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MUSCLE CELL AND TISSUE

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Notice

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Meet the editor

Associate professor Kunihiro Sakuma, Ph.D., currently works at the Research Center for Physical Fitness, Sports and Health in Toyohashi University of Technology. He is a physiologist working in the field of skeletal muscle. He was awarded sports science diploma in 1995 by 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 later was turned to the molecular mechanism and the attenuating strategy of sarcopenia (age-related muscle atrophy). Preventing sarcopenia is important for maintaining a high quality of life in the aged population. His opinion is to attenuate sarcopenia by improving autophagic defect using nutrient- and pharmaceutical-based treatments.

Contents

Preface

The skeletal muscle as an element of the movement system and as a highly differentiated tissue is in the focus of current scientific investigations.

Smooth muscle is found within the walls of blood and lymphatic vessels, the various tracts (e.g., gastrointestinal or respiratory), erector pili of skin, and iris of the eye. Intriguingly, the basic structure and function of smooth muscle cells is the same in different organs, but the activating stimuli differ substantially. Few events in science have captured the sustained interest of the nonscientific community as stem cells, tissue engineering, and regenerative medicine. Regenerative medicine is composed of attempts to change the course of chronic disease, in many instances regenerating failing organ systems lost due to age, disease, or damage. In order to complete tissue regeneration, various cells such as neuronal, skeletal, smooth, endothelial, and immune (e.g., macrophage) interact smoothly with each other. This book covers numerous topics such as stem cells, cell culture, biomaterials, epigenetics, therapeutics, and the creation of tissues and organs. Novel applications for cell and tissue engineering including cell therapy, tissue models, and disease pathology modeling are discussed. This book also deals with the functional role of autophagy in modulating muscle homeostasis and molecular mechanisms regulating skeletal muscle mass. The chapters can be of interest to graduate students, postdocs, teachers, physicians, and to executives in biotech and pharmaceutical companies, as well as researchers in the fields of molecular biology and regenerative medicine.

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Section 1

Vascular Remodeling

Chapter 1

Vascular Smooth Muscle as a Therapeutic Target in Disease Pathology

Andrew W. Holt and David A. Tulis

Additional information is available at the end of the chapter

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Abstract

Our circulatory system is composed of numerous elements that are responsible for transport of blood and delivery of essential nutrients and gases to vital downstream tissues. Among these components that make up our circulation is vascular smooth muscle (VSM), the primary muscular and contractile element of blood vessels and regulator of many blood vessel functions. This is of particular importance as cardiovascular disease (CVD), the number one killer of individuals in America and worldwide, is primarily vascular in origin. Logically, identifying and characterizing feasible targets that could control CVD are highly appealing and much desired. With this in mind and given its centrality in control of vascular physiology, VSM has gained wide attention as a plausible target to combat elements of CVD. This book chapter focuses on VSM as a potential therapeutic target against CVD and will provide overview of vascular anatomy and physiology and brief discussions about the pivotal roles of VSM in CVD pathology, the influence of abnormal blood flow mechanics and hemodynamics in CVD, neural control of VSM and the vasculature, and possible novel cellular and molecular signaling targets that could be used to control and/or minimize CVD. This chapter hopes to serve as a valuable resource for basic and applied scientists as well as clinicians interested in understanding the crucial roles that VSM plays in vessel physiology and pathology.

Keywords: Cardiovascular disease, cell signaling, cyclic nucleotides, hemodynamics, kinases, shear stress, vascular smooth muscle

1. Introduction

Our circulatory system is comprised of a vast network of cellular elements and organs that includes the heart, lungs, and vasculature composed of arteries, veins, and lymphatics. These integrated factors serve essential roles in controlling flow of blood and lymph and in the transport and delivery of essential nutrients, nutritive and vasoactive factors, and hormones and gases. Vascular smooth muscle (VSM), the primary functional constituent of blood vessels, serves critical regulatory roles of vessel relaxation and contraction to ensure adequate tissue blood flow and to maintain proper localized arterial blood pressures and perfusion of downstream tissues. These processes are elemental for normal vascular eutrophy and homeostasis and overall body health. Abnormalities in VSM anatomy and physiology, however, can contribute to a myriad of primary and secondary vessel pathologies. Moreover, adult blood vessels are normally contractile, quiescent, and static. However, under inimical conditions like those associated with cardiovascular disease (CVD), VSM cells undergo phenotypic alterations and revert to a growth-promoting, synthetic nature. In turn, this abnormal growth significantly contributes to the emerging cardiovascular disorder. These complications alone present significant health risks but also serve as confounding risk factors for associated cardiovascular complications including hypertension, hypercholesterolemia, diabetes, and metabolic syndrome. In fact, recent statements by the American Heart Association [1] and the World Health Organization [2] point to dysfunctional VSM as a primary underpinning behind CVD. Logically, therapeutically targeting VSM for clinical interventions aimed at controlling and hopefully eradicating CVD is highly significant and essential.

The term "cardiovascular disease" defines a wide range of disorders, diseases, and conditions that deleteriously affect the heart and blood vessels. If the heart is the primary organ affected then this can include heart failure, various myopathies, arrhythmias and/or conduction delays, valve complications, myocarditis and/or pericarditis. If the disorder is vascular in origin, then problems could consist of occlusive plaque formation and atherosclerosis, arteriosclerosis, coronary and/or peripheral artery disease (PAD), aneurysm formation, and/or restenosis and remodeling. There are numerous forms and manifestations of CVD as well as broad prognoses based on form and severity of the disorder. Abnormal biology of the vessel wall constitutes a major element in the pathogenesis of most forms of CVD [1], and therefore the vessel wall and particularly VSM makes a prime target for further discovery and potential therapeutic utility against CVD.

The overall goal of this chapter is to present some of the more recent and novel theories surrounding the primary vessel wall constituent, VSM, its importance in CVD, and its promise as a therapeutic target. Discussion will include succinct overviews of normal vascular elements and physiology with a focus on VSM, some common forms of CVD and their pathologies, the role of VSM dysfunction in CVD etiology, the influence of flow alterations and hemodynamic forces in vessel physiology and pathology, neural control of VSM and the vascular network, unique molecular and cellular signaling pathways that may offer innovative and precise targets for therapy in VSM, current therapeutic paradigms, and treatment strategies used to combat VSM pathobiology ranging from homeopathic lifestyle modifications to pharmaco-

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therapies to interventional vascular approaches. This chapter concludes with an overview of the therapeutic potential of VSM and its promise in the cardiovascular sciences. In all, this chapter promises to offer a valuable resource for basic, applied, and clinical scientists by providing a synopsis of the importance of VSM in normal vessel biology and vascular pathology and its utility as therapeutic target to combat CVD.

2. Overview of vascular anatomy and physiology

Upon leaving the heart, blood enters the vasculature which is comprised of diverse tissues and cell types. Conduit, large and small arteries, arterioles, capillaries, venules, veins, and lymphatics are basic elements of this system, each with unique characteristics and functions. The composition of the walls of each of these vessels also differs, based on size, location, and function, and this dictates the presence or absence of the three major vessel wall layers. Starting from the lumen that carries blood, the innermost blood vessel wall layer is the *tunica* (Latin for tunic, membrane, coat) *intima*, and this is formed by squamous vascular endothelial cells (VECs), a layer of connective tissue termed internal elastic lamina, and a basement membrane containing laminin, heparan sulphate proteoglycans and collagen, and often the glycosaminoglycan hyaluronan. The *tunica intima* provides a key, selectively permeable interface between flowing blood (which contains abundant growth factors and thrombogenic platelets) in the lumen and the highly thrombogenic components of the subintimal vessel wall. This important intimal layer is also largely responsible for controlling inflammation and medial VSM proliferation and migration, and these intimal VECs operate by eliciting crucial signals that, in communication with underlying VSM, help control blood vessel function. Hence, this layer is of utmost importance in maintaining normal vascular homeostasis. Interestingly, the endothelium of the *tunica intima* is continuous with the endocardium of the heart and is the only layer present in all blood vessels of all sizes and anatomical locations.

The thick and muscular middle layer of blood vessels is called *tunica media* and this is composed mainly of helical, spindle-shaped mononuclear VSM cells. The media also contains sparse macrophages and fibroblasts along with an interstitial matrix consisting of collagens; glycoproteins such as tenascin, vitronectin, and fibronectin; chondroitin sulphate proteoglycans including versican; and elastic laminae. The *tunica media* provides structural support to blood vessels and is the primary functional element that controls vascular constriction and dilation (and hence, blood flow) based on metabolic needs of the downstream tissues. VSM within the *tunica media* (in communication with intimal VECs) is predominantly responsible for maintenance of normal vascular physiology, yet dysfunction of medial VSM cells (and VECs) is a major contributor to the pathogenesis of CVD (discussed later). The outermost layer of blood vessels is termed *tunica externa* or adventitia, which is separated from the medial wall by the external elastic lamina and is made up of sparse VSM and nerve cells, fibroblasts, fat cells, and abundant connective tissue including elastin, collagen, and glycosaminoglycans that provide structural support as the extracellular matrix (ECM). In larger caliber vessels, the adventitia also contains unique small blood vessels termed *vasa vasorum* that feed nutrients to the thick muscular vessel wall (when the vessel wall cannot get adequate oxygen and nutrition simply

by diffusion from luminal blood). A schematic of blood vessel anatomy including these three critical layers is shown in Figure 1.

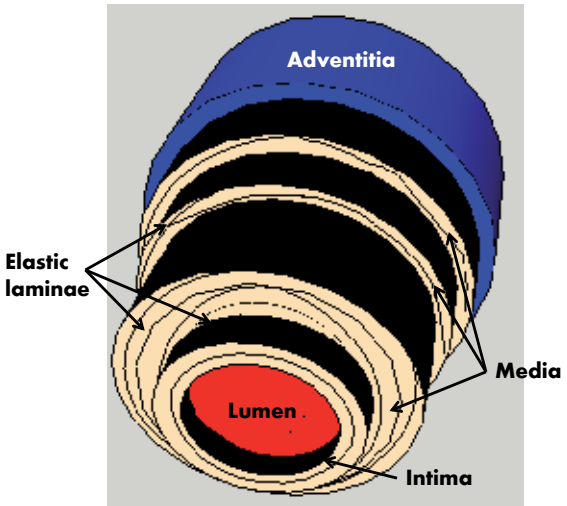

Figure 1. Blood vessel anatomy. The three primary circumferential layers of blood vessels include the innermost endothelium-rich *tunica intima*, the VSM-containing *tunica media*, and the outermost layer the *tunica externa* or adventitia. Elastic laminae exist between these layers as well as within the medial wall. These layers serve critical functions in maintaining normal blood flow and in providing key nutrients and gases to downstream tissues as well as in the removal of toxic by-products of metabolism. Dysfunction in their physiological abilities, however, can contribute to significant vessel disorders including uncontrolled growth which is foundational for the development of CVD.

Physiologically, blood vessels hold a critical place in our circulation between the heart, downstream tissues, and the lungs. As such, a major function of blood vessels is to provide blood flow complete with delivery of vital nutrients and gases (oxygen) to these essential tissues and removal of used metabolic by-products and gases (carbon dioxide) targeted for elimination via the lungs. Blood vessels also operate as highways for the distribution of secreted hormones and other endocrine or paracrine factors as well as white blood cells and/or platelets to their sites of action. Blood vessels also aid in the broader distribution of water, solutes, and heat throughout the system. In VSM-specific fashion, blood vessels have ability to regulate luminal caliber and hence, the amount of blood flow they carry, based on local and immediate metabolic needs of downstream tissues. This is of obvious importance for proper vascular function and tissue homeostasis and eutrophy. Blood vessels perform this critical function through abilities to constrict (vasoconstriction) and relax (vasodilation) through mechanisms fully dependent on functional medial VSM. Several other physiological aspects of blood vessels involve their critical roles in growth adaptations during wound healing or following surgical interventions or during vascular adaptations of exercise. These normal blood vessel growth responses can involve angiogenesis (formation of new blood vessels from existing vessels), vasculogenesis (de novo formation of new blood vessels), arborization or branching of existing vessels, and/or collateralization to provide a new blood supply to an existing vascular bed.

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3. Vascular smooth muscle

As mentioned, the primary structural, muscular, and functional unit of a blood vessel is the medial wall comprised primarily of VSM. In adults, medial wall VSM cells are normally highly differentiated and display a contractile phenotype (with abundant expression of contractile proteins) which enables their abilities to contract and relax. By virtue of vasoconstriction and vasodilation, VSM directly regulates lumen caliber and thus, arterial and venous tone and vascular resistance. These, in turn, control distribution of blood flow in tissue-specific fashion throughout the body. Mechanistically, VSM cells contain many thin actin filaments and relatively few thick myosin filaments, which are arranged along the long axis of these cells. In this arrangement, using the sliding filament theory and numerous states of actin and myosin cross-bridge phosphorylation, VSM utilizes slow force development to produce prolonged force maintenance with low energy utilization, thus helping in the maintenance of tetanic vascular (myogenic) tone and blood flow control. Thus, vasoconstriction and vasodilation represent major physiological functions of contractile VSM.

Under homeostatic unstimulated conditions, adult medial VSM cells are also predominantly quiescent and lack significant growth, "resting" in the nonproliferative G_0 phase of the cell cycle. In this differentiated state, VSM cells have low turnover and minimal proliferative or synthetic activities and are dedicated to their primary function of vasoconstriction/dilation. These cells, however, show considerable plasticity and are able to phenotypically switch between quiescent and synthetic phenotypes in response to local stimuli. So, following activation from a variety of agonists, circulating factors, and/or hormones or cytokines or as a result of injury or the onset of disease, these VSM cells dedifferentiate into noncontractile and synthetic cells with reduced expression of contractile proteins and increased capacity to proliferate, migrate, and produce ECM components. During this dedifferentiation process, the cytoskeleton becomes highly organized with defined F-actin filaments, nuclear hypertrophy, and enlarged Golgi. Along with these morphological changes these dedifferentiated cells become highly sensitive to stimulation by mitogenic and chemotactic factors including angiotensin II, platelet-derived growth factor, fibroblast growth factor, interleukins, and tumor necrosis factor alpha. These processes and vasoactive signals allow VSM cells to contribute significantly to the emerging vascular dysfunction through heightened proliferation, migration, and synthetic properties foundational to CVD. This conversion to a growth-promoting and synthetic embryonic phenotype represents a key event in the genesis and evolution of numerous cardiovascular pathologies including atherosclerosis, restenosis following bypass grafting or stent deployment, or aneurysmal disease as elements of CVD. Figure 2 shows a schematic of the major functions of a VSM cell under healthy or pathologic conditions.

4. Cardiovascular disease

As discussed, abnormal VSM growth is elemental for development and maintenance of CVD, which has historically ranked as the number one cause of morbidity and mortality in the United

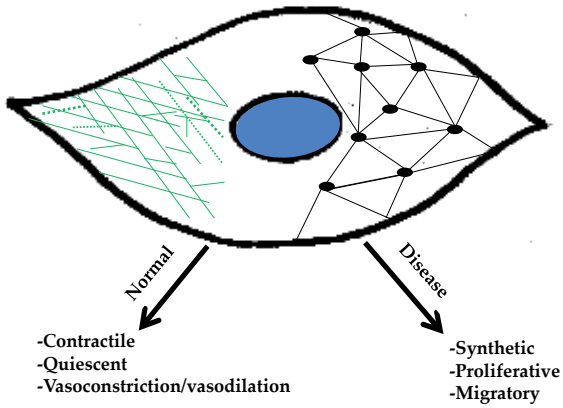

Figure 2. Vascular smooth muscle cell function. VSM cells are normally quiescent and operate via vasoconstriction/vasorelaxation to control local blood flows and pressures and downstream tissue perfusion. However, VSM dysfunction leads to dedifferentiation to an embryonic phenotype, and under these pathologic conditions VSM loses its contractile characteristics and becomes synthetic, proliferative, and migratory, serving as basis for many forms of CVD. Also shown in this schematic are cellular features of thick and thin filaments, dense plaques, and the cytoskeleton.

States and worldwide for many years [1,2]. Despite major advances in our knowledge of the numerous contributing mechanisms for CVD and potential therapeutic strategies against CVD, estimates suggest CVD-related deaths will continue to rise over the next 20 years or so [1,2]. Certainly, notwithstanding these significant basic science and clinical advances, failure in adequate clinical control of CVD highlights its complexity and points to the need for continued study of the underlying mechanisms of and potential routes for control of CVD.

Of the many forms of CVD perhaps the most common is an occlusive disorder termed atherosclerosis. Atherosclerosis is a gradual and progressive disease that involves combined influences of heightened inflammatory status, locally dysfunctional metabolism, abnormal vascular wall growth and remodeling, and the buildup of an occlusive plaque. As discussed, the involvement of uncontrolled VSM growth is a basic foundation of the evolution phase of atherosclerosis, and dedifferentiation of VSM cells into a growth-promoting and synthetic phenotype contributes largely to the emerging and growing plaque. This process of enhanced synthesis and proliferation of medial VSM cells can be slow and progressive and can occur over many decades and may not elicit observable symptoms. This pathology often eventuates in a stenotic plaque, either stable or complicated, which can then become jeopardized and lead to lumen obstruction and diminution of blood flow with clear repercussions of reduced oxygen and nutrient delivery to downstream tissues and diminished removal of toxic metabolites and gases. The plaque can also rupture and send microthrombi into the downstream circulation which can lodge in smaller blood vessels. If atherosclerosis occurs in the peripheral circulation, then these processes could manifest as tissue necrosis with perhaps loss of the affected tissue or limb. Of heightened significance, if this pathology occurs in the cerebral or myocardial circulation, this could result in a cerebral or myocardial infarct (stroke or heart attack) with critical and life-threatening implications.

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5. Blood flow, vascular hemodynamics and neural control

The cardiovascular system is a closed circuit comprised of the heart and an elaborate network of vessels in the systemic and pulmonary circulations. Biophysical forces and factors that govern transportation of essential gases and metabolic fuels and nutrients as well as waste products of metabolism are termed "hemodynamics" and are largely determined by local tissue and cellular supply and demand balance. Hemodynamics, especially in the systemic circulation, are regulated by intrinsic (ability of local tissues to regulate their blood supply) and extrinsic (reliance on neural mechanisms to distribute blood flow at the tissue level) neural control systems. These complex regulatory mechanisms rely on the ability of blood vessels to properly respond to chemical cues in order to efficiently orchestrate where and how much blood is being delivered and removed from various vascular beds. Dysfunctional intrinsic and extrinsic neural control systems compromise efficient oxygen and nutrient delivery to tissues and manifests in a variety of disease pathologies such as PAD, stroke, and/or heart attack.

As oxygenated blood leaves the left ventricle of the heart, it enters the aortic arch and exerts biophysical forces on the arterial walls. Driving pressure, transmural pressure, and hydrostatic pressure are all important forces that regulate blood flow and two major components of these forces are tensile stress and shear stress. Tensile stress is the perpendicular force exerted by flowing blood on the vessel wall and represents forces due to blood pressure [3]. In comparison, fluid shear stress is the force parallel or tangential to the vessel wall which corresponds to the frictional force of the blood in contact with the intimal endothelium [4]. These combined forces are critical for stimulating both intrinsic and extrinsic neural control mechanisms in the vasculature.

5.1. Autonomic nervous system and the vasculature

The autonomic nervous system is comprised of the complementary yet distinct sympathetic (SNS) and parasympathetic (PSNS) nervous systems. Under normal physiological conditions, these systems perform divergent regulatory functions on the vasculature. In general, the SNS typically acts to constrict blood vessels, effectively reducing blood flow, while the PSNS generally dilates blood vessels to increase flow. Autonomic dysfunction in the context of abnormally high blood pressure is typically the result of an overly active SNS. This dysfunction produces a constant neuronal discharge of sympathetic neurons resulting in tonic vasoconstriction of the VSM [5].

The SNS contains preganglionic sympathetic neurons in the ventral horn of the thoracolumbar spinal cord which project their axons just outside of the sympathetic trunk and synapse with postganglionic sympathetic neurons near its target organ. At the initial synapse, depolarization of the presynaptic neuron triggers tethering, docking, and fusion of vesicles containing the neurotransmitter Acetylcholine (Ach) to the axon hillock. Once Ach is released from the presynaptic neuron, it binds to an ionotropic, nicotinic, cholinergic receptor on the postganglionic neuron, resulting in depolarization on the postsynaptic cell. In the context of carotid artery blood flow, the postganglionic neuron projects onto, and synapses with, medial VSM cells. At the postganglionic synapse, the neurotransmitter that is released is primarily norepi-

nephrine (NE). In similar fashion, vesicles containing NE are released at the postganglionic synapse, yet they bind to metabotropic, adrenergic receptors on the VSM cells. VSM contains both alpha 1 and beta 2 adrenergic receptors. NE will preferentially bind to the excitatory alpha 1 receptors causing vasoconstriction and reduced blood flow. Since it is well documented that the carotid artery is tonically constricted, it is only after inhibition of this signal that the SNS is able to be turned off by the PSNS, thereby resulting in carotid artery dilation and enhanced cerebral blood flow. Essentially, the vasomotor area of the medulla is sending excitatory, efferent output to the thoracolumbar section of the spinal cord which is innervating and contracting carotid VSM. This action is executed until the vasomotor area receives an inhibitory signal from the Nucleus Tractus Solitarii (NTS), at which point the SNS is inhibited while the PSNS is activated.

In comparison to the constitutively active SNS, the PSNS is alternatively activated by the carotid sinus when the carotid artery pressure increases. This means that as luminal pressure exerts increased force on the vessel wall, the carotid sinus increases its firing frequency and amplitude, activating the PSNS and effectively inhibiting SNS tonic discharge. The purpose of its activation is to dilate the artery in an attempt to normalize or depress the increased pressure that is currently acting on the vessel walls. The PSNS projects its preganglionic neurons directly from the brainstem and/or sacral spinal cord towards, and in close proximity to, its target organ(s). At this synapse, the preganglionic neuron releases Ach in a similar fashion as that described for the SNS. The ensuing depolarization of the postganglionic neuron reaches the carotid artery and releases Ach again, yet the mechanisms of action at this second synapse are different than what occurs at the initial synapse. Here, Ach binds to beta 2, metabotropic, muscarinic receptors located on the endothelium adjacent to the VSM cells in the medial layer. After binding to the appropriate G-protein coupled receptor on the endothelium, a signaling cascade is initiated that results in an influx of intracellular calcium and the synthesis of nitric oxide (NO) from endothelial nitric oxide synthase (eNOS). NO can then stimulate broad and multifaceted actions on downstream processes including those associated with cyclic nucleotide signaling (discussed below).

5.2. Intrinsic neural control mechanisms

Arterial tensile stress elicits myogenic tone and primarily modulates mechanoreceptor feedback found in baroreceptors within conduit arteries (discussed below). Myogenic tone is the inherent and tonic contractile response of VSM to increased luminal pressures and is a critical component for determining vessel wall hypertrophy according to LaPlace's equation: $T = r \times P$ (T = tension; r = vessel radius; and P = pressure), whereby if vessel caliber or diameter remains constant, any increase in intravascular pressure will elicit an increase in wall tension. Since these two factors are proportional, the vessel wall thickens in order to help offset any experienced increase in wall tension. In the high pressure environment of the systemic vasculature failure to adapt can result in increased matrix metalloproteinase (MMP) activity, causing vessel wall thinning and ensuing aneurysmal formation [6]. The other key force in regulating blood flow is fluid shear stress. This tangential force is exerted on intimal endothelium and elicits VSM relaxation through canonical dilatory (largely NO-mediated) pathways.

The mechanical stimulation of intimal VECs by luminal blood flow activates nonselective cation channels (TRP channels) within the cellular plasma membrane [7]. The resulting increase in intracellular calcium facilitates activation of eNOS and yields NO as a by-product of L-arginine to L-citrulline conversion [8]. As a diatomic gas, NO freely diffuses across the VEC membrane and travels in paracrine fashion to abluminal VSM cells. Here, NO binds to the heme moiety on soluble guanylate cyclase (sGC) and dephosphorylates guanosine triphophosphate (GTP) to elicit pyrophosphate (PPi) and cyclic GMP. Two cyclic GMP molecules can then bind to the regulatory subunit of the serine/threonine (Ser/Thr) protein kinase G (PKG), which in turn phosphorylates a vast number of intracellular proteins to include ion channels, phospholamban, myosin light chain phosphatase, and other phosphorylatable targets including vasodilator-stimulated serum phosphoprotein (VASP), a topic of interest discussed later in this chapter. Ultimately, PKG activation results in the resequestration of calcium within the sarcoplasmic reticulum and induces VSM relaxation resulting in vessel dilation and increased blood flow.

5.3. Extrinsic neural control mechanisms

Of all our organs that receive systemic blood flow, the cerebral circulation is of critical importance. According to the American Heart Association, stroke is a leading cause of death and the leading cause of adult disability in the United States [1]. Ischemic stroke, hemorrhagic stroke, and transient ischemic attacks all result from failure of oxygenated blood to reach the brain. In order to maintain appropriate cerebral blood pressure and constant brain perfusion, numerous cardiovascular and neural control mechanisms exist that act cooperatively to ensure adequate and appropriate cerebral circulation. The carotid sinus baroreceptor reflex is partly responsible for maintaining and regulating blood flow to the brain through local and systemic regulation of blood pressures. The carotid sinus innervates the internal carotid artery just distal to the bifurcation of the common carotid artery. Here, the sensory fibers of the carotid sinus extend into the medial VSM layer of the internal carotid artery. These sensory nerve endings sense stretch (via geometric alteration of resident TRP channels) within the blood vessel and follow the baroreceptor reflex arc. The source of this stretch is typically caused by an increase in intraluminal pressure, which forces the vessel to expand in diameter in order to contain an elevated blood volume within a defined enclosed space (the vessel walls). These volume and pressure changes are also reflected as increased transmural pressure that is exerted by the freely flowing blood on the vessel wall and incidentally on the nerve terminals of the carotid sinus. Following stimulation, an excitatory signal is generated by an influx of cations entering the nerve terminal and the generation of a depolarization event. This afferent signal travels up the sinus nerve and connects to the glossopharyngeal nerve, which proceeds and terminates at the NTS in the brainstem. Once the signal has reached the brainstem, a series of excitatory or inhibitory interneurons communicate and are either stimulated or inhibited, which will differentially activate or inhibit one of the two divisions of the autonomic nervous system previously described. However, under abnormal conditions in the cerebral vasculature or carotid arteries, these control mechanisms may become compromised, thereby resulting in dysfunctional baroreceptor reflex mechanisms and compromised cerebral blood flow [5,9]. On

a broader scale, any obstruction or dysfunction at any point along this reflex arc may be instrumental in the pathogenesis of stroke and/or CVD as a whole [10].

5.4. Physiological blood flow profiles

Four types of physiological blood flow dominate the circulation: 1) pulsatile, 2) oscillatory, 3) laminar, and 4) turbulent [4,11]. Pulsatile and oscillatory blood flows are similar and result from periodic fluctuations in the upstream pumping of the heart in relation to downstream "vacuum" or pulling forces generated by the respiratory and skeletal muscles as well as changing metabolic demands. Since balance between laminar and turbulent flow is important for moderation of flow-mediated stresses and the development of CVD, ensuing discussion focuses on these two forms of blood flow. Continuous (unidirectional) laminar blood flow represents uninterrupted flow that occurs at or near the capillary level and is frequently studied in cell culture perfusion systems or computer modeling simulations. Laminar flow is characterized by layered blood flow in the absence of detectable blood velocity fluctuations or turbulence (e.g., eddies, whorls, and flow reversals). By convention, blood flow is laminar when Reynold's number (Re), a ratio of inertial to viscous forces of a solution, is below 2000, where Re=2rvp/n with r = radius, v = velocity, p = density, and n = viscosity. Re exceeds 3000 under conditions of turbulent flow. When Re is high, it means that the inertial forces outweigh the viscous forces of the liquid. Because stenotic vessels create a pressure drop distal to the site of occlusion, blood velocity increases at these sites and creates audible turbulence with extremely high Re values. Turbulent blood flow, then, is associated with changes in the layered context of flow as seen in laminar settings and is correlated with increased stresses on the vessel wall. Turbulent blood flow is also caused by branching or arborization of blood vessels (at bifurcations of arteries, for example) or by lesions or plaque located along the luminal wall which creates flow obstructions. Any local vascular region with a calculated Re > 3000 is associated with elevated risk for developing vascular occlusions (plaque) at that site.

Considering the importance of laminar flow in maintaining proper vascular homeostasis and turbulent or erratic blood flow in contributing to vascular dysfunction underlying CVD, we began studies aimed at investigating the influence of altered blood flow and flow-mediated shear stress on VSM migration. Using a newly developed in vitro flow apparatus with controlled fluid viscosity (ViscoLab4000) and a newly developed in vitro wound healing and migration assay (Ibidi microchannel VI.4), preexposure of rat VSM cells to 4 hours of elevated shear stress (10 dyn/cm^2) significantly reduced serum-stimulated migration compared to static controls (SC) with no observable changes in cell morphology through 16 hours (Figure 3). These early findings support our notion that elevated fluid shear stress protects against enhanced VSM cell migration as occurs in the setting of CVD.

These results demonstrate the importance of homeostatic levels of fluid shear in maintaining normal vessel function and are commensurate with other studies that have shown this time point and level of fluid shear stress to be physiologically relevant in reducing migration using non-in-situ migration assays [12,13]. It is important to accept the findings of these previous studies with some prudence since removing cells from their perfusion location and seeding them elsewhere can disrupt focal adhesions and other cytoskeletal arrangements formed in

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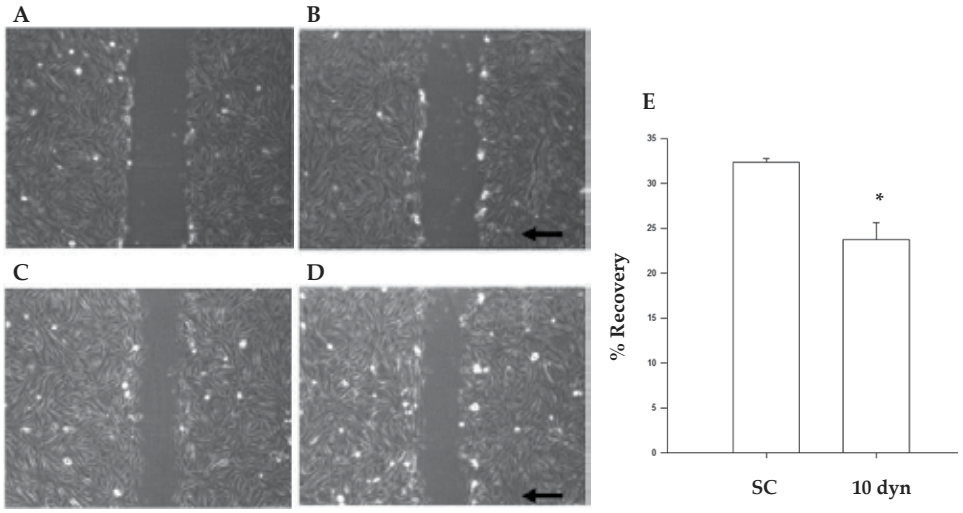

Figure 3. Fluid shear stress reduces migration of VSM cells. Using a newly developed laser capture microdissection (LCM)-assisted wounding assay to estimate cell migration, rat primary VSM cells exposed to increased shear stress show significantly reduced ability to migrate in response to serum compared to static control (SC) cells. Photomicrographs show confluent VSM cells exposed to a wounding scrape injury and treated with static flow (SC) or elevated flow (shear stress 10 dyn/cm^2) for 4 hours with continual exposure through 16 hours. **A** and **B** are photos taken after 4 hours pretreatment of 0 (SC) or 10 dyn/cm^2, and **C** and **D** represent photos taken after continual exposure for 16 hours. Arrows shown in **B** and **D** represent direction of flow. **E** shows measurements of percent recovery of the wound width normalized to time 0 for SC and flow-exposed cells.

response to fluid flow and that are necessary for proper cellular and cytoskeletal dynamics. Due to this shortcoming, we developed this novel wound-healing assay that utilizes an ultraviolet laser and LCM microscopy (Zeiss Palm Laser LCM) to precisely control and effectively denude adherent VSM cells from the perfusion channel substrate following exposure to fluid shear stress in a more physiological setting (without the aforementioned adverse confounding effects found in many traditional migration assays). This new technique allows the researcher to perfuse and injure cells in the same location without the deleterious effects of trypsinization and disruption of normal cellular architecture (including, notably, focal adhesions and cytoskeletal components). Channels are promptly washed with complete media prior to imaging at time zero. Migration time points are carried out up to 16 hours in order to prevent proliferation effects on wound closure rates. To further increase the accuracy and throughput of our migration measurements, we will use the motorized stage of the confocal microscope that is equipped with X, Y, Z coordinate control and on-stage incubator to perform simultaneous time-lapse analysis on multiple wounds.

As mentioned, one of the main functions of a healthy endothelial layer is to provide a key interface between blood flowing in the lumen and the underlying subintimal layer. When this functional endothelium becomes jeopardized, increased fluid shear stress experienced by the underlying medial VSM cells negatively contributes to arterial dysfunction. It has been shown that increased continuous laminar flow can improve re-endothelialization and attenuate VSM cell migration; however, in certain pathologies increased pulsatile and turbulent flow sensed

by exposed VSM and the presence of physical obstructions such as intimal plaque promotes VSM cell proliferation and migration as underpinnings of deleterious vessel growth and remodeling [14,15]. This pathogenic feed-forward mechanism has poor implications for proper vessel function and tissue perfusion.

6. Vascular endothelium

In addition to the essential VSM and autonomic neural mechanisms, a functional endothelium is equally important for appropriate blood flow control and modulation. VEC dysfunction is increasingly accepted as a common trait of nearly all forms of CVD and is often the initial insult in CVD pathogenesis. Though environmental and genetic factors consistently contribute to these disease states as well, the onset of vascular endothelial dysfunction can also be caused by smoking, hypertension, and/or diabetes [1]. Contrary to this evidence, Horvath and colleagues argue that arterial stiffness is not directly related to a properly functioning endothelium [9]. This is true in the sense that the mechanical elasticity of the vessel may compress or expand due to pressure changes alone, but a functional endothelium is nonetheless important for its contribution to baroreceptor function and is often a target for CVD prevention. It is well described that reduced NO bioavailability within the vasculature has a direct influence on VEC dysfunction [5]. Nonetheless, in the absence of an intact or functional endothelium, the VSM layer is still capable of receiving NO from alternate NOS isoforms independent of the endothelial layer. For example, inducible nitric oxide synthase (iNOS) either from circulating cells or platelets or from local adventitial nerves can provide VSM with bioavailable NO independent of endothelial contribution. Also, since iNOS is located within VSM it can intrinsically synthesize NO, in turn facilitating vasorelaxation in autocrine fashion. It should be noted though that the canonical pathway of vasodilation begins with VEC activation as described.

In sum, hemodynamics and intrinsic and extrinsic neural control of blood flow including sensory input to the carotid sinus and the ensuing baroreflex pathway are essential, yet can become compromised under many disease states. The causes of this dysfunction are not entirely understood, but research suggests that there is a direct link between autonomic output and neural integrity and vascular function/dysfunction and the establishment and/or maintenance of CVD. Thus, the preservation of the baroreceptor reflex within the carotid sinus as well as intrinsic/extrinsic neural control mechanisms are essential for ensuring adequate arterial blood supply to the brain in CVD prophylaxis.

7. Treatment strategies

Several treatment strategies currently exist to control and/or minimize symptoms associated with CVD. First, often the most prudent forms of CVD therapy involve lifestyle modifications, and many of these are based on known risk factors for CVD. Specific lifestyle choices may

already be in place in certain individuals in preventive/prophylactic measures and some of them may not be relevant to every CVD patient. Nonetheless, several examples of lifestyle modifications in either preventive fashion or following CVD diagnosis include appropriate weight control (keeping within the recommended BMI limits), particularly for increased waist-level fat or central fat (i.e., midline adiposity), appropriate control of diabetes and hypertension (if previously diagnosed), smoking cessation, low to moderate consumption of alcohol, an active lifestyle complete with regular moderate aerobic exercise, adequate and fitful sleep, and proper and well-balanced diet and nutrition [16]. In conjunction with these healthy lifestyle choices, the next line of defense against CVD often involves pharmacotherapy, also either already in place via prophylaxis (such as one-a-day baby aspirin, multivitamins including folate and B-complex) or prescribed against a form of CVD. Common pharmacotherapeutic strategies include antihypertension medication (beta-blockers, angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, diuretics), anticholesterol medications (statins), calcium signaling blockers, and agents to maintain proper insulin/glucose balance for diabetic patients. Antiplatelet therapies are also commonly prescribed for CVD patients. Following or concomitant with appropriate drug therapies can be surgical interventions to alleviate the problem. This can be of a variety of interventions including coronary artery or peripheral artery bypass grafting, transluminal angioplasty, and/or stent placement and deployment, depending on the nature of the complication. With many of these interventions, unfortunately, iatrogenesis or adverse side effects often occur, and the ensuing results can be even more significant than the original disorder.

8. Molecular and cellular signaling

Mechanistically, a wide variety of molecular and cellular signaling pathways and signal transduction processes have been theorized as serving key regulatory roles in the dedifferentiation and phenotypic conversion of VSM cells and subsequent vessel wall remodeling that leads to CVD. Among these, cyclic nucleotides and their predominantly kinase-driven downstream pathways represent significant regulatory factors in determining vascular physiology and pathology. Cyclic nucleotide signaling consists of purine and pyrimidine cyclic monophosphates, many of which have characteristics that allow them to serve as biologically important second messengers. The family of purine cyclic nucleotides consist of the well-known adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) and the lesser known inosine 3′,5′-cyclic monophosphate (cyclic IMP) and xanthosine 3′,5′-cyclic monophosphate (cyclic XMP). These signals operate primarily through protein kinase-driven phosphorylation events on downstream targets to exert functional control over a wide variety of cellular processes in a myriad of mammalian tissues. In the cardiovascular system, cyclic AMP and cyclic GMP are ubiquitous and are established as critical second messengers with abilities to regulate crucial homeostatic and pathophysiological functions. There also exists the less characterized family of cyclic pyrimidine nucleotides cytidine 3′,5′-cyclic monophosphate (cyclic CMP), uridine 3′,5′-cyclic monophosphate (cyclic UMP), and thymidine 3′,5′-cyclic monophosphate (cyclic TMP); however, their

physiological/pathophysiological importance in VSM and/or during CVD is not well understood.

As mentioned, several molecules exist in this wide family of cyclic nucleotides that are considered to be true second messengers. A second messenger is defined as being generated by a first-messenger-regulated enzyme, being activated by targeted effector proteins, exerting defined biological effects, being degraded by specific inactivation mechanisms, and whose effects can be duplicated by membrane-permeable analogues and/or bacterial nucleotidyl cyclase toxins. [17] Accordingly, both cyclic AMP and cyclic GMP are considered the traditional second messengers, being generated by membrane-bound and/or soluble cyclase enzymes, exerting many effects via intracellular kinases and/or nonkinase targets such as ion channels, have broad biological effects, are inactivated by a family of phosphodiesterases (PDEs), and whose effects can be mimicked by specific analogues or toxins. Also, new theories suggest that the pyrimidines cyclic CMP and cyclic UMP are in fact emerging second messengers in that they also fulfill these criteria [17,18].

The most recognized cyclic nucleotide second messengers are cyclic AMP and cyclic GMP which are synthesized and operate in similar mechanistic fashion. Cyclic AMP is generated through several different avenues including adenylate cyclase (AC) stimulation by direct agonists or following beta-stimulation or through G protein-coupled receptor activation. Following stimulation of AC, adenosine triphosphate (ATP) is dephosphorylated to produce cyclic AMP and PPi. Similarly, following activation of GC through natriuretic peptides (activating particulate GC) or from the gaseous ligands (NO) or carbon monoxide (CO) which activate soluble GC, GTP is dephosphorylated to yield cyclic GMP and PPi. Cyclic AMP and cyclic GMP operate largely through downstream phosphorylation events on Ser/Thr or tyrosine (Tyr) residues on target proteins and serve diverse roles in normal vascular physiology and homeostasis and during the pathogenesis of CVD. The preferred target proteins for cyclic AMP and cyclic GMP are protein kinase A (PKA) and PKG, respectively [19]. Much of the work from our lab over the past few years has focused on cyclic AMP and cyclic GMP and their abilities to target downstream phosphorylatable substrates mainly through PKA and PKG to elicit functional control over a variety of physiological and pathophysiological parameters elemental to CVD. Some of our latest studies have identified the Ser/Thr kinases PKA, PKG, protein kinase C (PKC), and the metabolic gauge AMP-activated protein kinase (AMPK) as biologically important regulators of VSM proliferation, migration, and chemotaxis; ECM and MMP balance; and cellular apoptosis or necrosis using a variety of experimental approaches and commercially available rodent VSM cells, primary rodent VSM cells, and human primary VSM cells. We have observed capacity of Ser/Thr-specific protein phosphatases (PPs) in moderating these kinase activities and in maintaining proper phosphorylative balance. We have also witnessed abilities of the Ser/Thr PPs to elicit control over VSM growth independent of direct kinase involvement. Additionally, many of these studies performed in cultured cells have been validated in whole animal models of injury-induced VSM growth, and these findings have verified biological ability of these cyclic nucleotide systems to operate in a whole body setting. Lastly, we have solidified these observations obtained in rodent models by recapitulating them in human primary coronary artery VSM preparations, thus

adding translational relevance to these basic science findings. Certainly, the capacity of cyclic AMP and cyclic GMP and their phosphorylatable kinase and PPi targets to control deleterious growth of VSM is significant and of particular importance for VSM-specific CVD. These observations provide important new perspectives on vessel wall biology and cell signaling and also highlight potential new targets that could be used to combat CAD and PAD and associated vascular occlusive disorders. Figure 4 depicts chemical structures of cyclic AMP and cyclic GMP, respectively.

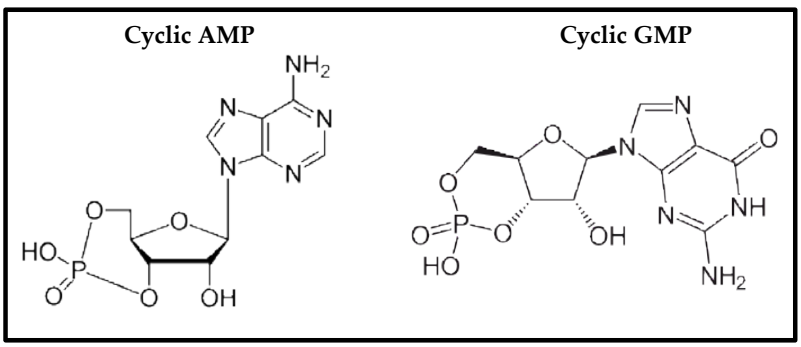

Figure 4. Chemical structures of cyclic AMP and cyclic GMP, respectively.

9. Vasodilator-stimulated phosphoprotein

Following generation of cyclic AMP and cyclic GMP and activation of their respective kinases PKA and PKG (among others, as discussed below), these kinases then target a variety of proteins and other factors to elicit functional consequences. Among these numerous bioactive targets is vasodilator-stimulated phosphoprotein or VASP. VASP belongs to the Ena/VASP Homology (EVH) family of cytoskeletal proteins that play essential roles in cellular dynamics and function. These proteins are involved in intracellular signaling processes and regulate "outside-in" communication via integrin–ECM interactions. VASP is comprised of an N-terminal EVH1 domain (targets focal adhesion and membrane domains), a proline-rich mid-region that binds to Src-homology 3 (SH3) domains, and WW domain-containing proteins that aid in Ser/Thr binding, and a C-terminal EVH2 domain that mediates tetramerization and actin/focal adhesion binding. Figure 5 shows a schematic of the primary VASP sequence including essential EVH1 and EVH2 domains.

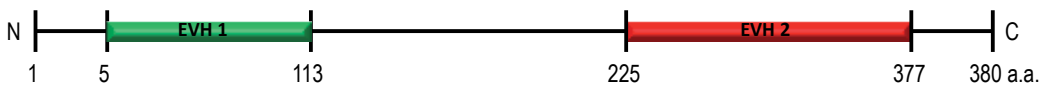

Figure 5. Schematic for the primary sequence of VASP complete with an N-terminal EVH1 domain (that targets focal adhesion/membrane domains) and a C-terminal EVH2 domain that targets focal adhesion/actin binding. (Adapted from [20])

VASP as a Ser/Thr-containing protein was originally characterized as a substrate for cyclic nucleotide-directed kinase-mediated phosphorylation signals, with cyclic AMP-driven PKA preferentially phosphorylating $VASP_{Ser157}$ and cyclic GMP-directed PKG primarily acting on $VASP_{Ser239}$ [21-24]. To date, at least four distinct Ser/Thr phosphorylation sites have been identified on VASP: Ser_{157} and Ser_{239} as well as Thr_{278} and Ser_{322} [25-27]. Interestingly, despite this original characterization of cyclic nucleotide-directed kinases "selectively" phosphorylating discrete Ser/Thr residues on downstream substrates including VASP, emerging reports from our lab and others have documented crosstalk and promiscuity of these and other Ser/Thr kinases to phosphorylate discrete residues on VASP. Recent observations from our laboratory in commercial [28] and primary VSM cells [19,29-31] as well unpublished findings in human primary coronary VSM cells document capacities of cyclic AMP and cyclic GMP to not only phosphorylate their respective canonical targets PKA and PKG but to also have abilities to phosphorylate other kinases as well. In these studies, findings reveal that cyclic AMP, stimulated via direct or indirect means, phosphorylates its accepted target PKA but also phosphorylates the preferred PKG target $VASP_{Ser239}$. Similarly, cyclic GMP, also activated directly or indirectly, phosphorylates its accepted target $VASP_{Ser239}$ as well as the supposed PKA substrate $VASP_{Ser157}$. We have also documented abilities of both cyclic AMP and cyclic GMP to stimulate members of the diverse PKC family [30]. Recently we observed that AMP kinase, another Ser/Thr enzyme, not only phosphorylates its canonical target $VASP_{Thr278}$ [32] but also has capacity to phosphorylate the preferred PKA target $VASP_{Ser157}$, yet interestingly without observable effects on $VASP_{Ser239}$ [33,34]. Recently it was reported that PKD, a downstream product of PKC and a Ser/Thr kinase involved in ECM receptor-mediated signal transduction pathways, phosphorylates both its reported $VASP_{Ser322}$ site as well as the PKA $VASP_{Ser157}$ site [35]. Considering this crosstalk among these kinases and the target VASP, it is important to note that while Ser/Thr and Tyr kinases can target a specific residue, these enzymes are also attracted to flanking residues alongside the known phosphoacceptor site. By this virtue, the catalytic cleft of the kinase interacts not only with its preferred Ser, Thr, and/or Tyr phosphoacceptor residue but also with their flanking regions, thereby binding to similar recognition sequences among similar substrate family members and in turn reducing their specificity for a particular residue. This kinase crosstalk then affords broad impact of upstream kinase signaling but also clouds precision of downstream targeting. Regardless of this observed promiscuity, these critical cyclic nucleotide/kinase/VASP signaling cascades elicit a vast array of significant biological effects in VSM and are of critical importance in vascular physiology and pathology.

Functionally, VASP operates as an anticapping protein and assists in the down-regulation of platelet adhesion molecules, promotes endothelial barrier protection, and assists in vessel structural integrity [36,37]. VASP was recently shown to protect against vascular inflammation and insulin resistance following exposure to high-fat diet [36]. In cancer research, VASP is implicated as a possible pharmacotherapeutic target aimed at controlling aberrant cancer cell migration [35,36]. In those studies, protection by VASP is attributed to its multiple phosphorylation residues (namely Ser_{157}, Ser_{239}, Thr_{278}, and Ser_{322}) that significantly and differentially impact its function as an actin-binding protein. Interestingly, like cancer cells, VSM cells rely on reorganization of their actin cytoskeleton as their primary mechanism of movement [38-40],

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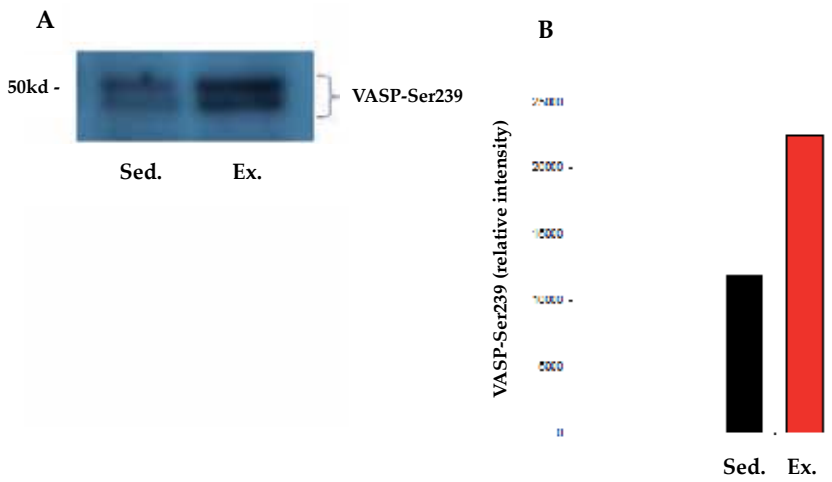

Figure 6. Exercise increases $VASP_{Ser239}$ in rat thoracic aorta (TA) segments following a 10 day treadmill running protocol. **A**: Tissue homogenates of TA sections were pooled from 4 animals per group (sedentary (Sed.) or exercised (Ex.)) and immunoprecipitation (IP) for $VASP_{Ser239}$ was performed. IP fractions were then subjected to gel electrophoresis followed by ECL Western blotting for $VASP_{Ser239}$. **B** represents results from densitometry for $VASP_{Ser239}$ from TA homogenates in Sed. or Ex. cohorts. Preliminary results show that exercise-induced shear stress approximately doubles $VASP_{Ser239}$ expression in rat TA sections compared to sedentary controls. These early results suggest that shear stress acts to reduce VSM migration via $VASP_{Ser239}$, which may serve as a mechanism underlying beneficial effects of exercise against CVD.

and so VASP serves as a plausible target for control of migration in the context of CVD. In our lab, we have recently examined site-specific phosphorylated VASP as a controller of VSM migration and proliferation in commercial and primary VSM cells [19,28-31]. In summary, results suggest that $VASP_{Ser239}$ acts primarily to control cell migration, yet $VASP_{Ser157}$ serves to regulate cell proliferation, both key components in CVD pathogenesis. Also, by virtue of its name, VASP is inherently involved in regulation of hemodynamics via its upstream activators (cyclic AMP/cyclic GMP/kinases) and downstream effectors. In the context of an endothelial-compromised (diseased) blood vessel with fenestration of the intimal–medial barrier and basement membrane, underlying VSM cells become exposed to elevated levels of blood flow and associated mechanical forces. In this regard, recent guidance from the American Heart Association [1] states physical activity as the first-line homeopathic therapy at ameliorating the symptoms associated with PAD, a main form of CVD. While it is known that exercise is beneficial for the circulatory system via formation of collaterals and arterialization of existing capillaries, our question of how hemodynamic forces alleviate PAD remains unanswered [41, 42]. Since exercise was recently shown to stimulate phosphorylated VASP [43], VASP is likely linked to beneficial outcomes observed following exercise. With this in mind, using a 10 day treadmill exercise regimen, preliminary observations show that hyperemic fluid shear stress has capacity to increase $VASP_{Ser239}$ in rat aorta segments compared to sedentary controls (Figure 6). This dilatory response can increase circumferential hoop stress and in the presence of an increased pressure head, consequently increase fluid shear stress. This effect can drastically alter the actin cytoskeleton within the cells of the exposed neointima [44,45] and perhaps then

serve to mitigate abnormal cellular migration and growth associated with CVD. Indeed, these early findings offer support for VASP and phosphorylated VASP species, as crucial cytoskeletal and signal transduction proteins, as capable of controlling deleterious vascular growth integral for CVD.

Indeed, VASP holds great promise regarding its ability to control VSM growth underlying vascular disorders but represents just one of the many end-targets of cyclic nucleotide-driven cellular signals. Vascular cyclic AMP and cyclic GMP and their Ser/Thr kinases also have capacity to exert regulatory control over synthetic TGF-beta signaling [19] as well as other cell-to-cell and focal adhesion/cytoskeletal proteins including gap junctional connexins [46,47], paxillin [34,48], G-actin and F-actin rations [34], and FAK [28,34]. We have also reported on capacities of these signals to regulate ECM-degrading MMPs as a potential route of action in VSM [34,49]. The broad influence of cyclic nucleotide signaling and its many end-target proteins including VASP and associated cytoskeletal components in VSM presents an attractive and biologically feasible therapeutic strategy aimed against basic elements of CVD and many forms of vascular dysfunction.

10. Summary and conclusions

The paramount importance of CVD on morbidity and mortality in the United States and globally is clear, yet inefficiencies in the clinical translation of basic science findings show that the underlying mechanisms of CVD are still not completely known and fully effective therapies against CVD are still needed. Indeed, despite ample basic and clinical research, CVD prevalence is estimated to increase 10% and the economic burden of CVD is projected to triple within the next 20 years with latest estimates that >40% of the adult US population will have some form of CVD by the year 2030 [50]. In our incessant search for possible targets to help control and possibly eliminate CVD, the pivotal influence of VSM growth in playing key roles in CVD pathogenesis is clear. We and others have focused our efforts on identifying and characterizing unique elements behind VSM biology and signaling under healthy as well as pathologic conditions, and findings to date reveal significant insights into many biochemical, molecular, and cellular elements foundational to CVD with perhaps that of cyclic nucleotide signaling at the forefront. Given its ubiquitous nature and multifaceted diverse functions, cyclic AMP and cyclic GMP and their downstream kinases and targets including VASP represent key elements capable of controlling deleterious vascular growth that serves as a basis for CVD. Only through persistent basic science and clinical investigation do we hope to fully understand these crucial factors that hold great promise in our seemingly never-ending struggle to combat and control CVD.

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Chapter 2

Vascular Wall-Resident Multipotent Stem Cells within the Process of Vascular Remodelling

Diana Klein

Additional information is available at the end of the chapter

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Abstract

Processes of new vessel formation are central events in tissue development and repair. Therein, sprouting endothelial cells and/or endothelial progenitor cells form immature blood vessels that lack coverage by pericytes and other mural cells. Subsequently, vascular remodelling takes place, in which association with mural cells (pericytes and smooth muscle cells, SMC) stabilizes these immature vessels resulting in normalization of the vascular structures. Vascular remodelling is a dynamic and strictly regulated process; an ordered remodelling seems to be critical for proper vascular development, maintenance and stability of the vessel wall. The molecular and cellular changes associated with this process and its importance for tumour growth remain elusive. Up to now, the origin of vascular wall cells in tumours and the molecular mechanisms that govern their recruitment and association with angiogenic endothelial cells (vascular stabilization) are not well understood. There is some evidence that pericytes and SMC might originate from multipotent mesenchymal stem cells. This chapter aims to explore the role of tissue-resident multipotent stem cells of mesenchymal nature (VW-MPSCs) which putatively reside in the adventitia of adult blood vessels within the process of vascular remodelling of tumour blood vessels as well as of molecular factors that regulate VW-MPSC differentiation into pericytes and SMC.

Keywords: Vascular wall-resident multipotent stem cell, vascular remodelling, vascular stabilization, tumour vascularization, postnatal vasculogenesis

1. Introduction

This chapter provides a summary of the current literature addressing the importance of vascular wall-resident multipotent stem cells within the process of vascular remodelling. First, the role of pericytes and smooth muscle cells (SMC) causing stabilization of angiogenic tumour vessels will be discussed at the molecular and cellular level. This stabilization phase is crucial for the survival of newly formed vessels, as immature vessels may rapidly become subject to regression and cell death when the angiogenic stimulus is removed. The second part of the chapter will focus on vascular wall-resident multipotent stem cells and evaluate the contribution of circulating progenitor cells versus vessel-resident stem cells in the generation of pericytes and SMC within the neovascularization process. Here, the hypothesis will be proved that tissue-resident multipotent stem cells which putatively reside within the vascular adventitia, rather than circulating multipotent stem cells, are the major source for pericytes and SMC in the vascular stabilization processes. Finally, the regulation of differentiation of vascular wall-resident multipotent stem cells into SMC will be discussed.

Aspects of vascular stabilization, e.g., some decisive factors for the mobilization of vessel-resident stem cells and differentiation into pericytes and SMC, may have the potential for clinically relevant applications in themselves. A better understanding of the molecular processes in these cells could lead to the identification of new therapeutic targets.

2. Pericytes and smooth muscle cells cause a stabilization of newly formed tumour vessels

Endothelia cover the innermost cell layer of the blood vessels. This continuous endothelium is made impermeable for substances dissolved in the blood by the formation of tight junctions in a first approximation. The necessary exchange of substances between blood and tissues is tightly controlled by a highly selective transport mechanism [1]. The uncontrolled cell growth which prevails in tumours results in a relative disparity between the tumour tissue and the sufficient formation of vascular structures. The initiation of tumour angiogenesis is associated with a structural destabilization of existing blood vessels. This causes an abnormally increased vascular permeability, i.e., the existing endothelium is fenestrated, and endothelial cells lose contact with one another and the underlying basal lamina. Finally, contact with the surrounding mural, peri-endothelial cells (pericytes for capillaries and SMC of larger blood vessels) is lost. This leads to the fact that the now mature and quiescent endothelial cells start to migrate and proliferate [2-4]. During angiogenesis, continuous endothelial cells (the particularly impermeable form of the endothelial cells) undergo phases where they are not continuous, so are discontinuous (angiogenic endothelial cells). Chemotactic stimuli and vascular active growth factors such as VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor) secreted by tumour cells induce mobilization and migration of angiogenic endothelial cells towards the tumour cells, which then build up new small blood vessels [5, 6].

During tumour angiogenesis, the hierarchical order of the blood vessels in large, medium and small blood vessels that is found in normal vasculature is lost. New vessel formation in the

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tumour is disordered in structure; chaotic vascular structures are formed with areas of apparent excess supply, in addition to areas with an undersupply of blood and in particular oxygen and nutrition. In addition, tumour vessels have peculiarities in their structure: tumour vessels run tortuously in the tissue, may end blindly (increased permeability of blood vessels), have arteriovenous shunts (shorting connections causing liquid transfer between normally separate vessels), or be directed opposite to the blood flow (heterogeneous perfusion of the tumour tissue). The endothelial lining is incomplete [4, 7, 8]. The newly formed vessel walls lack the smooth muscle elements in their walls, so that they cannot actively respond to physiological stimuli. For this reason, the newly formed angiogenic capillaries bear an increased risk of rupture. Because of these features, tumour vessels prove to be functionally inferior. This complicates the efficient administration of intravenous drugs in cancer therapy [9-12].

While angiogenesis describes new vessel formation by endothelial cells derived from preexisting vessels, postnatal vasculogenesis denotes vessel formation by assembly of endothelial and/or vascular progenitor cells [13, 14]. Thus, the active cellular component in these processes is granted by endothelial lineage cells, but neovascularization does not only depend on endothelial cell migration and proliferation with subsequent formation of endothelial tubes; it also requires pericyte coverage of vascular sprouts for vessel stabilization [15-18]. Thereby, the vascular network can mature by recruitment of pericytes as well as SMC to stabilize the immature tumour vessels (Figure 1).

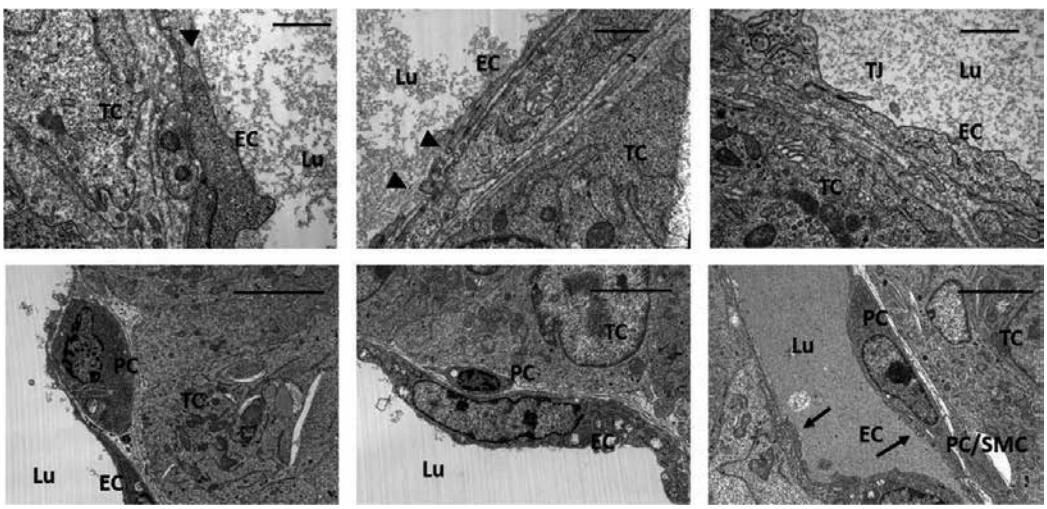

Figure 1. Ultrastructural analysis of angiogenic tumour vessels. Subcutaneously grown B16F10 melanoma tumours were removed 28 days after tumour induction and subjected to electron microscopic analysis. The presence of fenestrae (emphasized by arrowheads) in angiogenic endothelial cells (EC) corroborate the less mature and functional inferior phenotype of these tumour vessels. Upon vessel maturation, these fenestrae disappear (upper panel). Vascular remodelling can be further observed by association and integration of pericytes to the newly formed blood vessels, resulting in vascular stabilization and thus maturation of angiogenic endothelial cells (lower panel). At the structural level, the recruited pericytes are assembled into new capillaries and change cell morphology into a more flattened, smooth muscle cell-like phenotype. In some tumour vessels, vascular mural cells seem to be more regularly integrated into the wall of the new capillaries because of their tight contact with the endothelial cells, shown, for example, in their sharing the same basement membrane, thereby indicating vessel stabilization and maturation (arrows). On PC pericyte: SMC smooth muscle cell, TC tumour cell, Lu lumen. Scale bar 1μm upper panel, 5μm lower panel.

At the molecular level, for the expression of important signalling molecules or receptors, or cell adhesion molecules, there is a locally pronounced heterogeneity in the tumour vascular bed [19, 20]. For a long time, these findings were interpreted as if there were no restructuring processes (vascular remodelling) of newly formed blood vessels in terms of a re-stabilization in the tumour vascular bed. Recent findings, however, show that even tumour vessels undergo a reorganization in terms of vascular stabilization to a certain degree [21, 22]. Electron microscopy analyses indicate that partially stabilized blood vessels exist that differ in their architecture from the usual blood vessels next to structurally stabilized and mature blood vessels, so that angiogenic and less stabilized vessels are disordered and regarded as immature. In combination with the fact that tumours require blood vessels for progressive tumour growth, many new cancer therapies directed against the tumour vasculature (anti-vascular agents, anti-angiogenic agents) have been investigated. It was thought that these anti-angiogenic therapies could destroy the tumour vasculature to deprive the tumour of oxygen and nutrients. By contrast, it was shown that the process of vascular remodelling in tumours was affected during treatment with angiogenesis inhibitors [18, 23-26]. Besides a dramatic tumour regression observed some angiogenesis inhibitors, the tumours also became resistant to prolonged anti-angiogenic therapy. The tumour regression was histological, revealing a reduction in tumour vascularity observed during treatment predominantly as a result of the loss of less mature and highly proliferative small-calibre vessels. The remaining vessels were characterized by an increase in vessel diameter, and by the association and integration of pericytes and SMC leading to vascular stabilization in terms of vessel maturation, and thus a normalization of the vascular. This finally leads to an alternative hypothesis, that certain anti-angiogenic agents can also transiently normalize the abnormal structure and function of tumour vasculature to make it more efficient for oxygen and drug delivery [26]. Meanwhile, an extensive arsenal of anti-angiogenic compounds is available, and their effectiveness is currently being tested in numerous clinical studies. Bevacizumab is a humanized monoclonal anti-VEGF antibody which neutralizes any VEGF isoforms and prevents the interaction of VEGF with the corresponding receptors [27-29]. Clinical trials with bevacizumab show synergistic anti-tumour and chemotherapeutic effects. The results of histological examination of tumour tissue in clinical trials with anti-angiogenic substances showed a stabilization of tumour vessels, which was associated with a reduction in vascular density in the tumour tissue [11, 30-32].

In general, bevacizumab is used as first-line drug in combination with conventional chemotherapeutics in patients with metastatic colorectal cancer, unless contra-indicated. The continuation of bevacizumab beyond first-line progression is still controversial, due to a lack of prospective randomized evidence in this setting [33]. The clinical efficacy of angiogenesis inhibitors targeting vascular endothelial cells has not been as successful as initially hoped, and improved clinical outcomes have been observed in combination with chemotherapy or additional drugs for many types of human cancer. This may be at least partially due to the fact that anti-angiogenic therapy triggers vascular stabilization including pericyte coverage, and that pericyte coverage further impairs tumour vessel regression in response to anti-angiogenic treatment [34]. Furthermore, tumour vessels which are resistant to anti-angiogenic therapy are characterized by an increase in vessel diameter and a normalization of vascular structures.

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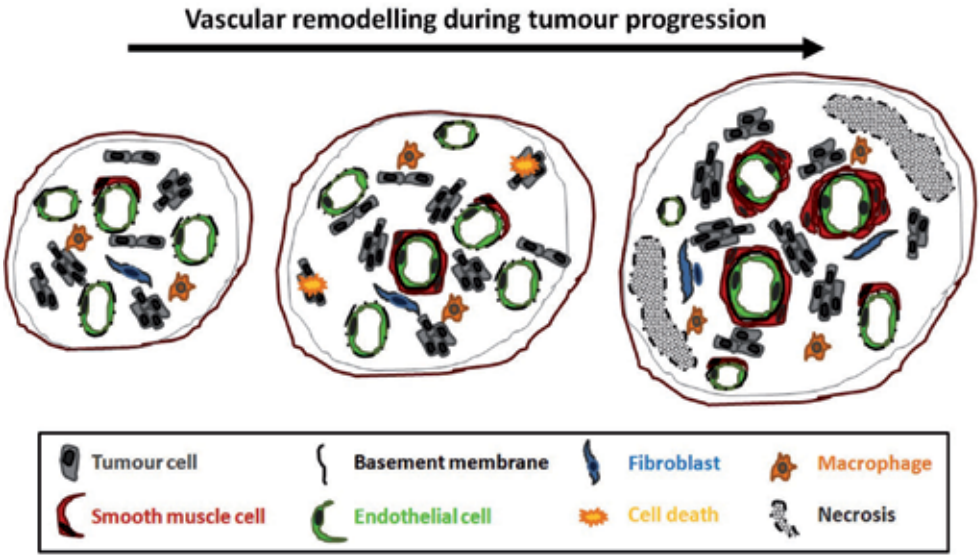

Figure 2. Scheme of the extensive remodelling of the tumour vascular bed by a partial structural stabilization of blood vessels upon tumour progression. Newly formed tumour vessels supply a highly dense network of immature and unstable vessels. Upon tumour progression, angiogenic vessels can mature by the association and integration of pericytes and smooth muscle cells preferentially in the tumour centre zone. This process is accelerated in tumour therapy when anti-angiogenic agents are applied. Further vessel maturation leads to vessels characterized by an increased diameter, a reduction of vascular density and mural cell integration, resulting in normalization of the vascular bed. This process is accompanied by an extensive necrosis of the neighbouring tumour areas while viable tumour cell rows are circularly arranged around stabilized large arteries.

This normalization is achieved by the recruitment and integration of mature pericytes in the vessel wall for capillaries as well as SMC for larger vessels (Figure 2). This process is accelerated in tumour therapy when agents that affect the formation of new vessels (anti-angiogenic agents) were applied [11]. In contrast, the presence of VEGF led to ablation of pericyte coverage on nascent vascular sprouts and vessel destabilization [35]. Thus, targeting pericyte recruitment, coverage and function in addition to endothelial cells may be suitable for promoting progress in anti-angiogenic tumour therapy [36, 37]. In addition, the use of angiogenesis inhibitors which lead to a normalization of tumour vessels in combination with conventional therapies such as radiation or chemotherapy should lead to enhanced efficacy of drug delivery and diminished toxicity [38-40].

3. Vascular wall-resident multipotent stem cells stabilize angiogenic tumour blood vessels by differentiation into pericytes and smooth muscle cells

Until some years ago, the bone marrow and endothelial cell compartment lining the vessel lumen (sub-endothelial space) were thought to be the only sources providing vascular

progenitor cells. Results published recently have identified the human vessel wall as a niche for stem cells [41-44]. Herein, the blood vessels themselves harbour progenitors and multipotent stem cells (vascular wall-resident EPCs, VW-EPCs and haematopoietic stem cells, HPCs), clearly indicating the presence of stem cell niches outside the bone marrow and the peripheral blood [45-48]. Arterial vessels have what is termed an adventitial regeneration zone, in which those various stem and progenitor cells reside (Figure 3). These cells are able to form vascular networks and are capable of differentiating into endothelial cells and CD68+ macrophages [43, 46, 49]. However, the blood vessel wall is made not only of endothelial but also peri-endothelial cells (pericytes/SMC) and adventitial cells. Thus, the adequate formation of new blood vessels under hypoxia, during ischaemia or in tumour neovascularization, depends on the presence and recruitment of these peri-endothelial in addition to endothelial cells. Accordingly, the stem cell niche "vasculogenic zone" also harbours mesenchymal stem cells (MSCs) [43, 46].

Pericytes play a central role in tumour angiogenesis and these cells significantly affect the success of anti-angiogenic therapies. Thus it is important to identify pericytes in different tumour entities [50, 51]. In capillaries, pericytes are in close contact with endothelial cells and share the same capillary basement membrane. Pericytes express alpha-smooth muscle actin (ACTA2) and thus they might have contractile properties. However, the origins of pericytes and of SMC in tumours, and the molecular mechanisms that govern their recruitment and association with tumour vessels, are not clear. Endothelial expression of the platelet-derived growth factor B (PDGF-B) was shown to trigger the recruitment of pericytes necessary for the remodelling of newly formed vessels in terms of vascular stabilization, so that immature vessels with or without pericytes are formed [52]. Using an 'in vitro angiogenesis' system, Nicosia and co-workers suggested that pericytes are formed by migration and de-differentiation of arterial SMC [53]. Interestingly, pericytes have been assumed to differentiate in situ from mesenchymal cells [54]. In line with the idea that pericytes might have their origin in MSCs, it has been shown that Sca-1-positive bone marrow (BM)-derived cells are recruited to the site of tumour progression using the RIP-Tag2 model of pancreatic cancer [55].

In line with these findings, several studies identified human vascular wall-resident CD44+ multipotent stem cells (VW-MPSCs) within the adult human vascular adventitia which were capable of differentiation into pericytes and SMC [45, 46, 56-59]. VW-MPSCs were shown to contribute to in vivo vessel morphogenesis by co-implantation of isolated VW-MPSCs and human umbilical cord vein endothelial cell (HUVEC) in a matrigel plug assay [46]. Within the plugs, implanted HUVEC formed blood perfused vessels. Co-implanted VW-MPSCs assembled at the new vessels and were differentiated into transgelin-positive/ACTA2-positive SMC/pericytes, undoubtedly confirming that VW-MPSCs have the capability to differentiate into pericyte/SMC and thus contribute to morphogenesis of new vessels under in vivo conditions. Electron microscopic analysis further demonstrated at the ultrastructural level that VW-MPSCs were not only aligned to new capillaries but were also regularly integrated into the wall of new capillaries; for example, EC and pericytes are enclosed by the same basal lamina. Thus, a crucial hypothesis concerning the vessel-resident stem cells is that these cells are the "first-line" cells, which are available on the basis of their anatomic location as the first point of contact for tumour cells and for tumour cell-secreted factors [43, 46, 60, 61]. Moreover, it is hypothesized that MPSCs or smooth muscle progenitors, resident in the vessel wall, would serve as a source for local recruitment of cells to stabilize new immature vessels constructed

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only by endothelial cells. Under vascular restructuring processes (remodelling) these VW-MPSCs associate with the newly formed blood vessels of the tumour and differentiate into pericytes and SMC, which results in a stabilization of the newly formed vessels (Figure 3).

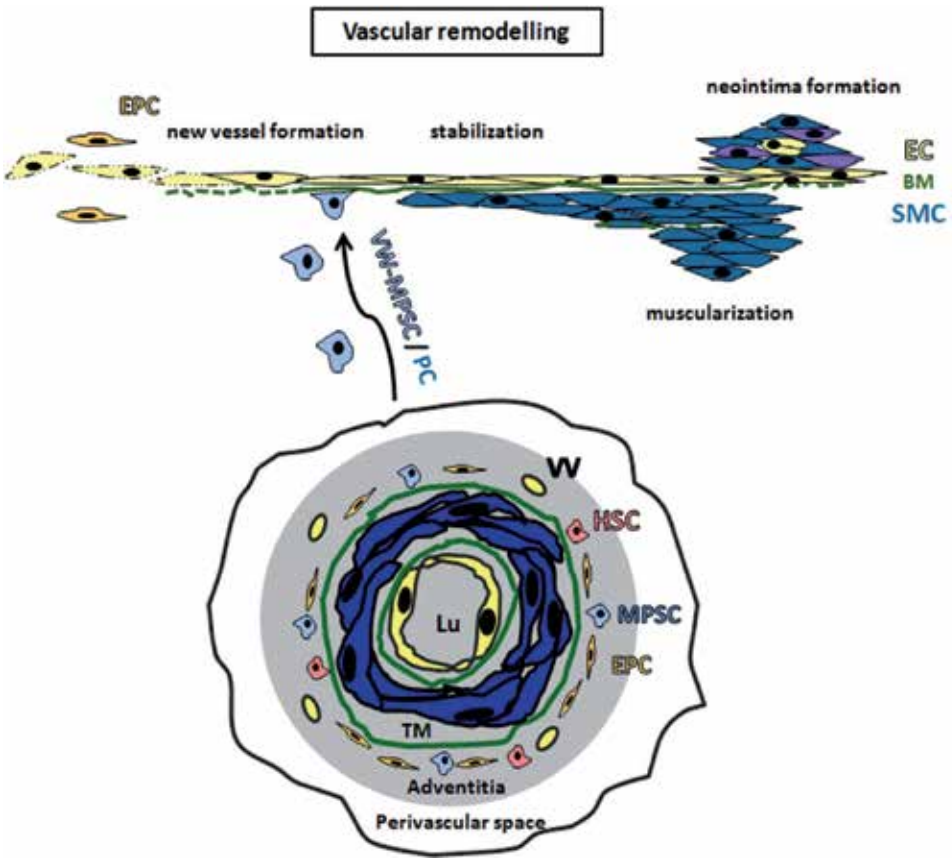

Figure 3. Vascular remodelling. New vessel formation by both angiogenesis and postnatal vasculogenesis is a prerequisite for tissue regeneration and several diseases including tumour progression and atherosclerosis. Vascular stabilization is achieved by the recruitment and integration of mature pericytes in the vessel wall for capillaries, as well as smooth muscle cells (SMC) for larger vessels. Intima, media (TM) and adventitia with vasa vasorum (VV) are fixed layers of the wall of large arteries and veins. The border between media and adventitia is marked by outer elastic membrane (green). The vasculogenic zone is a vascular mural zone located within the adventitia and close to the tunica media which harbours different subsets of vascular wall stem cells. The central hypothesis concerning vascular wall-resident multipotent MSCs (VW-MPSC) is that these cells serve as major source for pericytes and SMC for stabilization of new vessels, or repair of pre-existing vessels under physiological conditions. Localized within the vascular adventitia, which serves as an interface between the inner parts of the vessel wall, including blood flow and the surrounding tissue, the VW-MPSCs might serve as an important therapeutic target. Under vascular restructuring processes (remodelling), these MSCs associate with the newly formed blood vessels of the tumour and differentiate into pericytes and SMC, which results in a stabilization and thus normalization of angiogenic tumour blood vessels. VW-MPSCs' differentiation into SMC may be induced by tumours, inflammation and hypoxia in tissue areas around blood vessels, contributing to morphogenesis of new vessel walls (e.g., tumour vascularization, intimal lesions or neointima formation). In contrast to the direct action of MPSCs during tumour progression through becoming mobilized from their niche and subsequently differentiated at the site of injury, the protective effect of endogenous or exogenous applied MSCs could also be related to the modulation of paracrine characteristics of these cells. HSP, haematopoietic stem cell; EPC, endothelial progenitor cell; PC, pericyte; yellow, endothelial cells; green basement membrane and elastic membrane; blue, SMC.

Multipotent MSCs were intensively analysed using in vitro studies: optimized conditions were identified for their expansion and potential for differentiation along mesodermal lineages, e.g., into bone, fat, muscle and cartilage [62-65]. A frequently used source of MSCs is the bone marrow [66]. Here, only 0.01 to 0.001 % of the mononuclear cells in the BM are MSCs. Furthermore, human MSCs (hMSCs) can be obtained from umbilical cord blood, placental blood, foetal liver and adipose tissue [67-71]. It is further hypothesized that so-called permanent tissue stem cells exist in virtually every tissue type [72, 73]. In view of the fact that the blood vessels' area is a common structure of all tissues and organs, it is obvious that vessel-resident stem and progenitor cells may have great potential in biomedicine [46, 59, 74-76]. Together with the fact that tissue-specific stem cells differentiate predominantly into the tissue type from which they derive, vessel-resident (multipotent) MSCs may be particularly well suited to contribute to the formation of new vessels.

