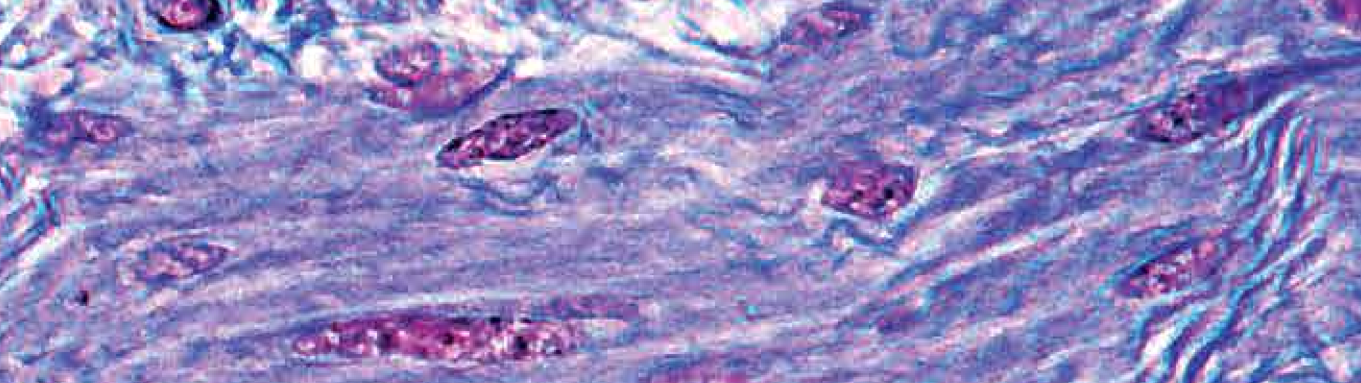

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Muscle Cell and Tissue

Current Status of Research Field

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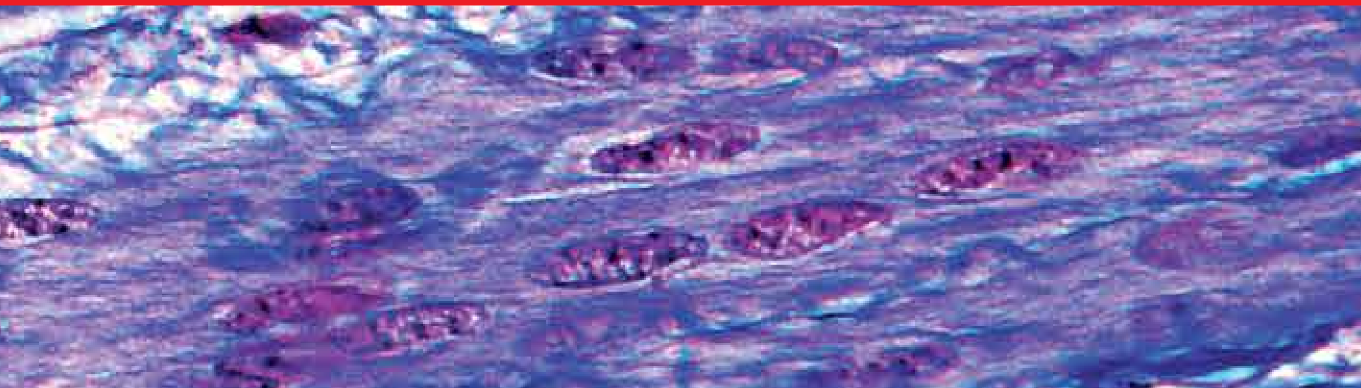

MUSCLE CELL AND TISSUE - CURRENT STATUS OF RESEARCH FIELD

Edited by **Kunihiro Sakuma**

Muscle Cell and Tissue - Current Status of Research Field
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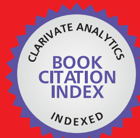

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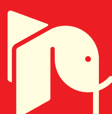

Meet the editor

Professor Kunihiro Sakuma, PhD, currently works at the Institute for Liberal Arts, Tokyo Institute of Technology. He is a physiologist working in the field of skeletal muscle. He received his sports science diploma in 1995 from the University of Tsukuba and started scientific work at the Department of Physiology, Aichi Human Service Center, focusing on the molecular mechanism of congenital muscular dystrophy and normal muscle regeneration. His interest was later changed to the molecular mechanism and attenuating strategy of sarcopenia (age-related muscle atrophy). His aim is to attenuate sarcopenia by improving autophagic defects using nutrient- and pharmaceutical-based treatments.

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Preface

Skeletal muscle tissue accounts for almost half of the human body mass. Muscle contractions of the skeletal muscle enable the body to move and maintain homeostasis. Human health is markedly affected by any deterioration in the material, metabolic and contractile properties of skeletal muscle. Muscle wasting and weakness such as cachexia, atrophy and sarcopenia are characterized by marked decreases in the protein content, myonuclear number, muscle fiber size and muscle strength. To attenuate various forms of muscle wasting, many researchers have investigated exercise-based, supplemental and pharmacological approaches. Our circulatory system is modulated by the heart, lungs and vasculature. These components serve crucial roles in controlling blood and lymph flow and in the delivery of gases, hormones and essential nutrients (i.e. glucose, fat, or amino acids). Vascular smooth muscle cells (VSMCs) are the most numerous cell types in blood vessels, where they are located in the medial layer of the vascular wall. VSMCs serve critical regulatory roles in the blood vessels, particularly for vasoconstriction, vasodilatation and synthesis of vascular extracellular matrix. Vascular remodeling is adaptive alternating process of vascular wall architecture and is caused by various stimuli such as vascular injury, oxidative stress and hemodynamic stress. VSMCs and endothelial cells (ECs) compose the arteries and have essential roles in vascular remodeling in conjunction with inflammatory cells. During vascular remodeling, the infiltration of macrophages and monocytes, synthetic or contractile phenotypic changes of VSMCs and the EC dysfunction promote vascular diseases such as atherosclerosis.

In order to complete tissue regeneration, various cells (neuronal, skeletal, and smooth) interact coordinately with each other. This book, *Muscle Cell and Tissue - Current Status of Research Field*, deals with current progress and perspectives in a variety of topics on the skeletal and smooth muscle, stem cells, regeneration, disease or therapeutics. Novel applications for cell and tissue engineering including cell therapy, tissue models and disease pathology modeling are introduced. This book also deals with the differentiation/de-differentiation process of vascular smooth muscle cells in health and disease. Furthermore, natural products to reverse metabolic syndromes are descriptively reviewed. These chapters can be interesting for graduate students, teachers, physicians, executives and researchers in the field of molecular biology and regenerative medicine.

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Section 1

Introduction

Chapter 1

Introductory Chapter: Current Status of Research Field in Muscle Tissue

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Additional information is available at the end of the chapter

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1. Introduction

Skeletal muscle tissue accounts for almost half of the human body mass. Muscle contractions of the skeletal muscle enable to move body and maintain homeostasis. Human health is markedly affected by any deterioration in the material, metabolic, and contractile properties of skeletal muscle. Skeletal muscle is a highly plastic organ that is modulated by various pathways controlling cell and protein turnover. Nowadays, the autophagy-dependent system and ubiquitin-proteasome signaling are well known as a major intracellular degradation system, and its appropriate function is crucial to health and muscle homeostasis. Indeed, muscle wasting and weakness such as cachexia, atrophy, and sarcopenia are characterized by marked decreases in the protein content, myonuclear number, muscle fiber size, and muscle strength [1]. Muscle wasting elicits a poor functional status and reduces the quality of life. Thirty-five percent of all cancer patients directly die because of cachexia and not from cancer. Different types of molecular triggers/catabolic factors such as pro-inflammatory cytokines and myostatin also seem to involve muscle wasting [2]. In contrast, mTOR- or serum response factor (SRF)-dependent signaling are positive regulators to promote protein synthesis and skeletal muscle-specific mRNA transcription. Interestingly, a functional defect in autophagy-dependent signaling in sarcopenic mice and humans are recently suggested [3, 4]. Such a condition accumulates the denaturing protein and nonfunctional mitochondria eventually result in the atrophy of sarcopenic muscle fibers because of the deterioration of homeostasis.

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2. Various therapeutic approaches for muscle wasting

To attenuate various forms of muscle wasting, many researchers have investigated exercise-based, supplemental, and pharmacological approaches. For example, the combination of resistance training and amino acid-containing supplements is thought to effectively prevent sarcopenia. In addition, myostatin inhibition for sarcopenic patients was successful in phase II trials, but the effect on muscular dystrophy is unclear. The administrations of ghrelin and megestrol acetate have shown good results against cancer cachexia [5]. Furthermore, recent studies [6, 7] indicated the possible application of novel supplements such as soy isoflavone and ursolic acid to prevent muscle atrophy in rodents. More recently, pharmacological treatment with fibroblast growth factor 19 markedly ameliorated two different type of muscle atrophy after aging and glucocorticoid treatment, probably via an obligate co-receptor for fibroblast growth factor 15/19, β-Klotho.

3. The function of smooth muscle cells

Our circulatory system is modulated of the heart, lungs, and vasculature. These components serve crucial roles in controlling blood and lymph flow and in the delivery of gases, hormone, and essential nutrients (i.e., glucose, fat, or amino acids). Vascular smooth muscle cells (VSMCs) are the most numerous cell types in blood vessels. They are located in the medial layer of the vascular wall, i.e., in the tunica media. The media also contains sparse fibroblasts and macrophages along with an interstitial matrix consisting collagens; chondroitin sulfate proteoglycans including versican; glycoproteins such as tenascin, vitronectin, and fibronectin; and elastic laminae. VSMCs serve critical regulatory roles of blood vessels, particularly for vasoconstriction, vasodilatation, and synthesis of vascular extracellular matrix. Adult blood vessels are normally contractile, static, and quiescent. However, under cardiovascular disease including atherosclerosis, hypertension, and diabetic angiopathy, VSMCs undergo phenotypic alterations and revert to a growth-promoting, synthetic nature. Indeed, after biochemical or mechanical damage to blood vessels, VSMCs undergo phenotypic modulation, characterized by increased proteosynthesis and by activation of the migration and growth of VSMCs [8, 9]. These changes often lead to severe damage to blood vessels, including stenosis and occlusion. Ischemia of the tissues supplied by the damaged vessels is then manifested by serious disorders, e.g., heart failure, brain stroke, or necrosis of leg tissues, which can result in amputation of the leg.

Vascular remodeling is an adaptive alternating process of vascular wall architecture and is caused by various stimuli such as vascular injury, oxidative stress, and hemodynamic stress [10]. VSMCs and endothelial cells compose the arteries and have essential roles in vascular remodeling in conjunction with inflammatory cells (macrophages, monocytes, leucocytes, and lymphocytes) [11]. During vascular remodeling, the infiltration of macrophages and monocytes, synthetic or contractile phenotypic changes of VSMCs, and the EC dysfunction promote vascular diseases such as atherosclerosis. Therefore, modulation of VSMC phenotype, maintenance of ECs, and regulation of inflammation in the vessel wall are important in arterial function and homeostasis.

This book deals with current progress and perspectives in a variety topic of skeletal and smooth muscle, stem cells, growth, regeneration, disease, biomaterials, or therapeutics. Novel applications for cell and tissue engineering including cell therapy, tissue models, and disease pathology modeling are welcomed. The molecular mechanism of hypertrophy and atrophy in muscle cell would be also discussed by linking with the signal pathway of protein synthesis and degradation.

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Section 2

Plasticity of the Skeletal Muscle

Chapter 2

The Role of Glucose and Fatty Acid Metabolism in the Development of Insulin Resistance in Skeletal Muscle

Sithandiwe Eunice Mazibuko-Mbeje,
Phiwayinkosi V. Dludla, Bongani B. Nkambule,
Nnini Obonye and Johan Louw

Additional information is available at the end of the chapter

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Abstract

The rapid rise in the prevalence of obesity and diabetes has significantly contributed to the increasing global burden of noncommunicable diseases. Insulin resistance is a major underpinning etiology of both obesity and type 2 diabetes. Insulin resistance is characterized by a reduced response of skeletal, liver, and fat tissues to the actions of insulin hormone. Although detailed mechanisms implicated in the development of insulin resistance remain plausible, skeletal muscles have been identified to play an integral role in the improvement of insulin sensitivity in the diseased state. The effective modulation of glucose and fatty acid metabolism in the skeletal muscle through exercise or by certain therapeutics has been associated with reversal of insulin resistance and amelioration of diabetes associated complications such as inflammation and oxidative stress. This chapter will briefly discuss the role of glucose and fatty acid metabolism in the development of insulin resistance in the skeletal muscle.

Keywords: skeletal muscle, insulin resistance, glucose and fatty acid metabolism

1. Introduction

According to the World Health Organization, type 2 diabetes mellitus (T2D) contributes to approximately 90% of all diabetes mellitus cases and is amongst the top 10 leading causes of death worldwide [1]. Symptoms such as enhanced thirst, polyuria, fatigue, and impaired wound healing are identified in those with T2D. The recent International Diabetes Federation (IDF) report projects an astonishing increase in cases of diabetes [2]. An estimated 425 million

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people are currently living with diabetes, with a projected 1.5 fold increase in the prevalence of diabetes, while a total of 629 million adults are expected to be diabetic by the year 2045 [2]. In Africa, the prevalence of diabetes is estimated at 16 million and is expected to rise to 41 million by 2045 [2]. Diabetes is characterized by hyperglycemia resulting from an inadequate production of insulin and insulin utilization in a T2D state. An unhealthy lifestyle such as lack of physical activity and a diet containing excessive fat content, including refined carbohydrates has been associated with an increased risk for developing insulin resistance (IR) [2, 3]. Carbohydrate-rich diets with a high glycemic index may contribute to obesity, impaired glucose tolerance, and hyperinsulinemia. This consequence can impair glucose and lipid metabolism, and accelerate the progression of IR. During a state of IR, insulin levels are elevated due to the rising glucose levels, but over time, a state of relative inadequate production of insulin can develop [2]. IR is regarded as one of the early phenotypes associated with development of obesity, and it is normally present in high-risk individual's years before development of T2D. Although maintenance of healthy lifestyle, as well as the use of hypolipidemic and hypoglycemic drugs, remains effective at attenuating insulin-resistant complications, the escalating incidence of the metabolic syndrome (MetS) warrants further exploration into pathological mechanisms implicated in the development of IR. Accumulative evidence suggests that effective modulation of energy substrates such as glucose and free fatty acids (FFAs) remains crucial in the amelioration of lifestyle diseases, including T2D [4–6]. This chapter will discuss the role of glucose and lipid metabolism in the development of IR.

2. Skeletal muscle and its role in modulating insulin resistance

Skeletal muscles are comprised of an intricate tissue, with diverse network of fibers, which have different mechanical and metabolic functions. Skeletal muscles contribute to approximately 40% of the total body weight and contain 50–75% of all body proteins [7]. Skeletal muscles account for more than 80% of insulin-stimulated glucose uptake [8], and using combined oral and intravenous glucose tolerance testing, Himsworth and Kerr were able to demonstrate that tissue-specific insulin sensitivity was lower in T2D individuals [9]. Therefore, IR in skeletal muscle has a major impact on whole-body metabolic homeostasis and it is the main element for the development of T2D. However, the underlying molecular mechanisms remain elusive. Several mechanisms that play a role in the development of IR in skeletal muscle have been proposed, and these include accumulation of intracellular lipid derivatives (diacylglycerol and ceramides) as a result of elevated plasma FFAs, oxidative stress, pro-inflammatory signals, and impaired gene transcription [8, 10]. Moreover, mitochondrial dysfunction has associated with IR [11]. The following section will focus on glucose regulation and fatty acid metabolism, in relation to the development of IR in skeletal muscle.

3. Glucose metabolism in skeletal muscle

Glucose is a monosaccharide used as a biological fuel during aerobic and anaerobic respiration or fermentation. Aerobic respiration is the most efficient means of glucose utilization, yielding

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32 molecules of ATP during the processes of glycolysis and oxidative phosphorylation. Glycolysis is the metabolic whereby glucose is metabolized into pyruvate or to lactate; this process yields higher capacity for ATP generation [12]. During glycolysis, glucose 6-phosphate is converted to fructose-6-phosphate by phosphohexose, and then to fructose-1,6-biphosphate by phosphofructokinase. This reaction is irreversible and is a major point of regulation during glycolysis. Energy utilization by adult skeletal muscle is tightly controlled, with muscle fibers having the ability to switch between different substrates for ATP production. This is highly dependent on the availability of energy substrates and the energy requirements [13, 14]. Skeletal muscles are able to utilize both glucose and FFAs as a source of ATP production. However, utilization of glucose and FFAs as a primary source of ATP production depends on the metabolic state of an individual, i.e., whether the individual is at a fed or fasting state [15]. During the fasting state, glucose uptake in skeletal muscle is reduced while plasma FFA levels are increased due to lipolysis in adipose tissue. This subsequently leads to the utilization of FFAs as the predominant source of ATP production [16]; whereas, during a fed state, plasma glucose levels are elevated, which stimulates insulin secretion and enhances glucose uptake by skeletal muscle. This also leads to reduced lipolysis in adipose tissue and a reduction in plasma FFAs. The ability of switching between substrates in the fasted and fed state is crucial in promoting skeletal muscle glucose oxidation. Consequently, it has been reported that muscle of insulin-resistant or diabetic subjects fails to switch between the substrates, showing metabolic inflexibility [8]. This metabolic inflexibility can result in impaired glucose and fatty acid metabolism, leading to the development of IR.

Several mechanisms, which include glucose transportation, are implicated in the regulation of skeletal muscle glucose metabolism and have been a therapeutic target for the reversal IR and improvement of skeletal muscle function. Briefly, postprandial glucose is transported actively across the plasma membrane by specific carrier proteins, which belong to the glucose transporter (GLUT) family. There are several types of glucose transporters located in the plasma membrane of myocytes. Each glucose transporter isoform plays a specific role in glucose transportation that is determined by its tissue distribution, substrate specificity, and transport kinetics [17]. Glucose transporter isoform 1 (GLUT1) is present in all cells and is largely responsible for regulating basal glucose and ensuring a steady influx of glucose into cells. The glucose transporter isoform 2 (GLUT2) is a high-Km glucose transporter expressed in hepatocytes, pancreatic beta cells, and the basolateral membranes of intestinal and renal epithelial cells. In contrast to other transporters, GLUT2 facilitates bidirectional glucose transport into and out of the cell [17]. GLUT3 is a low-capacity glucose transporter that is responsible for glucose uptake in neurons, while glucose transporter protein isoform 4 (GLUT4) is expressed exclusively in muscle and fat cells, and is responsible for increased glucose uptake into these tissues postprandially, thereby maintaining normoglycemia [17].

In skeletal muscle, insulin stimulation induces translocation of GLUT4 from intracellular vesicles within the cytoplasm to the plasma membrane and thereby increases glucose uptake [18, 19]. When insulin levels decrease in the blood and insulin receptors are no longer occupied, the glucose transporters are recycled back into the cytoplasm. Failure of GLUT4 to translocate to the plasma membrane results in IR [20]. The crucial step for effective modulation of GLUT4 translocation has been the binding of insulin or insulin-like growth factor 1 (IGF1) to its receptor or IGF1 receptor, leading to the activation of phosphatidylinositol-4,5-bisphosphate

3-kinase (PI3K)/protein kinase B (AKT) pathway. Activation of this pathway has been subject to ongoing research for its role in skeletal muscle tissue growth, and most importantly, in the regulation of insulin signaling [21]. This has been verified on various models showing that knockout of insulin receptor, PI3K and AKT genes, especially in skeletal muscle, is associated with growth retardation as well as with impairment of insulin action [22, 23]. Therefore, effective modulation of glucose transportation and activation of PI3K/AKT pathway remains important to improve glucose tolerance and also skeletal muscle function.

4. Fatty acid metabolism in skeletal muscle

FFAs are elongated hydrocarbon chains with a terminal carboxylate group. Apart from being one of the major sources of fuel in the body, FFAs can perform a number of other functions, including serving as building blocks for phospholipids and also acting as hormones as well as intracellular messengers [24]. FFAs can exist in unsaturated or saturated form, depending on the number of bonds the hydrocarbon chain contains. While unsaturated fats such as oleic acid, mostly available in vegetable oils, are considered beneficial to the body [25], saturated fats, namely palmitic acid, are associated with the development of IR [26]. For the latter, it is widely used in experimental models to induce IR [19, 27]. Exposure of cultured skeletal muscle cells to high palmitate concentrations has been linked with the activation of protein kinase C (PKC), one of the main enzymes involved in impaired insulin signaling [26, 28]. Briefly, by phosphorylating insulin receptor substrate 1, PKC can alter the whole downstream effect of insulin response, ultimately leading to impaired GLUT4 translocation and reduced glucose uptake in skeletal muscle. Evidence shows that PKC activation by 12-deoxyphorbol 13-phenylacetate 20-acetate is associated with a reduction in insulin-stimulated glucose uptake, whereas PKC inhibition with GF 109203X results in enhanced insulin action in cultured human skeletal muscle [29]. An abnormal reduction of glucose uptake in skeletal muscle, mainly due to an impaired switch in substrate preference, as explained by Randle [6], remains an important contributing factor to the development of IR and subsequent metabolic complications. Thus, it is a viable option to target glucose uptake improvement, concomitant to reducing glycogen stores to reverse IR in skeletal muscle.

Beta oxidation, the main catabolic process by which fats are broken down in the body, is another system crucial for the control of substrate switch within many cells, including skeletal muscle (**Figure 1**). Generally, during periods of fasting, a substrate switch occurs where FFAs become a predominant source for ATP production via beta oxidation [30]. Although FFAs are hydrophobic in nature and can passively diffuse across the lipophilic cell membrane, transporters such as plasma membrane fatty acid-binding protein (FABP), fatty acid transport protein 1 (FATP1), and cluster of differentiation 36 (CD36) are widely expressed in rodent and human skeletal muscle [31, 32]. By controlling entry of long chain fatty acids across the barrier of the inner mitochondrial membrane for subsequent beta oxidation, the carnitine shuttle system can influence skeletal muscle substrate switch. Some of the well-investigated components of the system include the malonyl-CoA sensitive carnitine palmitoyltransferase 1 (CPT1) that resides on the mitochondrial outer membrane (**Figure 1**).

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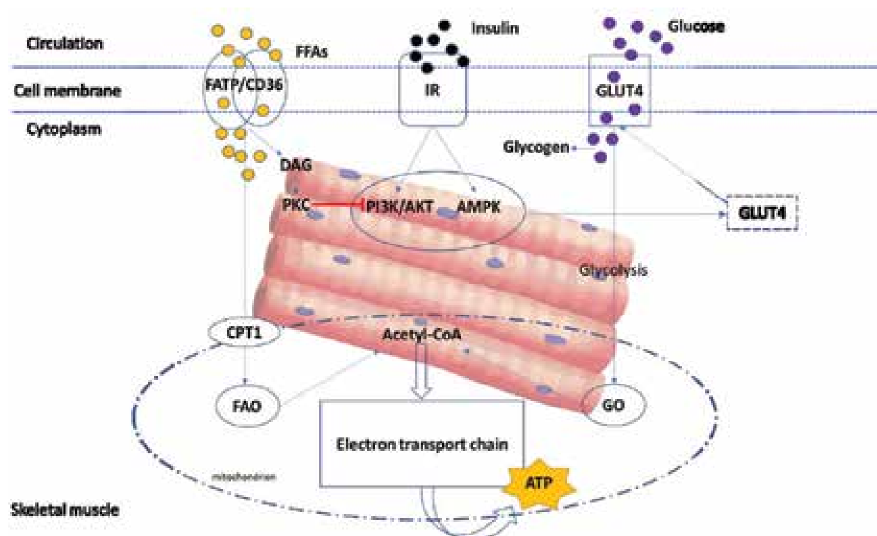

Figure 1. Glucose and free fatty acids (FFAs) are the predominant substrates that are oxidized to generate acetyl-CoA, which is then utilized by the electron transport chain to generate adenosine triphosphate (ATP). An insulin-resistant state is characterized by an impaired substrate utilization, a process termed metabolic inflexibility. Modulation of phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/AKT) and 5′ AMP-activated protein kinase (AMPK) signaling mechanisms are increasingly targeted by various pharmacological compounds to reverse insulin resistance and improve skeletal muscle function. Abbreviations: CD36: cluster of differentiation 36, CPT1: carnitine palmitoyltransferase 1, DAG: diacyl glycerol, FAO: fatty acid oxidation, FATP: fatty acid transport protein 1, GLUT4: glucose transporter isoform 4, GO: glucose oxidation, IR: insulin receptor, and PKC: protein kinase C.

Some polyphenols, including those from grape extracts, can influence muscle lipid metabolism by reducing CD36 and regulating CPT1 expression in high fat diet (HFD) fed rats, leading to upregulated GLUT4 protein expression and improved insulin signaling [33]. Similarly, metformin, a commonly used antidiabetic drug, has demonstrated increased capacity to reverse IR and improve skeletal muscle function through the modulation of CPT1 and 5′ AMP-activated protein kinase (AMPK) [34, 35]. Like PI3K/AKT, AMPK is also a target of ongoing research for its role in preventing metabolic disease through modulation of substrate metabolism in various tissues [36, 37]. Intracellular energy fluctuations, represented by changing AMP/ATP ratio, such as those identified in an IR state, remain monumental for the activation or deactivation of AMPK activity [38]. A number of natural products [39–41], including metformin, are known to activate AMPK, leading to the phosphorylation of acetyl-CoA carboxylase and to effective modulation of beta oxidation. However, the activity of AMPK is tissue specific and is tightly controlled in a T2D state, with its activation demonstrated to be important in reversing IR and improving signaling in skeletal muscle [19, 36].

5. Conclusions

Skeletal muscle forms the largest insulin-sensitive tissue in the body and remains the key site for insulin-stimulated glucose uptake. Glucose and FFAs are the prominent substrates

responsible for ATP production in the skeletal muscle. However, in an insulin-resistant state, the utilization of both glucose and FFAs is impaired, leading to abnormally enhanced intramuscular substrate storage. Modulation of the PI3K/AKT and AMPK signaling appears to be the driving mechanism responsible for the regulation of substrate metabolism, as well as associated downstream effects such as generation of oxidative stress. Interestingly, some pharmacological compounds such as metformin are known to exert their therapeutic effects through the modulation of these pathways, leading to improved control of energy substrates. Therefore, it is imperative that more research be directed at exploring signaling mechanisms implicated in the control of energy substrates, especially in the skeletal muscle, since it is known to be the major "hub" for energy metabolism.

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Conflict of interest

The authors report no conflict of interest. All authors are responsible for the content and writing of the paper.

Abbreviations

AKT	protein kinase B
AMPK	5′ AMP-activated protein kinase
ATP	adenosine triphosphate
CD36	cluster of differentiation 36
CPT1	carnitine palmitoyltransferase 1
FABP	fatty acid binding protein
FATP1	fatty acid transport protein 1
FFAs	free fatty acids

GLUT	glucose transporter
HFD	high fat diet
IDF	International Diabetes Federation
IGF-1	insulin-like growth factor 1
IR	insulin resistance
MetS	metabolic syndrome
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	protein kinase C
T2D	type 2 diabetes mellitus
UCP	uncoupling protein

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Molecular and Cellular Markers in Skeletal Muscle Damage after Acute Voluntary Exercise Containing Eccentric Muscle Contractions

Satu O.A. Koskinen and Maarit Lehti

Additional information is available at the end of the chapter

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Abstract

In eccentric muscle contraction, the muscle is lengthening while contracting. For example, in downhill walking, the thigh muscles are contracting eccentrically. It is well known that unaccustomed eccentric exercise causes pain and may lead to inflammation reactions on muscles few days after the exercise. The theme of the present chapter is molecular and cellular markers in skeletal muscle damage after voluntary exercise containing eccentric muscle contractions. The chapter contains three topics: In the first topic, the damaging process followed by regeneration is demonstrated with antibody stainings of connective tissue, plasma membrane, and cytoskeletal proteins. The second topic is infiltration of inflammatory cells in damaged skeletal muscle. Neutrophils are usually the first inflammatory cells mostly present in the injured tissues; however, neutrophils are not present in exercise-induced skeletal muscle damage. Finally, the relationship between skeletal muscle damage and systematic markers, serum creatine kinase and voluntary maximal force production, is described.

Keywords: skeletal muscle damage, eccentric exercise, infiltration of inflammatory cells, neutrophils, monocytes/macrophages, extracellular matrix

1. Introduction

In movements such as walking, running, and jumping, both eccentric (the muscle is actively lengthened/stretched) and concentric (the muscle is actively shortened) muscle contractions are present as the muscles undergo repeated stretching-shortening cycles. [1] Typical physical activities, which contain a great deal of eccentric contractions, are going down stairs, walking

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and running downhill, lowering weights, and the downward motion of squats and push-ups. It is fair to say that muscles contracting eccentrically produce "more for less." High mechanical muscle tension produced by eccentric muscle contraction is generated at lower metabolic cost and at a greatly reduced oxygen requirement compared to concentric muscle contraction. It has been reported that the oxygen requirement of submaximal eccentric cycling is only 1/6–1/7 of that for concentric cycling at the same workload [2]. Furthermore, eccentric training can increase the size and strength of muscles with very little demand on the cardiovascular system [3]. Therefore, eccentric training is potential training method for elderly and patients suffering from diseases that limit either the uptake or delivery of oxygen, e.g., chronic obstructive pulmonary disease or chronic heart failure. Any exercise that requires a significant increase in respiration and in cardiac output may not be only uncomfortable but also impossible for fragile individuals. However, it is not only fragile individuals who benefit from eccentric training: It is advantageous for anyone since less time is needed and training feels less strenuous compared to concentric type of training. As a disadvantage, unaccustomed eccentric exercise causes muscle pain and may lead to inflammation reaction on muscles for few days post-exercise. Likely, one bout of eccentric exercise induces protective effect against muscle pain and against skeletal muscle fiber injury for several weeks or even months [4].

Maximal eccentric exercise with the knee extensors or elbow flexors on an isokinetic dynamometer has frequently been used to induce and to study skeletal muscle damage in humans. A typical response to such high-force, single-joint eccentric exercise protocols is on average a 50% reduction of the force-generating capacity immediately post exercise, followed by gradual recovery over the next days or weeks [5]. There are clearly individual differences both in force-generating capacity immediately post exercise and in the length of the force recovery period [6, 7]. It is not known why the same amount of eccentric exercise induces skeletal muscle fiber damage, loss in force-generating capacity, and prolonged force recovery period for some individuals, whereas only short-term decrease in muscle force-generating capacity was observed in other subjects [6]. Prolonged recovery of muscle force is thought to be related to distortion of the myofibrillar structure and disturbed calcium homoeostasis and/or prolonged inflammatory response [5]. Consequently, it has been suggested that reduction in muscle force-generating capacity may be a valuable indicator for monitoring muscle damage following exercise [8]. Physiological adaptation takes place after a single bout of unaccustomed eccentric exercise by making muscles more resistant against structural changes from the second bout of eccentric exercise [9].

In the next sections, few examples are presented for visualizing skeletal muscle fiber injury in muscle biopsies after eccentric exercise. Heat shock protein 27 (HSP27) antibody staining shows abnormal sarcoplasmic staining pattern already during the first hour after the exercise. Plasma membrane protein (dystrophin)-negative skeletal muscle fiber indicates quite severe muscle damage, while basement membrane proteins laminin and type IV collagen are intact and are keeping the muscle fiber together. Counting the number of infiltrated inflammatory cells in a damaged skeletal muscle is often reported after different exercise protocols. However, antibodies used for counting neutrophils and monocytes/macrophages do not always exclusively recognize the studied leukocyte. Finally, the relationship between skeletal

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muscle damage and systematic markers, serum creatine kinase and voluntary maximal force production, is described.

2. Molecular markers for exercise-induced skeletal muscle damage

The first changes in the sarcoplasm of skeletal muscle fibers occur within few hours after the damaging exercise such as myofibrillar loss (**Figure 1D**). Furthermore, accumulation of mitochondria, local loss of myofilaments, Z line streaming (**Figure 1E**), swelling of mitochondria, disconnection of plasma membrane, swelling of sarcoplasmic reticulum, infiltration of inflammatory cells, complete loss of plasma membrane (**Figure 1F**), and disrupted muscle fibers may appear depending on how severe the damage is. In the next subsections, antibody stainings for HSP27, dystrophin, and type IV collagen are given as an example for visualizing changes after eccentric exercise in sarcoplasm, plasma membrane, and extracellular matrix, respectively.

2.1. Sarcoplasm of the skeletal muscle

In general, HSPs are considered to be the cellular protein quality control machinery. They can stabilize proteins during cellular damage, contribute to protein folding during increased protein synthesis, and protect proteins from aggregation. HSP27 can interact with actin and with many actin-binding proteins such as tropomyosin and troponin T [10]. HSP27 immunostainings in longitudinal sections of unexercised skeletal muscle fibers appear as fine lines (**Figure 2A**) indicating that HSP27 is localized to the Z-disks and/or I-band [11]. Immediately and 3 h after the exercise, in this case continuous drop jumping unilaterally on a sledge apparatus with a submaximal height until complete exhaustion, HSP27 immunostainings showed intensively stained and variable-sized clusters in both cross-sectional and longitudinal sections of skeletal muscle fiber HSP27 (**Figure 2C–F**) [12]. These stained clusters were probably formed due to translocation and accumulation of HSP27 on cytoskeletal/myofibrillar structures [11].

2.2. Plasma membrane of the skeletal muscle

Dystrophin is part of dystrophin protein complex, which transmits force laterally from contractile filaments to extracellular matrix through sarcolemma. Dystrophin is located beneath the sarcolemma, and it strengthens muscle fibers and protects them from injury. Immediately after forced lengthening contractions, the immunostaining of antibody against C-terminus of dystrophin fades out or disappeared partially before immunostaining of antibody against rod domain from few fibers in muscle sections from rat tibialis anterior muscle (**Figure 3**). This sequence of structural disturbance after eccentric exercise is interesting in relation to recent finding of new membrane-binding domain in dystrophin C-terminus [13]. In addition, proteinase-resistant regions in the rod domain of dystrophin make it more resistant against degradation.

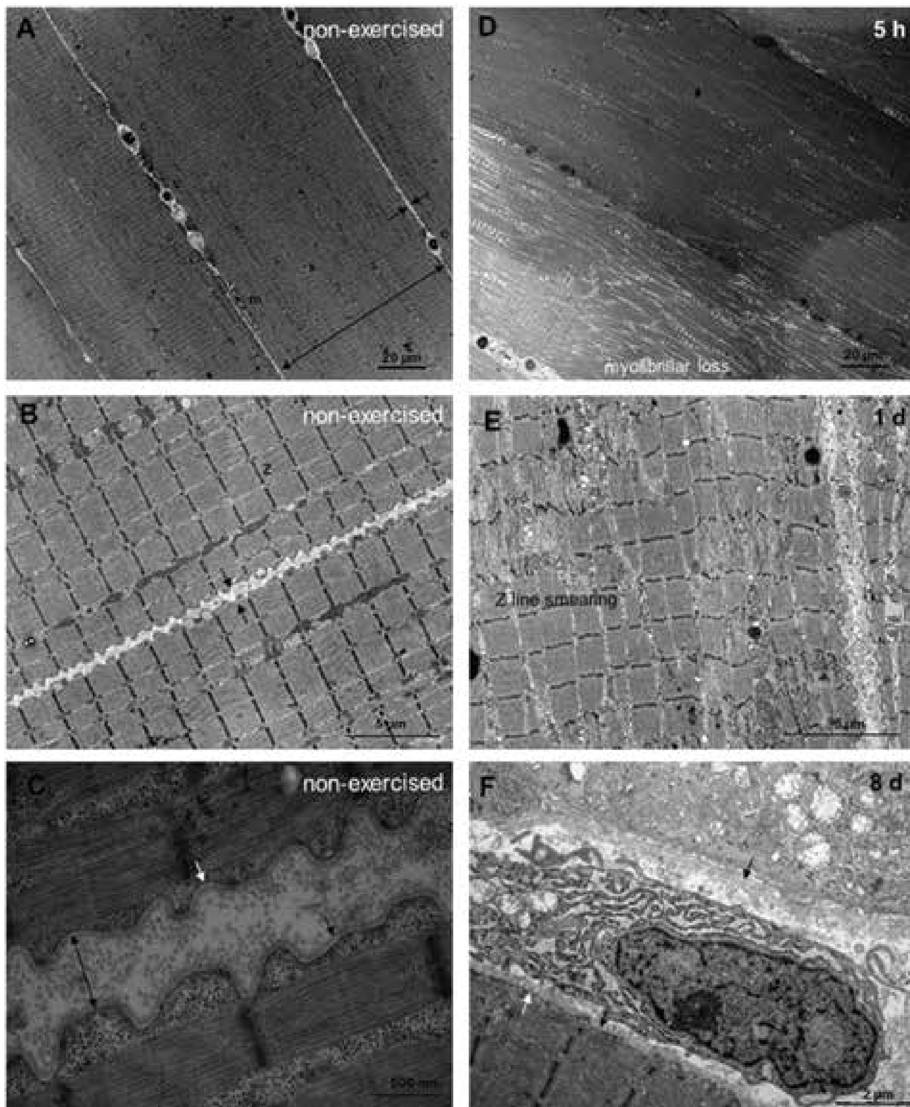

Figure 1. Electron micrograph of longitudinal sections of epoxy-embedded human skeletal muscle biopsy from vastus lateralis. (A–C) Non-exercised muscles. Skeletal muscle fiber (long two-headed arrow), endomysium (space between the two small arrows facing each other), myonuclei/satellite cell (m), capillary (c), plasma membrane is the fine dark line (white small arrow), basement membrane is the thick gray line (black small arrow). (D–F) Eccentric exercised muscle. (D) Five hours after the exercise, some myofibrils are lost in skeletal muscle fiber (lower left corner). (E) One day after the exercise, Z line smearing. (F) Eight days after the exercise, only basement membrane can be seen in damaged skeletal muscle fiber (upper small black arrow). Cell between two muscle fibers is probably fibroblast containing rough endoplasmic reticulum.

2.3. Basement membrane of the skeletal muscle

The extracellular matrix provides mechanical support for skeletal muscle fibers and plays an important role in force transmission. Fibrillar type I and III collagen are present in endomysium

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of skeletal muscle fibers (see **Figure 1C**, small gray dots between muscle fibers), whereas non-fibrillar type IV collagen is in basement membranes of skeletal muscle fibers (see **Figure 1C**, small black arrow) and capillaries. Type IV collagen is present in swollen, necrotic (**Figure 4**), and regenerated fibers, similarly as in undamaged skeletal muscle fibers. This suggests that basement membrane including type IV collagen holds on skeletal muscle fiber during the process of fiber damage, when the cytoskeleton is disrupted, contractile proteins are disorganized, and inflammatory cells are infiltrated [14].

2.4. What to remember about molecular markers for exercise-induced skeletal muscle damage

The first structural changes appear within few hours after eccentric exercise in sarcoplasm. This can be visualized using, for example, HSP27 immunostainings, which can be seen as intensively stained and variable-sized clusters (**Figure 2C–F**). During the next few days after the damaging exercise, antibody stainings for dystrophin fade out or disappear partially (**Figure 3**) and in severe damaged skeletal muscle fibers disappear totally (**Figure 4B**), whereas basement membrane including type IV collagen serves as a supportive structure during skeletal muscle fiber injury (**Figure 4A**) and regeneration.

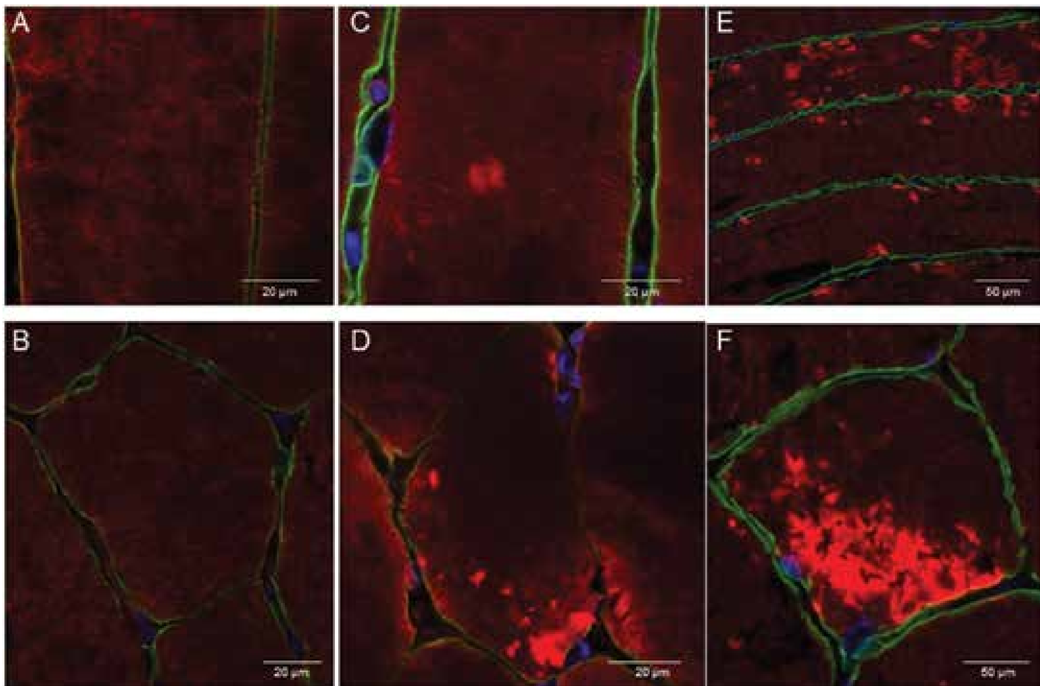

Figure 2. HSP27 (red) immunostaining as a marker for muscle damage. In biopsies obtained from human vastus lateralis before the exercise, no HSP27-stained clusters were observed (A and B). HSP27 was localized to the Z-disks of skeletal muscle fibers. After the exercise, intensively stained and variably sized clusters of HSP27 were observed immediately (C–E) and 3 hours post exercise (F). Dystrophin (green) immunostaining was used to visualize the borders of muscle fibers and DAPI (blue) stained nuclei. Published in Koskinen et al. [12].

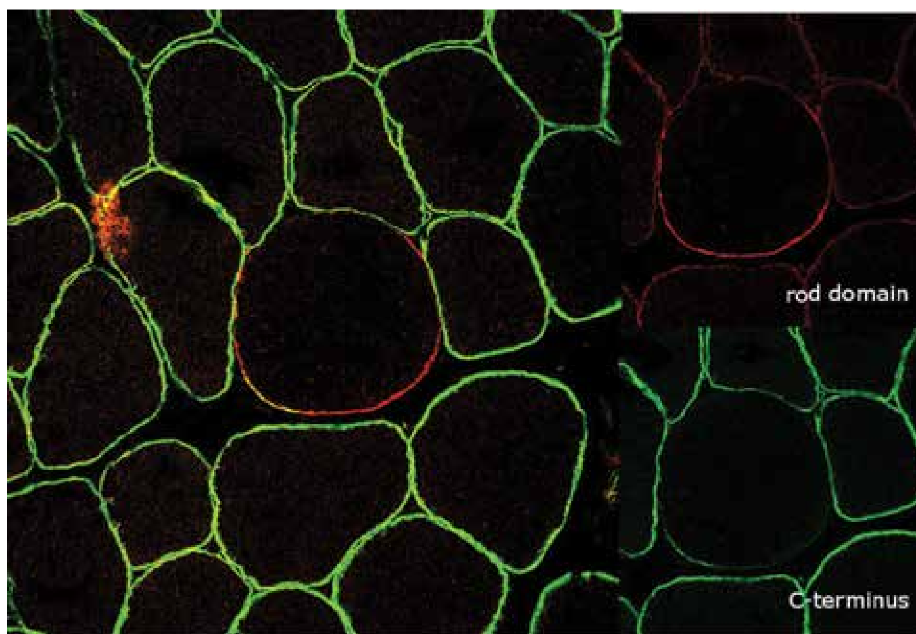

Figure 3. Immunostaining of antibody against C-terminus dystrophin (green) faded out or disappeared partially from individual muscle fiber (rat tibialis anterior muscle immediately after forced lengthening contractions), before than the staining for antibody against rod domain of dystrophin (red) that was still partially as bright as in non-exercised muscles.

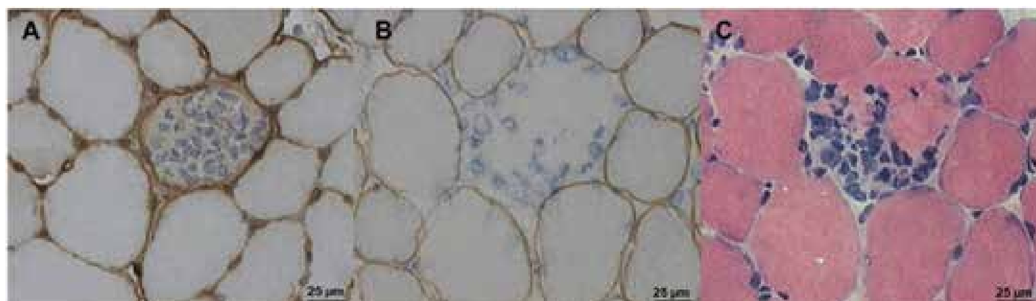

Figure 4. Injured skeletal muscle fiber in rat tibialis anterior muscle 2 days after forced lengthening contractions. Type IV collagen immunohistochemical staining is visible in the borders of basement membranes of skeletal muscle fibers (A), while dystrophin staining is negative (B). Infiltration of inflammatory cells inside the damaged fiber (D, hematoxylin–eosin staining). Bar = 25 µm. Published in Koskinen et al. [14].

3. Inflammatory cell markers in exercise-induced skeletal muscle damage

Immunostainings of leukocyte markers for neutrophils (CD11b, CD16, CD66b, neutrophil elastase, and myeloperoxidase) and for monocytes/macrophages (CD68) have been applied for localizing and counting the number of these inflammatory cells in human skeletal muscles

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after damaging exercise (e.g., see [15–20]). It is often concluded that the inflammatory cell reaction in skeletal muscle fiber injury caused by unaccustomed eccentric exercise is initiated by infiltration of neutrophils. However, the antibodies for CD11b, CD16, neutrophil elastase, and myeloperoxidase recognize also other leukocytes than neutrophils in leukocyte blood smears, whereas antibody for CD66b recognized only neutrophils (**Figure 5**) [6]. Therefore, the CD66b antibody is more suitable for detecting neutrophils in skeletal muscle sections than antibody for CD11b, CD16, myeloperoxidase, and neutrophil elastase. The CD68 antibody, the marker for monocytes/macrophages, recognized monocytes/macrophages and a portion of cells with bilobed nuclei (basophils or eosinophils) on leukocyte blood smears. In skeletal muscle biopsies after eccentric exercise, the CD68 antibody recognized more cell types than monocytes/macrophages [6].

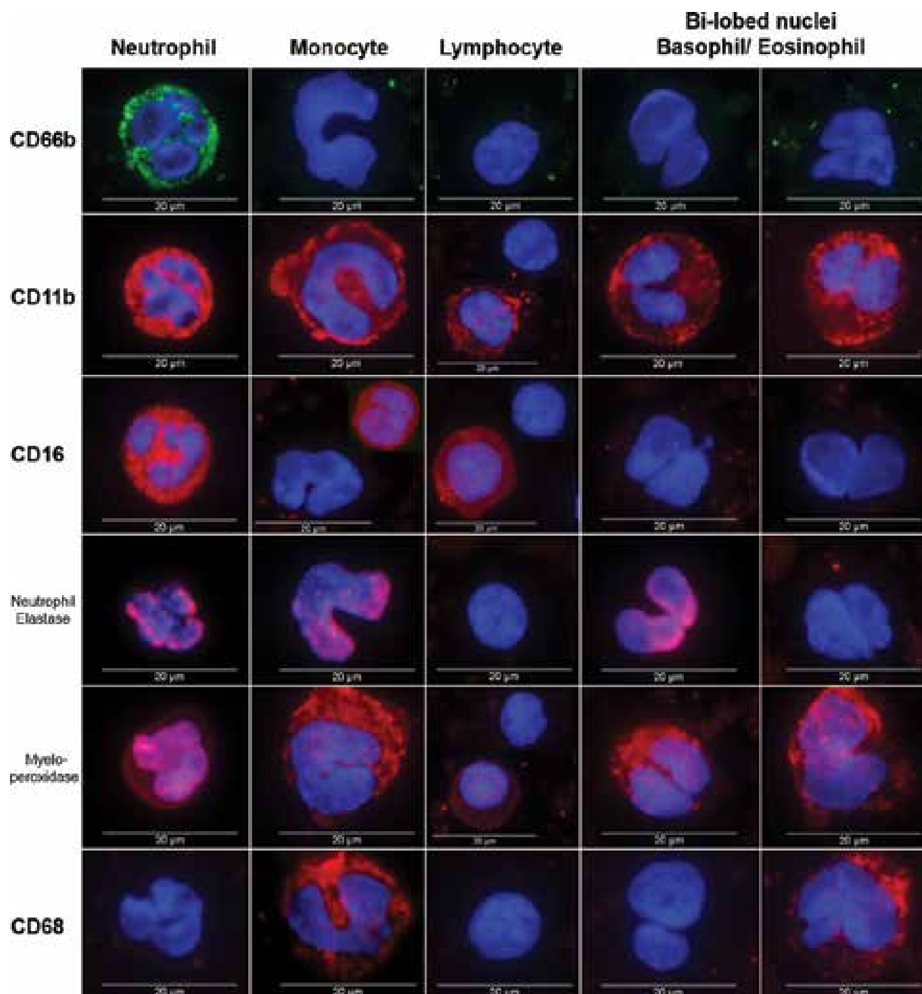

Figure 5. Immunostaining of CD11b, CD16, CD66b, CD68, myeloperoxidase, and neutrophil elastase antibodies on circulating leukocytes extracted from whole blood. Secondary antibody for CD66b Alexa Fluor 488 anti-mouse; for CD11b, CD16, CD68, and neutrophil elastase Alexa Fluor 594 anti-mouse; and for myeloperoxidase Alexa Fluor 594 anti-rabbit. Scale bar of 20 μm. Published in Paulsen et al. [6].

3.1. Neutrophils in damaged skeletal muscle

The number of CD66b-positive cells is very low in both exercised and non-exercised skeletal muscles [6]. As an example, only 34 of 122 biopsies from eccentrically exercised and non-exercised human biceps brachii muscles contained CD66b-positive cells. A closer examination showed that in 23 of these biopsies, the CD66b stained cells (varied from 1 to 4 CD66b-positive cells per 100 muscle fibers) were located inside capillaries or vessels (**Figure 6B**), attached to the wall of the vessels, or were detected in blood clots (**Figure 6A**). In the remaining 11 biopsies, single CD66b-positive cells (varied from 1 to 30 CD66b-positive cells per 100 muscle fibers) were observed in the endomysium of affected muscle fibers or in the sarcoplasm of damaged fibers. There was no consistent pattern regarding how CD66b-positive cells were distributed between exercised and non-exercised samples or between different time points. These results indicated that neutrophils were not involved in exercise-induced skeletal muscle fiber injury. Unusual high numbers of CD66b stained cells located in the endomysium or inside fibers may indicate trauma from the previous biopsy.

3.2. Monocytes/macrophages in damaged skeletal muscle

CD68-positive cell counts are widely used for indication of monocyte/macrophage infiltration in skeletal muscle biopsies after single bout of eccentric exercise (e.g., see [19]). However, it has been shown that the CD68 antibody recognized more cell types than monocytes/macrophages in human skeletal muscle biopsies after eccentric exercise [6]. The highest individual CD68-positive cell counts were related to skeletal muscle fiber injury, which was observed in exercised biopsies at 4 and 7 days after acute eccentric exercise. In these biopsies, CD68-positive cells typically occupied the entire sarcoplasm of damaged skeletal muscle fibers (**Figure 7A**). Therefore, monocytes/macrophages were probably the most prominent CD68-positive cell type in these biopsies. In addition, CD68-positive cells inside capillaries (**Figure 7C**) and vessels (**Figure 7D**) were most likely monocytes/macrophages. In the exercised biopsies without damaged skeletal muscle fibers, the determination of the monocytes/macrophages proportion of CD68-positive cells is not straightforward. Comparison of cells with similar appearance and location between light microscopy pictures of CD68-positive cells and transmission electron microscopy pictures

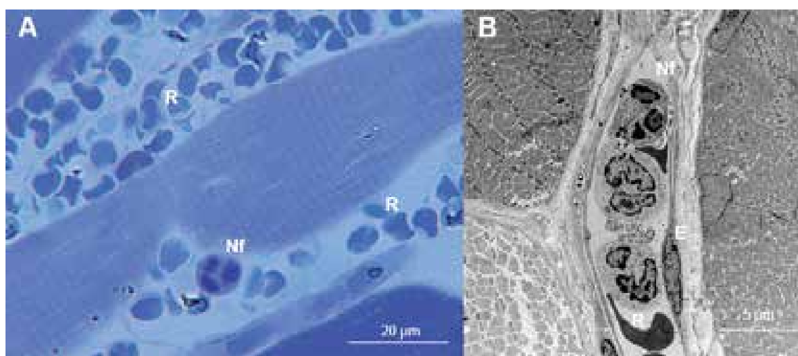

Figure 6. The nuclei of neutrophils are divided in few parts, which makes them easily recognizable. (A) Semi-thin toluidine blue stained section contains a cluster of red blood cells and single neutrophil (non-exercised skeletal muscle biopsy from human biceps brachii muscle). (B) Leukocytes inside a blood vessel (electron micrograph of epoxy-embedded exercised skeletal muscle biopsy from biceps brachii muscle). Endothelial cell (E), neutrophil (Nf), red blood cell (R).

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showed that the sarcoplasmic CD68 immunostaining observed in single cells aligned next to the laminin (**Figure 7B** arrow) was most likely satellite cells (in electron microscopy pictures, these cells were located between the plasma membrane and the basement membrane of skeletal muscle fibers). Furthermore, CD68-positive cells with long extensions located in endomysium (**Figure 7B** arrowhead) were probably fibroblasts or myofibroblasts. According to the observation from electron microcopy pictures, these types of cells contained prominent rough endoplasmic reticulum indicating cells with high capacity for protein synthesis such as fibroblasts/myofibroblasts. Both satellite cells and fibroblasts were most likely involved in the skeletal muscle adaptation to increased mechanical loading. The number of CD68-positive cells is not optimal to be applied as a quantitative value for monocytes/macrophages in human skeletal muscles.

3.3. What to remember about inflammatory cells in damaged skeletal muscle

Neutrophils are not involved in exercise-induced skeletal muscle fiber injury, although in other injured tissues neutrophils are usually the first inflammatory cells present. CD66b antibody seems to exclusively recognize neutrophils both in leukocyte blood smears and skeletal muscle biopsies. Numerous monocytes/macrophages are present in damaged skeletal muscle fibers, typically totally occupying the entire sarcoplasm of damaged fibers. CD68 antibody is not optimal to be applied as a quantitative value for monocytes/macrophages, because it recognizes also satellite cells and fibroblasts in skeletal muscle biopsies.

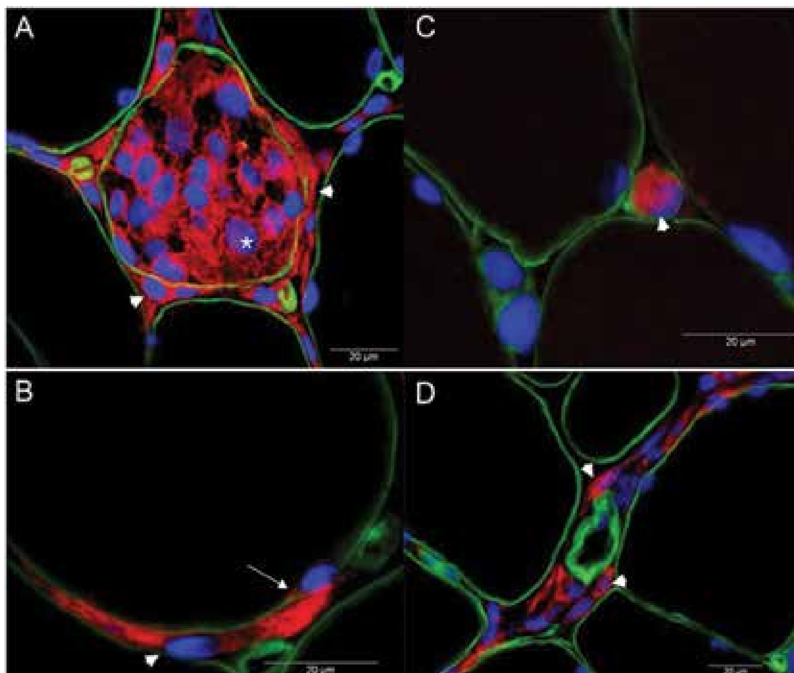

Figure 7. CD68 (red), laminin (basement membrane, green), and DAPI (nuclei, blue) staining on eccentric exercised human skeletal muscle. (A) Damaged skeletal muscle fiber infiltrated by CD68 stained cells (asterisk). In the endomysium, CD68 stained cells seemed to form a chain around necrotic fiber (arrow heads). (B) CD68 stained cells with long extensions in sarcoplasm (arrow) and in the endomysium (arrow head). (C) CD68 stained cell (arrow heads) inside capillary. (D) Vessel surrounded by CD68 stained cells. Scale bar of 20 μm. Published in Paulsen et al. [6].

4. Serum creatine kinase and voluntary maximal force production as indirect indicator for severity of skeletal muscle damage

Decrease in muscle force-generating capacity after eccentric exercise is a valuable tool as an indirect indicator for severity of skeletal muscle damage together with serum creatine kinase. As an example, 23 subjects were divided into 3 categories, mild (n = 6), moderate (n = 10), and severe (n = 7) effect of eccentric exercise, depending on the muscle force loss immediately after performing 70 maximal eccentric actions with elbow flexors on an isokinetic dynamometer and how fast muscle force was recovered during the following 7 days [6]. Muscle force loss immediately after the exercise was 64, 53, and 50% of pre-exercise in mild, moderate, and severe categories, respectively (**Figure 8**). After 7 days post exercise, muscle force was totally recovered in the category mild effect of eccentric exercise, whereas in the categories moderate and severe effect of eccentric exercise, force was still clearly below the baseline level (**Figure 8**).

The average values of serum creatine kinase (207, 228, and 140 U/l; mild, moderate, and severe, respectively) before eccentric exercise were similar in all three categories (**Figure 9**). The average values of serum creatine kinase changed the most in categories moderate and severe effects of eccentric exercise. The highest average creatine kinase values, 2929 and 10,266 U/l, in these categories were observed 4 days after eccentric exercise, whereas in the mild effects of eccentric exercise category, the highest average value, 538 U/l, was observed 7 days after the exercise.

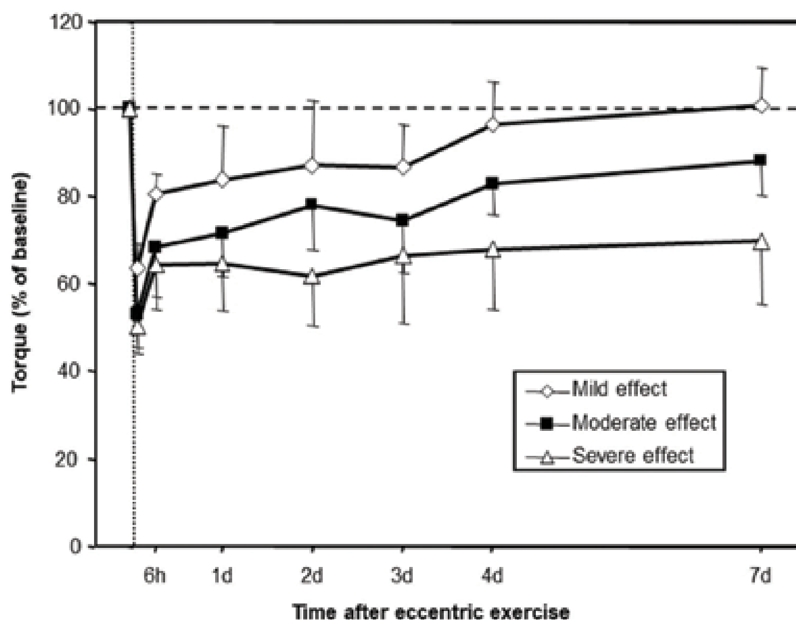

Figure 8. Post-exercise muscle force-generating capacity of the exercised arm. Subjects were divided into three categories, mild (n = 6), moderate (n = 10), and severe (n = 7) effect of eccentric exercise, based on the loss and the recovery of muscle force. Vertical dashed line indicates the time of eccentric exercise bout was performed and horizontal dashed line is the baseline for muscle force. Error bars are standard deviation. Published in Paulsen et al. [6].

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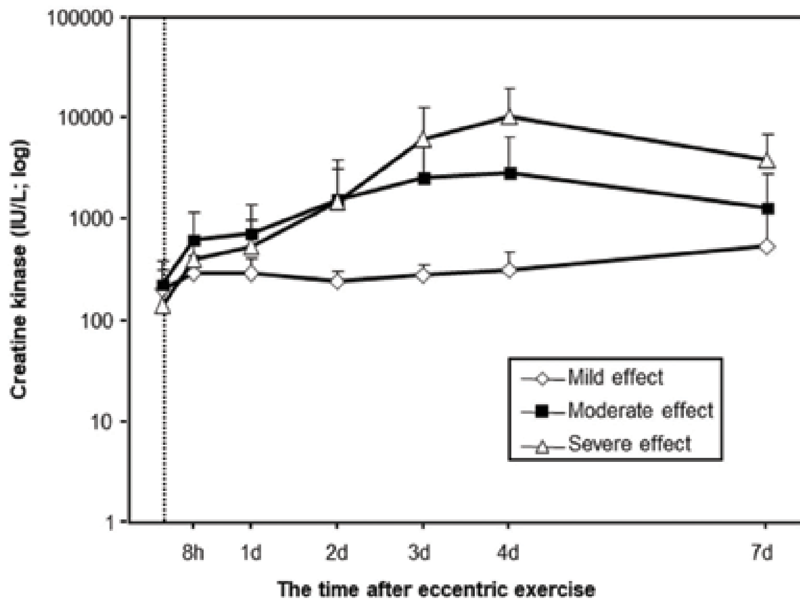

Figure 9. Serum creatine kinase. Subjects were divided into three categories, mild (n = 6), moderate (n = 10), and severe (n = 7) effect of eccentric exercise, based on the loss and the recovery of muscle force. Vertical dashed line indicates the time of eccentric exercise. Y-axis is logarithmic. Error bars are standard deviation. Published in Paulsen et al. [6].

Eight of total 23 subjects showed skeletal muscle fiber injury detected at light microscopy level as dystrophin-negative fibers and infiltration of CD68-positive cells at 4 or 7 days after the exercise. These subjects belonged to the categories moderate (five subjects of ten showed dystrophin-negative fibers) and severe (three subjects of seven showed dystrophin-negative fibers) effects of eccentric exercise. If the muscle force-generating capacity and serum creatine kinase have not totally recovered to the baseline level, it is most likely that the muscle is undergoing regeneration.

4.1. What to remember about indirect indicators for severity of skeletal muscle damage

Skeletal muscle fiber injury and prolonged regeneration process are probably the reasons for impaired peripheral muscle function after high-force eccentric exercise. Muscle force-generating capacity after single bout of eccentric exercise is a good indirect indicator of muscle damage in humans together with serum creatine kinase analysis. In the future studies, more attention should be paid for making sure that subjects' muscle force has recovered to the baseline level after the experiment is over.

Acknowledgements

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Conflict of interest

The authors declare they have no conflict of interest.

Abbreviations

HSP	Heat shock protein

Author details

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Chapter 4

Multinuclear Magnetic Resonance Spectroscopy of Human Skeletal Muscle Metabolism in Training and Disease

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Martin Krššák

Additional information is available at the end of the chapter

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Abstract

In this chapter, techniques and application of multinuclear (^{1}H, ^{13}C, and ^{31}P) *in vivo* magnetic resonance spectroscopy (MRS) for the assessment of skeletal muscle metabolism in health and disease are described. Studies focusing on glucose transport and utilization, lipid storage and consumption, handling of energy rich phosphates, and measurements of newly emerging noninvasive biomarkers, i.e., acetylcarnitine and carnosine are summarized. Muscle metabolism connections to exercise physiology and the development as well as possible treatment of metabolic diseases, such as obesity and diabetes are also discussed. Taken together, multinuclear *in vivo* MRS on humans helped to uncover defects in skeletal muscle metabolic pathways in insulin-resistant conditions; and to discover links between defects in mitochondrial activity/capacity and lipid metabolism, as well as defects in whole-body and/or muscle glucose metabolism. There is also to mention that several of the MR-derived readouts are affected by both training status and metabolic disease in a specific way, and thus could serve as potential markers of training status and metabolic flexibility.

Keywords: magnetic resonance spectroscopy, skeletal muscle, energy metabolism, training status, pathophysiology, glucose, lipids, diabetes mellitus, obesity, exercise

1. Introduction

Skeletal muscle is the key human tissue responsible for the body weight bearing and movement and plays a central role in whole-body energy metabolism. Even in the resting conditions

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skeletal muscle accounts for ~30% of metabolic rate of human body [1]. In particular, as the main target of insulin activity, skeletal muscle effectively regulates the glucose uptake, while serving also as a glycogen storage [2]. The extent to which skeletal muscle fulfills these roles is affected by many physiological and pathophysiological factors, which can change over time. Several diseases largely manifesting in skeletal muscle pathology have a rapidly increasing socioeconomic impact, as they start to affect not only the elderly, e.g., sarcopenia, but also young productive, population, e.g., insulin resistance and type 2 diabetes mellitus (T2DM). Insulin resistance and T2DM are rapidly reaching epidemic proportions worldwide and the associated treatment costs of T2DM also continue to grow. The cost of diabetes (with over 85% attributable to T2DM) was in 2012 over £1.5 milion an hour or 10% of the entire National Health Service budget for England and Wales [3]. In order to improve the understanding and clinical management of such disorders, it is vital to be able to assess muscle function and metabolism *in vivo* noninvasively, to support their diagnosis, monitor changes in tissue status during disease progression and interventions, and above all, to establish robust markers that can be used in disease prevention [4].

Magnetic resonance spectroscopy (MRS) represents an advanced noninvasive technology that allows for assessment of tissue metabolism in the healthy as well as diseased conditions [5]. In particular, MRS techniques are able to noninvasively monitor intramyocellular storage and turnover of important energy storage pools, namely lipids and glycogen. In addition, MRS is uniquely suited to quantitatively assess adenosine-triphosphate (ATP) production reactions in the muscle, i.e., mitochondrial oxidative phosphorylation, glycolysis, and creatine kinase activity. Among other things, proton (^{1}H) MRS is best suited to quantify intramyocellular lipid (IMCL) storage, carbon (^{13}C) MRS is optimal for glycogen reserves measurements and phosphorus (^{31}P) is ideal for investigations of ATP metabolism. This chapter briefly describes the basic principles and availability of these measurements, and further focuses on applications of MRS techniques for studying functional properties of skeletal muscle in health and disease. Obesity, type 2 diabetes mellitus, and skeletal muscle insulin resistance serve as good model for pathologic conditions, while the summary of MRS observable adaption to training is brought as positive control or contrast to aforementioned circumstances.

While most of the described methods and measurements have been introduced at lower field strengths 20–30 years ago [6–8], recent development in MR technology, namely the transition towards ultra-high field MR systems ($B_0 \geq 7$ T), meant significant improvements to *in vivo* MRS [9–13]. Next to the linear gain in signal-to-noise ratio (SNR), which can be translated to significantly shorten data acquisition time [12] or improved signal localization [14], higher field strength also provides improved spectral resolution, reducing metabolite overlapping, and thus, improving quantification accuracy. The increase in SNR is of particular importance to nonproton MRS, which is limited mainly by SNR in its applicability [15]. ^{31}P MRS benefits from additional increase in SNR per unit of time due to shortening of the T_1 relaxation of ^{31}P metabolites at 7 T [11]. MR systems ($B_0 \geq 7$ T) equipped with multinuclear broadband capabilities hold great potential for investigations of the not yet well-understood mechanisms of tissue metabolism.

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2. Methods of magnetic resonance spectroscopy

2.1. ^{1}H MRS

The main application of ^{1}H MRS that is used in exercise and nutrition research, just as often as in studying the etiology of insulin resistance and T2DM, is the quantification of intramyocellular lipids (IMCL) [16–18]. Among other typical uses of ^{1}H MRS belong: (a) detection of lactate (Lac) formation during exercise [8, 19–21]; (b) measurement of total creatine (Cr) content [22–24]; (c) assessment of muscle fiber orientation using dipolar coupling [25]; (d) measurement of intramyocellular metabolite diffusion [26]; and (e) the dynamic measurement of tissue (de)oxygenation using the signal of deoxymyoglobin (DMb) [27–29]. Furthermore, detection of resting muscle carnosine [30, 31] and acetylcarnitine (AcC) [32, 33] has been recently promoted as a promising use of ^{1}H MRS. An example of high resolved *in vivo* acquired ^{1}H MR spectrum of skeletal muscle is given in **Figure 1**.

2.1.1. Static examinations by ^{1}H MRS

Next to water, lipid accounts for the strongest signals in a ^{1}H spectrum of skeletal muscle at rest. However, even with optimal tissue selection, not all lipid signals in the spectrum are intramyocellular (IMCL). Fortunately, it is possible to differentiate between IMCL and extramyocellular

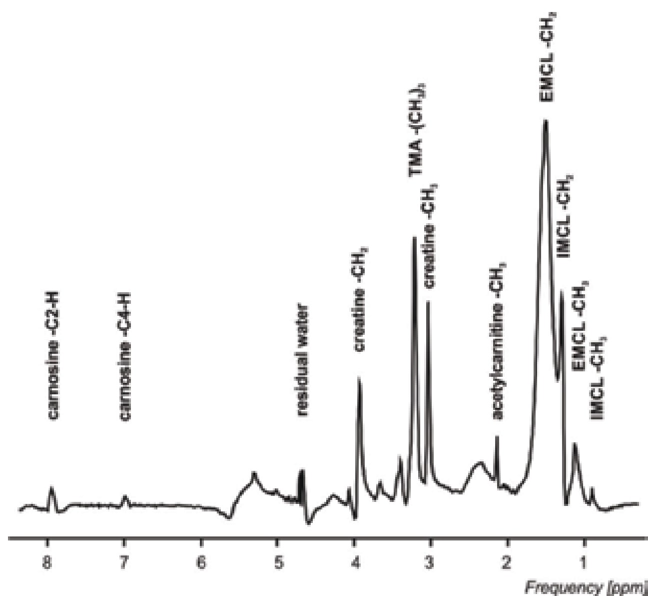

Figure 1. A representative ^{1}H-MRS spectrum from an athlete acquired from the vastus lateralis muscle at 7 T showing intramyocellular (IMCL) and extramyocellular (EMCL) lipids [0.9 and 1.1 ppm (CH_3 groups) 1.5 and 1.3 ppm (CH_2 groups)], AcC at 2.13 ppm, Cr at 3.03 and 3.9 ppm, trimethyl ammonium (TMA) groups of carnitine, AcC, and choline at 3.20 ppm, residual water peak at 4.7 ppm, removed in postprocessing, and carnosine spectral lines at 7 and 8 ppm.

lipids (EMCL). Inside myocytes, lipids form small droplets in the cytoplasm, whereas EMCLs are found layered between myocytes along the main muscle orientation, and are tubular in shape. This difference in spherical versus cylindrical geometry influences the bulk magnetic susceptibility of these lipid compartments making the differentiation possible [34, 35]. The IMCL/EMCL peak separation depends on the angular orientation of EMCL to the external magnetic field as a result of anisotropy effects [36] which results into maximum of 0.2 ppm frequency shift in case of fully parallel orientation [4], as is the case in tibialis anterior [25, 37].

In general, to maximize the acquired signal, MRS sequences with short echo time (TE) are often used for IMCL quantification [38–40]. This requires suppression of water signal and can also lead to broad resonances of various shapes and strong IMCL/EMCL overlap, which can cause inaccurate quantification of IMCLs [41, 42]. Contamination from subcutaneous adipose tissue or bone marrow can make this even more challenging. Moreover, if the water signal is to be used as an internal concentration reference, additional acquisition of water signal is necessary. Better separation of EMCLs and IMCLs and improved fitting of lipid resonances was suggested and observed when using an MRS acquisition with longer TEs [10, 42, 43]. This improved separation is a result of the different T_2 relaxation times of IMCL and EMCL resonances and the line width narrowing effect [10]. Thus, the long-TE acquisition has a major advantage in increased spectral resolution [10, 34] and provides the possibility to omit water suppression, reducing energy deposition in tissues. On the other hand, absolute quantification from the long-TE MR spectra requires precise T_2 relaxation correction, which can be inaccurate especially for signals with short T_2, i.e., water signal [10, 44]. Thus, an ideal acquisition combines a short TE measurement of water signal with long-TE detection of lipids [14].

Another muscle metabolite that greatly benefits from long-TE acquisition is acetylcarnitine (AcC). This relatively low concentrated metabolite fulfills a major role in translocation of long-chain fatty acids from cytosol to the mitochondrial matrix [45] and in maintaining pyruvate dehydrogenation activity [46], and is, therefore, of high interest in skeletal muscle research. The straight forward detection and quantification of AcC is challenging, due to the strong overlap of the 2.13 ppm line with lipid resonances, and the fact that the line at 3.20 ppm represents a combination of the trimethylammonium (TMA) groups of carnitine, AcC, and choline. Fortunately, the differences in T_2 relaxation times of AcC and lipids allow the detection of the 2.13 ppm line at rest, using long-TE ^{1}H MRS [32, 33].

The downfield region of the ^{1}H spectrum, i.e., left to the water signal, gets often overlooked as the detectable signals belong to low concentrated metabolites, e.g., carnosine, and can be easily mistaken for noise. This is very unfortunate, as carnosine is a pH-buffering metabolite that can be manipulated externally [47, 48]. The concentration of carnosine is mainly determined by muscle fiber type composition, with fast-twitch glycolytic fibers containing up to twice as much carnosine as slow-twitch oxidative fibers [49, 50]. In addition, chemical shift of carnosine is sensitive to pH, and thus, carnosine signal can be also used to assess intramyocellular pH [51, 52]. While it is possible to detect carnosine using clinical systems [30, 51], the increased SNR of ultra-high fields, provides high repeatability [31].

2.1.2. Dynamic examinations by ^{1}H MRS

While most of the metabolite signals can be observed in basal resting conditions, metabolic adaption to stress induces by exercise and/or ischemia may alleviate the visibility of specific

resonance lines. Of particular interest has been the formation of lactate (Lac) during exercise challenge or ischaemia [8, 19, 20, 53, 54], because lactate is the end-product of anaerobic metabolism and a source of free H^+, and thus, it plays an important role in skeletal muscle metabolism and pH regulation. Although ^{1}H MRS measurements of Lac were shown to be in good agreement with tissue extracts analysis [53], due to overlapping lipid signals, dipolar coupling and relaxation effects, quantification of Lac levels in skeletal muscle *in vivo* is extremely challenging, and thus, prone to inaccurate estimation [4].

It can often be unclear whether the measured results reflect real change in skeletal muscle metabolism or just manifest inadequate oxygenation state of the muscle. This query can be also answered by ^{1}H MRS, which can serve to noninvasively monitor the (de)oxygenation state of human skeletal muscle under stress through the measurement of deoxymyoglobin (DMb) [29]. Very low concentration of DMb is not an obstacle, as DMb resonates substantially downfield away from the typical spectral range, securing no overlap with other metabolites and has extremely short T_1 [55].

Formation of AcC during strenuous exercise and its slow decay after exercise has also been under investigation using ^{1}H MRS [56, 57]. While at lower fields, it is only the 2.13 ppm resonance line that gets resolved after strenuous exercise [56], 7T allows direct observation of split in resonance lines of AcC and carnitine in the TMA region, providing the option to quantify their ratio. Besides, the ^{1}H signal of AcC at this resonance is twice as strong, improving sensitivity of the measurement [57].

On the longer time scale of few 10 minutes during prolonged submaximal exercise and following recovery decrease and replenishment of IMCL pool can be observed [58, 59].

2.2. ^{13}C MRS

The presence of carbon nuclei in almost every organic structure, the nonzero spin of carbon-13 (^{13}C) nuclei, and a very wide chemical shift range of up to 200 ppm have made ^{13}C MRS well-suited for studies of molecular structure and biochemistry in cellular and animal models since the early days of biochemical MRS. The dynamic assessment of biochemical pathways in particular, forms the basis for the current application of ^{13}C MRS in humans.

Due to the different magnetic properties of ^{13}C compared to protons, the resonance frequency of ^{13}C at a given magnetic field is approximately one-quarter that of ^{1}H MRS. Although the natural abundance of carbon nuclei is very high in living tissues, i.e., almost matching the abundance of protons, the ratio of MR visible ^{13}C to MR invisible ^{12}C is extremely low (approx. 1:99). Lower gyromagnetic ratio and consecutively lower intrinsic sensitivity of ^{13}C MRS, together with lower natural abundance of ^{13}C nuclei leads to inherently low SNR, and thus, hampers the spatial and temporal resolution of ^{13}C MRS experiments. Techniques to increase low SNR of ^{13}C MRS include: (a) increased volume of interests and/or averaging of the MRS signal using a high number of repetitions, (b) elimination of the spin-spin coupling interaction between ^{13}C-nuclei and its coupled protons by the ^{1}H decoupling pulses in the period of ^{13}C signal acquisition; (c) the utilization of the ^{1}H-^{13}C magnetic interaction with polarization transfer techniques; (d) the use of a higher field-strength MR apparatus; and (e) increasing the abundance of the ^{13}C isotope by systemic infusion of ^{13}C-enriched metabolic substrates.

2.2.1. ^{13}C MRS natural abundance studies

The use of ^{13}C MRS for *in vivo* studies of skeletal muscle without artificial isotope enrichment is essentially limited to measurements of metabolites present at high concentrations, in particular glycogen and triglycerides [4]. Despite its high molecular weight, the glycogen C-1 resonance line is 100% MR visible [60, 61] due to the high intramolecular mobility of its glucose residues. Skeletal muscle glycogen is present at approximately 80–120 mM concentrations, depending on the muscle and physiological conditions [62–64]. Good reproducibility of natural abundance muscle glycogen measurements by ^{13}C MRS [65] favors the use of dynamic experimental protocols to assess the depletion of glycogen during exercise (**Figure 2**) and its resynthesis over the course of several hours during post-exercise recovery [58, 59, 66, 67].

2.2.2. ^{13}C MRC labeling studies

To overcome the low SNR due to low natural abundance of ^{13}C nuclei and increase the measurement sensitivity, it is common to use an isotope enriched infusion in ^{13}C MRS studies [4]. After an infusion of ^{13}C-labeled glucose under steady-state conditions, glycogen synthesis in skeletal gastrocnemius muscle has been quantified and correlated with whole-body carbohydrate consumption [7, 69, 70].

Another exciting use of ^{13}C MRS *in vivo* is the quantification of the flux through the tricarboxylic acid (TCA) cycle, which serves as a surrogate for the rate of mitochondrial oxygen consumption by the cellular respiration that is vital for skeletal muscle function. The labeling of substrates in the TCA by infusing [2-^{13}C]-acetate and observing the enrichment of the C4 position of glutamate, has been performed in muscle. These measurements can easily be combined with experiments in which undirectional flux through the skeletal muscle ATP-synthase is measured by means of ^{31}P saturation transfer [4].

