicine

Differentiated cells from iPS cells derived from the recipients are not rejected by the immune system upon transplantation, because they have the same immune markers as the recipient. Hence, it is expected that it will be possible to use iPS cells for cell therapy and regenerative medicine. Under this scheme, iPS cells are differentiated into the targeted cells, and the differentiated cells are then implanted into the diseased area where they can improve the patient's symptoms. Experiment procedures utilizing lab animals have already proven the effectiveness of this scheme for cell therapy. For example, in a rat model of Parkinson's disease, the implantation of dopamine-producing neurons that were differentiated from iPS cells led to a clear improvement in the symptoms (Hargus et al., 2010).

It is also possible to use an approach which is a combination of cell therapy and gene therapy. Sickle cell anemia is a genetic blood disorder. The patient's red blood cells are abnormally sickle-shaped, thus decreasing the oxygen transport ability of these patients' red blood cells compared to unaffected individuals. This abnormality is caused by a mutation in one gene. The mutation was repaired using gene therapy technology in iPS cells derived from model mice. The repaired iPS cells were differentiated into hematopoietic stem cells. The hematopoietic stem cells transplanted into the model mice started to generate normal red blood cells and cure the disease (Hanna et al., 2007). The effectiveness of these procedures has not yet been examined in humans. However, Geron and Advanced Cell Technology announced that they plan to start clinical trials of transplantation of cells derived from ES cells for spinal cord injury and muscular degeneration, respectively. The current advances in the differentiation induction technology are likely to facilitate human studies. For example, the three dimensional structure of the neural retina differentiated from mouse ES cells was recently demonstrated (Eiraku et al., 2011). The combinations of the various differentiation technologies will likely provide new sources and methods for regenerative medicine.

8.2 Concerns about using iPS cells for regenerative medicine

Before using iPS cells for clinical applications, the safety of iPS cells should be sufficiently verified. In the paper introduced previously about curing Parkinson disease model mice, the authors suggested several problems that need to be overcome before this strategy can be clinically used. The major problem was that the model mice transplanted with the cells differentiated from iPS cells eventually developed teratomas (Hargus et al., 2010). The formation of teratomas in donor mice was caused by the undifferentiated cells that were present in the differentiated cells used for transplantation. It will therefore be necessary to develop an efficient differentiation system that allows for the invariably selection of targeted somatic cells and complete removal of all residual undifferentiated cells. In fact, attempts have already been made to select or generate iPS cells which can easily be differentiated into targeted cells. Recently, our group demonstrated that iPS cells have various differentiation potentials, and we found that several iPS cell clones were highly resistant to neural differentiation (Miura et al., 2009). Additional, studies to identify the genes responsible for the resistance are currently underway.

In addition, it was reported iPS cells carry epigenetic memory of the original somatic cells during early passages. This memory affects their differentiation potential. For example, iPS cells from B cells differentiated into blood progenitor cells more efficiently than iPS cells derived from fibroblasts (Kim et al., 2010; Polo et al., 2010). The origin of iPS cells was therefore reflected in the differentiation potential of the iPS cells. Further accumulation of this knowledge will help create smooth path toward the clinical application of iPS cells.

8.3 Using iPS cells to understand pathological conditions and for drug discovery

Utilizing the advantages provided by the iPS cell technology, differentiated cells which are difficult to harvest from patients and culture in vitro can now be obtained in sufficient quantities for researchers to study the pathogenesis of diseases and to perform drug screening.

The first disease-specific iPS cells were established from patients with familial amyotrophic lateral sclerosis (ALS). However, the authors could not reproduce disease phenotypes using differentiated cells from ALS-iPS cells in vitro (Dimos et al., 2008). The first in vitro reproduction of a disease phenotype was achieved with iPS cells derived from a spinal muscular atrophy (SMA) patient. The motor neurons differentiated from SMA iPS cells exhibited the specific phenotype, such as a decreased number and size of neurons (Ebert et al., 2009). Recently, many disease models have been generated in vitro with iPS cells from patients with Familial dysautonomia (FD), myeloproliferative disorders, Dyskeratosis congenital, Leopard syndrome, Rett syndrome (REFS) and further diseases (Lee et al., 2009; Ye et al., 2009; Suneet et al., 2010; Carvajal et al., 2010; Marchetto et al., 2010).

The effects and side effects of drugs are generally tested using laboratory animals, primarily rodents. However, the effects of drugs are different between humans and animals, and such studies were one of obstacle to developing new therapeutic agents. Moreover, using laboratory animals is cost- and time-intensive. The ability to test new agents on specific types of cells will greatly facilitate research on drug effects and toxicity.

It was previously very difficult to collect sufficient amounts of targeted cells from patients for analyses until the iPS cell breakthrough. Many disease models using iPS cells will likely be established in the near future, because the safety of iPS cells (with regard to teratoma formation) is not an issue affecting basic research involving these cells. Therefore, iPS cell technologies will greatly facilitate our understanding of the pathogenesis of various diseases and will help in the development of novel treatments.

9. Conclusion

About sixty years ago, humans started to deeply and systematically investigate living things from a molecular point of view. The major purposes were to achieve a better understanding of the basic function of living things and to try to regulate and use these findings to enhance human lives. The biological systems improved by nature for several billion years are much more efficient than the engineered systems developed by humans. For example, fireflies emit thermal free light while producing their fluorescence, while electric lights produce heat. This indicates that biological systems are very efficient. Understanding and using these biological systems can therefore have a major impact on the quality of human life.

The development of iPS cells is a prime example of using such biological systems for human benefit. The development of iPS cells has demonstrated that the characteristics of differentiated cells could be changed artificially by employing appropriate factors and

methods. Recently, new direct reprogramming technologies have been developed, which allowed somatic cells to be directly reprogrammed into targeted somatic cells without involving iPS cells.

However, precisely what occurs during iPS cell inductionstill remains unclear. Human fertilized eggs differentiate into somatic cells for several months in the mother's womb. During the induction of iPS cells, the somatic cells are artificially induced to regress into pluripotent stem cells within just a few weeks. There is a possibility that abnormalities are accumulated in iPS cells due to artificial reprogramming stresses. It will be necessary to uncover the full mechanism of iPS cell induction, and many questions remain to be answered, including: Exactly what is happening during iPS cell induction? Can abnormalities of cells be caused by what is happening during iPS induction? Moreover, it is also important to evaluate the established iPS cells in comparison to ES cells. Such research will help pave the way for iPS cells to move from a scientific finding to a medical revolution.

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New Techniques in the Generation of Induced Pluripotent Stem Cells

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1. Introduction

Pluripotent stem cells have the ability to differentiate into cells of the three primary germ layer lineages, ectoderm, mesoderm and endoderm. The most studied type of pluripotent stem cells are embryonic stem cells (ESC), cells derived from the inner cell mass of embryos at the blastocyst stage of development (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). The pluripotent property of human embryonic stem cells (hESC) makes them useful for the development of cellular therapies to replace diseased or degenerated cells in the body. Moreover, hESC also possess the ability to propagate indefinitely *in vitro* while maintaining a normal karyotype, and thus can provide an unlimited source of cells for the development of cell replacement therapies (Pera et al., 2000). However, one of the major hurdles in hESC research has been the ethical implications of using stem cells derived from embryos. Furthermore, generation of patient-specific stem cell lines may overcome some of the issues associated with immuno-compatibility in cell replacement therapy.
The breakthrough studies conducted by Shinya Yamanaka's group demonstrated direct reprogramming of mouse or human fibroblasts back to pluripotent cells, creating so-called induced pluripotent stem cells (iPSC) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Studies of iPSC revolutionized stem cell research by creating a more reproducible method to generate sufficient amounts of patient-specific pluripotent cells and bypassing the ethical implications surrounding research utilizing human embryos. iPSC also provide an alternative approach to generate disease-specific lines for mechanistic studies in disease modeling, as well as high throughput screening for drug discovery or toxicology studies (Amabile and Meissner, 2009). As the area of iPSC research is rapidly evolving, this review aims to summarize and discuss the current techniques used for the generation of iPSC.

2. Reprogramming factors used in generation of induced pluripotent stem cells (iPSC)

The initial derivation of iPSC by Shinya Yamanaka's group was achieved by overexpressing four transcription factors first in mouse and then human fibroblasts, namely Octamer-binding transcription factor 4 (Oct4), Sex-determining region Y HMG box 2 (Sox2), Krüppel-like factor 4 (Klf4) and v-myc myelocytomatosis viral oncogene homolog (c-Myc), often

referred to as the 'Yamanaka factors' (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Alternatively, a study from James Thomson's lab identified a different combination of factors for the generation of human iPSC, using Oct4, Sox2, Nanog and Lin28 (Yu et al., 2007). Subsequent reports from many labs have contributed to a growing list of reprogramming factors used for iPSC generation, including Estrogen-related receptor beta (Esrrb), Sal-like 4 (Sall4), microRNAs (miRNA), simian virus 40 large-T (SV40LT) antigen and human telomerase reverse transcriptase (hTERT). This section will provide a background of our understanding of these reprogramming factors in regulating the cell fate of pluripotent stem cells and discuss their role during direct somatic cell reprogramming. Other strategies to enhance reprogramming efficiency will also be discussed, such as supplementation with small molecules as well as knockdown of p53, p21 and p16.

2.1 Oct4

Oct4 was one of the first transcription factors identified to be a master regulator of cellular pluripotency (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1989). During mouse development *in vivo*, Oct4 expression is restricted to the inner cell mass, primitive ectoderm and primordial germ cells (Pesce and Scholer, 2001). Similarly *in vitro,* ESC and embryonal carcinoma cells (ECC) have high expression of Oct4, which is reduced upon their differentiation (Assou et al., 2007; Rosner et al., 1990).

Although expression of Oct4 is fundamental for the maintenance of pluripotency and development of the inner cell mass in mice (Nichols et al., 1998), complex regulation of its precise level is required to prevent cells from differentiating into other lineages. A transient increase in endogenous Oct4 levels has been observed upon mesodermal differentiation of mouse embryonic stem cells (mESC) (Zeineddine et al., 2006). Furthermore in various over-expression studies, an increase in Oct4 expression can cause mESC to differentiate into endoderm, mesoderm and neuroectoderm lineages (Niwa et al., 2000; Shimozaki et al., 2003; Zeineddine et al., 2006). On the other hand, repression of Oct4 levels results in a loss of pluripotency and promotes trophectodermal differentiation in mESC and hESC (Matin et al., 2004; Niwa et al., 2000). Consistent with these studies, Oct4 has been shown to directly inhibit the expression of major trophectoderm differentiation regulators such as caudal type homeobox 2 (Cdx2) and Eomesdermin (Eomes) (Liu et al., 1997; Liu and Roberts, 1996; Niwa et al., 2005). Together these studies highlight the significance of the critical range of Oct4 level required to maintain ESC pluripotency (Niwa et al., 2000).

As a master regulator of pluripotency, Oct4 was one of the original four factors utilized by Yamanaka and colleagues in the generation of iPSC in both mouse and human (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). As seen in Oct4 over-expression and down-regulation experiments in ESC, the precise level of Oct4 in combination with other reprogramming factors is also essential for efficient generation of iPSC (Papapetrou et al., 2009). Using bicistronic vectors, Papapetrou *et al*. (2009) showed that a 3-fold higher expression of Oct4 compared to Sox2, Klf4 and c-Myc enhanced iPSC generation (Papapetrou et al., 2009). Interestingly, overexpression of Oct4 alone was sufficient to induce reprogramming in neural stem cells that already express high endogenous level of Sox2, c-Myc and Klf4 (Kim et al., 2009b; Kim et al., 2009c). To date, most protocols for generation of iPSC require ectopic expression of Oct4, underlying the important role of Oct4 during direct somatic cell reprogramming (Feng et al., 2009b). Recently, Heng *et al*. (2010) demonstrated that an orphan nuclear receptor Nr5a2 can functionally replace Oct4 in the generation of

mouse iPSC (Heng et al., 2010). However, the precise role of Nr5a2 in regulating cell fate in pluripotent stem cells remains unclear. Further research is also needed to confirm the ability of Nr5a2 to replace Oct4 in human iPSC generation.

2.2 Sox2

Sox2 is an important transcription factor in pluripotent stem cells as well as precursor cells of the neural compartment. It is expressed in the inner cell mass, epiblast and extraembryonic ectodermal cells during mouse embryo development (Avilion et al., 2003; Miyagi et al., 2004). Unlike Oct4, Sox2 expression is maintained in neural stem cells (Ellis et al., 2004; Graham et al., 2003) and over-expression of Sox2 favors neural differentiation in mESC (Kopp et al., 2008; Zhao et al., 2004). Sox2 is known to interact with several binding partners, including Oct4 in the maintenance of pluripotency (Yuan et al., 1995). In a genome-wide chromatin immunoprecipitation study in hESC, Sox2 and Oct4 were found to share many target genes, many of which are transcription factors important in development (Boyer et al., 2005).

Importantly, Sox2 was shown to be indispensable for maintaining pluripotency. Sox2 knockout mouse embryos are unable to form an epiblast and fail to develop past the implantation stage (Avilion et al., 2003). Down-regulation of Sox2 in mESC and hESC results in loss of pluripotency and differentiation towards the trophectoderm cell lineage (Adachi et al., 2010; Fong et al., 2008; Li et al., 2007; Masui et al., 2007). Somewhat surprising was the finding that expression of many Sox2 target genes were not affected by the loss of Sox2 (Masui et al., 2007). The authors in this study suggested potential compensation of Sox2 function by other members of the Sox family. Consistent with this idea, Nakagawa *et al*. (2008) demonstrated that Sox1, Sox3, Sox15 and Sox18 have the ability to replace Sox2 to some extent in iPSC generation (Nakagawa et al., 2008). To date, Sox2 and Oct4 remain to be the two fundamental reprogramming factors and are widely used in various protocols to generate iPSC. Similar to Oct4, it should be noted that ectopic expression of Sox2 can be omitted in the generation of iPSC, if the starting cell type expresses substantial levels of Sox2 (Utikal et al., 2009a).

2.3 Nanog

Nanog is a homeodomain protein that is widely considered as a master regulator for stem cell pluripotency (Chambers et al., 2003; Mitsui et al., 2003). Nanog expression is restricted to the inner cell mass, epiblast and primordial germ cells in the early embryo, as well as a number of pluripotent cells lines such as ESC, ECC and embryonic germ cells (Chambers et al., 2003). During embryo development, Nanog plays a role in suppressing Cdx2, a master regulator of trophectoderm differentiation, and in turn suppression of Cdx2 specifies the inner cell mass fate (Chen et al., 2009). Moreover, Nanog can also physically interact with Oct4 (Wang et al., 2006) and cooperates extensively with Oct4 and Sox2 to form an autoregulated core-transcriptional network that maintain stem cell pluripotency (Boyer et al., 2005; Chen et al., 2008; Kim et al., 2008).

Unlike Oct4 or Sox2, sustained expression of Nanog renders mESC and hESC resistant to differentiation (Chambers et al., 2003; Darr et al., 2006; Ivanova et al., 2006). However in the absence of Oct4, Nanog alone is not sufficient to maintain mESC self-renewal, suggesting that Nanog plays a subservient role in maintaining self-renewal (Chambers et al., 2003). This is also supported by evidence from Nanog knockdown studies. Although early studies

suggested that a reduction of Nanog resulted in differentiation of mESC and hESC (Fong et al., 2008; Hyslop et al., 2005; Ivanova et al., 2006; Mitsui et al., 2003), it was later discovered that transient down-regulation of Nanog can be reversible and does not necessarily mark commitment to differentiation (Chambers et al., 2007). In this respect, mESC can remain undifferentiated in the absence of Nanog, but are more prone to differentiation (Chambers et al., 2007).

Given the important role of Nanog in establishing cell pluripotency, it was somewhat surprising that the initial derivation of iPSC could be achieved without the ectopic expression of Nanog (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). However, Nanog has proved to be a valuable marker for identification of fully reprogrammed iPSC that are germline competent (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). Furthermore, ectopic expression of Nanog in combination with Oct4, Sox2, Klf4 and c-Myc seems to accelerate the reprogramming kinetics of somatic cells to iPSC, but has no effect on the overall reprogramming efficiency (Hanna et al., 2009).

2.4 Klf4

Klf4 and its family members have emerged as important regulators for maintaining pluripotency. Klf4 belongs to the Krüppel-like factor (Klf) family of zinc finger transcription factors. Klf4 can act as an oncogene or a tumor suppressor gene depending on the physiological context (McConnell et al., 2007; Rowland and Peeper, 2006). Klf4 is usually expressed in adult tissues that possess some degree of regenerative capability, including intestine, gut, skin and testis (Nandan and Yang, 2009). Li *et al*. (2005) provided the first evidence that Klf4 plays a role in regulating stem cell pluripotency, by showing that overexpression of Klf4 prevents differentiation of mESC into erythroid progenitors (Li et al., 2005). In conjunction with Oct4, Sox2 and c-Myc, Klf4 was among the first factors to be used to generate iPSC (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). However, the current view is that Klf4 acts only as a secondary factor to enhance somatic cell reprogramming, as iPSC can be generated without Klf4 using a different combination of reprogramming factors (Yu et al., 2007). Klf4 was later discovered to be an important component of the core transcriptional network that regulates expression of Oct4, Sox2, Nanog, Myc and also Klf4 itself (Chen et al., 2008; Kim et al., 2008). Furthermore, Klf4 can directly interact and cooperate with Oct4 and Sox2 to activate a subset of ESC specific genes, including Nanog and Lefty1 (Nakatake et al., 2006; Wei et al., 2009). Klf4 also acts to inhibit apoptosis by suppressing p53 (Rowland et al., 2005), which helps to reprogram somatic cells to a pluripotent state (See discussion below).

Importantly, other Klf family members including Klf1, Klf2 or Klf5, can substitute for Klf4 in iPSC generation (Nakagawa et al., 2008), which suggests that functional redundancies exist among the Klf family members in establishing cell pluripotency. This also explains the observation that Klf4 knockdown in mESC exhibited no obvious phenotype (Jiang et al., 2008; Nakatake et al., 2006), whereas triple knockdown of Klf2, Klf4 and Klf5 resulted in rapid differentiation of mESC (Jiang et al., 2008). However, isoform-specific functions of Klf4 and Klf5 are also observed. Previous studies showed that Klf5 knockout mice result in embryo lethality and defects in implantation (Ema et al., 2008), whereas Klf4 knockout mice are normal during early embryo development but die soon after birth due to loss of skin barrier function (Segre et al., 1999). Furthermore, Ema *et al*. (2008) demonstrated that knocking out Klf5 in mESC results in spontaneous differentiation. Although introduction of

Klf4 can rescue the spontaneous differentiation phenotype, proliferation is significantly decreased (Ema et al., 2008). Further studies are needed to dissect the precise roles of different Klf members in regulating cell pluripotency.

2.5 Lin28

Lin28 encodes for a cytoplasmic RNA binding protein that acts as a translational enhancer (Polesskaya et al., 2007). It was first identified as a heterochronic gene that regulates the developmental timing pathway in *Caenorhabditis elegans* (Moss et al., 1997). A previous transcriptome study has shown that Lin28 is a hESC-specific gene, suggesting that it may play a role in regulating stem cell pluripotency (Richards et al., 2004). However, functional studies of Lin28 in hESC and mESC yielded opposing results. In mESC, Lin28 knockdown resulted in decreased cell proliferation while overexpression of Lin28 enhanced cell proliferation (Xu et al., 2009a). In sharp contrast, Lin28 knockdown in hESC had no obvious phenotype, whereas Lin28 overexpression reduced proliferation and promoted extraembryonic endoderm differentiation (Darr and Benvenisty, 2009). Further studies are clearly needed to elucidate whether Lin28 has a different role in maintenance of pluripotent stem cells in mice and humans.

Lin28 was first used in combination with Nanog, Oct4 and Sox2 to generate human iPSC, acting as an enhancer for somatic cell reprogramming much like Klf4 (Yu et al., 2007). However, it remains unclear how Lin28 contributes to induction of pluripotency. It was demonstrated that Lin28 can block the processing of let7 microRNA family members, a group of pro-differentiation microRNA that also act as tumor suppressors (Melton et al., 2010; Viswanathan et al., 2008). Members of the let7 microRNA family have been shown to repress expression of oncogenes such as c-Myc and Ras. Hence, down-regulation of let7 by Lin28 could increase cell proliferation and drive cellular transformation (Viswanathan et al., 2009). Consistent with this idea, a recent study in mice demonstrated that Lin28 accelerates reprogramming kinetics by enhancing cell proliferation (Hanna et al., 2009). Another proposed mechanism of Lin28 action is that it can selectively regulate gene expression at a post-transcriptional level, enhancing translation of anti-differentiation mRNAs while degrading pro-differentiation mRNAs to maintain pluripotency. A previous study demonstrated that Lin28 can reside in polysomal ribosome fractions, in which mRNAs are translated (Balzer and Moss, 2007). Indeed, Lin28 has been shown to bind directly to Oct4 mRNA in hESC to facilitate translation via interaction with RNA helicase A (Qiu et al., 2010). Lin28 can also reside in P-bodies, in which mRNAs are degraded (Balzer and Moss, 2007). Therefore, it remains speculative that Lin28 may be able to selectively degrade certain pro-differentiation mRNA to support stem cell pluripotency. Further studies are needed to confirm this hypothesis.

2.6 c-Myc

The basic helix–loop–helix/leucine zipper transcription factor c-Myc has a well documented role in cellular transformation and tumor progression, by controlling cell cycle, apoptosis, protein biosynthesis and metabolism (Kendall et al., 2006; Patel et al., 2004). c-Myc has been shown to regulate its target genes through interactions with the transcription machinery, as well as exerting epigenetic regulation via interactions with chromatin remodeling complexes, DNA methyltransferases and histone modifying enzymes (Eilers and Eisenman, 2008). Subsequent studies also identified a critical role of c-Myc in mESC maintenance.

Overexpression of c-Myc enables mESC to be resistant to differentiation, whereas expression of a dominant negative form of c-Myc promotes differentiation (Cartwright et al., 2005). However, a functional study of c-Myc in hESC yielded rather different results. Overexpression of c-Myc drives hESC to apoptosis and differentiation into extraembryonic endoderm and trophectoderm (Sumi et al., 2007).

c-Myc was identified as one of the four 'Yamanaka factors' initially used to generate both mouse and human iPSC (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Other reports have also shown that c-Myc can be substituted by two other related family members, N-Myc and L-Myc, during somatic cell reprogramming (Blelloch et al., 2007; Nakagawa et al., 2008). Subsequent studies demonstrated that somatic cell reprogramming can be achieved without c-Myc, albeit with significantly reduced efficiency and slower kinetics (Nakagawa et al., 2008; Wernig et al., 2008). Furthermore, reactivation of c-Myc has been observed in iPSC following blastocyst incorporation, resulting in tumor formation in the chimeric mice (Okita et al., 2007). This finding raises concerns about the safety of using iPSC generated with c-Myc for clinical applications. Understanding the molecular mechanism of c-Myc contributions during somatic cell reprogramming may help identify alternative enhancers for iPSC generation that are less tumorigenic.

Recent studies have shed light on the function of c-Myc during somatic cell reprogramming. Genome-wide analysis of promoter binding demonstrated that c-Myc regulates a different set of target genes compared to other pluripotency factors Oct4, Sox2 and Klf4 in mESC and iPSC (Chen et al., 2008; Kim et al., 2008; Sridharan et al., 2009). This suggests that c-Myc may have a very different function than the other transcription factors associated with induction of pluripotency. One proposed function is that c-Myc acts to repress expression of somatic genes during the early reprogramming stage, a process that is necessary before the activation of pluripotency gene networks (Sridharan et al., 2009). Another proposed mechanism of action of c-Myc is that it may induce a cell cycle program that is necessary for self-renewal of stem cells, activating genes which promote proliferation (i.e. cyclin A, cyclin E or E2F) and repressing genes associated with growth arrest (i.e. p21, p27) (Vermeulen et al., 2003). Finally, c-Myc may exert epigenetic control by modifying the chromatin structure to become suitable for activation of the self-renewal gene program, thus allowing somatic cells to revert back to a pluripotent state (Knoepfler et al., 2006).

2.7 Esrrb

Esrrb belongs to a subfamily of orphan nuclear receptors that are closely related to estrogen receptors (Giguere, 2002). The natural ligands for Esrrb are currently unknown. Nevertheless, Esrrb and its family members can bind to DNA and function as transcriptional activators without exogenous ligands (Giguere, 2002). Most of our knowledge of the role of Esrrb in regulating self-renewal comes from studies in mice. Overexpression of Esrrb is sufficient to maintain self-renewal of mESC in conditions that favour differentiation, possibly by maintaining the level of Oct4 expression (Zhang et al., 2008). Also, knockdown of Esrrb level in mESC induces differentiation (Ivanova et al., 2006; Loh et al., 2006). These results identify Esrrb as a positive regulator of ESC pluripotency.

Indeed, Esrrb has been used as a factor to reprogram somatic cells back to a pluripotent state. In the presence of Oct4 and Sox2, Feng *et al*. (2009) demonstrated that Esrrb could replace Klf4 as an enhancer to reprogram mouse fibroblasts into iPSC, albeit with lower reprogramming efficiency (Feng et al., 2009a). Furthermore, another family member Esrrg

also possesses a similar reprogramming ability when used in conjunction with Oct4 and Sox2 (Feng et al., 2009a). Genome-wide analysis of promoter binding suggested that Essrb shares many target genes with Oct4 and Sox2. Further studies demonstrated that Esrrb is a binding partner for Oct4 and Nanog in mESC (van den Berg et al., 2010; Wang et al., 2006), but whether it physically interacts with Sox2 remains to be determined. In summary, Esrrb was found to have a partially overlapping role with Klf4 in enhancing somatic cell reprogramming in mice, by cooperating with other pluripotency factors Oct4 and Nanog. To date, it has not yet been determined whether Esrrb plays a similar role in human cells.

2.8 Sall4

Sall4 belongs to the family of *Spalt* transcription factors that are characterized by highly conservative C2H2 zinc-finger domains (Sweetman and Munsterberg, 2006). Mutations of Sall4 in humans results in Okihiro syndrome, a disease characterized by limb deformities and eye movement deficits (Kohlhase et al., 2002). Sall4 is highly enriched in ESC, and is one of the 'embryonic cell associated transcripts' identified by Shinya Yamanaka's group. A previous study indicated that knockdown of Sall4 promoted mESC differentiation, most notably into the trophectoderm lineage (Zhang et al., 2006). However, follow-up reports showed that Sall4-null mESC are able to remain pluripotent, albeit with impaired proliferation (Tsubooka et al., 2009; Yuri et al., 2009). A study by Yuri *et al*. (2009) also demonstrated high expression of trophectoderm markers in Sall4-null mESC (Yuri et al., 2009). These results suggest that Sall4 is not essential to maintain pluripotency in mESC, but rather functions to stabilize the stem cell phenotype by promoting proliferation and possibly repressing trophectoderm differentiation. Furthermore, it is becoming clear that Sall4 is an integral part of the autoregulatory transcriptional network of Oct4, Sox2 and Nanog in mESC (Lim et al., 2008; Yang et al., 2008). These results suggest Sall4 is a possible candidate reprogramming factor for induced pluripotency.

Recently, Wong *et al*. (2008) discovered that Sall4 can enhance the efficiency of reprogramming mouse fibroblasts through fusion with mESC (Wong et al., 2008). Sall4 also increases the reprogramming efficiency of mouse fibroblasts when used in combination with Oct4, Sox2 and Klf4 (Tsubooka et al., 2009). However, this enhancing effect of Sall4 in reprogramming is inconsistent in different human fibroblast cell types, possibly due to variations in endogenous levels of Sall4 in different samples (Tsubooka et al., 2009). Further studies of Sall4 in human pluripotent stem cells will clarify whether the role of Sall4 in regulating cell pluripotency is conserved between mouse and human.

2.9 miRNA

miRNA are small RNAs that provide post-transcriptional control of gene regulation. Once transcribed, primary miRNA undergo multiple processing steps to become mature miRNA that promote degradation or repress translation of target mRNA (Siomi and Siomi, 2010). A previous report provided evidence that miRNA play an important role in the inter-connected transcriptional network regulated by pluripotency factors Oct4, Sox2 and Nanog in mESC (Marson et al., 2008). The pluripotency factors Oct4, Sox2 and Nanog are able to bind and regulate expression of specific miRNA, activating ESC specific miRNA while repressing those associated with differentiation (Barroso-delJesus et al., 2008; Marson et al., 2008). It is believed that miRNA serve as a mechanism for Oct4, Sox2 and Nanog to fine-tune the expression level of their target genes.

Recently, Robert Blelloch's group described a subset of miRNA that play an important role in regulating the cell cycle of mESC, termed ESC-specific cell cycle regulatory miRNA (ESCC miRNA) (Wang et al., 2008b). These ESCC miRNA promote G1 to S transition in mESC by repressing expression of various cyclin E-Cdk2 inhibitors (Wang et al., 2008b). In a follow-up study, the same group demonstrated that ESCC miRNA, in particular mir-291-3p, mir-294 and mir-295, are able to enhance reprogramming efficiency when used in combination with Oct4, Sox2 and Klf4. These ESCC miRNA are found to be downstream effectors of c-Myc, and thus are able to act as substitutes for c-Myc albeit to a lesser extent (Judson et al., 2009). Another set of ESCC miRNA, the mir-302 cluster, is able to reprogram human melanoma and prostate cancer cells to ESC-like cells in the absence of any other reprogramming factors (Lin et al., 2008). However, it remains unclear whether miRNA on their own can reprogram normal human primary cells to obtain genuine iPSC, as the effectiveness of miRNA-based reprogramming strategies may be cell-type dependent. Alternatively, others have shown that suppression of pro-differentiation let-7 miRNA can also enhance reprogramming efficiency in mouse fibroblasts when used in combination with the Yamanaka factors (Melton et al., 2010). This result is consistent with the previous identification of Lin28 as a reprogramming factor for iPSC generation, which presumably acts by blocking the processing of the let-7 family of miRNA (see discussion above). Together, these results demonstrate opposing roles played by different miRNA in somatic cell reprogramming and identify miRNA as important regulators of cell pluripotency. Future research to screen for reprogramming effects of other miRNA members will prove helpful in deriving a more efficient somatic cell reprogramming method. For instance, a recent report showed that miRNA-145 can regulate expression of Oct4, Sox2 and Klf4 and represses pluripotency in hESC (Xu et al., 2009b). Therefore, it will be interesting to see whether miRNA-145 can contribute to somatic cell reprogramming.

2.10 SV40 LT antigen and hTERT

The SV40LT antigen and the catalytic subunit of hTERT are well documented for their roles in establishing immortalized cells. Overexpression of SV40LT and hTERT along with another oncogene Ras are sufficient to confer tumorigenic transformation of normal human cells (Hahn et al., 1999). One proposed mechanism by which SV40 functions in tumorigenesis is by perturbing cellular senescence pathways through suppression of p53 activity. As discussed below, reduced p53 activity during reprogramming has been shown to improve the efficiency. On the other hand, reduction in telomere length during the normal aging process results in replicative senescence and limits the cellular lifespan of human cells. Studies have shown that this can be prevented by ectopic expression of hTERT to drive cellular immortalization (Bodnar et al., 1998). High telomerase activity is observed in the vast majority of tumors and is vital for the progression of malignant tumor cells (Kim et al., 1994). Interestingly, a previous study has also shown that c-Myc overexpression can activate hTERT activity (Wu et al., 1999). Therefore, SV40LT and hTERT may be able to contribute to somatic cell reprogramming by activating a cell cycle program that is required for pluripotent cells, much like the role of c-Myc in induced pluripotency.

Two recent studies sought to enhance the efficiency of generating human iPSC by supplementing reprogramming factors with hTERT and/or SV40LT (Mali et al., 2008; Park et al., 2008). In the study by Mali *et al*. (2008), the reprogramming cocktails were supplemented with the SV40LT transgene and resulted in accelerated reprogramming kinetics and up to a 70-fold increase in reprogramming efficiency, depending on the

combination of reprogramming factors used (Mali et al., 2008). Similar results were obtained by Park *et al*. (2008) when hTERT and SV40LT were included in their reprogramming strategy (Park et al., 2008). Interestingly, while the addition of both SV40LT and hTERT was reported to increase cell proliferation, there were no viral integrations of either transgene in the genomes of the iPSC derived by this method (Park et al., 2008). This suggests that SV40LT and hTERT might be acting indirectly on supportive cells to enhance reprogramming. However, concerns remain about the safety of using SV40LT and hTERT in somatic cell reprogramming for clinical purposes. In this regard, future research studying whether iPSC generated using these two factors are more tumorigenic will help address this issue.

2.11 Silencing of the p53/p21/p16 pathway

One of the major roadblocks in iPSC generation is overcoming cellular senescence. p53 is known as the guardian of the genome and deregulation of p53 function promotes cell immortalization and bypasses cell senescence (Bond et al., 1994). Recent discoveries suggested that high-passage somatic cells with short telomeres show a dramatic decrease in the efficiency for iPSC generation (Marion et al., 2009; Utikal et al., 2009b). In this regard, bypassing cellular senescence may help improve the reprogramming efficiency of iPSC generation.

Key studies have shown that knockdown of senescence factors like p53, $p21^{CIP1}$ or $p16^{INK4a}$ enhances the efficiency of generating iPSC (Banito et al., 2009; Hanna et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Marion et al., 2009). Furthermore, p53 knockdown can be used to replace c-Myc and/or Klf4 in the Yamanaka factors (Hong et al., 2009; Kawamura et al., 2009). When used in combination with UTF1, a chromatin bound factor highly expressed in pluripotent stem cells, the addition of p53 further enhanced reprogramming efficiency by 100-fold when used with the Yamanaka factors (Zhao et al., 2008). Together these studies show that the p53 pathway not only acts as a roadblock for cancer but also for iPSC generation. As p53 is a major tumor suppressor, further studies will be required to evaluate the safety of p53 knockdown in iPSC generation before these iPSC can be used in clinical applications.

2.12 Small molecules used to enhance somatic cell reprogramming

It is becoming clear that reversion of somatic cells to a pluripotent state involves epigenetic changes to chromatin that allow different sets of genes to be expressed (Feng et al., 2009b). Several reports have investigated the use of small molecules to enhance current cellular reprogramming methods towards developing a completely transgene-free strategy. Huangfu *et al*. (2008) discovered that the addition of valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, can increase the efficiency and kinetics of reprogramming (Huangfu et al., 2008a). Moreover, two other HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA), can also improve the reprogramming efficiency, albeit to a lesser extent. This provides supporting evidence for the notion that chromatin modifiers can help to overcome the epigenetic barrier to achieving complete reprogramming. Furthermore, addition of VPA was also able to substitute for c-Myc and Klf4 during reprogramming, thus reducing the number of reprogramming factors required to derive iPSC (Huangfu et al., 2008b). Similar effects on enhancing reprogramming efficiency were also observed with DNA methyltransferase inhibitors, including azacytidine

(AZA) (Huangfu et al., 2008a) and RG108 (Shi et al., 2008a), as well as a histone methyltransferase inhibitor, BIX-01294 (Shi et al., 2008b). In addition, a calcium channel agonist, BayK864, was shown to enhance the effect of BIX-01294 and further improve the efficiency of reprogramming (Shi et al., 2008a). However, it remains unclear whether or not the enhancing effect of these small molecules is dependent on the cell type used for iPSC generation. Altogether, it is thought that histone deacetylase inhibitors (VPA, TSA, SAHA), methyltransferase inhibitors (AZA, RG108, BIX-01294) and possibly other, yet to be identified, chromatin modifiers may function by relaxing chromatin to allow ectopically expressed transcription factors to bind.

Other researchers have screened small molecule libraries to identify chemical compounds that can directly substitute for the known reprogramming factors. This led to the discovery of RepSox (replacement of Sox2), a small molecule used to substitute for Sox2 in the reprogramming strategy (Ichida et al., 2009). RepSox acts by inhibiting transforming growth factor-ß (TGF-ß) signaling, in turn increasing Nanog expression that ultimately promotes partially reprogrammed cells to become fully reprogrammed (Ichida et al., 2009). However, future research will need to address the specificity of RepSox in order to fulfill its potential in generating iPSC for clinical purposes.

Finally, an interesting study by Esteban *et al*. (2010) demonstrated that antioxidants, in particular vitamin C, also help to enhance somatic cell reprogramming when used in combination with Oct4, Sox2 and Klf4 (Esteban et al., 2010). During early stages of reprogramming, vitamin C is able to overcome, at least partially, the cellular senescence roadblock by down-regulating p53 to allow the conversion of partially reprogrammed cells into fully reprogrammed iPSC (Esteban et al., 2010). This study provides a natural alternative to synthesized small molecules and may be easier to obtain approval for clinical usage.

3. Techniques for delivery of reprogramming factors

Since the seminal iPSC work by Shinya Yamanaka and colleagues, the field has moved forward at a rapid pace. Significant progress has been made in identifying new strategies to enhance the reprogramming efficiency and new methods to improve clinical safety. In this section, we will discuss the current techniques employed to introduce the reprogramming factors required for iPSC generation. It is important to note that the reprogramming efficiencies discussed in this section are only subject to the context described in the particular studies. Actual efficiency can be highly affected by many factors including the cell type of origin, the reprogramming factors and enhancer molecules used, as well as the methods to calculate reprogramming efficiencies.