In general, abnormal vasculature is a hallmark of solid tumours. The exact quantification of tumour vessels is useful to evaluate prognosis, because the degree of angiogenesis is associated with tumour aggressiveness and clinical outcome [77]. Together with the fact that pericytes and SMC play a central role in vascular remodelling of tumour vessels, their recruitment and stable integration into stabilized tumour vessels may determine the success of anti-angiogenic therapies [78, 79]. Accordingly, future therapies targeting both endothelium and pericytes may favour progress in anti-angiogenic treatment for malignant tumours [80]. Thus, it is important to identify the origin and localization of pericytes and SMC in tumour tissues from cancer patients to gain a better understanding of their role in tumour growth and metastasis as well as to improve the outcome of anticancer therapies. Concerning the hypothesis that multipotent stem cells of mesenchymal nature (MPSCs) which express the (neural) stem cell marker nestin are the major source for pericytes and SMC in vascular stabilization processes, nestin-GFP transgenic mice were used in order to track MPSCs' contribution to the vascular remodelling processes. Nestin-GFP transgenic mice express GFP under the regulatory elements of the nestin promoter [81]. For transgene construction, the second intron of nestin gene was utilized, which was known to drive the expression in neural stem and progenitor cells. Furthermore, the 5′ upstream region (promoter region) in the transgene construct was included, the regulatory function of which is still unclear [82, 83]. Thus, these mice were ideally suited for the evaluation of the role of nestin-positive cells during the vascular remodelling of tumour blood vessels (Figure 4). Besides this, the BM tissue-resident nestin-GFP-positive cells are localized in the wall of mouse aortas and express nestin while lacking CD34 expression. Using arterial slice cultures of ex vivo isolates, these cells can be mobilized from their niche by factors secreted from cultured tumour cell lines, and are capable of differentiating into pericytes and SMC. In line with these results, Lin et al. have shown that tissue-resident MPSCs isolated from different anatomic locations gain the capacity to modulate the formation of vasculature by tightly surrounding newly formed microvessels as perivascular cells using a matrigel plug assay [84]. Furthermore, it has been demonstrated that human MPSCs derived either from the vascular adventitia or the bone marrow efficiently stabilized nascent blood vessels in vitro by functioning as perivascular precursor cells [46, 85]. Furthermore, vascular wall-resident nestin-GFP-positive cells can be isolated and cultivated. Primary cell cultures exhibited typical MSC characteristics. According to the guidelines, clonally expanded cells adhered on plastic,

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differentiated into adipocytes, chondrocytes and osteocytes under certain cell culture conditions [86]. These findings are in line with previous reports. Recently it has been elegantly demonstrated that nestin-GFP-positive cells in the BM are enriched in mesenchymal stem cell activities and are pericyte-like [87].

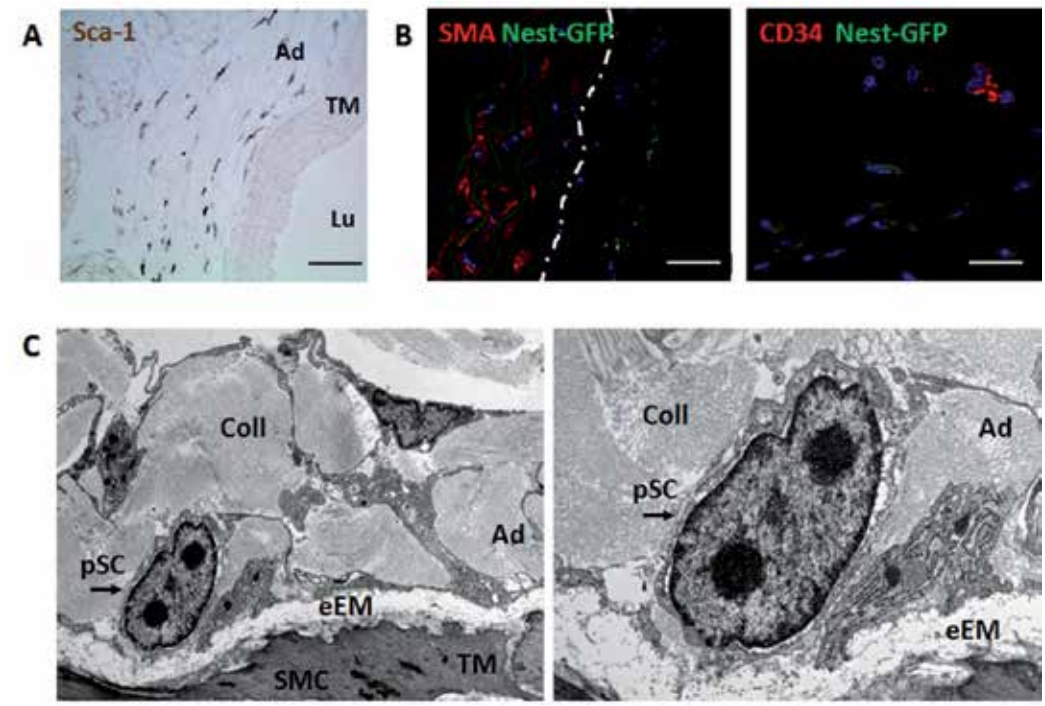

Figure 4. Nestin-GFP(+) multipotent cells are localized in the vasculogenic zone of murine aorta. (A) Immunohistological analysis of stem cell antigen-1 (Sca-1) expression in mouse aorta sections. Scale bar 100μm. (B) Immunofluorescence analysis of nestin-GFP-positive MSCs in their native niche was performed using double immunostainings on mouse aorta sections combining antibodies against GFP (green) and SMA or CD34 (red). Dotted line marks the border between media and adventitia of the aortic wall. Scale bar 20μm. (C) Electron microscopic analysis indicates the presence of undifferentiated cells (putative stem cells (pSC) in the vasculogenic zone (Ad) of the adventitia. eEM external elastic membrane, SMC smooth muscle cell, TM, tunica media, Coll collagen.

In order to determine the contribution of the tissue-resident MPSCs to the formation of tumour neovasculature, BM transplantation experiments were performed. Tissue-derived cells were tracked when wild-type BM cells were isolated from C57BL/6 mice and transplanted into lethally irradiated, age-matched, syngeneic, nestin-GFP transgenic recipients [81]. Tumours grown in reconstituted nestin-GFP transgenic mice which received wild-type BM showed that ACTA2-positive pericytes exclusively expressed GFP, demonstrating that nestin-GFP-positive pericytes derived from tissue-resident cells and not circulating (BM-derived) MSCs stabilize angiogenic vessels in tumours grown in those mice. In combination with intensive immunofluorescence analysis, these results strongly confirmed the hypothesis that nestin-GFP-positive MSCs are apparently involved directly in vascular remodelling processes in terms of vascular

stabilization, serving as a major source for pericytes and SMC. Thus, vascular wall-resident MSCs have to be considered in future strategies for anti-angiogenic tumour therapy. According to this idea, nestin expression of human colorectal adenocarcinoma metastases under clinical treatment with bevacizumab showed a prominent stabilization of tumour vessels by increased integration of nestin-positive pericytes and/or SMC into the vessel wall [81]. Mature vessels from the tumour's surrounding area or healthy tissue, by contrast, down-regulated nestin expression. Nestin expression had already been considered to be specific for developing vascular smooth muscle cells (VSMC), whereas differentiated, postmitotic VSMC were negative for nestin [88]. Conclusively, nestin-targeted therapy may suppress tumour proliferation via inhibition of neovascularization and vessel stabilization in numerous malignancies, including colorectal cancer and melanomas. Nestin, an intermediate filament protein, is reportedly expressed in repair processes, various neoplasms, and proliferating vascular endothelial cells [89, 90]. It was recently reported to be expressed in proliferating endothelial progenitor cells, but not in mature endothelial cells. Tumour endothelium-specific expression is thought to depend on the first intron of the nestin gene, whereas neural stem cell-specific and thus MSC-specific expression is usually regulated by the second intron [90]. Therefore, expression of nestin was described to be relatively limited in proliferating vascular endothelial cells and EPCs. Using another but similarly constructed nestin-GFP plasmid generated nestin-GFP transgenic mouse, nestin-positive pericytes have been identified as the progenitors of all Leydig cell phenotypes, indicating that vascular cell types, acting like adult stem cells, play a critical role in organ formation [91]. Thus, these findings confirm the idea that addressing pericytes, in particular by nestin-targeted therapy, may be suitable to selectively address newly formed and partially stabilized tumour blood vessels.

From the literature, it appears to still be controversial whether and to what extent BM-derived vascular progenitor cells or tissue-resident stem and progenitor cells contribute to neovascularization processes. BM-derived endothelial progenitor cells (BM-EPCs) have been shown to represent an alternative source of endothelial cells for adult neovascularization in the process defined as postnatal vasculogenesis [92, 93]. Thus, BM-EPCs might constitute a new and promising target for pro- or anti-angiogenic treatment strategies [94]. However, there is extensive variation about their contribution to tumour neovascularization of primary tumours, and the respective values range from 50 % incorporated BM-EPCs to undetectable numbers, demonstrating that the exact role of these cells in postnatal vasculogenesis is not quite clear [95-97]. These contradictory results may be due to the methodological difficulties in distinguishing BM-derived cells from intimately associated cells [94]. Furthermore, the effects of MSCs on tumour growth are still controversial. Interactions between MSCs and tumour cells might play an important role in tumour growth [98, 99]. Herein, MSCs have been shown to transmit their tumour-promoting activity via a paracrine mechanism of action: conditioned media derived from cultured BM-MSCs induced the expression of VEGF in tumour cells as well as the activation RhoA-GTPase and ERK1/2 [100]. Furthermore, BM-derived MSCs (also called mesenchymal stromal cells) have been reported to migrate to the site of tumour progression and to subsequently differentiate into carcinoma-associated fibroblast (CAF)-like cells, thereby representing tumour-promoting stromal cells. As CAFs express platelet-derived growth factor receptor

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(PDGFR) at a high level, a blockade of PDGF signalling pathways by imatinib treatment influenced the interaction between BM-derived MSCs and tumour cells in the tumour microenvironment and, hence, inhibited the progressive growth of colon cancer [101].

In general, considerable evidence is accumulating for the involvement of tissue-resident and in particular vessel-associated MPSCs in regenerative and pathological adult neovascularization [43, 102, 103]. In vitro experiments further suggested that proliferative SMCs are derived from the differentiation of multipotent vascular stem cell (MVSC) of the blood vessel wall instead of the de-differentiation of mature SMCs [104]. MVSCs-expressed markers including Sox17, Sox10 and S100β were cloneable, had telomerase activity, and differentiated into neural cells and mesenchymal stem cell (MSC)-like cells that subsequently differentiated into SMCs. In vivo experiments further demonstrated that MVSCs, rather than mature SMCs, repopulate the tunica media and form neointima after endothelial denudation injury [104]. Whether MVSCs were derived from the de-differentiation of mature SMCs was determined by lineage tracing using SM-MHC as a marker in SM-MHC-Cre/LoxP-enhanced green fluorescence protein (EGFP) mice [105, 106]. These studies support the hypothesis that vascular multipotent stem cells of a mesenchymal nature were activated and generated SMC by differentiation instead of a possible SMC de-differentiation of the vascular wall. We may conclude that, in addition to their above-described role in tumour vascularization, the aberrant activation and differentiation of vascular wall-resident multipotent stem cells may contribute the development of vascular diseases. These findings may have a transformative impact on vascular biology, vascular diseases and remodelling, and may lead to new therapies by using VW-MPSCs as a therapeutic target.

4. Regulation of differentiation of vascular wall-resident multipotent stem cells into smooth muscle cells

Epigenetic regulation was shown to play a crucial role in SMC differentiation [107]. High levels of histone modifications were found in promoters of SMC-specific genes as compared to undifferentiated embryonic stem cells [108, 109]. Of the epigenetic regulation mechanisms, histone acetylation, which is adjusted through acetyltransferases (HATs) and histone deacetylases (HDACs), primarily promotes the expression of target genes [110]. However, whether the differentiation of MSCs to SMCs was affected by such histone modifications remains unresolved. HDACs, however, can arrest stem cell proliferation and induce cell differentiation and apoptosis [111]. A histone deacetylase inhibitor (sodium butyrate) was further found to effectively promote rat BM-MSC differentiation into SMCs; a strategy that could potentially be applied in clinical tissue engineering and cell transplantation, for example for the treatment of bladder function disorders such as stress urinary incontinence [112, 113].

In order to identify molecular mechanisms governing the differentiation of the vascular wall-resident MPSCs into SMCs, cDNA microarray analyses on MPSCs isolated from human internal thoracic artery fragments in comparison to mature SMC of human aorta were performed (unpublished data). Among several genes being differentially expressed in VW-

MPSCs, the HOX genes HOXB7, HOXC6 and HOXC8 were found to be expressed in VW-MPSCs at a clearly higher level than in mature hAoSMC [60]. The HOX genes are a family of regulatory transcription factors that control the activity of other functionally related genes in the course of individual development, and are expressed variously in the adult organism [114]. Because of their central role in the development of body parts, limbs and organs, mutation of these genes can cause serious changes in body parts at points in the body that they do not physiologically occur, such as the conversion of complete limbs. In humans, so far, HOX-39 transcription factors have been identified in the four separate clusters (HOXA-D) that are located on four different chromosomes. Together with accessory factors, HOX proteins bind to specific DNA sequences in order to activate or repress genes [115]. HOX genes are thought to act as micromanagers orchestrating cell differentiation after embryonic development in many different cell types and developmental pathways [116]. In the adult, it is already known that colony-forming unit-fibroblasts (CFU-F) derived from different organs have characteristic HOX expression signatures that are heterogeneous but highly specific for their anatomical origin [117]. The topographic specificity of HOX code is maintained during differentiation, which indeed suggests that the pattern of expression is an intrinsic property of MSCs. Furthermore, stem and progenitor cells from mesodermal tissues have HOX-specific gene expression profiles. This so-called biological fingerprint can be used to differentiate functionally different MSC populations from bone marrow and umbilical cord blood [118]. Thus, HOX proteins have a role in specifying the cellular identity of MSC. A differential analysis of 39 HOX genes in vascular wall-resident MPSCs compared to terminally differentiated endothelial cells, SMC and less differentiated (pluripotent) embryonic stem cells showed that HOX family members HOXB7, HOXC6 and HOXC8 are overexpressed in the vessel-resident MPSCs. This suggests that these HOX genes are involved in the development and differentiation of the VW-MPSCs [60]. To gain further insights into the molecular role of these HOX genes for VW-MPSC differentiation as well as to identify potential downstream regulated genes of HOXB7, HOXC6 and HOXC8 activity, VW-MPSCs were transfected with HOXB7, HOXC6 and HOXC8-specific siRNAs both individually and in defined combinations using non-specific siRNAs as controls. Interestingly, silencing these HOX genes in VW-MPSCs significantly reduced their sprouting capacity and increased expression of the SMC differentiation and maturation markers transgelin (TAGLN) and calponin (CNN1), and the histone gene histone H1. Furthermore, the methylation pattern of the TAGLN promoter was altered, which clearly indicates a differentiation of VW-MPSCs to a more mature SMC phenotype. A restricted expression of HOX genes, in particular HOXB7, had already been reported in the 1990s to distinguish foetal from adult human SMC, whereby HOXB7 was expressed at markedly higher levels in embryonic vascular SMC as compared to mature SMC of adult vessels [119]. These data suggest that HOXB7 initiates a differentiation from multipotent cell type towards SMC, but stops the further differentiation of these cells into mature SMC. Further striking evidence is that H1 is also involved in the regulation of VW-MPSC differentiation into SMC [60]. H1 expression in VW-MPSCs is significantly enhanced upon differentiation towards SMC, as shown after gene silencing for HOXB7, HOXC6 and HOXC8, respectively. In general, H1 function can alter the chromatin structure and serves as both a positive

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and negative regulator of transcription, depending on the gene. H1 can further influence DNA methylation and regulate specific gene expression [120-122]. We may conclude that the interaction of H1 and HOXB7 might be a more specific mechanism regulating gene expression and differentiation of VW-MPSCs to SMCs and then to mature SMCs in physiological remodelling processes of the vessel wall and vascular diseases. Indeed, in human atherosclerotic lesions, where mature SMCs revert to a more immature and less contractile phenotype, HOXB7 mRNA was detected at a higher level than in normal artery wall [123]. An even closer relationship seems to exist between VW-MPSCs and mature SMCs. SMC differentiation is accompanied by enhanced ACTA2, TAGLN and CNN1 expression. TAGLN is expressed exclusively in smooth muscle-containing tissues of adult mammals, and is one of the earliest markers of differentiating SMCs [124]. While the expression of these markers is a common feature of SMC regardless of their anatomical position, it has been shown that even SMCs of different parts of adult arteries, e.g., aortic arch, abdominal aorta and femoral artery, exhibit different codes of HOX gene expression, indicating the close relation between HOX code and the anatomical positional identity of SMC in each part of the blood vessels [125].

Further candidate factors were reported to be important for MSC differentiation to SMC. The most prominent one is the morphogenetic transforming growth factor-beta (TGFb) [126]. TGFb stimulation alone is sufficient for the induction of a rapid SMC differentiation of MPSC and MSC-like cells [46, 127, 128]. Isolated VW-MPSCs exposed to exogenous TGFβ1 during culturing exhibited alterations in the gene expression profile in the form of significantly increased expression of the SMC markers TAGLN, hyaluronan and proteoglycan binding link protein (HAPLN), and thrombospondin 1 (THSP1) [46]. In embryonic stem cell-derived MSCs (hES-MCs), TGF-β-treatment resulted in SMC differentiation in a dose- and time-dependent manner as demonstrated by the expression of SMC-specific genes ACTA2, CNN1, and smooth muscle myosin heavy chain (SM-MHC) [127]. Mechanistically, TGFb-induced differentiation was Smad- and serum response factor/myocardin-dependent. Furthermore, the treatment of adipose tissue-derived MSCs (hASCs) with TGFb dramatically increased the contraction of a collagen-gel lattice and the expression levels of SMC-specific genes including ACTA2, CNN1, SM-MHC, smoothelin-B, myocardin and h-caldesmon, as well as causing an increased expression of vascular SMC-like ion channels, indicating differentiation of hASCs into contractile vascular SMCs [128]. Beside the direct action of growth and differentiation factors, either by direct, exogenous application to cultured MPSC and MSC-like cells, or by stimulation of vascular MPSC in their native niche (e.g., by tumour secretion), other factors were described as decisive for the SMC differentiation of vascular multipotent stem cells. The differential expression of these cell-type-specific factors seems to act more indirectly, and to prime the cell somehow to differentiate along the SMC lineage. The basic molecular mechanism behind these cell-type-specific factors remains elusive at present. A prominent EphA3 expression in endometrial spiral arterioles and surrounding stroma, but not in other human tissues, suggests EphA3 as a unique marker of perivascular MSCs that are implicated in rapid neovascularization and vascular remodelling [129]. This selective EphA3 expression was further observed in actively growing rather than established blood vessels in the vascular microenvironment of solid tumours. In addition, a strong expression of CD146 within a BM-MSC subpopulation

was associated with a commitment to a vascular smooth muscle cell lineage characterized by a strong up-regulation of calponin-1 and SM22α expression and an ability to contract the collagen matrix [130].

5. Concluding remarks

Angiogenesis and vasculogenesis are central events in tissue development and repair. Initially, sprouting endothelial cells form immature blood vessels that lack coverage by pericytes and other mural cells. Subsequently vascular remodelling takes place, in which association with mural cells (pericytes and SMC) stabilizes these immature vessels. Vascular remodelling is a dynamic and strictly regulated process, which is active in a variety of physiological processes, such as vessel growth, angiogenesis and wound healing. An ordered remodelling seems to be critical for proper vascular development and maintenance and is an absolute prerequisite to preserve the sensitive relationship between resilience and stability of the vessel wall. However, remodelling is also initiated during pathological processes, such as atherosclerosis, ischaemia, congenital vascular lesions, vasculotoxic therapies and tumour growth.

Organ-specific multipotent stem cell types are associated with the vessel wall, in particular within the so-called "vasculogenic zone" of the vascular adventitia. These findings together with the stem cell-supporting functions of endothelial cells suggest that the vascular wall provides niches for different somatic stem cell types within the sub-endothelial space and the vascular adventitia. It conformity with the niche function of the adventitial vasculogenic zone, the presence of Sca-1+ smooth muscle cell progenitors has been shown within this zone [131]. Furthermore, it was reported that a subset of CD34+ cells within the vascular adventitia has the capacity to differentiate into pericytes [132]. More recently, CD44(+)CD90(+)CD73(+)CD34(-)CD45(-) cells were identified within the adult human arterial adventitia, which were termed vascular wall-resident multipotent stem cells (VW-MPSCs) and were capable of differentiating into vascular SMC and pericytes under in vitro and in vivo conditions [46]. These cells reside predominantly in the vasculogenic zone of adult human blood vessels and contribute to maturation of newly formed vessels.

In general, tissue-specific stem cells differentiate mainly to the type of tissue from which they derive, indicating that there might be a certain code ("priming") within the cells determined by the tissue of origin. Furthermore, due to their anatomical localization it is believed the vessel-resident stem and progenitor cells are available as a first point of contact for the secreted factors from tumour cells (Figure 5). Without mobilization from the niche, VW-MPSCs express HOXB7, HOXC6 and HOXC8 at higher levels as compared to SMCs. These HOX genes suppress the expression of TAGLN and CNN1 in VW-MPSCs, essential factors of early SMC differentiation. This mechanism probably accounts for keeping the VW-MPSCs quiescent in the adventitial niche. In contrast, silencing of HOX genes alter the CpG methylation of TAGLN promotor resulted in increased TAGLN expression which induced VW-MPSC differentiation into SMC/pericytes [60]. Thus, as discussed here in detail, VW-MPSCs are directly involved in vascular remodelling processes as these cells represent the major source of pericytes and SMC during angiogenesis and vascular stabilization processes under physiological and pathological

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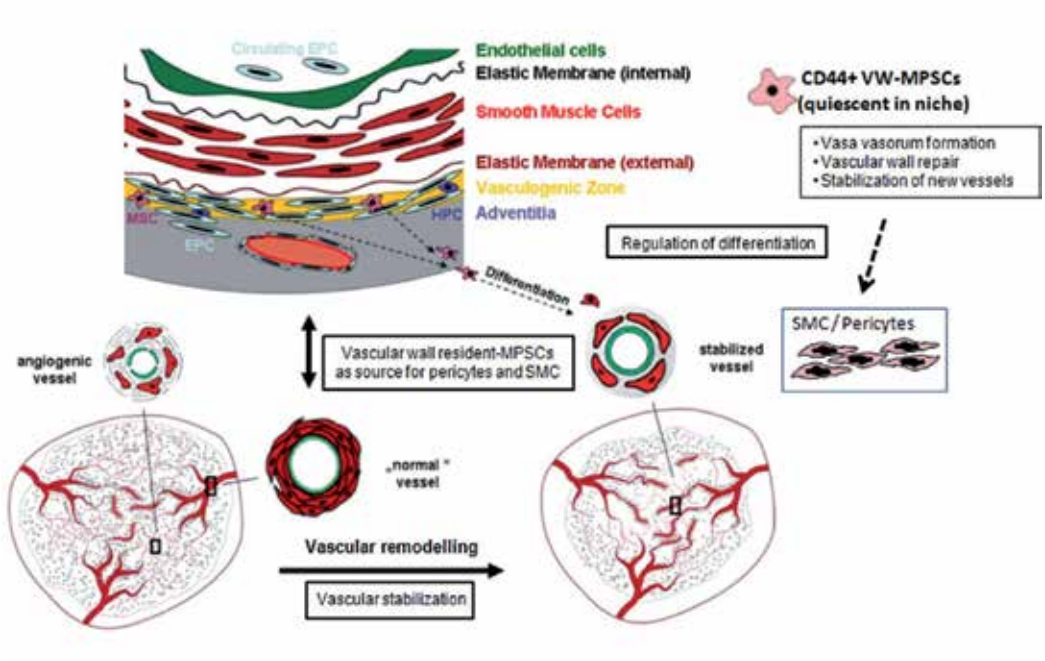

Figure 5. Vascular wall-resident multipotent stem cells (VW-MPSCs). VW-MPSC mobilization and differentiation into SMC may also be induced by signals released from, e.g., tumours, inflammation and hypoxia in tissue areas around blood vessels contributing to morphogenesis of the new vessel wall. It is hypothesized that VW-MPSCs are the "first-line" cells which were mobilized from their niche towards the tumour and activated to differentiate into pericytes and SMCs, which in turn stabilize angiogenic blood vessels. The molecular analysis of these stabilizing wall cells could yield new strategies for single-target genes, which could reduce the rate of drug-resistant tumours. Without mobilization from the niche VW-MPSCs express specific transcription factors at high levels. These genes suppress the expression of SMC-specific genes in VW-MPSCs. This mechanism probably accounts for keeping the VW-MPSCs quiescent in the adventitial niche. Tissue-resident MPSC and in particular VW-MPSCs, rather than circulating (BM-derived) multipotent stem cells, represent the major source of pericytes and SMCs during tumour vascularization. These findings on the origin of vascular-stabilizing pericytes and their function in tumour vascularization and remodelling provide a further basis for the design of novel strategies to improve anti-angiogenic therapies.

conditions. Therefore, these cells may be a promising target for counteracting vascular remodelling and related anti-angiogenic drug resistance.

In future investigations, a detailed molecular analysis of vascular wall-resident multipotent stem cells and of their differentiation into pericytes in response to tumour-secreted factors may be decisive to gain a better understanding of MPSC biology and differentiation. Particularly for cancer therapy, there is an urgent need to identify signalling molecules that are selectively regulated during the process of new vessel formation and/or subsequent vascular stabilization. Targeting of such molecules might also help to minimize anti-angiogenic drug resistance due to vascular stabilization. These investigations will provide basic knowledge for the design of innovative therapeutic strategies that target those vascular remodelling processes during cancer treatment that are associated with worse prognosis, for example, the generation of drug-resistant tumours.

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Chapter 3

Implications of MicroRNAs in the Vascular Homeostasis and Remodeling

Seahyoung Lee, Eunhyun Choi, Min-Ji Cha and Ki-Chul Hwang

Additional information is available at the end of the chapter

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Abstract

Vascular remodeling or arterial remodeling is a process of adaptive alteration of vascular wall architecture and leads to the endothelial cell (EC) dysfunction and synthetic or contractile phenotypic change of VSMCs, and the infiltration of monocytes and Macrophages that promotes vascular diseases including atherosclerosis. Recent findings have demonstrated that microRNAs (miRNAs) are involved in regulating gene expression at posttranscriptional level and disease pathogenesis. A change of miRNA expression profiles plays key roles in the gene expressions and the regulation of cellular functions. In this chapter, we summarize the vascular remodeling-related miRNAs and their functions in vascular biology.

Keywords: Vascular remodeling, vascular homeostasis, smooth muscle cells, endothelial cells, macrophage, microRNAs, vascular smooth muscle cell phenotypic switch

1. Introduction

Vascular remodeling (or arterial remodeling) is a process of adaptive alteration of vascular wall architecture and is caused by variety of environmental stimuli such as oxidative stress, vascular injury, and hemodynamic stress [1]. Endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) compose the arteries and play critical roles in vascular remodeling in conjunction with inflammatory cells such as monocytes or macrophages [2]. During vascular

remodeling, the EC dysfunction, synthetic or contractile phenotypic change of VSMCs, and the infiltration of monocytes and macrophages promote vascular diseases including atherosclerosis [3, 4]. Therefore, modulation of VSMC phenotype, maintenance of ECs, and regulation of inflammation in the vessel wall are important in arterial function and homeostasis.

Recent findings have been demonstrated that microRNAs (miRNAs) are involved in regulating gene expressions at posttranscriptional level and disease pathogenesis [5, 6]. Importantly, altered specific set of miRNAs is closely related to cell fate determination, tissue function, and homeostasis [7, 8], indicating that miRNAs plays key roles in the gene expressions and the regulation of cellular functions [9]. Consequently, the change of miRNA expressions can lead to VSMC phenotype switch, EC dysfunction, and the inflammatory response and lipid accumulation of macrophage in vascular pathophysiology [10–12].

In this chapter, we summarize the vascular remodeling-related miRNAs and the function of these miRNAs in vascular biology, and suggest novel therapeutic strategies for the treatment and/or prevention of vascular diseases via controlling the expressions of miRNA regulation.

2. MicroRNAs

MicroRNAs (miRNAs or miRs) are small noncoding RNA molecules approximately 18–25 nucleotides in length that participate in controlling gene expression at the posttranscriptional level [13]. MicroRNAs directly bind to 3 untranslated regions (3UTR) of target mRNAs, leading to translational inhibition and/or mRNA degradation [9]. One miRNA can suppress multiple target protein-coding mRNAs, which also means that a single protein can be regulated by numerous miRNAs [14]. Interestingly, about two-thirds of the coding-mRNA genes are regulated by miRNAs [15]. Since the first discovery of miRNAs in *Caenorhabditis elegans* in 1993, more than 1800 miRNAs in human have been identified and recorded in miRBase 21 (www.mirbase.org) [16].

The biogenesis of miRNAs are initiated by transcription from their genes by RNA polymerase II, which produces primary miRNAs (pri-miRNAs) with hundreds to thousands of nucleotides and single or multiple stem-loop-like structures [17]. After transcription, the pri-miRNAs are cleaved at ~70-nucleotide hairpin-shaped precursor miRNA (pre-miRNA) by RNase-III endonuclease (Drosha) and DiGeorge syndrome Critical Region 8 protein (DGCR8) complex [18]. The pre-miRNAs are transferred from nuclear to cytoplasm through the nuclear export protein exportin-5 [19]. In the cytoplasm, the pre-miRNAs are processed to produce mature miRNA duplex mediated by RNase-III endonuclease, Dicer: the passenger strand and the guide strand (~22 nucleotides long) [20]. The guide strand is loaded to RNA-induced silencing complex (RISC), and the passenger strand is degraded [21]. The miRNA–RISC complex recognizes and binds the miRNA based on the complementary match between the miRNA seed sequence (2–8 nucleotides in the 5' end) and the 3 UTR of the target mRNA, and this results in translational suppression and/or mRNA degradation (Figure 1) [22, 23].

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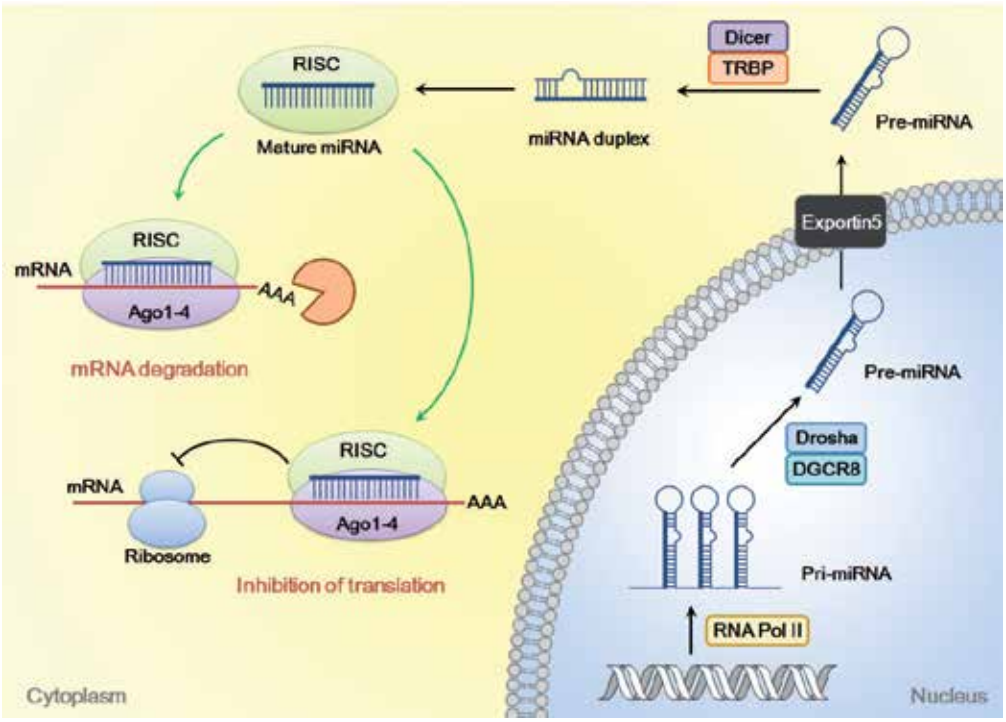

Figure 1. The biogenesis of miRNAs.

Increasing evidence suggests that miRNAs play important roles in a wide range of biological functions and processes, including but not limited to, development, growth, pathophysiology, regeneration, inflammation, stem cell fate regulation, and aging [24–29]. Because the changes of miRNA expression profiles are related to various human diseases, such as cardiovascular diseases, cancer, neuropathy, infection, and metabolic disorders [9, 30–34], the modulation of miRNA expressions consider as a novel therapeutic target and may inspire a new strategy for treatment of these diseases [35–37].

3. Vascular smooth muscle cells

The VSMCs of blood vessels in adult generally show a low proliferation rate and well-maintained homeostasis [38]. However, upon external stimuli, such as cytokines, blood flow rates, shear stress, hormones, injury, and inflammation, provoke the phenotypic switch of VSMCs from contractile to synthetic [2, 39]. The contractile or differentiated VSMCs express contractile markers, smooth muscle myosin heavy chain (SM-MHC), alpha smooth muscle actin (SM α-actin), and calponin; whereas the synthetic or dedifferentiated VSMCs show an increase of cell proliferation, migration, upregulation of extracellular matrix proteins,

such as collagen, elastin, and proteoglycans, and a decrease in the expression of contractile markers [16, 40]. The plasticity of VSMCs plays important roles in the vascular development and pathological/physiological vascular remodeling. The contractile phenotype is required to maintain the normal arterial wall structure and function, while the synthetic phenotype is involved in the development of atherosclerosis, restenosis after angioplasty, and hypertension [1, 39, 41].

The recent studies show that the regulation of VSMC phenotypic change is associated with the alteration of local environment as well as the modulation of gene expressions by miRNAs. Albinsson et al. reported that VSMC-specific deletion of Dicer-induced embryonic lethality at embryonic days 16 to 17 due to the thin and hypotensive vessel walls, impaired contractility, and hemorrhage [42]. In addition, the loss of Dicer in VSMCs of adult mice also showed a dramatic decrease in blood pressure, the impairment of contractile function, morphology, and phenotypic modulation [43]. These results strongly suggest that miRNAs are essential to vascular development and keep a balance of VSMC phenotype (Figure 2).

3.1. miRNAs in the contractile phenotype of VSMCs

3.1.1. miR-143 and miR-145

MiR-143 and miR-145 are the most well-known and enriched miRNAs in VSMCs and are in a bicistronic miRNA cluster on human chromosome 5 [44]. The significant role of miR-143 and miR-145 is to drive VSMC development and differentiation from human embryonic stem cells via targeting the stem cell pluripotency factors OCT4 (octamer-binding transcription factor 4), SOX2 [SRY (sex-determining region Y)-box 2], and KLF4 (Krüppel-like factor 4) [45]. After VSMC development, miR-143 and miR-145 are required for maintaining homeostasis of VSMCs through the suppression of multiple target proteins, such as KLF4, KLF5, E twenty-six (ETS)-like transcription factor 1 (ELK1), versican, several actin remodeling proteins, and angiotensin-converting enzyme. The transcriptional activation of miR-143 and miR-145 also leads to the expression of contractile genes [41, 46–48].

The maintenance of the VSMC contractile phenotype is modulated by several signaling pathways including serum response factor (SRF)/myocardin, transforming growth factor β (TGF-β), bone morphogenetic proteins 4 (BMP4), and Jagged-1 (Jag-1)/Notch signaling, which regulates the expression of miR-143 and miR-145 (Figure 3) [46]. The promoter region of miR-143 and miR-145 represents highly conserved *cis* elements and potential binding sites containing CArG box of SRF and Nkx2.5. In addition, myocardin and myocardin-related transcription factor A (MRTF-A), cofactors of SRF, synergistically and strongly activate the miR-143 and miR-145 transcription [49, 50]. The upregulation of miR-143 and miR-145 by TGF-β and BMP4 mediates the induction of SRF cofactors: TGF-β upregulates myocardin via the activation of parallel pathways of p38MAPK and SMAD dependent [51], whereas BMP4 stimulates the nuclear translocation of MRTF-A [52]. The Jag-1-mediated activation of Notch receptors is also activating miR-143 and miR-145 transcription [46]. Activation of Notch receptor by Jag-1 results in proteolytic cleavage and translocation to the nucleus of the Notch

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intracellular domain (NICD). In the nucleus, NICD forms complex with C promoter binding factor 1 (CBF1), which is binding to miR-143 and miR-145 promoter and increases in miR-145 and miR-145 expression [46].

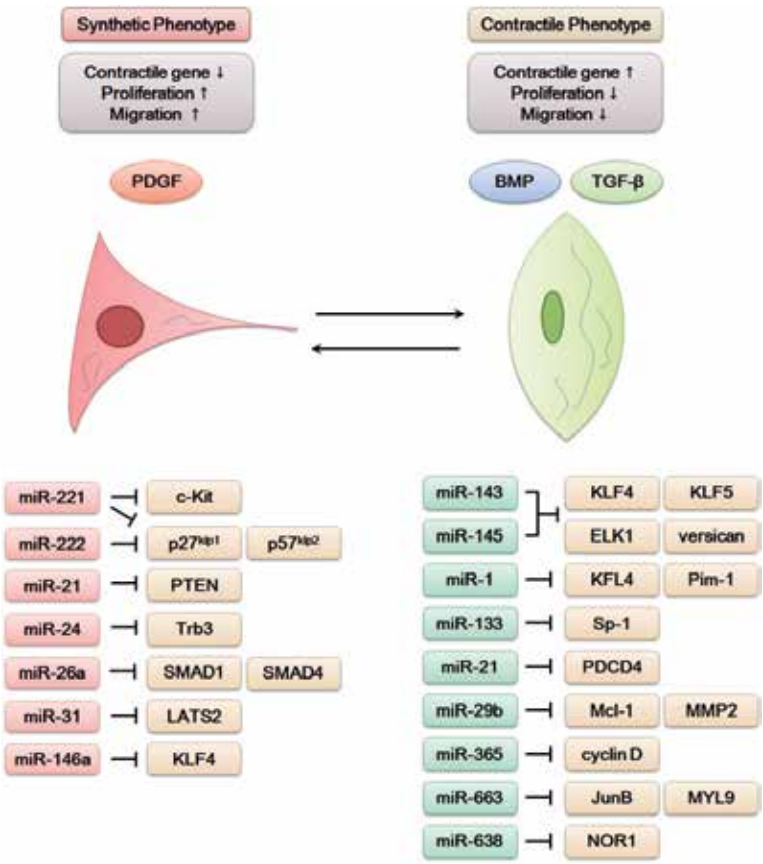

Figure 2. miRNAs in phenotypic switch of VSMCs.

The expression levels of miR-143 and miR-145 significantly decrease in animal vascular diseases models including apolipoprotein E knockout mice ($ApoE^{-/-}$), carotid artery ligation injury models, carotid balloon injury models, and in aortas from patients with aortic aneurism [53, 54]. MiR-143 and miR-145 knockout murine VSMCs showed a shift from contractile to synthetic state and the development of neointimal lesions [55], whereas the VSMC-specific overexpression of miR-143 and 145 increased the plaque stability and the expression of contractile proteins, and decreased macrophage infiltration, the expression of KLF4 and KLF5, and neointimal lesion formation [54, 56]. Altogether, these data indicate that miR-143 and miR-145 play an important role in the VSMC homeostasis and strengthen the VSMC contractile phenotype.

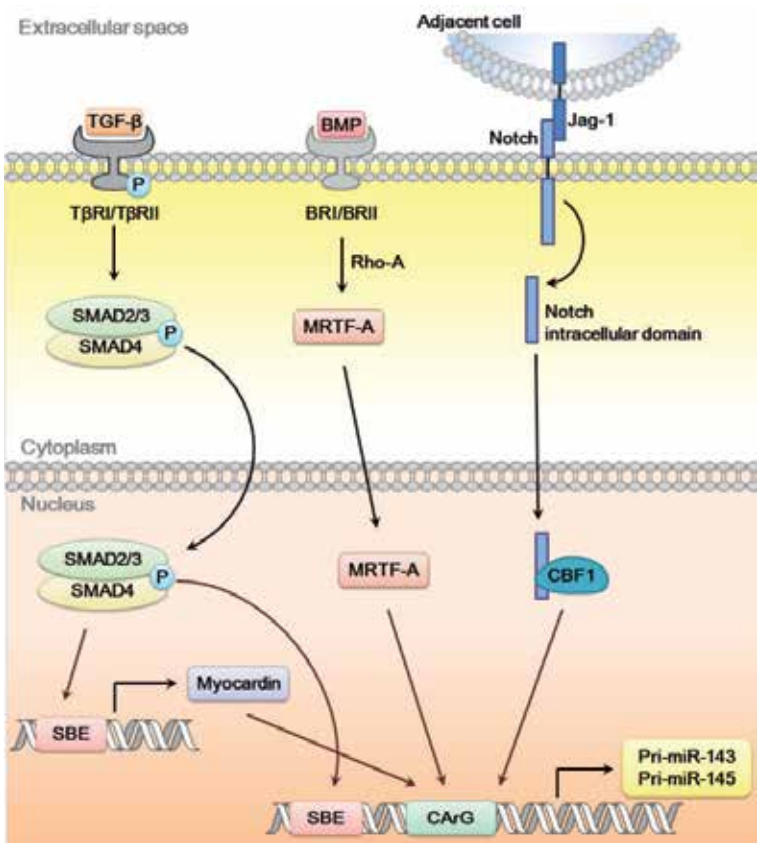

Figure 3. The signaling pathway of miR-143 and miR-145 expression.

3.1.2. miR-1 and miR-133

Similar to miR-143 and miR-145, miR-1 and miR-133 are also in bicistronic miRNA clusters and are induced by myocardin in VSMCs [57]. During differentiation of mouse ESC to VSMCs, the expression level of miR-1 is gradually increased, which results in the upregulation of VSMC-specific contractile proteins by repressing KLF4 [58]. Chen et al. demonstrated that overexpression of myocardin in VSMCs increased miR-1 expression and inhibited VSMC proliferation, which mediated the repression of Pim-1, a serine/threonine kinase [59].

The expression level of miR-133 is downregulated in proliferating VSMCs and after vascular injury. Overexpression of miR-133 reduces VSMC growth, but the downregulation of miR-133 induces VSMC proliferation *in vitro* [57]. Both the transcription factor Sp-1 (specificity protein 1) which regulates VSMC phenotypic switch and the actin-binding protein moesin that modulates VSMC migration are direct targets of miR-133. Accordingly, miR-133 overexpres-

sion in the rat carotid artery reduced neointimal hyperplasia, whereas anti-miR-133 increased VSMC proliferation and neointimal formation after balloon injury [57].

3.1.3. miR-21

The expression of miR-21 mediates TGF-β and BMP4 signaling pathway and promotes the contractile phenotype of VSMCs via downregulating programmed cell death 4 (PDCD4), a negative regulator of smooth muscle contractile genes [60]. Another target of miR-21 is the family of the dedicator of cytokinesis (DOCK) proteins, which promote VSMC migration by modulating the activity of small Rac1 GTPase [61]. On the other hand, Horita et al. reported that Fos-related antigen (FRA)-1, a direct target of miR-143, was a positive regulator of increased miR-21 expression [62]. The upregulation of miR-143 by SRF repressed FRA-1 expression, which led to the decrease of VSMC proliferation via the repression of miR-21 and subsequent increase of phosphatase and tensin homolog (PTEN), one of the target proteins of miR-21 [62]. The dual function of miR-21 in promoting both contractile and synthetic phenotype of VSMCs may suggest that diverse targets of miR-21 are involved in different biological processes depending on the cellular environments or context.

3.1.4. Other miRNAs

Our group has reported that miR-29b and miR-365 inhibit the proliferation and migration of VSMCs [63, 64]. The interleukin-3 (IL-3) is known to stimulate proliferation and migration in vascular diseases, and it downregulates miR-29b expression. Mir-29b significantly decreases the proliferation and migration of VSMCs through the inhibition of the signaling pathway related to Mcl-1 (myeloid cell leukemia 1) and MMP2 (matrix metallopeptidase 2). Consistent with the declined miR-29b expression in balloon-injured rat carotid arteries, overexpression of miR-29b by local oligonucleotide delivery can inhibit neointimal formation [64]. The proliferation of VSMCs by various stimuli, including platelet-derived growth factor (PDGF)-BB, angiotensin II (Ang II), and serum, led to the downregulation of miR-365 expression levels. The cell-cycle-specific cyclin D1 was found to be a potential target of miR-365; thus exogenous miR-365 overexpression reduced VSMC proliferation and proliferating cell nuclear antigen (PCNA) expression, blocking transition of G1/S [63, 65].

Li et al. reported that miR-663 is related to human VSMC phenotypic switch and the development of neointimal formation [66]. According to this particular study, overexpression of miR-663 upregulated VSMC differentiation marker genes and inhibited PDGF-induced VSMC proliferation and migration via targeting the transcription factor JunB and myosin light chain 9 (MYL9). Overexpression of miR-663 also dramatically attenuated the neointimal lesion formation in mice after carotid artery ligation [66]. In addition, the same group identified that miR-638 also is markedly downregulated by PDGF-stimulated human VSMCs, whereas upregulated in human VSMCs cultured in differentiation medium, a condition that inhibits proliferation [67]. Furthermore, the orphan nuclear receptor NOR1 (neuron-derived orphan receptor 1) was identified as a target of miR-638 and downregulation of NOR1 was implicated in the miR-638-mediated inhibitory effects on cyclin D1 expression, cell growth, and migration of PDGF-induced VSMCs.

3.2. miRNAs in the synthetic phenotype of VSMCs

3.2.1. miR-221 and miR-222

Similar to TGF-β and BMP4 promote VSMC contractile phenotype, PDGF induces VSMC phenotype switch from contractile to synthetic type [41]. Mir-221 and miR-222 are well characterized in VSMC phenotype switch and clustered on the X chromosome, and have same seed sequence [68]. Mir-221 and miR-222 are transcriptionally induced by activating PDGF pathway, which reduces the expression of contractile genes and promotes VSMC proliferation and migration [69]. In addition, the expression of miR-221 and miR-222 are elevated in neointimal lesion from balloon-injured rat carotid arteries [70]. The targets of miR-221 and miR-22 include $p27^{Kip1}$ and $p57^{kip2}$, both of which are a negative regulator of VSMC proliferation [70, 71]. Interestingly, miR-221 decreased the expression of c-kit, and this reduced c-kit expression subsequently repressed the expression of a VSMC-specific nuclear coactivator myocardin [69]. As the PDGF-treatment stimulated phenotype change of VSMCs, overexpression of miR-221 and miR-222 accelerated VSMC proliferation and migration, while downregulation of them attenuated VSMC proliferation and neointimal formation in rat carotid artery after angioplasty [70]. These data indicate that miR-221 and miR-222 have a crucial role in regulating VSMC phenotype change, and suggest their therapeutic potential in pathological vascular remodeling.

3.2.2. miR-146a

Recent study has shown that miR-146a is upregulated in rat balloon-injured arteries and serum-induced proliferative VSMCs [72, 73]. KLF4, a negative regulator of VSMC proliferation, was downregulated by miR-146a, and in turn, KLF4 repressed miR-146a expression through binding to CACCC (or GGGTG) elements on the miR-146a promoter. This negative feedback regulatory network between miR-146a and KLF4 regulates transcription of each other, controlling VSMC proliferation *in vitro* and vascular neointimal hyperplasia *in vivo* [72]. Dong et al. demonstrated that miR-146a upregulation is related to the downregulation of critical transcriptional factors such as the protein expression of nuclear factor-κBp65 (NF-κBp65) and PCNA. In contrast, miR-146a knockdown is linked to the increase in pro-apoptotic protein Bax expression [73]. Therefore, miR-146a is a novel regulator of VSMC proliferation in cardiovascular diseases.

3.2.3. miR-24

Both TGF-β and BMP4 signaling are important in maintaining the VSMC contractile phenotype, whereas PDGF signaling pathways can promote the synthetic phenotype conversion of VSMCs. Although these signaling pathways play key roles in VSMC phenotypic changes, the crosstalk between these pathways is not elucidating until quite recently. Chan et al. reported that despite of BMP4 stimulation, the contractile genes are downregulated by co-treatment with PDGF in human primary pulmonary SMCs (PASMCs) [74]. Furthermore, PDGF stimulation decreases the expression of Tribbles-like protein-3 (Trb3), an important modulator of the BMP and TGF-β signaling pathway. Interestingly, the result of Trb3 promoter-luciferase-

reporter assay showed that the downregulation mechanism of Trb3 expression by PDGF is associated with the effect of PDGF-induced miR-24, directly targeting 3URT of Trb3 mRNA, rather than PDGF stimulation [74]. The knockout endogenous miR-24 by antisense oligonucleotides restored Trb3 expression as well as attenuates PDGF-mediated synthetic activity, suggesting that PDGF-induced miR-24 expression which downregulated Trb3, and this miR-24-mediated downregulation of Trb3 is sufficient to induce synthetic phenotypic changes of VSMCs [74].

3.2.4. miR-26a

Leeper et al. demonstrated that serum-starved human aortic SMCs upregulates the expression of contractile marker genes, and miR-26a is significantly downregulated during serum-starvation and in murine abdominal aortic aneurysm (AAA) development models [75]. Overexpression of miR-26a promoted VSMC proliferation and migration as well as inhibited apoptosis, whereas downregulation of miR-26a induced contractile gene expression and reduced proliferation and migration. The potential targets of miR-26a include SMAD1 and SMAD4, members of TGF-β and BMP superfamily downstream signaling cascade. Consistently, knockdown of miR-26a led to SMAD1 and Samd4 upregulation and activates TGF-β and BMP signaling pathway. Thus, miR-26a is expected to actively participate in the regulation of VSMCs as an enhancer of cell growth and migration, and an inhibitor of apoptosis, modulating TGF-β and BMP signaling [75].

3.2.5. miR-31

Although miR-31 plays an important role in cancer cell growth and proliferation [76–78], the biological function of miR-31 in VSMCs remains unclear. Recently, Liu et al. found that miR-31 was an abundant miRNA in VSMCs of vascular walls, and was significantly upregulated in proliferative VSMCs and rat carotid arteries with neointimal growth after balloon injury [79]. The serum- or PDGF-induced VSMC proliferation is inhibited by miR-31 inhibitor, 2′OMe-miR-31 (the antisense oligonucleotide for miR-31 that is modified at each nucleotide by an O-methyl moiety at the 2′-ribose position). On the contrary, overexpression of miR-31 using the adenoviruses expressing rat miR-31 promotes VSMC proliferation [79]. The pro-proliferative effect of miR-31 is mediated by the direct inhibition of the large tumor suppressor homolog 2 (LATS2) expression, and is accelerated by the activation of mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) [79].

4. Endothelial cells

Vascular endothelial cells (ECs) cover the internal surface of blood vessels. The primary role of ECs is the maintenance of vessel wall permeability and function as a sensor for the altered physical and chemical signals by physiological or pathological processes including thrombosis, inflammation, and vascular wall remodeling [80–82]. The knockdown of Dicer in human ECs resulted in the change of several key regulator proteins of EC biology and angiogenesis,

such as TEK (Tie-2), KDR (VEGFR2), adhesion molecules and proteins, cytokine, chemokine, and IL-8. Furthermore, EC-specific knockdown of Dicer activated the endothelial nitric oxide synthase (eNOS) pathway, which reduces proliferation and capillary formation of EC, and the endogenous miRNA expression levels were also significantly altered [83]. These results indicate that altered miRNA expression in ECs affects the maintenance and functions of ECs (Figure 4).

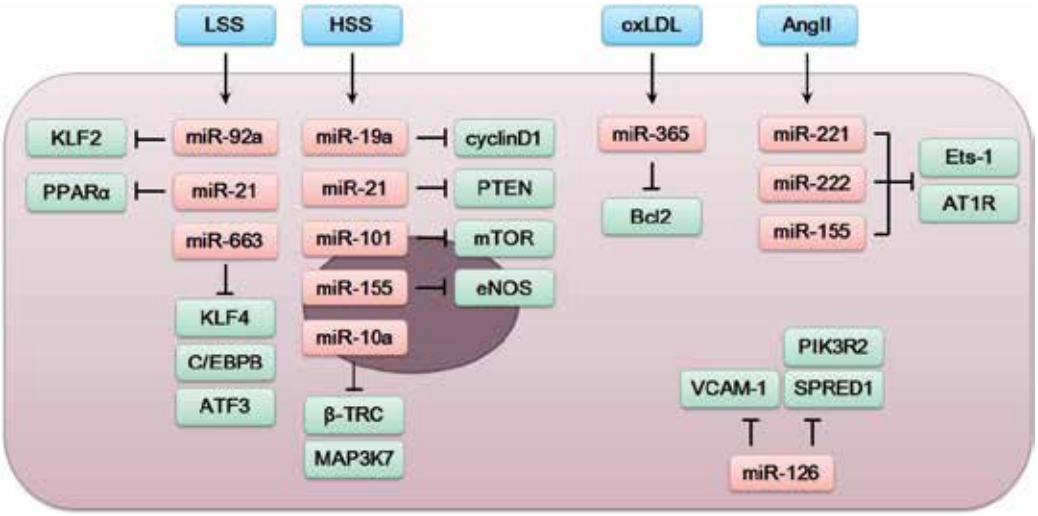

Figure 4. miRNAs in ECs regulating during vascular remodeling.

4.1. miR-126

Adhesion molecules in ECs are directly associated with leukocyte trafficking to the region of the injury and/or inflammation [84]. Generally, resting ECs do not express adhesion molecules, but activated ECs express adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), which plays an important role in modulating leukocyte trafficking and inflammation [85]. Based on microarray and Northern analysis, Harris et al. showed that miR-126 is the most frequently expressed in HUVECs as well as other origin ECs from dermal, brain, and vein [86]. *In silico* analysis suggests that VCAM-1 is one of the targets of miR-126. The upregulation of miR-126 by transfection of pre-miR-126 results in inhibiting TNF-α induced VCAM-1 expression, whereas the knockdown of miR-126 by antisense miR-126 oligonucleotide is causing TNF-α induced VCAM-1 upregulation in a dose-dependent manner. In addition, overexpression of miR-126 mediated a decrease of VCAM-1 downregulating leukocyte adherence to ECs. These data suggest that miR-126 regulates the expression of adhesion molecules and plays a crucial role in the control of vascular inflammation [86].

Fish et al. found that EC-specific miRNA miR-126 is also highly enriched in Flk-1 positive vascular progenitors from differentiating mouse embryonic stem cells in the embryonic body [87]. Nevertheless, overexpression of miR-126 using miR-126 mimic does not promote

endothelial differentiation of embryonic stem cells. The loss-of-function of miR-126 by a morpholino antisense to miR-126 shows that the EC migration and the formation/stability of capillary tubes are decreased compared to normal HUVECs. In addition, EC-targeted deletion of miR-126 results in vascular abnormalities such as vascular leakage, hemorrhaging, and embryonic lethality in both zebrafish [87] and mice [88], because of reduced EC growth, sprouting, and adhesion by decrease in angiogenic growth factor signaling. The pro-angiogenic function of miR-126 was due to its ability to repress two negative regulators of angiogenic process, namely sprouty-related EVH1 domain-containing protein 1 (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2) [87, 88]. Taken together, miR-126 is considered as new therapeutic implication for modulating vascular formation, function, leakage, and abnormal angiogenesis.

4.2. Endothelial Shear Stress (ESS) and miRNAs

The atherosclerotic lesions form at specific arterial regions such as the vicinity of branch points, the outer wall of bifurcations, and the inner wall of curvatures, where they show the disturbed flow and the change of hemodynamic forces, especially endothelial shear stress (ESS) [89]. ESS has been reported to regulate endothelial gene expression and promotes EC activation and atherosclerotic plaque progression and vascular remodeling [90, 91].

By miRNA microarray analysis using human umbilical vein endothelial cells (HUVECs), Ni et al. identified that the expression of miR-663 was mediated by low ESS (LSS) and LSS-induced miR-663 expression improved monocyte adhesion to ECs, but it had no effect on EC apoptosis. MicroRNA-663 has been reported to regulate several transcription factors related to inflammatory responses, such as KLF4, C/EBPB, and ATF3 [92].

In addition, miR-21 was also upregulated by LSS at transcriptional level in HUVECs through an increased activity and binding of c-Jun, a component of transcription factor activator protein-1 (AP-1), to the promoter region of miR-21 [93]. Mir-21 upregulated the expression of VCAM-1 and monocyte chemotactic protein-1 (MCP-1) by suppressing the peroxisome proliferators-activated receptor-α (PPARα), increasing adhesion of monocytes to ECs and pro-inflammatory responses [93].

MicroRNA-92a, a member of miR-17~92 cluster, was upregulated by LSS in HUVECs [94]. The decreased expression of miR-92a was correlated to the upregulation of KLF2 and subsequent KLF2-mediated eNOS and thrombomodulin (TM) expression and NO production, all of which are considered as potent anti-thrombotic, anti-adhesive, and anti-inflammatory properties [94, 95]. Therefore, LSS-induced miR-92a inhibited KLF2-mediated eNOS and TM expression that resulted in impaired EC functions.

In contrast to LSS, high ESS (HSS) appears to be protective against atherosclerosis, but the precise functions still remain unclear [96]. Mir-10a is one of the flow-induced miRNAs in ECs and is known to be downregulated in athero-susceptible regions of the inner aortic arch and aorto-renal branches than other regions [97]. The knockout of miR-10a led to IκB/NF-κB-mediated inflammation, which suggested anti-inflammatory role of miR-10a. Interestingly, two key regulators of IκBα degradation, mitogen-activated kinase kinase kinase 7 (MAP3K7;

TAK1) and β-transducin repeat-containing gene (β-TRC), are putative targets of miR-10a [97]. Therefore, miR-10a contributes to anti-inflammatory endothelial phenotype through the inhibition of pro-inflammatory molecules in athero-susceptible regions.

In addition, Qin et al. found that laminar shear stress induced miR-19a that suppressed cyclin D1 expression, leading to an arrest of cell cycle at G1/S transition [98]. Similar research by Chen et al. showed that miR-101 was also induced by laminar shear stress and cell cycle arrest at the G1/S transition and suppressed endothelial cell proliferation via targeting mTOR [99].

MicroRNA-155 is upregulated by prolonged HSS in HUVECs and it modulated endothelium-dependent vasorelaxation by repressing eNOS. Therefore, the inhibition of miR-155 improved endothelial dysfunction during the development of atherosclerosis [100].

MicroRNA-21 and miR-92a were induced by both LSS and HSS. Compared with LSS-induced miR-21, HSS-induced miR-21 decreased EC apoptosis and activated the NO pathway by targeting PTEN [101]. As mentioned earlier, downregulation of miR-92a led to upregulation of KLF2 and subsequent KLF2-mediated eNOS and TM, which facilitated the maintenance of EC homeostasis and functions against inflammation and pro-atherosclerotic effects [94, 95].

4.3. Angiotensin II (AngII) and miRNAs

Angiotensin II (AngII) has been implicated in the development and progression of cardiovascular diseases such as hypertension, atherosclerosis, and restenosis after vascular injury [102]. AngII-mediated intracellular singling is activated through its G-protein-coupled AngII Type 1 receptor (AT1R). In both VSMCs and ECs, AngII leads to vascular and endothelial dysfunctions by generating intracellular reactive oxygen species (ROS) and negatively regulating NO signaling pathway, respectively [103, 104]. Zhu et al. found that miR-155, miR-221, and miR-222 were highly expressed in both HUVECs and VSMCs, and a key endothelial transcription factor for inflammation and tube formation Ets-1 was a putative target of miR-155, miR-221, and miR-222 [105]. Interestingly, miR-155 also targeted AT1R, and AngII-stimulated HUVECs upregulates Ets-1 and its downstream genes, including VCAM1, MCP1, and FLT1 [105]. These results indicated that miR-155, miR-221, and miR-222 act as negative regulators to inflammatory response of ECs caused by AngII stimulation.

4.4. Oxidized low-density lipoprotein (oxLDL) and miRNAs

Oxidized low-density lipoprotein (oxLDL) induces EC death and dysfunction through the activation of NF-κB and AP-1 pathways, and it also activates pro-inflammatory response during the progression of atherosclerotic vascular diseases [2, 106]. Based on microarray and qRT-PCR analysis with oxLDL-stimulated HUVECs, Qin et al. found that four miRNAs are upregulating including miR-365 and miR-142-3p, whereas eleven miRNAs are downregulating including miR-590-5p and miR-33a in microarray, which expression levels are validated with qRT-PCR [107]. Using computational prediction algorithms (TargetScan, microRNA.org, and MicroCosm v5), the gene ontology (GO) database (www.geneontology.org), and KEGG pathway database (www.genome.ad.jp/kegg), the authors predicted and identified the functions of miRNA-targeted genes. As a result, miR-365 has been reported to be involved in

oxLDL-induced apoptosis of ECs by suppressing the anti-apoptotic protein Bcl-2 [107]. Therefore, the inhibition of miR-365 may reduce EC apoptosis and inhibit the pro-atherogenic progression caused by oxLDL.

5. Macrophage

The accumulation and activation of macrophages within the vascular wall are a crucial event in vascular remodeling and implicate the progression of atherogenesis, which is initiated by inflammation, a rise in circulating LDL levels, and the accumulation of oxLDL in macrophages [108, 109]. Inflammation triggers the recruitment and infiltration of monocytes and macrophage in atherosclerotic lesions, continuously accumulating lipids and oxLDL [110]. The macrophages respond to various inflammatory stimuli, which lead to the changes in expression of many genes and several miRNAs, including miR-155, miR-125a, miR-146a, and miR-146b [111, 112]. Although the microRNAs involved in oxLDL accumulation and inflammatory response in macrophages are currently unclear, considering the role of miRNAs in VSMCs and ECs during arterial remodeling, the idea of miRNAs that also contribute the macrophage functions does not seem to be illogical (Figure 5) [2].

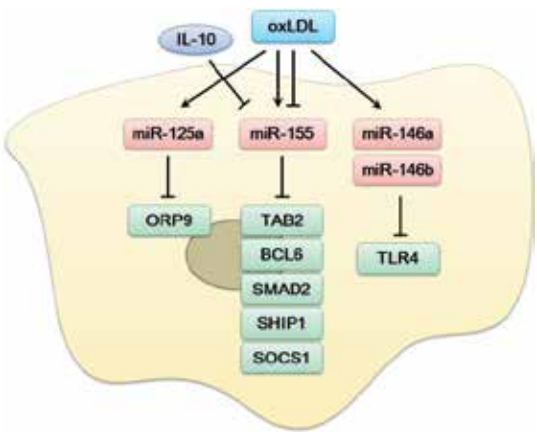

Figure 5. miRNAs in macrophages during vascular remodeling.

5.1. miR-155

The expression of miR-155 increased in human atherosclerotic lesions, but circulating miR-155 is known to be downregulated in patients with coronary artery diseases [113, 114]. During vascular remodeling, miR-155 is expressed in VSMCs, ECs, as well as activated macrophages [115]. The oxLDL and/or ESS promote the recruitment of monocytes into the arterial wall through ECs and then monocytes are differentiated toward macrophages and dendritic cells (DCs) [108]. MiR-155 is the most important miRNA in macrophage-mediated inflammation [116], and its expression is increased by several Toll-like receptor (TLR) ligands via myeloid

differentiation primary response gene (MyD88) and/or TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signaling in the inflammatory activation of macrophages [117]. In addition, the lipopolysaccharide (LPS) is also known to induce miR-155 in monocytes and DCs. LPS-induced miR-155 expression is mediated by the single-strand RNA-binding protein KH-type splicing regulatory protein (KSRP) in bone marrow-derived macrophages [118]. In activated primary human monocyte-derived DCs, LPS-induced upregulation of miR-155 inhibited Toll-like receptor/interleukin-1 (TLR/IL-1) inflammatory pathway as well as TAB2 (an adaptor in the TLR/IL-1 signaling cascade), attenuating IL-1β and inflammatory signals [119]. Furthermore, oxLDL and cytokine IFN-γ induced the expression of miR-155 that modulated the inflammatory response in macrophages by repressing BCL6, a negative regulator of pro-inflammatory NF-κB signaling. The attenuation of BCL6 was correlated to upregulation of chemokine CCL2, an activator of monocyte recruiting into atherosclerotic plaques [120]. MicroRNA-155 also regulates TGF-β signaling in macrophages by targeting SMAD2. Overexpression of miR-155 inhibited TGF-β induction of SMAD2 phosphorylation, which repressed TGF-β-dependent transcription of cytokines, such as IL-1β [121].

On the other hand, a potent anti-inflammatory cytokine IL-10 inhibited LPS-induced miR-155 expression via STAT3-dependent manner, which led to upregulation of the target proteins of miR-155 such as Src homology 2 domain-containing inositol-5-phosphatase 1 (SHIP1) and anti-inflammatory genes [122]. Moreover, LPS-activated PI3K/Akt1 pathway inhibited expressions of miR-155 and pro-inflammatory mediators [123]. Paradoxically, a small number of studies reported that miR-155 is also downregulated in oxLDL simulation and reduces lipid uptake and accumulation in macrophages [120, 124]. Therefore, miR-155 in macrophages maintains the balance between pro- and anti-inflammatory responses, which is influenced by the diseases context or extracellular microenvironments.

5.2. miR-125a

Similar to miR-155, upregulation of miR-125a seems to decrease lipid accumulation by directly targeting oxysterol-binding protein-like 9 (ORP9), but the functional role of miR-125a in oxLDL-stimulated monocytes and macrophage still remains unclear [125]. MicroRNA-125a has been reported to be upregulated through oxLDL in primary human monocytes, and inhibition of miR-125a increased the secretion of IL-6, TNF-α, IL-2, and TGF-β, possibly due to enhanced expression of scavenger receptors (LOX-1, CD68).

5.3. miR-146a and miR-146b

MicroRNA-146a/b is upregulated after stimulation with oxLDL in primary human monocytes [125]. MiR-146a and -b are located on different chromosomes, and differ by two nucleotides. Stimulation of TLR-2, 4, and 5 has been reported to induce miR-146a/b expressions in macrophages through NF-κB activation [126]. The oxLDL-mediated suppression of miR-146a promoted lipid uptake and cytokine release, most likely due to reduced suppression of TLR-4 (target of miR-146a) in macrophage [124]. Additionally, miR-146a and b are known to upregulated in human atherosclerotic plaques [127].

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6. Circulating miRNAs as biomarkers

In addition to the significance of miRNAs regulating gene expression at the intracellular regions, some miRNAs have been discovered in the extracellular body fluids including serum/plasma and bloodstream [128, 129]. Interestingly, the extracellular circulating miRNAs show remarkably stable and resistant against ribonucleases, freezing, boiling, low or high pH, and so on, which signifies that some protective mechanisms can countervail degradation [130–132]. The mature miRNAs are released from intracellular to extracellular environment via the complex with RNA-binding proteins such as Argonaute-2 (Ago2), binding to high-density lipoprotein (HDL), and loading into microvesicle bodies such as microvesicles, exosomes, or apoptotic bodies (Figure 6) [133–136].

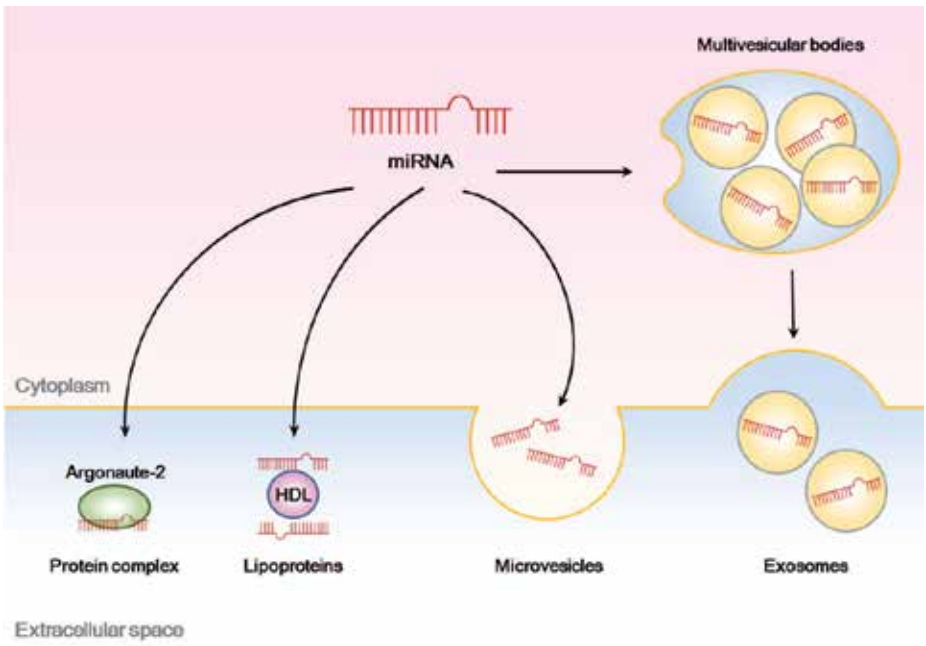

Figure 6. The cellular release mechanisms of miRNAs.

Although the cellular origin and function of the circulating miRNAs remain uncertain, their expression profiles are changed by fluid types and physiology or pathology conditions [137, 138]. The extraordinarily stable and tissue/diseases specific profiles of circulating miRNAs have been considered as promising biomarkers for diagnosis of diseases [139, 140].

Several studies suggest that circulating miRNAs may be taken into the recipient cells and regulate target gene expression [141–143]. In vascular diseases, a few studies showed that circulating miRNAs modulate the function of ECs [144, 145]. Zhang et al. found that the secretion of microvesicles containing miR-150 from human monocytic cells THP-1 is increased in the plasma of patients with atherosclerosis. Secreted monocytic miR-150 is transported into human microvascular endothelial cells (HMEC-1) and then promotes HMEC-1 migration via downregulating the miR-150 target gene c-Myb [144]. Likewise, Zernecke et al. showed that

EC-derived apoptotic bodies, containing miR-126, upregulates the production of CXC chemokine CXCL12, which promotes the recruitment of Sca-1^{+} progenitor cells and reduces the atherosclerotic lesion formation in ApoE$^{-/-}$ mice. These results suggest that circulating miR-126 in apoptotic bodies may have protective potential against atherosclerosis [145].

7. Conclusion

Vascular remodeling encompasses a series of complex biological pathways and involves the phenotype change of VSMCs, EC dysfunction, as well as macrophage activation. The alteration of VSMCs, ECs, and macrophage cellular functions is related to the various extracellular stimulus-dependent changes in transcriptional regulation, which is regulated by miRNAs. Thus, the identification of stimuli-dependent vascular remodeling which affects miRNA expression in different vascular cell types is imperative. The augmenting or inhibiting of the expression levels of specific miRNAs may provide opportunity for the development of miRNA-based therapeutic application to treating diverse vascular pathologies. In addition, extracellular circulating miRNAs have been reported to be altered under specific pathologic conditions, implicating their usage as biomarkers for specific diseases including cardiovascular disease. Although there are unsolved issues of the efficiency and safety of using miRNAs in diagnosis and therapy, accumulating evidence indicate that continuous research on the functions and mechanisms of miRNAs and the identification of a network between miRNAs and their targets is highly recommended and the results will expand our understanding of vascular remodeling and diseases.

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Section 2

Plasticity of the Skeletal Muscle

Chapter 4

Lifestyle and Aging Effects in the Development of Insulin Resistance — Activating the Muscle as Strategy Against Insulin Resistance by Modulating Cytokines and HSP70

Thiago Gomes Heck, Mirna Stela Ludwig, Analu Bender dos Santos and Pauline Brendler Goettems-Fiorin

Additional information is available at the end of the chapter

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Abstract

This chapter discusses about subclinical processes related to insulin resistance development that worsen the muscle metabolic functions, generated by factors such as lifestyle (bad quality food intake and sedentary behavior) and aging. Also discussed are the effects of regular physical exercise as a strategy to prevent the metabolic impairment in organisms, approaching since muscle subclinical molecular processes to the whole body's integrative physiology. Insulin resistance development includes modification in the pattern of inflammatory cytokines, heat shock proteins, tissue-specific defects in insulin action and signaling, oxidative stress and ectopic lipid deposition. The exercise is a known modulator of all parameters listed above and has important role in the regulation of "immune-metabolic" homeostasis from the muscle to the whole body. This chapter aims to present a new molecular approach related to the control of metabolism and encourage scientists and students to propose new strategies against insulin resistance and diabetes type 2 developments.

Keywords: Insulin resistance, exercise, heat shock proteins, cytokines, oxidative stress

1. Introduction

Metabolism can be defined as the sum of all reactions that occur in the whole body, from cells to complex system, with a multi-organ talk about energy transfer, signaling, and then regula-

tory pathways of metabolic status. One of the most important parameters of human metabolic homeostasis is glycemia that results from the availability and the utilization of nutrient sources. In this way, the cellular uptake and glycogenesis in skeletal muscle fibers have important implications in the regulation of blood glucose. This metabolic process was regulated by insulin that acts on muscle promoting the translocation of glucose transporter-4 (GLUT4), which is the most abundant insulin-dependent transporter in the cell membranes of the skeletal muscle, heart muscle, and adipose tissue, which leads to the uptake of glucose into the cell.

In this scenario, the muscle can be considered an important organ in glucose metabolism regulation, functioning at the same time as locus of start- and end-point of metabolic disorders related to glucose metabolism, and also the key organ of intervention strategies against insulin resistance. This chapter proposes an overview about effects of physical activity and exercise in the muscle as a strategy against insulin resistance. The physiological approach in this chapter is based on two major signaling pathways and biomarkers of muscle function, as well as its interaction with other cells and tissues—the cytokines and heat shock proteins (HSPs).

Skeletal muscle contains anatomic and physiological characteristics that represent different possibilities and functions in terms of metabolic properties and also in contractility and mitochondrial activity. There are three important factors that influence these characteristics: age, the dietary behavior, and the levels of physical activity. Together, these factors determine the muscle health status and capacity of physical performance, then, consequently, the whole body homeostasis. Aging, high caloric diet consumption, and sedentary behavior leads the progression of the muscle dysfunction that culminates in the loss of metabolic homeostasis, promoting dyslipidemia and glycemic alterations.

However, these outcomes are presented in the established metabolic disease, while many subclinical processes precede the onset of disease. Subclinical modifications also accom panied the undesired progression of metabolic disease, increased the incidence of comorbidities and increased hospital admissions. These silent subclinical effects, such as inflammation, oxidative stress, and molecular alterations, decreased gradually the individual health status and decreased the quality of life (Figure 1).

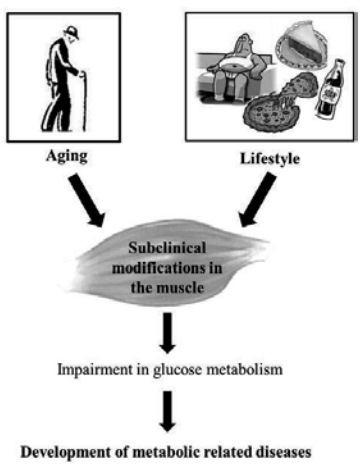

Figure 1. Muscle as the target of subclinical modification related to metabolic disease development.

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Thus, in this chapter we first discussed factors that worsen the muscle's subclinical metabolic functions, such as lifestyle (bad quality food intake and sedentary behavior) and aging; second we discussed about the effects of exercise as a strategy to prevent metabolic impairment, in the maintenance of muscle health, and to improve body homeostatic control, from the muscle's subclinical molecular processes to the whole body.

2. Lifestyle

A high dietary fat intake and low levels of physical activity characterizes much of the overall lifestyle. Surplus of fat intake is stored in many human tissues and these intracellular lipids serve as a rapidly available energy source during, for example, physical activity. Mainly in the sedentary condition, lipid excess leads to the development of modern diseases such as obesity and insulin resistance [1].

The consumption of high-fat diets (HFD) is associated with an excessive storage of fatty acids in the skeletal muscle [1]. The human (and also laboratory animals) body are composed of several muscles that contain slow-twitch (type I) fibers, which contain a high number of mitochondria and use oxidative metabolism as an energy source, and fast-twitch (type II) fibers, which generate energy mainly through glycolysis. High intake of hypercaloric or high-fat diets promotes a series of structural and metabolic changes that affect muscle capacity. An inadequate diet, such as HFD, induces muscle adaptations at molecular levels, promoting an increase in the proportion of oxidative fibers (type I fiber) by increasing the levels of the myosin heavy chain, slow fiber type protein, complexes of the oxidative phosphorylation, and the mitochondrial membrane composition. However, despite the increased oxidative fibers proportion, these modifications are insufficient to prevent impairments in oxidative metabolism [2]. The long-term HFD consumption promotes a decrease in the muscle mass and an increase in muscle triglyceride accumulation in parallel to the increased expression of biomarkers of mitochondrial metabolism such as succinate dehydrogenase complex subunit-myocytes and within the fascia surrounding skeletal muscle (increase in the intra and enzyme of β-oxidation), and the phosphorylation of acetyl-CoA carboxylase (ACC) (regulation of lipid synthesis). These alterations contribute to the morphological impairment known as myosteatosis or the ectopic skeletal muscle adiposity that represents fat infiltration within myocytes and within the fascia surrounding skeletal muscle (increase in the intra- and intermuscular fat content, respectively) [3]. Mounting evidence indicates that elevated intramyocellular lipid deposition is associated with diminished insulin sensitivity in the skeletal muscle, promoting insulin resistance. Since fiber type I have a higher capacity for "fat burning", studies have reported a negative association between adiposity and the relative percentage of type I fibers. In other words, more muscle oxidative capacity results in less adiposity [4]. The increase in type I fiber proportion represent an attempt to restore the energy homeostasis between the source and energy demand. If this adaptive response is insufficient, the human body is susceptible to metabolic dysfunction.

Insulin resistance in the skeletal muscle in humans is associated with decreased oxidative capacity of ATP synthesis and also related to the decrease of many genes expression. Genes that control mitochondrial activity, including peroxisome proliferator-activated receptor

gamma coactivator 1-alpha (PGC-1α), may indeed play a crucial role in the development of mitochondrial dysfunction, insulin resistance, and diabetes mellitus type 2 (T2DM) through the western lifestyle that is rich in hypercaloric or high-fat diets. Three days of HFD reduces PGC-1α protein levels by approximately 20% in humans and 40% in C57B1/6J mice after three weeks on an HFD treatment [5]. Sparks et al. [5] emphasized that HFDs in both humans and mice were associated with the reduction in the expression of genes involved in electron transport chain, nuclear genes encoding mitochondrial proteins (e.g., mitochondrial carrier proteins), and those involved in mitochondrial biogenesis (e.g., PGC1 and PGC), supporting the hypothesis that HFDs or high-fat flux explain the reduction in oxidative phosphorylation pathway (OXPHOS) genes seen in aging, the prediabetic state, and in overt diabetes.