Alternative approach for further improvement of signal-to-noise and localization is the application of so called indirect ^{13}C measurements, where high sensitivity and low chemical shift displacement of ^{1}H MRS is used for signal excitation and detection and chemical specificity is introduced exploiting magnetic interaction with coupling ^{13}C atoms. Proof of the principle for this approach has been demonstrated the measurements of fatty acid composition of human subcutaneous tissue [71], while application of similar methodology with the sensitivity enhancement by concomitant ^{13}C label infusion has been demonstrated in the study focused on postprandial lipid partitioning in liver and skeletal muscle in prediabetic and diabetic rats [72].

2.3. ^{31}P MRS

Skeletal muscle was the first human tissue studied by ^{31}P MRS *in vivo*, mainly because of its high metabolic activity, physiological importance, and relatively simple access [6, 73, 74]. ^{31}P MR spectra of skeletal muscle typically depict five major resonances from inorganic phosphate (Pi), phosphocreatine (PCr), and adenosine-triphosphate (ATP).

Other detectable ^{31}P metabolites include cell membrane precursors, i.e., phosphomonoesters (PMEs) combined from—phosphocholine (PC) and phosphoethanolamine (PE) and cell membrane degradation products, i.e., phosphodiesters (PDEs) in particular

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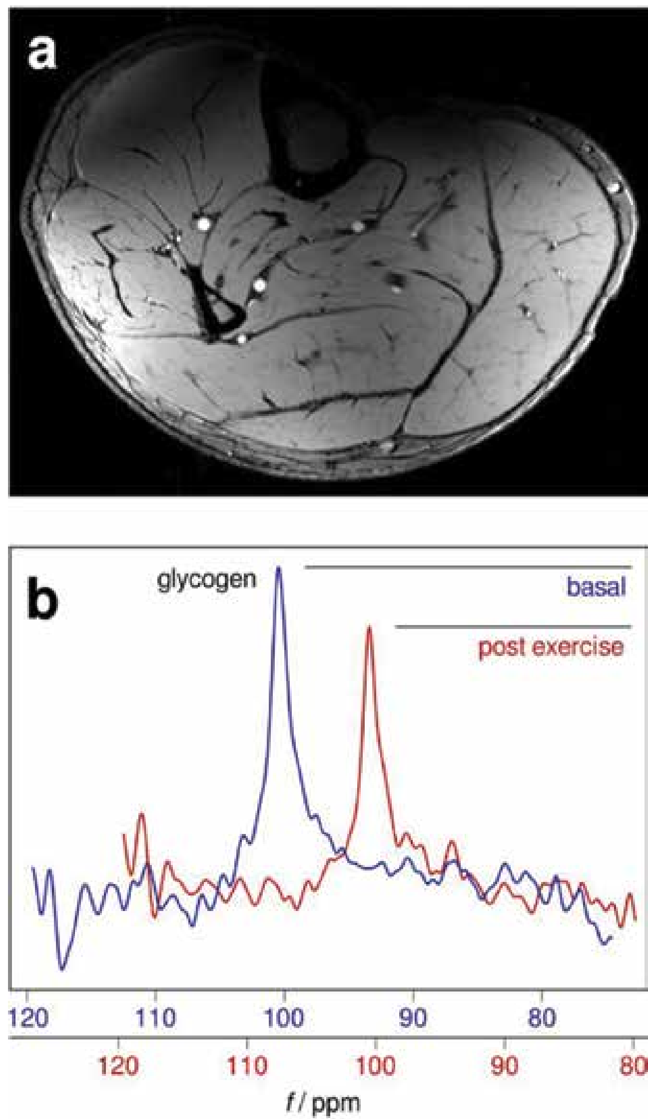

Figure 2. Transversal MRI of calf muscle (a) and natural abundance ^{13}C MR spectra acquired at 7T depicting glycogen signals from soleus and gastrocnemius muscle (b) (pulse-acquire block pulse MRS, acquisition time approx. 4 min). The glycogen signal is decreased after 90 s of toe raising exercise by approx. 30%. Adapted from Goluch et al. [68].

glycerolphosphocholine (GPC) and glycerol-phosphoethanolamine (GPE) [11] (**Figure 3**). Besides, using the chemical shift between PCr and Pi, intramyocellular pH can be calculated noninvasively [75].

Next to the analysis of resting ^{31}P MR spectra, for metabolite concentration determination, it is very frequent to obtain the ^{31}P MR spectra during exercise and consecutive recovery [6, 77]. Such dynamic ^{31}P MR experiments provide a measure of skeletal muscle oxidative metabolism, through quantification of mitochondrial capacity.

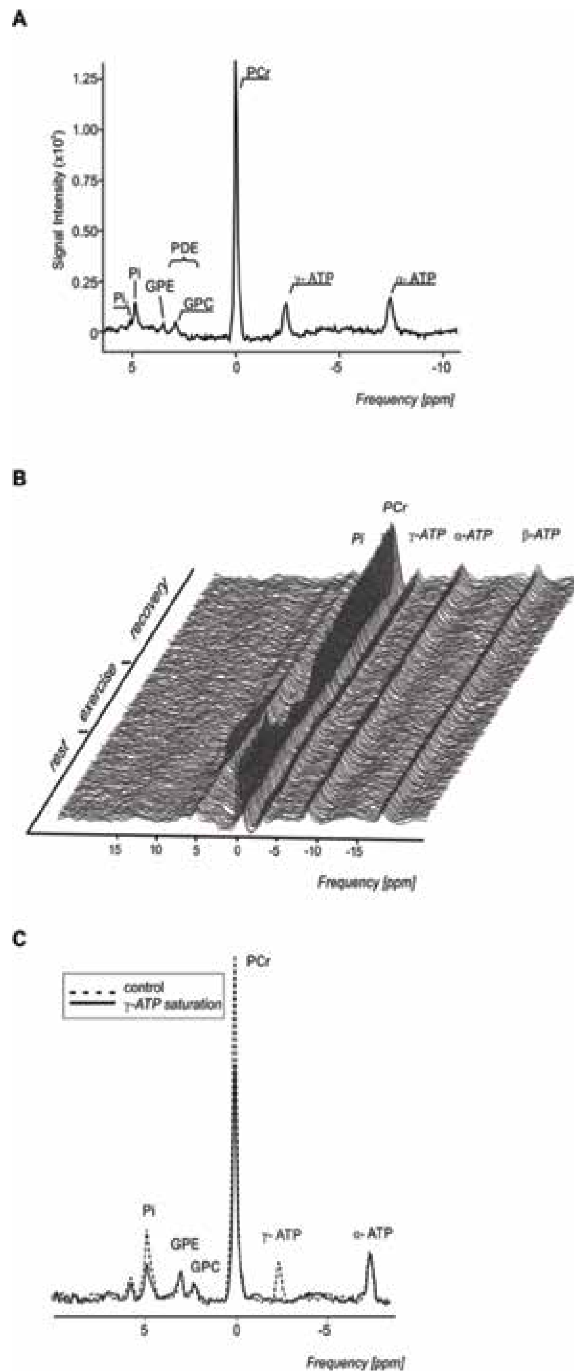

Figure 3. (A) A representative highly spectrally resolved static ^{31}P-MRS spectra acquired at 7T. (B) Time course of a ^{31}P MR spectra during a knee extension exercise with depicted depletion of the PCr signal and its subsequent resynthesis during the recovery period. (C) Saturation transfer spectra showing the effect of γ-ATP saturation, at approximately −2.48 ppm (solid line) on the Pi signal compared to the control experiment with saturation at approximately 12.52 ppm (dashed line). Adapted and reproduced from Klepochová et al. [76].

Alike ^{13}C MRS, ^{31}P MRS also has a lower gyromagnetic ratio in comparison to protons, and thus, suffers from lower intrinsic sensitivity. Therefore, SNR enhancing approaches, e.g., ^{1}H decoupling, at lower fields, or benefit from the SNR boost of higher magnetic fields are utilized [11, 12, 78].

2.3.1. ^{31}P MRS of resting muscle

The quantification of static ^{31}P-MR spectra was repeatedly exploited in the past to gather information about skeletal muscle fiber composition using the PCr/Pi ratio, however, the observed scattering in metabolite content is large and the final conclusions vary [79–83], thus severely limiting the reliability of these measurements [15].

On the other hand, ^{31}P MRS of skeletal muscle can provide valuable information about whole-body training status, metabolic health, and/or muscle integrity. In particular, the concentration of phospholipids-phosphodiesters seems to provide a valuable surrogate of metabolism or systemic muscle damage [82, 84–90]. At ultra-high fields (i.e., 7 T), or by using ^{1}H decoupling, the signal of main PDE in human skeletal muscle—GPC—can be separated and used directly rather than the total PDE signal [86]. Another very recent approach for the determination of skeletal muscle oxidative metabolism from resting ^{31}P MR spectra that profits from the increased spectral resolution of the ultra-high field systems (i.e., 7 T), is the assessment of alkaline pool of Pi signal (Pi_2) [91]. Based on its chemical shift (~5.1 ppm), relatively short T_1, and small contribution of extracellular space to skeletal muscle signal, the mitochondrial matrix has been recognized as the likely origin of this pool [91]. As such, it should be able to provide direct information about changes in mitochondrial density in response to training or defects of mitochondrial metabolism [15]. Thus far, Pi_2/Pi ratio was showed to be increased in the quadriceps of the trained subjects [92] and decreased in sedentary subjects [86] in comparison to normals, thus, supporting this hypothesis.

^{31}P MRS can also assess the reaction kinetics of energy metabolism through a technique called saturation transfer (ST). ST exploits the transfer of magnetization between nuclei that are in direct chemical exchange, thus estimating the unidirectional exchange rates and fluxes under steady-state conditions [4]. Unfortunately, ST experiment in skeletal muscle does not yield a net oxidative flux, as the measured flux contains a major glycolytic component and both turnover reactions operate close to equilibrium, i.e., the net rates of both glycolytic and oxidative ATP synthesis are low at rest [93]. On the other hand, the resting fluxes were correlated with parameters of oxidative metabolism [94, 95], and follow changes of oxidative metabolism observed in disease [96].

2.3.2. Dynamic ^{31}P MRS during exercise-recovery challenge

^{31}P MRS measured during muscle contraction and recovery, i.e., dynamic ^{31}P-MRS, can be used to observe the kinetics of intramyocellular pH and of the cytosolic concentrations of PCr, Pi, and ADP during perturbations of metabolic equilibrium. These measurements offer understanding of pH homeostasis, as well as insight into the oxidative ATP synthesis regulation driven by ATP demand. In short, to preserve stable ATP concentration, hydrolyzed ATP is resynthesized from PCr, causing PCr levels to decrease and Pi levels to increase during exercise. After the

challenge, the PCr buffer is restored primarily through oxidative phosphorylation allowing assessment of mitochondrial function [97]. The fitted PCr time recovery rate constant provides a good estimate on its own, however, it is pH dependent [98]. Using the calculated intracellular pH and consecutively the free ADP concentration [99], maximal oxidative capacity can be estimated providing a more robust parameter of mitochondrial capacity [15].

Unlike in static investigations, it is common to use only single spectral transient in dynamic examinations due to the high temporal resolution required (on the order of seconds) to sufficiently sample the PCr recovery time course. To boost the SNR for these experiments, highly sensitive surface receive coils are deployed and ^{31}P signal is "localized" by their restricted sensitivity volume. However, this type of localization does not allow to differentiate signals that originate from different anatomic and/or morphologic compartments, nor between muscle groups that are recruited differently in the performed exercise (e.g., soleus and gastrocnemius during plantar flexion [100–103]). Quantification of combined signal from differently active muscles significantly skews the measurement of mitochondrial capacity [103–105]. Over the last few years, many different localization techniques have been developed for dynamic ^{31}P MRS [103, 105–107], but as localization decreases available tissue volume and consecutively SNR, they are mainly used at ultra-high fields, i.e., 7T.

Examinations of skeletal muscle metabolism provide not only important information about muscle physiology, but can also be used to observe the effects of aging [108, 109] and/or to help define the training status [86, 110]. In addition, dynamic ^{31}P MR examinations can identify mitochondrial defects in muscular diseases and can uncover decreased oxidative metabolism of skeletal muscle.

3. Muscle MRS and training

Skeletal muscle demonstrates remarkable plasticity in functional adaptation and remodeling in response to contractile activity, i.e., exercise. Training-induced adaptations are reflected by changes in metabolic regulation, intracellular signaling, transcriptional responses and contractile protein and function [111]. Muscle mitochondrial density increases along with concomitant changes in organelle composition in just after 6 weeks of exercise training. Overall, the major metabolic consequences of the adaptations of muscle to endurance exercise are a slower utilization of muscle glycogen and blood glucose, a greater reliance on fat oxidation, and less lactate production during exercise of a given submaximal intensity [112]. Many of the named changes in skeletal muscles caused by exercise may be explored, identified, and potentially quantified by MRS (**Figure 4**). The effect of exercise can be studied from three angles: (i) direct comparison of differently trained subjects; (ii) exploration of acute exercise challenge effects; and (iii) longitudinal studies involving exercise intervention. The effect of dietary interventions on muscle metabolism and the role of MRS will also be discussed.

3.1. Metabolic differences in training status

Increased IMCL content has been reported in endurance-trained muscle indicating the switch to higher utilization and efficiency of fat oxidation, as during long-lasting exercise, IMCL stores are

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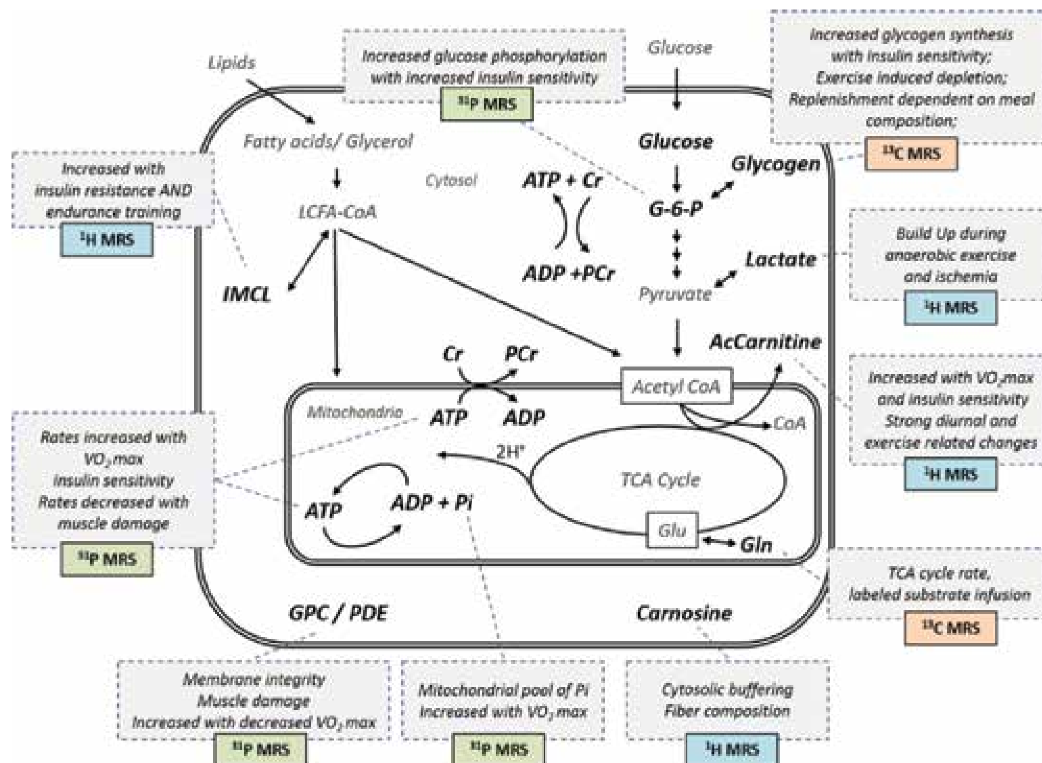

Figure 4. Summary of skeletal muscle metabolic processes exploitable by MRS. Linked in-figure legends denote observable effects, correlations with whole-body metabolic readouts, suggested mechanism in healthy trained, systemic metabolic disease or skeletal muscle myophaties/dystrophies and respective nucleus for MRS. Please note that several of the readouts are affected by both training status and metabolic disease and thus could serve as potential markers of training status and metabolic flexibility. Metabolites are abbreviated as follows: LCFA-CoA, long-chain fatty acid coenzyme A; IMCL, intramyocellular lipids; Cr, creatine; PCr, Phosphocreatine; ATP, adenosine triphosphate; ADP, adenosine diphosphosphate; Pi, inorganic phosphate; GPC, glycerophosphocholine; PDE, phosphodiester; Glu, glutamate; Gln, glutamine; TCA cycle, tricarboxyacid cycle; AcCarnitine, acetylcarnitine; Acetyl CoA, acetyl coenzyme A; G-6-P, glucose 6 phosphate.

utilized as an energy source, similarly to glycogen [113, 114]. The use of these substrates depends heavily on the exercise intensity, and both are replenished in the recovery phase post-exercise. Similarly to IMCL, glycogen levels are also elevated in endurance-trained subjects, which promote their fatigue resistance [115, 116]. The phenomenon of increased IMCL was also termed athlete's paradox, because increased IMCL observed in obese, sedentary subjects are indicative of insulin resistance [17]; however, insulin sensitivity is not impaired in endurance-trained people [18]. IMCL content differs between individual muscle groups, depending on muscle fiber composition. In particular, lower IMCL content has been found in predominantly glycolytic, fiber type II-tibialis anterior, gastrocnemius, and vastus lateralis compared to the predominantly oxidative, fiber type I-soleus and vastus intermedius muscles [117–119]. As the concentration of carnosine is also fiber composition dependent [48, 49], it is no surprise that explosive athletes have 30% higher carnosine levels in gastrocnemius muscle compared to a reference population, whereas it is 20% lower than normal in typical endurance athletes [120]. No significant difference has been reported in acetylcarnitine (AcC) concentration between endurance-trained and

untrained lean sedentary or obese sedentary volunteers [32, 121]. A recent study performed in trained and normally active subjects showed significant differences between AcC concentrations measured after overnight fast or after lunch [33]. This makes the comparison difficult and emphasizes the need for strict standardization of measurement time, dietary conditions, and physical activity (explained below) for the measurement of AcC/carnitine.

Endurance-trained athletes also have a higher volume of mitochondrial density, and, therefore, faster oxidative metabolism which is mirrored by faster PCr resynthesis following submaximal exercise [122]. Faster PCr resynthesis has been demonstrated in comparison to untrained [123–125], and even sprint-trained athletes reflecting superior oxidative metabolism function of endurance-trained subjects [122, 126]. Gradually decreasing training status is also mirrored in decreasing ^{31}P MRS derived measures of mitochondrial capacity and Pi_2/Pi ratio when comparing endurance-trained, lean sedentary and overweight-to-obese sedentary volunteers [86, 92, 110]. Sedentary lifestyle, if accompanied by overweight, type 2 diabetes mellitus or in connection to different muscle specific disease, gives also rise to higher PDE levels in skeletal muscle [84, 86]. Increased PDE levels, although to a much lesser extent, have been also reported in professional cyclists in comparison to normally trained men [85] and in long-distance runners compared to sprinters [82]. These increased PDE levels in highly trained or pathology hampered subjects can potentially indicate persistently damaged (and actively remodeling) muscles as the result of their training or disease. As yet, the connection of PDE to oxidative metabolism and/or muscle integrity is not completely understood.

3.2. Acute exercise challenge

From the metabolic point of view acute exercise challenge relates to changes of concentrations in energy storage pools, e.g., glycogen, lipids, or phosphocreatine, boost in the aerobic and anaerobic metabolism, lactate formation, following pH changes and effects on cell osmotic equilibrium.

From the MRS point of view: although carnosine concentration in gastrocnemius nor in soleus muscles could be influenced by a 1-h-long submaximal street run, the carnosine peak was shown to change in shape, demonstrating an exercise-induced change in pH [31]. The appearance of the second line of carnosine peak can potentially mirror the existence of two skeletal muscle compartments with different pH, possibly as a result of oxidative (slow-twitch) and glycolytic (fast-twitch) fiber composition. Acute exercise has been also shown to alter carnitine metabolism. Low-intensity exercise (below the individual's lactate threshold) does not cause significant changes in the MR detectable muscle carnitine pool, however, after only 10 min of high-intensity exercise, majority of muscle carnitine pool is redistributed to short-chain acylcarnitine. This redistribution is highlighted over a further 20 min of exercise and has long recovery period (over a 60-min) [45, 127]. Likewise, no changes in creatine (Cr) concentration were detected during exhaustive exercise, but a specific change in its methylene (Cr2) resonance line advocate for detection of compartmentation of Cr pool to bound and free sections [23].

Recently, high-intensity exercise challenge to the vastus lateralis muscle by performing squats continuously for 10 min also showed an increase in the AcC level and approximately 15 min after the cessation of exercise, AcC depletion or washout was observed [33]. Similar effect of increasing AcC levels was observed in trained and untrained subject after 30 min of cycle

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ergometer exercise. While, during 40-min recovery period, the AcC signal decayed rapidly in the trained group, it continued to rise in the untrained group [121]. Exercise that results in muscle glycogen depletion are followed by adenine nucleotide loss and muscle fatigue [116, 128]. Later on, depending on the diet and exercise regimen during the recovery, glycogen super-compensation can be seen. Comparing trained cyclists with untrained subjects, it has been shown that endurance-trained subjects resynthesize glycogen faster and are able to accumulate more muscle glycogen during the super-compensation period [116].

IMCL depletion can be observed during prolonged submaximal (60–70% of VO_2max) running or cycling [38, 58, 129, 130], but not during the sprints or repetitive bouts of strenuous exercise [129, 131], supporting the notion that increased IMCL stores serve as important energy reserves for endurance athletes. Following the exercise, repletion of IMCL stores was shown to be dependent on the diet composition in recovery period [16, 58, 130, 132].

3.3. Training interventions

Interventional studies focused on endurance training show an increase in IMCL after the intervention period of 4–6 weeks [133, 134]. On the other hand, 12 weeks of high-intensity training does not seem to have a similar effect [135]. This is potentially due to relative increase of type I oxidative muscle fibers during endurance training and the fact that IMCL concentration is fiber dependent, as discussed earlier. A recent overview of effects of a varying periods and different training types on the carnosine content in the vastus lateralis muscle showed that in most of them carnosine levels did not change after training. Only 8 weeks of power-training led to an increase of muscle carnosine levels [136]. Examining muscle glycogen resynthesis rate and levels after a glycogen-depleting exercise before and after 10 weeks of endurance training exposed higher glycogen concentration as well as an accumulation rate in trained than in untrained state [128], what is in good agreement with studies directly comparing trained and untrained subjects [116]. Eight weeks of endurance training also leads to lower PCr depletion and increased pH levels after exercise [137]. Similarly, the PCr resynthesis rate and muscle mitochondrial capacity can be improved by regular exercise [138].

3.4. Dietary interventions

Alternative approach to alter muscle metabolism without changing the physical activity pattern of an individual is a dietary intervention. This includes calorie restricting diets, carbohydrate loading, as well as substrate supplementation studies. Even very short, but rigorous calorie restriction in obese sedentary subjects leads to decrease in IMCL stores [139]. Although one could expect an improvement in muscular oxidative metabolism to accompany the IMCL reduction, it has been demonstrated using biopsies that mitochondrial capacity is unaltered by diet alone and can be improved only if combined with exercise intervention [140]. Creatine supplementation is often advertised as a tool to increase body mass in body building and physical sports [141]. An increase in total creatine and PCr levels in the muscle can be demonstrated [22], however no improvement in PCr resynthesis has been found after creatine supplementation [22, 142], off-putting the effect on muscle oxidative metabolism. Still, creatine supplementation leads to an increase in glycogen super-compensation [143], and thus can potentially be considered an affective ergogenic aid [141].

Increase in skeletal muscle glycogen super-compensation by carbohydrate loading due to the preceding depletion exercise was also detected in longitudinal study applying ^{13}C MRS [144]. Similar study setup where carbohydrate loading yielded glycogen super-compensation and insulin-stimulated glycogen synthesis as well as glucose-6-phosphate (G-6-P) accumulation was measured by $^{13}C/^{31}P$ MRS during hyperinsulinemic-euglycemic clamp confirmed the hypothesis that glycogen limits its own synthesis through feedback inhibition of glycogen synthase activity, as reflected by an accumulation of intramuscular G-6-P, which is then shunted into aerobic and anaerobic glycolysis [145]. Sequential ^{13}C MRS measurement could also show that caffeine ingestion 90 min before prolonged exercise did not exert a muscle glycogen-sparing effect in athletes with high muscle glycogen content [63].

4. Muscle MRS in metabolic and skeletal muscular disease

Variations in skeletal muscle metabolism are not only connected to training, but are also indicative of many health conditions. Whole-body metabolic disorders, e.g., insulin resistance, T2DM and metabolic syndrome are accompanied by impaired skeletal muscle metabolism [17, 84]. Similarly, skeletal muscle myopathies effect the metabolic health of skeletal muscles [146, 147]. The usability of MRS to monitor these two major groups of diseases influencing muscle metabolism will be discussed now.

4.1. Insulin resistance, T2DM and substrate over-abundance

Insulin-resistant states are characterized by hampered reactions of skeletal muscle to increased peripheral serum insulin concentrations. Insulin signaling, glucose transport and/or phosphorylation, glycogen synthesis, and glycolysis rates are reduced. Many ^{13}C MRS studies have characterized the defects in skeletal muscle metabolism in insulin-resistant states, including experimental manipulations. These studies revealed a ~60% decrease of insulin-stimulated glycogen synthesis in overt T2DM patients [7], as well as a comparable impairment in their lean insulin-resistant offspring [62, 148] and in obese nondiabetic insulin-resistant volunteers [149]. Similar ^{13}C MRS approaches have shown decreased postprandial skeletal muscle glycogen synthesis under normal physiologic conditions after a standard carbohydrate rich mixed meal regimen in T2DM patients [64]. In combination with ^{31}P MRS measurement focused on glucose phosphorylation, i.e., the formation of intramuscular glucose-6-phosphate [148], ^{13}C MRS measurements of intra- and extracellular glucose demonstrated that the lowered glucose transport is one of the main defects effecting whole skeletal muscle glucose metabolism in T2DM [150]. Excellent time resolution of labeled ^{13}C MRS measurements of skeletal muscle resynthesis following a depleting exercise could reveal early insulin independent and subsequent insulin dependent phases of this process [151], from which the latter, insulin dependent, is impeded in insulin-resistant offspring of individuals with T2DM [62].

Combined ^{13}C and ^{31}P MRS has also been used to monitor the effect of lifestyle changes and pharmacological insulin-sensitizing therapy on skeletal muscle glucose metabolism. One

bout of aerobic exercise normalized insulin-stimulated glucose fluxes along with the normalization of whole-body insulin sensitivity in insulin-resistant offspring of T2DM patients [152], while troglitazone treatment improved the skeletal muscle glucose transport and the glycogen metabolism of patients with T2DM [70, 153].

Unlike in endurance-trained volunteers, where IMCLs act as an important energy source for prolonged exercise [113], accumulation of ectopic lipids inside muscle cells in untrained subjects is detrimental. Starting with obesity, through the insulin resistance toward T2DM, IMCL have an increasing tendency, showing a clear correlation between IMCL and insulin sensitivity in sedentary subjects [17], making IMCL a very good indicator of metabolic defect. However, due to the fact that endurance training also leads to increased IMCLs, i.e., due to the athletes-paradox, high IMCL levels cannot be used as a marker of metabolic disorder on their alone. Muscle acetylcarnitine (AcC) levels measured at rest could be potentially used to tip the scales in the right direction, as it has been shown that while T2DM subjects have low muscle AcC concentration, endurance-trained subjects have high stores of muscle AcC [32]. Unfortunately, as the AcC levels are dependent on dietary status and physical activity [33], more studies accounting for these dependencies are required to support these initial findings. Multinuclear MRS studies have also revealed a link between IMCL accumulation measured by ^{1}H MRS and skeletal muscle glucose metabolism [17, 118, 154] assessed by ^{13}C and/or ^{31}P MRS, which has also been studied in different states of insulin resistance and physical fitness [155].

The role of free fatty acids (FFA) and amino acids (AA) serum over-abundance on skeletal muscle glucose metabolism has been investigated in studies simulating the metabolic conditions of T2DM in young healthy men. An experimentally induced increase in plasma FA concentrations showed that substrate over-abundance decreased glucose transport and phosphorylation [156–158], and impaired skeletal muscle glycogen synthesis [156], which precedes the decrease in whole-body glucose uptake in a dose-dependent manner [157]. The observed effect of over-abundance also holds true in various conditions of insulinemia [156–158], as well as with depleted skeletal muscle glycogen [159]. Measuring skeletal glucose transport/phosphorylation and glycogen synthesis in the skeletal muscle of young healthy men during an experimental AA challenge showed a direct effect of AA on glucose transport or phosphorylation [160] and reduced skeletal muscle glycogen synthesis. Substrate over-abundance and defects in lipid oxidation can lead to increased lipid accumulation inside the skeletal muscle. Exchange kinetics between Pi and ATP, measured by ^{31}P MRS ST, are also decreased in T2DM in basal and glucose/insulin challenged conditions [161] as well as in the presence of increased serum FFA in healthy volunteers and hyperinsulinemic-euglycemia [162]. Slower PCr recovery rate after exercise and lower mitochondrial capacity also accompanies obesity [86] and insulin resistance [163, 164]. Similarly, increased muscle PDE levels were found in T2DM and shown to correlate with insulin resistance [84]. However, the PDE dependence on age [86, 165] has to be taken into account when using PDE to compare different metabolic groups.

4.2. Skeletal muscle myopathies

Skeletal muscle pathologies are often characterized by muscle pain, weakness, and defects in skeletal muscle energetic metabolism. From the MRS point of view, changes in relative ^{31}P

metabolite concentrations, i.e., drop in PCr and increase in Pi, were observed in patients with mitochondrial myopathy [97] and Duchenne dystrophy [166]. Increased levels of PDE measured at rest can be indicative of congenital lipodystrophy [87], fibromyalgia [90, 167], or various muscular dystrophies [166, 168, 169]. Slower PCr recovery and decreased mitochondrial capacity was found in patients with chronic fatigue syndrome [170], as well as in patients with lipodystrophy [87]. Pathologic defects in muscle trimethylamine compounds-to-creatine ratio were found in facioscapulohumeral muscular dystrophy already prior to macroscopic muscle fat infiltration [171]. Furthermore, analytic *in vitro* MRS could detect alteration of lipid metabolism in patients with muscular dystrophy in early phase of the disease [172].

5. Summary

Summarizing the knowledge gained from skeletal muscle magnetic resonance spectroscopic studies, we can say that the combination of ^{1}H, ^{13}C, and ^{31}P MRS: (i) can measure intramyocellular lipids deposition, which can be either utilized as a useful energy source in endurance-trained athletes, or is an indication of metabolic disorder (athletes-paradox); (ii) enables quantification of acetylcarnitine that may help to resolve the athletes-paradox; (iii) can improve the knowledge about buffering capacities of skeletal muscle by observing changes in lactate and carnosine metabolism; (iv) can measure glycogen metabolism and glycogenic substrate flux in the skeletal muscle under various conditions; (v) can assess oxidative and nonoxidative energy fluxes in basal and exercise challenged conditions. Taken together, it has helped to uncover defects in skeletal muscle metabolic pathways in insulin-resistant conditions; and to discover links between defects in mitochondrial activity/capacity and lipid metabolism, as well as defects in whole-body and/or muscle glucose metabolism. There is also to mention that several of the MR-derived readouts are affected by both training status and metabolic disease, and thus could serve as potential markers of training status and metabolic flexibility.

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Conflict of interest

None of the authors or authors' institutions have any conflicts of interest to disclose.

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Section 3

The Characteristics of Neuromuscular Disease

Chapter 5

Skeletal Muscle Fiber Types in Neuromuscular Diseases

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Additional information is available at the end of the chapter

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Abstract

In the human body, there are 600 individual skeletal muscles that allow us to perform a variety of functions such as executing locomotive tasks, breathing, and moving our eyes. The ratio of fiber types within the muscle critically contributes to determine the function of these muscles. Significant changes of muscle fiber types occur not only in normal development; changes have also been observed under abnormal conditions in neuromuscular disorders. In this review, we describe how muscle fiber types are specified during embryonic myogenesis, what potential factors are involved in the changes of fiber type composition, and how fiber type variations are influenced by the pathological conditions under specific neuromuscular disorders. Understanding skeletal muscle at the individual fiber level aids in studying the normal physiology and the pathology of disease in human.

Keywords: muscle fiber type, neuromuscular disease, skeletal muscle, Type I, Type II

1. Introduction

Skeletal muscle is the most abundant tissue in human body. Skeletal muscle accounts for approximately 20% of our resting energy expenditure [1], and composes 30–40% of one's body mass [2] depending on their fitness level [3]. As a part of the musculoskeletal system, skeletal muscle is connected to the skeleton to form part of the mechanical system that moves the limbs and other parts of the body. While skeletal muscle refers to multiple bundles of cells called muscle fibers, the composition of the individual fibers is different between muscle types. In this review, we describe how muscle fiber types are specified during embryonic myogenesis, what potential factors would be involved in the changes of fiber type composition, and how fiber type variations are influenced by specific disease conditions. Knowing the functional role of how muscle fibers contribute to and are affected by skeletal muscle diseases

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aids in our understanding of the disease and provides insight to mechanisms of prevention, treatment, or cure of these conditions.

2. Skeletal muscle

Skeletal muscle plays important roles in the body that are concerned with movement, posture, and balance under voluntary control. Skeletal muscles are one of three major muscle types, the others being cardiac muscle and smooth muscle, and it is the most common of the three types of muscle in the body. As one component of the musculoskeletal system, skeletal muscle is attached to bones by tendons, and they produce all the movements of body parts in relation to each other. Unlike smooth muscle and cardiac muscle, skeletal muscle is under voluntary control. Similar to cardiac muscle, however, skeletal muscle is striated; it has long, thin, multinucleated fibers (known as myofibers).

3. Skeletal muscle development and fiber type specification

Skeletal muscle function seems to be maintained across mammals, but the composition of the individual fibers is different between muscle types [4]. Fiber type composition is initially defined in each muscle during embryonic myogenesis. In this section, we will go through the basic fundamentals of skeletal muscle development and fiber type specification (**Figure 1**).

3.1. Embryonic myogenesis

Studies investigating embryonic myogenesis have been extensively conducted in the embryos of zebrafish, chicken, and mice. After an embryo is generated, three germ layers (ectoderm, endoderm, and mesoderm) are formed. The mesoderm is characterized as paraxial, intermediate, and lateral mesoderm. The formation of skeletal muscle initiates from the paraxial mesoderm in early embryogenesis. In response to the signals from the notochord, neural tube, and surface ectoderm, the paraxial mesoderm forms segmented spheres termed somites. The somites are located as a pair on either side of the neural tube and the notochord and develop in a rostral-caudal direction. The somite is further specified as the dermomyotome, myotome, and sclerotome. The cells in the dermomyotome express the paired box transcription factors Pax3 and Pax7 [5, 6]. The cells in the dorsomedial and ventrolateral portions of the dermomyotome will give rise to the epaxial (primaxial) and hypaxial (abaxial) myotomes, respectively. Myf5-positive cells in the epaxial myotomes differentiate and form the trunk and back muscles. In contrast, MyoD-positive progenitors de-laminate and migrate from the hypaxial myotome into the developing limb as the source of limb muscles. MyoD and Myf5 are expressed in committed muscle cells, and are located in the myotome, which is form the maturation of dermomyotome lips [7–9]. The ventrolateral lip of the dermomyotome contributes to the hypaxial myotome, which is a source of precursor cells that form the trunk and thoracic vertebral column muscles. The dorsomedial lip of the dermomyotome contributes to the epaxial myotome, which is a source of muscles of the back. The process of myotome maturation originally initiates at the rostral part of the embryo and then extends to the tail [7].

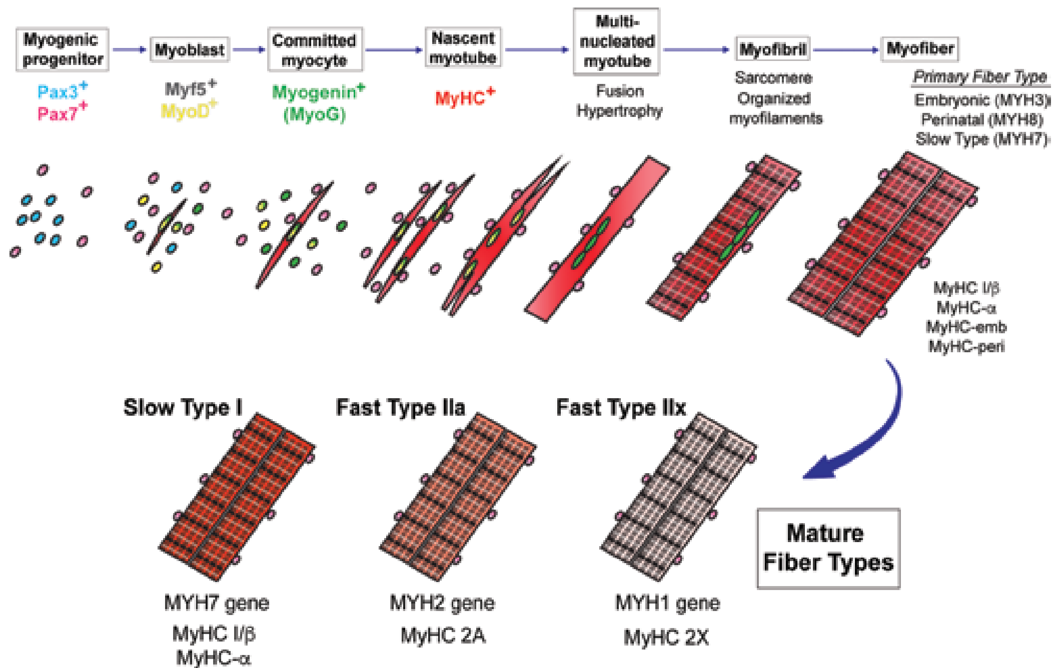

Figure 1. Skeletal muscle differentiation and fiber type specification. The terminal differentiation starts when Pax3+ and/or Pax7+ progenitors begin to express Myf5 or MyoD as committed myoblasts. Theses myoblasts gradually express myogenin (MyoG) and form single-nucleated nascent myotubes and multi-nucleated myotubes with myosin heavy chain (MyHC+). Actin, myosin, and elastic myofilaments are arranged to form organized sarcomeres within the myotubes. Primary myofibers express four isoforms of MyHC: MyHC I/β, MyHC-α, MyHC-emb and MyHC-peri. Development of fiber type continues as satellite cells differentiate and the fibers become innervated, forming mature fiber types. Different isoforms of myosin, MyHC I/β. MyHC-α, MyHC 2A, and MyHC 2x, are expressed. This figure is modified from Jiwlawat et al. [10].

3.2. Terminal differentiation and myofiber formation

The terminal differentiation of progenitors and myoblasts initiates when myogenic progenitors in the dermomyotome stop dividing and exit the undifferentiated stage [10]. The progenitors differentiate into committed myoblasts, and form nascent myotubes following the maturation of the myotome [11]. More specifically, Pax3 and/or Pax7-positive proliferating progenitors are withdrawn from the cell cycle once the differentiation step is initiated (**Figure 1**). These progenitors become committed myoblasts expressing Myf5 and/or MyoD and then form the nascent myosin heavy chain-positive myotubes with myogenin-positive nuclei.

Two waves of myotube formation occur during skeletal muscle development, and sequentially give rise to primary and secondary myotubes [12]. Primary myotubes are generated from fusion of early myoblasts, and then align between muscle tendons. Late-stage myoblasts proliferate on the surface of primary myotubes and fuse to form secondary myotubes, and motor axons initiate innervation to the myotubes [12]. At this point, primary and secondary myotubes express specific isoforms of myosin heavy chain (MyHC), which can be used to broadly define two distinct fiber types, slow-twitch Type I and fast-twitch Type II myofibers.

Primary myotubes preferentially express Type I fibers [13, 14], while Type II fibers appear later during myogenesis [15, 16]. Single-nucleated myotubes then fuse with the nearby myotubes to form multi-nucleated myotubes. Thick-myosin and thin-actin filaments within the myotube begin organization and form a sarcomere structure, which is the functional unit of muscle contraction. Well-organized sarcomeric structure gives rise to a striation pattern in myotubes, representing many chains of myofibrils.

3.3. Fiber type specification

Primary myogenesis starts during the embryonic stage, when somatic stem cells express the genes Pax3 and Pax7 (**Figure 1**). This transforms the cells into myogenic progenitors, which migrate from the dermomyotome to form myocytes and primary myofibers. At this point of embryonic myogenesis, three isoforms of myosin heavy chain are expressed; slow MyHC (MYH7), MyHC-emb (MYH3), and MyHC-peri (MYH8) [17]. These primary myofibers serve as a template for the skeletal muscle to mature and differentiate. Secondary myogenesis progresses as satellite cells differentiate, become innervated, and mature myofibers are formed. In whole, genetic influences and motor neuron innervation during developmental differentiation determines the fiber types that one is born with [17]. Fiber type ratios determined at birth are not concrete throughout one's life however, as skeletal muscle chemical properties can change over time to meet physiological or pathological demands.

4. Muscle fiber types

Skeletal muscle tissue in humans is heterogeneous, composed of a variety of molecules [4]. The main functional proteins and structures within the muscle are maintained, such as mitochondria network, myosin, actin and titin. Yet, the specific isoforms of the molecules and the concentration of each monomer differ between skeletal muscles all throughout the body. These heterogeneous tissues are a resultant factor of evolution which allows each muscle to have a specialized function. The size of each whole muscle is determined by both the number and the diameter of muscle fibers that compose it. Individual muscle fibers are multinucleated, with each nucleus controlling the protein type, myosin that is translated in its surrounding. This is known as a nuclear domain [18].

Myosin is the main protein within skeletal muscle, and the certain isoform that is expressed determines the rate at which the muscle contracts, as well as its physiological properties. Within a single sarcomere of a skeletal muscle fiber, myosin heads and actin interact to form cross bridges. ATP hydrolyzation via ATPase is responsible for the energy to cause cycling of the myosin head and actin connections, which ultimately causes the muscle contraction. The type of myosin expressed is one factor that ultimately determines the fiber type. There are 11 total isoforms of myosin known to mammals [4, 19], which when expressed in different ratios compose a fiber type with distinct physiological properties. As discussed above, there are two categories of adult muscle fiber types in humans; Type I and Type II fibers (**Figure 1**).

Type I and Type II fibers are classified based on their myosin isoform, velocity of contraction and presence of physiological enzymes [3]. Type I fibers are also known as slow oxidative. Compared to Type II, they contain a higher number of oxidative enzymes and a lower number of glycolytic enzymes. They are rich in mitochondria and have a great capillary network to perfuse the fibers [20]. This contributes to their oxidative capacity. Type I muscle fibers predominantly contain myosin isoforms MyHC I/β or MyHC-α, encoded by the gene MYH7 [17] and they contract slower and are more resistant to fatigue than Type II fibers. Because of their endurance properties, Type I fibers are commonly found in muscles mainly involved in posture, such as erector spinae, hamstrings, and gastrocnemius muscles.

Type II fibers on the other hand are fast to fatigue, as they have low oxidative capacity. These fiber types are recruited in short bursts of movement or power [3]. This is due to their greater maximal velocity of shortening, and abundance of glycolytic enzymes [3]. This in turn allows for quick energy utilization due to increased ATPase activity. There are two subcategories in human Type II fibers; Type IIa and Type IIx. Type IIa are classified as fast-oxidative glycolytic, a sort of combination between fast and slow contraction rates. Type IIx are fast glycolytic, having the fastest rate of contraction of all the human fiber types, yet the shortest time to fatigue. MyHC isoform genes MYH2 and MYH1 are expressed respectively in Type IIa and Type IIx fibers. The myosin protein isoforms present in each subtype are termed MyHC-2A and MyHC-2X [17].

Skeletal muscles are innervated by motor neurons which are responsible for the initiation of muscle contraction. Motor units are formed, consisting of a single alpha motor neuron that originates in the spinal cord that innervates a group of skeletal muscle fibers, all of the same fiber type. Changes in motor unit innervation of the skeletal muscle has shown to change the properties of fiber types innervated, therefore motor units too are contributors to the determinants of fiber type [21].

5. Factors influencing muscle fiber type composition

5.1. Physiological and pathological changes

Skeletal muscles have the property of plasticity. This means the composition of fiber types within a given skeletal muscle can change when under the influence of physiological changes such as mechanical stress and unloading. Further, abnormal health conditions caused by diseases and injuries also triggers significant changes of muscle fiber types [22]. The size and functional capacity of the muscle can be decreased upon injury, disease, or excess weight. As a result, scar tissue, connective tissue, or fat can take up mass that was once occupied by functional muscle [18]. When muscles become denervated, there is a tendency for slow to fast fiber transition [3]. This carries heavy implications for training status and disease state in humans [3, 23, 24].

5.2. Genetic and epigenetic controls

The physiological and pathological changes influence the levels of trophic factors, hormones, and nerve signaling associated with the muscle, which result in adaptive changes in muscle

fibers. The relative amounts of these factors and the extent of the changes that they can make are ultimately determined by genomic background and epigenetic control in individuals. The genes that one inherits controls and determines 40–50% of the ratio of Type I fibers within a muscle [3]. This means that physiological stressors can impact the plasticity of the muscles to a point, but in the end one's genetic make-up determines the extent to which the fiber types within the muscle can switch [3]. Like all cells in the body, the different fiber types contain the same genomic DNA sequence. MYH genes have been hypothesized to be clustered in a manner to facilitate temporal and spatial expression of these related genes [23]. Slow MyHC isoforms are located on chromosome 14, while chromosome 17 contains the fast and embryonic MYH genes in a cluster. The difference in gene expression, and resultant protein levels in a specific cell, are controlled by epigenetic mechanisms. As fiber types shift within a lifetime, the epigenetic profile within the cell is also affected, specifically in the amount of acetylation or deacetylation within the genome. This change is mostly seen within differentiating satellite cells, which are not fully mature [23]. Further, variations in expression levels of genes controlling systems such as mitochondrial biogenesis, glucose/lipid metabolism, cytoskeletal function, hypoxia, angiogenesis, and circulatory homeostasis would influence muscle fiber type. The frequency of alleles within a genome also impact the fiber type development [3]. Overall, there are many genetic factors at play such as single gene effects, gene–gene interactions and gene–environment interactions [3].

6. Muscle fiber types and musculoskeletal diseases

Neuromuscular diseases are caused by functional defects of skeletal muscles, directly via muscle pathology or indirectly via disruption of the nervous system. Most of these diseases are multi-facetted, and terminally result in wasting and atrophy of skeletal muscles. These abnormal conditions often lead to disabilities and complete loss of muscle function, with little to no cure. Pathology is best understood at the cellular level, and here we explore how the progression of the disease is involved in the changes of muscle fiber types, and how changes in fiber type may serve as a protective mechanism. Diseases covered in this chapter are mainly genetic in nature, having an uncontrollable disruption in cellular function that results in disease. This can either be inherited from previous ancestors or be sporadic in nature. This section will introduce several names of muscle and motor neuron diseases; however, this is not an exhaustive list.

6.1. Skeletal muscle diseases

6.1.1. Sarcopenia

Sarcopenia is a term that refers to the loss of lean body mass, particularly skeletal muscle, with an increase in aging [25]. This can be diagnosed through weakness within the body, difficulties walking, or dual-energy absorptiometry, which is a machine that tells the exact body composition of fat, bone mass and tissue. Sarcopenia at the individual fiber level is characterized by a loss of satellite cells associated with Type II fibers [18]. Organelles affected

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in the myofibers include a decreased amount of mitochondria, an alteration in the sarcoplasmic reticulum, and hindered excitation-contraction coupling. Both Type I and II fibers have shown to be affected by losing their maximal force in both men and women. This is attributed to a loss of myosin expression within the cell, or oxidation of the myosin protein which inhibits the formation of crosslinks [18]. Surprisingly, the expression levels of myosin isoform MYH7, that of slow muscle fibers, are not affected [26].

6.1.2. *Muscular dystrophies*

Muscular dystrophies are a group of muscle diseases that result in the wasting of skeletal muscles, caused by muscle fiber necrosis [27]. The dystrophies involve mutations in genes that encode functional proteins involved in dystrophin or enzymes that modify the dystrophin proteins [18]. These mutations affect velocity of cross bridge cycling of actin filaments on myosin and of particular interest, they change the quality and force production of Type I and Type II fibers [18]. Apoptosis and necrosis in fiber types are a trademark of the disease, with caspase 3 being a known apoptotic gene that is upregulated in muscular dystrophies compared to unaffected individuals [27].

In Duchenne Muscular Dystrophy, Type II muscle fibers are the first to be affected with Type I muscle fibers following late in the disease progression [26]. Remaining Type I fibers are not similar to those found in healthy muscle. Degeneration and regeneration of diseased fibers is hypothesized to take place, due to coexpression of fetal MYH and slow MYH genes in adult muscle fibers [28]. Since Type II fibers are the most commonly affected in Duchenne Muscular Dystrophy, it is thought that inducing the expression of Type I fibers will alleviate both the symptoms and progression of Duchenne Muscular Dystrophy. A similar trend was found in another type of muscular dystrophy, Facioscapulohumeral Muscular Dystrophy, as there is an early decrease in Type II fibers and an overall increase in the number of Type I fibers [29]. On the contrary, in myotonic dystrophy, Type I fibers are affected, as they atrophy more frequently and they lose a greater amount of force generation compared to Type II fibers [30–32]. One hypothesis for this fiber type susceptibility to disease states is variation to transcriptional control of muscle fiber type. Genetic manipulations and pharmacological interventions have shown the effect of fiber type switching on disease sates in mice [26]. For example, over expression of the transcriptional coactivator PPARGC1A rescues the cellular defects cause by the Dmd^{mdx} mutation via increased expression of Type I fiber contractile machinery and oxidative enzymes [26].

6.2. Motor neuron diseases

Motor neuron diseases are characterized by the progressive degeneration of motor neurons with subsequent functional loss. In the motor system, motor neuron axons carry the motor impulses from the spinal cord to the voluntary muscles. Innervation of alpha motor neurons from the central nervous system has a large part in determining the fiber type that is expressed within muscles. Motor units innervate muscle fibers in an “all or none” fashion, meaning a single motor unit innervates Type I and each subcategory of Type II fibers individually, and all the fibers that the motor unit innervate are of the same fiber type.

Co-expression of fiber types within a single muscle fiber has been seen in motor neuron diseases such as Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy [33]. In these diseases, specifically ALS, studying disease influence on skeletal muscles would provide valuable insight to the mechanisms of disease progression. Changes in muscle fiber types may occur at the early stage of diseases by reduced inputs from motor neurons, as disconnections between muscle and axon terminals have been observed in animal models of motor neuron diseases before symptom onset. Studying the disease onset in skeletal muscles has the potential to reveal the catastrophic pathology influence and the body's compensatory mechanisms to counteract disease progression [34].

Switches in muscle fiber type has been observed in patients in motor neuron diseases, however the switches cannot prevent the ultimate outcome: apoptosis and necrosis of individual muscle fibers [27]. Often, motor neuron diseases are diagnosed clinically via histochemical staining of muscle biopsies. Necrosis can be easily seen as fat or scar tissue under the microscope, but apoptosis is harder to identify due to the lack of inflammatory response from the body [27]. Denervation of the muscle results in upregulation of pro-apoptotic genes, such as bax and anti bcl-2, which are upregulated due to intrinsic cell stress. Muscle fiber atrophy is hypothesized to be caused by apoptosis induced degradation of a fiber's nuclei. This includes destruction of the nuclear lamina, the nuclear envelope, and DNA destruction [27].

6.2.1. Amyotrophic lateral sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is a fatal neurodegenerative disease caused by the selective loss of motor neurons in the spinal cord and brain stem. Motor neuron degeneration and neuromuscular junction denervation rapidly result in decreased motor function. Death typically results 3–5 years after diagnosis due to respiratory failure after loss of diaphragm control. About 90% of ALS cases occur sporadically; the remaining 10% are familial components. Approximately 70–80% of familial ALS have mutations of the Cn^{2+}/Zn^{2+} superoxide dismutase 1 (*SOD1*), *TDP43*, *FUS*, or *C90ORF72* genes [35].

Although a disease cause of sporadic ALS has not been specified, this disease is generally regarded as resulting from factors involving environment, lifestyle, aging, and genetic predisposition [36]. Several proposed pathological mechanisms of disease include protein misfolding and aggregation, glutamate excitotoxicity, oxidative stress, mitochondrial dysfunction, glial cell activation and related inflammatory processes, and axonal transport defects [37].

ALS causes motor neuron death and gradual denervation of skeletal muscles over time. This denervation causes loss of muscle function and muscular atrophy within affected cells, ultimately resulting in cellular death due to apoptosis. While many of existing ALS therapies are expected to promote motor neuron survival in the spinal cord or motor cortex [38], looking at the pathologies within skeletal muscle gives support for the "dying-back" hypothesis. This hypothesis states that irregularities within the skeletal muscle are the primary cause of ALS, denervating muscle and motor neuron [39]. Based on this hypothesis, possible contributions of skeletal muscles and neuromuscular junctions in ALS pathology have been proposed in recent studies. Specifically, our research group also reported that using stem cells to deliver growth factors directly into the skeletal muscle could restore motor function in a rat model of

familial ALS [40–42]. Our approach can sufficiently protect motor neurons by preventing the "dying back" of these cells from the skeletal muscle in ALS.

While the majority of ALS patients have limb onset, about 25% of cases eventually diagnosed with ALS have bulbar onset which strikes the corticobulbar area in the brain stem. This section controls muscles in the face, neck and head. Bulbar onset usually affects voice and swallowing first. Patients with bulbar onset have a correlation between the age of death and the loss of slow tonic fibers, although there is neither correlation seen in spinal onset ALS nor controls [34].

ALS pathology affects skeletal muscle in many ways, which seems to influence muscle fiber type changes. Autopsies show fiber atrophy, fiber grouping, fiber splitting, with increased fatty tissue and connective tissue [43]. Interestingly, unlike atrophy from exercise, ALS shows a fast to slow fiber type switch [39, 44]. In the hindlimb muscle (tibialis anterior muscle) of pre-symptomatic ALS model mice, there is denervation of the most forceful and fast to fatigue fibers Type IIB (only found in mice). This results in transitions to fast motor units with intermediate fatigue and fatigue resistant fibers. Although this transition is present, it is not a sudden change nor a complete loss of Type IIB fibers [44]. Biopsies taken from atrophied skeletal muscles in patients with ALS have shown that individual muscle fibers contain myosin isoforms corresponding to both fiber Types I and II, termed a mixed fiber type. An early pattern of denervation can be detected and has the potential to be used for diagnostic purposes. This pattern is individual fibers with a mixed fiber type and little fiber type grouping, all within an atrophying muscle [45].

It has been reported that specific muscle groups such as extraocular muscles are relatively spared from the disease phenotype in ALS [46]. Motility of the eye is often maintained in ALS patients [47] and autopsies have shown the extraocular muscles do have some muscle fiber pathology compared to control, but in relation to other ALS affected skeletal muscles in the body, the extraocular muscles were well preserved [43]. The pathology that was seen include change in fiber type composition, the cellular architecture, and decreased overall MyHC content. Embryonic MyHC was almost nonexistent in the extraocular muscles in those affected by ALS [43].

This preservation of extraocular skeletal muscle is accredited to the distinct fiber type composition within the extraocular muscles. Extraocular muscles have a unique myosin expression that is not found in skeletal muscles located other places of the body. Along with Type I and Type II fibers, a special myosin isoform, MyHC extraocular, is present and Type I fibers seem to express two separate forms of MyHC, of specific interest MyHC α cardiac. [43]. Embryonic MyHC has notable expression in the extraocular muscles, as healthy human controls show co-expression of embryonic MyHC in Type II fibers, while ALS patients had no embryonic MyHC expression [43, 48].

Although there is great speculation, the exact mechanism of why extraocular muscles are spared in ALS is unknown. However, one interesting hypothesis is the multiple innervations of slow tonic fibers serve as a protective mechanism against the neurodegenerative disease [48]. It has also been found that the motor neurons of the extraocular muscles have different surface markers than motor neurons found elsewhere in the body, suggesting they have

properties that make the neurons less susceptible to disease [49]. As an additional note, similar specific insusceptibility in the extraocular muscles has also been observed in Duchenne Muscular Dystrophy [50].

Another question is how sex influences fiber type specification in the muscle during ALS pathology. The exact etiology of ALS is still uncertain, but most epidemiological studies have shown a higher incidence of ALS in men than women. Interestingly, sexual dimorphism in disease onset and progression is also observed in rodent models of familial ALS [51, 52]. Although it is still uncertain whether such sexual differences are originated from the intrinsic difference in individual cells [53], further studies would be required to answer this question.

6.2.2. Spinal muscular atrophy

Spinal Muscular Atrophy (SMA) is a group of motor neuron diseases, which are autosomal recessive in nature. Each SMA type has a different clinical outcome, however all SMA types commonly demonstrate motor neuron degeneration caused by insufficient expression of a specific protein named Survival of motor neuron (SMN) [54]. The clinical severity of SMA ranges from I–IV, with IV being the least severe. I is infantile SMA that causes death early in childhood and IV involves some motor neuron loss, but allows for a normal life expectancy [55].

All cases of SMA result from reductions in levels of the SMN protein. Specifically, SMA is caused by deletion or mutation of the survival motor neuron gene (SMN1). The SMA disease is present in a spectrum of disease severities ranging from infant mortality, in the most severe cases, to minor motor impairment, in the mildest cases. The variability of disease severity inversely correlates with the copy number, and thus expression of a second, partially functional survival motor neuron gene, SMN2.

In type III SMA-induced mice, muscle atrophy resulted in a transition to slower, oxidative phenotype. This meaning that there were more Type I fibers in the soleus muscle and Type II fibers in fast twitch muscles transitioned to a more oxidative fiber type [54]. These same mice also had smaller motor neurons units than controls and the Type I motor neurons decreased in size as the disease progressed. Other studies that have used type III SMA-induced mice have shown to have increased fiber type grouping compared to wild type [56].

There has been evidence that these pathological changes in muscle fiber types can be reversed. Swimming aided the mice to regain more glycolytic fast twitch fibers, and restore Type I motor unit size close to wild type levels [54]. Running produced more Type I fibers compared to sedentary SMA mouse control [54] and was able to restore SMA fast fiber types. Upon completion of exercise intervention by type III SMA-induced mice, their structure and number of the Type I fibers were comparable to controls [54].

In humans, it has been shown that innervation of fibers in children with SMA (specifically Werdnig-Hoffmann disease) is incomplete. This results in atrophy of fibers and the inability of fetal MyHC to switch to adult Type I and Type II myosin. When this observation was tracked through childhood it showed that in infancy, there is a large increase in the number of Type I fibers, and no detectable Type II fibers by 20 months. This further emphasizes the need for motor neuron innervation for Type II fibers to prevail [4, 57].

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7. Conclusions

Muscle fiber type composition is primarily determined during development but will be altered by physiological and pathological conditions. Significant changes of fiber type composition have been identified in the muscles with a background of major neuromuscular diseases. To further understand the roles of muscle fiber composition in skeletal muscle development and diseases, additional studies using new research approaches may help us understand how muscle fiber type specification occurs during development and disease conditions. For instance, skeletal muscle cell culture derived from human pluripotent cell resources can provide a new tool to study how human skeletal myocytes differentiate into myotubes with specific fiber types in culture [58, 59]. These studies could highlight what specific mechanisms are involved in the significant changes of fiber type composition and ratio in the skeletal muscle during embryonic myogenesis and under disease conditions, and how these changes of muscle fiber types impact on muscle physiology and pathology.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Chapter 6

Hereditary Myopathies

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Additional information is available at the end of the chapter

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Abstract

Hereditary myopathies are inherited disorders primarily affecting the skeletal muscle tissue. These are caused by mutations in different genes-encoding proteins that play important roles in muscle structure and function. Skeletal muscle weakness and hypotonia are typical clinical manifestations in most of hereditary myopathies. Histological features such as fiber type disproportion, myofibrillar disorganization, and structural abnormalities are usually observed in muscle biopsies of non-dystrophic myopathies, while fibrosis, fiber regeneration, wasting, and atrophy are characteristic of dystrophic myopathies. However, similar histopathological features may overlap in different hereditary myopathies. This is how mutations in a same gene can lead to different forms of hereditary myopathies and a same myopathic phenotype can derive from defects in different related genes making difficult a specific diagnosis. In this regard, understanding all aspects of hereditary myopathies can facilitate a better diagnosis and treatment. In this chapter, we offer a review of some of the most prevalent hereditary myopathies, highlighting clinical, histological, and molecular aspects of these muscle disorders.

Keywords: hereditary myopathy, muscle disease, congenital myopathy, muscular dystrophy

1. Introduction

Hereditary myopathies are a heterogeneous group of inherited diseases primarily affecting the skeletal muscle tissue. These are caused by mutations in genes encoding proteins critical for muscle structure and function, with X-linked, autosomal-recessive or -dominant inheritance pattern. Hereditary myopathies include several forms of dystrophic and non-dystrophic disorders with a wide spectrum of genetic, biochemical, histological, and clinical features. A common characteristic

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is the presence of hypotonia and progressive or non-progressive muscle weakness. The onset of hereditary myopathies is commonly at birth, although they may become evident later in childhood or adulthood. Clinical severity is variable being the early-onset forms usually more severe [1]. Diagnosis of hereditary muscle diseases involve physical and neurological evaluation, electromyography and nerve conduction studies (EMG and NCS), magnetic resonance imaging, blood tests including creatine kinase levels (CK), which typically rises in muscle damage and histopathological makers in muscle biopsies [1]. Advances in molecular genetics have allowed identifying an increasing number of genes linked to different forms of hereditary myopathies in the last decades. With this, it has become evident that mutations in a same gene can lead to more than one pathological and clinical phenotype as well as the same pathological feature can result from mutations in different genes. Given this overlap in genetic, clinical, and histological features, the use of different approaches is critical for a proper diagnosis.

This chapter aims to summarize clinical, histological, and molecular aspects of some inherited forms of muscle disease, providing a general overview of the most prevalent hereditary myopathies, including congenital, mitochondrial, and metabolic myopathies, myotonia, and muscular dystrophies. The information discussed in this chapter is resumed in **Table 1**.

Hereditary myopathy	Genes/proteins	Mechanism affected	Histopathological features	Clinical features
Non-dystrophic				
Nemalin myopathy	*NEB*/nebulin *ACTA*/skeletal muscle alpha-actin *TPM3*/alpha-tropomyosin-3 *TPM2*/beta-tropomyosin-2 *TNNT1*/troponin T1 *CFL2*/cofilin-2 *KBTBD13*/Kelch-repeat-and-BTB-domain-containing-13 *KLHL40*/Kelch-like-family-member-40; KLHL4 *KLHL41*/Kelch-like-family-member 41 *LMOD3*/leiomodin-3 *MYPN*/myopalladin	Structural disorganization of sarcomere units	Rod inclusions	Hypotonia, proximal weakness, respiratory insufficiency in the most severe cases
Core myopathy (Central and multi-minicore)	*RYR1*/ryanodine receptor *SEPN1*/selenoprotein N	EC-uncoupling Defects in Ca^{2+} and redox homeostasis	Cores and/or multiminicores	Hypotonia, proximal weakness, delayed motor milestones and orthopedic complications. Malignant hyperthermia susceptibility
Centronuclear myopathy/ myotubular myopathy (XLMTM)	*MTM1*/myotubularin *DNM2*/dynamin-2 *BIN1*/amphiphysin-2 *RYR1*/ryanodine receptor channel	Defects in membrane remodeling and intracellular trafficking	Nuclei centralization Necklace fibers	Proximal and distal muscle weakness, delayed acquisition of motor milestones, difficulty to walk

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Hereditary myopathy	Genes/proteins	Mechanism affected	Histopathological features	Clinical features
	TTN/titin *SPEG*/striated muscle preferentially expressed protein kinase			and climb stairs. Facial muscles involvement typically presenting ptosis and ophthalmoplegia. Persistent hypotonia and respiratory muscles failure in the most severe cases of XLMTM.
Congenital fiber-type disproportion myopathy	*TPM3*/alpha-tropomyosin-3 *ACTA*/skeletal muscle alpha-actin *RYR1*/ryanodine receptor channel	Defects in acto-myosin interaction Defects in EC-coupling	Prevalence and hypotrofia of type 1 fibers (5-40% smaller than type 2)	Mild to severe weakness mainly in shoulders, upper arms, hips, and thighs musculature and in face muscles; orthopedic complications and joint contractures . Respiratory muscle hypotonia is observed in some cases.
Myosin storage myopathy	*MYH7*/slow-skeletal/β-cardiac myosin heavy chain	Defects in myosin folding Impaired sarcomere thing filament assembly	Hyalin bodies	Hypotonia, proximal muscle weakness, delayed acquisition of motor milestones, respiratory insufficiency secondary and cardiac involvement may occur in some cases.
Mitochondrial myopathy	Cytochrome b/cytochrome c oxidase Q10 Coenzyme *TK2*/thymidine kinase *POLG1*/polymerase gamma 1	Dysfunction of the respiratory chain and energy production	Sub-sarcolemmal and intermyofibrillar accumulation of mitochondria; "ragged blue/red fibers"	Typically are multisystem disorders with predominant involvement of muscles and nerves. Myalgia, fatigue, exercise intolerance, proximal and distal muscle weakness and slowly progressive paresis of the extra ocular muscles. Marked hypotonia, respiratory muscle weakness and

Hereditary myopathy	Genes/proteins	Mechanism affected	Histopathological features	Clinical features
				feeding difficulty in most severe cases.
Metabolic myopathy (glycogen storage disease)	*GAA*/lysosomal enzyme acid α-glucosidase *AGL*/glycogen-debranching enzyme *PYGM*/myophosphorylase	Defects in glycogen hydrolysis and energy production	Basophilic/PAS positive vacuoles cytosolic inclusions	Generalized muscle weakness and hypotonia, fatigue and exercise-induced myalgia, cardiac and respiratory failure in most severe cases
Metabolic myopathy (lipid storage disease)	*SLC22A5*/carnitine transporter OCTN2 *ETF*/electron-transfer flavoprotein *ETFH*/ETF-dehydrogenase *PNPLA2*/adipose triglyceride lipase	Fatty acid dysmetabolism and defects in energy production	Increased number and size of lipid droplets and neutral-lipid containing vacuoles inside muscle fibers	Hypotonia, muscle weakness and cardiomyopathy systemic disorders are often observed including encephalopathy, hepatomegaly, hypoglycemia, and metabolic acidosis
Congenital myotonia	*ClC1*/skeletal muscle chloride channel	Reduced chloride conductance Enhanced sarcolemmal excitability	Predominance and hypotrophy of type 2 fibers, increased endomysial connective tissue and tubular aggregates are usually observed	Myotonia, myalgia, transient episodes of generalized weakness and muscular hypertrophy
Paramyotonia congenita	*SCN4A*/alpha-subunit of the skeletal muscle sodium channel	Abnormal persistent sarcolemmal sodium currents	-	Early-onset generalized weakness and "myotonic discharges" and extreme sensitivity to cold
Dystrophic				
Myotonic dystrophy (DM1/DM2)	*DMPK*/myotonin-protein-kinase (CTG repeat expansion) *ZNF9*/zinc finger 9 (CCTG repeat expansion)	Deregulation of RNA-binding proteins, toxic nuclear foci and impair gene expression	Central nuclei, type 1 fiber atrophy, regenerating fibers, fibrosis and adipose deposition. Atrophic type 2 fibers with pyknotic nuclear clumps are specifically observed in DM2	Muscle wasting, progressive weakness, myotonia, cataracts, and multi-organ involvement affecting heart, brain, and endocrine system
Duchenne and Becker muscular dystrophy	*DMD*/dystrophin	Defects in DGC , mechanical stress during muscle contraction, sarcolemmal damage and	Muscle fiber necrosis, regeneration, fibrosis and atrophy, nuclei internalization, Inflammatory	Early-onset dystrophy, delayed acquisition of motor milestones and rapid progression of muscle weakness

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Hereditary myopathy	Genes/proteins	Mechanism affected	Histopathological features	Clinical features
		abnormal Ca^{2+} homeostasis	response and elevated serum levels of CK are typically observed	that usually leads to wheelchair needing.
LGMD1	*MYOT*/myotilin *LMNA*/lamin A/C *CAV3*/caveolin3 *DNAJB6* *DES*/desmin*TNPO3*/transportin3 *HNRPDL*	Defects in sarcomere integrity, nuclear maintenance and gene regulation among others	Muscle fiber necrosis, regeneration, fibrosis and atrophy, nuclei internalization, Inflammatory response and elevated serum levels of CK are typically observed	Proximal and distal weakness, calf hypertrophy, cramps associated to exercise and respiratory and cardiac involvement in some cases
LGMD2	*CAPN3*/calpain3 *DYSF*/dysferlin alpha-sarcoglycan beta-sarcoglycan gamma-sarcoglycan delta-sarcoglycan *TCAP*/telethonin *TRIM32* *FKRP*/fukutin-related-protein *POMT1*/O-mannosyl-transferase 1 *FKTN*/fukutin *POMT2*/O-mannosyl-transferase 2 *POMGnT1*/O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1 *DAG1*/dystroglycan gene *TTN*/titin *ANO5*/anoctamin5 *PLEC1*/plectin *DES*/desmin *TRAPPC11*/transport protein particle complex 11 gene *GMPPB*/GDP-mannose pyrophosphorylase B *ISPD*/isoprenoid synthase domain containing *GAA*/lysosomal enzyme acid α-glucosidase *LIMS2*/*PINCH2*/senescent cell antigen-like-containing domain protein 2 gene *POPDC1*/Popeye-domain-containing 1 *TOR1AIP1*/lamina-associated polypeptide 1B *POGLUT1*/O-glucosyltransferase 1	Defects in sarcomere organization and maintenance, defects in DGC function and sarcolemmal repair, impaired intracellular trafficking , among others	Raising in serum CK, nuclei internalization wasting and regeneration of muscle fibers, inflammatory infiltrates in some cases	Progressive weakness and atrophy of the shoulder and pelvic girdle musculature with cardiac and respiratory muscles involvement in some cases

Hereditary myopathy	Genes/proteins	Mechanism affected	Histopathological features	Clinical features
Congenital muscular distrophies	*LAMA2*/merosin/laminin-α2 chain *COL6A1*/*COL6A2*/*COL6A3*/ collagen 6A *POMT1*/O-mannosyl-transferase 1 *POMT2*/O-mannosyl-transferase 2	Defects in DGC function and cell matrix integrity	Variation of fiber size, whorled and split fibers, nuclei internalization increase of connective and adipose tissue	Generalized hypotonia and predominantly proximal weakness, joint contractures, cardiomyopathy, respiratory failure and central nervous system involvement, retinal and brain malformations in the most severe cases
Facioscapulohumeral musular dystrophy	*DUX4*/double homeobox 4	Toxic "gain of function" of the normally repressed transcriptional regulator DUX4	Dystrophic features including fibrosis, muscle fiber hypertrophy , central nucleation and endomysial inflammation	Slowly progressive asymmetric and descending weakness, initially affecting face (facio), scapula (scapulo) and upper arms (humeral), followed by weakness of the distal lower extremities and pelvic girdle
Emery-dreifuss muscular dystrophy	*EMD*/emerin *LMNA*/lamin A/C	Nuclear envelope defects, impair in gene expression, cell signaling and chromatin architecture	Dystrophic features such as fiber size disproportion, nuclei internalization, increase of endomysial connective tissue, necrosis and regeneration are usually observed. Reduced expression of emerin or lamin A/C in muscle, fibroblasts or blood	Slowly progressive muscular weakness, joint contractures, spine rigidity and heart disease

Table 1. Genetic, histological, and clinical aspects of hereditary myopathies.

2. Congenital myopathies

Congenital myopathies are genetic neuromuscular disorders characterized by typical histo-pathological alterations including type-1 fibers predominance and hypotrophy and presence of structural abnormalities such as rod-inclusions and cores, among others [1]. Their clinical

course is usually non-progressive or slowly progressive and their prognosis is mainly determined by the involvement of respiratory muscles. Unlike muscular dystrophies, patients with congenital myopathy typically exhibit normal or discretely increased levels of CK [2]. The onset of the disease generally occurs in the neonatal period and it has an estimated incidence of 1:25,000 live births [1].

Clinically, congenital myopathies manifest with heterogeneous features such as generalized weakness, hypotonia, hyporeflexia, and poor muscle bulk. Congenital myopathies also presents with dysmorphic characteristics, secondary to the myopathy such as *pectus carinatum* (a chest malformation characterized by a protrusion of the sternum and ribs), scoliosis, joint-contractures, foot deformities, high-arched palate, and elongated facies [3]. Different mutations in a same gene can cause different phenotypic forms of congenital myopathy, whereas mutations in different genes can induce muscle diseases with overlapping clinical and histological features, making difficult a specific diagnosis. However, based on the histological markers observed in muscle biopsies, congenital myopathies can be divided in five forms: nemaline myopathy, core myopathy, centronuclear myopathy, fiber-type disproportion myopathy, and myosin storage myopathy [1].

2.1. Nemaline myopathy

Nemaline myopathy (NM) is one of the three major types of congenital non-dystrophic myopathies with an estimated incidence of 1:50,000 [4]. Based on the severity and the onset of the disease, NM can be divided in different subtypes ranging from severe forms with neonatal-onset, which is usually lethal in the first months of life, to less severe forms with onset in the childhood or adulthood [5] Clinically NM courses with hypotonia, weakness of proximal skeletal muscles, including facial and neck flexor muscles that can lead to respiratory insufficiency and death in the most severe cases [4]. Less severe forms of NM exhibit a static or slowly progressive weakness of the distal limbs, trunk, and facial muscles with a delay in the acquisition of motor milestones. Cardiac muscles are usually not affected in NM [4].

Histologically, NM characterizes by the presence of **nemaline-bodies** or small "**rod-like inclusions**." These are thread-shape structures that stain red or purple by the modified Gömöri's trichrome staining [1] (**Figure 1**). Rod-inclusions vary from 1 to 7 μm of length and 0.3 to 2 μm of width and, mainly, consist of **actin and alpha-actinin accumulation**, apparently product of an alteration of the ratio of actin-binding proteins and their interaction [6]. At the light microscopy, nemaline bodies appear like clusters localized at the cytoplasm and often at the periphery of the muscle fibers, although they also can be located in the nucleus making difficult to identify. At the ultrastructural level, rod inclusions appear like electron-dense bodies at the Z-bands [1]. The presence of rod-like structures in the sarcomere apparatus difficult the contractibility of muscles. It has been suggested that NM-linked mutations in proteins that compose the sarcomere affect the arrangement of muscle fibers, hindering the typical slid of the fibers during movement, and causing muscles to be unable to efficiently contract [7] (**Figure 2**). However, although the presence of nemaline bodies is required for the diagnosis of NM, they are just a product and are not necessarily the cause of the disease. In this regard, myofibrillar dissociation and smallness appear to be a primary defect causing impaired

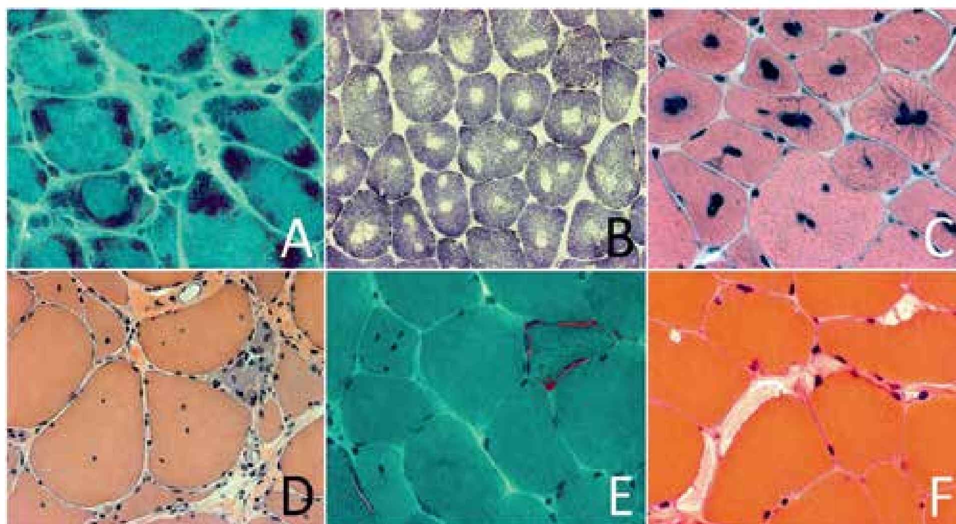

Figure 1. Histopathological markers in hereditary myopathies. (A) Modified Gömöri's trichrome stain showing rods in nemaline myopathy. (B) SDH staining in central core disease due to RYR1 mutations. Note that cores are frequently eccentric and that there are two or more in several fibers. (C) HE staining in a case of DNM2-related centronuclear myopathy. Notice multiple centralized nuclei in some fibers and the radiating strands of intermyofibrillary network. (D) Dystrophic changes in Emery-Dreyfus muscular dystrophy. There is a large variability on the size of the fibers, multiple nuclei internalizations, increase of endomysial connective tissue, and foci of necrosis-regeneration, which define the dystrophic pattern. (E) Modified Gömöri's trichrome stain showing a ragged red fiber in mitochondrial myopathy. (F) HE stain in McArdle's disease. At the subsarcolemmal level large chromophobe vacuoles containing glycogen are shown. Pictures in A and B are courtesy of Dr. Norma B. Romero, Institute of Myology, Paris, France; picture in panel C is courtesy of Prof. Anders Oldfors, University of Gothenburg, Gothenburg, Sweden.

contractility and muscle weakness in NM [5]. Moreover, nemaline bodies can be observed in other myopathies and in muscle degeneration related to aging [6].