3.1 Integrating Viral Vectors

3.1.1 Retroviral vectors

Retroviruses are efficient gene delivery vectors widely used in a broad range of dividing cell types. They can integrate into the host cell genome to produce continuous transgene expression. However, slow dividing or non-dividing cells are extremely resistant to retroviral transduction and the random sites of transgene integration can lead to unpredicatable genetic mutations within the genome and aberrant transgene expression. The initial derivation of iPSC utilized retroviral-mediated introduction of Oct4, Sox2, Klf4 and c-Myc to convert mouse fibroblasts back to a pluripotent state (Takahashi and Yamanaka, 2006), and subsequently human iPSC derived from adult dermal fibroblasts,

fetal and neonatal cells (Lowry et al., 2008; Park et al., 2008; Takahashi et al., 2007). Also, it was observed that retroviral mediated expression of transgenes were silenced during the iPSC reprogramming process (Hotta and Ellis, 2008). Even with a low efficiency (0.001%-0.5%), these pioneering studies revealed a potential alternative to the controversial use of ESC as a cell source for cellular transplantation therapies.

3.1.2 Lentiviral vectors

At the same time Yamanaka and colleagues reported the generation of human iPSC by retroviral transduction, Yu *et al*. (2007) demonstrated successful derivation of human iPSC using lentiviral methods to deliver a different set of factors Oct4, Sox2, Nanog and Lin28 (Yu et al., 2007). Lentiviruses, a subclass of retroviruses, offer the capability of high-efficiency infection in both dividing and non-dividing cells with stable expression of the transgenes and low immunogenicity. These distinguishing characteristics allow lentiviral vectors to be used for reprogramming a broader range of somatic cell types. However, lentiviruses integrate randomly into the host genome, similar to other retroviruses, which may hinder the use of iPSC generated using these methods for clinical applications. Initial derivation of human iPSC using lentiviral transduction yielded reprogramming efficiencies of 0.01%, significantly lower than previous retroviral methods (Yu et al., 2007). Subsequent improvements have been made by using lentiviral vectors to deliver SV40T, UTF1 or p53 shRNA to supplement the reprogramming cocktails, resulting in a 70-100 fold increase in reprogramming efficiency (Mali et al., 2008; Zhao et al., 2008). Moreover, reporter and antibiotic selection casettes have also been incorporated into lentiviral vectors to aid in the isolation of iPSC (Hotta et al., 2009a; Hotta et al., 2009b).

Previous studies indicated that transient expression of reprogramming factors is sufficient to activate the endogenous pluripotent gene program to allow for direct cell reprogramming (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). This led to the development of inducible lentiviral vectors for direct cell reprogramming. Inducible lentiviral methods provide for improved temporal control over the levels of reprogramming factor expression and have been used to study the timing of reprogramming as well as the molecular changes that occur during the process. This system relies on inclusion of an additional vector that constitutively expresses the reverse tetracycline transactivator (rtTA). In the presence of the drug doxycycline, the rtTA functions to drive expression of the reprogramming factors, while in the absence of doxycycline the reprogramming transgenes are not expressed. Utilizing this type of inducible system, it has been established that exogenous transgene expression is necessary for 8-12 days to induce reprogramming of mouse fibroblasts and dispensable thereafter (Brambrink et al., 2008; Maherali et al., 2008; Stadtfeld et al., 2008a). Moreover, when doxycycline was removed eight days after initial transduction, the partially reprogrammed cells were unable to survive in the ESC growth conditions due to incomplete reactivation of their self-renewal programs. This provides a useful system to select for cells that have completely reverted back to a pluripotent stem cell state.

A major obstacle encountered when attempting to transduce cells with multiple viruses is that only a small proportion of the total cells will become infected with all the viruses. During reprogramming of somatic cells, those cells infected with few viruses may fail to become reprogrammed; leading to a low reprogramming efficiency. In this regard, development of a system to express the transgenes from a single vector may substantially improve the efficiency. One method to express the four transgenes from a single promoter is to insert the self-cleaving 2A and 2A-like sequences between each cDNA sequence

(Donnelly et al., 1997). These 2A sequences act by triggering ribosomal skipping that result in expression of each sequence in a stoichiometric fashion. Importantly, efficient polycistronic expression by 2A-mediated separation of transgenes was achieved in hESC (Hasegawa et al., 2007). This strategy was recently applied to derive mouse and human iPSC using a single lentiviral vector expressing Oct4, Sox2, Klf4, and c-Myc (Carey et al., 2009; Shao et al., 2009; Sommer et al., 2009). One of the advantages of using polycistronic vectors is that it reduces the difficulty of handling multiple lentiviral vectors for different reprogramming factors. Moreover, the mouse and human iPSC generated using this method had less viral integration sites compared to those developed using lentiviral delivery with individual gene. Indeed, the results showed as few as a single viral integration was sufficient for reprogramming (Carey et al., 2009; Shao et al., 2009). Minimizing the number of viral integrations reduces the risk of tumorigenesis and genomic instability. Also, the use of polycistronic vectors ensures expression of all four factors in the transduced cells. However, reprogramming efficiencies using this method were significantly lower (0.05%) than previous methods and reprogramming kinetics were also notably slower. One possible explanation for this low reprogramming efficiency is that the reprogramming factors are required to be expressed at an optimal stoichiometry in order to achieve efficient direct cell reprogramming (Papapetrou et al., 2009). Further studies are needed to clarify if the use of polycistronic vectors is an ideal technique to generate iPSC.

3.2 Non-integrating and excisable approaches

A major concern with employing retroviral or lentiviral-based methods to derive iPSC is random, uncontrollable integrations of the foreign transgenes into the host chromosomes. While many of these integrations prove harmless to the cells, residual viral portions have been shown to contribute to tumor formation. Moreover, it has been suggested that viral integrations may be a possible cause for some of the gene expression and differentiation potential differences observed between blastocyst-derived ESC and iPSC. These differences could affect the interpretation of results during mechanistic studies and, due to the safety concerns, severely limit the clinical applicability of these genetically modified cells. The previous approaches were extremely inefficient processes and most required multiple integration sites to induce reprogramming. Therefore, many groups have focused on developing novel, non-integrating methods for deriving iPSC. Some of these methods include the use of non-integrating vectors, excisable vectors, as well as RNA- or protein-based reprogramming.

3.2.1 Adenoviral vectors

The use of adenoviral vectors is advantageous for somatic cell reprogramming as they lack the machinery to integrate into the host's genome. This allows for high-level expression of exogenous genes with a low risk of integration of viral transgenes into the host genome. The viral titer becomes diluted after every cell division, which allows transient expression of the transgenes. On the other hand, multiple rounds of infections can achieve prolonged expression of transgenes, but it can be difficult to control gene expression levels. Reprogramming somatic cells with adenoviral vectors was first reported by Stadtfeld *et al* (Stadtfeld et al., 2008b). Using adenoviral vectors, mouse and human iPSC were generated using the Yamanaka factors in various donor cell types, albeit with a low reprogramming efficiency as compared to integrating viral vectors (0.0001%-0.001%) (Stadtfeld et al., 2008b;

Zhou and Freed, 2009). Similarly, a low reprogramming efficiency was also observed using polycistronic adenoviral vectors (Okita et al., 2008). One explanation for this is that it is difficult to maintain high enough transgene levels for multiple days to allow for reprogramming in many of the cells. Also, roughly 20% of the transduced cells were tetraploid, a phenomenon which was not seen in retroviral or lentiviral induced iPSC (Stadtfeld et al., 2008b). The reason for this observed tetraploidy is not clear, but it was suggested that cellular fusion or the presence of a rare aneuploid cell population in the starting culture may account for this result. Therefore, further research is needed to refine the use of adenoviral vectors in reprogramming somatic cells back to pluripotency.

3.2.2 Plasmids

Another non-integrating approach to transiently express reprogramming factors is the use of conventional plasmids. Previous reports have successfully generated integration-free mouse iPSC using polycistronic plasmids to express the Yamanaka factors (Gonzalez et al., 2009; Okita et al., 2008). However, a substantial amount of iPSC colonies contained integration of the transgenes. Therefore, screening of transgene integration sites is still necessary for iPSC generated using this technique to ensure their safety for clinical purposes. Furthermore, multiple rounds of transfection are required to sustain transgene expression at the level required to induce reprogramming and the observed reprogramming efficiency remained significantly lower than seen with the retroviral vectors (Gonzalez et al., 2009; Okita et al., 2008). Improvements to this plasmid-based method were made by the use of a polycistronic nonviral minicircle plasmid to reprogram human adult adipose stem cells (Jia et al., 2010). Minicircle DNA offers a higher transfection rate and is diluted at a slower rate than conventional plasmids when the cells divide. As a result, fewer rounds of transfections are required to generate iPSC. Using this method, Jia *et al*. (2010) generated integration-free human iPSC with a reprogramming efficiency of 0.005%, an efficiency still much lower than the integrating viral methods (Jia et al., 2010).

Episomal plasmids are another non-integrating method used to reprogram somatic cells into iPSC. Unlike conventional plasmids where transient transgene expression is gradually depleted after each cell division, episomal plasmids can be stably established in a number of cell types by drug selection and removed when the drug selection is withdrawn. Using this technique, Yu *et al*. (2009) generated a polycistronic episomal vector to co-express seven transgenes to reprogram human foreskin fibroblasts into iPSC (Yu et al., 2009). It was observed that different positioning of the transgene in the polycistronic vector resulted in varying reprogramming efficiencies, with the highest efficiency achieved being 0.006%. In summary, these studies provide proof-of-principle of the derivation of human iPSC free of genomic integration. However, all plasmid-based methods used to date yield a lower reprogramming efficiency compared to integrative viral methods, possibly due to difficulties in sustaining high transgene expression. Further research combining enhancing factors, such as small molecules, may help improve the reprogramming efficiency of these plasmid-based methods.

3.2.3 Cre recombinase /loxP system

Early non-integrating methods, such as those utilizing adenoviral and plasmid introduction of reprogramming factors, were substantially less efficient than the retroviral methods. Excisable integrating vectors offer a plausible reprogramming strategy to overcome the

shortcomings of both the integrating retroviral and the transient expression methods. These excisable systems allow high initial transgene expression followed by subsequent removal of exogenous factors. Soldner *et al*. (2009) used inducible lentiviral vectors to derive human iPSC from fibroblasts collected from Parkinson's disease patients followed by Cre-recombinase mediated excision of the viral transgenes (Soldner et al., 2009). In this study, a constitutively active reverse tetracycline transactivator lentivirus was infected along with doxycycline-inducible lentiviruses for expression of the reprogramming factors, Oct4, Sox2, Klf4 and c-Myc. The inducible lentiviruses were engineered to contain a loxP site in the 3′ LTR that becomes duplicated into the 5′ LTR during viral replication, producing loxP sites flanking the transgenes. Subsequent expression of Cre-recombinase by electroporation allows the transgenes to be excised. Using this technique, the authors reported successful derivation of integration-free human iPSC (Soldner et al., 2009). Interestingly, the authors also demonstrated that the gene expression profile of these iPSC are more similar to hESC following transgene excision, suggesting that residual integrated reprogramming factors perturb the transcriptional profile of human iPSC (Soldner et al., 2009). Although the Cre-loxP system is the most efficient recombination system known, screening of integration-free iPSC clones is still required, as the authors reported that only sixteen out of 180 clones analyzed were integration-free following excision of transgene. Moreover, Cre-mediated excision of the transgenes does not remove the loxP sites, which raises concern of the possibility of disruption of endogenous gene expression. In this regard, a recent report by Chang *et al*. (2009) demonstrated successful generation of integration-free human iPSC where residual loxP sites did not interrupt expression of any genes or other functional sequences (Chang et al., 2009).

3.2.4 piggyBac transposon-based system

Unlike the Cre-loxP system, the advantage of transposon-based system is that transposases can remove all exogenous transposon elements from the host DNA. In particular, the piggyBac transposons have been demonstrated to be an efficient system for excisable gene delivery, delivering up to 10kb DNA fragments (Ding et al., 2005). Following transfection of the piggyBac transposons, transient expression of the transposase enzyme catalyzes the insertion or the excision event (Fraser et al., 1996). The advantage of the piggyBac transposon system is that it can be completely removed from the host genomes without altering the DNA sequences at the integration sites (Wang et al., 2008a). This led to the development of piggyBac-based reprogramming strategy that allowed generation of mouse and human iPSC using tetracycline-inducible or polycistronic expression of reprogramming factors (Kaji et al., 2009; Woltjen et al., 2009; Yusa et al., 2009). A high reprogramming efficiency of 2.5% was reported in the generation of mouse iPSC using this technique (Kaji et al., 2009). Furthermore, the integration sites were sequenced to confirm that excision of transgenes did not alter the host genome. Therefore, the piggyBac transposon system represents an efficient non-integrative approach to generate iPSC. However, excision of the transgenes by the transposase may still lead to micro-deletions of the genomic DNA (Wang et al., 2008a), which could hinder the clinical application of iPSC generated using this method.

3.2.5 RNA and protein-based reprogramming

Since all of the reprogramming strategies highlighted above create the risk of unexpected genetic modifications, many groups have begun to devise ways to reprogram somatic cells

in the absence of genetic modification. Yakubov *et al*. (2010) recently developed a reprogramming technique by transfecting RNA synthesized *in vitro* from cDNA of Oct4, Lin28, Sox2, and Nanog to generate iPSC from human fibroblasts (Yakubov et al., 2010). This method harnesses the power of the endogenous translational machinery for proper protein folding and post-translational modifications. Also, this method of generating iPSC eliminates the risks associated with the use of viruses and DNA transfection methods. However, at least five consecutive transfections were necessary to reprogram these human fibroblasts as the transfected RNAs have a limited half-life. Finally, the reprogramming efficiency (0.05%) remained lower than those observed for integrating viral methods. Further characterization is also needed to confirm the pluripotency of the iPSC-like cells generated using this method and to prove the feasibility of RNA-based reprogramming.

An alternate approach to somatic cell reprogramming without genetic modification is using protein transduction. Importantly, a previous study demonstrated that protein tagged with a C-terminus poly-arginine domain allows efficient protein transduction through the cell membrane (Matsushita et al., 2001). Using direct protein delivery of reprogramming factors, two groups have report successful derivation of iPSC with the Yamanaka factors (Kim et al., 2009a; Zhou et al., 2009). Zhou *et al*. (2009) was the first to use recombinant reprogramming factors tagged with the poly-arginine domain to generate mouse iPSC. This virus-free and DNA-free method yielded a reprogramming efficiency of 0.006% with the use of an enhancer molecule VPA (Zhou et al., 2009). In addition, Kim *et al*. (2009) used whole cell extract from human embryonic kidney cells overexpressing the Yamanaka factors for the generation of human iPSC, yielding a reprogramming efficiency of 0.001% (Kim et al., 2009a). Both studies generated iPSC without any genetic modification, making them suitable for clinical applications. This direct protein transduction method also eradicates the need to screen for integration-free iPSC, thus shortening the time required for generating clinical grade iPSC. However, the reprogramming efficiency achieved with this method is still far lower than those obtained with viral mediated reprogramming. Moreover, multiple rounds of treatment are required during the reprogramming process as the recombinant proteins become degraded overtime. Nevertheless, these studies proved the feasibility of protein-based reprogramming methods and improvements to this technique could be important for final clinical application of iPSC.

4. Conclusions

The clinical potential of hESC for cell replacement therapies and for studying human diseases is undeniable. However, the use of these cells has been constantly burdened by both ethical (destruction of human embryos) and practical concerns (lack of available embryos, difficulties with generation of histo-compatible hESC). The conversion of somatic cells into pluripotent cells may overcome many of these roadblocks. Large numbers of embryos are no longer needed to create banks of patient-matched lines, since cells can now be harvested directly from each patient to create iPSC that are genetically identical and immune-compatible. Since the initial derivation of iPSC using the four Yamanaka factors, a growing list of reprogramming factors have been identified that either permit or enhance the reprogramming process. Moreover, researchers have begun to study other ways to improve the reprogramming efficiency, including the use of miRNA, small molecules, and several different methods to overcome barriers that prevent direct somatic cell reprogramming.

The first generation iPSC methods involved integration of viral transgenes, but the clinical necessity for deriving iPSC without these viral transgenes has pushed many researchers to develop alternative methods. The use of inducible polycistronic lentiviral vectors has already evolved to the utilization of excisable Cre-loxP and piggyBac expression systems, non-integrating plasmids, and recombinant proteins and RNA transfection as tools for generating iPSC. Advances in these non-viral, non-integrating methods will presumably continue until a method is discovered that can, with high efficiency, be used to derive patient-specific iPSC lines in a technically simple manner which can be adopted by many researchers and clinicians. In this regard, pursuing the development of protein-based and small molecule-based reprogramming methods may be most beneficial. However, these methods are still at an early stage and further improvements are needed to achieve high reprogramming efficiencies. Interestingly, a recent report utilized lentiviral vectors to deliver three transgenes and demonstrated direct conversion of mouse fibroblasts into neuronal like cells, bypassing the need of reprogramming back to a pluripotent state (Vierbuchen et al., 2010). Further research is needed to develop similar non-viral methods and to translate these techniques to human cells for clinical applications. Many methodologies used in the derivation of iPSC can be applied to study direct conversion of a somatic cell into another cell type of interest. However, the disadventagous of such direct reprogramming of a somatic cell into another lineage is that the reprogrammed cells are terminally differentiated and are not proliferating. Therefore, this reprogramming strategy is not ideal for large scale production to yield cells for the development of cell replacement therapies. In this regard, direct reprogramming of a somatic cell to a progenitor stage, where the progenitor cells remain proliferative, may prove advantageous. Recent studies also showed that iPSC retain an 'epigenetic memory' of their origins, where differentiation of iPSC to their tissue of origin is more efficient than other lineages (Kim et al., 2010; Polo et al., 2010). This could be used as a strategy to derive efficient protocols for differentiating iPSC to a particular cell type of interest. In summary, the field of stem cell biology was radically altered by the derivation of iPSC. Since their generation, the field has moved forward at a staggering speed, in large part due to the potential of iPSC to transform modern medicine as well as our understanding of human development.

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Generation of ICM-Type Human iPS Cells from CD34$^+$ Cord Blood Cells

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1. Introduction

One of the major technical hurdles for clinical application of embryonic stem (ES) cells or induced pluripotent stem (iPS) cells is formation of teratomas by undifferentiated cells after transplantation. In addition, iPS cells have their own safety concerns such as an increased chance of tumorigenicity caused by chromosomal instability or alteration during the reprogramming process (1). Since the first report of mouse iPS cell generation by retroviral vectors (2), several non-integrating vector systems have been examined in pursuit of "safer" iPS cell generation methods. These approaches include adenoviruses (3), Sendai viruses (SeV) (4, 5), Cre-excisable viruses (6), the piggyBac transposition system (7, 8) conventional plasmids (9), the oriP/EBNA1-episomal vector (10), direct protein delivery methods (11, 12) or small molecule delivery methods (13, 14).

A number of cell sources for generating human iPS cells have been reported, including dermal fibroblasts (15), keratinocytes (16), peripheral blood cells (17), adipose tissue (18), and cord blood (CB) cells (19). The three germ layer differentiation potential of these established iPS cells has been demonstrated. However, it is not clear which cell source is best for generating "standard" iPS cells, as differentiation preferences of established iPS cells reflect the epigenetic status of the original cells (called "epigenetic memory") (20).

Recently, several groups reported new insights into two distinct stages of pluripotency in ES cells. These stem cell stages consist of the inner cell mass (ICM) of blastocyst type (ICM type-cells or naïve cells), and epiblast type stem cells (EpiSCs or prime cells) (21). Mouse 129 or C57/BL6 mouse ES cells are the ICM type: "true" pluripotent stem cells representing pre-implantation blastocysts that contribute to chimerism and demonstrate germ line transmission when placed back into blastocysts. They can also be grown in single cell suspension. In contrast, the "EpiSCs" or "prime" ES cells represent post-implantation stage epiblasts. They retain the potential of three germ line differentiation *in vitro*, but are incapable of contributing to chimerism and cannot survive after single cell cloning. Human ES cells or iPS cells seem to correspond to the EpiSCs with respect to colony morphology

and gene expression prolife (22, 23), but can be converted to the naïve stem cell stage by cultivation (24) or constitutive activation of *KLF2/KLF4* genes (25). In this report, we demonstrate an easier and safer reprogramming method for the direct establishment of ICM-type human iPS cells from fresh or frozen CB cells using temperature-sensitive SeV vectors, which facilitates confirmation of removal of the SeV construct at a single cell level.

2. Experimental procedures, materials, and methods

All experimental protocols were reviewed and approved by the ethical committee of the Riken Center for Developmental Biology (CDB), the Foundation for Biomedical Research and Innovation (FBRI), Asagiri Hospital, and the animal experiment committee of FBRI.
Fresh CB was supplied by Asagiri Hospital. $CD34^+$ cells were purified from mononuclear cells (isolated from fresh CB with Lymphoprep TM (Cosmo Bio Co., Tokyo, Japan)) using a human CD34 Micro Bead kit and Auto Macs columns (Miltenyi Biotec) in accordance with the manufacturer's instruction. We also used frozen $CD34^+$ CB cells obtained from Riken RBC (Tsukuba, Japan). $CD34^+$ cells were cultured in hematopoietic culture medium (HC media) [serum free X-VIVO 10 (Lonza, Basel Switzerland) containing 50 ng/mL IL-6 (Peprotech, London UK), 50 ng/mL sIL-6R (Peprotech), 50 ng/mL SCF (Peprotech), 10 ng/mL TPO (Peprotech), 20 ng/mL Flt3-ligand (R&D system, MN)] (4) for one day prior to viral infection. SNL76/7 feeder cells (European Collection of Cell Culture, Salisbury, UK) were treated with 100 μL of mitomycin C solution (1 mg/mL) (Nacalai Tesque, Kyoto, Japan) in 10 cm dishes for three hours to generate mitomycin C treated-SNL 76/7 feeder cells (MMC-SNL). They were seeded on 24-well plates (Becton Dickinson, Tokyo, Japan), or in six-well plates, or in 60 mm dishes in naïve human ES cell culture medium. Fifty mL of naïve human ES cell medium was prepared by mixing 24 mL DMEM/F12 (Invitrogen; 11320), 24 mL Neurobasal (Invitrogen; 21103), 0.5 mL of x100 nonessential amino acids (Invitrogen), 1 mL B27 supplement (Invitrogen; 17504044), and 0.5 mL N2 supplement (Invitrogen; 17502048). The medium also contained 0.5 mg/mL of BSA Fraction V (Sigma), penicillin-streptomycin (final x 1, Invitrogen), 1 mM glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), 1.0 μM PD0325901 (Stemgent), 3.0 μM CHIR99021 (Stemgent), 10 μM forskolin (Sigma) and 20 ng/mL of recombinant human LIF (Millipore; LIF1005). Prime human iPS cells were cultured with prime human ES cell medium [DMEM/F-12 (SIGMA) containing 20% KSR (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% NEAA (Invitrogen), 0.1 mM 2-ME (Invitrogen), and 4 ng/mL bFGF (Peprotech)]. The medium was changed every day. Passage of human ES cell-like cells was previously described (26). The split ratio was routinely 1:3 or 1:4.

2.1 Viral infection and generation of ICM-type iPS cells

Temperature-sensitive Sendai viral vector constructs integrating the four Yamanaka factors (SeV18+*OCT3/4*/TS7, SeV18+*SOX2*/TS7, SeV18+*KLF4*/TS7, and SeV(HNL)c-MYC/TS7) were supplied by DNAVEC Corp. The $CD34^+$ cells were thawed and cultured for one day in HC media in six-well plates at a density of 2×10^4 cells/two mL/well before the infection with SeV. The thawed $CD34^+$ cells (1×10^4), or an equivalent number of freshly isolated $CD34^+$ cells, were transferred to 96-well plates in 180 μL of hematopoietic cell culture medium with 20 μL of viral supernatant containing two m.o.i. each of the five SeV constructs (SeVTS7-*OCT3/4, -SOX2, -KLF4, -c-MYC, -GFP*). The medium was replaced by fresh medium the following day and infected cells were cultured another four days. At this point, 1×10^4

infected CB cells were seeded and cultured on confluent MMC-SNL cells in six-well plates in human naïve ES cell medium supplemented with PD0325901, CHIR99021, recombinant human LIF (rhLIF) and forskolin under hypoxic conditions (MCO-5M, SANYO Japan, 5% O_2, 5% CO_2 at 37° C). Dome-shaped naive ES cell like-colonies were picked up between fourteen and nineteen days, suspended as single cells, seeded on MMC-SNL and cultured with naïve ES cell medium. The second passage colonies were subjected to heat treatment (38° C for three days) and then passaged again for detection of remaining SeV constructs by RT-PCR and immunostaining with anti-SeV (HN) antibody. SeV-free colonies were transferred to a normal oxygen environment (MCO-5M, SANYO Japan, 20% O_2, 5% CO_2 at 37° C) and cultured on MMC-SNL cells with prime human ES cell medium shown in Fig 1.

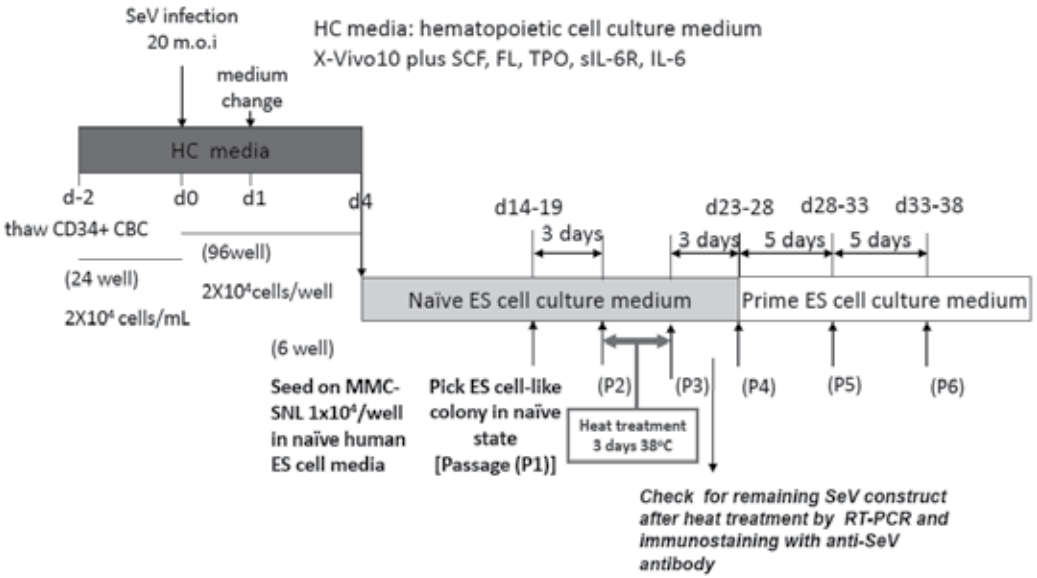

Fig. 1. Schema for generating naive and prime iPS cell from CB cells with SeV vectors.

2.1.1 Optimized culture conditions for naïve iPS cells

$1X10^4$ SeV-infected $CD34^+$ CB cells were transferred onto various numbers of pre-seeded MMC-SNL cells in 60 mm dishes (from 1.0×10^5 to 2.0×10^6). Cells were cultured for 14 days in naïve human ES cell medium either under hypoxic or normoxic conditions. The emergent colonies were fixed and stained for ALP activities. The number of colonies stained positively for ALP activities was scored.

The naïve ES cell-like colonies were picked up 14 to 19 days after seeding on SNL in naïve ES cell medium under 5% O_2 culture conditions. These cells were subjected to heat treatment at 38 °C for three days at passage two in the naive state. After heat treatment, prime ES cell-like colonies were passaged (passage three) and checked for residual SeV constructs by RT-PCR and immunostaining with anti-SeV antibody. Then, the virus-free cell clumps from passage three were cultured in prime human ES cell medium under 20% O_2 culture conditions. Viral-Free (VF) iPS cell colonies were passaged two or three times and then tested for further appraisal of differentiation potential of the reprogrammed cell clones. We tried to induce pluripotency in adherent cells derived from $CD34^+$ cells in Table 1.

Cell source	Vector	Infected cell numbers	Infectivity	Substrate on cuture plate	Numbers of ES-like colony	iPS cell clones characterized
Fresh $CD34^+$ CB	SeV	$1.0x10^4$	20	MMC-SNL cells	5	5

Clone #	RT-PCT (undifferentiation)	RT-PCT (differentiation)	IHC	Teratoma	Karyotype
#24	✓				
#30	✓		✓		
#35	✓	✓	✓	✓	
#36	✓				
#37	✓	✓	✓	✓	✓

✓: performed

Table 1. Efficiency of induction of iPSC clones from cord blood cells with SeV vectors.

2.1.2 Alkaline phosphatase and immunohistological staining

Naive ES cell like- and prime ES cell like-colonies were stained with leukocyte alkaline phosphatase kit (VECTOR, Burlingame, CA) in accordance with the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde followed by immunostaining with a series of antibodies. Nuclei were stained with DAPI (1:1000, SIGMA). Photomicrographs were taken with a fluorescent microscope (Olympus BX51, IX71, Tokyo) and a visible light microscope (Olympus CKX31).

Expression of CD34 and CD45 in mononuclear cells (MNC) from CB was determined by flow cytometry (middle). $CD34^+CD45^{low+}$ cells (0.2%) and $CD34^-$ $CD45^+$ cells were fractionated by cell sorting and both were infected with SeV carrying four factors and GFP. Phase contrast microscopic and fluorescence photographs of $CD34^+$ cells (right) and $CD34^-$ mononuclear cells (left) the day after infection are shown in lower panels.

We found that the GFP^+ population was selectively found in the $CD34^+$ fraction the day after SeV infection (Fig. 2). This fraction corresponds to hematopoietic stem cells or progenitors, as reported elsewhere (27).

2.1.3 Determination of SeV construct in naïve ES cell-like cells

The remaining SeV constructs in naïve ES cell-like colonies were determined by RT-PCR and immunostaining. Using four temperature-sensitive Sendai viral constructs (SeV TS7) integrating Yamanaka's transcription factor quartet (*c-MYC, KLF4, OCT3/4 and SOX2*), we were able to generate ES cell-like colonies from $CD34^+$ $CD45^{low+}$ CB cells. The protocol for generating iPS cells from CB cells with temperature-sensitive SeV vector is shown in Fig. 1. Naïve ES cell-like colonies were generated by culturing cells in naïve human ES cell medium under hypoxic conditions (5% O_2). Merged dome-like colonies were picked up three weeks after SeV infection and subjected to heat treatment at 38°C to reduce the amount of residual SeV constructs. Remaining SeV constructs were detected by RT-PCR and immunostaining with anti-SeV antibody. Then, the cell clumps of "naïve" virus-free cell clones were transferred to conventional prime human ES cell medium and cultured under normoxic

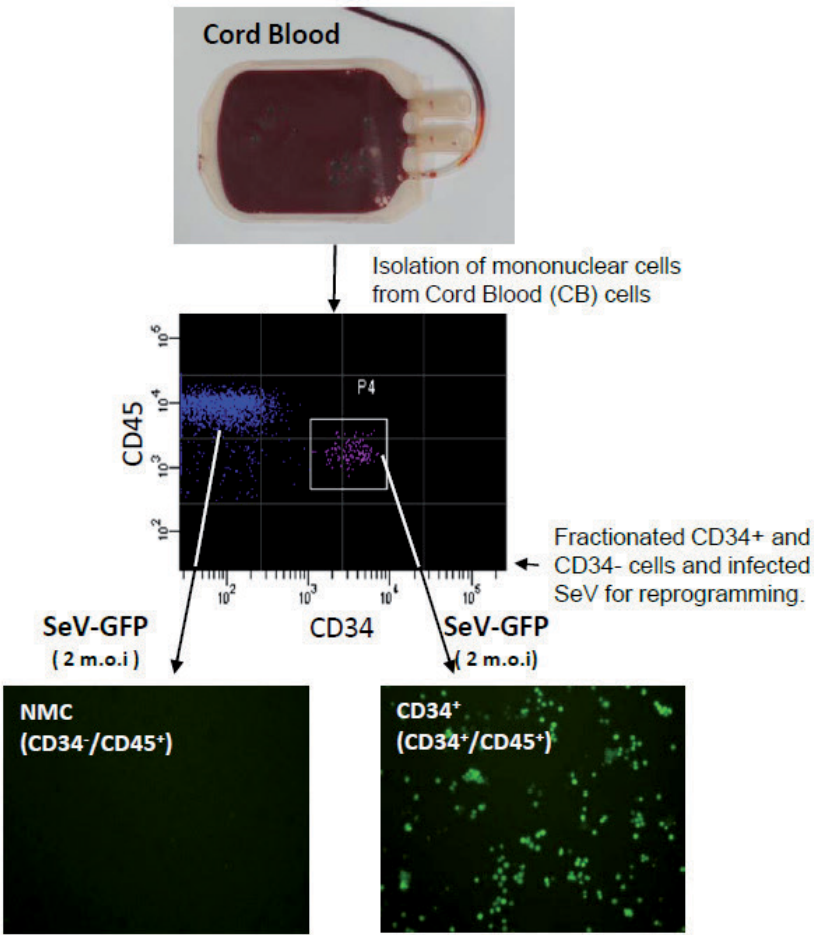

Fig. 2. SeV selectively infects the CD34+ fraction of CB cells.

conditions (20% O_2) to convert cells to "prime" virus-free ES cell-like cells. We cannot maintain the naïve state for more than five passages due to the instability of pluripotency in naïve culture conditions and the tendency for spontaneous differentiation. In contrast, pluripotency in the prime state (like conventional human ES cells) was stable and we could maintain prime ES or iPS cells for more than 50 passages. Therefore, further appraisal of the differentiation potential of the reprogrammed cells was done in the prime state (Fig. 4A,B).

Naïve ES cell-like clones from a single cell suspension were examined. Like mouse ES cells, emergent dome-like colonies (P = 1) started to express SSEA-1 in the naïve stage (Fig. 3C, lower left), but its expression ceased after shifting to the prime state (Fig. 3C, lower right). Expression of pluripotency-related molecules in the prime state was examined by immunostaining with a set of antibodies (Table 2). The presence of SeV constructs in the naïve reprogrammed cells was examined by RT-PCR at the single cell level (Fig. 3D). Heat-treated naïve clones that were free of SeV constructs under hypoxic conditions (5% O_2) were transferred to prime culture with a normoxic atmosphere (20% O_2). These virus-free ES cell like-clones were expanded in conventional prime human ES cell culture for further appraisal of the differentiation potential.

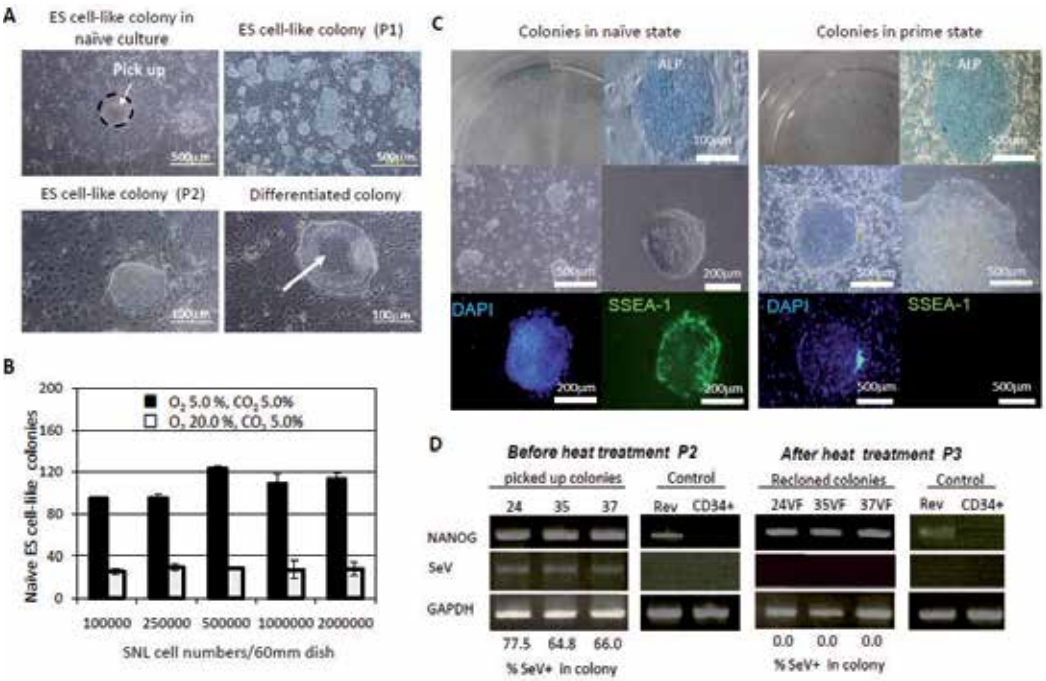

Fig. 3. Elimination of SeV constructs as determined by RT-PCR and generation of naïve or prime iPS cells.