Ciapaite et al. [6] suggest that the consumption of unhealthy obesogenic HFDs in combination with a sedentary lifestyle may create a vicious cycle by impairing skeletal muscle function and decreasing exercise potential, which may lead to further aggravation of obesity and skeletal muscle dysfunction. The adaptation response to dietary lipid overload occurs by fiber-type-specific mechanisms, leading to differential impairment of fast-twitch and slow-twitch skeletal muscle contractile function. Fast-twitch fibers suffered impairment in mitochondrial ATP production and Ca^{2+} homeostasis, and slow-twitch fibers have changed the sarcomere composition and force production. Together, changes in both types of fiber related to the consumption of HFD affect the functionality and muscular performance [6].

Muscle metabolic function can also be impaired by increasing visceral fat accumulation. This fat is more lipolytic (rapidly turned over) than subcutaneous fat and less sensitive to the anti-lipolytic effect of insulin. As abdominal fat develops in obesity related T2DM, the adipocytes release non-esterified fatty acids, many inflammatory products and reactive oxygen species (ROS). When non-esterified fatty acids accumulate in cells they undergo β-oxidation, forming acetyl-CoA that enters the Krebs cycle. The excessive amount of free radical formed in this situation requires a protective response against oxidative stress—decreased entry of glucose into the cell to avoid more free radical formation by glucose metabolism. Thus, indirectly, whole body adiposity inhibit the phosphorylation of tyrosine in insulin receptor substrate 1 (IRS-1) as a 'protective mechanism' that down-regulates insulin sensitivity in the muscle [7]. In the same direction, fat accumulation inside muscle cells may lead to the entry of fatty acids into the mitochondria where they are prone to ROS production [1]. Considering that the muscle tissue plays a key role in the regulation of metabolism, especially concerning glucose levels, disturbances on functionality and in the redox state in the muscle might be related to diabetogenic effects.

Adipose tissue insulin resistance and dysfunctional lipid storage in adipocytes are sentinel events in the progression toward metabolic dysregulation with obesity [8]. Many of the complications of obesity are due to a chronic subclinical inflammation. Gene expression profiling of the obese phenotype revealed a differential regulation of many pro-inflammatory genes. In part, a significantly higher number of macrophages are present in obese adipose tissue explain the increase in pro-inflammatory status [9]. Thus, a major determinant for many obesity-induced implications is the low-grade inflammation of the enlarged adipose tissue and the persistent release of inflammatory adipokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [10, 11]. The basis for this view is that increased circulating levels of several

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markers of inflammation, both pro-inflammatory cytokines and acute-phase proteins, are elevated in the obese [11].

The most crucial step in insulin signal transduction, the phosphorylation of the IRS-1 can be blunted by both TNF-α and free fat acids conferring insulin resistance in target tissues. Also, several serine kinases are involved in this impaired insulin signaling as c-jun amino terminal kinase (JNK) that is potently induced by TNF-α and free fat acids. Interestingly, increased intramyocellular lipid levels is correlated to insulin resistance with no significant changes in TNF-α, IL-6 or adiponectin concentrations, suggesting that a dysregulation in muscular fatty acid oxidation per se may mediate insulin resistance by mitochondrial defect in oxidative phosphorylation [9]. Thus, systemic inflammation may participate in insulin resistance development but muscle metabolism impairment can be crucial to T2DM installation.

Conditions of tissue stress, such as oxidative stress, inflammation, and molecular alterations, could develop initial compensatory responses of cytoprotection, such as the expression of HSP70. The severity of the metabolic state (measured by glycemia, glucose intolerance, obesity, or insulin resistance) promotes modification in the muscle and adipose HSP70 expression. The initial impairment and moderate glucose intolerance promotes an increase in HSP70 content in adipose tissue and no modification in the muscle [12] as an initial adaptative response, while obesity plus T2DM have an decrease in both muscle and adipose HSP70 content [13]. Since HSP70 expression can inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and JNK dependent mechanisms that promote insulin resistance, blunted heat shock response can be interpreted as an additional silent effect of HFD consumption and sedentarism. These results support the hypothesis that an increase in visceral fat, closely associated with the lifestyle (high-fat intake and sedentarism) promotes subclinical effects that is associated with the development of muscle insulin resistance [14] (Figure 2).

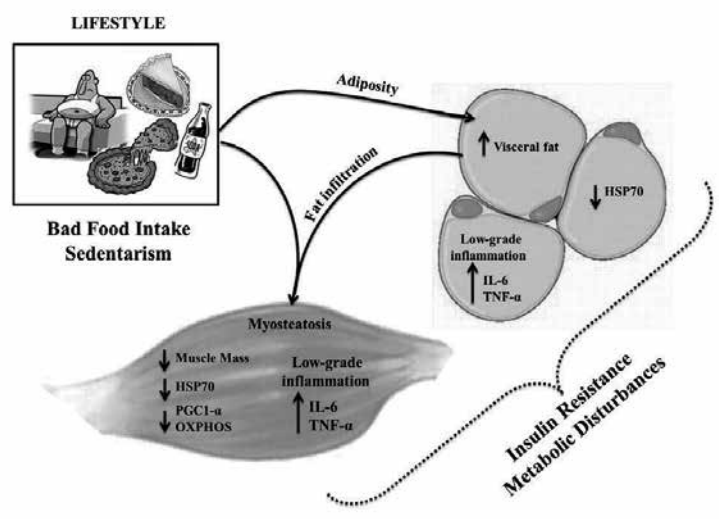

Figure 2. Lifestyle promotes molecular and metabolic alterations in muscle and adipose tissue, promoting insulin resistance.

Another fact is that increased extracellular HSPs levels (mainly 70kDa isoforms as eHSP72) are correlated with oxidative damage and stress in diabetes and in obesity. Moreover, the content of the plasma eHSP72 is higher in T2DM obesity compared to DM or only obese subjects, suggesting eHSP70 levels as biomarker of glucose homeostasis unbalance [15]. Together, eHSP70 and pro-inflammatory cytokines represent a link between metabolic and immune related events. During severe stress response that can cause insulin resistance, we can observe the action of inflammatory cytokines such as IL-1 and TNF-α [16]. Under hypoglycemic conditions, as a part of the homeostatic stress response, HSP70 is secreted to the bloodstream and may be purely a danger signal to all the tissues of the body for the enhancement of immune and metabolic surveillance state or actively participate in glycemic control under stressful situations [17]. Additionally, eHSP70 can bind receptors in immune cells that induce pro-inflammatory cytokine release.

3. Aging

Aging can be understood as a natural result between destructive processes that act on cells and organs over a lifetime and the responses that promote homeostasis, vitality, and longevity [18]. Aging is associated with the decline of a number of physiological systems, such as when they reach critical levels of functionality. The time of this decline depends on variables such as genetics, development, metabolism, and lifestyle [19].

The sarcopenia in the elderly is a significant public health problem because it leads to gradual slowing of movement, increasing the risk of injury, and loss of independence. The mass and muscle strength are lost due to a decline in neuromuscular transmission, the structure, function, metabolism, and muscle performance. Muscle, bone, and their metabolic needs must be considered as a single entity. Muscle strength, energy balance, and bone health influence the ability to perform physical tasks of daily life and the ease with which these tasks can be performed determines the degree of independence that an individual can keep up with advancing age [19].

Cross-sectional studies indicate that muscle mass decreases with aging in the early third decade [20] and that approximately 10%–15% of the mass is lost between the ages of 20 and 50. In women with menopause, muscle mass decreases at an accelerated rate of approximately 1% per year, affecting muscle strength and bone integrity, with a negative impact on activities of daily living [21]. In addition, older, postmenopausal women are more likely to develop abdominal adiposity and highest lipase activity, which is associated with increased systemic levels of inflammatory cytokines and free fatty acids [22].

Similar to what happens in obese individuals, elderly individuals also exhibit elevated levels of inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1), TNF-α and C-reactive protein (CRP) in the extracellular space (e.g., serum and plasma). This pro-inflammatory profile has no acute characteristic such as after acute traumas (sepsis, stroke, e.g.), but is a low-grade chronic inflammation present in elderly individuals that has been defined as "inflammaging." Epidemiological studies have shown that "inflammaging" is a risk factor in

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the accelerated decline of muscle mass and strength, and these changes in muscle performance can be a critical step in mediating the causal link between "inflammaging" and disability [23].

The pathogenesis of T2DM is established by a vicious cycle of metabolically induced inflammation, impaired insulin responsiveness, and loss of homeostatic signaling. Approach in metabolic, pro-inflammatory cytokines may influence the state of the insulin response and can lead to insulin resistance in acute or chronic form. Adipocytes and macrophages secrete inflammatory cytokines (e.g., TNF-α) that activate the serine-threonine kinases JNK and inhibitor of κB kinase (IKK-β) in insulin sensitive organs, such as liver, skeletal muscle, and adipose tissue. JNK and IKK-β both impair the function of the insulin receptor and interfere with downstream signaling [24].

Cytokines released into the bloodstream can bind to receptors and activate intracellular signaling pathways, thus facilitating the activation of phosphorylation of JNK and inhibiting the signal transduction of insulin. JNK is also activated by stress and fatty acids [25]. Situations of inflammatory stress can also affect the process of regulating the availability of non-insulin dependent glucose as the activation of AMP-activated protein kinase (AMPK) enzyme sensitive to intracellular energy status [25, 26]. This pathway is frequently studied in the animal model of insulin resistance (or in some cases T2DM) by high fat diet protocols (Goettems-Fiorin et al., manuscript in preparation). These studies of insulin resistance show that several structural and metabolic changes in the muscle are correlated with increased levels of fasting glucose and/or response to glucose intolerance during glucose tolerance test (GTT).

Aging and tissue degeneration also involve the accumulation of damage to cellular macromolecules. Chemical damage due to oxidative stress, glycation, and the addition of sugar residues has the capacity to modify both DNA and proteins. Situations of inflammatory stress, as described above, are able to activate the expression of genes that perform the cytoprotection in many tissues, especially when we talk about the muscle tissue. The muscle, which is rich in protein chains, uses the expression of HSPs to repair the damage that can be induced by both the consumption of high-fat diets and aging.

It has been assumed that HSPs, particularly 70kDa (HSP70) levels, generally decrease during normal aging processes. As HSP70 functions to chaperone cytosolic proteins to allow appropriate refolding or degradation by ubiquination pathways, these reductions in HSP70 have been implicated in the aging process, as cells accumulate oxidation products without adequate cellular protection [27]. Age-dependent decline in the heat shock response is also observed in muscle tissues. In these tissues, vigorous contraction leads to the induction of HSP that is cytoprotective in nature. These effects are severely blunted in the muscles of older animals and aging humans, suggesting that decline in muscle mass and force generation may be related to loss of HSP expression [18].

The leak of HSP70 expression in muscles of older subjects represents a failure to exercise adaptation, in terms of both structural and metabolic characteristics. Loss of heat shock factor 1 (HSF1) activity and expression, the transcription factor of HSP70, which is at the same time the result and consequence of obesity and aging, induce an impairment of muscle mass maintenance and insulin signaling (Figure 3). The relevance of HSP70 expression in the muscle

can be identified since the pro-inflammatory mediators JNK and NF-κB can be inhibited by the HSP70 expression, which is low in age, T2DM, obesity, and finally in sedentary subjects, suggesting that active muscle can be a non-pharmacological option to prevention or repair this "meta-inflammation" process that harm the human health.

Older adults with diabetes are vulnerable to accelerated loss of lean body mass. Declines in lower extremity muscle mass in particular, can be associated with decreased muscle strength and lead to muscle weakness, poor lower-extremity performance, and mobility loss, all of which have been reported in persons with diabetes [28]. Aging also produces muscle changes such as decrease in type II fibers, decline in oxidative capacity of type IIa and I fibers, and forming a favorable development of metabolic alterations. During aging, changes in the morphological structure of the skeletal muscle and also in the number and function of mitochondria are observed in the decline in this tissue. Studies have consistently shown that PGC-1α, a transcription factor that promotes mitochondrial biogenesis, decline with aging and in many age-related chronic diseases suggesting that such decline may explain the progressive mitochondrial dysfunction with aging [23].

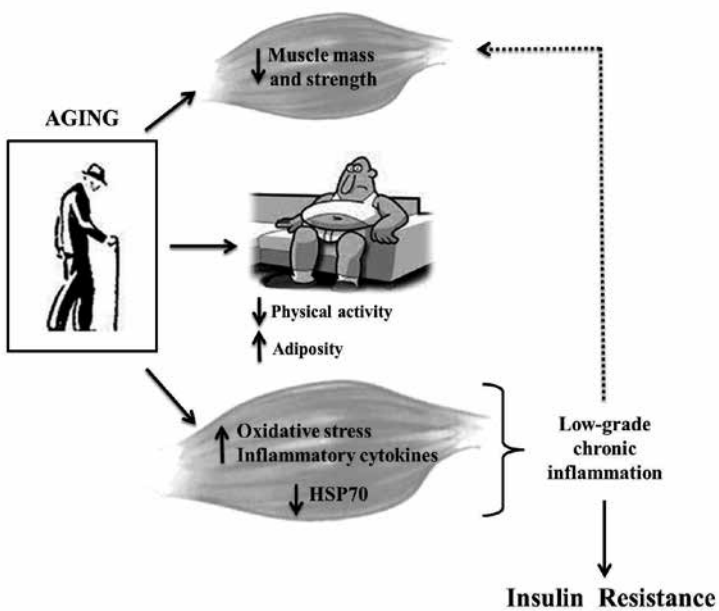

Figure 3. Aging alterations in the muscle related to insulin resistance.

As previously described, both the lifestyle and aging promote body changes, which may be directly related to skeletal muscle dysfunctions. Changes in composition and functional capacity of type 1 and type 2 fibers, which reduce the mass and muscle strength, occur due to molecular disorders affecting the muscle, whether caused by the high consumption of HFD and physical inactivity or by aging. Myosteatosis, reduction of PGC-1α development of a low-grade inflammatory with increased expression of pro-inflammatory cytokines such as TNF-

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α and IL-6, and reduction of HSPs, represent the body's response to aging is quite similar to lifestyle (hipercaloric food intake and sedentarism). The term "inflammaging", described in several studies highlights the low-grade inflammatory condition in the aging process, with an increase of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) that promotes the activation of JNK and IKK-β in the muscle interfering with the sensitivity to insulin (Figure 3). Thus, many subclinical process of insulin resistance development occurs in the muscle and activating this organ can be an important strategy to obtain a healthy aging process.

4. Exercise

Human lifestyle changed from daily strong physical activity patterns of hunter-gatherer societies (about 1,000–1,500 kcal/day with 3–4 h/day of moderate-to-vigorous physical activities) to digital industrialized society with dramatic reductions in daily activity levels. Physical inactivity is a "modern" human behavior that increases the risk of many adverse health conditions, including the development of diseases, such as T2DM and metabolic syndrome, decreasing the life expectancy. Although nowadays health professionals and population has knowledge about the necessity of exercise practice) to avoid many diseases, the definition of sedentarism still lacks a consensual definition [29, 30].

From ancient physicians, including Hippocrates and Plato, scientists believed in the benefits of physical activity for human and animal health. Classical epidemiological studies, as performed by Morris et al. [31], showed comparative evidences about the risk of sedentary routine to the cardiovascular system. However, in quantitative analyses, many protocols and descriptions were used in the sedentarism research field to classify an inactive person. "Active" definition may be based on total energy expenditure since an increase in daily energy expenditure (aprox.150 kcal) can promote health benefits. Other definition is about accumulative activities during one week, since less than 30 minutes of moderate-intensity physical activity represent also a cardiovascular risk factor. Recently, it was estimated that physical inactivity causes 7% of the burden of disease from T2DM and causes 9% of premature mortality, or more than 5 million of deaths [30]. If the percentage of inactivity decreases at least 10% in the world's population, it is estimated that around more than one million deaths could be averted per year.

A recent meta-analysis has reported that exercise training is associated with a decline in glycosylated hemoglobin (HbA1c) levels. This effect is presented in aerobic, resistance, or combined aerobic and resistance training modes and also is compared to reductions achieved by commonly used oral antidiabetic medications. Three times a week of an exercise program with more than 30 minutes and at moderate intensity (above 50% VO_2) is sufficient to reduce 10% in HbA1c levels mainly in insulin resistance subjects, according to the Diabetes Association in the past decades [32]. However, recent data suggest that structured exercise training of more than 150 minutes per week is associated with greater HbA1c declines than that of 150 minutes or less per week and that physical activity advice is also associated with lower HbA1c, but only when combined with dietary advice [33]. Current opinion appointed that the volume of exercise training (and not the type or intensity of exercise) is a major determinant of glycemic

control in patients with T2DM. Reduction in HbA1c is associated with exercise frequency in supervised aerobic training, and with weekly volume of resistance exercise in supervised combined training promotes better results in the reduction of HbA1c [34]. Furthermore, a dose response can be estimated to exercise benefits with longer life expectancy for those who accumulate more than 450 minutes of exercise per week [35].

For elderly individuals, combined training (resistance and aerobic) performed twice a week promotes similar muscular adaptation (strength and thickness) and some similar cardiovascular adaptations when compared to three times per week, suggesting that it is applicable as an exercise prescription for aged people to improve the adherence to an health life style. [36]. Regular exercise can block the aging-associated increase in sympathetic nervous system activity to peripheral tissues (probably an adaptation to improve energy consumption). Chronically augmented sympathetic stimulation promotes reductions in the peripheral blood flow and vascular conductance and thus can also contribute to the metabolic dysfunction, by increasing glucose intolerance and insulin resistance. Each exercise session possibly substitutes the necessity of an increase in the sympathetic activity induced by aging and then stimulates thermogenesis to prevent increasing adiposity [37]. In this way, daily physical activity is able to promote weight loss without caloric restriction and also can reduce obesity, particularly abdominal obesity, and insulin resistance in men. Exercise programs without weight loss reduce abdominal fat and prevent further weight gain, preventing insulin resistance [38].

The epidemiologic and clinical trials results listed above exist because exercise represents a physical stress that challenges human body homeostasis. In response to this stressor, autonomic nervous system and the hypothalamic-pituitary-adrenal axis are known to react and to participate in the maintenance of homeostasis. It is well known that exercise induces several changes on both immune system [39, 40] and endocrine system. Cytokines may be modulated by the secretion of hormones from the hypopituitary-hypothalamus axis and in an integration cross-talk, hypopituitary-hypothalamus axis is also modulated by immunologic status by secretion of cytokines, showing an important neuroendocrine-immune loop in the human body [41, 42]. Skeletal-muscle fibers also can produce several hundred secreted factors, including proteins, growth factors, cytokines, and metallopeptidases, with such secretory capacity increasing during muscle contractions or after exercise training. Muscle-derived molecules exerting either paracrine or endocrine effects are termed “myokines” and are strong candidates to make up a substantial fraction of the exercise “polypill” effect to the whole body [37].

These signaling actors can be influenced by multiple factors including mode, duration, and intensity of exercise. Changes are proportional to exercise intensity and duration of exercise, although the effect of intensity is more marked, as showed by impaired immune functions and high secretion of hormones after an acute bout of vigorous exercise [39]. Some adaptations from regular training appears to be related to modified circulating hormones, as cortisol, insulin and glucagon, and also by alterations in the pro/anti-inflammatory cytokine balance (IL-2/IL-10 ratio and more th1/th2 lymphocytes cytokines ratio).

Thus, chronic moderate exercise improves immune functions [43] by the induction of an anti-inflammatory environment with each bout of exercise promoted. The IL-6 is expressed in high

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amounts in active muscle and is the first cytokine released into the circulation during exercise, followed by increased anti-inflammatory cytokines as IL-10 [44, 45]. Also, each exercise session promotes an increase in ROS production that result in improved antioxidant defense in the muscle, plasma, and other tissues [37]. In parallel, some studies demonstrated that exercise is a physiological stimulus that promotes an increase in the muscle HSP70 expression and eHSP70 concentration (plasma), influenced by the intensity [46] and duration [47]. Increased intracellular HSP70 expression in leukocytes can be associated to less TNF-α plasma concentration after an exercise session [48]. Thus, regular exercise can shift the whole body from a pro-oxidant and pro-inflammatory state to an equilibrium induced by molecular, redox, hormonal, and "immune-metabolic" adaptations.

Associated to the progression of cardiovascular disease was observed higher levels of inflammatory markers such as IL-6 and CRP that are related to reductions in nitric oxide (NO) concentrations caused by reduced eNOS activity [49]. These extracellular pro-inflammatory signaling is increased in obese and type two diabetic people. Curiously, higher eHSP70 content in the bloodstream is also observed in these individuals, connecting immune-inflammatory events that promote a pro-atherogenic profile. eHSP70 levels were associated with multiple biomarkers of the acute-phase reaction, inflammation, and endothelial-cell activation, indicating the presence of a complex stress response that involves immune-metabolic signaling by eHSP70, by interaction of eHSP70 with cell surface receptors in immune cells. The release of these proteins promotes the bind to Toll-like receptor 4 (TLR4)/CD14 receptors, resulting in endothelial cells expressing adhesion molecules in smooth muscle cells leading to proliferation, and in macrophages inducing a range of proinflammatory cytokines [50].

The elevation of eHSP70 levels could be an important integrative response, from/to immunologic/metabolic center of homeostasis control in response against physiological disorder or disease [14, 17, 51, 52]. Whereby healthy people have low plasmatic levels of eHSP70, the association of these proteins with illness, disease progression, and mortality were hypothesized, as well as longevity and health parameter status were attributed to this protein [53]. Interestingly, in the last years, from Walsh et al. (2001) [54] to present [55-57], some studies investigated eHSP70 concentration in response to exercise. However, eHSP70 released from immune cells during exercise can be an immune signal from the periphery to the central nervous system (e.g. hypothalamus) leading to the "fatigue sensation/behavior". In other words, after eHSP70 concentrations had reached a critical level (not known yet), higher exercise loads or duration would be dangerous to the whole body [51]. Then, eHSP70 can be a signal to impose fatigue sensation and shutdown of exercise, avoid a pro-inflammatory state to maintaining homeostatic, metabolic, and hemodynamic equilibrium.

In the bloodstream, eHSP70s and cytokines might participate in the physiologic responses of physical exercise, as chemical messengers released during an effort. Increased levels of eHSP70 in the plasma during exercise may participate in the fatigue sensation, also acting as a danger signal from the immune system [51]. On the other hand, intracellular HSP70 (iHSP70) synthesis is necessary for homeostasis maintaining in the muscle and other tissues. In the intracellular

millie, these proteins have molecular chaperone action of such proteins, limiting protein aggregation, facilitating protein refolding, and maintaining structural function of proteins. iHSP70 have further been demonstrated to provide cytoprotection by anti-apoptotic mechanisms, inhibiting gene expression and regulating cell cycle progression [58]. Intracellularly activated HSP70 are anti-inflammatory by avoiding protein denaturation and excessive NF-kB activation that may be damaging to the cells. Thus, iHSP70 act as a suppressor of NF-kB pathways (inhibiting TNF-α expression), an important anti-inflammatory role of HSP70 family proteins [51].

The chaperone function of iHSP70 is more than microscopic measurements of laboratory research field. Muscle disuse result in muscular atrophy that is represented by the decrease in muscle mass, fiber cross sectional area, and total myofibrillar protein content. In this situation, contractile protein breakdown exceeds protein synthesis. Moreover, in atrophied muscle occurs an increase in the proportion of fibers containing the fast myosin heavy chain by the transformation from the slow myosin heavy chain (MyHC-I/β) to the fast myosin heavy chain (MyHC-IId/x). As early as 18 hours and as late as 18 days after muscle disuse, it is possible to measure a decrease in iHSP70 in the soleus muscle [59]. Interestingly, previous heat treatment is a strategy to induce iHSP70 expression in the muscle and this molecular adaptation results in the maintenance of muscle mass during a 7-day period of immobilization [60]. In this way, iHSP70 expression appears to have no full protective effect on muscle mass, fiber cross sectional area, and total myofibrillar protein content, but prevents the decrease of MyHC-I/β and the increase of MyHC-IId/x induced during the atrophy process [61]. These evidences suggest that HSP70 can inhibit a key signaling pathway for atrophy in muscle cells preventing the muscular atrophy.

Heat treatment also has been tested in humans. Short wave diathermy therapy is a clinical strategy that means to increase deep heating of tissues with higher water content. This strategy may promote a 58% increase of HSP70 expression in *vastus lateralis* [62]. It is possible that previous heat treatments cannot reduce markers of muscle damage but is able to reduce muscular pain, preserve strength, and improve range of motion following exccentric contractions. Curiously, there is a gender difference in heat shock response in both basal and induced by exercise iHSP70 levels, with men showing low pre-exercise levels and an attenuated iHSP70 response. The gender difference may be explained by the effects of estrogen modulation in heat shock response [62].

If the disused muscle is in trouble, the reuse of the musculature may represent many stages of soreness. After immobilization, the reload process to the muscle implies in newest molecular adaptations. If less required muscle is submitted to a challenge, the iHSP70 expression increased greatly (~200%) in the first two weeks of reload process and the return to basal levels (above disuse levels) early as eight weeks [59, 63]. This effect is accompanied by an increase in the percentage of slow type I MHC fibers (MyHC-I/β). Although many factors appear to be related to the down- and upregulation of iHSP70, the expression of this protein is closely related to the morphological and functional changes of muscle cells.

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Exercise-induced expression of iHSP70 in the skeletal muscle has a major role in restoring muscle metabolic functionality as it provides cytoprotection to damaged cells. An iHSP70 inducing ability has been shown as a result in different protocols of exercise, including eccentric, concentric not damaging, and aerobic or resistive, all of which are capable to induce intramuscular HSP70 expression [64, 65]. Although time and intensity of the physical effort are determinant factors to increase of intramuscular iHSP70, its rise may be detectable just 2 h after the onset of an acute exercise session, when HSPA1A mRNA expression peaks [54]. Moreover, exercise-induced iHSP70 presents a time and intensity dependence [46].

Since iHSP70 family members promote the facilitation of protein transport into the mitochondria, allowing and improving structural integrity of the organelle during fast energy flow, iHSP70 content has been correlated with an increase in the oxidative capacity of muscle cells. Several studies have demonstrated the relationship between high iHSP70 levels in the skeletal muscle and increased activity of mitochondrial enzymes after a short training period [66]. On the other hand, decreased mitochondrial function is known to be associated with the accumulation of intramyocyte triglycerides (and its byproducts), insulin resistance and diabetes. For this additional reason, exercise-induced iHSP70 expression can lead to improvements in metabolite oxidation and, consequently, insulin sensitivity.

Thus, exercise training or regular physical activity tend to reduce the stressful impact of each exercise session and improve glucose uptake and storage, antioxidant capacity, associated with the increase in the iHSP70 content in muscle [67, 68]. In fact, increased iHSP70 expression has been demonstrated to participate in cell signaling that prevents insulin resistance [67]. Since iHSP70 family members promote the facilitation of protein transport into the cytoplasm environment and help the cell to maintain the structural integrity of the organelles and proteins involved in cross-bridge contraction, iHSP70 can help prevent the loss of muscle mass, the decrease in the type II fiber content, and the loss of metabolic capacity as oxidative metabolism and thus improving glucose usage by the active muscle [66], which is important both in obese and aging conditions.

Exercise also promotes several redox related adaptations. The increase in ROS levels and adenosine monophosphate (AMP), signaling the activation of the enzyme AMPK to stimulate the catabolism of substrates in producing new ATP [69]. In this way, AMPK in skeletal muscles contributes to the maintenance of normoglycemia by two mechanisms—most glucose uptake occurs by increasing the translocation of GLUT4 and by reducing peripheral insulin resistance. In addition, this enzyme acts on hypothalamic functions by modulating the events related to hunger and satiety. Thus, plays a key role in the regulation of cellular metabolism and in the maintenance of energy homeostasis [70].

During a moderate aerobic exercise session the skeletal muscle is able to uptake glucose without insulin signaling and increase fatty acid mobilization to use as an energy source. Belonging to the family of enzymes activated by cellular stress caused primarily by ATP depletion, AMPK is responsible for the regeneration of ATP levels via oxidation of fatty acids and glucose [71]. When the AMPK is activated, it is capable of inhibiting both enzymes, the 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoA reductase) and ACC. In the liver, the inactivation of the malonyl CoA ACC concentrations is insufficient to inhibit CPT1. Thus, there

is a predominance of the β-oxidation of fatty acids synthesis, and the production of energy outweighs the expense [72, 73]. Considering intervention strategies for T2DM, exercise improves muscular oxidative capacity and, consequently, insulin sensitivity, up regulating the expression of PGC-1α in healthy subjects. In many rodent models, the increase of the OXPHOS is coordinated by PGC-1α, which increases the transcriptional activity of PPARγ, reducing the effects that promote insulin resistance.

The predominant oxidative metabolism in type I fibers, which are modified by the action of aerobic training at moderate or high intensity, may be a key point to glucose metabolism regulation. There is an increase in the number of mitochondria and GLUT4 vesicles, thus promoting the acceleration of oxidative metabolism with increased ROS production accompanied by increased antioxidant defense capacity. Increase in antioxidant enzyme activity observed in superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities, and also in glutathione (GSH) content, represent oxidative stress preventive adaptations induced by exercise training. The redox protection has direct impact in the maintenance of insulin sensitivity in the muscle [74, 75]. These changes may be triggered or stimulated by increased HSP70 expression [76].

Regular exercise promotes the acute increase of blood flow and shear stress and, in turn, improves the NO bioavailability, hence increasing the endothelium-dependent vasodilatation. This NO effect occurs in parallel to the decrease in pro-inflammatory biomarkers after exercise training. This improvement in NO and decrease in pro-inflammatory markers could represent one of the most important mechanisms of cardioprotection induced by regular exercise that prevent comorbidities associated to T2DM. However, the exercise intensity seems to be a crucial variable to future studies. iHSP70 expression depends on exercise intensity (level of physical challenge, measured by workload or time), while many other adaptations could not be influenced by exercise training intensity. We believe that exercise can induce both intracellular and extracellular HSP70, but promotes equilibrium in HSP70 signaling to the whole body in T2DM [77, 78].

5. Conclusion

Exercise is a known modulator of all parameters listed above: glycemia, eHSP70, iHSP70, IL-6, TNF-α, and ROS. Intracellular and extracellular HSP70 have different roles in the regulation of "immune-metabolic" homeostasis as well as cytokines. Equilibrium is also obtained by exercise in redox state by improving antioxidant defenses in the muscle and between pro/anti-inflammatory cytokines production. Reduction or prevention of obesity by active muscles can be considered key to the process. By metabolic and anti-inflammatory effects, exercise represents a good and safe strategy against insulin resistance induced by age and lifestyle factors (Figure 4).

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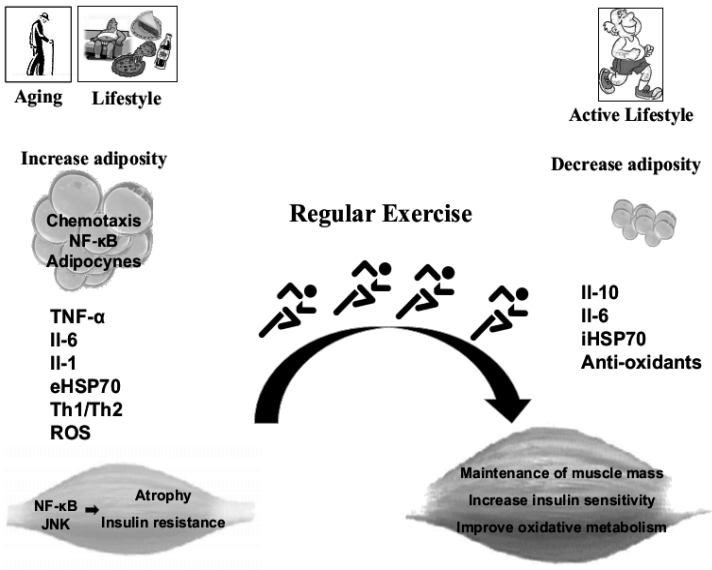

Figure 4. Exercise as a strategy against muscle insulin resistance induced by aging and lifestyle.

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Chapter 5

Importance of Plasma Membrane Nanodomains in Skeletal Muscle Regeneration

Beata Pająk and Arkadiusz Orzechowski

Additional information is available at the end of the chapter

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Abstract

Numerous studies showed the importance of skeletal muscle plasma membrane (sarcolemma) in the control of skeletal muscle biology. The emphasis in this review is on the sarcolemmal bioactive lipids decisive for survival, proliferation, differentiation, and function of skeletal muscle cells with the particular concern on muscle stem cells (resident satellite cells, RSC) responsible for muscle regeneration. Nowadays, it is obvious that cholesterol (CHOL), basic component of the lipid rafts (LR) through the control of assembled dystrophin–glycoprotein complexes (DGC), directs muscle fiber contractile properties. Another phospholipid, phosphatidylserine (PS), is a component of the inner plasma membrane leaflet, even though it allows the fusion of myoblasts when exteriorized. Sphingolipids, such as ceramide, sphingosine, sphingosine-1-phosphate, and ganglioside GM3, are important signaling molecules in the charge of RSC activation, their motility, and commitment to particular lineage (myoblasts and myofibroblasts). Phosphoinositides and phosphatidylinositol-4,5-biphosphate (PIP2) specifically establish protoplasmic platforms for protein interactions essential for cell viability and mitochondrial activity. Additionally, both prenylation and palmitoylation of certain proteins (i.e., heterotrimeric G proteins) determine their biological activity in signal transduction from G-protein coupled receptors (GPCR). Isoprenoids are therefore crucial for the recruitment and metabolic responses of RSC to physiological and pathological stimuli. Finally, iatrogenic modifications of sarcolemma with hydroxylamines and their derivatives lead to increased resistance of muscle cells to apoptotic stimuli and slow progression of some skeletal muscle dystrophies.

Keywords: sarcolemma, lipids, satellite cells, nanodomains, skeletal muscle regeneration

1. Introduction

Skeletal muscle growth and regeneration is dependent on the activation of mitotically quiescent resident cells known as skeletal muscle satellite cells (SC) located beneath the basal lamina (integral part of basement membrane) on the plasma membrane (sarcolemma) of adult skeletal muscle fibers. Activated by muscle injury including work overload (i.e., weight lifting), satellite cells proliferate making myogenic precursor cells (myoblasts) that migrate to the site of injury and after withdrawal from the cell cycle fuse collectively or with damaged fibers. The fusion process is mediated by plasma membrane proteins, some of which are the receptors for intermediate of lipid metabolism such as sphingosine 1-phosphate (sphingolipid, S1P). A great deal of plasma membrane surface and integral proteins at the extracellular site is glycosylated and prenylated, the processes indispensable for intracellular protein transport (from endoplasmic reticulum to Golgi apparatus, and from trans-Golgi network to the plasma membrane) as well as for lateral and vertical protein translocation within sarcolemma. In adult skeletal muscle, the self-renewing capacity of satellite cells contributes to muscle growth, and regeneration-associated hypertrophy as skeletal muscle-specific adaptation to workload. Hypertrophy also occurs in satellite cell-depleted skeletal muscle, although in this case neither increase in myonuclei in satellite cell-depleted fibers nor the muscle regenerates after $BaCl_2$-induced severe muscle damage [1]. Accordingly, the biochemistry and structural modifications of plasma membrane are seemingly indispensable for the commitment of satellite cells and their progeny of myoblasts to skeletal muscle renewal. In this review, we hypothesized that changes in the sarcolemmal composition of proteome, glycoproteome, and/or lipidome are the major determinants of satellite cells and muscle fibers to regenerate skeletal muscle. From the experiments and clinical observations related to statin-induced myopathy [2–4] as well as the successful efforts aiming to correct plasma membrane integrity by the modification of skeletal muscle plasma membrane fluidity, we conclude that closer examination of the plasma membrane composition and structural organization might shed more light on the molecular mechanisms of satellite cell commitment to muscle rejuvenation.

2. Skeletal muscle growth and regeneration

Nowadays, it is obvious that skeletal muscle growth and regeneration is firmly linked to the activity of satellite cells adjacent to extrafusal and intrafusal muscle fibers. Intact skeletal muscle encloses satellite cells in the quiescent state, with a dense nuclear chromatin (heterochromatin), fine rim of the cytoplasm, and little organelles. Covered by a thin layer of basement membrane, the satellite cell rests closely applied to the sarcolemma of the muscle fiber (Figure 1). The notion that satellite cells donate nuclei to a growing or regenerating fiber, one at a time, is widely known from a half of the century [5]. Although quiescent in normal skeletal muscle, satellite cells (named by Mauro, [6]) become activated and recruited to the cell cycle when there is a requirement to increase myonuclear number [7]. In response to signals accompanying skeletal muscle injury, denervation, exercise, or work overload, the activation reverses the morphology of satellite cells to lower chromatin density (euchromatin), expanded cytoplasm,

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and additional organelles [8–9]. Several lines of evidence suggest numerous molecules including hormones, growth factors, cytokines, and reactive species as potent incentives in the activation of satellite cells, yet it is still not clear how these muscle progenitors become receptive to the stimuli. Rearrangement of plasma membrane lipids, proteins, and their glycosyl and lipid conjugates might be considered as possible beginning of satellite cell commencement to sense some of the signals. Despite great biological and clinical interest, our knowledge of *in vivo* N-glycosylation sites – a prerequisite for detailed functional understanding – is still very limited [10]. Similarly, the conception of plasma membrane lipidome input to sense and transduce the signals for the activation of satellite cells is limited [11]. Thus, any endeavor intended to decipher the details and mechanisms hidden behind the dynamic changes of plasma membrane organization is an attractive approach with promising perspective for future clinical application in the treatment of skeletal muscle myopathies.

3. Sarcolemma

Plasma membrane in skeletal muscle has several exclusive features related to the structure and composition as well as unique characteristics of membranoskeleton. Nonetheless, some properties are fairly common for any membrane as phospholipids spontaneously form lipid bilayers in aqueous environments due to the amphipathic nature of the molecules with a highly hydrophobic "tail" (acyl chains) and hydrophilic "head" (glycosyl or phosphatidyl) moieties. Lipids in membranes are distributed disproportionately accounting for substantial differences in the extracellular vs. intracellular face of plasma membrane (PM). Anyway, a given membrane has a stable and specific membrane composition dependent on cell type and organelle, and any changes are observed only in certain physiological situation or pathological anomaly. The asymmetry of the external leaflet of PM (exoplasmic) is featured by highly enriched in choline-containing lipids such as phosphatidylcholine (PC) and sphingomyelin (SM), whereas the cytoplasmic leaflet (protoplasmic) is rich in phosphatidylethanolamine (PE), phosphatidylserine (PS), and other phospholipids [phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP), and phosphatidylinositol-4,5-biphosphate (PIP2)]. The latter participates in cell signaling. Despite cross-sectional diversity, membranes posses lateral asymmetry in basal, lateral, and apical regions [12–13]. Lipids are also capable of displaying different phases under different conditions (mesomorphism). Some lipids, however, as cone-shaped lysophosphatidylcholine (LPC) or PE can form nonlamellar finite structures such as spherical micelles or tubular structures in membranes [14]. Nonlamellar prone lipids are involved in membrane fission and fusion processes with the aim of enzymes such as scramblases, flippases, and floppases. Finally, intact PM is extremely elastic as it reseals after mechanical rupture allowing for the separation of cell fragments (i.e., synaptosomes).

As thin (5–10 nm) lipid bilayer in eukaryotes, PM plays several tasks emerging beyond defining simple cellular boundary. It can organize complex tools for transportation (ion channels, transport proteins, pumps, and invaginations for macromolecules) or molecular sensing (receptors, Figure 2). PM is also essential to control intercellular communication (flow of information) through highly dynamic microdomains acting as platforms for molecule inter-

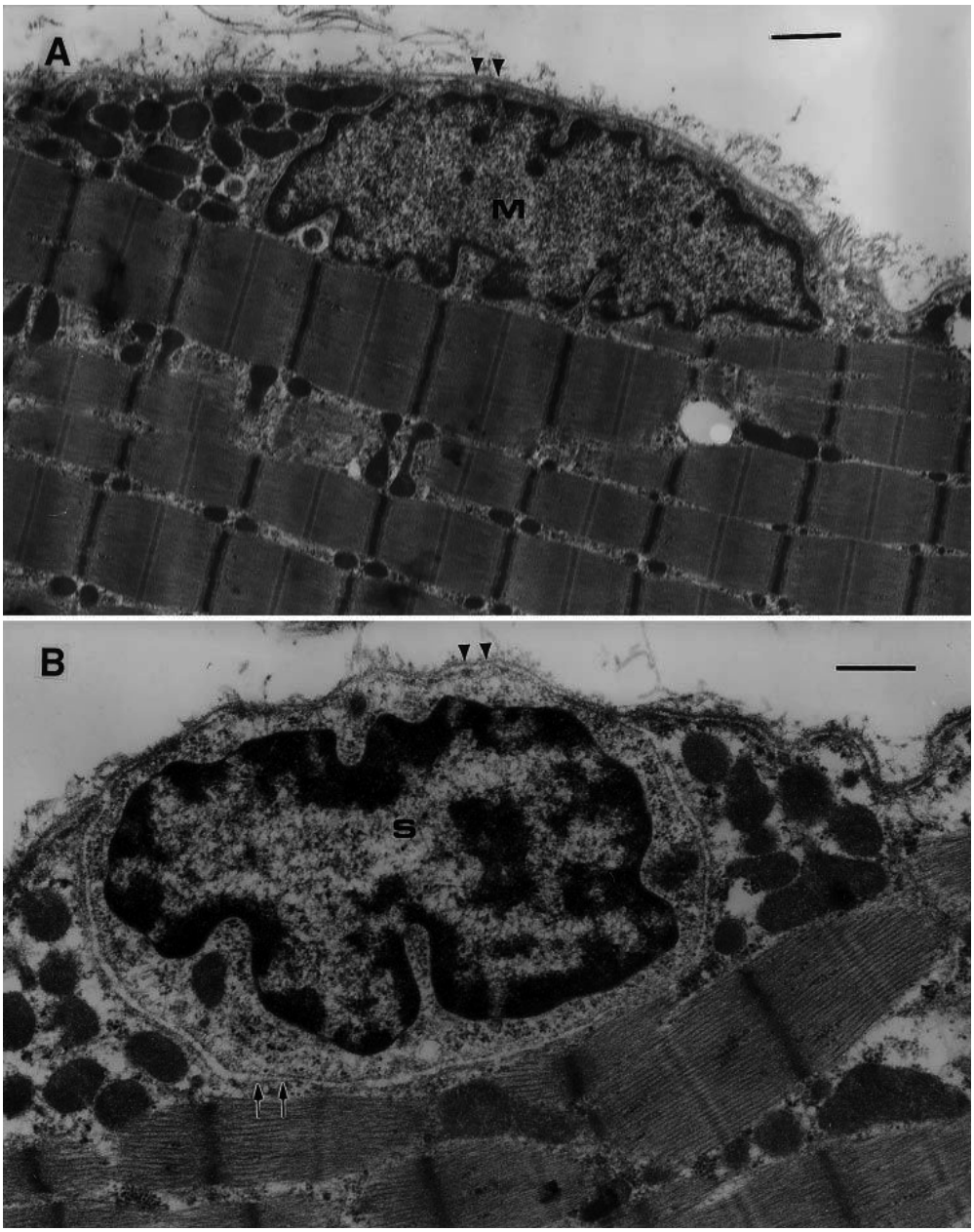

Figure 1. Electron micrograph of a typical myonucleus (*A*) and a muscle satellite cell (*B*). Muscle satellite cells (S) were identified by their location inside the basal lamina (arrowheads) and outside the sarcolemma (arrows) and an independent cytoplasm. In contrast, a myonucleus (M) is located inside the sarcolemma of the muscle fiber and does not contain an independent cytoplasm. Bar, 1 mm., reprinted from Sinha-Hikim et al. 2003 [133].

actions. Skeletal muscle cell plasma membrane is specifically adapted to resist consequences of muscle fiber shortening during contraction. Additionally, sarcolemma periodically invagi-

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nates, giving rise to the transverse tubule (TT) network liable for sensing depolarization by dihydropyridine receptor (DHP) essential to trigger of calcium flux evoked by activation of ryanodine receptor (RY). Dystrophin–glycoprotein complex (DGC), also known as dystrophin-associated protein complex (DAC), is embedded in sarcolemma (found in other cell types such as astrocytes) and plays paramount role in the aforementioned actions (Figure 3). It is composed of several proteins including, dystrophin (DP), dystrobrevin (DB), syntrophin (SP), dystroglycans (α- and β-DG), and sarcoglycans (α-, β-, δ-, and γ-SG). These proteins are assembled in order to transmit lateral force during isotonic twitch. Subsarcolemmal protein assemblies circumferentially aligned in schedule with the Z-disk or peripheral myofibrils are also known as costameres (assemblies of costameric proteins, Figure 4). They physically couple force-generating sarcomeres with the sarcolemma in striated muscle fibers and are thus considered a pitfall of skeletal muscle, a critical component of striated muscle morphology which, when compromised, is thought to directly contribute to the development of several distinct myopathies also termed sarcolemmopathies (Figure 5). Costameric proteins are found in a cholesterol (CHOL) rich membrane fraction pointing to lipid rafts (LR) as spatial localization of DGC [15]. Actually, LR are clustered at the level of DP complex through laminin-mediated interaction with dystroglycans [16]. Furthermore, CHOL depletion uncouples β-DG from sarcolemmal domains with related impairment of mechanical activity of skeletal muscle [17] (Figure 6), whereas DP repeats which interact with membrane lipids strengthen the sarcolemma providing flexible support to muscle fiber membranes (Figure 7) [18]. Additionally, DGC spatial organization is vital for physical interface between SP and sodium channels or neuronal nitric oxide synthase (nNOS), although not at the same time. DGC is regularly distributed along sarcomeres and aligned mainly with Z-disks to connect actin cytoskeleton with extracellular matrix (ECM) protein laminin (Figure 8). Laminin together with collagen IV, fibronectin, perlecan, entactins, agrin, and glycosaminoglycans is a component of basement membrane. Extrinsic protein β-dystroglycan is the laminin receptor and binds membrane-spanning (integral) α-dystroglycan that mediates interactions with DP and DB [19]. At the focal adhesions facing Z-lines, the integrin receptors (α7β1) connect fibronectin/laminin with actin filaments of sarcomeres (Figure 9). Tallin, vinculin, and paxillin are intermediate filaments forming a lever that hooks up integrins with thin filaments [20, 21]. Caveolin-3 is the muscle-specific form of caveolin found mainly as intrinsic (inner leaflet) membrane protein at the sarcolemma and TT [22]. The functions of caveolin-3, β-DG, DP, and SG are controlled by cholesterol and sphingolipid concentrations in the lipid rafts and caveolae [17–18, 23]. In striated muscle, signal transduction through cellular membranes can be regulated by the interaction of the cytoskeleton with caveolae (C) – caveolin-enriched membrane domains [24]. Mounting evidence demonstrates that lipids themselves regulate the location and activity of many membrane proteins, as well as defining membrane microdomains (lipid rafts, caveolae, and coated pits) that serve as spatiotemporal platforms for interacting signaling proteins [11]. Lipid rafts (single lipid raft is approximately 50 nm in diameter) are defined as detergent insoluble glycolipid (GL)-enriched planar domains (detergent resistant membranes, DRM) highly enriched in cholesterol (CHOL), SM, glycosphingolipids (GSL), and glycosylphosphatidylinositol (GPI)-anchored proteins. To sum up, membrane lipids are classified into three major groups: glycerol-based lipids (glycosyl-glycerides and phospholipids), sphingolipids (SL) with sphingoid-base backbone (SM and GSL), and cholesterol. Similar LR may differ in

their size (fusion and fission), as well as in the proportions of lipids and proteins, somewhat modified by pathophysiological processes or nutritional and/or pharmacological interventions. Caveolae are dissimilar to LR as they are deficient in (GPI)-anchored proteins and poor in CHOL but rich in caveolins, the structural proteins assembled to stabilize membrane invaginations [25]. In extreme situations, PM may be subjected to disintegration, protein misfolding, and aggregation, and finally profound dysfunction causes cell death by necrosis. How these nanodomains are segregated within plasma membrane is a matter of debate, although cholesterol molecules establish closeness of PL, GL, GSL, SM, and proteins. Cholesterol (alcohol) acts as "glue" with hydroxyl group that combines with the phosphate head of phospholipids, whereas the hydrophobic steroid section works together with phospholipids acyl chains. Growing body of evidence points to physicochemical forces (intermolecular forces including electrostatic interactions, hydrogen bonds, Van der Waals forces, and hydration forces) which determine asymmetric geometry of membranes both laterally and in cross-section. Moreover, integral proteins also influence lipid structure in the membrane. One might bear in mind that according to lipidomics, more than 1000 different lipid forms are to be found in plasma membrane. To meet the requirements of fluidity, membrane components are also subject to considerable qualitative and quantitative seasonal changes adjusted by the cell [26]. It is determined by the environmental conditions (i.e., cold vs. heat) but also by needs of adaptation such as hyperplasia/hypertrophy or resistance to different types of stress (shear stress, oxidative stress including irradiation). In either case, physicochemical properties of membranes have to facilitate cell signaling and motility. In turn, cell signaling and motility are influenced by the glycosylation status of PM proteins, both integral represented by receptors and peripheral because they are heavily modified on the external leaflet.

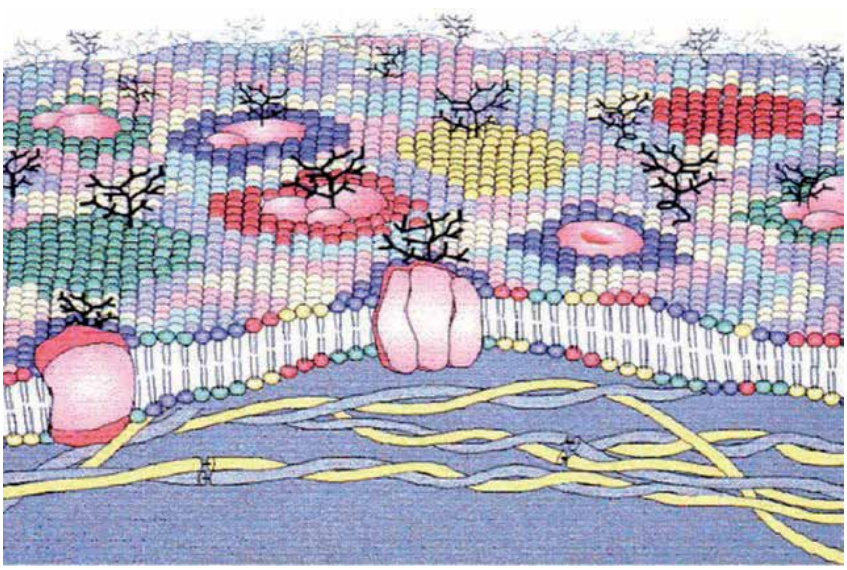

Figure 2. Schematic illustration of a biomembrane, depicting membrane lipid asymmetry as well as microdomains enriched in particular lipids and those induced by membrane proteins, reprinted from Escriba et al. [12].

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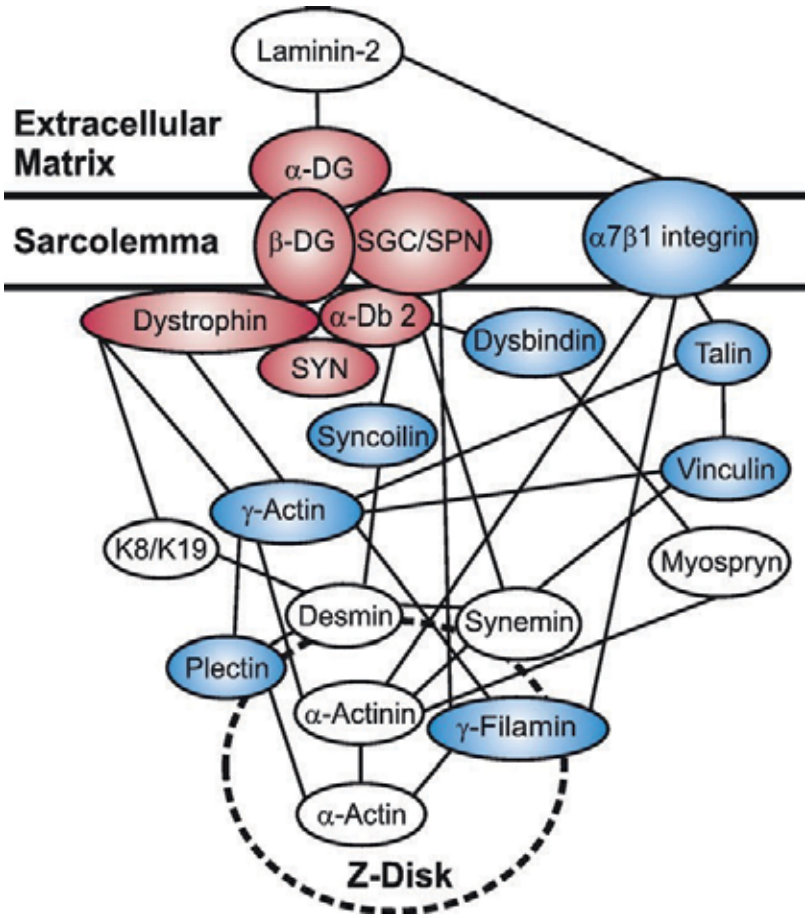

Figure 3. The dystrophin–glycoprotein complex network. Shown in red are the constituents of the core dystrophin–glycoprotein complex, which copurify as a highly stable complex from skeletal muscle and which show greatly decreased abundance in dystrophin-deficient muscle. α-Dystroglycan and β-dystroglycan (α-DG, β-DG); the sarcoglycan complex (SGC); sarcospan (SPN); α-dystrobrevin-2 (α-Db 2); syntrophin (SYN). Also shown are structural proteins that interact directly with components of the dystrophin–glycoprotein complex, their direct binding partners, and their location within striated muscle cells. Cytokeratins 8 and 19 (K8/K19). Proteins highlighted in blue are present at increased levels when dystrophin is absent, reprinted from Ervasti 2007 [23].

4. Statins and statin-induced myopathy

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase reversible inhibitors, became the most frequently prescribed drugs in modern societies used clinically to improve the lipid profile of hyperlipidemic patients, thereby decreasing the incidence of primary or secondary ischemic cardiac events [27–28]. The primary mechanism of action of statins is to lower CHOL levels by the inhibition of mevalonate formation, the rate limiting step in the cholesterol biosynthesis [29]. Pleiotropic effects of statins which seem to be independent of the

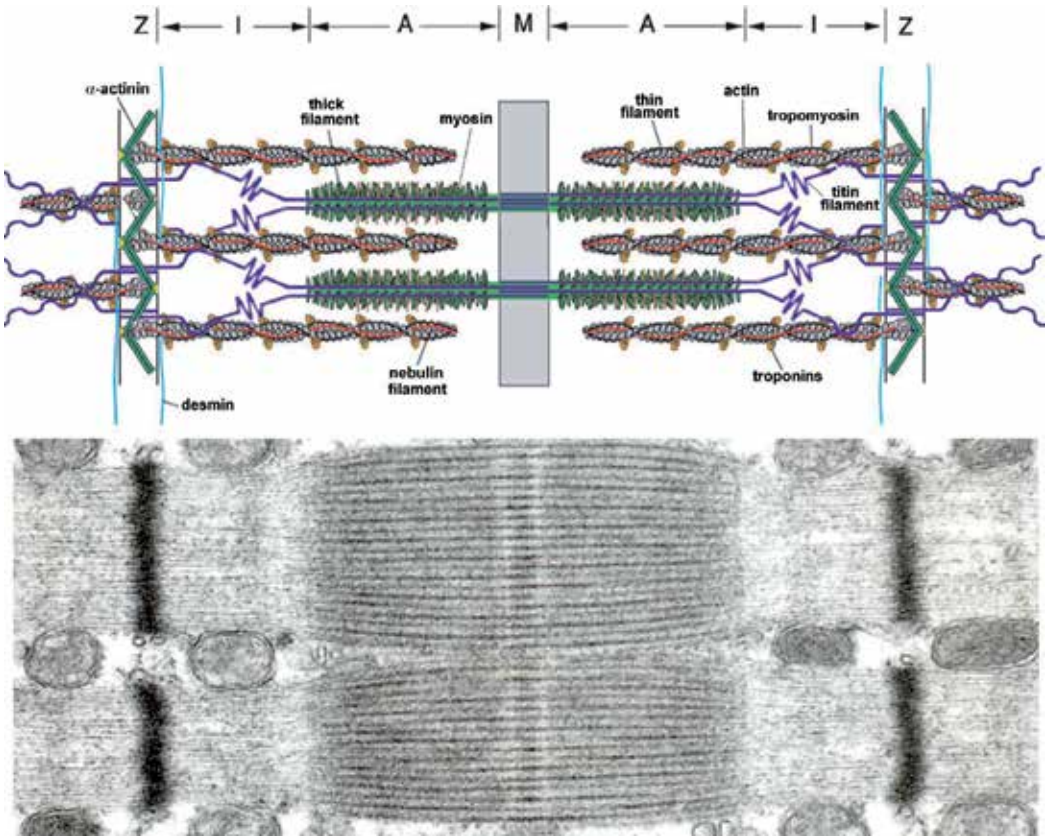

Figure 4. *Top*: Simplified model of two muscle sarcomeres in parallel. The sarcomere is composed of the thin (mostly actin) filaments, the thick (mostly myosin) filaments, and the giant filamentous molecule titin. The thin filaments are anchored in the Z-line, where they are cross-linked by α-actinin. The thick filament is centrally located in the sarcomere and constitute the sarcomeric A-band. The myosin heads, or cross-bridges, on the thick filament interact with actin during activation. Titin spans the half-sarcomeric distance from the Z-line to the M-line, thus forming a third sarcomeric filament. In the I-band region, titin is extensible and functions as a molecular spring that develops passive tension upon stretch. In the A-band, titin is inextensible due to its strong interaction with the thick filament. *Bottom*: Electron microscopy photograph of the ultrastructural organization of sarcomeres in parallel, reprinted from Ottenheijm et al. 2008 [134].

inhibiting effect on CHOL formation have also been reported [30–31], although statin-induced release of nitric oxide (NO) and prostaglandins (PGI2) does not explain side effects of statins on skeletal muscle cells (NO stimulates myogenesis, while PGI2 inhibits platelet activation). Even if statins are in general well tolerated and are safe for almost all patients, they were reported to induce different grades of myopathy in a significant part of the population, ranging from mild myalgia to morbid rhabdomyolysis [32–33]. There are risk factors for developing statin-induced myopathy (SIM), the significant component of statin intolerance during statin treatment, such as advanced age, excessive exercise, and multisystem disease as renal or hepatic insufficiencies, diabetes, or hypothyroidism [34]. Impaired metabolism of statins, pharmacokinetic interactions, and genetic effects are all probable causes of statin-induced

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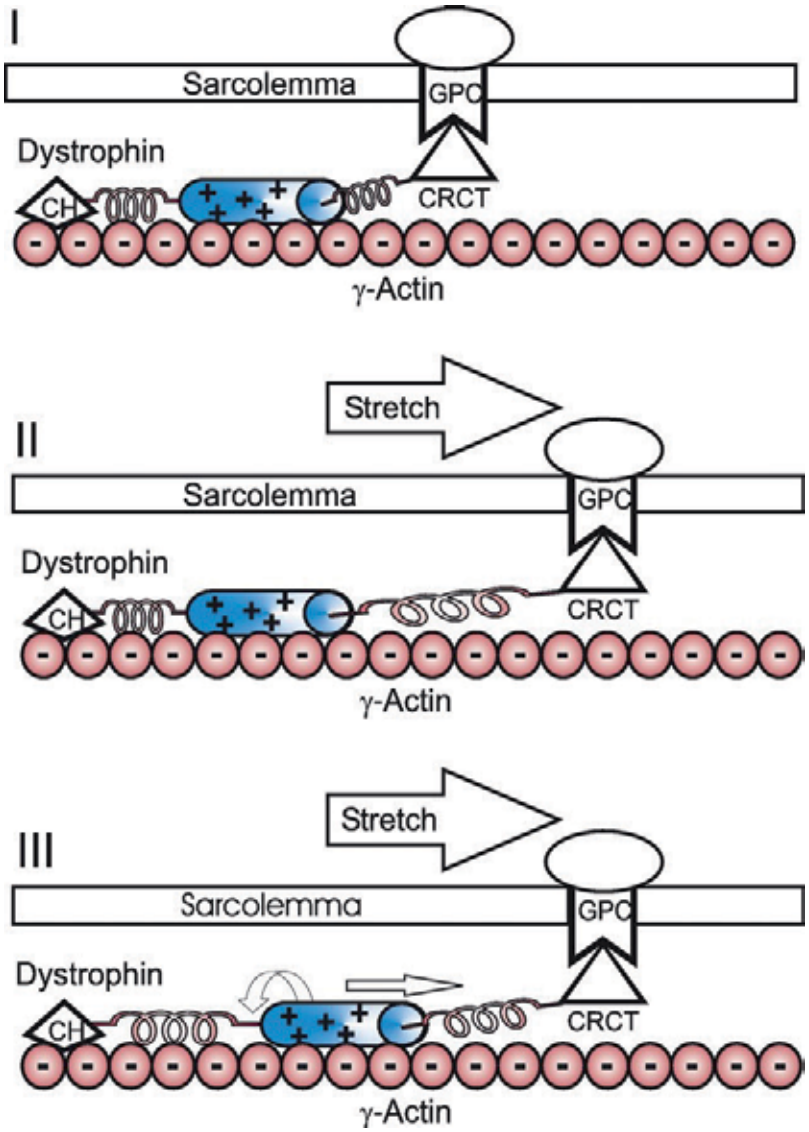

Figure 5. Dystrophin as a molecular shock absorber. Shown is a hypothetical model for how dystrophin may function to dampen elastic extension during muscle stretch. (I) Relaxed muscle. (II) Muscle stretch imposes forces that uncoil spring-like elements within repeats 1–10 and 18–24. (III) Electrostatic interaction of basic actin-binding repeats 11–17 with acidic actin filaments dampens extension of the spring-like elements. The "nonspecific" electrostatic interaction between the basic spectrin repeats and actin filaments is optimal because it does not require a specific orientation for interaction and would allow sliding between dystrophin and actin. As muscle rapidly shortens during contraction, the electrostatic interaction of the basic actin-binding repeats with acidic actin filaments would also serve to dampen elastic recoil, reprinted from Ervasti 2007 [23].

myotoxicity (muscle toxicity), although the molecular mechanism has not yet been elucidated in full. The most frightening clinical adverse effect is drug-induced rhabdomyolysis (0.1–0.5% in patients treated with pravastatin) the frequency of which is further increased by coadmi-

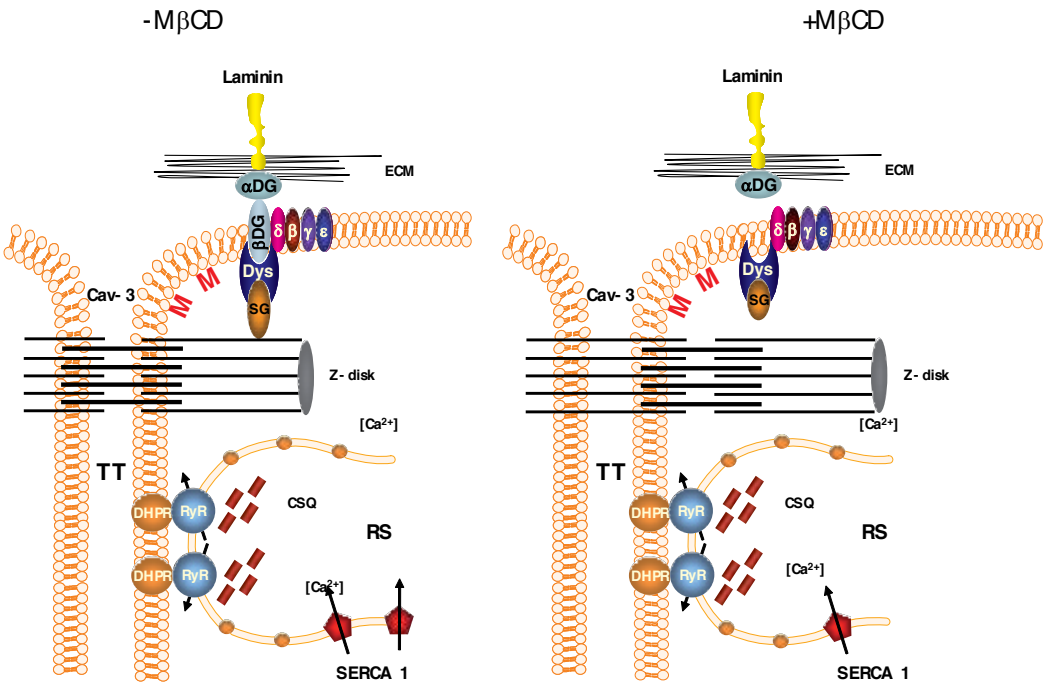

Figure 6. A model of the effect of membrane cholesterol depletion on the sarcolemmal distribution of β-DG. The sarcolemmal lipid rafts close to the clear opening of the TT-membrane are enriched in cholesterol, GM1 (ganglioside M1), Cav-3 (caveolin-3), and β-DG (dystroglycan). The schematic diagram illustrates the diminished contact between β-DG and Dys (dystrophin) in the presence of MβCD (methyl β-cyclodextrin); the β-DG/Dys interaction is essential for lateral force transmission. SERCA1 (sarcoplasmic reticulum calcium ATPase) and RyR (ryanodine receptor (SR Ca^{2+} channel)) function normally. SL, sarcolemma; SR, sarcoplasmic reticulum; EM, extracellular matrix; LR, lipid raft; Dys, dystrophin; SG, sarcoglycan; CSQ, calsequestrin, adapted from Vega-Moreno et al. 2012 [17].

nistration of fibrates [35]. Reductions in skeletal muscle membrane CHOL were initially thought to account for the range of myopathic reactions. Additionally, the lowering the isoprenoid levels has been suggested to contribute to these pathologies as protein prenylation, and the potential consequences of a generalized insufficiency of this form of protein modification [36] are important for the activity and anchorage of plasma and other membrane proteins (nuclear envelope, dystrophin–glycoprotein complex, cytoskeletal G-proteins, etc.).

There is growing interest to decipher the molecular mechanism of the statin-induced myopathy, both by scientific community and pharmaceutical companies. One in three people over the age of 45 is taking a statin to reduce heart attack risk, the HMG-CoA antagonist with three orders of magnitude greater affinity to bind and subsequently to inhibit HMG-CoA reductase activity than that of natural substrate (HMG-CoA). Two in five women taking the statin are weaker than before, with one in ten reporting they felt “much worse”. As statins became the most frequently prescribed drugs to prevent cardiovascular crisis alongside with the effort to pace physical activity, the issue how to protect from statin-induced myalgia, myositis, and rare cases of rhabdomyolysis is of great concern. In fact, due to muscle toxicity, an estimated 5–

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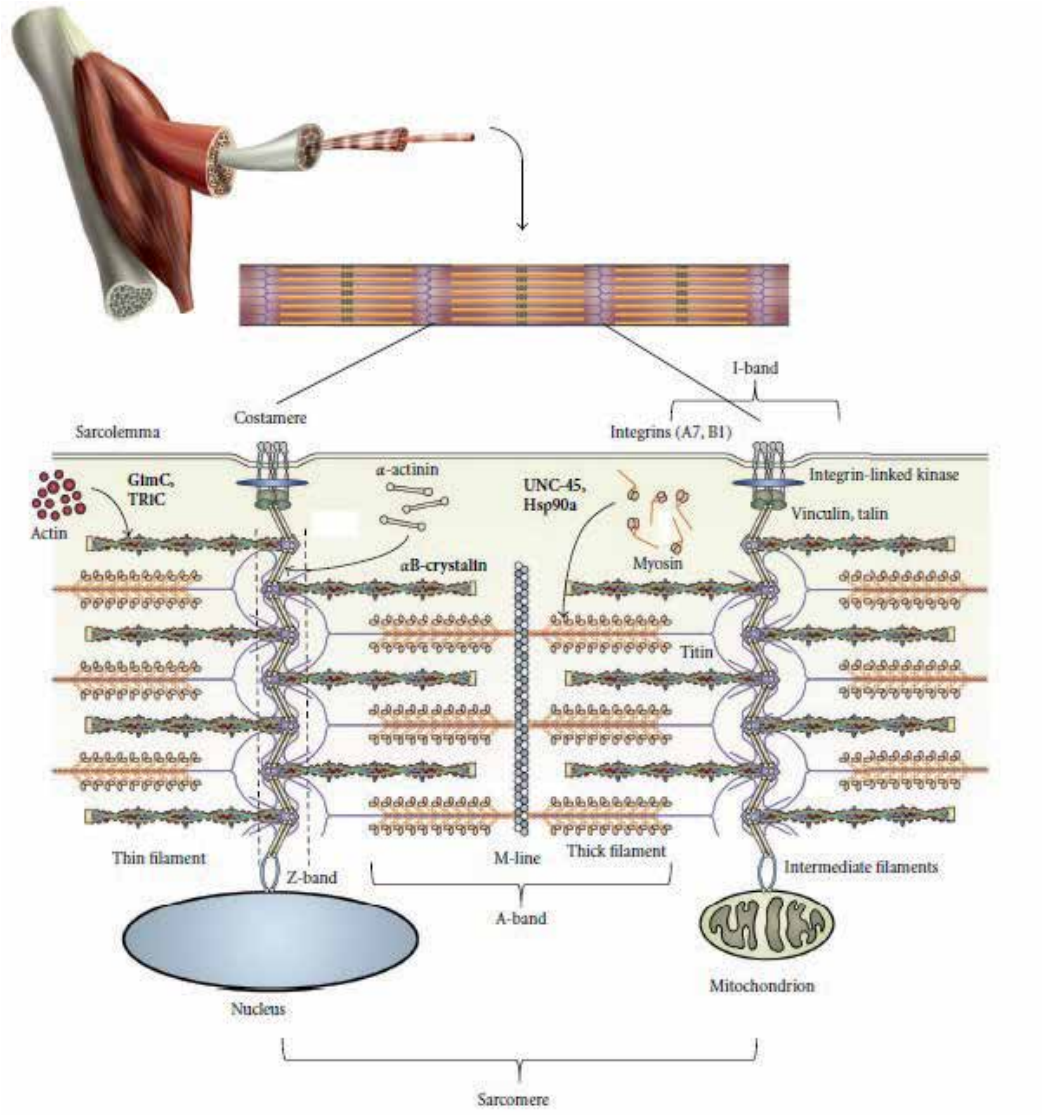

Figure 7. Schematic diagram of the sarcomere and costamere protein complexes of striated muscle cells. Major components of the mature sarcomere and costamere are shown, along with the cytoskeletal and motor filament systems, in context with the sarcolemma and organelles of syncytial myocytes. Known chaperone or cochaperone molecules are shown in bold, along with their substrates. Arrows indicate regions where chaperone-mediated protein folding is essential to incorporate polymeric filament proteins, adapted from Sparrow and Schock 2009 [135], reprinted from Myhre and Pilgrim 2012 [136].

10% of patients discontinue statin use due to myopathic symptoms. Reports of myositis and myopathic symptoms increase with increased statin dose [37], with different classes of statins, or when statins are coupled with other drugs [38], and with exercise [33]. The mechanistic underpinning of statin myopathy are believed to be multifactorial and partially attributed to

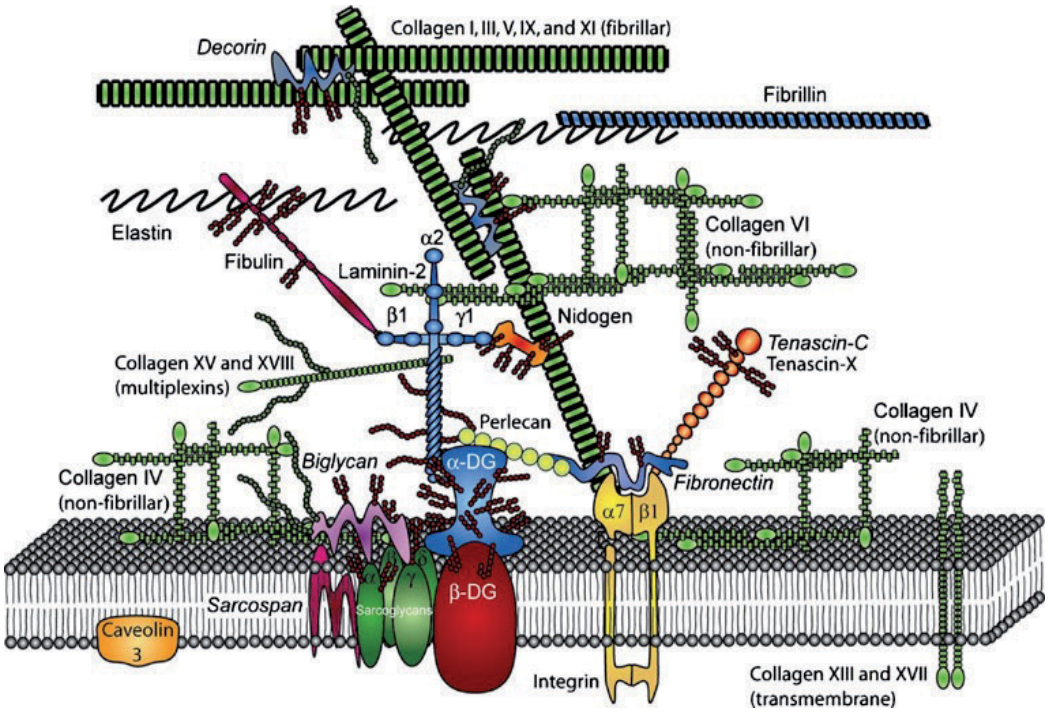

Figure 8. This figure shows the structure of the costamere and known molecular interactions. Below the membrane bilayer shown is the intracellular space and above it is the extracellular space. In the intracellular space, the costamere is attached to the contractile proteins through dystrophins (for the dystrophin glycoprotein complex, DGC), vinculin, talin, and paxilin (for the integrin complexes; not shown). In the extracellular space, both DGCs and integrin complexes bind to the components of the basal lamina that is attached to the rest of the extracellular matrix that consists mostly of fibrillar collagens, reprinted from Voermans et al. 2008 [137].

the regulatory effects of statins on apoptosis of muscle cells [39] and proliferation [3]. As skeletal muscle resident satellite cells (RSC) represent physiological reserve of undifferentiated muscle progenitors, it is obvious that activation followed by proliferation and migration are crucial in muscle adaptation to mechanical overload and regeneration from injury [40]. Thus, if statins impair RSC activation these processes could not be initiated. To tackle the problem of reduced CHOL concentration in plasma membrane and associated changes in the function of LR in muscle cells seems to be fundamental. The focal point is LR, where changes (biochemical and morphological) are presumably attributed to the consequences of disturbed cell signaling. As HMG-CoA reductase activity is ubiquitous, while the side effects of statins are confined to skeletal muscle, it is suggested that muscle tissue is featured by unique response to lower CHOL. RSC are targeted by CHOL depletion and the consequences are cumulative as muscle growth is stopped at the initiation phase (signal transduction). This assumption is supported by the data obtained from sarcolemma examination (single molecule microscopy and molecular studies), which demonstrate the isolation and downregulation of LR and the recruitment of mitochondrial oxidative phosphorylation system during myogenesis [41–42]. CHOL and GSL/SL are also present in the membranes of cellular organelles such as ER and

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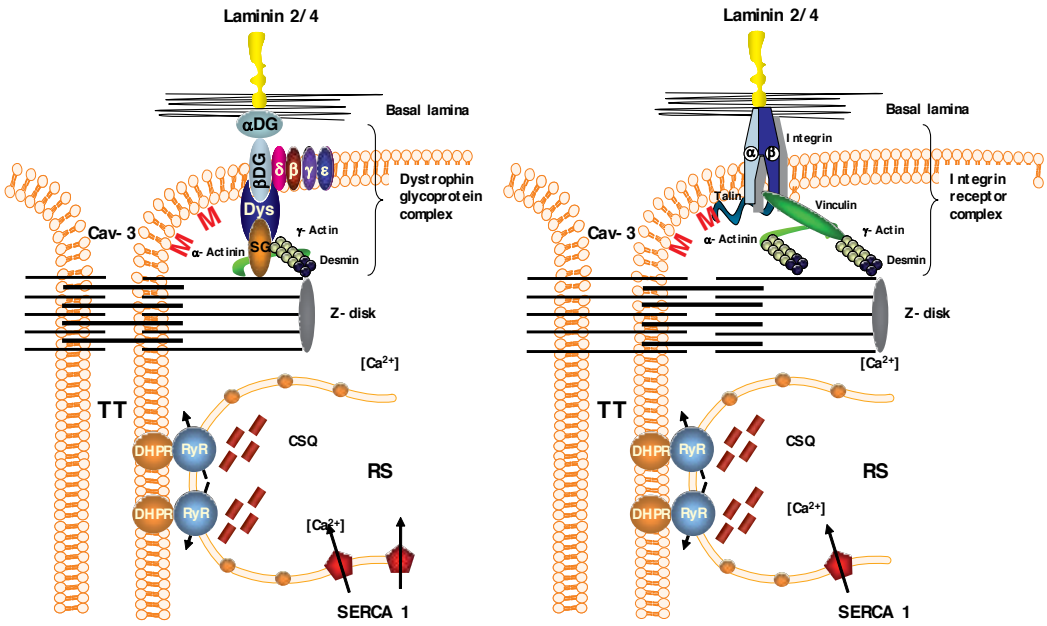

Figure 9. Schematic representation of a costamere and the focal adhesion complex (FAC). Two laminin receptors, a dystrophin/glycoprotein complex and an integrin receptor complex, are among the sarcolemmal structures that link the contractile apparatus of muscle fibers with the surrounding basal lamina. Components of both receptors, i.e., both dystrophin and the integrin-associated cytoskeletal proteins (talin, vinculin, α-actinin), colocalize in subsarcolemmal complexes which connect through γ-actin and the intermediate-filament proteins desmin and vimentin to the Z-disk of skeletal muscle fibers, adapted from Patel and Lieber 1997; Rybakova et al., 2000 [138–139], adapted from Fluck et al. 2002 [20].

Golgi complex (GC). Their role, however, remains obscure as methods to study effects of CHOL depletion in organelles are limited. The main concern should be placed on the sarcolemma and mitochondria, since these organelles control both muscle growth and development [43, 44].