NM is caused by mutations in 11 genes encoding proteins that compose or regulate the sarcomere thin filaments. These are **nebulin** (*NEB*) [8], **skeletal muscle alpha-actin** (*ACTA1*) [9], **alpha-tropomyosin-3** (*TPM3*) [10], **beta-tropomyosin-2** (*TPM2*) [11], **troponin T1** (*TNNT1*) [12], **cofilin-2** (*CFL2*) [13], **Kelch-repeat-and-BTB-domain-containing-13** (*KBTBD13*) [14], **Kelch-like-family-member-40** (*KLHL40*) [15], **Kelch-like-family-member 41** (*KLHL41*) [16], **leiomodin-3** (*LMOD3*) [17] and **myopalladin** (*MYPN*) [18]. Most of these genes encode sarcomeric proteins that are critical for the structural organization and function of the contractile apparatus. Therefore, NM-causing mutations can directly affect these functions, resulting in skeletal muscle weakness. The most frequent mutated gene is **nebulin** (*NEB*) which account for over 50% of the NM cases, all of them inherited in an autosomal-recessive way [8]. **Nebulin** is a giant actin-binding protein whose large size is proportional to the thin filament length. Nebulin C-terminal region is anchored into the Z-disks and its N-terminal extends to the thin filament pointed-end, acting as a "ruler" of the thin filament length for the sarcomere assembly during myofibrillogenesis [19]. A great diversity of nebulin isoforms has been described, which differ among various striated muscles types, developmental stages, and diseases [20]. Most nebulin mutations causing NM result in truncations or internal deletions and in a

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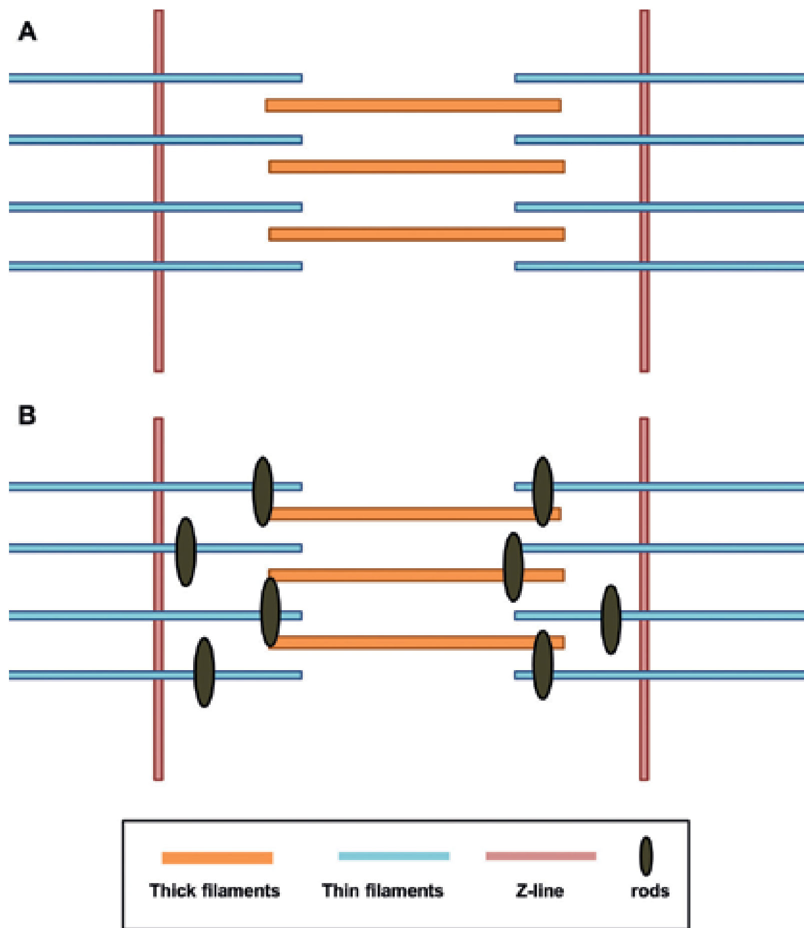

Figure 2. Rod inclusions in the sarcomere. (A) A healthy sarcomere is schematized. (B) Rod inclusions in the sarcomere are schematized. Rods are clustered near to the Z-line in the sarcomere of nemaline myopathy patients, affecting the sarcomere arrangement and hindering contraction.

reduction in the diversity of nebulin isoforms [6, 20], likely shortening thin-filaments length during myofribillogenesis and disrupting muscle development.

Mutations in *ACTA1* gene are linked to around 20% of the NM cases [9] which are predominantly inherited in an autosomal-dominant way [1]. Mutations in skeletal alpha-actin that cause NM and other congenital myopathies are spread over different domains affecting primary actin functions such as binding and hydrolysis of nucleotide, folding, F-actin polymerization and stability, or its interaction with actin-binding proteins [21, 22].

Less prevalent mutations in *TPM3* and *TPM2* account for approximately 2–3% of the NM cases [1]. Tropomyosins are coiled-coil proteins that polymerize along actin filaments providing stability and regulating the binding of the myosin heads to the thin actin filaments in the sarcomere (cross-bridges), in a Ca^{2+}-dependent way. NM-linked mutations in **beta-tropomyosin-2** have shown to

induce a reduction in actin-affinity and Ca^{2+} sensitivity [23] and to change the position of tropomyosin in the actin filaments, disorganizing the assembly of the actomyosin complex, reducing its ATPase activity and leading to contractile dysfunction [24]. NM-causing mutations in TPM3 have shown to suppress the expression of the slow **alpha-tropomyosin-3** [25], likely deregulating myosin-actin interaction and impairing the force-generating capacity of the sarcomere.

Mutations in the *TNNT1* are less likely cause of NM. The **troponin complex** (troponin C, troponin I, and troponin T) blocks the actin-myosin interaction, preventing contraction in resting muscles. Specifically, **troponin T** binds to tropomyosins regulating the interaction of the troponin complex with thin actin filaments. NM causing mutations in TNNT1 are mainly recessive, and produce loss of the expression of troponin T in skeletal muscles [26] and reduction of tropomyosin-binding affinity [27] likely impairing regulation of the muscle contraction.

Mutations in the *CFL2* gene have shown to cause NM [13]. **Cofilin2** is a skeletal muscle-specific actin-depolymerizing factor, and NM-linked mutations significantly reduce the cofilin2 expression levels affecting actin dynamics likely causing its accumulation in the nemaline bodies [13].

More novel mutations in proteins that form part of the BTB/Kelch family have been linked to several forms of NM. BTB/Kelch proteins are involved in a broad variety of cellular processes including cytoskeleton modulation, gene transcription, ubiquitination, and myofibril assembly. Dominant mutations in **Kelch-repeat-and-BTB-domain-containing-13** (*KBTBD13*) produce a mild form of NM with nemaline rods and core lesions ([14]. Autosomal-recessive mutations in **Kelch-like-family-member-40** (*KLHL40*), which seems to be critical for myogenesis and muscle maintenance, cause a severe form of NM that includes fetal akinesia [15]. Recessive mutations in **Kelch-like-family-member 41** (*KLHL41*) associate with a severe NM phenotype with neonatal death; NM-linked mutations cause destabilization of KLHL41 structural domains and reduce its protein levels in skeletal muscle [16].

Leiomodin-3 (*LMOD3*) is a skeletal muscle-specific member of the tropomodulin family and colocalizes with sarcomere thin actin filaments. Mutations in LMDO3 produce a severe form of NM that manifests with absence of fetal movements, generalized hypotonia, and respiratory insufficiency. NM mutations abolish LMOD3 expression in skeletal muscle tissue of NM patients [17].

Mutations in **myopalladin** have been also recently identified to cause relatively mild forms of NM with slowly progressive muscle weakness [18]. Myopalladin is a sarcomere protein localized at the I-bands and Z-line that interacts with several sarcomeric components, including nebulin, regulating sarcomere assembly [18]. NM-linked mutations in *MYPN* are loss of function mutations that markedly decrease the full-length protein levels likely affecting the maintenance of the sarcomeric organization [18].

2.2. Core myopathy

Core myopathies (CM) are heterogeneous congenital muscle diseases that present with hypotonia and weakness of proximal muscles with a static or slow-progressive clinical course. CM is the most common form of congenital myopathy [28]. Histologically, CM is characterized by the presence of "**cores**," large areas of abnormal myofibrillar arrangement and sarcomeric

disorganization, devoid of mitochondria, and oxidative-enzyme activity, which are mainly found in type-1 muscle fibers (**Figure 1**). Cores can be single or multiple; and based on biopsy observations, CM can be classified as **central core disease** (**CCD**) or **multiminicore disease** (**MmD**) [28]. In CCD, single cores are centrally or eccentrically located along the longitudinal axis of type 1 muscle fibers (**Figure 1**), while in MmD numerous short core lesions localize diffusely throughout type 1 or type 2 muscle fibers [29]. Cores and rods lesions can occur together in "**core-rod myopathy**," a variant of NM [30]. Dominant-inherited CCD typically courses with hypotonia and motor developmental delay in the childhood, presenting fetal akinesia in the most severe cases [28]. Most mild forms manifest with myalgia, proximal weakness with hip, girdle, and axial muscles involvement. Orthopedic complications including hips dislocation, scoliosis, and foot deformities are also typical in CCD patients [28]. Clinical manifestations of MmD are highly variable. These range from a severe and most prevalent neonatal form, which include axial muscle weakness, spinal rigidity, respiratory impairment, and cardiac failures [31], to milder forms that course with generalized muscle weakness predominantly affecting pelvic girdle [31].

CCD and MmD are caused by mutations in genes encoding two proteins involved in the excitation-contraction (E-C)-coupling, calcium homeostasis, and redox regulation in muscle fibers, these are the skeletal-muscle **ryanodine receptor** (*RYR1*) [32] and the **selenoprotein N** (*SEPN1*) [31]. RyR1 is a functional calcium release channel that plays a critical role in the E-C coupling by releasing calcium from the sarcoplasmic reticulum in response to conformational changes induced by the activation of the voltage-sensing dihydopiridine receptor DHPR [33]. Most mutations in RyR1 gene are autosomal-dominant and associate with a CCD phenotype [34]. Autosomal-dominant mutations in RyR1 mainly localizes in the C-terminal region, which encodes the calcium release channel pore of the ryanodine receptor protein and in the N-terminal region that includes the "foot" structure that interacts with DHPR [35]. An important number of dominant mutations in RyR1 also associate to malignant hyperthermia susceptibility (MHS), a pharmacogenetic predisposition to severe and potentially lethal episodes induced by halogenated anesthetic agents (halothane, isofluorane) and succinylcholine [36]. MHS-linked mutations cause a hyperactive RyR1 channel that release excess of calcium to the sarcoplasm [37], initiating a cascade of events that induce hypermetabolism, increased CO_2 production and O_2 consumption, acidosis, muscle rigidity, tachycardia, and tachypnea among others [36]. Although not all patients with MHS exhibit a muscular affection, the majority of CCD patients are susceptible to malignant hyperthermia. Patients with MmD and centronuclear myopathy also exhibit predisposition to MHS linked to RyR1 mutations [36]. Recessive mutations in RyR1 predominantly associate with MmD and are distributed evenly throughout the gene [35], but can also cause other phenotypes with less well defined cores on muscle biopsy [38]. Different molecular mechanisms related to RyR1 mutations have been suggested to underlay core myopathy. For instance, the "leaky channel hypothesis" suggests that MHS- and CCD-linked mutations confer hypersensitivity to the RyR1 channel, increasing its activity; thus, affecting Ca^{2+} homoeostasis by depletion of the intracellular Ca^{2+} stores [39]; CCD-mutations have also shown to impair the Ca^{2+} permeation through RyR1 channel after activation, affecting the E-C coupling ("E-C uncoupling hypothesis") [40] and MmD-linked mutations have been associated with a reduction of the RyR1 protein expression levels [41].

Additionally to RyR1-related forms, mutations in SEPN1 associate with approximately 50% of the cases of the most prevalent form of MmD [28]. Selenoprotein-N is a sarcoplasmic glycoprotein implicated in several processes including antioxidant defenses and calcium homeostasis [42]. This is part of the selenoproteins family, which characterize for containing selenocysteine aminoacids (Sec). Incorporation of Sec to the polypeptide chain in selenoproteins occurs due to a "redefinition" of the stop-codon UGA during translation, which requires a Sec insertion sequence (SecIS) in the non-translated 3'UTR region and a Sec redefinition element (SRE) located adjacent to the UGA codon. Myopathy causing mutations in SEPN1 affects the Sec insertion efficiency, decreasing the expression of selenoprotein-N, and leading to a deficiency of the protein [43]. Mutations in SEPN1 have been also pointed as causative of congenital muscular dystrophy with rigid spine (RSMD), a rare neuromuscular disorder characterized by early spine stiffness and respiratory deficiency [44].

2.3. Centronuclear myopathy

Centronuclear (CNM) myopathy is a heterogeneous group of congenital myopathies clinically manifested by myalgia, fatigability and progressive weakness and atrophy of distal skeletal muscles [45]. Histological markers of the disease are the presence of abnormally high number of muscle fibers with a central rather than peripheral nuclei distribution (**Figure 1**), predominance, and atrophy of type 1 fibers, and a radial arrangement of the sarcoplasmic strands on oxidative stains [45]. Different forms of CNM have been described according to the inheritance pattern and clinical manifestations. The X-linked recessive form, called **myotubular myopathy** (**XLMTM**), presents as a severe myopathy with marked hypotonia and generalized muscle weakness in newborn males and exhibit a poor prognosis with the most of patients dying within the first months of life as a consequence of respiratory failure [46]. A late-onset myotubular myopathy has been also reported, which presents with milder symptoms during childhood that worsen after the first or second decade of life and that histologically characterizes by the presence of "**necklace fibers**," a basophilic ring deposit following the contour of the cell in which myonuclei are aligned [47]. XLMTM is mainly caused by mutations in the *MTM1* gene encoding **myotubularin**, an ubiquitously expressed lipid phosphatase that specifically dephosphorylates phosphatidylinositol-3-phosphate and phosphatidylinositol-3,5-bisphosphate [48]. Myotubularins are implicated in several cell process including endocytosis, membrane trafficking, autophagy, and cytoskeletal dynamics [48, 49] and seem to be critical for skeletal muscle maintenance as shown in MTM1-deficient mice [50]. More than 200 different XLMTM-linked mutations in myotubularin have been described to date with most of the mutations predicted to affect its expression and enzymatic activity [51–54]. A mouse model of XLMTM exhibit T-tubules disorganization, a depression in the sarcoplasmic Ca^{2+} release by a reduction in the RyR1 levels and consequent defects in EC-coupling [55].

A classical autosomal-dominant form of CNM, which accounts about 50% of the CNM cases, is caused by mutations in the *DNM2* gene encoding **dynamin-2** [56]. The spectrum of dynamin-2-related CNM severity varies from mild, with late-onset, to severe with neonatal onset [57]. Mild and moderates forms of DNM2-related CNM manifests with delayed motor milestones, specially walking and climbing stairs, distal muscle weakness, ptosis, and ophthalmoplegia.

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More severe early-onset forms courses with generalized weakness and hypotonia, scoliosis, Achilles tendon contractures, and jaw opening among other symptoms [56]. Dynamin-2 is a large GTP-ase that expresses ubiquitously in different tissues and participates in various intracellular processes including endocytosis, exocytosis, membrane trafficking, and actin cytoskeleton remodeling among others [58–60]. CNM-causing mutations in dynamin-2 are clustered in structural domains involved in dynamin's oligomerization (middle domain) and lipid-binding (PH domain) [57]. These mutations have shown to increase dynamin basal GTP-ase activity and enhance oligomerization [61–63]. The impact of CNM-linked mutations on dynamin-2-dependent processes has not yet been fully understood. In non-muscle cells both, reduction [64, 65] and absence of effect [66] in clathrin-mediated endocytosis has been reported. Skeletal muscles of CNM patients exhibit abnormal cytosolic accumulation of dynamin-2 and other endocytic proteins [67], while impaired actin remodeling and actin-mediated trafficking was reported in a rodent mammalian model of CNM [68].

Mutations in the *BIN1* gene, encoding **amphiphysin 2**, cause an autosomal-recessive form of CNM, which presents with a large clinical variability from severe to moderate phenotypes [46]. Currently, BIN1-related CNM manifests with a delay in the acquisition of motor milestones, difficult to walk, run and climb stairs, diffuse muscle weakness and atrophy and facial involvement including diplegia, ptosis and ophthalmoplegia [56]. Amphiphysin 2 is a ubiquity expressed protein that belongs to the BAR-domain family, which acts as sensor of the membrane curvature [69]. At late stages of the clathrin-mediated endocytosis, amphiphysin2 bind to the invaginated membranes and recruits other proteins to the endocytic machinery including dynamin-2 [70]. CNM-causing mutations in BIN1 localizes in its BAR domain affecting its capabilities to tabulate membranes and in its SH3 domain, producing a partial truncation that eliminates its interaction with dynamin-2. The later suggests that mutations in amphiphysin-2 disrupt the formation and maintenance of the T-tubule network by impairing membrane remodeling, leading to CNM [71].

CNM-causing autosomal-recessive mutations have been also reported in the gene encoding *RyR1* [72, 73] and *TTN* gene, encoding **titin** [74]. Clinically, RyR1-related CNM patients exhibit early hypotonia, motor developmental delay, proximal and proximal, facial and ocular muscle weakness. Histologically, they show a variable prominence of central nuclei, type-1 fiber predominance and a wide range of intermyofibrillary abnormalities [72, 74]. Most CNM-linked mutations in RYR1 result in reduced expression of the ryanodine receptor channel [75] likely suggesting defects in the EC-coupling. **Titin** is a giant protein (the largest one known) important in the contraction of the striated muscle. It connects the Z line to the M line in the sarcomere forming a third filament system important for the structural integrity of the myofibril and for the passive tension in stretched muscle fibers [76]. CNM-causing mutations in titin produce degradation and truncated versions of the protein in patients [74], likely affecting muscle stiffness and contractibility. Mutations in titin also associate with cardiomyopathies [77] and muscular dystrophy [78].

Recently, mutations in the *striated muscle preferentially expressed protein kinase SPEG*, a myotubularin-interacting protein, have been related to myotubular centronuclear myopathy [79, 80]. Mutations in *SPEG* cause phenotype that range from mild forms of CNM with moderate hypotonia and weakness to more severe forms with cardiac involvement [79, 80].

2.4. Congenital fiber-type disproportion myopathy

Congenital fiber-type disproportion myopathy (CFTDM) is defined by an abnormal disproportion between the size of type-1 (slow) and type-2 (fast) muscle fibers, with the type 1 fibers found to be at least 35–40% smaller than the type 2 ones [81]. This is a critical point for the diagnosis since other myopathic conditions manifests with fiber type disproportion. Clinically, CFTDM patients experience mild to severe muscle weakness mainly affecting shoulders, arms, hips, and thighs. Orthopedic affections such as lordosis, scoliosis, and joint contractures are usually observed. Approximately, 30% of CFTDM patients exhibit respiratory muscle hypotonia, requiring breathing assistant. Face muscles can also be affected producing long face, high-arched palate, ptosis, and ophthalmoplegia [1]. Genetically, the most well-established causes of CFTDM are mutations in *TPM3*, *RYR1*, and *ACTA1* genes [81]. Mutations in *TPM3* are the most common cause of CFTDM accounting between 20 and 50% of the diagnosed cases [81, 82]. Almost all TPM3 mutations associated with CFTDM are dominant missense changes and are predicted to impair the interaction between alpha-tropomyosin and actin [82] likely affecting acto-myosin interaction in the cross-bridges cycle and impairing a proper muscle contraction [83]. Recessive mutations in **RYR1** have shown to cause CFTDM accounting 10–20% of the CFTDM families. The most specific clinical indication of RyR1-related CFTDM is the presence of ophthalmoplegia and a dramatic disproportion in the size of type 1 fibers (being 50–84% smaller than type2 fibers) [84]. CFTDM-causing mutations reduce RyR1 protein expression levels [84], probably impairing channel conductance and EC-coupling. Mutations in *ACTA1* linked to CFTDM are less probable compared to ACTA1 mutations in nemaline myopathy, and account approximately 5% of the CFTDM patients [85]. How mutations in ACTA1 produce fiber type disproportion is uncertain since it is equally expressed in both, type 1 and type 2 fibers. The pathological mechanism of ACTA1-related CFTDM is also unclear, although it was reported that one mutation in a residue located in the external surface of alpha actin, in which a negatively charged residue is replaced by a non-charged one (D294V), impair actin-tropomyosin association, deregulating acto-myosin interaction, and leading to defects in muscle contraction [86]. Much less frequent causes of CFTDM are mutations in the genes encoding beta-tropomysoin-2 (*TPM2*), beta-myosin (*MYH7*), and selenoprotein-N (*SEPN1*) [81].

2.5. Myosin storage myopathy

Myosin storage myopathy (MSM) is a rare congenital myopathy caused by mutations in the gene encoding the **slow-skeletal/β-cardiac myosin heavy chain** (*MYH7*), a class II myosin and major component of the thick filaments. It is primarily expressed in heart but also in skeletal muscle type-1 fibers [87]. *In vivo*, myosin forms dimers of myosin heavy chains with two globular heads attached to a coiled-coil region known as the myosin rod. The head region is responsible for myosin's ATPase-activity and actin-binding, while the rod region allows the incorporation of myosin to the thick filaments [88]. *MYH7* mutations are spread along the different myosin's domains and according their location associate with different phenotypes. In this regard, mutations in the N-terminal globular head are linked to cardiomyopathies,

whereas mutations in the C-terminal rod associate to skeletal muscle myopathies such as MSM [89]. MSM-linked mutations in MYH7 have shown to alter myosin folding and stability, impairing sarcomere thick filaments assembly and integrity [88, 90]. MSM clinical phenotypes are highly variable between patients. It presents with childhood or adult-onset forms with static or slowly progressive clinical course. Hypotonia and proximal muscle weakness with delayed motor milestones are common features. Respiratory insufficiency secondary to the myopathy may occur, with the presence or not of cardiac involvement [1]. Histologically, MSM characterizes by the sub-sarcolemmal accumulation of β-myosin in type 1 fibers, which can be observed with hematoxylin-eosin and Gömöri trichrome stains as "**hyaline bodies**."

3. Mitochondrial myopathies

Dysfunctions of the respiratory chain, responsible for oxidative phosphorylation and ATP energy production in the inner mitochondrial membrane, cause **mitochondrial diseases**. These manifest as multisystem disorders with predominant involvement of muscles and nerves. When skeletal muscle is affected, the term **mitochondrial myopathy** is used. In isolated mitochondrial myopathy without involvement of other tissues, patients can exhibit myalgia, fatigue, exercise intolerance, proximal and distal muscle weakness, and elevated serum CK [91]. Other clinical manifestations include the **chronic progressive external ophthalmoplegia** (**CPEO**), in which a slowly progressive paresis of the extra ocular muscles is the most important phenotype [92] and **severe encephalomyopathy of infancy or childhood**, in which brain and skeletal muscle tissue are involved, producing marked hypotonia, respiratory muscle weakness, and feeding difficulty [93]. Mitochondrial myopathies may be caused by mutations in mitochondrial or nuclear DNA. Mitochondrial-DNA encoded **cytochrome b** [91] and **cytochrome c oxidase** [94, 95] are mutated in some forms of isolated mitochondrial myopathy. Mutations in nuclear DNA that produce deficiency of **Coenzyme Q10**, an important electron carrier of the respiratory chain also associate to mitochondrial myopathy [96]. Depletion of mitochondrial DNA in skeletal muscle, secondary to mutations in nuclear genes (mitochondrial depletion syndrome) can also cause mitochondrial myopathy. It mainly affects genes encoding proteins involved in the maintenance of the mitochondrial deoxy-ribonucleotide pool, such as **thymidine kinase** (*TK2*) [97] or proteins implicated in mitochondrial DNA replication such as the **polymerase gamma 1** (*POLG1*) [98]. Histologically, mitochondrial myopathy characterizes by a sub-sarcolemmal and intermyofibrillar **accumulation of mitochondria** in muscle fibers, which responds to compensatory mechanisms due to defects in the energy production. Upon Gömöri trichrome stain, proliferated mitochondria look as bright red masses against the blue background of the myofibers, defining the term "**ragged red fibers**" (**Figure 1**). Staining of succinate dehydrogenase (SDH) and cytochrome c oxidase (COX) are indicative of mitochondrial complexes activity. In SDH-positive biopsies "**ragged blue fibers**" can be observed. Staining pattern with normal fibers mixed with ragged blue/red COX-positive fibers is a histological marker of mitochondrial-DNA-related myopathy [99].

4. Metabolic myopathies

Metabolic myopathies result from defects in the metabolism of carbohydrates and lipids that primarily affect skeletal muscle. Defects in energy production are typically manifested by metabolic crisis with generalized muscle weakness, sometimes associated with cardiac and respiratory failure [100]. Metabolic myopathies can be classified as **glycogen storage** and **lipid storage diseases**. Among glycogen storage diseases is **glycogenosis type II** (**Pompe disease**), an autosomal-recessive disorder caused by mutations that lead to a deficiency of the **lysosomal enzyme acid α-glucosidase** (*GAA*), responsible for catalyze the hydrolysis of glycogen. Deficiency of GAA produces glycogen accumulation and disruption of tissue architecture in different tissues, especially in skeletal muscle [100]. The clinical phenotype of Pompe disease ranges from childhood-onset severe forms to mild adult forms [101]. The most classic childhood form manifests in the first months of life with severe myocardiopathy, generalized hypotonia and muscle weakness, feeding difficulties, and respiratory failure. The late-onset disease presents with progressive proximal and axial muscle weakness, leading to alteration in the posture and pattern of movements [100]. Enzyme replacement therapy allows diminishing the symptoms although untreated late-onset patients may worsen progressively eventually needing wheelchair and assisted ventilation [102]. Histological markers of Pompe disease are the presence of huge **basophilic vacuoles** inside muscle fibers in childhood-onset forms, and globular **cytosolic inclusions** with acid phosphatase activity in adult-onset forms of the disease [103]. Other glycogen storage diseases affecting glycogen degradation are **glycogenosis type III** (**Cori disease**), caused by recessive mutations in the *AGL* gene that lead to **glycogen debranching enzyme** deficiency [104] and **glycogenosis type V** (**McArdle disease**), caused by mutations in the *PYGM* gene that cause **myophosphorylase** deficiency [105]. Cori disease patients typically exhibit hypotonia and distal weakness and may present cardiac and hepatic failure [104]. McArdle disease patients presents with fatigue and exercise-induced myalgia. In some cases, patients can exhibit myoglobinuria and acute renal failure due to rhabdomyolysis, as well as higher susceptibility to malignant hyperthermia [106]. Large glycogen-containing vacuoles typically accumulate at the sub-sarcolemmal level in McArdle disease biopsies (**Figure 1**).

Lipid storage myopathies characterizes by abnormal lipid accumulation in muscle fibers due to fatty acid dysmetabolism. Different forms of lipid storage myopathy have been described among them **primary carnitine deficiency** (**PCD**), **multiple acyl-CoA dehydrogenase deficiency** (**MADD**) and **neutral lipid storage disease with myopathy** (**NLSDM**) [107]. PCD is caused by autosomal-recessive mutations in the *SLC22A5* gene that encodes the **carnitine transporter OCTN2**. Defects in OCTN2 lead to deficiency of carnitine and reduced transportation of long-chain fatty acids to the mitochondrial matrix, producing cytosolic lipid accumulation and a reduction in the ATP production for β-oxidation [107]. Clinically PCD manifests with a wide spectrum of symptoms including hypotonia, muscle weakness, and cardiomyopathy. Histopathological markers include elevated levels of CK and increased number and size of **lipid droplets**, especially in type 1 muscle fibers [107]. MADD is caused by deficiency of **electron-transfer flavoprotein** (**ETF**) or **ETF-dehydrogenase** (**ETFH**), two mitochondrial enzymes that act transferring high-energy electrons produced during the fatty acid β-oxidation by acyl-CoA-dehydrogenases to the respiratory chain. The clinical phenotype of MADD is

highly heterogeneous ranging from neonatal-onset forms that manifest with hypotonia, hepatomegaly, hypoglycemia, and metabolic acidosis and later-onset forms that present with proximal muscle weakness often with hepatomegaly, encephalopathy, and episodic lethargy. Like in PCD, muscle fibers of MAAD patients exhibit increased lipid droplets [107]. NLSDM is caused by mutations in the *PNPLA2* gene, encoding the **adipose triglyceride lipase** implicated in the catabolism of stored triglycerides to glycerol and non-esterified fatty acids [108]. Histopathological markers of the disease include **neutral-lipid containing vacuoles**, stained by oil-red O in neutrophils and monocytes as well as marked **triglyceride storage** and cytoplasmic **lipid droplets** in muscle sections [108]. NLSDM is clinically characterized by, either, childhood- or adult-onset proximal muscle weakness, typically affecting upper limbs, although distal asymmetrical muscle weakness may also occur, as well as elevated CK serum levels and cardiomyopathy in some cases [100].

5. Myotonia

Myotonia is a symptom associated to several neuromuscular disorders characterized by a prolonged contraction or rigidity of the skeletal muscles (delayed relaxation) after voluntary contraction or electrical stimulation. It is present in **congenital myotonia**, **paramyotonia congenita**, and **myotonic muscular dystrophy** among others muscular disorders. **Congenital myotonia** is a non-dystrophic disorder caused by loss-of-function mutations in the skeletal muscle chloride channel *ClC1* resulting in a reduced sarcolemmal chloride conductance [109]. ClC1 channels are critical players stabilizing resting membrane potential and promoting repolarization. Upon propagation of an action potential along T-tubules, an efflux of potassium ions occur to repolarize membrane potential. Due to the spatial confinement of T-tubules this efflux leads to an increase in extracellular potassium concentration which tends to produce an "after-depolarization" that is dampened by chloride conductance under normal conditions. Mutations in ClC1 reduce chloride conductance, enhancing the sarcolemmal excitability by accumulation of potassium ions in the transverse tubules [110]. This condition may trigger spontaneous action potentials explaining the persistent muscle contraction observed in myotonic patients. Congenital myotonia is classified in autosomal-dominant **Thomsen's disease** and autosomal-recessive **Becker's myotonia**. The first is a moderate form of myotonia with no progressive symptoms, allowing to the patients a relatively normal life expectancy. Becker's myotonia is an early-onset more severe form of the disease that presents with pronounced myotonia, myalgia, transient episodes of generalized weakness and muscular hypertrophy [110]. Predominance and hypotrophy of type 2 fibers and increased endomysial connective tissue are usually observed in biopsies of congenital myotonia patients. **Paramyotonia congenita** is a non-dystrophic muscular disorder caused by autosomal-dominant mutations in the *SCN4A* gene encoding the pore-forming **alpha-subunit of the skeletal muscle sodium channel** (**Nav1.4**) [111]. Nav1.4 mutations cause channel gain of function, producing abnormal persistent sodium currents that lead to the myotonic phenotype [111]. Paramyotonia congenita manifests with early-onset generalized weakness and "myotonic discharges" that produce an exacerbated stiffness by repeated muscle contraction.

Patients also exhibit an extreme sensitivity to cold that worsens the symptoms [111]. **Myotonic muscular dystrophy** is the most common cause of muscular dystrophy in adults and results from expression of RNAs that contain expanded nucleotide repeats in the 3′ untranslated region of two different genes leading to two forms of the disease. **Myotonic dystrophy type 1** (**DM1**) that results from an expansion of **CTG repeats** in the *DMPK* gene encoding **myotonin-protein-kinase** and **myotonic dystrophy type 2** (**DM2**) caused by an expansion of **CCTG repeats** in the **zinc finger 9** (*ZNF9*) gene. These mutant transcripts form **hairpins**, imperfect double-stranded structure that lead to deregulation of important RNA-binding proteins such as **muscleblind-like protein 1** (**MBNL1**), which are retained in nuclei forming toxic nuclear foci that impair gene expression [112]. Aberrant expansion of nucleotide repeats has shown to deregulate alternative splicing of pre-mRNA for **ClC1 channel**, affecting chloride conductance in skeletal muscle, leading to the myotonic phenotype [113]. Both DM1 and DM2 exhibit an autosomal-dominant inheritance pattern. Clinical presentation includes muscle wasting, progressive weakness, myotonia, cataracts, and multi-organ involvement affecting heart, brain, and endocrine system [112]. DM1 patients manifest more severe myotonia with prominent distal muscle involvement and a severe congenital form with mental retardation. DM1 is characterized by the phenomenon of "**anticipation**," by which the disease has an earlier onset and more severe course in subsequent generations [114]. In DM2 patients, proximal muscles are more affected; exhibiting milder myotonia with no "anticipation" [115]. Histological features in myotonic dystrophy include a high number of **internalized nuclei**, disproportion in fiber diameter with **type 1 fibers atrophy**, **basophilic regenerating fibers**, fibrosis, and adipose deposition. Specifically in DM2 biopsies **atrophic type 2 fibers** with pyknotic nuclear clumps are often observed [112, 116].

6. Muscular dystrophies

Muscular dystrophies (MD) are a heterogeneous group of neuromuscular disorders that result in progressive weakness and degeneration of skeletal muscles, affecting limbs, axial, and facial muscles. In some forms of the disease, heart and other organs are also affected [117]. The onset of MD is typically in early childhood, although the symptoms can appear in infancy up to middle age or later. The estimated incidence of MD is 1:2000 live births. Histopathological markers of MD are a diffuse **variation in the size of fiber types**, **necrosis** (with or without phagocytosis), **fiber regeneration**, **fibrosis**, and **atrophy**. **Inflammatory response** is present in some forms of MD (**Figure 1**). Elevated serum levels of **CK** are typically observed in MD patients [118]. MD are caused by mutations in genes that encode a wide variety of proteins, including transmembrane and membrane-associated proteins, extracellular matrix proteins, cytoplasmic enzymes, and nuclear envelope proteins [119]. More than 30 different forms of MD have been described, which differ in their genetic background, primarily affected muscles, the age of onset of the symptoms and the degree of weakness and progression. Some of the most common MDs are described below.

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6.1. Duchenne and Becker muscular dystrophy

Duchenne and Becker muscular dystrophies are two related X-linked recessive muscle disorders caused by mutations in the *DMD* gene encoding **dystrophin**, a critical component of the **dystrophin-glycoprotein complex** (**DGC**), a molecular scaffold that links fiber cytoskeleton to the extracellular matrix, providing mechanical stability to skeletal muscle [119]. Defects in DGC lead to mechanical stress during muscle contraction, producing sarcolemmal damage, abnormal Ca^{2+} homeostasis and consequent fiber necrosis [119]. Duchenne is the most common muscular dystrophy in childhood affecting approximately 1:3500 newborn males [117]. Duchenne-causing mutations usually lead to a pronounced reduction or complete absence of dystrophin, while Becker mutations have a less dramatic effect in dystrophin expression. Occasional muscle fibers positive for dystrophin can be found in about 50% of DMD patients called "revertant fibers," where dystrophin expresses in discrete domains with a "patchy distribution" that not influence the clinical phenotype [120]. Duchenne patients present early-onset dystrophy, delayed acquisition of motor milestones and a rapid progression of muscle weakness that usually leads them to need wheelchair by adolescence. Becker patients manifest milder symptoms with muscle weakness becoming later in childhood or in adolescence with a much slower progression. Both cases are usually associated with cardiomyopathy [121]. Insulin resistance and other metabolic alterations have also been observed in Duchenne and Backer patients [122].

6.2. Limb-girdle muscular dystrophies

Limb-girdle muscular dystrophies (LGMD) are a heterogeneous group of rare muscular disorders characterized by progressive proximal muscle wasting, predominantly affecting hip and shoulders. Clinical manifestations are broad, ranging from severe forms with rapid onset and progression to slowly progressive late-onset milder forms [123]. According to the inheritance pattern, LGMD can be classified in autosomal-dominant forms (LGMD1) and autosomal-recessive forms (LGMD2). Letters are added consecutively allowing to classify LGMD according to when individual genes were identified [117].

LGMD1 are usually adult-onset mild forms of the disease. Among them, **LGMD1A** is caused by mutations in the *MYOT* gene encoding **myotilin**, a Z-disk associated alpha-actinin-binding protein involved in the structural integrity of sarcomeres [123]. Patients exhibit proximal and distal weakness and occasional respiratory and cardiac involvement. **LGMD1B** is caused by mutations in the *LMNA* gene, encoding the nuclear membrane protein **lamin A/C**, implicated in several roles including mechanical maintenance of the nuclear membrane and gene regulation. Clinical manifestations of this "laminopathy" include proximal weakness, cardiac arrhythmias, and dilated cardiomyopathy [123]. **LGM1C** is caused by mutations in the *CAV3* gene that encodes **caveolin-3**, a muscle-specific sarcolemma protein component of caveolae membranes, specialized lipid rafts involved in plasma membrane maintenance, vesicular trafficking, and signal transduction [124]. Patients exhibit moderate proximal weakness, calf hypertrophy, and muscle cramps associated to exercise [123]. **LGMD1D** is caused by mutations in *DNAJB6*, a

member of the "DNAJ family," molecular chaperones involved in protein folding. **LGMD1E** associates to mutation in the *DES* gene encoding **desmin**, a component of the intermediate filaments that provides structural support to the sarcomere [123]. **LGMD1F** is caused by mutations in the **transportin 3** gene (*TNPO3*), a nuclear receptor for serine/arginine-rich proteins. Mutations in the RNA-processing protein **HNRPDL** cause **LGMD1G** [123].

Recessive LGMD are more frequent forms of the disease. Among them **LGMD2A** is the most prevalent LGMD worldwide accounting for up to 30% cases and is caused by mutations in the *CAPN3* gene encoding **calpain 3**, a Ca^{2+}-dependent non-lysosomal cysteine protease implicated in sarcomere organization and maintenance in mature muscle fibers. Most of the CAPN3 mutations result in autolysis and impaired proteolytic activity of calpain 3 [125]. Pathological features of the disease are progressive weakness and atrophy of the shoulder and pelvic girdle musculature, raised levels of serum CK and wasting and regeneration of muscle fibers in biopsy [126]. Recessive mutations in the *DYSF* gene encoding **dysferlin** cause **LGMD2B**. Dysferlin is a ubiquitous transmembrane protein implicated in Ca^{2+}-dependent resealing of the sarcolemma after injury [127]. Dystrophy-causing dysferlin mutations produce a drastic deficiency or complete absence of the protein; however, reduced dysferlin expression can also be observed in other muscular dystrophies secondary to mutations in other related genes [118]. LGMD2B account 15–25% of the LGMD2 cases and presents with slowly progressive proximal weakness that usually begin in the first or second decade of life and eventually lead to wheelchair dependence. Very highly elevated CK serum levels, nuclei internalization, muscle fibers necrosis and regeneration, and inflammatory infiltrates are typically observed. Other typical form of "dysferlinopathy" is **Miyoshi myopathy**, which is an adult-onset, more distal form that mainly affects posterior muscles of legs [117].

LGMD 2C-2F are referred as "**sarcoglycanopathies**" and are caused for loss of function mutations in the genes encoding **alpha**, **beta**, **gamma**, or **delta-sarcoglycans**, respectively. These are transmembrane proteins members of the **sarcoglycan-complex**, critical component of the DGC implicated in connecting cytoskeleton to the extracellular matrix providing mechanical stability to the skeletal muscle [128]. Sarcoglycanopathies have a childhood onset and typically manifest with rapid or slowly progressive proximal weakness involving both, cardiac and respiratory functions [123].

The "**dystroglycanopathies**" group a number of recessive **LGMD** (**2I, 2K, 2M, 2N, 2O, 2P**) linked to mutations in six genes implicated in the **glycosylation** of **alpha-dystroglycan**, critical component of the DGC: **fukutin-related-protein** gene (*FKRP*) linked to LGMD2I [129]; **O-mannosyl-transferase 1** gene (*POMT1*) linked to LGMD2K [130]; **fukutin** gene (**FKTN**) associated to LGMD2M [131]; **O-mannosyl-transferase 2** gene (*POMT2)* linked to LGMD2N [132]; protein **O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1** (*POMGnT1*) associated to LGMD2O [133] and the **dystroglycan gene** itself (*DAG1*) linked to LGMD2P [134]. Abnormal glycosylation of alpha-dystroglycan inhibits its binding to extracellular matrix proteins, impairing skeletal muscle stability. Dystroglycanopathy-causing mutations produce a wide spectrum of dystrophic phenotypes ranging from mild forms such as those observed in LGMD to more severe congenital muscular dystrophies (see below) [135].

LGMD2T is caused by mutations in the **GDP-mannose pyrophosphorylase B gene** (**GMPPB**), which catalyzes the conversion of mannose-1-phosphate and GTP to GDP-mannose, consequently

causing hypoglycosylation of α-dystroglycan [123]. LGMD2T patients exhibit dystrophy with intellectual disabilities [136]. Mutations in the **isoprenoid synthase domain containing** gene (*ISPD*) also impair dystroglycan glycosylation leading to **LGMD2U** and also to the severe **Walker Warburg syndrome** [137] (see below).

LGMD2J is caused by mutations in the gene encoding the giant sarcomeric protein **titin** (**TTN**), mainly clustered in the C-terminal M-line-linked region of the protein, interfering with its structure and function [138]. Mutations in the **anoctamin 5** gene (**ANO5**) cause **LGMD2L**, an adulthood-onset disease that presents with asymmetric muscle weakness, pain after exercise and elevated CK levels [123]. **LGMD2Q**, an early-onset non-progressive form of LGMD is caused by mutations in the gene encoding **plectin** (**PLEC1**) [123], a ubiquitously expressed protein linking actin microfilaments, microtubules and intermediate filaments [139]. Autosomal-recessive mutations in the **DES** gene (see above) determine **LGMD2R**, which may present cardiac involvement [123].

LGMD2S is caused by mutations in the **transport protein particle complex 11** gene (**TRAPPC11**), a transport protein involved in anterograde membrane trafficking from the endoplasmic reticulum (ER) to the ER-to-Golgi; this form shows childhood onset ataxia, and intellectual disabilities [143]. A form of adulthood-onset Pompe's disease has also been classified as **LGMD2V** [123] (see Section 4).

LGMD2W caused by mutations in the LIM and senescent cell antigen-like-containing domain protein 2 gene (**LIMS2/PINCH2**), that regulates cell shape and migration, presents as a childhood-onset LGMD2 with calf and tongue hypertrophy and severe quadriparesis [141].

Mutations in the **Popeye-domain-containing 1** gene (**POPDC1**) alter membrane trafficking and produce **LGMD2X** [142], which associates with atrio-ventricular conduction blockage [144]. Mutations in the torsinA-interacting protein 1 gene (**TOR1AIP1**) encoding the **lamina-associated polypeptide 1B** (LAP1B) cause **LGMD2Y** [142, 144]. **LGM2DZ** is caused by mutations in the **POGLUT1** gene [142] encoding **O-glucosyltransferase 1**, an enzyme involved in Notch-posttranslational modification and function, impairing muscle regeneration mediated by Notch. Patients exhibit typical features of LGMD2 and show reduced glycosylation of α-dystroglycan [145].

LGMD2G and **LGMD2H** are the rarest forms of LGMD2. LGMD2G is caused by mutations in the **TCAP** gene that encodes **telethonin**, a titin-interacting protein that links titin to other Z-disk proteins supporting sarcomere assembly and muscle stretching [146]. Clinical manifestations include adolescence-onset limb girdle weakness and cardiomyopathy susceptibility [123]. LGMDH is a late-onset disease characterized by proximal weakness and atrophy caused by mutations in the **TRIM32** gene encoding a ubiquitous E3 ubiquitin ligase involved in proteasome degradation of multiple targets, including actin [147].

6.3. Congenital muscular dystrophies

Congenital muscular dystrophies (**CMD**) include a number of neuromuscular disorders with onset at birth or early infancy that manifest with generalized hypotonia, hyperlaxitud and predominantly proximal weakness leading to pronounced head drop in most cases. Clinical course is slowly progressive but can evolve to severe retractions leading to skeletal deformations.

Usual cardiomyopathy and respiratory failure are observed and central nervous system involvement in the most severe cases [148]. Histological markers of CMD include abnormal variation of fiber size associated with split fibers, and in some cases with hypercontracted fibers, nuclei internalization and increase of connective and adipose tissue [148]. One of the most common CMD is **merosin-deficient-CMD** caused by mutations in the *LAMA2* gene encoding **laminin-α2 chain** (**merosin**) an extracellular matrix protein, major component of the basement membrane whose main function is link the extracellular matrix to the DGC. Mutations in *LAMA2* can result in a total or partial reduction of the protein levels, being the first cases more severe exhibiting progressive respiratory insufficiency, brain involvement and white matter abnormalities [149].

Other common forms of CMD are those related with defects in one of the three genes encoding **collagen 6A** (*COL6A1*, *COL6A2*, *COL6A3*). Collagen 6A is an important component of the extracellular matrix which forms a microfibrillar network that anchors the surface of cells with the interstitial connective tissue playing an important role in mediating cell matrix interactions due to its association with several matrix proteins [150]. Mutations in *COL6A* genes can cause three muscular disorders: **Ulrich-CMD**, **Bethlem myopathy**, and **myosclerosis myopathy**. Ullrich-CMD is a severe syndrome with neonatal onset that presents with hyperlaxity associated with proximal contractures in the spine (kyphosis), elbows, and knees and congenital hip dislocation. Children may never walk or walk and then lose this ability by the end of the first decade due to the progression of contractures and weakness. Respiratory function progressively declines over time leading to night-time respiratory failure and death [151]. Bethlem myopathy is a milder form with autosomal-dominant inheritance characterized by slowly progressive muscle weakness and wasting, distal hyperlaxity, and joint contractures and some patients exhibiting respiratory failures [153]. Myosclerosis myopathy is a rarest form of the disease with autosomal-recessive inheritance characterized by toe walking and calf contractures in childhood, and progressive contractures of all joints in the adult life [151].

Another severe form of CMD is the **Walker-Warburg syndrome**, a “dystroglycanopathy” mainly caused by recessive mutations in POMT1 and POMT2 [152]. It manifests with a dystrophic phenotype accompanied by retinal and brain malformations with most of the syndromic children dying in the first 3 years of life due to respiratory failure, seizures, hyperthermia, and ventricular fibrillation [153].

6.4. Facioscapulohumeral muscular dystrophy (FSHD)

FSHD is one of the most prevalent adult muscular dystrophies with an estimated incidence of 1:8000 live births worldwide [154]. It presents with slowly progressive asymmetric and descending weakness, initially affecting face (facio), scapula (scapulo), and upper arms (humeral), followed by weakness of the distal lower extremities and pelvic girdle [155]. Symptoms typically begin during the first or second decade of life [155]. There are no FSHD-specific histopathological markers in biopsy examination but dystrophic features such as fibrosis, muscle fiber hypertrophy, and central nucleation are present. Endomysial inflammation can be observed in up to one-third of FSDH biopsies [156]. Ninety-five percent of cases are inherited in an autosomal-dominant way and associated with a deletion of a key number of D4Z4 macrosatellite repeats in the 4q35 subtelomeric region in the chromosome 4 (**FSHD1**).

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The remaining 5% cases (**FSHD2**) have no deletion on chromosome 4q35 and have a variable inheritance pattern [155]. Loss of the D4Z4 repetitive elements leads to decreased methylation and opening up of the chromatin structure, allowing the expression of the *DUX4 gene encoding double homeobox 4*, a normally repressed transcriptional regulator. As reported by Lemmers and collaborators in 2010, the existence of single nucleotide polymorphisms in the region distal to the last D4Z4 repeat appears to create a poly-adenylation site that activates *DUX4*, leading to a "toxic gain of function" that cause FSHD1 disease [157]. In FSHD2 no deletions, but yet loss of methylation in the D4Z4 region of chromosome 4q35 also lead to *DUX4* abnormal expression [158] suggesting a common pathological mechanism in both FSHD.

6.5. Emery-Dreifuss muscular dystrophy

Emery-Dreifuss muscular dystrophy (EDMD) is an early-onset skeletal myopathy characterized by slowly progressive muscular weakness, joint contractures, spine rigidity, and heart disease [159]. Different types have been described, distinguished by their inheritance pattern in X-linked, autosomal-dominant, and autosomal-recessive forms. Overall prevalence of EDMD is unknown, although X-linked appear to be the most prevalent form affecting an estimated of 1:100,000 individuals [159]. EDMD is classified as a "laminopathy" caused mainly by mutations in the *EMD* gene encoding **emerin** and in the *LMNA* gene encoding **lamin A/C**. While *EMD* mutations associate to the X-linked form of EDMD, *LMNA* mutations are responsible for most of the autosomal cases [159]. Emerin and lamin A/C are important components of the nuclear envelope and defects in these genes could impair diverse functions including gene expression, cell signaling, nuclear structure and chromatin architecture [159, 160]. There are no clear histological markers in EDMD, but dystrophic features such as fiber size disproportion, nuclei internalization, increase of endomysial connective tissue, necrosis, and regeneration are usually observed (**Figure 1**). A reduced expression of emerin or lamin A/C in muscle tissue, fibroblasts or blood can usually confirm the diagnosis of EDMD [161].

7. Conclusions

An incredible large spectrum of hereditary myopathies has been described at date and only some of them have been commented in this chapter. Although hereditary myopathies are cataloged as "rare diseases" due to their relatively low prevalence, the sum of the different forms of hereditary myopathies makes these a relatively common health problem that affect the life quality of patients with complications that can lead to death in the most severe cases. Thanks to the improvement of technology, in the last decade, it has been possible to know a still growing number of genes causative of hereditary myopathies, which contributes to the classification and diagnosis of these disorders. Because defects in the same gene can be the cause of various hereditary myopathies and because the same myopathic phenotype can derive from mutations in different related genes, it is important to know and understand all aspects of the disease to give a successful diagnosis and an adequate management of the symptomatology.

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Section 4

Therapeutic Approaches for Muscular Disorder

Chapter 7

Making Skeletal Muscle from Human Pluripotent Stem Cells

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Additional information is available at the end of the chapter

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Abstract

Human pluripotent stem cells (hPSCs) proliferate in vitro for long periods without losing pluripotency and can be induced to differentiate into various cell types including skeletal muscle cells (SMCs). Human embryonic stem cells (hESCs) are generated from a preimplantation-stage embryo. Human-induced pluripotent stem cells (hiPSCs) are derived from somatic cells of both healthy donors and patients with muscle diseases of any age using reprogramming factors. Currently, there are two kinds of protocols to induce skeletal muscle from hPSCs. One type utilizes overexpression of a potent myogenic master regulator, MyoD, to directly induce skeletal muscle. Stepwise induction of skeletal muscle has also been reported by many research groups, but hiPSC-based cell therapy for muscular dystrophy is still experimental. On the other hand, hiPSCs derived from patients with muscle disease are widely used for disease modeling in vitro. Here, we review the recent literature on derivation of skeletal muscle from human pluripotent stem cells and discuss their application.

Keywords: muscular dystrophy, myoblasts, skeletal muscle, pluripotent stem cells, iPSC, Pax7, MyoD, myogenic differentiation, cell therapy, disease modeling

1. Introduction

When injured, skeletal muscle regenerates by activation and proliferation of its own stem cells: muscle satellite cells. Therefore, muscle satellite cells were expected to be a cell source for cell therapy for devastating muscular dystrophies. However, clinical trials in the 1990s

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were unsuccessful [1], possibly because myoblasts that had been expanded in vitro lost the high ability to fuse with the host's injured myofibers, indicating that improvement of muscle function requires a large quantity of myogenic progenitors with regenerative potential. Human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) [2] have almost unlimited proliferative potential and the ability to differentiate into the skeletal muscle lineage (reviewed in [3, 4]). Therefore, they are a promising source of new cells for cell therapy of muscle diseases such as muscular dystrophy (DMD). They are also useful for biological and physiological studies of human skeletal muscle.

Disease-specific hiPS cells are generated from patients' somatic cells (usually blood cells or skin fibroblasts) with almost the same efficiency and quality as cells from healthy donors [5]. Therefore, disease-specific-induced pluripotent stem (iPS) cells are being widely used to study the molecular mechanisms of these diseases and to screen potential drugs. As a method to directly derive skeletal muscle cells from hPSCs, MyoD-mediated reprogramming was established and is widely used. MyoD induces muscle cells in a relatively short period with high efficiency. In this chapter, we review the literature on derivation of skeletal muscle from human PSCs and discuss the next steps toward clinical applications. In the last part, we review the recent reports on successful disease modeling in vitro using patient iPS cells and discuss future directions.

1.1. Muscle stem cells

Muscle satellite cells were first identified by electron microscopy as a mononuclear cell between myofibers and the basal lamina and named by Mauro in 1961 [6]. Later, muscle satellite cells were shown to be skeletal muscle-specific stem cells in postnatal muscle [7]. Muscle satellite cells are activated upon muscle injury, proliferate as myoblasts, and fuse with each other or with regenerating myofibers to repair damaged muscle [8]. In 1989, Partridge et al. reported successful recovery of dystrophin expression in dystrophin-deficient mdx mice after direct injection of wild-type myoblasts [9]. Based on this finding, myoblast transfer therapy was performed on DMD patients in several hospitals. Unfortunately, the clinical trials failed to recover the muscle function of these patients. The majority of injected cells seemed to be lost within 48 h [1]. The results were unexpected at that time because endogenous muscle satellite cells themselves have high regenerative activity in situ and repair damaged muscle quickly. Later, researchers started to search for multipotent stem cells, which can be delivered systemically, engraft in muscle, and differentiate into myofibers. One of these cells is the mesoangioblalst, which showed an amazing ability to recover dystrophin expression in the muscles of dystrophic dogs after intra-arterial injection [10]. Mesenshymal stem/progenitor cells (MSC/MPC) are also expected to be a tool for regenerative medicine. They themselves do not differentiate into myofibers, but support muscle regeneration by paracrine effects [11]. The history of direct reprogramming in the muscle field is long, starting with the discovery of MyoD by Weintraub and his colleagues [12, 13]. MyoD powerfully converts non-muscle cells to skeletal muscle cells, but it is difficult to induce Pax7+ myogenic progenitors using MyoD alone. Recently Ito et al. reported that a combination of transcription factors (*Pax3*, *Mef2b*, and *Pitx1* or *Pax7*, *Mef2b*, and *Pitx1* for embryonic fibroblasts, and *Pax7*, *Mef2b* plus *MyoD* for adult fibroblasts) successfully induced transplantable myogenic progenitors from mouse fibroblasts [14]. Whether the same set of reprogramming factors can induce myogenic progenitors from human fibroblasts remains to be seen. Human iPS cells are relative newcomers in the muscle stem cell field. hiPSCs are pluripotent stem cells with almost

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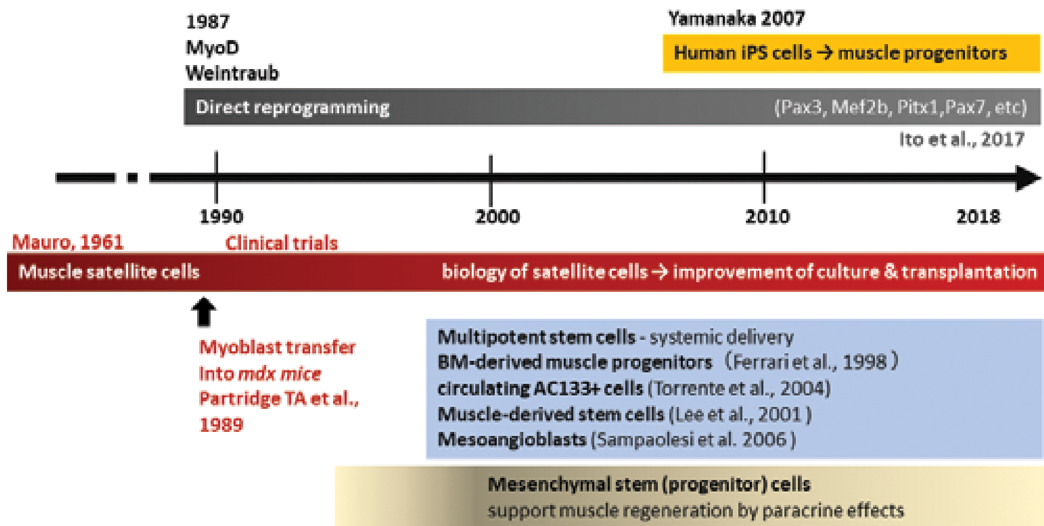

Figure 1. History of research on muscle stem cells and cell therapy for muscular dystrophies [2, 6, 10, 15–18].

equivalent properties to human ES cells, but can be derived from somatic cells such as skin fibroblasts [2]. Successful derivation of muscle cells from hiPS cells opened a new era of regenerative medicine for muscular dystrophies (**Figure 1**).

Muscle satellite cells were identified and named by Mauro in 1961 [6]. Direct reprogramming was reported for the first time in the skeletal muscle field in 1987 [15], but MyoD alone cannot induce myogenic progenitors. After a surprising report of BM-derived myogenic cells by Ferrari in 1998 [16], researchers looked for multipotent stem cells that can be delivered via the circulation. MSCs are modulators of muscle regeneration and widely used in regenerative medicine. Human iPS cells are a relative newcomer in the muscle stem cell field.

2. Myogenic induction by overexpression of myogenic transcription factors

MyoD: Weintraub and colleagues showed that overexpression of MyoD, a muscle-specific basic helix-loop-helix transcription factor, converted non-myogenic cells to muscle cells [13]. In 2014, Abujarour et al. reported efficient conversion of hiPSCs into muscle cells using a lentiviral vector-mediated doxycycline (DOX)-inducible MyoD overexpression system [19]. Sakurai and his colleagues used a PiggyBac transposon system to overexpress DOX-inducible MyoD in hiPSCs [20]. Importantly, the induced myotubes contracted on electrical stimulation [20]. MyoD induces skeletal muscle cells in a short period with high efficiency, but it cannot induce PAX7(+) myogenic progenitors. MyoD-induced skeletal muscle is now widely used for in vitro modeling of inherited muscle diseases.

Darabi et al. [21] reported derivation of engraftable muscle progenitors from hiPSCs by using a lentiviral vector encoding DOX-inducible PAX7. PAX7 expression was induced transiently in differentiating cells in a monolayer culture after a 7-day embryoid body (EB) culture [21].

Pax7-expressing GFP-positive cells purified by fluorescence-activated cell sorting (FACS) were then transplanted into the skeletal muscle of immune-deficient dystrophin-deficient mice, *NSG-mdx^{4Cv}*, and improved the muscle function of the mice. Whether myogenic cells induced by overexpression of Pax7 are suitable for cell transplantation therapy remains to be determined because transgenes have a risk of tumor formation. As far as the integration sites of the expression units in the genome and the expression of PAX7 are strictly regulated, myogenic progenitors expanded by PAX7 successfully regenerated damaged muscle of patients [22].

3. Stepwise induction of skeletal muscle by mimicking development

The majority of stepwise muscle induction protocols recently reported for human ESCs/iPSCs utilize a GSK3b inhibitor in common, myogenic growth factors (HGF, IGF-1, bFGF, EGF, etc.), and a serum-free medium (**Table 1**; [3]). For example, Chal et al. treated hiPS cells with CHIR-99021, which activates Wnt signaling, and LDN-193189, which prevents hiPSCs from differentiation into lateral mesoderm and induces differentiation into paraxial mesoderm. Treatment with these molecules of hESC cultures induced myogenin(+) myogenic cells with 25–30% efficiencies [34, 36, 39].

EB culture and sphere culture are often used to induce muscle progenitor cells (**Table 1**). Hosoyama et al. reported that hESCs/hiPSCs cultured as floating cell aggregates (termed EZ

Authors	Year	Journals	Cell types	Factors	Engraftment	Culture method/FACS sorting
Barberi et al.	2005	Plos Med	hESC	OP9 and C2C12 coculture		CD73(+) MSC sorting
Barberi et al.	2007	Nat Med	hESC	OP9 coculture, insulin		CD73(+) MSC sorting NCAM(+) cell sorting
Teng et al.	2010	J Cell Biochem	hESC	TGFβ inhibitor		EB culture
Ryan et al.	2012	Stem Cell Rev and Rep	hESC	FGF-2, All-trans RA		EB culture
Awaya et al.	2012	PloS One	hESC/hiPSC		yes	EB culture
Sakurai et al.	2012	PLoS One	hESC	LiCl, BMP4, ActivinA		2D culture
Xu et al.	2013	Cell	hiPSC	GSK3β inhibitor, FGF-2, forskolin	yes	EB culture
Leung et al.	2013	Biomacromolecules	hESC	C-PLC nanofibers, Wnt3a		2D culture
Borchin et al.	2013	Stem Cell Reports	iPSC	GSK3β inhibitor		2D culture c-met(+) cell sorting
Hosoyama et al.	2013	Stem Cell Transl Med	hESC/hiPSC	FGF-2, EGF		EZ sphere culture
Shelton et al.	2014	Cell Reports	hESC/hiPSC	GSK3β inhibitor, BMP, VEGF, Inhibinβ, FGF-2		EB culture
Chal et al.	2015	Nat Biotechnol	hESC/hiPSC	GSK3β inhibitor, BMP inhibitor		2D culture
Swartz et al	2016	Stem Cells Transl Med.	hiPSC	Transferrin, 1-thioglycerol, insulin, FGF-2, BMP4,PI3-kinase inhibitor , GSK3β inhibitor		2D culture
Chal et al.	2016	Nat Protoc	hiPSC	GSK3β inhibitor, BMP inhibitor, FGF-2, HGF, IGF-1	yes	2D culture
Caron et al.	2016	Stem Cell Transl Med	hESC	GSK3β inhibitor, Ascorbic acid, Alk5 inhibitor, Dex, EGFm insulin		2D culture
Hicks et al.	2018	Nat Cell Biol.	hiPSC	GSK3β inhibitor, BMP, VEGF, Inhibinβ, FGF-2 TGF-β inhibitor	yes	2D culture ERBB3(+) NGFR(+) cell sorting

Table 1. Papers reporting myogenic induction without transgenes from hPSCs [23–38].

spheres) in a medium developed for neural stem cells supplemented with bFGF and EGF efficiently differentiated into myogenic cells. After a 6-week floating culture, 40–50% of cells expressed PAX7, MyoD or myogenin [32]. Because dissociation of sphere cells into single cells during the induction process drastically reduces myogenic activity, direct cell-cell interaction might be essential for commitment of hPSCs to the skeletal muscle lineage.

4. Mimicking the muscle microenvironment

4.1. Importance of the extra-cellular matrix for myogenesis

The extra-cellular matrix (ECM) is an interactive environment having, apart from its simple role as a mechanical support or physical barrier, a role in signaling and providing a niche for the stem cell [40].

Collagens, laminins, fibronectin, or Matrigel have been used for in vitro studies in order to mimic the ECM of muscle cells in 2D or 3D culture systems because ECM is indispensable for skeletal muscle development [41]. Interestingly, native ECM obtained by decellularization of skeletal muscle stimulated muscle differentiation and a more rapid cell organization compared to single matrix glycoprotein culture [42], indicating again that ECM has functions in myogenesis. The use of a 3D fibrin-based hydrogel culture after production of myogenic cells with transient overexpression of Pax7 was recently reported to generate functional biomimetic skeletal muscle tissues from hiPSC-derived paraxial mesoderm cells for the first time [43].

The stiffness of the microenvironment has also been reported to regulate differentiation of myogenic cells [44, 45]. More precisely, in an in vitro context, 12 kPa stiffness was shown to give optimal results for muscle stem cells, compared to the harsh 10^6 kPa stiffness of a regular polystyrene plastic culture dish [45]. The impact of stiffness can be partially explained by the activation of pathways such as N-RAP, FAK, or PKC [44].

Some studies showed improvement of myoblast migration and differentiation through MMP-1 treatment [46], migration with MMP-13 [47], and fusion through MMP-7 overexpression [48], suggesting the importance of remodeling of the ECM for migration and differentiation of myogenic cells even in vitro.

4.2. Co-culture, exosomes, and miRNA

The interaction between myogenic cells and non-myogenic cells in muscle tissue may influence myogenesis either by interacting directly or by secretion of paracrine factors (**Figure 2**). Motor neurons were used to form neuro-muscular junctions [49] and further adapted to a 3D culture system [50]. The presence of motor neurons had positive effects on myotube maturation [50]. Co-culture with mesenchymal stem cells (MSCs) significantly enhanced the proliferation of muscle cells [51]. Co-culture of fibroblasts with myoblasts improved the alignment of the formed myotubes, but reduced differentiation [52]. Another paper, however, reported that the myoblasts formed longer, thicker myotubes with a mature phenotype in the presence of fibroblast-conditioning media, compared with control media [53].

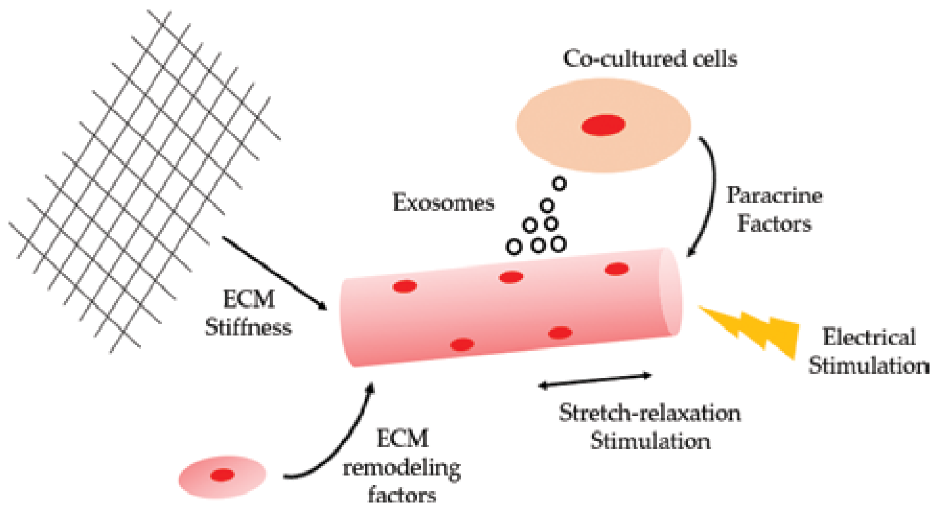

Figure 2. Summary of environmental inputs that promote differentiation of muscle cells.

Exosomes are major tools of cross talk between cells throughout the body and miRNAs are one of the elements they transport. Some miRNAs, such as miR-206, have been identified as promoters of skeletal muscle development and differentiation [54]. Therefore, miRNA-mediated induction of skeletal muscle from human iPSCs is a topic of great interest.

4.3. Differentiation of skeletal muscle cells through stimulation

Electrical: Another aspect of the muscle cell environment is the stimulation they are subjected to during the final steps of myogenesis. Exercise has been shown to have a great impact on muscle growth and differentiation in mice [55], and when electrically stimulated in vitro, C2C12 cells, a mouse myogenic cell line, showed similar responses to those of exercised skeletal muscle in vivo [56]. In the last couple of years, studies showed improvement of cardiac muscle differentiation of hiPSC by using electrical stimulation [57, 58].

Mechanical: Mechanical or stretch-relaxation stimulation has been shown to promote muscle growth and alignment in an electrical stimulus-independent manner [59]. Stretch-relaxation cycles have been shown to promote differentiation, muscle growth, fibers alignment, and overall organization [59–61]. Whether mechanical stimulation improves muscle induction from human iPS cells and their maturation remains to be determined even though results using cardiomyocytes are encouraging [58].

By mimicking the muscle microenvironment, it might be possible to increase the overall quantity, quality, and functionality of skeletal muscle cells produced from hiPSC.

5. Purification of myogenic cells

Most published protocols for muscle induction from human iPS cells generate a heterogenous cell population, including myogenic cells, undifferentiated cells, and non-myogenic cells. Non-myogenic cells are in many cases of neuronal lineage. Undifferentiated cells proliferate

actively and form tumors in the host muscle. Therefore, purification of myogenic progenitors is an important step for clinical use. Purification of myogenic cells would also facilitate the study of molecular pathogenesis and drug screening. To enrich myogenic cells by FACS, several iPS cell lines where the expression cassette of fluorescent proteins are inserted in the locus of myogenic regulators such as PAX7 [62] or Myf5 [63]. Combinations of myogenic cell-specific surface markers to enrich myogenic cells are also reported, for example, CD56 and CD82 [64], or CXCR4 (CD184) and C-MET [31]. We identified CD271 (NGFR) as a myogenic marker (submitted). M-cadherin antibody is also useful when the cells are dissociated into single cells by non-enzymatic treatment. Recently, Hicks et al. [38] identified ERBB3 (HER3) as a cell surface marker that enriches transplantable hiPSC-derived myogenic cells. To exclude neurogenic cells, CD57(HNK-1) is useful [31].

6. Myogenic progenitors for cell therapy

Cell source: Although cell therapy is a promising therapeutic approach to DMD and other muscular dystrophies, myoblast transfer therapy (MTT) in the early 90s failed to improve muscle function of DMD patients, possibly due to expansion of satellite cells *in vitro*. It was proposed that expansion of myoblasts in culture dishes reduced the regenerative activity of the injected cells. Incomplete immune suppression was also suggested as a cause of unsuccessful transplantation [1]. Because human iPSCs are highly proliferative, it might be possible to derive engraftable myogenic progenitors from hiPSCs on a large scale. iPSC-based cell therapy also allows the use of the patient's own cells (autologous cell transplantation) to avoid an immune response against engrafted cells. However, it takes a long time and is expensive to custom-make iPSCs from each patient and correct the disease-causing gene mutation. To solve this problem, HLA-homozygous donor-iPSC stocks are now being prepared (e.g., https://www.cira.kyoto-u.ac.jp/e/research/stock.html). It is expected that HLA-matched cells will greatly lower the risk of rejection. In addition, the safety of the cells in an iPS cell bank can be carefully examined in advance.

Tumorigenicity: A major concern of the use of hiPSCs for cell therapy is the tumorigenicity of hiPSC-derived cells. Tumor-forming cells could be roughly divided into two categories: residual undifferentiated cells and transformed cells. For undifferentiated iPS cells, purification of the differentiated cells with lineage-specific markers and elimination of undifferentiated cells using iPS markers would be effective. To avoid transformed cells, integration of the transgenes used for reprogramming into the genome should be carefully ruled out. Checking the integrity of the whole genome of parental iPS cells using genome-wide sequencing might be necessary. Prolonged culture of iPS cells should be avoided because long-term culture causes genomic abnormalities. Some groups propose, as a final line of defense, the use of a specific hiPSC line containing a HSV-tk gene [65] or an inducible Caspase-9 gene as a suicide gene, which would allow eradication of hiPSC-derived tumors in vivo after transplantation [66–69].

Quality control: For clinical use, (1) stable induction of myogenic progenitors in large quantity, (2) reduction of culture period and cost, and (3) establishment of a reliable system to monitor the quality and safety of the cells are all required. The monitoring system is especially important, because the cells change their properties during culture and it is difficult to keep the myogenic potential of the FACS-sorted cells high during culture.

7. Disease modeling in vitro using patient-derived iPSCs

During the past decades, researchers have generated numerous mouse models, such as knock-out mice to analyze the pathogenesis of muscle diseases. However, mouse models often fail to reproduce the phenotypes of patients. For example, dystrophic *mdx* mice, which carry a DMD-type mutation in the dystrophin gene, exhibit much milder dystrophic phenotypes than DMD patients. It also has happened that drugs proven to be effective in a mouse model have much less effect in human patients. This is why human iPS cells are expected to become a tool for disease modeling.

These days, iPSCs from many kinds of muscular diseases have been established. For example, Abujarour et al. generated iPSC lines from patients with DMD or Becker muscular dystrophies (BMD) [19]. Shoji et al. established DMD-iPSCs and reported abnormal calcium ion influx in DMD myotubes. Importantly, dystrophin expression was restored to DMD myotubes by an exon-skipping technique, and the calcium ion overflow was suppressed [70]. Choi et al. [71] reported that DMD-iPS cells showed aberrant expression of inflammation or immune-response genes and collagen genes, increased BMP/TGFβ signaling, and reduced fusion competence. Tanaka et al. [20] established iPSC lines from patients with Miyoshi myopathy. Patient-iPSC-derived myotubes showed defective membrane repair, and the authors rescued the phenotype by expression of full-length DYSFERLIN. Snider et al. [72] showed the expression of full length DUX4 in embryoid bodies from iPSCs with facioscapulohumeral dystrophy (FSHD). Caron et al. reported that FSHD1 myotubes were thinner, and the genes involved in cell cycle control, oxidative stress response, and cell adhesion were differentially regulated [37]. Du et al. and Ueki et al. [73, 74] reported that the CTG-CAG triplet repeats were expanded by passaging iPSCs derived from myotonic dystrophy type1 (DM1) patients. Yoshida et al. [75] generated iPSCs from a patient with infantile-onset Pompe disease that showed lysosomal glycogen accumulation, which was dose-dependently rescued by rhGAA.

These successful in vitro disease modelings using patient-iPSCs are encouraging and would be useful for screening new drugs. Because hiPSCs have unlimited proliferative potential, one can perform experiments repeatedly and screen potential drugs extensively even if it is a rare disease. It should be, however, also recognized that skeletal muscle cells derived from hPSCs are much more immature in gene expression, morphology, and function than real myofibers in the body. Therefore, to what extent patient-derived iPSCs can reproduce a disease phenotype in vitro is important. In addition, there are variations in differentiation propensity among hiPS cell lines [76], and it is important to confirm the reproducibility of the results. Patient-iPS cells whose mutated genes were corrected by genome-editing technique [77] would serve as good controls and help to validate the findings.

8. Conclusion

Skeletal muscle can be induced from hPSCs by direct reprogramming or a stepwise differentiation method. Disease modeling using patient-derived iPSCs is now widely used to elucidate disease mechanisms and to screen for drugs. For successful disease modeling, it is important

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to induce mature myofibers. For cell-based therapy, the protocols for induction of myogenic progenitors from hiPS cells and their purification have been almost completely established. However, to eliminate the risk of tumor formation by engrafted cells, more study is needed.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Notes/Thanks/Other declarations

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Chapter 8

A Novel Functional *In Vitro* Model that Recapitulates Human Muscle Disorders

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Abstract

Here, we aim to address the increasing need for a suitable human muscle *in vitro* model in order to advance in the knowledge of muscle pathophysiology and test novel therapies for muscle disorders. Our model is based on a simple 2D culture method that yields highly mature human myotubes under optimized environmental conditions. Culture conditions that produced functional and contractile human myotubes with an extended lifetime consisted in extracellular matrix overlay and addition of several trophic factors to the differentiation medium. In this work, we describe the generation of suitable models of muscular dystrophies (limb-girdle muscular dystrophy type 2A—LGMD2A and Duchenne) by silencing expression of key proteins in these myotubes. Western blot and immunocytochemical analyses demonstrated similar features between our knockdown human myotubes and dystrophic muscles in vivo, which support the general validity of our cellular models. We also found that both dystrophic models present higher resting cytosolic Ca^{2+} levels than controls, which support a common underlying deficit in calcium homeostasis. This novel human *in vitro* system would allow for high-throughput screening of new treatments for these muscular dystrophies as well as for other neuromuscular disorders. In addition, our model could be used to advance in our understanding of human skeletal muscle pathophysiology.

Keywords: human muscle model, LHCN-M2 cell line, calpain 3, dystrophin, myotube maturation, muscular dystrophy

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1. Introduction

Skeletal muscle comprises around 40% of the total body mass in adults, and it is composed of contractile multinucleated myofibers formed by the fusion of myoblasts. Muscle fibers are coated by an extracellular matrix (ECM), namely the basement membrane, which accounts for around 5% of the skeletal muscle mass. This matrix provides mechanical structure to myofibers during contractions, contributes elastic properties to the tissue, and partakes in the transmission of force from the myofiber to the tendon. It is well known that muscle homeostasis relies on the relation between the different cell types present in the muscle tissue and their microenvironment. Thus, communication between the ECM and muscle cells is essential for gene expression, cell proliferation, adhesion, and differentiation [1].

One of the *in vitro* systems most widely used to study muscle pathophysiology is the mouse myoblast culture, which differentiates into mature myotubes with spontaneous contractile capacity within 6–14 days [2, 3]. In contrast, human skeletal myotubes display lower maturation and survival capacity with reduced sarcomeric cross-striation, and they generally lack contractile capacity [4, 5]. Moreover, human myotubes display a high inter-individual heterogeneity with regard to myotube morphology, survival, and differentiation [6], which makes comparisons between healthy and diseased myotubes challenging. Furthermore, sample availability is a substantial limiting factor due to scarcity of muscle biopsies and limited proliferation of human myoblasts, particularly when isolated from dystrophic muscles [7].

On the other hand, in vivo mouse models do not often reproduce the severity of human disorders, probably due to a reduced regeneration capacity of human muscles compared to mice [8]. Also, different underlying pathophysiological mechanisms in humans and mice cannot be ruled out and, thus, therapies tested on mouse models may not necessarily yield the same results in human patients [9]. For instance, cerivastatin was released to the market as it was well tolerated in several animal models but caused fatal rhabdomyolysis in humans [10]. Therefore, it is becoming essential to generate a well-characterized and reproducible human *in vitro* model of skeletal muscle with pathophysiological relevance to human muscle diseases in order to advance in the generation of specific therapies.

Previous attempts in this line have been focused on promoting contractility of human myotubes, in order to increase maturation and mimic exercise and adaptive responses characteristic of skeletal muscle. Different strategies include exogenous application of electrical pulse stimulation to cultured myotubes [11–15], magnetic fields to induce myoblast differentiation, and co-culturing with spinal cord explants [16–19] or human motoneurons [20, 21] in order to achieve myotube innervation and subsequent maturation. More recently, spontaneous contractile activity has been observed in human myotubes by using a defined differentiation medium containing several trophic factors [22]. Other works have achieved improvement of survival, maturity, and attachment of human myotubes by modifying the extracellular microenvironment through addition of polymeric coatings or micro-patterned surfaces [23–27].

As an alternative, several studies on neuromuscular disorders are based on myotubes obtained from sources other than muscle biopsies, such as MyoD-converted fibroblasts

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[28, 29] or, more recently, human-inducible stem cells (induced pluripotent stem cells—iPSCs) derived to myoblasts [30, 31]. More recently, significant progress has been made toward engineering functional 3D human muscle systems using iPSCs [32, 33]. While these models overcome limitations regarding sample availability and they have demonstrated physiological relevance to humans, still they require special handling and significant resources in terms of costs and time. Immortalization of primary myoblasts from dystrophic patients seems to be a reasonable compromise among sample availability, relevance to human muscle, and cost-effectiveness [34].

In this chapter, we sought to generate a functional 2D human *in vitro* model of skeletal muscle. For this purpose, we have used the immortalized LHCN-M2 myogenic cell line that was obtained from the skeletal muscle of a healthy male donor [35]. This human myoblast cell line was selected due to its thorough characterization and availability to the scientific community and because it displays relatively high differentiation levels compared to other human cell lines. Thus, we thoroughly optimized culture conditions in order to obtain highly mature human myotubes with distinct sarcomeric patterning and spontaneous contractility. Then, we validated its relevance for the study of muscular dystrophies by silencing expression of dystrophin and calpain 3 proteins. We used shRNA-lentiviral infection to silence expression of dystrophin and calpain 3 proteins, whose deficiency causes Duchenne and LGMD2A muscular dystrophies, respectively. Relevance of these models was evaluated by analyzing expression of several genes and proteins involved in the pathological mechanisms of these muscular dystrophies. Lastly, Ca^{2+} homeostasis was analyzed by intracellular Ca^{2+} imaging in these myotubes. In this line, in a previous study, our group has already reported abnormal calcium homeostasis in the LGMD2A *in vitro* model.

2. Materials and methods

2.1. Antibodies

Primary antibodies were obtained from the following sources: 12A2-Calpain3 (NCL-CALP-12A2) (Leica Biosystems, Barcelona, Spain); Ryanodine Receptor type1 (MA3–925) (Affinity BioReagents, Golden, CO, USA); DHPRα2 (ab2864) (Abcam, Cambridge, UK); Actin (A2066), and Actinin (A7811) (Sigma-Aldrich, Madrid, Spain); Myosin Heavy Chain (MyHC A.1025) and Dystrophin (MANDYS1) (DSHB, Iowa City, IA, USA); Aldolase A (SC-12059) and α-sarcoglycan (SC-271321) (Santa Cruz Biotechnology, Heidelberg, Germany).