A: Emerging naïve ES cell–like colony. Dome-like colonies emerged and were picked up (top left). Naïve ES cell-like colonies were seeded on MMC-SNL cells (passage one: P = 1, top right). Naïve ES cell-like colony (P = 2, lower left). Cells in the center of the naïve colony (white arrow) started to differentiate at later passages (P = 6, lower right). **B**: The efficiency of generation of naïve ES cell-like colonies under hypoxic (black) and normoxic (white) conditions. The number of MMC-SNL cells seeded on 60 mm dishes and the number of ES cell-like colonies which emerged are scored on the X-axis and Y-axis, respectively. **C**: Staining of naïve ES cell-like colonies (left panels) and prime ES cell-like colonies (right panels). ALP staining of colonies on MMC-SNL (top left and right), phase contract observations of colonies on MMC-SNL (middle left) or Matrigel (middle right), colonies stained with DAPI (lower left) or immunostained with anti-SSEA-1 antibody (lower right). **D**: Detection of SeV construct in heat-treated clones by PCR. Picked colonies #24, #35, and #37 were subject to heat treatment (passage 2: P = 2) and subcloned. Subclones were named #24VF, #35VF, or #37VF (passage 3: P = 3). iPS cells generated from CB by retrovirus (ReV) and parent $CD34^+$ CB cell were used as negative controls. % SeV^+ in colony is the area positively stained with anti-SeV antibody divided by the total area of the colony calculated by two value recognition software (Adobe Photoshop). There was no difference in the frequency of emerging dome-shaped ES cell-like colonies in the naïve state from freshly isolated $CD34^+$ cells and from frozen $CD34^+$ cells.

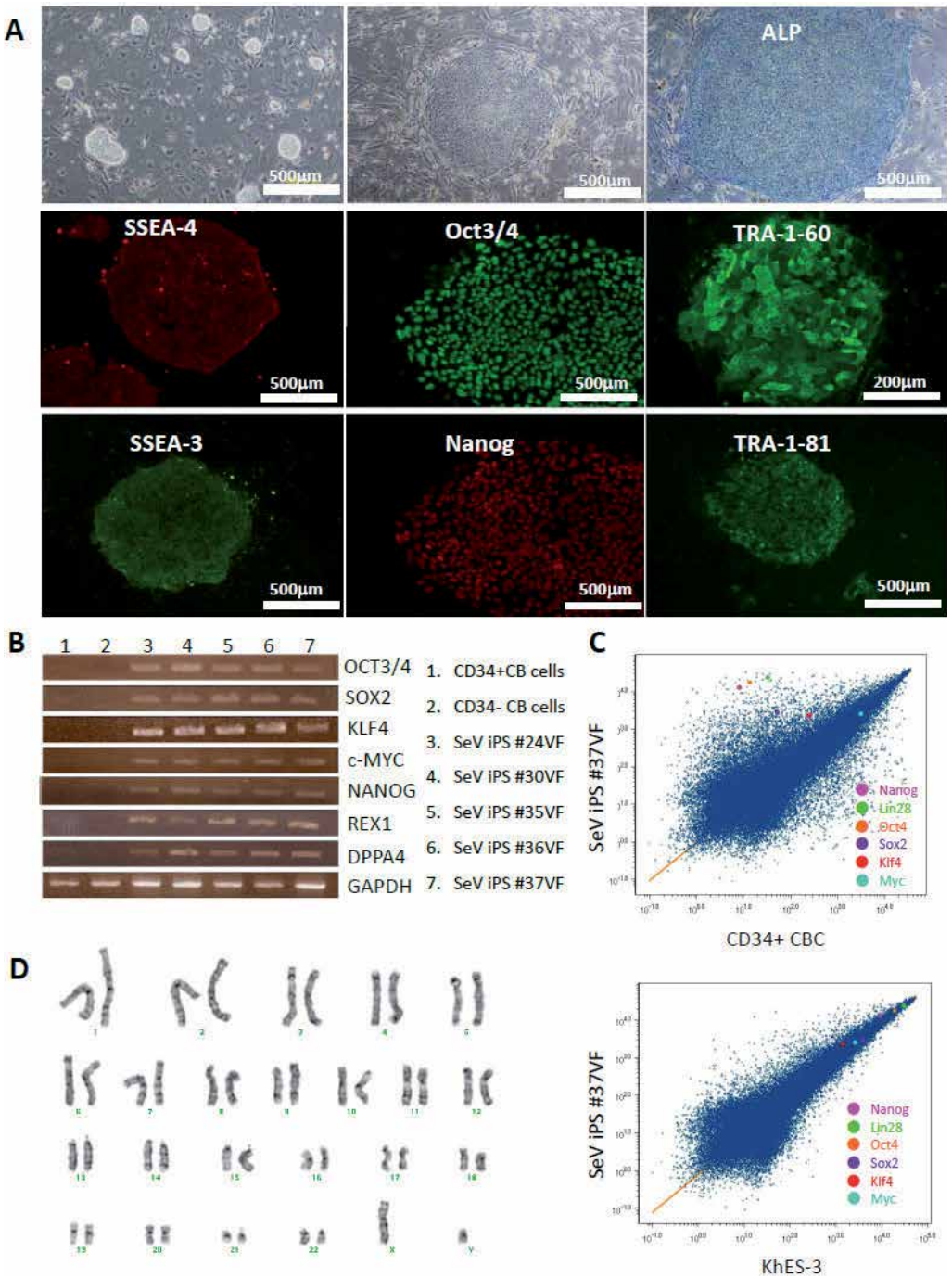

Fig. 4. Expression of pluripotency-associated genes and molecules in established SeV-free iPS cell clones.

A: Phase contrast images of a representative naive ES cell-like colony on MMC-SNL (P = 1: top left), after heat-treatment and recloning of a prime clone (SeV iPS #37VF, top middle) and its

ALP staining (top right). The expression of SSEA-4, Oct3/4, TRA-1-60, SSEA-3, Nanog and TRA-1-81 in the same prime clone (SeV iPS #37VF) was detected by immunohistochemistry. Alexa 594 (red) and Alexa 488 (green) conjugated secondary antibodies were used to visualize expression. **B**: Endogenous gene expression determined by RT-PCR. Sample description, pluripotency-associated genes, and lanes are indicated. $CD34^+$ and $CD34^-$ CB cells were used for controls. **C**: Gene expression comparison of SeV iPS #37VF vs $CD34^+$ CBC (upper panel) and SeV iPS #37VF vs human ES cell line KhES-3 (lower panel). Expression levels of pluripotency-related genes are marked in the panels. **D**: Karyotyping of SeV iPS #37VF.

Antibodies	supplier	Cat No	Dilution
anti-Oct4	Santa Cruz	sc-5279	1/ 100
anti-TRA-1-81	Chemicon	MAB4381	1/ 200
anti-TRA-1-60	Chemicon	MAB4360	1/ 200
anti-SSEA-3	Chemicon	MAB4303	1/ 200
anti-SSEA-4	Chemicon	MAB4304	1/ 200
anti-Nanog	Reprocell	RCAB0003P	1/ 1000
α-fetoprotein(AFP)	R&D	MAB1368	1/ 100
vimentin	Santa Cruz	sc-5565	1/ 200
α-smooth muscle actin(SMA)	SIGMA	A-2547	1/ 400
desmin	Dako	M0760	1/ 50
beta-III tubulin	SIGMA	T4026	1/ 200
GFAP	Santa Cruz	sc-6170	1/ 50
anti-SSEA-1	Santa Cruz	sc-21702	1/100
anti SeV HN	DNAVEC	IL4.1	1/100
Alexa Fluor 488 goat anti mouse	Invitrogen	A11001	1/ 1000
Alexa Fluor 594 rabbit anti mouse	Invitrogen	A11005	1/ 1000
Alexa Fluor 594 goat anti rabbit	Invitrogen	A11037	1/ 1000
DAPI	Invitrogen	D1306	5ug/ml

Table 2. List of antibodies used for immunostaining

2.2 Characterization of virus-free ES cell-like clones

2.2.1 Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was purified with RNeasy Mini kit (QIAGEN), according to the manufacturer's instructions. One μg of total RNA was used for reverse transcription reactions with PrimeScript RT reagent kit (TAKARA, Japan). PCR was performed with EXTaq (TAKARA, Japan). Total RNA from cell clones was extracted with the RNeasy minikit (QIAGEN). q-RT-PCR was performed with an ABI PRISM 7000 (Life Technologies Japan) using SYBR Premix EX Taq™ (TAKARA, RR041A) in accordance with the manufacturer's instructions. Primers are listed in Table 3.

2.2.2 Gene Chip analysis and karyotyping

Total RNAs from several established iPS cell clones, human ES cell line KhES-1, $CD34^-$ CB cells and $CD34^+$ CB cells were purified with RNeasy Mini kit (QIAGEN) and hybridized with human Gene Chip (U133 plus 2.0 Array Affymetrix) according to the manufacturer's

Primers			Size (bp)
hOCT3/4-F1165	GAC AGG GGG AGG GGA GGA GCT AGG	undifferentiated ES cell (endo)	144
hOCT3/4-R1283	CTT CCC TCC AAC CAG TTG CCC CAA AC		
hSOX2-F1430	GGG AAA TGG GAG GGG TGC AAA AGA GG	undifferentiated ES cell (endo)	151
hSOX2-R1555	TTG CGT GAG TGT GGA TGG GAT TGG TG		
hMYC-F253	GCG TCC TGG GAA GGG AGA TCC GGA GC	undifferentiated ES cell (endo)	328
hMYC-R555	TTG AGG GGC ATC GTC GCG GGA GGC TG		
hKLF4-F1128	ACG ATC GTG GCC CCG GAA AAG GAC C	undifferentiated ES cell (endo)	397
hKLF4-R1826	TGA TTG TAG TGC TTT CTG GCT GGG CTC C		
DPPA4-F	GGAGCCGCCTGCCCTGGAAAATTC	undifferentiated ES cell	408
DPPA4-R	TTT TTC CTG ATA TTC TAT TCC CAT		
REX1-F	CAG ATC CTA AAC AGC TCG CAG AAT	undifferentiated ES cell	306
REX1-R	GCG TAC GCA AAT TAA AGT CCA GA		
NANOG-F	CAG CCC CGA TTC TTC CAC CAG TCC C	undifferentiated ES cell	391
NANOG-R	CGG AAG ATT CCC AGT CGG GTT CAC C		
hGAPDH F	AAC AGC CTC AAG ATC ATC AGC	control	337
hGAPDH R	TTG GCA GGT TTT TCT AGA CGG		
hBRACHYURY-F1292	GCC CTC TCC CTC CCC TCC ACG CAC AG	mesoderm	274
hBRACHYURY-R1540	CGG CGC CGT TGC TCA CAG ACC ACA GG		
hPAX6-F1206	ACC CAT TAT CCA GAT GTG TTT GCC CGA G	ectoderm	317
hPAX6-R1497	ATG GTG AAG CTG GGC ATA GGC GGC AG		
hSOX17-F423	CGC TTT CAT GGT GTG GGC TAA GGA CG	endoderm	608
hSOX17-R583	TAG TTG GGG TGG TCC TGC ATG TGC TG		
SeV vector F15204	GGATCACTAGGTGATATCGAGC	SeV vectors	193
SeV vector R15397e	CATATGGACAAGTCCAAGACTTC		

Table 3. List of primers used to detect pluripotency-associated genes in reprogrammed cells.

instructions. Karyotyping of established iPS cells was reported by Nihon Gene Research Laboratories, Inc. (Sendai, Japan).

The expression of pluripotency-related molecules in the prime stage such as SSEA-4, SSEA-3, TRA-1-60, TRA-1-81, Oct3/4 and Nanog were detected by immunostaining (Fig. 4A). Endogenous expression of pluripotency-related genes was determined by RT-PCR (Fig. 4B). Total gene expression profiles of the established iPS clone SeV iPS #37VF are compared with human ES cell line KhES-3 or CD34+ cord blood cells (Fig. 4C). Karyotype of the established iPS cell clone SeV iPS #37VF is presented (Fig. 4D).

2.3 Differentiation assays of virus-free iPS cells *in vitro* and *in vivo*

2.3.1 *In vitro* differentiation assay

Established human ES cell-like clones were harvested using collagenase IV. Cells were transferred to six-well ultra-low attachment plates (Corning) and cultured in human prime ES cell medium without bFGF to form embryoid bodies (EB). The medium was changed every other day. The resulting EBs were transferred to gelatin-coated plates after eight days and cultured in the same fresh medium for another eight days. Three cell lines were tested for differentiation potential on gelatin coated dishes after EB formation (Fig. 5a). All of these

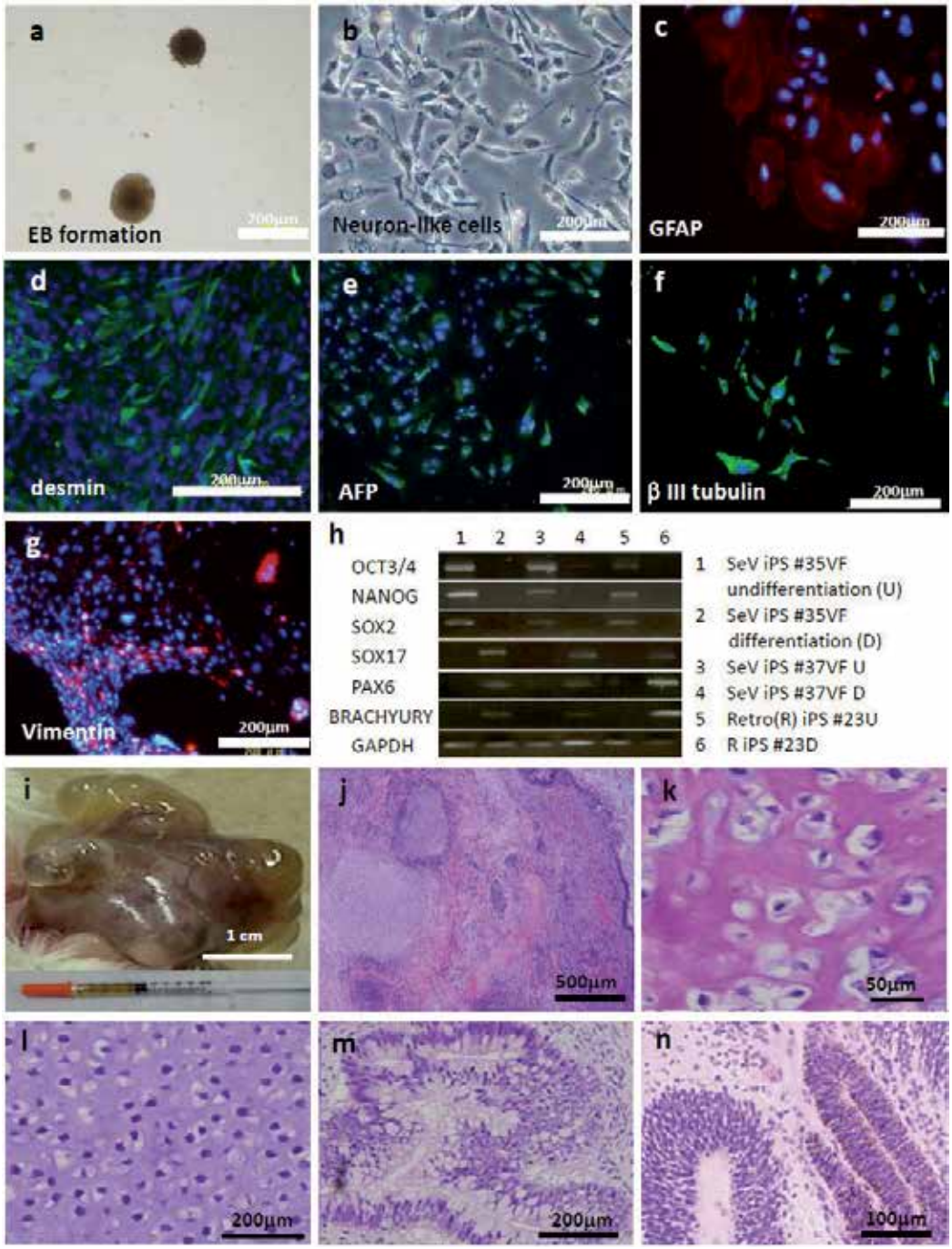

Fig. 5. *In vitro* and *in vivo* differentiation potentials of established iPS cell lines.
(**a-g**) Embryoid body-mediated differentiation of established iPS cells. All images shown are from cells derived from clone SeV iPS #35VF. Bright field images of embryoid bodies generated after eight days of culture (**a**). Embryoid bodies were transferred to gelatin dishes

and differentiated for a further eight days to induce either un-directed or guided differentiation (**b-g**). Phase contrast images of neuron-like cells (**b**) after differentiation on gelatin. Cells were fixed and stained with antibodies against GFAP (**c**), desmin (**d**), AFP (**e**), βIII-tubulin (**f**), and vimentin (**g**) to determine lineage-specific differentiation potential. (**h**) RT-PCR for lineage-specific differentiation of established iPS cell lines, SeV clones #35VF and #37VF. Retrovirally generated iPS cell clone R#23 from CD34+ CB cells was used for a control. (**i**) Teratoma formed from SeV iPS #35VF was injected into testis capsule. Teratoma had a cystic structure. The content of cysts is shown in the 1 mL syringe. (**j**) Hematoxylin and eosin staining of teratoma derived from iPS cells at low magnification. Histology showed derivatives of all three embryonic germ layers including bone-like (**k**: mesoderm), cartilage-like (**l**: mesoderm), gut-like epithelium (**m**: endoderm) and neural rosette-like (**n**: ectoderm) tissue.

clones were able to give rise to cells from all three germ layers as evidenced by cell morphology (Fig. 5b) and immunocytochemistry (Fig. 5c-g). Upon differentiation, the presence of gene expression characteristic of all three germ layers was determined by RT-PCR (Fig. 5h).

2.3.2 *In vivo* differentiation assay

One million iPS cells were injected beneath the testicular capsule of SCID mice (SLC Japan) for teratoma formation. Tumor formation was observed 60 - 80 days after cell transplantation. Tumor tissues were fixed with 4% formalin followed by hematoxylin and eosin staining. Two lines were tested for teratoma formation and both cell lines formed teratomas with a cystic structure (Fig. 5i). HE staining of teratoma tissues (Fig. 5j - 5n) showed differentiated tissues corresponding to all three germ layers.

3. Conclusions

Reprogramming of somatic cells with SeV vector without DNA integration is advantageous, as it reduces the chance for tumorigenicity caused by random genomic integration. Advantages of using SeV vector over other non-integrating reprogramming methods such as using adenovirus, episomal plasmid vectors, conventional plasmid vectors, or small molecule delivery systems include superior reprogramming capability with potent protein expression potential (13). The remaining concern in using SeV vector is how we can confirm the removal of potent SeV vectors from reprogrammed cells. In this report, we used the temperature-sensitive SeV vector TS7 to reduce the number of SeV-infected cells. In addition, we made use of a single cell cloning technique in the naïve state to confirm the absence of SeV vector constructs in the reprogrammed cells at a single cell level. Therefore, this cloning technique provides an ultimate solution for RNA virus vector-based reprogramming methodology.

The benefits of reprogramming somatic cells in the naïve state are not limited to a single cell cloning technology. It may provide answers to interesting questions like whether "standard" human iPS cells, having the correct epigenetic memory, can be generated by reprogramming somatic cells in the naïve state. Accumulation of epigenetic information before and after transferring to the naïve state would provide an answer to this question.

Several reports showed that iPS cells can be preferentially generated from the CD34+ fraction of CB cells and peripheral blood cells with retroviral vectors (19, 26). In our experiment, we also showed that the SeV TS7-GFP vector selectively infects freshly isolated

$CD34^+$ $CD45^{low+}$ cells and is able to reprogram this fraction. These data suggested that the use of SeV would facilitate the effective generation of iPS cells from CB cells. However, the molecule(s) responsible for SeV viral entry into the cell is elusive. Hemagglutinin-neuraminidase (HN), an envelope protein of Sev is reported to bind to sialomucin (28) and facilitate the cellular entry of virus. CD34 belongs to the sialomucin family. Although SeV is not able to infect $CD34^-$ cells from freshly isolated (non-cultured) CB cells, SeV is able to infect $CD34^-$ cells that have differentiated from $CD34^+$ CB cells after seven days of culture in hematopoietic cell culture media. With limited information, we cannot conclude that CD34 is the SeV entry molecule. Rather, it appears that a set of molecules other than CD34, expressed in $CD45^{low+}$ cells, might be responsible for it.

As a cell source for generating iPS cells, CB cells have certain advantages over other somatic cells. Unlike cultured cells or those obtained by biopsy from a variety of tissues at various ages, freshly isolated (non-cultured) $CD34^+$ CB cells are the youngest stem cell population available following birth. They also have distinct genetic and epigenetic profiles as hematopoietic stem cells and progenitors and lack genetic alternations like rearrangements or possible post-natal genomic damage caused by UV irradiation or chemical irritants. Furthermore, generating iPS cells from this fraction would facilitate our understanding of the reprogramming process, since the genetic profiles of the cell source and the reprogrammed cells are known. Another advantage of using cord blood cells would be the possibility of collaborating with the existing world-wide network of public cord blood banks. Extensive discussions concerning the conditions and ethical issues are necessary before such clinical applications are pursued. Nonetheless, the use of CB as a source for iPS cells is a realistic option for generating "bona fide" iPS cells for future clinical use.

4. Acknowledgment

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Modelling of Neurological Diseases Using Induced Pluripotent Stem Cells

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1. Introduction

Human embryonic stem cells (hESCs) were first established as an *in vitro* culture system (Thomson et al., 1998). hESCs are pluripotent cells that are able to self-renew and can differentiate into the three primary germ layers: endoderm, ectoderm and mesoderm. Specifically, hESCs can be used to generate gut epithelium, cartilage, bone, muscle, neuroepithelium, and embryonic ganglia (Zhang et al., 2001, Itskovitz-Eldor et al., 2000, Sottile et al., 2003, Thomson et al., 1998, Green et al., 2003). Since these hESC cell lines can be maintained for months in an undifferentiated state, they can be used as a stable resource to model human development in vitro. The conversion of hESCs to neural progenitors and subsequently to the three main neural lineages: neurons, astrocytes and oligodendrocytes was first demonstrated in 2001 (Reubinoff et al., 2001).

The recent advent (Takahashi and Yamanaka, 2006) of induced pluripotent stem cells (iPSCs) makes it possible to derive pluripotent stem cells from somatic tissue. iPSCs are derived by transfecting somatic cells (e.g. skin fibroblasts) with a select group of transcription factors; Sox2, Oct4, Myc and Klf4 to induce reprogramming of the genome over a period of 3-4 weeks. This breakthrough by Yamanaka and colleagues enables the modelling of human disease by, for example, taking a patient's skin cells, converting them to iPSCs and then differentiating it to any desired cell-type. Thus iPSC technology opens a new era of patient-specific disease modelling. Here, we review diseases of the nervous system that have been modelled using iPSC which includes; RETT syndrome (RTT), Familial dysautonomia (FD), Parkinson's disease (PD), Huntingtons (HD) and Amyotrophic Lateral Sclerosis (ALS). To provide some background to the neurological disease modelling studies by iPSCs we begin by reviewing protocols for neural induction (differentiation) of hESC.

2. Neural induction and neuronal differentiation of human embryonic stem cells (hESCs)

2.1 Neural induction of human ESCs

Several protocols, based on knowledge from developmental studies of embryogenesis and neurogenesis, have been developed to optimize yield and purity of neural progenitors from hESCs (Fig1). They can be categorized into two broad approaches. The first involves a

stepwise change of culture medium components followed by expansion of neural progenitors (Reubinoff et al., 2001). This protocol involves the long-term culture (3 weeks) of hESCs without replenishing the mouse embryonic fibroblast layer. These cells rapidly undergo morphological changes, forming neural rosettes. They are subsequently dissociated and replated in medium permissive for the growth and maintenance of NSCs (DMEM/F12, B27 supplement, EGF [epidermal growth factor], bFGF [basic fibroblast growth factor]). Similar to mouse ESCs, hESCs can also be differentiated as suspension cultures where they form cystic embryoid bodies (EBs). Formation of these three-dimensional spheres can recapitulate the microenvironment during embryo development, and are preferred over monolayer formats because it allows for up-scaling of differentiation cultures to a greater extent. However, differentiation of hESCs in this sphere format gives rise to issues such as heterogeneity (containing cell types pertaining to the three germ layers) and differential access to soluble factors, which translates to difficulties in optimizing the purity of the desired differentiated progenitors.

The second approach to induce neural lineage cells, is to co-culture hESCs with a variety of stromal cell types, such as the PA6 and MS5 (Kawasaki et al., 2000, Hong et al., 2008, Chimge and Bayarsaihan, 2010). Both PA6 and MS5 stromal cells were derived from murine bone marrow. This induction method is based on the knowledge that signals from mesodermal cells of the Spemann organizer (which develop into the notochord) can induce overlying ectoderm to neuroectodermal fate (Harland and Gerhart, 1997, Londin et al., 2005). This neural induction protocol was reported to generate up to 92% NCAM [neural cell adhesion molecule]–positive cells in 12 days. Given the ease and efficiency of this protocol it is not surprising that it is widely used. However, the neural-inducing effect of the stromal cells is not fully understood. PA6 cells can induce neural differentiation of hESCs in the absence of physical contact, but the conditioned medium of PA6 cells was unable to induce neural differentiation (Kawasaki et al., 2000). In addition, paraformalydehye-treated PA6 cells continued to exhibit neural-inducing activity, suggesting that the viability of these cells is not vital. In order to fine-tune the procedure to exclude animal products, a matrix material from the human amniotic membrane was found to support neural induction of hESCs with similar efficiencies to that of the mouse stromal cells (Ueno et al., 2006).

Given the caveats in the EB and co-culture methods, strategies were developed to maximize the induction of hESCs into neural lineage, using differentiating factors and monolayer subculture systems. The secretion of the bone morphogenic protein (BMP) antagonists, noggin and chordin, from the epidermal ectoderm of the *Xenopus* embryo was demonstrated to be essential for neural induction (Sasai et al., 1996). In addition, follistatin, an inhibitor of Activin signaling, was found to promote neural induction (Hemmati-Brivanlou and Melton, 1994). Hence, strategies used for increasing the yield of neural derivatives from hESCs include using inhibitors of BMP signaling such as Noggin (Itsykson et al., 2005), and the inhibitors of Activin/Nodal signaling such as the pharmacological inhibitor of Nodal signaling, SB431542 (Smith et al., 2008). Since both signaling pathways converge to downstream SMAD proteins, subsequent work further optimized the induction of neural lineage cells by using the inhibitors of both BMP and Nodal signaling (Chambers et al., 2009). However, Noggin, as a protein is expensive and may exhibit batch-to-batch variability. Recent findings report that substituting noggin with a small-molecule inhibitor of BMP, Dorsomorphin, can efficiently promote neural differentiation of hESCs and iPSCs (Morizane et al., 2010, Zhou et al., 2010). Other methods rely on genetic manipulations such

as generating nestin-EGFP hESC reporter lines to allow purification of hESC-derived neural progenitors (Noisa et al., 2010).

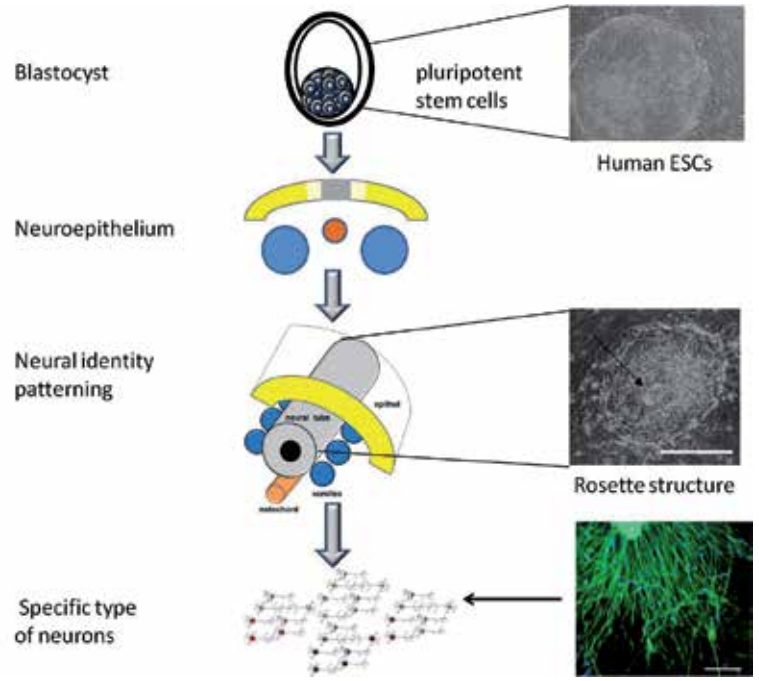

Fig. 1. Schematic representation of neural induction and patterning in *in vivo* and *in vitro*. 1) At early gastrulation, the notochord (orange) secretes BMP-antagonists, such as Noggin and Chordin to create a *gradient of BMP* activity (yellow). 2) Neuroepithelial (NE) cells that are proximal to the notochord become neural precursors (grey) as a consequence of low BMP activity; and the rest of the ectoderm becomes epithelial (yellow) or neural crest (not shown). The NE is patterned by many signals including Shh, RA, Noggin and Chordin (orange) that are secreted from the notochord; BMP (yellow) from the epithelium and FGF (blue) from the somites. 3) The cells in the NE differentiate to specific subtypes of neurons depending on the location in the neural tube and the factors that influence the differentiation. Neural induction and patterning of human ESCs can be induced *in vitro* as shown in the right panels (bright field micrograph or GFP labeled neuronal sub type).

The next aim, upon obtaining neural progenitors/stem cells, is to coax these cells into differentiation of various neuronal subtypes including the cholinergic neurons, dopaminergic neurons, motorneurons, and peripheral sensory neurons. Several methods using various combination of protein factors, co-culture systems and small molecules have successfully been utilized to obtain individual neuronal subtypes. The basic strategy for developing culture cocktails to obtain neuronal subtypes is to mimic signals from early developmental events during specification of the body axis of the embryo. Factors frequently used to induce neural progenitor differentiation include retinoic acid (RA), FGFs and Wingless-Int (Wnts) that can poise cells to differentiate to the neural lineage along the the anterior/posterior (A-P) axis (Hendrickx et al., 2009, Onai et al., 2009). Similarly, Sonic Hedgehog (Shh) and BMPs secreted from the ventral and dorsal neural

tube respectively are important for the precise patterning of multiple neuronal progenitors (Furuta et al., 1997, Litingtung and Chiang, 2000). Hence, the efficiency of deriving specific neuronal subtypes can be influenced by the mode of neural induction. The selection of the appropriate neural differentiation protocol is pertinent for the maximization of desired neuronal subtype.

2.2 Neuronal differentiation of human ESCs

Many neuronal subtypes have successfully been derived from hESCs (Fig1). Amongst them are the dopaminergic neurons, serotonergic neurons, peripheral sensory neurons, and cholinergic neurons (Bissonnette et al., 2011). Dopaminergic neurons are progressively lost in Parkinson's disease (PD), while the motorneurons are lost in amyotrophic lateral sclerosis (ALS). The factors and conditions used for successful derivation of dopaminegic neurons, motoneurons as well as other neuronal subtypes can be found in Table 1.

Shh and FGF8 were identified to be crucial for the specification of dopaminergic neurons *in vivo* (Ye et al., 1998) but were not directly effective *in vitro* (Stull and Iacovitti, 2001). This led to the search for other signals in promoting dopaminergic neuron differentiation. Currently, differentiation of human ESCs into midbrain dopaminergic neurons can be achieved through the formation of EBs and the use of various factors such as serum-free conditioned-medium from human hepatocarcinoma cell line HepG2, brain-derived neurotrophic factor (BDNF), ascorbic acid, transforming growth factor (TGF), and glial cell line-derived Neurotrophic factor (GDNF) (Cho et al., 2008a, Cho et al., 2008b, Park et al., 2004, Schulz et al., 2004, Yan et al., 2001). Particularly, TGFß can cooperate with Shh and FGF8 to increase the yield of dopaminergic neurons by enhancing their survival (Roussa et al., 2004); and GDNF was found to be important for the maintenance of motoneurons in the striatum proper (Nevalainen et al., 2010). Large-scale production of dopaminergic neurons from hESC was reported using the EB format with addition of Shh, FGF8 and ascorbic acid. 86% of totally differntiated cells were dopaminergic neurons. Secretion of factors by PA6 cells is important for dopaminergic differentiation (Vazin et al., 2008). PA6 secreted factors include stromal cell-derived factor 1 (SDF-1/CXCL12), pleiotrophin (PTN), insulin-like growth factor 2 (IGF2), and ephrin B1 (EFNB1).

The protocols used for the specification of spinal motoneurons from hESCs were derived from developmental studies. Here motoneurons were found to be induced by signals such as retionic acid from the caudal paraxial mesoderm (Guidato et al., 2003). Shh signaling from the notochord is also required for the induction of motoneurons (Lewis and Eisen, 2001). Neural induction is predominantly performed through the formation of EBs, using defined medium conditions. Because neuroectodermal cells differentiated from hESCs generally have a rostral character, motoneuron differentiation is then achieved by the caudalizing factor retinoic acid followed by the ventralizing factor Shh (Li et al., 2005). Small molecules that can activate the Shh signaling, purmorphamine and SAG (*a* chlorobenzothiophene-containing Hh pathway agonist), have been shown to be effective as substitutes of Shh in motoneuron differentiation (Li et al., 2008, Hu and Zhang, 2009, Wada et al., 2009). The use of Hb9 (homeobox gene selectively expressed in motoneurons) promoter driven GFP hESCs allows isolation of a fairly pure population of motoneurons (Singh Roy et al., 2005). Enrichment of derived motoneurons, up to 80%, was also shown to be achieved by gradient centrifugation in Biocoll (Wada et al., 2009), alleviating the need for genetic manipulations.

Neuronal Subtypes	Neural Induction Method	Factors for Final Differentiation	Done with iPS?	Reference
Dopaminergic neurons	50%-MedIIconditionedmedium, bFGF or DMEM/N2	DMEM/N2, GDNF, BDNF, 5% serum		(Schulz et al., 2004)
	EB with RA and bFGF	BDNF, TGF-α		(Park et al., 2004)
	EB, followed by bFGF and N2 supplement	Shh, FGF8		(Cho et al., 2008)
	PA6 co-culture	PA6, Shh, FGF8, GDNF		(Vazin et al., 2008)
	PA6 co-culture	PA6 co-culture or SDF-1/CXCL12, PTN, IGF2, EFNB1		(Vazin et al., 2009)
	MS5 co-culture	RA, Shh, FGF8, Wnt1	√	(Cooper et al., 2010)
Motoneurons	EB, then adherent in F12/DMEM, N2 supplement, heparin and bFGF	RA, then Shh		(Li et al., 2005)
	EB, bFGF	RA in DMEM/F12,N2, heparin, cAMP. Then Shh/purmorphamine, then GDNF, BDNF, IGF1		(Li et al., 2008)
	EB	RA, then Shh/purmophamine, then BDNF, GDNF, IGF, cAMP	√	(Hu and Zhang, 2009)
Peripheral sensory neurons	PA6 co-culture	Co-culture with PA6 stromal cells		(Pomp et al., 2008)
	MS-5 co-culture, followed by Shh, FGF8, BDNF, ascorbic acid	BDNF, GDNF, NGF (nerve growth factor), Dibutyryl-cAMP	√	(Lee et al., 2009)
Cholinergic neurons	Free-floating aggregates	BDNF, NT3, CNTF, NGF		(Nilbratt et al., 2010)
	RA, followed by free-floating aggregates	FGF8, Shh, BMP9		(Bissonnette et al., 2011)
Serotonergic neurons	RA with EB formation	5-HT, forskolin, acidic FGF, BDNF, GDNF		(Kumar et al., 2009)

Table 1. Factors used in differentiation of hESC-induced NSCs into the various neuronal subtypes.

2.3 Glial differentiation of hESCs

The two glial cell types of the nervous system, the astrocytes and oligodendrocytes, play supporting roles in the brain and peripheral nervous system. Astrocytes secrete various neurotrophic factors, are known to modulate oligodendrocyte myelination of neuronal axons (Moore et al., 2011), and modulate neurotransmitter levels through re-uptake and release mechanisms (Voutsinos-Porche et al., 2003). Evidence is emerging that implicates astrocytes in the pathogenesis of neurological disorders such as Rett syndrome (Maezawa et al., 2009) and ALS (Nagai et al., 2007). A protocol to obtain astrocytes includes exposure of adherent hESCs to cyclopamine, an inhibitor of hedgehog signaling, and subsequent culture in human astrocyte medium to generate a high percentage of nestin and glial fibrillary acidic protein (GFAP)-expressing cells (Lee et al., 2006).

Oligodendrocytes are cells that produce myelin sheaths that insulate axons of neurons, enabling saltatory conduction between the Nodes of Ranvier for rapid propogation of action potential. These cells are targets of severe developmental diseases such as Pelizaeus-Merzbacher disease, and demyelinating diseases such as multiple sclerosis and Charcot-Marie-Tooth (Bramow et al., 2010, Garbern, 2007, Sargiannidou et al., 2009). Dysfunctional oligodendrocytes in these diseases lead to disruptions in axonal transport. In spinal cord injury, demyelination of nerve fibres attributes to functional loss of neurons. So, myelin formation and insulation of neurons is crucial to restore functional network. Hence, it is important to source for pure poulation of glial (oligodendrocyte precursor) cells that can restore function of neurons by remyelination. A few reports and studies from Geron trial have shown the feasibility of using functional OPCs or neurotrophin expressing GRPs from directed differentiation of hESCs and their therapeutic potential at early time points after spinal cord injury (Keirstead et al., 2005, Cao et al., 2005).