5. Statin-induced myopathy and mitochondria

The development of statin-induced rhabdomyolysis is a morbid side effect of HMG-CoA reductase inhibition, occurring in less than 1 in 1000 statin-treated patients [45]. Even though occurrence of myopathy in statin-treated individuals has been estimated to range from 1 to 10%. Studies performed on rats confirmed that atorvastatin treatment reduces exercise capacity manifested by higher fatigability [46]. It is more common in statin users regularly exercised or statin-treated athletes pointing to the high correlation between the muscle contractive activity and muscle-associated complications of statin administration. Among several hypotheses raised to explain the aforementioned causal relationship between statins and physical exercise, growing body of evidence indicate both reactive oxygen species (ROS) and abnormal mitochondrial activity as probable inciting factors implicated in the deleterious effects of statins [47–48]. Actually, the inhibition of HMG-CoA reductase that hampers the

mevalonate pathway in addition to impaired cholesterol synthesis also reduces the synthesis of isoprenoids such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), intermediates affecting number of nonsteroid isoprenoids including coenzyme Q10/ubiquinone [49], and finally it adversely affects selenoprotein synthesis as well as the biosynthesis of dolichols, which are involved in the process of protein glycosylation (Figure 10). The latter mechanism is rate limiting by dolichyl phosphate, which acts as a donor of oligosaccharides in glycoprotein synthesis [50]. The importance of glycoproteins in skeletal muscle growth and regeneration is further emphasized by the examination of N-linked glycoproteome of C2C12 mouse myoblasts and myotubes [51]. It is clear from this study that approximately 128 (117 transmembrane, 4 glycosylphospatidylinositol-anchored, 5 ECM, and 2 membrane-associated) proteins were identified, while a few N-linked glycoproteins (including aquaporin-1 and β-sarcoglycan) were apparently of paramount value when myoblasts differentiate to myotubes. There was an evident decrease of aquaporin-1 and cadherin-2, whereas β-sarcoglycan expression level increased as the myoblasts fused and formed multinucleated syncytium. Ubiquinone is an important electron carrier between the complex I and complex III of respiratory chain in mitochondria. Thus, decrease in nonsteroid isoprenoids such as CoQ10 leads to the inhibition of complex I, incomplete reduction of oxygen at the level of cytochrome *c* oxidase (complex IV), and induces oxidative stress (augmentation of superoxide anion radical, hydrogen peroxide, and finally hydroxyl radical) postulated to cause myotoxicity of statins [48]. Myotoxicity is therefore at least in part the consequence of damage to lipids, proteins, and DNA, although lipids in cellular membranes are most likely targets of ROS (extensive lipid peroxidation). Additionally, limited access of proteins to prenylation impairs important lipid anchorages essential for PM attachment and function of a variety of proteins involved in cell signaling (i.e., heterotrimeric guanine nucleotide-binding protein-coupled receptors – GPCRs, GTP-binding small/G-proteins Rap1, Ras, Rac, and Rho).

6. LR partners control skeletal muscle regeneration

GPCRs, the most abundant PM receptors, regulate wide range of cellular processes through intracellular heterotrimeric G protein (GTP-binding protein). The latter acts as a signal transducer to control the activity of several catalytic proteins central to the message amplification and its intracellular broadening to effector proteins. Heterotrimeric proteins are composed of three subunits (Gαβγ). Agonist-mediated activation of GPCRs brings about conformational changes which lead to the exchange of guanosine diphosphate (GDP) for GTP on the Gα-subunit which then dissociates from Gβγ dimer. Now, Gα may translocate to the target protein(s), whereas Gβγ dimer inactivates the receptor through phosphorylation (it recruits G protein–coupled receptor kinase – GRK to inactivate the receptor). As GPCRs constitute the largest family of membrane receptors, there are at least 16 types of Gα subunits, 5 of Gβ subunits, and 12 types of Gγ subunits [52]. Gα signaling is stopped when GTP is converted to GDP by the intrinsic GTPase activity of the protein itself. In consequence, Gα-GDP is reassembled with Gβγ dimer and G-protein complex is reestablished. Importantly, target proteins for heterotrimeric G-proteins are membrane bound suggesting that lipid–

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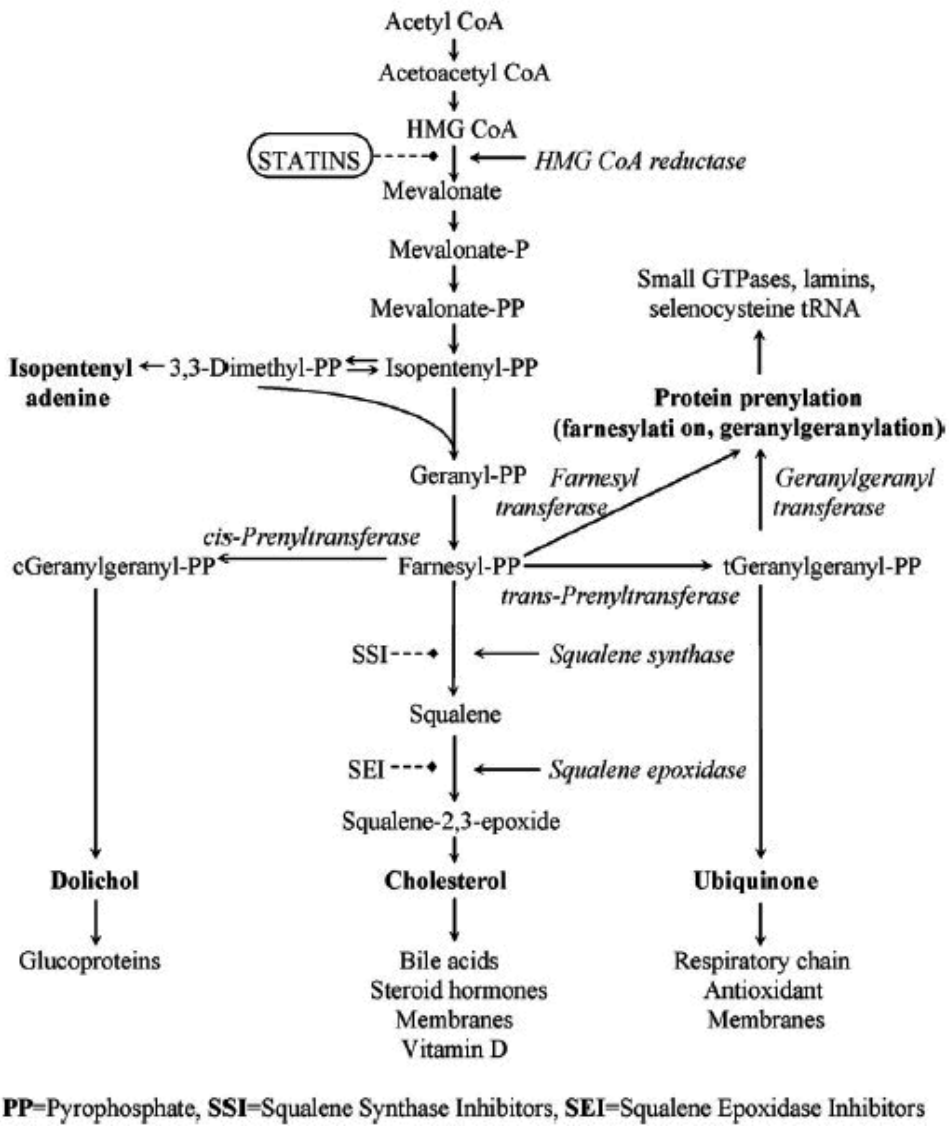

Figure 10. The biosynthetic pathway of cholesterol and other cometabolites, reprinted from Vaklavas et al. 2009 [58].

protein and/or lipid–lipid interactions are fundamental for G-protein-mediated signaling. Actually, all known Gγ proteins undergo isoprenylation on cysteine residues (either geranylgeranyl or farnesyl moieties) pointing to increased affinity to hexagonal nonraft phase (e.g., PE) of PM [53–54]. In contrast, Gα subunits are modified by myristoylation (Gα_i) and/or reversible palmitoylation (Gα) allowing them to get access to lamellar regions of PM (e.g., lipid rafts). It also explains how Gα subunits migrate to their cognate targets in LR after dissociation form G$\beta\gamma$ assembled to GPCRs. Minetti et al. [55] have showed in elegant study that skeletal muscle hypertrophy and differentiation are greatly influenced by signaling induced by

lysophosphatidic acid (LPA) acting on GPCRs which in turn activate a $G\alpha_i$ protein. Besides, $G\alpha_i$ enhanced muscle regeneration and caused switch to oxidative fibers and can act as a counterbalance to MuRF1 and MAFbx/atrogin-1. To sum up, lipid structures play active role in signal propagation with resulting localization of $G\alpha$ and $G\beta\gamma$ proteins.

Uncommon myopathic changes resultant from statin therapy offer the opportunity to gain new insight for the function of biochemical pathways downstream to HMG-CoA reductase in skeletal muscles. Irrespective of the type of statin treatment (hydrophilic or hydrophobic), the viability of skeletal muscle cells is considerably reduced, though the effect depends largely on the pharmacokinetic and pharmacodynamic properties of statins [56–57], while the signaling pathway(s) and molecular mechanisms are still not fully understood. Sometimes, signal transduction is dependent on small GTPase proteins that cycle between an inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound state. The posttranslational prenylation of these proteins occurs by the covalent addition of only two types of isoprenoids, FPP and GGPP, to cysteine residues at or near the C-terminus. Upon tyrosine kinase receptor activation, the prenylated (PM protoplasmic/inner leaflet attached) small GTPase protein Ras (MAPK kinase kinase kinase) binds GTP and becomes activated to initiate MAPK cascade ending with the stimulation of muscle cell growth (hyperplasia). In addition, small GTPase protein Rab1 (more than 60 Rab small GTPase isoforms have been identified) is involved in organelle biogenesis and intracellular vesicular trafficking [58]. Overall growth and survival signals depend on the activation of both protein receptor and nonreceptor tyrosine kinases.

Several lines of evidence suggest particular significance of IGF-1/PI3-K/AKT cascade in maintaining muscle cell growth and viability [59–61] likely through the suppression of FOXO-dependent activity of atrogin/MAFbx ubiquitin ligase gene required for the development of muscle atrophy [62–63]. Moreover, the statin-induced muscle damage is controlled by PGC-1α, a transcriptional coactivator that induces mitochondrial biogenesis and protects against the development of statin-induced muscle atrophy [64]. In *in vivo* model, simvastatin downregulated PI3-K/AKT signaling and upregulated FOXO transcription factors and downstream gene targets known to be implicated in muscle cachexia [63]. Insulin and IGF-1 are widely known agonists of their cognate receptors (IR and IGF-1R, respectively), although at concentrations higher than physiological cross-reactivity of insulin to IGF-1R and IGF-1 to IR were observed. On the other hand, LR have been shown to be platforms to initiate cellular signal transduction of IGF-1 and insulin-inducing skeletal muscle differentiation and hypertrophy. Notably, the impaired insulin/IGF-1 signaling [65–67] mimics the side effects of statin administration, whereas insulin and/or IGF-1 were reported to overcome statin-induced myopathy [61]. IR and IGF-1R with their intrinsic tyrosine kinase activities transduce the signal by recruiting insulin receptor substrate-1 (IRS-1) with its src-homology 2 domains (SH2) to the receptor phosphotyrosines. IRS-1 activates PI3-K/AKT/mTOR and Ras/Raf/MEK/ERK pathways, however, phosphoinositide 3-kinase (PI3-K) as a lipid kinase converts plasma membrane phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). The latter attracts kinases with pleckstrin homology domain (PH) downstream to PI3-K including phosphoinositide-dependent kinase 1 and 2 (PDK1, PDK2) and AKT. Depletion

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of CHOL and sphingolipids blocks IGF-1-induced AKT membrane recruitment, whereas LR reconstitution in CHOL- and sphingolipid-deficient cells restored AKT membrane recruitment and phosphorylation [68]. Thus, LR-localized PIP3 is essential for AKT membrane association, while AKT seems to promote formation of PIP3-containing rafts.

7. Isoprenoids limit skeletal muscle regeneration

While human and animal studies have demonstrated that statin treatment may reduce serum CoQ10 levels [2, 69], ubiquinone levels in human skeletal muscle do not appear to be affected by statins [61, 70]. Possible significance of some reactions and intermediates in CHOL synthesis indicate that GGPP and FPP as isoprene units play very important role in muscle cell survival and regeneration. The posttranslational prenylation of proteins such as heterotrimeric G proteins, small G-proteins, and lamins enables these proteins to anchor to cell membranes, whereas N-linked glycosylation of insulin and IGF-1 membrane receptors mediated by dolichols establishes their proper biological functions (sensitivity to ligands). Dolichols serve as carriers and situate the core oligosaccharide to be assembled to protein molecules. From some studies, it appears that it is mevalonate and isoprene units (GGPP and FPP) as their downstream intermediates rather than CHOL play the key role in statin-induced myotoxicity. Consequently, replacement of depleted mevalonate reversed the changes induced by statins, where squalene synthase or squalene epoxidase (steps in CHOL synthesis) inhibitors at concentrations sufficient to inhibit entirely CHOL synthesis did not affect muscle cell viability [71–72]. These observations point to cardinal role played by isoprenoids in physiology of skeletal muscle cells. Interestingly, statins which are sometimes harm to skeletal muscle cells do not act adversely on myocardium, a type of striated musculature. Substantial difference in the sensitivity of skeletal muscle cells and cardiomyocytes to the statin-induced myopathy could be related to dissimilar mechanisms that control viability of these cells [73]. Alternatively, it was postulated that Ca^{2+} homeostasis alterations could account for statin-induced muscular side effects [74, 75].

8. Ca^{2+} homeostasis and statin-induced myopathy

Acute application of simvastatin on human skeletal muscle fibers led to a large release of Ca^{2+} into the sarcoplasm [76]. The authors showed that mitochondrial Ca^{2+} efflux through both permeability transition pores (PTP) and Na^{+}/Ca^{2+}-exchanger (NCE) was a major initiator of the large sarcoplasmic reticulum (SR) Ca^{2+} release by affecting ryanodine receptor 1 (RyR1, Figure 11). Furthermore, the effects of simvastatin on Ca^{2+} homeostasis were not linked to the disturbed cholesterol synthesis pathway as GGPP and FPP treatments did not prevent statin-induced Ca^{2+} waves. They were caused by simvastatin-dependent fall in mitochondrial membrane depolarization and Ca^{2+} efflux to the cytoplasm. Next, Ca^{2+} was recaptured by the SR that in turn triggered the SR release of Ca^{2+} by at present unknown molecular mechanism as "calcium-induced Ca^{2+} release" is implausible since the main RyR isoform in mammalian

skeletal muscle is poorly sensitive to Ca^{2+} [77]. It is apparent from this study that mitochondria played critical role, and that Ca^{2+} efflux from mitochondria resulted from alteration of mitochondrial respiratory chain, as mitochondrial membrane potential (MMP) decreased with concomitant rise in NADH concentration. Nonetheless, with regard to these experiments some criticism has to be reserved, as simvastatin concentrations (50–200 μM) were much above the pharmacological range. Summing up, these observations point to ubiquitin/proteasome (UP) proteolysis and mitochondria as skeletal muscle target of statins, while the exact nature of their detrimental action (direct or indirect) remains to be elucidated. Recently, some interesting data were obtained from transcriptomic analysis of biopsies collected from atorvastatin-treated and exercised vs. nonexercised skeletal muscles of healthy volunteers. The authors complement severalfold rise in UP pathway gene expression in 8-hours eccentrically exercised vastus lateralis muscles baseline compared to the right leg after statin/placebo treatment [78].

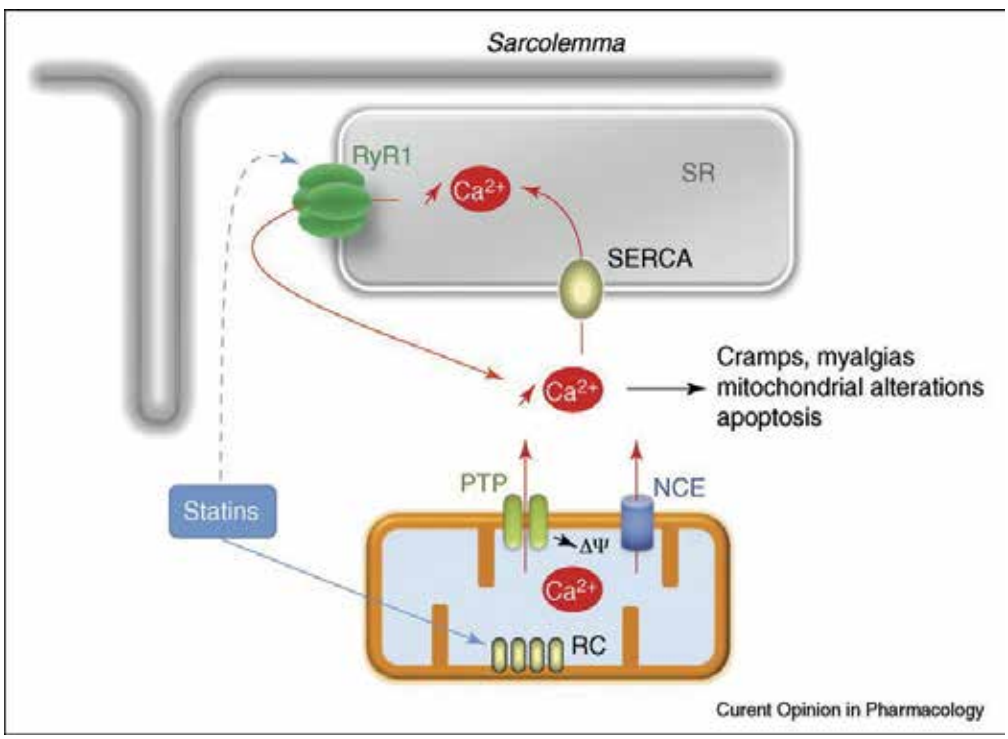

Figure 11. A possible direct pathophysiological effect of statins. Although statin myotoxycity may occur through the reduction of cholesterol synthesis, a direct effect of statins has been reported in vitro and in vivo in muscle fibers from both animal and human models. This diagram summarizes some of the most recent data suggesting a pathophysiological mechanism. Statins diffuse into muscle fibers and inhibit complex I of the mitochondrial respiratory chain (RC). This depolarizes the inner membrane (Dc) triggering a calcium release through the permeability transient pore (PTP) and sodium-calcium exchanger (NCE). This results in a first elevation of cytoplasmic calcium that will be partially uptaken by the sarcoendoplasmic reticulum calcium pump (SERCA) to the sarcoplasmic reticulum (SR). When overloaded, SR may spontaneously release calcium through the ryanodine receptor (RyR1) to generate a calcium wave. A direct effect of statins on RyR1 may not be excluded (dotted line). Impaired mitochondrial function and consequently calcium signaling may account for muscle symptoms, reprinted from Sirvent et al. 2008 [48].

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Deregulation of calcium ion (Ca^{2+}) homeostasis in mitochondria is indicated as initial step in cascade of events leading to statin myopathy [76, 79]. In several experiments carried out on C2C12 myoblasts and human muscle biopsies, statins impaired mitochondrial respiration and sensitized muscle fibers to calcium signaling. Accordingly, muscle fibers showed reduced level of ATP and higher frequency of Ca^{2+} waves [46, 79]. Disturbed regulation of Ca^{2+} homeostasis (elevated cytoplasmic concentrations) is known to trigger activity of calpains [80], whereas raise in ROS is associated with caspase cascade [81–82]. As RSC are enriched in SM, they obviously should have high LR/C representation. Modulation of SL modifications with sphingomyelinase inhibitors would provide closer look on LR/C impact cell viability/morbidity. Next to statins widely used as hypocholesteremic drugs, polyunsaturated fatty acids (PUFA) are frequently recommended to lower blood plasma concentration of low density lipoproteins (LDL) and triglycerides (TG). There is evidence that PUFA (n–3) synergize with statins in their positive effect, furthermore some PUFA (EPA and DHA) prevent statin-induced myopathy [83–84]. It was found that statins evoke endoplasmic reticulum stress (ERS) and ERS inhibitors as well as PUFA attenuate this response most likely through PPARγ-dependent mechanism. Detailed outline of PUFA protective effect has, however, not been explained. Scientific problem is to find out if LR contribute to the activation of RSC. Furthermore, if LR/C are essential to recruit RSC to enter the differentiation program, what would have been if LR are ablated? The study aimed to shed more light on the side effects of statin administration to skeletal muscle is urgently needed. Novelty of research should address etiology to abnormal function of sarcolemma in skeletal muscle cells of statin-treated subjects. We found only one report showing that CHOL depletion impaired muscle function [17]. CHOL is essential in holding together lateral assemblies of lipids in LR nanodomains. The latter are indispensable to cell signaling as they form platforms for signalosomes (proteins assembled in order to provide signal transduction from PM receptors). Their role has been corroborated for PI3-K/AKT and JAK/STAT but not Ras/Raf/MEK/ERK signaling pathway. We assume that former cascades (PI3-K/AKT and/or JAK/STAT) are involved in RSC activation while the latter are important to initiate proliferation. As muscle cell differentiation proceeds, the representation of SL in LR is subjected to additional modifications [85–88]. We guess these changes are related to the activation of sphingomyelinases and other SL converting enzymes. Ceramides and sphingosines were reported to frequently affect cell functions including proliferation, differentiation, and viability [89, 90], and other bioactive lipids are also important players in muscle growth and regeneration. At the same time, involvement of ROS and mitochondria, Ca^{2+} homeostasis, and proteolytic systems should be examined.

9. Skeletal muscle satellite cells and LR

Plasma membrane is not uniform in state of matter, i.e., fluid portion is represented by glycerophospholipids spontaneously mounted into lipid bilayer in disordered manner (Ld – liquid disordered). As mentioned before, in such membrane, numerous nanodomains known as lipid rafts contain sphingolipids and CHOL as well as lipid-modified integral membrane proteins. Nanodomains (Lo – liquid ordered) are buoyant in fluid portion of membrane and

have tendency to coalesce into larger platforms to form signalosomes essential for signal transduction [22]. Thus, in muscle cells deprived of mevalonate due to statin administration, one should expect lower level of LR/C and decreased availability of prenylated proteins (farnesylated and geranyl-geranylated). It is important to stress that muscle growth, adaptive hypertrophy, and regeneration are directly attributable to the PM representation of LR determined by CHOL and sphingolipids [87, 91] found in RSC. The mononucleated RSC are located beneath the basal lamina that surrounds multinucleated myofibers [6]. They are activated by signals from injured myofibers and macrophages to enter the cell cycle and produce myogenic precursor cells that then differentiate and fuse into multinucleated myotubes or existing myofibers [92]. The molecular mechanisms responsible for the transduction of such extracellular signals in satellite cells remain poorly defined, and the potential role of lipid-mediated signaling has not been previously considered in this context. There is an assumption that satellite cells are capable to rearrange PM composition in order to respond to extracellular signals and allow cell multiplication and migration which is followed by fusion. Actually, muscle cells were reported to change the lipid representation in PM according to the particular step of differentiation [87, 91, 93]. While phosphatidylserine (PS) is highly expressed during myoblast fusion [88], phosphatidylethanolamine (PE) is involved in cell motility [94]. Both mentioned are the glycerophospholipids of Ld phase located in the protoplasmic leaflet. On the other hand, sphingomyelin (SM) is found exclusively in the Lo phase where it forms LR nanodomains with other SL, GSL, CHOL, and proteins. CHOL is essential to organize LR as it helps both to position SL and GSL and provides the most advantageous energy status between Lo and Ld phase [95]. Nowadays, it is widely accepted that these nanodomains facilitate cytoplasmic signaling by acting to concentrate signaling molecules [96]. Additionally, SL metabolites, such as ceramide, sphingosine, and sphingosine 1-phosphate, are emerging as important regulators of a variety of cellular events, including cell proliferation, differentiation, and apoptosis [97–98]. With respect to SM, another important issue is that it is highly represented in PM of RSC but during satellite cell proliferation and subsequent differentiation it is almost undetectable [85]. One has to bear in mind that RSC as being stem cells undergo either symmetric or asymmetric division and that in the identical culture conditions they adopt characteristics consistent with a return to quiescent-like state [99]. Thus, it is apparent that certain activated satellite cells (ASC) by unknown mechanism are withdrawn from the cell cycle and they escape from the differentiation program. Cell decision whether to differentiate or not to differentiate is determined by the composition of PM and LR representation in particular. Under the appropriate conditions, SC differentiate into muscle cells with phenotype characterized by the accumulation of muscle contractile proteins and increased sensitivity to insulin. In these differentiated cells, insulin accelerates myogenesis [43]. Insulin initially stimulates proliferation, and subsequently, it stops cell divisions and stimulates metabolic pathways to promote protein synthesis. A clear explanation of how these signaling pathways elaborate such radically different physiological responses in this differentiated tissue has remained elusive, although compartmentalization and switching-off signaling molecules has been proposed [43]. Insulin activates their respective tyrosine kinase receptors to phosphorylate key residues on a "docking protein" or the receptor, respectively, which recruits multiple adaptor proteins. Recruited proteins include the GDP exchange factor Son of Sevenless (SOS), which activates the Ras/Raf/MEK/ERK mitogen-activated protein kinase cascade (mitogenicity), or the p85 regulatory subunit of PI3-kinase, which stimulates signaling pathways

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ultimately leading to AKT/PKB activation. PI3-K is strongly implicated in metabolic, but not mitogenic signaling in muscle cells, as is its downstream effector AKT [44]. In the latter paper, an important role of mitofusin 2 (Mfn2) protein has been shown as a partner and inhibitor of Ras. Thus, it become clear why studying the ability of different approaches to regulate numerous signaling molecules, statins and CHOL chelators, particularly PI3-K and AKT (PI3-K/AKT), are essential due to their relevance to anabolic metabolism in myotubes and LR reliance. Comparative studies concerning Ras/Raf/MEK/ERK and JAK/STAT would be crucial as these pathways are involved in muscle cell proliferation and they compromise muscle differentiation [100]. It is not clear if reactive oxygen species (ROS) are involved as a cause or an effect of disturbance [46, 79, 101]. Lower production of ATP would explain faster muscle fatigue observed during exercise in statin-induced myopathy [102]. Interestingly, appropriate muscle exercise (physical training) might protect skeletal muscles from undesired statin-induced side effects [103]. This is of particular importance as the widespread use of statins and more active lifestyle might foster the incidence of SIM.

10. Heat controls vital signaling molecules in plasma membrane

Although the molecular mechanisms that regulate the differentiation of satellite cells and myoblasts toward myofibers are not fully understood, cell membrane lipids and proteins that sense and respond to their environment must play an important role. Heat alters PM physical state into more fluidic form, and similar effect may be artificially induced by membrane fluidizers [104]. Interestingly, heat-induced hyperfluidization in animal PM is associated with the activation of the cholesteryl glucoside (CG) synthetase (glucosyltransferase) located in LR, the enzyme that catalyzes the transfer of the glucose moiety from glucose donor sphingolipid glucosylceramide to cholesterol [105]. CG production alters membrane physical properties and forms thermostable solid-ordered domains. Notably, CG and other steryl glucosides act as important lipid mediators in the process of heat shock factor-1 (HSF-1) activation. This transcription factor regulates the expression of heat shock proteins (HSPs), which are critical for the survival of cells [104]. Some authors showed that the alteration of membrane fluidity by heat or membrane fluidizer treatment causes the reorganization of lipid rafts linked to activation of heat shock response mediated by HSF-1 activation and HSP induction [106–108]. It is suggested by Kunimoto et al. [110–111] that PM fluidity leads to CG formation and the latter mediates HSF-1 activation and HSP induction. There is growing body of evidence that strategies aimed at increasing levels of HSPs may be successful in protecting cells in neurodegenerative diseases. At least in the animal model of amyotrophic lateral sclerosis (ALS), increasing HSP levels by treatment with arimoclomol (hydroxylamine derivative) delayed disease progression in mice [112, 113]. Another hydroxylamine derivative, namely, BGP-15 inhibited acetaminophen-induced caspase-independent apoptosis of hepatocytes [106]. The prime HSPs induced by heat stress or membrane fluidizers are HSP70 and HSP90. Importantly, HSP72 preserves muscle function and slows progression of severe muscular dystrophy [114]. Under normal circumstances, chaperone proteins involved in protein quality control can prevent protein aggregation by binding of misfolded proteins as soon as they are produced during translation or later during their organization into supramolecular structures, thereby assisting protein refolding or else in targeting for degradation [115].

11. Sphingolipids in skeletal muscle regeneration

Sphingolipids (SL) and cholesterol (CHOL) create LR in plasma membrane, but it is the biochemistry of SL that is apparently decisive for skeletal muscle biology including its growth and differentiation. PM sphingomyelin is a target for both acidic and neutral sphingomyelinases (aSMase and nSMase) bringing ceramide (Cer) as product. Ceramides are LR modulators believed to alter PM fluidity and favor receptor oligomerization [116]. Cer has been suggested to fulfill a second-messenger function but the evidence for this action is scarce and controversial, while support is emerging for its indirect impact on cellular signaling resulting from changes in membrane structure. Cer could be further converted to sphingosine by ceramidase, whereas sphingosine 1-phosphate is synthesized from sphingosine by a phosphorylation reaction catalyzed by the sphingosine kinases (SKs) SK1 and SK2, which are highly conserved enzymes activated by numerous stimuli including transactivation induced by IGF-1 [117]. From studies carried out on C2C12 myoblast cell line as progeny of mouse satellite cells, it is clear that IGF-1 evokes two mutually exclusive biological responses (hyperplasia vs. hypertrophy). This cytokine plays a key role in skeletal muscle regeneration as it is able to recruit satellite cells and stimulate myoblast proliferation and myogenic differentiation. As mentioned before, myoblasts must not fuse unless they are withdrawn from the cell cycle. How then, IGF-1 regulates two opposite responses? In recent years, the sphingosine 1-phosphate (S1P) attracts special attention with regard to physiology of resident skeletal muscle satellite cells as well as proliferating and differentiating myoblasts. First, several lines of evidence indicate significant role of S1P in skeletal muscle regeneration and repair [85, 117–122]. The extracellular action of S1P present in micromolar concentrations in peripheral blood is exerted by binding to five specific cell surface heterotrimeric G protein–coupled S1P receptors ($S1P_1$–$S1P_5$). In turn, S1P agonist levels are tightly controlled by the balance between biosynthesis catalyzed by SKs, reversible conversion to sphingosine mediated by specific and nonspecific lipid phosphatases, and S1P lyase (SPL)-dependent degradation. Second, S1P receptors are coupled to one or more G-proteins so they can elicit distinct and even contrasting final cellular effects (Figure 12). In skeletal muscle cells, major role is played by $S1P_1$, $S1P_2$, and $S1P_3$ receptors [117, 119–122]. Actually, in myoblasts, $S1P_1$ and $S1P_3$ receptors via SK activation negatively regulate the mitogenic effect elicited by IGF-1, whereas $S1P_2$ receptor is involved in myogenic effect of the growth factor (Figure 13). Thus, in myoblasts, SK/S1P axis upon IGF-1 action regulates two opposing biological effects – transducing its myogenic response on one side and inhibiting its mitogenic effect on the other. The IGF-1-dependent transactivation of S1P receptors was also observed in other cell types pointing to the conservation of the IGF-1/SK/S1P circuit in tissues other than skeletal muscle [123]. How IGF-1 does affect SKs leading to two divergent biological effects in skeletal muscle? Among the various signaling pathways activated by S1P in skeletal muscle cells, the activation of ERK1/2 and p38 MAPK, both identified as downstream effectors of $S1P_2$ in response to growth factors, was required for cell proliferation and the stimulation of myogenic differentiation, respectively [124]. The inhibition of ERK1/2 activity with specific U0126 metabolic inhibitor prevented SK1 phosphorylation induced by IGF-1, demonstrating that the activation of SK1 induced by the growth factor was mediated by ERK1/2 [116]. Similarly, the S1P-induced differentiation was prevented

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in myoblasts where p38 MAPK was inactivated by the overexpression of the dominant negative mutant or by the use of specific p38 MAPK inhibitors SB203580 and SB239063 [118].

	Cell Proliferation	Cell Migration
C2C12 Myoblasts	↓	↓
Activated Satellite Cells	↑	↑

Figure 12. Role of sphingosine 1-phosphate on cell proliferation and migration in myoblasts and activated satellite cells, adapted from Donati et al. 2013 [125].

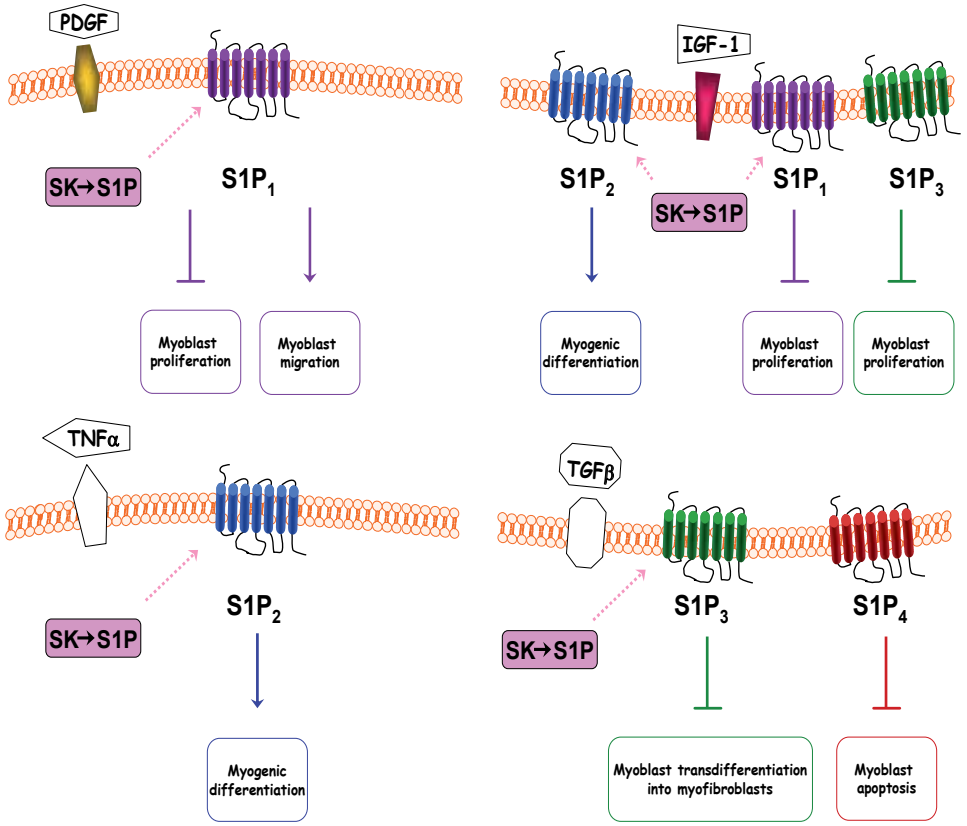

Figure 13. Schematic diagram of the biological actions evoked by S1PR transactivation by some growth factors in C2C12 myoblasts, adapted from Donati et al. 2013 [125].

One has to keep in mind, however, that S1P receptors are differently expressed in satellite cells, myoblasts, and muscle fibers, moreover their expression may vary in response to the action of particular factor. PDGF stimulates myoblasts proliferation and motility, while these effects are blocked by SK1/$S1P_1$ signaling axis [126]. In contrast, TGF-β1 was demonstrated to convey its detrimental profibrotic effect through $S1P_3$ receptors (Figure 13) [127]. These observations complement widely known activities of PDGF and TGF-β1 in wound healing. In the second intention healing as it is observed in the severe skeletal muscle injury or late stages of myopathy, the major role is played by myofibroblasts which cause fibrosis, a hallmark of in which myofibers are replaced by progressive deposition of extracellular matrix proteins [128]. The main task is therefore to facilitate skeletal muscle regeneration rather than repair, as the first one restores tissue structure and contractile function while the latter is limited to structural return. There are efforts observed to improve muscle healing by regeneration rather than fibrosis [129]. Collectively, taking into account the knowledge on how sphingosine 1-phosphate influences RSC and how it might prevent muscle fibrosis, it will be interesting to further investigate in this context the crosstalk between IGF-1 and S1P signaling pathway.

12. Concluding remarks

Primary stem cells in adult skeletal muscle known as satellite cells drive postnatal muscle growth and regeneration-associated hypertrophy. They reside beneath the basal lamina of the myofibers suggesting close contact between the adjacent cytoskeletons and chemical communication between the cells. One of the major unexplored areas of satellite cell biology is the identification of signals that are conferred from adjacent myofibers and the surrounding extracellular matrix. Equally important are soluble endocrine, paracrine, and autocrine factors that maintain satellite cells quiescent and control their preference to activate. For example, the maintenance of skeletal muscle requires notch signaling and greatly depends on delta upregulation for RSC activation [130]. In addition to the loss of notch activation, nonregenerating skeletal muscle produces excessive transforming growth factor (TGF)-β (but not myostatin), which induces unusually high levels of TGF-β pSmad3 and interferes with their regenerative capacity [131]. Thus a balance between endogenous pSmad3 and active notch controls the regenerative competence of muscle stem cells, and the deregulation of this balance in the old muscle microniche interferes with regeneration. The molecular mechanisms that regulate satellite cell quiescence, activation, and self renewal (asymmetric divisions) are not well understood, even though a possible clue for the ambiguous behavior of satellite cells could be associated with the membrane segregation of rafts and bioactive lipids such as PS that seems to accelerate myoblasts fusion into myotubes [132]. It seems plausible to affirm sarcolemmal differentiation as the leader constituent of stimulated skeletal muscle progenitors and subsequent populations of daughter cells (myoblasts, myotubes, and juvenile myofibers) involved in myogenic program. Lipid moiety of plasma membrane is not merely a boundary or the component in intercellular communication. Nowadays, it is widely accepted that specific lipids associate to form functional units (LR/C), creating substructures that actively modify its own composition including proteins and triggering a myriad of different signaling pathways. Lipid

segregation seems to account for the adaptability of skeletal muscle to a variety of stimuli, where the critical role is played by the myogenic signals represented by growth factors and cytokines. Cholesterol, isoprenoids, dolichols, and sphingolipids all contribute significantly to the physiological responses of skeletal muscle to injury. These bioactive lipids mediate most, if not all, of the signals elicited at the plasma membrane receptors. Recent advances in muscle research suggest key position occupied by sphingosine 1-phosphate, protein prenylation, and "caveolar" and "noncaveolar rafts" in skeletal muscle regeneration process.

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Chapter 6

Molecular Mechanisms Controlling Skeletal Muscle Mass

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Additional information is available at the end of the chapter

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Abstract

The interplay between multiple signaling pathways regulates the maintenance of skeletal muscle. Under physiological conditions, a network of interconnected signals serves to coordinate hypertrophic and atrophic inputs, culminating in a delicate balance between muscle protein synthesis and proteolysis. Loss of skeletal muscle mass, termed "atrophy," is a diagnostic feature of cachexia such as cancer, heart disease, and chronic obstructive pulmonary disease. Recent studies have further defined the pathways leading to gain and loss of skeletal muscle as well as the signaling events that induce post-injury regeneration. In this review, we summarize the relevant recent literature demonstrating these previously undiscovered mediators governing anabolism and catabolism of skeletal muscle.

Keywords: Skeletal muscle, hypertrophy, atrophy, mTOR, autophagy, myostatin

1. Introduction

In humans, skeletal muscle is comprises 40-50% of body mass and plays vital roles in locomotion, heat production during periods of cold stress, and overall metabolism. Skeletal muscles possess a highly plasticity in response to altered activity. Mechanical and metabolic demands elicit marked modifications of gene expression that could lead to gain (hypertrophy) or loss (atrophy) of muscle mass. Indeed, strength training induces marked hypertrophy of exercising muscles. Histochemical analyses clearly show a 10-30% increase in muscle fiber cross-sectional area after 10-12 weeks of resistance training in sedentary subjects [1].

Satellite cells are myogenic stem cells, accounting for 3-9% of the subliminal nuclei associated with adult normal muscle fiber, with the variation widely depending on animal species, age,

and muscle fiber type [2]. Satellite cells, existing between the basal lamina and the sarcolemma of the fiber, are normally found in a mitotically quiescent in adult muscles. When muscle is injured or mechanically stretched, satellite cells activate to enter the cell cycle. Activated satellite cells have been shown to migrate to the damaged site where they replicate DNA, divide, differentiate, and fuse with the adjacent muscle fiber or form new fibers [3].

It has been reported that satellite cells are activated in compensatory hypertrophy [3, 4], and addition of new nuclei to the growing fiber seems to be required for extreme hypertrophy. Since the myonuclear domain is constant in hypertrophied muscle after mechanical overloading, many satellite cells must be incorporated adjacent to muscle fibers. In fact, irradiation of satellite cells followed by a loading stimulus results in an attenuated increase in skeletal muscle mass and protein content [5]. Therefore, it is necessary for consecutive processes (the activation, proliferation, and differentiation of satellite cells) to elicit muscle hypertrophy in the case of mechanical overloading as well as normal growth. However, several researchers recently suggested satellite cell-independent muscle hypertrophy during mechanical overloading. In addition, some have debated whether the contribution of satellite cells to fiber hypertrophy in adult muscle is minor [6, 7].

In hypertrophied muscle, increasing protein synthesis and decreasing protein degradation are also important events. Phosphatidylinositol-3-kinase (PI3-K)/Akt/ mammalian target of rapamycin (mTOR) signaling has been shown to be crucial to protein synthesis [8, 9]. Mechanical stretching *in vivo* and *in vitro* activates serum response factor (SRF)-dependent signaling in skeletal muscle similar to smooth and cardiac muscles [10, 11]. In contrast, several possible mediators for muscle atrophy have been described. Many negative regulators are proposed to induce muscle atrophy by inhibiting protein synthesis and enhancing protein degradation in skeletal muscle. For example, the ubiquitin proteasome system (UPS) is thought to be a major contributor to many structural proteins [12]. The autophagy-lysosome system has been largely ignored despite the evidence that lysosomal degradation contributes to protein breakdown in atrophying muscles [13]. Recent studies demonstrated that autophagy is an important pathway for appropriate protein degradation in several neuromuscular disorders [14]. The group of Sandri et al. [15, 16] has shown that the autophagy-lysosome and UPS are coordinately regulated during muscle wasting. Furthermore, specific expression of mutant SOD (superoxide dismutase)1^{G93A} in skeletal muscle caused muscle atrophy and weakness mainly via autophagy activation [17]. In this chapter, we summarize possible candidates for proteins that regulate muscle hypertrophy and atrophy. In addition, we describe the possible modulators for switching, proliferation, and differentiation of satellite cells, a possible contributor to muscle hypertrophy.

2. Positive regulators of skeletal muscle mass

2.1. PI3-K/Akt/mTOR pathway

The serine/threonine kinase Akt regulates the translational level to be involved to a central pathway of hypertrophy. In muscle, Akt is activated by the upstream PI3-K, induced either by

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receptor binding or by integrin-mediated activation of focal adhesion kinase (FAK). PI3-K activates Akt, which then has the ability to phosphorylate and change the activity of many signaling molecules. Possible downstream regulators of Akt, mTOR, and glycogen synthase 3-β (GSK-3β) play a crucial role in the regulation of translation. Akt activates mTOR via phosphorylation and inactivation of tuberous sclerosis complex (TSC)-2. Subsequently, mTOR phosphorylates and activates the 70kDa ribosomal protein S6 kinase (p70S6K), which results in increased translation either directly or indirectly by activating initiation and elongations, elongation initiation factor (eIF)-2, eIF-4E [through eukaryotic initiation factor 4E binding protein (4E-BP)], and eEF-2. In addition, Akt also phosphorylates and inactivates GSK-3β, thereby activating translation via the initiation factor eIF-2B [18]. Other functions of Akt include the negative regulation of protein degradation by inhibiting forkhead box O (FOXO)-mediated proteasome activity.

2.1.1. Akt

Disruption of the Akt1 gene causes growth retardation and apoptosis [19], whereas deletion of Akt2 causes defects in glucose metabolism but not altered growth [20]. The striking effect of Akt1 on muscle size was demonstrated by the transient transfection of a constitutively active inducible Akt1 transgene in skeletal muscle *in vivo* [15, 21]. Downstream mediators (p70S6K, S6) of protein synthesis were activated, but satellite cells were not incorporated [21]. Akt1 transgenic muscles showed increased strength, showing that a functional hypertrophy was elicited [21]. Moreover, muscle mass was completely preserved in denervated transgenic Akt mice [22]. The effects of Akt on muscle mass regulation can be mediated by several different downstream effectors, including GSK-3β, mTOR, and FOXO.

2.1.2. mTOR and mTOR signaling complex (mTORC) 1

mTOR exists in two functionally distinct multi-protein signaling complexes, mTORC1 and mTORC2. In general, only signaling by mTORC1 is inhibited by rapamycin, and thus the growth regulatory effects of rapamycin are primarily exerted through the mTORC1 complex [23]. mTORC1 regulates several anabolic processes including protein synthesis, ribosome biogenesis, and mitochondrial biogenesis, as well as catabolic processes such as autophagy [23]. Two of the most studied mTORC1 targets are the 4E-BP1 and p70S6K, which both play important roles in the initiation of mRNA translation.

mTORC1 is activated in response to hypertrophic stimuli such as increased mechanical loading, feeding, and growth factors [24, 25]. In fact, hypertrophy induced by mechanical loading, insulin-like growth factor (IGF)-I, and clenbuterol is significantly, if not completely, blocked by rapamycin [25]. In addition, overexpression of constitutively active Akt activates mTORC1 signaling and induces hypertrophy through a rapamycin-sensitive mechanism [26]. These findings support the hypothesis that mTORC1 is sufficient to induce hypertrophy, however, the hypertrophic stimuli employed in these studies also induce signaling through PI3-K and Akt. Signaling through PI3-K/Akt can regulate mTOR-independent growth regulatory molecules [GSK-3β, tuberin (TSC-2), and FOXO]. However, it was not clear if signaling by mTORC1 was sufficient, or simply permissive, for the induction of hypertrophy.

To address this issue, overexpression of Ras homolog enriched in the brain (Rheb) was recently used as a means to induce a PI3-K/Akt-independent activation of mTORC1 [27]. Marked increases in protein synthesis and hypertrophy have been recognized in several muscles of Rheb abundant mice [27]. Stretch-induced activation of mTOR signaling was not abolished in the skeletal muscle of Akt1-/- mice [28]. Therefore, mechanically induced signaling through mTOR is not dependent on Akt. Furthermore, Akt-independent stimulation of mTOR may be regulated by phosphorylation of TSC-2. For instance, TSC-2 is inhibited by FAK in 293T cells [29] indicating that up-regulated FAK with increased loading could stimulate protein synthesis via TSC-2 inhibition. All these regulatory influences may explain the rise in the level of phosphorylated p70S6K. These results suggested that the activation of mTORC1 is indeed sufficient to induce hypertrophy, at least in part by increasing protein synthesis.

Although one of the most well-characterized upstream triggers of mTOR signaling in skeletal muscle is IGF-I, mechanical loading has been shown to activate mTOR by an IGF-I independent pathway involving PLD via its metabolite phosphatidic acid. More recently, Hornberger et al. [30] extended these initial findings by showing mTOR activation following eccentric contractions via PLD synthesis but not PI3-K-Akt activity. It has been shown that PLD1, but not PLD2, was a downstream effector of Rheb's activation of mTOR. In contrast, Vps34 is a class III PI3K previously shown to mediate amino acid activation of p70S6K by mTOR. In skeletal muscle, MacKenzie et al. [31] reported that high-resistance contractions increased Vps34 activity possibly in response to increased intramuscular leucine levels. In addition to Vps34, two groups reported the exciting discovery that the Rag family of GTPases was necessary and sufficient for amino acid activation of the mTOR pathway [32]. Therefore, mTOR is currently thought to be the major hub for the integration of an array of upstream signaling pathways which, when activated, ultimately result in increased translational efficiency [8]. Figure 1 summarizes the anabolic pathway (PI3-K/Akt/mTOR and SRF-dependent) regulating skeletal muscle mass.

2.2. Serum Response Factor (SRF)

SRF is an ubiquitously expressed member of the MADS (MCM1, Agamous, Deficiens, SRF) box transcription factor family, which binds the core sequence of SRF/CArG boxes [CC (A/T)6 GG] as homodimers. Functional CArG boxes have been found in several promoter regions of muscle-specific genes such as the skeletal α-actin and myosin light chain 1/3 genes. SRF-dependent signaling plays a major role in a variety of physiological processes, including cell growth, migration, and cytoskeletal organization [33]. Previous results obtained with specific SRF knock-out models by the Cre-LoxP system emphasize a crucial role for SRF in postnatal skeletal muscle growth and regeneration by modulating interleukin (IL)-4 and IGF-I mRNA expression [34]. More recently, Mokalled et al. [35] demonstrated that members of the myocardin family of transcriptional coactivators, MASTR and myocardin-related transcription factor (MRTF)-A, are up-regulated in satellite cells during muscle regeneration. In addition, skeletal muscle regeneration exhibited the impairment in mouse possessing double-knockout satellite cells (MASTR and MRTF-A). As proposed by Mokalled et al. [35], the promoting role on muscle regeneration seems to be attributable to both MASTR/myocyte enhancer factor 2 and/or MRTF-A/SRF complexes.

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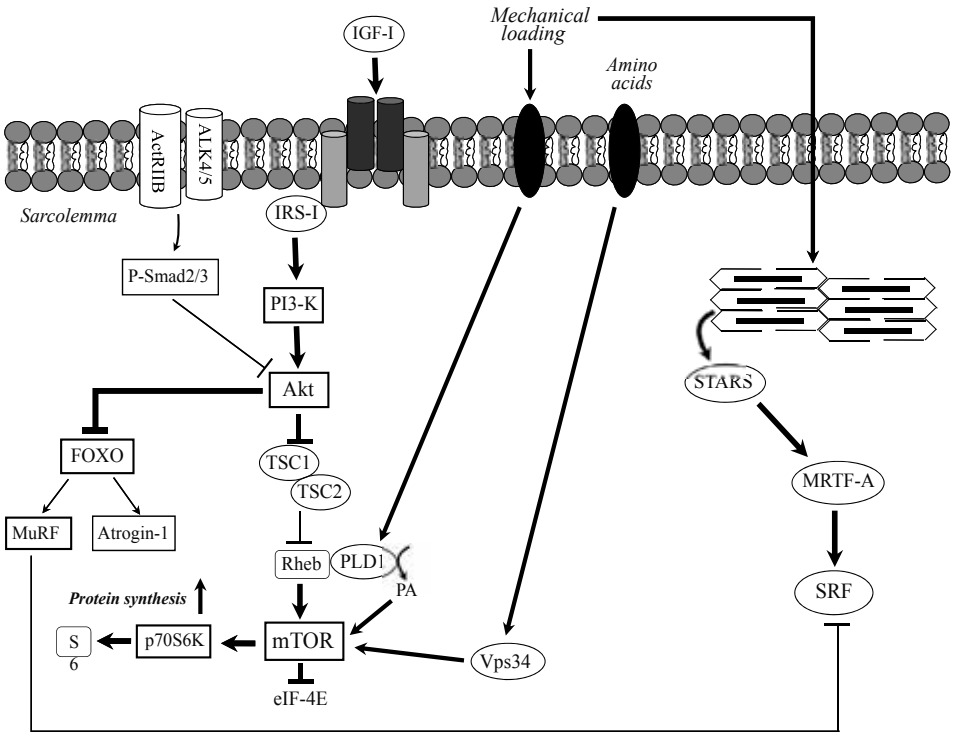

Figure 1. Anabolic pathway regulating skeletal muscle mass. The major anabolic pathway regulating protein synthesis in skeletal muscle is mTOR/TORC1 signaling. Upstream triggers (IGF-I, mechanical loading, amino acids) activate mTOR signaling through a number of different intermediary proteins such as Rheb, phospholipase D1 and its metabolite PA, and Vps34. Although myostatin signals through the ActRIIB-ALK4/5 heterodimer activate Smad2/3, reduced stimulation of myostatin in the presence of IGF-I and mechanical loading cannot block the functional role of Akt. Myosin-actin interaction by mechanical loading activates STARS /MRTF-A/SRF signaling. The accumulation of MuRF in muscle tissue during inactivity (hindlimb suspension, immobilization, etc.) is known to inhibit SRF-dependent transcription of muscle-specific genes. However, the functional role of SRF is not abrogated under such conditions, which lower MuRF expression because of marked inhibition of FOXO by abundant Akt. ActRIIB: activin receptor IIB; ALK4/5: activin-like kinase 4/5; eIF: eukaryotic initiation factor; FOXO: Forkhead box O; IGF-I: insulin-like growth factor-I; IRS-1: insulin receptor substrate-1; MRTF-A: myocardin-related transcription factor-A; mTOR: mammalian target of rapamycin; MuRF: muscle ring-finger protein; PA: phosphatidic acid; PI3-K: phosphatidylinositol 3-kinase; p70S6K: 70 kDa ribosomal protein S6 kinase; Rheb: Ras homolog enriched in brain; SRF: serum response factor; STARS: striated muscle activators of Rho signaling; TORC1: a component of TOR signaling complex 1; TSC: tuberous sclerosis complex. Data from Sakuma et al. [130]

It is proposed that the transcriptional activity of SRF is regulated by muscle ring finger (MuRF)-2 [36] and striated muscle activators of Rho signaling (STARS) [37]. At the M-band, the mechanically modulated kinase domain of titin interacts with a complex of the protein products of the atrogenes NBR1, p62/sequestosome 1 (SQSTM1), and MuRFs [36]. This complex dissociates under mechanical arrest, and MuRF-1 and MuRF-2 translocate to the cytoplasm and the nucleus [36]. One of the probable nuclear targets of MuRFs is SRF [36], suggesting that the MuRF-induced nuclear export and transcriptional repression of SRF may contribute to amplifying the transcriptional atrophy program. Thus, it is possible that MuRF-2

abrogates the synergistic transactivation of SRF and SRF-linked molecules *in vivo*. On the other hand, SRF activity is sensitive to the state of actin polymerization. G-actin monomers inhibit SRF activity, whereas polymerization of actin occurs in response to serum stimulation and RhoA signaling. Thus, signal inputs lower the ratio of globular actin to fibrillar actin liberating the binding of MRTF-A to globular actin resulting in the nuclear accumulation of MRTF-A and subsequent SRF-dependent gene expression [38]. It has been well established that overexpression of STARS contributes to the nuclear translocation of MRTF-A and MRTF-B [37, 39], and these factors activate SRF transcription.

2.2.1. The functional role of SRF during muscle hypertrophy

In adults, SRF activity could be important for the control of skeletal muscle mass. In fact, SRF also enhances the hypertrophic process in muscle fibers after mechanical overloading [40-42]. For example, Flück et al. [40] utilized a stretch-induced hypertrophic model, in which a weight equal to 10% of body weight was attached to the left wing of a rooster to induce enlargement of the anterior latissimus dorsi muscle. Gordon et al. [10] also indicated a significant increase in SRF protein in the soleus and plantaris muscles after 8 days of functional overload caused by surgical ablation of the gastrocnemius muscle in rats. In humans, 8 weeks of resistance training (leg presses, squats, and leg extensions) induced increases in SRF mRNA (3-fold) and nuclear protein (1.25-fold) in human muscle [41]. The same training also increases in the mRNA levels of several SRF-targeted molecules {alpha-actin, myosin heavy chain (MHC) IIa, and IGF-I [34]}. They proposed the induction of these molecules by SRF in human hypertrophied muscle, though they did not provide any direct evidence such as transcriptional activation by increased binding of SRF to the promoter region of alpha-actin, MHC IIa, and IGF-I. Although SRF would regulate proliferation and differentiation using different pathways, it would mainly activate the differentiation of satellite cells during muscle hypertrophy. Indeed, it was shown that, in mechanically overloaded muscles of rats, the SRF protein co-localized with MyoD and myogenin in myoblast-like cells during the active differentiation phase [42]. In this study, abundant SRF protein at 2 days was failed to be detected after mechanical overloading, when many proliferating satellite cells and/or myoblasts are expected to exist. In addition, the location of the SRF protein did not correspond with that of BrdU-positive satellite cells or ED-1 positive macrophages in the hypertrophied plantaris muscle [42].

As indicated earlier, by reducing the cytoplasmic concentration of monomeric G-actin, STARS promotes the nuclear translocation of SRF transcriptional co-activator-A and -B (MRTF-A and MRTF-B), resulting in an increase in SRF-mediated gene transcription [37]. A real-time PCR analysis conducted by Lamon et al. [41] demonstrated that increased mechanical loading from resistance training in humans caused significant increases in the upstream modulators of SRF (STARS mRNA; 3.4-fold, MRTF-A mRNA; 2.5-fold, MRTF-B mRNA; 3.6-fold, and RhoA protein; 2-fold). SRF seems to regulate the transcriptional facilitation of the alpha-actin promoter by the androgen receptor (AR) during muscle hypertrophy. Using male adult Sprague-Dawley rats, Lee et al. [43] showed an increase in AR protein of 106% and 279% after 7 and 21 days in mechanically overloaded plantaris muscles by surgical ablation of two synergistic muscles. Co-overexpression of either SRF or active RhoA with AR indicated a

synergistic 36- and 28-fold increase in the skeletal alpha-actin promoter activity. In contrast, cotransfection of AR, SRF, and active RhoA induced a 180-fold increase in skeletal a-actin promoter activity. Therefore, it is possible that intimate linkages among these three modulators induce alpha-actin expression in hypertrophied muscle *in vivo*. Intriguingly, experiments using C2C12 cells indicated that this AR coactivation for the alpha-actin promoter requires a co-expressed full-length SRF and SRF-binding site but not AR's direct binding to GRE sites [44].

More recently, Guerci et al. [45] investigated the functional role of SRF in adult mammalian muscle using $SRF^{flox/flox}$: HAS-Cre-ER^{T2} mice injected with tamoxifen. During the compensatory hypertrophy phase, growth was completely slow in the SRF-deleted plantaris muscle, demonstrating that SRF is necessary for overloaded-induced myofiber hypertrophy. Intriguingly, Guerci et al. [45] showed that the lack of SRF in myofibers affected satellite cell proliferation and fusion to the growing fibers. In their genetic mouse model, Cre recombinase is expressed only in myofibers but not in satellite cells. Furthermore, Guerci et al. [45] identified the secreted molecules mediating these paracrine effects and whose expression is under the control of SRF by using a global transcriptomic approach allowing the identification of genes activated by SRF. In SRF-deleted muscles, the overexpression of IL-6 is sufficient to restore satellite cell proliferation, but not satellite cell fusion and overall growth. Cox2/IL-4 overexpression rescues satellite cell recruitment and muscle growth without affecting satellite cell proliferation, identifying altered fusion as the limiting cellular events precluding the hypertrophic growth of SRF-deleted muscles. Guerci's excellent finding was further supported by Bruusgaard et al. [46], showing that the addition of nuclei precedes increased fiber size during compensatory hypertrophy and that this constituted the major cause of hypertrophy. However, the contribution of satellite cells to muscle hypertrophy has been a controversial issue [6, 47]. In fact, McCarthy et al. [48] suggested that satellite cell-depleted skeletal muscle undergoes extensive fiber hypertrophy after mechanical overloading. Therefore, further examination of SRF's role in muscle hypertrophy is needed.

2.2.2. Defects of SRF signaling with muscle wasting

Sarcopenia

Aging is associated with progressive declines in muscle mass, quality, and strength, a condition known as sarcopenia. Lean muscle mass generally contributes to ~50% of total body weight in young adults, but this value declines with aging, to just 25% at 75-80 years of age [49]. At the muscle fiber level, sarcopenia is characterized with specific type II fiber atrophy and fiber loss. Although the specific contribution of each is unknown, several possible signaling factors (Akt-mTOR, RhoA-SRF, and autophagy) have been proposed to contribute to age-related muscle atrophy [50, 51]. In fact, using crude and fractionated homogenates, our recent study has clearly demonstrated a blunted expression of SRF protein in the quadriceps and triceps brachii in aged mice [52]. Immunofluorescence microscopy also indicated the selective loss of SRF protein in the cell cytosol but not in satellite cells in sarcopenic mice. In addition, our data showed a decrease in MRTF-A mRNA (50-70%) and protein (76%) levels in only the nuclear fraction with age. Furthermore, we observed 40-60% decreases in the amount of STARS mRNA in the quadriceps and triceps brachii of 24-month-old mice [52].

A decrease of SRF expression achieved using a transgenic approach was found to accelerate the atrophic process in muscle fibers with age [53]. These SRF-deleted mice showed marked deposition of intermuscle lipid with aging. One morphologic aspect of sarcopenia is the infiltration of muscle tissue components by lipids, because of the increased frequency of adipocyte or lipid deposition [54] within muscle fibers. As with precursor cells in bone marrow, liver, and kidney, muscle satellite cells expressing the adipocytic phenotype increased with age [55], although this process is still relatively poorly understood in terms of its extent and spatial distribution. Lipid deposition may result from a net buildup of lipids due to the reduced oxidative capacity of muscle fibers with aging [54]. These lines of evidence clearly showed a defect of SRF-signaling in aged mammalian muscle.

2.3. Muscular disorder

SRF appears to be linked to the degenerative process during muscular dystrophy. Significant reduction in the amount of SRF has been observed [56], 40-50% and 50-65% at 2 and 12 weeks of age, respectively, in merosin-deficient congenital muscular dystrophy. Our immunohistochemical analysis demonstrated that mature normal mice exhibited an abundant SRF protein in the cytoplasm of several muscle fibers, while the *dy* mice did not. There is no direct evidence of a link between SRF disorders and the pathogenesis of disease in the skeletal muscle.

However, Lange et al. [36] observed that a mutation in the TK domain of titin, a possible modulator of SRF, disrupted Nbr1 binding and led to hereditary myopathy with early respiratory failure (HMERF). HMERF patient muscle biopsies revealed a Nbr1 diffusible localization, cytoplasmic p62/SQSTM1 aggregates, and the selective accumulation of MuRF-2 in centralized nuclei. Unfortunately, the localization of SRF has not been determined in the muscle of HMERF patients. In contrast, a natural dominant-negative form of SRF was demonstrated to be elevated in human heart failure [57]. The dominant negative SRF isoform potently inhibited SRF-dependent function, showing the biochemical phenotype seen in SRF-null mice [57]. In addition, a subsequent human heart failure study showed decreases in full-length SRF and elevated expression of a caspase-3-cleaved product of SRF [58]. A more recent review [59] proposed various disorders to be linked with the SRF mutation as shown by many reliable studies using cell-specific SRF-knockout phenotypes.

3. Negative regulators of skeletal muscle mass

3.1. Ubiquitin-Proteasome System (UPS)

The UPS is essential for protein degradation. The degradation of a protein via the UPS includes two steps: (1) tagging of the substrate by covalent attachment of multiple ubiquitin molecules and (2) degradation of the tagged protein by the 26S proteasome. The ubiquitination of proteins is regulated by at least three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). Consistent increases in two important E3 ubiquitin ligases (atrogin-1 and MuRF-1) gene expression have been observed in a wide range of in vivo

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models of skeletal muscle atrophy including diabetes, cancer, denervation, unweighting, or glucocorticoid treatment [60].

eIF 3 subunit 5 (eIF3-f) appears to be a key effector of atrogin-1 as targeted increases and decreases in eIF3-f levels cause skeletal muscle hypertrophy and atrophy, respectively [61]. Overexpression of atrogin-1 results in the polyubiquitination of MyoD and an inhibition of MyoD-induced myotube formation [62]. In contrast, the knockdown of atrogin-1 reversed endogenous MyoD proteolysis and the overexpression of a mutant MyoD, unable to be ubiquitinated, prevented muscle atrophy *in vivo* [62]. These results confirmed MyoD as a substrate of atrogin-1 during dexamethasone-induced myotube atrophy [63].

In contrast to atrogin-1, it appears that MuRF-1 mainly interacts with structural proteins. MuRF-1 binds to titin and potentially affects titin signaling. It also binds to and degrades MHC proteins following the treatment of skeletal muscle with dexamethasone. Additionally, MuRF-1 degrades myosin light chain 1 and 2 during denervation and fasting conditions [64]. These studies suggest that while numerous stimuli can activate both two atrogenes, the downstream pathways affected may be separate for each protein.

3.1.1. Adapatative changes in atrogin-1 and MuRF-1 in muscle atrophy models

UPS signaling in cachectic muscle

In many acute models of cachexia, including cancer, the UPS is thought to be fundamental in the process of muscle atrophy [60, 65]. Weight-losing mice bearing MAC16 adenocarcinoma exhibited the increased expression of mRNA for both α- and β-proteasome subunits in gastrocnemius muscle [66]. Several experimental models of cancer cachexia [e.g., AH-130, C26, Lewis lung carcinoma (LLC)] had increased UPS activity, as well as overexpression of atrogin-1 and MuRF-1 [67]. However, investigations in humans have failed to be conclusive. An earlier study suggested that the UPS plays a prominent role in the degradation of myofibrillar proteins, particularly in cancer patients with weight loss of > 10% [68]. In gastric cancer patients with average weight loss of 5.2%, increased UPS activity (determined by measurements of RNA and cleavage of specific fluorogenic substrates) was seen compared with that in controls, which was exacerbated by increasing tumor stage and weight loss [69]. In contrast, investigations of UPS activity in quadriceps muscle biopsies have shown levels similar to those in healthy controls in patients with lung cancer and weight loss < 10% (termed pre-cachexia) [70]. In a transcriptomic study of UGI patients, candidate genes, including FOXO and ubiquitin E3 ligases, were not related to weight loss. Another study in lung cancer patients with low weight loss demonstrated no change in the components of the UPS using Northern blotting, but there was a suggestion that the activity of the lysosomal pathway was increased [71]. Similarly, a more recent study also failed to show an increase of proteasome activity in muscle of esophageal cancer patients [72]. Although more descriptive studies are needed to determine whether UPS is activated in muscle in cancer cachexia, the adaptive manner of UPS components may differ between rodents and humans in cancer-cachectic muscles.

In chronic obstructive pulmonary disease (COPD) patients, the reported findings on the adaptive changes in the UPS in skeletal muscle are highly contradictory. Doucet et al. [73]

found significant increases in atrogin-1, MuRF-1, and FOXO1 mRNA in the muscle of COPD patients showing irreversible airflow obstruction [post-bronchodilator forced expiratory volume in one second (FEV_1) < 80% predicted and FEV_1/forced vital capacity (FVC) < 70%] by pulmonary function testing. They also observed an increase, albeit not significant, in the atrogin-1 protein content in the muscle of COPD patients. Such inconsistency in the protein levels of ubiquitin ligases was recognized in COPD patients by Natanek et al. [74]. In contrast, Natanek et al. [74] reported a significant decrease in atrogin-1 protein content in quadriceps in COPD compared with those in controls. Advanced cardiac heart failure (CHF) patients also did not exhibit high mRNA and protein levels of atrogin-1 in muscle [75], although CHF model mice with cardiac-specific overexpression of calsequestrin exhibited marked increases in atrogin-1 mRNA in both soleus and white vastus lateralis muscles [76]. In human studies, several investigators described a discrepancy between the mRNA expression level on atrogene and the extent of muscle atrophy [77]. Intriguingly, some recent studies have shown that atrogin-1 and MuRF-1 are not essential components for proteasome activity [78]. There have been few studies dealing with the protein levels of atrogin-1 and MuRF-1 in muscle wasting, particularly in human subjects.

UPS signaling in sarcopenic muscle

Atrogin-1 and/or MuRF-1 mRNA levels in aged muscle are reportedly increased or unchanged in humans and rats, or decreased in rats [50, 79, 80]. Even when the mRNA expression of these atrogenes increased in sarcopenic muscles, this was very limited (1.5- to 2.5-fold). Although mRNA levels of both ubiquitin ligases have been determined in several aged mammalian muscles, the analysis of protein levels did not correspond to age-related increases in the mRNA of ubiquitin ligases. For instance, the marked upregulation of phosphorylated Akt and FOXO4 have been observed in the gastrocnemius muscle of aged female rats [80]. These adaptations of protein levels probably contribute to the downregulation of atrogin-1 and MuRF-1 mRNA in aged muscle. In addition, Léger et al. [81], using human subjects aged 70 years old, demonstrated decreases in nuclear FOXO1 and FOXO3a in spite of no apparent age-related changes in the atrogin-1 and MuRF-1 mRNA. Interestingly, recent findings indicate that atrogin-1-knockout mice are short-lived and experience higher loss of muscle mass during aging than control mice [82], indicating that the activity of this E3 ubiquitin ligase is required to preserve muscle mass during aging in mice. Moreover, the muscle size of aged MuRF-1-null mice is preserved [83]. However, they exhibited a higher decay of muscle strength than controls. As indicated by Sandri et al. [82], chronic inhibition of these atrogenes should not be considered a therapeutic target to counteract sarcopenia because this does not prevent muscle loss but instead exacerbates weakness. Figure 2 summarizes a possible adaptation of atrogin-1 and MuRF-1 in sarcopenic muscle.

The adaptation of UPS in muscular dystrophy

Gene expression profiling in Limb-girdle muscular dystrophy (LGMD)2A showed overexpression of UPS-related genes [84, 85]. While the expression of atrogin-1 and MuRF-1 was not increased in mouse models of LGMD2A, FOXO1 was strongly upregulated and induced muscle atrophy in calpain-3-deficient mice [86]. More recently, LGMD2A patients have been shown to exhibit significantly higher expression of MuRF-1 protein but not atrogin-1 protein

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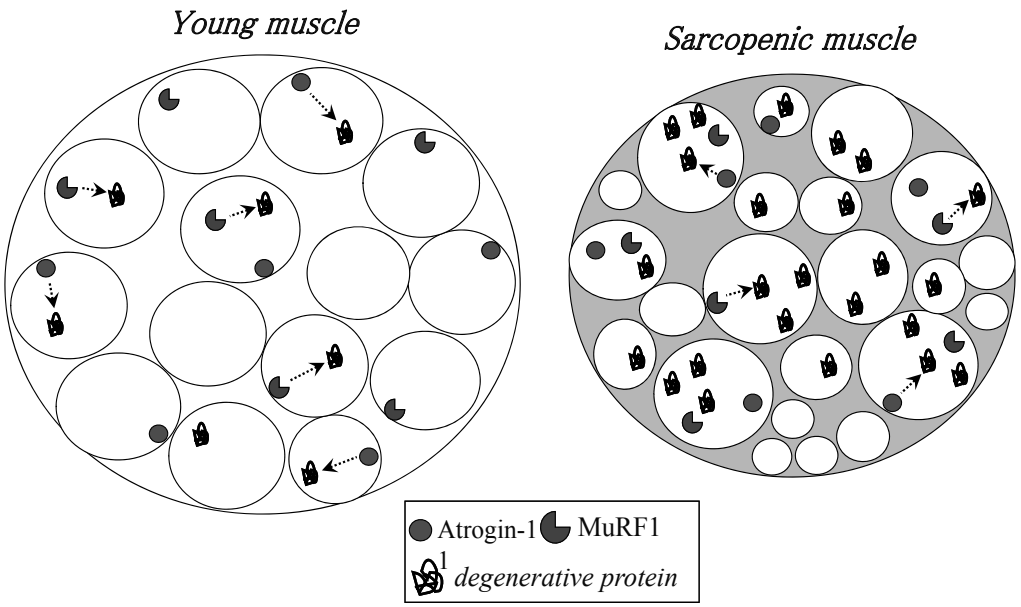

Figure 2. The comparison of ubiquitin-proteasome system between young and sarcopenic muscle. In contrast to young muscle, sarcopenic muscle exhibits no activation of atrogin-1 and MuRF-1-dependent signaling to destroy the degenerative proteins.

in skeletal muscle. LGMD2B is due to deficiency of the protein dysferlin. The loss of dysferlin causes failure in resealing of the membrane lesions generated during eccentric muscle contractions [88]. Similar to LGMD2A, dysferlinopathy patients exhibited more abundant mRNA and protein of MuRF-1 but not atrogin-1 [89]. Activation of UPS in dysferlinopathy has also been reported in cellular models (patient-derived muscle cells) [90]. UCMD is a common form of muscular dystrophy associated with defects in collagen VI, characterized by loss of muscle fibers and proliferation of connective and adipose tissues. More recently, Paco et al. [91] studied muscle biopsies of Ullrich congenital muscular dystrophy (UCMD) (n = 6), other myopathy [Duchenne muscular dystrophy (DMD)], calpain-3-deficient, Kearns-Sayre, and nemaline myopathy (n = 12), and control patients (n = 10) and found reduced expression of atrogin-1 and MuRF-1 mRNAs in UCMD cases.

Pharmacological inhibition of UPS appears to exert some beneficial effect on muscular dystrophy. Velcade, once injected locally into the gastrocnemius muscles of *mdx* mice, appears to increase the expression and membrane localization of dystrophin and members of the dystrophin-associated protein complex (DAPC) [92]. Treatment with Velcade (0.8 mg/Kg) over a 2-week period has been shown to reduce muscle degeneration and necrotic features, and to increase muscle size (gastrocnemius and diaphragm), in mdx muscle fibers [93]. In addition, Gazzerro et al. [93] observed that Velcade administration generates many myotubes and/or immature myofibers expressing embryonic myosin heavy chain in mdx muscle, probably due to upregulation of myogenic differentiating modulators.

3.2. Autophagy-dependent signaling

Macroautophagy (herein autophagy) is a catabolic process that involves the bulk degradation of cytoplasmic components by interacting a lysosome [94, 95]. This process is characterized by the engulfment of part of the cytoplasm inside double-membrane vesicles (autophagosomes). Autophagosomes subsequently fuse with lysosomes to form autophagolysosomes in which the cytoplasmic cargo is degraded. The turnover of most proteins, biological membranes, and whole organelles such as mitochondria and ribosomes is mediated by autophagy [96].

Autophagy represents an extremely refined collector of altered organelles, abnormal protein aggregates, and pathogens, similar to a selective recycling center [97]. The selectivity of the autophagy process is conferred by a growing number of specific cargo receptors such as p62/SQSTM1 and Nix (Bnip3L) [98]. These adaptor proteins are equipped with both a cargo-binding domain, with the capability to recognize and attach directly to molecular tags on organelles. At the same time, these adaptor proteins bind a microtubule-associated protein light chain LC3)-interacting region domain to recruit and bind essential autophagosome membrane proteins. Three molecular complexes mainly regulate the formation of autophagosomes: the LC3 conjugation system and the regulatory complexes governed by unc51-like kinase-1 and beclin-1. The conjugation complex is composed of different proteins encoded by autophagy-related genes (Atg). The Atg12-Atg5-Atg16L1 complex, along with Atg7, plays an essential role in the conjugation of LC3 to phosphatidylethanolamine, which is required for the elongation and closure of the isolation membrane.

The UPS and the lysosomal-autophagy system in skeletal muscle are interconnected [15, 16]. Both these studies identified FOXO3 as a regulator of these two pathways in muscle wasting [Fig. 3]. FOXO3 is a transcriptional regulator of the atrogin-1 and MuRF-1. FOXO3 modulates the expression of autophagy-related genes in mammalian skeletal muscle and C2C12 myotubes [16]. Masiero et al. [99] found an intriguing characteristic using muscle-specific autophagy knockout mice, which exhibit fiber atrophy, weakness, and mitochondrial abnormalities. Autophagy-dependent protein degradation seems to be also modulated by tumor necrosis factor (TNF) receptor associated factor 6 and peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) [100, 101]. Wenz et al. [101] recognized no significant age-related increase in the ratio of LC3-II to LC3-I in MCK-PGC1α mice. Therefore, PGC1α would attenuate the autophagic process probably through increased anti-oxidant defense and mitochondrial biogenesis.