2.2. Culture conditions

LHCN-M2 immortalized human were generated in the Platform for Immortalization of Human Cells (Myology Institute, Paris, France), as previously described [35]. LHCN-M2 myoblasts were grown in Skeletal Growth Medium (SGM, C-23060, Promocell, Heidelberg, Germany) on 12-mm coverslips (Thermo Scientific, Madrid, Spain) coated with 0.5% gelatin (G1890, Sigma-Aldrich). At confluence, cells were washed with Dulbecco's phosphate-buffered

saline—DPBS (Life Technologies, Madrid, Spain) and 200 μl of Extracellular Matrix Gel (E6909, Sigma-Aldrich) diluted at 1:3 ratio in Dulbecco's Modified Eagle's Medium (41966-029, Life Technologies) was added. Cultures were incubated at 37°C for 30 min in order to let extracellular matrix proteins self-assemble and produce a thin overlay of ~1 mm covering myoblasts. Then, differentiation medium was added to cultures (DM1, **Table 1**) and half of the medium was replaced every 3 days. In some experiments, a differentiation medium with trophic factor was added to cultures (DM2, **Table 1**), when myoblasts started to fuse and form large multinucleated myotubes (at 3–4 days post-differentiation). See **Figure 2A** for schematics. Similar results were obtained with myoblasts grown in proliferation medium (**Table 1**), instead of commercial SGM. Culture media reagents were obtained from the following sources: Fetal Bovine Serum (FBS, 10270-106), Newborn Calf Serum (NCS, 16010-159), GlutaMAX-I (35050-038), MEM (51200-038), Medium 199 (M199, 31150-022), Neurobasal A medium (10888-022), B27 supplement (17504-44), mouse laminin (23017-015), bFGF (13256-029), and Gentamicin (15750-037) were from Life Technologies; human sonic hedgehog (Shh, 1845-SH-025), IGF-1 (4326-RG), CNTF (557-NT), and agrin (550-AG-100) were obtained from R&D Systems (Abingdon, UK); EGF (AF-100-15), BDNF

Proliferation Medium (SGM)	Differentiation Medium 1 (DM1)	Differentiation Medium 2 (DM2)
Skeletal Growth Medium	DMEM	Neurobasal A Medium
FBS 20%	Insulin 10μg/mL	B27 Supplement 1x
Glutamax 1x	ApoTransferrin 100μg/mL	Glutamax 1x
Gentamicin 50μg/mL	Gentamicin 50μg/mL	BDNF 20ng/mL
		Shh 50ng/mL
		IGF-1 10ng/mL
		CNTF 5ng/mL
		NT-3 20ng/mL
		Laminin 4μg/mL
		Agrin 100ng/mL
		Gentamicin 50μg/mL

Table 1. Culture media used for human myoblast growth and differentiation.

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(450-02), and NT-3 (450-03) from Peprotech (London, UK); insulin (I6634), human apo-transferrin (T1147), Fetuin (F2379), HEPES (H3375), and dexamethasone (D2915) were obtained from Sigma-Aldrich.

2.3. Lentiviral infection

Lentiviral particles were produced by Inbiomed (San Sebastian, Spain) from plasmid DNAs TRCN0000003494 (shCapn3), TRCN0000053243 (shDyst) and SHC002 (NS-shRNA) (Sigma-Aldrich). Human immortalized LHCN-M2 myoblasts were seeded at approximately 25% of confluence and the next day, cells were infected at MOI 5 with 4 μg/ml polybrene (H9268, Sigma-Aldrich). Myoblasts were selected and expanded in SGM with 1 μg/ml puromycin (A11138-03, Life Technologies) for 7 days, as previously described [36].

2.4. Western blot

Proteins from human myotubes grown on 24-well plates were lysed directly with 75 μl of reducing loading buffer and resolved in 4–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-rad, Madrid, Spain). A total of 10 μl of protein homogenates were loaded per lane, corresponding to ~10–15 μg of protein. Membranes were stained with Ponceau-S to verify similar total protein loaded. Proteins were transferred onto nitrocellulose membranes, blocked with 5% nonfat milk, and 2% goat serum (Sigma-Aldrich) in Tris-buffered saline with 0.1% Tween 20 (TBST). Membranes were incubated with primary antibodies overnight at 4°C and the following day, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies diluted 1:40,000 for 1 h (Santa Cruz Biotechnology). Chemiluminescent signal detection was performed with Supersignal West Dura kit (Thermo Scientific).

2.5. Immunocytochemistry

Myotubes grown onto 12-mm glass coverslips were fixed in acetone for 3 min. Cells were washed with phosphate-buffered saline (PBS) and preincubated for 1 h with 2% normal horse serum, 2% bovine serum albumin, and 0.5% Triton X-100 in PBS. Cells were incubated overnight at 4°C with primary antibodies and then washed and incubated with Alexa Fluor 488-, CY3-, or CY5-conjugated secondary antibodies (1200, Life Technologies). Coverslips were mounted with Prolong Gold antifade reagent with 4′,6-diamidino-2-phenylindol (DAPI) (Life Technologies). Samples were visualized on an inverted epifluorescent microscope (Nikon Ti-S) and images were acquired with a high-resolution camera (ORCA Flash 2.8, Hamamatsu).

2.6. Myotube viability determination

Myotube viability was determined with the cytotoxicity assay Cytotox96 (Promega, Madrid, Spain), which quantifies lactate dehydrogenase (LDH) activity. Myoblasts were seeded in gelatin-coated 96-well plates, grown to confluence and differentiated. LDH was measured in 3–4 replicates at several time points during the differentiation process, in both supernatants and cell lysates. For this assay, myotubes were directly lysed with 20 μl of 0.1% Triton X-100 in

2 M Tris solution. Myotube viability was calculated from the ratio between LDH released to the medium to total intracellular LDH in myotube lysates. Percentage of myotube viability at 7 and 10 dpd was normalized to levels at 5 dpd (100%).

2.7. Myotube differentiation and maturation

Total creatine kinase (CK) activity has been previously used to determine levels of myotube differentiation and maturation [37]. CK activity was determined with the colorimetric kit CK-NAC (Thermo Scientific) at several days during myotube differentiation. Cells were grown and differentiated in 96-well plates and directly lysed with 20 μl of 0.1% Tris 2 M Triton solution. Myotube differentiation levels were expressed as the fold increases in CK activity normalized to levels obtained at 3 dpd.

2.8. Total protein quantification

Total protein was quantified with Bradford protein assay (Bio-rad). Myotubes differentiating in 96 well plates were lysed with 20 μl of 0.1% Triton X-100 in 2 M Tris solution.

2.9. Calcium imaging

Myotubes grown on glass coverslips were loaded with 4 μM Fura 2-AM for 30 min at 37°C. Cells were incubated in Ringer buffer (125 mM NaCl, 5 mM KCl, 6 mM glucose, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 25 mM HEPES, pH 7.4) for 20 min at room temperature to remove non-hydrolyzed fluorophore and complete de-esterification of the dye. Experiments were performed at 37°C under continuous perfusion (2 ml/min) with Ringer buffer using an ECLIPSE Ti-S/L100 microscope (Nikon) equipped with a 20X S-Fluor objective and attached to a lambda-DG4 illumination system. Image acquisition was performed using an Orca-Flash 2.8 camera (Hamamatsu) with the Nis-Elements AR software. Variations in Ca^{2+} levels over time are determined by the ratio between the fluorescence intensities at 340 and 380 nm excitation wavelengths.

2.10. RT-qPCR

mRNA levels of *CAPN3*, *GRP78*, *CHOP*, *HERP*, X-box binding protein-1(*XBP1*), and spliced *XBP1* (*sXBP1*) were quantified using complementary DNA (cDNA) synthesized from DNAse-treated RNA obtained from control (shCtrl) and Capn3-deficient LHCN-M2 myotubes (shCapn3). qPCR was performed and analyzed with the 7900HT Real-Time PCR System (Applied Biosystems), using SyberGreen master mix as previously described [36, 38]. Measurements were performed in triplicates in three different cultures, and the results were normalized to a normalization factor based on the geometric mean of four reference genes: *creatine kinase* (*CK*), *DHPRα1*, *dmd*, and *HPRT1*. The primer sequences are shown in **Table 2**. Primers were designed using Primer Express software (Thermo Fisher), and specificity was ensured with reverse e-PCR online software (www.ncbi.nlm.nih.gov/projects/e-pcr/reverse.cgi).

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Gene	Forward Primer	Reverse Primer
C-FLIP	TCCTTCAAATAACTTCAGGCTCCATA	GGATTTCTTCACTGGTTCTTGTTGA
CAPN3	GAAAAGAGGAACCTCTCTGAGGAA	CGAAGATGATGGGCTTGGTT
CHOP	CTCCTGGAAATGAAGAGGAAGAAT	TGCTTGTGACCTCTGCTGGT
CK	GAAGCTCTCTGTGGAAGCTCTCA	CCTTCTCCGTCATGCTCTTCA
DHPRα1	GCCATCTCCGTGGTGAAGAT	CACTGCACCACGTGCTTCA
DYST	ACAGGGCAAAAACTGCCAAA	CGCAGTGCCTTGTTGACATT
GRP78	AGAAGGTTACCCATGCAGTTGTTACT	CTCATAACATTTAGGCCAGCAATAGTT
HERP	CAAGGTGGCTGAATCCACAGA	GCCTTAAACCATCACTTGAGGAAT
HPRT1	CATGGACTAATTATGGACAGGACTGA	TGAGCACACAGAGGGCTACAA
sXBP1	GCTGAGTCCGCAGCAGGT	CCCCACTGACAGAGAAAGGGAGG
XBP1	GCAGGTGCAGGCCCAGTTGTCAC	CCCCACTGACAGAGAAAGGGAGG

Table 2. Primers sequences used for qPCR experiments.

2.11. Statistical analyses

Data are presented as mean ± SEM. An independent *t*-test with a significance level of 0.05 was applied to test differences between two groups. For multiple group comparisons, one-way analysis of variance (ANOVA) test was applied with Tukey's post hoc test.

3. Results

3.1. Extracellular matrix overlay increases attachment and results in mature LHCN-M2 human myotubes

LHCN-M2 myoblasts were grown on gelatin-coated tissue culture plates and differentiated following standard protocols (see Section 2). Differentiation medium (DM1, **Table 1**) was added when myoblasts reached confluence, and at 3 days post-differentiation (dpd) first myotubes were observed. In our hands, detachment of myotubes started at 7 dpd and almost no myotubes survived after 10 dpd (**Figure 1**). Among different cultures, a substantial variability in myotube survival and differentiation was observed within this period. We hypothesized that myotube maturation was hindered by deficient attachment and, thus, we aimed to optimize myotube attachment by

addition of a suitable protein scaffold. We analyzed the effect of extracellular matrix gel (ECM) on myotube attachment, since previous studies have indicated that addition of ECM proteins to human myotubes facilitates muscle cell attachment and organization [39]. Also, we anticipated that enriching extracellular architecture would provide additional support during myotube contraction. Thus, an ECM gel secreted by Engelbreth-Holm-Swarm mouse sarcoma cells was added to myotube cultures in order to provide both protein components and a suitable scaffold. Several dilutions and protocols were tested, and best results were obtained with a 1-mm layer of ECM gel diluted 1:3 in Dulbecco's Modified Eagle's Medium, which was added to confluent myoblasts (see Section 2). Similar improvements in myotube attachment, survival, and maturation were observed using the analogous protein mixture BD Matrigel Basement Membrane Matrix diluted 1:3 (data not shown). Specifically, we found that addition of an ECM overlay to myoblasts

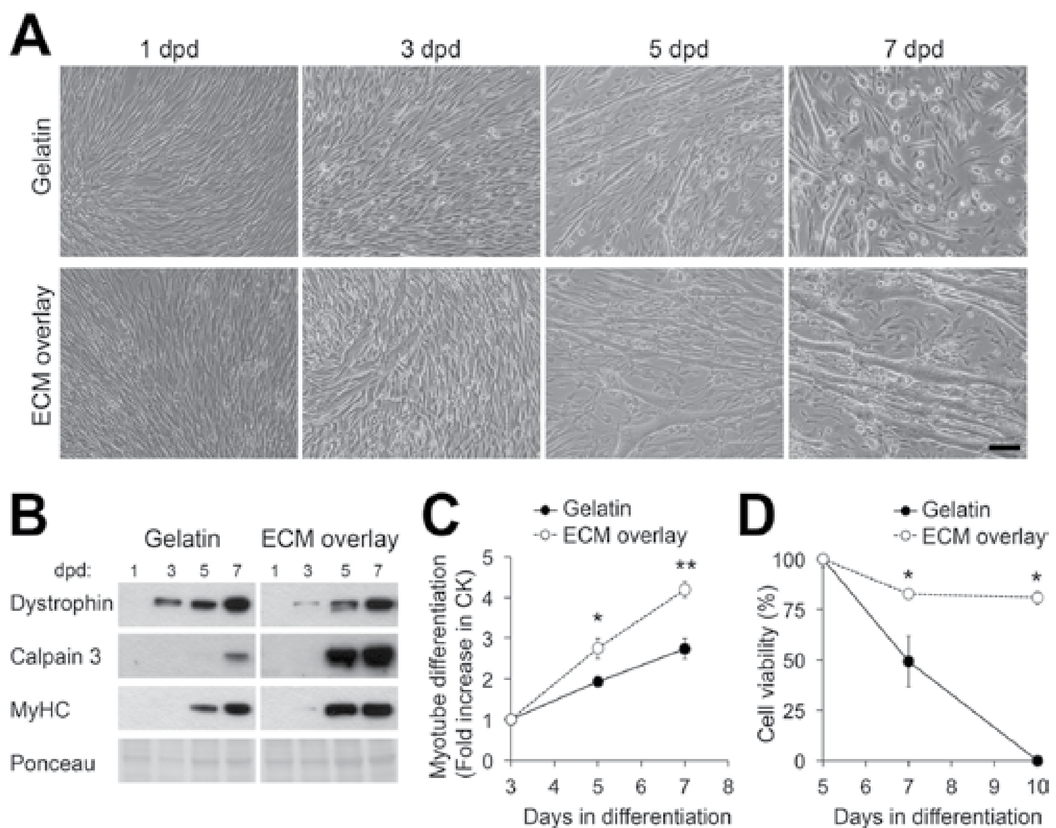

Figure 1. Addition of an extracellular matrix overlay enhances human LHCN-M2 myotubes survival and maturation. (A) Representative bright field images of myotubes at several days post-differentiation (1–7 dpd) in standard culture conditions (upper row) or differentiated under a ~1 mm ECM overlay (lower row). (B) Western blot analysis of skeletal muscle differentiation markers (1–7 dpd) under standard conditions (left panel) and with an ECM overlay (right panel). (C) Quantification of myotube differentiation levels, expressed as average fold increase in creatine kinase (CK) activity. Addition of an ECM overlay (white circles) before differentiation resulted in a significant increase in myotube maturation levels compared to standard condition (black circles). (D) Myotube viability was quantified at 5, 7, and 10 dpd. Addition of an ECM overlay (white circles) resulted in a significant increase in myotube viability compared to standard condition (black circles). ECM overlays promoted attachment and survival of myotubes beyond 10 dpd. All data expressed as mean ± SEM, $n = 4$ *$p < 0.05$, **$p < 0.005$). ECM, extracellular matrix gel; dpd, days post differentiation. Scale bar = 40 μm.

significantly extends the culture lifetime, promoting development of mature striated myotubes that survive for up to 12–14 dpd (**Figure 2**). In addition, we often observed spontaneous contractile activity in cultures with the longest lifetimes (Video 1, https://www.intechopen.com/download/index/process/151/authkey/84bcd15620efa9dacbd8d3c504b4f45a). Analysis of specific skeletal muscle proteins by Western blot revealed that compared to standard culture conditions, myotubes differentiated under ECM overlays display higher levels of dystrophin, calpain 3, and myosin heavy chain (MyHC, **Figure 1B**), and expression of these proteins was detected at earlier stages of differentiation. Effect of ECM overlays on human myotube differentiation was quantified by measuring the increase in creatine kinase (CK) enzyme activity, which is a sensitive marker of differentiation [37]. We found that addition of an ECM overlay to confluent myoblasts significantly increased CK activity at both 5 and 7 dpd (**Figure 1C**; $p < 0.05$). Finally, effect of ECM overlays on myotube survival was quantified at 5, 7, and 10 dpd by measuring lactate dehydrogenase (LDH) released to the medium from dying myotubes. We found that addition of an ECM overlay significantly increased cell viability at 7 dpd compared to standard conditions ($p < 0.05$). At 10 dpd, most of the myotubes are detached in standard cultures, whereas myotubes differentiated under ECM overlays retained ~80% cell viability compared to levels at 5 dpd (100%; $p < 0.001$, **Figure 1D**). These data support our theory that providing an appropriate protein scaffold to human myotube cultures results in higher attachment and survival and, as a consequence, it produces a significant increase in the maturation levels of these myotubes, which develop spontaneous contractile capacity.

3.2. Optimization of differentiation medium to further improve survival and maturation of human myotubes

Presence of trophic factors during differentiation may further stimulate maturation of LHCN-M2 human myotubes, as previously reported for primary human myoblasts [22]. Therefore, we designed a new differentiation medium that included several factors with reported activity for promoting myotube survival and maturation, such as agrin and neurotrophins [16, 40]. This new differentiation medium (DM2, **Table 1**) was added to myotubes cultured under ECM overlays when large myotubes first appeared, which was usually at 3–4 dpd (**Figure 2A** for schematics). A few days after DM2 addition, there was an obvious increase in myotube density compared to myotubes differentiated in DM1 (**Figure 2B**, 7–9 dpd). This was confirmed by total protein content quantification, which revealed significant 2- to 3-fold increases at 5, 7, and 10 dpd in myotube cultures differentiated in DM2 compared to DM1 (**Figure 2C**). Around 12–14 dpd, most of the myotubes differentiated in DM2 predominantly displayed a rhythmic and vigorous contractile activity and myotube lifetime was usually extended beyond 15 dpd (Video 2, https://www.intechopen.com/download/index/process/151/authkey/84bcd15620efa9dacbd8d3c504b4f45a). Moreover, most myotubes differentiated in DM2 displayed well-defined sarcomeric patterns and peripherally aligned nuclei at 14 dpd (**Figure 2B**). Next, we analyzed the developmental expression profiles for a range of myogenic proteins throughout myotube differentiation in both media (**Figure 2D**). Overall, myotubes differentiated in DM2 showed higher maturation as observed by higher expression levels of dihydropyridine receptor (DHPR), ryanodine receptor 1 (RyR1), and MyHC. This was supported by significantly higher CK activity levels in myotubes differentiated in DM2 compared to DM1, at 10 dpd and beyond (**Figure 2E**). Finally, quantification of released LDH

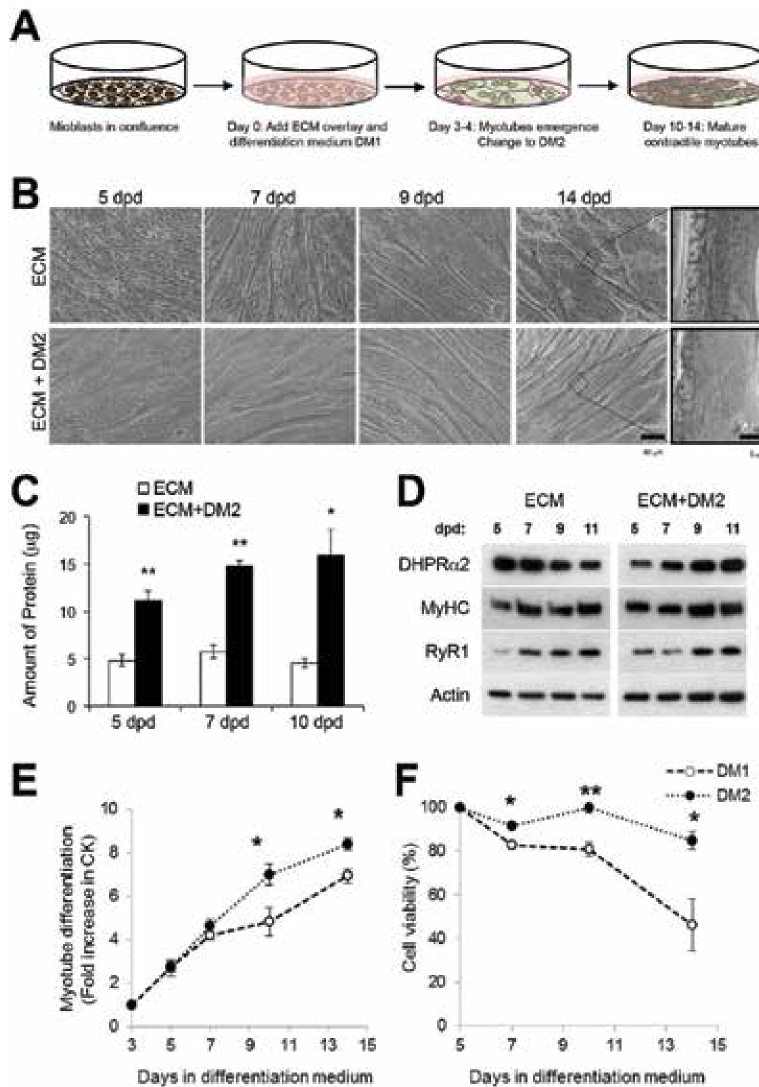

Figure 2. Differentiation medium with trophic factors further increases LHCN-M2 myotubes density, survival, and maturation. (A) Schematics of the optimized differentiation protocol. Human LHCN-M2 myoblasts were seeded onto gelatin-coated coverslips and grown until confluence. Medium was removed and 200 μl of ECM (1:3 dilution) was added over myoblasts. A gel scaffold was allowed to form by incubating 30 min at 37°C. Myoblasts were differentiated in standard differentiation medium (DM1) until multinucleated myotubes emerged (around 3–4 dpd). Then, this medium was substituted by a differentiation medium with trophic factors (DM2). At 10–14 dpd, mature, cross-striated, and contractile myotubes were observed. (B) Representative bright field images of LHCN-M2 myotubes differentiating under ECM overlay. Upper row images represent myotubes differentiating in standard medium, while lower row images depict myotubes where DM2 differentiation medium was added at 3 dpd. (C) Protein quantification from myotubes differentiating under ECM overlays at 5, 7, and 10 dpd. DM2 differentiation medium (black bars) promoted a significant increase of protein content compared to standard medium (white bars). (D) Western blot analysis of several myogenic proteins shows higher maturation of myotubes differentiating under ECM overlay with DM2 (right) compared to standard medium (left). (E) Addition of DM2 significantly increased myotube maturation levels, as expressed as average fold increase in creatine kinase (CK). (F) Addition of DM2 significantly increased myotube viability. At 14 dpd, viability of myotubes differentiated in DM2 was preserved (white circles), while it was markedly reduced in myotubes differentiated in standard medium (black circles). Data are expressed as mean ± SEM, n = 4 $*p < 0.05$, $**p < 0.005$. Scale bar = 40 μm; 5 μm in inset. DM2, differentiation medium 2; dpd, days post differentiation.

showed that myotubes cultured in DM2 displayed significantly higher viability levels than the ones in DM1 (**Figure 2F**). These results demonstrate that among the different conditions analyzed in this study, the combination of ECM overlays and DM2 medium constitute the most suitable environmental conditions for the generation of highly mature and functional human myotubes with a more extended lifetime.

3.3. Silencing gene expression in highly mature LHCN-M2 myotubes as a relevant human model of muscular dystrophies

We aimed to generate relevant human cellular models of muscular dystrophies by silencing specific proteins in LHCN-M2 myoblasts in combination with the optimized myotube differentiation protocol described earlier. Thus, we used lentivirus carrying short hairpin RNAs (shRNAs) specific for human calpain 3 (shCapn3) or dystrophin (shDyst), in order to generate cellular models of limb-girdle muscular dystrophy type 2A (LGMD2A) and Duchenne muscular dystrophy (DMD), respectively. Myoblasts infected with lentivirus carrying none-silencing shRNAs were used as controls (shCtrl). Infected LHCN-M2 myoblasts were selected with puromycin for more than 10 days and resistant myoblasts were expanded and used for subsequent analyses after verification of knockdown efficiency. We noticed that when compared to noninfected LHCN-M2 myoblasts, those subjected to lentivirus infection and subsequent selection resulted in myotubes with a reduced contractile capacity, although a number of myotubes with spontaneous contractility were observed.

We found that lifetime of shCtrl and shCapn3 myotubes extended up to 14 dpd, whereas shDyst myotubes showed obvious early detachment, and they were unable to survive beyond 12 dpd (**Figure 3A**). Quantification of myotube viability by measuring released LDH confirmed that viability of shDyst myotubes was significantly diminished at 11 dpd compared to shCtrl myotubes (68.13 ± 3.65 vs. 97.66 ± 1.20%; $p < 0.005$). In contrast, viability of shCapn3 myotubes (97.00 ± 1.03% at 11 dpd) did not show any difference compared to controls throughout differentiation (**Figure 3B**). Also, no obvious differences in myotube maturation levels were observed between control (shCtl) and dystrophic myotubes (shDyst and shCapn3), as quantified by fold increase in CK activity (**Figure 3C**). Likewise, no marked differences in cell morphology were observed between dystrophic and control myotubes (**Figure 3A**).

We have already reported a preliminary characterization of our LGMD2A model in a recent study [36]. In summary, we achieved an efficient silencing of Capn3 expression in human myotubes that resulted in a concomitant reduction of sarco/endoplasmic reticulum Ca^{2+}-ATPase and RyR1 protein levels (**Figure 4A**). Thus, our results are in line with previous studies showing that SERCAs and RyR1 protein levels undergo significant reduction in mouse models [36, 41] as well as in muscle samples from LMGD2A patients [36, 42]. Furthermore, we have found that the expression of the antiapoptotic factor cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP), was downregulated by 25% in Capn3-deficient myotubes. This result supports a previous study performed in LGMD2A biopsies claiming that c-FLIP expression is regulated by Capn3 via NF-κβ signaling [43]. Since the Capn3-deficient cellular model displays multiple alterations of sarcoplasmic reticulum (SR) homeostasis such as reduction of SERCA and RyR1 proteins, as well as abnormal calcium homeostasis, we next sought to determine whether SR-stress pathways were upregulated in

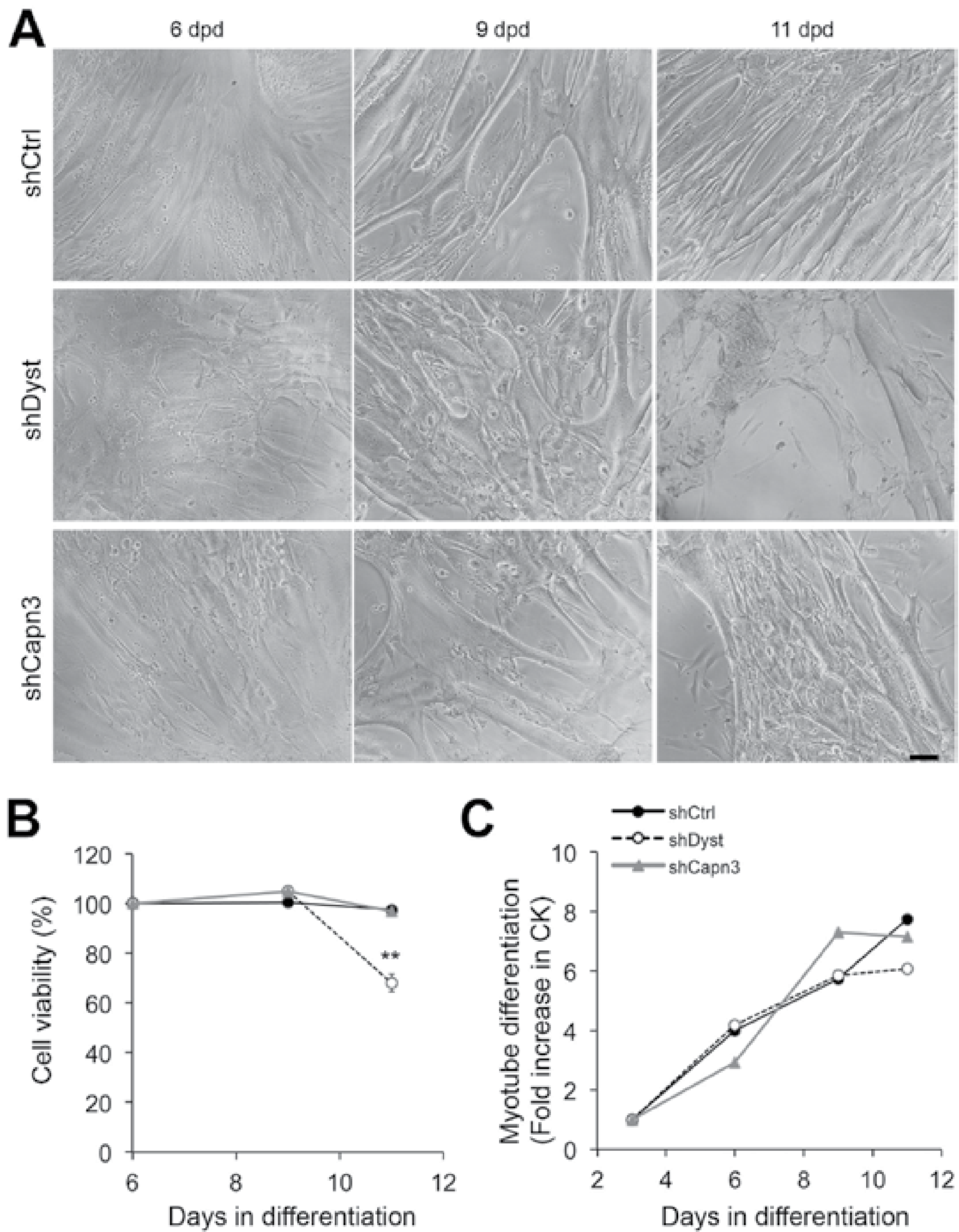

Figure 3. Generation of human cellular models of muscular dystrophies by shRNA lentiviral transduction. (A) Representative bright field images of LHCN-M2 myotubes infected with lentivirus-carrying control non-silencing (shCtrl), dystrophin (shDyst), or calpain 3 (shCapn3) shRNAs, at 6, 9, and 11 dpd. At 11 dpd, obvious detachment of shDyst expressing myotubes was observed. (B) At 11 dpd, viability of shDyst expressing myotubes (white circles) was significantly reduced to 60% as opposed to shCtrl (black circles) or shCapn3 (gray triangles) expressing myotubes, which showed no obvious decrease in their viability. (C) Similar maturation levels were observed among myotubes expressing shCtrl, shDyst, or shCapn3 throughout 11 days of differentiation, as quantified by fold increase in creatine kinase (CK) activity. Data expressed as mean ± SEM, $n = 3$ *$p < 0.05$, **$p < 0.005$). Scale bar = 40 μm.

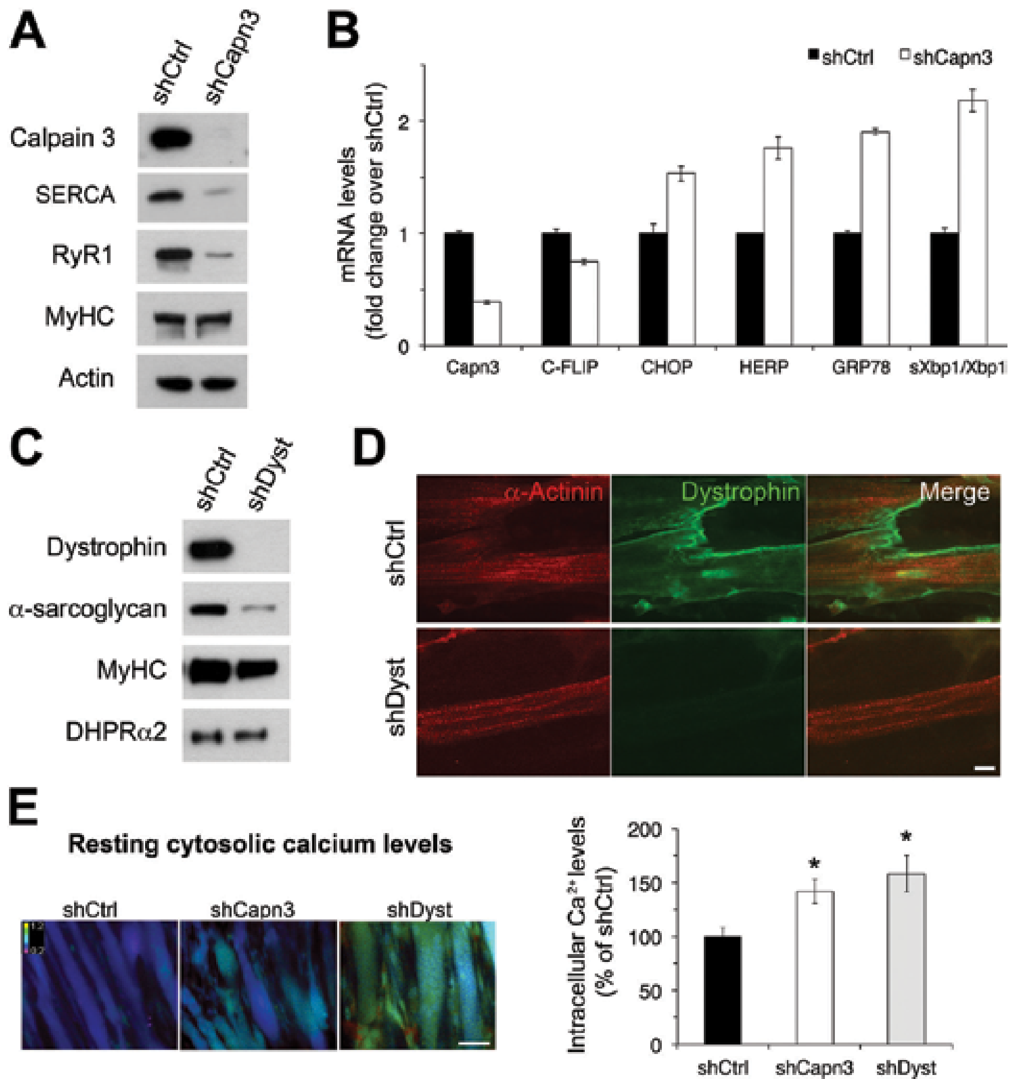

Figure 4. Validation of the human cellular models for muscular dystrophies. (A) Western blot analysis in LHCN-M2 myotubes expressing control non-silencing shRNA (shCtrl) and calpain 3 shRNA (shCapn3). Efficient silencing of calpain 3 expression was confirmed in shCapn3 myotubes. Compared to controls, calpain 3-deficient myotubes displayed a concomitant reduction of SERCA and RyR1 protein levels, while MyHC and actin levels remained unchanged. (B) Analysis of mRNA expression levels in the LGMD2A model. CAPN3, c-FLIP, and several genes involved in sarcoplasmic reticulum stress (CHOP, GRP78, HERP, and XBP1) in the LGMD2A model. Calpain 3-deficient myotubes present a concomitant decrease of c-*FLIP* mRNA levels. Compared to controls, shCapn3 myotubes show increased mRNA levels of several genes associated with sarcoplasmic reticulum stress, such as *CHOP*, *HERP*, *GRP78*, and the spliced *XBP1*/*XBP1* ration. (C) Western blot analysis in LHCN-M2 myotubes expressing control non-silencing shRNA (shCtrl) and dystrophin shRNA (shDyst). Efficient silencing of dystrophin expression was confirmed in shDyst myotubes. Compared to controls, shDyst myotubes displayed a reduction of α-sarcoglycan, while levels of MyHC and DHPRα2 remained unchanged. (D) Double immunostaining of α-actinin (red) and dystrophin (green) in control (shCtrl) and dystrophin knockdown (shDyst) myotubes. Scale bar = 10 μm. (E) Resting intracellular Ca^{2+} levels of control (shCtrl), calpain-deficient (shCapn3), and dystrophin-deficient (shDyst) myotubes were measured with ratiometric dye Fura-2 AM. Left: Representative pseudocolored images showing the F340/F380 fluorescence ratio recordings. Right: Quantification analysis shows that both dystrophic models, shCapn3 (142 ± 11%) and shDyst myotubes (158 ± 17%), present significantly higher resting intracellular calcium levels than controls (100 ± 8%). Data expressed as mean ± SEM. 200–500 myotubes were analyzed from at least $N = 3$ independent experiments; $*p < 0.05$ vs. shCtrl. Scale bar = 25 μm.

our model. Indeed, we observed overexpression of SR-stress-related genes such as *GRP78*, *CHOP*, *HERP*, and spliced *XBP1* in shCapn3 myotubes (**Figure 4B**) [44–46]. SR stress leads to the activation of the unfolded protein response (UPR), which involves splicing of X-box binding protein-1 (*XBP1*) mRNA. After sustained UPR, the transcriptional factor CCAAT/-enhancer-binding protein homologous protein is induced, which leads to caspase activation and apoptosis [47]. Our results indicate that Capn3 deficiency results in significant SR stress that leads to activation of programmed cell death, which is likely caused by reduced protein levels of RyR1 and SERCAs. Therefore, skeletal muscle fiber apoptosis due to SR stress may be a crucial event in the pathological mechanism of LGMD2A patients.

In the DMD model, we verified efficient knockdown of dystrophin in shDyst myotubes by Western blot and immunocytochemistry (**Figure 4C** and **D**). Immunocytochemical analysis revealed a predominant sarcolemmal distribution of dystrophin in shCtrl myotubes, which is indicative of high maturation of myotube cultures. As expected, dystrophin was mostly undetectable in shDyst myotubes (**Figure 4C** and **D**). Next, we sought to assess expression levels of α-sarcoglycan, a member of the dystrophin-dystroglycan complex, since this protein has been repeatedly found diminished in muscle biopsies from patients with Duchenne muscular dystrophy [48–50]. Consistent with these reports, we found that shDyst myotubes display a marked reduction of α-sarcoglycan levels compared to shCtrl myotubes (**Figure 4C**).

Finally, we sought to analyze Ca^{2+} homeostasis capacity of dystrophic myotubes, since several studies have shown that calcium dysregulation is involved in several forms of muscular dystrophy [51]. Indeed, as we have previously reported [52], resting intracellular calcium levels were significantly increased in Capn3-deficient myotubes by 42% compared to control myotubes (**Figure 4E**). Interestingly, we observed an even higher increase in the resting cytosolic calcium levels of shDyst myotubes (by 58%) compared to control myotubes. Overall, our findings regarding Ca^{2+} homeostasis and expression/localization of proteins involved in the pathological mechanisms of muscular dystrophies indicate that our human cellular models are suitable for the study of these muscular disorders, and they may be particularly helpful as a drug-screening platform for compounds targeting calcium homeostasis.

4. Discussion and conclusions

In this work, we present a novel tool for the study of muscular dystrophies and other genetic muscle disorders, which is based on the previously described human immortalized myoblast cell line LHCN-M2 [35]. This is a well-characterized and widely used human myogenic cell line that displays a similar in vivo and *in vitro* behavior to the one observed in primary myoblasts [52–55].

Since most of the relevant proteins involved in the pathogenesis of muscular dystrophies are expressed at late stages of myogenic differentiation, first we optimized culture conditions of LHCN-M2 cells to achieve highest levels of myotube maturation and survival. This protocol could also be suitable for other human cell lines as well as for primary human myoblasts. Furthermore, we have generated human cellular models of LGMD2A and Duchenne muscular dystrophies by combining these culture conditions with long-term gene silencing of calpain 3 and dystrophin in LHCN-M2 myoblasts. Compared to previous *in vitro* human

systems, our model is inexpensive, straightforward, and does not require special equipment. In addition, it overcomes several limitations of other human *in vitro* models such as sample availability, heterogeneity among different samples, and lack of physiological relevance. In this regard, our cellular models display several features characteristic of muscles from patients with muscular dystrophies, which validates their relevance to these disorders. In addition, as shown here, as well as in our previous study [36], the high maturity of these myotube cultures allows for localization analysis of proteins involved in the pathogenesis of muscular dystrophies as well as calcium transients recording. Our results regarding generation of highly differentiated human myotubes are consistent with a previous study describing human satellite cell differentiation under serum-free culture conditions [22]. Indeed, both procedures accomplish highly mature human myotubes capable of spontaneous contraction without neural innervation. However, our model has several advantages over previous methods. First, the use of the LHCN-M2 cell line allows for unlimited sample and higher homogeneity among experiments, since human primary myoblasts or satellite cells display a wide heterogeneity in terms of proliferation capacity and fusion into myotubes [56]. Also, substantial differences in human myotube survival and response to extrinsic factors have been observed among primary cultures from different samples [6]. Cellular senescence is an additional limitation of human primary myoblasts, which typically restricts *in vitro* myoblast proliferation to a maximum of 15–20 divisions. In addition, this proliferative potential decreases dramatically in dystrophic conditions such as Duchenne muscular dystrophy [7, 57].

As additional advantages of our model, instead of complex polymeric coatings or micro-patterned surfaces [22–25], we use a simple overlay of ECM gel to provide a suitable scaffold that results in increased myotube attachment, maturation, and survival. In fact, addition of this scaffold is sufficient to induce contraction capability in LHCN-M2 myotubes, without the need of neural innervation or addition of trophic factors. However, when an optimized differentiation medium with trophic factor was added to ECM overlaid cultures, contractile capacity displayed by myotubes was notably widespread throughout the whole culture.

Once myotube differentiation was optimized, shRNA-lentiviral infection was performed in order to silence expression of proteins relevant to muscular dystrophies. In particular, we accomplished efficient long-term knockdown of calpain 3 and dystrophin in LHCN-M2 myotubes, whose deficiency in skeletal muscle cause, respectively, Duchenne and LGMD2A muscular dystrophies. Remarkably, these proteins are expressed at late stages of myotube differentiation, so highly mature myotubes are essential to study deficiency of these proteins. While infected myotubes did not display a widespread contractile capacity as the one shown by noninfected myotubes, high maturation levels were demonstrated in both control and dystrophic myotubes by the presence of a striated actinin pattern. Most importantly, calpain 3 and dystrophin-deficient LHCN-M2 myotubes recapitulate key features of LGMD2A and Duchenne muscular dystrophies, such as reduced expression and abnormal localization of several proteins involved in their pathological mechanisms. In particular, dystrophin-deficient myotubes differentially showed reduced attachment, likely due to dystrophin's main function as an anchor between extracellular matrix and the contractile apparatus [58]. Overall, our findings validate the relevance of our novel *in vitro* human muscle model to LGMD2A and Duchenne muscular dystrophies. In addition, maturation of the cellular model may be further enhanced, if needed, with chronic electrical stimulation, as reported in previous works [12, 13].

Current research is focusing on novel therapeutic strategies for muscular disorders, but there is an obvious lack of appropriate *in vitro* human models to test these strategies. Animal models are often used for in vivo preclinical testing before moving forward to clinical trials. However, many examples in the literature indicate that therapies tested on animals may not necessarily yield the same outcomes in humans. Our novel human cellular model of muscular dystrophies described in this study allows testing of new therapies and treatments in a system more representative of human muscle pathologies, which may be used to complement preclinical studies performed in animal models. While this model has been validated for muscular dystrophies, we expect that the methodology described in this work could be also applicable to primary or immortalized human myoblasts to study other muscle disorders.

In conclusion, here we present a relevant human *in vitro* system that offers multiple possibilities for the study of skeletal muscle pathophysiology. Our model could be easily adapted to obtain cellular models for other neuromuscular diseases, and it may be further customized using current technological advancements in genomic editing (transcription activator-like effector nucleases—transcription activator-like effector nucleases (TALEN), Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9 (CRISPR/Cas9)), exon skipping or 3D tissue engineering in order to generate more refined *in vitro* muscle models. Low variability of the system would allow its use for high throughput screening of potential therapeutic drugs for muscular diseases, with an emphasis on compounds targeting calcium homeostasis.

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Conflict of interest

The authors declare that they do not have any competing or financial interests.

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Chapter 9

Role of Stem Cells and Extracellular Matrix in the Regeneration of Skeletal Muscle

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Additional information is available at the end of the chapter

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Abstract

Adult skeletal muscle has a remarkable capacity to initiate a rapid and extensive repair process after damage due to injury or degenerative disease. Although satellite cells are the primary skeletal muscle stem cells, there are many reports of non-satellite cell populations with myogenic capacity resident within skeletal muscle. The activity of muscle-resident stem cells during the regeneration process is tightly controlled through the dynamic interactions between intrinsic factors within the cells and extrinsic factors constituting the muscle stem cell niche. The extracellular matrix (ECM) in skeletal muscle plays an integral role in force transmission, structural maintenance, and regulation of stem cell niche. ECM interacts with stem cells either directly by binding cell surface receptors or indirectly through growth factor presentation, and maintains a balance between their quiescence, self-renewal, and differentiation. These interactions are reciprocal since the stem cells can remodel the niche and secrete or degrade ECM components. Natural ECM scaffolds, derived from decellularized tissues can influence stem cell activity both *in vitro* and *in vivo* and are widely being investigated for skeletal muscle repair. In this chapter, we discuss the regenerative potential of stem cell populations and ECM bioscaffolds in the treatment of skeletal muscle injury and disease.

Keywords: stem cells, extracellular matrix, regeneration, trauma, decellularization

1. Introduction

Skeletal muscle injuries are common in military service members and professional athletes and can range from minor sprains and contusions to severe lacerations and penetrating trauma.

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The type and severity of muscle injury greatly influences the healing outcome. While skeletal muscle can regenerate very well following minor injuries, severe penetrating trauma involving physical loss of muscle tissue is well beyond the innate capacity for repair [1, 2]. In acute and self-healing muscle injuries, the typical muscle repair process consists of the destruction and inflammatory phase (1–3 days), the repair phase (3–4 weeks), and the remodeling phase (3–6 months). Extracellular matrix (ECM) deposition can be observed within a week post-injury and is primarily due to the activity of fibroblasts in response to locally produced mediators such as transforming growth factor beta 1 (TGF-β1) [3]. In chronic muscle injuries the inflammatory phase persists for weeks and the deposition of ECM proceeds more rapidly than myogenesis [4, 5]. A major impediment to the complete recovery of skeletal muscle post-injury is the development of fibrosis, defined as an abnormal and chronic over proliferation of ECM components [6]. If unresolved, fibrotic tissue can interfere with stem cell activity and myofiber regeneration. Furthermore, since fibrotic tissue lacks the elasticity and contractile properties of native skeletal muscle, patients with muscle fibrosis are dramatically more likely to suffer subsequent muscle injuries, each worsening muscle damage and fibrosis [7]. Fibrotic tissue deposition in the absence of muscle regeneration reduces both muscle strength and range of motion, impeding physical rehabilitation.

In skeletal muscle, fibrosis is also associated with muscular dystrophies. These degenerative diseases are characterized by muscle inflammation and wasting, which compromises patient mobility [1]. Duchenne muscular dystrophy (DMD) is a severe and progressive form of muscular dystrophy that may cause premature death due to respiratory and cardiac failure [8]. Despite continuing efforts, there are currently no effective therapies for severe muscle trauma or DMD. Recently, many novel therapeutic strategies focusing on muscle stem cells and ECM have emerged, as discussed below, and their efficacy is under evaluation in both preclinical and clinical studies.

2. Stem cells for skeletal muscle regeneration

The skeletal muscle microenvironment is heterogeneous, with diverse cell populations that can be influenced by local structural and biochemical cues. Skeletal muscle is endowed with a remarkable capacity for regeneration, primarily due to the reserve pool of muscle-resident satellite cells. The anatomic location of satellite cells is in proximity to vasculature where they interact with other muscle-resident stem/stromal cells such as mesenchymal stem cells (MSCs) and pericytes through paracrine mechanisms (**Figure 1**) [9, 10]. A variety of other stem cell populations have also been identified in skeletal muscle including, side population cells [11, 12], fibro/adipogenic progenitors [13, 14] and interstitial stem cells [15]. These cell types share many features with MSCs such as multipotency and cell surface marker expression and are known to undergo proliferation in response to muscle injury [16]. Additionally, muscle-resident or marrow-derived hematopoietic stem cells (HSCs) are known to rapidly colonize skeletal muscle post-injury [9]. Here, we review the contribution of satellite and non-satellite stem cells to muscle repair in animal models of injury and disease.

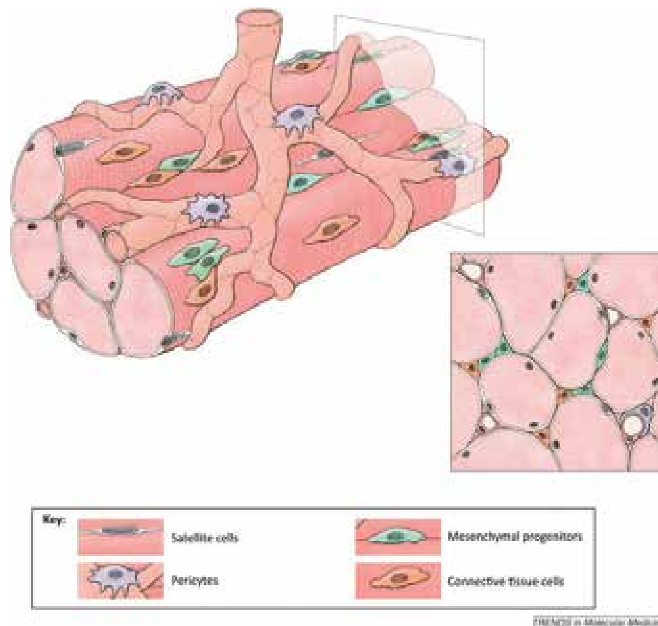

Figure 1. Satellite cells (white) constitute the major muscle stem cell population that reside beneath the basal lamina near the vasculature. Other muscle-resident stem cell populations such as pericytes (purple) and mesenchymal progenitors (green) contribute to muscle repair and regeneration. Reproduced with permission from Elsevier Ltd. Pannerec A, Marazzi G, Sassoon D. Stem cells in the hood: the skeletal muscle niche. Trends in Molecular Medicin; 2012;**18**(10):599-606. DOI:10.1016/j.molmed.2012.07.004.

2.1. Satellite cells

Satellite cells ($Pax7^+$) reside in between the basal lamina and the sarcolemma in a quiescent state. They express integrin $\alpha 7\beta 1$ receptors that bind with laminin in the basal lamina and M-cadherin adhesion molecules that interact with the sarcolemma [9]. In response to injury, they undergo asymmetrical cell-division and give rise to transit-amplifying cells and myoblasts that can either fuse with each other or with existing myofibers to initiate repair. The indispensable role of satellite cells in skeletal muscle regeneration has been well-documented using selective depletion of the $Pax7^+$ cell population [17–20]. Interestingly, these studies also highlighted a role of $Pax7^+$ satellite cells in the inhibition of fibrosis development by negative regulation of muscle-resident fibroblasts.

Besides Pax7, several markers have been used for the isolation of satellite cells such as VCAM-1 [21], α7 integrin [22], M-cadherin [23], and nestin [24]. However, it is unclear if these cell surface markers allow for the isolation of the same stem cell population. While the transplantation of autologous satellite cells seems like an effective therapeutic strategy, several challenges have been identified with this approach. For instance, the regenerative capacity of cultured satellite cells is significantly lower than freshly isolated cells [25]. Poor survival and migration of transplanted cells, as well as undesirable immune reactions, have also been

reported [26, 27]. To circumvent these issues, alternative modes of satellite cell delivery have been attempted in preclinical studies. Collins et al. transplanted single intact myofibers into radiation-ablated muscles and demonstrated that as few as seven satellite cells associated with a single myofiber could generate >100 new myofibers containing thousands of myonuclei [28]. Other groups have shown beneficial effects of single fiber transplantation in preclinical models of aging [29] and heart failure [30]. However, the isolation of single myofibers is challenging and requires a high degree of technical skill and expertise as well as the need for regulatory approval. An alternative approach is to deliver bundles of minced muscle fibers to transfer not just satellite cells but also other cell types with an intact ECM. Corona et al. have repeatedly demonstrated appreciable muscle regeneration by transplantation of minced muscle autografts in both rodent [31–33] and porcine [34] models of volumetric muscle loss (VML).

A scaffold-based strategy for satellite cell encapsulation and delivery has also been attempted in various studies. Rossi et al. reported improvement in muscle structure, the total number of new myofibers as well as muscle function by delivering satellite cells encapsulated in a hyaluronan-based photocrosslinkable hydrogel to partially ablated tibialis anterior muscles [35]. Recent work by Corona et al. has evaluated the myoconductive properties of collagen gels [36], and laminin-111 supplemented hyaluronic acid hydrogels [37] by co-delivering them with minced muscle autografts in rodent models of VML. However, none of these strategies were able to augment the regenerative potential of minced muscle autografts, possibly due to an exacerbated immune response to the implanted materials or due to the inability of the materials to influence satellite cell activity.

2.2. Mesenchymal stem cells

MSCs are a multipotent stem cell population located in several adult tissues. From a tissue engineering standpoint, MSCs exhibit several attractive features such as self-renewal, multipotency, immunomodulation, and the secretion of a wide variety of pro-regenerative growth factors and cytokines. While MSCs can undergo differentiation into osteogenic, chondrogenic, adipogenic, and myogenic lineages *in vitro* under specific culture conditions, their capacity for site-specific differentiation post-transplantation into injured tissues is controversial [16]. Regardless, 646 on-going clinical trials are currently investigating the therapeutic efficacy of MSCs for a wide-range of different conditions in various tissues (source: clinicaltrials.gov).

Muscle-resident MSCs, isolated as Sca1$^+$ CD45$^-$ cells, are reported to be non-myogenic, as they do not form myotubes in culture or fuse with myofibers *in vivo*. However, intramuscular delivery of this cell population can increase Pax7$^+$ satellite cell quantity, new fiber synthesis, myofiber hypertrophy, and arteriogenesis in eccentrically exercised mouse hindlimb muscles [38–40]. Muscle-derived MSCs are also known to secrete a wide variety of growth factors in response to exercise *in vivo* [38] and mechanical strain *in vitro* [41, 42]. Overall, these studies indicate that muscle-resident MSCs serve as a crucial component of the cellular niche that can facilitate muscle regeneration.

In clinical and preclinical studies, bone marrow remains the most commonly utilized source for the isolation of MSCs. Intramuscular delivery of bone marrow-derived MSCs (BMMC) into rodent models of crush trauma has improved muscle function in a dose-dependent

manner [43, 44]. Additionally, systemic delivery of BMMC via intra-arterial transplantation was also reported to restore muscle functional capacity in a rodent model of crush trauma. Since systemically delivered cells were not found in the traumatized muscle tissue, the authors speculated that the observed improvements in muscle function were likely due to the secretion of soluble factors by the transplanted cells [45]. Pumberger et al. [46] delivered BMMC with recombinant growth factors in an alginate cryogel in traumatized soleus muscles. This biomaterial driven approach enhanced paracrine signaling in MSCs, which resulted in improved muscle function, remodeled scar tissue, and increased the formation of new myofibers.

Corona et al. worked with a lineage depleted (Lin^-) fraction of bone marrow cells to increase the concentration of stem and progenitor cells in the isolated cell population. In a mouse model of ischemia-reperfusion (I/R) injury, the Lin^- bone marrow-derived stem cells were injected intramuscularly. While the cells survived transplantation for up to 1 month after injury, they did not improve muscle function [47]. In a subsequent study, these cells were delivered intravenously in a mouse model of I/R injury to avoid potential muscle damage from intramuscular injections. This approach resulted in stem cell homing to the injured leg and improvements in muscle regeneration as well as function [48]. However, the exact mechanism of repair or the contribution of specific stem cell subsets remains unclear.

In a muscle model of repeated laceration injury, BMMC were suspended in Matrigel and transplanted in the soleus muscles [49]. The BMMC treated muscles showed fewer fibers with a centrally located nucleus, and larger muscle fiber cross-sectional area compared to non-treated muscles, but no differences in fibrotic tissue deposition were observed. The study did not determine whether the BMMC participated in muscle regeneration directly by differentiating into myogenic cells, or indirectly through the secretion of angiogenic and pro-regenerative soluble mediators. In another study, it was demonstrated that transplantation of BMMC suspended in a fibrin matrix in a rodent model of muscle laceration restored muscle function. Again, the cells did not differentiate into or fuse with skeletal myofibers, signifying an alternate mechanism for repair [50]. Currently, the trophic support provided by MSCs (via the release of soluble growth factors and cytokines) is increasingly being considered as the primary mechanism for tissue repair and regeneration. This prevailing hypothesis has provided the underlining foundation for a plethora of clinical trials.

2.3. Adipose-derived stem cells

Adipose-derived stem cells (ASCs) are morphologically and phenotypically similar to MSCs and have gained increased popularity in regenerative medicine because large quantities can be easily isolated using minimally invasive procedures [51]. ASCs are reported to exhibit myogenic potential *in vitro* [52]. Myogenic progenitors derived from ASC cultures have successfully engrafted into skeletal muscle, promoted myofiber synthesis, and restored dystrophin expression in dystrophic (*mdx)* mice [53]. Besides improving muscle regeneration, ASCs can also modulate inflammation and fibrosis to ameliorate the dystrophic phenotype in mouse models [54]. To combat fibrosis development in injured dystrophic muscles, ASCs were delivered in combination with an anti-fibrotic medication called losartan. The combined treatment was able to downregulate TGF-β1, inhibit fibrosis, and simultaneously

improved muscle regeneration and hypertrophy [55]. In another study, transplantation of ASCs alleviated skeletal muscle fibrosis induced by high dose radiation by suppressing the level of TGF-β1 [56]. In a VML model, ASCs were delivered to the defect site in a collagen hydrogel. The results showed that this treatment approach accelerated muscle repair and vascularization while simultaneously reducing inflammation and fibrosis [57]. Collectively, these studies suggest that ASCs can support muscle repair and regeneration either directly by giving rise to myogenic progenitors or indirectly by attenuating inflammation and fibrosis.

2.4. Hematopoietic stem cells

HSCs are multipotent stem cells that continuously replenish all classes of blood cells including both the lymphoid and the myeloid lineages. Cells of the adaptive immune system comprise the lymphoid lineage while cells of the innate immune system, megakaryocytes, and erythrocytes comprise the myeloid lineage [58]. Adult bone marrow, cord blood, and mobilized peripheral blood are the primary sources of HSCs used in clinical transplantation protocols for the treatment of cancer and other blood or immune disorders [59].

The first report of bone marrow-derived cell-mediated muscle regeneration *in vivo* was published in 1998 by Ferrari et al. [60]. Since then several studies have repeatedly shown that bone marrow-derived cells can contribute to myogenesis and muscle regeneration. For instance, Gussoni et al. [61] showed that HSCs derived from wild-type mice could participate in myogenesis and partially restore dystrophin expression in lethally irradiated *mdx* mice following intravenous transplantation. LaBarge et al. [62] demonstrated that following irradiation-induced depletion of satellite cells, cells from a bone marrow transplant could occupy the satellite cell niche and contribute to myofiber regeneration. In a recent report, Goldman et al. [63] transplanted bone-marrow derived mononuclear cells in a rat model of VML. The transplanted cells contributed to limited *de novo* muscle fiber regeneration, without any significant changes in myogenic gene transcription and muscle function.

Other studies have highlighted the role of injury in HSC-mediated myogenic repair. It has repeatedly been shown that HSCs contribute to myogenic events only in response to damage or injury, but do not participate in myofiber repair under normal physiological conditions. For instance, Corbel et al. [64] showed that although the fusion of hematopoietic progenitors with myofibers could occur at low frequency under normal physiological conditions, this capacity increases significantly with muscle damage. Polesskaya et al. [65] observed a 10-fold increase in the numbers of cells coexpressing the cell surface markers CD45 and Sca1 following cardiotoxin injury in skeletal muscle. It was further demonstrated that while the $CD45^+$ cell population from uninjured muscle did not differentiate into myogenic cells, the $CD45^+$ cells from injured muscle readily underwent myogenic differentiation *in vitro.* The myogenic commitment of this cell population was induced by the stimulation of the Wnt signaling cascade.

In contrast, Camargo et al. [66] suggested that in response to muscle injury, myeloid inflammatory progenitors derived from HSCs are recruited to the site of muscle damage and undergo stochastic fusion with regenerating myofibers. Due to these conflicting reports, it

remains unclear if the HSCs can give rise to myogenic cells in response to environmental cues or if they undergo nuclear reprogramming post-fusion with a damaged myofiber.

The hematopoietic capacity of adult muscle-resident stem cells has also been investigated. Jackson et al. [67] isolated muscle-resident stem cells by enzymatic digestion and co-transplanted them into irradiated mice with whole bone marrow cells. After 6–12 weeks the recipient mice showed engraftment of muscle-derived cells in the bone-marrow and reconstitution of all the major blood cell lineages. Therefore, endogenous adult stem cells resident in skeletal muscle may represent a multipotent stem cell population capable of giving rise to both blood and muscle tissue.

Taken together, these studies indicate that adult stem cells may have differentiation potential beyond their tissue of origin. It further calls into question the influence of environment versus lineage in the commitment and differentiation of stem cell populations and highlights a previously unrecognized potential for plasticity in tissue-resident adult stem cells.

2.5. Pericytes

Pericytes are perivascular stem cells that encase and form intimate connections with adjacent capillary endothelial cells [68]. They influence the migration, proliferation, permeability, and contractility of endothelial cells and play essential roles in various stages of angiogenesis [69–71]. These cells can also regulate vascular diameter and capillary blood flow by producing both vasoconstriction and vasodilation within capillary beds [72]. Two major subpopulations of pericytes have been identified: type 1 ($Nestin^{-}NG2^{+}$) and type 2 ($Nestin^{+}NG2^{+}$). While type 1 pericytes are reported to contribute to fat accumulation, type 2 pericytes are known to support new muscle formation [73].

Recent reports have suggested an indispensable role of pericytes in the postnatal growth of skeletal muscle [74, 75]. Type 2 pericytes have been identified in the satellite cell niche and are believed to be the primary population involved in muscle formation [73]. Kostallari et al. [76] showed that pericyte depletion could result in significant myofiber hypotrophy with a slight increase in a total number of $Pax7^{+}$ satellite cells. *In vitro* co-culture studies demonstrated that pericytes could promote both myogenic differentiation and quiescence in satellite cells through the secretion of insulin-like growth factor 1 (IGF-1) and angiopoietin 1 (ANGPT1), respectively [76]. Dellavalle et al. [75] showed that vascular pericytes are bi-potent, as they give rise to both the smooth muscle layer of blood vessels and skeletal muscle fibers, during postnatal growth in mice. They also demonstrated that pericytes express myogenic markers only in differentiated myotubes, and when transplanted into immunodeficient dystrophic (*scid-mdx*) mice, pericytes can generate dystrophin-positive myofibers [77]. However, the environmental or biochemical signals that regulate the myogenic differentiation of pericytes remain unknown. This study also demonstrated that pericytes delivered systemically can cross the vessel wall to colonize skeletal muscle—a feature that is absent in satellite cells. Lorant et al. [78] were able to corroborate these findings by injecting human perivascular stem cells into cryoinjured muscles of *scid* mice. The cells were able to integrate into the host injured tissue and were associated with the production of structural proteins expressed in differentiated myofibers.

Meng et al. also attempted to replicate these studies by delivering muscle-derived pericytes intra-arterially in *scid-mdx* mice [79]. Surprisingly, their results showed that pericytes did not contribute to muscle regeneration. They attributed the discrepancies between their findings and previous data to the differences in cell-isolation and culture protocols, animal models, and outcome measurements. Therefore, these studies suggest that while pericytes may represent a useful cell population for future cell therapy of musculoskeletal disorders, more comprehensive studies are needed to establish a clear and definitive role for these cells in muscle regeneration and repair.

3. Extracellular matrix for skeletal muscle regeneration

The ECM in skeletal muscle is critical for tissue development, structural support, and force transmission [10]. The main components of ECM are largely conserved across animal species [80, 81]. The ECM of skeletal muscle is organized into three layers; the endomysium layer surrounds the individual muscle fibers, the perimysium surrounds the bundles of muscle fibers known as fascicles, and the epimysium surrounds the entire muscle (**Figure 2**). Collagen type I is predominant in the perimysium, whereas collagen type III is prevalent in the endomysium

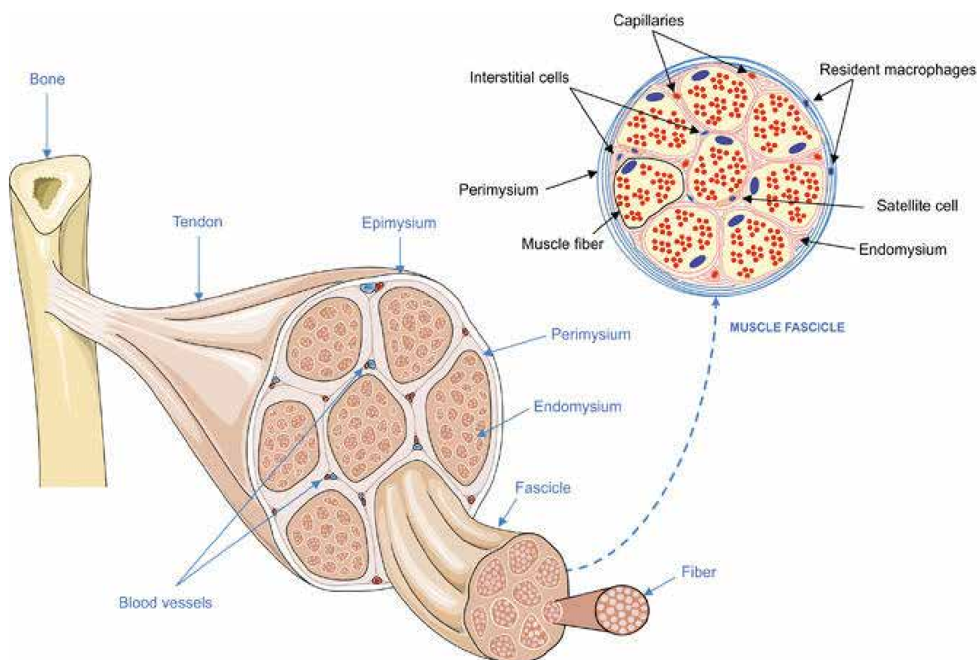

Figure 2. Skeletal muscle consists of muscle fibers bound by connective tissue. The outermost sheath of connective tissue that wraps around the muscle is called the epimysium. Bundles of muscle fibers, called fascicles, are enclosed by the perimysium and each muscle fiber is covered in a thin connective tissue layer called the endomysium that contains the extracellular fluid and nutrients to support the muscle fiber. Reproduced with permission from Springer Nature. Sciorati, C., et al., Cell death, clearance and immunity in the skeletal muscle. Cell Death and Differentiation. 2016;**23**(6):927-937. DOI: 10.1038/cdd.2015.171.

and the epimysium [82]. The basement membrane is a specialized layer of ECM between the sarcolemma of a muscle fiber and the surrounding endomysium [83]. Laminin and collagen type IV form the principal ECM components of the basal lamina layer [84]. The reticular lamina, located below the basement membrane, is composed mainly of fibrils of collagens (type I, III, and VI) and fibronectin in a proteoglycan-rich gel [83, 85].

Two major transmembrane protein complexes in the muscle fiber membrane are responsible for force transmission from the outer ECM to the inner cytoskeleton: the dystroglycan/sarcoglycan complex and the integrin α7-laminin complex [86]. These linkages are crucial for the stabilization of sarcolemma during contraction and are important for normal muscle function and strength. The importance of these linkages is demonstrated by the fact that defects in these molecules can result in the development of muscular dystrophy [87, 88]. A list of essential extracellular and intracellular components identified in skeletal muscle is provided in **Table 1**.

Stem cells in skeletal muscle are sensitive to biochemical and biophysical cues provided by the ECM [89]. For instance, loss of regenerative capacity in laminin-deficient (*dy/dy*) mice, as well as enhanced satellite cell activity with laminin-111 supplementation in vivo, suggest a pivotal role for this ECM protein in the regulation of stem cell function post-injury [90–92]. Similarly, the absence of collagen VI in Col6a$^{-/-}$ mice can impair satellite cell self-renewal and repair following injury [93, 94]. Conversely, stem cells can also influence ECM composition. While fibroblasts are considered the leading contributor of ECM production in the skeletal

Key extracellular and intracellular components of skeletal muscle
Collagens
Fibril forming: collagen types I, III, V, VI Network forming: collagen types IV Fibril associated: collagen types XII and XIV Multiplexins: collagen types XV and XVIII
Glycoproteins
Laminin, fibronectin, and nidogen
Proteoglycans and glycosaminoglycans
Heparan sulfate, chondroitin sulfate, dermatan sulfate, decorin, biglycan, perlecan and agrin
Matricellular proteins
Osteopontin, secreted protein acidic and rich in cysteine (SPARC), thrombospondin, and tenascin-C.
Matrix remodeling enzymes
Matrix metalloproteinases (MMP)-1,2,9,13, and Tissue inhibitors of matrix metalloproteinases (TIMPs)-1-3
Contractile and structural proteins
Actin, myosin, tropomyosin, troponin, titin, α-actinin, nebulin and dystrophin

Table 1. The major extracellular and intracellular components responsible for regulating skeletal muscle function [126].

muscle, stem cells including satellite cells and MSCs can also produce several ECM constituents including collagen, laminin, fibronectin, and matrix metalloproteinases [12, 42, 95–97]. Thus, ECM not only serves as a supportive framework for skeletal muscle, but also dynamically regulates resident cell activity in a way that can direct tissue development, repair, remodeling, and function [89].

3.1. ECM scaffolds for skeletal muscle repair

Challenges associated with cell-centric therapies such as low survival, poor engraftment, inadequate supply of donor cells, and culture-induced changes in differentiation potential have motivated the development of acellular ECM-based tissue engineered therapeutic strategies. Biologic ECM scaffolds are prepared by the removal of cellular antigens and are at the forefront of tissue engineering strategies for muscle repair. These decellularized scaffolds preserve the ultrastructure and composition of the ECM [81] and are known to contain basement membrane structural proteins, growth factors, and glycosaminoglycans (GAG). Therefore, these scaffolds possess the potential to recruit endogenous host cells while evading the problems associated with the delivery of exogenous cells such as cellular apoptosis, immunogenicity and ineffective delivery [98, 99].

In preclinical models, these scaffolds are reported to support vascularization and functional recovery post-VML injury [100–105]. While some studies have used skeletal muscle [102, 106] as the source of ECM, other studies have created decellularized scaffolds from dermis [104, 107], porcine urinary bladder (UBM) [108–110], and small intestinal mucosa (SIS) [104, 111, 112] for skeletal muscle tissue engineering. Additionally, scaffolds composed of single ECM proteins such as collagen [113], laminin-111 [37, 114], and cell-derived ECM such as Matrigel™ [115, 116] have also been used for skeletal muscle repair and regeneration.

Degradation products of ECM scaffolds are known to exert chemotactic and mitogenic effects on multipotential progenitor cells *in vitro* [117] and *in vivo* [118]. The recruitment of several stem cell populations such as Sca1$^+$ cells [32, 119], perivascular stem cells [120, 121], pluripotent adult progenitors (Sox2$^+$) [122], CD133$^+$ progenitor cells [123], and neural stem cells [124] has been reported post-implantation of ECM scaffolds in animal models. However, the ability of these scaffolds to recruit sufficient quantities of satellite cells to promote their activity in the VML defect region has not been conclusively established [32, 109]. For instance, Corona et al. reported that co-delivery of decellularized urinary bladder matrix negatively affects the regenerative capacity of minced muscle autografts in a rodent model of VML [108]. Using a similar approach, Kasukonis et al. implanted an allogeneic decellularized skeletal muscle scaffold with minced muscle autografts in a rat model of VML [125]. In contrast to the findings reported by Corona et al., they demonstrated significant improvements in muscle mass and peak contractile force.

3.2. Clinical application of ECM scaffolds

Recent work has also described encouraging clinical outcomes following the implantation of decellularized scaffolds in patients with VML injuries [126]. In a five patient cohort study, the VML injured muscle compartment was reconstructed with a commercially available ECM

bioscaffold (MatriStem, Acell Inc.) [98]. Within 24–48 hours after surgery, each patient was placed in an aggressive and exhaustive physical therapy program, as the application of a mechanical load during the ECM remodeling phase is reported to promote cellular infiltration, angiogenesis, and connective tissue reorganization and alignment. While this strategy promoted perivascular stem cell infiltration and angiogenesis at the site of scaffold implantation, significant muscle regeneration was not observed. Histological images indicated the presence of small islands of desmin$^+$ and myosin heavy chain (MHC$^+$) myofibers separated from the adjacent healthy muscle by collagenous connective tissue. Six months after surgery, three of five subjects showed improvement in the functional outcomes [98].

In a subsequent report [127], six patients were implanted with MatriStem (UBM-ECM, Acell Inc.), and an additional seven patients were implanted with BioDesign (SIS-ECM, Cook Medical) or XenMatrix (dermis-ECM, C.R. Bard). Similar to the previous study, post-surgical physical therapy was initiated between 24 and 48 hours following surgery. In addition to perivascular stem cell infiltration and desmin$^+$ islands of regenerated myofibers, this study also reported the presence of neurogenic cells (β-III tubulin$^+$) at the remodeling site. Ultrasound imaging revealed complete resorption of the BioDesign and MatriStem scaffold materials by seven months. Out of 13 patients, 7 had improvement from pre-surgical maximum strength with an average force production change of ~15.2% at 6–8 weeks post-surgery. By 24–28 weeks, an average improvement of ~37.3% was reported.

In the aforementioned studies, due to the lack of significant muscle regeneration, the improvements in muscle function could be attributed to both "functional fibrosis" [102] and physical rehabilitation [128]. Although the exact mechanism by which ECM scaffolds support tissue remodeling is unclear, it has been associated with recruitment of stem/progenitor cells. While these studies do not provide conclusive evidence of a causal relationship between recruitment of stem cells and improved functional outcomes, the reported findings suggest that ECM-based scaffolds are worthy of investigation as a viable treatment option for traumatic muscle injuries. Future studies could augment the regenerative potential of these scaffolds by simultaneous delivery of autologous stem cell populations or a more targeted rehabilitation program.

4. Conclusion

Muscle regeneration and recovery is a complex process that involves several different stem cell populations and ECM components. While the delivery of stem cells in injured or dystrophic muscles has been associated with improvements in muscle repair and function, the exact mechanism by which these cells contribute to muscle repair is unclear. Future studies should focus on identification of the intrinsic and extrinsic regulatory mechanisms that govern satellite and non-satellite cell differentiation and trophic factor secretion during the muscle regeneration process. Additionally, the mechanical and biochemical cues provided by key ECM components that can promote or dysregulate stem cell activity should be examined. This information will be crucial in the discovery of biomaterial-based strategies to augment the stem cell mediated muscle repair.

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Chapter 10

Skeletal Muscle as a Therapeutic Target for Natural Products to Reverse Metabolic Syndrome

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Additional information is available at the end of the chapter

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Abstract

Natural compounds, especially polyphenols have become a popular area of research mainly due to their apparent health benefits. Increasing the phenolic content of a diet, apart from its antioxidant benefit, has a beneficial effect on signaling molecules involved in carbohydrate and lipid metabolism. These effects could potentially protect against metabolic syndrome, a cluster of metabolic complications such as obesity, insulin resistance and type 2 diabetes that is characterized by a dysregulated carbohydrate, and lipid metabolism. Research continues to investigate various natural compounds for their amelioration of impaired signaling mechanisms that may lead to dysregulated metabolism to find means to improve the life expectancy of patients with metabolic syndrome. In this chapter, a systematic search through major databases such as MEDLINE/PubMed, EMBASE, and Google Scholar of literature reporting on the ameliorative potential of commonly investigated natural products that target skeletal muscle to ameliorate metabolic syndrome associated complications was conducted. The selected natural products that are discussed include apigenin, aspalathin, berberine, curcumin, epigallocatechin gallate, hesperidin, luteolin, naringenin, quercetin, resveratrol, rutin, and sulforaphane.

Keywords: skeletal muscle, metabolic syndrome, insulin resistance, type 2 diabetes, natural products

1. Introduction

A considerable amount of interest has been placed on the discovery of novel naturally occurring plant-derived compounds for the treatment and prevention of various diseases. Bioactive

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compounds of plant origin have long been shown to possess strong ameliorative properties against various communicable and noncommunicable diseases [1, 2]. For example, since its traditional use during the 1950s, artemisinin, an antimalarial *qinghao* derived lactone, has been the leading therapy for the treatment of *Plasmodium falciparum* malaria worldwide [3]. Similarly, the traditional use of galegine, an alkaloid isolated from *Galega officinalis*, led to the discovery of biguanide class of antidiabetic medications such as metformin [4]. Agents such as metformin are effective at lowering blood glucose levels and combating complications associated with insulin resistance (IR), the major characteristic of the metabolic syndrome [5]. However, the continued rise in the mortality of diabetic patients warrants an investigation into alternative therapies to reduce the burden of noncommunicable diseases. Naturally derived compounds such as polyphenols are increasingly explored for their therapeutic potential to reverse IR and thus decrease the risk of developing the metabolic syndrome. This may eventually lead to an increased life expectancy of diabetic individuals [6]. Thus, due to its modulatory effect of glucose and lipid metabolism, skeletal muscle has been a target to a growing number of therapeutic interventions in an effort to reverse IR and improve the management of metabolic syndrome [7, 8]. Here, we systematically assessed the available literature on the ameliorative potential of some of the prominent natural products against IR associated complications. A systematic search was conducted on all major databases such as MEDLINE/PubMed, EMBASE, and Google Scholar, for available literature reporting on the ameliorative properties of some of the prominent natural compounds including apigenin, aspalathin, berberine, curcumin, epigallocatechin gallate, hesperidin, luteolin, naringenin, quercetin, resveratrol, rutin, and sulforaphane against IR related to the development of metabolic syndrome. The search was conducted from inception until the end of January 2018, gray literature such as abstract proceedings and pre-prints were also included. There were no language restrictions implemented while review articles were screened for primary findings.

2. Apigenin

Apigenin (PubChem CID: 5280443) is a natural flavone (4′,5,7-trihydroxyflavone) with the molecular formula $C_{15}H_{10}O_5$ (MW 270.24 g/mol) that is abundantly present in fruits and vegetables, including parsley, chamomile, and celery (**Figure 1**) [9]. Apigenin was identified as the main yellow dye compound in the flowers of *Delphinium Zalil* as early as the 1890s [10], and its bioavailability and metabolism profile has been studied as far back as the 1970s [11].

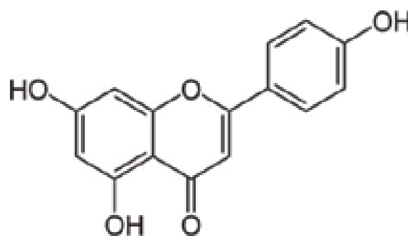

Figure 1. The chemical structure of apigenin (4′,5,7-trihydroxyflavone).

Although pharmacokinetic studies show that apigenin has low bioavailability [12, 13], this compound has been detected in rat plasma after intravenous bolus administration [14], and it was demonstrated that human intestinal microbiota might contribute to its metabolism [15]. The known metabolites of apigenin detected in the urine of rats consist of p-hydroxyphenylpropionic acid, p-hydroxycinnamic acid, and p-hydroxybenzoic acid metabolites [11] while it is known glucosides include apiin, apigenin, vitexin, isovitexin, and rhoifolin.

In relation to its biological activities, increasing studies have demonstrated that apigenin displays a broad spectrum of anticarcinogenic properties as reviewed by Sung et al. [16]. Some of the well-studied mechanisms associated with the chemo-preventative capabilities of apigenin include its anti-inflammatory activity, its ability to suppress cell proliferation and oxidative stress, as well as its modulatory effect of autophagy and apoptosis [16, 17]. Interestingly, similar mechanisms have also been implicated in the development and aggravation of IR and its related complications. In a recent study, Jung et al. [18] showed that in addition to reducing circulating free fatty acids (FFAs), total cholesterol, and apolipoprotein B levels, apigenin modulated transcriptional factors linked with the development of obesity and related metabolic disturbances in high fat diet (HFD)-induced mice. This study showed that apigenin upregulated the expression of genes responsible for the regulation of beta-oxidation, oxidative phosphorylation, as well as electron transport chain and cholesterol homeostasis, which are all essential target sites for the control of substrate usage in cells. Although limited studies are reporting on its effect on skeletal muscle, two recent studies have shown that apigenin can regulate skeletal muscle function. For instance, Choi et al. [19] showed that this flavone improved mitochondrial function and exercise capacity by reducing the expression of atrophic genes such as RING-finger protein-1 and Atrogin 1 in mice fed HFD. Jang et al. [20] demonstrated that in C2C12 cells and skeletal muscle of C57BL/6 mice, this flavone promoted hypertrophy and myogenic differentiation by regulating protein arginine methyltransferase 7 (Prmt7)-peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)-G protein-coupled receptor 56 (GPR56) pathway, as well as the Prmt7-p38-myoD pathway. Although additional studies are required to further assess the impact of apigenin in the modulation of metabolic disease-related complications through the regulation of skeletal muscle function, the two aforementioned studies suggest that this flavone has a potential to protect against skeletal muscle weakness associated with metabolic complications.