Oligodendrocytes undergo multiple stages of differentiation, from oligodendrocyte progenitors, to pro-oligodendrocytes, to non-myelinating oligodendrocytes, and finally myelinating oligodendrocytes (Reviewed in Miller, 2000). A protocol by Hu *et al*. 2009 provides an excellent example of the procedure for obtaining oligodendrocyte progenitors. Briefly, hESC-derived neural progenitors are treated with RA and Shh for patterning into the ventral spinal progenitors, followed by FGF2 for inhibition of differentiation into motoneurons. Oligodendrocyte progenitors were induced with the addition of factors promoting for survival and proliferation of oligodendrocytes – transferrin, progesterone, sodium selenite, putrescine, triiodothyronine (T3), neurotrophin 3 (NT3), platelet-derived growth factor (PDGF), cyclic adenosine-monophosphate (cAMP), insulin growth factor-1 (IGF-1) and biotin. Other factors shown to promote oligodendrocyte differentiation of hESCs include hepatocyte growth factor (Hu et al., 2009) and extracellular matrix protein vitronectin (Gil et al., 2009). Oligodendrocytes have also been shown to be derived from iPS cells (Czepiel et al., 2011).

3. iPSC technology and implications for neurodegenerative diseases

Neurological diseases are conditions that affect the central and peripheral nervous system. At present pharmacological interventions for many neurological diseases, especially the degenerative conditions, are limited and predominantly restricted to alleviation of symptoms. Finding drugs for treatment of neurological disorders represents one of the critical goals of medical research today. The recent discovery of iPSC technology (Takahashi and Yamanaka, 2006) opens the possibility to generate patient

specific models of human disease. The generation of patient specific cells using iPSC technology will be a powerful resource for both cell therapy and drug screening (Fig2). iPSC is particulary important for neurological diseases as there are limited cellular models of the nervous system.

3.1 X-linked diseases

A disproportionately large number of disease conditions have been associated with the X chromosome because the phenotypic consequence of a recessive mutation is revealed directly in males for any gene that has no active counterpart on the Y chromosome. Thus, although the X chromosome contains only 4% of all human genes, almost 10% of diseases with a mendelian pattern of inheritance have been assigned to the X chromosome (307 out of 3,199). 168 X-link diseases have been explained by mutations in 113 X-linked genes(Ross et al., 2005).

While males carry a single X chromosome, females have two and hence two copies of each gene. Yet, as one of their X chromosomes is inactive in each cell, females also have only one working X chromosome in each cell. Thus, females havea mosaic of cells that express either the paternal X allele or the maternal X allele. This cellular mosaicism gives females a big advantage over males in the context of X-linked diseases. When an X-linked gene is mutated, the normal cells can partly compensate for the cells that express the mutant allele as a result from cell elimination or by functional compensation (Migeon, 2007). In males, on the other hand, all cells express the mutant gene and therefore usually show much more severe symptoms.

Because the process that inactivates X chromosomes is random with respect to parental origin of the X, usually half of the female cells contain a working X chromosome from the father while the other half contain a working X chromosome from the mother. By reprogramming fibroblasts from female with X-linked disease one can generate both the perfect pair of control (expressing the normal allele) and experimental (expressing the mutant allele) cell types for investigation of the disease phenotype (see schematic Fig. 3). These isogenic control and mutant iPS-derived neurons represent a promising source for modelling X-linked diseases.

3.2 Rett syndrome (RTT)

RTT is one of the most common causes for mental retardation in females, affecting 1 in *10000* live female births. It was first reported in 1966 by the neurophysiologist Dr. Andreas Rett (Rett, 1966). The large majority of RTT cases are caused by sporadic or from germline mutations within the coding sequence of the X-linked methyl CpG binding protein 2 **(**MeCP2) gene (Amir et al., 1999) and therefore the mutation cannot be detected by simple screening of the parents. Females with classic RTT appear normal from birth until 6–18 months of age, but then they fail to acquire new milestones and enter a period of regression during which motor and language skills are lost. These females show a large diversity of symptoms, that appear progressively (reviewed in Chahrour and Zoghbi, 2007) until they reach a plateau, suggesting that the condition does not involve progressive neurodegeneration (Sun and Wu, 2006). The postnatal onset of symptoms might be explained by the increase in MeCP2 levels in cortical neurons throughout normal development (Akbarian et al., 2001, Balmer et al., 2003, Jung et al., 2003).

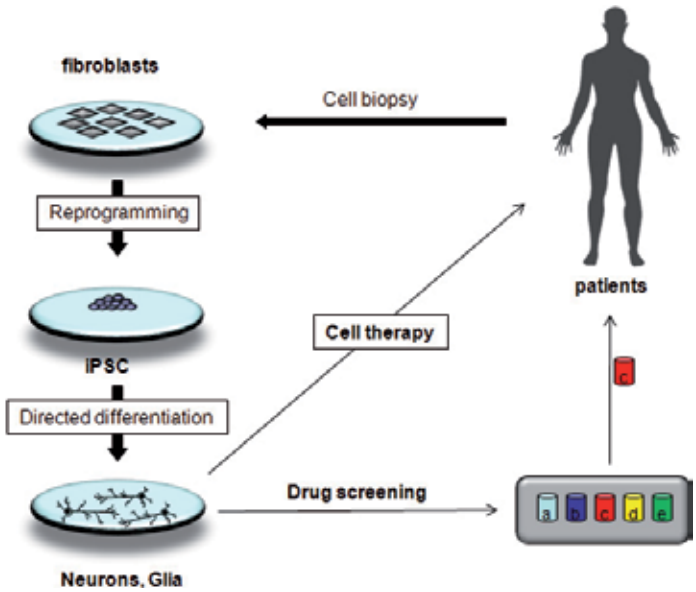

Fig. 2. The potential of iPSCs technology. Cell sample can be easily obtained from patients and cultured *in vitro*. These cells can then be reprogrammed into iPSCs and further be differentiated into the afflicted cells. Cellular phenotype is assessed by measuring cell properties (i.e. neural morphology, maturation of synapses, cell survival under stress, etc). Once a distinct disease related phenotype is identified, drug screening platform can be developed to test their potential to reverse these phenotypes (in this example, component 'c' is a potential candidate (see Fig. 3) and can be used for further tests *in vivo*). Another usage for this technology is cell therapy - transplantation of the cells back to the patient.

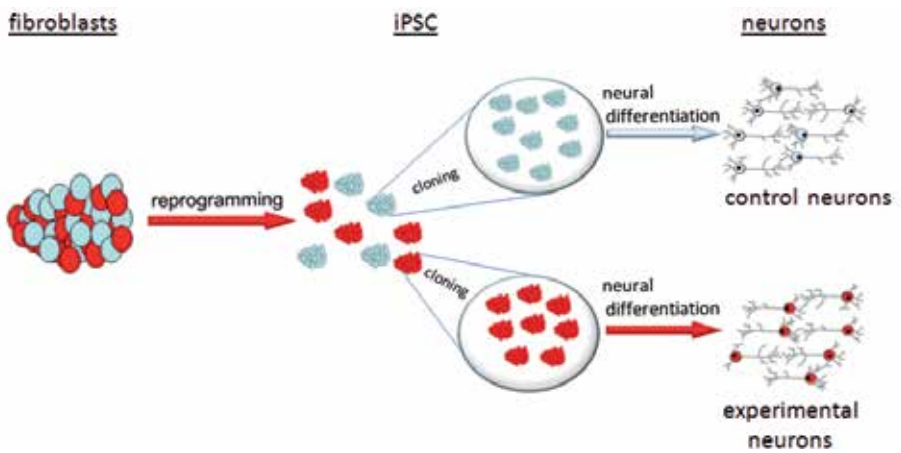

Fig. 3. *In vitro* disease modeling for X-linked diseases. Schematic explaining reprogramming of mosaic cell population from a female with X-linked disease, such as RTT, both iPSC clones expressing the mutant allele ($Xa^{mut}\ Xi^{wt}$) and iPSC clones expressing the wild-type ($Xa^{wt}\ Xi^{mut}$) can be expanded and further differentiated into the afflicted cells (i.e. neurons). The wild type neurons, can be used as a perfect control for the mutant neurons for further studies, such as screening of molecules that might potentially reverse the symptoms.

Although MeCP2 is expressed in a large variety of cells, the most afflicted cells in RTT are neurons. In agreement, a specific knockout in the central nervous system revealed the same spectrum of symptoms as the full mouse knockout. Several specific neurons were studied in the context of RTT. For example, pyramidal glutaminergic neurons, which show obvious morphological differences when they express the mutation (see Fig. 3), can account for the mental retardation seen in RTT; dopaminergic neurons can account for the motor function; neurons in the Amygdala can affect learning and memory; and hypothalamus neurons can affect feeding, aggression and stress.

In females, the major source for phenotypic variability is the pattern of X chromosome inactivation (XCI) (Bourdon et al., 2001). Females with classical RTT are usually a balanced mosaic with regard to MeCP2 expression (Adler et al., 1995), whereby half of their cells express the wild-type MeCP2 allele and the other half express the mutant MeCP2 allele (Shahbazian et al., 2002). However, sometimes, by chance, the XCI pattern favors expression of the wild-type allele. Such 'lucky' females might show milder symptoms and even to be asymptomatic(Sirianni et al., 1998). Another source for phenotypic variability is the type of mutation in the MeCP2 gene. Over 300 different mutations in the gene MeCP2 were identified, which are the major source of the phenotypic variability in males with RTT. Thus generation of iPSCs from multiple patients might shed more light on the correlation between mutation type and symptoms as well as the function of different domains in MeCP2 protein. In addition, there are important implications for drug screening, as some drugs might treat patients with certain mutations but not others. For example, aminoglycosides antibiotics, such as gentamicin, can increase wild type MeCP2 expression levels in affected neurons by skipping a premature stop codon by bind to the 16S rRNA, impairing ribosomal proofreading (Kellermayer, 2006, Marchetto et al., 2010). Thus, disease modeling for multiple patients will allow us to tailor specific drugs for each patient.

Initially, RTT was thought to be a neurodegenerative disease, however, the decrease in axondendritic arborization and impaired development of dendrites (Fig. 4) suggest that RTT is a disorder featuring an arrest in neuronal development (Hagberg, 1985, Belichenko et al., 1994, Armstrong, 1992, Armstrong, 2001). Furthermore, no obvious cell death is seen in RTT, and this therefore begs the question of whether restoring MeCP2 expression would restore normal neuronal function and reverse the resulting disease phenotypes. In a seminal work, Guy et al (2007) provide evidence supporting the feasibility of disease reversibility in mouse models of RTT (Guy et al., 2007). They created a mouse in which endogenous MeCP2 is silenced by insertion of a Lox-Stop cassette and can be conditionally activated through Cre-mediated deletion of the cassette. The MeCP2 lox-Stop allele behaved as a null mutation, and its activation was controlled by a tamoxifen-inducible Cre transgene. Gradual tamoxifen injection reversed the late-onset neurological phenotype of adult MeCP2-lox-Stop/+; cre heterozygotes, indicating that MeCP2-deficient neurons are not permanently damaged, since MeCP2 activation leads to robust abrogation of advanced neurological defects in both young and adult animals. This work establish that consequences of MeCP2 loss of function are reversible, and suggest that the neurological defects in RTT, and other MeCP2 disorders, are not impervious to therapeutic possibilities. Indeed, several molecules, have delayed the onset of RTT-like symptoms in animal models, and enhanced survival rates (Chang et al., 2006, Ogier et al., 2007). Potential molecules for treatment of RTT are BDNF, IGF-1 and NGF. A molecule with promise in RTT therapy is BDNF. There are phenotypic similarities between MeCP2- and BDNF-null mice, including a reduction in brain weight and hindlimb

clasping. Over expression of BDNF in MeCP2-null mouse brains delayed the onset of RTT-like symptoms, and enhanced survival rates (Chang et al., 2006, Ogier et al., 2007). More specifically, BDNF overexpression rescued the hypoactivity in wheel running exhibited by MeCP2 knockout mouse, and the low frequency of action potential firing observed in their cortical neurons (Chang et al., 2006); and treatment of MeCP2 null mice with AMPAkines (which increases BDNF mRNA and protein levels) rescued the irregular respiratory patterns exhibited by MeCP2. However, the therapeutic utility of BDNF is hampered by its poor efficiency at crossing the blood–brain barrier. Nevertheless, a therapeutic intervention in humans might thus arise from identifying an agent similarly capable of stimulating synaptic maturation.

An *in vitro* disease modeling for RTT was established by Marchetto et al (2010). In this study they reprogrammed fibroblasts from RTT patients into iPSCs, and further differentiated them into neurons. When analyzed, these neurons showed lower synaptic density, simpler morphology with less branching and smaller cell body. Furthermore, by using electrophysiological methods they showed that RTT neurons have a significant decrease in frequency and amplitude. This is in agreement with studies on RTT mice models and on postmortem brain tissues from patients. This RTT disease model was used to screen molecules, such as IGF-1. Like BDNF, IGF-1 is widely expressed in the CNS during normal development (D'Ercole et al., 1996), strongly promotes neuronal cell survival and synaptic connections. Indeed, IGF-1 treatment leads to a partial rescue of RTT (Tropea et al., 2009).

In their study, Marchetto et al (2010) investigated the use of IGF1 and gentamicin in iPSC-derived neurons carrying a MeCP2 mutation. While IGF1 treatment increased synapse number in some clones, it stimulated glutamatergic RTT neurons above normal levels. Gentamicin was used to rescue neurons derived from iPSCs carrying a nonsense MeCP2 mutation by increasing full-length MeCP2 levels in RTT neurons, rescuing glutamatergic synapses. Thus, this *in vitro* disease model can be used to screen therapeutic candidate molecules.

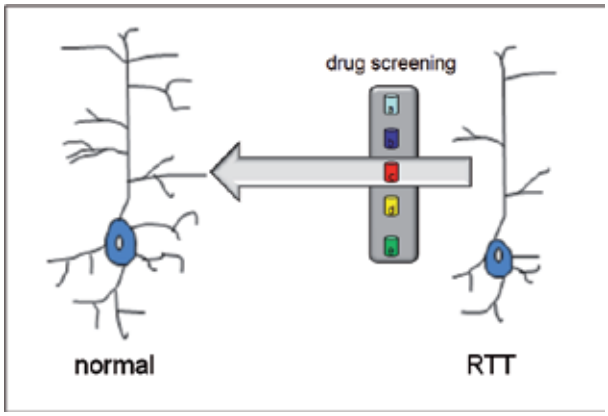

Fig. 4. Schematic representation of drug screening for pyramidal neurons carrying MeCP2 mutation. In RTT, the cell body is small and there is reduced dendritic branching (Armstrong 1995), fewer synapses, and reduced spine density compared to the control. These morphological differences can be an indicator for drug screening. In this example, component 'C' reverses the impaired morphology of the RTT neurons and therefore can be further studied in RTT animal models as a potential drug to reverse the RTT symptoms.

3.3 Familial dysautonomia

Familial dysautonomia (FD), also known as Riley–Day syndrome or HSAN-III is a neurodevelopmental disorder, caused by a mutation in the I-k-B kinase complex-associated protein (*IKBKAP*) gene on chromosome 9. This condition is affects 1 in 3,700 Eastern European Jewish ancestry (Ashkenazi Jews), but rare in the general population. The disease was first reported by Conrad Milton Riley and Richard Lawrence. It is a fatal autosomal recessive disease characterized by the degeneration of sensory and autonomic neurons (Slaugenhaupt et al., 2001, Axelrod et al., 2002), resulting in variable symptoms including: insensitivity to pain, inability to produce tears, poor growth, and labile blood pressure (episodic hypertension and postural hypotension). Future parents can be screened for the mutation and if both parents are shown to be carriers by genetic testing, there is a 25% chance that the child will produce FD. The point mutation in the *IKBKAP* gene results in a tissue-specific splicing defect with various levels of exon 20 skipping and reduced levels of normal IKBAP protein (Slaugenhaupt et al., 2001, Anderson et al., 2001). The ratio between the normal and mutant transcripts affects the severity of the disease.

An iPSC-based model for FD was established by Lee et al (Lee et al., 2009). FD-iPSC-derived neural crest showed reduced migration as well as decreased rate of neurogenesis. Furthermore, when they screened the afflicted cells with candidate drugs, they found that kinetin resulted in a marked reduction of the mutant *IKBKAP* splice form, associated with an increase in normal *IKBKAP* levels. This treatment increased the percentage of differentiating neurons but it did not affect the migration potential of the neural crest. Thus FD-iPSC model is an example of gaining insight about early progession of a neuro-developmental disease.

3.4 Parkinson's disease

Parkinson's disease (PD) is one of the most widely studied neurodegenerative disease caused by the progressive degeneration of midbrain dopaminergic neurons. Although the aetiology of PD remains enigmatic, partially attributed to complex environmental factors, and mutations in several genes (LRRK-2, Parkin, DJ-1, PINK1, ATP13A2, alpha-synuclein, GBA) have been found to give rise to PD-like pathogenesis. However, these genetic factors contribute to only a small fraction of PD cases, impeding significant progress in understanding PD pathogenesis completely. Moreover, it was found in PD patients who had received neuronal grafts through transplantation that the cells which were previously young and healthy developed α–synuclein and ubiquitin-positive Lewy bodies after more than a decade. This led to the proposal of several mechanisms such as inflammation, oxidative stress, excitotoxicity and growth-factor deprivation that may have substantial impact on the propagation of PD (Brundin et al., 2008). Thus, it would be necessary to re-examine the disease pathology by mimicking characteristics of the disease under these varied conditions or in combination with known genetic defects since the benefits of cell transplantation in PD trials is still uncertain. This objective may soon be achievable as Soldner et al (2009) demonstrated that iPSCs could be derived from patients with idiopathic PD. Furthermore, the iPSCs could be directed to differentiate into dopaminergic neurons through EB formation in the presence of FGF2, FGF8 and Shh. Coupled with the possibility of deriving large-scale functional dopaminergic neurons; via the generation of homogenous spherical neural masses (Cho et al., 2008), the ultimate goal of creating patient-specific neuronal cells for transplantation therapy may soon be in the pipeline. It is reassuring to note that neurons

reprogrammed from mouse fibroblasts were able to integrate functionally into the fetal brain and improve the locomotor behavior in at least a rat model of PD upon transplantation into the adult brain (Wernig et al., 2008). However, whether these transplanted cells will succumb to the same fate in the long-term as observed in the post-mortem human PD trails remains to be investigated.

3.5 Huntington's disease

Huntington's disease (HD) is caused by CAG repeats in the N-terminus of the gene encoding Huntingtin protein. Like Lewy proteins in Parkinson's disease patients, the expression of aberrant polyglutamine-containing molecules in HD leads to massive loss of medium spiny neurons in the striatum and neurons in the cortex as the disease progresses. Despite substantial efforts invested in various cellular and animal models to understand the disease, the mechanisms implicated in the selective cell death of neurons remains unclear and there is no cure at present. One major hurdle is the lack of appropriate human samples carrying the genetic mutation for HD which would offer the most ideal system for investigating the process of neurodegeneration. With the advent of iPSC technology, HD-specific iPSCs were generated (Park et al., 2008). Thereafter, HD specific NSCs (nestin+/PAX6+/SOX1+/OCT4-) were obtained and these were subjected to differentiation conditions combining morhogens (SHH and DKK1) and neurotrophins (BDNF) to induce neurons of the striatal lineages (Zhang et al.). Importantly, these neurons contained the same CAG expansion as the mutation in the HD patient in which the iPSC line was established. Thus, a valuable resource is now available to search for drugs that can reduce the toxicity of polyglutamine.

3.6 Amyotrophic lateral sclerosis

Another seminal study to interrogate a deliberating disease, arising from the progressive degeneration of motor neurons of the spinal cord using the iPSC technology is amyotrophic lateral sclerosis (ALS) (Dimos et al., 2008). In this landmark study, skin fibroblasts produced directly from an 82-year-old elderly woman patient, diagnosed clinically with ALS and a SOD1 mutation were reprogrammed successfully into the pluripotent state. These patient-specific iPSCs showed strong alkaline phosphatase activity and exhibited markers (SSEA-3, SSEA-4, TRA1-60 and TRA1-81) and a transcriptional profile (*REX1*, *FOXD3*, *TERT*, *NANOG* and *CRIPTO*) that are comparable to pluripotent hESCs. Furthermore, these cells could form EBs and were capable of differentiating into cells of the germ layers. Most importantly the iPSCs were directed specifically towards both motor neurons and glia fates, enabling further exploration of either a cell autonomous (such as the amount of SOD1 in motor neurons) or non-cell-autonomous (such as the role of neighboring astrocytes, microglia and oligodendrocytes) function of the disease as implicated in rodent models (Boillee et al., 2006, Yamanaka et al., 2008). Thus, the iPSC technology essentially reversed the patient's history to allow the onset and progression of ALS to be captured in culture for drawing mechanistic insights.

In addition to ALS, Park and co-workers (Park et al., 2008) managed to single-handedly generate reprogrammed cells from patients with a range of genetic diseases that were either Mendelian or complex in inheritance. The diseases in which iPSCs were derived include: adenosine deaminase deficiency-related severe combined immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher disease (GD) type III, Duchenne

(DMD) and Becker muscular dystrophy (BMD), Parkinson disease, Huntington disease, juvenile-onset, type 1 diabetes mellitus (JDM), Down syndrome (DS)/trisomy 21 and the carrier state of Lesch-Nyhan syndrome. Similar to the set of assays used in the characterization of the ALS iPSCs, these disease-associated iPSC lines were all confirmed to be pluripotent and capable of multi-lineage differentiation. Moreover, these human iPSCs produced teratomas in immunodeficient Rag2−/−γC−/− mice, the golden standard for testing pluripotency. Taken together, these efforts undoubtedly demonstrated the feasibility of the iPSC technology to reprogram somatic cells from a variety of diseased patients. And in the process, provided valuable source of material that pave the way for unraveling disease mechanisms and customized cellular therapies tailored for the individual.

4. Conclusion

Research on hESCs has allowed the development of specific protocols for generation of neural progenitors and differentiated neural lineages. These protocols can now be applied to iPSCs to enable the generation of specific neurons, astrocytes and oligodendrocytes. The availability of samples from relevant donors carrying different mutations will also facilitate modelling of neuronal diseases by reprogramming somatic cells. A 'mutation library' for a particular disease can then serve as a platform for the tailoring of specific drugs for specific patients. Furthermore, disease modelling from multiple patients might provide additional insights on the pathogenesis of the disease; particularly RTT in which specific mutations are associated with variable severity in clinical symptoms. Another advantage in reprogramming is that the clinical history of every donor is known. This will allow us to model diseases in which the genetic component is not known such as Alzheimer and Parkinson's. Thus the use of iPSCs technology will herald a new era in the study of neurological diseases.

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Part 8

Pluripotent Alternatives - Other Cell Sources

22

Very Small Embryonic/Epiblast-Like Stem Cells (VSELs) Residing in Adult Tissues and Their Role in Tissue Rejuvenation and Regeneration

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1. Introduction

Embryonic development and later rejuvenation of adult tissues are regulated by a population of stem cells (SCs) that, by undergoing self-renewal, maintain their own pool and, by giving rise to differentiated progenitors, replace cells used up during life (Ratajczak et al., 2007). Thus, SCs are guardians of tissue/organ integrity and regulate the life span of an adult organism. The most important SCs, from a regenerative potential point of view, are pluripotent stem cells (PSCs). According to their definition, such cells must meet certain *in vitro* and *in vivo* criteria. PSCs must: i) give rise to cells from all three germ layers; ii) complete blastocyst development; and iii) form teratomas after inoculation into experimental animals.

The SC compartment shows a high degree of hierarchy (Hayashi & Surani, 2009). In embryonic development, the most primitive stem cells are the fertilized oocyte (zygote) and the first blastomers in the morula. These cells are called totipotent, possessing the ability to give rise to both embryo and placenta. The developing morula gives rise to the blastocyst, where PSCs are found in the inner cell mass (ICM). These cells may give rise to all three germ layers of the developing embryo; however, they have lost the ability to differentiate into placenta. The PSCs at this stage can be expanded *ex vivo* as immortalized embryonic stem cell (ESC) lines (Evans & Kaufman, 1981).

After implantation of the blastocyst, PSCs from the blastocyst ICM give rise to pluripotent epiblast stem cells (EpiSCs) that will form the entire embryo proper (Brons et al., 2007; Tesar et al., 2007). During the gastrulation process, cell lineage determination programs are initiated and EpiSCs respond to the signals from surrounding extra-embryonic tissues, which leads to their differentiation into several types of tissue-committed stem cells (TCSCs) (Ratajczak et al., 2007). TCSCs are monopotent (unipotent), which means they are restricted in their differentiation potential to cells for one tissue only (e.g., epidermis, intestinal epithelium, liver, skeletal muscles, or lympho-hematopoietic). TCSCs terminate expression of pluripotent genes and, at the same time, turn on lineage-specific molecular programs.

The first population of SCs, which at around embryonic day 7.25 (E7.25) become specified in the proximal epiblast, are primordial germ cells (PGCs), and alkaline phosphatase (AP)-

positive PGCs grow into extra-embryonic mesoderm at the base of the allantois as an appendage arising from around the posterior primitive streak (Surani et al., 2007). These cells transcribe pluripotency-related genes, such as *Oct-4, Nanog,* and *Sox2,* and are the only population of SCs that maintains expression of these genes during gastrulation. When PGCs are cultured over murine embryonic fibroblasts and exposed *ex vivo* to three growth factors (kit ligand, leukemia inhibitory factor, and basic fibroblast growth factor), they continue to proliferate and form large colonies of embryonic germ cells (EGCs), which, like ESCs, can be expanded indefinitely (Matsui et al., 1992). At around E12.5, PGCs arrive at the genital ridges, lose their markers of pluripotency, and initiate their commitment to becoming gametes (oocytes and sperm).

As mentioned above, SCs show a developmental hierarchy (Hayashi & Surani, 2009), and PSCs that emerge during embryogenesis give rise to more differentiated SC populations with the ability to self renew, but with a more limited ability for multilineage differentiation (Surani et al., 2007). Evidence is accumulating that differentiation potential is regulated by epigenetic reprogramming (Surani et al., 2007). PSCs from the ICM show global DNA demethylation, which results in i) activation of the X chromosome, ii) expression of germ-line, lineage-characteristic genes (e.g *Stella, Mvh, Dazl,* and *Sycp3*), and iii) expression of repetitive sequence families (e.g. *LINE1, SINE,* and *IAP*). After implantation of the blastocyst in the uterus, ICM-derived PSCs give rise to EpiSCs and again methylate i) the X chromosome, ii) promoters for genes characteristic of PSCs in the ICM (*Rex-1* and *Stella*), and iii) repetitive sequences (Hayashi et al., 2008). Whereas most EpiSCs undergo further differentiation into TCSCs by stable repression of promoters for pluripotent-specific genes, some EpiSCs in the proximal epiblast (precursors of PGCs) revert to a state that resembles ICM PSCs by undergoing genome-wide DNA demethylation (Hayashi & Surani, 2009). This leads to re-activation of the X chromosome and promoters for germline-lineage genes and repetitive sequences.

Unlike differentiated somatic cells, PSCs commonly express the pluripotency core transcription factors (TFs) such as Oct-4, Nanog, and Sox2 (Kim et al., 2008). These TFs form the pluripotency core circuitry by reinforcing the expression of genes involved in keeping PSCs in an undifferentiated state and, at the same time, repressing their differentiation. The biological significance of these core TFs has been experimentally proven by the generation of inducible pluripotent stem cells (iPSCs), in which fully differentiated somatic cells can be reprogrammed into ESC-like stem cells after transduction by so-called Yamanaka factors (*Oct-4, Sox2, Klf4,* and *cMyc*) (Takahashi & Yamanaka, 2006; Wernig et al., 2007).

Another hallmark of pluripotency is the presence of transcriptionally active chromatin structures at the promoters of core TFs due to methylation and histone modifications of promoter DNA (Cedar & Bergman, 2009). Thus, promoters for core TFs in PSCs are de-methylated and highly enriched for histone codes associated with active transcription, such as acetylated histones and trimethylation on lysine 4 of histone 3 (H3K4me3). In addition to the expression of pluripotency core TFs, undifferentiated PSCs also exhibit specific epigenetic marks called bivalent domains (BDs) (Azuara et al., 2006; Boyer et al., 2006; Lee et al., 2006; Stock et al., 2007). In BDs, the transcriptionally active H3K4me3 code coexists with repressive histone codes, such as trimethylated lysine 27 in histone 3 (H3K27me3). The BDs are mainly detected in the promoter regions of homeodomain-containing developmental master TFs, such as *Dlx-, Irx-, Lhx-, Pou-, Pax-,* and *Six*-family proteins. Due to the overwhelming effect of the transcription-repressive activity of H3K27me3, the transcription of BD-controlled genes is transiently repressed to prevent premature differentiation.

However, in response to developmental stimuli, BDs in promoters of these genes are switched into the monovalent type that promotes transcription. Therefore, both positive (expression of Oct-4-Nanog-Sox2) and negative (repression of differentiation-inducing TFs by bivalent domains) mechanisms are indispensable for controlling the pluripotent state of PSCs.

As mentioned before, PSCs are detected only during very early embryonic development and they disappear after differentiation into TCSCs and germline cells (Niwa, 2007). However, recent evidence has accumulated demonstrating that PSCs may reside in adult tissues and are able to differentiate into TCSCs (Ratajczak et al., 2007). These cells have been variously described in the literature as i) multipotent adult progenitor cells (MAPCs), ii) marrow-isolated adult multilineage-inducible (MIAMI) cells, iii) multipotent adult (MA) SCs, or iv) OmniCytes (Beltrami et al., 2007; D'Ippolito et al., 2004; Jiang et al., 2002; Pochampally et al., 2004). Thus, the physical presence of PSCs in adult tissues may better explain stem cell plasticity, according to which TCSCs are purportedly plastic and can trans-dedifferentiate into SCs for other tissues.

However, several questions remain to be addressed regarding these rare PSCs. First, the developmental origin of these cells is unresolved. As shown in **Figure 1,** PSCs during embryogenesis/gastrulation may become eliminated after giving rise to TCSCs, or conversely, they may survive among TCSCs and serve as a back-up/reserve source for these cells. Thus, it is important to elucidate whether PSCs found in adult tissue cells are functional under steady-state conditions or are merely remnants from developmental embryogenesis that reside in a dormant state in adult tissues. Second, is the question of

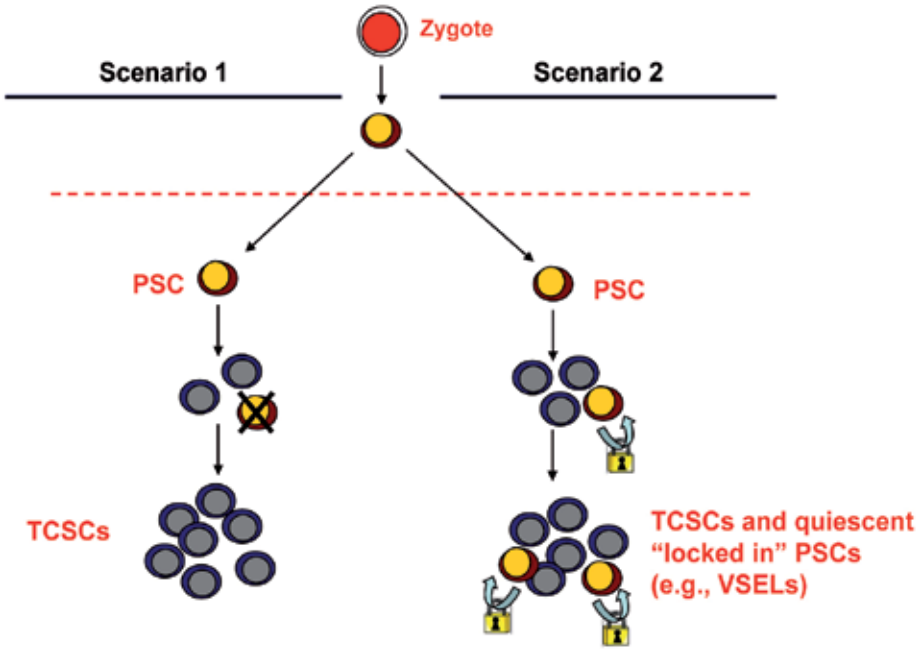

Fig. 1. **Developmental specification of PSCs into TCSCs. Scenario I:** During embryogenesis/gastrulation, PSCs are eliminated after giving rise to TCSCs and PGCs. **Scenario II:** PSCs survive and serve as a back-up/reserve source of TCSCs.

whether the dormant state of these cells is regulated by cell-intrinsic epigenetic reprogramming similar to other PSCsduring embryogenesis. Finally, there is the question of whether their dormant state is influenced by microenvironmental cues, such as their (i) location in non-physiological niches, (ii) exposure to inhibitors, or (iii) deprivation of appropriate stimulatory signals. The answer to these questions could be key to successful application of these adult-tissue-derived PSCs in the clinical setting. In this chapter, we will discuss these issues in more detail.

2. Very small embryonic-like stem cells (VSELs) residing in adult tissues

Recently, our group purified a population of very small embryonic-like stem cells (VSELs) from BM by employing a multiparameter fluorescence-activated cell sorter (FACS) (Kucia et al., 2006b). These rare Sca-1$^+$Lin$^-$CD45$^-$ cells reside in several adult murine organs (*e.g.*, brain, liver, skeletal muscles, heart, and kidney) (Zuba-Surma et al., 2008) and recently were detected also in human umbilical cord-blood and mobilized peripheral-blood (Kucia et al., 2006a; Paczkowska et al., 2009; Wojakowski et al., 2009). VSELs are very small in size (~3–6 μm) and express pluripotent markers, such as Oct-4, Nanog, Rex-1, and SSEA-1 (Kucia et al., 2006b). They are morphologically, similarly to ESCs, possessing large nuclei containing unorganized chromatin (euchromatin) and exhibiting a significantly higher nuclear/cytoplasm (N/C) ratio and a lower cytoplasmic area than hematopoietic stem cells (HSCs). The true expression of *Oct-4* and *Nanog* in BM-derived murine VSELs was recently confirmed by demonstrating transcriptionally active chromatin structures for both *Oct-4* and *Nanog* promoters (Shin et al., 2009). If cultured under a C2C12 myoblast feeder layer, freshly isolated VSELS form spheres corresponding to embryoid bodies (EBs). These VSEL-derived spheres (VSEL-DSs) contain primitive SCs that, after replating into tissue-specific differentiation media, are induced to differentiate into cells from all three germ layers (Kucia et al., 2008a). From experiments with mouse models, it has been proposed that VSELs are mobilized into peripheral blood in response to injury and circulate to the organ of injury in an attempt to enrich and regenerate damaged tissues (e.g., following heart infarct or stroke) (Kucia et al., 2008b; Paczkowska et al., 2009; Wojakowski et al., 2009). This physiological mechanism probably plays a significant role in the regeneration of some small tissue and organ injuries; however, further studies are needed to demonstrate that these cells do in fact home to the damaged organs.

3. Molecular signature of VSELs residing in BM

To investigate the relationship between VSELs and embryonic PSCs (e.g. embryonic stem cells [ESCs], epiblast stem cells [EpiSCs], primordial germ cells [PGCs], and embryonic germ cells [EGs]), we employed several molecular strategies to evaluate VSEL molecular signatures (**Figure 2**). Highly purified Sca-1$^+$Lin$^-$CD45$^-$ VSELs from murine BM were evaluated for i) expression of pluripotent genes, epiblast/germ line markers, and developmentally crucial imprinted genes; ii) the presence of BDs; and iii) reactivation of the X chromosome in female VSELs.

3.1 VSELs express PSC genes

PSCs express the essential pluripotency TF *Oct-4*. The importance of this TF is well-established by the fact that transduction with *Oct-4* is obligatory in several protocols for

generating iPSCs. We found that VSELs express Oct-4 at both the mRNA and protein level (Kucia et al., 2006a). However, recently some doubts have been raised about whether cells isolated from adult tissues express these embryonic genes, and it has been suggested that positive PCR data showing *Oct-4* expression may be due to amplification of *Oct-4* pseudogenes (Lengner et al., 2007; Liedtke et al., 2007). Thus, to prove true expression of the *Oct-4* gene in VSELs, we investigated the epigenetic state of the *Oct-4* promoter. Our DNA methylation studies of the *Oct-4* promoter using bisulfite sequencing revealed that it is hypomethylated in highly purified Sca-1$^+$Lin$^-$CD45$^-$ VSELs, similarly to cells isolated from ESC-derived EBs (28% and 13.2%, respectively) (Shin et al., 2009). Next, to evaluate the state of histone codes for the *Oct-4* promoter, we performed the chromatin-immunoprecipitation (ChIP) assay to verify its association with acetylated histone 3 (H3Ac) and dimethylated lysine 9 of histone 3 (H3K9me2), the molecular marks for open- and closed-type chromatin, respectively. By employing the carrier-ChIP assay (using the human hematopoietic cell-line THP-1 as carrier) we found that *Oct-4* promoter chromatin is associated with H3Ac and its association with H3K9me2 is relatively low (Shin et al., 2009). We also evaluated the epigenetic state of another core TF, *Nanog,* and observed that its promoter has a higher level of methylation in VSELs (~50%). However, in quantitative ChIP experiments performed in parallel, it was confirmed that the H3Ac/H3K9me2 ratio favors transcription and supports its active state (Shin et al., 2009). Based on these results, we conclude that VSELs truly express *Oct-4* and *Nanog*. Of note, we also reported that VSELs express several other markers of PSCs, such as SSEA-1 antigen, as well as *Sox2* and *Klf4* TFs (Shin et al., 2010b).