3.2.1. Adaptation of autophagy-linked signaling during muscle atrophy

A possible contribution of autophagic signaling to cachexia

As for cancer cachexia, earlier results obtained on muscles isolated from cachectic animals led us to rule out a substantial role for lysosomes in overall protein degradation [102]. In contrast, an elevation of total lysosome protease activity has been observed in the skeletal muscle and liver of tumor-bearing rats [103]. In addition, increased levels of cathepsin L mRNA have been reported in the skeletal muscle of septic or tumor-bearing rats, whereas cathepsin B gene expression has been shown to be enhanced in muscle biopsy samples obtained from patients

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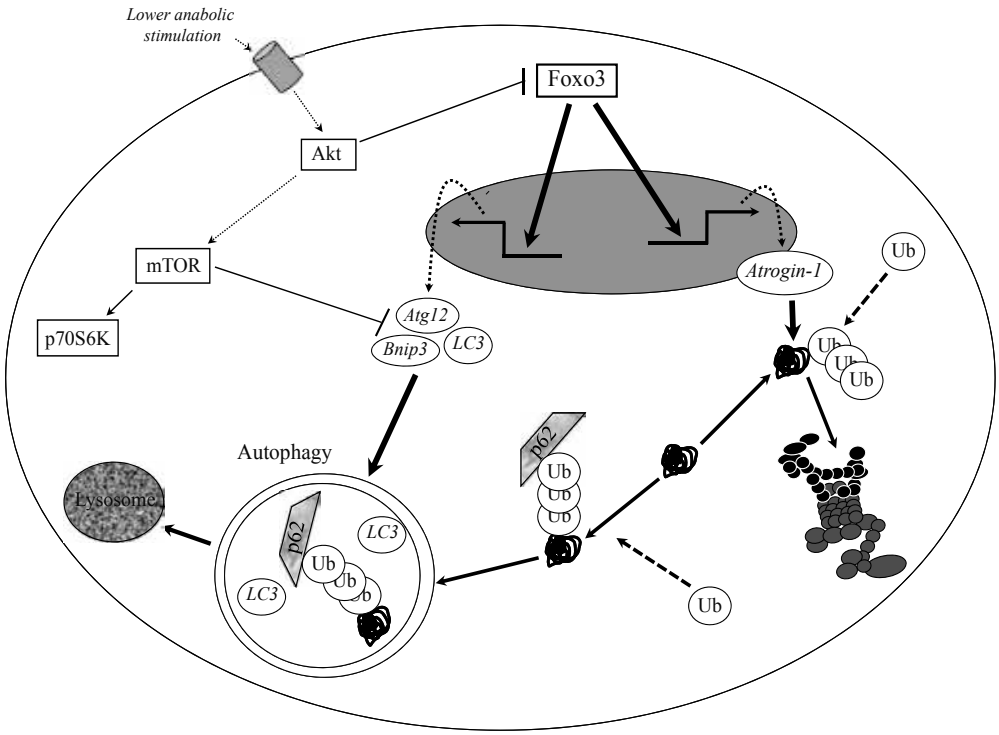

Figure 3. Contribution of the proteolytic pathways to muscle atrophy during catabolic conditions. In catabolic conditions such as denervation, cancer, and fasting, an atrophy program is induced to degrade muscle proteins and organelles. Proteins can have a double fate, being recognized and removed by the proteasome or docked to the autophagosome. In the latter case the chains of polyubiquitins are interacting with the p62. These proteins have also a domain for the interaction with LC3 therefore bringing the ubiquitinated proteins to the growing autophagosome. Less anabolic stimulation (IGF-I, mechanical loading, amino acids, etc.) reduces the amount of activated Akt, not promoting protein synthesis by activating the mTOR/p70S6K pathway. Lower Akt activity also does not block the nuclear translocation of FOXO3 to enhance the expression of autophagy-related genes (Bnip3, LC3, Atg12) and Atrogin-1 and the consequent protein degradation. FOXO: forkhead box O; IGF-I: insulin-like growth factor-I; LC3: microtubule-associated protein light chain; mTOR: mammalian target of rapamycin; p70S6K: 70 kDa ribosomal protein S6 kinase; Ub: ubiquitin. Data from Sakuma et al. [130]

with lung cancer [71, 104]. Furthermore, a few general observations suggested that autophagy can be activated in the muscle of animals bearing LLC or colon 26 (C26) tumor [105, 106]. More recently, Penna et al. [107] investigated whether autophagy signaling was elevated in muscle using three different models of cancer cachexia. They observed marked increases in the levels of beclin-1, p62/SQSTM1, and LC3B-II (the lipidated form; a reliable marker of autophagosome formation) in muscle in C26-bearing mice. In addition, Penna et al. [107] evaluated autophagic markers in the gastrocnemius muscle of rats bearing Yoshida AH-130 hepatoma or of mice transplanted with LLC. Several autophagic markers were upregulated in the muscle of these two cancer cachexia rodent models, although there was some difference in the adaptive manner. Furthermore, OP den Kamp et al. [70] indicated that the levels of both LC3B-I and -II proteins but not LC3B mRNA were significantly increased in the vastus lateralis muscle of

patients with lung cancer cachexia. Esophageal cancer patients also appear to exhibit higher LC3B-II/I ratios and levels of cathepsin B and L expression in muscle [72]. Since they did not detect a significant change of proteasome, calpain, or caspase 3 activity in the muscle of these patients, they considered that the autophagic-lysosomal pathway is the main proteolytic system in the muscle in esophageal cancer cachexia

The functional importance of autophagy in the pathogenesis of lung disease in COPD patients has recently been demonstrated by Chen et al. [108] who described significant increases of autophagy in clinical lung samples taken from COPD patients. LC3B, beclin-1, Atg7, and Atg5 were all upregulated, and autophagosome formation was visualized using electron microscopy. In addition, Ryter et al. [109] have also described increased autophagy in clinical specimens of the lung from patients with COPD. They showed the increased expression and activation of autophagic regulator proteins (i.e., LC3B, beclin-1, Atg5, Atg7) in lung. Similar evidence of increased autophagy was observed in mice subjected to chronic inhalation of cigarette smoke [108] and in lung epithelial cells exposed to aqueous cigarette smoke extracts [110]. Taking these findings together, autophagy seems to be activated in the lungs as a stress response. To date, little research has been completed on the contribution of the autophagy system to protein degradation and loss of skeletal muscle mass in COPD patients. Using muscle biopsy samples obtained from severe COPD patients with marked atrophy [forced expiratory volume in 1 s (FEV1) value of 35±2% of predicted], Plant et al. [111] demonstrated that there was no difference in the levels of beclin-1 and LC3 transcripts in the quadriceps muscle of patients with COPD compared with those in control individuals. On the basis of these results, Plant et al. [111] concluded that autophagy is not activated in muscles of COPD patients. However, they assessed the degree of autophagy by measuring mRNA levels only. More recently, Guo et al. [112] performed a pilot experiment using Western blot and real-time PCR mRNA measurements to evaluate autophagy-related gene expression of muscle biopsies obtained from cases of severe COPD. These experiments revealed significant increases in the intensity of LC3B-II protein in muscle of COPD patients compared with that in control subjects. In addition, they also observed significant increases in beclin-1 and p62/SQSTM1 protein levels in muscle biopsies of COPD patients indicating the activation of autophagy. More complete elucidation of the functional role of autophagy in muscle of COPD patients remains to be determined, but some research in this field has been undertaken. It is probable that the activation of autophagy in the muscle of COPD patients is modulated by several factors, such as oxidative stress, inflammation, malnutrition, and therapeutic medication, as proposed in an excellent systematic review by Hussain and Sandri [113].

One original study investigated the relationship between CHF and autophagy signaling in skeletal muscle [114]. It was suggested that there is a difference in the manner of autophagic adaptation between soleus (slow-type) and plantaris (fast-type) muscles by using rats with myocardial infarction. In fact, the transcription levels of GABARAPL-1 and Atg7 were increased in the plantaris but not the soleus muscle. However, the expression levels of other autophagic markers (beclin-1 and Atg12) did not change significantly. In addition, an autophagy-activating marker (LC3B-II/I) also did not increase in both muscles. However, there have been no studies examining the autophagy in muscle in cases of CHF. It remains to be elucidated

whether CHF includes autophagic activation in skeletal muscle similar to muscle in cancer cachexia and COPD.

A possible contribution of autophagic signaling to sarcopenia

Autophagic defect has been described for invertebrates and higher organisms during normal aging. Inefficient autophagy has been attributed a major role in the age-dependent accumulation of damaged cellular components, such as undegradable lysosome-bound lipofuscin, protein aggregates, and damaged mitochondria [115]. The function of the autophagy/lysosome system of protein degradation seems to decline during aging in the *Drosophila* skeletal muscle [116]. Senescent *Drosophila* muscle exhibits the progressive accumulation of the aggregates of poly-ubiquitin protein. Intriguingly, overexpression of the FOXO upregulates the many autophagy-related genes, preserves the function of the autophagy pathway, and prevents the accumulation of poly-ubiquitin protein aggregates in sarcopenic *Drosophila* muscle [116]. Several investigators reported autophagic changes in aged mammalian skeletal muscle [101, 117, 118]. Compared with those in young male Fischer 344 rats, amounts of beclin-1 were significantly increased in the plantaris muscles of senescent rats [117]. Using Western blot of fractionated homogenates and immunofluorescence microscopy, we recently demonstrated the selective induction of p62/SQSTM1 and beclin-1 but not LC3 in the cytosol of sarcopenic muscle fibers in mice [119]. In addition, we also observed a significant smaller p62/SQSTM1-positive muscle fibers in aged muscle compared to the surrounding p62/SQSTM1-negative fibers [119]. In contrast, aging did not influence the amounts of Atg7 and Atg9 proteins in rat plantaris muscle [117]. More recently, Wohlgemuth et al. [117] clearly showed a marked increase in the amount of LC3 in muscle during aging using analysis of Western blot. However, they failed to detect an aging-related increase of the ratio of LC3-II to LC3-I, a better biochemical marker of ongoing autophagy. In addition, we failed to detect marked increases in LC3-I and LC3-II (active form) proteins in aged quadriceps muscle [119]. In contrast, a significant increase in the ratio of LC3-II to LC3-I during aging has been demonstrated in the biceps femoris muscle of wild-type mice [101]. None of the studies determining the mRNA expression level of autophagy-linked molecules found a significant increase with age [117, 118]. Not all contributors to autophagy signaling seem to change similarly at both mRNA and protein levels in senescent skeletal muscle. Therefore, sarcopenia may include a partial defect of autophagy signaling, although more exhaustive investigation is needed in this field. Intriguingly, more recent study [120] using biopsy samples of young and aged human volunteers clearly showed the age-dependent autophagic defect such as the decrease in the amount of Atg7 protein and in the ratio of LC3-II/LC3-I protein. Figure 4 summarizes a possible adaptation of autophagy-linked molecules (LC3 and p62/SQSTM1) in sarcopenic muscle.

Autophagic signaling in muscular dystrophy

Inhibition/alteration of autophagy contributes to myofiber degeneration leading to accumulation of abnormal (dysfunctional) organelles and of unfolded and aggregation-prone proteins [94, 99], which are typical features of several myopathies [121, 122]. Generation of Atg5 and Atg7 muscle-specific knockout mice confirmed the physiological importance of the autophagy system in muscle mass maintenance [99, 123]. The muscle-specific Atg7 knockout mice are characterized by the presence of mitochondrial abnormality, accumulation of polyubiquiti-

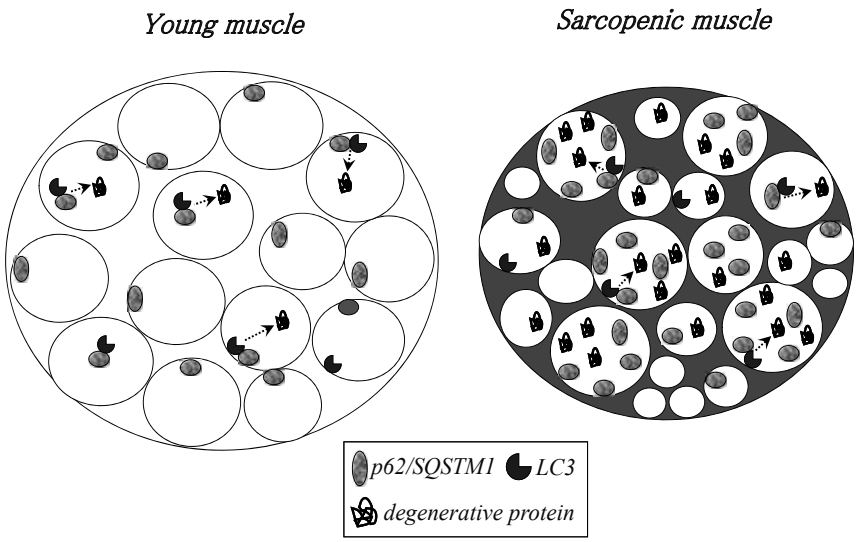

Figure 4. The comparison of an autophagy-dependent system between young and sarcopenic muscle. In contrast to young muscle, sarcopenic muscle exhibits abundant p62/SQSTM1 proteins with no activation of LC3, showing apparent autophagy defects, which cannot destroy the degenerative proteins.

nated proteins, and sarcomere disorganization [99]. In addition, the central role of the autophagy-lysosome system in muscle homeostasis is highlighted by lysosomal storage diseases (Pompe disease, Danon disease, and X-linked myopathy). These diseases are a group of debilitating muscle disorders characterized by alterations in lysosomal proteins and autophagosome buildup [124]. Intriguingly, the accumulation of autophagic vacuoles inside myofibers is recognized in all of these myopathies because of defects in their clearance.

Various muscular dystrophies also exhibit the apparent defect of autophagy-dependent signaling. The first evidence of impaired autophagy in these models was provided by studies in mice and patients with mutations in collagen VI [125]. Mutations that inactivate Jumpy, a phosphatase that counteracts the activation of VPS34 for autophagosome formation and reduces autophagy, are associated with centronuclear myopathy [126]. De Palma et al. [127] have described a decreased expression of autophagic regulator proteins (i.e., LC3 II, Atg12, GABARAPL-1, Bnip3) in dystrophin-deficient mdx mice and DMD patients. In addition, starvation and treatment with chloroquine, potent inducers of autophagy, did not activate autophagy-dependent signaling in both tibialis anterior and diaphragm muscles of mdx mice [127]. Furthermore, mdx mice and DMD patients exhibited an unnecessary accumulation of p62/SQSTM1 protein, which was lost after prolonged autophagy induction by a low-protein diet [127]. A similar block in autophagy progression was described in lamin A/C null mice [128]. LGMD2A muscles showed up-regulation of p62/SQSTM1 (2.1-fold) and Bnip3 (3-fold) mRNA and slightly increased LC3-II/LC3-I protein ratio and p62/SQSTM1 [87]. Conversely, laminin-mutated (*dy*/*dy*) animals displayed excessive levels of autophagy, which is equally detrimental [129]. These findings suggest that the defect of autophagy signaling has a central role in the degenerative symptoms in various types of muscular dystrophy.

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4. Conclusions and perspectives

Recent progress has significantly expanded our understanding of the molecular mechanisms that regulate skeletal muscle protein synthesis and degradation. Despite this, considerably more research is required to fully elucidate the many different mechanisms that potentially regulate these two processes. Successful identification of common regulatory molecules/pathways will greatly aid our understanding of how different types of stimuli promote changes in skeletal muscle mass. The Akt/mTOR/p70S6K pathway and SRF-dependent signaling have been considered to be major contributors to protein synthesis and muscle-specific transcription, respectively [11, 23]. Over the past decade, studies using rodent muscles have indicated that atrogin-1 and MuRF-1 contribute to the protein degradation in muscular wasting [60]. More recent studies using human muscle do not necessarily support such a role for these atrogenes [77]. It seems that the disorganization of the autophagy system accelerates the muscular disorder with age (sarcopenia) in rodents and human.

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Chapter 7

Autophagy, a Highly Regulated Intracellular System Essential to Skeletal Muscle Homeostasis — Role in Disease, Exercise and Altitude Exposure

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Additional information is available at the end of the chapter

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Abstract

Autophagy is an evolutionarily conserved intracellular system that selectively eliminates protein aggregates, damaged organelles, and other cellular debris. It is a self-cleaning process critical for cell homeostasis in conditions of energy stress. Autophagy has been until now relatively overlooked in skeletal muscle, but recent data highlight its vital role in this tissue in response to several stress conditions. The most recognized sensors for autophagy modulation are the adenosine monophosphate (AMP)-activated protein kinase (AMPK) and the mechanistic target of rapamycin (MTOR). AMPK acts as a sensor of cellular energy status by regulating several intracellular systems including glucose and lipid metabolisms and mitochondrial biogenesis. Recently, AMPK has been involved in the control of protein synthesis by decreasing MTOR activity and in the control of protein breakdown programs. Concerning proteolysis, AMPK notably regulates autophagy through FoxO transcription factors and Ulk1 complex. In this chapter, we describe the functioning of the different autophagy pathways (macroautophagy, microautophagy, and chaperone-mediated autophagy) in skeletal muscle and define the role of macroautophagy in response to physical exercise, a stress that is well assumed to be a key strategy to counteract metabolic and muscle diseases. The effects of dietary factors and altitude exposure are also discussed in the context of exercise.

Keywords: Cachexia, Endurance exercise, Hypoxia, Proteolysis, Sarcopenia

1. Introduction

Skeletal muscle exhibits remarkable adaptive capabilities in response to various stimuli such as loading conditions (resistance training, microgravity), contractile activity (electrical stimulations, endurance exercise), environmental factors (altitude exposure), or nutritional interventions. To access this great capacity, a plethora of quantitative and functional adaptations are involved. Changes in the size of adult muscle, in response to these external stimuli, are mainly due to the growth of individual muscle fibers rather than an increase in fiber number [1].

The control of muscle mass is dependent upon a balance between anabolic and catabolic processes. Hypertrophy is associated with increased protein synthesis, while atrophy is characterized by increased degradation of muscle proteins and/or a decrease in protein translation. The initiation of protein synthesis is mainly mediated by a signaling pathway in which the mammalian/mechanistic target of rapamycin complex 1 (MTORC1), a multiprotein complex composed of MTOR (mammalian/mechanistic target of rapamycin), RPTOR (regulatory-associated protein of MTOR), mLST8/GβL (MTOR-associated protein LST8 homolog), DEPTOR (DEP domain containing MTOR-interacting protein), and PRAS40 (proline-rich Akt substrate of 40 kDa) [2,3]. MTORC1 by phosphorylating its substrates S6K1 (ribosomal protein p70S6 kinase 1) and 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1) controls skeletal muscle protein translation and hypertrophy [4-7]. The Insulin signaling pathway leads to the activation of MTORC1 through the activation of the kinases PI3K (phosphatidylinositol 3-kinase), PDK1 (phosphoinositide-dependent kinase-1), and Akt. Akt, also known as protein kinase B (PKB), inactivates tuberous sclerosis complex 1/2 (TSC1/2), promoting MTOR activation by Rheb-GTP [8-10]. Akt also phosphorylates and inactivates the glycogen synthase kinase 3β (GSK3β), resulting in the activation of the eukaryotic translation initiation factor 2B (eIF2B) and increased protein synthesis [5,11].

Muscle atrophy leads to a state of weakness and emaciation of the body, which is encountered, for example, in the terminal phase of certain diseases or chronic infections such as cancer, AIDS, diabetes, bacterial infections, and nerve degeneration [12]. Muscle atrophy is also observed during aging, immobilization, and stress or trauma to the muscle and is associated with increased proteolysis. Protein degradation is essentially modulated by two conserved pathways: the ATP-dependent ubiquitin-proteasome system and the autophagy pathways.

The first one has been particularly involved in the degradation process after the discovery of two E3 ubiquitin ligases (E3 ligases), MAFbx/atrogin-1 (muscle atrophy F-box) and MuRF1 (muscle RING finger-1), which are both overexpressed in various models of atrophy (fasting, cancer, diabetes, immobilization, and other stresses) [13,14]. The invalidation of these proteins confers a resistance to certain types of induced atrophy, suggesting a critical role in the catabolism for the ubiquitin-proteasome pathway [13]. The function of E3 ligases is to ubiquitinate specific proteins to target them for recognition by the 26S proteasome where they are eliminated. Other E3 ligases like zinc-finger protein 216 (ZNF216), the mitochondrial E3 ubiquitin protein ligase 1 (Mul1), and the tripartite motif-containing protein 32 (Trim32) have been shown to play an important role in skeletal muscle atrophy [15-17].

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The autophagy signaling, which constitutes the second pathway, is important for maintaining cell metabolism and organelle turnover. It involves the degradation of substrates by hydrolases into a vesicle called lysosome [18]. Recent evidence demonstrates cross talk and cooperation between the ubiquitin-proteasome system and autophagy [19,20]. The importance of this pathway in skeletal muscle was long much neglected, and autophagy was thought to be a nonselective degradation system. However, it is now well recognized that autophagy machinery is critical for muscle homeostasis and organelle turnover in response to cellular stress like physical exercise or hypoxia [21]. Importantly, the energy sensor AMPK (5'-adenosine monophosphate-activated protein kinase) has been involved in the regulation of both protein translation pathway and protein degradation systems, with a particular interest in the regulation of autophagy program for the last few years. The present chapter focuses on the role of autophagy in skeletal muscle homeostasis. We will describe the functioning of the autophagy signaling pathways (i.e., macroautophagy, microautophagy, and chaperone-mediated autophagy) and detail the regulation of macroautophagy by both AMPK and MTOR, with the final goal to discuss the potential applications of recent discoveries concerning autophagy-related pathologies. The involvement of autophagy in response to physical exercise (acute and chronic exercise) and altitude exposure is thereafter discussed.

2. Autophagy, an intracellular system that delivers cytoplasmic components to lysosome for degradation

2.1. The autophagy pathways

2.1.1. Description of the system

Autophagy has been discovered during nutrient privation and can be referred to as "self-eating" as cells degrade their own constituents to maintain cellular homeostasis in response to various injuries like starvation and hypoxia and in pathological conditions, including cancer, muscular dystrophy, and neurodegenerative diseases. One purpose of the starvation-induced autophagy is to degrade materials to provide amino acids and free fatty acids in order to preserve metabolism and ATP levels when extracellular nutrients reach hazardous low levels [22]. Moreover, autophagy also eliminates protein aggregates as well as unwanted and dysfunctional organelles. The term autophagy embraces macroautophagy, microautophagy, and chaperone-mediated autophagy that we will describe hereafter.

Macroautophagy and microautophagy are conserved from yeast to humans, and these processes were originally described as bulk degradation mechanisms. However, these two processes can be selective for targeting different organelles, and we distinguish mitophagy selective for degradation of mitochondria; pexophagy, selective for degradation of peroxisomes; xenophagy, selective for degradation of intracellular bacteria and virus; reticulophagy, selective for endoplasmic reticulum; heterophagy, selective for substances taken in by phagocytosis; golgiphagy, selective for Golgi apparatus; ribophagy, selective for ribosomes; crinophagy, specific for the contents (proteins, peptides) of secretory granules; glycophagy, selective for glycogen; and lipophagy, selective for lipid droplets [23-29]. Among these different varieties of

autophagy, mitophagy has been the most studied in the last decade, and this process involves notably two Parkinson's disease factors, the RING-between-RING E3 ligase Parkin and the mitochondrial kinase PINK1 (PTEN-induced putative kinase protein 1), PTEN being "phosphatase and tensin homolog" [30,31]. After mitochondrial potential depolarization, PINK1 promotes Parkin activation through phosphorylation of its ubiquitin-like domain [32]. In addition to these factors, the mitochondrial E3 ligase Mul1 can also be involved in mitophagy by the degradation of the mitochondrial fusion protein Mfn2 (mitofusin-2) and the stabilization of the dynamin-related protein 1 (DRP1), resulting in mitochondrial fragmentation [33].

Autophagy by providing a turnover of the cellular components plays a central role in the homeostasis of the cell. It is a key mechanism by which a starving cell reallocates nutrients from unnecessary to more-essential processes. Macroautophagy, microautophagy, and chaperone-mediated autophagy lead cytoplasmic substrates inside lysosomes in which their contents are digested by a battery of acidic hydrolases [34]. Four essential ubiquitous proteases have been identified: the cathepsins B, D, H, and L [35,36]. High levels of these cathepsins are expressed in tissues exhibiting high rates of protein turnover like the kidney, spleen, liver, or placenta, while low concentrations of cathepsins are found in tissues with lower protein turnover as skeletal muscles [37-40]. Similar enzymatic properties were reported for different muscles, independent of their metabolic and contractile type. However, their concentrations differ according to the fiber type. Indeed, slow-twitch oxidative muscles exhibit higher levels of cathepsins than the fast-twitch glycolytic muscles [35], suggesting a more important activity of this system in oxidative muscle. This data is in agreement with the fact that oxidative muscles present a more important protein turnover and a greater translational activity than glycolytic muscles.

Although skeletal muscle expresses cathepsins B, D, H, and L, they play distinctive roles. During fusion of myoblasts into myotubes, several groups have reported an increase in the expression and activity of lysosomal cathepsins, in particular cathepsin B [41-46]. Several studies showed that the expression of cathepsin L is induced during various forms of skeletal muscle atrophy including starvation [12,36,47-49]. Increase in cathepsin D activity has been reported in muscles of dystrophic mice and chicken and in muscles of patients with Duchenne muscular dystrophy [35,50].

2.1.2. Microautophagy

Microautophagy is localized directly at the level of the lysosome which directly engulfs cytosol components by invagination, protrusion, and/or elimination of the lysosomal limiting membrane. It is implicated in the degradation of long half-life proteins in numerous cell types and does not respond to classical stimuli inducing chaperone-mediated autophagy and macroautophagy [51]. In contrast to macroautophagy, microautophagy has not been extensively studied in skeletal muscle, and its functions in muscle proteolysis have to be more characterized [35].

2.1.3. Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) is a selective form of autophagy that has only been described in mammalian cells to date [52,53]. In this form of autophagy, only cytosolic proteins that possess the consensus pentapeptide Lys-Phe-Glu-Arg-Gln (KFERQ) are recognized. The

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KFERQ-like sequence is recognized by the heat-shock cognate protein of 73 kDa (hsc73) then targeted to lysosomes for degradation. This targeting needs the binding of the complex protein substrates hsc73 to the lysosome-associated membrane protein type 2A (LAMP-2A) and to a multi-molecular chaperone complex including hsp40, hsp70, and hsp90 at the cytosolic side of the lysosomal membrane [54]. LAMP-2A is a glycoprotein present at the lysosomal membrane which acts as a CMA receptor. The substrates are then unfolded and translocated across the lysosomal membrane with the hsc73 protein. Unlike other forms of autophagy, CMA is very selective in substrate degradation and cannot eliminate organelles [55-57].

The use of an antibody directed to the KFERQ amino acid sequence substrates showed that proteins containing this sequence are conserved in skeletal muscle during starvation, while they are degraded in the liver and heart [58]. Moreover, the absence of consensus sequence in most myofibrillar proteins indicates that this degradation pathway is not implicated in their degradation. However, Nishino and colleagues showed that LAMP-2 deficiency is the primary defect in human Danon disease, a pathology characterized by myopathy and cardiomyopathy with massive accumulation of autophagic vacuoles [59,60].Thus, as microautophagy, CMA has to be more characterized in the context of muscle atrophy.

2.1.4. Macroautophagy

Macroautophagy, often referred to as autophagy or autophagosome-lysosome system, is an evolutionarily conserved intracellular system that coordinates and oversees the degradation of damaged organelles as mitochondria, peroxisomes, or ribosomes, intracellular pathogens, and unused long-lived proteins [61]. More than 30 autophagy-specific genes (Atgs) have been identified and are known to facilitate the sequestration of cytoplasmic substrates into a double-membrane vesicle called autophagosome or autophagic vacuole. Atgs are essential mediators of autophagy, by controlling the formation of the autophagosome. Autophagosome fuses with lysosome to form an autolysosome (also called autophagolysosome).

2.2. Macroautophagy: Autophagosome formation

2.2.1. Initiation

Macroautophagy (autophagy) is initiated in response to a multitude of factors including nutrient deprivation and oxidative stress. The activation of autophagy during muscle wasting was shown by the accumulation of autophagosomes in muscles of fasted transgenic GFP-LC3 mice [62]. Studies showed that during starvation-induced atrophy, FoxO3a (forkhead box class O3a) regulates the transcription of several Atgs, including Atg4B, LC3B (microtubule-associated protein 1A/1B-light chain 3B), Beclin-1, Vps34 (vacuolar protein sorting 34)/PI3K class III, Gabarapl1 ($GABA_A$ receptor-associated protein-like 1), Atg12, and Ulk2 (unc-51-like kinase 2) [63,64]. Initiation of the autophagy processes involves the activation of the unc-51-like kinase 1 (Ulk1, also called Atg1 in yeast)/Atg13/FIP200/Atg101 and the Beclin-1/Vps34 complexes. These proteins operate in conjunction with several Atgs to mediate the assembly of the autophagosomal membrane [65-68]. Ulk1 also phosphorylates Beclin-1 at Ser-14 following amino acid withdrawal, and this stage is necessary for the Vps34 lipid kinase

activation and full autophagy induction [69]. In yeast and mammalian cells, Atg1 or Ulk1 (respectively) activity is suppressed under nutrient-rich conditions by MTORC1 (phosphorylation of Ulk1 at Ser-757) [65,70-73]. In addition, MTOR inhibition and its subsequent dissociation from Ulk1 are critical for Beclin-1/Vps34 complex activation by Ulk1 [69].

2.2.2. Maturation of autophagosomes

The maturation and completion of the autophagic vacuole is facilitated by a ubiquitin-like conjugation-signaling cascade that culminates with the binding of phosphatidylethanolamine (PE) to the cytosolic form of LC3 (LC3-I) to form LC3-II. LC3, a mammalian homolog of yeast Atg8, is a protein with a molecular mass of 17 kDa that is distributed ubiquitously in mammalian tissues. Two other LC3 homologs are $GABA_A$ receptor-associated protein (Gabarap) and Golgi-associated ATPase enhancer (GATE-16). LC3-phosphatidylethanolamine conjugate (LC3-II) is recruited to the autophagosomes [74,75]. LC3-II plays here a structural role that allows the elongation and the formation of the mature autophagosome. The mature autophagosome fuses with the lysosome to form an autolysosome (Fig.1). Concomitantly, LC3-II in autolysosomal lumen is degraded by lysosomal hydrolases; thus, turnover of the autophagosomal protein LC3-II reflects autophagic activity [76]. LC3 also interacts with the autophagy adapter p62/SQSTM1 (sequestosome 1), which contains multiple apparent protein-protein interaction domains. p62 binding to LC3 permits the entry of ubiquitinated cargo into the autophagosome [77]. In addition, the Atg12-Atg5-Atg16 complex plays an important role in the maximal promotion of LC3 lipidation and autophagy induction [78].

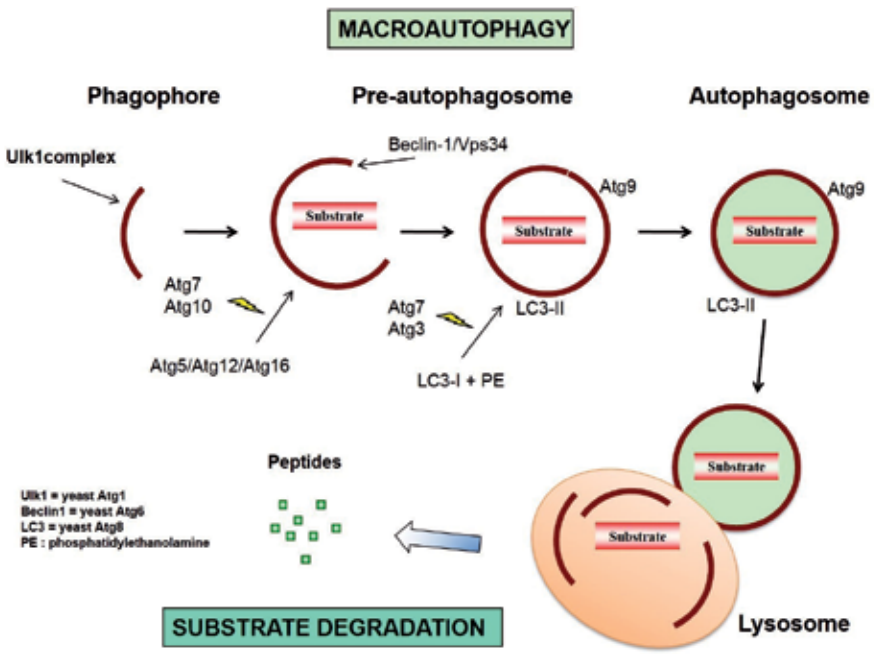

Figure 1. Processing of the macroautophagy system

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2.3. Implication of autophagy in cell homeostasis and disease

Autophagy plays a prominent role in the maintaining of cell homeostasis by selectively eliminating protein aggregates, damaged organelles, and other nonactive cellular debris [79,80]. This process is required for normal cellular function, but it is increasingly apparent that it can have both beneficial and detrimental effects on cells and tissues, depending on the origin of its activation [81]. Physiological function of basal autophagy in maintaining tissue homeostasis has been demonstrated in several tissues as the brain, liver, heart, striated muscle, intestine, pancreas, and adipose tissue [82-89]. Furthermore, exciting reports suggest that autophagy may contribute to counteract the deleterious effects of aging by limiting the deposition of aggregated proteins and damaged mitochondria [90-92]. By blocking apoptosis, autophagy preserves cell survival by providing endogenous metabolites when exogenous substrates are lacking [93]. Thus, at regular levels of activation, autophagy may represent the first step to restore homeostasis. However, when the autophagic capacity is submerged, apoptosis takes over [94]. Nevertheless, the relationship between autophagy and apoptosis appears to be extremely complex, and additional data are necessary to clarify the situation, especially in the context of disease.

In numerous pathologies as diabetes, obesity, cancer, heart failure, and neurodegenerative, infectious, and inflammatory disease, autophagy activity is affected [34,95,96]. For instance, it has been reported that the lack in Beclin-1 expression decreases autophagy flux and leads to increased risk of breast and prostate cancers [97]. However, the systematic beneficial effect of autophagy should be tempered. Thus, the role of autophagy in cancer is ambivalent, and this process can be involved in both the promotion and the prevention of this disease. In the first stage of the malignancy (i.e., tumor initiation), inhibition of autophagy may allow the growth of initial cancerous cells, and thus autophagy can act as a suppressor of cancer [98,99]. When cancer is established, transformed cells may need autophagy to survive, especially in nutrient-limiting condition [100]. In addition, for patient undergoing treatment such as chemotherapy, cancer cells could use autophagy to protect themselves from the stress induced by the therapy. Other reports indicate also that glycogen storage disease type II (also called Pompe disease) – an autosomal recessive metabolic disorder – is a pathology attributable in part to mutations of Atgs. In this disease, muscle and nerve cells are damaged by an accumulation of glycogen into the lysosomes caused by a deficiency of the lysosomal acid alpha-glucosidase enzyme [101,102]. In many neurodegenerative disorders such as Parkinson's, Huntington's, and Alzheimer's diseases, accumulation of autophagic vesicles has been also observed [103-105]. Regarding Alzheimer's disease, it has been reported that Atg7 influences the accumulation of amyloid β (Aβ) peptides, resulting in aggregation into plaques in the brain [106]. In this model, autophagy seems to participate to the disease progression since it is involved in the generation of Aβ peptides.

In summary, by removing misfolded proteins and abnormal organelles, autophagy can be considered as a critical mechanism for cell protection. On the contrary, by destroying excessive fraction of cytosol and organelles, too high levels of autophagy represent a side mechanism responsible of the initiation of pathologies [94,107].

2.4. The roles of autophagy in skeletal muscle

Compared to other tissues like the liver or pancreas, autophagy in skeletal muscle exhibits a low protein turnover and a small size of autophagosomes. These peculiar characteristics have probably constituted a brake for the detection of autophagy in this tissue for a long time. Associated to the use of transgenic mice expressing LC3 fused with GFP, autophagy process can be now easily visualized [62]. Conversely to liver or pancreas in which autophagy is activated transiently for a few hours, in skeletal muscle, autophagy can be activated for several days [62]. As it was shown in other tissues, muscular autophagy is activated by nutrient deprivation or by the absence of growth factors [108].

Although it was reported that the mRNAs coding for Atgs are present in abundance in skeletal muscle [109], the role and the regulation of basal autophagy have been poorly characterized in this tissue until recently. In order to assess the function of autophagy in skeletal muscle, Masiero and colleagues performed experiments on mice deprived of the Atg7 gene, a gene necessary to the unfolding of the autophagy program [86,110]. Importantly, mice showed obvious signs of muscular weakness and atrophy exacerbated during ageing. Mice presented an accumulation of degraded proteins and free radicals, a deterioration of the internal cellular structures, and an activation of the apoptotic program. The authors clearly defined that inhibition of basal autophagy does not protect from skeletal muscle atrophy induced by denervation or starvation, but on the contrary, contributes to its degeneration. Similar muscle alterations have been obtained in muscle-specific $Atg5^{-/-}$ mice [87], confirming the necessity to have regular autophagic flux in the cells, even during atrophy.

In many conditions varying from fasting, denervation, inactivity, microgravity, various pathologies as cancer, AIDS, sepsis, diabetes, cardiac failure, and myopathies, autophagy is overactive and pathologic, thus leading altered metabolism and muscle loss [81,108,111]. Contribution of autophagy to muscle loss begins to be clarified with the use of different animal models and innovative techniques. Inactivation of autophagic flux by LC3 silencing partially prevents FoxO3-mediated muscle atrophy [63] and atrophy caused by the expression of mutant SOD1 G93A in skeletal muscle [112]. In another model, atrophy induced by L-type calcium channel (DHPR) inactivation is linked to the expression of autophagic genes including LC3, Vps34, BNIP3 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3), and cathepsin L (for the lysosome) [113]. During sepsis, an upregulation of autophagy was found in parallel to mitochondrial injury and decreased biogenesis, especially in locomotor muscles [114]. Reactive oxygen species overproduction by altered mitochondria is now considered as a critical signal for the promotion of skeletal muscle autophagy, thus finding an opening to practical prospects for treatment of disease [115]. Accordingly, antioxidant supplement can lead to an inhibition of skeletal muscle autophagy through a reduction of oxidative stress and an increase in antioxidant capacity [116]. Taken together, these recent results confirm that excessive activation of autophagy is a critical factor for muscle wasting determinism, and strategies attempted to control autophagy level will be promising.

Data concerning sarcopenia are also specific. Sarcopenia is an age-related loss of muscle mass and strength, which is associated with increased autophagy, apoptosis, and exacerbated proteolysis [117]. Elevated peroxisome proliferator-activated receptor-coactivator α (PGC-1α) expression in muscle during aging contributes to reduce the proteolytic activity associated

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with atrophy. In sarcopenia, attenuation of the degradative processes and the maintenance of mitochondrial function contribute to the preservation of muscle integrity [118].

In summary, as already described for cancer cells, autophagy in muscle is a complex process that can be, according to its activation level, beneficial or deleterious. During disease, the systematic question is to determine whether autophagy plays a protective function, has a causative role, or is a result of the disease process itself.

3. AMPK and skeletal muscle autophagy

3.1. The AMP-activated protein kinase

AMPK is a serine/threonine protein kinase highly conserved through evolution. AMPK is a heterotrimeric complex composed of a catalytic subunit (AMPK-α) and two regulatory subunits (AMPK-β and AMPK-γ). Humans have seven genes encoding AMPK subunits (α1, α2, β1, β2, γ1, γ2, γ3) that can form at least 12 αβγ heterotrimers, increasing the diversity of its functions [119]. The catalytic α subunit contains the threonine phosphorylation site that upon phosphorylation leads to AMPK activation [120]. AMPK acts as a sensor of cellular energy status by regulating several intracellular systems including glucose and lipid metabolisms and mitochondrial biogenesis [121]. Thus, AMPK activation leads to increased glycolysis flux [122] and fatty acid oxidation [123-126] and on the contrary, to an inhibition of glycogenogenesis [127,128] and cholesterol and fatty acid biosynthesis [129-131]. The enzyme also increases the expression of PPARα (peroxisome proliferator-activated receptor α) target genes and PGC-1 leading to mitochondrial biogenesis and enhanced oxidative metabolism of muscle cells [132,133]. AMPK is activated by a large variety of cellular signals that decrease cellular ATP levels and increase AMP in response to different kinds of stress like electrical-stimulated muscle contraction, exercise, hypoxia, and heat shock or under conditions of nutrient deprivation [124,134,135]. The recognized enzymes in the regulation of AMPK under energy stress conditions are LKB1 (liver kinase B1), CaMKK (calmodulin-dependent protein kinase kinase), and TAK-1 (transforming growth factor beta-activated kinase 1).

3.2. Regulation of skeletal muscle autophagy by AMPK

The role of AMPK in protein turnover has been clearly defined in recent years. AMPK has been involved in the control of protein synthesis and the repression of skeletal muscle mass by inhibiting MTOR activity [136,137] in two ways: AMPK phosphorylates TSC2 (tuberous sclerosis complex 2) at Thr-1227 and Ser-1345 and RPTOR at Ser-722 and Ser-792, leading to a reduction of MTORC1 activity. Several studies also showed that AMPK increases protein degradation through the modulation of the ubiquitin-proteasome and the autophagosome-lysosome pathways [138,139]. AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside, an AMPK activator) treatment increases the expression of the E3 ligases MAFbx/Atrogin-1 and MuRF1 in muscles cells. In addition, increase of autophagic flux by AMPK has been reported in several muscle models as C2C12 myoblasts, C2C12 myotubes, and primary myotubes [138,139]. Two major signaling pathways were characterized in AMPK-induced muscular autophagy (Fig.2).

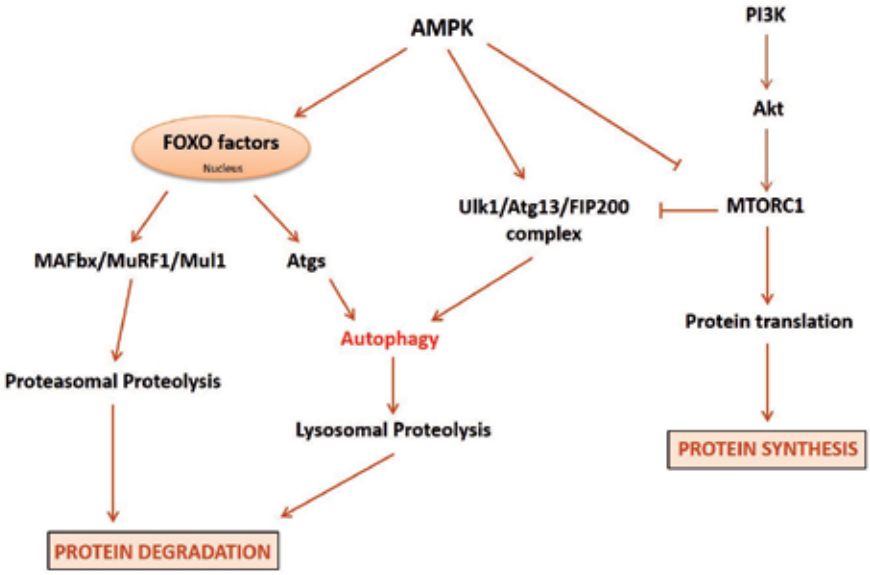

Figure 2. Role of AMPK in skeletal muscle protein turnover

The first one concerns the activation of the forkhead box class O proteins (FoxO), notably involved in the regulation of protein breakdown, energy metabolism, and mitochondrial turnover [140]. FoxO factors also play an important role in exercise-induced angiogenesis by limiting it during the first days of training program [141,142]. Activation of FoxO3a by AMPK leads to an increase in several Atgs expression, including LC3-II and Gabarapl1 that act as promoters of autophagosome fabrication [139]. AMPK directly interacts with FoxO3a and phosphorylates it on Ser-588, a residue known to lead to FoxO3a activation [139,143]. The upregulation of several Atgs by FoxO factors have been described in *Drosophila* larval fat body [144], mammalian cardiomyocytes [145], hepatocytes [146], and colorectal cancer cells [147]. Regarding the regulation of FoxO3a subcellular localization in muscle cells, while long treatments (i.e., 24 h) with AMPK activators do not change FoxO3a nuclear content, an increase in the total protein level is notable after 30 min. With a short time course (30 min–6 h), the activation of AMPK by AICAR leads to a relocalization of FoxO3a into the nucleus [139]. Tong and colleagues reported that AICAR treatment causes FoxO3a nuclear relocation through a decrease in FoxO3a phosphorylation at Thr-318/321 [148]. However, Greer and colleagues have reported an increase of FoxO3a transcriptional activity without any change in the nuclear content of the factor after AMPK activation by 2-deoxyglucose (2DG) in HEK293T cells [143]. These data strongly suggest that FoxO3a relocalization into the nucleus is not necessarily required to increase its transcriptional activity. A possibility is that AMPK may also control FoxO3a protein stability.

The second pathway involves modulation of the Ulk1 complex. A multiprotein complex composed of AMPK, MTORC1, Ulk1, FIP200, and Atg13 has been identified in muscle cells (Fig.3) [139]. These data fit with the model found in other cell types showing that, under basal

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conditions, MTORC1 prevents autophagy by interacting with Ulk1 [72]. Under nutrient-rich conditions, phosphorylation of Ulk1 by MTORC1 represses Ulk1 kinase activity and its ability to interact with Atg13 or FIP200; thereby, it coordinates the autophagy response [65,149]. In muscle cells, activation of AMPK (by AICAR treatment) or inhibition of MTORC1 (by Torin1 treatment or amino acid privation) removes AMPK, MTOR, and RPTOR from Ulk1 [139]. These events are known to induce the Ulk1-dependent phosphorylation of Atg13 and FIP200, leading to the initiation of autophagy [70].

Proteomics screens of autophagy [150] and a co-immunoprecipitation study performed in HEK293T cells [151] showed that AMPK interacts with both Ulk1 and Ulk2. In muscle cells, Ulk1 also acts as an interacting partner of AMPK, and Ser-467 site identified by Egan and colleagues is also phosphorylated by AMPK [139]. Ulk1 phosphorylation by AMPK may participate to conformational changes and thus disrupts the interaction between Ulk1 and MTORC1, in agreement with the suppression of MTORC1 anti-autophagy activity in the Ulk1 complex [72]. Moreover, Ulk1 phosphorylation by AMPK may directly activate Ulk1 kinase activity. Indeed, in vitro studies showed that Ulk1 is highly phosphorylated and that purified Ulk1 can phosphorylate itself and requires autophosphorylation for stability [152]. In mammals, Ulk1 phosphorylation by AMPK is critical for mitochondrial homeostasis and cell survival during starvation [153]. In summary, AMPK regulates Ulk1 activity by decreasing MTORC1 activity and by phosphorylating Ulk1 [121].

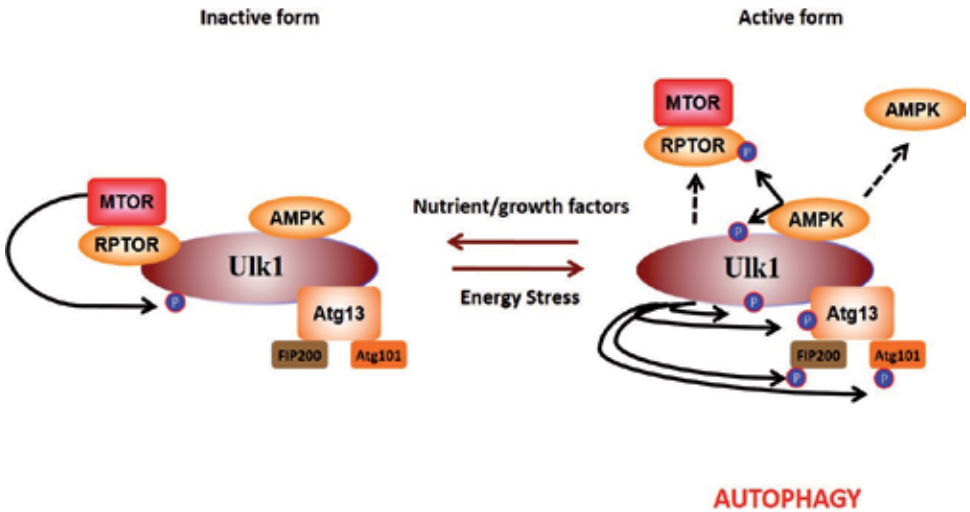

Figure 3. The Ulk1/Atg13/FIP200/MTORC1/AMPK complex

Time-course studies have been performed in muscle cells in order to better understand the dynamics of UlK1 complex following autophagy induction [139]. Interestingly, AMPK dissociates from Ulk1 3 h after AICAR treatment [139]. In agreement with these observations, in HeLa cells, AMPK is associated with Ulk1 only under nutrient-rich condition, and it dissociates from Ulk1 5 min after starvation [154]. Thus, in normal condition, Ulk1 is associated

with AMPK; upon AICAR treatment, the complex remains stable for 3 h and then dissociates. As suggested by another group, Ulk1 dissociation from AMPK could permit to Ulk1 complex to be more active [154]. Conversely, this dissociation can constitute a negative regulatory feedback as proposed by Loffler et al. [155]. The authors showed that Ulk1 could mediate phosphorylation of AMPK on the regulatory subunits, constituting an inhibitory feedback control. Further works have to define the molecular mechanisms for these events, especially in skeletal muscle.

4. Autophagy, exercise and altitude exposure

Attractive data concerning the role of autophagy during exercise are emerging. Autophagic vacuole formation during physical exercise was observed for the first time by Salminen and coworkers in 1984 with electron microscopy [156]. Nevertheless, there were no further studies on the topic until recently. In the last decade, data supporting the importance of autophagy in muscle homeostasis in response to exercise have been numerous, starting with a study from Bonaldo's team that showed that mice presenting impaired autophagy develop severe muscle weakness (i.e., accumulation of defective mitochondria, exacerbated apoptosis, muscle degeneration, and atrophy) [157]. Thereafter, other studies highlight that chronic inactivation of autophagy leads to a loss of metabolic effects related to exercise and drastic decreases in endurance performance [158].

Concerning autophagy modulation in response to acute exercise in humans, studies by Jamart and colleagues [159] were the first to demonstrate a raise of autophagy-regulatory genes and autophagic flux markers after ultraendurance exercise. By showing that AMPK and FoxO3a regulate in a coordinated way autophagy and ubiquitin-proteasome pathways during ultraendurance exercise, the authors gave an important picture of the cross-regulation of both degradation pathways in response to long-lasting endurance exercise [160]. Regarding more common endurance exercises, the modulation of muscle protein turnover, autophagy, and mitochondrial dynamics markers has been investigated thereafter in mice in response to different exercises conducted or not until exhaustion. Endurance exercise quickly initiates the autophagy pathway through Ulk1 activation resulting in an increase of autophagic flux, especially near exhaustion [161]. A rise in the phosphorylation of DRP1, a GTPase essential for mitochondrial fission, quickly occurred during exercise without any change in the expression of fusion markers (OPA1 and Mfn2). These data are consistent with an increase in mitophagy (i.e., the degradation of mitochondria by autophagy) since exacerbated fission can lead to mitochondrial fragmentation. Noteworthy, exercise decreases the activity of the main protein synthesis pathway (i.e., Akt/MTOR signaling pathway), from 90 min of moderate exercise (40–50 % of VO_{2max}), concomitantly to an increase in the phosphorylation of a marker of endoplasmic reticulum stress (eiF2α Ser-51) [161]. Others studies reported an increase of endoplasmic reticulum stress by the evaluation of the content of the double-stranded RNA-activated protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), the ER stress-induced transcription factor C/EBP homologous protein (CHOP), and the X-box binding protein 1 (XBP1s), in response to both ultraendurance [162] and moderate-intensity exercise [163]. However,

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reticulophagy has not been studied yet in skeletal muscle, especially in response to exercise. Further works are needed to clarify the possible clearance of important organelles, such as ribosomes or endoplasmic reticulum, during physical exercise. Exercise promotes better "cell health"; it would be not surprising to discover that exercise increases the turnover of such organelles like it is strongly suggested for mitochondria [21].

The rise of autophagy is essential to prevent mitochondrial damage during endurance exercise. Although acute inhibition of autophagy prior to exercise seems to not significantly affect performance, it leads to accumulation of dysfunctional mitochondria and augmentation of oxidative stress especially during eccentric contraction [164]. Thus, autophagy has a critical role in mitochondria quality control during acute exercise. In addition, autophagy is highly involved in exercise training-induced adaptations. Autophagy-deficient mice present attenuated improvement of endurance capacity in response to endurance training. In parallel to lower increases of basal autophagy flux, these mice show attenuated raises of mitochondrial content and angiogenesis [165], explaining the poor response to the training program.

The dietary factors have to be considered in autophagy response to acute exercise since essential amino acids (EAA) or carbohydrate (CHO) intake modulate protein turnover. Jamart et al. (2014) found that exercise performed in the fasted state permits a higher raise in autophagic flux indexes compared with the fed state. Concerning resistance exercise, few works showed a depression of autophagy markers after such an exercise like the study by Fry et al. conducted in humans [166]. In addition, autophagic flux markers can be depressed following EAA and carbohydrate (CHO) ingestion after resistance exercise [167]. To date, autophagy seems non-critical for muscle adaptations to resistance training [161]. However, an exception occurs during aging in which both endurance and resistance training are able to reverse the drop of autophagy regulatory proteins that occurs [168,169] (Fig.4).

Little is known regarding protein turnover pathways, especially autophagy, in response to exercise performed during altitude exposure in humans. Such an environment can induce a state of hypoxia that is exacerbated according to the level of altitude considered. Hypoxia results in decreased oxygen availability and leads to several hormonal, cardiorespiratory, and muscular adjustments in order to preserve cell homeostasis. Long-lasting hypoxia can cause a diminution of skeletal muscle mass and a reduction of muscle oxidative capacity. In agreement with an alteration of protein synthesis flux, hypoxia impairs the overload-induced increase of the PI3K/Akt/MTOR signaling pathway in rats [170]. Regarding degradation pathways in humans, while ubiquitin-proteasome system seems not positively modulated by environmental hypoxia, an upregulation of skeletal muscle autophagic flux markers has been found during acute normobaric hypoxia (10.7 % O_2) and after exercise conducted in such an environment [171,172]. Another study investigated the effects of acute high-altitude exposure (at 5,300 m altitude) in the course of the Caudwell Research Expedition to Mt. Everest [173]. The authors notably found an upregulation of heat shock cognate 71 kDa protein involved in chaperone-mediated autophagy and a reduction of protein translation markers. Taken together, these studies seem to highlight a preventive role of autophagy for energy expenditure and an activation of chaperone-mediated autophagy during acute high-altitude exposure. However, the effects of chronic altitude exposure on autophagy and its combination with training remain to be characterized to date. Chronic hypoxia leads to a change in oxidative

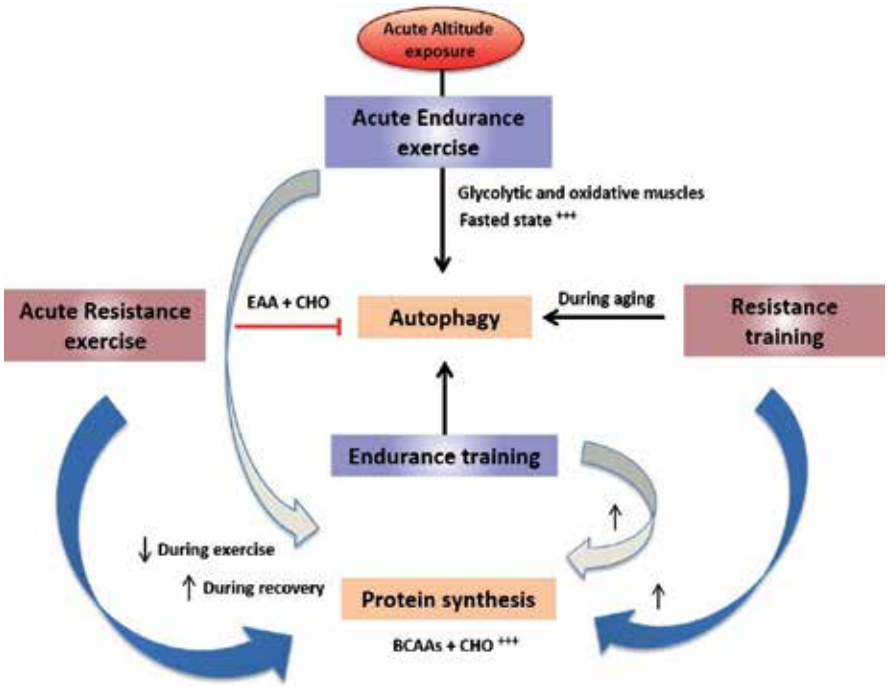

Figure 4. Exercise and autophagy in skeletal muscle. Adapted from Sanchez AMJ et al. 2014. Autophagy is essential to support skeletal muscle plasticity in response to endurance exercise. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology 307(8): R956-R969 [21]. Essential amino acids (EAA); carbohydrate (CHO)

metabolism [174,175]; it is likely that autophagy, especially mitochondrial autophagy, be amended during such an exposure. Consistent with this, experiments performed in cells showed that mitochondrial autophagy is induced by chronic hypoxia through HIF-1 (hypoxia-dependent factor-1) and BNIP3, and this regulation constitutes a preventive response that is necessary to avoid accumulation of reactive oxygen species and cell death [176]. The effects of exposition to moderate altitude (i.e., 1,500–3,000 m) have also to be definite since it concerns a larger population (athletes, general tourist population, and highlanders) compared to high altitude.

Research of this type is leading to a better understanding of the autophagy-mediated turnover of organelles like mitochondria in response to exercise and altitude exposure. Finding optimal training strategies represents an important objective to enhance exercise adaptations in both athletes and patients with metabolic or muscle diseases, including COPD (chronic obstructive pulmonary disease) that can cause systemic hypoxia and loss in muscle capability.

5. Conclusions

In summary, autophagy represents a crucial mechanism for cell homeostasis and cell survival. Autophagy constitutes a recycling process that degrades used or flawed internal structures

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into amino acid, enabling cells to survive in difficult circumstances. The identification of Ulk1 as a direct target of AMPK represents a significant step toward understanding how cellular energy stress regulates autophagy machinery in muscle cells. Based on the data presented here, it is clearly conceivable that the autophagy pathway must be considered in muscle pathologies such as sarcopenia and myopathies. Fine molecular studies on AMPK/FoxO3a and AMPK/Ulk1 axes will offer new and promising strategies in the treatment of muscular loss associated not only to several pathologies like cancer, AIDS, and neuromuscular diseases but also to age-related disorders. Further investigations must be conducted in order to better understand whether exercise-induced autophagy can also prevent skeletal muscle diseases. In addition, autophagy is involved in skeletal muscle adaptation to endurance exercise in a healthy population. While autophagy is important to prevent mitochondria alteration and exacerbated oxidative stress in response to severe acute exercise, its role in the improvement of endurance capacity has also been demonstrated in response to endurance training. In addition, the feeding pattern and the environment, especially altitude exposure, have to be considered since they represent additional modulators of protein turnover and autophagy pathway. Regular exercise is associated with an enhanced quality of life and represents the most profitable way to limit metabolic disorders and the loss of muscle mass. As a result, these research directions are of major interest in the battle against a wide range of diseases and have to be sustained.

List of abbreviations

4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; Aβ, amyloid β; AIDS, acquired immune deficiency syndrome; AMPK, 5'-adenosine monophosphate-activated protein kinase; Atg, autophagy-specific gene; BCL2, B-cell lymphoma 2; BNIP3, BCL2/adenovirus E1B 19 kDa-interacting protein-3; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase; CHO, carbohydrate; CHOP, C/EBP homologous protein; CMA, chaperone-mediated autophagy; COPD, chronic obstructive pulmonary disease; DEPTOR, DEP domain-containing MTOR-interacting protein; EAA, essential amino acids; eIF2B, eukaryotic translation initiation factor 2B; eIF2α, eukaryotic initiation factor 2α; FIP200, focal adhesion kinase (FAK) family-interacting protein of 200 kDa; FoxO, forkhead box class "other" O; Gabarapl1, $GABA_A$ receptor-associated protein-like 1; GATE-16, Golgi-associated ATPase enhancer; GSK3β, glycogen synthase kinase 3β; hsc73, heat-shock cognate protein of 73 kDa; HIF-1, hypoxia-dependent factor-1; IGF-1, insulin-like growth factor-1; LAMP-2A, lysosome-associated membrane protein 2A; LC3, microtubule-associated protein 1A/ 1B light chain 3; LKB1, liver kinase B1; MAFbx/atrogin-1, muscle atrophy F-box; Mfn2, mitofusin-2; mLST8/GβL, MTOR-associated protein LST8 homolog; MTOR, mechanistic/mammalian target of rapamycin; MTORC1, mechanistic/mammalian target of rapamycin complex 1; Mul1, mitochondrial E3 ubiquitin protein ligase 1; MuRF1, muscle RING-finger protein-1; OPA1, optic atrophy 1; p62/SQSTM1, sequestosome 1; PDK1, phosphoinositide-dependent kinase-1; PE, phosphatidylethanolamine; PERK, double-stranded RNA-activated protein kinase R (PKR)-like endoplasmic reticulum kinase; PGC-1, peroxisome proliferator activator receptor γ coactivator-1; PI3K, phosphatidylinositol 3-kinase; PINK1, PTEN-induced putative kinase protein 1; PKB/Akt,

protein kinase B; PPARα, peroxisome proliferator-activated receptor α; PRAS40, proline-rich Akt substrate of 40 kDa; PTEN, phosphatase and tensin homolog; Rheb, ras homologous enriched in brain; RPTOR, regulatory-associated protein of MTOR, complex 1; ROS, reactive oxygen species; S6K1, p70S6 kinase 1; TAK-1, transforming growth factor β-activated kinase 1; Trim32, tripartite motif-containing protein 32; TSC21/2, tuberous sclerosis complex 1/2; Ulk, unc-51-like kinase; Vps34, vacuolar protein sorting 34; XBP1s, X-box binding protein 1; ZNF216, zinc-finger protein 216

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Section 3

Atherosclerosis

Chapter 8

Cell Composition of the Subendothelial Aortic Intima and the Role of Alpha-Smooth Muscle Actin Expressing Pericyte-Like Cells and Smooth Muscle Cells in the Development of Atherosclerosis

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Additional information is available at the end of the chapter

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Abstract

The cell composition of the human arterial intima has been intensely studied but is still not well understood. The majority of cell population in normal and atherosclerotic intima is represented by cells expressing smooth muscle α-actin, which are thought to be smooth muscle cells. Some antigens, which are absent in medial smooth muscle cells, were detected in intimal smooth muscle cells. In particular, using 3G5 antipericyte antibody, presence of stellate-shaped pericyte-like resident cells in normal and atherosclerotic human aortic intima has been found. In all analyzed aortic tissue specimens, 3G5+ cells were found to account for more than 30% of the total intimal cell population of undiseased intima. In the atherosclerotic lesions, the number of 3G5+ cells becomes notably lower than that in undiseased intima. The use of 2A7 antibody that identifies activated pericytes revealed the presence of 2A7+ cells in atherosclerotic plaques, while no 2A7+ cells were detected in normal intima. The strongest correlation was established between the number of pericyte-like cells and the content of intimal lipids. The correlation coefficients between the number of pericyte-like cells and collagen content and intimal thickness were greater than the correlation coefficients for smooth muscle cells. On the basis of these findings, pericyte-like cells but not smooth muscle cells or other cell types have been declared to be the key cellular element driving the formation of atherosclerotic lesions. The present chapter aims to detail the abovementioned issues. The present chapter also aims to promote a view that α-smooth muscle actin+ pericyte-like cells represent the key players in the development of atherosclerotic lesions.

Keywords: Smooth muscle cells, Pericyte-like cells, 3G5 antigen, 2A7 antigen, Arterial wall, Intima, Atherosclerosis, Atherogenesis

1. Introduction

The cell composition of the human arterial intima has been intensely studied but is still not well understood [1]. The majority of cell population in normal and atherosclerotic intima is represented by cells expressing α-actins, which are thought to be smooth muscle cells [2]. According to the current paradigm, smooth muscle cells show striking plasticity responding to microenvironment signals and thus can be presented by different phenotypes [3-7]. Immune-inflammatory cells also reside in the intima of healthy arterial walls [8]. Resident immune-inflammatory cells (macrophages, lymphocytes, and dendritic cells) represent only a minority of the subendothelial cell population, but their proportion increases during the development of atherosclerotic lesions, reaching up to 20% of the total cell content [9].

Some antigens, which are absent in medial smooth muscle cells, were detected in intimal smooth muscle cells [10-13]. In particular, using 3G5 antipericyte antibody, presence of stellate-shaped pericyte-like resident cells in normal and atherosclerotic human aortic intima has been found [10, 11]. In all analyzed aortic tissue specimens, 3G5+cells were found to account for more than 30% of the total intimal cell population of undiseased intima. In the atherosclerotic lesions, the number of 3G5+ cells becomes notably lower than that in undiseased intima [11]. The use of 2A7 antibody that identifies “activated” pericytes revealed the presence of 2A7+ cells in atherosclerotic plaques, while no 2A7+ cells were detected in normal intima [12]. The strongest correlation was established between the number of pericyte-like cells and the content of intimal lipids [12]. The correlation coefficients between the number of pericyte-like cells and collagen content and intimal thickness were greater than the correlation coefficients for smooth muscle cells. On the basis of these findings, pericyte-like cells but not smooth muscle cells or other cell types have been declared to be the key cellular element driving the formation of atherosclerotic lesions [14].

The present chapter aims to detail the abovementioned issues. The present chapter also aims to promote a view that α-smooth muscle actin+ pericyte-like cells represent the key players in the development of atherosclerotic lesions.

2. Structural organization of human aortic intima

The wall of large arteries consists of three layers, namely, the tunica intima, the tunica media, and the tunica adventitia. Furthermore, the tunica intima of the adult human aorta consists itself of two layers separated by distinct boundaries [15-20]. The muscular-elastic layer, adjacent to the media (also called the Jores' layer), is separated from the media by the internal

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elastic lamina. The innermost intimal layer, adjoining the arterial lumen, is separated from the muscular-elastic layer by the internal limiting membrane. This innermost intimal layer is also called as elastic-hyperlastic [17], connective-tissue [19, 21-24], juxtaluminal [20], or proteoglycan-rich layer [21]; it is located between the internal limiting membrane and the endothelial lining (Figure 1).

It is well known that the thickness of the intima is greater in atherosclerotic lesions than in grossly normal areas, with intimal thickness reaching the maximum in atherosclerotic plaques. In fatty streak, the thickness of the muscular-elastic layer is the same as in uninvolved intima, while in atherosclerotic plaque, it is only 11% greater than in the normal intima [25]. In contrast to the muscular-elastic layer, the thickness of the proteoglycan-rich layer increases considerably in atherosclerotic plaque, forming an intimal protrusion into the lumen, which reduces blood flow through the aorta. On average, in fatty streak, the thickness of the proteoglycan-rich layer is almost two times and in the plaque almost four times as high as that in uninvolved intima [25]. Sometimes, the thickness of the proteoglycan-rich layer in the plaque can be 10- to 20-fold as high as that in a normal vessel [25].

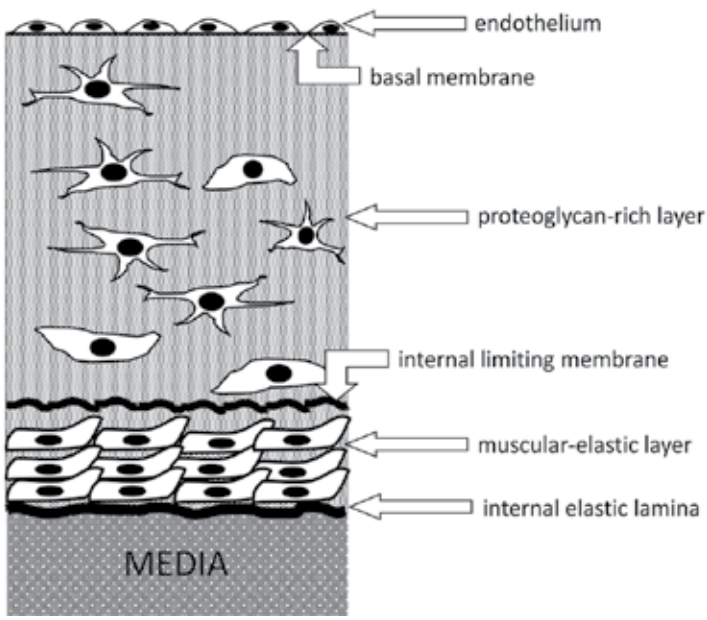
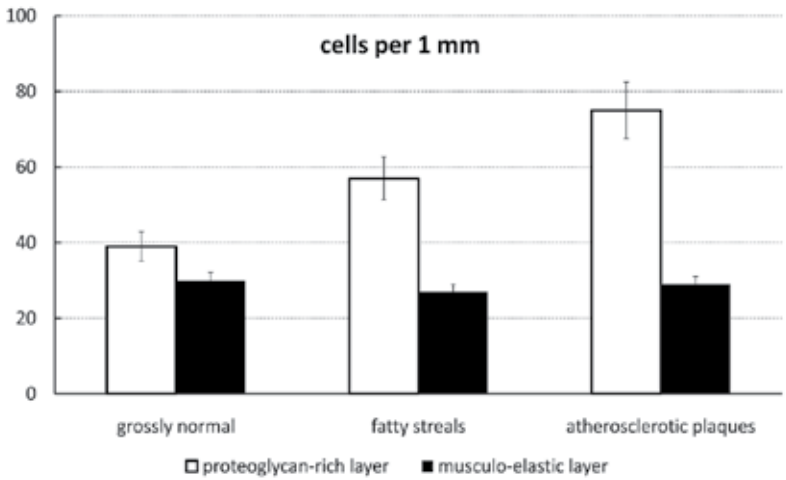


Figure 1. The intima is separated from the media by the internal elastic lamina. The innermost proteoglycan-rich sublayer of the human intima is separated from the muscular-elastic layer, adjacent to the media, by the internal limiting membrane. Intimal layers differ from each other in cell arrangement and by intercellular matrix organization. The muscular-elastic sublayer consists of several rows of longitudinally arranged elongated cells separated by elastic fibers, also longitudinally oriented. The connective tissue fibers of the proteoglycan-rich layer have no definite orientation to the vessel axis, and its cell population is morphologically heterogeneous, as was described as far as in the nineteenth century [24]. Histological and histochemical observations showed that in the normal intima, the muscular-elastic layer contains much more elastic fibers than the proteoglycan-rich layer, while the proteoglycan-rich layer contains more collagen and reticulin fibers [20, 23]. The layers differ from each other by the composition of glycosaminoglycans [20].

3. Cellular composition of normal and atherosclerotic intima of the human aorta

Cell numbers in the intimal layers of grossly normal areas and atherosclerotic lesions have been determined after alcohol-alkaline dissociation of tissue [26]. In the proteoglycan-rich layer of a fatty streak and an atherosclerotic plaque, the number of cells was found to be 1.5- and 2-fold higher than that in undiseased intima, while in the muscular-elastic layer of atherosclerotic lesions, the number of cells was found to remain practically unchanged in comparison with the normal intima [25].

Similar results were obtained during analysis of aortic cross sections [25]. Cell number in the muscular-elastic layer of uninvolved and atherosclerotic intima is similar, while the number of cells in the proteoglycan-rich layer of atherosclerotic lesions is twofold higher than in normal intima (Figure 2). Thus, atherosclerotic manifestations in the vascular wall coincide with an increase in the cell number of the proteoglycan-rich layer.



Cell number in the intimal layers was determined in suspension after alkali-alcohol dissociation on fixed tissue [25].
PG - proteoglycan-rich layer of the intima, ME - musculoelastic layer of the intima.
* - significant difference from normal, p<O.05

Figure 2. Cell number in different layers of the human aortic intima.

What are the cells populating different layers of the intima and what are the differences between these cells?

Cellular composition of the intima of human arteries has been studied for more than one century [27-30]. In classic works in this field, a special attention has been paid to the fact that the cell population inhabiting vascular walls is heterogeneous and consists of two subpopulations: resident vascular cells and round cells that are morphologically similar to peripheral blood monocytes and lymphocytes (inflammatory cells). In those studies, it was noted that

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resident subendothelial cells differ from typical smooth muscle cells of the media [28, 29]. These cells were referred to as intimal fibroblasts [27], mesenchymal reserve cells [28], pericytes or vascular cambium [30], etc. However, the application of electron microscopy studies led to the concept that *intimacytes* of major arteries are modified smooth muscle cells [31, 32]. Figure 3 shows typical ultrastructural features of cells that represent the main population among the subendothelial cells. The structural features of these cells include the presence of individual myofilaments and bundles of myofilaments in cell cytoplasm, the presence of "dense bodies" seen in the cytoplasm along the cell-surrounding membrane, and the presence of basal membrane surrounding the cell membrane extracellularly, a combination suggesting smooth muscle nature of these cells [31-33].

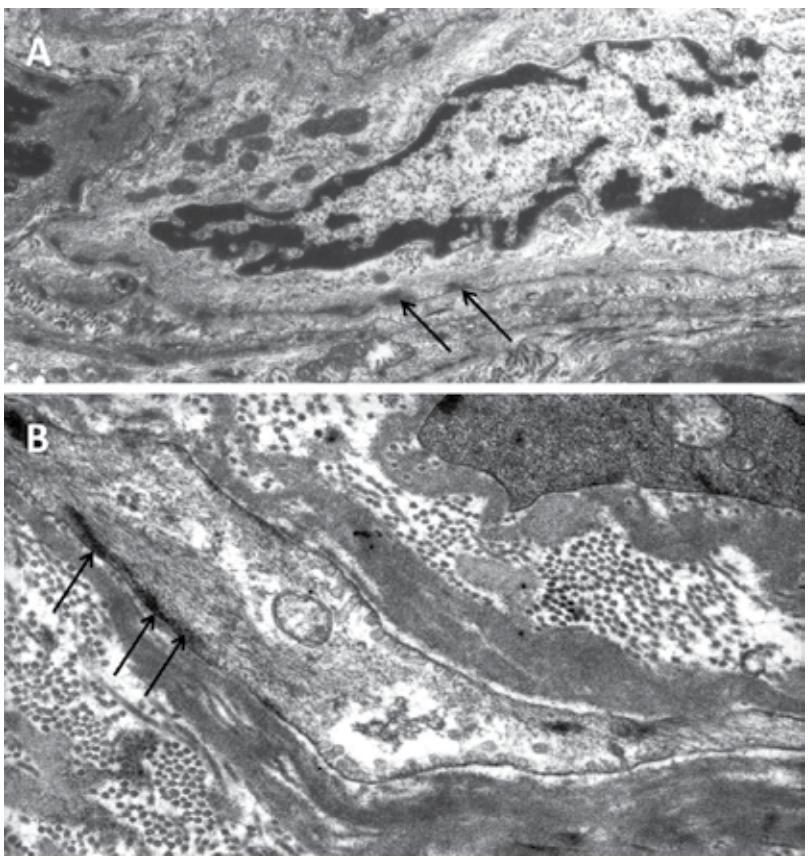

Figure 3. Electron micrographs showing ultrastructural appearance of *intimacytes* most frequently seen in the subendothelial space of undiseased aorta (**A**, **B**) (up to 90% of the total cells of the tunica intima of the undiseased aorta). Image (**A**): cell body; image (**B**): cell process. In (**A**, **B**), note the presence of myofilaments and the basal membrane; arrows show "dense bodies," a reliable ultrastructural criterion for the identification of smooth muscle cells. The presence of myofilaments and the basal membrane further supports the identification of the cells as smooth muscle cells.

4. Immunocytochemical typing of subendothelial cells

The use of antibodies specific to various types of mesenchymal cells has provided additional information regarding the cellular composition of normal and atherosclerotic intima. In addition to cells that expressed smooth muscle α-actin [34-37], immune-inflammatory cells such as macrophages [38-42], lymphocytes [40, 42-47], mast cells [48], and dendritic cells [49] were identified in the aortic intima.

Using a set of cell type-specific cytochemical markers, cells of undiseased and atherosclerotic intima have been characterized (Table 1). The majority of cell population in normal and atherosclerotic intima was found to be represented by cells expressing smooth muscle α-actin. About two-thirds of cells in the muscular-elastic layer expressed smooth muscle α-actin, while in the proteoglycan-rich layer, the proportion of these cells was lower (Table 1). Immune-inflammatory cells (lymphocytes and macrophages) were confined preferentially to the juxtaluminal part of the proteoglycan-rich layer. Their proportion was found to increase in atherosclerotic lesions, reaching 20% of the total cell content.

Examined area	Positively stained cells, %						
	Smooth muscle α-actin	**CD45**	**CD68**	**3G5**	**2A7**	**Inflammatory cells (CD45+CD14)**	**Resident cells**
Grossly normal [0][a]	47.6±2.3 (4)[b]	2.2±0.4 (3)	3.9±0.4 (5)	31.3±7.0 (4)	0.0±0.0 (3)	5.5±1.2 (8)	97.3±0.6 (8)
Initial lesions [I]	47.2±3.1 (3)	6.2±1.2 (4)	6.1±1.4 (4)	6.3±1.0* (3)	1.2±0.3 (3)	9.6±1.4* (6)	90.9±1.2 (6)
Fatty streaks [II]	42.2±3.1 (4)	5.0±0.9* (3)	13.2±0.8* (5)	11.7±2.0* (8)	3.0±0.7 (3)	13.4±1.5* (10)	89.4±1.1 (10)
Fibrolipid plaques [Va]	47.0±10. 9 (5)	6.2±1.8* (9)	13.1 ±2.3* (4)	5.0±0.7* (5)	27.0±3.1 (3)	18.7±2.0* (8)	84.7±1.4 (8)
Fibrotic plaques [Vc]	ND	ND	ND	ND	ND	6.4±1.9 (6)	94.9±1.6 (6)

[a]: Lesion type according to AHA Council on Atherosclerosis [50] is indicated in the square brackets.

[b]: The number of cases examined is indicated in the round brackets.

*: Significant difference from the percent of positively stained cells in grossly normal areas, $p<0.05$.

ND: Not determined.

Table 1. Immunocytochemical identification of cells in human aortic intima

The presence of pericyte-like resident cells in normal and atherosclerotic human aortic intima have been demonstrated [10, 11], using 3G5 antipericyte antibody [51]. The antigen for 3G5

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antibody represents O-sialoganglioside of the plasma membrane of brain capillary pericytes [51], which are known as a very stable, slowly renewed cell population [52]. It has been stated that this antigen is typical for quiescent pericytes [51]. Previously, 3G5-positive pericyte-like cells were identified in bovine aorta, in human undiseased intima, and in complicated atherosclerotic plaques with ectopic osteogenesis [10]. These cells account for more than 30% of the total intimal cell population (Table 1). On cross sections, cells bearing 3G5 antigen were found to present only in the uppermost subendothelial layer of the intima. On *en face* preparations, 3G5-positive cells with long processes form a network immediately under the endothelium (Figure 4) [1, 11-14]. The subendothelial localization of pericytes suggests that in the aorta and large arteries, 3G5-positive cells perform the functions similar to those of capillary pericytes [1, 11].

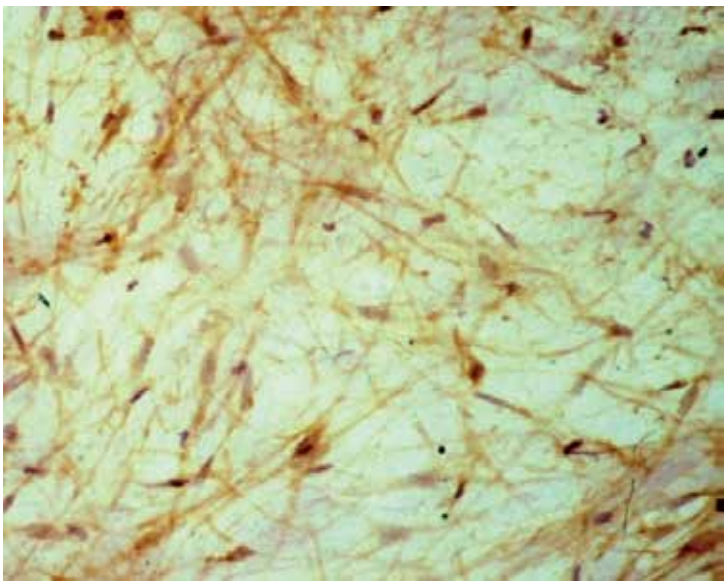

Figure 4. Positive cells by means of their long processes form a network immediately under the luminal endothelium in undiseased intima. *En face* specimen of the human aorta; immunohistochemistry.