3. Aspalathin

Aspalathin (PubChem CID: 11282394) is a natural *C*-glucosyl dihydrochalcone (3′-β-D-glucopyranosyl-2′,3,4,4′,6′- pentahydroxydihydrochalcone) with the molecular formula $C_{21}H_{24}O_{11}$ (MW 452.412 g/mol) (**Figure 2**) [21]. Although aspalathin was known to be uniquely found in rooibos [22], recent evidence has shown that this *C*-linked dihydrochalcone glucoside can be detected in trace amounts in two other species of *Acacia pendula* [23]. Aspalathin is considered to have a poor bioavailability profile in different experimental settings as reviewed by Muller et al. [24] and Johnson et al. [25]. While Stalmach et al. [26], using high-performance liquid chromatography-mass spectrometry method, showed that *O*-methyl-aspalathin-*O*-glucuronide

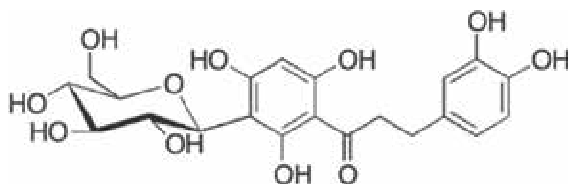

Figure 2. The chemical structure of aspalathin (3′-β-D-glucopyranosyl-2′,3,4,4′,6′- pentahydroxydihydrochalcone).

and eriodictyol-*O*-sulfate were the main metabolites excreted following ingestion of rooibos extract containing 10-fold higher levels of aspalathin in human subjects. In addition, a recent study by Bowles et al. [27] showed that aspalathin can be absorbed and metabolized to mostly sulfate conjugates detected in the urine of mice. However, additional evidence is required to establish the pharmacokinetic profile of aspalathin.

Relevant to its biological activity, the initial evidence demonstrated that aspalathin possess strong antioxidant properties by scavenging 2,2-diphenyl-β-picrylhydrazyl (DPPH) radical in vitro [28]. This effect was important since experimental and clinical studies support the notion that drug compounds that enhance intracellular antioxidant properties can further exhibit a wide range of beneficial effects against the development of metabolic syndrome [29]. In addition to its robust antioxidant activity [28, 30–34], aspalathin can ameliorate inflammation [35–39], protect cardiac cells exposed to high glucose concentrations [40–44], and also display glucose lowering properties [45–50]. In addition to work by our group [46, 48], studies conducted by Kawano et al. [51] and Son et al. [50] have reported on the effect of pure aspalathin or an aspalathin rich green rooibos extract on the signaling mechanisms that regulate glucose and lipid metabolism in skeletal muscle. Activation of 5' AMP-activated protein kinase (AMPK), an important kinase in the regulation of energy production, as well as increasing the expression and translocation of glucose transporter (GLUT) 4 have been the key molecular targets by aspalathin in the skeletal muscle. Thus, although additional evidence such as assessing the therapeutic effect of this dihydrochalcone on skeletal muscle biopsies of insulin-resistant human subjects is still necessary, its aforementioned potential to target AMPK, and improve glucose uptake is of major importance for future therapeutic development.

4. Berberine

Berberine (PubChem CID: 2353) is a quaternary alkaloid (5,6-Dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium) with the molecular formula $C_{20}H_{18}NO_4^+$ (MW 336.37 g/mol) that is present in several plants including *Hydrastis canadensis*, *Xanthorhiza simplicissima*, *Phellodendron amurense*, and *Berberis aristata* (**Figure 3**) [52]. Berberrubine, thalifendine, demethyleneberberine, and jatrorrhizine are some of the major metabolites detected in plasma following the administration of berberine in rats, with the liver and intestinal bacteria identified to participate in the metabolism, and disposition of this compound in vivo [53]. Although a number of factors, including it being hydrophilic in nature and its containment of quaternary ammonium groups contribute to the low bioavailability of berberine [54]. Interestingly, the absorption of berberine in the small intestine can be enhanced by d-α-tocopheryl polyethylene glycol 1000 succinate [55].

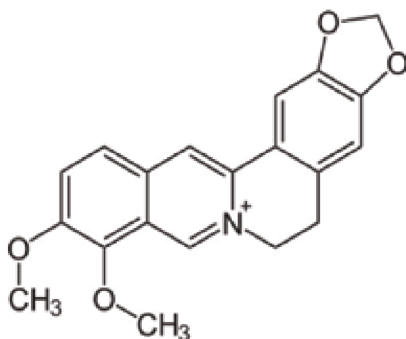

Figure 3. The chemical structure of berberine (5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium).

Therefore, further research is required to better understand and inform on mechanisms that can add to our current knowledge on the bioavailability of berberine, which is crucial in improving its efficacy in vivo.

Berberine has a long history of medicinal use in traditional Chinese and Native American medicine [56] and has demonstrated a number of beneficial effects against metabolic complications, including amelioration of IR. Berberine demonstrated an enhanced effect to reduce body weight and raise plasma triglyceride levels while improving glucose tolerance and insulin action in both type 2 diabetic (*db*/*db*) mice and in FHD fed rats [57]. Interestingly, similar to aspalathin, an increase of glucose uptake through activation of AMPK as well as enhanced translocation of GLUT4 in skeletal muscle remains important in the ameliorative potential of berberine against IR [58–62]. However, it has been reported that berberine can alter muscle metabolism by altering mitochondrial function, resulting in the development of muscle atrophy in normal, and diabetic (*db*/*db*) mice [63]. Although the results were not in human subjects, these findings remain relevant since loss of muscle mass is an important feature that occurs in type 2 diabetic patients, especially in older individuals [64]. These results suggest that precaution should be taken when using these quaternary alkaloids, especially considering the toxicity of high doses [65]. In addition to acting by targeting the mitochondria [65], another mechanism by which berberine can reverse IR include downregulating toll-like receptor 4 (TLR4)/inhibitor of nuclear factor kappa-B kinase subunit beta (IKKbeta)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inflammation signaling pathway, leading to reduced inflammation [66].

5. Curcumin

Curcumin, also known as diferuloylmethane (PubChem CID: 969516; (1E,6E)-1,7-Bis (4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione), is a major polyphenolic derivative of turmeric (*Curcuma longa*) with the molecular formula $C_{21}H_{20}O_6$ (MW 368.39 g/mol) (**Figure 4**) [67]. A single oral dose administration of curcumin can lead to the detection of its metabolites, glucuronide, and sulfate conjugates in plasma of human subjects [68]. Although is considered to have a safety profile, curcumin displays poor bioavailability profile that is coupled with quick metabolism and systemic removal [69]. However, recent developments such as blocking of metabolic pathways by

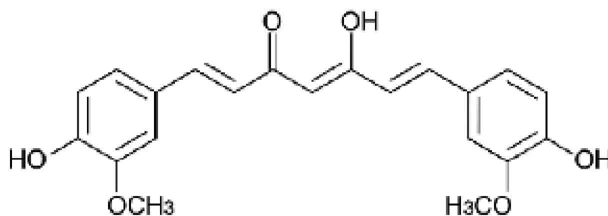

Figure 4. The chemical structure of curcumin ((1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione).

concomitant administration with other agents, conjugation, and modification of structure, as well as modulation of route and medium of administration are some of the explored approaches to improve the bioavailability of curcumin as reviewed by Prasad et al. [70]. Indeed, increasing research over the past 30 years has focused on exploring the pharmacokinetics, safety profile, and efficacy of this natural product in order to enhance its therapeutic profile in humans [71].

An increasing number of reviews has been published to keep track of the cumulative literature informing on the therapeutic potential of curcumin, including anticancer, antioxidant, anti-inflammatory, and antibacterial activities [70–72]. Relevant to its effect on skeletal muscle function. A study published in 2005 by Farid et al. [73] showed that curcumin failed to inhibit NF-κB activity, leading to its inability to ameliorate loss of muscle mass in the soleus. However, in a follow-up study published in 2008, curcumin presented enhanced effect in blocking sepsis-induced muscle proteolysis, at least in part by inhibiting NF-κB, and p38 activities in rats [74]. In L6 or C2C12 myotubes exposed to high palmitate concentrations as a model of IR, curcumin reversed IR by increasing glucose and FFA oxidation, at least in part by mediating LKB1-AMPK pathways, as well as suppressing insulin receptor substrate 1 (IRS-1) Ser^{307} and protein kinase B (AKT) phosphorylation [75–77]. Although similar evidence has been supported by in vivo experiments on skeletal muscle tissue of either diabetic or nondiabetic rodents [75, 77], curcumin displays an enhanced capacity to protect against oxidative stress associated complications by improving mitochondrial biogenesis, and other antioxidant mechanisms [78–81]. This involves activation of the nuclear factor (erythroid-derived 2)-like 2 (NRF2) [82], an essential intracellular antioxidant response element that is a target of various natural products aiming to reduce metabolic disease-associated complications.

6. Epigallocatechin gallate

Epigallocatechin gallate (PubChem CID: 65064) is an ester of epigallocatechin and gallic acid ([(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate, with the molecular formula $C_{22}H_{18}O_{11}$ (MW 458.375 g/mol), that is abundantly found in tea (**Figure 5**) [83]. Due to the popularity of green tea and as one of its major components, epigallocatechin gallate remains one of the highly consumed polyphenolic compounds [84]. Although it is detectable in its original form in human plasma after oral administration [85], epigallocatechin gallate is considered to have very low oral bioavailability profile as reviewed by Mereles and colleagues [86]. Although additional evidence is required to improve its

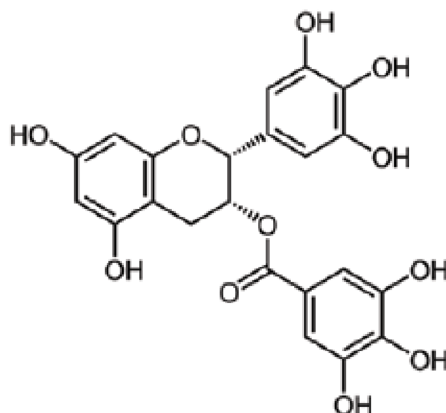

Figure 5. The chemical structure of epigallocatechin gallate ([(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl] 3,4,5-trihydroxybenzoate).

bioavailability, there has been an extensive exploration of this polyphenolic compound for its chemopreventive properties. Among the 10 polyphenols present in green tea, epigallocatechin gallate was found to exhibit the most antiproliferative and antiapoptotic effects [87].

It has already been established that epigallocatechin gallate can ameliorate complications linked with the development of the metabolic syndrome, by improving insulin sensitivity in both obese rodents and patients [88–90]. The enhanced therapeutic effect of this catechin has been associated with the modulation of various signaling pathways, including targeting of genes involved in cell survival, FFA regulation, mitochondrial energetics, intracellular antioxidant response, and others as reviewed by Singh and colleagues [91]. A number of studies have demonstrated several mechanisms associated with the ameliorative effect of epigallocatechin gallate on IR and associated complications in skeletal muscle. In addition to strengthening muscle integrity [92–94], accumulative data has been presented that this catechin can improve insulin sensitivity by enhancing glucose uptake, reduce lactate concentrations, enhancing mitochondrial capacity and stimulating beta-oxidation in cultured cells, or rodents as well as obese human subjects [95–100]. Inhibition of oxidative stress, activation of AMPK, increased expression of PGC-1α, NAD-dependent protein deacetylase sirtuin-1 (SIRT1), nuclear respiratory factor 1, medium chain acyl coA decarboxylase, uncoupling protein 3 (UCP3), AKT, and peroxisome proliferator-activated receptor alpha (PPARα) are some of the mechanisms targeted by epigallocatechin to enhance skeletal muscle function in a diseased state [101–104].

7. Hesperidin

Hesperidin (PubChem CID: 10621) is a flavanone glycoside ((2S)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxy-2,3-dihydrochromen-4-one) with the molecular formula $C_{28}H_{34}O_{15}$ (MW 610.565 g/mol) that is present in high amounts in citrus fruits (**Figure 6**)

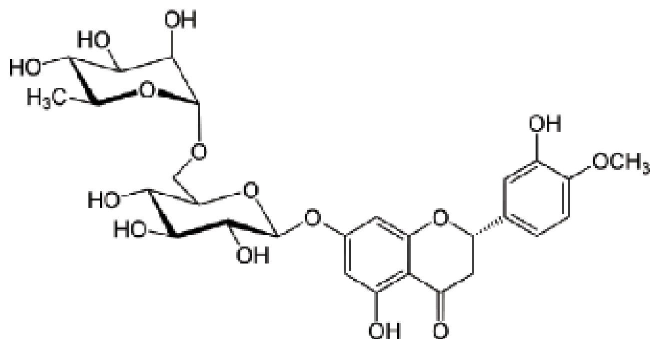

Figure 6. The chemical structure of hesperidin ((2S)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxy-2,3-dihydrochromen-4-one).

[105]. Although it has a low bioavailability due to the rutinoside moiety attached to the flavonoid [106], hesperidin can be converted to glucuronides and sulfoglucuronides, which have been shown to be excreted in urine nearly 24 hours after the orange juice ingestion [107]. In a randomized controlled trial, Nielsen et al. [108] demonstrated that removal of the rhamnose group to yield hesperetin-7-glucoside improved the bioavailability of the aglycone hesperetin. Suggesting that additional interventions are required to improve the bioavailability of citrus flavonoids such as hesperidin.

Increasing data has supported the notion that hesperidin possesses increased potential to lower raised blood glucose and lipid levels in various models of type 2 diabetes [109–111]. When administered in rats subjected to swimming exercise, this citrus flavonoid improved the biochemical and antioxidant profile of the animals [112]. This compound may induce its therapeutic effect through the regulation of genes implicated in insulin signaling such as insulin receptor substrate 1, GLUT2/4, and those linked with lipid metabolism, including sterol regulatory element–binding protein 1c (SREBP-1c), fatty acid synthase (FAS) and acetyl-CoA carboxylase [113]. Although data on its effect on skeletal muscle is currently limited, it can reverse IR by reducing muscle glycogen content and ischemia–reperfusion injury while promoting myogenic differentiation through the activation of MyoD-mediated myogenin expression in cultured cells and animals [109, 114, 115].

8. Luteolin

Luteolin (PubChem CID: 5280445) is a flavone glycoside (2-(3,4-Dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone) with the molecular formula $C_{15}H_{10}O_6$ (MW 286.239 g/mol) that is rich in various dietary sources such as fruits, vegetables, and teas (**Figure 7**) [116]. As with most flavonoids, during its metabolism luteolin is broken down to its glucuronides, which can eventually pass through intestinal mucosa as shown by Yasuda and colleagues [117]. Although studies reporting on the pharmacokinetic profile of luteolin in human subjects are limited, this flavone is quickly absorbed in rats and can be detected in urine and feces while showing a slow elimination rate [118]. Furthermore, luteolin from peanut hull extract can be easily absorbed compared to the pure compound, with its absorption more efficient in the

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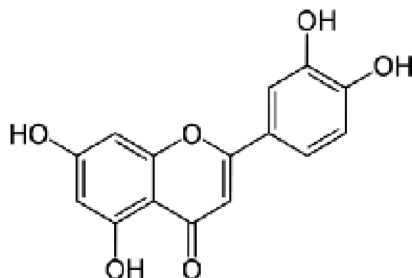

Figure 7. The chemical structure of luteolin (2-(3,4-dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone).

jejunum and duodenum than in the colon and ileum [119]. Alternatively, luteolin-loaded solid lipid nanoparticles prepared by hot microemulsion ultrasonic technique can also improve the solubility and increase the compound concentration in plasma of rats [120].

In addition to its strong antioxidant effects [121], in vitro experiments have provided evidence that luteolin possesses chemopreventive and anti-inflammatory properties [122, 123]. Hydroxyl groups and 2–3 double bond remain key structural features of luteolin that are linked to its enhanced therapeutic effect [124]. Recent studies show that this flavone attenuates hepatic steatosis and IR by upregulating PPARγ protein expression and activating AMPKα1 signaling, which may be linked to the improvement in circulating FFA levels in diet-induced obese mice [125, 126]. However, only a few studies have reported on the effect of luteolin on the skeletal muscle. Available literature has reported on its effect in preventing lipopolysaccharide-induced muscle atrophy, oxidative stress-induced tissue injury and inflammation, partly through regulation of atrogin-1/MAFbx expression, and c-Jun N-terminal kinases (JNK) phosphorylation reported on [127–129].

9. Naringenin

Naringenin (PubChem CID: 932) is a flavanone (5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one) with the molecular formula $C_{15}H_{12}O_5$ (MW 272.256 g/mol) that is also predominantly found in citrus fruits (**Figure 8**) [130]. The chemical structure of naringenin comprises three hydroxy groups at the 4′, 5, and 7 carbons while its glycoside, naringin contains an additional disaccharide neohesperidose that is linked via its carbon end. Although naringenin can be

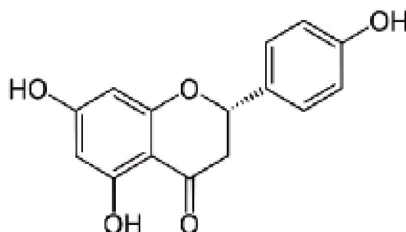

Figure 8. The chemical structure of naringenin (5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one).

detected as monoglucuronides in plasma and urine after ingestion of orange fruit juice in human subjects [131], the bioavailability of naringenin can be influenced by its glycosidic moiety. Felgines et al. [132] demonstrated that kinetics of absorption of naringenin and naringenin-7-glucoside was similar. In addition, naringenin-7-rhamnoglucoside exhibited a delay in its intestinal absorption, resulting in decreased bioavailability after ingestion in rats. On the other hand, complexation of naringenin with hydroxypropoyl-β-cyclodextrin has been another viable alternative to improve the bioavailability of naringenin, which is important to enhance its therapeutic potential [133].

Naringenin is among the well-studied citrus flavonoids shown to prevent complications associated with IR and the metabolic syndrome. Its role in preventing the deterioration in skeletal muscle mass and protecting against metabolic associated complication is summarized. In low-density lipoprotein (LDL) receptor–null ($Ldlr^{-/-}$) mice fed HFD, this flavanone reduced fasting hyperinsulinemia, improved glucose utilization and increased insulin sensitivity through regulation of SREBP-1c–mediated lipogenesis [134]. It stimulated glucose uptake but failed to have a significant effect on basal or insulin-stimulated AKT phosphorylation while significantly increasing AMPK phosphorylation/activation in cultured L6 myotubes [135]. Bhattacharya and colleagues showed that naringenin stimulates glucose uptake, indicating a dependence on GLUT4 activity as well as phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and/or p38MAPK activity [136]. Maintenance of muscle mass by reducing muscle diacylglycerol content, improving hyperinsulinemia, promoting phosphorylation of p38/MAPK via estrogen receptor beta (ERβ), lowering reactive oxygen species (ROS) production, and enhancing tyrosine phosphorylation are other mechanisms associated with protective effect of naringenin in either cultured cells or in vivo animal models [137–140].

10. Quercetin

Quercetin (PubChem CID: 5280343) is classified as a flavonol (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) with the molecular formula $C_{15}H_{10}O_7$ (MW 302.238 g/mol) that is abundantly found in various fruits and vegetables (**Figure 9**) [141]. Quercetin is one of the most abundant dietary flavonoids that is rapidly metabolized to glucuronides and sulfates that can be detected in plasma and urine [142]. Although oral bioavailability of quercetin remains

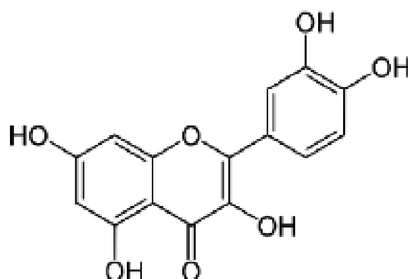

Figure 9. The chemical structure of quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one).

low, the type of sugar moiety attached to its structure may affect its absorption. This has been as demonstrated with quercetin glycosides from onion which have a higher absorption rate compared to apple-derived quercetin [143, 144]. Quercetin-4′-*O*-glucoside and quercetin-3-*O*-rutinoside (rutin) are one the accomplished glycosides of quercetin, and their absorption rate and extent can be influenced by plant matrix as demonstrated by Graefe and colleagues [145]. However, it is clear that further investigations into improvement strategies for pure quercetin aglycone are required to improve the therapeutic potential of this flavonol.

Quercetin exhibits a wide range of biological functions. Although Stewart et al. [146] failed to show any beneficial effect of quercetin against IR in diet induced-obese mice, other researchers have shown that this flavonol plays a major role in modulating several signaling pathways to reverse metabolic syndrome and improve skeletal muscle function, either in vitro on cultured cells or in vivo in animals and samples from human subjects [148–168]. In L6 myotubes and skeletal muscle of genetical modified (*ob*/*ob*) mice, quercetin improved insulin sensitivity by increasing GLUT4 expression [147]. Several studies using different experimental models have also demonstrated the positive effect of quercetin in improving skeletal muscle insulin sensitivity through enhanced uptake of glucose, and reducing oxidative stress or inflammation-induced damage, with modulation of tumor necrosis alpha (TNF-α), AKT, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and AMPK as prime pathways involved in the process [148–158]. The therapeutic potential of quercetin extends to its preventative effect against ischemia–reperfusion injury, as well as strengthening muscle fibers through the modulation of calcium homeostasis, and enhancing intracellular antioxidants [159–168].

11. Resveratrol

Resveratrol (PubChem CID: 445154) is a phytoalexin stilbenoid (3,5,4′-trihydroxy-trans-stilbene) with the molecular formula $C_{14}H_{12}O_3$ (MW 228.247 g/mol) that is present in abundant amounts in various food sources such as grapes, blueberries, and red wine (**Figure 10**) [169]. Upon ingestion, resveratrol can be metabolized to form conjugated sulfates and glucuronides, namely resveratrol monosulfate, monosulfate dihydroresveratrol, and monoglucuronide dihydroresveratrol, as reviewed by Gambini and colleagues [170]. Although the bioavailability of resveratrol is considered low, it can vary depending on the method of administration and type of dietary source ingested [171]. The dimethyl ether analog of resveratrol, pterostilbene,

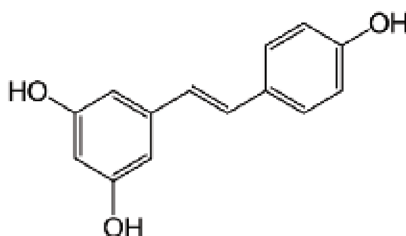

Figure 10. The chemical structure of resveratrol (3,5,4′-trihydroxy-trans-stilbene).

has been shown to exhibit a higher bioavailability, in terms of total plasma levels of both the parent compound and metabolites than does resveratrol [172]. However, Li et al. [173] showed that intravenous and oral pharmacokinetic characteristics of trans-resveratrol can be improved through encapsulating with PP123 self-assembling lecithin-based mixed polymeric micelles. Suggesting that alternative methods to improve the bioavailability of resveratrol are required, which may translate to enhanced therapeutic potential in vivo.

Resveratrol has displayed a variety of antidiabetic effects in rodent models. In addition, resveratrol attenuates thermal hyperalgesia, cold allodynia, as well as raised serum lipid levels [174–176]. In diabetic individuals, resveratrol administration is associated with significantly improved glucose and insulin control [177]. The systematic search of evidence linking resveratrol and IR in skeletal muscle revealed up to 18 studies published between 2007 and 2017, with 9 papers produced between 2016 and 2017, suggesting that this phytoalexin stilbenoid is increasingly explored for therapeutic effect against metabolic associated complications. Although Williams and colleagues showed no effect on insulin signaling pathways [178], stimulation of glucose uptake by resveratrol in cultured C2C12 cells or skeletal muscle has been linked with activation of extracellular signal-related kinase/p38/PI3K [179]. Its effect in promoting glucose uptake and improving insulin sensitivity was also associated with increased NAD-dependent protein deacetylase sirtuin-1 (SIRT1) expression, activation of AMPK while abolishing phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), JNK, and IκB kinase α/β (IKKα/IKKβ) [180–190]. Other documented beneficial effect of resveratrol includes inhibiting ischemia–reperfusion injury through its potent antioxidant properties [191], reducing cell proliferation through upregulating PGC-1α [192], promoting muscle regeneration and attenuating the impact of ROS [193], and elevated forearm skeletal muscle mitochondrial capacity [194].

12. Rutin

Rutin (PubChem CID: 5280805) is a glycoside combining the flavonol quercetin and the disaccharide rutinose (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α-L-rhamnopyranosyl-(1→6)-β- D-glucopyranosyloxy]-4H-chromen-4-one) with the molecular formula $C_{27}H_{30}O_{16}$ (MW 610.521 g/mol) that is found in many plants and fruits, as well as tea infusions (**Figure 11**) [195]. Upon oral administration, rutin can be metabolized into sulfates and glucuronides of quercetin that are detected in blood, whereas unchanged forms of rutin and quercetin were not detected [142, 196]. Although quercetin glycosides from onions demonstrate an enhanced absorptive capacity than pure aglycones [143, 144], some studies have showed that rutin has a lower oral absorption rate than quercetin [142, 197]. However, as with the use of natural deep eutectic solvents [198], alternative methods to improve the absorptive capacity of rutin is tested to improve therapeutic effect in vivo.

Like quercetin, rutin exhibits a wide variety of biological properties, mostly attributed to its strong antioxidant properties [199, 200]. It is accomplished that rutin displays enhanced potential

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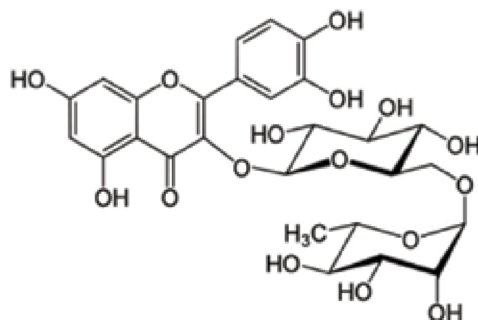

Figure 11. The chemical structure of rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyloxy]-4H-chromen-4-one).

to improve insulin sensitivity by regulating genes involved in glucose and lipid metabolism such as GLUT4, PPARγ, and tyrosine phosphatase 1B in cultured cells or skeletal muscle of rodents [201–204]. However, from the study by Zyma et al. [205], that demonstrated that rutin induces conformational changes in the myosin structure of skeletal muscle of rabbits accompanied by an increase in ATPase activity, accumulative evidence has supported muscle strengthening capacity of this polyphenol. For example, Su et al. [206] presented data showing that rutin promoted skeletal muscle endurance capacity by modulating markers of mitochondrial biogenesis such as PGC-1α and SIRT1 expression in ICR mice subjected to a weight-loaded forced swim test. These findings were further supported by data showing that rutin increased the mitochondrial size and mitochondrial DNA content as well as gene expression related to mitochondrial biogenesis, such as PGC1-α, NRF-1, transcription factor A, and SIRT1 [207, 208].

13. Sulforaphane

Sulforaphane (PubChem CID: 5350) is an isothiocyanate (1-isothiocyanato-4-methylsulfinylbutane) with the molecular formula $C_6H_{11}NOS_2$ (MW 177.28 g/mol) that is found in cruciferous vegetables such as cabbages, broccoli, and brussels sprouts (**Figure 12**) [209]. Although sulforaphane displays a dose-dependent pharmacokinetic behavior, as higher doses show reduced absorptive potential, lower doses of the compound can be rapidly absorbed in rats following intravenous administration, with the absolute bioavailability being able to reach 82% [210]. In human subjects consuming fresh broccoli sprouts or the broccoli sprout extract, with each estimated to provide 200 μmol sulforaphane daily, the

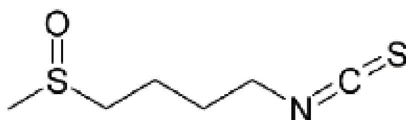

Figure 12. The chemical structure of sulforaphane (1-isothiocyanato-4-methylsulfinylbutane).

compound metabolites were found to be three times higher in plasma and urine of sprout consumers, suggesting enhanced sulforaphane absorption from sprouts [211]. Therefore, dietary form and dosing schedule of sulforaphane may influence impact absorption and therapeutic potential in human subjects.

Sulforaphane has received a considerable interest due to its ability to simultaneously control multiple cellular targets involved in various metabolic complications. For instance, in rats fed HFD, this isothiocyanate has displayed an enhanced hypoglycemic potential as well as the elevation of GLUT3 expression in the cerebral cortex and hypothalamus, leading to improved glucose tolerance [212]. Other studies [213, 214] have supported the beneficial effect of sulforaphane or its stable precursor glucoraphanin, to reverse IR, mostly through its robust antioxidant properties. In skeletal muscle, sulforaphane has exhaustive exercise-induced muscle damage, reducing muscle glycogen content, and enhanced exercise endurance capacity through inhibition of pro-inflammatory response and enhancing antioxidant response by upregulating NRF2 expression [215–220].

14. Conclusions

Natural compounds have gained popularity for their potential beneficial effect to fight metabolic diseases due to their less adverse effect compared to synthetic drugs. Furthermore, natural compounds serve as a valuable source for the discovery of new drugs. Currently, knowledge shows that natural compounds can ameliorate IR, however, the gap in scientific evidence of plant-derived therapeutic benefits still exist due to the slow rate of translation of animal studies findings into human clinical trials. In this chapter, evidently reported the great potential and the future promise of natural compounds for the management and treatment of metabolic disorders, specifically IR, obesity, and T2D. Therefore, further research is required to assess the use of natural compounds alone or in combination with well know antidiabetic drugs might result in synergistic and enhanced effects in combating metabolic diseases.

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Abbreviations

AKT	protein kinase B
AMPK	5' AMP-activated protein kinase
ATP	adenosine triphosphate
CD36	cluster of differentiation 36
DPPH	2,2-diphenyl-β-picrylhydrazyl
ERβ	estrogen receptor beta
FAS	fatty acid synthase
FFA	free fatty acid
GLUT	glucose transporter
HFD	high fat diet
IR	insulin resistance
IRS-1	suppressing insulin receptor substrate 1
JNK	c-Jun N-terminal kinases
LDL	low density lipoprotein
LKB1	serine/threonine kinase 11
MAPK	mitogen-activated protein kinase
MW	molecular weight
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NRF2	nuclear factor (erythroid-derived 2)-like 2
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PPAR	peroxisome proliferator-activated receptor
Prmt7	protein arginine methyltransferase 7
SIRT1	NAD-dependent protein deacetylase sirtuin-1
SREBP-1c	sterol regulatory element–binding protein 1c
T2D	type 2 diabetes mellitus
UCP	uncoupling protein

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Section 5

The Characteristics of Vascular Smooth Muscle Cells

Chapter 11

Vascular Smooth Muscle Cell

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Additional information is available at the end of the chapter

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Abstract

Vascular smooth muscle cells (VSMCs) are the stromal cells of the vascular wall and are responsible for regulating arterial tone, blood pressure, and blood supply of the tissues. VSMCs display diversity in function and phenotype depending on their location within the arterial tree (large conduit vs. small resistance vessels), their embryologic origin, and their organ-dependent microenvironment. The heterogeneity of VSMCs is regulated by multiple mechanisms including intracellular signaling and changes in the VSMC micro-environment. Genetic disorders and extrinsic stimuli-induced dysfunction in VSMCs are associated with age-related vascular pathogenesis and vascular diseases, and thus are considered as a potential therapeutic target.

Keywords: vascular smooth muscle cell, blood vessel, circulation, blood pressure, stiffness

1. Introduction

Vascular smooth muscle cells (VSMCs) are the main cellular components of the normal blood vessel walls, interweaving with elastic fiber layers to form the vascular media that provides structural integrity. VSMCs play an important role in the regulation of blood pressure and blood distribution to various tissues of the body through dynamic contraction and relaxation in response to vasoactive stimuli such as hormones, metabolites and neurotransmitters. Morphological and biochemical studies have revealed that two distinct phenotypes of VSMCs co-exist in the vessel wall, which are the differentiated contractile and the synthetic proliferative phenotypes. These two phenotypes of VSMCs are dictated by their environmental and functional requirements and also reflect differing patterns of gene expression [1–3].

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The contractile VSMCs are characterized by specific contractile proteins, ion channels, and cellular surface receptors that regulate the contractile process. Synthetic VSMCs, also called secretory VSMCs, are characterized by significant proliferation and migration activity, such as the production of a large amount of extracellular matrix during development, in response to the physiological changes (such as long-term exercise and pregnancy) and pathological injury (such as under the conditions of inflammation, hypertension, diabetes) [4]. It has been shown that the different phenotypes of VSMCs can reversibly switch, but a nonreversible change from the contractile to the synthetic phenotype is a prerequisite for the progression of vascular disease [4]. This chapter will summarize the current state of our knowledge on the origin and the ultrastructure of the VSMCs, and the mechanisms underlying the change in the VSMC phenotypic switch. We will also outline the current progress on the role of the VSMC dysfunction in the development of the vascular diseases and the therapeutic potential of the manipulation of the VSMC gene expression in these diseases.

2. Origin of VSMCs

Heterogeneity within the blood vessels is critical to cardiovascular function. In order to meet distinct physiological requirements, different regions of the vasculature exhibit different physical properties. Early studies have shown that the VSMCs of the proximal large vessels (which comprises the arch of the aorta, the common carotids, the common pulmonary trunk, and the brachiocephalic artery) are derived from the neural crest (NC) (ectomesenchymal smooth muscle) [5, 6], while VMSCs of the distal vessels (which includes the abdominal aorta and the right and left carotid arteries) are derived from the mesenchyme [7, 8]. At the region of the interface of these vessels, VSMCs are derived from mixed origins, both of the ectomesenchymal and mesenchymal.

There is abundant evidences showing that the embryonic origin of the VSMCs plays an important role in vascular biology and in the response to the stimuli response [9, 10]. Firstly, the different embryonic origins of VSMCs reflect different gene expression patterns [11]. Secondly, differentiation of VSMCs from embryonic stem cells through NC- or mesoderm-lineages showed that VSMC characteristics are programmed largely based on embryonic origin [12]. These distinct embryonic origin differences also converge in the adult vessels [13]. As noted earlier, the VSMCs of the proximal aorta arise from two distinct embryonic origins: the NC and the somatic mesoderm [14, 15]. This juxtaposition of the VSMCs from different embryonic origins in the aorta contributes to the specific ability of the aorta to respond to high local pressure/force loading and various chemical stimulation as well as neuronal–hormonal–regulation to meet the physiological requirement of circulation. In addition, VSMCs from different embryonic origins may also be responsible for some specific pathogenesis of vascular diseases. For example, studies have shown that when vessels that are prone to atherosclerosis are placed in a vascular region that does not typically develop atherosclerosis, they retain their predisposition to disease [16]. This evidence suggests that individual VSMC characteristics may be linked to embryonic origin. However, definitive evidence that embryonic origin dictates vascular phenotype has not been fully elucidated [17].

While the embryonic origins of many VSMC populations are known, the exact nature of the VSMC precursor remains elusive. It has been indicated that VSMC progenitors may arise from distinct embryonic sources including the splanchnic mesoderm [18], somatic mesoderm [15, 19], neural crest (NC) [14, 20], mesothelial [21], and other embryonic cell types [22]. In the aorta, splanchnic mesodermal cells are first recruited and differentiate into VSMCs. Before the splanchnic mesoderm cells completely encircle the dorsal aorta, the cells are displaced by the somatic mesodermal cells. Differentiation of these somatic mesodermal cells begins in the ventral anterior end of the vessel. Differentiation then proceeds around the circumference of the vessel and down the length of the aorta toward the diaphragm. Meanwhile, the cardiac NC migrates down the pharyngeal arches to invade the aortic sac. A subset of the cardiac NC participates in septation of the truncus arteriosus into the aortic arch and the pulmonary trunk [23]. The rest of the cardiac NC remains in the pharyngeal arch arteries and become the VSMCs of the aortic arch and the arteries of the head and neck [20]. The border that forms between the NC-derived VSMCs of the ascending aortic arch (aAo) and mesoderm-derived VSMCs of the descending aorta (dAo) is maintained throughout development and into adulthood [14, 20]. Once cells encircle the aorta and differentiate into VSMCs, they undergo a closely regulated process of layer formation within the media.

3. The structure and ultrastructure of VSMCs

Under physiological conditions, VSMCs mostly express the contractile phenotype with a spindle-like shape with a length of 50–200 μm, a width of 2–8 μm. The nucleus is located in the center surrounded by smooth endoplasmic reticulum and mitochondria. The cytoplasm is rich in thick and thin myofilaments, with every thick myofilament surrounded by 15 thin myofilaments. The thick myofilaments and thin myofilaments are aggregated into myofilament units, also known as systolic units. The intracellular thin filaments are connected by dense bodies. The adjacent dense bodies are connected by intermediate filaments to form a smooth muscle network. Thin filaments and cell membranes are connected by dense patches. Smooth muscle cells are surrounded by reticulated fibrous connective tissue, including extracellular matrix secreted by VSMCs, which interlaces individual cells into clusters to be functional units [24].

3.1. The thick filaments

The diameter of thick filaments is 8–16 nm, which is a myosin dimer. The myosin superfamily is subdivided into 18 categories based on their conserved motor domain and systemic development. Type II is the constituent protein of thick filaments found in multiple subtypes of striated muscle, myocardium and smooth muscle. The smooth muscle subtype is encoded by the same gene, with selective splicing producing myosin monomer SMA and SMB [25]. The SMB type is more expressed in phasic contractile smooth muscle such as in the bladder and smooth muscle of the small intestine [26]. The SMA type is more abundant in tensile contractile smooth muscle. Smooth muscle myosin has a molecular weight of about 50 kDa, containing

two heavy chains and four light chains. Each heavy chain has a carboxyl-terminal tail and an amino-terminal head, containing approximately 2000 amino acids, approximately 20 kDa. The tails of the two heavy chains are wound in the form of α-crimping spirals to form the skeleton of the thick filaments [25]. The heads of the two heavy chains are separated, face the thin filaments, and form part of the transverse bridge. Myosin is hydrolyzed by trypsin to produce about 350 kDa heavy meromyosin and 150 kDa light meromyosin. Hydrolyzed myosin can be produced by proteases such as papain to generate fragment S1 and S2. The fragment S2 is a helical structure. The fragment S1 is the head of myosin and can be divided into a motor domain and lever arm. The motor domain contains an actin binding site and a nucleotide binding site [27]. The lever arm contains binding site of convert domain, myosin light chain 17 (MLC17) of 17 kDa, and myosin light chain 20 (MLC 20) of 20 kDa. The torsion zone is a site where relative rotation occurs between the motor domain and lever arm to relatively slide actin and myosin. MLCl7 is located near the torsion zone and related to the structural stability of the lever arm. MLC20 is located near the junction of S1 and S2 [27] .

3.2. The thin filaments

Thin filaments, which consists of acting, are 5–8 nm in diameter and 1 μm in average length. Actin is the most abundant protein in eukaryotic cells and accounts for 20% of total protein weight in muscular cells. Actin monomers, which called globular actin (G-actin), are dumbbell-shaped. Microfilaments are formed by the conglomeration of actin monomers into large multimers, which are called fibrous actin (F-actin) [28]. Monomeric actin consists of 375 amino acid residues with a molecular weight of 42 kDa which has three binding sites, one for ATP binding and two for myosin binding. Actin maintains its polymer in a dynamic, polar state by hydrolyzing ATP. Some proteins are closely related to the smooth muscle filament's function including tropomyosin, caldesmon and calponin [17, 29]. VSMCs expressed at least five tropomyosin subtypes with subtype α displaying the highest abundance. By regulating the binding of other proteins to actin filaments, tropomyosin affects the interaction between actin and myosin and multimerization of actin [30]. The calponin protein is a 34 kDa-sized protein that is present in smooth muscle and non-muscle tissue, and is primarily calponin-l in smooth muscle, which may reduce muscle contraction by inhibiting the myosin ATP enzyme [31].

3.3. The skeleton protein

The skeleton protein plays an important role in maintaining cellular shape, intracellular organelle location, intracellular vesicle trafficking, cell migration, and division [32]. Like all eukaryotic cells, VSMCs mainly contain three skeleton proteins: microfilaments, intermediate filaments and microtubules. The microfilament has a diameter of about 4 nm and consists of linear polymerization of actin. The filaments are connected to each other through dense bodies to form a network structure and are connected to the cell membrane through dense spots. There are four types of actin isoforms in smooth muscle: α-smooth muscle actin, β-non-muscular actin, γ-smooth muscle actin and γ-cytoplasmic actin, all of which are distinct gene products [33, 34]. In VSMC, α- smooth muscle actin is the main subtype interacting with myosin to produce contraction. Approximately 60% of large arterial vascular actin is α-smooth

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muscle type, 20% is β-non-muscle actin and the remainder is γ-smooth muscle and γ-cytoplasmic actin. Γ-smooth muscle actin is mainly confined to the gastrointestinal muscles. Studies have shown that γ-cytoplasmic actin is confined to the cell cortex, α-actin serpentine longitudinal full-length cells, and β-actin borders dense plaques. The diameter of the intermediate filament is about 10 nm, which is involved in maintaining the three-dimensional structure of the cell, maintaining the proper position of the organelle in the cytoplasm, and participating in the transfer of the membrane receptor signal to the nucleus [35]. The intermediate filaments of VSMC are in the shape of the crest, and the periphery is often accompanied by a dense body [36]. It is abundantly expressed during development and decreased with cell maturation. The intermediate filaments of differentiated vascular smooth muscle are mainly composed of vimentin and desmin [37, 38]. Vimentin, which is generally found in cells of mesenchymal origin, is the major intermediate filament types of aortic smooth muscle [37]. Skeletal proteins make the different components of the VSMCs an organic three-dimensional structure, which is a dynamic process that produces adaptive changes based on changes in cell function.

3.4. The sarcoplasmic reticulum

The sarcoplasmic reticulum, also known as sarcoplasmic reticulum, is a specialized smooth endoplasmic reticulum in muscle cells; a phospholipid bilayer forming a capsular network, which stores a large amount of Ca^{2+} [39]. At rest, the sarco/endoplasmic reticulum Ca^{2+} − ATPase (SERCA) transport the cytosolic Ca^{2+} into the sarcoplasmic reticulum through hydrolyzing ATP. The inositol (1,4,5)-triphosphate receptor (IP3R) and ryanodine receptor (RyR) channel release Ca^{2+} into the cytoplasm when excited and thus play an important role in the regulation of contraction and relaxation [40, 41]. SERCA is a type of sarcoplasmic reticulum transmembrane Ca^{2+} transport ATP enzyme, which can transport two Ca^{2+} per one ATP hydrolyzation. Three major subtypes are known: SERCA1, SERCA2 and SERCA3. SERCA2a is presented in the myocardium, skeletal muscle and multiple smooth muscle cells. The sarcoplasmic reticulum releases calcium ions to the cytoplasm through the IP3R and RyR Ca^{2+} channels. IP3R is a membrane glycoprotein complex composed of inositol trisphosphate-activated Ca^{2+} channels. It consists of four subunits with a molecular weight of approximately 300 kDa. The ratio of IP3R and RyR in vascular smooth muscle is 3:1 to 4:1. The RyR can cluster in the sarcoplasmic reticulum near the cellular membrane, so that the local release of Ca^{2+} is at a high concentration [40].

4. The VSMC phenotypes and their regulations

4.1. The phenotypes of VSMCs

Although the primary function of VSMCs in the adult animal and human is contraction, VSMCs maintain considerable plasticity throughout life and can exhibit a phenotypic switch during normal development, the repair of vascular injury, and in disease states [42, 43]. During development, VSMCs exhibit a secretory phenotype that is distinct from the spindle-shaped mature, contractile phenotype present during physiological conditions in the adult. Secretory

VSMCs contain a large number of organelles involved in protein synthesis, whereas the main component of contractile smooth muscle cells is myofilaments.

Secretory VSMCs show high proliferation rates, apparent migration activity and strong extracellular matrix synthesis [42]. These extracellular matrixes include collagen, elastin, proteoglycan, cadherin, and integrin. At the developmental stage, VSMCs form a large number of gap junctions with endothelial cells, a process that is critical for vascular maturation [44]. In contrast, the contractile phenotype of VSMCs is very low in proliferation and the migration activity and synthesis of extracellular matrix are also low. The expression of some marker proteins is different in different phenotypic smooth muscle cells, for example, PDGF-a, intercellular adhesion molecule 1 (ICAM1), I-caldesmon, osteopontin, matrix Gla protein (MGP), collagen 1 and connexin43 decrease gradually in the process of VSMC transition from secretory to contractile type [45, 46]; whereas αl-, β1- and α7 integrins, transcriptional co-activation factors myocardin, cadherin, α-smooth muscle actin, desmin, smooth muscle protein 22α (SM22α), carboxypeptidase-like protein, smooth muscle calponin, h-calmodulin binding protein, aortic preferentially expressed gene1 (APEG1) cysteine-rich protein 2 (CRP2) gradually increased during VSMC transition from secretory type to contractile type [47–49] .

Importantly, calcium signaling varies between the two phenotypes. Ca^{2+} signals controlled by large conductance K^+ channels $K_{Ca}1.1$, voltage-gated L-type Ca^{2+} channels and RyR are associated with the transcription of differentiated contractile protein markers while signals controlled by intermediate conductance Ca^{2+}-activated K^+ channels ($K_{Ca}3.1$) and TRPC channels are associated with the transcription of pro-proliferative protein markers [50]. Furthermore, the expression levels of intracellular Ca^{2+}release channels, Ca^{2+}-activated proteins and pumps are also altered during VSMC phenotype switching: the synthetic VSMCs lose the RyR3 and the SERCA2a pump and reciprocally regulate isoforms of the ca^{2+}/calmodulin-dependent protein kinase II [50]. Changes in calcium signaling molecules as a result of phenotypic switches reflect changes in the function of the VSMCs as contractility is substituted for proliferation.

Currently, smooth muscle myosin heavy chain (SM-MHC) and smoothelin are two marker proteins that identify the contractile phenotype of smooth muscle. SM-MHC was found only in smooth muscle cells in *in vivo* studies and was observed only in VSMCs during embryonic development; its detection sensitivity is high in cultured vascular smooth muscle. Additionally, phenotypic switching of SMCs in response to PDGF BB *in vitro*, or vascular injury *in vivo* has been shown to be associated with a loss of activating histone modifications at gene loci encoding SMC marker genes, but retention of additional markers such as H3K4 methylation [51]. It was postulated that epigenetic mechanisms may allow for the “cell lineage memory” during the reversible phenotypic switching of VSMCs [51].

4.2. The regulation of VSMCs phenotypes

The mature skeletal muscle is terminally differentiated, that is, the its ability to be changed is limited. However, the mature VSMC has a strong plasticity, with significant and reversible phenotypic changes occurring when the local environment changes. The VSMCs can quickly transform from a contractile phenotype to a secretory phenotype in response to injury.

Significantly, when the injury is repaired and the local environment returns to normal condition, VSMCs can regain the contractile phenotype. Thus, the regulatory mechanisms have to be reversible.

Although the molecular mechanisms controlling the VSMCs phenotypes switching have not been fully understood and the signaling pathways involved under different conditions may be variant, epigenetic mechanisms have been suggested as a possible explanation of the reversibility of VSMC switching. For example, microRNA 663 and micro RNA 133 have been identified as modulators of VSMC phenotypic switching [52, 53]. In addition, it has been widely-accepted that serum response factor (SRF)-mediated signaling pathways play an important role in the regulation of VSMC phenotype changes [54]. The SRF belongs to the MADS box transcription factor superfamily, through which the cis-element CArG box binding regulates the transcription of smooth muscle marker genes and is closely related to the phenotype of smooth muscle [54]. SiRNA-mediated *SRF* suppression affected the expression of established SRF target genes such as *SMA* or *SM22α* and decreased both F-actin formation and cell migration [54]. The binding state of SRF and myocardin under normal oxygen conditions promotes the transcription and contraction phenotype of smooth muscle marker genes. The transcriptional coactivator myocardin and the nuclear transcription factor Elk1 competed for the same binding site on the SRF. The binding state of SRF and myocardin in pulmonary vascular smooth muscle under normal oxygen conditions promotes the transcription and contraction phenotype of smooth muscle marker genes, and the expression of myocardin decreases under hypoxia, leading to enhanced binding of SRF to Elk1 and smooth muscle orientation [55]. Notably, the transcription of smooth muscle cell markers can also be under epigenetic control [56]. Inhibition of histone acetyl transferases (HATs) and histone deacetylases (HDACs) decreases the activity of the promoters of SMC marker genes [57].

The expression of myocardin is down-regulated during hypoxia, leading to enhanced binding of SRF. Elevated PDGF-BB can also promote Elk1 phosphorylation and Elk1-substituted myocardin binding to SRF through the ras/raf/mek/erk kinase pathway, promoting conversion to the secretory phenotype [58]. Activation of RhoA is essential for smooth muscle specific transcriptional up-regulation. RhoA activates multimerization of actin via ROCK and then promotes the translocation of myocardin-related transcription factor (MRTF) into the nucleus and binds to SRF, stimulating smooth muscle marker gene transcription and conversion to a contractile phenotype [59]. Rho-dependent MRTF nuclear translocation is one of the key regulation mechanisms of smooth muscle cell differentiation.

5. VSMCs dysfunction in vascular pathogenesis and diseases

5.1. Intrinsic VSMC mechanical property and aging-induced aortic stiffness

An increase in vascular stiffness is a common vascular pathogenesis of aging and of aging-related cardiovascular disease and has been assumed to be caused by molecular changes of the ECM and the dysfunction of endothelial cells in elastic arteries. It was not until recent years,

with the use of two unique techniques, atomic force microscopy (AFM) [60] and a reconstituted tissue model [60], that it was discovered that both the intrinsic mechanisms in VSMCs and the alterations in VSMC-ECM interaction contribute to the increased aortic stiffness in the old non-human primates [21]. The underlying mechanisms involve the increased expression and polymerization of α-smooth muscle actin (a stress fiber-specific isoform of actin for VSMCs), microtubules and myosin light chain kinase (MLCK), and also the increased expression of adhesion molecule β1-integrin and its binding to fibronectin. It was also suggested that the oscillatory behavior of VSMC elasticity and adhesion are affected differently during aging, which may link these events to changes in vascular stiffness. However, the molecular mechanisms are still not fully understood [61].

5.2. VSMCs and hypertension

Hypertension is one of the most common cardiovascular diseases, which eventually results in heart, renal failure or stroke. Although most of previous studies focused on the changes in the ECM and impaired endothelial control [62–64], increasing evidences indicate that VSMCs play an important role in the development of hypertension. It has been shown that arterial hypertension is accompanied by the proliferation and migration of VSMCs [65, 66]. One of the most typical features of vascular remodeling in the course of hypertension includes thickening of the middle layer and intima and the increase of the ratio of wall thickness to lumen. These changes are mainly found in the small arteries of hypertension, mainly due to hypertrophy and proliferation of VSMCs and the migration of VSMC into the intima. In addition, recent studies confirmed that the increased intrinsic stiffness of VSMCs from hypertensive aorta contribute to the aortic stiffening and high blood pressure. The underlying mechanisms are involved in the upregulation of ROCK-SRF/myocardin and α-smooth muscle actin signaling in the VSMCs. It is noteworthy that a heterogeneity of mechanical properties in VSMCs between the large aorta and downstream distal arteries in the hypertensive model was shown, which is accompanied with a parallel regional difference of the SRF/myocardin signaling pathway. These observations further support the concept that the different origins of VSCMs plays a role in the development of hypertension. Furthermore, a most recent study from the same group also indicate that VSMCs from hypertensive aorta are able to contribute to hypertensive vessel changes by interrupting synthesis and degradation as well as organization of ECM through the regulation of activity of lysyl oxidase (LOX) and integrin β1. Importantly, targeting the stiffening of VSMCs effectively lowered aortic stiffness and blood pressure which revealed a promising therapeutic potential of anti-hypertension treatment in the future [28, 59].

5.3. VSMCs and atherosclerosis

Studies have been shown that VSMCs play a complex role in the formation of atherosclerosis, including increased matrix synthesis, production of multiple proteases, and changes in vascular contractility, in which the proliferation and apoptosis of VSMCs play a major role in the process of intima thickening and formation of atherosclerotic plaque [67]. Prior to the development of atherosclerosis the VSMCs maintain a stable phenotypical features and showed low

proliferation. As atherosclerosis developed, the VSMC phenotype changes to a more proliferative nature, with reduced contraction, increased proteoglycans, but reduced expression of the typical smooth muscle markers [68–70]. The biological effects of VSMCs were discovered during the *in vitro* culturing of these cells and they vary with different subtypes of different species, such as the spindle-shaped smooth muscle cells (S-SMC), the epithelioid-smooth muscle cell (E-SMC) and rhomboid- smooth muscle cell (R-SMC) in animal models [71–73]. The S-SMC exhibit features of the contractile phenotype, showing high expression of α-SMA, desmin, and SM-MHC [74]. The E-SMC and R-SMC, on the other hand, exhibit the synthetic phenotype that had significantly higher proliferation and migration proficiency. The S-SMC is more sensitive to vascular contractile factors (including endothelin 1, angiotensin II, etc.) than E-SMC. Studies on the biological behavior of different smooth muscle subtypes have led to a deeper understanding of the phenomenon of deposition of smooth muscle cells in the subendothelial cells during the course of atherosclerosis. As atherosclerotic plaque grow, apoptosis in mainly macrophages and VSMCs have been detected in the plaques [75]. It has been suggested that macrophages play a role in inducing the apoptotic process in the VSMCs which may trigger plaque rupture [76, 77]. Interestingly dying VSMCs are associated with inflammation as the apoptotic process induce release of cytokines such as IL-1 and IL-1β from the dying or apoptotic VSMCs [78]. Chronic VSMC apoptosis promotes the progression of atherosclerosis.

5.4. VSMCs and aortic aneurysm

Aortic aneurysm (AA) is a life-threatening condition where a bulge forms in the aortic wall. There are abdominal and thoracic AA. The basic structural unit of the aortic wall is the two layers of elastic fibers clamped by the VSMCs to form a sandwich structure. This structure allows the blood vessel wall to have a good contractile strength and elasticity. The abnormality of its composition and function may lead to AA. The development of AA can begin with the degradation of ECM within the media due to a proteolytic process, which loosens the wall tension created by the ECM and VSMCs [79]. Mucoid degeneration in the aortic media can induce the disappearance of VSMCs. Loss of VSMCs inhibits the clearance of proteolytic enzymes, leading to replacement by vacuoles, proteases, apoptotic cells and modified glycosaminoglycans. This state leads to the progression to chronic dilatation and development of thoracic AA [79]. Like atherosclerosis, phenotypic switching and VSMC apoptosis also influence the development and progression of AA [80]. VSMCs express NADPH oxidases isoforms, which then regulates the proliferation migration and apoptosis of the VSMCs [81, 82]. This implicates that oxidative stress also play an influential role in the development of AA. The mutations in some cytoskeletal proteins, such as MYH11, is associated with cellular contractions, may cause AA [83]. The rigidity and function of the cytoskeletal proteins is important to the function of the VSMCs. In the blood vessel wall of patients with MYH11 mutations, the axis of the cytoskeleton/membrane integrin-extracellular matrix is disrupted, there is accumulation of proteoglycans, breakage of elastic fibers and reduction in the number of VSMCs. The typical characteristics of AA include the medial membrane degeneration, abnormal VSMC arrangement and proliferation of epithelial vasodilatation [79]. In addition to systolic function, VSMCs are also capable of trans-differentiation and secretion under mechanical and

biochemical stimuli, such as the secretion of a variety of matrix proteins through the interaction with integrins, G protein-coupled receptors (GPCR) and disks on the cell membrane surface [84].

6. Pharmacological targets of VSMCs

Since VSMCs play a critical role in the cardiovascular diseases such as aortic stiffening, hypertension, atherosclerosis and aortic aneurysm, they have become a therapeutic target in the treatment of these diseases. For example, atorvastatin, one of the most effective drugs for treating cardiovascular diseases, suppresses tacrolimus-stimulated VSMC proliferation via down-regulation of β-catenin, ERK1/2, and cyclin B. Tropoelastin is shown to regulate VSMC phenotypic switch and inhibit VSMC proliferation and migration [22]. Drugs, such as paclitaxel and rapamycin which are eluted in the coronary artery stent inhibit the proliferation of VSMCs and significantly reduces the restenosis rate. In addition, new basic investigation on the VSMCs in aortic stiffness during aging and hypertension also provides new targets for the treatment of vascular diseases. For example, two anti-stiffening treatment of VSMC that act either through the inhibition of ROCK-SRF/myocardin signaling by Rock inhibitor (Y-27632), or through the inhibition of the SRF/myocardin signaling (CCG-100602) have been shown to be able to reduce the aortic stiffness and high blood pressure in hypertension. These suggest that VSMCs can be a promising target of the treatment of hypertension [28, 59]. The successful preclinical application of these VSMC-targeted interventions underscores the promising prospective of medications targeting VSMC. In fact, Plumericin inhibits proliferation of VSMCs by blocking STAT3 signaling via S-glutathionylation, highlighting the feasibility of clinical manipulation of VSMCs [85, 86].

7. Conclusions

As summarized in the **Figure 1**, VSMCs are not only the dominant components of the medial layer of blood vessels, but also important endocrine cells which secret various signaling factors promoting the arterial remodeling in the case of pathological stimuli. Multiple factors including embryonic origin, regional mechanical load, pathological stimuli and genetic mutations mediate the gene expression of VSMCs through different signaling pathways which involve the VSMC membrane receptors, calcium channels, miRNAs, DNA methylation, and histone modification. This results in the regulation of VSMC phenotypes, the expression of stiffness-related proteins, and ECM production. These changes subsequently affect VSMCs stiffness, migration, and proliferation, as well as ECM remodeling, thus, playing a role in normal vascular physiology and diseases. Although the mechanisms involved in vascular diseases remain largely unknown, SRF/myocardin mediated signaling pathways have been identified as a key mechanism within the developmental of vascular diseases through their regulations on the VSMC stiffness, phenotypic switching and ECM remodeling. Targeting VSMC is a

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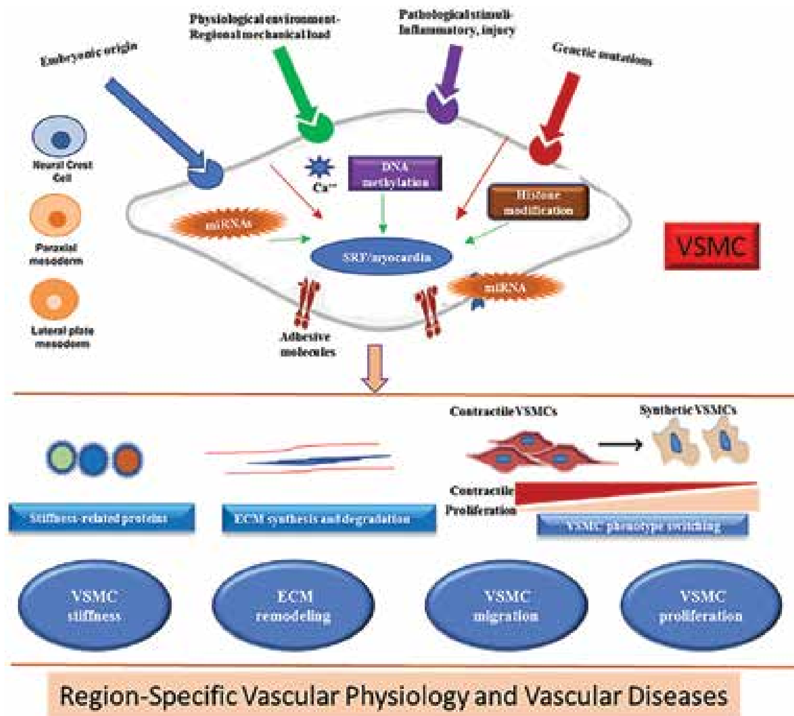

Figure 1. Summary of the role of VSMCs in normal vascular physiology and in the development of vascular diseases. Multiple factors including embryonic origin, regional mechanical load, pathological stimuli and genetic mutations mediate the gene expression of VSMCs through different signaling pathways which involves the VSMC membrane receptors, calcium channels, miRNAs, DNA methylation, and histone modification. This results in the regulation of VSMC phenotypes, the expression of stiffness-related proteins, and ECM production. These changes subsequently affect VSMCs stiffness, migration, and proliferation, as well as ECM remodeling, thus, playing a role in vascular normal physiology and diseases.

promising therapeutic of hypertension, atherosclerosis and aortic dissection/aneurisms and other related diseases.

8. Future directions

The pathological changes, especially the phenotypic switching of VSMC, are the most important mechanisms and characteristics of various cardiovascular diseases, including hypertension, atherosclerosis and aortic dissection/aneurisms. Medications targeting VSMCs have been clinically prescribed in the treatment of these diseases. SMC-specific drugs may be achieved through the different approaches: (1) identifying genes/proteins targets that differentially regulate VSMC phenotype changes and identifying markers of synthetic phenotype; (2) identifying genes/proteins that target intrinsic mechanical properties of the VSMCs and developing inhibitors of the proteins; (3) exploring non-coding RNAs, including microRNA, long- or short-non-coding RNA and other epigenomic alterations such as DNA methylation and

histone regulation that differentially regulates VSMC function; (4) identifying growth factor/hormones that have differential cellular effects on VSMC; and (5) combined usage of multiple drugs to achieve distinct functions in ECs and VSMCs.

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Conflict of interest

None.

Notes/Thanks/Other declarations

None.

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Chapter 12

The Role of Vascular Smooth Muscle Cells in the Physiology and Pathophysiology of Blood Vessels

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Additional information is available at the end of the chapter

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Abstract

Vascular smooth muscle cells (VSMCs) play important roles not only in the physiological functions of the blood vessels, such as vasoconstriction, vasodilatation and extracellular matrix production, but also in the pathogenesis of vascular diseases, particularly atherosclerosis and hypertension. VSMCs are mostly of mesodermal origin, although some are of neuroectodermal origin, for example, VSMCs present in the aorta and in blood vessels arising from the aortic arch. VSMCs of neuroectodermal origin are implicated in defects of cardiovascular morphogenesis, such as bicuspid aortic valve, coarctation of the aorta, patent ductus arteriosus and tetralogy of Fallot. The origin, location in the vascular tree, gender, species, strain and age influence the phenotype of VSMCs and their propensity to migration and growth. In a healthy adult organism, VSMCs have a quiescent and differentiated contractile phenotype characterized by early markers (e.g., SM α-actin, SM22-α), intermediate markers (h-caldesmon, calponin) and late markers (SM myosins, smoothelin) of VSMC differentiation. However, after blood vessel injury, surgery or explantation *in vitro*, VSMCs undergo a phenotypic modulation to synthetic phenotype, which endows them with high activity in migration, growth and proteosynthesis. These features can lead to stenosis or to obliteration of the vascular lumen and impaired blood supply to various tissues and organs.

Keywords: blood vessels, smooth muscle cells, contractile phenotype, synthetic phenotype, phenotypic modulation, vascular diseases, atherosclerosis, hypertension, developmental pathology

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1. Introduction

Vascular smooth muscle cells (VSMCs) are an important component of blood vessels. The cells are located in the medium part of a blood vessel, that is, *tunica media*, where they are oriented in a circle around the vascular lumen and form numerous layers. In medium vessels, there are up to 40 layers of VSMCs, and in large vessels, there are up to 60 layers. As the medium part of the blood vessel wall, the *tunica media* is located between the *tunica intima* and *tunica adventitia*, and is separated from these parts by the *lamina elastica interna* and the *lamina elastica externa*, respectively. The *tunica intima* (also referred to as *tunica interna*) contains a semipermeable monolayer of endothelial cells and forms the luminal part of a blood vessel, contacting the blood. The *tunica adventitia*, that is, the exterior part of a blood vessel (also referred to as *tunica externa*), contains fibroblasts, nerves and, in bigger vessels, also small blood vessels supplying the vascular wall, called *vasa vasorum*. In addition, the *tunica adventitia* anchors blood vessels to the adjacent tissues (**Figure 1**) [1]. This structure is similar in arteries and in veins; in veins, the *tunica media* is usually thinner, due to the lower blood pressure in the venous bed, and the *tunica intima* in some veins contains valves in order to keep blood flowing in a single direction (**Figure 2**) [2]. The smallest blood vessels are capillaries, which lack the three classical layers of a blood vessel wall. They consist only of a fine tubular structure built of endothelial cells, which is surrounded by pericytes, which are somewhat like VSMCs and are phenotypically similar to VSMCs (**Figure 3**) [3].

VSMCs play important roles in the physiological functioning of blood vessels and in pathological changes in them. In healthy blood vessels of an adult organism, VSMCs ensure that the blood vessels contract and relax, and in this way, they make a marked contribution to the regulation of blood circulation. In healthy adult blood vessels, VSMCs are in a quiescent nonproliferative phenotype, referred to as contractile phenotype. This phenotype is characterized by abundant contractile fibers containing VSMC-specific contractile proteins, such as α-isoform of actin and the SM-1 and SM-2 myosin heavy chain isoforms, and other specific proteins

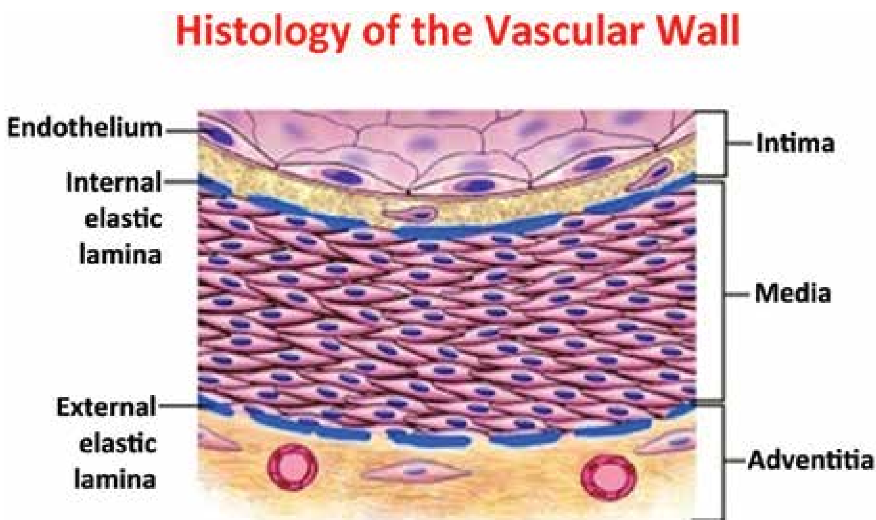

Figure 1. A scheme of the anatomy of an arterial wall. Available from [1].

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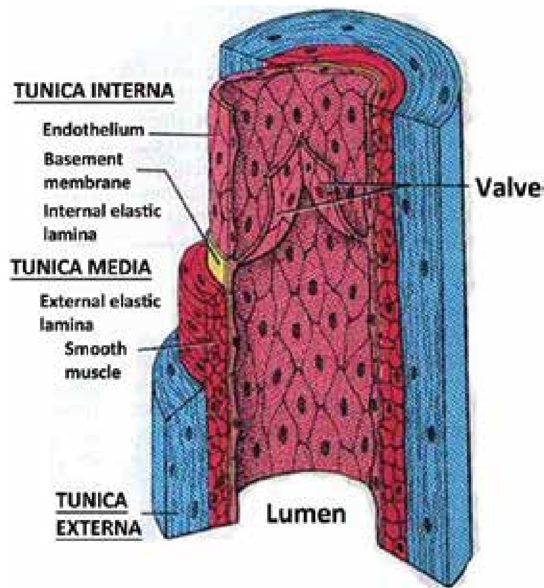

Figure 2. A scheme of the anatomy of a venous wall. Available from [2].

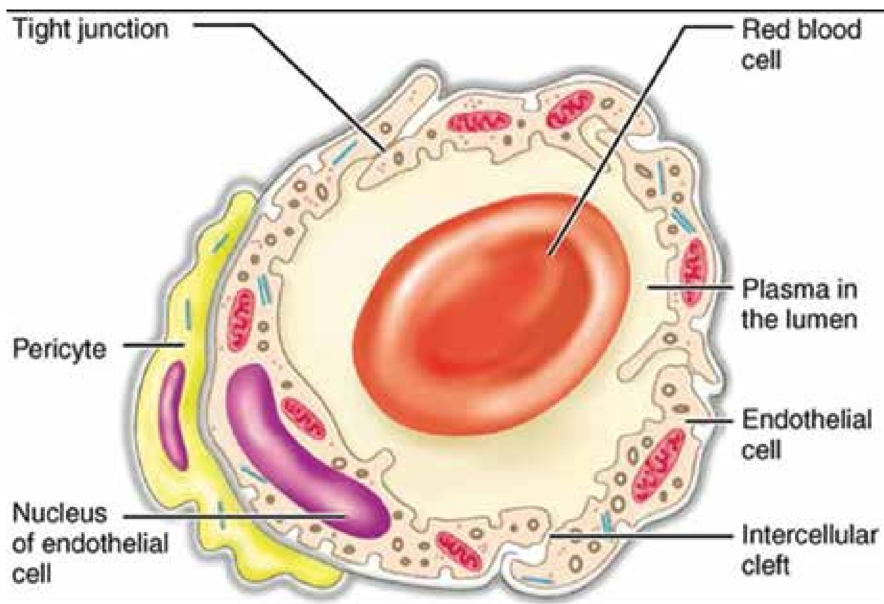

Figure 3. A scheme of the anatomy of a capillary. Available from [3].

associated with the contractile apparatus [4–8]. Under pathological conditions accompanying the onset and development of vascular diseases, VSMCs undergo a process referred to as phenotypic modulation, that is, they switch from the contractile phenotype to the synthetic phenotype, characterized by a loss of contractile filaments and associated molecules, and by increased formation of organelles associated with proteosynthesis (**Figure 4**) [9]. VSMCs of

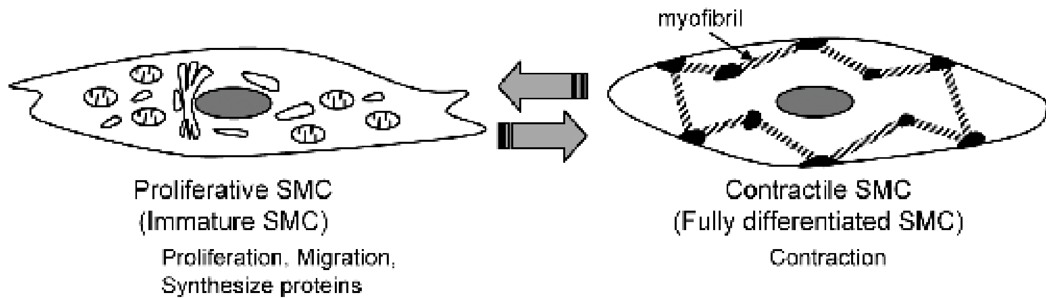

Figure 4. Transition between the contractile and synthetic phenotype in VSMC [9].

synthetic phenotype are active in migration and growth. This can lead to intimal thickening, formation of atherosclerotic plaques, thickening of the blood vessel wall during hypertension, and finally to stenosis or full obliteration of the vascular lumen [4–6, 9–11]. Similar changes in VSMCs also occur after vascular surgery and when VSMCs are cultivated *in vitro*, particularly under conventional static conditions and in standard serum-supplemented media [6, 7, 12].

This chapter summarizes the most important knowledge on the role of VSMCs in the physiological behavior and in pathological alterations of blood vessels, on the contractile and synthetic phenotype of VSMC, and on the implication of these cells in developmental disorders of the cardiovascular system, atherosclerosis and hypertension. This chapter includes the author's own experience in her studies of gender-related differences in the migration and proliferation of VSMCs, and in studying the role of VSMCs in vascular remodeling during hypoxic pulmonary hypertension.

2. Vascular smooth muscle cell origin and its role in vascular pathology

2.1. Sources of VSMCs

The VSMCs arise from two main sources: the mesoderm and the neuroectoderm, that is, the ectoderm of the neural crest. Most of the VSMCs in the vascular tree are of mesodermal origin, and are formed mainly from mesenchyme, that is, a type of connective tissue found mostly during the development of an embryo [13]; or from mesothelium *via* the epithelial-to-mesenchymal transition [14].

VSMCs differentiated from neural crest cells reside in the cardiac outflow tract, the ascending aorta, the aortic arch, the proximal thoracic aorta, the brachiocephalic trunks, the common carotid arteries, the internal and external carotid arteries and subclavian arteries, and also in the blood vessels of the facial structures and the forebrain [15–17]. The pericytes in regions supplied by these vessels are also of neuroectodermal origin [18]. Neural crest cells also differentiate into adventitial fibroblasts. However, the endothelial cells of all the vessels are of mesodermal origin [15, 19]. In addition, the coronary and pulmonary arteries and the descending aorta remain devoid of neuroectodermal VSMCs and contain only mesodermal VSMCs, similarly as the remaining vessels in the body [20].

The factors that regulate the differentiation of neural crest cells into VSMCs include the Notch and Hippo signaling pathways, fibronectin, transforming growth factor-β (TGF-β), Smad2, and myocardin-related transcription factor B.

The Notch plays a critical, cell-autonomous role in the differentiation of neural crest precursors into smooth muscle cells both *in vitro* and *in vivo.* Mutations in components of the Notch signaling pathway result in defects of the cardiac outflow tract [21]. For proper Notch signaling, Hippo signaling is required. Neural crest-specific deletion of the Hippo effectors Yap and Taz produces neural crest precursors that migrate normally, but fails to differentiate into VSMCs [22]. In addition, Notch signaling is regulated by fibronectin 1 (Fn1), which is synthesized by the neural crest cells (NCCs) and mediates the morphogenesis of the aortic arch artery. The Fn1 signals are delivered into NCCs through integrin $\alpha_5\beta_1$ adhesion receptors and lead to the differentiation of NCCs into VSMCs [23].

TGF-β plays a controversial role in VSMC differentiation. On the one hand, TGF-β induces the differentiation of VSMCs from NCCs. This differentiation is mediated by Smad2, a transcription factor which is required for TGF-β-induced nuclear translocation of myocardin-related transcription factor B (MRTFB). MRTFB enhanced the binding of Smad2 to a promoter of the expression of genes encoding differentiation markers of VSMCs [24, 25]. On the other hand, in mature VSMCs of neural crest origin, TGF-β increased the synthesis of DNA, which is known to be associated with a loss of differentiation markers in VSMCs. At the same time, TGF-β inhibited the growth of VSMCs of mesodermal origin. This dual effect of TGF-β was explained by the different composition of TGF-β receptors in VSMCs of different origin. In neuroectodermal VSMCs, subunit II is non-glycosylated, while in mesodermal VSMCs, subunit II of this receptor is fully glycosylated [26].

2.2. The role of VSMC origin in developmental pathology

Proper differentiation of neural crest cells into VSMCs is required for normal cardiovascular morphogenesis. If this differentiation is defective, various cardiovascular disorders can occur, for example, bicuspid aortic valve, coarctation of the aorta, patent ductus arteriosus, tetralogy of Fallot, aneurysm of the thoracic aorta, and intracranial aneurysm.

Bicuspid aortic valve (BAV) is associated with the decreased expression of MYH11, the gene encoding the myosin heavy chain in VSMCs of neural crest origin, impaired contraction of these cells and decreased TGF-β signaling based on phosphorylation of SMAD2. In addition, patients with BAV are at higher risk of developing aneurysms of the thoracic aorta than patients with tricuspid aortic valve [27].

The major role in the pathogenesis of coarctation of the aorta in humans is attributed to deregulation of the Forkhead Box C1 (FOXC1) transcription factor or its downstream genes [28]. FOXC1 is also involved in the pathogenesis of ocular diseases, particularly glaucoma. FOXC1 dysfunction causes disruptions in basement membrane integrity and lower resistance of cells to cell death in response to oxidative stress [29, 30]. In addition, patients with BAV and coarctation of the aorta are more prone to developing an intracranial aneurysm [31].

Patent ductus arteriosus, that is, a temporary fetal vessel that bypasses the lungs by shunting the aortic arch to the pulmonary artery, can result from insufficient proliferation, differentiation

and contractility of a specific smooth muscle subpopulation that shares a common neural crest precursor with cardiovascular melanocytes [32].

Tetralogy of Fallot (TOF) is a serious congenital disorder characterized by a ventricular septal defect, overriding aorta, right ventricular outflow tract obstruction (i.e., pulmonary stenosis) and right ventricular hypertrophy. TOF has a polygenic origin, being caused by a combination of deleterious mutations in genes essential for apoptosis and cell growth, for the assembly of the sarcomere, and also for the neural crest and the secondary heart field, that is, the cellular basis of the right ventricle and its outflow tract. Numerous genetic abnormalities are associated with TOF. They include mutation of the gene encoding myosin binding protein C3 (MYBPC3) [33], mutations and polymorphism of the gene encoding vascular endothelial growth factor (VEGF), which regulates vasculogenesis and angiogenesis [34, 35], mutation of the gene encoding Neuropilin1 (NRP1), a membrane co-receptor of VEGF [36], and mutation of the gene encoding Jagged1 (JAG1, also designated as CD339), that is, a cell surface protein interacting with receptors in the mammalian Notch signaling pathway [37], and also trisomy of chromosome 21, that is, Down's syndrome [33].

3. Vascular smooth muscle cells in healthy adult vessels

In healthy adult blood vessels, the vast majority of VSMCs are in a quiescent non-proliferative phenotype. This phenotype is usually defined as contractile, while the phenotype of proliferating VSMCs has been widely referred to as synthetic. However, the population of quiescent nonproliferating VSMCs is highly heterogeneous, varying dynamically from primarily contractile cells to synthetic cells specialized in extracellular matrix production [38]. In addition, the blood vessel wall contains abundant stem/progenitor cells, which are largely responsible for VSMC accumulation in the intima during vascular remodeling, such as neointimal hyperplasia and arteriosclerosis [10, 39–41]).

The degree of VSMC differentiation can be determined by the level of expression of specific markers of VSMC differentiation at mRNA and protein level. Markers of VSMC differentiation have been divided into early, mid-term and late, according their appearance during embryonic development [42], during restoration of differentiated phenotype of VSMC [7] or during differentiation of stem cells toward VSMCs [43, 44]. For example, during the early stage of differentiation of embryonic stem cell-derived embryoid bodies, SM α-actin is the first to be detected, followed by myocardin, SM22-α and smooth muscle myosin heavy chain (SM-MHC). The expression of SM-α actin, myocardin, SM22-α and SM-MHC was found to begin on day 0, 8, 11, 13, respectively, during early embryonic vascular development [42].

Early markers of VSMC differentiation include SM α-actin, myocardin and SM22-α [7, 43, 45]. Mid-term markers are h-caldesmon and SM-calponin [46–50], although these markers have been designated in other studies as early markers [44]. Late markers are desmin, meta-vinculin, SM-1 and SM-2 isoforms of myosin heavy chain, and smoothelin [7, 43, 44]. Markers of VSMC differentiation, stained by immunofluorescence, are demonstrated in **Figure 5** [8, 51–57].