3.2 Expression of epiblast markers

As a result of the epigenetic reprogramming that occurs during implantation of the blastocyst, EpiSCs exhibit transcription profiles different from ESCs. For example, the expression of *Nanog, Sox2,* and *Stella* is reduced in EpiSCs through DNA methylation of their promoters (Surani et al., 2007). In functional assays, EpiSCs, unlike ESCs, show a highly restricted capacity to complement blastocyst development (Brons et al., 2007; Tesar et al., 2007). However, pluripotent EpiSCs may differentiate *in vivo* into TCSCs, which during embryogenesis orchestrate organogenesis and later in adult life are involved in rejuvenation of tissues and organs. Like EpiSCs, highly purified BM-derived Oct-4$^+$ VSELs do not complement blastocyst development and therefore cannot enable *in vitro* differentiation into cells from all three germ layers. Therefore, we have hypothesized that VSELs may be epiblast-derived precursors of TCSCs (Ratajczak et al., 2010). To investigate the similarity between VSELs and EpiSCs, we examined the expression of genes that are characteristic of EpiSCs (*Gbx2, Fgf5,* and *Nodal*) and ESCs from the blastocyst ICM (*Rex-1*) in adult BM-derived VSELs. It is known that *Gbx2, Fgf5,* and *Nodal* are upregulated in EpiSCs, but are expressed at lower levels in ESCs isolated from the ICM (Hayashi et al., 2008). In contrast, the level of *Rex-1* transcripts is highly expressed in ICM cells. We found that VSELs highly express *Gbx2, Fgf5,* and *Nodal*, but express the *Rex-1* transcript at a low level compared to the established murine ESC cell line, ESC-D3. This suggests that VSELs are more differentiated than ICM-derived ESCs and share several markers with more differentiated EpiSCs (Shin et al., 2010b).

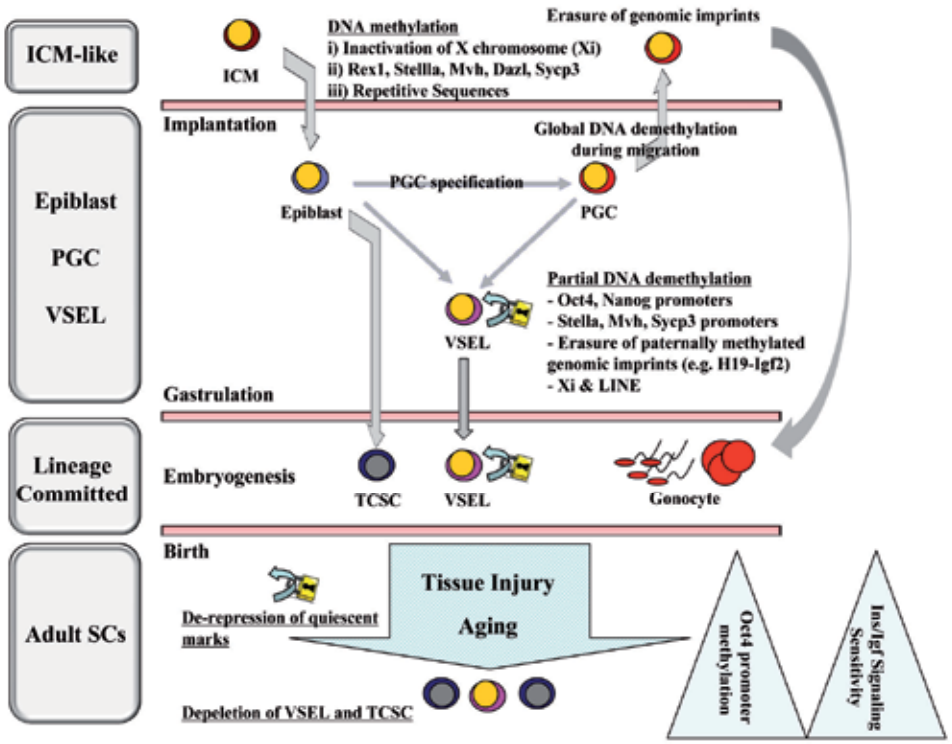

Fig. 2. **Epigenetic modification of VSELs during embryogenesis and aging.** Epigenetic modifications control the differentiation potential of SCs during embryogenesis and aging. The DNA methylation processes during development of ICM-derived epiblast SCs specify them to TCSCs. However, at the beginning of gastrulation, the proximal epiblast-specified PGCs can reset their epigenetic profile to one that characterizes ICM-derived PSCs. Subsequently, during PGC migration to the genital ridges, global DNA demethylation leads to the erasure of genomic imprints. Consistent with the hypothesis that VSELs originate from an epiblast-derived PGC population, they show a PGC-like epigenetic profile, including partial DNA demethylation of several pluripotency, germ-line, and genomic imprints. These epigenetic profiles of developing VSELs are retained after their deposition into adult tissues. This parent-specific reprogramming of the genomic imprinting of VSELs deposited in adult tissues (e.g., BM) functions as i) a "lock-in mechanism" to prevent their inappropriate proliferation and ii) a mechanism to restrict their sensitivity to Ins/Igf signaling. During the aging process, while residing in adult tissues, VSELs exposed to oxidative stress or chronic Ins/Igf signaling de-repress locked-in genomic imprints and progressively methylate DNA in the Oct-4 promoter (Ratajczak et al 2011a). As a result of these epigenetic changes, the total number and pluripotency of VSELs decreases with age (Ratajczak et al. 2008), which leads to impaired tissue regeneration and rejuvenation.

3.3 Expression of germline markers

During gastrulation, EpiSCs lose expression of core pluripotency TFs. On the other hand, during specification of proximal epiblast EpiSCs into PGCs, the expression of these early embryonic genes is re-activated by resetting epigenetic programs in these cells (Hayashi &

Surani, 2009). Thus, PGCs reset epigenetic marks to an ICM-like state, which results in re-activation of pluripotency and germline-related genes (Hayashi & Surani, 2009). The specification of PGCs is initiated at E7.25 by expression of germline master regulators, such as *Fragilis, Blimp1,* and *Stella,* in response to signals from extra-embryonic tissues (Surani et al., 2007). At this time, around 40 proximal epiblast-derived PGCs are detected. At E8.5, PGCs enter back into the embryo proper through the primitive streak and start migration through the hindgut endoderm and mesentry to the aorta-gonads-mesonephros (AGM) region, and at around E11.5 they reach the genital ridge in which PGCs differentiate into monopotent gametes (sperm and egg). Our data indicate some relationship between VSELs and PGCs (Shin et al., 2010b). Accordingly, VSELs are reported to highly express genes that are involved in germ-line specification of the epiblast (e.g., *Stella, Prdm14, Fragilis, Blimp1, Nanos3,* and *Dnd1*). The expression of Stella, Blimp1, and Mvh has been confirmed at the protein level by immunostaining. Furthermore, the *Stella* promoter in VSELs is partially demethylated and displays transcriptionally active histone modifications (H3Ac and H3K4me3) and is less enriched for transcriptionally repressive histone marks (H3K9me2 and H3K27me3) (Shin et al., 2010b). It can be concluded that VSELs express several germline-specific genes and display a *Stella* promoter chromatin structure that is characteristics of germline specification. VSELs also highly express *Dppa2, Dppa4,* and *Mvh*, which characterize late migratory PGCs; however, they do not express *Sycp3, Dazl,* and *LINE1* genes that are expressed in post-migratory PGCs (Maatouk et al., 2006; Maldonado-Saldivia et al., 2007). Thus, our results *in toto* support the conclusion that VSELs deposited into murine BM show some similarities in gene expression and epigenetic signatures to epiblast-derived migratory PGCs (at ~E10.5–E11.5).

3.4 VSELs are marked by BDs

As mentioned above, in undifferentiated ESCs, most of the homeodomain-containing developmental TFs are repressed by BDs (Bernstein et al., 2006), which are chromatin structures in which transcriptionally opposite histone codes physically co-exist in the same promoter. In undifferentiated ESCs, BD epigenetic codes at the promoters of these TFs are temporarily repressed, preventing their premature differentiation. During differentiation, the transient repressive epigenetic marks in these TFs become monovalent and thereafter activate or repress expression of the appropriate TFs. Our preliminary data indicate that murine VSELs display BDs in the promoters of several homeodomain-containing developmental TFs (*Sox21, Nkx2.2, Dlx1, Lbx1h, Hlxb9, Pax5,* and *HoxA3*). The presence of transcriptionally active histone codes, such as H3K4me3, physically coexisting with repressive histone codes, such as H3K27me3, was confirmed by employing the carrier-ChIP assay (submitted for publication).

3.5 VSELs from female mice partially activate an X chromosome

The process of X-chromosome inactivation is mediated by expression of the large noncoding RNA *Xist,* which is transcribed on the inactivated X chromosome. Coating of the X chromosome to be inactivated by spreading of Xist RNA induces the silenced chromatin structure (Payer & Lee, 2008). As already mentioned above, it is well known that female PSCs (*e.g.*, murine and human ESCs isolated from the blastocyst ICM, as well as PGCs) reactivate the X chromosome that was inactivated after fertilization, and, as a result, female PSCs display two equivalently activated X chromosomes (Surani et al., 2007). Our initial

studies in murine female VSELs show that these cells partially reactivate the inactivated X chromosome. As mentioned above, female murine VSELs partially hypermethylate the *Xist* promoter (~80%), unlike somatic cells which show 50% DNA methylation. This result strongly suggests that murine VSELs, like ESCs and PGCs, can undergo the process of X chromosome reactivation (submitted for publication).

4. Developmental origin of VSELs

Taking into consideration all the molecular signatures characteristic of VSELs, we propose that VSELs are epiblast-derived PSCs deposited early during embryogenesis in developing organs as a potential reserve pool of precursors for TCSCs. Thus, VSELs have an important role in tissue rejuvenation and regeneration (Shin et al., 2010a). Because of the gene expression profile and epigenetic state of the core TFs, expression of epiblast and germline genes suggests that VSELs deposited in adult BM originate from migratory PGCs that have gone astray from the "orthodox" migration route.

From the second trimester on, VSELs are easily found as a Sca-1^{+}Lin^{-}CD45^{-} population in murine fetal liver, which is the main embryonic hematopoietic tissue. VSELs that emerge in fetal liver (FL-VSELs) follow the developmental route of hematopoietic stem cells (HSCs) and subsequently colonize BM together with HSCs (Zuba-Surma et al., 2009). FL-VSELs and their BM-derived counterparts express a similar pattern of pluripotent and epiblast/germline genes at the mRNA and protein levels. Accordingly, the promoters for *Oct-4, Nanog,* and *Stella* show significant DNA demethylation and enriched histone modifications for an open, transcriptionally active structure in these promoters (Shin et al., 2010a).

Mounting evidence also indicates that PGCs could be related to HSCs, another population of highly migratory SCs (De Miguel et al., 2009). In support of this notion, the first primitive HSCs appear in the extra-embryonic tissues in yolk sac blood islands at the time when proximal epiblast-specified PGCs enter the extra-embryonic mesoderm (Mikkola & Orkin, 2006). In addition, the appearance of definitive HSCs in the AGM region of the embryo proper coincides with migration of PGCs to the genital ridges through the AGM (De Miguel et al., 2009). Furthermore, PGCs isolated from murine embryos have been proven to be able to grow HSC colonies while, on the other hand, robust hematopoietic differentiation has been observed in some classical germline tumors (Kritzenberger & Wrobel, 2004; Ohtaka et al., 1999; Rich, 1995; Saito et al., 1998; Woodruff et al., 1995). All this suggests developmental overlap between PGCs and HSCs.

On the other hand, VSELs share several characteristics with both PGCs and HSCs. In particular, VSELS i) share several BM- and FL-derived markers characteristic of the epiblast/germ line (Shin et al., 2010b), ii) follow the developmental route of HSCs (Zuba-Surma et al., 2009), and iii), in appropriate culture conditions, can also be differentiated toward the hematopoietic lineage. All of this suggests that VSELs are the most primitive murine BM-residing population of SCs and function as precursors for long-term repopulating HSCs (Ratajczak et al., 2011b).

Thus, PGCs, HSCs , and VSELs together form a unique highly migratory population of interrelated SCs that may be envisioned as a kind of 4th (highly migratory) germ layer. Due to this unique developmental origin, VSELs show characteristic epigenetic reprogramming and gene expression in stemness, germline, and imprinted genes (as described below) that maintain their pluripotency, but also prevent inappropriate proliferation and teratoma formation (Shin et al., 2009).

5. Epigenetic changes of imprinted genes that regulate VSEL pluripotency

Unlike ESCs, highly purified BM-derived Oct-4$^+$ VSELs do not proliferate *in vitro* if cultured alone and do not grow teratomas *in vivo*. On the other hand, cells from VSEL-DSs have restored their proliferation potential, demonstrating that their quiescent state can be modulated. This suggests that VSELs are a quiescent cell population and that some mechanisms must exist to prevent their unleashing of proliferation and teratoma formation. Like VSELs, PGCs in cultures freshly isolated from embryos proliferate only for a few days before disappearing, either because they differentiate or die (De Felici & McLaren, 1983). They also neither grow teratomas nor complement blastocyst development (Surani et al., 2007). However, when PGCs are cultured over a feeder layer supplemented by a specific combination of growth factors, they continue to proliferate and can be reprogrammed into EGCs (Shamblott et al., 1998; Turnpenny et al., 2003). Therefore, it is possible that these two SC populations employ a similar molecular mechanism to regulate their pluripotency and to prevent cell proliferation.

The hallmark of epigenetic reprogramming during PGC development is erasure of genomic imprinting (Surani et al., 2007), which is an epigenetic process ensuring paternal-specific, mono-allelic expression of imprinted genes (Reik & Walter, 2001). Around 80 imprinted genes (expressed from maternal or paternal chromosomes only) have been reported in the mouse genome and their proper mono-allelic expression regulates totipotency and pluripotency of the zygote and developmentally early SCs, respectively. Furthermore, most imprinted genes, such as insulin-like growth factor 2 (*Igf*2), H19, Igf2 receptor (*Igf2R*), and $p57^{KIP2}$ (also known as *Cdkn1c*) are directly involved in embryo development. Since the majority of imprinted genes exist as gene clusters enriched for CpG islands, their expression is coordinately regulated by the DNA methylation state of CpG-rich cis-elements known as differentially methylated regions (DMRs) (Delaval & Feil, 2004). The differential methylation state of DMRs is mediated by DNA methyltransferases (*Dnmts*), depending on the parental allele of origin. Depending on the developmental period of methylation, there are two types of DMRs: "primary DMRs" are differentially methylated during gametogenesis and "secondary DMRs" acquire allele-specific methylation after fertilization. So far, 15 primary DMRs have been identified in the mouse genome. Interestingly, most DMRs are methylated at the maternal allele and only three DMRs (at *Igf2-H19, Rasgrf1,* and *Meg3* loci) are paternally methylated (Kobayashi et al., 2006). In addition to DNA methylation of DMRs, histone modifications also contribute to monoallelic expression of imprinted genes (Fournier et al., 2002; Mager et al., 2003).

Shortly after PGC specification at E7.25, PGCs initiate epigenetic reprogramming programs, resulting in global DNA demethylation and changes in histone modifications (Seki et al., 2007). As a result, epigenetic marks for genomic imprinting in both parental chromosomes are erased during migration into the genital ridge and new genomic imprints are established during differentiation into gametes in a sex-dependent manner. The erasure of genomic imprints could be a mechanism to protect the Oct-4-expressing germline SCs from uncontrolled expansion and teratoma formation. For example, while the nuclei of early migrating PGCs at E8.5–9.5 can be successfully used as donors for nuclear transfer, nuclei from post-migratory PGCs after E11.5 are incompetent to support full-term development (Yamazaki et al., 2003).

Since VSELs, as discussed above, share similar molecular signatures as PGCs, we have proposed that VSELs, like PGCs, modify methylation of imprinted genes, which prevents

them from unleashing proliferation and may explain their quiescent state in adult tissues. Indeed, as shown in **Figure 3** and **Table 1**, VSELs freshly isolated from murine BM erase the paternally methylated imprints (e.g., *Igf2-H19* and *Rasgrf1* loci), while they hypermethylate the maternally methylated ones (e.g., *Igf2* receptor (*Igf2R*), *Kcnq1-p57^{KIP2},* and *Peg1* loci). Because paternally expressed imprinted genes (*Igf2* and *Rasgrf1*) enhance embryo growth and maternally expressed genes (*H19, p57^{KIP2},* and *Igf2R*) inhibit cell proliferation (Reik & Walter, 2001), the unique genomic imprinting pattern observed in VSELs demonstrates growth-repressive imprinting in these cells. As coordinated with genomic imprinting reprogramming programs, VSELs highly express growth-repressive imprinted gene transcripts (*H19, p57^{KIP2},* and *Igf2R*) and downregulate growth-promoting ones (*Igf2* and *Rasgrf1*), which explains the quiescent state of VSELs (Shin et al., 2009). Importantly, all the growth-repressive patterns of genomic imprinting are progressively recovered during the formation of VSEL-DSs, in which SCs proliferate and differentiate. These results suggest that epigenetic reprogramming of genomic imprinting should maintain the quiescence of Oct-4$^+$ VSELs deposited in the adult body and protect them from premature aging and tumor formation. Therefore, the modulation of mechanisms controlling genomic imprinting in VSELs is crucial for developing more powerful strategies to unleash the regenerative potential of these cells for efficient employment in the clinical setting.

6. VSELs and ageing

Tissue regeneration depends on the proper function of SCs, and we envision that aging can be partially explained by a decline in the regenerative potential of VSELs (Ratajczak et al., 2008). In support of this notion, the number of VSELs in murine BM gradually declines with age, ranging from 0.052 ± 0.018% at 2 months to 0.003 ± 0.002% at 3 years of age (Kucia et al., 2008a). Furthermore, the frequency of VSEL-DS formation decreases with age, thus little VSEL-DS formation was observed in cells isolated from older mice (>2 years). Accordingly, VSELs from older mice (2 years) also show lower expression of pluripotency master regulators, such as *Oct-4, Nanog, Sox2, Klf4,* and *cMyc,* while the *Oct-4* promoter in VSELs becomes hypermethylated with age and shows a closed chromatin structure (Ratajczak et al., 2011a). The age-dependent decrease of the pool size and function of VSELs in BM may explain the decline of regeneration potential during aging. This hypothesis has been further corroborated by looking for differences in the content of these cells among BM mononuclear cells (BMMNCs) in long- and short-lived mouse strains. The concentration of VSELs was much higher in the BM of long-lived (e.g., C57B6) compared to short-lived (DBA/2J) mice (Kucia et al., 2006b).

We have also reported that while long-lived Laron dwarf mice, with low levels of circulating Igf1, have a higher number of VSELs in BM compared to normal littermates (Ratajczak et al. 2011a), short-lived bovine-growth-hormone-expressing transgenic mice, with high circulating Igf1 levels, prematurely deplete this population of PSCs (submitted for publication). These observations suggest interesting links between high caloric uptake, increases in chronic insulin/insulin growth factor signaling, and premature depletion of VSELs.

In support of this linkage, it is well known that changes in insulin/insulin-like growth factors (Ins/Igf) signaling molecules play a crucial role in aging. In particular, insulin-like growth factor 1 (*Igf1*) signaling negatively regulates the life span of animals, from worms

and flies to mammals (Russell & Kahn, 2007), whileIgf1 and insulin level in blood is regulated positively by caloric uptake (Piper & Bartke, 2008). Overall, the genomic imprinting reprogramming in VSELs leads to impaired Ins/Ingf signaling due to i) downregulation of expression of *Igf2*, ii) upregulation of expression of *Igf2R,* which serves as a molecular sink for Igf2, and iii) a decrease in expression of *Rasgrf1,* which is a small GTP exchange factor (GEF) for Ins/Igf signal transduction. This suggests that the epigenetic mechanism governing the VSEL quiescent state regulates the sensitivity to Ins/Igf signaling and could, if overactivated, lead to premature depletion of primitive VSELs in tissues (Ratajczak et al., 2011a).

Thus, high chronic calorie uptake, followed by high plasma insulin and Igf-1 levels may, over time, prematurely deplete VSELs from adult organs and thus accelerate aging.

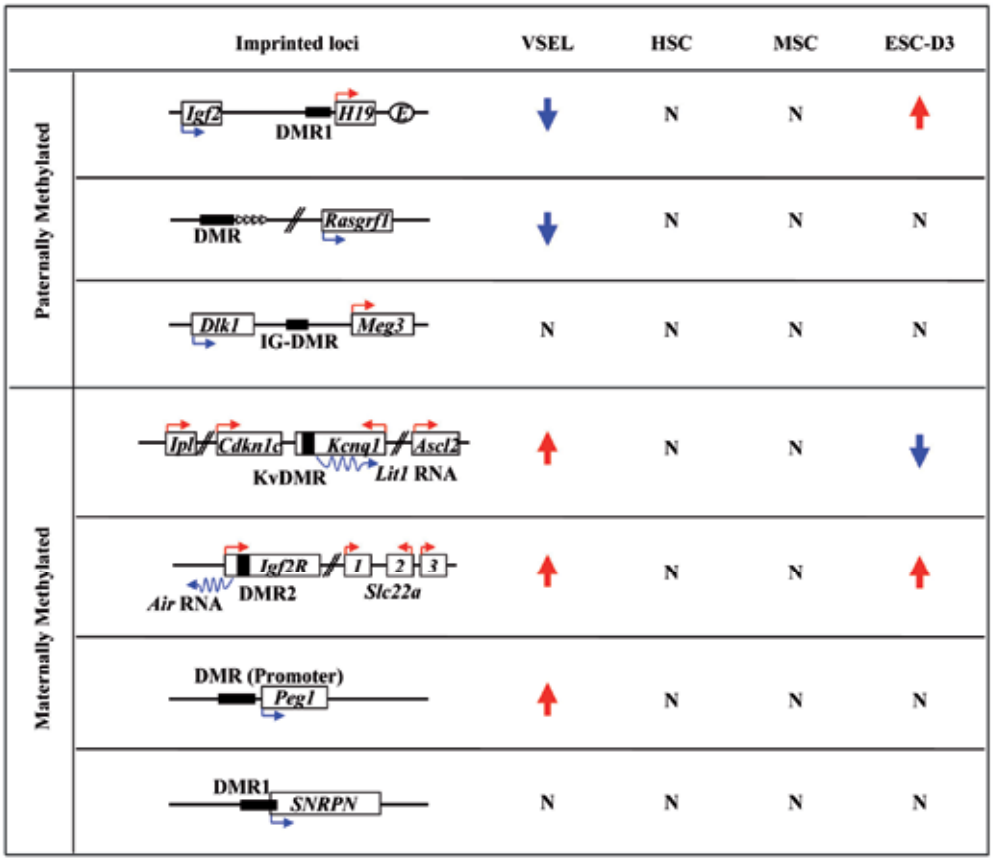

Fig. 3. **The unique DNA methylation pattern and expression of imprinted genes in VSELs.** Arrows in the schematic diagram of paternally (Igf2-H19 and Rasgrf1) or maternally (Igf2R and Kcnq1) methylated loci indicate the transcriptional activity of the indicated gene. Red (up) and blue (down) arrows indicate upregulated and downregulated gene expression, respectively. M = maternal chromosome, P = paternal chromosome. DMR1, IG-DMR, KvDMR, DMR2 = DMRs for Igf2-H19, Rasgrf1, Kcnq1, and Igf2R loci, respectively.

Imprinted gene	Expression	Proliferation	VSEL
Igf2	Pat	+	↓
Rasgrf1	Pat	+	↓
Dlk1	Pat	+	N
Air	Pat	+	↓
Lit1	Pat	+	↓
H19	Mat	–	↑
Meg3	Mat	–	N
Igf2R	Mat	–	↑
$p57^{KIP2}$/Cdkn1c	Mat	–	↑
SNRPN	Mat	–	N

Table 1 . Expression profiles of crucial imprinted genes in murine VSELs.

The expression level of paternally (pat) or maternally (mat) expressed imprinted genes in murine BM-derived VSELs is indicated by red-up (up-regulated) and blue-down (down-regulated) arrows. The effect of the indicated imprinted genes on cell proliferation is marked as '+' (proliferation promoting) or '–' (repressing).

7. Regeneration potential of VSELs *in vivo*

To address the most important question of whether these primitive VSELs could be efficiently employed in the clinic, we have tested their potential role in several *in vivo* tissue-regeneration animal models. First, VSELs can be specified *in vivo* into mesenchymal stem cells (MSCs). Accordingly, in the first study by Taichman *et al*, VSELs isolated from GFP^+ mice were implanted into SCID mice, and 4 weeks later the formation of bone-like tissues was observed (Taichman *et al*., 2010). Second, freshly isolated BM-derived VSELs from GFP^+ mice were injected into the hearts of mice that had undergone ischemia/reperfusion injury. After 35 days of follow-up, VSEL-treated mice exhibited improved global and regional left ventricular (LV) systolic function (as determined by echocardiography) and attenuated myocyte hypertrophy in surviving tissue (as determined by histology and echocardiography) when compared with vehicle-treated controls (Zuba-Surma et al., 2010). Finally, we observed that VSELs, if plated over the supportive OP9 cell line, give rise to

colonies of $CD45^+CD41^+Gr\text{-}1^-Ter119^-$ cells that, when transplanted into wild-type animals, protected them from lethal irradiation and differentiated *in vivo* into all major hematopoietic lineages (e.g., $Gr\text{-}1^+$, $B220^+$ and $CD3^+$ cells) (Ratajczak et al., 2011b). Thus, we propose that VSELs are a population of BM-residing PSCs that give rise to long-term-engrafting hematopoietic SCs.

8. Conclusion

In the past few years, several attempts have been made to purify a population of PSCs from adult tissues. We propose that the VSELs described by our team play a physiological role in rejuvenation of the pool of TCSCs under steady-state conditions. VSELs developmentally originate from epiblast-derived migrating PGCs and they could be deposited in adult organs early in development as a reserve pool of primitive SCs for tissue repair and regeneration. Therefore, VSELs share several molecular signatures with epiblast and migrating PGCs with respect to gene expression and epigenetic programs. Based on the developmental origin of VSELs, their proliferation, like PGCs, is controlled by the DNA methylation state of some of the developmentally crucial imprinted genes (e.g. *H19, Igf2,* and *Rasgrf1*). During the ageing process, proliferation-repressive epigenetic marks progressively disappear, resulting in the increased sensitivity to Ins/Igf signaling and, concomitantly, to depletion of the primitive VSEL population. The decrease in the number and pluripotency of these cells will affect pools of TCSCs and have an impact on tissue rejuvenation and life span. Furthermore, VSELs can be specified into several tissue-residing TCSCs (e.g. MSCs, HSCs, and cardiac SCs) in response to tissue/organ injury. Therefore, VSELs isolated from adult tissues are an alternative and ethically uncontroversial source of SCs for regenerative medicine. However, to successfully employ VSELs for this purpose, it is very important to establish experimental protocols for reprogramming of the growth-repressive genomic imprinted state of VSELs into the regular somatic pattern to unleash their regenerative potential.

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10. Conflicts of interest statement

The authors declare that they have no competing financial interests.

11. References

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23

Multipotent Dental Stem Cells: An Alternative Adult Derived Stem Cell Source for Regenerative Medicine

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1. Introduction

The pluripotent nature of embryonic stem cells (ESCs) makes them amenable for regenerative therapies because they can differentiate into cells that form all tissue types within the body (Zandstra and Nagy, 2001). The potential drawbacks to the use of ESCs for cellular therapies include the obvious ethical dilemmas of obtaining ESCs, the potential of cancer or tumor formation and the risk of immunogenic rejection (Wobus and Boheler, 2005). Therefore adult stem cell sources with multipotent and pluripotent potential have been sought as an alternative for ESCs including mesenchymal stem cell (MSCs) and tissue-derived specific stem cells.

Interestingly, the isolation of a population of dental stem cells derived ectodermally from the neural crest (NC) have been shown to be multipotent and give rise to multifarious cell types that result in the development of many of the body's organs or tissues (Huang et al., 2009a; Huang et al., 2009b). Stem cells extracted from dental tissues including dental pulp, periodontal ligament, apical papilla and dental follicle precursor cells have an expansive differentiation potential with respect to mesodermal and ectodermal lineages. Currently there are six types of dental stem cells that are well characterized and described both in vitro and in vivo (Gronthos et al., 2000; Huang et al., 2009a; Karaöz et al., 2010; Miura et al., 2003; Morsczeck et al., 2005; Seo et al., 2004; Sonoyama et al., 2006).

Some dental stem cells lines have been shown to express ESC markers Oct4, Nanog, Sox2 and Klf4 and NC markers p75, Sox10, Slug and Nestin suggesting that dental stem cells may be able to become many of the same tissues as ESCs (Huang et al., 2009a). Further, dental stem cells have been shown to differentiate into neurogenic, adipogenic, cardiomyogenic, chondrogenic, myogenic and osteogenic lineages (Huang et al., 2009a; Karaöz et al., 2010; Miura et al., 2003; Seo et al., 2004; Sonoyama et al., 2006; Zhang et al., 2006). Since dental stem cells have been shown to differentiate into a multitude of cell types, their potential for use in tissue regeneration may be boundless.

We are currently using dental stem cells to investigate the mechanisms of mechanotransduction elicited during dynamic cyclic compression for chondrogenesis. Our long term goal is to develop technology and protocols utilizing dental stem cells and biomechanical force for reparative medicine and tissue regeneration of cartilage. This review

will discuss the most current findings in tissue engineering with respect to dental stem cells both for whole tooth regeneration and potential use in future stem cell therapies.

2. Characteristics and sources of stem cells

2.1 What is a stem cell?

The general properties that define a stem cells are: 1. Stem cells are cells that are clonogenic and have the ability for self-renewal; 2. Stem cells are unspecialized cells that when correctly stimulated have the ability to differentiate into specialized cell types (Blau et al., 2001; Bongso and Fong, 2009).

There are two broader categories of stem cells: embryonic stem cells (ESCs) and adult stem cells. Embryonic stem cells are derived from the blastocyst stage of a developing embryo (Fortier, 2005; Thomson et al., 1998) and are capable of forming all three germ layers (ectoderm, endoderm, and mesoderm)(Bongso and Fong, 2009). Harvesting ESCs requires the destruction of the embryo (Lanzendorf et al., 2001) which leads to ethical dilemmas when obtaining these cells. The use of adult stem cells avoids these ethical issues. Adult stem cells have been obtained from multiple tissues including bone marrow (Pittenger et al., 1999), adipose tissue (Zuk et al., 2001), muscle (Deasy et al., 2001), umbilical cord tissue (Schugar et al., 2009), intestine (Wong, 2004), and skin (Blanpain et al., 2004). While most of this book focuses on the embryonically derived stem cells, this chapter focuses on the adult or postnatal stem cells with special emphasis on those derived from dental tissues.

2.2 Stem cell potency

Stem cell potency refers to the ability of the cell to differentiate into specific tissue type(s). Totipotent is defined as the ability to differentiate into any of the cell types of the entire organism, both embryonic and adult cell types (Dannan, 2009; Mummery et al., 2011; Smith, 2006). An example of a totipotent cell is a fertilized egg cell because it is able to differentiate into embryonic, extra-embryonic (ie. placenta) and adult tissues of the entire organism (Alison et al., 2002; Dannan, 2009; Mummery et al., 2011). Pluripotent stem cells have the potential to differentiate into all cell lineages including the three germ layers: ectoderm, mesoderm, or endoderm but not the extra-embryonic tissues (Alison et al., 2002; Dannan, 2009; Fortier, 2005; Mummery et al., 2011; Smith, 2006). ESCs are considered the gold standard of stem cells because of their pluripotency and their ability to be maintained indefinitely in culture (Thomson et al., 1998). Pluripotency has also been demonstrated in adult stem cells including bone marrow mesenchymal stem cells (BMMSCs) (Jiang et al., 2002). Multipotent stem cells have the ability to differentiate into multiple cell lineages that can form more than one tissue type (Alison et al., 2002; Mummery et al., 2011; Smith, 2006). Mesenchymal stem cells (MSCs) derived from adipose tissue are an example of a multipotent stem cell and are able to differentiate into multiple tissues of the mesodermal lineage including bone, fat and cartilage (Zuk et al., 2002).

2.3 Bone marrow mesenchymal stem cells

Stem cells obtained from bone marrow are a major source of adult MSCs and are widely studied. Bone marrow stromal cells can be harvested from bone marrow by mechanical disruption, but the cell suspension will contain both hematopoietic stem cells and BMMSCs (Bianco et al., 2001). BMMSCs are isolated as colony forming unit-fibroblasts (CFU-Fs) from the bone marrow cell suspension. In order to separate the two types of stem cells, the cell

suspension is cultured in vitro at low density. A small number of BMMSCs will adhere to the plate and begin to form colonies while the non-adherent hematopoietic cells are then removed by repeat washings (Bianco et al., 2001; Chamberlain et al., 2007). BMMSCs isolated in this manner are capable of 20–25 passages in vitro without significant changes to the cell phenotype (Bianco et al., 2001; Conget and Minguell, 1999). Gronthos et al. (2003) further showed that BMMSCs could be isolated from bone marrow aspirates by determining which CFU-F colonies were highly reactive to the antibody STRO-1 (STRO-1^{Bright}) and also reactive to the antibody VCAM-1 (VCAM-1^{+}). These new studies isolated BMMSCs in bone marrow aspirates based on STRO-$1^{Bright/}$ VCAM-1^{+} cell surface markers by florescence activated cell sorting (FACS) and were capable of proliferating up to 40 population doublings (Gronthos et al., 2003).

BMMSCs show a great level of plasticity and have shown the potential to differentiate into multiple tissue types in vitro including muscle, adipose, cartilage, bone, connective tissue, neurons and endothelial cells (Gronthos et al., 2003; Pittenger et al., 1999; Woodbury et al., 2000; Young and Black, 2004). Interestingly, when transplanted into immunodeficient mice, BMMSCs undergo osteogenic differentiation in vivo and form bone (Kuznetsov et al., 1997).

In 2006, the minimum criteria to define human multipotent mesenchymal stromal cells was established as: Cells that are plastic adherent in standard culture; Cells that have the ability to differentiate in vitro into osteoblasts, adipocytes and chondroblasts; Cells that express the cell surface markers CD73, CD90 and CD105 in 95% of the cell population as determined by flow cytometry and lack the expression (≤ 2% positive) of CD14, CD34, CD45 or CD11b, CD79a or CD19 and HLA class II (Dominici et al., 2006).

2.4 Dental stem cells

Dental stem cells are an alternative source of adult stem cells that are easily accessible by tooth extraction with a local anesthetic or when a primary tooth is replaced. This section discusses where dental stem cells arise during tooth formation and the types of tissue they form. We also characterize the many types of the dental stem cells utilized in research today and compare the utility of dental stem cells versus BMMSCs.

There are six types of human dental stem cells that have been well described in the literature: 1. Dental pulp stem cells (DPSCs) (Gronthos et al., 2000); 2. Stem cells isolated from human exfoliated deciduous teeth (SHEDs) (Miura et al., 2003); 3. Stem cells derived from human natal dental pulp (hNDPs) (Karaöz et al., 2010); 4. Periodontal ligament stem cells (PDLSCs) (Seo et al., 2004); 5. Stem cells isolated from the apical papilla (SCAPs) (Sonoyama et al., 2008); 6. Stem cells isolated from dental follicle precursor cells (DFPCs) (Morsczeck et al., 2005).

Within the body, MSCs have been localized to perivascular niches (Crisan et al., 2009; Kolf et al., 2007) and recent studies have also shown that dental stem cells are also localized to perivascular niches within the tooth structure (Chen et al., 2006; Shi and Gronthos, 2003). Dental stem cells arise from dental mesenchyme which has early interaction with the neural crest during normal tooth development (Huang et al., 2009b). Therefore, dental stem cells may display characteristics of both mesoderm and ectoderm due to their ectomesenchymal origins (Huang et al., 2009b).