It has been suggested that pericytes contribute to various pathophysiological processes associated with microcirculation dysfunctions, including diabetes, inflammation, wound healing, hypertension, tumor growth [52-54], and also regulate endothelial functions, including endothelial cell proliferation and ion and molecule exchange [53-57]. At the present time, the major predisposition of pericytes is assumed as precursors for other cells of mesenchymal origin, including smooth muscle cells [54], osteoblasts [56-59], chondrocytes [54], and adipocytes [60]. This is consistent with the earlier concepts that consider subendothelial cells as pluripotent cambial cells [30] and mesenchymal reserve cells [28]. The network formed immediately under the luminal endothelium by pericyte-like cells has been suggested to play a role in immune function of the arterial wall, especially taking into account that pericyte-like cells express HLA-DR antigen [1].

A comparison of the number of pericyte-like cells in atherosclerotic lesions with the number of pericyte-like cells in undiseased intima revealed that in atherosclerotic lesions, the number of the cells possessing 3G5 antigen is much lower than that in undiseased intima (Figure 5).

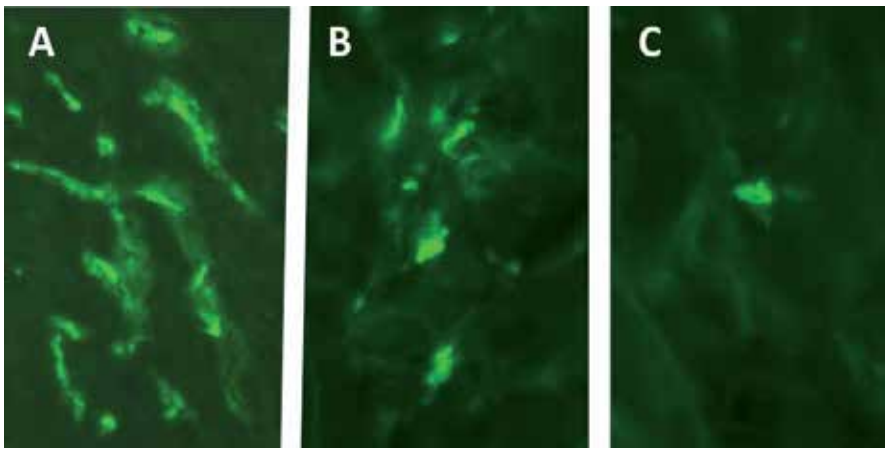

Figure 5. Visualization of 3G5+ cells in normal (undiseased) intima (**A**), in fatty streak (**B**), and in atherosclerotic plaques (**C**) by means of immunofluorescent analysis.

This could suggest that the number of pericyte-like cells in atherosclerotic lesions decreases. However, the results obtained in experiments with the use of antipericyte antibody 2A7, which recognizes another pericyte antigen, argue with such a suggestion. 2A7+ cells were found to appear in atherosclerotic plaques, while 2A7+ cells are absent in normal intima (Table 1). Anti-2A7 represents an antibody against melanomaassociated high-molecular-weight antigen (HMW-MAA), which is a chondroitin sulfate proteoglycan (also termed as melanoma proteoglycan), which is also present on pericytes in the areas of active angiogenesis (in granulation tissues in healing wounds, synoviitis, etc.) [61]. 2A7 is expressed by “activated” pericytes capable of proliferating [61].

The above-described findings led to a question: are 3G5 and 2A7 pericytic antigens expressed by the same cell or by different cell types? In order to answer this question, a culture of human brain pericytes was used for experiments. It has been found that in human brain pericytes, about 40% of cells express 3G5 antigen and 80% of cells express 2A7 antigen. Simultaneous staining for both antigens revealed 80% of positively stained cells indicating that all cells having 3G5 antigen expressed 2A7 antigen; however, there was a population of pericytes that expressed only 2A7 antigen. As was mentioned above, 2A7 and 3G5 antigens were described on the cells in different functional states: 3G5 antigen is typical for "quiescent" but 2A7 for "activated" pericytes, respectively. Activated 2A7-positive pericytes are capable of intense replication. It can be hypothesized that most of the cultured pericytes are "activated" (as a result of serum stimulation); therefore, only a part of these preserves 3G5 antigen, which is typical for quiescent pericytes.

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It is well known that lipid accumulation is one of the most pronounced manifestations of atherosclerosis. Lipid accumulation may influence the expression of pericyte antigens in the subendothelial cells. In fact, a 1.5- to 2-fold increase in the intracellular lipid content induced by modified low-density lipoproteins (modLDL) leads to a decrease in the proportion of 3G5-posilive cells (Figure 6). The total cell number remains unchanged, which indicates that a decrease in the proportion of 3G5+ cells does not result from selective death of these cells but is due to disappearance of 3G5 antigen caused by intracellular lipid accumulation. This occurs in atherosclerotic lesions, where the number of 3G5-positive cells is lower than in normal intima. It can be suggested that the functional state of the pericyte-like cells in the proteoglycan-rich layer of the intima is changed upon formation of the atherosclerotic lesions, which is accompanied by alterations in antigenic expression.

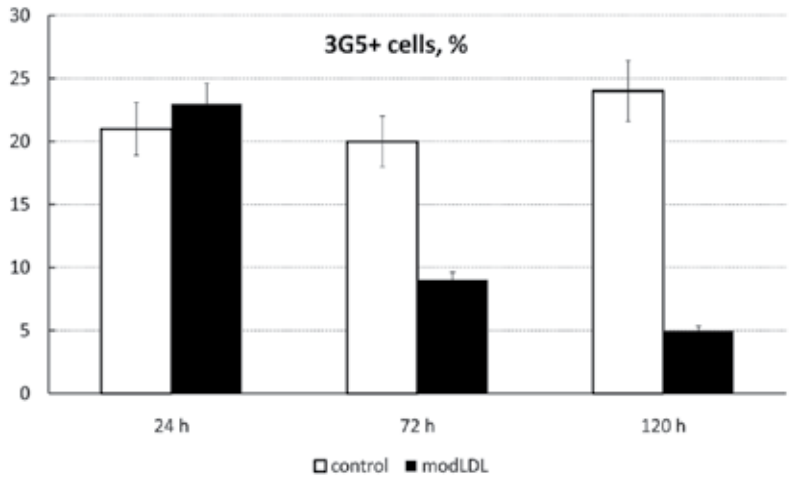

Figure 6. The effect of intracellular lipid accumulation on the expression of 3G5 pericyte-associated antigen in cultured cells from undiseased human aortic intima. On day 4, cells were washed with sterile PBS and incubated for 24, 72, hand 120 h in Medium 199 with 10% LDS (control), Medium 199 with 10% LDS + 100 μg/ml native LDL (not shown), or Medium 199 with 10% LDS +100 μg/ml modified (desialylated) LDL (modLDL). After incubation, cells were washed with PBS and stained with antibody against 3G5 pericyteassociated antigen. Total cell number and number of positively stained cells were calculated in each cell culture in triplicates. The data are representative of the three experiments performed. The data obtained with native LDL were similar to results without LDL and were not shown.

5. Inflammatory and resident cells

Immunocytochemical analysis of the cellular composition of the intimal layers showed that the cell population of the muscular-elastic layer is homogeneous, consisting predominantly of smooth muscle cells. These cells are similar to the medial smooth muscle cells, the majority of which react with antimuscle α-actin antibodies. The proteoglycan-rich layer of the intima is populated by resident and inflammatory cells. In the atherosclerotic lesions, the proportion of resident cells expressing smooth muscle α-actin is similar to that in uninvolved intima, while

the proportion of inflammatory cells increases, but these cells do not become predominating. Besides the smooth muscle cell antigen, resident intimal cells express pericyte antigens as well as the macrophage-associated antigen CD68.

What are the specific features of resident intimal cells? Morphological heterogeneity of intimal population consisting of processed cells of various shapes was described by Langhans [27], Schonfelder [62], Schlekunov [30, 63], Khavkin [64], Geer and Haust [19], and others. Elongated bipolar cells typical of the media predominate in the population of the muscular-elastic layer. At the same time, the cells of proteoglycan layer markedly vary in shape: they are elongated and stellate, with a variety of intermediate shapes. The presence of processed star-like cells [27] is the major characteristic of the proteoglycan-rich layer.

The morphological forms of resident subendothelial cells (elongated and stellate) have been described in primary cultures of enzyme-isolated cells from normal and atherosclerotic human aorta [65]. Elongated cells have a long body without processes or with small side processes. They are packed in compact cell layers, express α-actin, and have a well-developed contractile apparatus [65]. Stellate (pericyte-like) cells have a round body with three or more processes. They are unevenly distributed in the loose connective-tissue matrix of the intima. In addition to α-actin, some juxtaluminally located cells express 3G5 and 2A7 pericytic antigens and CD68 macrophage-associated antigen [13], a scavenger receptor [39]. ln atherosclerotic lesions, the cytoplasm of these cells is filled with lipids (foam cells) [66]. Electron microscopic studies revealed considerable numbers of synthetic organelles in these cells [14]. In the atherosclerotic lesions, the number of stellate cells increases sixfold while the total number or cells and the number of elongated cells increase only twofold. Thus, both quantitative and qualitative changes occur in cellular composition of the intima underlying atherosclerotic lesions.

6. Stellate cells and atherosclerosis-related manifestations

Is there any relationship between changes in cellular composition and the well-known manifestations of atherosclerosis such as intimal thickening and accumulation of lipids and collagen? The strongest correlation was established between the number of stellate cells, on the one hand, and the content of cholesteryl esters and total lipids, on the other [14]. The correlation coefficients between the number of stellate cells and collagen content and intimal thickness were somewhat lower; nevertheless, they remained greater than the correlation coefficients for elongated cells and the total cell content [14].

Stellate cells are located in the close proximity to endothelial lining; they are the first barrier for the compounds entering the vascular wall from the blood. This may account for the great extent of the involvement of the stellate cells in atherosclerotic manifestations in the vascular wall [1, 14].

The direct correlation between the number of stellate cells and atherosclerotic manifestations raised a number of questions. Why do cells become stellate? Are stellate cells specific to cell type or does this shape reflect the functional state of a cell? In the human body, there are other

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tissues containing stellate cells, for example, neurons and other nervous cells, dendritic cells of lymphatic follicles, Ito cells in the liver, mesangial cells in renal glomeruli, etc. [1]. These cells serve predominantly communicative functions via a system of their processes [1]. A similar function can be ascribed to stellate cells of subendothelial intima, which form an integrating communication network by their long branching processes [1].

In primary cultures, intimal cells are generally flattened and polygonal. In these cell cultures, arborization, i.e., formation of stellate cells, using cAMP elevators, was induced [12]. Almost all cells derived from the proteoglycan-rich layer become stellate, while the content of stellate cells is not higher than 50% in cultures from the muscular-elastic layer and about 10% in cultures from the media [12].

Arborization is accompanied by redistribution of connexin 43 (Cx43), a major protein of specific cell-to-cell contacts (gap junctions). This protein is localized on cell surface in specific structures, so-called Cx43 plaques. On nonarborized cells, small Cx43 plaques are unevenly distributed over the cell surface. In addition to small Cx43 plaques, on arborized (stellate) cells, large Cx43 plaques are localized predominantly on the ends of the cellular processes in the areas of the cell-to-cell contacts [12].

Thus, an increase in the intracellular cAMP content leads to arborization of subendothelial intima cells. Changes in the cell shape are accompanied by redistribution of connexin 43 plaques and, probably, by increase of the rate of the intercellular communication via gap junctions. This suggests that the stellate shape is important for the formation of cell-to-cell contacts in the intima. Subendothelial intimal cells differ from medial smooth muscle cells, which are poorly or not arborized, by the ability to form these contacts. Therefore, it is reasonable to suggest that subendothelial intimal cells are a specific cell type distinct from typical smooth muscle cells.

7. Cellular network in the intima

Based on the observations of *en face* preparations of the intima, Schonfelder [62] has suggested that stellate intimal cells are joined into a common network. He supposed that empty spaces between the interlaced cellular processes are the channels reaching deep into the intima.

The concept of a common cellular network in the intima of the human aorta was confirmed later [67]. Using scanning electron microscopy, it was shown that all cells in the proteoglycan-rich layer of uninvolved intima are interconnected in the horizontal plane and form a common network [67]. The cells are also joined to each other in the vertical plane. Thus, a three-dimensional cellular network is formed. All cell processes contact with the processes or bodies of other cells. Consequently, the proteoglycan-rich layer cannot be regarded as a sum of separated cells but as a unified cellular system. Empty spaces between the processes filled with the connective tissue matrix were also observed [67], as described by Schonfelder [62].

Cells of the muscular-elastic layer do not form a network characteristic of stellate cells in the proteoglycan-rich layer. Scanning electron microscopy showed that most of the cells have a

bipolar elongated shape characteristic of medial smooth muscle cells. In the muscular-elastic layer, densely packed cells form strata oriented at a small angle to each other [67].

A continuous vertical gradient in the ratio between the number of stellate subendothelial cells and typical elongated smooth muscle cells has been discovered [67]. The number of elongated cells decreases from the muscular-elastic layer to the endothelium, while the number of network-forming stellate cells increases.

In atherosclerotic lesions, the cellular system of the intima undergoes considerable changes. In fatty streaks, many stellate cells are generally laden with lipids [14, 25, 66]. This may account for an increase in their dimensions and formation of surface bleb-like protrusions by lipid droplets and vesicles. Vesicles in the extracellular space were also found; this suggests that these vesicles are propagated by gemmation (budding) from the surface of the cells and cell processes. Vesicle gemmation from the ends of the cell processes can obviously lead to the degradation of intercellular contacts and dissociation of the common network. In atherosclerotic plaques, changes in the cell system are more pronounced. In the superficial layers of the connective tissue cap, stellate cells are always settled as separate cells or small groups. Sometimes, these cells contain lipid inclusions. In deep layers of the intima, the number of processed cells increases, but they do not form a network. Interestingly, a three-dimensional cellular network typical of uninvolved intima was found in the intima next to the plaque shoulders displaying no visible atherosclerosis-related changes.

8. Intercellular communication

Thus, it was demonstrated that the cellular network is disintegrated in the proteoglycan-rich layer of atherosclerotic lesions [67]. Eventually, this disintegration results in complete separation of cells in an atherosclerotic plaque. It is an established fact that the regulation of cells forming highly differentiated tissue systems occurs via specialized cell-to-cell contacts, gap junctions [68, 69]. It was demonstrated that these contacts play an important role in the regulation of tissue homeostasis, providing the transport of cell metabolites, second messengers, and other biologically active molecules from cell to cell without entering the extracellular space. The presence of these specialized contacts is a specific feature of the differentiated cell systems with a high degree of intercellular integration [68-73]. Stellate shape of intimal cells and their interaction via the cellular processes may substantially contribute to the vascular wall regulation. It can be hypothesized that functional disturbances in gap junctions are one of the causes of atherosclerosis-related disintegration of cellular networks formed by the proteoglycan-rich layer intimacytes [73].

The degree of intercellular communication via gap junctions can be assessed by expression of proteins forming these contacts. Connexin 43 (Cx43) is the major protein of these contacts. This protein is localized on cell surfaces in so-called connexin plaques [73]. A primary culture of aortic cells was used to elucidate the causes of reduced intercellular communication in atherosclerotic lesions. In addition to the identification of Cx43, another approach can be employed in a cell culture, namely, the transfer of fluorescent dye from the injected cell to neighboring cells. Fluorescent dye is specifically distributed only via gap junctions, and the

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rate of the communication is evaluated by the number of contacting fluorescent cells. The rate of intercellular communication in cell cultures with various densities has been estimated [73]. It was revealed that both approaches correlated very closely with each other. It was found that the intensity of intercellular communication in cultures derived from grossly normal areas is 1.5-fold higher than that in cultures obtained from atherosclerotic lesions [73].

The presence of lipid-laden cells resembling so-called foam cells was a specific feature of cultures derived from atherosclerotic lesions [73]. Assuming that there is a relationship between the content of intracellular lipids and the rate of the intercellular communication, fluorescent dye was injected into cells with and without visual lipid inclusions. The rate of gap junctional communication of cells without lipids was similar to that of cells cultured from grossly normal areas [73]. The rate of communication of foam cells was twofold lower than that of lipid-free cells. The number of Cx43 plaques per lipid-laden cell was lower than per cell without lipids [73]. It can be suggested that lipid accumulation is a possible cause of reduced intercellular communication in the intimal cell system.

On the basis of the above data, it can be concluded that stellate cells are the principal cell type of the proteoglycan-rich layer. Stellate cells of subendothelial intima differ from typical medial smooth muscle cells. In contrast to elongated smooth muscle cells, they have several long processes capable of forming a network. Stellate cells have poor contractile organelles but possess a well-developed rough endoplasmic reticulum. These cells express a number of markers (pericyte antigens 3G5 and 2A7, macrophage antigen CD86, scavenger receptor) that are not expressed by typical smooth muscle cells. These markers may determine functional peculiarities of stellate cells. The number of stellate cells increases in arteriosclerotic lesions and strongly correlates with the major manifestations of atherosclerosis. In uninvolved intima, the cells of the proteoglycan-rich layer contact with each other by their processes forming a three-dimensional network. In atherosclerotic lesions, the rate of intercellular communication decreases and the contacts between cells are impaired, presumably as a result of lipid accumulation.

9. Atherosclerotic manifestations on the cellular level

The main manifestations of atherosclerosis are associated with alterations on the cellular level, namely, lipid accumulation leads to the formation of foam cells. Local increase in the cell number is a result of cell proliferation or migration. Accumulation of the connective tissue matrix is a consequence of synthesizing activity of the cells.

10. Lipidosis (intracellular lipid accumulation)

In grossly normal areas of the intima, lipids are accumulated predominantly in the extracellular space. Initial atherosclerotic lesions are characterized by the presence of cells with lipid inclusions [29]. The proportion of cells with lipid inclusions in uninvolved intima and in atherosclerotic lesions has been determined [66]. Fatty streaks have the highest content of lipid-

laden cells (up to 25%); these cells are located in the upper part of the proteoglycan-rich layer, comprising approximately two-thirds of it. By contrast, in atherosclerotic plaques, most of the cells with lipid inclusions are located in one-third of the proteoglycan-rich layer adjacent to the internal limiting membrane. In the muscular-elastic layer, the proportion of lipid-laden cells is the highest in atherosclerotic plaques, remaining not higher than 5%. Cells with lipid inclusions were found among all cell types of the aortic intima; however, their proportion among stellate cells reaches 30%, which is considerably higher than that among other cell types.

11. Proliferation (hypercellularity)

Cell number estimated on cross sections and in the suspensions obtained by alcohol-alkaline dissociation is twofold higher in atherosclerotic lesions compared to uninvolved intima [25]. The maximum cell number was revealed in lipid-rich lesions. In pronounced lipidrich lesions (fatty streaks and fibrolipid plaques), where the cell number is maximal, the number of both resident and inflammatory cells is increased. As the bulk of the intimal cell population (84-93%) is made of resident cells, the changes in the number of resident cells determine the increase in the cell content in atherosclerotic lesions. It should be noted that the cell number of fibrotic plaque is significantly lower than that of lipidrich atherosclerotic lesions.

For the identification of proliferating cells antibody to cyclin (proliferating cell nuclear antigen - PCNA), a protein expressed in the S-phase of cell cycle was used [74]. The number of proliferating cells in the lipidrich atherosclerotic lesions (fatty streak and fibrolipid plaques) is 10- to 20-fold higher than in uninvolved intima. The number of proliferating cells in fibrous plaques was found to be lower than in fatty lesions, being significantly higher than in uninvolved intima (Figure 7).

The proliferative index (the ratio between proliferating cells and the total cell number) for resident cells was considerably higher in all atherosclerotic lesions than in uninvolved intima [9, 74]. The maximum proliferative index (eight times as high as that in uninvolved intima) was recorded for fibrous plaques. Proliferative index of inflammatory cells is higher than that for resident cells; however, it is similar in uninvolved intima and atherosclerotic lesions and is comparable to that of peripheral blood leukocytes [9, 74]. From these findings, it can be suggested that the changes in cellularity of arterial intima is the result of resident cell proliferation and, probably, the migration of inflammatory cells. A “splash” of proliferative activity of resident cells takes place in lipid-rich lesions (fatty streaks and fibrolipid plaques). Proliferation of inflammatory cells was observed in the vascular wall too; however, proliferative index of inflammatory cells does not change in atherosclerotic lesions. Presumably, proliferative activity of inflammatory cells is a background process in atherosclerotic lesions and reflects the changes in the number of inflammatory cells as a result of their migration into subendothelial intima from blood stream. In contrast to resident cells, proliferative activity of inflammatory cells is not stimulated in atherosclerosis.

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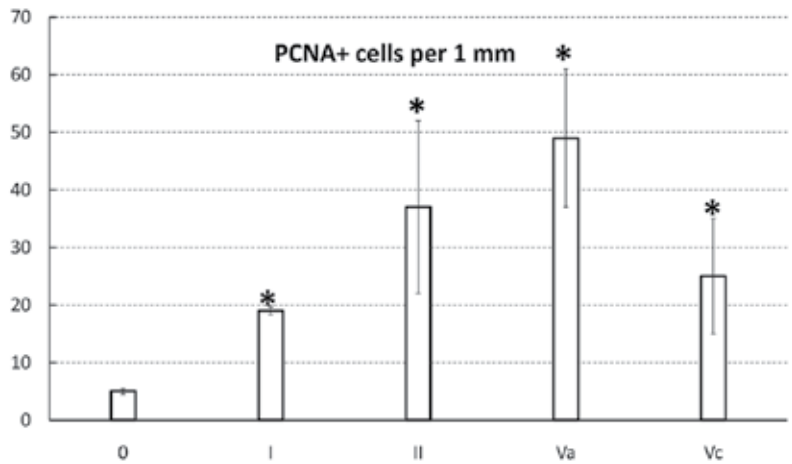

0 – grossly normal (4), I – initial lesions (5), II – fatty streaks (4), Va - fibrolipid plaques (5), Vc - fibrotic plaques (6). The number of autopsy cases is indicated in the brackets.
*- significant difference from 0, $p<0.05$.

Figure 7. PCNA-positive cells in human aortic intima. On cross sections of human aortic intima, immunocytochemical identification of PCNA-positive cells was performed. Total cell number and number of cells with positively stained nuclei were calculated on each tissue section and the data from the same atherosclerotic lesions combined. Reproduced from Orekhov AN, Andreeva ER, Mikhailova IA, Gordon D. Cell proliferation in normal and atherosclerotic human aorta: proliferative splash in lipid-rich lesions. Atherosclerosis 1998;139(1):41-8 [74], with permission from Elsevier.

12. Fibrosis (synthesis and accumulation of extracellular matrix)

Accumulation of the extracellular matrix and formation of the connective tissue cap are the most clinically significant atherosclerotic manifestations. The total collagen content increases preferentially in the proteoglycan-rich layer but not in the muscular-elastic layer [25]. In uninvolved intima, cells producing collagen type I, the main interstitial collagen accumulated in atherosclerotic plaques, were not found [53]. In the proteoglycan-rich layer of atherosclerotic lesions, collagen-producing cells account from 6% (initial lesions) to 18% (fatty streaks) of total cell population [53]. These findings agree with immunohistochemical data on the localization of various collagen types in atherosclerotic lesions [25, 75, 76]. It was demonstrated that collagen is accumulated predominantly in the juxtaluminal intima.

The formation of the initial atherosclerotic lesions coincides with the emergence of cells producing type collagen I, the major interstitial collagen, which is accumulated in the cap of an atherosclerotic plaque. Formation of fatty streaks is accompanied by a considerable increase in the proportion of collagen-producing cells. Fibrollpid plaques have the maximum content of collagen-synthesizing cells, while in fibrous plaques the proportion of these cells is significantly lower, being comparable to that in initial lesion. Thus, in the sequence initial lesions - fatty streak - fibrolipid plaque fibrous plaque, a “splash” of synthetic activity of cells with the maximum in lipid-rich lesions (fatty streaks and fibrolipid plaques) has been detected [53].

Hypersecretion of extracellular matrix may be a cause or a consequence of disintegration of the intimal cellular system in atherosclerotic lesions. In initial lesions, where the cellular network is preserved, collagen-producing cells are not numerous and are integrated into a network [53]. The network is destroyed in fatty streaks and atherosclerotic plaques. The proportion of collagen-producing cells in these lesions is higher than in initial lesions, and the cells are located at the sites of the network disintegration [53]. This suggests that synthetic activity of cells increases considerably after they have lost contact with neighboring cells.

13. Mechanisms of cellular lipidosis

Bearing in mind that the "splash" of proliferative activity and collagen production occur in lipid-rich lesions, it can be suggested that there is a relationship between the main atherosclerotic manifestations: lipid accumulation (lipidosis), proliferation, and secretion of the extracellular matrix (fibrosis). Since the proliferative activity and the collagen-synthetic activity are the highest in the zones of lipidosis, it was important to find out how the accumulation of lipids in cells (cellular lipidosis) is related to other manifestations of atherosclerosis, namely, proliferation and fibrosis. To investigate this relationship, lipid accumulation was induced in cultured cells with maximum adequacy to cellular lipidosis occurring in the vascular wall [77, 78].

Disturbances in cell metabolism of lipids may be one of the causes of intracellular lipid accumulation. In the cells cultured from atherosclerotic lesions, the rate of the synthesis of main classes of lipids is higher than that in cells cultured from uninvolved human aortic intima [79]. The rate of lipid synthesis is directly correlated with the intracellular lipid content: the higher intracellular excess of fat, the higher the intensity of lipid metabolism in atherosclerotic cells [79]. However, these findings provide no answer to the following question: what is the cause of lipid metabolism dysfunction in the vascular wall that led to excessive lipid accumulation in vascular cells?

Low-density lipoprotein (LDL), the major lipid carrier in the blood, is the main candidate for the source of lipids that overload cells of atherosclerotic lesions. Considerable efforts have been concentrated on induction of lipid accumulation in cultured cells by adding LDL to the culture medium. However, most attempts failed: even high concentrations of LDL in culture medium induced no lipid accumulation [80]. This is due to reverse regulation of cholesterol metabolism in the cell, when the number of specific LDL receptors on the cell surface decreases in response to increased intracellular cholesterol content, which prevents LDL internalization via the receptor pathway [81].

It was demonstrated in several laboratories that intracellular lipid accumulation can be induced by LDL-containing insoluble complexes, such as LDL associated with glycosaminoglycans, proteoglycans, fibronectin, collagen, elastin, and other components of the arterial wall connective tissue matrix [77, 80, 82-92]. This pathway for intracellular lipid accumulation is quite possible, since all conditions are present in the vascular wall.

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In addition, LDL association (formation of complexes including several LDL particles) is a necessary and sufficient condition for the accumulation of intracellular lipids [86, 87]. Presumably, LDL associates, like LDL-containing insoluble associates, which are essentially large particles, are internalized into the cell not via the receptor pathway but by nonspecific phagocytosis. This may result in unregulated lipid deposition and lipid overload. Native LDL generally does not form associates, while chemically modified lipoprotein readily associates, forming rather large particles. It has been shown that all known chemical modifications of the lipoprotein, including naturally occurring multiple-modified LDL, stimulate LDL association [86-88].

Atherogenic modified LDL circulating in the blood, but not native LDL, induces lipid accumulation in cultured cells [88]. Modifications occur in protein, carbohydrate, and lipid moieties of an LDL particle; as a result, its size, charge, density, immunogenicity, and many other properties change [89]. As mentioned above, lipid accumulation caused by multiple-modified LDL is observed only when modified LDL forms associates. Similar to native LDL, unassociated modified LDL does not induce intracellular lipid accumulation [77]. A direct and strong correlation was established between the ability to induce lipid accumulation and the size of LDL associate [88].

Multiple-modified LDL has been isolated from the blood of patients with assessed atherosclerosis and from healthy subjects. However, the content of modified LDL in the blood of patients with atherosclerosis of any localization is considerably higher than in healthy subjects [90]. The positive correlation was revealed between the blood level of modified LDL and atherogenic potential of blood serum, i.e., its ability to induce intracellular lipid accumulation [90].

14. Relationship between lipidosis and other atherosclerotic manifestations

Blood serum and LDL isolated from blood of atherosclerotic patients capable to induce intracellular lipid accumulation can also lead to other atherogenic manifestations in cultured cells of subendothelial human intima [91]. Preincubation of cells with atherogenic serum or LDL, causing intracellular lipid accumulation, also stimulates cell proliferation and synthesis of collagen, glycosaminoglycans, and total protein [91, 93]. Thus, blood serum and LDL of atherosclerotic patients have a broad spectrum of atherogenic properties, causing all main manifestations of atherosclerosis: enhancement of cell proliferation, fibrosis, and lipidosis. Direct contact of modified LDL with cells is not necessary for stimulation of proliferation and production of the connective tissue matrix components. Increased proliferative activity and extracellular matrix production are preserved for several days after removal of atherogenic LDL from the culture medium [91]. This finding suggests that not the interaction between LDL and cells but rather the consequences of this interaction, i.e., lipid accumulation, which coincides with increased cell proliferation and extracellular matrix production, are the crucial aspect in the realization of atherogenic potential at the cellular level.

As mentioned above, lipid accumulation affects the integrity of cellular network in human aortic intima. In the lipid-rich lesions, where the content of lipid-laden and foam cells is high, rupture of the network have been observed. It should be noted that cells not contacting with their neighbors are often overladen with lipids [67].

A decrease in the rate of intracellular communication between cultured cells caused by modified LDL which induce intracellular lipid accumulation may account for the decreasing of cell contacts and their disruption. In fact, incubation of subendothelial intimal cells with modified LDL results in accumulation of intracellular lipids with a parallel reduction of the degree of intercellular communication [73]. It is reasonable to suggest that the reduction of cell-to-cell contacts is a consequence of lipid accumulation.

15. Atherogenic manifestations and cyclic nucleotides

Simultaneous and unilateral atherogenic modifications leading to accumulation of intracellular lipids, enhanced proliferation, and hypersecretion of extracellular matrix point to a relationship between these processes. This relationship may occur at the level of cellular regulation, since all these processes are regulated by cyclic nucleotides, universal cellular regulators. It is well known that cyclic 3',5'-adenosine monophosphate (cAMP) inhibits cell proliferation, lipid synthesis and interactions between LDL and cell receptors, and synthesis and secretion of proteins (e.g., collagen and glycosaminoglycans) but stimulates hydrolisis of lipids. On the other hand, cyclic 3',5'guanosine monophosphate (cGMP) has opposite effects on these processes [94, 95].

It was found that cAMP content in atherosclerotic cells (cells populating atherosclerotic lesions) is two- to eight-fold lower than in normal intimal cells, while cGMP content in atherosclerotic cells is more than twofold higher than in normal cells [94, 96]. It was demonstrated that changes in the content of cyclic nucleotides are associated with altered activity of enzymes involved in their synthesis and degradation (cyclases and phosphodiesterases, respectively) [97]. For example, the activity of adenylate cyclase, an enzyme synthesizing cAMP, in fatty streak is lower than in uninvolved intima, being the lowest in atherosclerotic plaque. At the same time, the content of phosphodiesterase, an enzyme hydrolyzing cAMP, remains unchanged, which accounts for cAMP drop in atherosclerotic cells [97]. On the other hand, the activity of guanylate cyclase, an enzyme that synthesizes cGMP, is higher in fatty streak than in uninvolved intima, while the activity of phosphodiesterase is unchanged [97]. In atherosclerotic plaques, the situation is different: the activity of guanylate cyclase is the same as in uninvolved intima, while the activity of cGMP phosphodiesterase is much lower [97]. These changes clearly explain increased cGMP content in atherosclerotic lesions.

On the basis of numerous data, it can be stated that a decrease in the intracellular content of cAMP and an increase in that of cGMP in atherosclerotic lesions should lead to the stimulation of the main atherosclerotic manifestations at the cellular level, i.e., cell proliferation, lipid accumulation, and extracellular matrix production [95].

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A question arises: what are the causes of the atherosclerosis-related changes in the intracellular contents of cyclic nucleotides? Upon incubation of human aorta intimal cells with modified LDL, a substantial lipid accumulation and parallel cAMP decrease and an increase of cGMP increase were observed, i.e., the exact changes in cyclic nucleotides occurring in atherosclerotic lesions (Table 2). It can be suggested that changes in the cyclic nucleotide system are caused by intracellular lipid accumulation.

	cholesterol	**cAMP**	**cGMP**
natLDL	105±14	90±10	96±10
modLDL	259±31	52±7*	254±26*

The data are presented as percent of control (cells without LDL).

*: Significant difference from the control, $p<0.05$.

Table 2. The effect of modLDL-induced intracellular cholesterol accumulation on the cAMP and cGMP content in human aortic intimal cells

Bearing in mind the data on cyclic nucleotides, a more precise scheme for the development of atherosclerotic manifestations at the level of subendothelial cells can be proposed. In the intima, modified LDL forms associates or LDL-containing complexes which activate phagocytosis. This reduces cell-to-cell contacts. Since modified LDL is internalized not by the regulated receptor pathway, lipids are accumulated in intimal cells, which decreases intracellular content of cAMP and increases that of cGMP. This stimulates lipid accumulation, cell proliferation, and extracellular matrix secretion. Presumably, these events occur at the early stages of atherogenesis. Assuming that further development of an atherosclerotic lesion proceeds in the same direction, intracellular lipid content, cell proliferation, and extracellular matrix production should constantly increase. This argues with the observations, cited above, that the intensity of atherosclerotic manifestations decreases in pronounced fibrotic atherosclerotic lesions in comparison with more early lipid-rich lesions and prompts further search for causes of the bell-shaped change in cellular lipidosis as well as in proliferative and synthetic activity observed in atherosclerosis.

16. Concluding remarks

The above-presented data allows us to suggest sequence events responsible for the initiation and the progression of atherosclerotic process. Modified LDL or/and their associates enter subendothelial intima from the blood. LDL associates or LDL-containing complexes stimulate phagocytosis, which reduces intercellular communication. LDL enters the cells via a pathway other than regulated receptor lipoprotein uptake and induces intracellular lipid accumulation. This lowers cAMP and elevates cGMP, stimulating lipid accumulation, cell proliferation, as well as synthesis and secretion of the extracellular matrix. Such a situation is typical of initial

atherosclerotic changes in the intima. Further accumulation of lipids internalized by phagocytosis leads to isolation of cells, disruption of cell-to-cell contacts, and disintegration of the cellular network characteristic of uninvolved intima. Foam cells laden with lipids appear. Further lipid accumulation in intimal cells and destruction of cell-to-cell contacts stimulate cell proliferation and extracellular matrix production. This is typical of pronounced lipid-rich lesions (fatty streak and fibrolipid plaque). Secreted collagen and other components of the extracellular matrix surround the matrix-producing cells and isolate them from other neighboring cells; this eventually leads to disintegration of the cellular network. The intensity of the major atherosclerosis-related processes (intracellular lipid accumulation, cell proliferation, and extracellular matrix production) decreases considerably. This occurs in a fibrous plaque.

Proceeding from this concept, intracellular lipid accumulation induced by modified LDL is the crucial event of atherogenesis. Lipid accumulation stimulates the major atherosclerotic manifestations; at the same time, associates of modified LDL destroy the cellular network in the intima by stimulating phagocytosis. This leads to the formation of pronounced lipid-rich lesions, namely, fatty streaks or fibrolipid plaques, where the intensity of atherogenic manifestations at the cellular level is maximal.

The intensity of cell functions activated in atherosclerosis is similar in uninvolved intima and atherosclerotic plaques. However, there is a principal difference in the state of cellular systems in these zones. Although functionally the cellular system in a fibrous plaque is similar to that of uninvolved intima, structurally it is changed to the extent indicating an irreversible transformation. Therefore, transformation of fibrous plaque to uninvolved intima is impossible. Clinically, it is unlikely that fibrous plaque is dangerous, since it is a stable, low-activity formation.

The situation is completely different in lipid-rich lesions, specifically in a fibrolipid plaque (atheroma). This is an unstable lesion with maximally or near-maximally active cellular processes. Clinically, these lesions are most dangerous, since they rapidly develop, and their growth leads to so-called rupture of the plaque which is accompanied by critical local changes in homoeostasis resulting in thromboembolic events, which is the cause of vascular catastrophes such as myocardial infarction, stroke, sudden death, etc. However, fibrolipid plaques and especially initial lesions are probably reversible, since changes in their cellular system presumably are not irreversible. Based on the suggestion that lipid accumulation is the key event in the initiation and development of atherosclerotic lesions, one may assume that prevention of lipid accumulation and removal of excessive fat from cells are most effective approaches to the prevention and reversion of atherosclerotic lesions preceding the fibrous plaque.

The above-provided data is indicative that α-smooth muscle actin+ pericyte-like cells represent the key players in the development of atherosclerotic lesions. Obviously, further studies are needed to understand the mechanisms of interaction between α-smooth muscle actin+ pericyte-like cells with other cell types during atherogenesis and evaluate how functioning of α-smooth muscle actin+ pericyte-like cells affects immune-inflammatory processes in atherogenesis. The involvement of pericyte-like cells in the regulation of immune-inflammatory

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processes in atherosclerosis is supported by a finding of widespread expression of HLA-DR antigen by the vast majority of cells residing in the arterial subendothelial space [98].

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Chapter 9

Dynamic Interplay Between Smooth Muscle Cells and Macrophages in Vascular 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) and monocytes/macrophages represent major players in atherosclerotic vascular diseases. In addition to physiological and pathological roles of each cell type in atherosclerosis, dynamic interplay between SMCs and monocytes/macrophages may contribute to the pathogenesis of atherosclerosis more critically than previously understood. Activated macrophages accelerate pro-atherogenic functions of SMCs in vascular lesions. Activated SMCs promote additional accumulation of pro-inflammatory macrophages through expression of chemoattractants. More recent evidence suggests the interchangeability between SMC and monocyte/macrophage lineages. Future efforts to understand such dynamic interactions between SMCs and macrophages may provide novel insight into the pathogenesis of vascular disease and the development of new classes of medical solutions.

Keywords: atherosclerosis, smooth muscle cells, macrophages, inflammation

1. Introduction

Atherosclerosis is a complex multifactorial disorder and involves various cell types, including vascular smooth muscle cells (SMCs), macrophages, lymphocytes, neutrophils, and endothelial cells [1]. Evidence has established that each cell type changes its phenotype in response to a microenvironmental cue. SMC phenotype ranges from the undifferentiated state to differentiated state during vascular development [2]. Vascular lesions in adults also exhibit such

phenotypic diversity of SMCs, which often mirrors their functions (e.g., contractile vs. synthetic SMCs). The role of lesional SMCs appears to vary depending on the disease context and stage of the disease. The production of extracellular matrix by SMCs often contributes to the lesion development [3, 4], but also may exert beneficial effects (e.g., stabilizing the fibrous cap of atherosclerotic plaques) [5, 6]. SMCs often reside in vascular lesions in close proximity to macrophage clusters, and appear to be influenced by factors released from inflammatory cells. Particularly, macrophages in the lesion may promote activation and pro-atherogenic functions of vascular SMCs.

In the middle of the nineteenth century, German pathologist Rudolf Virchow made significant contributions to cardiovascular medicine. He identified the formation of tunica intima as a key atherosclerotic change and suggested that the contents of the intima promote expansion of matrix components [4, 7]. He further indicated that infiltrated leukocytes may contribute to the pathogenesis of atherosclerosis. Numerous studies have subsequently unraveled the role of immune cells, leading to a widely accepted theory that atherosclerosis is a complex chronic inflammatory disorder [1, 4, 8].

Coronary and cerebrovascular atherosclerosis underlies life-threatening complications such as acute myocardial infarction and stroke. Major risk factors, including dyslipidemia, accelerate the development of atherosclerotic changes in arteries. Elevated levels of low-density lipoprotein (LDL) cholesterol in the circulating blood cause dysfunction of endothelial barriers and infiltration of circulating leukocytes (e.g., monocytes) into the artery wall. Monocytes differentiate into macrophages within the subendothelial space. Accumulating LDL undergoes oxidative modifications in the arterial wall (oxidized LDL), which is then recognized and taken up by macrophages, leading to the accumulation of lipid-laden foam cells. Foam cells secrete proinflammatory mediators that facilitate lipoprotein retention and maintain vascular inflammation [9, 10]. To minimize the risk of atherosclerotic complications, primary and secondary prevention strategies seek to control risk factors such as hyperlipidemia. LDL-lowering drugs (e.g., 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors or statins) reduce the onset of acute complications of atherosclerosis.

Bone marrow-derived progenitor cells, including endothelial progenitor cells (EPCs) and smooth muscle progenitor cells (SMPCs), also may serve as a source of atherosclerosis-related cell lineages [11, 12]. Pioneering work suggested that these progenitors differentiate into mature and functional endothelial cells (ECs) and SMCs, respectively, in physiological and pathological settings [11, 13]. However, their functional contributions to atherogenesis remain unclear [14-17]. The evidence also indicated that some intimal SMC may originate from circulating monocytes or their subset [14, 18, 19]. In contrast, other lines of evidence have proposed an opposite direction of transdifferentiation of SMC into macrophage or macrophage-like cells [20-25].

In this chapter, we address the functions and interplay of SMCs and monocytes/macrophages present within the pathological arterial wall. We also discuss emerging concepts of the interchangeability of these two cell lineages. Better understanding of these complex biologies may provide important insight into the mechanisms and new therapeutic strategies for vascular diseases.

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2. The many faces of SMCs in vascular disease

SMCs exerts various functions during development, in normal homeostasis in adults, and in the pathogenesis of vascular diseases [2]. While vascular tissues develop, SMC need to migrate, and produce selected proteins that contribute to these functions. After birth, SMC are highly specialized in contraction, which is their main role in normal homeostasis for regulation of vessel tone and diameter to control of blood pressure and flow. Adult SMCs thus express specific contractile proteins, ion channels, and signaling molecules that are unique to this muscle compared with other muscles, including the skeletal muscle and cardiac muscle [2, 3]. Such SMC-selective or -specific genes thus serve as markers of SMCs. These include the proteins that comprise the contractile apparatus including SM α-actin, SM myosin heavy chain (SM-MHC) isoforms SM1 and SM2, h1-calponin, SM22α, and smoothelin [3]. However, some of these SMC markers are expressed, at least transiently, in other cell types during development, tissue repair, or disease states, while SM-MHC isoforms are specific to SMCs [2]. Therefore, SM-MHC isoforms are the most definitive markers for SMC.

Accumulation of SMC in the subendothelial space contributes to the formation of the intima, as a precursor of the future atherosclerotic plaques. The process appears to involve migration and proliferation of activated SMCs. Where those SMCs originate from is an interesting question that we discuss later. The ability of SMC to migrate and proliferate in vascular lesions often associates with their phenotypic modulation from a contractile type to a synthetic one, as characterized by loss of contractile proteins (e.g., SM-MHC) and the increased synthesis of matrix proteins (e.g., collagen) [2, 26-29]. Reduced expression of SMC-specific proteins also indicates their decreased state of differentiation. Platelet-derived growth factor (PDGF), released by platelets on the surface of dysfunctional endothelium, and activated macrophages, may participate in the loss of SMC differentiation marker genes and promote their migration and proliferation [30, 31, 32].

Collagens are major products of SMCs. The balance of collagen production by SMCs and degradation by macrophage-derived matrix metalloproteinases (MMPs) may positively or negatively contribute to the mechanisms of vascular diseases. Depending on the context, the presence of vascular SMCs within atherosclerotic plaques may be beneficial. Particularly, accumulation of fibrillar collagen in the fibrous cap of atherosclerotic plaques may be protective. Macrophage expression of collagenases of the MMP family or reduced SMC due to cell death may impair the integrity of plaques, leading to physical disruption ("rupture") and thrombosis [5, 6, 33]. Libby et al. claimed in the early 1990s that the pro-inflammatory T cell cytokine IFNγ inhibits collagen I and III synthesis by human SMCs [34]. Interestingly, IFNγ has become known to promote pro-inflammatory macrophage activation (the so-called M1 polarization). This may indicate that a pro-inflammatory microenvironment promotes plaque instability, another example of SMC-macrophage crosstalk participating in the pathogenesis of acute cardiovascular complications. There are other examples for the beneficial or adverse role of collagen production by SMCs in vascular disease. After stent implantation, migrating SMCs cover the luminal surface of stent struts, as a healing process. But SMCs often overgrow, resulting in restenosis, which remains the most prevalent complication of percutaneous coronary intervention.

3. Existence of immature SMCs in vascular disease

SMCs exhibit the diversity of phenotypes in the vascular lesions. A panel of antibodies for SMC differentiation markers enables to identify a wide spectrum of their phenotypes in experimental and human vascular diseases. In the arterial lesions after acute mechanical injury and in chronic atherosclerotic plaques of experimental animals, intimal SMC often express lower levels of SM-MHC isoforms SM1 and SM2, whereas the medial SMC usually stain positively for SM-MHC and α-actin. While SM1 expression appears at the late stage of vascular development, SM2 expression increases later, particularly after birth [2, 35-37]. SM2 thus serves as a sensitive marker of mature SMC. Aikawa et al. demonstrated that intimal SMC of the rabbit aorta 4 months after mechanical injury and the initiation of a high-cholesterol diet showed an immature phenotype as gauged by decreased SM2 immunoreactivity (Figure 1, top panels) [38]. Interestingly, dietary cholesterol lowering for 8 or 16 months induced the increased expression of SM2 in intimal SMC to the levels similar to those of medial SMC (Figure 1, bottom panels). Such SMC plasticity may depend on microenvironmental cues. Alternatively, different microenvironment may affect the balance of SMC subpopulations. Thus, the recovery of SM2 expression by lipid lowering may result from either "redifferentiation" of the same group of intimal SMC, the expansion of a subset of SMC with a mature phenotype, or the repopulation of immature SMC by mature SMC.

In human coronary arteries, SMC begin to lose their SM2 expression even in the physiological intima of young adults with no obvious signs of atherosclerotic changes such as macrophage accumulation [28]. In more advanced plaques, immature SMCs often exist in areas where macrophages accumulate. In Figure 2, SM2 was undetectable in SMC identified by SMα-actin and SM1. Co-existence of intraplaque microvessels and macrophages may indicate active recruitment of circulating monocytes into this area. A pro-inflammatory microenvironment produced by activated macrophages via the release of IL-1β, PDGF-BB, and other factors and production of oxidative stress may have promoted phenotypic modulation of SMC in this region. As we discuss later, some of these immature SMC may have originated from a subset of monocytes. Alternatively, some SMCs may have transdifferentiated into macrophage-like cells. Understanding of such crosstalk and potential interchangeability between SMCs and macrophages may provide important insight into the identification of new therapeutic targets. Of note, "redifferentiation" or "repopulation" of intimal SMC after mechanical injury also occurs in human coronary arteries. The differentiation state of SMC reduces a first few months after percutaneous coronary intervention while it increases over time [27]. The key question is where those intimal SMCs originate from.

4. The role of SMC-derived foam cells

Pathological changes in the atherosclerotic intima include increased modification of lipoproteins (e.g., oxidized LDL) and SMC uptake of modified lipoproteins. Foam cell formation by SMCs, in addition to macrophage-derived foam cells, may represent a pivotal

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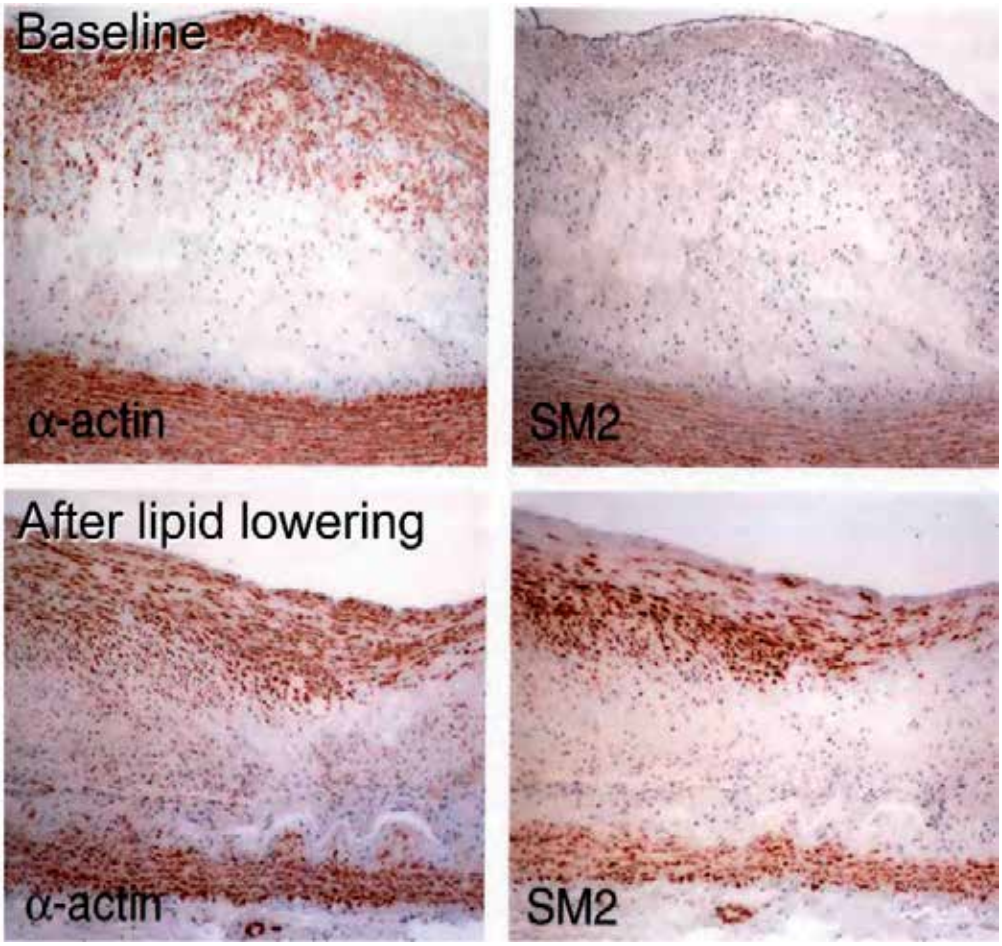

Figure 1. Accumulation of immature SMC in the arterial intima. Top panels: SM α-actin is expressed in a wide range of differentiated states of SMC, while mature SMCs produce SM2. In the atherosclerotic rabbit aorta 4 months after mechanical injury and the initiation of a high-cholesterol diet, medial SMC stained positively for SM α-actin and SM2. SM2 was undetectable in intima SMC identified by SM α-actin antibody, indicating their immature phenotype. Bottom panels: Dietary cholesterol lowering for 16 months increased SM2 immunoreactivity in the intima. The data indicate highly plastic nature of SMCs ("re-differentiation"). Alternatively, a repopulation of intimal cells by a more mature SMC subset may have resulted in increased SM2-positive cells, although their origin is unknown.

step in the transition of physiological intimal thickenings into nascent atherosclerotic lesions. [39]. Atherogenic lipoproteins also promote SMC growth by modulating calcium (Ca2+) signaling [40, 41]. SMC foam cell formation may, in part, result from the increased uptake and impaired clearance of lipids [42]. SMCs within intimal thickenings express increased levels of receptors regulating endocytosis of modified lipoproteins, including scavenger receptor A (SR-A), CD36, lectin-type oxidized LDL receptor 1, and low-density lipoprotein receptor-related protein 1 (LRP1) [43]. In parallel, the expression of ATP-binding cassette transporter A1 (ABCA1) and apolipoprotein A1, key molecules for reverse cholesterol transport, decreases in intimal SMCs [39].

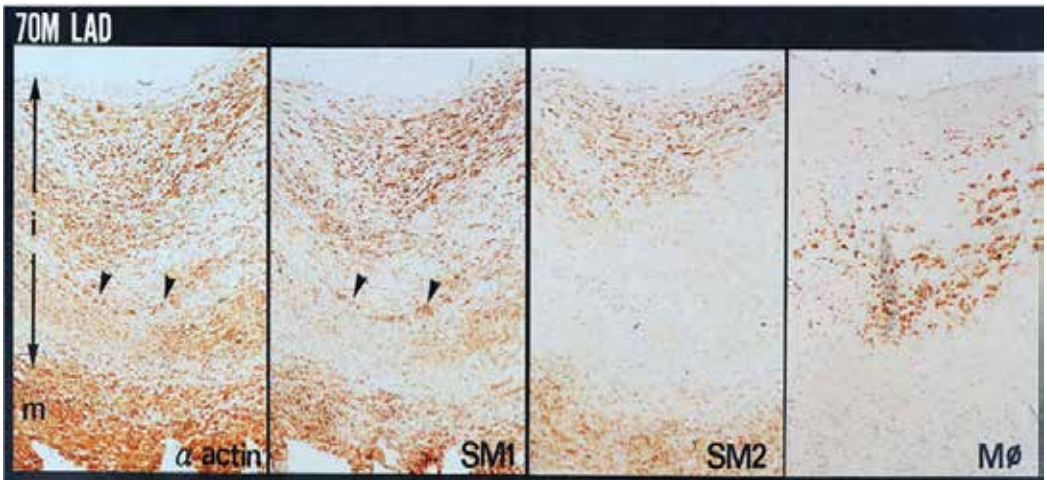

Figure 2. Decreased SMC differentiation in macrophage-rich regions of human coronary arteries. Immunohistochemistry of SMC differentiation markers in the left ascending coronary artery (LAD) of a 78-year-old male had developed the thickened intima. SM α-actin covers a wide spectrum of SMC differentiation. SMC-specific myosin heavy chains isoforms are definitive markers of differentiated SMC. In particular, SM2 identifies mature SMC. In this atherosclerotic human artery, medial SMC stained positively for all three markers, suggesting an apparently "normal" phenotype. Intima SMC exhibited the diversity of SMC phenotype. Some SM α-actin expressing cells were not immunoreactive for SM1 and SM2 antibodies. Especially, many SMC did not express the detectable level SM2 in the area where many macrophages (CD68) accumulated. They may have undergone phenotypic modulation ("de-differentiation") due to a pro-inflammatory microenvironment induced by neighboring macrophages. As an alternative possibility, some of these SM2-negative cells probably co-expressed SM α-actin, SM1, and CD68, an intermediate phenotype between SMC and macrophage lineages. Of note, intraplaque microvessels surrounded by macrophages may have recruited a subset of activated monocytes as precursor cells for smooth muscle–like cells of the bone marrow origin.

In lipid-laden intimal SMCs, cholesterol accumulation induces cell death. SMC death and subsequent necrosis promote a series of pro-inflammatory events: the release of pro-inflammatory cytokines including monocyte chemo-attractant protein-1 (MCP-1) and IL-1β from the dying and surrounding SMCs [44]; migration and proliferation of adjacent SMCs [45], MCP-1-mediated monocyte infiltration. MCP-1 and IL-1β modulate SMC phenotype, growth, and MMP production [46]. Such a cascade of events accelerates a positive feedback loop of vascular inflammation. To assess the anti-inflammatory and anti-atherosclerotic effects of a monoclonal anti-human IL-1β antibody, a randomized, placebo-controlled trial entitled CANTOS is currently ongoing in high-risk cardiovascular patients [47].

5. The origin of intimal SMC

As discussed, SMCs participate in the development of atherosclerotic plaques and the onset of acute thrombotic complications. Lesional SMCs show dynamic changes in their phenotypes depending on the disease context and the stage of each disease. Where do they come from? Many studies have led to the traditional theory that intimal SMC originate from the tunica media via proliferation and migration. More recently, several lines of evidence have indicated

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that intimal SMC or intimal cells that possess phenotypes similar to SMC (often called "SM-like cells") may originate from sources other than the media, including circulating precursors, adventitial cells and local stem cells [15, 32]. The relative contribution of each of these sources, however, remains obscure, and may also depend on the disease context in humans, and models or species in experimental animals [48]. Figure 3 illustrates possible sources of intimal SMC or SM-like cells.

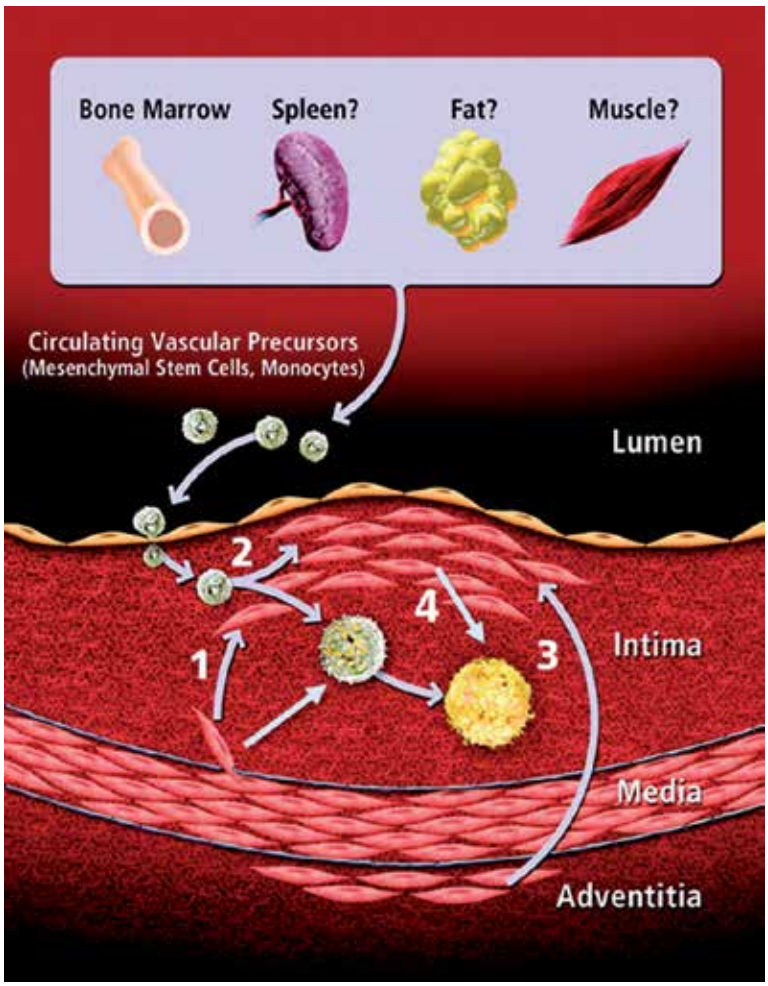

Figure 3. Potential sources of intimal smooth muscle cells (SMCs) 1) In response to injury or inflammatory stimuli, medial SMC undergo phenotypic modulation, migrate into the subendothelial space, and form the tunica intima. Resident progenitor cells in the media may contribute to intima formation. **2)** Circulating progenitor cells such as mesenchymal stem cells and activated monocytes may engraft in the intima and contribute to lesion development. Other organs, including the spleen, fat, and skeletal muscles may also release SMC progenitors. **3)** The adventitia may contain SMC precursors (resident stem cells), and contribute to intima formation, particularly after mechanical injury. **4)** Potential transdifferentiation of SMCs into macrophage- or macrophage-like cells may also contribute to atherogenesis. (Modified from Ref. 48 by Fukuda et al.)

Recent cell lineage studies have indicated that some bone marrow–derived cells express SMC-specific markers, while SMCs can display proteins associated with macrophages [15, 32]. For instance, a subpopulation of circulating monocytes may become SM-like cells in the intima [14], whereas SMC can transdifferentiate into macrophage-like cells. [25]. Therefore, the origin and fate of SMC in vascular lesions are not so clear as we traditionally thought [49]. We need to overcome several challenges to explore seemingly complex, intertwined mechanisms [32]. The limited availability of lineage-tracing methods that enable to identify the specific origin of SMC cells, particularly in disease contexts where cells tend to reduce the expression of differentiation markers. Therefore, some lesional SMCs may not be identified (false negative). As suggested by many studies, cell types other than SMCs in vascular lesions can express SMα-actin (false positive). According to a study by Caplice et al. up to 10% of cells in advanced atherosclerotic lesions of human coronary arteries expressing SMα-actin are of the myeloid lineage [24]. TGF-β or thrombin may induce macrophage expression of SMC markers including SMα-actin and SM22α [50, 51].

6. Monocytes and macrophages in vascular disease

In atherosclerosis research, monocytes and macrophages have called particular attention. Cardiovascular risk factors, such as dyslipidemia, hypertension, smoking, and diabetes mellitus, promote sustained activation of monocytes and macrophages. Activated monocytes and macrophages critically contribute to multiple processes of atherogenesis from the initiation to the acute onset of devastating complications [52-54]. Dyslipidemia provokes monocytosis by expanding the pro-inflammatory subset of circulating monocytes [55]. These inflammatory monocytes attach to vascular EC and invade the subendothelial space. Monocytes then differentiate into macrophages, although how recruited monocytes are retained and differentiate into macrophages remains incompletely understood [55, 56]. Monocytes recruitment to the subendothelial space mediated by chemokines, such as MCP-1 [57, 58] and C-C chemokine-5 (CCL5) [59], and adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on activated ECs, initiates the early processes of atherogenesis [60, 61]. The secretion of collagenolytic MMPs by atherosclerotic plaque macrophages may weaken the protective fibrous cap, causing plaque disruption and thrombosis [62-65].

7. Monocytes, subsets, and functions in atherosclerosis

Several subsets may exist in circulating monocyte in mice and humans. At least two distinct subsets of mouse monocytes are identified via differential expression levels of Ly-6C, CX3CR1, and CCR2 [66]. One subset exhibiting Ly-6C^{high}CX3CR1^{low}CCR2^{+} is designated as pro-inflammatory monocytes, and the other subset with Ly-6C^{low}CX-3CR1^{high}CCR2^{-} as resident or patrolling monocytes [67, 68]. Human monocytes are often classified into three subsets based on the expression levels of CD14 and CD16 [69]. The classical subset CD14^{++}CD16^{-}CCR2^{high} may correspond to mouse Ly-6C^{high} pro-inflammatory monocytes, whereas the nonclassical

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subset $CD14^{+}CD16^{++}CCR2^{low}$ may be equivalent to mouse $Ly\text{-}6C^{low}$ patrolling monocytes [70-72]. The intermediate subset is identified as $CD14^{++}CD16^{+}$ cells [73]. $CD14^{++}CD16^{-}CCR2^{high}$ cells appear to preferentially enter sites of atherosclerosis [74, 75]. In mice, $Ly\text{-}6C^{high}$ monocytes in peripheral circulation were induced by hypercholesterolemia [76]. Similarly, in human, elevated $CD14^{++}CD16^{-}$ monocyte levels are associated with an increased risk of cardiovascular events [77]. Human monocyte subpopulations are also associated with the status of atherosclerosis [78, 79].

In addition to the influence of phenotypes of infiltrating monocytes, as macrophage precursors, microenvironmental cues in the vascular wall may determine macrophage phenotype. In vitro studies have established the paradigm of macrophage heterogeneity. IFNγ induces a pro-inflammatory phenotype of macrophages (M1). IL-4 promote non/anti-inflammatory macrophages (M2) [74, 80]. The in vivo significance of such macrophage polarization has driven many investigations in the contexts of cardiovascular and metabolic disorders [81-86]. More specific terms, M(IFNγ) and M(IL-4), rather than M1 and M2, have been recently proposed [87].

Hyperlipidemia causes the generation of monocytes in bone marrow through medullary hematopoiesis and thus induces monocytosis. Hematopoietic stem and progenitor cells, however, may relocate to the splenic red pulp and differentiate into $Ly\text{-}6C^{high}$ monocytes [88]. The spleen as a monocyte pool may participate in further accumulation of monocytes/macrophages in peripheral organs such as atherosclerotic plaques. The fate and function of $Ly\text{-}6C^{low}$ monocytes also remain incompletely understood. These cells may patrol the endothelium for injury and infection and also promote wound healing [89].

8. Roles of macrophages in the development and progression of atherosclerosis

The major functions of lesional macrophages include removal of excessive lipids such as oxidized LDL and glycolaldehyde-LDL. Macrophages internalize oxidized lipids through scavenger receptors and become lipid-laden foam cells. Activated macrophage foam cells produce potent chemoattractants, such as MCP-1, which recruit additional monocytes/macrophages from the circulating blood, accelerating the positive feedback loop of vascular inflammation. The imbalance between uptake and removal causes excessive cholesterol ester accumulation and apoptosis in lesional macrophages. Due to the lack of negative feedback mechanisms of scavenger receptor expression, macrophages cannot limit the uptake of lipids and largely depend on the cholesterol efflux to maintain cellular lipid homeostasis [42]. In early atheroma, neighboring macrophages take up apoptotic macrophages (efferocytosis) [90-92]. As atherosclerosis progresses, however, efferocytosis becomes impaired [93], leading to secondary necrosis. Due to the macrophage release of their cellular contents (e.g., debris, oxidized lipids, proinflammatory mediators), secondary necrosis amplifies pro-inflammatory response and develop the necrotic core within the lesions.

The balance between proinflammatory and anti-inflammatory populations of accumulating macrophages may determine the development of atherosclerotic plaques [53]. Active pro-

inflammatory responses of macrophages may destabilize atherosclerotic plaques. The production of MMPs by macrophages may degrade collagen in the fibrous cap and make plaques susceptible for plaque rupture and thrombosis. Fukumoto et al. and Deguchi et al. used genetically altered mouse strains to provide the first in vivo direct evidence for the role of collagenases of the MMP family for the loss of fibrillar collagen within the intima [94, 95].

As mentioned, emerging data have proposed the heterogeneity of macrophages. A subpopulation of T lymphocytes (Th1 cells) secretes such as IFNγ, IL-2, IL-12, and TNFα and promotes the activation of macrophages toward a pro-inflammatory phenotype (M1). Th2 cytokines (e.g., L-4 and IL-13) induce an alternative form of activation toward a non/anti-inflammatory (M2) phenotype. The balance of such macrophage polarization (M1/M2 balance) may affect plaque outcome [71]. The high M1/M2 ratio in atherosclerotic plaques may induce lesion formation and plaque vulnerability [80, 96]. The evidence has identified switching of macrophage phenotypes from M1 to M2 during the regression of atherosclerosis or in response to anti-inflammatory therapies [84, 97]. Proinflammatory M1 macrophages also induce SMC proliferation [98].

Alternatively activated M2 macrophages may generally exert anti-atherogenic effects. M2 cells suppress Th1 inflammatory responses. TGF-β released by M2 macrophages may inhibit the recruitment of inflammatory cells and the development of atherosclerosis [99]. M2 macrophages also release IL-10, which inhibits the production of inflammatory cytokines from T lymphocytes and other macrophages. M2 macrophages suppress inflammatory milieu by clearing apoptotic cells and tissue debris [100, 101]. During the repair process after tissue injury, M2 cell may promote fibrosis [102], [103]. This action may potentially be beneficial in plaque instability via thickening the fibrous cap [104].

While the M1/M2 paradigm has clear relationships between stimulators and downstream effects and has thus served as a useful mechanistic model, the evidence suggests that macrophages are more diverse. In particular, M2 may further contain various forms of macrophage activation, e.g, M2a to M2d [104]. Mox macrophages develop in response to atherogenic phospholipids and have lower phagocytotic and chemotactic capacity than do conventional M1 and M2 cells [104, 105]. Mhem cells, induced by intraplaque hemorrhage, often associate with atherothrombotic complications [105, 106]. CXC chemokine ligand 4 induces M4, a recently proposed subtype of atherogenic macrophages [107]. The heterogeneity of macrophages thus seems to be more complex than previously proposed. Furthermore, in vivo functional significance of each macrophage subpopulation remains incompletely understood. Recently, new terms more specific to each stimulator were proposed, e.g. M(IFNγ), M(LPS), M(IL-10), and M(IL-4) [87].

9. Smooth Muscle Progenitor Cells (SMPCs)

Studies have identified circulating SM progenitor cells (SMPCs) and EPCs that can acquire SMC-like or EC-like phenotypes in mouse and human: [11, 13, 108]. These cell types share similar surface markers and functions with myeloid cells [109, 110] and SMCs and ECs,

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although their origin, identity, and physiological and pathological functions remain unclear. Many studies have identified BM-derived cells that express SMC or EC-specific genes within vascular lesions.

SMPCs, identified as circulating BM-derived cells, enter blood vessels and acquire phenotypes expressing SMC marker genes, particularly SMα-actin [13, 19, 111, 112]. Atherosclerotic vessels in patients who received sex-mismatched BM transplantation contain donor-derived smooth muscle-like cells, suggesting the possible involvement of circulating SMPCs in the lesion development [24]. Functions of SMPCs in the process of atherogenesis, however, remain obscure. SMPCs may promote inflammation and plaque instability by producing cytokines and MMPs [113]. SMPCs may participate in pathological angiogenesis [109]. In contrast, atheroprotective effects of SMPCs remain unknown. Further studies are needed to understand the role of SMPCs in the vascular disease.

Challenges in identification of SMC lineage are in part caused by their outstanding plasticity during the development and pathological processes. Non-SMCs also express SMC differentiation markers other than SM-MHC. For instance, ECs, fibroblasts/myofibroblasts, and macrophages express SMα-actin in certain conditions [2]. Iwata et al. used multiple SMC differentiation markers to analyze BM-derived smooth muscle-like cells [14]. BM-derived SMα-actin-immunopositive cells in vascular lesions in mice did not express the definitive SMC lineage marker, SM-MHC [14]. As mentioned, SM-MHC expression reduces in intimal SMCs after vascular injury and increases over time [27, 38] (Figure 1). Twelve months after vascular injury in mouse arteries, when resident SMCs had fully recovered SM-MHC expression, BM-derived cells did not express SM-MHC. Instead, the BM-derived SMα-actin-positive cells expressed markers of monocytes/macrophages. Moreover, we found that adoptively transferred CD11b+Ly-6C+ BM monocytes expressed SMα-actin in the injured artery (Figure 4). Interestingly, increased expression of inflammatory genes and MMPs in these BM-derived SMα-actin+ cells indicated their potential role in the remodeling processes [14]. These results suggest that an activated monocyte population can become SM-like cells in atherosclerotic lesions, which may promote plaque instability. Future investigations will further evaluate the origin and functionality of SMC and macrophage lineages in vascular lesions [114].

10. Circulating fibrocytes

Fibrocytes, BM-derived mesenchymal progenitors [115, 116], coexpress markers of hematopoietic stem cells, the monocyte-lineage, and fibroblasts. Fibrocytes may participate in various diseases, including inflammatory bowel diseases, allergy, and pulmonary and liver fibrosis. They produce extracellular matrix components as well as matrix-degrading enzymes and further differentiate into myofibroblast-like cells [117, 118]. Human fibrocytes also express genes, including Tall-like receptor 4, IL-1β, CCL2, CCL3, CCL7, CCL22, and C5aR, suggesting that they mediate inflammatory responses [119]. They may originate from CD14+ BM–derived monocytes in humans [119] and from the Gr1+CD115+CD11b+ monocyte population in mice [120], suggesting that circulating fibrocytes are a transitional cell population between mono-

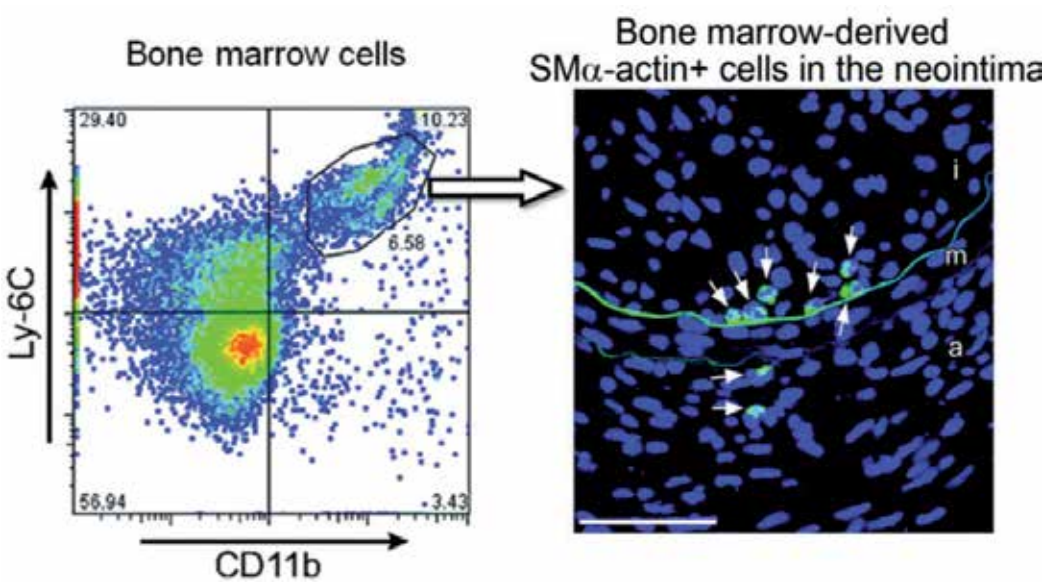

Figure 4. Bone marrow CD11b+Ly-6C+ cells migrate to the arterial wall and express SM α-actin. CD11b+Ly-6C+ cells within the bone marrow of SM α-actin-EGFP mice were sorted and adoptively transferred into wild-type mice with femoral arteries that had been subjected to wire-induced arterial injury. Four weeks after the injury, EGFP+ cells corresponding to CD11b+Ly-6C+-derived SM α-actin+ cells (arrows) were found in the walls of the injured vessel. Scale bar=50 μm. i Indicates intima; m, media; and a, adventitia (Modified from Ref 14 by Iwata et al.).

cytes and fibroblasts. Considerable overlaps exist in the gene expression profiles among human monocytes, macrophages, fibrocytes, and fibroblasts [121]. Human fibrocytes also may differentiate into cells with characteristics of adipocytes, chondrocytes, and osteoblasts [122, 123]. In human peripheral blood, 0.1% to 0.5% of nucleated cells are circulating fibrocytes that express type 1 and 2 collagens, vimentin, and SMα-actin [124]. Because no single marker can unequivocally identify fibrocytes, the combined use of collagen and other surface markers, including CD34, CD45, and CD68, is a common approach. More recent studies have used a combination of CD45RO, 25F9, and S100A8/A9 or CD49.

The fibrous cap of human atherosclerotic lesions contains fibrocytes expressing procollagen I and CD34 [125]. Subendothelial SMα-actin–positive myofibroblasts expressing the monocyte marker CD68 have been found in lipid-rich areas of the atherosclerotic intima in human aorta [126]. The overexpression of TGF-β1 resulted in the increased accumulation of fibrocytes in atherosclerotic plaques of Apoe-/- mice [127]. The pro-inflammatory monocyte subset $CD14^{+}$ $^{+}CD16^{-}CCR2^{high}$ may be precursors of fibrocytes. The expression profile of marker genes indicates considerable overlaps between fibrocytes, SMPC, smooth muscle–like cells, and monocytes/macrophages, suggesting the importance to clarify the relationship in lineages and functions between these cell types to unfold intertwined mechanisms for atherosclerosis and provide insight into the development of new classes of therapeutics.

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11. Macrophage influence over SMC activation

Macrophages and SMCs play pivotal roles in vascular diseases. The evidence suggests the interplay between these cell lineages. Macrophages promote SMC activation. In vitro co-culture studies showed macrophage-derived PDGF promotes SMC growth [128]. IL-6 released by macrophages promotes SMC MMP-1 production [129]. Several in vivo studies identified macrophage MCP-1 and its receptor CCR2 depletion as instigators of SMC activation [130-132]. Macrophage affects SMC differentiation through mechanisms of PDGF-BB, a major macrophage product. PDGF-BB suppresses SMC differentiation markers as gauged by the expression of SM α-actin, SM-MHC, and α-tropomyosin [30]. In a rabbit model of atherosclerosis, lipid lowering decreases the accumulation of macrophages expressing PDGF-B, and concomitantly increases the differentiated state of intimal SMCs, as gauged by increased expression of the SM-MHC isoform SM2 [26]. While these intimal SMCs regained SM2 expression, they decreased MMP expression. As discussed, more recent evidence indicates that BM-derived cells in the circulating blood (e.g., a subset of activated monocytes, SMPC) may differentiate into smooth muscle–like cells and contribute to the development of vascular lesions and the onset of their clinical complications [14].

12. SMC transdifferentiation into macrophages

In addition to the potential existence of intimal SMC or SMC-like cells of monocytes origin, accumulating evidence suggests transdifferentiation of SMC into macrophage lineage. Rong et al. demonstrated that cholesterol or oxidized LDL loading of SMCs suppresses SMC marker expression, but induces macrophage markers (e.g., CD68, Mac-2, and ABCA1) and phagocytic activity in cultured SMCs, raising the possibility that some macrophages within lesions may originate from SMC [20]. Bentzon et al. presented evidence that a significant fraction of lesional cells of Apoe-/- mice immunopositive for the macrophage marker Mac2 are not of the bone marrow origin [21]. Medial SMCs may undergo clonal expansion, loose classical SMC marker expression, and convert to macrophage-like cells in mouse atherosclerotic plaques [22]. Immunohistological analysis of human coronary atherosclerotic lesions demonstrated that a subpopulation (>40%) of macrophage foam cells (CD68 and oil-red O positive) co-express SMα-actin [23]. Caplice et al. showed that more than 90% of SM α-actin-expressing cells within lesions are not of the hematopoietic cell lineage [24]. More recently, Vengrenyuk et al. demonstrated that cholesterol loading converts vascular SMCs into cells similar to macrophage foam cells via the mechanisms dependent on micro RNA-143/145, and the transcription factor myocardin, and its co-activator serum response factor, responsible for SMC differentiation [25]. These studies indicate more dynamic crosstalk between SMC and monocytes/macrophage lineages than traditionally thought (Figure 5).