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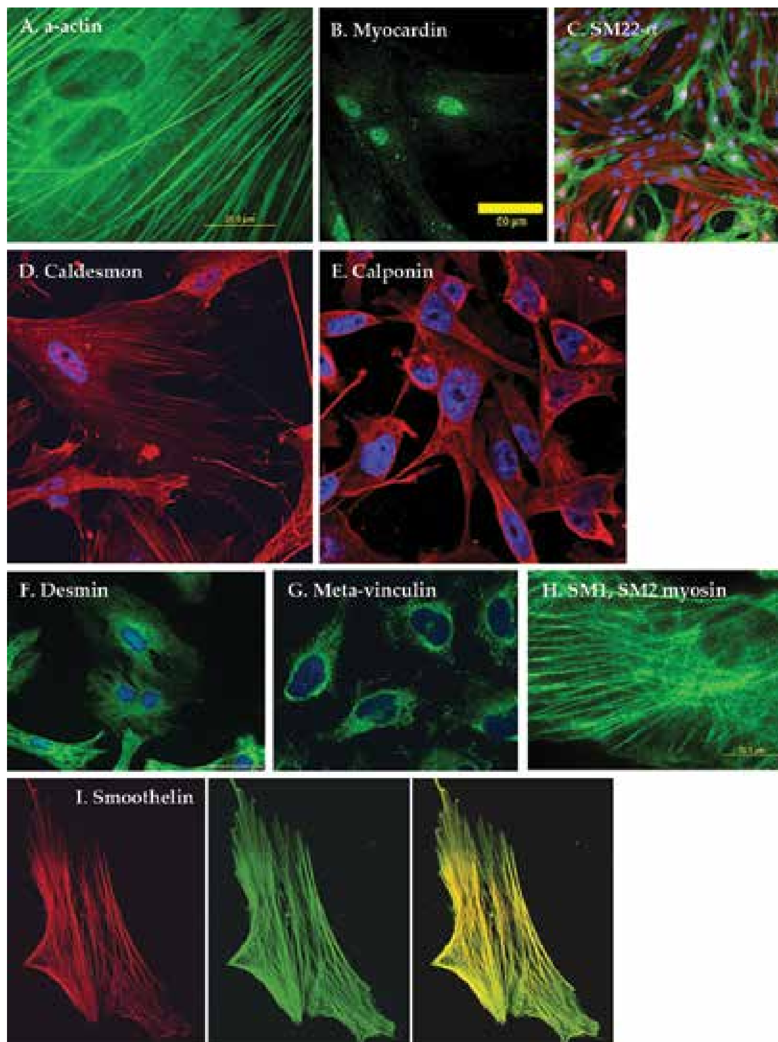

Figure 5. Markers of early (**A–C**), intermediate (**D, E**) and late (**F–I**) differentiation of VSMCs. **A–C, F–H**: Green fluorescence; **D, E, I**: Red fluorescence. I: Co-localization of smoothelin with alpha-actin: Left: Smoothelin, center: α-actin, right: Merge. A: [51], B: [52], C: [53], D: [54], E: [55], F: [56], G [57], H: [51], I: [8].

SM α-actin is the earliest known marker of VSMC differentiation, but its expression alone does not provide definitive evidence for a smooth muscle lineage [43]. SM α-actin is located in thin filaments of VSMCs, together with tropomyosin, troponin and calponin. Alpha-actin is the predominant isoform in the VSMCs of a healthy adult vessel. Other actin isoforms in VSMCs include β- and γ-actin, but they are minority components in adult physiological VSMCs, being localized predominantly in immature VSMCs during development or in dedifferentiated VSMCs in diseased vessels [4, 5, 9, 58].

Myocardin is a transcription factor essential for VSMC-specific differentiation. It is a transcriptional coactivator of the serum response factor involved in cell cycle regulation, apoptosis, cell growth and cell differentiation. Myocardin induces the expression of SM α-actin,

SM22-α, calponin and SM-MHC. Mice lacking myocardin die during embryogenesis from a lack of differentiated VSMCs. During supraphysiological mechanical load, for example during hypertension and prolonged stretching of VSMCs *in vitro*, myocardin is translocated from the nuclei to the cytoplasm and is degraded by the proteasome, which led to phenotypic modulation of VSMCs toward the synthetic and proliferative phenotype [9, 59–62].

SM22-α is an actin-binding protein of the calponin family that is involved in calcium-independent smooth muscle contraction [45, 63]. SM22-α interacts directly and co-localizes with F-actin, and it therefore participates in the organization of the actin cytoskeleton in differentiated VSMCs. SM22-α facilitates the assembly of actin filaments into bundles, enhances the contractility and the mobility of VSMCs, and maintains the contractile phenotype in VSMCs [64]. Disruption of SM22-α is involved in osteochondrogenesis in arterial diseases and also in vascular inflammation [45, 65]. SM22-α attenuated vascular inflammation by suppressing the IKK-IκBα-NF-κB signaling cascades [65].

Caldesmon is a cytoskeletal protein interacting with actin, tropomyosin, myosin, calmodulin and phospholipids. Due to alternative splicing of one gene, calmodulin occurs in two isoforms, namely high molecular weight (89–93 kDa) caldesmon isoforms (h-caldesmon), and low molecular weight (59–63 kDa) caldesmon isoforms (l-caldesmon). H-caldesmon is present in adult and fully differentiated smooth muscle cells, while l-caldesmon is found in non-muscle cells and in de-differentiated smooth muscle cells. H-caldesmon is also a marker of tumors, for example of soft tissue tumors of the skin. All isoforms are potent inhibitors of the actin-tropomyosin activated myosin MgATPase. Smooth muscle caldesmon, together with tropomyosin, is a mediating factor for Ca^{2+}-dependent inhibition of smooth muscle contraction [66, 67].

Calponin is another actin filament-associated regulatory protein expressed in smooth muscle cells and in many types of nonmuscle cells. It occurs in three isoforms, that is, calponin 1, 2 and 3, encoded with three homologous genes, CNN1, CNN2 and CNN3, respectively. All three isoforms inhibit actin-activated myosin ATPase and stabilize the actin cytoskeleton. Calponin 1 is specifically expressed in smooth muscle cells and plays a role in fine-tuning smooth muscle contractility. Similarly as in caldesmon, the interaction of calponin with actin inhibits actomyosin MgATPase activity. Calponin 2 is expressed both in smooth muscle cells and in nonmuscle cells, and it regulates multiple actin cytoskeleton-based functions. Calponin 3 participates in actin cytoskeleton-based activities in embryonic development, myogenesis and neuronal plasticity [68–70]. Another important role of calponin is its tumor-suppressing effect. The levels of calponin 1 and 2 have been found to be decreased in tumor cells, and transfection of these cells with gene encoding calponin 1 reduced their growth and malignancy. The level of calponin 2 in the serum of patients can also be used as a biomarker of tumor diseases, for example, breast cancer [70, 71].

Desmin, together with vimentin, forms intermediate filaments in VSMCs. During the development and specialization of cells toward smooth muscle cells, desmin replaces vimentin as the predominant component of intermediate filaments. Desmin is upregulated during differentiation of VSMCs from stem cells, for example, human bone marrow-derived mesenchymal stem cells (MSCs) [72] and also from embryonic mesothelial cells *via* epithelial-to-mesenchymal transition [14]. Desmin and smooth muscle myosin were expressed together in the cells, and their acquisition appeared indicative of the terminal differentiation of smooth muscle [73]. However, the amount of desmin was found to be much lower in VSMCs than in smooth muscle cells of the digestive, respiratory, and urogenital tract, and also much lower

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than the amount of vimentin in VSMCs [58]. For example, rat aortic smooth muscle cells contain 51% of vimentin alone-positive cells, 48% with both vimentin and desmin and 1% with desmin alone [4].

Meta-vinculin is a high-molecular form of vinculin, that is, a protein of the focal adhesion plaques associated with integrin adhesion receptors in cells [74]. Meta-vinculin and vinculin are co-localized in focal adhesion plaques [75]. Together with SM-MHC, meta-vinculin is considered as a marker of well-differentiated contractile VSMCs [76, 77]). During phenotypic modulation of VSMCs, for example, in venous grafts used as aortocoronary bypasses [75], in human coronary arteries affected by arteriosclerosis [78] or during cultivation of VSMCs [75], the content of meta-vinculin in VSMCs decreases, and meta-vinculin can fully disappear from cells [75]. In cell culture conditions, the content of meta-vinculin in VSMCs can be reestablished when the cells reach confluence [79]. During hypoxic pulmonary hypertension induced in newborn calves, proliferation occurred almost exclusively in the meta-vinculin-negative VSMC population rather than in the VSMC population expressing meta-vinculin [80].

SM-1 and SM-2 isoforms of myosin heavy chain (SM-MHC) are located in thick filaments of fully differentiated VSMCs. In intact adult rat thoracic aorta, the ratio of SM-1:SM-2 is 80:20 both for mRNA and for protein [7]. VSMCs also contain the non-muscle (NM) isoform of myosin heavy chain, which is present, for example, in fibroblasts, macrophages, lymphocytes and platelets [81, 82]. The expression of myosin heavy chain isoforms in VSMCs is highly variable. The same VSMC cell can contain all three isoforms of myosin heavy chain or only SM myosins [7, 83]. This leads to a functional diversity of VSMCs, which have a fast contractile gene program, giving rise to a phasic smooth muscle phenotype, or a slow contractile gene program, giving rise to a tonic smooth muscle phenotype [84]. During vascular diseases, such as atherosclerosis [74], after vascular surgery, for example, coronary angioplasty [85] or after explantation of VSMCs *in vitro* [7], the expression of SM-1 myosin, and particularly of SM-2 myosin, decreases rapidly, while the content of NM myosin increases.

Smoothelin (SMTN) is a cytoskeletal protein present exclusively in contractile smooth muscle cells. The SMTN family of proteins consists of two isoforms of SMTN, namely SMTN-A, SMTN-B, and the SMTN-like protein 1 (SMTNL1). The SMTN-A isoform is located predominantly in visceral smooth muscle cells, while the SMTN-B isoform is located in vascular smooth muscle cells, and SMTNL1 is located in both visceral and vascular smooth muscle cells. SMTN-A and SMTNL1 are associated with the contractile apparatus in VSMCs, and their main function is to increase the contraction potential of VSMCs, to contribute to vascular adaptations in various physiological and pathological conditions, such as pregnancy, exercise training or hypertension and to contribute to the maintenance of the contractile phenotype [86, 87].

4. Vascular smooth muscle cells in vascular pathology

Typical examples of vascular diseases accompanied by vascular remodeling are atherosclerosis and hypertension. These two diseases have several common features, mainly thickening of the vessel wall and narrowing of the vascular lumen, which is due, among other reasons, to the activation of migration, growth and proteosynthesis in VSMCs.

4.1. Atherosclerosis

The onset of atherosclerosis is usually associated with mechanical or biochemical damage to the endothelial cell layer. Mechanical damage occurs during hypertension or during vascular surgery. Biochemical damage is due to the presence of various harmful compounds in the blood, for example, reactive oxygen species (ROS), nicotine or products of nonenzymatic glycation, and also due to elevated concentrations of originally physiological biomolecules, such as glucose, cholesterol or homocysteine. Biochemically damaged endothelium becomes thrombogenic, permeable and immunogenic. Platelets adhering to the endothelium then release platelet-derived growth factor (PDGF), a potent mitogen and chemoattractant for VSMCs [88, 89]. The production of PDGF-like molecules and other growth factors, for example VEGF, is also activated in the VSMCs themselves [90]. Other examples of growth factors that can penetrate into the vascular wall from the blood through the damaged endothelial barrier, and that can stimulate the proliferation of VSMCs, include epidermal growth factor (EGF), fibroblast growth factor (FGF) [91], nerve growth factor (NGF) [92], insulin-like growth factor-1 (IGF-1) [93] and TGF–β [11]. In addition to growth factors, other blood-borne molecules that can penetrate into the blood vessel wall can change the composition of its extracellular matrix (ECM) and can influence the behavior of VSMCs, include albumin, globulins including immunoglobulins, hemoglobin, lipoproteins, fibrinogen, fibrin, thrombin and thrombospondin [94–98].

Damaged endothelium is also prone to inflammatory activation. Endothelial cells increase their expression of adhesion molecules of immunoglobulin and selectin families, namely intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial-leucocyte adhesion molecule-1 (ELAM-1) [6, 11]. These molecules, present on the membrane of endothelial cells, bind the cells of the immune system, namely leucocytes, lymphocytes, monocytes, macrophages and mast cells. These cells are then stimulated by chemotactic factors to migrate inside the vascular wall. The chemotactic factors include interleukins (e.g. interleukin-1), tumor necrosis factors (TNFs, e.g. TNF-α) and monocyte chemoattractant protein-1 (MCP-1), and are produced in VSMCs, endothelial cells and inflammatory cells [11, 99]. In addition, immunoglobulin adhesion molecules, such as VCAM-1, can be expressed directly by VSMCs [100]. Another potent source of inflammatory cells penetrating inside the vascular wall is *tunica adventitia*, particularly its *vasa vasorum* [40, 101]. As concerns infiltration of the vascular wall with inflammatory cells, atherosclerosis has been considered as a specific type of inflammation [4, 102, 103]. In addition, the inflammatory cells produce proteolytic enzymes, such as chymase, tryptase and metalloproteases (MMPs, e.g. MMP-2, MMP-9 and MMP-13), which degrade the ECM and liberate the VSMCs from their proliferation control exerted by ECM [11, 104, 105]. The inflammatory cells also produce ROS, which can cause cell death in high concentrations. In low concentrations, however, they can stimulate phenotypic modulation and proliferation of VSMCs directly or indirectly by damage to the vascular ECM [11, 106]. Macrophages, like VSMCs, can proliferate within the damaged vascular wall [4, 107] and can also store lipids and form so-called foam cells (**Figure 6**) [11].

Under all these pathological conditions, VSMCs in the damaged vascular wall undergo phenotypic modulation, migrate to the *tunica intima* and proliferate. The proliferation of VSMCs can be very massive and can lead to considerable stenosis or to full obliteration of the vascular lumen. For this reason, early investigators of atherogenesis compared the proliferation

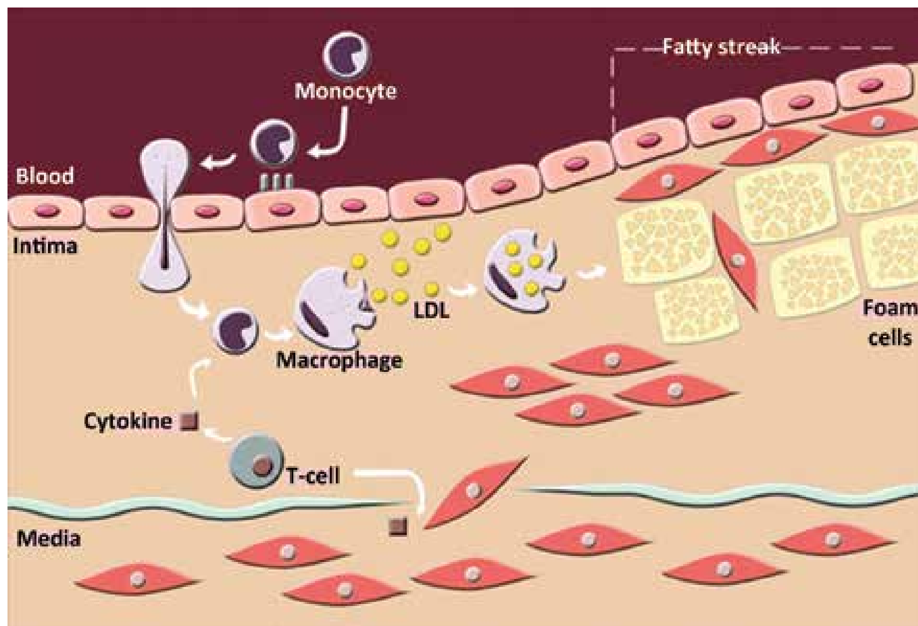

Figure 6. A scheme of the process of atherogenesis [11].

of VSMCs to the growth of tumor cells and considered atherosclerotic plaques as "benign tumors of VSMCs" [108, 109].

During phenotypic modulation, the late markers of VSMC differentiation are the first to be lost, while the early markers can persist even in migrating and proliferating cells. For example, smoothelin-B is the first smooth muscle cell marker that disappears when vascular tissues are compromised, for example, in atherosclerosis or restenosis [8]. Smoothelin was not detected in primary or long-term smooth muscle cell cultures, which simulate the pathological conditions in damaged blood vessels *in vivo* [86]. Similarly, in cultured VSMCs derived from the rat thoracic aorta and grown in 10% serum for 3–5 days to sub-confluence, the expression of SM-1 myosin at mRNA level decreased by 30% and the expression of SM-2 myosin at mRNA level decreased by 80%. At the protein level, SM-1 myosin was detectable at a reduced level in confluent cells, whereas SM-2 myosin was absent in confluent cells. Cultivation of confluent cells in serum-free media, that is under conditions used for restoration of the contractile phenotype, had little or no effect on SM-1 or SM-2 myosins at the mRNA level. In contrast, the level of SM α-actin, the earliest marker of VSMC differentiation, decreased both at the mRNA level and at the protein level. However, it did not fully disappear in these cells, and growth arrest by serum withdrawal or by high cell population density led to renewed SM α-actin expression in these cells [7]. In addition, phenotypically modulated VSMCs synthesize a spectrum of ECM molecules, which is altered quantitatively and qualitatively. For example, these VSMCs synthesize higher amounts of collagen type I, III and V, elastin and glycosaminoglycan, particularly chondroitin sulfate A/C and dermatan sulfate [6, 110]. In addition, phenotypically-modulated VSMCs synthesize ECM molecules, which are typical for osteoblasts and are involved in matrix calcification, for example, osteopontin and osteonectin [111, 112]. These VSMCs also showed increased expression and

DNA-binding activity of a transcription factor named Core binding factor alpha1 (Cbfa1) [111], expression of Runt-related transcription factor-2 (Runx2) [113], and increased expression and activity of alkaline phosphatase, an enzyme involved in matrix mineralization [111].

4.2. Hypertension

Two types of hypertension should be distinguished, namely **systemic hypertension** and **pulmonary hypertension**. Systemic hypertension can be a cause or a consequence of atherosclerosis, while pulmonary hypertension does not depend on the systemic blood pressure and has specific causes of its own, mainly pulmonary hypoxia.

Both types of hypertension include phenotypic modulation and proliferation of VSMCs, that is hyperplasia, which means an increasing number of VSMCs. In addition, **systemic hypertension** is characterized by the hypertrophy of VSMCs, that is an increase in VSMC volume. This hypertrophy is often associated with polyploidy, mainly tetraploidy and, to a lesser extent, also octoploidy. The polyploidization of VSMCs has been attributed to so-called "incomplete growth stimulation," which is sufficient only for DNA synthesis and the onset of mitosis, but not for subsequent karyokinesis and cytokinesis. Incomplete growth stimulation has been attributed to an increased level of contractile agonists, such as angiotensin II, arginine vasopressin (also known as antidiuretic hormone), adrenaline, bradykinin and serotonin, which act as weak mitogens for VSMCs [114, 115]). Incomplete mitosis without karyokinesis and cytokinesis is referred to as endomitosis [116]. If only cytokinesis is absent, binucleated or multinucleated, VSMCs are formed. If only DNA synthesis occurs, without complete mitosis, the process of cell polyploidization is referred to as endoreduplication. The increase in volume and ploidy in VSMCs has often been referred to as "specific hypertrophy," and has been considered by some investigators as a physiological response of VSMCs to mechanical loading during blood circulation, that is, as a certain type of VSMC differentiation. Polyploid VSMCs are more effective in synthesis of contractile proteins and mechanically resistant ECM proteins, and occur even under physiological conditions. For example, the aorta of healthy young rats contains 8–10% of polyploid VSMCs. However, during hypertension and with increasing age, the number of polyploid VSMCs can reach several tens % [114, 116, 117]. For this reason, the polyploidy of VSMCs has been proposed as a biomarker of senescence [118]. In addition, contractile proteins, such as actin and myosin, are not synthesized in proper isoforms typical for differentiated VSMCs. In other words, polyploidy of VSMCs appears to be associated with decreased-to-absent expression of muscle-specific proteins [119]. Accordingly, the maximal force per cross-sectional area generated by the hypertrophic smooth muscle in aorta from hypertensive rats was lower than in normal rat aorta [120]. In addition, increased synthesis of ECM proteins (e.g., collagen III, fibronectin) can lead to increased stiffness of the blood vessel wall, which further worsens the hypertension [115].

The main cause of **pulmonary hypertension** is alveolar hypoxia, which is due to a lower concentration of oxygen in the atmosphere, for example, at high altitudes [121] or in experimental isobaric or hypobaric hypoxic chambers [122–124]. Alveolar hypoxia also occurs during obstruction of airways, for example, during bronchial asthma [125–126] and chronic obstructive pulmonary disease (including pulmonary emphysema) [121, 127, 128], during interstitial fibrosis, which hampers the diffusion of oxygen from the alveoles to the capillaries [121, 128], during sterile and microbial inflammations in lungs [124, 126], during thromboembolism in pulmonary arteries [129] and also during extrapulmonary diseases, such as liver diseases [130] and cardiac diseases [131]).

Pulmonary hypertension leads to phenotypic modulation and hyperplasia of VSMCs [121, 132, 133], but usually not to VSMC polyploidization. Surprisingly, the number of tetraploid VSMCs in pulmonary arteries even decreased, as revealed by flow cytometry of pulmonary arterial medial cells obtained from calves exposed to hypoxia in a hypobaric hypoxic chamber [122]. Tetraploid VSMCs were found in the pulmonary arteries of Eker rats, that is an animal model of somatic mutations in the tuberous sclerosis complex-2 (TSC2) gene [134].

Other factors associated with pulmonary hypertension include vasoconstriction [121, 131], damage to VSMCs by ROS [121], synthesis of ECM [132], degradation of ECM proteins by proteases [104, 105, 123], infiltration of the vascular wall with immunocompetent cells, particularly mast cells [104, 105], and inflammatory activation of VSMCs [132, 133, 135]. In addition, not only VSMCs in the *tunica media*, but also fibroblasts in the *tunica adventitia* migrate, proliferate and undergo inflammatory activation, and they therefore contribute to vascular remodeling [133, 136].

4.3. Other factors influencing the phenotype and proliferation of VSMCs

The propensity of VSMCs to phenotypic modulation and activation of migration and proliferation can also be influenced by their origin, their location in the vascular system, species, strain, breeding conditions, age and gender.

As mentioned earlier, VSMCs are of **neuroectodermal and mesodermal origin**. These two types of VSMCs respond in a different manner to various factors playing roles in the pathogenesis of vascular diseases acquired in adulthood, for example, atherosclerosis. For example, the *tunica media* of the aortic arch composed of VSMCs of neural crest origin calcified significantly earlier than the *tunica media* of the descending aorta composed of mesoderm-derived VSMCs [137]. Fluid shear stress, another factor contributing to the development of vascular diseases, inhibited the proliferation of mesodermal VSMCs but induced the proliferation of neuroectodermal VSMCs by increasing the expression of cyclin D1 (which mediates cell cycle progression from the G1 phase to the DNA-replicative S phase), by downregulating the cell cycle inhibitor p21 and by activating the Akt pathway in a manner dependent on phosphoinositide 3-kinase [138]. In addition, mesodermal VSMCs derived from avian embryonic vessels expressed about 10 times more SM α-actin and tropoelastin than neuroectodermal VSMCs [139].

An example of **regional differences** in VSMC growth is the higher incidence of polyploid VSMCs in the aorta and in other big elastic arteries than in smaller muscular arteries, for example, mesenteric arteries [114, 115]. A possible explanation is the lower mechanical load of big arteries, leading to relatively little damage to the endothelial barrier and lower permeability of this barrier for mitogens from blood. Another explanation is that elastin keeps the VSMCs in a contractile quiescent state, and these VSMCs are therefore less responsive to growth stimulation and undergo incomplete mitosis [140, 141]. Other examples include higher resistance of human VSMCs from the internal mammary artery to dedifferentiation and induction of migration and proliferation in comparison with VSMCs from other arteries, particularly VSMCs from coronary arteries [142], and a higher propensity of human arterial VSMCs than of venous VSMCs to form atherosclerotic lesions [143]. In spite of this, phenotypic alterations to venous VSMCs appear to be critical for the development of primary varicose veins. The VSMCs of varicose veins showed a lower expression of desmuslin, an intermediate filament protein, which

resulted in decreased expression of SM α-actin, SM-MHC and smoothelin, disassembly of actin stress fibers and increased levels of collagen synthesis and MMP-2 expression [144]. Phenotypic modulation, migration and proliferation of VSMC intima also occurs in vein grafts implanted in arterial position, for example, as an aortocoronary bypass. The VSMCs in vein grafts decreased their expression of myocardin, SM-1 and SM-2 myosins [61] and meta-vinculin [74]. Phenotypic modulation and proliferation of VSMCs in vein grafts can be attenuated by a perivascular drug delivery system releasing sirolimus and preventing distension of the vein grafts [145, 146], and also by transduction of VSMCs with microRNA-145-encoding plasmids [61].

However, it should be pointed out that, in addition to regional differences in the VSMC phenotype and growth, the VSMC population of **the same vessel** is highly heterogeneous, containing a wide spectrum of VSMCs varying from primarily contractile phenotype to synthetic cells specialized in extracellular matrix production, and also less-differentiated progenitor cells [10, 38–41].

Species-specific differences have been found, for example, in the expression of isoforms of arginase, that is an enzyme that stimulates VSMC proliferation and collagen deposition, and thus implicated in the vascular damage during atherosclerosis and during systemic and pulmonary hypertension. Specifically, rat VSMCs expressed isoform I of arginase, while human VSMCs expressed only arginase II [147]. Another example is a species-specific difference in VSMC-endothelial cell interaction. In co-culture with endothelial cells, the proliferation of VSMCs derived from human aorta was inhibited, while the proliferation of bovine aortic VSMCs was stimulated [148].

Strain-specific differences in VSMC growth have been studied mainly in rats. It was found that polyploidization of aortic VSMCs was the highest in the Wistar-Kyoto (WKY) strain of rats (i.e., normotensive inbred rats related to spontaneously hypertensive rats, SHR); it was intermediate in SHR (genetically hypertensive rat), and it was lowest in Sprague-Dawley rats (i.e., normotensive outbred rats) and in Fischer rats (i.e., normotensive inbred rats). Nonarterial cells (venous VSMCs and lung cells) from WKY and SHR remained essentially diploid, suggesting that polyploidization is also tissue-specific [149]. At the same time, the proliferation, that is hyperplasia, of VSMCs derived from the aorta of SHR rats was markedly higher than that found in aortic VSMCs from WKY rats [150]. The propensity of VSMCs to migration and proliferation can also be influenced by **breeding conditions**. In our earlier study, the migration and proliferation of cultured aortic VSMC derived from Wistar rats raised under conventional conditions was higher than in cells from Wistar rats raised under specific pathogen-free (SPF) conditions [151].

The phenotype and growth of VSMCs can also be influenced by the **age of the organism**. The proliferation activity of fetal and neonatal VSMCs is higher, and their differentiation status is physiologically lower, than in adult VSMCs [152, 153]. In adult organisms, increased age is associated mainly with negative factors affecting VSMCs, for example, oxidative damage, DNA damage (including telomere attrition), mitochondrial dysfunction, apoptosis, proinflammatory secretory phenotype associated with the loss of VSMC differentiation markers, such as SM α-actin and SM22-α, increased expression of transcription factors Msx2 and Runx2 and of bone morphogenetic protein-2, that is markers of osteoblast transition of VSMCs, and increased sensitivity of β-adrenoceptors, which are implicated in the inhibition of cellular

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proliferation [154–157]. These changes in VSMC behavior caused by donor age can be further magnified by proliferative aging of VSMCs during their cultivation *in vitro* [156].

Gender plays an important role in the propensity of VSMCs to migration and proliferation. It is generally known that estrogens decrease the proliferative activity of VSMCs, while androgens increase it. This is considered as the main cause of the higher incidence of cardiovascular diseases in male organisms. However, VSMCs from males proliferated faster even without the actual presence of sex hormones in the cell culture medium. For example, VSMCs isolated from the thoracic aorta of adult male rats migrated earlier from the explants and proliferated faster than their female counterparts [158, 159]. These differences were enhanced in a serum-free medium [160] and after repeated passaging of VSMCs [161], and were also apparent in newborn rats [162] and in both WKY rats and SHR rats [150]. These differences have been explained by prenatal synthesis of androgens initiated by the expression of specific genes in the SRY locus on the Y chromosome. Among others, androgens increase the sensitivity of VSMCs to adrenergic hormones, which persists throughout life, and also without the actual presence of physiological levels of androgens [163, 164].

5. Conclusion

Vascular smooth muscle cells (VSMCs) are physiological and the most numerous component of the arterial and venous wall, and they ensure vasoconstriction and vasodilatation and other functions, such as synthesis of extracellular matrix. However, VSMCs are also implicated in vascular disorders, such as defects of cardiovascular morphogenesis, atherosclerosis, and systemic and pulmonary hypertension. The VSMCs in healthy adult blood vessels are in the quiescent contractile state, characterized by specific markers of VSMC differentiation namely SM α-actin, myocardin and SM22-α (early markers), h-caldesmon and SM calponin (intermediate markers) and desmin, meta-vinculin, SM-1 and SM-2 isoforms of myosin heavy chain and smoothelin (late markers). However, in pathologically changed blood vessels, VSMCs lose their differentiation markers, activate migration and proliferation and increase proteosynthesis. This VSMC behavior can lead to remodeling of the vascular wall, including stenosis and obliteration of the vascular lumen.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 13

The Dichotomy of Vascular Smooth Muscle Differentiation/De-Differentiation in Health and Disease

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Additional information is available at the end of the chapter

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Abstract

Vascular smooth muscle cells (SMCs) are thought to display cellular plasticity by alternating between a quiescent 'contractile' differentiated phenotype and a proliferative 'synthetic' de-differentiated phenotype in response to induction of distinct developmental pathways or to local micro-environmental cues. This classic de-differentiation and re-programming process is associated with a significant loss in the expression of key SMC differentiation marker genes for a large number of proliferative vascular diseases *in vivo* and in sub-cultured cells *in vitro*. Regarded as essential for vascular regeneration and repair *in vivo*, phenotypic modulation represents a critical target for therapeutic intervention. However, recent evidence now suggests that this process of vascular regeneration may also involve differentiation of resident vascular stem cells and the accumulation of stem cell-derived myogenic, osteochondrogenic and macrophage-like phenotypes within vascular lesions *in vivo* and across sub-cultured SMC cell populations *in vitro*. This review summarises our current knowledge of vascular regeneration, de-differentiation and re-programming of vascular SMCs, and focuses on the accumulating evidence of a putative role for stem cell-derived progeny and the evolving dichotomy of the origin of SMC-like cells during intimal-medial thickening and the progression of arteriosclerotic disease.

Keywords: smooth muscle cells, differentiation, re-programming, vascular stem cells, atherosclerosis, arteriosclerosis, intimal-medial thickening, Cre-LoxP, lineage tracing, epigenetics

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1. Introduction

Vascular smooth muscle cells (SMCs) comprise the medial layer of arterial and venous blood vessels. Through their contraction or relaxation, SMCs control vascular tone and blood flow, thereby playing a fundamental role in the regulation of blood pressure and the delivery of dietary nutrients and oxygen throughout the body [1]. Historically, SMCs are widely reported to have significant cellular plasticity. They are capable of de-differentiation to a more synthetic state and undergo re-programming to myogenic, osteochondrogenic, and macrophage-like phenotypes [2, 3]. This phenotypic switch also occurs when SMCs are cultured *in vitro* [4], and is commonly associated with vascular injury and disease [5], and hypertension [6] *in vivo* that leads to a (re)stenosis of the vessel lumen. In particular, SMCs within arteriosclerotic lesions exhibit proliferative, migratory and extracellular matrix (ECM) secretory capacities, indicative of their phenotypic switch to a de-differentiated phenotype [7]. In diseases such as atherosclerosis, SMCs can also assume a foam cell phenotype, typical of the sub-intimal macrophage-derived foam-like cells following exposure to cholesterol and oxidised LDL *in vitro* [8] and *in vivo* [9]. This display of different phenotypic identities is due to an inherent ability to respond effectively to numerous micro-environmental cues and extracellular and intracellular stimuli [5]. Moreover, this phenotypic switch can occur in response to aberrant signalling inputs and changes in contractile SMC gene expression associated with vascular pathology and requires integration of key transcriptional, metabolic and ultrastructural programs [10]. Indeed, several growth factors [platelet-derived growth factor BB (PDGF-BB), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and transforming growth factor beta 1 (TGF-β1) are important regulators of SMC phenotype in part by modulating autophagic activity [11].

Notwithstanding the 40-year-old phenomenon of SMC de-differentiation and phenotypic switching and the significant evidence of a putative role for de-differentiated SMCs in vascular disease [12, 13], there is now compelling evidence to suggest that lesional cells within the adventitial, medial and (neo)intimal layers of arteriosclerotic vessels may also be derived from resident vascular stem cells following iatrogenic injury in rodent models [14–18] and in human arteriosclerotic tissue [19, 20]. Indeed, recent lineage tracing analysis of genomic marked stem cells supports this contention [18, 21, 22]. There is also evidence that circulating progenitor cells home to sites of vascular injury with subsequent differentiation into various cell lineages [16, 17, 23]. Within the early vasculature, progenitor cells are recruited to an endothelial tube and traverse intermediate stages of phenotypic adaptation during development from embryonic to adult SMCs [24]. These progenitors remain within the vessel wall throughout life with the potential to become synthetic SMC-like cells and other cell types within the local vascular micro-environment of diseased vessels [25].

Therefore, a major driver of phenotypic change (whether de-differentiation/re-programming of differentiated SMCs and/or differentiation of stem cells and the generation of stem-cell derived progeny) is the relative level of various transcriptional and post-transcriptional regulatory effectors of myogenic, vasculogenic, osteochondrogenic and macrophage-like phenotypes [13, 26–28]. This is further influenced by the modulatory role of various microRNAs (miRs) [29], and other lineage-restricted regulatory effectors [30] that impact on SMC differentiation *in vivo* and in cultured cells *in vitro* [31].

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2. Differentiated vascular smooth muscle cells (SMCs)

SMCs provide important structural integrity for stabilisation during embryonic and postnatal development and facilitate the distribution of blood throughout the adult circulation (**Figure 1**) [32]. Differentiated adult contractile SMC express a number of cell-specific contractile genes for this purpose including myosin heavy chain 11 (Myh11), calponin 1 (Cnn1) and alpha actin (Acta2) that encode for proteins critical to regulating vessel diameter dynamically via contraction and relaxation in response to key vasoactive stimuli [13]. They have a low rate of turnover (less than 0.1%) within the normal healthy vessel wall [32] and are closely associated with a dynamic ECM that provides important structure thereby promoting a quiescent, non-migratory phenotype that facilitates cell contraction [33]. SMCs themselves provide all the surrounding matrix proteins (collagen, elastin) required for this structural support. Endothelial cells (ECs) connect to SMCs through myoendothelial junctions (MEJs) that penetrate the internal elastic lamina [34] and facilitate transport of solutes and other molecular mediators between SMC and the overlying endothelium [34].

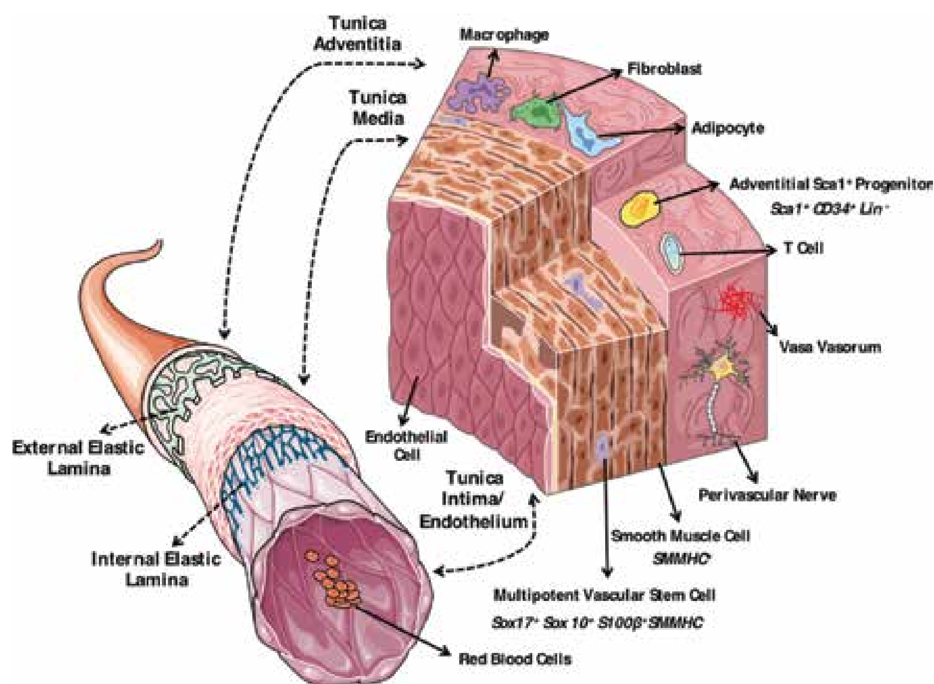

Figure 1. Schematic diagram of a murine blood vessel wall consisting of a tunica intima (endothelial), tunica media (SMCs) and tunica adventitia (fibroblasts) housing differentiated cells in addition to various undifferentiated resident stem cell populations including medial Sox10/Sox17/S100β$^+$ multipotent vascular stem cells (MVSCs) and adventitial Sca1$^+$ progenitors [19, 23].

3. Transcriptional control of SMC differentiation genes

Control of SMC differentiation is a complex process involving the co-operative interaction of many different key factors [10]. Vascular SMC differentiation is determined primarily by serum

response factor (SRF) and myocardin (MYOCD) (**Figure 2**) [36]. They collectively directly promote SMC differentiation through binding to CArG elements and activation of the corresponding SMC differentiation genes. The level and activity of SRF-MYOCD dictates the transcriptional switch for a growing number of SMC differentiation genes [37]. A common feature of these genes is the presence of a 10 base pair cis-element known as a CArG box (consensus is CCW6GG, where W can be either A or T). These CArG sequences are recognised and bound by SRF and many of the genes restricted to SMC are dependent upon SRF activity. SRF recruits a number of coactivators that modulate the binding of CArG-SRF around those SMC genes to maintain cellular homeostasis. MYOCD, a cardiac- and SMC-restricted gene selectively transactivates CArG-containing contractile genes through a physical association with the MADS domain of SRF [38]. SRF-MYOCD is highly active over genes containing multiple CArG elements, yet the presence of multiple CArG sites does not always indicate functionality in SRF-MYOCD [39] as additional coding information in DNA may attenuate SRF-MYOCD transcriptional activation, probably through structural changes in CArG-SRF leading to sub-optimal MYOCD binding. The putative role of MYOCD in dictating SMC phenotype is confirmed by several loss-and-gain-of-function studies that demonstrate any non-SMC type overexpressing MYOCD is converted into a SMC-like state [40]. There are two other related myocardin genes [Myocardin-related transcription factor A (MRTFA) and B (MRTFB)] that have similar SRF-dependent functions and are widely expressed but are under different control processes than MYOCD [41].

In addition to SRF-MYOCD complexes, microRNAs (miRs) represent another class of regulatory effectors of SMC differentiation (**Figure 2**) [40, 42]. These non-coding RNAs function as molecular regulators by lowering protein levels through mRNA degradation, mRNA de-adenylation, or translational repression [43]. Several miRs have been defined in SMCs (miR-143/145, miR-1, miR-21, miR-221, miR-146a, miR-24, and miR-26a) [44] but the major regulatory effector for SMC differentiation is the miR-143/145 bicistronic cluster [31]. The miR-145 targets include the Kruppel-like factor 4 (KLF4) and 5 (KLF5) to direct SMC differentiation and are sufficient to promote myogenic differentiation while reducing the proliferative response to growth factors [31]. In addition, there are several parallel transcriptional

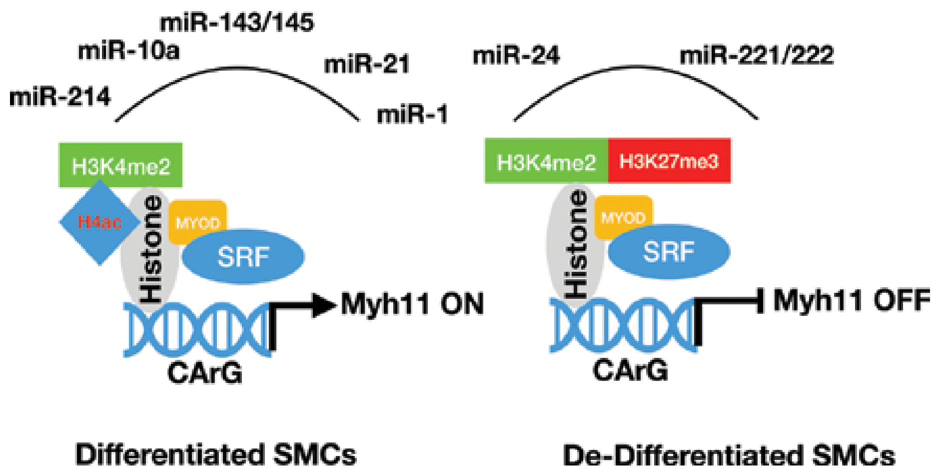

Figure 2. Cartoon summarising the transcriptional regulation of the Myh11 promoter by SRF, MYOCD and miRs at CArG sites in mature SMCs and phenotypically modulated de-differentiated SMCs [35].

(or post-transcriptional) pathways that control SMC differentiation including the histidine-rich calcium-binding protein (HRC) gene that lacks functional CArG elements but contains a conserved binding site for the MEF2 family of transcription factors [45]. Similarly, (Heart And Neural Crest Derivatives Expressed 2 (HAND2), intracellular Notch receptor domains (NICD), SMADs and a large number of zinc finger-containing transcription factors have also been implicated in SRF-MYOCD independent control of SMC-specific genes [42, 46].

4. Signalling pathways that control SMC differentiation

The process of SMC differentiation is dependent on diverse stimuli that include growth factors, ECM, miRs, epigenetic modifiers, and mechanical forces [14]. The most widely recognised stimulators of SMC differentiation are TGF-β1 [47], PDGF-BB [48] and the Notch [49] and Hedgehog [50, 51] signalling pathways. TGF-β1 is a potent multifunctional soluble cytokine that exists in at least three isoforms, TGF-β1, -2 and -3. *In vivo*, loss-and-gain-of function studies clearly associate TGF-β ligands with early embryogenesis, vasculogenesis, angiogenesis, haematopoiesis and cell adhesion [47]. TGF-β signalling can also cause nonhereditary disorders like atherosclerosis and cardiac fibrosis [47]. *In vitro*, TGF-β1 promotes vascular myogenic differentiation of embryonic stem cells (ESCs) and the maturation of mural cells by positive regulation of SMC differentiation genes through the Smad2 and Smad3 dependent pathways and the Notch signalling pathway [52–54]. PDGF is another key modulator of SMC differentiation. It exists in five isoforms (PDGF-A, PDGF-B, PDGF-C, PDGF-D and PDGF-AB homo- or hetero-dimmer including PDGF-AA, PDGF-BB and PDGF-AB), and is mainly derived from platelets upon activation. PDGF-BB transduces its signal via specific tyrosine kinase receptors, PDGFR-α and PDGFR-β and acts as a potent mitogen. PDGF-BB promotes myogenic differentiation of stem cell antigen 1 $(Sca1)^+$ progenitor stem cells via PDGFR-β-mediated signalling [23]. Both TGF-β1 and PDGF-BB can also have a negative effect on SMC differentiation by the repression of SMC markers [48, 55]. These effects are cell density dependent and are mediated by Smad3 and ETS1, respectively.

Notch and hedgehog ligands are integral to SMC differentiation and arterial identity during development [56, 57]. Notch signalling can either promote [52, 53] or inhibit [54, 58] SMC differentiation depending on the origin of the SMC and/or progenitor [59]. Although the precise mechanism(s) of Jagged-1/Notch-induced SMC differentiation is still poorly understood, a number of studies have systematically investigated the molecular pathways leading to the pro-differentiation and pro-proliferative effects of Notch signalling in SMCs [49, 60]. In a similar manner, Hedgehog signalling promotes SMC growth via a Notch-dependent mechanism [51], while hedgehog ligands promote SMC differentiation of SMC progenitors [50, 55, 61, 62].

5. Epigenetic control of SMC differentiation genes

Epigenetics refers to heritable changes in gene expression that occur independent of changes in the genomic sequence due to environmental influences [63]. Epigenetic mechanisms play an important role in the regulation of chromatin structure and remodelling during SMC

differentiation (**Figure 3**). Chromatin is composed primarily of genomic DNA and protein. The nucleosome is the fundamental unit of chromatin encompassing 146 base pairs of DNA wrapped around an octamer of histone proteins. This octamer contains two copies each of histones H2A, H2B, H3, and H4. The histone N-terminal tails are not bound to the nucleosome core and may undergo post-translational modifications including acetylation, phosphorylation, ubiquitination, and ADP-ribosylation [63]. Post-translational modifications of histone proteins alter chromatin conformation and thereby control the manner in which key transcription factors bind DNA, resulting in the activation or silencing of gene transcription. Two of the most extensively studied epigenetic changes during SMC differentiation are histone modifications (which alter the packaging of the chromatin) and DNA methylation (occurring at the 5′-cytosine in CpG dinucleotides).

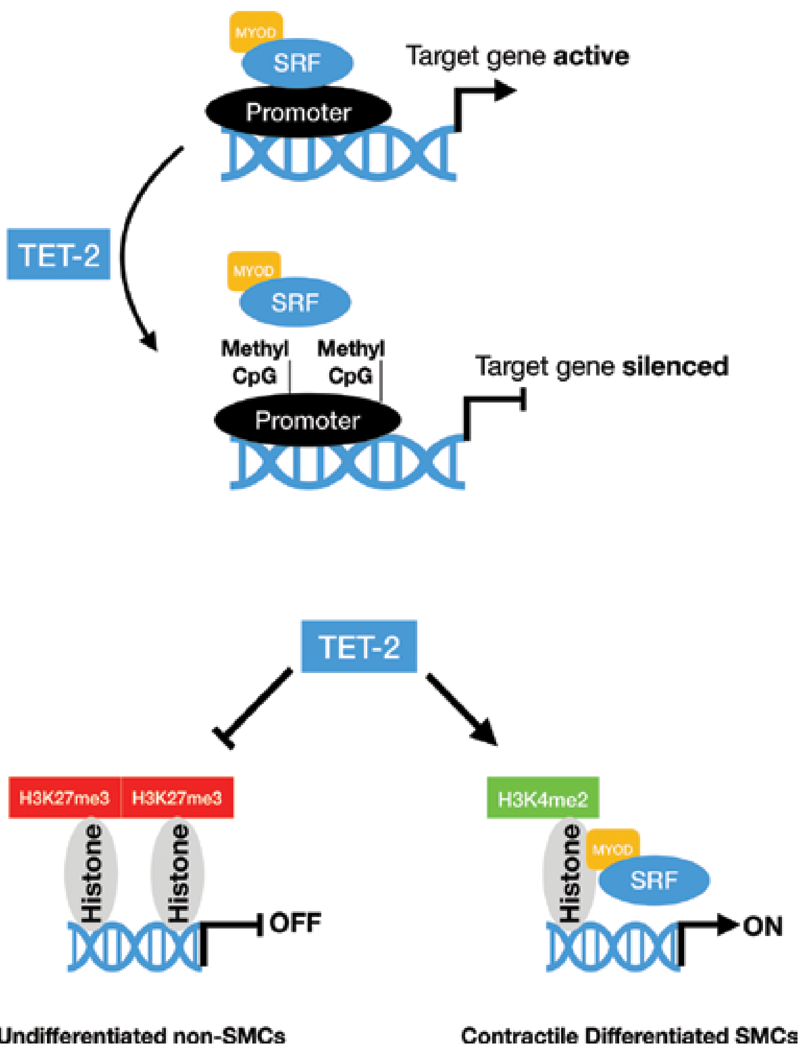

Figure 3. Cartoon summarising the epigenetic regulation (histone modifications and demethylation of DNA) of SMC promoters at CArG sites in mature SMCs and undifferentiated non-SMCs [10].

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Histone modifications alter the higher order chromatin structure through attraction or repulsion of charged histone tails thereby regulating nucleosome density and the accessibility of various cis promoter–enhancer control elements. For instance, changes in chromatin accessibility, mediated in part by histone modifications, render SMC marker genes permissive for subsequent activation by the SRF/myocardin complex during SMC differentiation [64]. Histone acetylation is a potent mechanism of gene activation and occurs on Myh11 and Acta2 gene loci early in the process of myogenic differentiation from SMC precursors. SRF binds only to the SMC marker genes that have been enriched in histone acetylation. Moreover, inhibition of histone acetyl transferases (HATs) or expression of histone deacetylases (HDACs) leads to decreased SMC marker gene promoter activity in cultured SMCs [58, 65]. In addition, there is also a marked enrichment of the histone modification, di-methylation of lysine 4 on H3 (H3K4me2), on key SMC marker genes, including Myh11, Acta2, and Transgelin (Sm22α), in both mature SMC [66] and more notably SMC progenitor cells committed to myogenic differentiation [60]. The methylation of H3K4 is partially attributed to the recruitment of WD repeat-containing protein 5 (WDR5) and the associated histone lysine methyltransferase SET/MLL by paired-like homeodomain transcription factor 2 (Pitx2) to SMC promoters in early stages of differentiation [67]. In contrast, this enrichment was absent in ESCs and non-SMC cells [66]. Moreover, H3K4me2 enrichment may occur in the absence of binding of SRF to CArG elements facilitating a tethering of MYOCD to H3K4me2-modified histone tails that stabilise binding of the SRF–MYOCD to CArG regions (**Figure 3**) [66]. A novel *in vivo* assay combining in situ hybridization and a proximity ligation assay provides further evidence that the H3K4me2 mark at the Myh11 locus is restricted to differentiated SMCs *in vivo* [68]. Similarly, a histone H3 lysine 4 mono-methylation (H3K4me1) catalysed by the Set7 lysine methyltransferase is considered a further hallmark of transcriptionally active chromatin [69]. Recent transcriptional network analyses has revealed that SMC differentiation genes are also subject to Set7-mediated regulation [69]. Hence, several cell-specific epigenetic mechanisms govern the expression of cellular markers during SMC differentiation.

DNA methylation is the most widely characterised epigenetic modification linked to gene silencing [70]. In mammalian cells, DNA methylation occurs at the 5′ position of the cytosine ring through the actions of the DNA methyltransferases DNMT1, DNMT3A and DNMT3B [70]. Several *in vivo* and *in vitro* studies have reported a role for DNA methylation and gene silencing of some key SMC differentiation genes during disease progression and changes in SMC phenotype. However, more evidence is required to determine whether DNA methylation directly plays a causal role in this process (**Figure 3**) [71]. Nevertheless, the mechanisms of DNA demethylation, albeit controversial have recently been described during SMC differentiation and centre on the putative role of the ten-eleven-translocation (TET) family of enzymes that oxidise 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) in SMC [72]. Through the DNA repair pathway and thymine-DNA glycosylase (TDG), 5hmC is then converted to unmethylated cytosine, leading to DNA demethylation and gene activation. TET-2 is the predominant isoform in human coronary artery SMC cultures, and is highly enriched in smooth muscle tissues. TET-2 expression increased following myogenic differentiation and was significantly reduced following PDGF-BB-stimulated de-differentiation. Parallel loss-and-gain-of-function studies confirm the putative role TET-2 induction of SMC differentiation genes (MYOCD, Myh11, Acta2, and Sm22α) by modifying histone methylation

(H3K4me2) contributing to regulation of these genes [72]. Notably, while 5hmC was initially thought to serve only as a transient intermediate in the process of demethylation [73], this epigenetic mark may persist in quiescent, differentiated SMC *in vivo* to play a role in self-renewal and lineage commitment [71].

6. De-differentiated vascular smooth muscle cells (SMCs)

When vascular regeneration occurs after iatrogenic injury, de-differentiated SMCs are thought to promote Intimal-medial thickening (IMT) and participate in the formation of neointima by decreasing the expression of contractile proteins and increasing proliferation, migration and matrix protein synthesis [74, 75]. Similarly, during various disease states such as arteriosclerosis and atherosclerosis, the recruited SMCs also acquire a synthetic de-differentiated phenotype in the course of lesion formation [12]. Indeed, IMT is present in human arteries before atherosclerosis develops, particularly in the atherosclerosis-prone arteries such as coronary arteries and aorta [76].

The paradigm of de-differentiation, phenotypic switching and re-programming of SMCs was first proposed to explain the phenotypic changes that occur when differentiated contractile medial SMCs are isolated and grown in culture [2, 3].

PDGF-BB is considered the major stimulus for SMC de-differentiation *in vitro* [48]. Sub-cultured SMCs from a variety of species lose their expression of SMC differentiation markers (MYH11, CNN1 and SM22α) and acquire an extensive rough endoplasmic reticulum/Golgi system to facilitate SMC migration and proliferation concomitant with an increase in cytoskeletal proteins such as non-muscle myosin (Myh10) and vinculin [2]. Decreases in microRNAs such as miR-143/145 can also occur resulting in a less contractile phenotype [31]. The expression of MYOCD is further consistently reduced in several *in vitro* models of cultured SMC when compared to fresh aortic tissue [36] while ectopic expression of MYOCD results in partial recovery of the SMC differentiated phenotype in phenotypically modified SMCs [77]. SMC phenotypic switching is therefore primarily a function of reduced MYOCD expression with little understanding of the molecular mechanisms underlying this phenomena *in vitro* or *in vivo*.

Epigenetically, SMCs in culture have been extensively characterised following treatment with PDGF-BB and other stimulators of SMC de-differentiation [10]. Although the acetylation of histones is diminished during the de-differentiation process, the H3K4me2 epigenetic mark at the Myh11 locus persists through SMC phenotypic modulation [68], albeit at a lower level when compared to fresh aorta [60], concomitant with reduced expression of the SMC differentiation marker, Myh11 (**Figure 3**) [60]. Importantly, the maintenance of this epigenetic mark in cultured SMCs *in vitro* has been widely used to purport that SMCs in culture are derived from a differentiated parent population *ex vivo* [68].

Another important factor that dictates SMC phenotypic switching is the transcription factor KLF4. KLF4 factors are expressed in phenotypically modulated SMCs and bind to G/C-rich cis elements found in SMC marker gene promoters. KLF4 is not expressed in differentiated SMCs in normal blood vessels but is rapidly induced following vascular injury [78]. The effects of KLF4 in suppressing SMC differentiation marker gene expression are mediated through

epigenetic changes associated with transcriptional silencing, including reduced H4 acetylation mediated through KLF4-dependent recruitment of HDAC2 and HDAC5, and nearly complete loss of SRF binding to the SMC promoter CArG elements within intact chromatin. PDGF-BB mediates compaction of chromatin at SMC differentiation gene loci through KLF4 recruitment of HDAC2, HDAC4, or HDAC5 to CArG regions on the Acta2 and Myh11 promoters, thereby reducing histone acetylation and inhibiting the accessibility of this region to the transcription factors MYOCD, SRF, and MRTF [48, 66]. Overexpression of KLF4 in cultured SMCs also results in profound activation of multiple induced pluripotential stem (iPS) cell pluripotency factors, including the POU domain transcription factor, Oct4 and the transcription factor, SRY (sex determining region Y)-box 2 (Sox2), but not Nanog, suggesting that SMC phenotypic switching involves the activation of multiple pluripotency genes in addition to KLF4 [79].

Differentiated medial SMCs also undergo dramatic phenotypic changes following vascular injury comparable to the changes observed in sub-cultured SMCs (e.g., reduced SMC differentiation marker expression) [2, 3, 80]. Following injury, the cytoskeleton becomes perturbed resulting in defective organisation of cytoskeletal-contractile proteins, SMC apoptosis and the release of SMC- and matrix-associated growth factors concomitant with plasma- and platelet-derived factors that impact on the surviving SMCs [81]. Platelet-derived factors (e.g., PDGF-BB) are considered crucial to SMC phenotypic switching and re-programming of differentiated SMCs *in vivo* through binding and activation of surface receptors present on SMCs [35]. These structural, molecular and physiological changes in medial SMCs are considered pivotal to the development of neointimal lesions following vascular injury.

It is clear that SRF levels do not significantly change in SMCs following arterial injury but reflect an association with different coactivators (such as the ETS domain-containing protein ELK1) that direct new programmes of immediate early gene expression (e.g., c-fos, jun) [42, 82]. These genes are not normally expressed in differentiated SMCs as SRF is bound to MYOCD but result in the secondary activation of delayed response genes, including growth factors that act in an autocrine/intracrine fashion to stimulate SMC cell cycle entry and migration [82]. The significant decrease in SMC differentiation marker gene expression following vascular injury results from a decrease in MYOCD and miR-145 both of which are reduced following vascular injury [83, 84]. This change in MYOCD stabilises ELK1 binding to SRF and is thought to facilitate SRF binding to a different set of CArG-dependent genes that promote phenotypic switching of the remaining medial differentiated SMCs following injury. Thus, SRF in neointimal de-differentiated cells may engage a new set of CArG-dependent genes different from those in medial SMCs [42]. Indeed, over-expression of MYOCD mitigates against SMC phenotypic switching following vascular injury [77]. Similarly, KLF4 mediates its effects at least in part by inducing epigenetic changes of SMC marker gene loci associated with the formation of heterochromatin and transcriptional silencing [10]. Moreover, gene expression profiling of differentiated SMCs versus neointimal 'de-differentiated' cells has revealed distinct molecular phenotypes between these two cell populations [85]. In particular, a subset of differentiated SMCs is thought to revert and re-programme to a more primitive phenotype characterised by the expression of so-called 'embryonic' genes (e.g., tropoelastin, osteopontin, PDGF-BB) that promote growth and migration of SMCs typical of intimal medial thickening [85, 86].

Vascular injury-induced de-differentiation *in vivo* is accompanied by significant changes in the epigenetic profile of these cells. Specifically, there is a decrease in H3 acetylation at the Sm22α

promoter concomitant with the binding of a complex consisting of KLF4, ELK1, and HDAC2 [87]. This complex was contingent on a G/C repressor element found in many CArG-dependent SMC genes. Similarly, a transient decrease in H4 acetylation has been reported at the Acta2 and Myh11 promoters following injury [66]. The importance of this epigenetic change is confirmed using HDACs inhibitors that attenuated SMC dedifferentiation and neointima formation following injury [88]. A further common feature of SMC phenotypic switching following vascular injury *in vivo* in murine models is the persistence of H3K4me2 mark on specific SMC gene promoter loci despite silencing of these genes following injury due to the loss of SRF-MYOCD binding, the formation of heterochromatin, and the loss of H3/H4 hyper-acetylation [10]. Recent studies have since confirmed that neointimal cells from human lesions retain the H3K4me2 mark at the Myh11 promoter suggesting that these cells may also be derived from a differentiated medial SMC that re-programmed following phenotypic switching [68]. This raises the possibility, if true, that H3K4me2 may serve as a mechanism of epigenetic cell lineage memory, i.e., a mechanism for phenotypically modulated SMCs to remain permissive for de-differentiation and re-programming during reversible phenotypic switching [89].

Indirect evidence from several groups has strongly supported the apparent contribution of mature differentiated SMCs that undergo phenotypic switching during the progression of IMT and arteriosclerotic lesions, including neointima formation after endothelial injury, vein graft arteriosclerosis and native atherosclerosis [10]. The more compelling recent lineage tracing studies using tamoxifen-inducible Myh11-CreER mice to mark Myh11 differentiated SMCs before injury have provided further evidence of SMC phenotypic switching and re-programming *in vivo* [7, 90, 91]. These data are consistent with the longstanding view that differentiated SMCs undergo injury-induced SMC phenotypic switching with onset of cell proliferation. However, many cells not of SMC origin have also been identified within atherosclerotic lesion [9].

Collectively, these data suggest that SMCs may acquire mechanisms that reactivate certain pluripotency gene networks as a means of increasing their cellular plasticity and enhancing regenerative processes critical for survival following injury [10].

7. The role of resident vascular stem cells in intimal-medial thickening (IMT)

The accumulation of 'de-differentiated' SMC-like cells within the intima was initially proposed as a key event in the development of arteriosclerosis [92]. As outlined above, compelling support for this view comes from recent lineage tracing studies of genomic marked Myh11 differentiated SMCs using Cre-LoxP transgenic animals (**Figure 4**) [7, 68, 90, 91]. A discrete subpopulation of Myh11 medial SMCs appear responsible for the vast majority of neointimal cells within the vessel wall following injury or disease [7]. If correct, the existence of such a subpopulation is to be expected since the majority (>70%) of medial cells are lost by apoptosis following mechanical-induced injury [93, 94]. This early apoptotic response is considered vital for the progression of IMT since inhibition of SMC apoptosis results in a significant reduction in IMT, independent of re-endothelialization [95].

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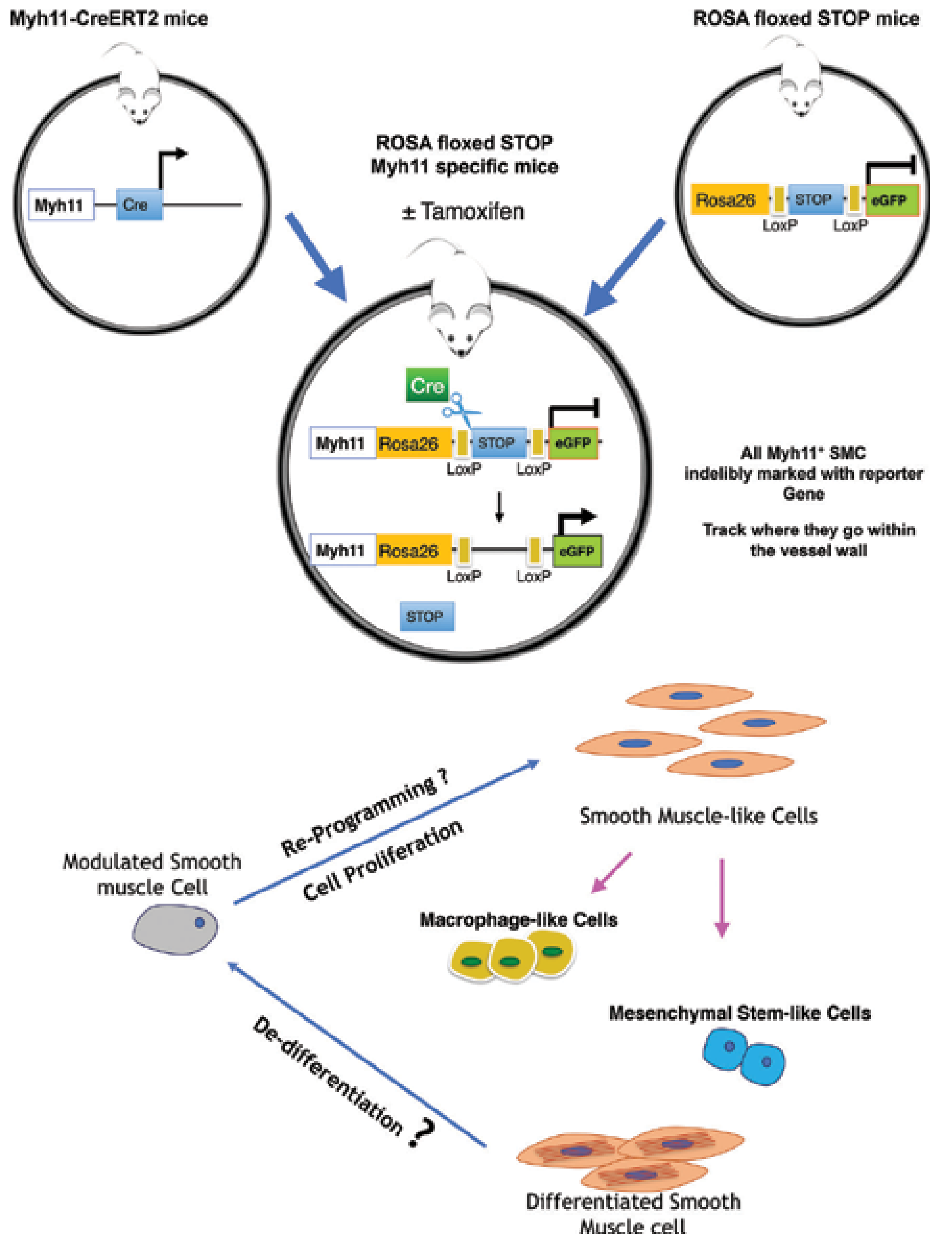

Figure 4. Cartoon summarising lineage tracing analysis of Myh11 marked differentiated SMCs using inducible Myh11-CreERT2-Rosa26-eGFP transgenic reporter mice in support of de-differentiation of mature SMCs and the paradigm of de-differentiation and phenotypic switching of SMCs *in vivo* to explain IMT [7, 91].

A key unanswered question is whether it is solely the differentiated subpopulation of SMC within the vessel, or whether there is also a contribution from resident vascular stem cells? If the latter, resident vascular stem cell niche(s) could give rise to alternative cell types during IMT and arteriosclerotic disease progression, and in doing so re-programme chromatin architecture

to facilitate the appearance of new cell lineages [89]. This stem cell hypothesis remained speculative due to the absence of robust genetic fate mapping data. However, recent studies whilst controversial, provide compelling evidence that the mobilisation and recruitment of

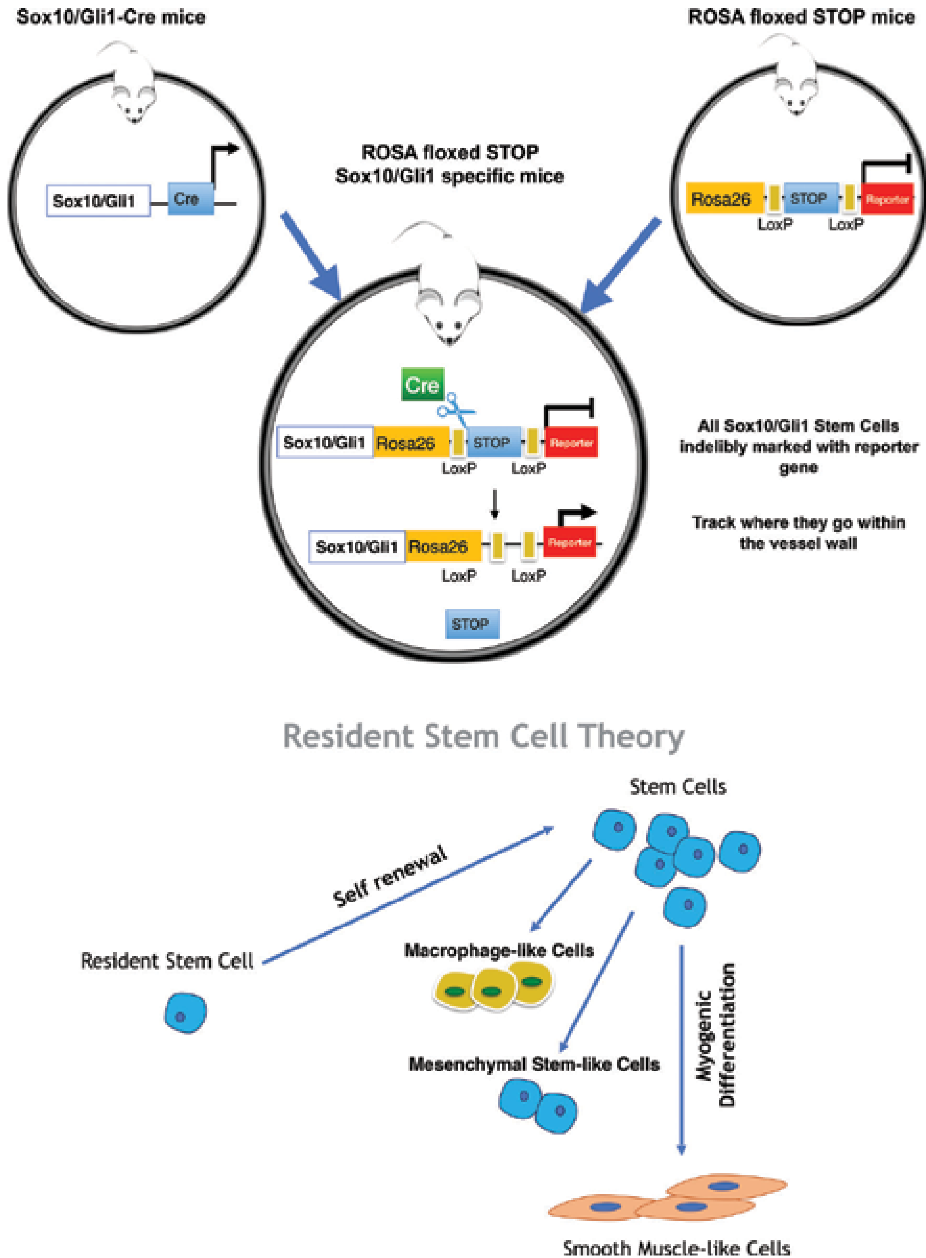

Figure 5. Cartoon summarising lineage tracing analysis of Sox10 and Gli1 marked stem cells using constitutive Cre-LoxP transgenic reporter mice in support of stem cell derived myogenic progeny and the paradigm of myogenic differentiation of resident vascular stem cells *in vivo* to explain IMT [18, 21, 22].

stem/progenitor cells present within the vessel wall and accumulation of their progeny also significantly contribute to IMT and vascular remodelling (**Figure 5**) [18, 19, 21, 22].

Initial transplant studies using green fluorescent protein (GFP) bone-marrow (BM) from GFP transgenic mice addressed the potential role of circulating progenitors and the contribution of BM-derived SMC-like cells to lesion formation [96]. Further human transplant studies demonstrate that SMC-like cells of donor origin enrich in coronary atherosclerotic plaques when compared with the healthy controls. These data initially supported the contention a circulating progenitor origin for neointimal cells in primary atherosclerosis [97]. However, subsequent studies using apolipoprotein E (ApoE)$^{-/-}$ mice transplanted with BM from Myh11–Cre/ROSA26R/ApoE$^{-/-}$ transgenic mice clearly demonstrated that very few neointimal SMC-like cells of atherosclerotic lesions were marked as Myh11 positive, thereby ruling out a BM origin for neointimal SMC-like cells at least in atherosclerotic lesions [98].

Meanwhile, several studies have since demonstrated the presence of multipotent vascular stem cells within the normal vessel wall [19, 23, 99–104] and the appearance of discrete stem cell markers, stem cell antigen-1 (Sca1), SRY-related HMG-box 10 (Sox10), SRY-box 17 (Sox17), S100 calcium binding protein B (S100β), and haematopoietic cell E- And L-Selectin ligand (CD44) on neointimal and medial cells *in vivo* following vascular injury and IMT [18, 19, 105], and in human lesions [106]. Moreover, sub-cultured SMC express these same stem cell markers *in vitro* [107, 108]. While de-differentiation and re-programming of SMCs to a more plastic phenotype could account for some of these observations, recent lineage tracing studies that selectively genomic marked resident vascular stem cells provide compelling evidence in support of the 'stem cell hypothesis' [18, 21, 22].

8. The role of adventitial stem cells

The outermost connective tissue surrounding blood vessels is called the adventitia [109]. It contributes significantly to a variety of disease pathologies, including IMT, atherosclerosis and restenosis [109, 110]. In 2004, Hu et al., were first to describe Sca1, CD117/stem cell factor receptor (c-kit), CD34 and Flk1 vascular progenitor cells within the adventitia, particularly in the region of the aortic root, that differentiate into SMCs and participate in lesion formation in vein grafts [23]. Subsequent studies identified resident vascular progenitor cells in the border zone of adventitia and media in human arteries and veins (**Figure 1**) [99, 102]. Adventitial Sca1 stem cells differentiate into other types of cells participating in vascular lesions, including osteogenic progeny [111] and macrophage colony-forming units [112]. More recent studies using lineage tracing analysis have suggested that these adventitial Sca1 cells are derived from Myh11 genomic marked differentiated SMCs and are major contributors to adventitial remodelling [113]. Indeed, the generation of adventitial vascular progenitor cells from differentiated SMCs may be a normal physiological process that contributes to the vascular stem cell pool and plays an important role in arterial homeostasis and disease [113]. The vasa vasorum and surrounding connective tissue in adult thoracic aortic adventitia is considered a niche for these progenitor cell populations in human vessels that facilitates their role as myogenic progenitors with the potential for multi-lineage progression in atherosclerosis and

IMT following injury [20]. Importantly, recent lineage tracing studies that genomic mark and track these adventitial cells following iatrogenic injury using Gli1-Cre transgenic mice provide compelling evidence for their specific role in IMT and neointimal formation in mice [21, 22]. These studies clearly demonstrate that Sca1, CD105, CD29, and CD34 positive adventitial progenitors cells contribute to neointima formation after acute femoral artery injury and support the idea that resident perivascular mesenchymal stem cell (MSC)-like cells represent a major source of SMC-like cells in vascular lesions [21, 22].

9. The role of medial stem cells

The presence of resident vascular stem cells within the medial layer of the vessel wall has also been recently established (**Figure 1**). These cells are Sca1, c-kit(−/low) Lin-CD34(−/low) and undergo myogenic (SMC) and vasculogenic (EC) differentiation *in vitro* in response to PDGF-BB/TGF-β1 and VEGF, respectively [104]. Similarly, Sca1, Oct4, Stro-1 and Notch-1 positive mesenchymal-like stem cells have been reported that lack haematopoietic or endothelial markers but exhibit myogenic, adipogenic and chondrogenic potential [103]. In 2012, Tang et al., reported on the existence of a Myh11 negative, Sox10, Sox17, S100β, nestin (Nes) positive neuroectodermal medial stem cell population in various human and rodent vessels that may give rise to the majority of SMC-like cells within lesions following injury [19]. Sox10 is expressed in neural crest stem cells during embryonic development and controls their multipotency [114]. Moreover, in normal vessels, Sox10 stem cells are sparse and primarily located within the medial and adventitial layer [115]. Using Myh11-Cre transgenic mice to mark and track medial SMCs, these lineage tracing studies purported that medial SMCs are in fact terminally differentiated and incapable of phenotypic transition during vascular injury and disease [19]. However, these studies proved controversial since they relied on tracking a Myh11 negative population that might represent failed cre-mediated recombination, silencing of the lineage tracing gene, and/or technical loss of the reporter marker [116]. In addition, since isolation of these cells required complete removal of the adventitia prior to medial explant, it is possible that residual adventitial Sox10 cells remained on the external elastic lamina (EEL) and migrate from medial explants during culture. Nevertheless, follow-up lineage tracing analysis using Sox10-cre/Rosa-loxP-LacZ mice supported their original conclusion and confirmed that a resident $Sox10^{+}$ multipotent vascular stem cell (whether from the medial or adventitial layer) is an important source of SMC-like cells during IMT following iatrogenic injury [18].

10. The evolving dichotomy about the origin of Neointimal cells

Numerous research groups, some mentioned above, have attempted to define the specific cell population that gives rise to IMT and the progression of arteriosclerotic disease [117]. Data in support of the classic theory of SMC de-differentiation and re-programming has provided compelling evidence that neointimal cells are derived from a discrete subpopulation of medial differentiated SMCs; those studies employed robust lineage tracing analysis [7, 91], clonal SMC expansion in aggregation chimeras [118], and *in situ* epigenetic profiling of the

stable SMC epigenetic mark, H3K4me2 [9, 68]. However, parallel lineage analysis of marked resident vascular stem cells has clearly demonstrated that adventitial and/or medial progenitor stem cells also play a significant contributory role [18, 21, 22]. Given the innate heterogeneity of medial SMCs in culture, it is not surprising that there exists such a dichotomy. Although significant progress has been made in understanding the molecular mechanisms and signalling pathways that dictate myogenic differentiation of stem cells into SMCs [59], a key issue of molecular switching of differentiated SMCs has yet to be fully established because most of the molecular analysis that controls SMC differentiation was performed on stem cells and/or sub-cultured SMCs *in vitro* [89]. This may explain some of the controversy about the origin of neointimal cells since the acute changes in phenotype of freshly isolated SMCs over time and the fate of genomic marked medial SMCs in culture as models of SMC de-differentiation have both been recently investigated [4, 18].

Sandison and colleagues tracked native medial SMCs continuously post isolation for up to 4 days in culture using time-lapse imaging to determine if de-differentiation and phenotypic switching can give rise to different functional behaviours of SMCs *in vitro* [4]. Their studies indicate that differentiated SMCs are capable of altered functional behaviour by acutely converting from a contractile phenotype to a migratory one capable of phagocytosis [4]. However, when genomic marked differentiated medial SMCs from Myh11-Cre/Rosa-loxP-RFP transgenic mice were isolated, grown and sub-cultured *in vitro* using standard protocols, Myh11 marked cells were lost over time and replaced by a $Sox10^{+}$ population [18]. These data strongly suggest that sub-cultured SMCs routinely used in culture are not in fact derived from medial differentiated SMCs that undergo de-differentiation and re-programming [18], but instead are derived from a Sox 10 positive population that outgrows medial SMCs [18]. Interestingly, several commercial SMC lines also exhibit a similar stem cell phenotypic expression profile in culture (i.e., Sox10, Sox17, S100β) [107, 108]. Moreover, when sub-cultured SMCs were interrogated using vibrational Raman spectroscopy, their photonic signature was notably similar to stem cell-derived myogenic progeny *in vitro* [60]. Collectively, these data suggest that sub-cultured SMCs routinely used to assess the process of SMC de-differentiation *in vitro* are not derived from differentiated medial SMCs but rather from a Sox10 progenitor stem cell present in primary isolates that predominates the culture population over time [18].

Further evidence for this paradigm comes from epigenetic profiling of medial SMCs in culture [10]. Medial SMCs are enriched for the stable SMC epigenetic mark, H3K4me2, at the Myh11 locus by chromatin immunoprecipitation (ChIP) analysis of cell populations [60] and by using an *in situ* hybridization and proximity ligation assay of individual cells [68]. However, when these cells are isolated and grown in culture (up to passage 3), the level of H3K4me2 enrichment within the population is significantly lower compared with fresh aortic SMCs [60] with up to 80% of individual cells in early passage reported negative for the H3K4me2 mark at the Myh11 promoter [64, 68]. If this H3K4me2 epigenetic mark is truly stable in de-differentiated cells, as previously reported [66], these data further reinforce the likelihood that sub-cultured SMCs lacking this mark are not derived from medial differentiated SMCs as originally thought [66]. The presence of this stable H3K4me2 epigenetic mark at the Myh11 promoter has also been used to confirm the presence of de-differentiated SMCs *in vivo* within murine and human arteriosclerotic lesions [9, 68]. However, since the acute reduction in Myh11 promoter activity recovers in SMC-like cells following injury [119] and because stem

cell-derived progeny acquire this H3K4me2 mark at the Myh11 promoter following myogenic differentiation *in vitro* [60, 66], it is likely that that stem cell-derived myogenic progeny also enrich for the H3K4me2 mark *in vivo*.