2.4.1 Mammalian tooth formation

A mature tooth is comprised externally of hard structures of enamel, dentin and cementum and internally possesses a soft dental pulp (Figure 1). Tooth formation or odontogenesis is a

complex process involving multiple tooth-associated cell types. Odontogenesis occurs as a tooth bud is formed from an aggregation of embryonic cells. These cells have ectodermal and ectomesodermal origins from the first branchial arch and the neural crest respectively (Ten Cate, 1998; Tucker and Sharpe, 2004). Tooth development has three stages. 1. The bud stage, where epithelial cells begin to proliferate into ectomesenchyme and condense in the jaw forming the tooth bud. 2. The cap stage, where ectomesenchmyal cells aggregate and begin to surround and enclose the epithelial cells which invaginate further into the mesenchyme and form the dental follicle, the enamel organ or cap and the dental papilla (Slatter, 2002; Ten Cate, 1998; Tucker and Sharpe, 2004). The dental follicle is of ectomesodermal origins and forms a sac surrounding the developing tooth that supports the tooth prior to eruption. The enamel organ is of ectodermal origins and eventually forms the enamel, whereas the dental papilla is of mesodermal origins and eventually forms the primary dentin and the pulp. 3. The bell stage, where the tooth undergoes extensive differentiation with the epithelial cells differentiating into ameloblasts and mesenchymal cells differentiating into odontoblasts. After the bell stage, the hard structures are formed with ameloblasts forming enamel while odontoblasts form dentin (Figure 1). Secondary dentin aids in root formation. Later in tooth development further differentiation of the dental follicle occurs with the formation of cementoblasts, fibroblasts and osteoblasts to form the cementum, the periodontal ligament and bone respectively (Figure 1).

2.4.2 Sources of dental stem cells

2.4.2.1 Dental Pulp Tissue

The soft dental pulp is located in the middle of the tooth surrounded by the harder structures of the tooth including dentin, cementum and enamel (Figure 1). The dental pulp contains a mix of cell types including fibroblasts which form the extracellular matrix and collagen and odontoblasts that form reparative dentin (Gronthos et al., 2002; Liu et al., 2006). The dental pulp region also contains nerve fibers and blood vessels and is accessible to external stimuli through the apical foramen (Figure1). Three types of stem cells have been identified from dental pulp tissue: DPSCs, SHEDs and hNDPs. DPSCs are present in the pulp of the adult tooth, whereas SHEDs are only present in the pulp of primary teeth or "baby teeth." Lastly, hNDPs are a unique type of dental pulp stem cells isolated only from the pulp of newborn teeth. Very few newborns are born with teeth, approximately one in every two to three thousand births (Leung and Robson, 2006), so hNDPs are very rare.

2.4.2.1.1 Dental Pulp Stem Cells

DPSCs are a heterogeneous population of cells that were first isolated by Gronthos et al. (2000) and exhibited some characteristics of BMMSCs, including the production of fibroblast-like cells that were clonogenic and had a high proliferation rate. Interestingly, DPSCs had a higher proliferation rate than BMMSCs (Gronthos et al., 2000) . DPSCs also had a similar protein expression pattern to BMMSCs in vitro including vascular adhesion molecule 1, alkaline phosphatase, collagen I, collagen III, osteonectin, osteopontin, osteocalcin, bone sialoprotein, α-smooth muscle actin, fibroblast growth factor 2 and the cell surface marker CD 146 (Gronthos et al., 2000). Immunohistochemistry staining further showed that like BMMSCs, primary cultures of DPSCs did not stain for the cell surface markers CD14, CD34, and CD45 or other markers including MyoD, neurofilament, collagen II , and peroxisome-proliferator activated receptor γ-2 (Gronthos et al., 2000).

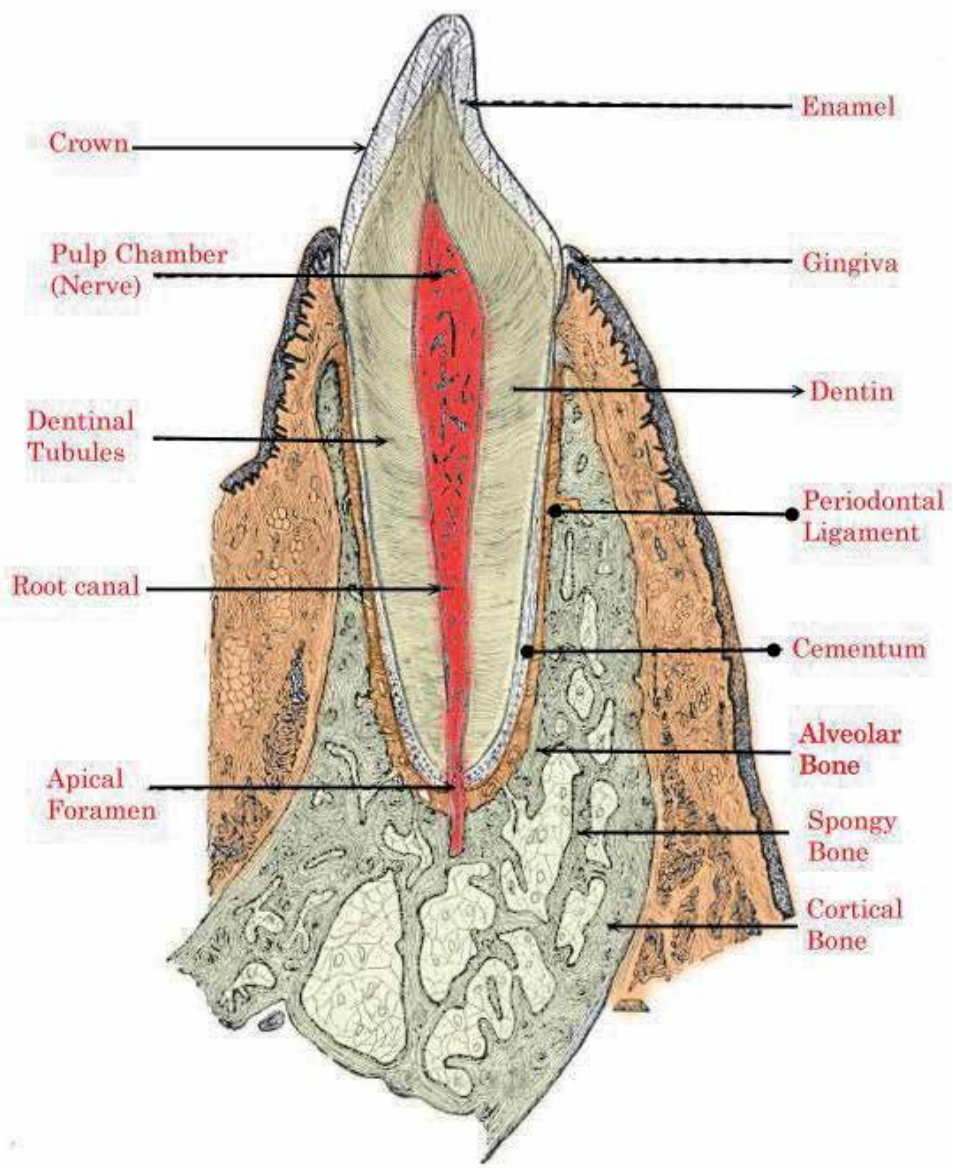

Fig. 1. Mature tooth anatomy. Image copied with permission from Dr. Martin S. Spiller, D.M.D. from DoctorSpiller.com.

Recently FACS has been used to sort DPSCs based on cell surface markers which found that in addition to the markers identified above, DPSCs expressed the following: CD9, CD10, CD13, CD29, CD44, CD49d, CD59, CD73, CD90, CD105, CD106, CD166 and STRO-1(Lindroos et al., 2008; Nam and Lee, 2009). Further, DPSC did not express CD14, CD 31, CD 45 (Nam and Lee, 2009) (Summarized in Table 1).

When cultured under osteogenic conditions DPSCs were capable of forming calcified deposits sparsely throughout the culture; these results were unlike BMMSCs which formed sheets of calcium deposits (Gronthos et al., 2000). In vivo transplantation of DPSCs into immunocompromised mice resulted in the production of a dentin-pulp-like complex with a collagen matrix containing blood vessels and lined with odontoblasts (Gronthos et al., 2000) suggesting that DPSCs are multipotent. Further studies also found DPSCs to be multipotent, capable of differentiating into myoblasts, osteoblasts, odontoblast-like cells, chondrocytes, adipocytes and neural cells (Gronthos et al., 2002; Liu et al., 2006; Pierdomenico et al., 2005; Zhang et al., 2006).

Letter name	BMMSCs	DPSCs	SHEDs	hNDPs	PDLSCs	SCAPs	DFPCs
CD9	+	+			+		+
CD 10		+		-	+		+
CD 13	+	+	+	+	+	+	+
CD 14	-	-	-		-	-	
CD 18						-	
CD 19		-					
CD 24		-			-	+	
CD 29		+	+		+	+	+
CD 34	-	-	-	-	-	-	-
CD 44	+	+	+	+	+	+	+
CD 45	-	-	-	-	-	-	-
CD 53							+
CD 59		+			+		+
CD73	+	+	+	+	+	+	+
CD 90	+	+	+	+	+	+	+
CD 105	+	+	+		+	+	+
CD 106	+	+	+		+	+	+
CD 146	+	+	+	+	+	+	+
CD 150						-	
CD 166		+	+	+	+		+
STRO-1	+	+	+		+	+	+

Table 1. Cell surface markers expressed in dental stem cells compared to bone marrow mesenchymal stem cells as determined by flow cytometry. Table adapted from Karaöz et al. 2011, Rodriguez-Lozano et al. 2011, Huang, G.T. et al. 2009, Nam and Lee 2009, Lindroos et al. 2008 and Shi et al. 2005. BMMSCs, bone marrow mesenchymal stem cells; DPSCs, dental pulp stem cells; SHEDs, stem cells from human exfoliated deciduous teeth; SCAPs, stem cells from apical papilla; DFPCs, dental follicle precursor cells; hNDPs, stem cells derived from human natal dental pulp; + = marker present; - = marker absent.

2.4.2.1.2 Stem Cells from Human Exfoliated Deciduous Teeth

SHEDs are found in the pulp of the naturally exfoliated deciduous teeth or "baby teeth." When the permanent tooth erupts from the gums the deciduous tooth in displaced. SHED cells were first isolated by Miura et al. (2003) from the remnant pulp in the crown of human deciduous incisors of children 7-8 years old.

Similar to DPSCs, SHEDs met the criteria to be defined as a stem cell population as they were highly proliferative, capable of self-renewal and had the ability to differentiate into multiple cell types (Miura et al., 2003). SHEDs also had a fibroblast-like morphology similar to DPSCs. However, SHEDs were capable of a greater number of population doublings and had a higher proliferation rate than both BMMSCs and DPSCs (Miura et al., 2003). SHEDs have also been isolated and identified as immature dental pulp stem cells (IDPSCs) (Kerkis et al., 2006) and found to express embryonic stem cell markers Oct-4 (POU transcription factor), Nanog, stage specific embryonic antigens (SSEA-3, SSEA-4), and tumorigenic recognition antigens (TRA-1-60, TRA-1-81). SHEDs have also been shown to express neural stem cell markers SRY (sex determining region Y)-box 2 (Sox-2), nestin, and ATP-binding cassette, subfamily G, member 2 (ABCg2)(Morsczeck et al., 2010).

SHEDs were further characterized using FACS as having the following cell surface markers: CD13, CD29, CD31, CD44, CD73, CD90, CD105, CD146, CD166, and STRO-1 and similar to BMMSCs and DPSCs, SHEDs did not express CD14, CD34 or CD45 (Kerkis et al., 2006; Morsczeck et al., 2010; Pivoriuunas et al., 2010; Shi et al., 2005; Wang et al., 2010) (Summarized in Table 1).

A distinguishing feature of SHEDs not demonstrated for DPSCs is SHEDs formed sphere-like clusters when cultured in neuronal differentiation media (Miura et al., 2003). While Miura et al. (2003) demonstrated that SHEDs could differentiate into neural cells, adipocytes and odontoblasts, Kerkis et al. (2006) showed that SHEDs also had chondrogenic and myogenic potential. SHEDs were shown to express chondrogenic markers Sox-9, type II collagen and type X collagen when cultured for 14 days with bone morphogenic protein 2 (BMP2), a chondrogenic signaling protein in the TGFβ family (Koyama et al., 2009). Interestingly, Koyama et al. (2009) did not find any expression of the chondrogenic markers in their untreated populations of SHED cultures.

When SHED were transplanted into immunocompromised mice they exhibited an osteoinductive capacity in vivo but were not able to regenerate the dentin-pulp-like complex that DPSCs cells were able to form (Miura et al., 2003). Kerkis et al. (2006) also showed that when SHEDs were transplanted into immunocompromised mice via intraperitoneal injection they engrafted into the lungs, liver, spleen, brain and kidney and the tissue formed by the SHEDs was indistinguishable from the host tissue for liver, spleen, brain and kidney (Kerkis et al., 2006).

2.4.2.1.3 Stem Cells Derived from Human Natal Dental Pulp

Natal teeth are deciduous teeth that arise in newborns that are smaller than primary teeth and have little or no root development (Leung and Robson, 2006). Karaöz et al. (2010) isolated and characterized hNDPs from the remnant pulp of natal teeth. A small number of hNDPs adhered to plastic in culture and displayed a fibroblast-like spindle shaped morphology that eventually became flattened in later passages (Karaöz et al., 2010). Similar to DPSCs, hNDPs had a higher proliferation rate the BMMSCs, were clonogenic, and had the ability to differentiate into multiple cell types, satisfying the criteria to be classified as a stem cell population.

Using flow-cytometry Karaöz et al. (2010) showed that like BMMSCs, hNDPs expressed CD13, CD44, CD73, CD90, CD146, and CD166 but did not express CD14, CD31 or CD45 (Summarized in Table 1). Stem cells derived from human natal dental pulp expressed many of the same cell surface markers seen in DPSCs and SHEDs (Table 1).
Cultures of NDPs with chondrogenic, osteogenic, adipogenic, myogenic and neurogenic media expressed the appropriate differentiation markers associated with their culture media. Further, the multipotent nature of hNDPs was demonstrated by their differentiation in vitro into chondroblasts, osteoblasts, adipocytes, myoblasts and neuro-glial-like cells respectively (Karaöz et al., 2010). Interestingly, hNDPs expressed detectable levels of the embryonic stem cell markers Rex-1, Oct4 and Nanog as well as the transcription factors Sox-2 and FoxD3 suggesting that these cells display some of the characteristics for pluripotency (Karaöz et al., 2010).

2.4.2.2 Periodontal Ligament Stem Cells

The periodontal ligament is the part of the tooth derived from the neural crest and made of soft connective tissue that resides between the cementum and the alveolar bone of the jaw (Figure 1). It is responsible for anchoring and supporting the tooth within the tooth socket. The periodontal ligament is composed of a heterogeneous population of cells containing fibroblasts, osteoblasts and cementoblasts (Bartold et al., 2000; Gay et al., 2007; Lekic et al., 2001; Seo et al., 2004; Shimono et al., 2003). Early studies have suggested that the PDL tissue had regenerative or repair abilities when an injury was incurred by the periodontal tissue (Reviewed in Bartold et al. (2005) and Shimono et al. (2003)).
Periodontal ligament stem cells were first isolated by Seo et al. (2004) from impacted third molar adult teeth. Immunohistochemical staining of PDLSCs stained positive for early mesenchymal stem cell markers STRO-1 and CD146 suggesting that these cells has similar stem cell characteristics to BMMSCs (Seo et al., 2004). PDLSCs demonstrated other characteristics of BMMSCs and DPSCs including fibroblastic-like cell morphology that adhered to plastic and formed clonogenic cell clusters with the ability to differentiate into multiple cell types (Seo et al., 2004). PDLSCs had a higher proliferation rate than BMMSCs, but similar rate to DPSCs after 24 hours in culture (Seo et al., 2004).
Multipotent human PDLSCs cells have been characterized using FACS sorting and were shown to express the following cell surface markers: CD9, CD10, CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD106, CD146, CD166 and STRO-1 (Feng et al., 2010; Lindroos et al., 2008; Shi et al., 2005; Wada et al., 2009). Like BMMSCs, DPSCs and SHEDs, PDLSCs do not express CD14, CD31 or CD45 (Shi et al., 2005)(Summarized in Table 1). PDLSCs have also been shown to express the embryonic stem cell markers Oct4, Sox-2, Nanog and Klf-4 and neural crest markers Nestin, Slug, p75 and Sox-10 (Huang et al., 2009a).
Like BMMSCs and DPSCs, PDLSC's formed calcium deposits when cultured in osteogenic media, however, unlike BMMSCs and DPSCs these deposits were sparsely distributed though out the culture (Gay et al., 2007; Seo et al., 2004). Increased protein expression of osteoblastic/cementoblastic markers alkaline phosphatase, bone sialoprotein, matrix extracellular protein, osteocalcin and TGFβ receptor 1 was observed after osteogenic induction (Seo et al., 2004). PDLSCs were also capable of differentiation into adipocytes as demonstrated by the formation of oil red O positive droplets and the upregulation of adipocyte specific transcripts after 21-25 days of culture in adipogenic inducing media (Gay et al., 2007; Seo et al., 2004). Gay et al. (2007) showed that PDLSCs could undergo chondrogenic differentiation in vitro after 21 days culture.

When transplanted into immunocompromised mice PDLSCs formed a cementum/PDL-like structure with attached collagen fibers (Seo et al., 2004). However, despite the expression of osteogenic / cementoblastic markers in vitro (Gay et al., 2007; Seo et al., 2004), PDLSCs were unable to form dentin or bone in vivo (Seo et al., 2004). By implanting PDLSCs into immunocompromised rats with periodontal defects, Seo et al. (2004) were able to show that PDLSCs were capable of periodontal tissue repair.

2.4.2.3 Stem Cells from the Apical Papilla

SCAPs were first isolated by Sonoyama et al. (2006) from impacted wisdom teeth of adults aged 18-20. The apical papilla is a part of the soft tissue found at the apices of the immature permanent tooth that eventually becomes the pulp tissue in the mature tooth (Huang et al., 2009b; Sonoyama et al., 2006; Sonoyama et al., 2008). Histological characterization of the apical papilla by Sonoyama et al. (2008) showed that the apical papilla is separate from the pulp canal and apical cell rich zone of the immature tooth.

SCAPs expressed the early mesenchymal stem cell markers STRO-1 and CD146 suggesting that these cells were a stem cell population. Further characterization of this cell population showed that SCAPs formed adherent fibroblastic cell cultures that were clonogenic and capable of over 70 population doublings with the ability to transform into odontoblastic/ osteoblastic, adipogenic, chondrogenic and neural cell types (Abe et al., 2007; Sonoyama et al., 2006; Sonoyama et al., 2008). Sonoyama et al. (2006) also showed that SCAPs had a greater proliferation rate and population doubling than DPSCs isolated from the same tooth. SCAPs were also distinct from DPSCs with respect to expression levels of survivin, telomerase and the cell surface marker CD24, all which are thought to be associated with cell proliferation (Sonoyama et al., 2006).

Analysis of cell surface markers by flow cytometry showed that SCAPs expressed CD13, CD24, CD29, CD73, CD90, CD105, CD106, CD146, CD166 and STRO-1 but did not express CD14, CD18, CD34, CD45, or CD150 (Abe et al., 2007; Sonoyama et al., 2006) (Summarized in Table1).

In vitro culture of SCAPs, DPSCs and BMMSCs showed that SCAPs and DPSCs had similar osteo/dentinogenic potential to BMMSCs, but had a weaker response to adipogenic differentiation than BMMSCs (Sonoyama et al., 2008). After neural induction, immunostaining showed that SCAPs expressed the following neuronal markers: βIII tubulin, glial fibrillary acid protein, glutamic acid decarboxylase, nestin, neuronal nuclear antigen, neuronal filament M, neuron-specific enolase and 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (Abe et al., 2007; Sonoyama et al., 2008). When transplanted into immunocompromised mice SCAPs underwent in vivo differentiation into odontoblasts which regenerated the dentin-pulp-like structure and connective tissue (Sonoyama et al., 2006).

2.4.2.4 Dental Follicle Progenitor Cells

As described above, the dental follicle is the ectomesodermal tissue surrounding the developing tooth that leads to the formation of cementoblasts, periodontal ligament and osteoblasts. Morsczeck et al. (2005) isolated human DFPCs from the dental follicle area of impacted wisdom teeth and noted that a small number had stem cell characteristics. DFPCs formed clonogenic, fibroblastic-like colonies in culture that adhered to plastic (Morsczeck et al., 2005). Like SHEDs, DFPCs expressed neural stem cell associated markers Sox-2, nestin, and ABCg2 (Morsczeck et al., 2010).

Interestingly, multipotent DFPCs have been reported in mice and rats that are capable of undergoing osteogenic, adipogenic, chondrogenic and neurogenic differentiation (Luan et al., 2006; Yao et al., 2008). However, only osteogenic differentiation has been demonstrated consistently for human DFPCs in vitro (Honda et al.; Kémoun et al., 2007; Lindroos et al., 2008; Morsczeck et al., 2005). For human DFPCs neural induction has also been demonstrated by Morsczeck et al. (2010) but conflicting results for adipogenic and chondrogenic differentiation have been observed (Honda et al.; Kémoun et al., 2007; Lindroos et al., 2008).

Immunohistochemistry and FACS sorting have shown that DFPCs express the following cell surface markers: CD9, CD10, CD13, CD29, CD44, CD53, CD59, CD73, CD90, CD105, CD106, CD146, CD166 and STRO-1 but do not express CD34 or CD45 (Lindroos et al., 2008; Morsczeck et al., 2010; Yagyuu et al., 2010).

When Morsczeck et al. (2005) transplanted human DFPCs into severe combined immunodeficiency (SCID) mice they saw an increase in bone sialoprotein, osteocalcin and collagen I expression in vivo but did not see any evidence of cementum or bone formation. However, transplantation of mouse DFPCs into SCID mice demonstrated that DFPCs were capable of regenerating the PDL in vivo (Yokoi et al., 2007). Recently, when cryopreserved DFPCs were transplanted into immunocompromised rats, a mineralized tissue structure was formed in vivo containing cementocyte/osteocyte cells, but the exact identity of the tissue type could not be determined as dentin, cementum or bone (Yagyuu et al., 2010).

3. Tissue engineering

3.1 Dental stem cells in tissue engineering and regenerative medicine

When tissues become damaged or non-functional tissue engineering is used to replace, repair or restore damaged tissue in the body (Levenberg and Langer, 2004). Tissue engineering and regenerative medicine requires an abundant cell source capable of differentiation into the required tissue. Therefore, stem cells with their ability to self-renew, proliferate and differentiate make an ideal cell source for this type of tissue repair and replacement (Barrilleaux et al., 2006).

Whole tooth regeneration is the goal of many researchers and much of tissue engineering involving dental stem cells is used to reconstruct or repair damaged and diseased dental tissue (Dannan, 2009; Huang et al., 2009b; Shi et al., 2005; Sonoyama et al., 2006; Yen and Sharpe, 2008; Yokoi et al., 2007). When a patient's dental pulp cavity becomes infected or diseased, often the entire pulp is removed and replaced with a filling. Due to the ability of DPSCs to form the dentine-pulp-like complex in vivo, it has been suggested that this may soon be an option for regenerative therapy of teeth (Caton et al., 2010). SHEDs also have shown potential for regenerating the dental-pulp-like tissue in vivo when transplanted into immunocompromised mice (Cordeiro et al., 2008) and therefore may be useful for future regenerative endodontic procedures. Further, Seo et al. (2004) showed that PDLs participated in periodontal tissue repair and formed a PDL/cementum-like complex when transplanted into immunocompromised mice suggesting that we will soon be able to regenerate tissues surrounding the teeth. Unfortunately one of the challenges remaining for whole tooth regeneration is that we are currently unable to regenerate human enamel (Mitsiadis and Papagerakis, 2011).

Two significant advancements in the area of whole tooth engineering are the ability to generate dental tissue structures in vitro and the ability to deliver these dental stem cells in vivo (Cordeiro et al., 2008; Yen and Sharpe, 2008). An important development in tissue engineering is the use of hydroxyapatite/ tricalcium phosphate (HA/TCP) particles and other carrier particles that allow dental stem cells cultured in vitro and delivered in vivo (Caton et al., 2010; Sharma et al., 2010). Also important to for dental tissue engineering is developing appropriate biodegradable scaffolds that can be seeded with stem cells for use in transplants and that provide the correct 3D space for differentiation (Caton et al., 2010; Dannan, 2009; Huang, 2009; Sharma et al., 2010; Yen and Sharpe, 2008). Scaffolds are made from both synthetic polymers like polylactic acid (PLA), polyglycolic acid (PGA), polylacticco-glycolic acid (PLGA), and polycaprolactone (PCL)) or natural polymers like collagen, fibrin, polysaccharides and alginates (Sharma et al., 2010). PGA fibers have been shown to be useful for engineering dental pulp-like tissue (Bohl et al., 1998).

Dental stem cells are also used to repair or supplement other types of tissues including bone, heart and neuronal tissue (d'Aquino et al., 2009; Gandia et al., 2008; Huang et al., 2009b; Wang et al., 2010). The potential of stem cells to regenerate bone tissue was demonstrated in a study by d'Aquino et al. (2007). These researchers showed that in vivo transplantation of human DPSCs in woven bone chips or polymer scaffolds into immunocompromised rats resulted in adult bone formation complete with de novo synthesis of blood vessels (d'Aquino et al., 2007). Potential treatment with DPSCs has also been tested using cardiac tissue in rats that have been subjected to a myocardial infarction. After transplanting DPSCs into the site of infarction via injection, there was a decrease in the size of the infarct and increased vessel formation near the infarct (Gandia et al., 2008). Interestingly, SHED cells have been used by researchers to produce dopaminergic neuron cells (Wang et al., 2010) to alleviate the effects of Parkinson's disease in rats. Wang et al. (2010) used a two-step induction protocol to stimulate SHED cells to form neurospheres which were then treated with a neurogenic cocktail to stimulate their differentiation into dopaminergic neurons. The formation of neurons by SHED cells suggests that dental cells may become an invaluable resource for neurodegenerative disease therapies. Another suggested application of stem cell therapy for SHEDs is for the treatment of wound healing (Nishino et al., 2011). Using a mouse model, SHEDs were transplanted into an excisional wound and were found to accelerate healing after 5 days when compared to control (Nishino et al., 2011). The potential use of dental stem cells has become even more viable for tissue regeneration and other therapies with the recent advances in cryopreservation. These advances allow proliferation and long term storage of these cells for future cell therapy treatments while maintaining their differentiation potential (Ding et al., 2010; Papaccio et al., 2006; Seo et al., 2005; Woods et al., 2009; Zhang et al., 2006).

3.2 Dental stem cells and dynamic compression

Our lab is exploring the area of cellular based tissue engineering and in particular the effects of dynamic cyclic compression on chondrogenesis in two types of human dental stem cells, PDLSCs and SHEDs.

Earlier work in our lab on biomechanical force has shown that short intervals of cyclic compression cause rabbit BMMSCs to up-regulate TGFβ (Huang et al., 2005). TGFβ3 has been shown to induce chondrogenic differentiation of BMMSCs in vitro (Barry et al., 2001) and the extracellular signal-related kinase (ERK) 1/2 signal transduction pathway of

mitogen activated protein kinases (MAPKs) has been implicated in this process (Lee et al., 2004). The application of dynamic mechanical compression has been shown to induce chondrogenic differentiation of stem cells via an autocrine signaling pathway (Huang et al., 2005). Interestingly, just two hours of cyclic compression applied to BMMSCs stimulated TGFβ gene expression and the expression of both of its receptors (Huang et al., 2005). This stimulation in turn resulted in an up-regulation of the early response genes c-Fos and c-Jun as well as chondrogenic specific genes Sox-9, aggrecan and collagen type II (Huang et al., 2005).

Our lab has developed a line of adult dental stem cells derived from the PDL that are multipotent and express some ESC markers (Huang et al., 2009a). Using in vitro cultures in chondrogenic media we were able to show that after two weeks in culture with TGFβ3, PDLSCs increased expression of the chondrogenic markers collagen II and aggrecan (Huang et al., 2009a). Further, we see that PDLSCs express chondrogenic markers when subject to dynamic cyclic compression in a custom built bioreactor. After applying 15% strain at 1 Hertz for four hours we see a two to three fold increase in PDLSCs chondrogenic gene expression of Sox-9 and aggrecan as well as a 50% increase in ERK1/2 activity (Fritz, 2009). These results suggest that human PDLSCs, like BMMSCs, subject to dynamic cyclic compression require the ERK1/2 signaling pathway for chondrogenic expression (Fritz, 2009).

Recently we examined the effects of shorter durations of dynamic cyclic compression on SHEDs. As in previous experiments (Fritz, 2009; Pelaez et al., 2009), fibrin gel constructs were cast into 1.5-mm deep and 8-mm diameter Teflon molds set on top of a clean microscope slide. We loaded 1 X 10^7 SHEDs into 85 μL fibrin gel mixture containing 5 U/mL thrombin in PBS and 40 mg/mL fibrinogen in high-glucose DMEM. The fibrin gel constructs were allowed to solidify for one hour before removing from the Teflon mold and then placed in fibrin gel media containing high-glucose DMEM, 1% penicillin/streptomycin and 1× ITS supplement (BD Biosciences, San Jose, CA) and 0.0875 IU/mL aprotinin from bovine lung (Sigma-Aldrich, St. Louis, MO) for 24 hours in a water-jacketed incubator at 37°C and 5% CO_2. The compression chambers were loaded with the fibrin gel constructs and 650 μL of fibrin gel media. Fibrin gel constructs were then subjected them to 1Hertz dynamic cyclic compression with 15% strain in a custom built bioreactor placed in a water-jacketed incubator at 37°C and 5% CO_2. Twelve samples for each treatment were subjected to 0 minutes, 15 minutes, 30 minutes, 60 minutes and 240 minutes of compression. Fibrin gel constructs were removed from the bioreactor and flash frozen in liquid nitrogen.

Messenger RNA expression was determined using methods similar to Fritz (2009) and Pelaez et al. (2009). Briefely, fibrin gel constructs were homogenized using a IKA Ultra Turrax® T8 Homogenizer in 1 mL TRIzol (Invitrogen, Carlsbad, CA) and RNA was extracted according to manufacturers recommended protocol. Purified RNA concentration was quantified on a NanoDrop ND-1000 spectrophotometer. Reverse transcription of mRNA was performed using MultiScribe™ Reverse Transcriptase (Applied Biosciences, Foster City, CA) according to the manufacturer's suggested protocol. Quantitative real-time PCR was performed in a MxPro 3005P machine (Stratagene) using SYBR® Green PCR Master Mix (Applied Biosciences, Foster City, CA) according to manufacturer's suggested protocols. All samples were run in triplicate. GADPH was used as a reference gene. Fold changes of the chondrogenic genes (Sox-9, c-fos, and TGFβ3) were calculated from the log-transformed C_T values and expressed relative to the No Compression treatment group using

a modification of the delta-delta C_T method (Livak and Schmittgen, 2001; Vandesompele et al., 2002).

Protein was extracted from the fibrin gel scaffolds and levels of p-ERK assessed using the methods described in Fritz (2009). Briefly, fibrin gel constructs were homogenized in 1 mL of RIPA cell lysis buffer 2 (Enzo Life Sciences Int'l, Inc., Plymouth Meeting, PA) plus 0.5 µL/mL protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 1 mM phenymethanesulfonyl fluoride (Sigma-Aldrich, St. Louis, MO). The homogenate was kept on ice for 40 minutes and vortexed every 10 minutes. The homogenate centrifuged and the remaining supernatant was saved for protein analyses. The level of p-ERK 1/2 in each sample was determined using [pThr^{202}/Tyr^{204}]Erk1/2 EIA kit (Enzo Life Sciences Int'l, Inc., Plymouth Meeting, PA). Manufacturer suggested protocols were performed for the EIA assays and all samples were analyzed in duplicates.

All data are reported as mean ± S.E.M. (N=number of samples). 0 minutes, 15 minutes, 30 minutes, 60 minutes and 240 minutes of compression were compared level of p-ERK 1/2 and for mRNA expression of Sox-9, c-fos, and TGFβ3. Significant differences were determined by using a One-way ANOVA in Sigma Stat 3.00 (SPSS Inc.).

The effects of dynamic compression on chondrogenesis and ERK 1/2 signal transduction was observed (Figure 2). As expected, we saw an increase in the levels of the early response gene c-fos after 60 minutes of dynamic compression (Figure 2). Surprisingly, we did not see any change in the other early response gene c-jun (Data not shown) as seen in BMMSCs described above. We also saw an increase in the gene expression of both TGFβ3 and Sox-9 after 30 minutes of dynamic compression which lasted for at least 60 minutes of compression (Figure 2). Unlike the PDLSCs response to dynamic compression, SHEDs did not express Sox-9 after four hours. Further, we noticed and increase in the phosphorylation of ERK 1/2 as early as 15 minutes which was sustained for at least 60 minutes, but was no longer elevated after 4 hours (Figure 2).

SHEDs clearly have a different response to dynamic compression than PDLSCs. Like PDLSCs we see an early rise in the phosphorylation of ERK 1/2 suggesting that this signal transduction pathway is responding to compressive forces in SHEDS. The rise in TGFβ3 likely triggers the chondrogenic differentiation. Similar to PDLSCs, we see an increase in Sox-9, a transcription factor for chondrocyte differentiation, suggesting that SHEDs are indeed beginning to undergo chondrogenic differentiation. However, unlike PDLSCs, we did not see any aggrecan expression in our experiment which arises later in chondrogenic differentiation. Therefore we can suggest that SHEDs, like PDLSCs, respond to compressive force by undergoing chondrogenic differentiation and this is likely mediated though the ERK 1/2 signal transduction pathway. However, although chondrogenic differentiation is triggered within 30 minutes by dynamic compression, it is not completed during these short time intervals. This may be due to the decrease in TGFβ3 gene expression after 4 hours, which may be required to maintain chondrogenic differentiation in the dynamically compressed constructs as there was no supplementation of TGFβ3 in the media. Interestingly, the control samples did not show any gene expression for aggrecan but these samples were maintained in media without any supplemental TGFβ3. Koyama et al. (2009) did not show any chondrogenic gene expression in control cultures of SHEDs, but felt it may be due to the fact that some of their cultures were infected. We would suggest repeating that experiment to determine if SHEDs cultures express any of the chondrogenic markers without TGFβ3 supplementation.

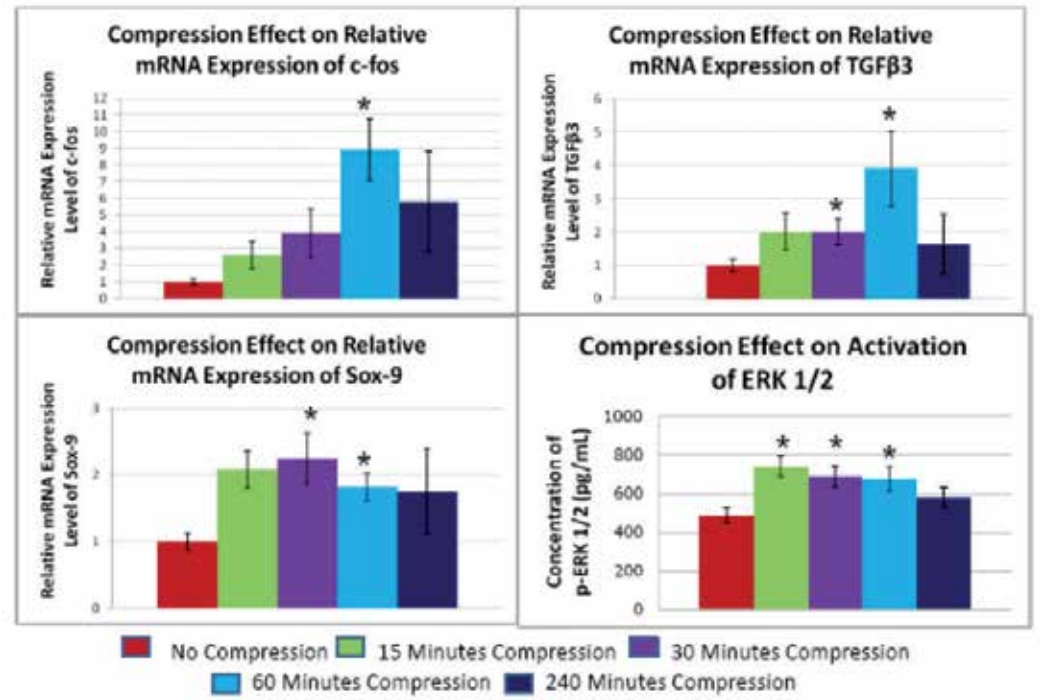

Fig. 2. The effects of different durations of dynamic cyclic compression (15% strain) on relative messenger RNA expression of c-fos, TGFβ3 and Sox-9 and on the phosphorylation level of ERK 1/2. All treatments N=6. Values are means ± S.E.M.; *P < 0.05, significantly different from No Compression treatment.

4. Dental stem cells versus embryonic stem cells

There are advantages and disadvantages for using dental stem cells compared to the using embryonic stem cells for tissue engineering and regenerative medicine. One advantage of using dental stem cells compared to ESCs is in the ease of obtaining these cells as they can be obtained from tissue during a standard tooth extraction or through loss of primary teeth (Huang et al., 2009b). ESCs are obtained from the inner cell mass of the embryoblast (Biswas and Hutchins, 2007) which requires access to the early embryo. An advantage of using embryonic stem cells is their pluripotent potential and their ability to differentiate into all three germ layers (Alison et al., 2002; Mummery et al., 2011; Thomson et al., 1998). Studies on dental stem cells have only shown them to only be multipotent (Gronthos et al., 2000; Karaöz et al., 2010; Miura et al., 2003; Morsczeck et al., 2005; Seo et al., 2004; Sonoyama et al., 2006). Interestingly, one PDL cell line has shown a broad differentiation potential and expresses some of the pluripotent markers expressed by ESCs (Huang et al., 2009a). A previous disadvantage of culturing ESCs is that they require a mouse embryonic feeder layer but recent studies have shown that ESCs can be cultured using human serum and human feeder cells and these cultures can be maintained for an extensive period of time (Ellerström et al., 2006). Dental stem cells do not require a feeder layer but only have a limited number of passages (See above).