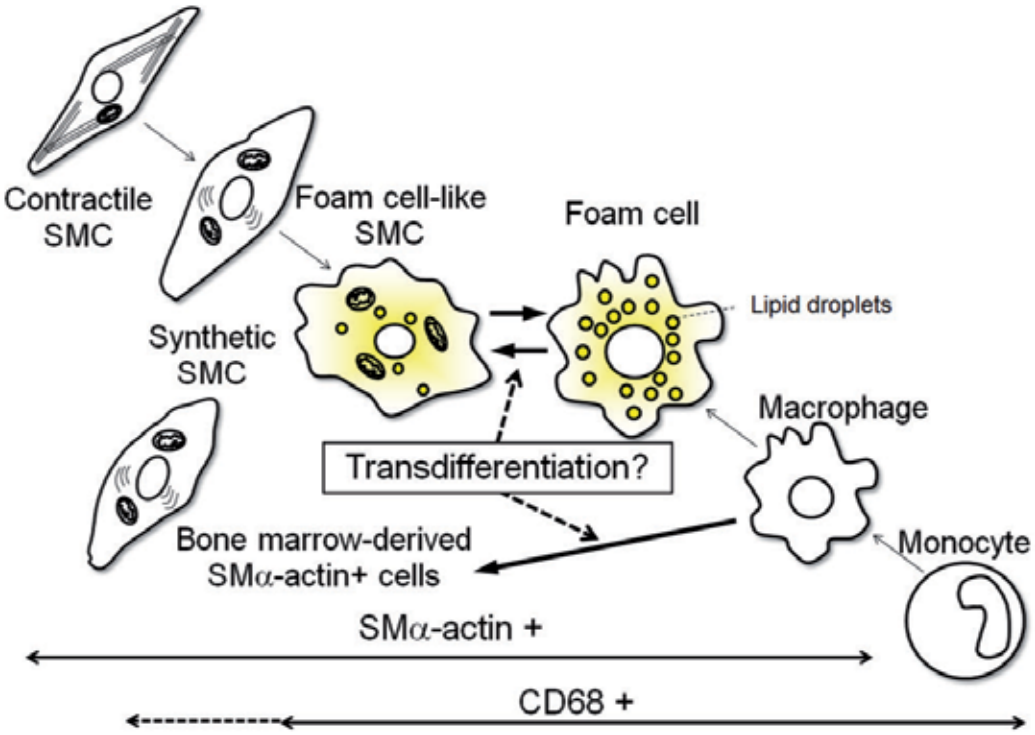

Figure 5. Possible transdifferentiation processes between SMC and macrophage lineages in the atherosclerotic plaques. The evidence suggests possible transdifferentiation between cells in the SMC and monocyte/macrophage lineages within the plaque. Various studies have demonstrated this concept in two directions: from SMC to macrophage foam cells (Ref. 20-25) and from monocytes to smooth muscle–like cells (Ref. 13, 14, 18, 19, 111,) and addressed its potential contribution to the pathogenesis of vascular disease.

13. Conclusions and future perspective

The evidence has used cutting-edge technologies, particularly in mouse models, to propose the substantial heterogeneity of SMCs and monocytes/macrophages. Due to technical difficulties in identifying SMC and monocyte/macrophage lineages in lesions, addressing the origin and the functionality of each cell type remains challenging. Lineage tracing of lesional cells in humans particularly requires highly sophisticated technologies. Gomez et al. recently reported a rigorous method with detection of histone modification at specific gene loci of SM-MHC gene [133]. Such specific cell lineage tracing methods will serve as powerful tools to provide insights into the crosstalk between SMCs and macrophages in human atherosclerosis. In addition, the use of multidisciplinary strategies, involving in vitro models, animal experiments, human samples, and more systemic approaches such as network analysis may help to unfold complex mechanisms for human atherogenesis. In addition, such strategies may identify new classes of therapeutic targets for atherosclerosis and its devastating complications

such as myocardial infarction and stroke, and may help to evaluate the effects of new therapeutics.

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Skin Pigmentation

Chapter 10

Current Challenges in Understanding the Story of Skin Pigmentation — Bridging the Morpho-Anatomical and Functional Aspects of Mammalian Melanocytes

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Additional information is available at the end of the chapter

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Abstract

Melanocytes are specialized dendritic melanin producing pigment cells, which have originated from the pluripotent embryonic cells and are termed as neural crest cells (NCC). The primary locations of these cells are basal layer of epidermis and hair follicles. Besides this, they are also found in the inner ear, nervous system, and heart with spatial specific functions. There are other cells able to produce melanin but of different embryonic origin (pigmented epithelium of retina, some neurons, and adipocytes). Melanocytes of the epidermis and hair are cells which share some common structural features but in general they form biologically different populations living in unique niches of the skin. Ultra structurally, melanocytes differ from each other on the basis of their locations and function. Principal function of epidermal melanocytes is photoprotection and thermoregulation by packaging melanin pigment into melanosomes and delivering them to neighboring keratinocytes. It is unfair to think that melanocytes reap all the glory for their role in pigmenting the skin and providing it critical protection against UV damage. They probably play a significant role in diverse physiological functions and their particular functions in all target places are much wider than the melanin synthesis only. Alternation in any structure and function of these pigmentary cells affects the process of pigmentation/melanogenesis which leads to pigmentary disorders like hyperpigmentation or hypopigmentation.

Keywords: Melanocytes, ultrastructure, receptors, pigmentation, disorders

1. Introduction

The biology of skin pigmentation plays a critical role in assorted physiological faculties from lower to higher vertebrates, like social interaction, camouflage, mimicry, sexual display as well as distinct racial coloration as seen in human beings. The pigmented biopolymer referred to as melanin is predominant in contributing color to skin, hair, and eyes of mammals. Melanin destined for pigmentation is produced in melanosomes, which are exclusively synthesized within melanocytes and in retinal pigment epithelial (RPE) [1]. Melanocytes are the key components of the pigmentary system because of their ability to produce melanin. These cells are found at many locations throughout the body. Mammalian melanocytes can be classified as "cutaneous" (follicular and epidermal) and "extracutaneous" (e.g., choroidal, cochlear) [2]. Though all of them have derived from pluripotent, NCC and have the capacity to synthesize melanin, their distinct task in all target places are much broader than the melanin synthesis solely.

The existence of melanocytes is not only restricted to the epidermis but they are also found in other locations of the human body such as hair, iris, part of heart, nervous system, inner ear, etc. Here it is important to notice that ability of melanin synthesis is not confined to melanocytes only, but also other cells such as pigmented epithelium of retina, epithelia of iris and ciliary body of the eye, some neurons and adipocytes, which can also generate melanin [3]. Two types of melanocytes have been found: first are the differentiated melanocytes of neural crest origin which are also present at various locations within the body and the other are the RPE cells, originated from the outer layer of the optic cup of brain [4]. The RPE cells perform a critical role in the phagocytosis, that is, turnover and renewal of shed photoreceptor membrane as well as maintenance of normal visual functions [5].

Melanocytes and their production of melanin pigment have important roles in determining the physiology of mammal skin. They synthesize melanin inside a special membrane bound organelle, termed as melanosomes. Melanosomes are transferred via dendrites to surrounding keratinocytes where keratinocytes arrange them to form a critical protective barrier (known as supranuclear "caps") to shield the DNA from UV radiation. The anatomical relationship between keratinocytes and melanocytes is known as "the epidermal melanin unit" and it has been estimated that each melanocyte is in contact with 36 keratinocytes in the basal and suprabasal layers [6].

The amount and type of melanin produced, that is, eumelanin or pheomelanin, as well as its eventual distribution in the epidermis, dramatically affects visible color, which ultimately determines the various functions of the pigment, such as photoprotection. "Normal" pigmentation is regulated by more than 250 genes and which function during the development, migration, survival, proliferation, and differentiation of melanocytes from their precursors, that is, melanoblasts [7].

The present chapter is proposed to provide morphological and ultra structural details of melanocytes, found in different locations of the body along with their significant functions. Here we will also highlight some miscellaneous functions of melanocytes, other than melanin

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production. The present chapter will provide significant knowledge on the basics of mammalian melanocytes from origin to different stages of development along with their specific markers. Signaling pathways of skin melanocytes along with the diseases associated with their disruption have also been elaborated. Concurrently, their etiologies along with the ultrastructural details of pigmentary disorders and the role of receptors have also been discussed.

2. Origin and development of melanocytes

The embryonic development of melanocytes provides an opportunity to better understand the story of skin pigmentation and its related skin diseases. Melanocytes derived from the pluripotent, NCC are also known as the fourth embryonic layer [8]. In addition to melanocytes, they give rise to neurons and glial cells, adrenal medulla, cardiac cells, and craniofacial tissue.

Originating from the border between the dorsal neural tube and overlying ectoderm, NCC appears following closure of the neural tube during neurulation. Induction of the neural crest population requires the action of several transcription factors including; Msx1, Pax3 (paired-box 3), FoxD3, Zic1, Snail2, AP-2, and Sox10 (sex-determining region Y (SRY)-box 10), microphthalmia induced transcription factor (MITF), endothelin 3, and endothelin receptor B (EDNRB) [9,10]. Expression of these factors is in turn regulated by Wnt and Bone Morphogenic Protein (BMP) signalline [11]. These proteins and signaling pathways provide integrated spatial and temporal signals to create the proper environment for development and migration [12].

NCC migrates extensively around the embryo, during which they differentiate into specialized cell types. The fate of the NCC depends on environmental factors they meet on the migratory pathways. At the trunk, from somite eight to twenty eight, NCC emerge after an epithelio-mesenchymal transition (EMT), proliferate extensively and follow two main migration paths; the dorso-lateral and the dorso-ventral pathways. The cells that migrate along the dorso-lateral pathway, between somite and ectoderm, are thought to be the main source for melanocytes while dorso-ventrally migrating cells give rise to the peripheral nervous system and adrenal medulla [13].

Melanoblasts, which are the precursors of melanocytes, migrate, proliferate, differentiate, and spread to their final destinations in the basal layer of epidermis and hair follicles; however, precise distribution of melanocytes varies among various species [14]. Cell type–specific markers are useful tools to demystify the development of certain cell types. Tyrp-2/Dct is reported to be a specific marker of melanoblasts development [15]. It has been reviewed by [16] that the markers for the precursors for melanocytes are: a tyrosinase kinase receptor KIT (c-kit) and transcription factors such as MITF, SOX10, Pax3. Melanocytes are usually identified by their expression of melanocyte-specific proteins, for example tyrosinase (TYR), TYRP1, DCT, Pmel17/gp100, MART-1, and/or MITF (Figure 1).

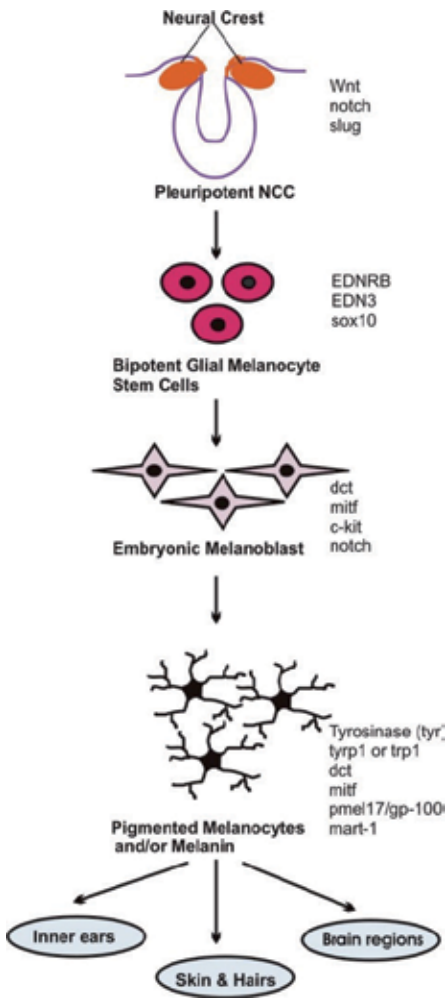

Figure 1. Embryonic development of melanocytes from NCC. Melanocytes originate from the pluripotent NCC which in turn develop from dorsal most point of neural tube. Wnt, slug, and notch are the specified marker genes involved in the development pathway of early NCC. The bipotent stem cells of melanocytes differentiate into specialized cells termed as melanoblast with its specific marker genes. Melanoblasts are the precursor of melanocytes which migrate, proliferate, differentiate, and spread to their final destination in epidermis and hair follicles, stria vascularis of inner ears, the uveal track of eyes, substantia nigra, and locus coeruleus of brain and finally evolve into the mature melanocytes.

3. Morpho-anatomical aspects of mammalian melanocytes

The pigment cells of lower vertebrates/mammals (melanophores/melanocytes) are specialized type of smooth muscle cells, which due to their intracellular movement of melanin granules, control skin color. Several studies have been conducted on the effects of various hormones, factors, and pharmacological agents on vertebrate pigment cells and it has been widely

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accepted that they are controlled by either nerves alone or by hormones or by a combination of both [17–20]. Several cytokines, growth factors and other receptors have also been established to support the transformation of migrating melanoblasts to differentiated functional melanocytes. Receptors from a long time have evaded scientific investigations and are present on melanophores/melanocytes. These mysterious protein molecules mediate a host of physiological and pharmacological actions through endogenously as well as exogenously applied drugs. Receptor systems participate in a very coordinated manner in mediation of responses of color change [21]. Cellular receptors of adrenergic, cholinergic, histaminergic, and serotonergic nature have been found to regulate pigmentation via melanin displacement in various animal melanocyte models [22–24].

A number of researches have revealed that there is a close association between cell shape and function. Cell form can be affected by the topography of the surface, where the cells are full-grown, each in cell culture as well as in an animal [25]. Depending on the localization, two types of melanocytes can be identified in the skin. These are (a) epidermal melanocytes occurring in the stratum basal of interfollicular epidermis and (b) follicular melanocytes residing within the hair follicle. Various electron microscopic, histochemical studies have been carried out in order to find out the ultrastructural details of melanocytes present in epidermis, hair follicles, uvea of eyes, etc.

3.1. Morpho-Anatomical Details of Epidermal Melanocytes

a. Morphological Structure of Epidermal Melanocytes

The epidermis is a stratified squamous epithelium consisting mainly of cells with two different origins: keratinocytes and melanocytes, of which keratinocytes form the 90–95% of epidermis. Melanocytes comprise from 5% to 10% of the cells in the basal layer of epidermis. Epidermal melanocytes are thin, elongated cell with branched structures, consisting of a central cell body and long numerous branches, or dendrites through which they interact with the adjacent keratinocytes of the basal epidermal layer [26]. Kemkemera *et al.* [27] have examined the cell shape of normal melanocytes (M-C) and of melanocytes from the skin of one neurofibromatosis type 1 patient (M-NFS) *in vitro*. They have reported that all cell types are bipolar and orient parallel to the grooves on the structured surface part, while all melanocytes remain distributed randomly. It has been reported by Valia [28] that although the size of melanocytes can vary, they are typically 7 μm in length. In human body variable pigmentation is seen due to variation in melanocyte population in different regions of the body. However there is no sexual or racial variation in the density of melanocytes in the skin. Thus the cellular activity is considered as the main contributor to racial differences in skin pigmentation [29].

b. Ultrastructural Details of Epidermal Melanocytes

At the ultrastructural level the melanocyte is a distinctive cell. It was noticed by some workers that the melanocyte is free of the fibrillar material (tonofibrils, desmosomes, and tonofilaments) typical of the keratinocytes, and that intercellular bridges between them and the other cells of the epidermis are also absent. The nucleus of a melanocyte is smaller and more deeply basophilic than that of a basal keratinocyte. Also the presence of dendrites makes it distin-

guished from neighboring keratinocytes [30]. Dendrites of melanocytes are revealed more effectively with silver salts that stain the melanin black; they then are seen to arborize in all directions among neighboring keratinocytes and even extend into the uppermost part of the dermis. Unlike the keratinocyte, mitochondria are abundant in melanocytes. Micro fibrils, as distinct from tonofilaments, are seen in the cytoplasm. Unlike tonofilaments they show no tendency to form bundles and are often seen as parallel arrays of fine filaments. The Golgi apparatus is usually prominent and the endoplasmic reticulum is well developed.

The characteristic organelle of the melanocytes is the melanosome. Melanosomes are produced inside the melanocytes, and they pass through several developmental stages, starting in the middle of the melanocyte, migrating to the outer edge of the cell through the dendrites. In 1971, the following four stages (see Figure 2) in the development of the melanosome were recognized by Fitzpatrick *et al.* [31].

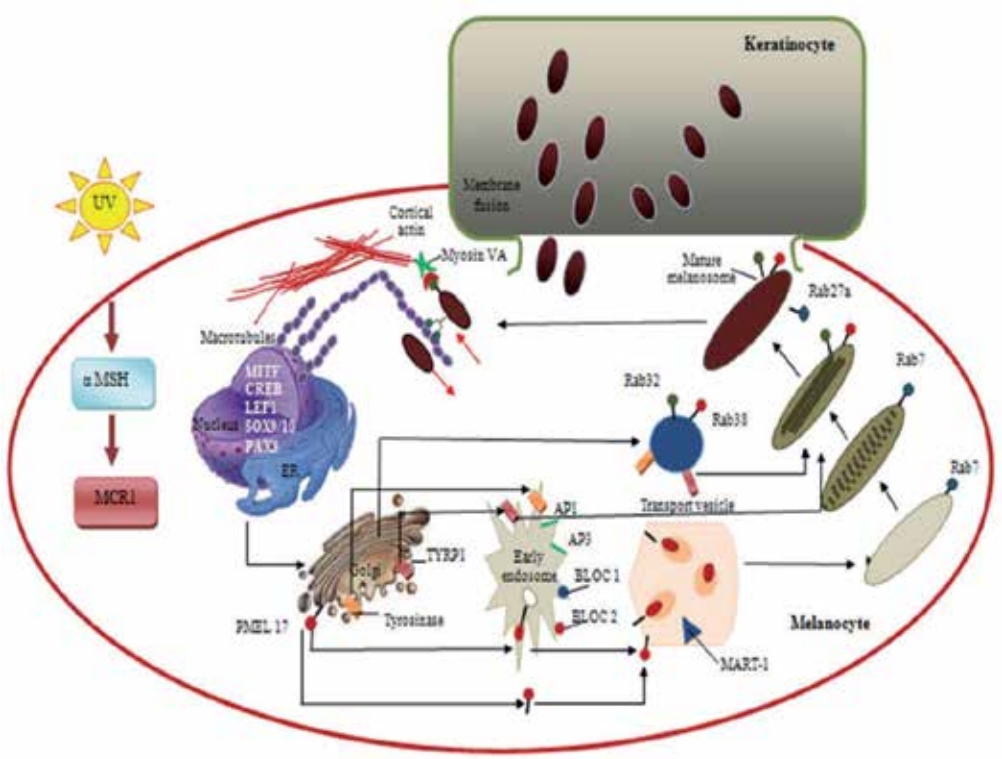

Figure 2. Development and maturation of melanosomes. The synthesis, maturation, and translocation of melanosomes to the keratinocytes are the result of complex regulatory processes involving various sub-cellular organelles, enzymes, proteins, cytoskeleton, intracellular signal transduction pathways, etc. Stage I premelanosomes are ovoid and have poorly defined protein matrix assumed to be originated from the endoplasmic reticulum in which tyrosinase is transferred from Golgi body through vesicular transport. Stage II premelanosomes are tyrosinase positive and have fibrillar matrix but no deposition of melanin takes place in this stage. Stage II premelanosomes matures to Stage III melanosomes in which synthesis of melanin takes place and hence it was seen as partial electron dense structure. Finally, stage IV melanosomes evolved as complete electron dense structure in which the fibrilliar matrix become indistinguishable due to complete deposition of melanin.

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Stage I. The Stage I melanosomes or premelanosomes likely develop form endoplasmic reticulum (ER). They have an amorphous matrix and display internal vesicles that form as a result of membrane invagination. Premelanosomes already contain the glycoprotein Pmel17 (gp100), but it requires further processing to become a component of the final fibrillar matrix. These filaments have a distinct periodicity of 100 A.

Stage II. The organelle is oval and shows numerous membranous filaments, with or without cross linking, having a distinct periodicity. Although no active melanin synthesis takes place in this stage, they already contain the enzyme tyrosinase.

Stage III. The internal structure, characteristic of Stage II has become partially obscured by electron-dense melanin due to deposition of melanin in its fibrillar matrix.

Stage IV. The oval organelle is electron-opaque without discernible internal structure in routine preparations.

Weiss and Zelickson [32] reported that melanocytes containing melanosomes of several developmental stages were present in the epidermis of 15-day-old C57BL16 mouse embryo and subsequently increased in number. Stage I melanosomes are seen as spherical vesicles near the Golgi apparatus. The other stages are usually seen scattered singly throughout the cytoplasm though there is a preponderance of Stage III and Stage IV melanosomes in the dendritic processes. If preservation is good, a distinct unit membrane can be seen surrounding the internal structure of the organelles.

Later, Hach [33] divided the melanosomes into two basic groups based on their ultrastructure: (1) elliptical (Ovoid) lamellar or fibrilliar melanosomes with a protein matrix arranged into coiled lamella or fibrils or rolled up sheets arranged parallel to the long axis of the organelle; (2) spherical globular melanosomes characterized by a granular appearance possibly due to a sponge-like architecture of their structural proteins with melanin stuffing the empty space. Melanocytes may produce eumelanosomes or pheomelanosomes at different times, switching from one to the other.

Schraermeyer [34] reviewed a comparison between eu- and pheomelanogenesis and further revealed that the earliest form of melanosomes is identical. The vasiculo-globular bodies are involved in melanosomal constituents [35]. In pheomelanogenesis, pheomelanosomes are smaller (~0.7 μm in diameter), ovoid to sub-spherical in shape, and their glycoprotein matrix appears disorganized and loose as lamellae are not formed, but these bodies fuse with each other to form an amorphous matrix on complete differentiation of melanosomes. Mature eumelanosomes in eumelanogenesis are large, (~0.9–0.3 μm), typically elliptical in shape with rounded ends and contain a highly structured fibrillar glycoprotein matrix required for eumelanin synthesis [34]. Melanosomes are numerous in melanocytes of Negroids and Caucasoid individuals, with a dark complexion though they are seen without difficulty in the melanocytes of those with a fair skin. They are ovoid or rod shaped bodies measuring 0.4–1.0 μm in length and 0.1–0.5 μm in diameter.

As lower vertebrates share similar mechanism of skin pigmentation with human beings, they have outstanding potential as phylogenetical tools to demystify and better understand their

role in pigmentation biology and its evolutionary significance. Several detailed elucidations have been done on the fine structure of melanophores of various classes of vertebrates like teleosts and lung fishes [36–38], larval amphibians, Indian toad [39], and reptiles [40], which have revealed remarkably consistent fine structural features of these melanosomes synthesizing cells in order to explain their role in color changes. In the cytoplasm of the epidermal and sub-epidermal melanophores, well developed nucleus, tubular type of vesicular mitochondria, Golgi body, vesicular endoplasmic reticulum with oval to elliptical melanosomes of various degree of melanization have been found to remain randomly scattered [39], resembling similar ultrastructural features of mammalian melanocytes. From these studies it is concluded that the presence of immature, pre-melanosomes to highly electron dense, mature melanosomes showing progressive degrees of melanization from stage I to stage IV melanosomes, in lower vertebrate melanophores is similar to mammalian melanocytes with same mode of development [41,42]. Hence, the ultrastructure of melanophores of these animal models appears to have a treasure of information, which may have physiological links with the human melanosomes from phylogenetic and therapeutic points of view.

c. Intracellular Site of Melanin Synthesis in Epidermal Melanocytes

In 1976, it was reported by Hunter [43], that the information gathered from electron microscopy, electron microscopic cytochemistry, autoradiography, and cell particle fractionation supports the view that tyrosinase is synthesized on the ribosomes. It is then transferred via the rough endoplasmic reticulum (RER) to the Golgi apparatus, from where it is channeled via tubular elements to a focal dilatation of the smooth endoplasmic reticulum in which the coiled melanosomal matrix has independently formed. Melanisation of the structural protein can then take place and once this is completed, the connection with tubular system is severed [44,45].

3.2. Morpho-anatomical details of hair follicular melanocytes

a. Morphological Description of Hair Follicular Melanocytes

Hair bulb matrix is the principal site for the fully developed follicular melanocytes, which differs from epidermal melanocytes by a larger size, larger dendrites containing more developed Golgi and RER and producing two to four fold larger melanosomes [46]. Also, they have a supplement of fewer keratinocytes in contrast to epidermal melanocytes (30–40 keratinocytes) [47]. These melanogenically active melanocytes along with the neighboring immature pre cortical keratinocyte constitute the follicular melanin unit, which usually consists of one melanocyte for every five keratinocytes in the hair bulb region, but in the basal epithelial layer next to the dermal papilla the ratio is 1:1 [48]. Pigmentation of hair is regulated by this follicular melanin unit [10].

Hair structure is divided into three morphological components: the multicellular cuticle sheath, the fibrous cortex, and the medulla. Follicular melanocytes in the fully developed hair follicle (HF) are localized in distinct anatomic compartments based on their differentiation status. Formation of hair begins with the inward folding of epidermis into the dermis. The dermis forms thickening from below and the edge of the inward folding comes to enclose it.

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These dermal thickenings mature into the dermal papilla, while the adjacent parts of inward folding forms the hair bulb (Figure 3). The hair follicular melanocytes derived from epidermal melanocytes are highly dendritic cells and colonize the hair matrix, lower half of the hair bulb. Hair bulb melanocytes secrete stage IV melanosomes into keratinocytes consisting of hair bulb and as a result hair cortex and medulla develops [49].

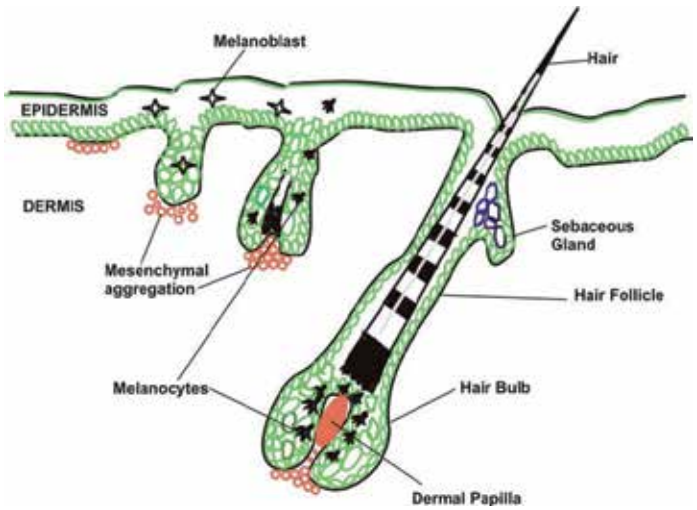

Figure 3. Developmental stages of mammalian hair follicles. Formation of hair begins with the inward folding of epidermis into the dermis. The dermis forms thickening from below and the edge of the inward folding comes to enclose it. These dermal thickenings mature into the dermal papilla, while the adjacent parts of inward folding forms the hair bulb. Hair bulb melanocytes secrete stage IV melanosomes into keratinocytes consisting of hair bulb and as a result hair cortex and medulla develops.

In mice, the process of morphogenesis of hair follicle structures is cyclic [50] and called hair growth cycle [47]. The hair growth cycle consists of three stages: resting (telogen); growth (anagen); and regression (catagen). Anagen phase is divided into six sub stages (anagen I–VI). Melanogenesis is strictly coupled to the growth phase of the hair growth cycle (anagen III–VI). Melanogenesis ceases early in catagen and is completely absent in telogen [51]. During early anagen, melanocyte stem cells residing in the lower permanent portions of the hair follicle are recruited for the regeneration of melanogenically active follicular melanocytes. This melanocyte stem cell progeny on activation migrated out from niche, amplifies and differentiates into pigmented melanocytes. The vacant niches are again repopulated by the melanocyte stem cells and remain quiescent till the next early anagen [52]. Towards the end of anagen the number of identifiable melanocytes decreases and melanocytes lose their dendrites, shrink and become less pigmented, and then they disappear in catagen [47]. However, a small number of dedifferentiated cells (melanoblasts) positive to the combined dopa-premelanin reaction still remain in the hair bulbs.

Melanocytes occurring in hair follicles outer root sheath (ORS) express tyrosinase related protein-2 (TRP-2) and relatively weak tyrosinase related protein-1 (TRP-1). Unlike the bulge

melanocytes, they display proliferative activity during the early and mid-anagen stage of hair cycle. They represent the differentiating melanocytes. Melanocytes, which actively produce melanin in the hair follicle, are located in the hair matrix above the dermal papilla. They express all melanogenic enzymes – tyrosinase (TYR), TRP1, and TRP2 – and proliferate only during mid-anagen [53].

b. Ultrastructural Details of Hair Follicular Melanocytes

The ultra structure of amelanotic melanocytes from human hair follicles is different from that of epidermal melanocytes, and these characteristics determine the functional nature of amelanotic melanocytes. The fine-structure observation of the cultured melanocytes revealed round or elliptical shape, single huge nucleus and the karyotheca were double layered. The clear euchromosomes and meager heterochromosomes were observed within the nucleus. The cytoplasm was furnished with several organelles, such as unit membranous melanosomes of similar size, RER, ribosomes, as well as mitochondria were also present. While the Golgi apparatus in the cells was not seen easily, the electron dense melanin granules were concentrically deposited within the melanosomes [54]. The hair melanosomes are two to four times larger than the epidermal melanosomes. It has been reviewed by Ortonne and Paul [55] that the different stages of hair cycle of hair melanocytes have their own unique ultrastructure features. In hair follicles of C57 black mice, early anagen (growth) stage of hair cycle extensive growth occurred in melanocytes where cytoplasm volume increased, with increase in dendricity, elaborate RER, and Golgi zone along with increase in size and number of melanosomes. However, during catagen and telogen stage of hair cycle some degenerative changes takes place in melanocyte, with only a few small premelanosomes. The volume of cytoplasm decreases with less well developed Golgi complex and RER as well as nuclei with prominent heterochromatin patterns. Hair melanocytes transfer melanosomes to follicular epithelial cells. Medullary cells receive their melanin from melanocytes in the upper part of the hair bulbs similar to the way described for epidermal melanin unit. Melanosomes are also transferred to immature corticle cells. Due to their larger size, melanosomes are usually distributed singly, whatever the ethnic background.

Many studies have been carried out to demystify the ultrastructural aspects of hair melanin pigmentation. Earlier, it was revealed by Jimbow *et al.* [56] that human red hair, specified as pheomelanic, its melanocytes contain spherical melanosomes with vasiculo-globular and proteinaceous matrices on which melanin deposition is spotty and granular. Melanin granules are smaller and less numbered in blonde than in dark-hair human subjects [57]. Melanosomes are not fully melanized even in the dendritic processes of melanocytes. Whereas ultrastructure of follicular melanocytes of black hair is identical to the melanocytes in the epidermis of negroids as they observed to contain typical ellipsoidal melanosomes, at various stages of melanization and large melanosomes are transferred singly to the neighboring keratinocytes [58]. In brown hair, the follicular melanocytes also contain all the developmental stages of melanosomes. Lighter brown hairs have smaller melanosomes [59]. In the melanocytic zone of the senile gray hair bulb, the number of melanocytes appears normal or reduced [60]. These cells show little melanogenic activity and contain very few melanosomes.

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3.3. Morpho-anatomical aspects of ocular melanocytes

Ocular melanocytes are found in the uveal tract, which consist of cells of the choroid, iris, ciliary body [61]. The RPE is a monolayer that lies between photoreceptors and choroid. Melanin is produced in RPE and choroid of eyes. Melanocytes and RPE cells have a different embryonic origin and development [62]: While RPE cells emerge from the neural tube, melanocytes are neural crest–derived cells. Consequently some differences exist between melanocyte and RPE melanogenesis [63]. Ocular melanocytes are in contact with only each other, and they do not transfer their melanosomes. The RPE is a monolayer of pigmented cells of neural tube origin in the background of the eye. Together with the endothelial cells of the chorio-capillaris it forms the blood-retinal barrier [64]. RPE cells are cubical and highly polarized. Basal infoldings and apical microvilli serve as enlargement of the surface. The shape of mature RPE melanosomes differs from that of melanocytes in being oval [65].

The typical oval shaped melanosomes of the RPE are located in the microvilli, and round shaped melanosomes in the cytoplasm near the nucleus. In spite of this RPE cells and the epidermal melanocytes have been described to follow the standard pathway of melanogenesis. It was reported that they exploit the similar melanogenic proteins and the fine structural characterization of both the epidermal melanocytes and pre-natal RPE cells confirmed the presence of distinct melanosomes of various stages (I–IV) of development [66]. Schraemeyer [67] reported that the formation of classical premelanosomes in RPE have been induced by illumination. Ocular melanin content differs among species. Stages II–IV melanosomes have been found in the RPE of squirrels [68]. Although melanogenesis in RPE and choroid seems to follow a common pathway, the RPE of adult hamsters and probably all the vertebrates, contains unique melanosomes never present in the melanosomes of choroid. These are large, spherical melanosomes with loosely packed melanofilaments. Whereas the high numbers of melanin granules are spindle shaped in the RPE, they are smaller and more spherical in the choroid. The large type melanosomes often fuse with earlier-stage melanosomes and have been described as late immature melanosomes in the prenatal RPE of humans. In the RPE melanosomes contain melanofilament ordered concentrically as are the membrane in phagosomes [69]. In the RPE of chicken, parallel filaments which appeared to have the same structure as those forming the framework of melanosomes were frequently found in phagosomes [70]. Ultrastructural similarities between phagosomes and melanosomes have also been reported. In the RPE of cattle, phagosomes were found that contain an electron dense melanin-like material that was not autofluroscent and therefore not lipofuscin. Additionally electron dense vasiculo-globular bodies (10–100 nm) were found in phagosomes during disk membrane degradation as well as within melanin granules [71].

4. Functional aspects of melanocytes

Although melanocytes are distinguished for their ability of skin pigmentation, but this is possibly not the single occupation of these pigment bearing cells. In fact, melanocytes have plentiful enzymes which are proficient in biochemical, genetical, and functional association to the immune system as well as antimicrobial protection [72].

4.1. Cutaneous pigmentation

Cutaneous pigmentation is the product of two crucial events: biosynthesis of melanin granules within melanocytes and their subsequent transfer to the neighboring keratinocytes. Though the quantity of melanocytes in all human races is basically invariable, the amount, size, and the way in which melanosomes are spread within keratinocytes may differ [1]. The quantity of melanin granules of human epidermal melanocytes is diverse not only among different skin types but also among different locations of the skin from the same person [73]. This diversity is vastly controlled by gene expression of melanosomal proteins, which regulate the complete activity and expression of melanosomal proteins inside the particular melanocytes [74]. It has been noticed that those melanocytes having low quantity of melanin pigment, produce TYR very slowly but degrade it very rapidly, than melanocytes having higher quantity of melanin pigment and TYR activity [75]. Indeed, darkly pigmented skin possesses several single, large (0.5–0.8 mm in diameter), elliptical, highly electron dense melanosome particles of Stage IV. However, the lighter skin pigmentation is linked with smaller (0.3–0.5 mm in diameter), immature, less dense melanosomes of stages II and III which remain clustered in membrane-bound groups (FIGURE 4) [76]. These varied configurations of melanosome stages or types as well as distribution exist since birth and are not influenced by external factors (such as sun exposure). They are liable for wide range of skin tones [26].

Characteristic features	Darkly pigmented skin	Lightly pigmented skin
Stage of melanosomes	Stage IV	Stage II & III
Size (Diameter)	0.5-0.8 mm	0.3-0.5 mm
Number of melanosomes per cells	>200	< 20
Transport to keratinocytes	Single	Group of 2-20
Rate of degradation	Fast	Slow
		

Figure 4. Variations in human skin pigmentation due to different types of melanosomes within epidermal melanocytes.

4.2. Protection from Ultraviolet Radiations (UVR)

The principal function of melanin is to protect the skin from harmful effects of sunlight, a task accomplished by its capability to scatter and absorb ultraviolet light [77]. So the primary role of melanocytes is to act as natural sun block. Adaptive pigmentation or tanning is the natural

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physiologic response of the skin against exposure of UVR. Melanin bearing melanocytes, situated in the basal layer of epidermis have vital tasks in the skin's barrier function by preventing damage by UVR. In the basal layer of the epidermis, melanin pigment creates a protective cover over the nuclei of keratinocytes; in the outer layers, they are more evenly distributed. Melanin absorbs UV radiation, thus protecting the cell's nuclei from DNA (deoxyribonucleic acid) damage [78]. UV radiation induces keratinocyte proliferation, leading to thickening of the epidermis [79]. Hence, it is concluded that the tanning involves both an increase in the amount of melanin pigment and epidermal thickening. Both changes serve to increase melanin accumulation in the epidermis, so that the skin is better protected against subsequent UV exposures [80].

4.3. Determination of eye color

The external origin of eye color is determined by the distribution and quantity of melanocytes in the uveal tract of the eye. The amount of melanocytes does not vary between eye colors [81], but it is the intracellular melanin content, types, and its packaging within the melanosomes that vary, which provides a wide variety of eye colors. There are two forms of melanin pigment particles (skin and hair melanocytes) produced during melanogenesis and both occur in the iris of the humans [82,83]. The quantity and types of melanin in the iridal melanocytes vary with iris color. However, unlike the skin and hair in which melanin is produced continuously and secreted, in the eye the melanosomes containing the pigment are retained and accumulate in the cytoplasm of the melanocytes within the iris stroma. White light entering the iris can absorb or reflect a spectrum of wavelengths giving rise to the three common iris colors: blue, green-hazel, and brown. Even though blue colored eyes have same number of melanocytes but they posses low melanin pigment and few melanosomes; green-hazel color of irides are the outcome of average number of melanin granules as well as melanosome number; and brown colored iridies are the product of huge numbers of melanin granules along with melanosome particles [84].

4.4. Other incomparable abilities of melanocytes

Melanocytes are now beginning to take more and more fascinating roles in other tissues of the body. The existence of melanin and melanocytes are not limited to the skin, hairs, and RPE [85] only, besides, they are reported to be present in the leptomeninges, stria vascularis of the cochlea, in the heart, as well as in the substantia nigra and locus coeruleus of the brain. They have also been verified to function in hostile regions of our body, such as adipose tissues. In stria vascularis of the cochlear melanocytes remains present as intermediate cells. The stria intermediate cells play crucial role in the initiation of endolymph-mediated action potentials that is essential for normal hearing. Melanin granules produced by melanocytes in the inner ear even play important roles in body balance [86].

Extracutaneous melanocytes located in the brain may have several neuroendocrine functions. Pigment granules found in brain are known as neuromelanin, consists of giant, dense eumelanin covered pheomelanin core which may also include aliphatic and peptides [87]. Neuromelanin is predominantly confined to dopaminergic neurons of substantia nigra as well as in

the locus coeruleus, and ultimately gets accumulated in the human substantia nigra with age [88]. Several researches have favored the view that neuromelanins have a defensive role by binding/elimination of reactive oxygen species (ROS) along with metals that would otherwise be extremely toxic to neurons [89]. In a recent study, it has been reported that nearly all brain tissues hold significant quantity of neuromelanin, which is supposed to play crucial tasks in reducing organ toxicity [90].

Interestingly, it has been observed that melanocytes are also situated in the valves and septa of heart [91]. It was further hypothesized that the origin of cardiac melanocytes may take place from the same precursor population as that of skin melanocytes because of its dependency on the same signaling molecules which is needed for the appropriate development of skin melanocytes [92], but their exact function in this location is still unclear. The synthesis of melanin is not always beneficial, either in heart or in other tissues, for example, in the lungs where in an unusual ailment known as Lymph angioleio myomatosis (LAM) [93], the muscle cells slip back into their developmental stages along with the expression of melanocytes like specific markers Pmel17, tyrosinase, etc. The resulting production and accumulation of melanin in lung tissues is eventually lethal.

Randhawa *et al.* [94] reported that the biosynthesis of melanin also occurs in the adipose tissue of morbidly obese humans. It was also assumed that the ectopic synthesis of melanin in the cytosol of obese adipocytes possibly provide a balanced mechanism to work as anti-inflammatory factor as well as it decreases the oxidative damage. In the course of enhanced deposition of cellular fat, adipocytes become more exposed to endogenous apoptotic signals especially with ROS, which could be counteracted by ectopically produced melanin. In addition, adipocytic melanin may also suppress the secretion of proinflammatory molecules [3].

5. Etiology of pigment cell disorders

Skin color is a very important social and cultural human characteristic. Even a minor alteration of cutaneous pigmentation can result in a major esthetic concern, with psychological implications. Skin color is primarily due to the pigment melanin, which is produced by melanocytes, and normal pigmentation is dependent on the normal structure and function of these cells. Any defect affecting the complex process of skin pigmentation may result in pigmentary disorder, which may be either (a) hyperpigmentary or (b) depigmentary/hypopigmentary.

5.1. Pathology and ultrastructure of hyperpigmentary disorders

Hyperpigmentary skin disorders may be defined as enhanced pigmentation of the skin above the normal level. Such increase in melanin production may occur in the epidermis, dermis, or both. Epidermal hyperpigmentary disorders exclusively occur due to the increase of melanin pigment and are characterized by "brown hyperpigmentation" because of their brown color. They may be associated with (melanocytic epidermal hyperpigmentation) or without (melanotic epidermal hyperpigmentation) an actual increase in the melanocytes number. On the other hand, in dermal hyper pigmentary diseases, collectively known as "blue hyperpigmen-

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tation" or circuloderma, either melanin pigments (melanin dermal hyperpigmentation) or non-melanin pigments (non-melanin dermal hyperpigmentation) are involved [95]. Generally hyperpigmentary disorders can be congenital or acquired [96].

a. Melanotic Melanoma

The incidence of malignant melanoma is continuously increasing worldwide. The detailed fine structure of melanotic melanoma cells studied by Wellings *et al.* [97] was of the pale melanocyte variety. It has been reviewed by Polnikorn [98] that there is increased number of epidermal and follicular melanocytes (approx. 30%) in the melanotic lesions. Melanotic melanocytes were either round, elliptical or at times irregular and possessed numerous cytoplasmic microvilli. The ratio of the nucleus to the cytoplasm was low. The nuclei, which contained light chromatin, were usually ovoid, with a definite indentation on one side, thus giving the cell an appearance of polarity. Some of the nucleoli contained masses of fine granules and had clear round areas within them. The endoplasmic reticulum consisted of slender sac-like forms with lamellae and vesicles, which infrequently were slightly swollen. The endoplasmic reticulum often was found surfaced with ribosomes, but some de-granulated forms of it were also observed. The Golgi apparatus and centrioles occupied an extensive part of the cytoplasm of most cells and usually were located immediately adjacent to the indented part of the nucleus, when such was present. In the immediate vicinity of the Golgi area, numerous vesicles, premelanosomes and immature melanosomes at various stage of development, dense areas of ribosomes and many mitochondria were present; however, melanosomes in various stages of development also were observed in areas of the cytoplasm far from the Golgi apparatus [99].

The intermediate vesicle, which originated in the Golgi apparatus, underwent changes which ultimately led to the fully-developed melanosome. The most striking feature of pigmented melanoma cells of humans was the occurrence of numerous melanosomes that varied considerably in size and shape, particularly the spheroidal melanosomes with a diameter ranging between 200 nm and 500 nm. Some of the melanosomes showed ellipsoidal profiles and melanosomes showing a granular structure were common, whereas lamellar melanosomes were seen less frequently. As the melanosome became completely electron dense due to the deposition of melanin granules, the pigment tended to disturb the arrangement and to leak into the cytoplasm [100]. The cultures of pigment cell lines were observed to have lower growth rate and viability in comparison to the non-pigmented cell lines. Extremely pigmented cells died and discharged their distinct melanin granules into the medium. The "amelanotic" melanoma cells were examined to have minimal synthesis as well as faulty development of premelanosomes. Also, it was noticed that they did not possess the distinct fibrillar structure and had less deposition of melanin as reported in the "melanotic" melanoma cells. It seems that the formation of discrete fibrillar pattern may be the fundamental step for the normal synthesis and deposition of melanin along with the development of melanosomes [101].

5.2. Pathology and ultrastructure of hypopigmentary disorders

Hypopigmentary/depigmentary disorder of the skin is characterized by the reduction/complete loss of skin pigmentation. Usually they may be of two types: (a) melanopenic

(melanocytopenic), which is related to the melanin pigment, and (b) non-melanopenic, which is not related to the melanin pigment. Melanopenic hypopigmentation mainly occurs due to the disruption in the complex process of skin pigmentation. Non-melanopenic hypopigmentary disorders may be associated with anemia, edema, and Raynauld's phenomenon.

a. Vitiligo

Vitiligo is a hypopigmentary disorder of the skin in which cutaneous and ocular melanocytes are selectively destroyed that result in loss of pigmentation. It affects 1–2% of the population including both sexes and all races equally [102]. Multiple theories have been proposed, including genetic, neural, biochemical, viral, and autoimmune mechanisms. However, an autoimmune mechanism has been proposed as the most accepted cause of vitiligo. In previous studies, it has been demonstrated that most important feature in vitiligo is alternation of the melanocyte ratio at the dermal-epidermal junction [103,104]. The long dendritic, melanin granules filled, dopa positive melanocytes were found to be prominent in the outer peripheries of vitiligo lesions [105].

Study conducted by Mohamed and El-Saman [106] on vitiligo patients using light and electron microscopy, showed complete loss of melanin pigment granules in the epidermis, mononuclear cellular infiltration in the dermis, and marked positive ICAM-1 expression over keratinocytes in the epidermis and around endothelial and inflammatory cells in the dermis of the vitiligo sections. The above authors have reported that the biopsies of vitiligous skin samples exhibited significant ultrastructural changes of degenerative nature in the keratinocytes. This was conspicuous by the presence of electron dense cytoplasm with vacuolization and fragmentation of keratin tonofilaments. Large, irregular indented nuclei were also observed in the biopsies. Besides, the cytoplasm of keratinocytes becomes completely deficient of melanosomes in contrast with the control group. Dilated intercellular spaces (edema) and loss of intercellular junctions with or without remnants were also observed. Complete absence of melanocytes and obvious presence of lymphocytes in the basal layer and dermis were evident. These findings give strong fine structural evidence that the vitiligo leads to the degenerative changes in the structure of melanocytes.

We have recently shown that active ingredients of plants like *psoralea corylifolia,* (psoralen), *nigella sativa* (thymoquinone), *piper nigrum* (piperine), and *withania somnifera* (withaferin), *berberis vulgaris* (berberine) all have powerful melanogenic (skin darkening) properties and are excellent activators of melanosomal receptors, which when properly stimulated can cause skin darkening in some of the animal models studied in our laboratory [107–112]. Whether these have a similar role in mammalian melanocytes and specific cell lines is yet to be determined at the cellular level. Therefore, it could be an interesting possibility to further explore the herbal ingredient-activated melanosomal receptor signaling cross talk within the melanogenetic pathways, where different receptors participate in skin pigmentation and its dysfunctions.

6. Conclusion

The morphoanatomical activities of the melanocytes have evolved to enable them to perform specific functions with great efficiency: the regulated production and distribution of melanin

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within the epidermis. It is unfair to think that melanocytes reap all the glory for their role in pigmenting the skin and providing it critical protection against UV damage. They also play a significant role in diverse physiological functions such as phagocytosis, vision, hearing, balancing and several neuroendocrinal functions. Ultrastructural diversity is found in melanocytes, based on their specific locations like type of melanin produced (pheo- or eu-melanin), shape, size, and number of melanosomes. These structural differences result in functional differences as well, such as the cutaneous and hair pigmentation. There is increasing evidence that melanocytes are associated with pathophysiological circumstances as well. Diverse effects such as hyperpigmentation and hypopigmentation are associated with malfunctioning of the pigmentary system of unknown etiology. Further study of these organelles may not only furnish insights into the underlying intricate mechanisms of pigmentation, but could also yield therapeutic tools for skin disorders.

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Section 5

The Regulation of Smooth Muscle Tone

Chapter 11

Ca^{2+} Dynamics and Ca^{2+} Sensitization in the Regulation of Airway Smooth Muscle Tone

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Additional information is available at the end of the chapter

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Abstract

Airway smooth muscle tone is ultimately generated by phosphorylation of myosin light chain, which is regulated by the balance between concentrations of Ca^{2+} and sensitivity to Ca^{2+} in the cytosolic side. The former is due to the Ca^{2+} influx passing through ion channels (Ca^{2+} dynamics), leading to activation of myosin light chain kinase, and the latter is due to Rho-kinase (Ca^{2+} sensitization), leading to the inactivation of myosin phosphatase. Alterations to contractility and to the proliferative phenotype, which are influenced by Ca^{2+} dynamics and Ca^{2+} sensitization, are involved in the pathophysiology of asthma and chronic obstructive pulmonary disease (COPD). Ca^{2+} dynamics are mainly due to store-operated capacitative Ca^{2+} influx and receptor-operated Ca^{2+} influx, and partly due to L-type voltage-dependent Ca^{2+} (VDC) channels. Large-conductance Ca^{2+}-activated K^+ ($K_{Ca,}$ $BK_{Ca,}$ Maxi-K^+) channels are activated by G_s connected to β_2-adrenoceptors, whereas these channels are inhibited by G_i connected to M_2 muscarinic receptors. VDC channel activity regulated by K_{Ca} channels contributes to not only functional antagonism between β_2-adrenoceptors and muscarinic receptors but also to synergistic effects between β_2-adrenoceptor agonists and muscarinic receptor antagonists. Moreover, an increase in Ca^{2+} influx via the K_{Ca}/VDC channel linkage causes airflow limitation and β_2-adrenergic desensitization. In contrast, an increase in sensitivity to Ca^{2+} via Rho-kinase causes airflow limitation, airway hyperresponsiveness, β_2-adrenergic desensitization, and airway remodeling. These airway disorders are characteristic features of asthma and COPD. K_{Ca} channels are regulated by trimeric G proteins (G_s, G_i), and Rho-kinase is regulated by a monomeric G protein (RhoA). Therefore,

Ca^{2+} dynamics due to G proteins/K_{Ca}/VDC channel linkage and Ca^{2+} sensitization due to RhoA/Rho-kinase processes are therapeutic targets for these diseases.

Keywords: Ca^{2+}-activated K^+ channels, β_2-adrenoceptors, G proteins, Rho-kinase, Intrinsic efficacy

1. Introduction

Contractility of airway smooth muscle is involved in airflow limitation, which is implicated in the pathophysiology of asthma and chronic obstructive pulmonary disease (COPD). Spasmogens act on trimeric G protein-coupled receptors (GPCRs), such as acetylcholine (ACh), histamine, leukotrienes, and prostaglandins, to mediate airway smooth muscle contraction. Airway smooth muscle tone is ultimately regulated by the activation of myosin light chain (MLC); MLC is phosphorylated via myosin light chain kinase (MLCK) and dephosphorylated via myosin phosphatase (MP). Activation of MLCK contracts airway smooth muscle mediated by Ca^{2+}-dependent mechanisms, which is due to increased concentrations of intracellular Ca^{2+} ($[Ca^{2+}]_i$) via a Ca^{2+} influx through Ca^{2+} channels (Ca^{2+} dynamics). In contrast, inactivation of MP contracts airway smooth muscle by Ca^{2+}-independent mechanisms, which are due to an increase in the sensitivity to Ca^{2+} via Rho-kinase, a protein affected by RhoA, a monomeric G protein (Ca^{2+} sensitization) [1]. RhoA/Rho-kinase processes are widely distributed in tissues including the respiratory system and regulated by agonists for GPCRs.

β_2-adreneoceptor agonists and muscarinic receptor antagonists counteract spasmogen-induced contraction with reducing $[Ca^{2+}]_i$ (antagonizing Ca^{2+} dynamics). β_2-adrenoceptor agonists also suppress airway smooth muscle contraction by reducing sensitivity to Ca^{2+} (antagonizing Ca^{2+} sensitization) [1, 2]. Inhibition in both Ca^{2+} dynamics and Ca^{2+} sensitization is involved in the effects of β_2-adrenoceptor agonists against spasmogen-induced contraction. Moreover, β_2-adrenoceptor agonists relax airway smooth muscle via 3'-5'-cyclic adenosine monophosphate (cAMP)-dependent protein kinase (protein kinase A: PKA), leading to inactivation (phosphorylation) of MLCK. Large-conductance Ca^{2+}-activated K^+ ($K_{Ca,}$ BK_{Ca}, Maxi-K^+) channels are markedly activated by PKA-induced phosphorylation [3, 4, 5, 6] and G_s-induced action (G, a stimulatory trimeric G protein of adenylyl cyclase) [4, 5, 6, 7]. K_{Ca} channels are activated by β_2-adrenoceptor agonists via G_s and suppressed by muscarinic receptor agonists via G_i, an inhibitory trimeric G protein of adenylyl cyclase [7, 8]. Since K_{Ca} channels have a large conductance of outward currents and exist innumerably on the cell membrane in airway smooth muscle [9], the opening of these channels also regulates airway smooth muscle tone mediated by membrane potential-dependent Ca^{2+} influx (Ca^{2+} dynamics), such as L-type voltage-dependent Ca^{2+} (VDC) channels [10]. Therefore, not only Ca^{2+} signaling (Ca^{2+} dynamics and Ca^{2+} sensitization) but also K_{Ca} channels play a key role in the functional antagonism between β_2-adrenoceptors and muscarinic receptors in airway smooth muscle.

Alterations of contractile phenotype, i.e. hyperresponsiveness to contractile agents (airway hyperresponsiveness) or hyporesponsiveness to relaxant agents (β_2-adrenergic desensitiza-

tion), occurs due to intrinsic or extrinsic factors involved in the pathophysiology of asthma. Dysfunctional contractility, which is a characteristic feature of patients with asthma, may depend on Ca^{2+} signaling (Ca^{2+} dynamics and Ca^{2+} sensitization) and K_{Ca} channels [1, 6, 11, 12]. Furthermore, airway smooth muscle cells have the ability to change the degree of various functions, such as contractility, proliferation, migration, and synthesis of inflammatory mediators [1, 13, 14]. The plasticity from a contractile phenotype to other phenotypes (proliferation, migration, or secretion of chemical mediators) may enhance airway inflammation, leading to airway remodeling, which is also characterized in asthma. This phenotype change may also be associated with Ca^{2+} signaling (Ca^{2+} dynamics and Ca^{2+} sensitization) and K_{Ca} channels.

Ca^{2+} signaling and K_{Ca} channels involved in the regulation of airway smooth muscle tone may be therapeutic targets in asthma and COPD [1, 6, 10, 11, 12, 15]. To elucidate the cause of the pathophysiology in asthma and COPD, and to establish a rational bronchodilator use for these diseases, the mechanisms underlying the regulation of airway smooth muscle tone via β_2-adrenergic and muscarinic receptors were examined by using physiological techniques such as single-channel recording in tracheal smooth muscle cells, isometric tension recordings of isolated tracheal smooth muscle and simultaneous recording of isometric tension and F_{340}/F_{380} in Fura-2–loaded tracheal smooth muscle. In this chapter, the functional characteristics of airway smooth muscle involved in alterations of contractile and proliferative ability are focused on Ca^{2+} signaling (Ca^{2+} dynamics and Ca^{2+} sensitization) mediated by G protein/K_{Ca}/VDC linkage and RhoA/Rho-kinase processes.

2. Mechanism of airway smooth muscle tone

Contractile agonists acting on GPCRs cause contraction of airway smooth muscle with increasing $[Ca^{2+}]_i$ mediated by Ca^{2+} influx passing through Ca^{2+} channels (Ca^{2+} dynamics). When ligands are connected to the GPCRs, receptor-operated Ca^{2+} (ROC) influx is activated [16], and Ca^{2+} is released from sarcoplasmic reticulum (SR) via the production of inositol-1,4,5-triphosphate (IP_3). This Ca^{2+} release activates store-operated capacitative Ca^{2+} (SOC) influx (Figure 1) [17]. Moreover, VDC channels are mainly activated by membrane depolarization under the condition of high K^+ at the extracellular side. Ca^{2+} influx passing through VDC channels contributes to high K^+-induced contraction. In contrast, VDC is partly involved in the GPCR-mediated Ca^{2+} influx [10]. An increase in $[Ca^{2+}]_i$ enhances the binding of Ca^{2+} to calmodulin (CaM), a calcium-binding messenger protein. MLCK activity is augmented by a Ca^{2+}-CaM complex (Ca^{2+}/CaM), and MLC is phosphorylated (activated) by MLCK [18], leading to contraction of airway smooth muscle (Ca^{2+}-dependent contraction: Ca^{2+} dynamics) [10, 17, 19]. After activated MLC is dephosphorylated (inactivated) by MP, contraction is reversed to relaxation (Figure 1). On the other hand, contractile agonists activate RhoA mediated by stimulating GPCRs. RhoA is activated by binding to GTP (RhoA-GTP: active form of RhoA). Rho-kinase is activated by RhoA-GTP, and MP is phosphorylated by Rho-kinase (MP inactivation) (Figure 1) [20, 21]. MP is also phosphorylated by CPI-17, which is another potential

mediator regulated by protein kinase C [22]. Since MLC activity is sustained, not suppressed, by loss of MLC dephosphorylation via inactivation of MP, airway smooth muscle tone is enhanced without increasing $[Ca^{2+}]_i$ (Ca^{2+}-independent contraction: Ca^{2+} sensitization) [19, 23]. Airway smooth muscle tone is regulated by the degree of MLC phosphorylation mediated by both MLCK and MP activity. Alterations of contractile phenotype, which are due to both Ca^{2+} dynamics and Ca^{2+} sensitization, have clinical relevance to airflow limitation, airway hyperresponsiveness, and reduced responsiveness to β_2-adrenoceptor agonists (β_2-adrenergic desensitization), which are implicated with the pathophysiology of obstructive pulmonary diseases, such as asthma and COPD [1].

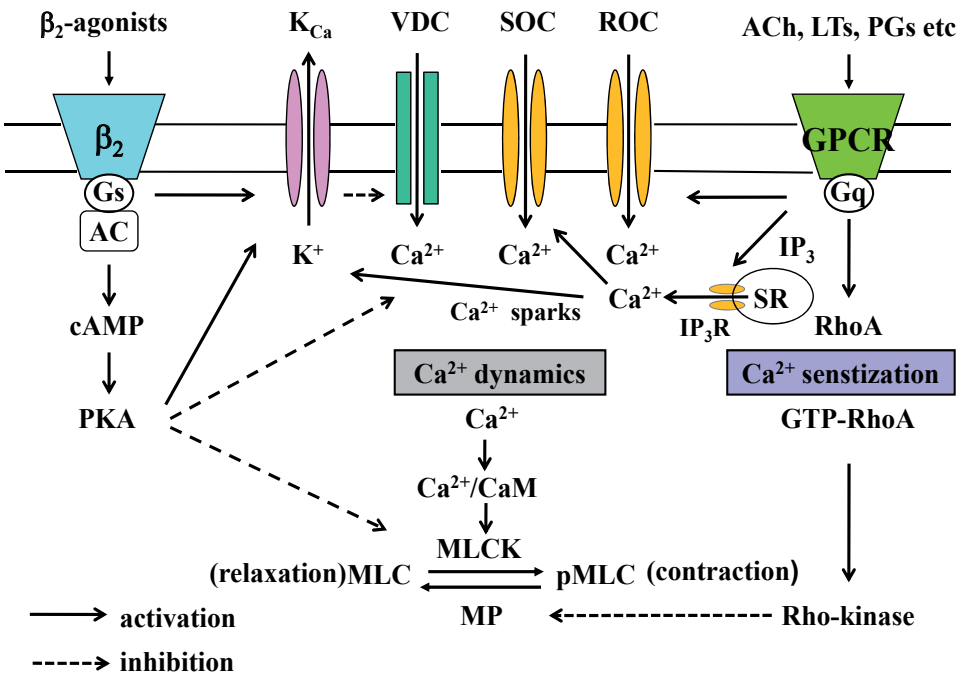

Figure 1. Role of Ca^{2+} dynamics and Ca^{2+} sensitization in the regulation of airway smooth muscle tone. Ca^{2+} signaling via Ca^{2+} dynamics and Ca^{2+} sensitization contributes to the functional antagonism between β_2-adreneceptor agonists and contractile agonists (such as histamine, ACh, LTs, and PGs), acting on GPCRs. MLC phosphorylation (pMLC), which is regulated by a balance between MLCK and MP, is fundamental for controlling contraction in airway smooth muscle. GPCR-related agents cause Ca^{2+} influx by activating ROC and cause Ca^{2+} release from SR by producing IP_3. The latter process induces Ca^{2+} influx via activating SOC. An increase in intracellular concentrations of Ca^{2+} mediated by these processes enhances the binding of Ca^{2+} to CaM. A Ca^{2+}–CaM complex (Ca^{2+}/CaM) augments MLCK activity, leading to MLC phosphorylation (Ca^{2+} dynamics: Ca^{2+}-dependent mechanisms). On the other hand, contractile agonists activate RhoA by acting on G-protein–coupled receptors. Rho-kinase activated by GTP-RhoA phosphorylates (inactivates) MP, leading to MLC phosphorylation (Ca^{2+} sensitization: Ca^{2+}-independent mechanisms). ACh: acetylcholine, LTs: leukotrienes, PGs: prostaglandins, β_2: β_2-adrenoceptors, GPCRs: G-protein–coupled receptors, AC: adenylyl cyclase, ROC: receptor-operated Ca^{2+} influx, SOC: store-operated Ca^{2+} influx, IP_3: inositol-1,4,5-triphosphate, SR: sarcoplasmic reticulum, PKA: protein kinase A, CaM: calmodulin, MLCK: myosin light chain kinase, MLC: myosin light chain, MP: myosin phosphatase, K_{Ca}: large-conductance Ca^{2+}-activated K^+ channels, VDC: L-type voltage-dependent Ca^{2+} channels Illustrated based on ref. [1]

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3. Airway smooth muscle tone regulated by K_{Ca} channels

3.1. Characteristics and physiological roles of K_{Ca} channels

3.1.1. Structure of K_{Ca} channels

K_{Ca} channels are composed of a tetramer formed by pore-forming α-subunits along with accessory β-subunits, and these channels are activated by increased membrane potential and increased $[Ca^{2+}]_i$. The α-subunit is ubiquitously expressed by mammalian tissues and encoded by a single gene (Slo, KCNMA1) [24, 25]. The α-subunit transmembrane domains comprise seven membrane-spanning segments (S0-S6) with extracellular loops and share homology with all voltage-gated K^+ channels with six transmembrane domains (S1-S6) and a pore helix. S1-S4 are arranged in a bundle that forms the voltage-sensing component, and S5-S6 and pore helices contribute to form the pore-forming component and the K^+ selective filter [26]. The C-terminal tail confers the Ca^{2+}-sensing ability of the K_{Ca} channels, involving a pair of Ca^{2+}-sensing domains that regulate the conductance of K^+ (RCK), i.e., RCK1 and RCK2 [27]. Although the Ca^{2+} sensor of the K_{Ca} channels has high specificity for Ca^{2+}, other factors including divalent cations also influence the opening of these channels. Magnesium (Mg^{2+}) enhances activation of these channels via a distinct binding site in the voltage sensor and RCK1 domain [28]. On the other hand, intracellular protons (H^+) attenuate the opening of the K_{Ca} channels [9, 29]. K_{Ca} channels associate with modulatory β-subunits, which are expressed in a cell-specific manner and have unique regulatory actions on these channels. The β-subunits bring about diversity of the K_{Ca} channels. There are four distinct β-subunits, β1-4, which are encoded by KCNMB1, KCNMB2, KCNMB3, and KCNMB4. These β-subunits in the K_{Ca} channels consist of two transmembrane domains with intracellular N- and C-termini and a long extracellular loop. The β1 subunit was the first β-subunit to be cloned and is primarily expressed in smooth muscle [30].

3.1.2. Electrical characteristics of K_{Ca} channels

K_{Ca} channels are densely distributed on the cell membrane in airway smooth muscle cells and have a large conductance (about 250 pS in a symmetrical 135-150 mM K^+ medium) [31, 32, 33], as compared to other K^+ channels. In freshly isolated human bronchial smooth muscle cells, single currents of the K_{Ca} channels were also recorded in cell-attached patches, inside-out patches, and outside-out patches [34, 35]. These channels have a conductance of about 210 pS in symmetrical 140 mM K^+ medium. K_{Ca} channels are highly selective for K^+ despite their large conductance [36]. Ca^{2+} sensitivity may be increased by intracellular Mg^{2+}, as is the case in vascular muscle [37]. Effects of intracellular pH (pH_i) on K_{Ca} channels have been studied in rabbit tracheal muscle by using inside-out patches [9]. K_{Ca} channel activity was markedly inhibited by intracellular acidification, by reducing the sensitivity to Ca^{2+} and also by shortening the open state of the channel. On the other hand, intracellular alkalization had an opposite effect (increasing Ca^{2+} sensitivity and lengthening the open state of the channel). Single-channel currents of K_{Ca} channels in guinea pig and canine tracheal muscle, studied in

outside-out patches, were reversibly blocked by external application of charybdotoxin (ChTX) or iberiotoxin (IbTX), selective antagonists of K_{Ca} channels. This effect was not a result of reduced current amplitude; rather, it was caused by reducing the open-state probability (nPo), the fraction of the time during which the channel is open [7, 38]. In bovine trachealis, externally applied tetraethylammonium (TEA, 1 mM) strongly reduced the amplitude of single K_{Ca} channel current, different from the effects of ChTX (100 nM) on these channels without affecting current amplitude [32]. The effect of ChTX was also reversible. In contrast, the K_{Ca} channels were not affected by 4-aminopyridine (4-AP, 1 mM) applied internally or (2 mM) externally.

3.1.3. Physiological role of K_{Ca} channels

Typical action potentials have not been found in airway muscle under physiological conditions. This lack of action potentials is believed to be due to a marked increase in K^+ conductance of the plasma membrane upon depolarization [39]. Thus, when the K^+ conductance of the membrane is reduced by blocking K^+ channels, one would expect an increase in excitability. In airway smooth muscle that is only weakly excitable, spontaneous phasic contractions can be initiated along with electrical activities by applying K^+ channel blocking agents, such as TEA, 4-AP, ChTX and IbTX [40]. Some of these contractions are accompanied by electrical activity. These observations suggest that outward K^+ currents passing through K_{Ca} channels may be functioning in an important regulatory role in these smooth muscle cells [41].

In excitation-contraction coupling of smooth muscle cells, local increases in Ca^{2+} concentrations occur due to focal releases of Ca^{2+} through ryanodine receptors (RyR) from the sarcoplasmic reticulum (SR), termed Ca^{2+} sparks [42]. Hundreds of K_{Ca} channels are opened by the Ca^{2+} sparks from SR close to the sarcolemma, leading to spontaneous outward currents (STOCs) (Figure 1). The coupling of ryanodine-mediated Ca^{2+} sparks to K_{Ca} channel-mediated STOCs is enhanced by the β_1 subunit, resulting in hyperpolarization of smooth muscle cells and the subsequent reduction of Ca^{2+} influx and initiation of muscle relaxation. In K_{Ca} channel β_1 subunit knockout mice, tracheal contraction induced by carbachol (CCh), a muscarinic receptor agonist, was enhanced as compared to wild-type mice, and not only the single channel activity of K_{Ca} channels in an inside-out patch but also STOCs in a whole cell configuration were markedly attenuated in tracheal smooth muscle cells of knockout mice as compared to wild-type mice [43]. IbTX (30 nM) enhances contraction induced by methacholine (MCh), a muscarinic receptor agonist, and verapamil, an inhibitor of VDC, suppresses the effect of IbTX on tension, demonstrating that K_{Ca} channel inhibition augments contraction via a Ca^{2+} influx through VDC channels [10].

3.2. Stimulatory regulation of K_{Ca} channels by β_2-adrenergic receptor agonists

3.2.1. cAMP-dependent phosphorylation

The involvement of cAMP-dependent processes in K_{Ca} channel regulation has been examined in rabbit tracheal smooth muscle cells by using single-channel recording. In the presence of cAMP and adenosine triphosphate (ATP, 0.3 mM), application of PKA (10 units/ml) to the

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cytosolic side of inside-out membrane patches reversibly increased the nPo of K_{Ca} channels without changes in the amplitude of single-channel currents, and the recovery from this activation was significantly delayed by okadaic acid, an inhibitor of protein phosphatases [3]. A similar effect was observed with the catalytic subunit of PKA (10 units/ml), indicating that phosphorylation of a K_{Ca} channel protein enhances the open state of the channel [3, 4]. External application of isoprenaline (0.2 μM), a β_2-adrenoceptor agonist, and okadaic acid (10 μM) also increased the activation of K_{Ca} channels in the cell-attached patch-clamp configuration, and the recovery from this activation was also significantly delayed by okadaic acid (Figure 2A) [3]. In Xenopus oocytes, similar results were observed in β-adrenergic action [44]. Moreover, external application of forskolin (10 μM), a direct activator of adenylyl cyclase, increased the K_{Ca} channel activity in tracheal smooth muscle cells [45]. These results are in accordance with results obtained in cultured smooth muscle cells of rat aorta using isoprenaline (10 μM), forskolin (10 μM), and dibutyryl cAMP (100 μM) in cell-attached patches and by using PKA (0.5 μM) and cAMP (1 μM) in inside-out patches [46]. These findings demonstrate that β_2-adrenoceptor agonists augment K_{Ca} channel activity via PKA-mediated phosphorylation in airway smooth muscle.

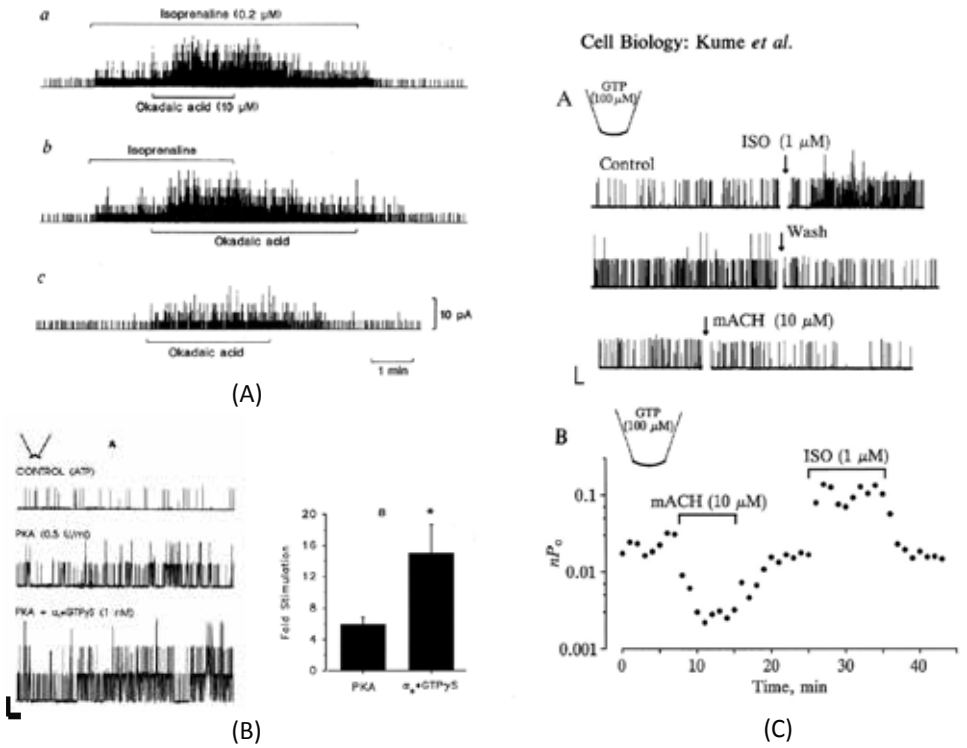

Figure 2. Stimulation and inhibition of K_{Ca} channels by β_2-adrenoceptor and muscarinic receptor agonists in single-channel recording of tracheal smooth muscle cells. A: A continuous recording of the effects of external application of isoprenaline (0.2 μM) and okadaic acid (10 μM) on K_{Ca} channels in a cell-attached configuration held at −40 mV. Isoprenaline increased K_{Ca} channel activity, and okadaic acid enhanced the effects of isoprenaline on these channels (up-

per trace). The time course for washing out the effects of isoprenaline was markedly prolonged in the presence of okadaic acid (middle trace). These results demonstrate that K_{Ca} channel activity is regulated by phosphorylation via PKA. Okadaic acid augmented K_{Ca} channel activity, demonstrating that phosphatase activity is still intact in this experimental condition (lower trcae). B: A continuous recording of the effects of PKA and α_s*GTPγs on K_{Ca} channels in an inside-out patch held at 0 mV (left panel). PKA (0.5 U/ml) maximally increased K_{Ca} channel activity, and addition of the α_s*GTPγs (1 nM) enhanced K_{Ca} channel activity prestimulated by PKA (0.5 U/ml), indicating that α_s activates K_{Ca} channels independent of PKA. Calibration bars, 3 pA and 4 s. Fold stimulation of channel activity are shown under the condition of addition of PKA (0.5 U/ml) and subsequently by addition of α_s*GTPγs (1 nM) (right panel). C: A continuous recording of the effects of ISO (1 μM) and mACH (10 μM) on K_{Ca} channels in an outside-out patch held at 0mV. External application of ISO increased K_{Ca} channel activity, whereas, following washout, mACH decreased this channel activity (upper trace), indicating that K_{Ca} channels are key molecules for the functional antagonisms between these two receptors. Calibration bars, 3 pA and 10 s. Relationship between nPo and time for an experiment similar to the upper trace with agonists added in reverse order (lower trace). PKA: protein kinase A, α_s: α-subunit of G_s, which is stimulatory G protein of adenylyl cyclase, ISO: isoprenaline, mACH: methacholine, K_{Ca}: large-conductance Ca^{2+}-activated K^+ channels, nPo: open−state probability, U: unit. Cited from ref. [3, 4, 7].

3.2.2. Membrane-delimited activation by G_s 30 nM

Activation of K_{Ca} channels by isoprenaline is mediated by the α-subunit (α_s) of the stimulatory G protein of adenylyl cyclase (G_s), independent of cAMP-dependent protein phosphorylation [4, 7]. In porcine, canine and ferret tracheal muscle cells, isoprenaline increased the activation of K_{Ca} channels in outside-out patches when guanosine triphosphate (GTP, 100 μM) was present at the cytosolic side of the patch. A similar increase in K_{Ca} channel activity was also observed even when phosphorylation was inhibited by the nonmetabolizable ATP analog, adenosine 5′-[β, γ-imido] diphosphate (ATP [β; γ NH], AMP-PNP (1 mM)) [4, 7]. In inside-out patch configuration with a patch pipette containing isoprenaline (1 μM), nonhydrolyzable GTP analog guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S, 100 μM) similarly potentiated the K_{Ca} channel activity. The recombinant α_s proteins preincubated with GTP-γ-S (α_s*GTPγS, 100-1000 pM) increased the channel activity in a concentration-dependent manner when applied to the cytosolic side of inside-out patches [7]. The maximum effects of α_s*GTPγS were observed at 1000 pM, and the nPo of K_{Ca} channels was augmented to approximately 16-fold. On the other hand, α_s preincubated with guanosine 5′-O-(2-thio-diphoshate) (GDP-β-S) had no effect on these channels. These results indicate that the K_{Ca} channels are directly activated by α_s (membrane-delimited action) and that cAMP-dependent phosphorylation is not required. A direct action of G_s protein on the K_{Ca} channels has also been demonstrated in channels from rat or pig myometrium incorporated into planar lipid bilayers, by using GTP-γ-S and AMP-PNP [47]. β_2-adrenoceptor agonists act on smooth muscle without the intracellular signal transduction processes (the cAMP-PKA pathway).

3.2.3. Dual regulation by cAMP-dependent and -independent processes

To examine whether receptor-channel coupling could occur in β_2-adrenergic action on K_{Ca} channels, isoprenaline was applied to outside-out patches in the presence of GTP (100 μM) and AMP-PNP (1 mM), the competitive ATP inhibitor, in porcine tracheal smooth muscle cells (Figure 3) [4]. Isoprenaline (1 μM) markedly activated K_{Ca} channel activity without an alteration in current amplitude and returned to the control level within 5 min after drug washout. The nPo of the channels was increased to approximately fivefold in the presence of isoprenaline. This result was roughly equivalent to the level of channel stimulation previously

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reported in outside-out experiments in the absence of ATP, but without AMP-PNP. Consistent with a membrane-delimited, G protein-dependent coupling mechanism, addition of guanine nucleotides to the cytosolic side stimulated K_{Ca} channel activity in inside-out patches exposed to isoprenaline on the external side. Internal application of GTP (100 μM) also led to a marked increase in K_{Ca} channel activity; the nPo of the channels was increased to an approximately equivalent fold, as compared to the experimental condition when isoprenaline was applied to the outside-out patches in the presence of GTP. Stimulation of channel activity resulted in an apparent shift in the relationship between voltage and nPo by 10-15 mV after the addition of 100 μM GTP.

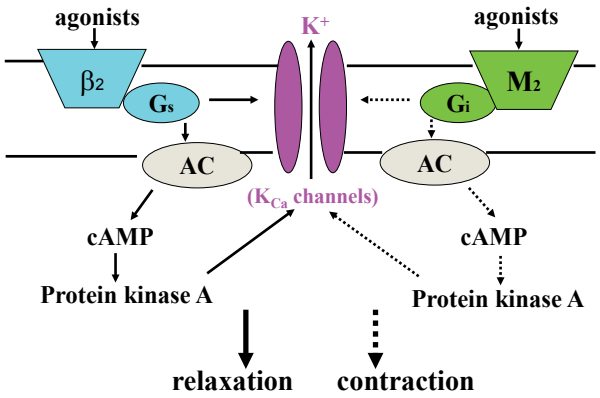

Figure 3. Dual pathway and dual regulation of K_{Ca} channels in the functional antagonisms between β_2-adrenoceptors and muscarinic receptors. At least two mechanisms are involved in activation of K_{Ca} channels following the β_2-adrenoceptor activation; one is mediated through cAMP-dependent channel phosphorylation and the other through direct, cAMP-independent regulation by G_s protein (dual pathway). In contrast, in the muscarinic suppression of K_{Ca} channels, G_i proteins connected to M_2 receptors are involved (dual regulation). The relationship between G proteins and K_{Ca} channels, i.e. the G_s/K_{Ca} stimulatory linkage and the G_i/K_{Ca} inhibitory linkage, may play a key role in the functional antagonisms (relaxation, contraction) between β_2-adrenoceptors and muscarinic receptors in airway smooth muscle. β_2: β_2-adrenoceptors, M_2: M_2 muscarinc receptors, AC: adenylyl cyclase, G_i: inhibitory G protein of adenylyl cyclase, G_s: stimulatory G protein of adenylyl cyclase, PKA: protein kinase A, K_{Ca}: large-conductance Ca^{2+}-activated K^+ channels. Illustrated based on ref. [3, 4, 7, 53].

Stimulation of K_{Ca} channels by the catalytic subunit of cAMP-dependent PKA was examined in inside-out patches. K_{Ca} channel activity was progressively stimulated by a cumulative dose-response protocol (PKA between 0.0005 and 5.0 units/ml). The maximum level of K_{Ca} channel stimulation by PKA was observed at either 0.5 or 5.0 units/ml (approximately sevenfold stimulation). At peak effect, the mean stimulation was approximately 7-fold, which was substantially less than the approximately 16-fold stimulation previously observed for 1 nM α_s*GTPγS. To examine the dual pathway of β_2-adrenoceptor/channel coupling, inside-out patches were stimulated to near maximum with PKA (0.5 unit/ml). This concentration was chosen since it provided near maximal stimulation in all patches and a stable stimulation of channel activity over time. Following incubation with PKA for 5 min, α_s*GTPγS (1 nM) was added. K_{Ca} channels were potently activated by the addition of recombinant α_s protein after

stimulation by the near maximally effective concentration of PKA (Figure 2B) [4]. PKA produced an approximately 6-fold stimulation, and addition of α_s produced an approximately 15-fold increase over baseline channel activity (Figure 2B). The fold stimulation produced during the condition of combined PKA and α_s application was more than twice as great as the maximal fold stimulation that could be produced by PKA alone, suggesting that PKA and α_s affect the channels independently.