The ultimate dichotomy arises from the outcomes of the most recent lineage tracing analyses that independently marked and tracked both Myh11/SM22α/Acta2 differentiated SMCs [7, 91, 120], and Sox10/Gli1 stem cells [18, 21, 22] following vascular injury. Both series of cell fate mapping studies concluded that the vast majority of neointimal cells are derived from either one source or the other. Differences in the animal models deployed (carotid ligation vs. femoral injury vs. ApoE mice) [121], the extent of the endothelial damage and the level of disruption to the internal elastic lamina to facilitate movement of differentiated SMCs, may in part account for these differences [59, 122]. However, recent studies assessing tamoxifen (Tm)-inducible Cre recombinases activity in mice may also offer some important clues [123]. While the efficiency of inducible Cre-loxP recombination is readily evaluated with reporter strains, the precise length of time that Tm induces nuclear translocation of CreER(Tm) and subsequent recombination of a target allele following cessation of Tm treatment is rarely assessed. It is clear that the doses of Tm commonly used to induce Cre-loxP recombination in transgenic mice to track differentiated SMCs may continue to label a significant number of cells for weeks after Tm treatment if the Myh11 promoter is active, thereby confounding the interpretation of time-sensitive studies using Tm-dependent models [123]. It is widely accepted that Myh11 promoter activity is initially lost after vascular injury but recovers in neointimal cells to near normal levels within 7–14 days [119]. Hence, it is highly likely that stem cells that acquire an active Myh11 promoter following myogenic differentiation will also be marked as Tm may still be present with the tissue [123]. In this context, resident vascular stem cells that are not originally marked following Cre-loxP recombination using Myh11-CreERT2 transgenic mice could become marked when the Myh11 promoter becomes active 7 days post injury and Tm is still present with the vessel wall to drive the recombination and mark the stem cell. Importantly, most studies using Tm to induce Cre-loxP recombination and mark differentiated SMCs wait 5–14 days before injury; yet Tm is known to remain in tissues for up to 4 weeks [123]. For most studies using $ApoE^{-/-}$ mice, the high fat diet-induced vascular injury is routinely initiated immediately after the Tm treatment ceases [9, 79] raising the likelihood that stem cells acquiring an active Myh11 promoter following myogenic differentiation will also be marked as Tm may still be present with the tissue [123]. Hence, the length of time that Tm-induced Cre-LoxP recombination occurs following cessation of Tm treatment needs to be empirically and routinely evaluated before these possibilities can be disregarded. In a similar manner, medial differentiated SMCs that are not marked using the constitutively active Sox10-Cre or Gli1-Cre transgenic mice to track stem cells could in theory also acquire the mark if the respective promoters driving the cre recombinases become active post injury during a re-programming event. It would be prudent to clarify this in future studies, since many of these elements are widespread in experimental arteriosclerosis research.

There is a clear need for more rigorous lineage tracing studies using Tm-inducible Cre recombinases to track resident stem cells. In this context, we have recently reported using Sca1-eGFP transgenic mice that eGFP positive cells predominate the lesion following injury [105] and that these cells also express S100β [124]. Moreover, lineage tracing analysis using a Tm-inducible

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Cre recombinase driven by S100β promoter confirms that the majority of neointimal cells post injury are derived from a S100β parent population present within the adventitia prior to injury and not present within the medial layer where differentiated SMCs are located [124].

11. Concluding remarks

Our understanding of de-differentiation and re-programming of SMCs continues to evolve since the seminal work of Julie Chamley-Campbell and Gordon Campbell [2, 3]. Initially it was thought that aberrant proliferation of SMCs after phenotypic switching exclusively drove IMT. At the same time, it was acknowledged that SMCs were also protective in advanced lesions, preventing fibrous cap rupture and promoting plaque repair. However, more recent studies using lineage tracing, loss-and-gain-of-function, and epigenetic profiling have changed the landscape [12]. In this context, SMC differentiation and re-programming may also account for the appearance of osteochondrogenic and macrophage-like phenotypes within vascular lesions [17, 92].

Furthermore, whilst still controversial, resident vascular stem/progenitor cells are beginning to be recognised as potentially important players in the development of IMT and the pathogenesis of atherosclerosis. Regardless of the source of the progenitor stem cell (circulating, adventitial, or medial), the impact of the local micro-environment and the relevant cues dictating the pattern of gene expression and behaviour of these cells warrants further investigation. In this context, many of the signalling molecules and molecular switches that are known to impact on the generation of stem cell-derived myogenic, osteochondrogenic and macrophage-like phenotypes have all been implicated in lesion development [42]. For instance, Notch and Hedgehog signalling proteins are known to control stem cell fate and are also upregulated within vascular lesions [49, 51, 125] when either SMC re-programming and/or stem cell-derived differentiation down various lineages is presumed to occur. Inhibition of these pathways ameliorates IMT confirming their putative role in vascular pathology [125, 126]. While Notch-dependent lateral inhibition signalling may promote a particular fate but prevent surrounding cells from doing the same ensuring that not all medial SMCs de-differentiate and re-programme, its putative role in controlling adventitial and/or medial stem fate cannot be disregarded [52]. Further studies that define the specific cell populations contributing to IMT under different circumstances, and the molecular controls involved in their regulation, will add greatly to our overall understanding of vascular pathology and our ability to successfully target these cells therapeutically.

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Section 6

Vascular Smooth Muscle Cells and Tissue Engineering

Chapter 14

Vascular Smooth Muscle Cells (VSMCs) in Blood Vessel Tissue Engineering: The Use of Differentiated Cells or Stem Cells as VSMC Precursors

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Additional information is available at the end of the chapter

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Abstract

Vascular smooth muscle cells (VSMCs) play important roles in the physiology and pathophysiology of the blood vessels. In a healthy adult organism, VSMCs are quiescent, but after a blood vessel injury, they undergo phenotypic modulation from the contractile phenotype to the synthetic phenotype, characterized by high activity in migration, proliferation and proteosynthesis. This behavior of VSMCs can lead to stenosis or obliteration of the vascular lumen. For this reason, VSMCs have tended to be avoided in the construction of blood vessel replacements. However, VSMCs are a physiological and the most numerous component of blood vessels, so their presence in novel advanced vascular replacements is indispensable. Either differentiated VSMCs or stem cells as precursors of VSMCs can be used in the reconstruction of the *tunica media* in these replacements. VSMCs can be obtained from blood vessels (usually from subcutaneous veins) taken surgically from the patients and can be expanded in vitro. During in vitro cultivation, VSMCs lose their differentiation markers, at least partly. These cells should therefore be re-differentiated by seeding them on appropriate scaffolds by composing cell culture media and by mechanical stimulation in dynamic bioreactors. Similar approaches can also be applied for differentiating stem cells, particularly adipose tissue-derived stem cells, toward VSMCs for the purposes of vascular tissue engineering.

Keywords: vascular replacements, adipose tissue-derived stem cells, transforming growth factor-beta, bone morphogenetic protein-4, mechanical loading, dynamic bioreactors, smooth muscle cell differentiation, tissue engineering, regenerative medicine

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1. Introduction

VSMCs are the most numerous cell types in blood vessels, where they are located in the medial layer of the vascular wall, that is, in the *tunica media*. These cells are necessary for the physiological functioning of blood vessels, particularly for vasoconstriction, for vasodilatation and for synthesis of vascular extracellular matrix. These cells are also implicated in pathological changes in blood vessels during atherosclerosis, hypertension, diabetic angiopathy and other vascular disorders. After biochemical or mechanical damage to blood vessels, VSMCs undergo phenotypic modulation, that is, they make the transition from their original quiescent contractile phenotype to a synthetic phenotype, characterized by increased proteosynthesis and by activation of the migration and growth of VSMCs [1–4]. These changes often lead to irreversible damage to blood vessels, including stenosis and occlusion. Ischemia of the tissues supplied by the damaged vessels is then manifested by serious disorders, for example, heart failure, brain stroke or necrosis of leg tissues, which can result in amputation of the leg.

Low patency of arteries can be treated by balloon angioplasty or by endarterectomy. However, in cases of severe blood vessel damage, vascular replacements need to be implanted, usually in the form of bypasses spanning the damaged region of the original vessel.

Vascular bypass grafts can be obtained from four sources: autologous, allogenous, xenogenous or artificial. Autologous grafts, that is, grafts derived from the patient, have the drawbacks of limited availability, donor site morbidity, burden to the patient due to additional surgery and, in the case of implantation of a vein into an arterial position, also mechanical mismatch. Allogeneous transplants, that is, transplants derived from the same species, or xenogenous transplants, that is, transplants derived from a different species, are associated with a risk of immune rejection, disease transmission and, when they are fixed in glutaraldehyde, also potential release of cytotoxic molecules [5, 6]). In view of these problems, artificially constructed vascular grafts have been considered as very promising for future applications.

Artificial grafts currently used in clinical practice are made of synthetic polymers, namely polyethylene terephthalate (PET), expanded polytetrafluoroethylene (ePTFE) and, in some cases, also polyurethane [5, 7, 8]. The first generation of these prostheses was constructed as cell-free, that is, without the reconstruction of any layer of the natural blood vessel. However, the inner surface of the prosthesis attracted cell types participating in thrombus formation, immune reaction and prosthesis restenosis, that is, thrombocytes, inflammatory cells (leucocytes, lymphocytes, monocytes, macrophages), and also VSMCs. VSMCs migrated on the prosthesis mainly from the sites of the anastomosis of the graft with the original vessel and were prone to excessive proliferation. In addition, precursors of VSMCs, originating from the bone marrow and circulating in the blood, can adhere to the inner surface of the prosthesis and can proliferate [9]. All these events can lead to considerable stenosis, obliteration and failure of vascular prostheses, especially medium-diameter vascular grafts (up to 8 mm in diameter) and small-diameter vascular grafts (up to 4 mm in diameter). Attempts have therefore been made to cover the luminal surface of the prosthesis with a confluent, phenotypically mature and semi-permeable endothelial cell layer, which is considered optimal for preventing thrombosis, inflammatory cell adhesion and VSMC hyperplasia [5, 7, 8].

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However, in advanced vascular replacements, it is necessary to reconstruct not only the endothelial cell layer, that is, the main component of the *tunica intima*, but also the other layers of the vascular wall, particularly the *tunica media* with VSMCs as the physiological component of natural blood vessels. It is necessary only to control the proliferation activity of these cells precisely and to direct them toward a differentiated quiescent contractile phenotype. In modern tissue engineering, it is also desirable to differentiate stem cells toward VSMCs, particularly stem cells derived from adipose tissue, which is relatively easily accessible and is available in sufficient quantities [10, 11].

This chapter summarizes our own experience and the experience of other authors in re-differentiating VSMCs on vascular constructs via appropriate cultivation substrates, the composition of cell culture media, cell–cell interaction and mechanical stimulation in dynamic bioreactors. Similar approaches have also been applied for differentiating stem cells, particularly adipose tissue-derived stem cells, toward VSMCs for the purposes of vascular tissue engineering.

2. Use of differentiated VSMCs in blood vessel tissue engineering

As mentioned earlier, attempts have been made to reconstruct the *tunica intima* on artificial vascular replacements, and these replacements have been used sporadically in clinical practice [7, 8]. At the same time, the *tunica media* has been reconstructed only rarely in vascular replacements, due to the tendency of VSMCs to proliferate excessively, and these attempts still remain at the experimental level. However, as was mentioned earlier, the presence of the *tunica media* enhances the functionality of artificially constructed blood vessels, if the VSMCs gain their quiescent contractile phenotype [12]. This phenotype is usually lost during the expansion of VSMCs after they have been harvested from blood vessels obtained surgically from patients. The contractile phenotype can be restored by an appropriate structure and composition of the scaffolds, by appropriate composition of cell culture media, by appropriate cell–cell interactions and by appropriate mechanical stimulation of VSMCs in dynamic cell culture systems, especially if the factors mentioned here are applied in combination.

2.1. Structure and composition of the scaffolds

As concerns the structure of the scaffolds, three-dimensional (3D) porous scaffolds are more physiological than two-dimensional (2D) scaffolds, because 3D scaffolds better mimic the architecture of the native *tunica media* and enable a multilayered arrangement of VSMCs [13, 14]. The differentiation response of VSMCs to the uniaxial stress generated by a dynamic cell culture system was more pronounced in 3D scaffolds than on 2D scaffolds [15].

As concerns the chemical composition of the scaffolds, attempts are being made to fabricate these scaffolds from degradable materials, such as synthetic polymers (e.g., polylactides, polyglycolides, polycaprolactone and their copolymers), natural polymers (collagen, elastin, fibronectin, laminin, fibrin) and combinations of these materials [14–20]. Degradable scaffolds are used for vascular tissue engineering, because the scaffolds will gradually be removed and replaced by a newly regenerated vascular tissue. In addition, some natural polymers maintain the VSMCs in a differentiated contractile phenotype, for example, elastin and proteins of the

cell basement membrane, namely type IV collagen and laminin, while other natural polymers, such as fibronectin and vitronectin, stimulate the phenotypic modulation of VSMCs toward the synthetic phenotype and VSMC migration and proliferation [18, 21, 22]. The role of type I collagen is ambiguous. Polymeric fibrillary type I collagen in a healthy blood vessel keeps the VSMCs in their quiescent state, but degraded or denatured type I collagen supports VSMC proliferation [21, 23]. Scaffolds obtained by decellularization of various tissues, including blood vessels, recently emerged as very promising structures for cardiovascular tissue engineering. After decellularization, the tissues lose most of their immunogenicity and could even be used for xenogeneic transplantation. At the same time, these scaffolds retain their original biochemical composition and mechanical properties [11, 12, 19, 20, 24].

2.2. Composition of the cell culture medium

Another important issue in the reconstruction of the *tunica media* is the composition of the cell culture medium. In the initial phase of colonization of the scaffolds with VSMCs, the migration and proliferation of these cells and their synthesis of ECM molecules is desirable, and therefore a standard serum-supplemented medium can be used. At the same time, the scaffolds should be seeded with a high number of VSMCs in order to shorten their migratory and proliferative phase as much as possible. It is known that the confluence of VSMCs and the development of cell–cell contact support the re-differentiation of VSMCs toward the contractile phenotype [25]. When the scaffolds are well populated with VSMCs, it is necessary to achieve the quiescent differentiated contractile phenotype of VSMCs. For this purpose, chemically defined serum-free or serum-low media are used, for example, media supplemented with transforming growth factor-β (TGF-β) [26–28] or with heparin [29]. At the same time, heparin supports endothelialization of the prosthesis [30], which also contributes to the development of the contractile phenotype in VSMCs, for example, by producing sulfated heparin-like glycosaminoglycans [2, 18, 31], nitric oxide [32, 33] and by developing contacts between VSMC and endothelial cells, that is, myoendothelial gap junctions [27].

2.3. Interactions of VSMCs with endothelial cells and with other VSMCs

VSMCs co-cultured in direct contact with endothelial cells showed more pronounced differentiation toward the contractile phenotype (manifested by increased expression of contractile proteins, that is, SM1 and SM2 isoforms of smooth muscle myosin heavy chain, calponin 1 and smooth muscle α-actin) than VSMCs co-cultured with endothelial cells without direct contact with these cells. This effect was mediated by connexin 43 (Cx43), an important component of myoendothelial gap junctions. Inhibition of gap junctional communication pharmacologically or by knock down of Cx43 in endothelial cells blocked TGF-β signaling and VSMC differentiation [27]. However, the gap junctions between VSMCs are a more controversial issue. On the one hand, an increased number of these junctions and upregulation of Cx43 have been shown to be associated with undesirable VSMC proliferation and vascular diseases. On the other hand, when increased expression of Cx43 in VSMCs was induced by TGF-β1, these cells enhanced the expression of smooth muscle α-actin (SM α-actin), calponin and SM1 myosin heavy chain, that is, markers of VSMC differentiation toward the contractile phenotype [34].

In comparison with other connexins, for example, Cx37, Cx43 is highly mechanosensitive. The exposure of human coronary artery smooth muscle cell to shear stress of 5 dyn/cm^2, but not

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to physiological shear stress of 12 dyn/cm^2, caused the dysfunction of Cx40/Cx43 heterotypic myoendothelial gap junctions, which may be replaced by homotypic Cx43/Cx43 channels and induced the transition of VSMCs to the synthetic phenotype, which was manifested by decreased expression of smooth muscle myosin heavy chain (SM-MHC) and calponin and by increased release of platelet-derived growth factor-BB (PDGF-BB). At the same time, the VSMCs under shear stress of 5 dyn/cm^2 were randomly oriented, while under shear stress of 12 dyn/cm^2, these cells were aligned in the flow direction [35].

In our experiments, we investigated the effects of periodical uniaxial stretching of VSMCs on the Cx43 expression. Mechanical stimulation of VSMCs was performed using STREX equipment (B Bridge International, Ltd). VSMCs were seeded in flexible silicone chambers coated with type I collagen and fibronectin. After a 2-day static culture, the VSMCs were subjected to stretch at a frequency of 0.5 Hz and an amplitude of 5%. After a further period of 48 h, the frequency was changed to 1 Hz. The changes in Cx43 expression were tested by qRT-PCR. At near-physiological conditions (frequency of 1 Hz and amplitude of 5%), the expression immediately rose almost 5 fold, with the maximum in the first 30 min. At a lower degree of stimulation (at a frequency of 0.5 Hz and an amplitude of 5%), the maximal expression was delayed to about 60 min, and it was considerably lower. For longer time periods, the expression of Cx43 decreased again (**Figure 1**). VSMCs were also stained by immunofluorescence to show the changes in the arrangement and the distribution of the contractile protein SM α-actin. After stretching, the SM α-actin was more intensely stained than in the control static culture and was organized into filaments, especially in cells after 24 h of stretching (**Figure 2**).

2.4. Mechanical loading of VSMCs in a dynamic culture system

In general, dynamic cultivation of VSMCs is an important tool for restoring the contractile differentiated phenotype of these cells [16, 19]. It has been shown repeatedly that differentiation of VSMCs requires pulsatile stress and cyclic strain, that is, components of the hemodynamic stress to which blood vessels are exposed in vivo [36].

As concerns **pulsatile stress**, rabbit aortic VSMCs were seeded onto rubber-like elastic, three-dimensional poly(lactide-*co*-caprolactone) scaffolds and were exposed to a pulsatile flow of

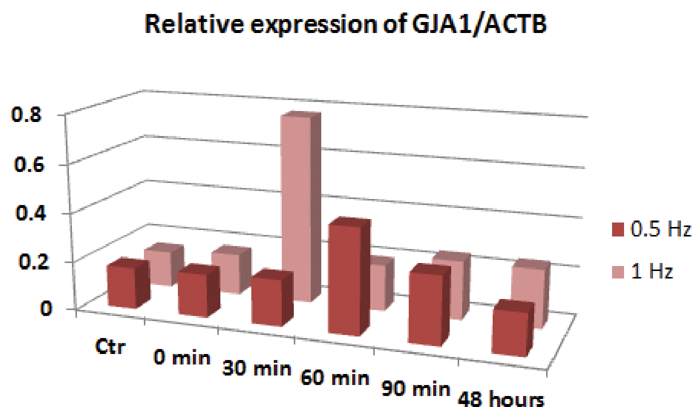

Figure 1. Relative mRNA expression of connexin 43 (GJA1) in rat aortic smooth muscle cells after uniaxial stretching in the STREX dynamic cell culture system (B bridge international, ltd.) for 0–48 h at a frequency of 0.5 Hz (dark) or 1 Hz (light).

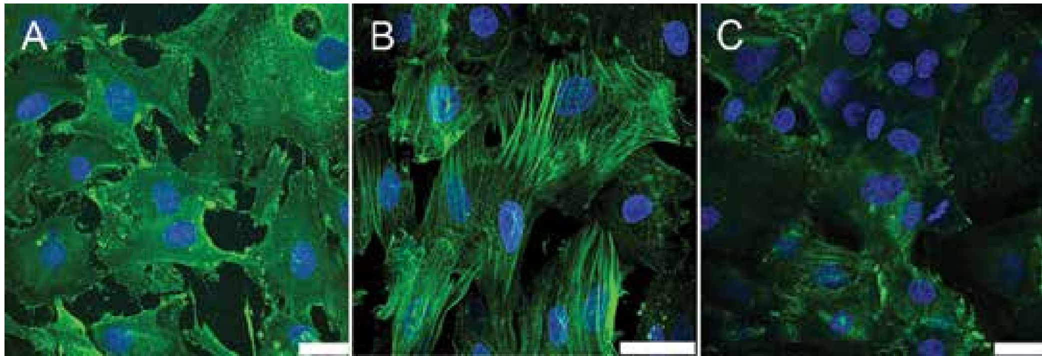

Figure 2. Immunofluorescence of SM α-actin in rat aortic smooth muscle cells in 2-day-old cultures after exposure to uniaxial stretching at a frequency of 1 Hz for 4 h (A), 24 h (B) and in control cells without stretching (C). Leica SPE confocal microscope (DM 2500 CSQ V-VIS), obj. 63×. Scale bar = 25 μm.

the culture medium (flow rate 130 ml/min, pressure 25 mmHg with a pulse of 1 Hz, amplitude of radial distention 5%, exposure 8 weeks). The pulsatile strain and the shear stress enhanced the VSMC proliferation and collagen production. However, at the same time, the expression of SM α-actin, an early marker of VSMC differentiation, was upregulated 2.5-fold in comparison with the value in VSMCs under static conditions, and the VSMCs were aligned in a direction radial to the distending direction, that is, similarly as in native blood vessels in vivo, whereas the VSMCs were randomly oriented under static conditions [37].

The behavior of VSMCs in a pulsatile bioreactor can be further modulated by the presence or absence of endothelial cells. Endothelial cells were seeded on the opposite side of a porous polycarbonate membrane and were placed in contact with a collagen gel containing VSMCs. The presence of the endothelial cells increased the VSMC size and the expression of the contractile proteins, namely SM α-actin and SM-MHC. Absence of endothelial cells decreased the expression of SM α-actin and SM-MHC without affecting the size of the VSMCs. The proliferation of VSMCs was not affected by the presence or absence of endothelial cells [38].

As concerns **cyclic strain**, collagen-based gels laden with primary human umbilical artery VSMCs were exposed to a 10% cyclic strain at 0.5 Hz for 5 days. Cyclic stimulation promoted cell-driven collagen matrix bi-axial compaction, enhancing the mechanical strength of the strained samples with respect to the static controls. Moreover, cyclic strain had a positive effect on VSMC behavior: the cells maintained their contractile phenotype and spread uniformly throughout the thickness of the walls of collagen-based tubular structures [39].

The effect of cyclic strain can be further modulated by the presence of various growth factors. For example, VSMCs in a 3D collagen type 1 matrix were exposed to a 10% circumferential strain at a frequency of 1 Hz. These conditions increased the gel compaction and the VSMC proliferation, which was further enhanced by adding PDGF into the cell culture medium. Conversely, the addition of TGF-β strongly inhibited cell proliferation and increased the expression of SM α-actin [40]. In a study by Yao et al. [41], rat aortic VSMCs in 70% confluence and after starving in a Dulbecco's Modified Eagle Medium (DMEM) without serum for 24 h were subjected to cyclic strain of 10% elongation at 1.25 Hz for 24 h in the Flexercell Tension

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Plus system. The strain stimulated the secretion of TGF-β1 by VSMCs and upregulated the expression of contractile phenotype markers in these cells, namely smooth muscle protein 22-α (SM22-α), SM α-actin and calponin.

The parameters of the cyclic strain also strongly modulate the VSMC response. For example, rat aortic VSMCs were exposed to cyclic strains in vitro with defined parameters, that is, 5% strain, considered as physiological, and 15% strain, considered as pathological. Both types of strain had a frequency of 1.25 Hz and were applied for 24 h. The results showed that 15% strain significantly increased VSMC migration and proliferation in comparison with 5% strain [42].

3. Use of stem cells as a source of VSMCs for blood vessel tissue engineering

Stem cells have emerged as a promising resource for advanced tissue engineering, including vascular tissue engineering. Differentiated VSMCs are often obtained from aged and polymorbid patients. These cells show lower proliferation potential than is desirable, as the harvested cells need to be expanded in cell culture conditions. In addition, the VSMCs also show a higher tendency toward senescence. Another consideration is that these VSMCs are mostly of venous origin because it is easier and less invasive to isolate subcutaneous veins than arteries. However, venous VSMCs have different properties from those of arterial VSMCs, for example, they are adapted for lower pressure and slower blood circulation in the vein system.

Stem cells are a component of the blood vessels themselves, where they are distributed throughout the entire vascular wall, that is, in the subendothelial space of the *tunica intima*, in the *tunica media* and also in the *tunica adventitia*. Their primary function is postnatal vasculogenesis and regeneration of the vascular wall after injury, but they can also be a cell source for vascular tissue engineering [43–45]. However, harvesting stem cells and isolating differentiated VSMCs are associated with similar problems [46].

Other sources of stem cells with the potential to be differentiated into VSMCs are human pluripotent stem cells, obtained from embryonic tissues [47, 48] and induced pluripotent stem cells (iPSCs) [49–51]. However, the use of these cells, although promising, is associated with ethical and legal issues in human embryonic stem cells and with a risk of potential tumorigenicity of iPSCs. These complications can be overcome by the use of stem cells isolated from extrafetal tissues, for example, placenta [52] and umbilical cord [53] or by the use of stem cells from adult tissues, such as bone marrow [36, 54, 55], epidermis, namely hair follicles [56] or skeletal muscle [57]. In addition, adult stem cells can be applied in autologous form. However, harvesting the adult tissues mentioned here is often invasive and painful, and the tissues are obtained in relatively small quantities. Consequently, adipose tissue-derived stem cells (ASCs) seem to be the most promising source because the adipose tissue, located subcutaneously, can be obtained by a less invasive method, that is, liposuction, and in relatively large quantities.

ASCs have been used relatively widely for experimental vascular tissue engineering. The main tools for differentiating ASCs toward VSMCs include composing cell culture

media and exerting mechanical stress in dynamic cell culture systems, similarly as for the re-differentiation of VSMCs. Examples of results obtained by various authors [10, 11, 58–67] are summarized in **Table 1**.

Author	Scaffolds	Medium supplement	Cultivation system	Obtained VSMC markers
Rodríguez et al. [10]	Uncoated tissue culture polystyrene dishes or dishes coated with laminin or collagen	Medium MCDB 131 with 1% FBS plus 100 units/ml of heparin	Static	SM α-actin, calponin, caldesmon, SM22-α, SM-MHC, smoothelin
Kim et al. [58]	Tissue culture polystyrene	Angiotensin II	Static	SM α-actin, calponin, h-caldesmon, SM-MHC
Kim et al. [59]	Tissue culture polystyrene	Bradykinin	Static	SM α-actin
Kim et al. [60]	Tissue culture polystyrene	Thromboxane A_2 mimetic U46619	Static	SM α-actin, calponin, SM-MHC, smoothelin
Nincheri et al. [61]	Tissue culture polystyrene, microscope slides coated with gelatine	Sphingosine 1-phosphate	Static	SM α-actin, transgelin, cytoskeletal F-actin assembly, Ca^{2+} currents
Wang et al.. [62]	Tissue culture polystyrene dishes	TGF-β1, BMP-4	Static	SM α-actin, SM22-α, calponin, SM-MHC
Aji et al. [63]	Tissue culture polystyrene	TGF-β1, BMP-4	Static	SM α-actin, SM22α, calponin, SM-MHC
Elçin et al. [64]	8-chamber slides (Labtek)	TGF-β1, BMP-4, angiotensin II	Static	SM α-actin, calponin, h-caldesmon SM-MHC
Lachaud et al. [65]	Tissue culture polystyrene dishes	EGF	Static	SM α-actin, calponin, caldesmon, SM22α, desmin, SM-MHC, smoothelin-B
Wang et al. [66]	Polyglycolic acid mesh	TGF-β1, BMP-4	Pulsatile stress	SM α-actin, calponin, SM-MHC
Harris et al. [11]	Decellularized saphenous vein	angiotensin II, SPC, TGF-β1	Bioreactor generating: Tension Compression Pressure Perfusion	calponin, caldesmon, SM-MHC
Rashidi et al. [67]	Plasma-treated silicon membranes with collagen I	TGF-β1	Cyclic strain	SM α-actin, SM22-α, h-caldesmon, calponin3

TGF-β1: transforming growth factor-β1; BMP-4: bone morphogenetic protein-4 (a polypeptide belonging to the TGF-β superfamily); SM α-actin: α-isoform of smooth muscle actin; SM22-α: smooth muscle protein 22-α; SM-MHC: smooth muscle myosin heavy chain; Transgelin: actin cross-linking/gelling protein in fibroblasts and smooth muscle cells; SPC: sphingosylphosphorylcholine; EGF: epidermal growth factor.

Table 1. Culture conditions for differentiation of ASCs into VSMCs and the obtained markers of differentiation.

For our experiments, the ASCs were isolated from lipoaspirates obtained from patients by liposuction under their informed consent and ethical approval. Lipoaspirates of subcutaneous adipose tissue were taken from three different regions, that is, the abdominal region and the inner or outer side of the thighs. Liposuction was performed under low negative pressure (−200 mmHg) and under high negative pressure (−700 mmHg). The ASCs were then harvested by a method originally described by Estes et al. [68], with a slight modification described in our earlier study [69].

3.1. Differentiation of ASCs toward VSMCs by the composition of the cell culture medium

In our first set of experiments, we attempted to optimize the composition of the cell culture media in a conventional static cell culture system in order to differentiate the ASCs toward VSMCs. First, three types of culture media were tested, namely a DMEM medium (Sigma-Aldrich, Cat. No. D5648) with 10% of fetal bovine serum (FBS), SmGM®-2 Smooth Muscle Growth Medium-2 BulletKit® (SMGM, Lonza, USA, Cat. No. CC-3182) and Endothelial Growth Medium-2 (EGM-2, Lonza, USA, Cat. No. 3162). These media alone, that is, without additional supplementation, did not promote the differentiation of ASCs into VSMCs. Therefore, we supplemented the media with transforming growth factor-β1 (TGF-β1; 2.5 ng/mL, Abcam) and with bone morphogenetic protein-4 (BMP-4; 2.5 ng/ml, Sigma-Aldrich) because this combination of growth factors showed greater differentiation efficiency than TGF-β1 or BMP-4 alone or in combination with angiotensin II [64]. The addition of TGF-β1 into SMGM-2 and EGM-2 media caused rapid proliferation and subsequent detachment of the ASCs. The differentiation experiments were therefore performed with DMEM +2% of FBS + TGF-β1 + BMP-4. The addition of TGF-β1 increased the proliferation of ASCs in comparison with DMEM +2% FBS without any supplement. When the ASCs were cultured with TGF-β1 and BMP-4 for three days, immunofluorescence staining revealed the formation of SM α-actin-containing filaments and an increasing number of calponin-positive cells (**Figure 3A-C**). In later culture intervals (days 14–17), cells with slight positivity for desmin and sporadic SM-MHC-positive cells were also detected. Cells cultured without the supplements only sporadically contained SM α-actin filaments or calponin (**Figure 3D** and **E**). In our experiments, we observed individual differences in proliferation and differentiation among the ASCs from various patients and also among the cells taken from the same patient but from different regions of the body.

3.2. Differentiation of ASCs toward VSMCs by the composition of the cell culture medium and by mechanical load

In our second set of experiments, we studied the differentiation of ASCs toward VSMCs by combining cell differentiation media with mechanical load. The blood pumped by the heart generates several mechanical stimuli on the arterial wall, such as the wall shear stress affecting endothelial cells, and also the pressure force and the cyclic strain stress. These types of stimuli promote or accelerate the differentiation and the phenotypic maturation of ASCs and other stem cells into VSMCs [36, 55, 66]. In order to simulate the effects of these mechanical stimuli, we have developed a unique dynamic cultivation system. This system consists of special cultivation chambers

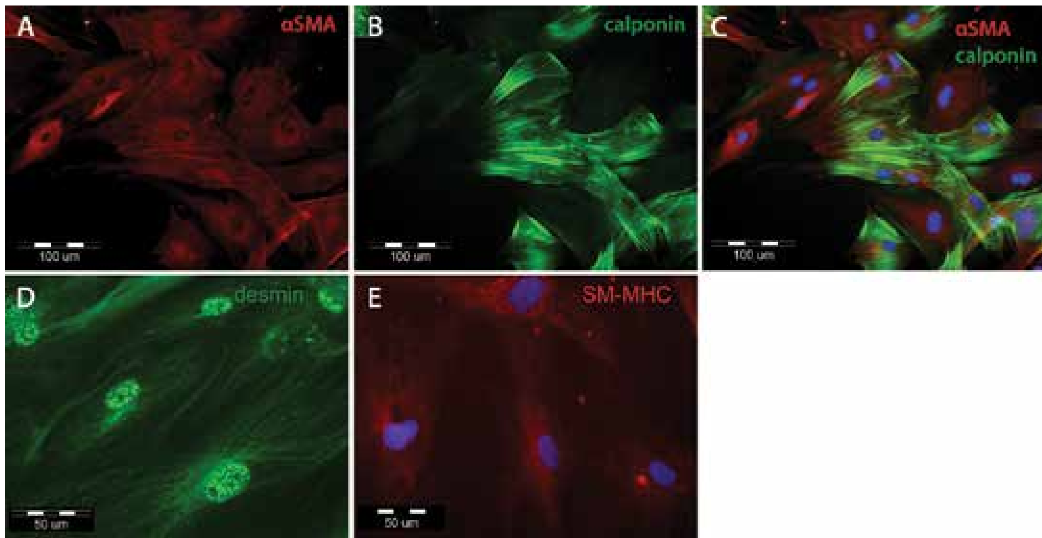

Figure 3. Immmunofluorescence staining of SM α-actin (red) and calponin (green) in ASCs on day 3 of differentiation (A–C) in a medium containing TGF-β and BMP-4. Immunofluorescence staining of desmin (D) on day 14 and of SM-MHC (E) on day 17. Cell nuclei are visualized with Hoechst #33258 (blue). Olympus IX 71 microscope, objective ×20 and ×40, scale 100 μm and 50 μm, respectively.

and a pressure generation system. The design of the chamber allows the use of rigid substrates (glass) or flexible substrates (cast silicone). A rigid substrate is used for evaluating the effect of the pressure force. A flexible substrate simulates an elastic arterial wall. The pressure force that is applied mimics the dilatation and constriction of the arterial wall by generating cyclic strain stress. To improve their hydrophilicity, the substrates are plasma treated. In addition, these substrates can be coated with collagen or fibrin gels to improve the adhesion and the initial proliferation of the cells. After cell seeding, the chamber is hermetically sealed to allow controlled stimulation (**Figure 4**). The pressure generation is maintained by a computer-controlled custom-built linear syringe pump. A pressure-based feedback-controlling algorithm is implemented to

Figure 4. Cultivation chambers used for mechanical stimulation (left). The use of transparent surfaces allows microscopic live-cell imaging (right).

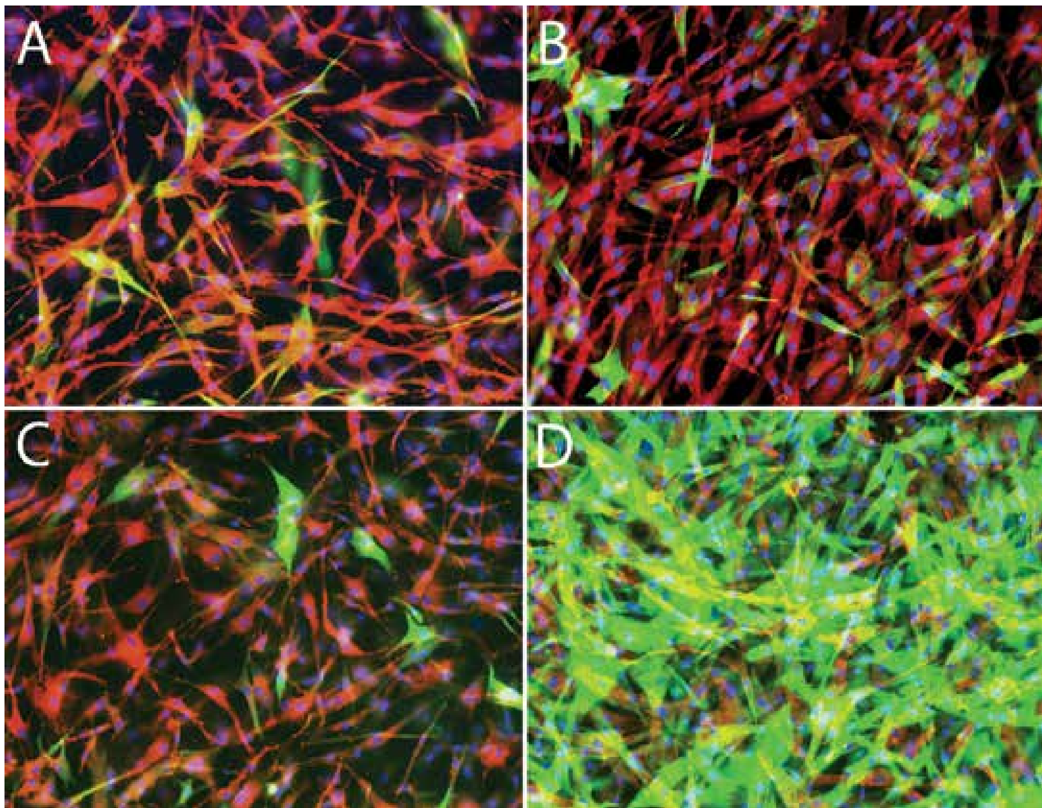

Figure 5. Immunofluorescence staining of SM α-actin (red) and calponin (green) in ASCs cultured embedded in a fibrin gel on glass under static conditions (A, C) and under dynamic conditions (B, D), using the system depicted in **Figure 4**. A, B: 3 days of cultivation; C, D: 7 days of cultivation. Cell nuclei are counterstained with Hoechst #33258. IX71 Olympus microscope, DP71 digital camera, obj. × 10.

maintain stable conditions. Pressure pulses are generated between two set points that simulate systolic and diastolic pressure. Maximum pressure can be set up to 300 mmHg (40 kPa), with a maximum pulse rate of up to 180 beats per minute (3 Hz).

Our experimental results, obtained in the dynamic culture system described above, indicated positive effects of pressure stimulation on the differentiation of ASCs toward VSMCs. ASCs in low passages 2–4, with initial density of approx. 70,000 cells/cm^2, were cultured in high glucose DMEM medium (Sigma-Aldrich, Cat. No. D5648), supplemented with 2% FBS, TGF-β1 (2.5 ng/mL, Abcam) and BMP-4 (2.5 ng/ml, Sigma-Aldrich) for 3 or 7 days under static culture conditions or under dynamic pulse pressure stimulation. This stimulation was set to physiological 120/80 mmHg (15.9/10.6 kPa) and pulse rate simulating 60 beats per minute (1 Hz). The cell culture medium was replaced after 3 days. The ASCs were stained for SM α-actin (Sigma-Aldrich, Cat. No. S2547), an early marker of VSMC differentiation, and for calponin (Abcam, Cat. No. ab46794), an intermediate marker of VSMC differentiation, and the cell nuclei were counterstained with Hoechst #33258. Pressure loading supported ASC proliferation after 3 days (**Figure 5B**) and after 7 days (**Figure 5D**). This was manifested by a higher cell population

density than in the static culture (**Figure 5A** and **C**). The ASCs were positively stained for SM α-actin on all samples. Moreover, increased numbers of cells positively stained for calponin were found in ASCs cultured in fibrin gel under pulse pressure on day 7 (**Figure 5D**). This suggests that the differentiation of ASCs into VSMCs in the presence of TGF-β1 and BMP-4 was significantly enhanced by dynamic pressure loading.

3.3. Differentiation of ASCs in co-culture with vascular endothelial cells

In our third set of experiments, we studied the behavior of ASCs in co-culture with vascular endothelial cells. ASCs are known to possess the ability to stimulate endothelial cells to form capillaries. In a co-culture model of ASCs and endothelial cells, the ASCs in close contact with endothelial cells differentiated after 7 days into pericyte-like cells, which stained positively for SM α-actin and stabilized the wall of newly formed capillaries (**Figure 6**). Similar results were achieved in a study by Rohringer et al. [70], who co-cultured ASCs

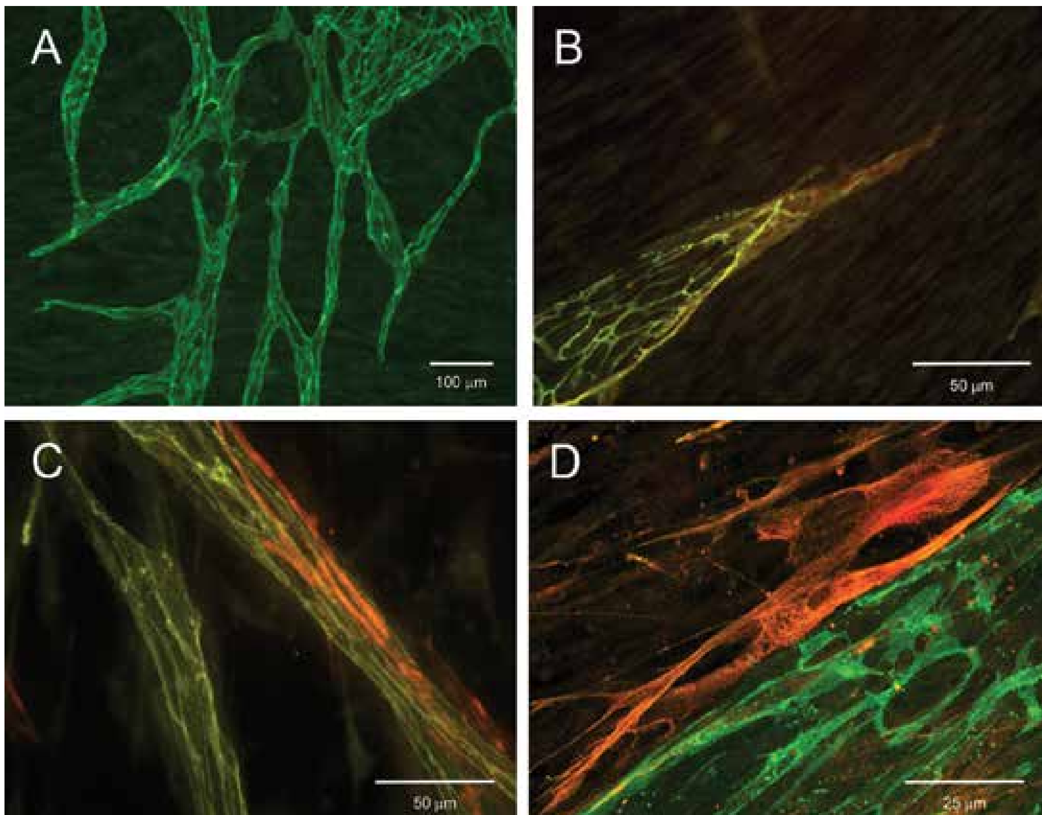

Figure 6. Capillary formation in the co-culture model of ASCs with endothelial cells. A: Vascular network formed by endothelial cells in the co-culture model, endothelial cells stained against VE-cadherin. B: Detail of vascular sprouting; VE-cadherin in green, CD146 (a marker of pericytes) in red. C: Stabilization of capillaries by perivascular cells after 7 days of co-culture; VE-cadherin in green, SM α-actin in red. D: Detail of the close contact between perivascular cells and endothelial cells; VE-cadherin in green, SM α-actin in red. Nikon Ti-E inverted fluorescence microscope with a CARV II confocal scanner.

and vascular endothelial cells in a fibrin gel and demonstrated that the proximity of ASCs and endothelial cells stimulated the formation of tubular structures by endothelial cells, which were stabilized by ASCs developing the characteristics of pericytes. Recent studies have documented similarities between mesenchymal stem cells and pericytes. Pericytes are contractile cells that are in close contact with endothelial cells in capillaries and serve to control the blood flow. When grown in vitro, pericytes express similar surface antigens as ASCs (CD73, CD90 or CD105) [71] and lack hematopoietic markers (CD45) and endothelial markers (CD31, von Willebrand factor, VE-cadherin). Pericytes are also capable of multipotential differentiation, for example, adipogenic, osteogenic, chondrogenic and myogenic differentiation [72]. However, the level of CD146, which is considered to be a marker of pericytes, differs greatly among different isolations of ASCs (in our experience from 0.5–90%). CD146 (also known as MCAM, S-endo-1, MUC18 or P1H12) is not expressed solely in pericytes. It is also considered to be a marker of endothelial progenitor cells and endothelial cells. It was recently shown that CD146 acts as a receptor for Wnt5a and regulates cell migration [73] or that it is involved in controlling the formation of the blood–brain barrier, where it ensures communication between endothelial cells and pericytes [74]. In the co-culture model of endothelial cells with ASCs, it remains elusive whether every ASC that is in close contact with an endothelial cell can act as a pericyte, or whether pericytes form a subpopulation of the heterogeneous population of ASCs with a specific, irreplaceable function.

4. Conclusion

Vascular smooth muscle cells (VSMCs) are the most numerous component of the arterial and venous wall, and they ensure the physiological functions of blood vessels. Under pathological conditions, however, VSMCs lose their differentiation markers, which is accompanied by activation of migration and proliferation of these cells. This can lead to stenosis or obliteration of the injured blood vessels. For this reason, VSMCs were not included in the early generations of vascular replacements, which were either cell-free or pre-endothelialized in vitro. Reconstruction of *the tunica media* containing VSMCs remains at the experimental level. The *tunica media* can be reconstructed with the use of differentiated VSMCs taken from blood vessels (usually subcutaneous veins), isolated surgically or with the use of stem cells, which is a more advanced approach. Various types of stem cells have been used for differentiation into VSMCs and for constructing vascular replacements, including embryonic stem cells, induced pluripotent stem cells, stem cells from extrafetal tissues and stem cells isolated from adult tissues, such as bone marrow, skeletal muscle, epidermis and adipose tissue. Adipose-tissue derived stem cells (ASCs) seem to be the most promising source of VSMCs because they can be isolated in relatively large quantities, by relatively non-invasive methods (liposuction) and in autologous form. Differentiation of ASCs into VSMCs can be induced by appropriate scaffolds (preferably three-dimensional and compliant) by appropriate composition of the cell culture media (e.g., a low-serum medium supplemented with TGF-β1 and BMP-4) and particularly by mechanical stimulation in dynamic cell culture systems generating pulsatile stress, cyclic strain and pressure stress. In co-cultures with endothelial cell forming tubular structures, ASCs form pericyte-like cells.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 15

Methods and Model Systems Used to Study Pregnant Human Uterine Smooth Muscle

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Additional information is available at the end of the chapter

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Abstract

Successful pregnancy necessitates that the human uterus is maintained in a relaxed, quiescent state for the majority pregnancy, before ultimately transforming to a contractile phenotype capable of powerful, coordinated contractions to facilitate parturition. The exact mechanisms that regulate this transition are yet to be fully understood, and as such, we still do not understand the molecular mechanisms that trigger the onset of human labor. This is in large part due to the ethical considerations associated with human pregnancy, which, outside of clinical trials, primarily limits human studies to *in vitro* investigations on cell lines and biopsied tissues. Researchers have therefore devised numerous model systems for investigating pregnant human uterine smooth muscle, which have played vital roles in elucidating the fundamental biology and key regulatory pathways that underpin the transition from quiescence to contractility. This chapter describes in detail, those methods and model systems used to study pregnant human uterine smooth muscle, and explores the challenges associated with these model systems.

Keywords: smooth muscle, parturition, cell lines, tissue pieces, tissue strips

1. Introduction

Parturition requires that myometrial smooth muscle undergo a phenotypic transition, remaining quiescent for the majority of gestation and then transforming to a tissue capable of generating forceful, coordinated contractions to expel the fetus and the placenta [1–3]. Characterizing the regulation of key myometrial genes is essential to understanding normal human birth, as well as obstetric complications, including preterm labor. Outside of clinical trials, researchers are primarily limited to observational studies of human pregnancy, and as such, rely heavily on *in vitro* models of the uterus for performing investigational studies. Human cell cultures

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are a valuable *in vitro* tool, used to gain insight into numerous physiological and pathological processes; however, concerns have been raised about the lifespan of cultured primary cells, as well as their ability to remain representative of the tissue of origin. In an attempt to address the limited lifespan of cultured primary cells, immortalized cell lines have been developed by transfecting telomerase expression vectors into primary cells. Nevertheless, multiple studies have shown that immortalization could cause fundamental changes in the cells, and therefore many studies opt to use primary cells at low passage numbers instead. More recently, 3D cell culture models have been developed to more closely mimic the *in vivo* environment of the cells. Although these 3D cell cultures recreate tissue environments, they still have multiple limitations, such as cost, long production times and often the need for specialized equipment. Incubation of tissue *ex vivo* as small pieces (explants) or strips models the *in vivo* phenotype more closely and has emerged as a popular experimental model for interrogating myometrial biology. Nonetheless, recent evidence indicates that pregnant human smooth muscle tissue undergoes rapid phenotypic changes during these *ex vivo* studies. The consequences are considerable in that findings made using 'non-laboring' tissue may in fact have been attained using tissue that had spontaneously transitioned to a laboring phenotype throughout the course of the study. Model systems of myometrium that are in a state of flux have the capacity to confound results when researchers seek to elucidate the trigger(s) for labor. This chapter describes in detail, methods and model systems used to study pregnant human uterine smooth muscle, and explores the challenges associated with these model systems.

2. Preterm birth

Preterm birth is defined by the World Health Organization as birth occurring before 37 completed weeks of gestation [4]. Estimates of global rates of preterm births suggest that of the 135 million live births worldwide in 2010, 14.9 million babies were born preterm, representing a preterm birth rate of 11.1% [5, 6]. In recent decades, the rate of preterm birth has continued to rise in most countries, despite advances in medical technology as well as the introduction of medical interventions designed to reduce preterm birth [5, 7–9]. This is alarming given that preterm birth is a leading cause of neonatal death, responsible for 44% (2.761 million) of the 6.3 million deaths of children who died before age 5 years in 2013 [10]. Additionally, survivors of preterm birth often suffer both short- and long-term morbidities. Short-term morbidities include respiratory distress syndrome, necrotizing enterocolitis, intraventricular hemorrhage, and patent ductus arteriosus, while long-term morbidities include cerebral palsy, mental retardation and learning impairment, visual and hearing problems, as well as possible increased risk of cancer [11–13]. These translate into enormous economic and societal cost [14]. The economic costs include immediate expenses associated with neonatal intensive care, as well as the long-term costs of ongoing management of disabilities and diseases [14, 15]. The societal costs include families experiencing the sudden loss of a newborn, or stressful hospitalization of newborns that often extends for months, followed by lifelong support of children with varying severities of ongoing disease(s) [14]. Achieving decreased rates of preterm birth with improved neonatal outcomes would therefore have enormous implications at the community level. Nevertheless, little progress has been made addressing preterm birth due to a poor understanding of the underlying mechanisms that initiate myometrial contractions. As

such, the development of *in vitro* methods and model systems for studying pregnant human myometrium is critical to determining the regulatory mechanisms that underpin the onset of both term and preterm labor.

3. Human myometrial smooth muscle cell culture models

Human myometrial smooth muscle cell (SMC) cultures have been used extensively to study myometrial biology and researchers have been able to elucidate many aspects of fundamental biology, such as the regulation of gene expression and signaling pathways. In SMC cultures, myometrial biopsies are typically obtained from the lower uterine segment of term singleton pregnancies during Cesarean section, dissected into 1 mm^3 pieces and washed in a saline solution, such as Hanks' Balanced Salt Solution, to remove excess blood. Myometrial pieces are enzymatically digested down to single cells (collagenase XI, collagenase IA). The digestion suspension is then repeatedly passed through a fine sterile pipette to disperse myometrial cells. Enzymatic digestion is stopped by addition of 5% fetal bovine serum (FBS) to the medium. Myometrial cells are then purified using a cell sieve (pore diameter of ~70 μm) and centrifugation, before ultimately being plated into culture flasks containing smooth muscle medium, supplemented with 5% FBS and antibiotic-antimycotic. The isolated SMCs are typically assessed by Trypan blue exclusion method to determine cell viability. The myometrial SMCs are incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for growth and expansion, as required (**Figure 1**).

Casey *et al.* [16] established first human myometrial SMCs in monolayer culture in 1984 and reported that the cells were stable for up to a year with no morphological changes. Nevertheless, concerns were raised about the lifespan of cultured primary cells, and Hayflick and Moorhead [17] reported that cell populations are only capable of dividing a fixed number of times before reaching replicative senescence. In an attempt to address the limited lifespan of cultured primary cells, numerous strategies have been used to develop myometrium-like cell lines with greater proliferative potential.

Perez-Reyes *et al*. [18] immortalized human SMCs *in vitro* by infecting the cells with a retroviral vector that contained the E6/E7 open reading frames of human papillomavirus type 16. The immortalized SMCs had significantly increased growth rates, compared to non-immortalized control cells, and there were no signs of senescence with long-term passage [18]. These first retrovirally infected SMC lines can be in continuous tissue culture for more than 1 year [18]. However, in addition to having an increased growth rate, cell size was decreased and there were alterations in α-smooth muscle actin (α-SMA) filament distribution and staining intensity [18].

Previous studies have demonstrated that human telomerase reverse transcriptase (hTERT) can stabilize telomere length and prolong cellular proliferative capacity [19–23]. Therefore, Condon *et al*. [24] developed a SMC line from hTERT-infected myometrial cells to investigate the complex molecular, hormonal and cellular processes associated with the myometrium. hTERT-infected myometrial cells were able to be in continuous culture for more than 10 months [24]. Furthermore, these cells exhibited a number of markers of SMCs, including α-SMA, smoothelin, *h*-caldesmon and calponin expression, as well as genes associated with reproductive function and parturition,

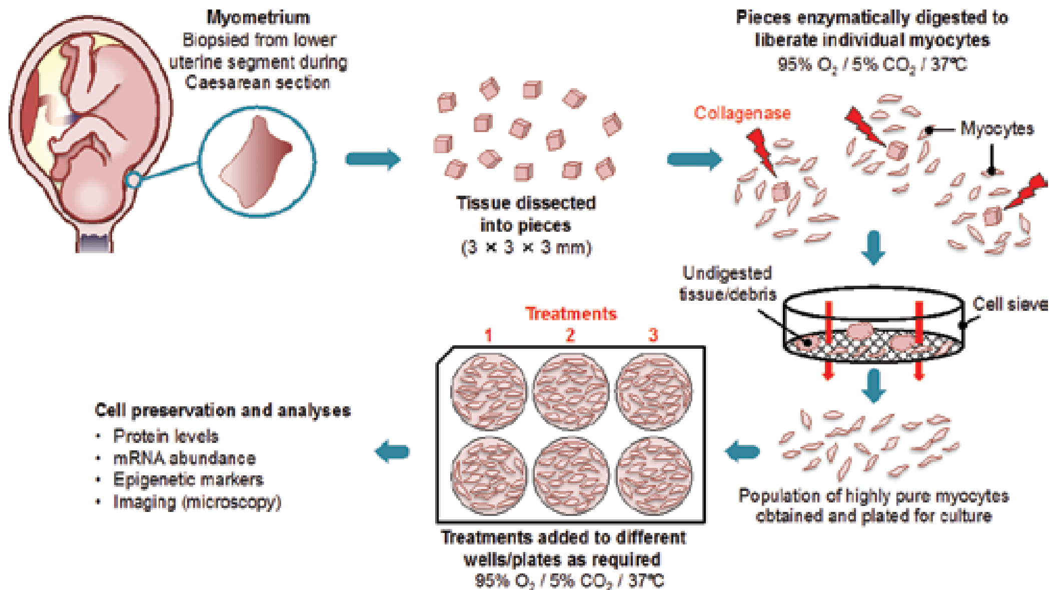

Figure 1. Human myometrial smooth muscle cell culture model. Human myometrial samples are obtained from lower uterine segment during elective Cesarean section of singleton pregnancies. The samples are cleared of serosa, fibrous or damaged tissue and visible blood vessels, and dissected into small pieces. The myometrial pieces are enzymatically digested down to single cells (collagenase XI, collagenase IA). Myometrial cells are then purified using a cell sieve and plated into culture plates containing smooth muscle medium (95% O_2/5% CO_2/37°C). The cultured myometrial cells are then subjected to desired treatments or experimental conditions, after which cells can be snap frozen or preserved for subsequent analyses.

including the oxytocin receptor (*OXTR*), estrogen receptor 1 (*ESR1*) and progesterone receptor (*PGR*) [24]. Additionally, hTERT-infected myometrial cells were responsive to both oxytocin and estrogen, thus further implying that they are a suitable *in vitro* model for investigating the molecular mechanisms that regulate uterine SMC gene expression [24].

Since hTERT cell lines have become popular among researchers, Soloff *et al*. [25] compared phenotypes of three telomerase-immortalized myometrial cell lines with the matching cells in primary culture, from which the immortalized cells were derived. Out of more than 10,000 expressed genes, only 1% of genes consistently exhibited expression changes in the telomerase-immortalized cell lines [25]. Furthermore, the comparison between primary and telomerase-immortalized cells revealed no significant differences in signaling pathways, such as epidermal growth factor (EGF)-stimulated phosphorylation of the EGF receptor, insulin-stimulated Akt phosphorylation, oxytocin and lysophosphatidic acid-stimulated extracellular signal-regulated kinase (ERK) 1 and 2 phosphorylation, myosin light chain (MYL) phosphorylation, and interleukin-1 induction of IκBα degradation [25]. Although this study found no significant difference between immortalized and primary cell lines, it has been suggested that immortalization could cause fundamental changes in the cells [26], and as such many studies use primary cells only at low passages (passage five and lower) [27].

Mosher *et al*. [26] established primary cultures of human myometrial cells isolated from paired upper and lower segment uterine biopsies for 10 passages and determined the expression of smooth muscle markers, fibroblast markers, contractile proteins or labor-associated

proteins over time. It was found that both upper and lower segment human myometrial cells stably expressed smooth muscle markers (α-SMA, calponin, caldesmon, tropomyosin) and fibroblast markers (vimentin, 1B10) to at least 10 passages [26]. Interestingly, comparison of paired upper and lower segment myometrial cells revealed that mRNA levels for Connexin 43 (*GJA1*), Prostaglandin-endoperoxide synthase 2 (*PTGS2*) and vimentin (*VIM*) were significantly higher in lower segment cells compared to upper segment cells [26]. These findings supported the concept of a functional regionalization of the upper and lower segment of the human uterus and indicated that both upper and lower segments should be examined in order to garner maximum insight into the mechanisms underlying human parturition. Furthermore, both cell populations retained their ability to respond to inflammatory stimuli from passage 1 through to passage 10, as demonstrated by increased expression of *PTGS2* and release of the pro-inflammatory chemokine, *CXCL8*, following treatment with the interleukin-1β (IL-1β) [26]. Mosher *et al*. [26] concluded that primary myometrial cells are viable and responsive for at least 10 passages, and therefore represent a useful tool for investigating human parturition. Nevertheless, this study did not to compare each passage with the fresh tissue to ascertain whether the cells were truly representative of the tissue of origin.

Recently, a study by Zaitseva *et al*. [28] compared gene expression profiles between myometrial and fibroid tissues, as well as SMCs isolated from these tissues that were cultured for up to three passages. It was found that 2055 genes were differentially expressed between all groups (fresh myometrial tissue, fresh fibroid tissue, myometrial SMCs at passage 0, fibroid SMCs at passage 0, myometrial SMCs at passage 3 and fibroid SMCs at passage 3) [28]. A total of 128 genes were found to be significantly different between fresh myometrial and fibroid tissues [28]. More than 1100 genes were significantly different between fresh tissues and cultured SMCs, with 648 genes common between both myometrial and fibroid SMCs at passage 0 and passage 3 [28]. These findings indicate that culture conditions significantly changed the gene expression profile of myometrial and fibroid SMCs, decreasing differences between the cells *in vitro* compared to the cells *in vivo* [28]. Furthermore, Zaitseva *et al*. [28] examined expression of *ESR1* and *PGR* in fresh myometrial and fibroid tissues, as well as cultured SMCs. Expression of both receptors was significantly decreased in cultured SMCs compared to fresh tissues [28]. Zaitseva *et al*. [28] concluded that myometrial and fibroid cell cultures provide a vital tool to study the uterus, but nevertheless, *in vitro* studies must be carefully planned and assessed to provide significant results.

4. Stretch-based culture systems

Throughout normal pregnancy the uterus increases several-fold in size by both hyperplasia and hypertrophy to accommodate the growing fetus and placenta [29, 30]. In 1982, Manabe *et al*. [31] applied mechanical stretching of the uterus to seven term patients who were not in labor by inserting and inflating a 150 mL rubber balloon. All seven patients showed significant cervical softening, as well as initiation and progress of labor [31]. Furthermore, analysis of amniotic fluid revealed that the uterine stretch initiated increased release of prostaglandin (PG) F (PGF) [31]. Based on these results, the *in vitro* model of pregnancy-induced uterine stretch has been used in multiple studies to investigate the effect of stretch on human SMCs [29, 30, 32, 33].

The *in vitro* model of pregnancy-induced uterine stretch involves growing SMCs in 6-well culture plates with a flexible collagen growth surface. The plates are placed in a computer-driven Flexercell strain unit, which generates a vacuum in order to deform the flexible growth surface. Through deforming the growth surface, cultured cells can be subjected to static stretch of 6, 11 or 16% for 1 or 6 h (**Figure 2**). Using this model, Sooranna *et al*. [30] investigated the effect of stretch on PG synthesis in non-pregnant, pregnant non-laboring and pregnant laboring primary human SMCs. Non-pregnant and pregnant laboring SMCs had significantly higher *PTGS2* mRNA levels than pregnant non-laboring SMCs [30]. When stretch was applied, *PTGS2* mRNA expression significantly increased in all three groups [30]. In additional studies using pregnant non-laboring SMCs, 6 h of stretch increased PTGS2 protein levels, increased PGI_2 metabolite and PGE_2 concentrations in the media, as well as decreased $PGF_{2\alpha}$ metabolites in the media [30]. Furthermore, following stretch there was increased activator protein-1 (AP-1) nuclear protein DNA binding activity in pregnant non-laboring SMCs [30]. These results provided evidence that increased PTGS2 activity, following stretch of human myocytes, occurs through activation of the AP-1 system [30].

Terzidou *et al*. [32] investigated the effect of stretch on the expression of *OXTR* mRNA and DNA binding of AP-1, CCAAT/enhancer binding protein (C/EBP)β, and nuclear factor-κB (NF-κB) transcription factors in non-pregnant, pregnant non-laboring and pregnant laboring primary

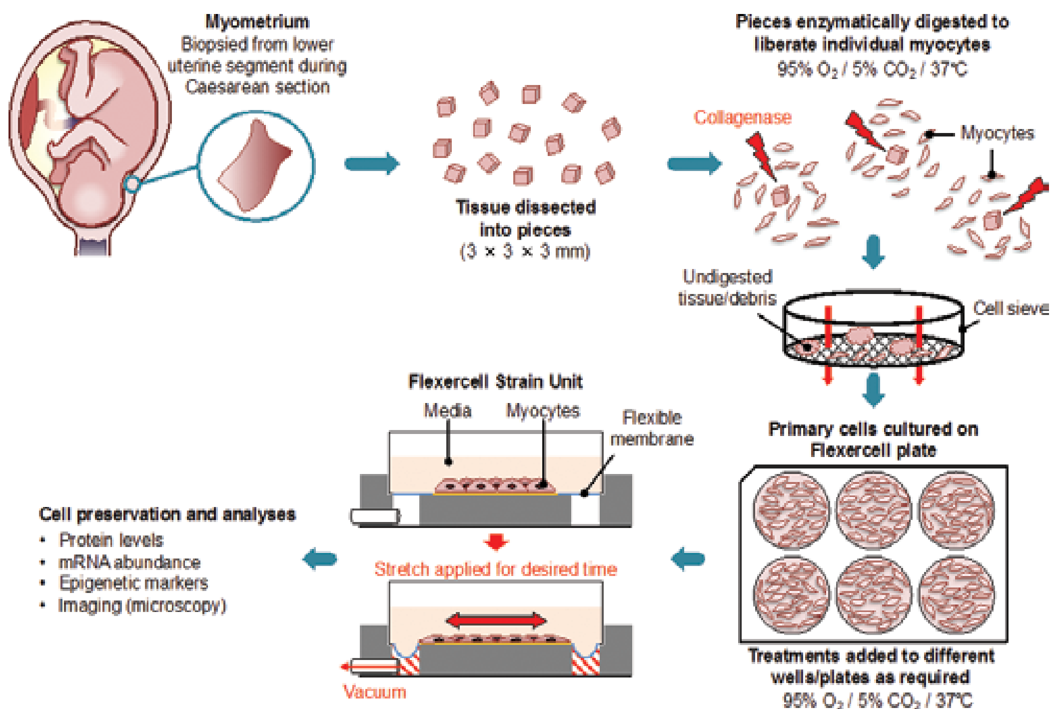

Figure 2. Flexercell stretch-based culture system. Primary myometrial cells, prepared from myometrial biopsies, are plated into 6-well culture plates with a flexible collagen growth surface (95% O_2/5% CO_2/37°C). The plates are placed in a computer-driven Flexercell strain unit capable of generating a vacuum that deforms the flexible growth surface. Through deforming the growth surface, cultured cells can be subjected to varying degrees of stretch, as well as treated with exogenous agents. The cells can then be snap frozen or preserved for subsequent analyses.

human SMCs. Pregnant laboring SMCs had significantly higher *OXTR* mRNA expression compared to non-pregnant and pregnant non-laboring SMCs [32]. Application of stretch to pregnant non-laboring human myocytes significantly increased *OXTR* mRNA levels and increased *OXTR* promoter activity [32]. Conversely, stretching of non-pregnant and pregnant laboring human myocytes did not affect *OXTR* mRNA levels [32]. Increased promoter activity within the pregnant non-laboring cells was associated with increased DNA binding of C/EBPβ and AP-1 [32]. Overexpression of C/EBPβ led to increased *OXTR* promoter activity [32]. Based on these results, the authors concluded that stretch of pregnant non-laboring SMCs increases *OXTR* expression through increased C/EBPβ DNA binding [32]. More importantly, these results imply that stretch contributes to the increase in *OXTR* expression at the time of labor during human parturition [32].

Loudon *et al*. [29] investigated the effect of stretch on the expression of interleukin-8 (IL-8) in non-pregnant, pregnant non-laboring and pregnant laboring primary human SMCs. Previous studies have shown that labor is associated with increased synthesis of pro-inflammatory cytokines, including IL-8 [34, 35]. Pregnant laboring SMCs have significantly higher *IL-8* mRNA expression than non-pregnant and pregnant non-laboring SMCs [29]. The application of stretch, via the Flexercell strain unit, significantly increased *IL-8* mRNA expression in myocytes from all three groups [29]. Furthermore, stretch-induced increase in *IL-8* mRNA expression was concurrent with increased IL-8 levels in the culture supernatant, as well as increased promoter activity [29].

Previous pregnancy-induced uterine stretch studies have shown that *PTGS2* and *IL-8* mRNA expression increases *in vitro* following mechanical stretch of uterine SMCs [29, 30]. Therefore, Sooranna *et al*. [33] further investigated whether IL-1β and mechanical stretch increase the myometrial expression of *PTGS2* and *IL-8* through mitogen-activated protein kinase (MAPK) activation, and whether these effects were synergistic. Pregnancy-induced uterine stretch increased *PGHS-2* and *IL-8* mRNA expression through ERK1/2 and p38 MAPK pathways [33]. Likewise, IL-1β increased *PGHS-2* mRNA expression through ERK1/2 and p38 MAPK pathways [33]. However, IL-1β increased *IL-8* mRNA expression through the ERK1/2 pathway only [33]. Furthermore, there was no evidence of a synergistic effect of IL-1β and stretch on *PTGS2* and *IL-8* mRNA expression [33].

These studies using the *in vitro* model of pregnancy-induced uterine stretch demonstrated that mechanical stretch is able to increase expression of various genes. Namely, stretch increased *PTGS2* and *IL-8* expression through MAPK-dependent signaling, while *OXTR* expression was increased through a MAPK-independent mechanism [29, 30, 32, 33]. As such, Lei *et al*. [36] investigated whether progesterone was able to inhibit stretch-induced MAPK activation, as well as *PTGS2* mRNA expression and protein synthesis [36]. Pre-incubation of primary SMCs with progesterone did not inhibit stretch-induced ERK1/2 activation or *PTGS2* mRNA expression [36]. Additionally, it was determined that mechanical stretch did not modify the ability of progesterone to modulate progesterone-responsive gene expression, activate a progesterone response element or inhibit IL-1β-driven *PTGS2* mRNA expression [36]. Mechanical stretch was found to decrease *PR-T* and *PR-B* mRNA expression via NF-κB activation; however, stretch does not appear to inhibit progesterone action [36]. These findings, derived from the *in vitro* model of pregnancy-induced stretch, suggest that stretch is not responsible for the functional progesterone withdrawal observed with the onset of human labor [36].

In a similar approach, Dalrymple *et al*. [37] used the Flexercell strain unit to subject SMCs to tonic stretch of 25% for 1, 4 and 14 h. Mechanical stretch for 14 h increased basal calcium entry, as well as cyclopiazonic acid-induced calcium/manganese entry [37]. Furthermore, prolonged tonic stretch increased transient receptor potential canonical (TRPC) channel gene expression and protein levels [37]. TRPC proteins are known components of store-operated calcium entry and are expressed in human myometrium during pregnancy [38–40]. Since an increase of calcium entry leads to increased contractility of smooth muscle, this study reveals a possible pathway by which uterine function can be modified in response to the growing fetus throughout the pregnancy [37].

The studies discussed thus far have utilized human primary SMCs to investigate the effects of stretch. Recently, Lee *et al*. [41] used hTERT cells to investigate whether stretch facilitates peripheral leukocyte extravasation into the term myometrium through the release of various cytokines by uterine myocytes. In the study, hTERT cells were grown on flexible-bottom culture plates and static stretch of 25% was applied for 24 h [41]. It was found that mechanical stretch resulted in secretion of multiple cytokines and chemokines, including IL-6, IL-12p70, migration inhibitory factor (MIF), C-X-C motif ligand (CXCL) 8, CXCL1, granulocyte colony-stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor subunit B (PDGF-bb) [41]. Furthermore, stretch-induced cytokines increased leukocyte adhesion to the endothelium of the surrounding uterine microvasculature by stimulating the expression of endothelial cell adhesion molecules, as well as by directing the transendothelial migration of peripheral leukocytes [41]. These results provide evidence for the mechanical regulation of leukocyte migration from the uterine blood vessels toward the myometrium [41].

Up until now, majority of *in vitro* cell-based assays have utilized traditional two-dimensional (2D) culture, where cells are grown as a monolayer on a flat surface. Although 2D culture has proven to be an effective method for cell-based studies, and researchers have been able to elucidate many aspects of fundamental biology, the limitations of 2D culture have gradually been recognized. In the *in vivo* environment, the majority of cells are surrounded by other cells and extracellular matrix in a three-dimensional (3D) arrangement. In recognition of this, studies are increasingly exploring the application of 3D culture techniques in an effort to more closely mimic the *in vivo* environment of the cells.

5. 3D culture models

3D culture is the growing of cells in an artificial environment that permits cells to grow and interact with their surroundings in all three dimensions. Often this involves growing SMCs on or in a scaffold comprised of extracellular matrix proteins, which is designed to resemble the *in vivo* tissue environment. Through mimicking the *in vivo* environment, the scaffold permits cells to attach, attain the right configuration and migrate or differentiate. Additionally, the 3D platform can provide cells with an environment comprising the growth factors, cytokines and some extracellular matrix proteins produced by the cells during their growth. Over the past several years, immense effort has been put into the creation of various 3D cell culture platforms, for instance protein gels such as Matrigel and collagen, which reconstruct extracellular

matrix composition [42]; polymer scaffolds, which mimic tissue structure and material properties [43]; hanging drop spheroids, which use water tension in liquid droplets to aggregate cells into spheroids [44–46]; round bottom plates, which use plate geometry to aggregate cells in spherical bottom wells [46]; as well as nano-patterned plates. Although these 3D cell culture platforms recreate tissue environments to varying degrees, they still have technical and cost limitations; for instance, long production times, the need for specialized equipment, as well as the produced 3D structures primarily being limited to spheroids, as opposed to more complex shapes that would better enable investigations into uterine smooth muscle contractility, such as rings or strips [47].

Research using primary SMCs to develop 3D culture systems was first performed by Young *et al*. [48] in 2003. Young *et al*. [48] performed single- and two-mesh experiments to study human myometrial physiology. In the single-mesh experiments, myometrial SMCs were seeded onto a polyglactin-910 (Vicryl) mesh after several passages [48]. In order to avoid contact with the plastic dish, each mesh was transferred to another culture dish after several days and suspended [48]. SMCs grew into and filled the pores of the mesh by repetitive proliferation, retraction, and proliferation [48]. A confluent, 3D tissue was attained 10–14 days after the initial seeding of the mesh [48]. In the two-mesh experiments, myometrial SMCs were seeded onto two layers of mesh and then cultured as described above [48]. SMCs not only grew into and filled the pores of each mesh, but also bridged between the two layers of mesh [48]. A confluent, 3D tissue was attained 2–3 weeks after the initial seeding of the mesh [48]. In the single-mesh experiments, average membrane potential of the cells was -35 ± 6 mV, and the thickness of the tissue was 9–40 μm (1–8 cells thick) within the pores of the mesh. In the two-mesh experiments, the bridging SMCs were able to sustain a tension of 5 g/cm^2 before separation of the two meshes occurred, as well as coordinated contractions of 40–200 cells [48]. SMCs grown in 3D using Vicryl mesh would therefore provide a model system for investigating the physiology of cell-to-cell interactions in human myometrium.

Malik *et al*. [49] developed human myometrium 3D cultures using a 3D collagen gel and examined the response of these cultures to external stimuli. Myometrial cells in 3D culture maintained the *in vivo* characteristics of the tissues they were derived from, as well as the characteristic fusiform of the SMCs [49]. That is, the cells exhibited long, tapering ends, resulting in a highly spindle-shaped phenotype, which stained positive for smooth muscle-specific α-actin and F-actin fibrils [49]. Previous studies have shown that TGF-β3 treatment of myometrial cells in 2D culture resulted in up-regulation of various extracellular matrix genes [50, 51]. During 3D culture of myometrial SMCs, TGF-β3 treatment produced a similar up-regulation of extracellular matrix genes, thus demonstrating that the cells in 3D culture were similarly bioactive [49]. These results indicate that cells in 3D culture can be used to investigate aspects of human parturition and to assess the effectiveness of various treatments.

More recently, Souza *et al*. [52] used magnetic 3D bio-printing to create more complex shapes, such as rings, to imitate uterine smooth muscle. The process involved magnetizing primary SMCs with a biocompatible nanoparticle assembly consisting of gold, iron oxide, and poly-L-lysine, then aggregating the cells into hollow rings using magnetic forces [52]. Once aggregated, the cells interacted and built extracellular matrix to recapitulate native tissue environments [52]. The bio-printed uterine rings contracted immediately after they were

removed off the magnet, which is consistent with the nature of the SMCs [52]. Furthermore, the tocolytic compounds, indomethacin and nifedipine, dose-dependently inhibited contractions in the myometrial smooth muscle rings [52]. Magnetic 3D bio-printing therefore constitutes a novel model system that can serve as a valuable toolfor investigating human parturition [52].

A recent development in the sphere of 3D culture is the use of biological scaffolds derived from decellularized tissues and organs. Decellularization involves devoiding a tissue or organ of its inhabiting cells, leaving behind the extracellular matrix scaffold of the original tissue. This naturally occurring 3D biologic scaffold can be then recellularized with primary SMCs that will differentiate into the original type of tissue (**Figure 3**). This approach has been successfully used in animal studies as well as in human clinical applications [53–59], and more recently, in myometrium [60]. Young *et al*. [60] used tissue-engineering techniques to reconstruct allo- and xeno-neo-myometrium from isolated rat and human myocytes and

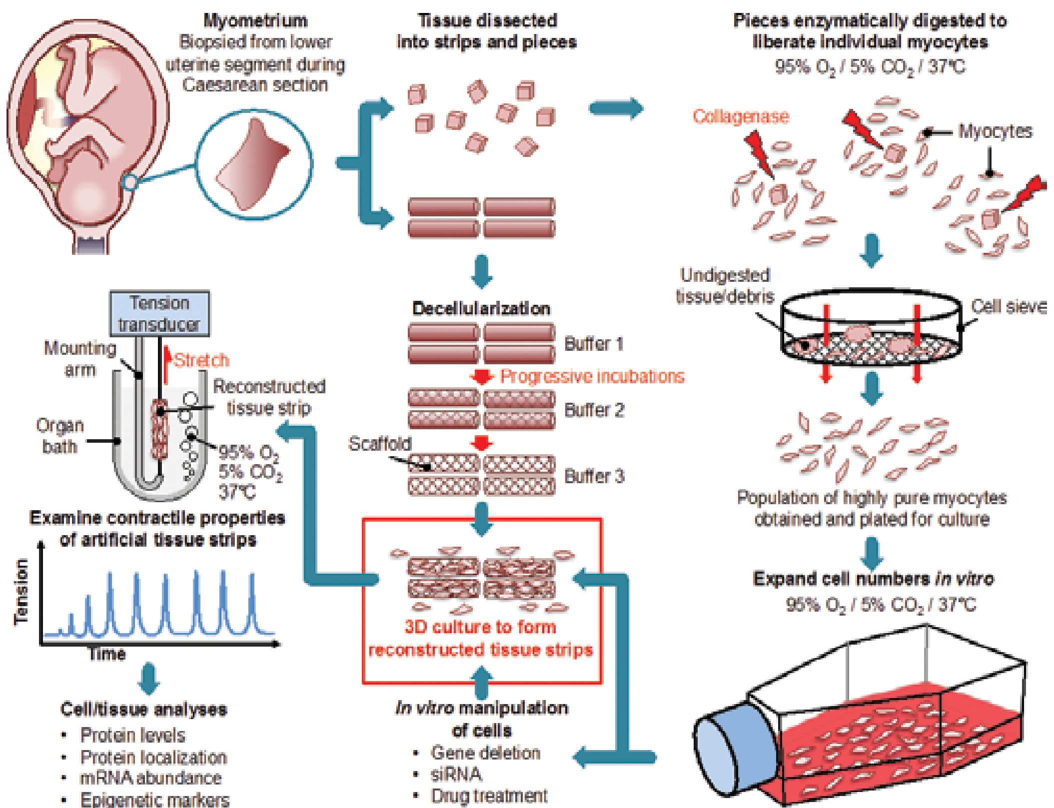

Figure 3. Application of 3D culture and decellularization to reconstruct tissue strips. Human myometrial biopsies are utilized to prepare primary myocyte cultures, as well as dissected into strips. Primary myocyte number is amplified using traditional 2D culture, while the tissue strips undergo decellularization to obtain scaffolds. Isolated primary myocytes are then seeded back into the scaffolds and the 3D culture maintained. Once the myocytes have permeated the scaffold, the contractility of the reconstructed tissue strips can be examined using contraction bioassays. To investigate signaling networks, primary myocytes can be modified while in 2D culture to observe the effect on contractility. The reconstructed strips can also be subjected to desired treatments or experimental conditions. The strips can then be snap frozen or preserved for subsequent analyses.