Many of the problems encountered with stem cells delivery and scaffold choice are the same for both dental stem cells and ESCs. The use of dental stem cells or ESCs for tissue engineering or regenerative repair often produce similar results and many of the same types of problems arise. Currently whole tooth regeneration is not possible using ESCs or dental stem cells. Similar to dental stem cells, human ESCs have been used to form osteoblasts both in vitro and in vivo and had the capacity to form mineralized tissue (Bielby et al., 2004). Like DPSCs, ESCs have been used to repair cardiac function in infarcted myocardium. Human ESCs were injected into an infarcted mouse myocardium and were shown to improve cardiac function after four weeks but this improvement was not maintained after three months (van Laake et al., 2008). ESCs have also been used to explore neuronal regeneration for patients affected with Parkinson disease. Using the monkey as a primate model, ESCs have been used to generate dopaminergic neurons to aid in the relief of Parkinson disease (Takagi et al., 2005).

5. Concluding remarks

This review shows that dental stem cells are a viable alternative to embryonic stem cells for regenerative medicine. Dental stem cells are easily obtainable from the dental pulp of teeth and from other dental tissues which are often discarded as waste. Further, dental stem cells, like BMMSCs, form clonogenic, highly proliferative, multipotent cell populations in vitro and maintain their differentiation potential in vivo. Dental stem cells also show potential for cell therapy with respect to whole tooth regeneration. More work needs to be done to optimize the use of dental stem cells for use in cell therapies of other tissue types in the future including bone and cartilage formation. The potential of dental stem cells as an alternative choice to embryonic stem cells seems realistic for future stem cell therapies and regenerative medicine.

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Pluripotent Stem Cells from Testis

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1. Introduction

1.1 Importance of Spermatogonial Stem Cells (SSCs)

Spermatogonial stem cells (SSCs) are undifferentiated germ cells that balance self-renewing and differentiating divisions to maintain spermatogenesis throughout adult life. This is a productive stem cell system that produces millions of spermatozoa each day while also maintaining rigorous quality control to safeguard germline integrity. SSCs are the only adult stem cells that are capable of self renewal and that differentiate to produce haploid cells transmitting genes to next generation. In the recent years, derivation of multipotent embryonic stem (ES)-like cells, which are capable of differentiating to three germinal layers, from SSCs in the testis, has been reported. This is of immense importance in medicine, basic science and animal reproduction and overcomes ethical issues pertaining to the use of human embryos for research. Since the population of SSCs in the testis is very low (< 1%), the identification of markers that are specifically expressed in SSCs aids in their efficient isolation. The characteristics of SSCs and ES-like cells in culture, and differential expression of genes in both these cell types can provide better understanding. In the present chapter, we would describe and compare the expression of SSC-specific markers in vivo and in vitro. We will also review the in vitro culture conditions of SSCs and characteristics of ES-like cells that differentiate from SSCs. This would enhance our perceptive of these special cells that has opened new avenues for stem cell researchers.

1.2 Origin of Spermatogonial Stem Cell pool

SSCs arise from gonocytes in the postnatal testis, which originally derive from primordial germ cells (PGCs) during foetal development. PGCs are a transient cell population that is first observed as a small cluster of alkaline phosphatase-positive cells in the epiblast stage embryo at about 7–7.25 days post psot coitum (dpc) in mice (Phillips et al., 2010). The specification of PGC is dependent on the expression of BMP4 and BMP8b from the extraembryonic ectoderm (Ginsburg et al., 1990; Lawson et al., 1999; Ying et al., 2001). During the formation of the allantois, the PGCs are passively swept away from the original position before they start migrating via the hindgut to arrive at the indifferent gonad (genital ridge) between 8.5 and 12.5 dpc in mice. PGCs replicate during the migratory phase and approximately 3000 PGCs colonize the genital ridges (Bendel-Stenzel et al., 1998). In the

male gonad at about 13.5 dpc, PGCs give rise to gonocytes, which become enclosed in testicular cords formed by Sertoli precursor cells and peritubular myoid cells. Gonocyte is a general term that can be subcategorized into mitotic (M)-prospermatogonia, T1-prospermatogonia and T2-propsermatogonia (McCarrey 1993). M-prospermatogonia are located in the centre of the testicular cords, away from the basal membrane and continue proliferating until about 16.5 dpc of mouse development when they become T1-prospermatogonia and enter the G0 stage of mitotic arrest (McLaren 2003; Tohonen et al., 2003). Gonocytes resume proliferation during the first week after birth (marking their transition to T2-prospermatogonia), concomitant with migration to the seminiferous tubules basement membrane (Clermont & Perey 1957). T2-prospermatogonia that colonize the basement membrane give rise to the first round of spermatogenesis as well as establish the initial pool of SSCs that maintains spermatogenesis throughout postpubertal life (Kluin & de Rooij 1981; McCarrey 1993; Yoshida et al., 2006).

1.3 Dynamics of SSCs

The kinetics of sperm production were first described in rodents (Oakberg 1956) with the knowledge of the presence of adult stem cells, such as hematopoietic stem cells (HSCs), researchers hypothesized that germ cell differentiation in the testis required a stem cell population. The presence of a stem cell population responsible for continual sperm production in the testis was demonstrated in 1994 (Brinster & Zimmermann). They documented the first successful spermatogonial stem cell (SSC) transplantation in mice resulting in donor-derived spermatogenesis. SSCs are part of a subset of male germ cells called undifferentiated spermatogonia (Caires et al., 2010). This subset includes A_{single} (As) spermatogonia that are thought to be the SSCs and their progeny cells A_{paired} (Apr) and $A_{aligned}$ (Aal) spermatogonia (figure 1). SSCs are a self-renewing population of adult stem cells capable of producing progeny for a continual production of sperm by sexually mature males. They help in maintaining a constant supply of undifferentiated spermatogonia, which are critical to the initiation of spermatogenesis and the long-term production of sperm. One must be careful to distinguish between spermatogonial stem cells (SSC) and spermatogonia in general. The term "spermatogonia" is used collectively to refer to a continuum of cells ranging from those resting on the basement membrane (known as either $A_{isolated}$ or A_{single} spermatogonia), to proliferating sub-populations (A_{paired} to $A_{aligned}$, spermatogonia) to differentiating sub-populations [A_{1-4}, Intermediate and Type B spermatogonia]. The differentiating stages are committed to enter spermatogenesis, but whether the $A_{isolated}$ cells represent the only spermatogonia with a true stem cell nature is not yet fully clear. The differentiation of germ cells from diploid undifferentiated spermatogonia to mature and haploid spermatozoa is supported by the Sertoli cells of the seminiferous epithelium. The formation of the SSC population is dependent on the associated niche in the microenvironment in the seminiferous tubules of the testis. This niche environment supplies factors and provides interactions crucial for the survival and development of SSCs. It is usually composed of adjacent differentiated cells, the stem cells themselves, and the extracellular matrix surrounding these cells. The somatic cells produce factors, which aid in the extrinsic regulation of the SSC self-renewal/differentiation process. SSCs represent a model for the investigation of adult stem cells because they can be maintained in culture, and the presence, proliferation and the loss of SSCs in a cell population can be determined with the use of a transplantation assay.

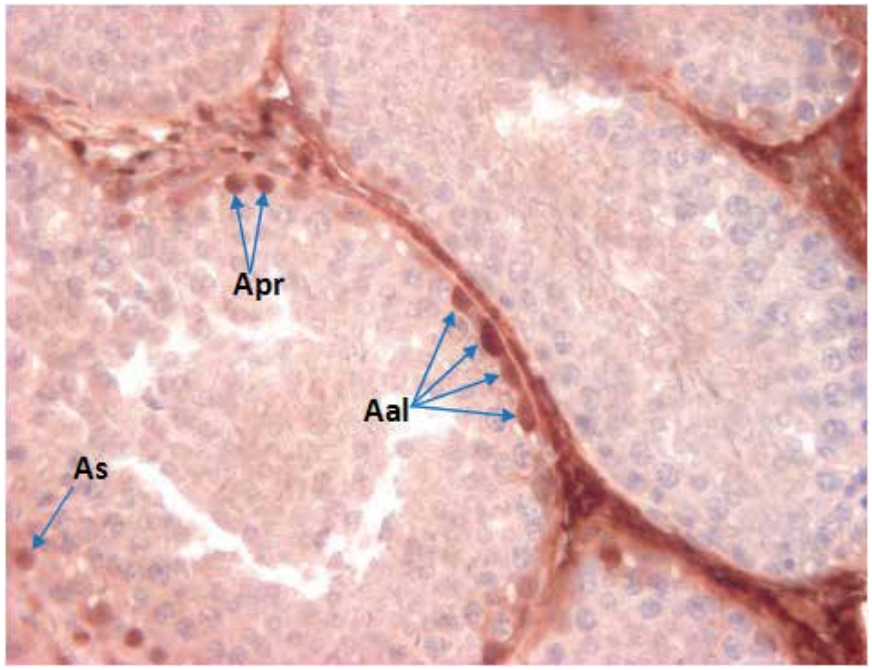

Fig. 1. Adult pig testis section stained with anti-UCHL-1 antibody. Spermatogonia are present as, A_{single} (As), A_{paired} (Apr) and $A_{aligned}$ (Aal) at the basement membrane of seminiferous tubule. The As spermatogonia are thought to be the spermatogonial stem cells (SSCs).

2. Identification of SSCs

2.1 Markers of SSCs

Exploring markers for spermatogonia help to identify the stem cell pool in the testis. Certain rodent markers for germ cells and SSCs such as VASA, DAZL, PLZF, and GFRα1are also of monkey (Hermann et al., 2007) and in other species. VASA expression marks spermatogonia in sheep (Borjigin et al., 2010), buffalo (Goel et al., 2010b) and bull (Fujihara et al., 2011) testis. It has been demonstrated that GFRα1 is a marker for mouse SSCs and probably their progeny (Meng et al., 2000; Buageaw et al., 2005; Hofmann et al., 2005; Naughton et al., 2006; He et al., 2007), and that KIT is a characteristic for the more differentiated spermatogonia, including type A_{1-4} spermatogonia (Yoshinaga et al., 1991). KIT expression is shown to mark pig gonocytes (Goel et al., 2007) and spermatogonia (Dirami et al., 1999). PLZF marks pig (Goel et al., 2007) and sheep (Borjigin et al., 2010) gonocytes and type-A spermatogonia. Because there is not a unique marker available to distinguish the SSCs and other undifferentiated spermatogonia, called A_{pr} and A_{al}, it is helpful to use two or three antibodies to characterize their phenotypes. The GFRα1 and POU5F1 antibodies stain the

same subset of mouse spermatogonia (He et al., 2007). POU5F1 also marks spermatogonia in buffalo (Goel et al., 2010b) and bull (Fujihara et al., 2011). GPR125 is also believed to be a marker for mouse spermatogonial stem/progenitor cells (Seandel et al., 2007). It is speculated that some of these markers as mentioned above will also be applicable to human spermatogonia. It has been recently shown that GPR125 may be a marker for human SSCs, as it is for mouse SSCs (He et al., 2010).

A comparison of the markers for spermatogonia and their progenitors in human and rodents indicates that these spermatogonia share many but not all phenotypes. In rodents, α6-integrin (CD49f), β1-integrin (CD29), and Thy-1 (CD90) are surface markers for mouse spermatogonial stem/progenitor cells (Shinohara et al., 1999; Kubota et al., 2003). THY-1 also marks bovine spermatogonia (Reding et al., 2010). CD9 is a surface marker for mouse and rat spermatogonial stem/progenitor cells (Kanatsu-Shinohara et al., 2004a). GFRα1 and RET are co-receptors for GDNF and markers for spermatogonial stem/progenitor cells (Buaas et al., 2004; Costoya et al., 2004; Buageaw et al., 2005; Hofmann et al., 2005; Naughton et al., 2006). In human, α6-integrin is expressed in spermatogonia and their progenitors and was used to isolate and purify human spermatogonial cells by magnetic-activated cell separation (Conrad et al., 2008). Other rodent surface markers, such as CD90, GFRα1, and CD133, were also used to select human spermatogonia by magnetic-activated cell separation (MACS) and comparable results to α6-integrin were obtained (Conrad et al., 2008). PLZF is characterized as a hallmark for mouse spermatogonial stem/progenitor cells (Buaas et al., 2004; Costoya et al., 2004). In adult monkey, the expression of PLZF is confined to the A_{dark} and/or A_{pale} spermatogonia (Hermann et al., 2007). GPR125 has been demonstrated to be expressed in mouse spermatogonia and their progenitors (Seandel et al., 2007), and it is recently reported that GPR125 is also present in human spermatogonia (Dym et al., 2009). UCHL-1 is known to express in spermatogonia of mice (Kwon et al., 2004), monkey (Tokunaga et al., 1999) and humans (He et al., 2010). UCHL-1 protein expression is present specifically in the spermatogonia of domestic animal testes such as bull (Wrobel et al., 1995; Herrid et al., 2009), pig (Luo et al., 2006; Goel et al., 2007), sheep (Rodriguez-Sosa et al., 2006) and buffalo (Goel et al., 2010b). UCHL-1 protein expression is also specific to spermatogonia of wild bovids (Goel et al., 2010a). UCHL-1 also marks spermatogonia in the testis of Indian mouse deer (*Moschiola indica*) and slender loris (*Loris tardigradus*) (unpublished data). It is therefore likely that the expression of UCHL-1 is conserved in a variety of species. Collectively, the above studies suggest that some spermatogonial markers are conserved between rodents and humans and other species. In contrast, some other rodent markers for spermatogonia and their progenitors are not applicable to human and other species. This can be illustrated by the fact that α1-integrin (CD29), a marker for rodent spermatogonial stem/progenitor cells, is not expressed in human spermatogonia but present in spermatocytes, spermatids, and spermatozoa in normal human testis (Schaller et al., 1993). Another example is that POU5F1 (Oct-4), a marker for mouse spermatogonial stem/progenitor cells (Ohbo et al., 2003; Ohmura et al., 2004; Hofmann et al., 2005), is not detected in adult human spermatogonia (Looijenga et al., 2003). POU5F1 shows a rather unique expression pattern in pig testis where spermatogonia show a weak staining, however, strong expression is present in differentiating germ cells such as spermatocytes and spermatids (Goel et al., 2008). Similarly, KIT is regarded as a marker for mouse differentiating spermatogonia (Yoshinaga et al., 1991; Schrans-Stassen et al., 1999; Dolci et

al., 2001), but it is undetected in human spermatogonia (Rajpert-De Meyts et al., 2003). Notably, some human markers for spermatogonia are also not applicable to rodents. As an example, the TSPY protein is preferentially expressed in elongated spermatids but not in spermatogonia of adult rat testis (Kido and Lau, 2006), unlike the expression pattern of the TSPY in adult human spermatogonia (Schnieders et al., 1996). Other rodent markers, including CD9 (Kanatsu- Shinohara et al., 2004b), CDH1 (Tokuda et al., 2007), neurogenin3 (Yoshida et al., 2004, 2007), RET (Naughton et al., 2006), and STRA8 (Giuili et al., 2002), were demonstrated to be expressed in spermatogonia and their progenitors; however, whether these rodent markers are present in human spermatogonia remains to be clarified. Similarly, some human markers, such as CD133 (Conrad et al., 2008), CHEK2 (also known as chk2 tumor suppressor protein) (Bartkova et al., 2001; Rajpert-De Meyts et al., 2003), and NSE (Neurone-specific enolase) (Rajpert-De Meyts et al., 2003), are also awaiting further studies to explore whether they are present in rodent spermatogonia and their progenitors. Such investigations would uncover further similarities and/or differences in spermatogonial phenotypes between human and rodents.

2.2 Functional assay of SSCs

Transplantation of isolated germ cells from a fertile donor male into the seminiferous tubules of infertile recipients can result in donor-derived sperm production. Therefore, this system represents a major development in the study of spermatogenesis and a unique functional assay to determine the developmental potential and relative abundance of spermatogonial stem cells in a given population of testis cells. The application of this method in farm animals has been the subject of an increasing number of studies, mostly because of its potential as an alternative strategy in producing transgenic livestock with higher efficiency and less time and capital requirement than the current methods such as microinjection of genes into fertilized eggs and somatic cell nuclear transfer.

Germ cell transplantation (GCT), also referred to as spermatogonial stem cell (SSC) transplantation, is a powerful technology first introduced in 1994 by Brinster and colleagues. Although initially developed using a mouse model, GCT has important applications in the study and manipulation of spermatogenesis in many species. In this method, testis cells obtained from a fertile donor male are transferred into the seminiferous tubules of infertile recipient testes, where donor-derived sperm production can occur, allowing the recipient to sire progeny (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994). In essence, donor SSCs deposited in the lumen of the recipient seminiferous tubules are allowed by the Sertoli cells to migrate to the basolateral compartment of the tubule, to proliferate, form new colonies and initiate donor-derived spermatogenesis (Nagano et al., 1999; Ohta et al, 2000). Following the original introduction of GCT in mice (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994), the technique was also successful in rats (Jiang & Short, 1995;Ogawa et al., 1999b), monkey (Schlatt et al., 2000a) and goat (Honaramooz et al., 2003). In laboratory rodents, GCT not only provides a unique opportunity for gaining a new insight into spermatogenesis and the biology of the stem cell niche, but also presents a unique functional bioassay to test the competence of putative SSCs. Furthermore, GCT also offers a new strategy for preservation of male fertility and an alternative approach for generation of transgenic animals (Brinster 2002, 2007).

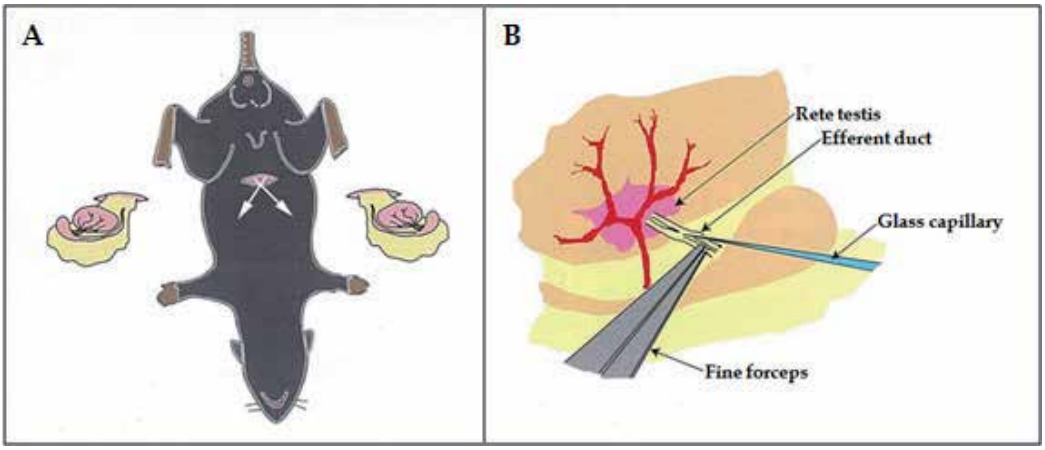

Fig. 2. Germ cell transplantation (GCT) in mice. (A) Germ cell depleted mice testes are extoriated through a ventral incision. (B) The testicular cell suspension is injected into seminiferous tubules through the efferent duct using a fine glass capillary.

Rather surprisingly, cross-species (xenogeneic/heterologous) transplantation of testis cells from donor rats and hamster into recipient mice resulted in complete rat (Clouthier et al., 1996) and hamster (Ogawa et al., 1999a) spermatogenesis. This development sparked an interest in the idea of using the laboratory mouse as a universal recipient model for testis cells from different donor species. However, GCT from genetically more distant donor species, including farm animals, into mice only resulted in colonization or proliferation of SSCs but not in complete spermatogenesis (Nagano et al., 2001, 2002; Dobrinski et al., 2000; Kim et al., 2006). We have recently shown that spermatogonia from endangered wild bovid (the Indian black buck; *Antelope cervecapra L.*) were able to colonize the recipient mice testis following GCT, however, showed no signs of proliferation or differentiation (Goel et al., 2011). This block in differentiation of donor germ cells is believed to be due to the incompatibility of donor germ cells and mouse Sertoli cells (Shinohara et al., 2003). Although GCT from nonrodent species into the mouse testis did not result in complete spermatogenesis, it is still the only available bioassay for detecting the colonization potential of SSCs in a given population of donor testis cells from any species (Dobrinski et al., 1999a, b, 2000). Interestingly, when (rather than transferring isolated testis cells into the seminiferous tubules) small fragments of testis tissue were transplanted under the back skin of recipient mice, complete donor-derived spermatogenesis was observed from a wide range of donor species, including farm animal species (Honaramooz et al., 2002, 2004; Schlatt et al., 2002b; Oatley et al., 2005; Rathi et al., 2006, 2008; Arregui et al., 2008; Ehmcke et al., 2008; Nakie et al., 2010).

3. Pluripotential ability of germ stem cells in testis

3.1 Introduction

In the years 2006 and 2007, many articles were published, where somatic skin cells could be reprogrammed to ES-like cells, the so-called induced pluripotent stem (iPS) cells. Each report on the induction of pluripotency in mouse and human skin fibroblasts used retroviral delivery of key pluripotent stem cell genes such as, Oct-4 (Pou5f1), Sox2, c-Myc, and Klf4

(Takahashi and Yamanaka, 2006; Hanna et al., 2007; Meissner et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007). In the second step, transformed iPS cells were identified and selected by expression of pluripotent markers including Nanog (Okita et al., 2007; Wernig et al., 2007) or Oct-4 (Wernig et al., 2007), or by ES-specific morphology (Meissner et al., 2007). These iPS cells had unique characteristics as they were germ-line competent and indistinguishable from ES cells derived from the embryo at the epigenetic level. Additionally, recent work has demonstrated that patient-autologous skin iPS cells can be genetically modified and used after differentiation by induction to cure a mouse model of sickle cell anemia (Hanna et al., 2007). Although this research paves the way toward stem cell therapy, it seems to be impractical for the iPS cells to be used in clinical application because of their instability and potential retroviral infections (Dym et al., 2009).

As a result, it is essential and necessary to figure out more physiological methods to induce pluripotency from adult somatic cells or adult stem cells. More recent efforts have been taken to generate iPS cells from neural stem cells using only two transcription factors, Oct-4 and either c-Myc or Klf4 (Kim et al., 2008). It has also been demonstrated that iPS cells can be produced from adult cells by non-integrating adenoviruses transiently expressing Oct-4, Sox2, Klf4, and c-Myc (Stadtfeld et al., 2008). However, it is still possible that these factors could somehow find their way into the genome of the iPS cells. Therefore, reprogramming of adult cells without the use of oncogenes would be very useful and safer as a means to produce ES-like cells. The proof of principle that spermatogonial stem cells/progenitor cells could be reprogrammed to pluripotent cells by biochemical means alone was first shown by Shinohara and colleagues (Kanatsu-Shinohara et al., 2004b). However, they could not derive ES-like cells from SSCs of adult mice. Guan et al. (2006) demonstrated that mouse adult spermatogonia, possibly the spermatogonial stem cells and/or their progeny, were able to reprogram biochemically to pluripotent ES-like cells. This was confirmed in mouse by (Seandel et al., 2007) showing that adult spermatogonia and/or their progenitors could indeed form pluripotent ES-like cells. Golestaneh and others (Conrad et al., 2008; Kossack et al., 2008; Golestaneh et al., 2009) have recently demonstrated a similar phenomenon in male germ cells and spermatogonia in the human testis. It is important to note that the SSCs/progenitor cells appear to reprogram spontaneously to pluripotency when the cells are removed from their niche and when ES cell media is added. Thus, SSCs/progenitor cells have a great potential to be used as a safe means to generate ES-like cells that eventually can be used for clinical therapies of human diseases. Human ES cells are pluripotent stem cells that have the potential to differentiate into all the types of cell lineages and tissues in the body, and thus they are ideal cell sources for cell transplantation and gene therapy. However, the major concern is the ethical issues associated with obtaining human ES cells from IVF clinics. The human iPS cells have major advantages over human ES cells because there are no ethical issues involved and, more importantly, the iPS cells appear to be similar to ES cells in morphology, proliferation, and pluripotency, as evaluated by teratoma formation and chimera contribution. In contrast, the iPS cells have some disadvantages, e.g., safety is a major concern due to the potential of cell transformation or tumor formation because of the oncogenes from the transfected iPS. It may be possible to overcome these issues by generating pluripotent stem cells without oncogene transfer directly from spermatogonial stem cells by testicular biopsy. Thus, the generation of human ES-like cells from SSCs may offer a safer means of obtaining pluripotent stem cells than from the iPS cells. The identification of human SSCs is now especially important in view of the discrepancy between the Skutella report (Conrad et al., 2008) and the report by the Reijo-

Pera group (Kossack et al., 2008). The Skutella group concluded that the ES-like cells derived from human spermatogonia (Spga) were in fact as pluripotent as true embryonic stem (ES) cells. The Reijo-Pera group noted that their cells differ from true ES cells in gene expression, methylation, and in their ability to form teratomas. Comparisons are difficult because the Skutella group used isolated Spga to get their ES-like cells, whereas the Reijo-Pera group used the entire testis biopsy without separating the Spga. It is possible that ES-like cells derived from isolated spermatogonial stem cells yield superior ES-like cells compared to using whole testis, but this remains to be determined. There are now three means to generate human pluripotent stem cells: (1) from a fertilized embryo as the traditional method; (2) from adult somatic cells through iPS technology; and (3) from adult SSCs and/or their progeny. One major advantage of the third approach is that the production of the ES-like cells is spontaneous, unlike method 2, where several genes, some cancer causing, is employed. Thus, human SSCs and/or their progeny have a great potential for cell- and tissue engineering-based medical regeneration for various human diseases. It is possible that in the near future men could be cured of their diseases using a biopsy from their own testes (Dym et al., 2009).

3.2 Culture of SSCs and induction of ES-like colonies

Kanatsu-Shinohara et al. (2004b) cultured SSCs in such a way that these cells propagated themselves, while retaining their capacity to repopulate a recipient mouse testis upon transplantation. A special medium, which was designed to culture hematopoietic stem cells, has been used for culture and contained several growth factors, including GDNF. In this culture system, a feeder layer is first formed that is composed of the contaminating somatic cells of the neonatal testis. Then, after 2 weeks and two passages, mitomycin-treated mouse embryonic fibroblasts (MEFs) are used as a feeder layer. During the first weeks of culture, the formed colonies consisted of SSCs, but, within 4-7 weeks, colonies that morphologically resembled ES cell colonies formed. Further work indicated that these colonies were indeed composed of multipotent ES-like cells. In order to maintain the multipotent character of these ES-like cells, they were subsequently cultured under a standard ES cell culture conditions in medium containing 15% fetal calf serum and LIF. Under these conditions, the cultured SSCs could not be propagated because of the lack of GDNF. ES-like colonies could only be obtained when the starting population of SSCs was derived from neonatal mice; when it was derived from older mice, ES-like colonies did not appear. However, cultures of SSCs derived from adult *p53* (*Trp53*)-null mice did produce ES-like colonies. P53 is involved in the cellular response to DNA damage and a lack of P53 increases the chances of teratoma development. Possibly, P53-deficient SSCs are more capable of undergoing the transition into ES-like cells.

An essentially similar protocol was followed by Seandel et al. (2007), except that this group used inactivated testicular stromal cells consisting of a mixture of $CD34^+$ peritubular cells, α -smooth-muscle-actin-positive peritubular cells and cells positive for the Sertoli cell marker vimentin, as a feeder layer because they had less success using MEFs. By this method, ES-like colonies only appeared after more than 3 months in culture, more slowly than reported by Kanatsu-Shinohara et al. (2004b). A substantially different approach was taken by Guan et al. (2006). Their starting material was derived from 4- to 6-week-old mice and they did not use the stem cell medium described by Kanatsu-Shinohara et al. (2004b) but simply Dulbecco's Modified Eagle's Medium (DMEM) with serum and added GDNF, in which testicular cells were initially cultured for 4 to 7 days. These cells were then sorted for the

expression of STRA8 and subsequently cultured in DMEM under various conditions, but without adding GDNF. Colonies of ES-like cells formed when LIF was added to the medium and/or when the cells were cultured on a feeder layer of MEFs. The ES-like cells were further expanded by culture on MEFs and the addition of LIF.
Hu et al. (2007) cultured germ cells of prepubertal mice under conditions that favour osteoblast differentiation and reported the emergence of cells that had characteristics of osteoblasts after several weeks in culture. In this system, there was no period of culture with added GDNF. Finally, Boulanger et al. (2007) employed no culture step at all. This group transplanted cells isolated from adult mouse seminiferous tubules, together with mammary cells, into mammary fat pads to obtain the differentiation of SSCs into mammary epithelial cells.
We have shown that gonocytes from neonatal pig testis can attain multipotency during short-term culture (Goel et al., 2009). Freshly isolated gonocytes were found to have either weak or no expression of pluripotency determining transcription factors, such as *POU5F1*, *SOX2* and *C-MYC*. Interestingly, the expression of these transcription factors, as well as other vital transcription factors, such as *NANOG*, *KLF4* and *DAZL*, were markedly upregulated in cultured cells. The formation of teratomas with tissues originating from the three germinal layers following the subcutaneous injection of cells into nude mice from primary cultures confirmed their multipotency.
Taken together, it does not seem that a very specific approach is required to obtain the transformation of SSCs into ES-like cells. This transformation can occur on different feeder layers and even without a feeder layer, if LIF is added to the culture medium. Furthermore, the culture medium also does not seem to play a decisive role in the transformation of SSCs into ES-like cells, as the groups of Kanatsu-Shinohara et al. (2004b) and Seandel et al. (2007) used a specific stem cell medium, whereas Guan et al. (Guan et al., 2006) used DMEM. All three groups added GDNF to the culture, either continuously (Kanatsu-Shinohara et al., 2004b; Seandel et al., 2007) or only at the start (Guan et al., 2006). However, to obtain the transformation of SSCs into cells of another lineage, it might not be necessary for them to become ES-like cells first. Putting the SSCs in an osteoblast-inductive environment in culture (Hu et al., 2007) or transplanting them into a mammary gland-inductive environment in vivo (Boulanger et al., 2007) might be enough for these cells to change their lineage. This rather suggests that SSCs are restricted to the spermatogenic lineage owing to the seminiferous tubular environment in which they reside. Once outside of this environment, they can switch to another lineage depending on the particular niche in which they are placed.

3.3 Gene expression in SSCs and ES-like cells

A crucial question is what changes in gene expression accompany the transition from a cultured SSC to an ES-like cell? In this respect, it is interesting to study the possible changes in the expression of those genes that can transform a fibroblast into an ES-like cell, that is *Myc, Oct4* (*Pou5f1*), *Sox2* and *Klf4* (Takahashi and Yamanaka, 2006; Wernig et al., 2007), in SSCs and in the ES-like cells derived from them. Kanatsu-Shinohara et al. (2008b) found that all four pluripotency genes are already expressed at low levels in cultured SSCs, although no NANOG (Kanatsu-Shinohara et al., 2004b) or SOX2 protein expression was found in these cells. In ES-like cells, the expression of these four genes is much increased. In addition to these pluripotency genes, the ES cell markers such as stage-specific embryonic antigen-1

(SSEA-1; FUT4) and, to a low level, Forssman antigen (GBGT1), were induced in the ES-like cells and, as in ES cells, high levels of alkaline phosphatase (AP) were also found (Kanatsu-Shinohara et al., 2004b). Guan et al. (2006) assayed expression patterns in SSCs cultured under conditions that induced these cells to become ES-like cells. In this situation, it is difficult to categorize these cells as being either SSCs or ES-like cells as they might be in an in-between state. In these SSCs/early ES-like cells, *Oct4, Nanog* and *SSEA1* were expressed (Guan et al., 2006). Indeed, the level of expression of *Nanog* and *SSEA1* suggests that these cells were already on their way to becoming ES-like cells. Seandel et al. (2007) also studied gene expression levels before and after the transition of cultured SSCs to ES-like cells. *Oct4* was present in both cell types, but *Nanog* and *Sox2* were strongly induced in ES-like cells, whereas the early spermatogonial markers *Stra8, Plzf* (*Zbtb16*), *c-Ret* and *Dazl* became inhibited. Besides these specific studies, Kanatsu-Shinohara et al. (2008b) also carried out a microarray study and found that a great many genes changed their expression levels during the transition from being a cultured SSC to an ES-like cell. Among these genes, over a 100 were induced more than 5-fold in ES-like cells as compared with cultured SSCs, and another 100 were inhibited more than 5-fold in ES-like cells. Clear differences between the patterns of genomic imprinting are also seen between cultured SSCs and ES-like cells. Kanatsu-Shinohara et al. (2004b) studied the imprinting pattern of three paternally (*H19, Meg3* and *Rasgrf1*) and two maternally (*Igf2r* and *Peg10*) imprinted regions in cultured SSCs and in the ES-like cells derived from them. Cultured SSCs show a completely androgenetic (paternal) imprinting pattern at the differentially methylated regions (DMRs) of these genes and loci; the DMRs of *H19* and *Meg3* are completely methylated and that of *Igf2r* is demethylated. By contrast, in the ES-like cells, the paternally imprinted regions are methylated to different degrees and the maternally imprinted regions (*Igf2r* and *Peg10*) are rarely methylated. Interestingly, the methylation patterns that are seen in the ES-like cells are not the same as those seen in proper ES cells, as the DMRs in ES cells are generally more methylated than those in ES-like cells, including the maternally imprinted regions. Furthermore, both Kanatsu-Shinohara et al. (2004b) and Seandel et al. (2007) reported that most of the ES-like cells obtained had a normal karyotype and that there was no evidence of clonal cytogenetic abnormalities. However, recently, Takahashi et al. (2007) did find some SSC-derived ES-like cells that were trisomic for chromosomes 8 or 11, which is a common chromosomal abnormality in ES cells.

In conclusion, the transition from cultured SSCs to ES-like cells is accompanied by extensive changes in gene expression, during which three of the four pluripotency genes (the exception being *Oct4*, which is already expressed in mouse SSCs) become expressed at higher levels, along with many other genes. Furthermore, changes occur in the genomic imprinting patterns of these cells as they undergo this transition. Although the ES-like cells acquire the expression of ES cell-specific genes, the expression pattern of these genes in ES-like cells is not identical to that seen in normal ES cells, with differences evident, for example, in the expression of brachyury, *Gdf3*, Forssman antigen, *Nog* and *Stra8*.

4. Applications of SSCs

4.1 Restoration of fertility

SSC transplantation may have application for treating male infertility in some cases. For example, high-dose chemotherapy and total body radiation treatment of cancer can cause permanent infertility. While adult men can cryopreserve a semen sample prior to their

oncologic treatment, this is not an option for pre-adolescent boys who are not yet making sperm. Using methods similar to those already established for other species, it may be possible for these young cancer patients to cryopreserve testis cells or tissue prior to cancer treatment and use those tissues to achieve fertility after they are cured (Orwig & Schlatt, 2005; Goossens et al., 2008; Hermann et al., 2009). Recently, a non-human primate model of cancer survivorship to test the safety and feasibility of SSC transplantation in a species that is relevant to human physiology has been established (Hermann et al., 2007). Although SSC transplantation is not yet ready for the human fertility clinic, it may be reasonable for young cancer patients, with no other options to preserve their fertility, to cryopreserve testicular cells (Schlatt et al., 2009). Ginsberg and co-workers have been cryopreserving testicular tissue for young cancer patients since 2008 and report that this intervention is acceptable to parents and that testicular biopsies caused no acute adverse effects (Ginsberg et al., 2010). A human SSC culture system would be particularly useful in this setting because a few SSCs could be obtained in a small biopsy and expanded to a number sufficient for transplantion therapy.

4.2 Genetic modification

4.2.1 Genetic modification of rodents

The establishment of SSCs led to the development of a new strategy for generating a genetically modified animal as an alternative or a potentially superior method to the conventional ES-based technology. First, SSCs are infected or transfected with a viral or plasmid vector carrying a drug-resistant gene. Individual clones of drug-resistant SSCs cells are selected and expanded in vitro. After DNA analysis, genetically modified SSCs are transplanted to infertile mice. In theory, half of spermatozoa that developed from the SSCs carry the transgene. Finally, recipient male mice are crossed with female mice to yield heterozygous transgenic mice with a theoretical success rate of 50%. In the conventional ES cell-based technology, the frequency of heterozygous transgenic mice depends on the properties of chimera mice. In some cases, no germline chimera can be found in the chimera population. In this respect, SSCs which are committed to spermatogenesis are advantageous. When SSCs were transfected with a plasmid vector bearing a drugresistant gene and used for the production of transgenic mice based on this method, approximately 50% of offspring derived from a drug-resistant clone maintained a transgene (Kanatsu-Shinohara et al., 2005). Mice lacking a specific gene (i.e. occludin) by homologous DNA recombination was then successfully produced (Kanatsu-Shinohara et al., 2006). Homozygous mutant mice showed signs of chronic gastritis, osteoporosis and a loss of acidic granule in the salivary glands, similar to the occludin knockout mice generated using ES cells (Saitou et al., 2000). From these finding, it was demonstrated that SSCs can be used for gene targeting in a similar manner to ES cells.

4.2.2 Application to generation of knockout animals in domestic species

The method for generating genetically modified mice based on ES cell technology has become a conventional experimental technique, and contributed to the functional analyses of many genes. Under current culture conditions, SSCs grow slower than ES cells. For example, clonal expansion from a single transfected SSCs requires mixing with non-transfected SSCs to maintain cell densities during drug selection. Thus, SSC-based transgenic technology is not yet useful as ES cell-based technology. However, SSC-based

technology is potentially applicable to animal species other than mouse, and this is the greatest advantage of this technology. At present, ES cell-based transgenesis is feasible only in mice, and cloning by somatic cell nuclear transfer is extremely difficult or impossible due to the low proliferation potential of somatic cells and the poor success rates of nuclear transfer. If SSC culture conditions specific for animal species are established, animal transgenesis may become feasible in other species. Specifically, SSCs are derived from the testes of rat, hamster and cattle (Hamra et al., 2005; Ryu et al., 2005; Aponte et al., 2008; Kanatsu- Shinohara et al., 2008c). Hamsters have been historically difficult to manipulate genetically; however, the SSC technology may provide a good animal model. Bovine SSCs may be useful for the cattle industry as an important application. In particular, rats are important experimental animals that are larger than mice and have been used in various research areas. Therefore, SSC technology will be highly beneficial if it allows unlimited transgenesis in rats. Transplantation of SSCs is an essential step in SSC-based animal transgenesis and is technically challenging. The efficiency of this method is low in large animals (Ogawa et al., 1999b). Development of technologies for xenogeneic transplantation and in vivo spermatogenesis, in addition to the determination of culture conditions, are anticipated for universal application to various species in the future. With regard to xenogeneic transplantation from rats to mice, normal rat offspring were successfully born after transferring rat spermatids and spermatozoa developed in the mouse testes into rat oocytes by in vitro microinsemination (Shinohara et al., 2006; Kanatsu-Shinohara et al., 2008a).

4.3 Regenerative therapy

Recent advances in cellular therapies have led to the emergence of a multidisciplinary scientific approach to developing therapeutics for a wide variety of diseases and genetic disorders. Although most cell-based therapies currently consist of heterogeneous cell populations, it is anticipated that the standard of care needs well-characterized stem cell lines that can be modified to meet the individual needs of the patient. Extensive research in the area of regenerative medicine is focused on the development of cells, tissues and organs for the purpose of restoring function through transplantation. The general belief is that replacement, repair and restoration of function is best accomplished by cells, tissues or organs that can provide the appropriate physiological/metabolic functions more efficiently than any mechanical devices, recombinant proteins or chemical compounds. Several cell-based strategies are currently being investigated, including cell preparations from autologous parenchyma or established cell lines, as well as cell therapies derived from a variety of stem cell sources such as bone marrow or cord blood stem cells, embryonic stem cells, as well as cells, tissues and organs from genetically modified animals. Several lines of evidence have suggested extensive proliferation activity and pluripotency of germline stem cells, including SSCs. These characteristics provide new and unprecedented opportunities for the therapeutic use of SSCs for regenerative medicine.

Guan et al. (2006) succeeded in developing a procedure for the isolation and purification of SSCs from adult mouse testis. They were able to isolate and culture these cells in culture medium containing the precise combination of cellular growth factors needed for the cells to reproduce themselves *in vitro*. These cells were characterized with regard to their molecular profiles and these were compared via molecular profiling of embryonic stem cells using a stem cell array which contains relevant genes related to stem cell metabolism. The results

indicate that SSCs share many molecular characteristics with embryonic stem cells. On the cellular level, SSCs resemble embryonic stem cells; they form an embryoid body structure after 2 weeks of culture. Stem cell potential of isolated SSCs was examined using the transplantation technique. This method allowed SSCs to recolonize the seminiferous tubules of germ cell-depleted mice and regenerate spermatogenesis. These cells are able to differentiate into various cell types of three germ layers *in vitro* (Guan et al., 2006, 2007). In contrast to ESCs, the use of SSCs for cell transplantation will allow establishment of individual cell-based therapy, because the donor and recipient are identical. In addition, any ethical problems are avoided.This approach provides an accessible *in vitro* model system for studies of mammalian gametogenesis, as well as for developing new strategies for reproductive engineering, infertility treatment and establishment of regenerative therapy.

4.4 Conservation biology

Thus far, most works on technologies of assisted reproduction in males have focused on mature spermatozoa. Successful semen collection, cryopreservation, and thawing techniques have been determined for a number of species. Advancements in the handling and storage of mature sperm have revolutionized the practice of both human and veterinary clinical reproductive medicine. In addition, these innovations have changed the nature of agriculture and agricultural economics, as artificial insemination has often supplanted natural breeding in intensive production regimens. Research on mature sperm is limited in that there are no cell culture systems that support spermatogenesis *in vitro*; therefore, sperm can only be obtained as primary cells from reproductively mature individuals. The stem cells that will produce sperm, on the other hand, are present in early neonates. Soon after birth in most species, gonocytes migrate to the basement membrane of the seminiferous cords; at this point in time the gonocytes transition into spermatogonia.

The development of reproductive technologies based on SSCs could preserve the breeding potential of males that die prior to puberty. This can be of great importance when the genetic contribution of a single individual could have significant impact on a long-term viability of a population. Examples of this would include attempts to preserve the genetic information represented in offspring of founder individuals in captive breeding programs, or attempts to propagate individuals with diseases that preclude natural mating. In addition, because SSCs can be maintained in culture, these cells are similar to ESCs in providing an opportunity for genetic manipulation that is not present in the terminally-differentiated spermatozoa. Efforts to take advantage of the attributes of SSCs have thus far focused on two technologies: spermatogonial stem cell transplantation (SSCT) and testis xenografting.

5. Conclusions

SSCs are the novel source of pluripotent stem cells that have an advantage over the iPS cells in numerous ways. Firstly, pluripotent cells from SSCs do not require the addition of any foreign genes as they are spontaneously generated in culture. Secondly, since pluripotent cells from SSCs are derived without viral transduction, they provide a safer alternative to iPS cells. Finally, SSCs and SSC-derived pluripotent cells can find immediate application in the field of regenerative medicine, fertility restoration, and genetic modification of mice and large animals and in the conservation of endangered species.

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Amniotic Fluid Stem Cells

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1. Introduction

The amniotic fluid or liquor amnii, was first isolated and studied during the beginning of the 20th century (Brace et al., 1989). More recently, in the 1960s and 1970s there was an increased interest in characterization and culture of the cells contained in the amniotic fluid (Huisjes HJ, 1970; Marchant GS, 1971). Nevertheless, most all of these studies were directed at using amniotic fluid, and the cells contained within, for determining the health of the fetus during development, or to provide a general characterization of the amniotic fluid. Although the discovery of stem cells, in particular bone marrow stem cells, occurred in the 1960's, it was not until recently that the possibility of isolating stem cells from the amniotic fluid was investigated. Amniotic fluid stem cell isolation and characterization is therefore fairly recent, dating back to the early 1990's (Torricelli et al., 1993).

The study of amniotic fluid-derived stem cells (AFDSCs) has captured the attention of researchers and clinicians for several reasons. First, AFDSCs can be collected during amniocentesis and isolated from material that would be otherwise discarded. Therefore, their use is not subject to the ethical debate that surrounds the use of embryonic stem cells. Second, like other fetal derived stem cells, storage of AFDSCs is easy and achieved at minimal costs. AFDSC populations can be easily expanded, and have shown the capability of being stored over long periods of time with no adverse effects (Da Sacco et al., 2010). Furthermore, the "banking" of AFDSCs from developing fetuses, may guarantee a source of stem cells with a matching immune profile to that of the recipient. Most importantly, the extensive characterization of a specific subset of AFDSCs positive for the marker c-Kit+ (De Coppi et al., 2007), have displayed no tumor formation following transplant into an animal model, even after several months. These cells, known simply as amniotic fluid stem cells (AFSC) have been at the forefront of AFDSC research and will be discussed in depth later. Finally, as a source of stem cells collected before birth AFDSCs may become an invaluable source of stem cells for direct treatment of various genetic disorders treatable in utero (Turner CG et al., 2009).

The potential applications and implications of AFDSCs in regenerative medicine and therapeutic treatments are significant, however; AFDSCs research is still in its infancy and much work is required to properly characterize AFDSCs and determine their effectiveness. In this chapter, we describe the different AFDSCs that have been isolated to date, list their characteristics, and provide an overview of the different organs in which AFDSCs have been used in vitro or in vivo to develop this stem cell population into a viable therapeutic strategy.

2. The amniotic cavity

The amniotic fluid is contained in the amniotic cavity that, in humans, starts forming as early as seven days post fertilization, and is delimited by a membrane called amnion. The formation of the amniotic cavity is a result of the cavitation of the epiblast. The amnion is formed by the cells of the epiblast, by the side facing the cytotrophoblast. This is the first appearance of the amniotic ectoderm, and at this stage it is still a continuum of the portion of the epiblast that will form the embryo. The amnion formation is completed at fourteen days post fertilization and is constituted of two layers: the amniotic ectoderm (inner layer facing the amniotic fluid) and the amniotic mesoderm (outer layer). The amnion has the important function of protecting the embryo and controlling the composition and the volume of the amniotic fluid. In humans, after seventeen weeks of gestation the amnion becomes surrounded and fused with another membrane, the chorion, and is therefore incorporated into the placenta. At the beginning of the formation of the amniotic cavity, active transport of solutes from the amnion, followed by passive movement of water, comprise the amniotic fluid.

In mice the amniotic cavity starts forming at embryonic stage E0.5 as a result of apoptotic events in the epiblast. At this stage, there is still the presence of a proamniotic cavity and the amnion that will start forming during gastrulation, is not yet defined. At approximately day E7.5 the amniotic cavity is formed and one day later the embryo starts the rotation process. At the end of the rotation, the embryo will be surrounded by the amnion. Surrounding the amnion are two more membranes, the visceral yolk sac and most externally the parietal yolk sac (Kaufman MH, 1992). These membranes represent three distinct layers surrounding the mouse embryo. Differently from human, in mice, the amnion does not fuse with the chorion and is not included in the placenta.

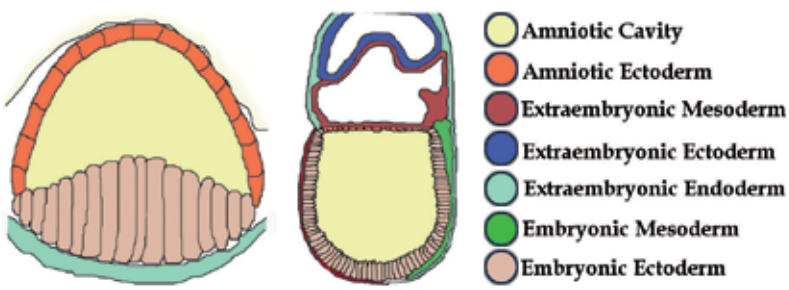

Fig. 1. Amniotic cavity formation

Twelve days post fertilization the human amniotic cavity is delimitated by the amnion (that at this stage is composed by the amniotic ectoderm) and the embryonic ectoderm (left). In the 7.5-day mouse embryo (right) the amnion is formed by the amniotic mesoderm and the amniotic ectoderm.

In mammals the embryo is immersed in the amniotic fluid contained inside the amniotic cavity. In human (left) the cavity is delimited by the amniotic ectoderm and the amniotic mesoderm that constitute the amnion, and by the chorion. The amniotic ectoderm is in direct contact with the amniotic fluid. In mouse (right) the amnion is surrounded by two extra membranes, the visceral and parietal yolk sac.

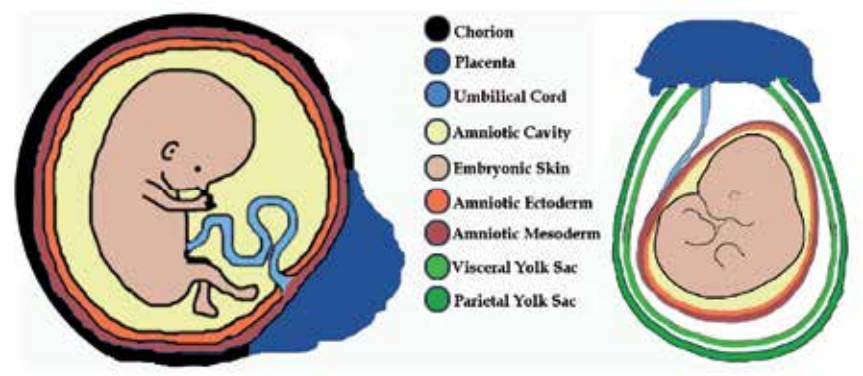

Fig. 2. Extra-embryonic membranes

3. The amniotic fluid

The amniotic fluid is the liquid present in the amniotic cavity and is constituted of about 98% water. This volume and composition change continuously during the different gestational stages. The volume of the amniotic fluid at the beginning of the pregnancy is multiple times the volume of the fetus, but at the end of gestation, at forty weeks, it will represent only a quarter of the volume of the fetus. Early during development, when the fetus has not yet started urination and deglutition, the plasma from the mother is surmised to play an important role in the composition of the amniotic fluid, and even though the mechanism is not completely understood, active transport of solutes is probably present between the amnion into the amniotic cavity, therefore creating a gradient for water recruitment (Bacchi Modena A and Fieni S, 2004). The exchange of fluid through the skin that occurs until keratinization is also an important contributor to the osmolarity of the amniotic fluid. After keratinization, urination, swallowing and secretion due to breathing events also contributes to the composition of the amniotic fluid. Urine start to be part of the composition of the amniotic fluid at about eight weeks and its amount will increase during gestation, reaching a flow rate of up to 900 ml/day at the end of gestation (Lotgering FK et al., 1986). Similarly, at approximately eight weeks, the fetus begins swallowing and secreting material including lung fluid and urine. Secretion of lung fluid is due to an active transport of chloride through the epithelium of the lung (Adamson TM et al., 1973). Sampling of amniotic fluid at later stages of the pregnancy is used to monitor lung development via the presence or absence of surfactant lipids and proteins secreted into the amniotic fluid.

The cells present in the amniotic fluid have both embryonic and extraembryonic origins. Approximately forty years ago, researchers attempted to characterize these cells by cytological and biochemical parameters (Morris HHB et al., 1974). Early characterization distinguished four epithelial cell types in the amniotic fluid: large eosinophilic cells, large cyanophilic cells, small round cyanophilic cells, and polygonal eosinophilic cells (Huisjes HJ, 1970). Today we know that most of the cells of the amniotic fluid are derived from the skin, digestive, urinary (Fig. 3) and pulmonary tracts of the fetus and from the surrounding amnion. We also know that the proportion and type of cells changes continuously during the different gestational stages. Some cells may also be derived from the mother, passing through the placenta into the fluid itself. The size of the cells contained in the fluid can

range from 6um to 50um and the shape can vary notably from round to squamous in morphology (Siddiqui and Atala, 2004).

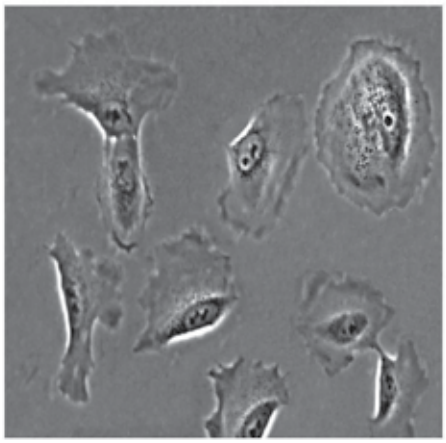

Fig. 3. Kidney amniotic fluid cells

Amniotic fluid contains cell populations derived from several different tissues. Pictured above is a population of cells isolated using kidney specific markers (40X magnification).

4. Amniotic fluid-derived stem cells

AFDSCs belong to the group of stem cells present in extra embryonic tissues; all sharing the feature of belonging to material that is discarded after birth or that can be collected during amniocentesis. Besides the amniotic fluid, the amnion, umbilical cord and placenta have shown to contain stem cells that can be isolated at birth (Bailo M et al., 2004; Banas RA et al, 2008; Brunstein CG et al., 2006; Fukuchi Y et al., 2004).

The first studies of AFDSCs, were completed using mesenchymal amniocytes isolated from sheep. These cells showed the ability to expand in vitro and to integrate into a scaffold (Kaviani A et al., 2001). In the following years, the identification of cells expressing the marker Oct4 (Prusa et al., 2003), or co-expressing Oct4, CD44 and CD105 (Tsai et al., 2004) were discovered in amniotic fluid. More recently a clonal population of AFDSCs derived from human and mouse were isolated and characterized (De Coppi P et al., 2007). These cells named AFSCs, were isolated through positive selection for the marker CD117 (or c-Kit), and represented 1% of cells derived from amniocentesis. AFSCs express the marker of "stemness", Oct4, and the embryonic stem cell (ESC) marker SSEA-4. Furthermore AFSCs express markers characteristic of mesenchymal and neural stem cells such as CD29, CD44, CD73, CD90, and CD105. Interestingly, these cells are negative for markers of hematopoietic stem cells such as CD34 and CD133.

Recently, a screen for the expression profile of cells present in the amniotic fluid was reported (Da Sacco S et al., 2010). This screening analyzed cells obtained from human amniotic fluid between gestational weeks 15 to 20 and showed that markers such as Oct4 and CD117 are stably expressed during gestation. Furthermore, while markers for ectoderm are stably expressed during gestation, markers for the early endoderm and mesoderm are more abundant during early gestation and tend to disappear after 17 to 18 weeks. During

the same time, organ specific markers start to become highly expressed. A full proteome analysis (Tsangaris G et al., 2005) using bi-dimensional gel electrophoresis and mass spectrometry, has allowed the identification of specific proteins expressed in the cells present in the amniotic fluid. This analysis has confirmed that amniotic fluid contains a heterogeneous population of cells, both differentiated and with characteristics of stem cells. In the following paragraphs a detailed description of the approaches used to differentiate AFDSCs into various lineages is presented.

When considering the use of amniotic fluid stem cells for regenerative medicine and various therapeutic interventions, clinicians and researchers agree that the ease of amniotic fluid stem cell isolation and culture make them attractive candidates for further research and development. As mentioned previously, amniotic fluid stem cells are isolated from samples of amniotic fluid collected during routine amniocentesis. This routine procedure (Fig. 4.) occurs during weeks 16-20 of a pregnancy, where approximately 10-20 milliliters of amniotic fluid is collected and split into two samples (Trounson, 2007). One sample serves as the test sample to screen for genetic and gestational abnormalities, while the other sample serves as a back up. When the back-up sample is no longer needed, some diagnostic laboratories donate this "medical waste" to research laboratories for stem cell isolation and further research. Throughout this entire process, neither the mother, nor the fetus is harmed, making the collection of these cells ethically neutral.

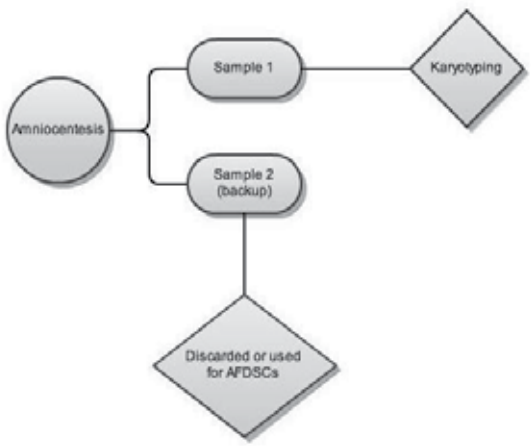

Fig. 4. Diagram for Amniotic Fluid-Derived Stem Cells isolation

The use of AFDSCs for the treatment of congenital anomalies has great potential, but in most cases is still far from clinical applications. Nerveless there is at least one case in which cells derived from amniotic fluid have been successfully used for tissue engineering. Mesenchymal cells isolated from amniotic fluid have been expanded in vitro using a chondrogenic medium and than seeded into a biodegradable scaffold and maintained in a rotating bioreactor (Kunisaki SM et al., 2006). The cells used in this report were not specifically analyzed for pluripotency or selected for specific markers, and were considered progenitor cells by the authors. Being a mixed population of cells they likely contained both committed lineages and AFDSCs, but most importantly they were able to differentiate into cartilage in vitro into a three-dimensional scaffold and maintain these characteristics for as long as fifteen weeks.

5. Amniotic fluid stem cells

Within amniotic fluid are a menagerie of cells previously described as AFDSCs, however approximately 1% of the cells contained within the fluid have been identified and designated as amniotic fluid stem cells (AFSC). AFSCs represent the most characterized clonal population of pluripotent stem cells isolated from amniotic fluid. AFSCs can be isolated by immunoselection with magnetic microsphere or FACS for the receptor for stem cell factor (c-Kit or CD117). The clonal origin of these cells was tested by integration of a single provirus (CMMP-eGFP) and analysis of subclones. Analysis of the subclones grown at limiting dilution maintained the signature integration at the same position (a 4 kilobase BamH1 fragment). The sublones were able to differentiate into lineages representative of the three embryonic germ layers. After isolation AFSCs will grow slowly for about one week (this phenomenon differs in AFSCs isolated at different gestational stages), and will then start to proliferate faster following this initial 'lag-phase' (Siddiqui MM and Atala A, 2004). AFSCs grow in absence of feeder layer when plated on Petri dishes and have a doubling time of about 36 hours (De Coppi et al, 2007). The isolated population can then be cultured quite readily on plastic or glass. If maintained at a sub-confluent state, AFSCs do not differentiate. Clones should be cultured in medium containing α-minimal essential medium supplemented with 20% Chang-B and 2% Chang-C solutions, 20% fetal bovine serum (FBS), 1% L-glutamine, and 1% antibiotics. Clones should be periodically monitored for the presence of a correct karyotype, and for the expression of specific markers such as Oct4, SSEA4, CD29, CD44, and the absence of markers such as CD45, CD34, and CD133 (see De Coppi et al., 2007 for a complete list of specific markers). AFSCs are pluripotent and can be differentiate in vitro into several lineages (De Coppi et al., 2007; Siddiqui MM and Atala A, 2004). Numerous groups have reported the high renewal capacity of these cells without differentiation or loss of telomere length (Da Sacco et al., 2010).

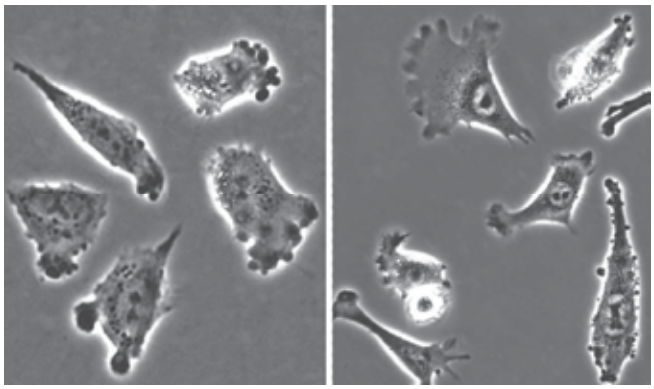

Fig. 5. Amniotic Fluid Stem Cells

Human amniotic fluid stem cells (left) and mouse amniotic fluid stem cells (right) that were isolated via selection for the surface marker CD117. Both cells have similar phenotypes (40X magnification).

5.1 Differentiation of amniotic fluid stem cells

C-kit positive amniotic fluid stem cells are pluripotent and have been successfully differentiated into all three germ layer cell types: endoderm, ectoderm and mesoderm.

From these pluripotent cells, various phenotypes have been derived in vitro. Osteogenic, endotheilial, hepatic, neurogenic, adipogenic and myogenic progenitor cell lines are a few of the lineages derived to date. Derivation of these lines has been verified by morphogenesis, phenotypic analysis and a litany of biochemical assays for characteristic of each cell type. Culture and manipulation of these cells into various progenitors has become so streamlined, that various standard protocols have been established (Delo et al., 2006). Although a significant milestone, differentiation of AFSC into various lineages in vitro is quite distinct from the in vivo potential, use and efficacy of these cells. Transplantation of these cells into a living system, or the use of these cells to create a functional organ hinge on the ability of these cells not simply to survive in vivo, however; success is dependant on the physiological functionality of these cells to perform within the anatomy. The future of regenerative medicine and cellular therapy hinges on this principle, and not surprisingly, AFSC have also shown remarkable capabilities in vivo in numerous organs.

5.1.1 Hematopoietic system

AFSC expressing CD117+ and Lin-, derived from both human and mouse, have been shown to have hematopoietic potential (Ditadi A et al., 2010). These cells were capable of differentiating into erythroid, myeloid, and lymphoid lineages in vitro as well as in vivo, in the peripheral blood of irradiated mice. Furthermore, single cells analysis was able to assess the expression of several genes important during different stages of hematopoietic differentiation.

5.1.2 Brain

A fully mature neural differentiation remains to be tested for cells derived from amniotic fluid. Neural differentiation was fist reported during the initial identification of AFSCs (De Coppi et al., 2007). Subsequently, a study for the differentiation of AFSCs into dopamine neurons (Donaldson AE et al., 2009), showed that AFSCs express specific markers of neural progenitors and immature dopamine neurons, but were unable to fully differentiate in vitro or in vivo. Analyzing other cell lines isolated from amniotic fluid (McLaughlin D et al., 2006) it was shown that phenotypic characteristics of dopaminergic neurons are present, while markers for other neurons, like cholinergic, GABAergic, and adrenergic were absent or had a weak expression.

5.1.3 Bone

AFSC cultured with an osteogenic medium, can secrete alkaline phosphatase and produce mineralized calcium, characteristic of functional osteoblasts. Furthermore, when implanted into an immunodeficient mouse, AFSC where able to produce mineralized tissue in vivo (De Coppi et al., 2007). A comparison between AFDSCs and bone morrow-derived stem cells (MSCs), has shown that while MSCs undergo a faster differentiation, AFDSCs can maintain and increase the mineralization for a longer period (Peister A et al., 2011).

5.1.4 Kidney

AFSC therapy in the kidney is progressing quickly and is arguably at the forefront of AFSC research. Research groups using AFSC in kidney have not only been able to demonstrate the ability of AFSC to populate the kidney and form renal structures, but also to protect the kidney during injury and aid in the regeneration of renal tissue. The groundbreaking

studies, which follow, paved the way for much of the other organ specific experimentation, in particular, that of the lung.

In the embryonic kidney, AFSC have been shown to differentiate into tubular and glomerular structures and express characteristic kidney cell markers and genes (Perin et al., 2007). In this study, metanephric kidneys were isolated from embryonic mice, microinjected with approximately 1000 CM-dil labeled c-kit positive AFSC and placed on a membrane for cultivation in an incubator. What is remarkable is that even though the embryonic kidney was not fully formed at the beginning of the experiment, labeled AFSC were seen to integrate into developing C and S-shaped structures at day 5, and at day 6, integrated into tubular and glomerular structures. Reverse transcriptase-PCR for human kidney specific genes, not previously expressed by the AFSC, identified expression of ZO-1, claudin and glial-derived neurotrophic factor were detected. This experiment showed the ability of AFSC to survive within developing tissue, engraft into that tissue, differentiate into the appropriate cell type and aid in the population of an organ.

Furthermore, it has recently been discovered that AFSC injected into the acutely injured kidney stimulate the release of anti-inflammatory cytokines and attenuate pro-inflammatory signaling greatly reducing apoptosis and allowing for proliferation and repopulation of injured epithelia (Perin L. et al., 2011). In this study, nude mice, deprived of water for a period of 22 hours, were given an intramuscular injection of a 50% hypertonic glycerol solution in water. This type of injury induces rhabdomyolysis-related acute tubular necrosis (ATN) ultimately resulting in renal dysfunction. Following intrarenal injection of $1.2x10^6$ cells, AFSC were observed, via luciferase, to persist at the site of injury most notably at 48 and 72 hours, with persistence in the kidneys for up to 6 days. Additionally, analysis of the cytokine milieu showed the markedly different expression patterns of cytokines at 14 days post transplant. Mice with ATN only, and no AFSC transplant, showed a general trend of increased pro-inflammatory cytokines and decreased anti-inflammatory cytokine expression. On the other hand, mice with ATN and intra-renal injection of AFSC demonstrated that the anti inflammatory cytokines increased over the 14 day study period, while pro-inflammatory cytokines decreased. In another study after glycerol-induced acute kidney injury (Hauser PV et al., 2010) a comparison between mesenchymal stem cells (MSCs) and AFSCs has shown that while MSCs where mainly inducing proliferation, AFSCs had an antiapoptotic effect. Thus, these data suggests that AFSCs responds in a paracrine manner in response to injury and/or stress, and modulation of immune signaling is what contributes to the alleviation of symptoms associated with the injury.

5.1.5 Lung

In utero, the developing lungs of the fetus are filled with fetal lung liquid which is actively secreted into the amniotic fluid. In the late gestational period, surfactant produced by the fetal lungs contributes to the composition of amniotic fluid and can be measured to determine the developmental stage of the surfactant system within the fetal lungs. Thus, it makes sense that when looking for regenerative therapies for lung tissue, AFSC are a logical source.

In our preliminary transplantation studies, it was found that c-kit positive AFSC can incorporate into mouse embryonic lung and express human lung epithelial cell markers (Carraro, et al., 2008). In the same study, following naphthalene injury in nude mice, and intravenous transplantation of $1x10^6$ AFSC, cells were observed to preferentially remain at the site of injury when compared to uninjured controls when visualized via luciferase assay.

Additionally, following oxygen injury in the lung it was observed that AFSC appear to exhibit alveolar epithelial type II phenotypes, widely surmised to be a lung epithelial stem cell, suggesting that once in the lung these cells are stimulated to differentiate in response to injury. Furthermore, in vivo, the efficiency of AFSC diapedesis, integration and expression in upper and lower airway epithelia is increased following injury. After oxygen injury, AFSC were observed to be taken into the SP-C positive alveolar epithelial lineage, whereas after naphthalene injury AFSC are taken up into the CC10-positive Clara cell lineage. AFSC presence persisted in the lung after injury, but decreased over time. Although integration into the adult lung following injury is a relatively rare event, additional therapeutic mechanisms displayed by these cells, such as the modulation of the inflammatory milieu and their differentiation into type II lineages demonstrate great potential in the stimulation of lung repair mechanisms.

Lung researchers have also begun investigating the potential of seeding AFSC on a scaffold to regenerate tissue for transplantation. Due to the overwhelming shortage of donor lungs, and the inability of modern medicine to effectively treat or halt many progressive lung diseases such as idiopathic pulmonary fibrosis, research focus has shifted to the bioengineering of functional lung tissue. Decellularization of lungs, where all cells are removed from the extracellular matrix of an organ, has become an investigational target. In 2010 a whole lung decellularization method and tissue engineering study using neonatal lung epithelia was reported (Peterson et al., 2010). What is remarkable about this study is that while it has long been known that epithelial cells seeded on a decellularized lung matrix were capable of forming alveolar epithelia, this study demonstrated the functionality of the regenerated tissue. The decellularized, repopulated and regenerated lungs were transplanted into a rat and were able to support short-term perfusion and gas exchange. In another study, researchers were able to seed not only epithelium, but also endothelium as well on a decellularized rat lung. Following transplantation, blood gas analysis of the engineered lung demonstrated that the lung was capable of gas exchange (Ott et al., 2010). Thus decellularized lung matrix seems currently to be the most promising scaffold for whole lung regeneration and the possibility of using an autologous source of stem cells such as AFSC to repopulate the scaffold could have great potential in the future.

5.1.6 Heart

The use of AFSC as a regenerative therapy for cardiac disease and congenital disorders has shown the efficacy of transplanted cells providing both cardio protective potential, as well as the engineering of various cardiac components such as valves and tissue (Bollini et al., 2011; Schmidt et al., 2007; & Hilkfer et al., 2011).

The engineering of heart valves, obtained from normal human amniotic fluid samples, sorted via positive selection for the CD133 molecule, was elegantly demonstrated in 2007 (Schmidt et al., 2007). Both CD 133 positive and negative cell populations were cultured in media that caused differentiation towards endothelial phenotypes. CD133+ cell populations showed the ability to produce functional endothelial cells indicated by the expression of eNOS and CD141, while CD133- cells displayed a more mesenchymal phenotype. CD133- cells were then seeded on biodegradable PGA leaflets that were positioned within a mold to form a valve structure. After 14 days, CD133+ cells were seeded onto the scaffold as well. While regeneration of both extracellular matrix and endothelial layers were generated, functional testing revealed that the heart valves were sufficiently functional only under low-pressure conditions. This failure to perform at

physiological levels was not due to the scaffold material, which displayed linear properties prior to being seeded with cells, but instead was a result of the incomplete formation of collagen suggesting that the method of seeding and culture upon the biodegradable scaffolding needs to be optimized further to be able to transplant these engineered valves into patients. In an acute myocardial infarction model, ischemia, produced via ligation of the left anterior descending coronary artery, was followed with intravenous transplantation of AFSC and reperfusion of the heart for 2 hours. Animals treated with $5x10^6$ cells intravenously, showed a significant decrease in infarct size and number of apoptotic cardiomyocityes when compared to control animals administered saline alone (Bollini et al., 2011). Staining to determine the localization and viability of transplanted AFSC showed that two hours post transplant, cells localized to the lung, spleen and heart. AFSC within the heart co-stained for epithelia vWf and α-SMA, suggestive of the potential of these cells to commit to endothelium and smooth muscle following transplant. Long term retention and engraftment in the injured myocardial tissue did not occur however. The secretion of thymosin beta 4 in vitro, a cardio protective factor, suggests that the transplantation of AFSC in this model exert a paracrine effect.

5.2 Why use AFSC in regenerative medicine?

When selecting a stem cell population for use in a regenerative or therapeutic capacity, there are a myriad of factors that need to be considered. The pluripotentiality, the ability of the cells to differentiate into different germ layers and tissue types, is of fundamental importance if one is isolating cells to treat diseases or developmental deficiencies in which progenitor cells within the patient are compromised or overwhelmed. Additionally, the plasticity of the cells and their ability to differentiate to repopulate different populations within an organ, and repopulate them correctly is crucial. Furthermore, the behavior of the cells after injection must be carefully studied and characterized. Tumorogenicity, immunogenicity and the propensity to form teratomas and further exacerbate a disease state can rule out various cellular therapies simply due to risk. To date, amniotic fluid stem cells have demonstrated the ability to meet all of these criteria and behave remarkably well in a regenerative and therapeutic capacity. Amazingly pluripotent, less immunogenic, and not prone to teratoma formation, AFSCs have quickly risen near the top of the list of stem cell therapies to continue developing.

Furthermore, recently induced pluripotent stem (iPS) cells have been prepared from cells derived from amniotic fluid (AF-iPS), and have shown high efficiency of transformation and colony formation after just six days (Li C et al., 2009). Although not fully understood, this is probably due to the presence of an epigenetic status closer to the embryonic state (Galende E et al., 2010). Reprogramming of somatic cells using the four specific factors, Oct4, Sox2, Klf4, and c-Myc has the potential to provide pluripotent stem cells specific for patients, thus AF-iPS seem to be more easily reprogrammed to pluripotency compared to adult cells or cells from neonates.

6. Conclusion

The studies outlined in this chapter demonstrating the capability that AFSC have shown in vitro and in vivo show that AFSC are viable targets for regenerative medicine and for future therapeutic treatment strategies. Although the relatively early stage of AFSC research limits

a full understanding of the behaviors, properties and characteristics of these fascinating cells, research to date demonstrates two important mechanisms of action that need to be investigated further.

First, AFSC have the potential to serve as an in vivo treatment to stimulate endogenous cell populations, repopulate injured tissue or ameliorate inflammatory or disease states. These properties are advantageous when dealing with disease or injury states in which there is enough functional tissue remaining in an organ to drive repopulation. The only caveat to endogenous cellular stimulation is that the remaining tissue (that is being stimulated) must be functional, meaning that it is free of genetic disorders or mutations. If remaining tissue within an organ meets these standards, exogenous AFSC transplantation can be used to stimulate endogenous progenitors to repopulate, protect progenitor or other cell types from further injury, or AFSC may be driven to differentiate to repopulate this tissue, as was indicated in the aforementioned embryonic studies.

Second, AFSC have the potential to engineer whole organs in vitro to be transplanted into a recipient. This strategy is advantageous in situations where, for whatever reason, enough functional tissue does not remain to repopulate with a cell transplant. Whole organ re-engineering, perhaps the holy grail of regenerative medicine, involves a symphony of factors, events and coordinated expression patterns to form intricate niche structures including endothelium, epithelium, extracellular matrix and so on. The engineering of a whole organ will in fact require a much deeper understanding of these cells as signaling cascades and response elements need to be coordinated to engineer every cell type within a specific organ. However the recent findings of Kajtsura et al (2011) support the our notiion that the genome within a single stem cell type may prove to be sufficiently plastic to simultaneously derive all of the cell lineages required for complex organ repair or engineering.

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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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