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scaffolds. Allo-neo-myometrium was made from components of the same species (rat SMCs with rat scaffold; human SMCs with human scaffold), while xeno-neo-myometrium was made across species (human SMCs with rat scaffold) [60]. Myocytes were isolated using collagenase digestion, while scaffolds were isolated using ethanol/ trypsin protocols [60]. Isolated myocytes were amplified using monolayer culture and then cultured back into the scaffolds [60]. When human myocytes were cultured into human scaffold (allo-neo-myometrium), the myocytes overgrew each other after prolonged culture; nevertheless, they then separated, retracted, and never attained a thick layer of myocytes on the scaffold surface [60]. Rat myocytes cultured into rat scaffolds (allo-neo-myometrium) only formed multicellular layers on the surface following artificially created defects in rat scaffold [60]. Human myocytes grown on rat scaffold (xeno-neo-myometrium) formed multicellular layers on the surface [60]. These surface multicellular layers were thick, and more importantly, bundles of cells were observed to depths of 500 μm within the rat scaffold [60]. This xeno-neo-myometrium revealed structural integrity, good cellularity, as well as excellent cellular viability [58]. Interestingly, isometric contractility experiments revealed that human myocytes on rat scaffold (xeno-neo-myometrium) produced coordinated contractions, while human myocytes cultured into human scaffold (allo-neo-myometrium) did not [60]. The construction of neo-myometrium, prepared through the recellularization of decellularized scaffolds, is a model system that enables researchers to examine the role of extracellular matrices in contractility [60]. Additionally, through performing genetic manipulation of the SMCs prior to recellularization, the approach may enable researchers to investigate the role of different genes/proteins in myometrial contractility. Despite being an exciting avenue for future exploration, the production of neo-myometrium is complex, and to date, researchers have primarily utilized myometrial tissue strips or pieces to investigate the transition between uterine quiescence and contractility.

6. Tissue strip models

A model system frequently used to study pregnant human myometrium is the contraction bioassay, which uses strips of myometrium to study contractility *ex vivo*. Due to being freshly isolated intact pieces of tissue, *ex vivo* tissue strips arguably represent the *in vivo* phenotype more closely than other model systems. During the contraction bioassay, myometrial biopsies are typically obtained from the lower uterine segment of term singleton pregnancies during Cesarean section then dissected into strips (approximately 10 × 2 × 2 mm). The myometrial strips are connected to force transducers and lowered into organ baths containing a salt solution, such as Krebs–Henseleit buffer, which is continuously bubbled with 95% O_2/5% CO_2 and maintained at 37°C (pH 7.4). The contraction assay apparatus enables passive tension to be applied to individual strips. As a general protocol, myometrial strips are equilibrated for the first hour, during which the buffer is exchanged every 10 min and tension (1 g) is re-applied to strips after each wash. Following the equilibration period, strips are then left to develop spontaneous rhythmic contractions *ex vivo*. Once spontaneous rhythmic contractions have developed, the myometrial tissue strips can be used to analyze the effect of treatments on contractility in real-time [61–63], as well as capture dynamic events that occur during contraction and relaxation, such as protein phosphorylation [64, 65] (**Figure 4**).

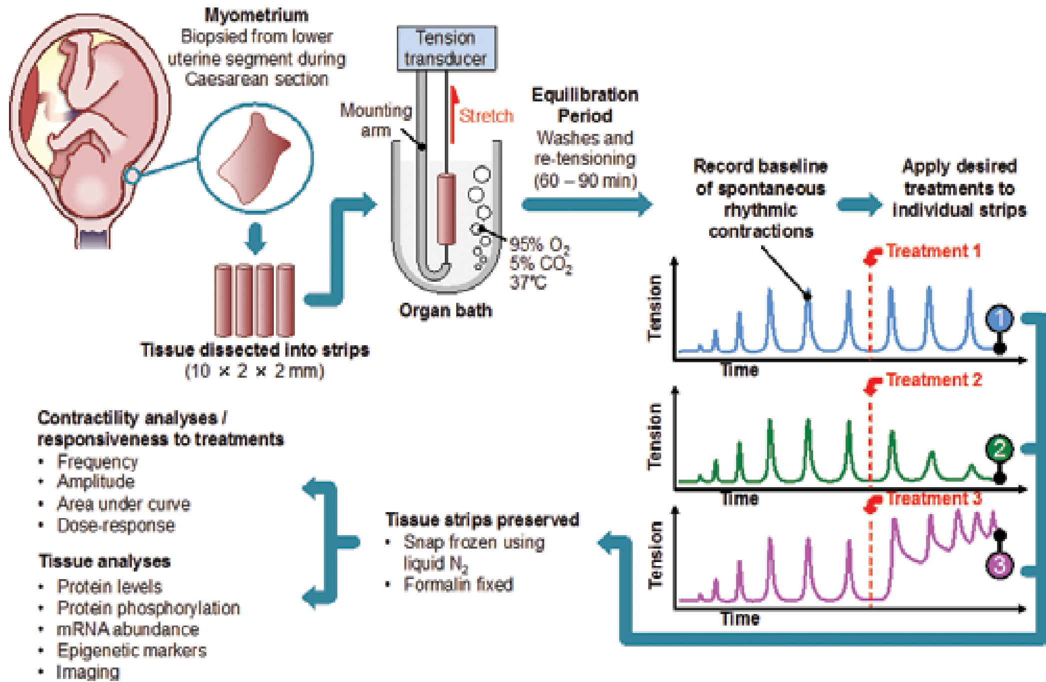

Figure 4. Myometrial tissue strip contraction bioassay. Human myometrial samples, collected during Cesarean section, are cleared of serosa, fibrous or damaged tissue and visible blood vessels then dissected into strips (~10 × 2 × 2 mm). The strips are then connected to a force transducer and lowered into organ baths containing a salt solution, which is continuously gassed with 95% O_2/5% CO_2 and maintained at 37°C (pH 7.4). The strips are equilibrated for the first hour, with washes and re-tensioning every 10 minutes. Strips are then left to develop spontaneous rhythmic contractions *ex vivo*. Upon recording a baseline of consistent contractions, desired treatments are applied to individual strips and the effects recorded. For subsequent analyses, strips can be snap frozen or preserved at the completion of the study, or at specific stages in relation treatment or contraction status.

Multiple studies have used myometrial tissue strips to analyze the effect of various treatments on contractility. Ruddock *et al.* [66] exposed contracting myometrial strips to progesterone or 17-alpha-hydroxyprogesterone caproate (17P) to determine whether these hormones directly inhibit human uterine contractility *in vitro*. Progesterone dose-dependently-inhibited uterine contractility, while 17P dose-dependently-stimulated contractility [66]. Similarly, Anderson *et al.* [61] also found that progesterone inhibited uterine contractility *in vitro*, however, in contrast to Ruddock *et al.* [66], they reported that 17P had no effect on uterine contractility [61]. Sexton *et al.* [67] also reported that 17P did not affect the contractility of pregnant or non-pregnant human myometrial strips. Although bioassay evidence suggests that 17P does not affect human uterine contractility directly, numerous clinical trials have showed that administration of 17P is successful in preventing preterm delivery in high-risk women [68–70]. This positive clinical outcome could be mediated through long-term genomic pathways that are not imitated during comparatively short-term organ bath studies. During organ bath studies, Anderson *et al.* [61] found that myometrial contractility began to decrease after 8 h incubation, presumably due to cell death and lack of energy substrates in the tissue; however, longer term studies by Young *et al.* [71] have shown that

culturing myometrial strips under tension can maintain spontaneous contractility and oxytocin responsiveness for up to 7 days.

Numerous tocolytics have also been tested using the contraction bioassay. Baumbach *et al*. [62] performed studies investigating the effects of a variety of contraction blocking drugs on term, nonlaboring myometrium *in vitro,* in both the presence and absence of progesterone. Progesterone alone had little inhibitory effect on contractility [62]. Nifedipine and indomethacin both significantly inhibited myometrial contractility alone, and to a greater extent when combined with progesterone [62]. These results indicated that combinations of progesterone with nifedipine or indomethacin might help in the prevention of preterm birth [62]. Another group also performed *in vitro* studies using myometrial strips to determine the effect of progesterone and nitric oxide alone or in a combination [72]. Previous study had shown that nitric oxide inhibits human myometrial contractions *in vitro* [73]. The combination of progesterone with nitric oxide was significantly more effective at inhibiting uterine contractility than progesterone alone or nitric oxide alone [72]. These findings suggest that administration of progesterone in combination with nitric oxide could be more successful in the treatment of preterm labor than either agent alone and demonstrates that the contraction bioassay is an effective model for elucidating synergisms between different tocolytics. This could translate into the development of more effective strategies for the prevention of preterm birth.

Recently, Paul *et al*. [74] developed the first drug delivery system targeting the pregnant uterus for preventing preterm birth. The team created liposomes loaded with various tocolytics, including nifedipine, salbutamol and rolipram, as well as the contraction-promoting agent, dofetilide, and conjugated to an antibody that specifically recognized an extracellular domain of the oxytocin receptor (OTR) [74]. Utilizing the contraction bioassay, Paul *et al*. [74] demonstrated that OTR-targeted liposomes loaded with nifedipine, salbutamol or rolipram consistently abolished human myometrial contractions *in vitro*, while OTR-targeted liposomes loaded with dofetilide increased contraction duration [74]. Non-targeted control liposomes loaded with these agents had no effect on contractility *in vitro* [74]. Paul *et al*. [74] also utilized the contraction bioassay to demonstrate that targeted liposomes were similarly effective against mouse uterine tissue *in vitro*.

The contraction bioassay model can also be used to examine tissue responsiveness and contractility after short- or long-term pre-treatment of myometrial strips. Fetalvero *et al*. [75] incubated myometrial tissue strips for 48 h in the presence or absence of the prostacyclin analog, iloprost. Initially, tissue strips displayed irregular and infrequent spontaneous contractions; as such, oxytocin had to be added to initiate rhythmic contractions [75]. The study revealed that long-term pre-treatment of myometrial strips with iloprost resulted in increased contractile responsiveness to oxytocin [75]. These results suggest that endogenous myometrial prostacyclin could play an important role in regulating myometrial activation, a crucial step in the initiation and progression of parturition [75]. Tyson *et al*. [63] revealed that corticotropin-releasing hormone (CRH) inhibited contractility in preterm and term myometrial strips *in vitro*. Pre-treatment of term myometrial tissue strips for 1 h with progesterone significantly increased CRH-induced relaxation [63]. Furthermore, pre-treatment of term myometrial tissue strips for 1 h with rolipram, a selective phosphodiesterase-4 inhibitor, significantly increased both CRH- and salbutamol-induced relaxation [63]. This implies that CRH might be a facilitator in the transition of the myometrium from relaxation to contraction.

In a novel application, Paul *et al*. [64] adapted the contraction bioassay to study the molecular regulatory events associated with contraction and relaxation in term pregnant human myometrium. In order to examine rapid phosphorylation and de-phosphorylation events that occur in phase with contractions, myometrial tissue strips were snap frozen at specific stages during the development of spontaneous contractions, including: (1) prior to the onset of any contractions, (2) at peak contraction and (3) during maximum relaxation between individual contractions [64]. The results showed that Caldesmon and ERK 1/2 were both phosphorylated during contractions and de-phosphorylated during periods of relaxation [64]. This application of the contraction bioassay provided a unique insight into phasic phosphorylation events that occur during spontaneous rhythmic myometrial contractions in humans. The study emphasized the importance of considering the contractile status of the tissue (contracted versus relaxed) during experimental design and interpretation of results [64]. Hudson *et al*. [65] applied the methodology developed by Paul *et al*. [64] to measure phosphorylation of MYL kinase (MYLK) and myosin phosphatase (MYLP) during spontaneous and oxytocin receptor (OXT)-stimulated phasic myometrial contractions *in vitro*. MYLK is activated by calcium-calmodulin, while MYLP is inhibited by phosphorylation of its myosin-binding subunit (MYPT1) by calcium-independent mechanisms [65]. Hudson *et al*. [65] showed that in fresh human myometrial tissue strips, spontaneous and OXT-stimulated phasic contractions were associated with Rho-associated kinase (ROCK)-dependent increases in phosphorylation of MYL and MYPT1 [65]. These findings provide new insight into uterine physiology and increase our understanding of the control of human myometrial activity.

While tissue strip models enable the real-time assessment of contractile properties of the myometrium, assessment of contractility is not always necessary. For such studies, preparation of myometrium as small pieces, often called explants, is an attractive alternative as it represents a similar *ex vivo* model, while avoiding the complexity of subjecting the tissue to stretch.

7. Tissue pieces/explant models

Explants are pieces of tissue cut from an animal or plant and used to initiate a culture. The term 'explant' is perhaps incorrectly applied to human myometrium, at least in the context of this model, in that the small myometrial pieces are not used to establish an ongoing culture. Rather, the tissue pieces are used as an *ex vivo* model on which to directly perform relatively short-term studies (<7 days). For this reason, tissue 'pieces' is perhaps more appropriate, and will be utilized hereafter. When utilized to study human parturition, tissue piece models typically involve the collection of non-laboring or laboring pregnant human myometrium during Cesarean section delivery, which is then dissected into small pieces (~3 × 3 × 3 mm) and incubated in Petri dishes or multi-well plates containing a nutrient media, such as Dulbecco's Modified Eagle's Medium (DMEM) [76–79]. The tissue pieces are then subjected to desired treatments or experimental conditions, after which the tissue pieces are snap frozen or preserved for subsequent analyses. Utilizing the tissue piece model, researchers can examine the effect of multiple treatments on a broad range of endpoints, including gene expression, protein levels and epigenetic modifications, as well as section the tissue to perform visualization studies (**Figure 5**). The number of treatments and endpoints that can be examined is limited only by

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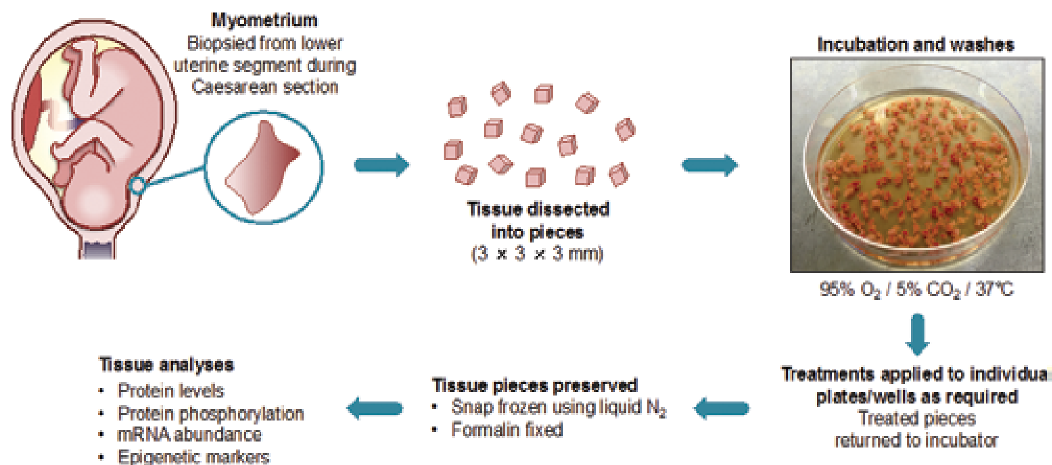

Figure 5. Myometrial tissue piece culture model. Human myometrial samples, collected during Cesarean section, are cleared of serosa, fibrous or damaged tissue and visible blood vessels, then dissected into pieces (~3 × 3 × 3 mm). The tissue pieces are incubated in Petri dishes containing a nutrient media (95% O_2/5% CO_2/37°C). Tissue pieces are then subjected to desired treatments or experimental conditions, after which the tissue pieces are snap frozen or preserved for subsequent analyses.

the amount of biopsied tissue available. The versatility and relative simplicity of the model has led to its extensive application to study human parturition. Fortunately, evidence indicates that the tissue piece model is more representative of myometrium *in vivo* than other *in vitro* models.

Georgiou *et al*. [80] compared gene expression in fresh myometrium, which was frozen at the time of Cesarean section, to *ex vivo* myometrial pieces, passage 4 primary myometrial cells, and hTERT myometrial cells. While whole-genome transcriptome analysis revealed that none of the *in vitro* models overlapped with the fresh tissue, in a principal component analysis plot [80], the gene expression profile of the tissue pieces most closely resembled that of the fresh tissue [80]. Upon direct comparison; 1444 genes varied between the *ex vivo* tissue pieces and fresh tissue; 3840 genes varied between passage 4 primary myometrial cells and fresh tissue; and 4603 genes varied between hTERT myometrial cells and fresh tissue [80]. A total of 555 genes varied commonly upon comparing all three *in vitro* groups (*ex vivo* tissue pieces, passage 4 primary myometrial cells and hTERT myometrial cells) to fresh tissue [80]. Georgiou *et al*. [80] validated the microarray results by examining genes associated with reproductive function and parturition, including *PTGS2*, *OXTR*, *PGR* and *GJA1*, as well as genes associated with the smooth muscle phenotype, including alpha smooth muscle actin (*ACTA2*) and MYL kinase (*MYLK*) [80]. *PGR*, *OXTR* and *GJA1* mRNA expression did not vary significantly between the *ex vivo* tissue pieces and fresh tissue [80]. In contrast, *PGR* mRNA expression significantly decreased in both passage four primary myometrial cells and hTERT myometrial cells, compared to fresh tissue [80]. Moreover, in passage four primary myometrial cells, *OXTR* and *GJA1* mRNA expression significantly decreased and increased, respectively, compared to fresh tissue [80], while *PTGS2* mRNA expression significantly increased in both tissue pieces and hTERT myometrial cells, compared to fresh tissue [80]. This was consistent with the gene ontology analysis, which indicated that there was an increase in genes associated with inflammation in all three *in vitro* models compared to fresh tissue [80]. *ACTA2*

and *MYLK* mRNA expression remained unchanged in *ex vivo* tissue pieces, compared to fresh tissue, whereas both were significantly decreased in hTERT myometrial cells, compared to fresh tissue [80]. Georgiou *et al*. [80] therefore concluded that myometrial *ex vivo* tissue pieces represent a superior model for studying human parturition over that of cell culture models.

It is important to note, however, that while evidence indicates that *ex vivo* tissue piece models are more representative of *in vivo* tissue than other models, recent studies demonstrate that *ex vivo* tissue pieces are not invulnerable to culture-induced changes. Ilicic *et al*. [81] examined whether myometrial tissue pieces undergo changes in key parturition-associated genes (*ESR1*, *PTGS2* and *OXTR*) upon being incubated for 48 h *ex vivo* (DMEM, 95% O_2/5% CO_2, 37°C, pH 7.4). Compared to fresh term non-laboring myometrium, the abundance of *ESR1* and *PTGS2* mRNAs significantly increased after 48 h incubation [81], while *OXTR* expression, which was high in the fresh non-laboring tissue, significantly decreased after 48 h incubation [81]. These changes are of importance as myometrial expression of *ESR1* [76, 82] and *PTGS2* [83, 84] increase with the onset of labor, while OXTR expression has been reported to decrease with advanced labor [84–87]. The gene expression changes observed during *ex vivo* incubation of term non-laboring myometrial tissue pieces are therefore consistent with the tissue transitioning to a labor-like state.

In light of these findings, Ilicic *et al*. [88] further examined whether myometrial tissue pieces undergo culture-induced changes in *PGR* isoform expression that are consistent with transition to a pro-contractile, labor-like phenotype. It was revealed that progesterone receptor A (*PR-A*) mRNA abundance increased after just 1 h culture, whereas progesterone receptor B (*PR-B*) mRNA abundance remained constant [88]. The net effect was a statistically significant increase in the *PR-A*/*PR-B* expression ratio after just 6 h of *in vitro* incubation [88]. This was consistent with previous reports that PR-A protein abundance increased during labor, while levels of PR-B were not altered by labor status [89]. Furthermore, it has been shown that the onset of labor is associated with increased abundance of *PR-A* mRNA, and an increase in the *PR-A*/*PR-B* expression ratio in term human myometrium [82]. The observation that *PR-A* mRNA abundance and the *PR-A*/*PR-B* expression ratio significantly increased during *in vitro* incubation is therefore once again consistent with the non-laboring tissue pieces transitioning to a labor-like state as a consequence of *in vitro* conditions [88]. Performing *in vitro* experiments using myometrium that is in a state of flux is not ideal, and raises the possibility that the relevant literature may contain findings reported for ‘non-laboring’ tissue that were in fact obtained with myometrium that was actively transitioning, or had already transitioned to, a laboring phenotype. To address this, the authors sought to identify culture conditions that could be implemented to maintain a non-laboring state, whereby *ex vivo* myometrial tissue pieces retained high *OXTR* mRNA expression, low *ESR1*, *PTGS2* and *PR-A* mRNA expression, as well as a low *PR-A*/*PR-B* expression ratio (<1; a *PR-B* dominant state), thereby providing a more appropriate *in vitro* model for conducting studies into myometrial biology [81, 88]. Adding progesterone to culture media prevented the culture-induced increase in *ESR1*, but failed to prevent culture-induced increases in *PTGS2* [81]. Culture-induced decrease in *OXTR* expression was prevented by supplementing media with PMA, however, this was concurrent with further up-regulation of *PGTS2* expression toward a laboring phenotype [81]. Culture-induced increase of *PTGS2* expression *in vitro* was not counteracted by any of the treatments examined, including

supplementation with NF-κB inhibitors [81]. A combination of progesterone and estrogen prevented the culture-induced increase in *PR-A*, but failed to prevent significant increase in the *PR-A*/*PR-B* expression ratio as *PR-B* expression was also reduced [88]. Stretch blocked the effects of steroids on *PR-A* expression [88]. Perhaps most importantly, supplementing media with the histone deacetylase inhibitor (HDACi), trichostatin A (TSA), prevented the culture-induced functional progesterone withdrawal phenomenon by maintaining a low *PR-A*/*PR-B* expression ratio, consistent with maintenance of a non-laboring phenotype in the *ex vivo* tissue pieces [88]. Similarly, Georgiou *et al*. [80] attempted to mimic physiological conditions as closely as possible by supplementing culture media with steroids and the adenylyl cyclase agonist, forskolin, but found that this had no effect on maintaining *PGR* levels *in vitro*. Culture-induced changes in *ESR1*, *PTGS2*, *OXTR* and *PGR* expression could not be controlled simultaneously [81, 88]. The implications are potentially significant as findings made using 'non-laboring' tissue may in fact have been generated using tissue that was no longer representative of the non-laboring phenotype, which could affect experimental outcomes.

Interestingly, rapid transition away from the non-laboring phenotype could be a requisite for contraction bioassay models. That is, upon suspending strips of freshly isolated term, non-laboring myometrium in organ baths, connected to force transducers, the strips initially show little to no contractility. Spontaneous contractions then progressively develop over time, ultimately becoming consistent and rhythmic after 1–2 h. This suggests that the tissue strips may be rapidly transitioning away from a non-laboring phenotype toward a labor-like phenotype [61, 63–67, 71, 74]. Additional studies are necessary to determine whether this indeed is the case.

Model	Advantages	Disadvantages
Primary myometrial smooth muscle cell culture model	• Cost effective • Straightforward/easy procedure • Easy downstream processing (cell extraction, imaging of cells)	• Not representative of 3D *in vivo* environment • Reduced cell-to -cell interaction • Short period of viability • Low biological relevance • Susceptible to contamination
Immortalized myometrial smooth muscle cell culture model	• Can be maintained for prolonged periods • Extended and indefinite growth *in vitro* • Cost effective • Increased growth rate • Easy downstream processing	• Not representative of 3D *in vivo* environment • Immortalization alters cell characteristics and functions • Low biological relevance • Susceptible to contamination • Decreased cell size
Stretch-based culture model (Flexercell)	• Mimics *in vivo* stretch • Defined and controlled deformations of cells *in vitro* • Straightforward/easy procedure	• Not representative of 3D *in vivo* environment • Reduced cell-to -cell interaction • Low biological relevance • Susceptible to contamination

Model	Advantages	Disadvantages
3D culture model	• Depicts 3D environment • Cell-to-cell interaction • Cells interact with extracellular matrix • More likely to represent the *in vivo* phenotype	• Expensive • Long production times • Large array of scaffold materials to trial and optimize • Requires specialized equipment • Lacks complex shapes • Long downstream processing • Extraction and imaging of cells hampered by culture depth
Tissue strip model (contraction bioassay)	• Representative of *in vivo* phenotype in the short-term • Multiple treatments can be assessed • Real-time assessment of contractility • Effects of treatments observed in real-time	• Tissue-to-tissue variation • Organ baths not sealed, therefore susceptible to contamination • Decreased myometrial contractility following prolonged incubation • Complex procedure • Requires expensive specialized equipment (organ bath contraction apparatus, analysis software) • Limited capacity to manipulate cells prior to the contraction assay
Tissue pieces model	• More representative of the *in vivo* phenotype than 2D cultured cells • Cost effective • Straightforward/easy procedure • Controlled culture conditions • Multiple treatments can be assessed simultaneously	• Tissue-to-tissue variation • Long downstream processing (mRNA/protein isolation, tissue sectioning and imaging) • Unable to assess stretch/contractility as an endpoint

Table 1. Advantages and disadvantages of model systems used to study pregnant human uterine smooth muscle.

Regardless of the whether uterine smooth muscle cells and tissues are in a state of flux during experimentation, it is apparent that each model system is associated with key advantages and disadvantages (**Table 1**). These include factors such as cost, ease of use, relevance to the *in vivo* situation, ability to be coupled with other techniques, downstream processing and more, and should be taken into consideration when selecting model systems.

8. Conclusion

Successful pregnancy necessitates that the uterus is maintained in a relaxed, quiescent state for the majority of pregnancy, before being transformed to a contractile and excitable

phenotype to facilitate parturition. Despite our rapidly advancing knowledge of myometrial biology, the exact mechanisms that regulate parturition are not yet understood. Characterizing the complex interactions that form the key regulatory pathways controlling uterine quiescence, contractility and the transition between the two states is therefore essential to understanding normal human birth, as well as obstetric complications, including preterm labor. *In vitro* models, such as myometrial SMC lines and *ex vivo* tissues, have been important tools for investigating these complex interactions. Nevertheless, recent evidence has shown that both primary cell lines and *ex vivo* tissues undergo culture-induced changes in expression of key myometrial genes. For that reason, further studies are warranted to determine appropriate culture conditions that could prevent or attenuate the changes, thus providing researchers with a stable platform on which to investigate myometrial biology. Until then, researchers should remain mindful of the limitations of myometrial cell and *ex vivo* tissue models, and be cautious when interpreting the relevance of results toward understanding human parturition.

Conflict of Interest

The Authors declare that there is no conflict of interest.

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Section 7

Cardiac Myocytes

Morphology of Right Atrium Myocytes

Marina L. Bugrova

Additional information is available at the end of the chapter

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Abstract

The chapter of this book is devoted to characteristics of the ultrastructure and function of secretory cardiac myocytes of the right atrium in norm and the experimental pathology in rats. The data were obtained at various models, such as clinical death, renovascular hypertension in rats, and in the isolated Langendorff-perfused rat hearts. We investigated the effect of the drug Mexidol on morphological and functional characteristics of endocrine cardiac myocytes in rats and in the isolated heart. Quantitative data on intracellular structures of atrial myocytes were presented. We estimated the accumulation of the atrial natriuretic peptide in granules and excretion into the sarcoplasm in secretory cardiac myocytes. The results were obtained using the methods of transmission electron microscopy and immunocytochemistry. The study makes an important contribution to histology and improves the understanding of the function of the heart as an endocrine organ. This chapter is intended for students of medical and biological universities and for experts.

Keywords: atrial secretory (or endocrine) cardiac myocytes, atrial natriuretic peptide (ANP)

1. Introduction

The concept of the heart's endocrine function was described by A.A. Galoyan with coauthors in 1967–1971 years [1]. Later, the ultrastructure of atrial secretory cardiac myocytes was described, and the atrial natriuretic peptide (ANP) contained in the granules of the myocytes was identified [2]. Currently, about 100 bioactive substances synthesized by atrial cells were determined [1].

Endocrine (or secretory) cardiac myocytes have not only well-developed contractile structures such as in ventricular myocytes but also electron-dense granules in the sarcoplasm.

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Examining the heart morphology under the influence of various factors, the researchers focus on the myocardium left ventricle. It is widely accepted that the key role in the development of numerous cardiac pathologies is given to the ventricular contractile myocytes [3]. The role of atria is given less attention than ventricles, but their dilation determines the development of chronic heart failure and arrhythmia [4].

Studies of secretory atrial myocytes have scientific and practical importance, as these cells are the main source of production and storage of ANP [1, 2]. Peptide has a hypotensive effect due to the diuretic, natriuretic actions, and the suppression of the renin-angiotensin-aldosterone system [5, 6]. ANP is released from granules after tension of the heart wall [7], under the influence of hypoxia and neurohumoral factors [8]. The peptide inhibits the growth of smooth myocytes, endothelial cells, and the activity of fibroblasts [9]. The peptide is involved in the differentiation of cardiac myocytes [10], reduces hypertrophy [11], has anti-inflammatory effect, and so on [12]. The definition of ANP concentration in the blood has diagnostic and prognostic value [13]. Synthetic peptide is used in cardiology [14]; therefore, the study of interaction with drugs is an actual problem.

Despite more than 30 years of research, the question of "hormonal paradox" awaits a solution. It demonstrates the absence of a hypotensive effect of ANP in hypertension of different etiologies [6]. The role of ANP in the pathogenesis of cardiovascular diseases is ambiguous [8].The contradictory data of the study could partially be associated with the use of different methods for the determination of ANP. The few research works of atrial cardiac myocytes are devoted to their morphology only [15] or to the quantitative assessment of the hormone content without analyzing the ultrastructure of cells [16].

Morphometry of immunocytochemical-labeled granules in atrial myocytes with using the transmission electron microscopic analysis of the myocardium allows to investigate the localization of ANP along with changes in the ultrastructure of cells. It also evaluates the intensity of granulopoiesis in norm and in experimental pathology.

Special drugs for the correction of metabolic disorders caused by hypoxia are used in the intensive care unit. One such of drugs, Mexidol (ethylmethylhydroxypyridine succinate), is used in Russia. The neuro- and cardioprotective actions of the drug in the post-reperfusion period (PRP) were studied [17]. Mechanisms of the influence of the drug on the accumulation and excretion of ANP in secretory granules of myocytes have not been investigated.

Thus, the study does not give a complete picture of the morphological and functional features of secretory cardiac myocytes in different conditions. Therefore, we have applied histological techniques to study the right atrium and experimental models for the investigation of this type of cardiac myocytes in this work. This approach gives the possibility to quantitatively assess the dynamics of cardiomyocytes, granulopoiesis, and contents of ANP in norm, experimental pathology, and after the injection of Mexidol. The study makes a significant contribution to the discovery of the mechanism of the endocrine function of the heart.

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2. Materials and methods

Experiments were carried out on white outbred Wistar male rats (n = 180) weighing 200–220 g. We used various models, such as clinical death [18], renovascular hypertension [19], and the Langendorff-perfused rat heart [17].

Clinical death (10 min) was induced using the method described by Korpachev [14]. Rats were anesthetized with Nembutal (25 mg/kg) and intubated; then, the cardiovascular fascicle was clamped with a special L-shaped hook without opening the chest. The heart completely stopped at 2–4 min after clamping. Before the start of resuscitation, 0.1% epinephrine solution (0.1 mL) was administered endotracheally. Resuscitation was performed by external cardiac massage and artificial respiration. We investigated experimental rats after 60 min and 60 days of post-reperfusion period. In experiments with Mexidol, the drug was administered after intraperitoneal resuscitation for the first hour, every 20 min [17].

Renovascular hypertension was induced using the method described by Kogan [19]. Rats were anesthetized, and then the artery of the left kidney was ligated. After 30 days of the procedure, hypertension was developed.

The model of a Langendorf isolated heart was used with the saline Krebs-Henseleit solution of the following composition (mmol/L): NaCl—130; KCl—4; $NaH_2PO_4 \cdot 2H_2O$—1.1; $NaHCO_3$—24; $MgCl_2$—1; $CaCl_2 \cdot 2H_2O$—1.8; glucose—5.6. The solution was saturated with Carbogen (95% O_2, 5% CO_2), with the pH of 7.3–7.4 at a temperature of 37°C. Two refrigerators were used to switch to perfusion with Mexidol: one with Krebs-Henseleit control solution and the other one with Mexidol in the dose of 25 mg/kg added [17].

For electron microscopy analysis, samples were taken from the right atrium of intact and experimental animals. The heart tissue was fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4), post-fixed in 1% osmic acid, dehydrated in ascending alcohols, and embedded in epon and araldite mixture according to the standard protocol. Cellular localizations of atrial natriuretic peptide was detected on ultrathin sections of the right atria using primary polyclonal anti-ANP (rabbit anti-atrial natriuretic factor (1–28) (rat), Peninsula Laboratories, LLC, Bachem) and secondary antibodies (Protein-A/Gold (15 nm), EM Grade, Electron Microscopy Sciences).

Ultrathin sections were analyzed under a Morgagni 268D (FEI) transmission electronic microscope. Morphometric analysis of the areas occupied by mitochondria, sarcoplasmic reticulum, myofibrils, and sarcoplasm of cardiomyocytes was performed using AnalySIS software.

In secretory myocytes of the right atrium, the number of immunodeficiency granules with ANP using the classification was evaluated [19]: counted granules of A-type ("reserving peptide") with a well-defined membrane and osmiophilic content, and B-type ("releasing peptide") without a membrane and with a less electron-dense content (**Figure 1**).

The results were evaluated using Mann–Whitney test and Spearman correlation coefficient. The differences were significant at $p \leq 0.05$. Data in tables are presented as mean (M) ± standard deviation (SD).

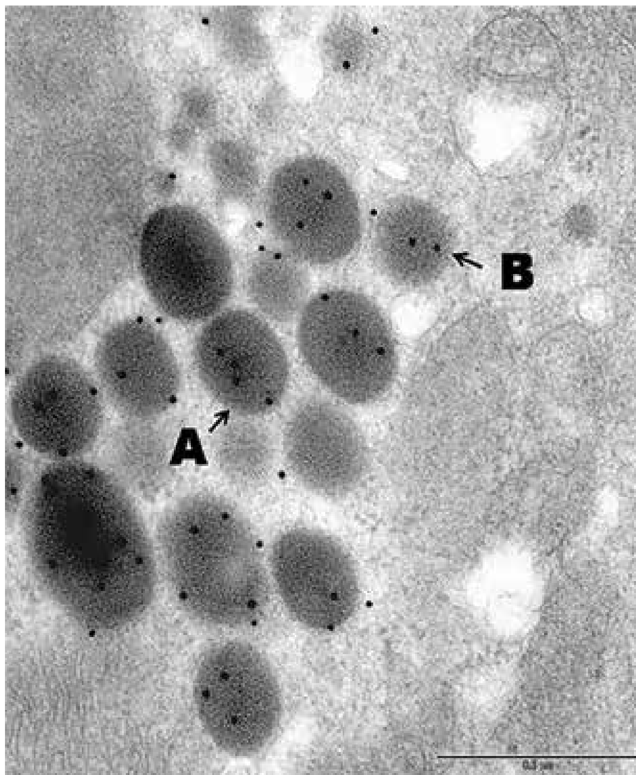

Figure 1. Immunocytochemical detection of ANP in the granules of right atrial cardiac muscle cells in rat. A and B, granules of A and B types, respectively. ×71,000.

3. Results

3.1. Secretory atrial myocytes in normal rats

Atrial secretory or endocrine cardiac myocytes differ from the contractile cardiac muscle cells by the presence of secretory granules in sarcoplasm (**Figure 2**).

The specific localization of this type of myocytes in the right atrium is not found. Cells with granules are mixed with the cells without those. Endocrine cardiomyocytes may differ from each other by the number of granules. We assume that all atrial myocytes have the potential ability for secretory function.

The most part of granules are localized in the perinuclear space near the Golgi complex and contain immunoreactive material of atrial natriuretic peptide (ANP). The quantitative distribution of A- and B-type granules with ANP was 63 and 37% in secretory cardiac myocytes (**Table 1**).

Quantitative values of areas occupied by myofibrils, mitochondria, sarcoplasmic reticulum, and sarcoplasm of atrial cardiac myocytes of intact animals are presented in **Table 2**.

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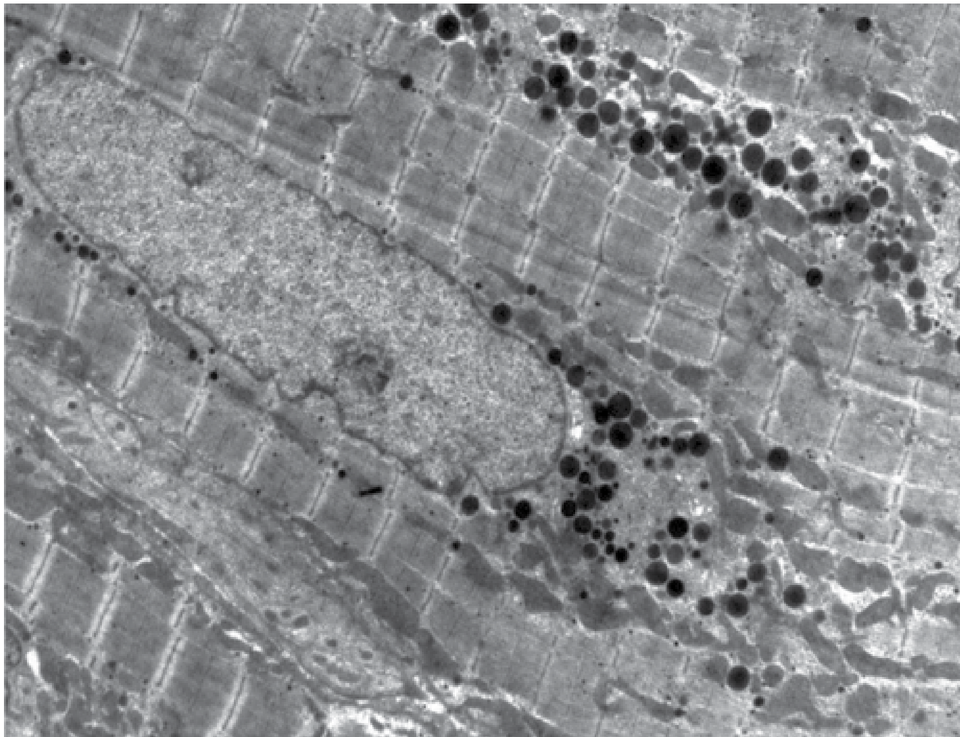

Figure 2. Cardiomyocytes of the right atrium of the intact rat. ×4400.

In the previous study, we revealed the individual peculiarities in the right atrium and left ventricle of intact animals [20]. The areas occupied by various organelles in atrial secretory cardiomyocytes include mitochondria (23%), myofibrils (46%), sarcoplasmic reticulum (1%), and sarcoplasm (30%). Similarly, in ventricular cardiomyocytes, the area was distributed as follows: mitochondria (33%), myofibrils (56%), sarcoplasmic reticulum (0.5%), and the sarcoplasm (10.5%).

Experimental conditions	A-granules	B-granules	Total number of granules
Intact rats	65.75±19.49	38.90±19.63	104.65±33.41
60-min PRP	85.64±20.78*	56.48±17.00*	142.12±36.53*
Langendorff-perfused rat heart	88.54±19.22*	42.17±14.53	130.71±29.79*
Langendorff-perfused rat heart after 10-min ischemia	99.97±33.40*	65.93±23.36*	165.90±55.08*
60 days of PRP	105.17±28.27*	54.71±19.66*	159.88±44.55*
Renovascular hypertension	71.45±24.84	23.75±10.58*	95.20±32.82

Note: $p < 0.05$ in comparison with *the intact animals (Mann-Whitney test).

Table 1. Content of A and B granules containing ANP—granules in atrial cardiac myocytes in experiment (number of granules in visual field, M ± SD).

Ultrastructural element	Intact rats	60-min PRP	60 days of PRP	Renovascular hypertension
Mitochondria	6.82±2.14	7.58±2.13	7.34±1.69	7.,51±1.62
Myofibrils	15.50±2.79	15.70±4.51	17.65±2.41*	14.86±2.55
Sarcoplasmic reticulum	0.31±0.22	0.50±0.29*	0.55±0.36*	0.27±0.30
Sarcoplasm	9.77±2.90	8.91±3.38	6.87±1.56*	9.13±2.61

Note: $p < 0.5$ in comparison with *the intact animals (Mann–Whitney test).

Table 2. Areas occupied by ultrastructural elements of cardiac myocytes in the right atrium in experiment (μm^2, M ± SD).

These data attest to individual functional differentiation of the myocytes: in comparison with ventricular cardiomyocytes, the atrial endocrine ones were characterized by less developed contractile and the energy-providing apparatuses (according to the area occupied by myofibrils and mitochondria) as well as an increased content of the synthetic apparatus (reflected by sarcoplasmic reticulum) and greater sarcoplasm areas [20].

3.2. Secretory atrial myocytes after 60-min post-reperfusion period

After 60 min of post-reperfusion period, when blood stream had been restored, we revealed the heterogeneity of myocytes. Most of the cells were without any changes, some were with degenerative disorders. The areas occupied by mitochondria, myofibrils, and myofibril-free sarcoplasm in atrial secretory cardiomyocytes did not differ significantly from the corresponding values of intact rats. By contrast, sarcoplasmic reticulum area increased by 61% (**Table 2**). Some areas of myocardium with interstitial edema were identified (**Figure 3**). The evident damages were not observed in most of the cells. It should be noted from the previous study that the ventricular cardiomyocytes changed more severely than the atrial ones [20].

After 60 min of post-reperfusion period, submicroscopic examination of the myocytes of the right atrium revealed a pronounced increase in the content of ANP containing A- and B-granules by 30 and 45%, respectively, whereas the total content of secretory granules increased by 36%. These data attest to intensive accumulation and secretion of ANP (**Table 1**). There was a loose positive correlation between the total number of ANP-storing granules and SR area ($r = 0.37$). The study reports upregulation of granule formation in atrial cardiomyocytes via receptors associated with G proteins (Go and Gq), which trigger $Ca2^+$-activated (SK4) potassium channels residing in the sarcoplasmic reticulum [21]. The calcium ions activate protease corin, which converts ANP precursor (pro-ANP) to mature and active ANP [22]. However, no correlation between the total number of granules and the areas occupied by mitochondria or myofibrils was revealed.

According to our previous study [23], the applied heart rate variability (HRV) analysis and arterial pressure (AP) measurement enabled to conclude the following: within the first minutes of post-reperfusion period, a short-term AP increase and the activation of

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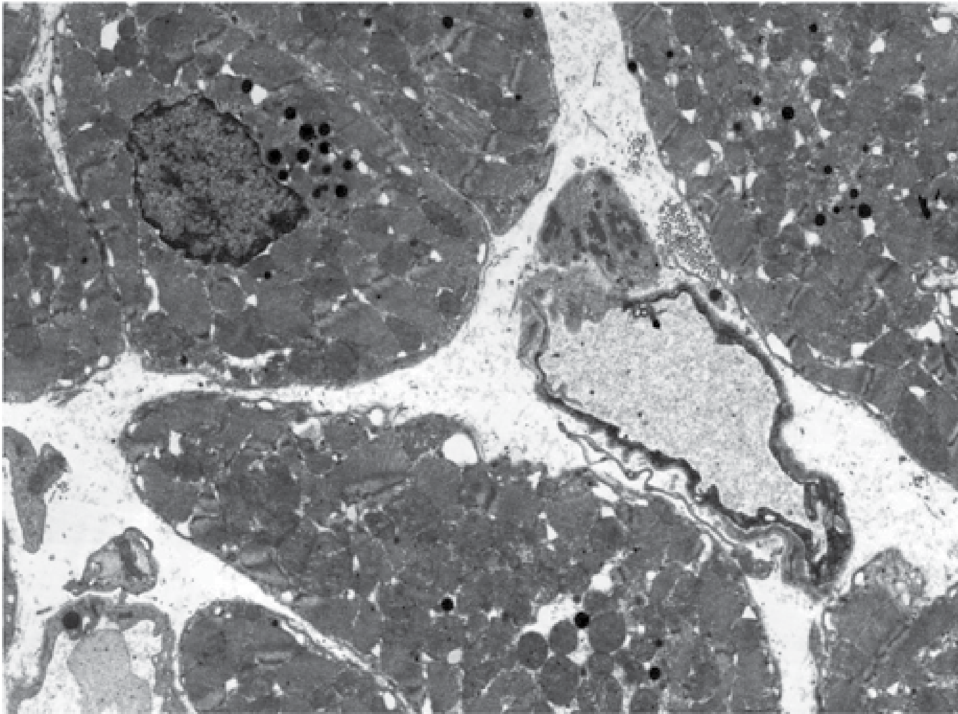

Figure 3. Cardiac myocytes of the right atrium after 60 min of post-reperfusion period: The area of myocardium with interstitial edema, ×8900.

sympathoadrenal, pituitary-adrenal, and rennin-angiotensive systems had no effect on ANP synthesis and secretion in the right atrial myocytes. On the 60th min of post-reperfusion period, a high intensity of ANP synthesis, and accumulation and secretion in atrial myocytes were associated with a stimulating effect of hypoxic and ischemic factors during this period [24].

3.3. Secretory atrial myocytes in Langendorff-perfused rat heart

According to the study and our own research, the heart starts the autonomous functioning after 60 min of post-reperfusion period [24, 25]. The influence of external neurohumoral factors on morpho-functional characteristics of secretory cardiomyocytes was studied in Langendorff-perfused rat heart.

According to Arjamaa and Nikinmaa [26], the myocardium of isolated perfused heart is experiencing a small hypoxia due to the lower oxygen content in the solution compared to the blood. The cardiac myocytes of the isolated heart mainly retain their structure and have adaptive changes under the influence of hypoxia: expanded sarcoplasmic reticulum. We found the small intercellular edema (**Figure 4**).

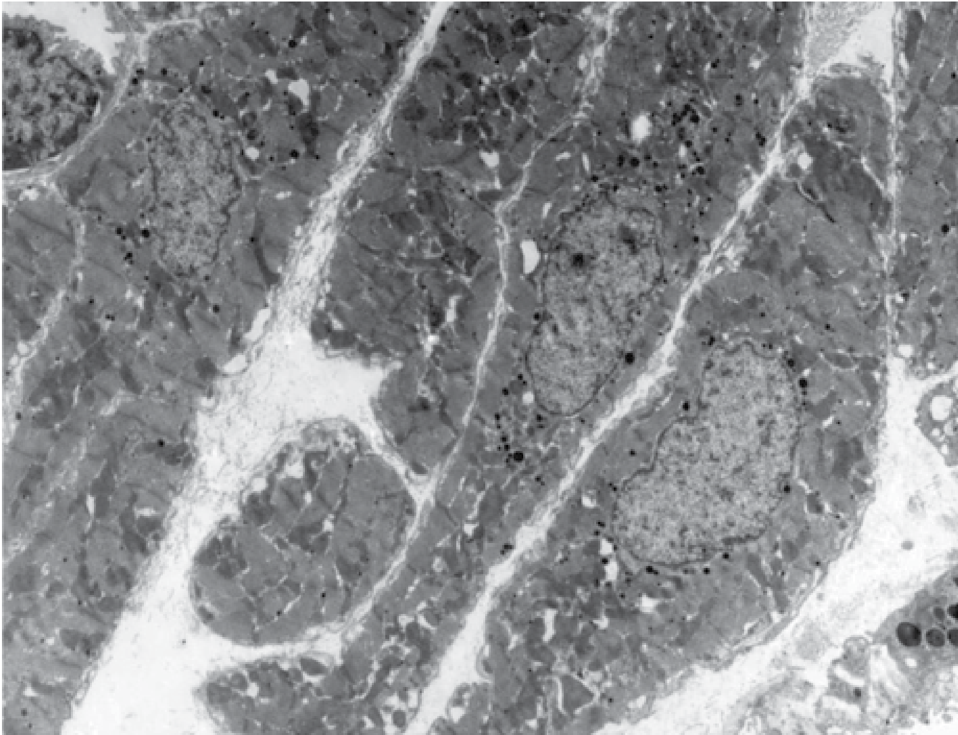

Figure 4. The atrium of isolated rat heart with the intercellular edema: Cardiac myocytes with expanded sarcoplasmic reticulum. ×2800.

The accumulation of ANP in atrial myocytes of isolated heart enhanced: number of A-type of granules increases at 35% and the total number of granules on 25% in comparison with indicators of intact rats (**Figure 5**). According to the authors [26], hypoxia provokes an increase in the transcription of the peptide due to the activation of HIF—"hypoxia inducible factor."

We investigated the contribution of ischemia and reperfusion in the change of ultrastructure and granulopoiesis in atrial myocytes using an experiment with simulations of 10-min ischemia and reperfusion in an isolated heart. There are intracellular destructive changes in comparison with the control group of isolated hearts. Almost all myocytes have condensed or vacuolated form of mitochondria: the dilatation of the sarcoplasmic reticulum. Lysis of myofibrils of cardiomyocytes was observed in some cells (**Figure 6**).

A 10-min period of ischemia and reperfusion stimulates the accumulation and excretion of ANP in endocrine myocytes isolated heart. An increase in the granules A-type by 13%, B-type by 56%, and the total number of granules by 27% compared with the control group of isolated heart is shown (**Table 1**, **Figure 5**). It should be noted that quantitative data and changes in the ultrastructure of cardiac myocytes are similar to the characteristics of rats after 60 min of post-reperfusion period.

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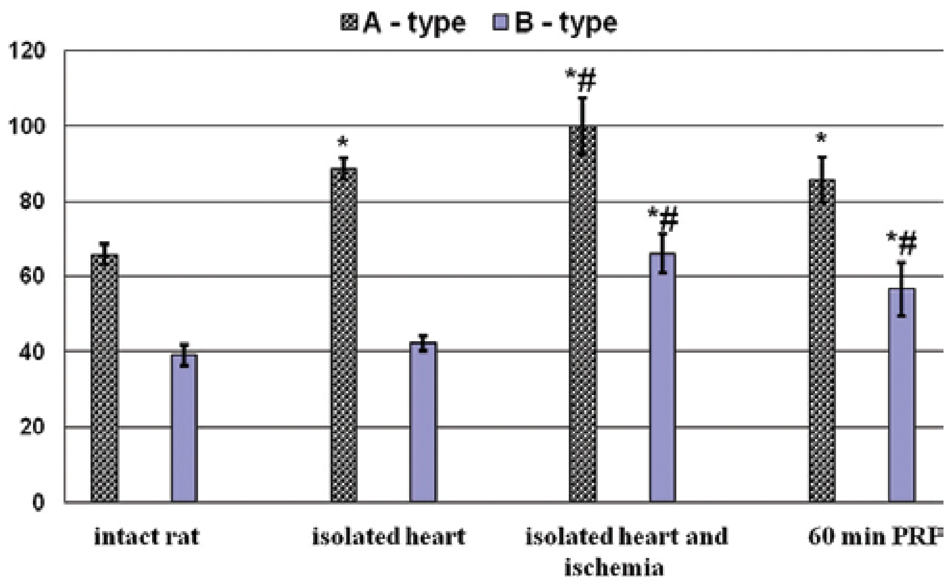

Figure 5. Quantitative distribution of the granules with ANP in the intact rat hearts, isolated perfused hearts, isolated perfused hearts after 10-min ischemia, and rat after 60-min PRP (post-reperfusion period). Asterisk indicates significant differences from intact animals; hash indicates significant differences from isolated heart; $p < 0.05$ (according to Mann-Whitney test).

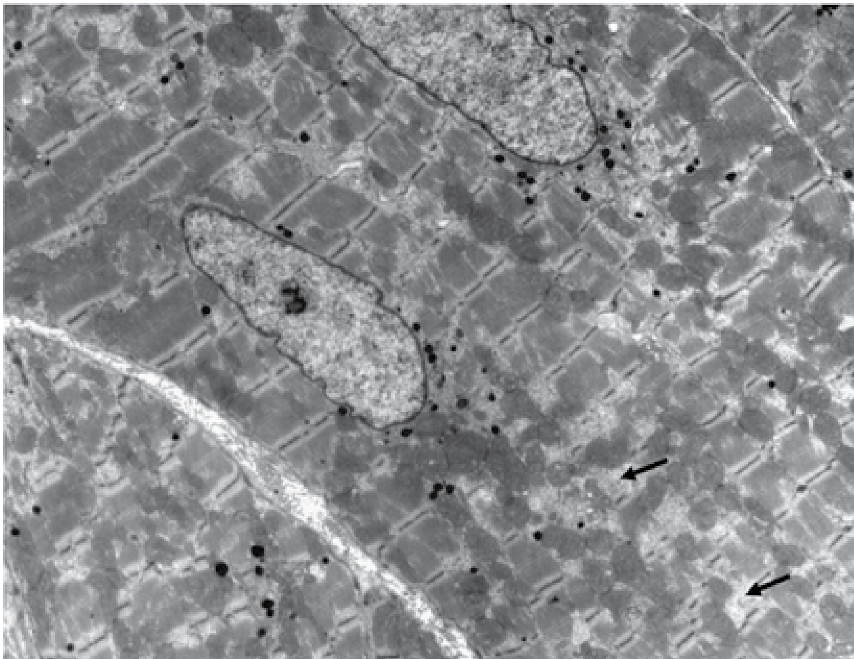

Figure 6. The atrium of isolated rat heart after 10-min ischemia: Cardiac myocytes with lysis of myofibrils (arrows). ×3500.

Thus, granulopoiesis and changing the accumulation and excretion of ANP in secretory cardiac myocytes occur regardless of external neurohumoral factors after 60 min of post-reperfusion period. The processes are influenced by ischemia and reperfusion. This study confirms the existence of a functional isolation of the heart after 60 min of post-reperfusion period.

3.4. Effects of Mexidol on secretory atrial myocytes

The effect of Mexidol used in the correction of ischemic myocardial damage on the secretory myocytes was investigated in rats after 60 min of post-reperfusion period and in Langendorff-perfused hearts (control group and after 10 min of ischemia period and reperfusion).

The nuclei of atrial cardiomyocytes contain euchromatin and nucleoli, the expanded Golgi complex, and an increased number of granules of glycogen in the sarcoplasm in the group after 60 min of post-reperfusion period with the injection of Mexidol (**Figure 7**).

The area of the sarcoplasmic reticulum does not differ from the values of intact animals. The increase of the mitochondrial area of cardiac myocytes by 16% is believed to indicate the increase of functional activity (**Table 2**). Researchers have observed this state of mitochondria in suspension during aeration, in the addition of adenosine triphosphate [27], and in aerobic respiration in the cells [28].

The positive effect of Mexidol on the synthetic activity of secretory cardiac myocytes is manifested by the increase of the content of ANP in comparison with the control group: granules

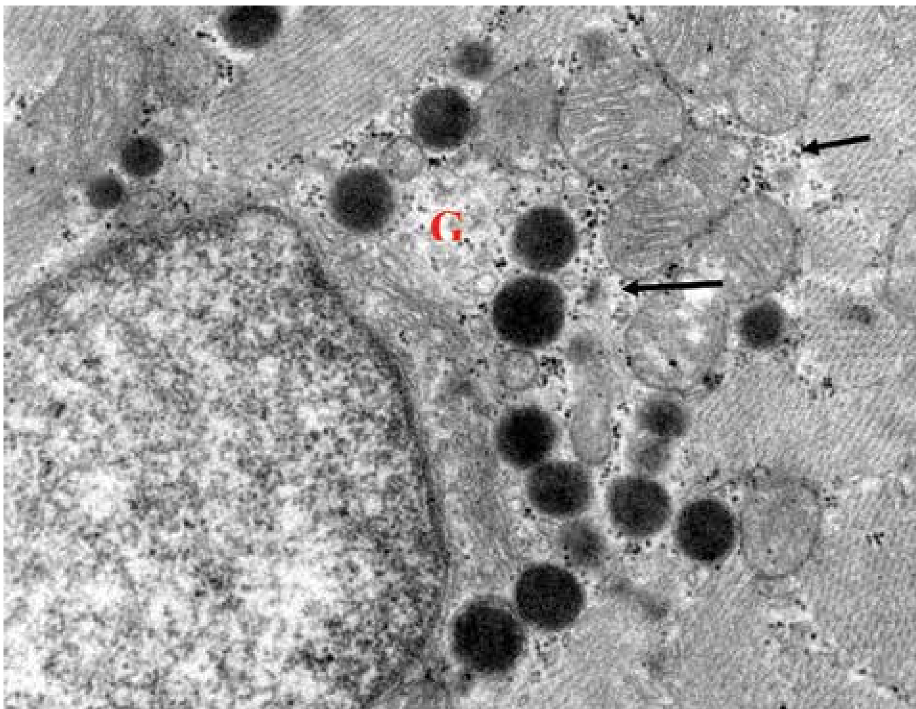

Figure 7. Cardiac myocytes of the right atrium after 60 min of post-reperfusion period with the injection of Mexidol: the expanded Golgi complex (G) and an increased number of granules of glycogen (arrows). ×18,000.

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A-type was increased by 38%, B-type by 42%, and the total number of granules by 37% (**Table 1**). A direct correlation between the total number of granules and the area of mitochondria (r = 0.44) allows us to consider the increased synthesis of adenosine triphosphate to promote granulopoiesis in myocytes.

Previously, it was shown that the heart after 60 min of post-reperfusion period is in a state of functional isolation. The effect of Mexidol on the endocrine cardiac myocytes in the conditions of complete isolation of the heart and under the influence of factors of ischemia/reperfusion was investigated in isolated hearts and after 10-min period ischemia and subsequent reperfusion.

The morphological picture was similar to the group after 60 min of post-reperfusion period with the injection of Mexidol. The number of granules containing the ANP was more than in the control myocardium of isolated hearts: granules of A-type by 33%, B-type 53%, and the total number by 39%. A dramatic increase in the A- and B-type granules indicated a beneficial effect of Mexidol on ANP formation and release in the isolated rat heart. Apparently, it was related to the cytoprotective effect of the drug, which manifested itself on the myocardium ultrastructure as a high content of glycogen cytogranules in the sarcoplasm and sarcoplasmic reticulum without dilatation cisterns. The identified increase in the average value of the mitochondria area with the preservation of membrane structures and matrix indicated the energized state of the organelles that arise, according to the authors [27, 29], at media aeration, with oxidation substrates or ATP added. The membrane-protecting effect, improvement, and preservation of high-energy compounds synthesis with Mexidol administration had a positive impact on energy input processes of ANP formation and release (**Figure 8**).

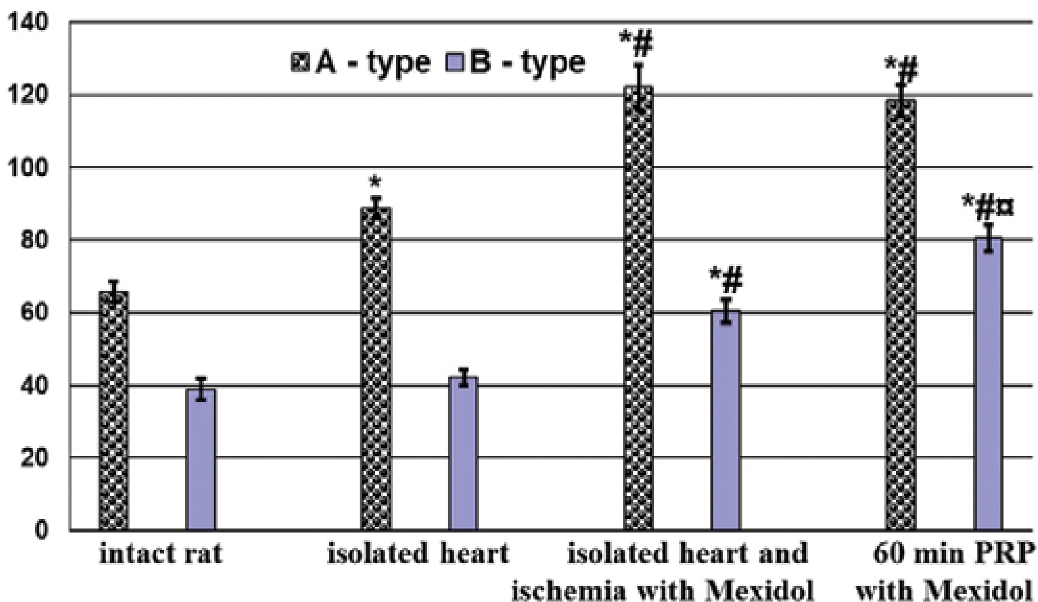

Figure 8. Quantitative distribution of the granules with ANP in the intact rat hearts, isolated perfused hearts, isolated perfused hearts after10-min ischemia with Mexidol administration, and rat after 60-min PRP with Mexidol administration (post-reperfusion period). Asterisk indicates significant differences from intact animals; hash indicates significant differences from isolated heart; $p < 0.05$ (according to Mann-Whitney test).

According to the research data [24, 25], ANP introduced into the ANP perfusion solution has a cardioprotective effect on the cardiomyocytes of an isolated perfused heart. The ANP effect on the electrophysiological heart function is also known [30]. It is put into effect in two ways in the isolated heart: (1) directly, through the autonomic nervous system (according to the authors, ANP depresses the sympathetic and activates parasympathetic component of the autonomic nervous system; (2) through calcium canals: ANP weakens the calcium flow into the cell, inhibiting ICaL canals. Herewith, the cyclic guanosine monophosphate (cGMP) activated by the peptide facilitates the performance of calcium ATPases which carries intracellular calcium into the sarcoplasmic reticulum and reduces the risk of calcium overload. Besides, ANP is shown [31] to prevent the so-called electrical remodeling leading to atrial fibrillation.

Thus, we found a positive effect of Mexidol on the ultrastructure of secretory cardiac myocytes, granulopoiesis, and the secretion of ANP after exposure to ischemia and reperfusion in body rats and in the isolated hearts. The revealed effect of Mexidol perhaps discovers another mechanism of its cardioprotective action and can be used in pharmacology and medicine.

3.5. Secretory atrial myocytes after 60 days of post-reperfusion period

After 60 days of post-reperfusion period, the morphological diversity of the myocytes of the atrium was revealed (**Figure 9**). We found the cardiac myocytes without visible changes and cells with some degenerative changes in the nuclei or appeared apoptotic bodies (**Figure 9**).

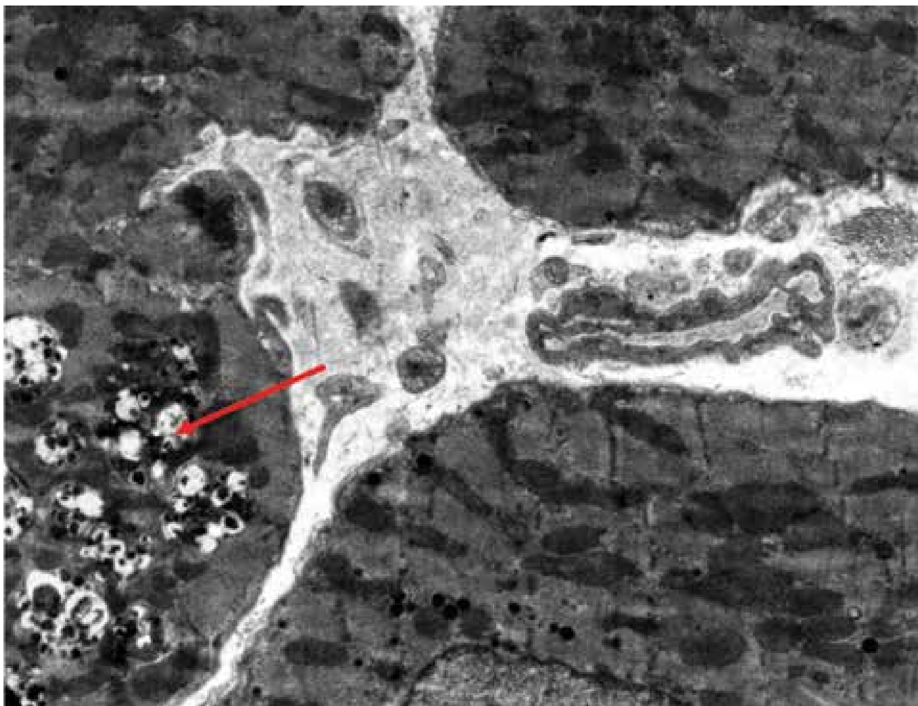

Figure 9. The right atrium after 60 days of post-reperfusion period: apoptotic bodies in the myocyte (arrow). ×5600.

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We identified condensed forms of mitochondria or organelles with the enlightenment of the matrix and disorientation of cristae (**Figure 10**).

The areas of myofibrils and the sarcoplasmic reticulum increased in right atrial cardiomyocytes by 14 and 77%, respectively, while the area occupied by mitochondria did not differ from the intact value. In parallel, the area of myofibril-free sarcoplasm decreased by 30% in comparison with the initial value (**Table 2**). There were a large number of granules with immunoreactive label to ANP. At this, the content of A- and B-granules increased by 60 and 41%, respectively, whereas the total content of granules increased by 53% in comparison with the intact values (**Table 1**). The A:B granule content ratio was 66:34%. A moderate positive correlation was revealed between the total content of secretory granules with ANP and the sarcoplasmic reticulum area ($r = 0.36$). By contrast, there was no correlation between the total content of granules and the area occupied by mitochondria or myofibrils. An enhanced endocrine activity of the secretory cardiomyocytes was observed against the background rise of arterial pressure by 23% [23], which in the view of some researches upregulates not only secretion but also the synthesis of ANP [15, 21].

In previous studies [20], we revealed a hypertrophy of ventricular cardiomyocytes and an increase in the area occupied by the connective tissue in the interstitial space. According to the researchers, these structural changes in the myocardium eventually provoke myocardial remodeling [4]. Taking into account the data of the study, ANPs secrete in response to the increase in the synthetic activity of fibroblasts and/or hypertrophy of cardiac myocytes [6, 11].

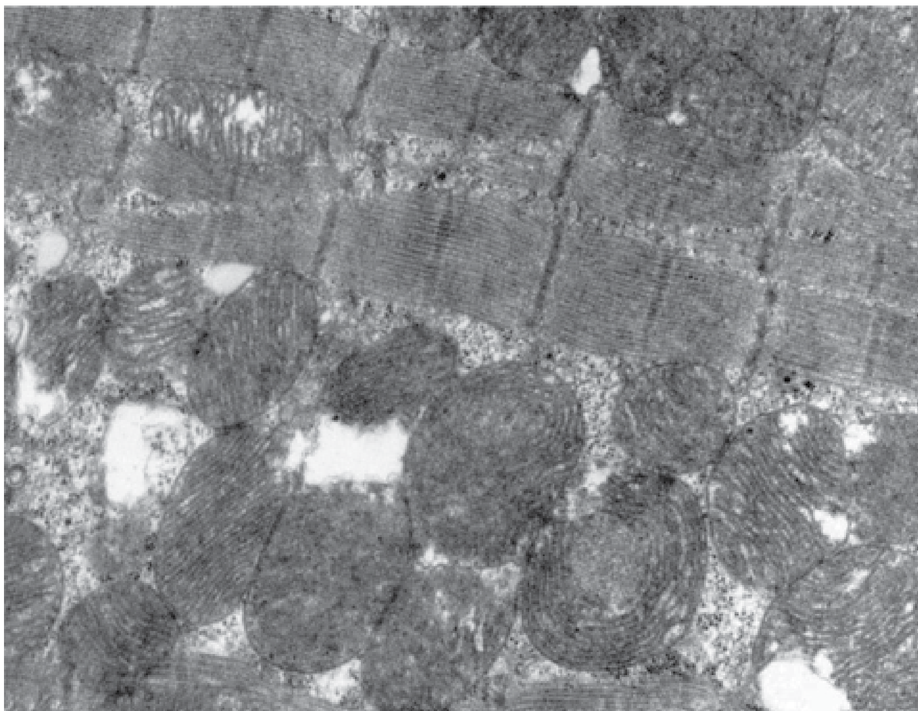

Figure 10. Enlightenments of the matrix and disorientation of cristae of mitochondria in the myocyte of the right atrium after 60 days of post-reperfusion period. ×22,000.

Thus, after 60 days of post-reperfusion period, we observed some pronounced changes in the ultrastructure of the secretory atrial cardiac myocytes and an increase in the accumulation and excretion of ANP in their granules. These processes are accompanied by a high blood pressure, an increase in the area occupied by the connective tissue in the myocardium, and the hypertrophy of the ventricular myocytes.

3.6. Secretory atrial myocytes in different types of the arterial hypertension

Some scientists believe the ambivalence of the role of secretory cardiac myocytes and ANP in the development of cardiovascular diseases accompanied by an increased blood pressure to be present [11]. A scientific interest to the comparison of the content of peptide in the endocrine myocytes in hypertension being formed on different experimental models appeared. We investigated the structure and granulopoiesis in myocytes with renovascular hypertension, developed in 30 days after ligation of the left renal artery and compared the data of hypertensive animals after 60 days of post-reperfusion period.

We have identified both similarities and differences in the experimental groups. Heterogeneity of cardiac myocytes was found in both groups, but after 60 days of post-reperfusion period, we noted morphological signs of apoptosis (**Figure 9**). In renovascular hypertension, we found mitochondria with vacuoles and myofibrils lysis in myocytes (**Figure 11**).

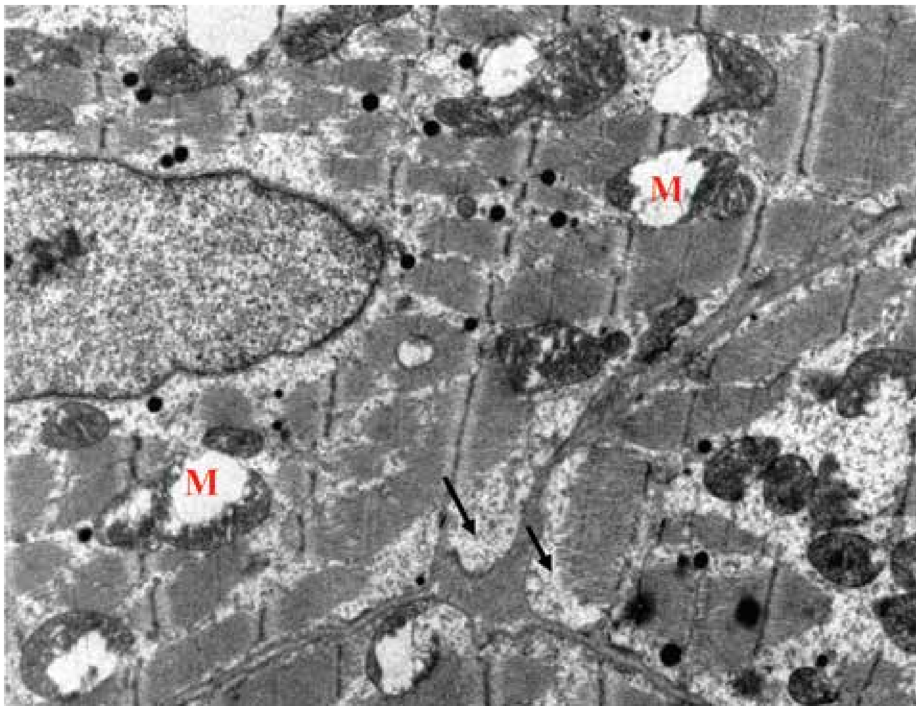

Figure 11. Cardiac myocytes of the right atrium in renovascular hypertension: mitochondria with vacuoles (M) and myofibrils lysis (arrows). ×7100.

Quantitative characteristics of the ultrastructure changes were different (**Table 2**). The table shows the area occupied by mitochondria, myofibrils, the sarcoplasmic reticulum, and the sarcoplasm to be not significantly different from the values of the intact animals.

There is hyperplasia of mitochondria in cardiomyocytes after 60 days of post-reperfusion period. We revealed mitochondrion destruction in renovascular hypertension (**Figure 11**). Vacuoles were noticed in cardiomyocytes of both groups. According to the study, these ultrastructural changes indicate a destabilization of the energy metabolism in the myocytes of rats in renovascular hypertension [3]. Morphological picture indicates compensatory processes in cells after 60 days of post-reperfusion period [4]. The area occupied by the sarcoplasmic reticulum of cardiomyocytes is more increased after 60 days of post-reperfusion period than in the group with renovascular hypertension (**Table 2**).

There is a difference of granulopoiesis in atrial myocytes of experimental groups (**Table 1**, **Figure 12**). In renovascular hypertension, the number of A-granules and the total content of granules were the same as the intact values. B-granules decreased by 39%.

Thus, the content of ANP is not increased in myocytes of rats in renovascular hypertension. After 60 days of post-reperfusion period, we revealed the intensive synthesis and secretion of ANP and have shown a positive correlation between the area occupied by the sarcoplasmic reticulum and the total number of granules (r = 0.36).

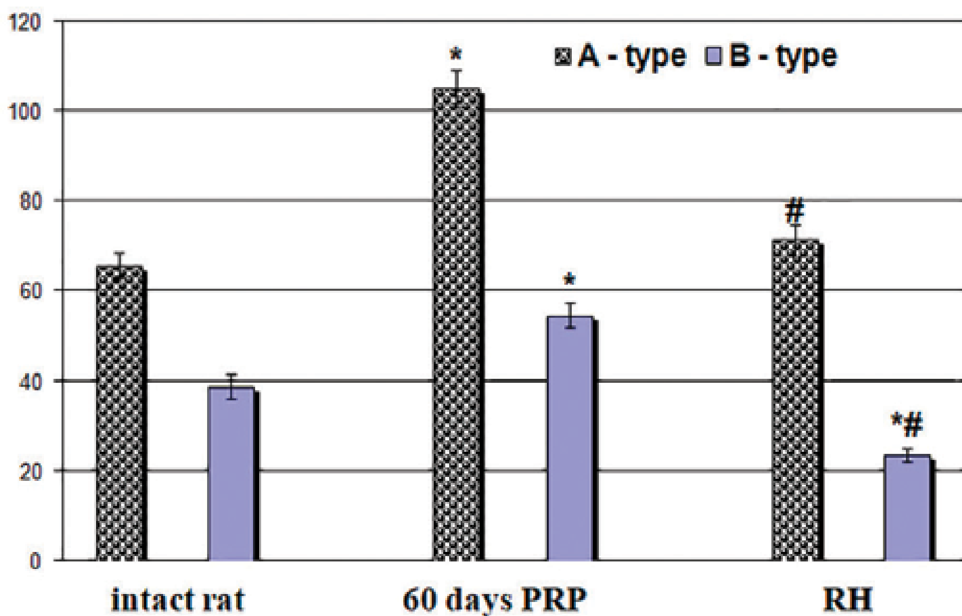

Figure 12. Quantitative distribution of the granules with ANP in the intact rat hearts, rat after 60 days of post-reperfusion period (PRP) and rat with renovascular hypertension (RH). Asterisk indicates significant differences from the intact animals; hash indicates significant differences from the rats after 60 days of PRP; $p < 0.05$ (according to Mann-Whitney test).

The hypertrophy of cardiomyocytes was detected in both experimental groups [32], but the area occupied by the connective tissue does not increase in renovascular hypertension in contrast to the 60 days of the post-reperfusion period (**Figures 6** and **7**).

Thus, the comparison of models of renovascular hypertension and 60 days of post-reperfusion period shows various ultrastructural changes of secretory myocytes and the content of ANP in their granules. The granulopoiesis in atrial myocytes depends on the combination of factors, such as a high blood pressure, the hypertrophy of cardiac myocytes of the left ventricle, and the area occupied by the connective tissue in the myocardium.

4. Conclusion

The study identified the morphological characteristics of secretory cardiac myocytes of the right atrium in male Wistar rats in norm and in experimental cardiovascular pathology.

The certain regularity of localization of this type of myocytes in the right atrium is not detected, so we assume that all atrial myocytes have the potential ability for secretory function.

Experiments on models of clinical death, renovascular hypertension, and in Langendorff-perfused rat heart allowed detecting features of granulopoiesis in atrial myocytes under the influence of pathological factors of ischemia/reperfusion and in high blood pressure.

We found a direct correlation between increasing the area occupied by the sarcoplasmic reticulum or mitochondria and the increased number of granules with ANP. The increase in blood pressure is not always the main stimulus for the formation and secretion of the peptide in myocytes of the right atrium. The granulopoiesis can be activated by a certain combination of factors influencing the ultrastructure of the secretory myocytes.

The process of formation and secretion of ANP in the granules of endocrine cardiac myocytes occurs without the involvement of extracardiac factors after 60-min post-reperfusion period.

Factors of ischemia/reperfusion stimulate the accumulation and secretion of ANP in the granules of the myocytes in isolated rat hearts.

In the research, we revealed a significant positive effect of Mexidol on the ultrastructure, granulopoiesis, and secretion of ANP from granules into the sarcoplasm of secretory cardiac myocytes of the right atrium in Langendorff-perfused hearts and in rats at the early post-reperfusion period. The cardioprotective property of Mexidol can be realized indirectly by activating the synthesis and secretion of ANP in the myocytes of the right atrium.

The changes of the ultrastructure of secretory myocytes of the right atrium and the intensity of the accumulation and secretion of ANP vary considerably in hypertension of different genesis. After 60 days of post-reperfusion period, increased granulopoiesis and secretion of the peptide are associated with an increase in the area of sarcoplasmic reticulum and integrity of mitochondria. These processes are accompanied by the increase of the total area occupied by the connective tissue in the intercellular space in the myocardium.

However, the functional activity of myocytes of the right atrium does not increase despite high blood pressure and hypertrophy of ventricle cardiac myocytes in renovascular hypertension. Based on these data, we put forward the concept of the dominant role of structural reorganization of the myocardium resulting in a change of the ultrastructure of secretory myocytes and the secretion of atrial natriuretic peptide, localized in the granules, in hypertension.

The study makes a significant contribution to the understanding of the peculiarities of ultrastructural organization of endocrine cardiac myocytes of the right atrium containing atrial natriuretic peptide. It is necessary to underline the role of their granulopoiesis in the regulation of complex mechanisms of the heart in normal and pathological conditions.

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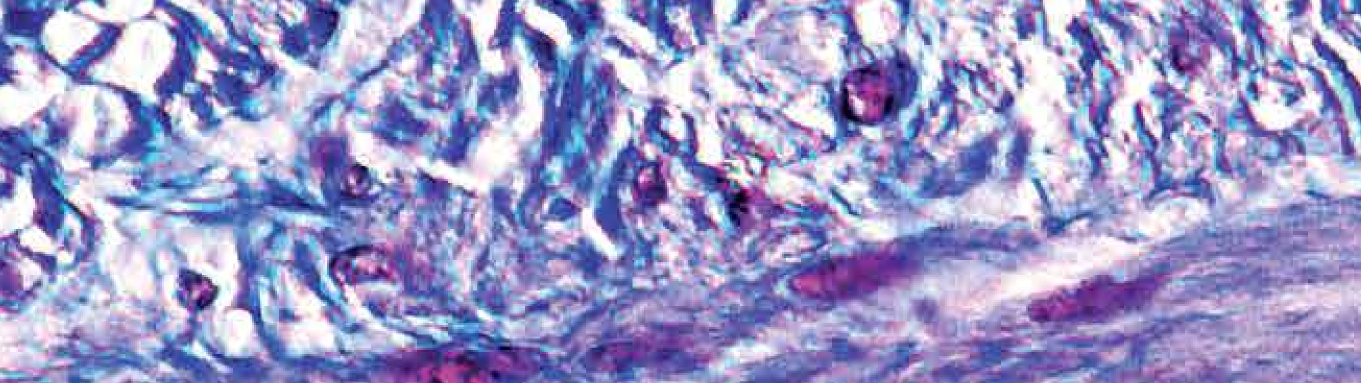

Edited by Kunihiro Sakuma

In order to complete tissue regeneration, various cells (neuronal, skeletal and smooth) interact coordinately with each other. This book, *Muscle Cell and Tissue - Current Status of Research Field*, deals with current progress and perspectives in a variety of topics on the skeletal and smooth muscle, stem cells, regeneration, disease or therapeutics. Novel applications for cell and tissue engineering including cell therapy, tissue models and disease pathology modeling are introduced. This book also deals with the differentiation/de-differentiation process of vascular smooth muscle cells in health and disease. Furthermore, natural products to reverse metabolic syndromes are descriptively reviewed. These chapters can be interesting for graduate students, teachers, physicians, executives and researchers in the field of molecular biology and regenerative medicine.

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