The gating kinetics of K_{Ca} channels were quantitatively examined by monitoring the effects of stimulation of channel activity by isoprenaline (outside-out patches) and by PKA and α_s (inside-out patches) at the level of channel open-time kinetics [4]. K_{Ca} channel open-times were well fit by the sum of two exponentials of mean duration τ_1 and τ_2, similar to previous reports [8, 9, 48]. The effect of α_s on open-time kinetics was remarkably similar to that produced by isoprenaline on open-time kinetics; that is, α_s did not alter the mean lifetimes, but increased the proportion of long open-time events. In contrast, the major kinetic effect of PKA was on open-state time constants, resulting in an increase in the mean duration of the long openings. The effect of PKA on channel kinetics was distinct from that of α_s, consistent with distinct or independent modulatory effects at the channel protein.

3.2.4. Role in relaxation by β_2-adrenergic receptor agonists

Airway smooth muscle relaxation produced by β-adrenoceptor activation is generally accompanied by membrane hyperpolarization, observed with intracellular microelectrodes in guinea pig, dog, and human tracheal muscles [49, 50], for which activation of K_{Ca} channels is thought to be responsible for the relaxation, as described earlier. This idea is supported by the observations in guinea pig and human trachealis that the relaxation by noradrenaline (1 μM) against CCh-induced contraction was nearly blocked by ChTX (50 nM) and that the concentration-relaxation curves to β_2-adrenoceptor agonists, such as isoprenaline and salbutamol, were selectively shifted to the right by ChTX [51, 52]. The relaxant effect of forskolin on MCh-induced contraction was also attenuated in the presence of ChTX, similar to isoprenaline [45]. Therefore, an increase in K_{Ca} channel activity may contribute to airway smooth muscle relaxation induced by β_2-adrenoceptor agonists and cAMP-related agents. After G_s activity was irreversibly augmented by incubation with cholera toxin (2 μg/ml) for 6 h in guinea pig trachea, MCh-induced contraction was significantly attenuated, and this effect by G_s was reversed in the presence of ChTX (100 nM) [53]. The G_s/K_{Ca} stimulatory linkage may also be involved in β-adrenergic relaxation in airway smooth muscle.

3.3. Inhibitory regulation of K_{Ca} channels by muscarinic receptor agonists

3.3.1. Membrane-delimited inhibition by G_i

When MCh (50 μM) was applied to outside-out patches of porcine or canine tracheal muscle cells, the nPo of the K_{Ca} channel was markedly decreased without changes in the amplitude of single-channel currents [8, 54]. The decreased nPo is due to a reduction in channel open times,

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probably reflecting a decrease in the Ca^{2+} sensitivity of the channel. The muscarinic inhibition of K_{Ca} channels, similar to that found in airway smooth muscle, has been reported for the circular muscle of canine colon. The inhibition of K_{Ca} channels through muscarinic activation in guinea pig and swine tracheal muscle cells may be partly responsible for the prolonged suppression by ACh of STOCs following a transient increase [55, 56]. This suppression has been observed in longitudinal muscle cells of the rabbit jejunum. As discussed by Saunders and Farley, this inhibition is difficult to explain by the depletion of intracellular Ca^{2+} stores, because it occurs even with elevated Ca^{2+} concentrations. In the porcine and canine trachealis, the inhibition of K_{Ca} channels produced by muscarinic stimulation was potentiated by cytosolic application of GTP (100 μM), and strong, irreversible, potentiation was obtained with GTP-γ-S (100 μM) [8]. On the other hand, when GDP-β-S (1 mM) was applied to the cytosolic side, muscarinic inhibition was not observed. Incubation (4-6 h) of airway smooth muscle cells with pertussis toxin (0.1-1.0 μg/ml), which blocks signal transduction through ADP ribosylation of G_i, the inhibitory G protein of adenylyl cyclase, abolished the channel inhibition by MCh, without reducing channel activity in the control state [8]. The G_i/K_{Ca} inhibitory linkage may be involved in the muscarinic action in airway smooth muscle.

3.3.2. Dual regulation by G_s and G_i

As described earlier, K_{Ca} channels are markedly activated by β_2-adrenoceptor agonists; in contrast, K_{Ca} channels are markedly suppressed by muscarinic receptor agonists via G proteins (Figure 3). The activation process is mediated by the stimulatory G protein, G_s; in contrast, the suppression process is mediated by the inhibitory G protein, G_i (dual regulation). To demonstrate the functional antagonistic, hormone-linked stimulatory and inhibitory regulation of K_{Ca} channels by G proteins at the single-channel level, isoprenaline and MCh were sequentially applied to identical outside-out patches under the condition of physiologic Ca^{2+} concentration and GTP (100 μM) [7]. External application of isoprenaline (1 μM) markedly increased K_{Ca} channel activity, and following drug washout this channel activity reversed to baseline; then, external application of MCh (10 μM) markedly decreased this channel activity (Figure 2C). Receptor-linked stimulatory and inhibitory modulation of K_{Ca} channels was not sequentially dependent as shown by an experiment in which this channel activity was inhibited by MCh and then activated by isoprenaline. Consistent with these outside-out experiments, internal addition of guanine nucleotides stimulated K_{Ca} channels when isoprenaline was present at the extracellular side in inside-out patches, and conversely, guanine nucleotides suppressed the channel activity when MCh was present at the extracellular side in inside-out patches [7]. These results indicate that the functional antagonism between β_2-adrenergic and muscarinic action converges on a single K_{Ca} channel current. Therefore, K_{Ca} channel activity plays a key role in the regulation of airway smooth muscle tone.

3.3.3. Role in contraction by muscarinic receptor agonists

After incubation of tracheal smooth muscle with pertussis toxin (1.0 μg/ml for 6 h), MCh-induced contraction was significantly attenuated, and this effect by pertussis toxin was

reversed in the presence of ChTX [53]. The G_i/K_{Ca} inhibitory linkage may be involved in the muscarinic-induced contraction in airway smooth muscle. From a functional point of view, it would be favorable to reduce the K^+ conductance of the plasma membrane to produce excitation by agonists such as ACh. G_i protein couples with the M_2 subtype of muscarinic receptors, leading to an inhibition in cAMP. These M_2 receptors exist on the surface of airway smooth muscle cells. A selective M_2 receptor antagonist (AF-DX 116, a benzodiazepine derivative) suppressed MCh-induced contraction in a concentration-dependent manner and potentiated relaxation induced by isoprenaline and forskolin in MCh-precontracted tracheal muscle [53]. AF-DX116 had no effect on isoprenaline-induced relaxation when the preparation was precontracted with histamine. The functional antagonism between isoprenaline (or forskolin) and M_2 receptor stimulation may not only be simply mediated by inhibition of adenylyl cyclase through the M_2 receptors but also be exerted by the direct inhibition of K_{Ca} channels by pertussis toxin–sensitive G_i protein through activation of muscarinic receptors, since there is evidence that the activation of K_{Ca} channels is involved in the relaxation induced by forskolin and isoprenaline. Furthermore, M_2 receptors inhibited the activity of K_{Ca} channels via dual pathways of a direct membrane-delimited interaction of $G\beta\gamma$ and activation of phospholipase C/protein kinase C [57]. In K_{Ca} channel $\beta1$ subunit knockout mice, CCh-induced contraction and membrane depolarization in tracheal smooth muscle were enhanced as compared to wild-type mice, and these augmented effects of CCh were inhibited in the presence of AF-DX116 [43, 58]. These results indicate that the K_{Ca} channel $\beta1$ subunit plays a functional role in opposing M_2 muscarinic receptor signaling.

3.3.4. Regulation of K_{Ca} channels by other factors (cGMP, protein kinase C)

3.3.4.1. NO, cGMP

Nitric oxide (NO), which is primarily generated by nitric oxide synthase (NOS) in the endothelium, causes relaxation of vascular smooth muscle cells via hyperpolarization of the cell membrane [59, 60]. NO also augmented the K_{Ca} channel activity in vascular smooth muscles, and NO-induced vasodilation was attenuated by blockade of the K_{Ca} channel activity [61]. The NO/3'-5'-cyclic guanosine monophosphate (cGMP) pathway plays an important role in relaxation of smooth muscle including vessels and airways. K_{Ca} channels were markedly enhanced by cGMP-mediated processes, suggesting that activation of these channels leads to cGMP-induced relaxation of smooth muscle [62, 63]. The K_{Ca} channel α-subunit null mice had increased vascular smooth contraction as compared to wild-type mice [64]. This phenomenon was due to an impaired response to cGMP-dependent vasorelaxation, indicating that the K_{Ca} channel is an important effector for cGMP-mediated action. Protein kinase G (PKG) was involved in this activation of K_{Ca} channels via the NO/cGMP pathway [65, 66]. Activation of K_{Ca} channels via dopamine receptors occurs through PKG and mediates relaxation in coronary and renal arteries [67]. PKG may be cross-activated by cAMP to stimulate K_{Ca} channels [68]. Moreover, dual pathways of K_{Ca} channel modulation by NO have been demonstrated; these pathways are the PKG-dependent pathway [69] and the direct activation of NO with the

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channel protein [70]. Since the stimulatory effect of NO on K_{Ca} channels was abolished by knockdown of the β-subunit with antisense, the β-subunit acts as a mediator of NO [71].

3.3.4.2. Protein kinase C

K_{Ca} channels are activated via phosphorylation of their channels by PKA and PKG, as described earlier. However, the effects of protein kinase C (PKC) on these channels are still controversial. PKC enhanced the activity of K_{Ca} channels in rat pulmonary arterial smooth muscle [72]. In contrast, PKC reversed cAMP-induced activation of these channels [73]. The phosphorylation by PKC acts on K_{Ca} channels via direct inhibition and also acts as a switch to influence the effects of PKA and PKG [74, 75]. In addition to these pathways, c-Src and tyrosine kinase suppressed the activity of the K_{Ca} channels in coronary and aortic myocytes [76], whereas cSrc-induced phosphorylation augmented these channels in HEK 293 cells [77].

3.3.4.3. Redox and ROS

Reactive oxygen species (ROS) synthesized in endothelial and smooth muscle cells exert physiological and pathophysiological effects on smooth muscle via altering intracellular reduction and/or oxygen (redox) status [78]. The redox state influences the gating of K_{Ca} channels [79]. However, the effects of redox are complex. Preferential oxidation of methionine increased the activity of K_{Ca} channels, whereas oxidation of cysteines reduced the channel activity [80, 81]. K_{Ca} channel activity was enhanced by hydrogen peroxide (H_2O_2) in pulmonary arterial smooth muscle, resulting in vasodilation mediated by membrane hyperpolarization [82]. Hydrogen peroxide (H_2O_2) may directly bind to K_{Ca} channels to regulate them, or it may activate these channels via the phospholipase A_2-arachidonic acid pathway and metabolites of lipoxygenase [83]. On the other hand, H_2O_2 caused contraction of tracheal smooth muscle in a concentration-dependent fashion and elevation of $[Ca^{2+}]_i$ [84]. Moreover, peroxynitrite (OONO-), an oxidant generated by the reaction of NO and superoxide, caused contraction of the cerebral artery by inhibiting K_{Ca} channel activity [85].

3.3.4.4. Arachidonic acid

Arachidonic acid and its metabolites such as 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) play an important role in the regulation of vascular smooth muscle tone. Arachidonic acid and EETs caused vasodilation mediated by increasing K_{Ca} channel activity [86, 87]. In airway smooth muscle, 20-HETE also caused relaxation with membrane hyperpolarization via activation of K_{Ca} channels [88]. On the other hand, 20-HETE is a vasoconstrictor. K_{Ca} channel activity was inhibited by 20-HETE, and this phenomenon is mediated by PKC [89]. The vasoconstriction induced by 20-HETE was also attenuated by increasing K_{Ca} channel activity [89]. Acute hypoxia reduced the generation of 20-HETE, and subsequently the inhibitory action of 20-HETE on K_{Ca} channels was removed in cerebral arterial smooth muscle cells [90].

3.4. Synergistic effects between muscarinic and β_2-adrenergic receptors

The combination of muscarinic receptor antagonists with β_2-adrenoceptor agonists has pharmacological rationale as a bronchodilator therapy for COPD [91]. In the human airway, muscarinic contraction is more resistant to β_2-adrenoceptor–induced relaxation than that induced by other contractile agonists [92]. Muscarinic receptors and β_2-adrenoceptors are unevenly distributed in the human airways. β_2-adrenoceptors were increased in the distal airways: segmental bronchus < subsegmental bronchus < lung parenchyma [93]. M_3 receptors are expressed more exclusively in segmental than subsegmental bronchus; in contrast, the M_2 subtype is widely distributed throughout the airways, while the M_1 subtype is found only in parenchyma [93]. These findings may explain why combined inhalation of a muscarinic antagonist and a β_2-adreneceptor agonist causes greater bronchodilation than monotherapy [94]. Furthermore, characteristic interactions between muscarinic receptors and β_2-adrenoceptors are involved in prejunctional modulation of ACh release from parasympathetic nerve endings [95] and intracellular signaling cross-talk at the adenylyl cyclase/PKA level [96], resulting in synergistic effects on relaxation of airway smooth muscle. K_{Ca} channel activity may contribute to these interactions between these two receptors; however, little is known about the detailed underlying mechanisms.

In isometric tension recordings of guinea pig tracheal smooth muscle, indacaterol (1 nM), a long-acting β_2-adrenoceptor agonist, modestly inhibited MCh-induced contraction (1 μM) (Figure 4A). When glycopyrronium bromide (10 nM), a long-acting muscarinic receptor antagonist, was applied in the presence of indacaterol (1 nM), the relaxant effect of glycopyrronium bromide was significantly augmented (Figure 4A) [97]. The value of percent relaxation for the combination of indacaterol with glycopyrronium bromide was more than the sum of that for each agent. Similar results were observed between indacaterol (1 nM) and glycopyrronium (3-30 nM) [97]. Moreover, similar results were also observed between other β_2-adreneceptor agonists, such as salbutamol and procaterol, and other muscarinic receptor antagonists, such as atropine and tiotropium (our unpublished observation). These results indicate that the combination of muscarinic receptor antagonists with β_2-adrenoceptor agonists causes a synergistic inhibition against muscarinic contraction. This phenomenon was observed in isolated human bronchus [98]. This synergistic effect was enhanced after exposure to pertussis toxin (1 μg/ml) or cholera toxin (2 μg/ml) for 6 h; in contrast, the effect was attenuated in the presence ChTX (100 nM) or IbTX (30 nM). A reduction in this synergistic effect induced by ChTX or IbTX was reversed to the control response in the presence of verapamil (Figure 4B) [99]. Inactivation of the G_i/K_{Ca} inhibitory linkage and activation of the G_s/K_{Ca} stimulatory linkage are involved in this synergistic effect between muscarinic receptor antagonists and β_2-adrenoceptor agonists in airway smooth muscle (Figure 3) [4, 7, 53]. Moreover, the K_{Ca}/VDC channel linkage is also involved in this synergistic effect. On the other hand, synergistic effects did not occur between β_2-adrenoceptor agonists and theophylline in airway smooth muscle (our unpublished observation). Although the clinical relevance of this result is still unknown, this result may provide evidence that combination therapy between muscarinic receptor antagonists and β_2-adrenoceptor agonists is an effective bronchodilator therapy for COPD [100].

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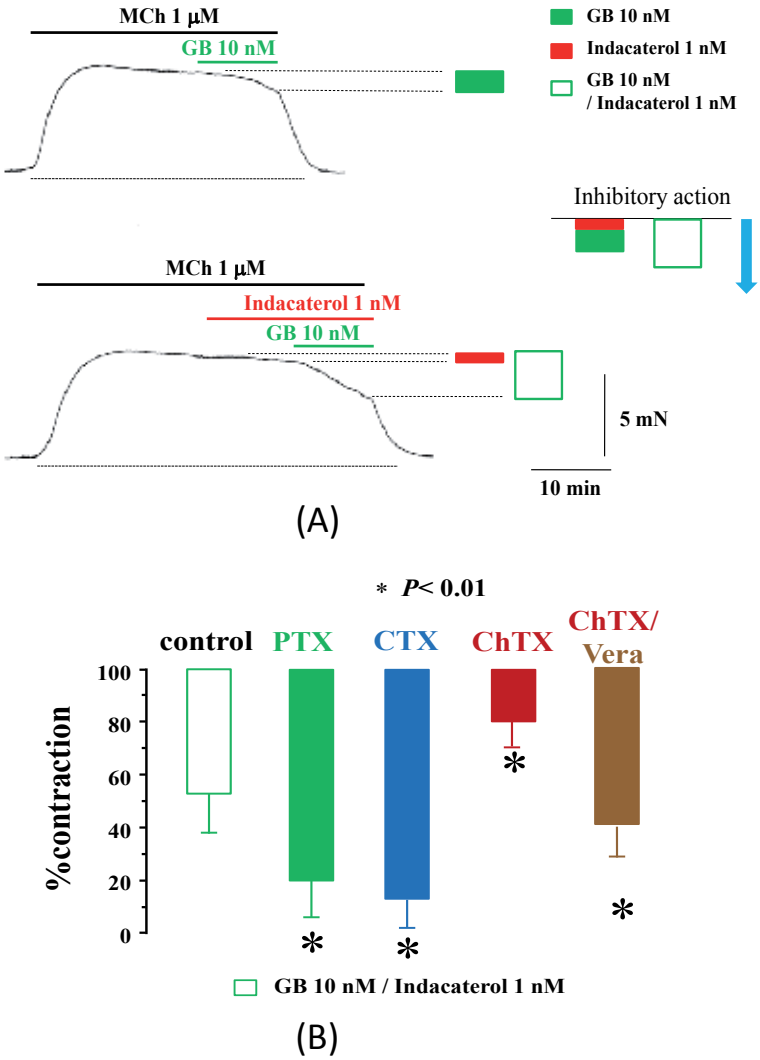

Figure 4. Synergistic action in combination of β_2-adrenocetor agonists with muscarinic receptor antagonists against tracheal smooth muscle contraction. A: Left panel: A typical example of the inhibitory effects of GB (10 nM), a long-acting muscarinic receptor antagonist (LAMA) against MCh (1 μM)-induced contraction (upper trace). A typical example of the inhibitory effects of equimolar amounts of GB in the presence of indacaterol (1 nM), a long-acting β_2-adrenoceptor agonist (LABA) against MCh-induced contraction (1 μM) (lower trace). Right panel: Percent inhibition of combining GB with indacaterol is greater than the sum of each agent, demonstrating synergistic action between β_2-adrenoceptor agonists and muscarinic receptor antagonists. B: The percent inhibition of GB (10 nM) with indacaterol (1 nM) against MCh-induced contraction (1 μM) was markedly augmented after incubation with PTX (1 μg/ml) and CTX (2 μg/ml) for 6 h. In contrast, the percent inhibition was significantly attenuated in the presence of ChTX (100 nM), and this ChTX-induced effect was reversed to the control level by addition of Vera (1 μM). These results indicate that the G proteins (G_i, G_s)/K_{Ca} channel linkage and the K_{Ca}/VDC channel linkage contributed to this synergistic action, similar to the mechanisms shown in Figures 1, 3. GB: glycopyrronium bromide, MCh: methacholine, PTX: pertussis toxin, CTX: cholera toxin, ChTX: charybdotoxin, Vera: verapamil, K_{Ca}: large-conductance Ca^{2+}-activated K^+ channels, VDC: L-type voltage-dependent Ca^{2+} channels. Cited from ref. [97, 99].

4. Airway smooth muscle tone regulated by Ca^{2+} dynamics

4.1. Membrane potential-independent Ca^{2+} dynamics

In simultaneous recordings of isometric tension and $[Ca^{2+}]_i$ in fura-2-loaded tissues of tracheal smooth muscle, various spasmogens including contractile agonists acting on GPCRs augment the tone of airway smooth muscle with elevated $[Ca^{2+}]_i$ in a concentration-dependent fashion (Figure 1) [19, 101]. However, even though contraction fully occurs, these agents cause a modest depolarization of the cell membrane in a microelectrode experiment, indicating that airway smooth muscle contracts by Ca^{2+} influx via membrane potential–independent pathways. These Ca^{2+} dynamics with a modest depolarization are involved in Ca^{2+} influx through SOC and ROC [16, 17]. Depletion of the SR Ca^{2+} stores by thapsigargin, an inhibitor of the SR Ca^{2+}-ATPase, caused an increase in $[Ca^{2+}]_i$ and contraction, demonstrating Ca^{2+} entry through SOC [17]. Because SOC was not inhibited by nifedipine, an inhibitor of VDC, VDC is not involved in SOC. Under the condition that SOC is fully activated, MCh and histamine caused further increases in $[Ca^{2+}]_i$ and tension, demonstrating Ca^{2+} entry independent of SOC and VDC (non-SOC) [17]. The Ca^{2+} influx and contraction via non-SOC was inhibited by Y-27632. In contrast, Y-27632 did not affect SOC.

4.2. Membrane potential–dependent Ca^{2+} dynamics

In fura-2–loaded tissues of tracheal smooth muscle, verapamil caused an inhibition of MCh-induced contraction with reduced $[Ca^{2+}]_i$; however, relaxant effects of verapamil are not so dramatic, indicating that VDC is partly involved in contraction mediated by GPCRs. IbTX enhanced MCh-induced contraction with elevation of $[Ca^{2+}]_i$. These effects of IbTX on tension and $[Ca^{2+}]_i$ are antagonized by verapamil [10], demonstrating that K_{Ca} channel inhibition results in contraction with elevation of $[Ca^{2+}]_i$ induced by opening VDC channels via depolarization of the cell membrane, whereas channel activation results in relaxation with reduction of $[Ca^{2+}]_i$ induced by closing VDC channels via hyperpolarization of cell membrane.

When $[Ca^{2+}]_i$ is increased by Ca^{2+} entry via various pathways described earlier (Ca^{2+} dynamics), the activity of MLCK is enhanced via CaM, leading to contraction via phosphorylation of MLC (see Section 2). In airway smooth muscle, alteration of contractility regulated by Ca^{2+} dynamics is involved in the pathophysiology implicated in asthma and COPD, such as airway limitation, airway hyperresponsiveness, and β_2-adrenergic desensitization. It is useful to suppress Ca^{2+} dynamics for improving these pathological conditions in the airways.

4.3. Effects of Ca^{2+} release from the SR

K_{Ca} channels were activated by ACh (30 μM), substance P (0.1 μM) or IP_3 (2.4-20 μM), as well as by caffeine (5 mM), suggesting that the activity was due to Ca^{2+} released from intracellular stores. These activations with the agonists and IP_3 were markedly and reversibly reduced by heparin (50-100 μg/ml), which inhibits IP_3 binding to its receptors in the SR. Furthermore, in cultured human bronchial smooth bradykinin (0.01-1 μM), an inflammatory mediator caused bronchoconstriction and activated K_{Ca} channels in a concentration-dependent manner; the

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augmented currents were inhibited by heparin (10 μg/ml) [102]. Ca^{2+} release from the SR via stimulation of IP_3 receptors causes an increase in the activation of K_{Ca} channels in smooth muscle including airways and vessels. Two pathways participate in Ca^{2+} release from the SR, the RyR pathway and the IP_3 receptor pathway. In smooth muscle cells, the IP_3 receptor is more abundant than the ryanodine receptor and reacts to IP_3, which is generated from the activation of GPCRs and phospholipase C.

5. Airway smooth muscle tone regulated by Ca^{2+} sensitization

5.1. Characteristics and physiological role of RhoA/Rho-kinase

Although an increase in $[Ca^{2+}]_i$ plays an important role in the contraction of airway smooth muscle (Figure 1) [18], it is generally considered that muscarinic receptor agonists and histamine increase tension at a constant $[Ca^{2+}]_i$. This phenomenon is referred to as Ca^{2+} sensitization [103, 104] and is mediated by a G protein-coupled mechanism. Rho is a monomeric G protein that belongs to the Ras superfamily. The Rho family makes up a major branch that contains Rho, Rac, and CdC42. Rho has isoforms of A-G; however, most of the function is described based on studies of RhoA. RhoA exhibits both GDP/GTP binding activity and GTPase activity, and it acts as a molecular switch between a GDP-bound inactive state (GDP-RhoA) and a GTP-bound active state (GTP-RhoA). When cells are stimulated with G protein–coupled receptor agonists, receptor tyrosine kinases and higher concentrations of potassium chloride (KCl), GDP-RhoA is converted to GTP-RhoA. RhoA and Rho-kinase are widely distributed to many organs, including the respiratory system. Rho-kinase (160 kDa) is an effector molecule of RhoA [105, 106]. Rho-kinase activated by GTP-RhoA interacts with MP and hinders MP activity by phosphorylating threonine 696 and 853 of myosin phosphatase targeting subunit 1 (MYPT1), a myosin-binding subunit [107, 108]. Rho-kinase has effects on contraction due to Ca^{2+} sensitization, stress fiber formation due to actin (cytoskeletal) reorganization, cell migration, and cell proliferation [20, 109]. These processes are implicated in the major pathophysiological characteristics of asthma and COPD, such as airflow limitation, airway hyperresponsiveness, β_2-adrenergic desensitization, eosinophil recruitment and airway remodeling [1].

5.2. Role of RhoA/Rho-kinase on contraction

Y-27632, a pyridine derivative, was developed as a specific Rho-kinase inhibitor. Y-27632 suppresses Ca^{2+} sensitization and relaxes vascular smooth muscle to treat hypertension in rats [21]. The effects of Y-27632 on MCh-induced contraction were analyzed by using strips of guinea pig airway smooth muscle treated with fura-2. Y-227632 suppressed contraction induced by agonists, such as MCh, histamine, prostaglandins, and leukotrienes, in a concentration-dependent manner, but there was no significant decrease in $[Ca^{2+}]_i$ [19]. Recently, it has been demonstrated that MYPT1 is an effective protein for Rho-kinase action on MP in airway smooth muscle cells and that Y-27632 inhibits the phosphorylation of MYPT1 in a concentration-dependent manner [108, 110]. Fasudil hydrochloride (HA-1077), a specific inhibitor of

Rho-kinase, is used clinically to suppress cerebral vasospasm following subarachnoid hemorrhage [111]. Alteration of contractility of airway smooth muscle regulated by Ca^{2+} sensitization is also involved in airflow limitation, airway hyperresponsiveness, and β_2-adrenegic desensitization [1].

6. Role of Ca^{2+} dynamics and Ca^{2+} sensitization in airway disorders

6.1. Airflow limitation (contraction)

Airway smooth muscle contraction due to muscarinic receptor agonists (ACh, MCh and CCh), histamine, prostaglandins or leukotrienes is involved in airflow limitation, which is a characteristic feature of asthma and COPD (Figure 5). These agonists cause contraction of airway smooth muscle with increasing $[Ca^{2+}]_i$ by Ca^{2+} dynamics via Ca^{2+} entry passing through SOC, ROC, and partly VDC. Sphingosine 1-phosphate (S1P: a bioactive lysophospholipid) [108], tryptase (trypsin-like neutral serine-class protease) and SLIGKV (non-enzymatic activator of protease-activated receptor 2, PAR2) [112] released from mast cells induce airway smooth muscle contraction with increasing $[Ca^{2+}]_i$. Since clinical studies have demonstrated that S1P and tryptase may be involved in the pathophysiology of asthma, these substances have been examined as novel mediators. ATP is released from injured airway epithelium during the inflammatory processes implicated in asthma. Extracellular ATP also causes contraction of airway smooth muscle with increasing $[Ca^{2+}]_i$ [113]. Furthermore, oxidative stress and mechanical stress are related to the pathophysiology of not only COPD but also asthma. 8-iso-prostaglandin $F_{2\alpha}$, an isoprostane [114], and hydrogen peroxide (H_2O_2) [84] produced by oxidative stress contract airway smooth muscle by increasing $[Ca^{2+}]_i$.

As described earlier, Y-27632 inhibited the contraction induced by spasmogens such as MCh, histamine, prostaglandins, and leukotrienes, which are involved in the pathophysiology of asthma and COPD, in a concentration-dependent manner, with no significant decrease in $[Ca^{2+}]_i$ in strips of guinea pig airway smooth muscle treated with fura-2. Furthermore, Y-27632 also inhibited the following types of contraction in a concentration-dependent manner with a modest effect on $[Ca^{2+}]_i$: contraction due to S1P and tryptase released from mast cells; contraction due to isoprostanes and Hydrogen peroxide (H_2O_2) produced by oxidative stress; and contraction due to ATP synthesized in injured airway epithelium. Spasmogens, which are implicated in the pathophysiology of asthma and COPD, cause force generation in airway smooth muscle via both Ca^{2+} influx and Ca^{2+} sensitization [115]. Force maintenance is due to Ca^{2+} sensitization induced by Rho-kinase [116]. PKC, which is an intracellular signal transduction pathway for GPCR activation, also contracts airway smooth muscle mediated by both Ca^{2+} dynamics and Ca^{2+} sensitization [22].

These findings indicate that a contractile phenotype in airway smooth muscle cells is altered by the inflammatory processes related to obstructive pulmonary diseases, such as asthma and COPD, via both Ca^{2+} dynamics and Ca^{2+} sensitization, leading to the airflow limitation (bronchoconstriction) associated with these diseases (Figure 5).

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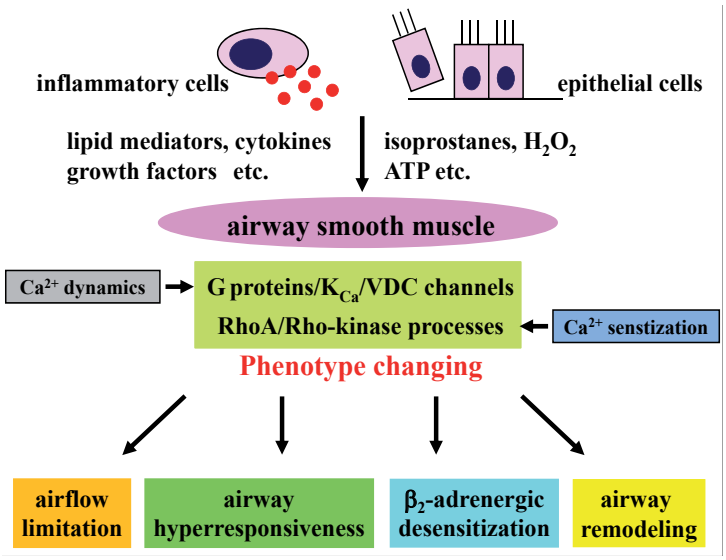

Figure 5. Involvement of G proteins/K_{Ca}/VDC channel linkage (Ca^{2+} dynamics) and RhoA/Rho-kinase processes (Ca^{2+} sensitization) in the pathophysiology of asthma and COPD. Chronic exposure to lipid mediators, cytokines, and other substances related to asthma and COPD, which are released and synthesized from inflammatory cells and epithelial cells in airways, affects airway smooth muscle functions via the G proteins/K_{Ca}/VDC channel linkage due to Ca^{2+} dynamics and RhoA/Rho-kinase processes due to Ca^{2+} sensitization. These inflammatory processes cause not only alterations of contractility but also changing to proliferative phonotype in airway smooth muscle, referred to as a phenotype change. The former phenomenon is attributed to airflow limitation, airway hyperresponsiveness, and β_2-adrenergic desensitization; the latter phenomenon is attributed to airway remodeling via cell proliferation and migration. Therefore, G proteins/K_{Ca}/VDC channel linkage and RhoA/Rho-kinase processes are involved in almost all of the principal mechanisms of asthma and COPD. These pathways involved in Ca^{2+} dynamics and Ca^{2+} sensitization are molecular targets for therapy of these diseases. VDC: L-type voltage-dependent Ca^{2+} channels, K_{Ca}: large-conductance Ca^{2+}-activated K^+ channels. Illustrated based on ref. [1].

6.2. Airway hyperresponsiveness

Airway hyperresponsiveness is a characteristic feature of asthma, and it is essential for the diagnosis and severity assessment of asthma. Airway hyperresponsiveness is also observed in some patients with COPD. This airway disorder is clinically defined as increased responsiveness to muscarinic receptor agonists (ACh and MCh) and histamine. Airway hyperresponsiveness is mediated by various inflammatory stimulations involved in the pathophysiology of asthma, such as antigens, chemical mediators, cytokines, and eicosanoids. In a postmortem study of airway smooth muscle strips of patients with asthma, the response to histamine and ACh was greater than in healthy individuals [117]. In human airway smooth muscle passively sensitized with human asthmatic serum, contraction due to histamine is significantly elevated [118]. When airway smooth muscle is exposed for an extended period of time to interleukin (IL)-5, IL-13, IL-17, or tumor necrosis factor $(TNF)_{\alpha}$, which are released from inflammatory cells and epithelial cells in airways, contraction due to muscarinic receptor agonists and KCl is significantly increased [119, 120, 121]. This enhancement of contraction induced by TNF_{α} may be involved in Ca^{2+} sensitization via RhoA/Rho-kinase [110]. In the presence of a lower concentration of leukotriene C_4, KCl-induced contraction is markedly

augmented in porcine tracheal smooth muscle, and this enhanced contraction due to KCl is attenuated by Y-27632 [122]. When airway smooth muscle is exposed to S1P released from mast cells or ATP released from damaged epithelial cells, contraction in response to MCh is markedly increased after exposure to S1P or ATP, and its augmented contraction is suppressed by Y-27632 in a concentration-dependent manner [108, 113, 123]. Furthermore, pre-treatment of 8-iso-prostaglandin E_2, an isoprostane, causes an increased response to CCh in airway smooth muscle, and its augmented contraction is suppressed by Y-27632 [124]. These observations indicate that airway hyperresponsiveness is caused by direct interactions among inflammatory cells, airway epithelial cells and airway smooth muscle cells and that Ca^{2+} sensitization based on Rho-kinase–induced MYPT1 phosphorylation contributes to the airway hyperreactivity [107, 108]. Suppression of geranylgeranyltransferase, which is involved in the activation of RhoA, also reduces hyperresponsiveness in mouse bronchus [125]. Alterations of Ca^{2+} regulatory mechanisms in airway smooth muscle may play a key role in this phenomenon. Therefore, the pathophysiology of asthma (inflammatory processes involved in this disease) and alterations in the mechanical properties directly affect the function of airway smooth muscle cells via the RhoA/Rho-kinase processes. In airway smooth muscle cells, this phenotypic change for contractility induced by not only Ca^{2+} sensitization but also cytoskeleton reorganization (cell stiffness) may cause an augmented response to spasmogens [1, 126, 127]. Lung resistance in response to MCh was increased in mice sensitized by allergen challenges, as compared with control mice (airway hyperresponsiveness). Fasudil hydrochloride (HA-1077), an inhibitor of Rho-kinase, suppressed the augmented response to MCh by allergen challenges [128]. On the other hand, Ca^{2+} dynamics (Ca^{2+} mobilization) also contributes to altering the contractile phenotype of airway smooth muscle, leading to augmented responsiveness to spasmogens [129]. Moreover, acidification of esophageal lumen increases the contractile response to ACh and KCl in guinea pig trachealis mediated by activation of VDC channels and Rho-kinase [130], indicating that both Ca^{2+} dynamics and Ca^{2+} sensitization play key roles in airway hyperresponsiveness (Figure 5).

6.3. Desensitization of β_2-adrenergic receptors

After β_2-adrenoceptors are excessively activated, responsiveness to an agonist is attenuated. This phenomenon is referred to as desensitization of β_2-adrenoceptors. The phosphorylation of β_2-adrenoceptors, which leads to desensitization via uncoupling G_s from the receptors, is mediated by two types of protein kinases, cAMP-dependent PKA and cAMP-independent protein kinases such as β_2-adrenergic receptor kinase (βARK) [131]. PKA-induced phosphorylation, which is produced by exposure to a low concentration of β_2-adrenoceptor agonists, leads to heterologous desensitization (a nonspecific reduced response to other agonists involving cAMP) [132]. On the other hand, βARK-induced phosphorylation, which is produced by exposure to a high concentration of β_2-adrenoceptor agonists, leads to homologous desensitization (a specific reduced response to β_2-adrenoceptor agonist) [133]. These phenomena also occur in tracheal smooth muscle, including human tissues [10, 134, 135, 136]. β_2-adrenergic desensitization occurs after continuous [134, 135, 136] or repetitive administration [10, 135, 136] of β_2-adrenoceptor agonists or after exposure to substances related to the inflammatory processes in asthma, including inflammatory cytokines such as IL-1β [137],

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growth factors such as transforming growth factor (TGF)-β1 [138] and platelet-derived growth factor (PDGF) [139], lipid mediators such as lysophosphatidylcholine (Lyso-PC), a lysophospholipid produced by phospholipase A2 [140], and S1P [141], or PAR2 agonists such as tryptase and SLIGKV [112]. Therefore, desensitization of β_2-adrenoceptors in airway smooth muscle is an extremely important phenomenon that occurs due to both the treatment and the pathophysiology of asthma. Reduced responsiveness to β_2-adrenoceptor agonists after excessive or repeated exposure to these agonists was prevented when G_s linked to β_2-adrenoceptors was irreversibly activated by pre-treating airway smooth muscle with cholera toxin (2 μg/ml) for 6 h [134, 135, 142, 143] (Figure 6A). On the other hand, in the presence of ChTX or IbTX, this β_2-adrenergic desensitization was markedly enhanced [134, 135]. Inactivation of the G_s/K_{Ca} channel linkage plays an important role in β_2-adrenergic desensitization (Figures 5, 8).

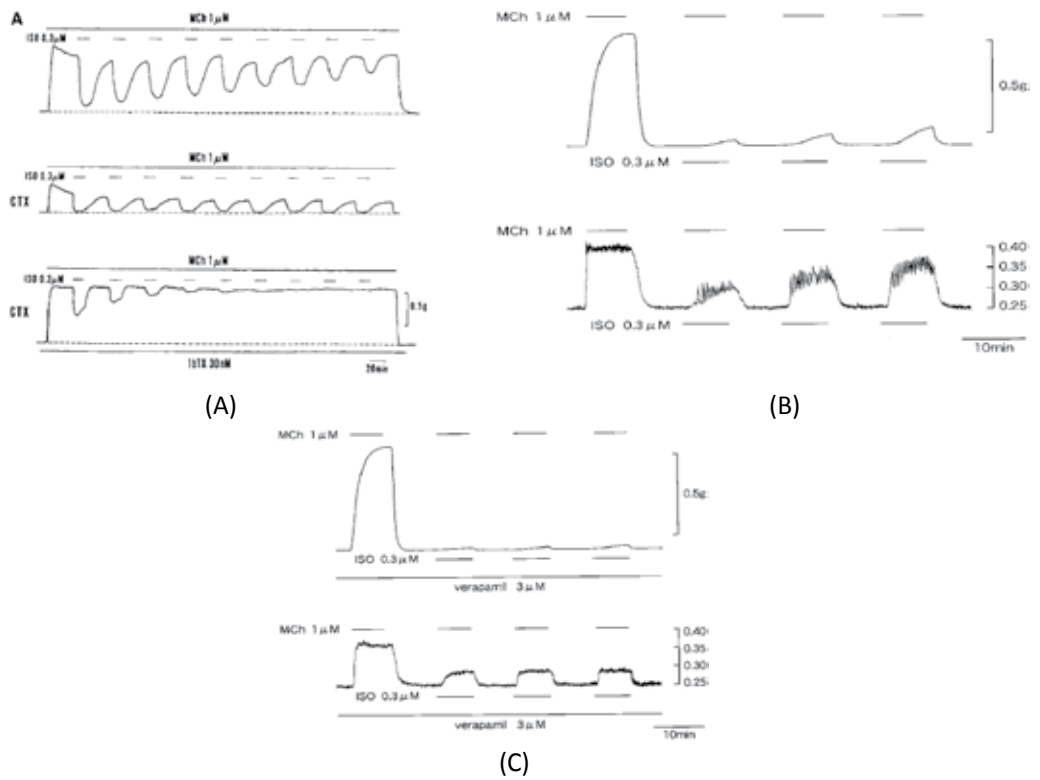

Figure 6. Inhibitory effects of G_s/K_{Ca} channel linkage on the β-adrenergic desensitization after repeated exposure to a β-adrenoceptor agonist in isometric tension recording of tracheal smooth muscle. (A) A typical example of repeated application of ISO (0.3 μM) to tissues precontracted by MCh (1 μM) at intervals of 20 min under the following experimental conditions: control (upper trace), preincubation with CTX (2 μg/ml) for 6 h (middle trace), and preincubation with CTX and in the presence of IbTX (30 nM) throughout the experiment (lower trace). The G_s/K_{Ca} channel stimulatory linkage is involved in the prevention of β_2-adrenergic desensitization. (B) A typical example of simultaneously recorded isometric tension (upper trace) and $F_{340}/_{F380}$ (lower trace) after repeated exposure to MCh (1 μM) with ISO (0.3 μM) in fura-2–loaded tissues of tracheal smooth muscle in guinea pigs. (C) A typical example of simultaneously recorded isometric tension (upper trace) and F_{340}/F_{380} (lower trace) after repeated exposure to MCh (1

μM) with ISO (0.3 μM) in the presence of verapamil (3 μM) in fura-2–loaded tissues similar to (B). Ca^{2+} dynamics via the K_{Ca}/VDC channel linkage are involved in β_2-adrenergic desensitization. ISO: isoprenaline, MCh: methacholine, CTX: cholera toxin, IbTX: iberiotoxin, K_{Ca} channels: large-conductance Ca^{2+}-activated K^+ channels. Cited from ref. [10, 135].

6.3.1. Ca^{2+} dynamics

In fura-2–loaded tissues of guinea pig tracheal smooth muscle, the relaxant effect of isoprenaline on MCh-induced contraction was gradually attenuated with increasing $[Ca^{2+}]_i$ following repeated exposure to isoprenaline with MCh for 10 min every 30 min [10, 135] (Figure 6B), and this reduced responsiveness to isoprenaline was avoided by pre-exposure to cholera toxin or the addition of verapamil with no change in $[Ca^{2+}]_i$ [10] (Figure 6C). In contrast, after repeated exposure to forskolin, db-cAMP and theophylline, the relaxant effect of these cAMP-related agents was not diminished with no change in $[Ca^{2+}]_i$ (homologous desensitization) [10, 135]. Furthermore, after exposure to PDGF for 15 min, the relaxant effect of isoprenaline against MCh-induced contraction was markedly attenuated with increasing $[Ca^{2+}]_i$, and this reduced responsiveness to isoprenaline was reversed by verapamil [139]. The relaxant effects of not only β_2-adrenoceptor agonists but also forskolin are markedly attenuated with elevated $[Ca^{2+}]_i$ after exposure to growth factors, such as TGFβ_1 and PDGF (heterologous desensitization) (Figure 8). In contrast, the relaxant effects of db-cAMP and theophylline are not diminished after exposure to TGFβ_1 and PDGF. These results indicate that β_2-adrenergic desensitization occurs via dysfunction of the receptor/G_s/adenylyl cyclase processes in airway smooth muscle and that the cAMP-independent pathway is involved in this phenomenon [3, 4. 7, 8]. These results indicate that the Ca^{2+} influx passing through VDC is involved in β_2-adrenergic desensitization and that VDC activity may be augmented by dysfunction of the G_s/K_{Ca} channel stimulatory linkage (Figures 5, 8).

6.3.2. Ca^{2+} sensitization

In fura-2–loaded tissues of guinea pig tracheal smooth muscle, the inhibitory effect of isoprenaline against MCh-induced contraction following continuous exposure to Lyso-PC [140] was markedly attenuated with no changes in $[Ca^{2+}]_i$ (Figure 7A). This reduced responsiveness to isoprenaline was reversed to the control response by application of Y-27632 in a concentration-dependent manner (Figure 7B). In contrast, the relaxant effect of cAMP-related agents such as forskolin, theophylline, and db-cAMP, was not diminished after exposure to Lyso-PC (homologous desensitization). Similar to Lyso-PC, reduced responsiveness to isoprenaline was observed with no changes in $[Ca^{2+}]_i$ after the exposure of tracheal smooth muscle to tryptase and SLIGKV [112] and S1P [141]. The relaxant effects of forskolin were not attenuated after exposure to tryptase and SLIGKV; in contrast, the relaxant effects were markedly diminished after exposure to S1P, indicating that the receptor/G_s/ adenylyl cyclase process is also involved in the dysfunction of β_2-adrenoceptors in airway smooth muscle. cAMP activity may still be intact under this condition of excessive stimulation of β_2-adrenoceptors. Furthermore, in the presence of bisindolylmaleimide, a membrane-permeable inhibitor of PKC, reduced responsiveness to isoprenaline is not prevented after exposure to an agonist [134, 135, 140]. These observations indicate that after exposure to these lipid mediators and PAR 2 agonists, tolerance

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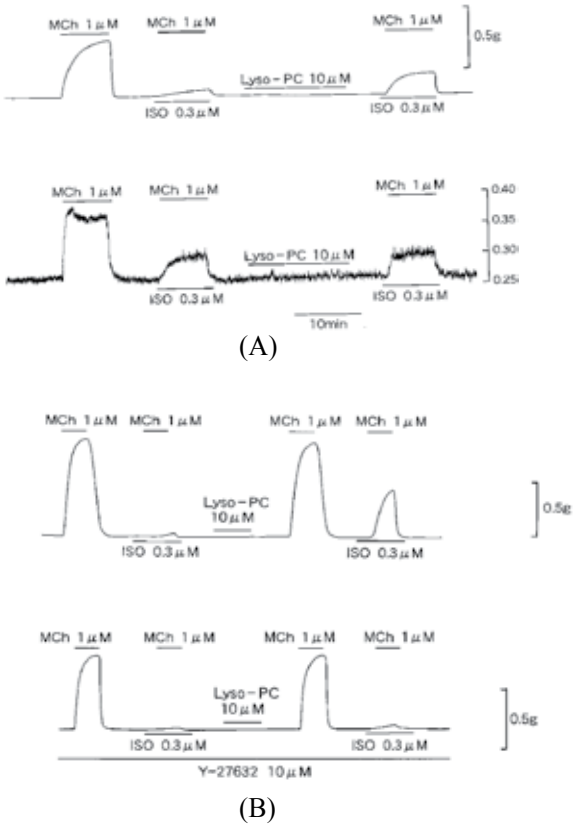

Figure 7. The effects of Ca^{2+} sensitization mediated by RhoA/Rho-kinase on β-adrenergic desensitization in tracheal smooth muscle. A: A typical example of simultaneously recorded isometric tension (upper trace) and F_{340}/F_{380} ratio (lower trace) induced by MCh (1 μM) with ISO (0.3 μM) inhibition before and after exposure to Lyso-PC (10 M) for 15 min. Pretreatment with Lyso-PC attenuates ISO-induced relaxation without elevating $[Ca^{2+}]_i$, indicating that Ca^{2+} sensitization is involved in β_2-adrenergic desensitization. B: A typical example of the inhibitory effects of ISO (0.3 μM) on MCh-induced contraction (1 μM) before and after exposure to Lyso-PC (10 μM) for 15 min in the absence (upper trace) and presence (lower trace) of Y-27632 (10 μM) throughout the experiments. Y-27632 inhibits β_2-adrenergic desensitization induced by Lyso-PC, indicating that Ca^{2+} sensitization via RhoA/Rho-kinase processes is involved in this phenomenon. MCh: methacholine, ISO: isoprenaline, Lyso-PC: lysophosphatidylcholine. Cited from ref. [140].

to β_2-adrenoceptor agonists occurs due to Ca^{2+} sensitization via the RhoA/Rho-kinase processes, not via PKC. This β_2-adrenergic desensitization is caused by elevated sensitization to intracellular Ca^{2+} based on G_s inactivation and Rho-kinase activation, although little is known about the functional relationship between G_s and RhoA/Rho-kinase (Figures 5, 8).

6.3.3. Intrinsic efficacy

The potency of a β_2-adrenoceptor agonist depends on its receptor affinity and intrinsic efficacy. Intrinsic efficacy (intrinsic activity) refers to the ability of an agent to activate its receptors without regard for their concentration. Some agonists completely activate receptors, but others only partially activate them. The former are referred to as full agonists, and the latter are

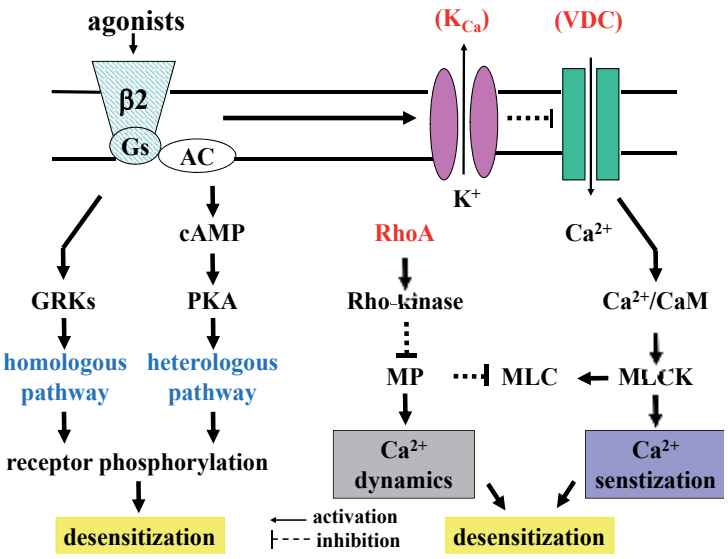

Figure 8. Role of Ca^{2+} dynamics and Ca^{2+} sensitization in the desensitization of β_2-adrenoceptors in airway smooth muscle. Phosphorylation of β_2-adrenoceptors is essential for reduced responsiveness to their agonists. There are two pathways in the mechanisms of β_2-adrenergic desensitization: 1) cAMP-independent phosphorylation of their receptors via members of the GRK family such as βARK (homologous desensitization), and 2) cAMP-dependent phosphorylation of their receptors via PKA (heterologous desensitization). Inactivation of G_s, which is linked to β_2-adrenoceptors, is involved in desensitization of the receptors mediated by Ca^{2+} dynamics and Ca^{2+} sensitization. Impairment of the stimulatory linkage between G_s/PKA and K_{Ca} channels causes an increase in the membrane potential, leading to Ca^{2+} influx passing through VDC channels (Ca^{2+} dynamics: Ca^{2+}-dependent mechanisms). On the other hand, impairment of the inhibitory correlation between G_s/PKA and RhoA/Rho-kinase processes causes an increase in Rho-kinase activity, leading to a reduced MP activity (Ca^{2+} sensitization: Ca^{2+}-independent mechanisms). β_2: β_2-adreneceptors, AC: adenylyl cyclase, GRK: G protein-receptor kinase, βARK: β-adrenoceptor kinase, PKA: protein kinase A, MLCK: myosin light chain kinase, MLC: myosin light chain, MP: myosin phosphatase, K_{Ca}: large-conductance Ca^{2+}-activated K^+ channels, VDC: L-type voltage-dependent Ca^{2+} channels. Illustrated based on ref. [1, 2, 10, 112, 134, 135, 136, 138, 139, 140, 141, 142, 145, 146].

referred to as partial agonists. Moreover, partial agonists are subclassified as weak partial agonists, which have lower efficacy, and strong partial agonists, which have higher efficacy [144, 145]. Intrinsic efficacy was measured indirectly as a physiological response (changes in smooth muscle relaxation determined by isometric tension recording in vitro) [145]. The ratio of the intrinsic efficacy of any two β_2-agonists is expressed as a fraction between 0 and 1 by concentration-inhibition curves, taking that of adrenaline as 1. The order of efficacy (the maximal percent relaxation against 10 μM MCh-induced contraction) was as follows: isoprenaline = adrenaline > indacaterol, formoterol, procaterol > salbutamol > salmeterol > tulobuterol [97, 145] (Table 1); these efficacies are similar to the values measured by changing the level of intracellular cAMP [144]. Isoprenaline behaves as a full agonist, and other agonists behave as partial agonists. Isoprenaline caused β_2-aderergic desensitization greater than that of other agonists, indicating that excessive activation of a full agonist leads to reduced responsiveness to β_2-adrenoceptor agonists in airway smooth muscle [134, 135, 136, 142, 145]. In contrast, tulobuterol, which is the weakest partial agonist, caused a modest reduction in response to an agonist, even in cases of excessive exposure to tulobuterol [146].

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β_2-adrenoreptor agonists	The maximal inhibition (1 μM MCh)	(10 μM MCh)	classification
isoprenaline	100.0	100.0	Full agonists
adrenaline	100.0	100.0	
indacaterol	100.0	72.3	Strong partial agonists
formoterol	100.0	68.9	
procaterol	100.0	66.8	
salbutamol	78.1	48.2	Weak partial agonists
salmeterol	59.4	32.1	
tulobuterol	34.6	20.6	
propranorol	0	0	antagonists

Table 1. Intrinsic efficacy of β_2-adrenoceptor agonists. Values of intrinsic efficacy of β_2-adrenoceptor agonists were measured as a physiologic response in airway smooth muscle. The values of intrinsic efficacy were expressed as the maximum percent inhibition for each β_2-adrenoceptor agonist against MCh-induced contraction (1 and 10 μM) in guinea pig tracheal smooth muscle. MCh: methacholine. Cited from ref. [1, 97, 145].

6.4. Airway remodeling

Airway inflammatory reactions involving activated eosinophils act on the epithelium, subepithelium, and smooth muscle layers and bring about characteristic structural changes in the airways. Subepithelial fibrosis results from the deposition of collagen fibers and proteoglycans under the basement membrane (thickening of the airway wall). This phenomenon is known as airway remodeling, which is thought to be related to asthma severity. Airway smooth muscle contributes to airway remodeling by mass formation via cell proliferation and migration [147, 148]. Unlike normal cells, increased airway smooth muscle cell proliferation in patients with asthma is not suppressed by glucocorticosteroids because of CCAAT/enhancer-binding protein (C/EBP)-α deficiency in airway smooth muscle cells [149].

6.4.1. Cell proliferation

Factors facilitating the proliferation of airway smooth muscle cells are roughly divided into the following two groups: 1) ligands (polypeptide growth factors) of tyrosine kinase receptors (RTKs), such as epidermal growth factor (EGF) and PDGF, and 2) ligands (contractile agents) of GPCRs, such as leukotriene D_4, thromboxane A_2 and endothelin. When ligands bind to growth factor receptors, tyrosine kinase is first activated, followed by Ras and extracellular regulated kinase (ERK)1/2, to transmit information to the nucleus [150]. Next, via cyclin D1 activation, DNA synthesis and cell proliferation occur [151]. In addition to this main pathway for smooth muscle proliferation, cross-talk between RTKs and GPCRs is mediated by phos-

phatidylinositol 3-kinase (PI3K), p70S6 kinase, and glycogen synthase kinase-3 (GSK-3) [150, 152]. The involvement of the Rho family (RhoA, Rac and Cdc42) in the control mechanisms of airway smooth muscle cell proliferation has not been sufficiently clarified. EGF- and PDGF-induced cell proliferation is not suppressed by inactivation of RhoA/Rho-kinase signaling [126]; in contrast, the activation of RhoA, not Rac or cdc42, causes the proliferation of human bronchial smooth muscle cells that have been stimulated with serum. This proliferative reaction is suppressed by Y-27632, C3 exoenzyme, and simvastatin, a HMG-CoA reductase inhibitor, which attenuate proliferation via the geranylgeranylation of RhoA [153]. Another factor, M_2 muscarinic receptor, facilitates the proliferation of airway smooth muscle cells [154, 155]. A recent clinical trial has demonstrated that an antagonist of VDC channels inhibits airway remodeling in patients with severe asthma [156]. Therefore, Ca^{2+} influx via VDC channels is enhanced since K_{Ca} channel activity is attenuated by G_i when MCh is applied to airway smooth muscle [7, 8]. These results indicate that both Ca^{2+} dynamics and Ca^{2+} sensitization contribute to the proliferation of airway smooth muscle cells (Figure 5).

6.4.2. Cell migration

Cell migration is a characteristic function of inflammatory cells, fibroblasts and smooth muscle cells, and it plays an important role in various pathophysiological environments, such as inflammatory cell infiltration and airway smooth muscle hyperplasia [157]. Migration of airway smooth muscle cells is enhanced by the extracellular matrix [158]. Cell migration occurs due to contraction involving actin, myosin reactions and actin reorganization. Since RhoA/Rho-kinase signaling is the most important factor controlling the cytoskeleton of airway smooth muscle cells and other cells [159], this pathway may control the migration of airway smooth muscle cells via changes in the cytoskeleton. Hence, RhoA/Rho-kinase may be involved in airway remodeling mediated not only by cell proliferation but also by cell migration. Urokinase, PDGF, leukotriene and lysophosphatidic acid facilitate the migration of human airway smooth muscle cells [160, 161, 162, 163]. Moreover, heat shock protein, PI3K, p38 mitogen-activated protein kinase, prostaglandin D_2, and IL-13 facilitate airway smooth muscle migration [160, 164, 165]. Y-27632 significantly suppresses the increased migration of airway smooth muscle cells, due to PDGF or leukotriene stimulation [161, 162], indicating that RhoA/Rho-kinase signaling (Ca^{2+} sensitization) plays an important role in controlling cell migration (Figure 5). On the other hand, Ca^{2+} dynamics regulate the migration of airway smooth muscle cells and inflammatory cells. Ca^{2+} influx via SOC channels contributes to PDGF-induced cell migration of airway smooth muscle [166], and increasing $[Ca^{2+}]_i$ via other mechanisms also causes substance P–induced cell migration of airway smooth muscle [167] (Figure 5). Since IL-13 enhances Ca^{2+} oscillation in airway smooth muscle cells, cell migration induced by IL-13 may be regulated by Ca^{2+} dynamics [168].

6.4.3. Interaction between airway smooth muscle and inflammatory cells

As described earlier, contractility of airway smooth muscle is altered by tryptase and S1P, which are released from mast cells, and Lyso-PC, which is synthesized in the membrane of various inflammatory cells [108, 112, 140, 141]. Ca^{2+} sensitization by RhoA/Rho-kinase

processes contributes to this phenomenon. When sensitized mice are subjected to allergen challenges, eosinophil infiltration is markedly increased in the airways. In allergen-challenged mice, pretreatment with Rho-kinase inhibitors such as Y-27632 or fasudil hydrochloride (HA-1077) markedly suppressed an increase in eosinophil recruitment in the airway in a dose-dependent manner [128]. The actions of Lyso-PC are mediated by RhoA/Rho-kinase, leading to β_2-aderenergic desensitization [140], and administration of Lyso-PC to guinea pigs enhances eosinophil recruitment and resistance in the airways [169]. The effects of S1P are also mediated by RhoA/Rho-kinase processes, leading to airway hyperresponsiveness [108] and remodeling [170]. S1P increased mRNA and protein expression of vascular cell adhesion molecule (VCAM)-1 when S1P is applied to pulmonary endothelial cells, leading to eosinophil infiltration to the airways, and this upregulation of VCAM-1 is attenuated by C3 exoenzyme and Y-27632 [171]. Y-27632 reduces not only the number of eosinophils but also macrophages and neutrophils in an animal model of allergic asthma [172]. Ca^{2+} sensitization via RhoA/Rho-kinase processes contributes to recruitment of inflammatory cells to the airways.

Therefore, Ca^{2+} sensitization by RhoA/Rho-kinase processes [1, 173, 174, 175] and Ca^{2+} dynamics by ion channels including VDC and SOC [6, 11, 12, 176] may be a therapeutic target for obstructive pulmonary diseases including asthma.

7. Conclusions

Ca^{2+} signaling, which is due to Ca^{2+} dynamics and Ca^{2+} sensitization, contributes to alterations of contractility that lead to airway disorders (airflow limitation, airway hyperresponsiveness, and β_2-adrenergic desensitization), which are characteristic features of asthma and COPD. Ca^{2+} dynamics and Ca^{2+} sensitization also facilitate the proliferation and migration of airway smooth muscle via changing to proliferative phenotype. A recent report has indicated that bitter taste receptor stimulation causes relaxation of airway smooth muscle via activation of K_{Ca} channels [177]. Hence, Ca^{2+} dynamics due to G proteins/K_{Ca}/VDC channels and Ca^{2+} sensitization due to RhoA/Rho-kinase processes may be therapeutic targets for asthma and COPD, and research in these areas may provide novel strategies in the development of bronchodilators for these diseases.

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Section 6

Therapeutic Approaches for Muscular Disease

Chapter 12

Research on Skeletal Muscle Diseases Using Pluripotent Stem Cells

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Abstract

The generation of induced pluripotent stem cells (iPSCs), especially the generation of patient-derived pluripotent stem cells (PSCs) suitable for disease modelling *in vitro*, opens the door for the potential translation of stem-cell related studies into the clinic. Successful replacement, or augmentation, of the function of damaged cells by patient-derived differentiated stem cells would provide a novel cell-based therapy for skeletal muscle-related diseases. Since iPSCs resemble human embryonic stem cells (hESCs) in their ability to generate cells of the three germ layers, patient-specific iPSCs offer definitive solutions for the ethical and histo-incompatibility issues related to hESCs. Indeed human iPSC (hiPSC)-based autologous transplantation is heralded as the future of regenerative medicine. Interestingly, during the last years intense research has been published on disease-specific hiPSCs derivation and differentiation into relevant tissues/organs providing a unique scenario for modelling disease progression, to screen patient-specific drugs and enabling immunosupression-free cell replacement therapies. Here, we revise the most relevant findings in skeletal muscle differentiation using mouse and human PSCs. Finally and in an effort to bring iPSC technology to the daily routine of the laboratory, we provide two different protocols for the generation of patient-derived iPSCs.

Keywords: Pluripotent stem cells, Myogenic differentiation, Disease modelling, Patient-specific induced pluripotent stem cells, Muscular dystrophy

1. Introduction

Regenerative Medicine aims to restore the loss of function in tissues and organs due to any cause (trauma, stress, aging, or disease) by the replacement of dysfunctional structures with competent cells, tissues, or organs. In order to achieve this goal Regenerative Medicine takes advantage of different forefront methodologies, such the use of stem cells, gene therapy, and tissue engineering among others.

1.1. Human embryonic stem cells (hESCs)

The isolation and derivation of hESCs by Thompson and colleagues in 1998 attracted significant attention in the Regenerative Medicine field [1]. Indeed, regenerative cell transplantation therapies have been expected to treat incurable diseases, such as spinal cord injury [2], neurodegenerative disease [3], heart failure [4,5], diabetes [6], and retinal disease [7].

Nowadays, clinical application of hESCs still shows many concerns regarding the use of human embryos, tissue rejection after transplantation, and tumour formation. However, hESCs possess the dual ability to proliferate indefinitely without phenotypic alterations, and more importantly, to differentiate, theoretically, into all cell types in the human body. These qualities suggest extensive utility of hESCs in applications varying from the definition of differentiation protocols, to the generation of drug screening platforms for disease treatment. Thus, hESCs represent an ideal source for understanding skeletal muscle development and disease, such skeletal muscle.

1.2. Induced pluripotent stem cells (iPSCs)

In 2006 Professor Shinya Yamanaka and colleagues [8] showed for the very first time, that by introducing different transcription factors the epigenetic status of somatic cells could be reverted to pluripotency. In particular, the Japanese team ectopically induced the expression of specific transcription factors related with embryonic stem cells (ESCs) biology, generating in a period of only 30 days, cells that were identical to mouse ESCs (mESCs) in terms of self-renewal capacity, expression of endogenous pluripotency-related factors, and *in vivo* and *in vitro* differentiation potential to give rise to cells belonging to the three germ layers of the embryo (ectoderm, mesoderm, endoderm). This discovery was awarded with the Nobel Price of Medicine in 2012 to Professor Shinya Yamanaka.

While, at first, somatic reprogramming was described using mouse embryonic fibroblasts, the Japanese team could show that also a reduced formula of the original "Yamanaka cocktail" could be used to reprogram human somatic cells towards human iPSCs (hiPSCs) [9]. Since 2007 different research groups, including us, have shown that iPSC technology can be applied to reprogram a huge variety of human somatic cells, independently of their embryonic origin [10–13]. Interestingly, during the last years the generation of protocols avoiding the use of lentiviral or retroviral vectors for the expression of Yamanaka factors has involved the definition of novel strategies for hiPSCs generation, including the use of recombinant proteins [14,15], episomal vectors [16], or mRNAs [17,18], among others [13]. Thus, the generation of

hiPSCs, especially the generation of patient-derived iPSCs suitable for disease modelling *in vitro*, opens the door for the potential translation of patient-derived iPSCs into the clinic. Successful replacement or augmentation of the function of damaged cells by patient-derived differentiated stem cells would provide a novel cell-based therapy for skeletal muscle-related diseases.

Satellite cells (SCs), the adult stem cell pool in skeletal muscle, are often compromised in patients with muscle dystrophies (MDs). Over the last decades the understanding of the transcription factors and intrinsic and extrinsic signals that govern SCs or terminally differentiated myogenic cells have represented a good starting point for the definition of protocols for the generation of myogenic cells from PSCs (both from mouse and human ESCs and iPSCs). In the same manner, the generation of patient-derived cell platforms can help us to develop experimental strategies toward generating muscle stem cells, either by differentiating patient-specific iPSCs or by converting patient's somatic cells towards myogenic cells (transdifferentiation). Overall, the possibility to generate disease-free patient iPSCs can help us to identify which are the mechanisms driving muscle disease, and more importantly, to develop new compounds for treating MDs (Figure 1).

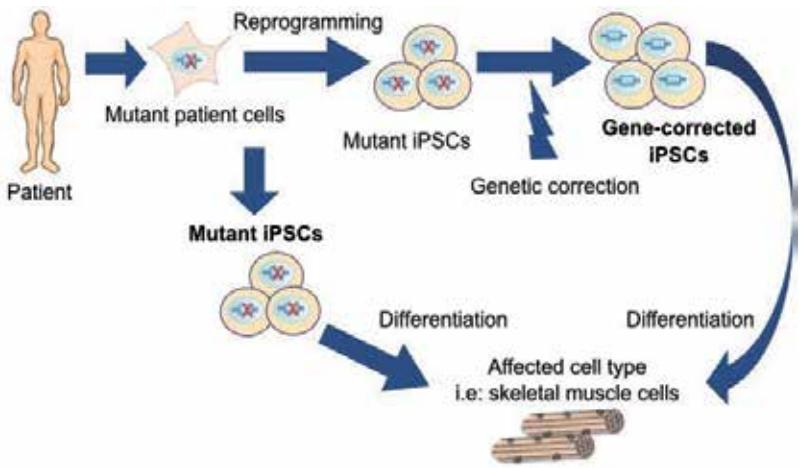

Figure 1. Patient iPSCs represent an unprecedented tool for the generation of i*n vitro* platforms for disease modelling and the definition of protocols for PSCs differentiation. The correction of the genetic defect(s) leading to disease may help to understand the molecular and cellular mechanisms driving disease gestation and progression, and more importantly, to identify novel mechanisms leading to muscle regeneration.

2. General approaches to induce *in vitro* differentiation of pluripotent stem cells (PSCs)

Both mouse and human PSCs are routinely cultivated in the presence of feeder layers. PSCs grow on the feeder layers as colonies (Figure 2). Generally, human and mouse PSCs are

enzymatically dissociated with trypsin, acutase, or dispase to obtain a suspension of single cells, which is then transferred for subculture and expansion or differentiation purposes. For mouse PSCs, LIF can substitute for feeder layers. However, since LIF is not effective for human PSCs, in the last years different chemically defined media have been generated in order to sustain human PSCs culture and expansion in feeder-free substrates.

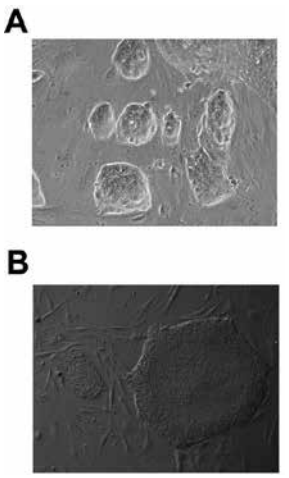

Figure 2. PSCs are typically maintained in mitotically inactivated supportive cells. A) Mouse iPSCs cultured on top of irradiated mouse embryonic fibroblasts grow in tight colonies that are further trypsinized for subculture or differentiation purposes. B) Human iPSCs cultured on top of human irradiated dermal fibroblasts grow as colonies with defined borders.

As an option for culturing human PSCs without feeder cells, Matrigel™ has proven to be a useful alternative enabling the stable culture of human PSCs. Moreover, we have also shown that Matrigel™ allows the generation of hiPSCs without animal-derived feeder cells [19]. Since Matrigel™ was derived from Engelbreth-Holm-Swarm mouse sarcoma cells [20], other types of matrices which do not contain animal-derived agents have been tested and used as feeder-cell substitutes for the successful maintenance and generation of human PSCs; such as CellStart [21,22], recombinant proteins [23–25], and synthetic polymers [26,27].

The culture media used in the early generation of hESCs contained fetal bovine serum [1]. In order to remove unspecific agents that might cause the differentiation of hESCs, knockout serum replacement (KSR) has now been established as a defined material for maintaining hESCs [28] and is also traditionally used for hiPSC generation [9,12,29,30]. In this regard, mTeSR1 medium was developed as a chemically defined medium for maintaining human PSCs [31]. Importantly, in the last years several authors have reported the generation of commercially developed *xeno*-free media for maintaining hiPSCs, and such media have already been used successfully for iPSCs generation. These media include: TeSR2 [32], NutriStem [33], Essential E8 [24], and StemFit [34].

When factors that sustain PSCs stemness are deprived from the media, PSCs spontaneously differentiate into derivatives of the three embryonic germ layers. This capacity has been

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profited for more than 30 years in order to direct PSCs to the desired cell product. In this regard, up to day, an infinite number of protocols have been established to promote the development of the cell type of interest.

The following are basic strategies to induce *in vitro* differentiation of PSCs cells:

a. **Embryoid Bodies' (EBs) formation**: In contrast to monolayer cultures, EBs are spherical structures that allow PSCs culture in suspension (Figure 3). The three-dimensional structure, including the establishment of complex cell-adhesions and paracrine signaling within the EB microenvironment, enables differentiation and morphogenesis. For that reason, the first protocols for muscle differentiation took advantage of EB induction from mESCs, followed by different periods of exposure to specific cell culture media in which serum, mitogenic factors, and essential substrates (such as amino-acids or glutamine) were formulated. In that manner, those first assays proved the feasibility of mESCs to give rise to myogenic cells, setting the bases for the definition of robust protocols for the differentiation of muscle cells from human PSCs. Up to day, most of the protocols for the generation of myogenic cells from PSCs make use of the differentiation of EBs derived from either wild type or transgenic PSCs.

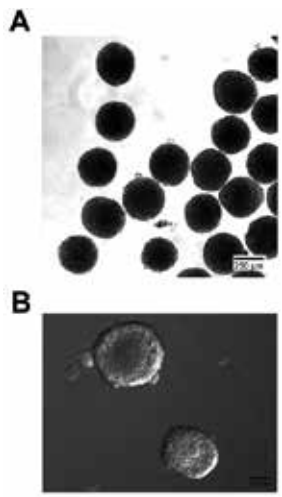

Figure 3. PSCs are capable to differentiate into cells belonging to the three somatic germ layers of the embryo. The generation of EBs from PSCs is a common method for producing different cell lineages for further purposes. A) EBs from mouse iPSCs grown in suspension. B) EBs derived from human iPSCs grown in suspension.

b. **Modification of medium composition**: Monolayers of PSCs and also EBs have been traditionally subjected to changes in nutrient composition, (i.e, reduction/increase of serum concentration, addition/removal of a growth factor or addition/removal of cytokines, among others) in order to induce their differentiation towards the desired cell type. These changes are conducted in order to promote changes on gene expression profiles and cell proliferation rates. In this manner, by means of relatively simple methods, PSCs are artificially guided towards the desired cell type. Although these methodologies have proven low efficiency yields for specific cell types (i.e., motorneurons, hepatic cells; among

others), they are extremely valuable when combined together with PSCs in which the expression of master factors critical for differentiation are under the control of hormones (i.e., tamoxifen inducible reporters) or antibiotics (i.e., puromycin, or hygromycin, among others). The control of expression of the specific transcription factor of choice (i.e., MyoD1) together with the addition of specific molecules mimicking tissue development [i.e., insulin like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF)] has demonstrated good results when differentiating mouse or human PSCs towards myogenic cells.

c. **Genetic manipulation of PSCs**: Forced expression of transcription factors can direct differentiation of PSCs toward specific lineages. In the last years, the generation of platforms for transgene expression in PSCs has emerged as one of the most potent tools for PSCs differentiation. Whereas the first studies took advantage of exogenous gene expression systems (i.e., lentivirus or retrovirus), nowadays the use of integrative vectors are limited, since they incur uncertain risks for potential cell-based therapeutic applications [35]. In this regard, the use of excisable vectors (i.e., transposons; [36,37], or mRNAs [17]) offer an unprecedented opportunity for the derivation of differentiated PSCs suitable for regenerative medicine.

d. **Use of extracellular matrix (ECM) and signaling molecules**: Unlike *de novo* embryonic muscle formation, muscle regeneration in higher vertebrates depends on the injured tissue retaining of an ECM scaffolding that serves as a template for the formation of muscle fibers [38]. In this regard, the interaction between cells and ECM via integrins determines the expression of signaling molecules that affect PSCs differentiation [39]. Of note, when mouse iPSCs have been cultured in the presence of matrigel, myogenesis (this is, proliferation of myoblasts and further fusion into myotubes) has been positively induced [40]. Similar results have been observed when using collagen-based matrix for the differentiation of human iPSCs expressing a Dox-inducible expression cassette of MyoD1 [41]. For the organization and alignment of muscle fibers not only the composition of the ECM but also its anisotropic architecture are essential. To address this, a number of strategies have been developed to organize myotubes: topography-based approaches based on the use of nanofibers, [42], microabrated surfaces [43], and microcontact printing of ECM proteins such as fibronectin [44] and vitronectin [45]. In a complementary approach, biochemical cues have also been introduced to promote cell alignment and differentiation. By using inkjet bioprinting, spatially defined patterns of myogenic and osteogenic cells were derived from primary muscle-derived stem cells (MDSCs) as a response to BMP-2 patterning [46]. The combination of topographical and biochemical signaling has also been explored by coating sub-micron polystyrene fibers with either FGF-2 or BMP-2 to provide spatial control of cell fate and alignment in order to mimic native tissue organization [47]. The vast majority of these works present cells to static microenvironments. Latest trends point out the relevance of presenting cells to spatially and temporally dynamic microenvironments [48]. Surfaces with gradient concentrations of growth factors (BMP-2 and BMP-7) have shown to successfully drive cell differentiation [49,50]. Although not yet a reality, these strategies appear a promising way to direct the differentiation of PSCs [51].

e. **Use of biomatrices mimicking skeletal muscle niche:** Tissue engineering approaches have been used to design synthetic and natural 3D scaffold materials to mimic the structural, biochemical, and mechanical properties of the stem cell niche [52,53]. Synthetic

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and natural scaffolds have been designed to provide support for muscle growth and allow myofibroblasts to rebuild their native ECM. Natural scaffolds based on ECM proteins such as fibrin and collagens have been used to form hydrogels for musculoskeletal tissue engineering [54–56]. Commercially available ECM substitutes such as Matrigel™ hydrogels are also showing promising results in the differentiation of PSCs towards cardiomyocytes [57]. However, current ECM protein-based scaffolds are limited by their immune rejection and scaling up technologies. Synthetic scaffolds, which can be fabricated with ideal architectures at the nanoscale, pore sizes and mechanical properties, represent an advantageous solution to mimic the 3D ECM microenvironment (Figure 4). Technologies such as electrospinning, which allows organizing the polymers into thin sheets of fibrous meshes, are promising in this field [58,59]. Recently, it has been proved the reprogramming of mouse fibroblasts onto cardiomyocyte-like cells on polyethylene glycol (PEG) hydrogels functionalized with laminin and RGD peptides [60]. This opens new perspectives toward the use of custom-engineered synthetic scaffolds in the differentiation of PSCs to muscle cells. Finally, the use of acellularized tissue scaffolds is also being explored in muscle regeneration. They offer a native ECM with the optimal biochemical and mechanical properties for cell culture and preserve the architectural features of the tissue. Their use as a matrix supporting the commitment of cardiac muscle cells has been recently reported, thus showing the potential of this approach [61].

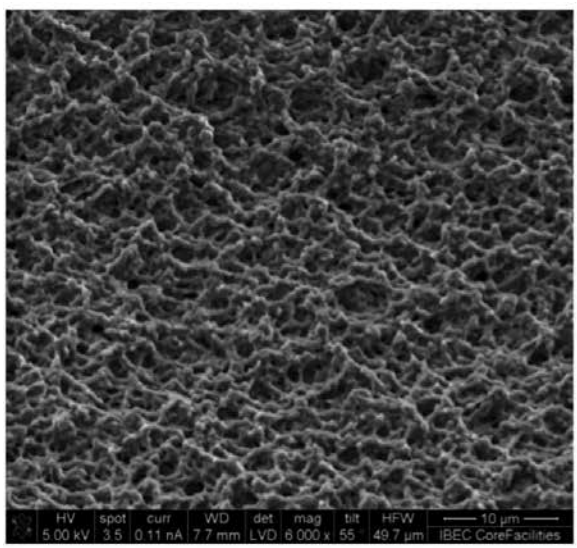

Figure 4. Bio-inert polyethylene glycol (PEG)-based hydrogels have been designed as the scaffold substrate for biomimetic matrices supporting muscle migration in three dimensions. The picture shows PEGDA hydrogel (MW 550 KDa) cross-linked by UV light.

f. Co-culture with supportive cells (feeder cells): The co-culture of mouse and human PSCs (either as monolayers or EBs) with feeder cells has been traditionally used in order to induce PSCs differentiation [39]. The stromal cell line OP9 [62,63], which is derived from

newborn mouse calvaria, supports hematogenesis [64,65]. The preadipose cell line PA6 [66] promotes neural differentiation of mouse and human PSCs [10,11,67]. In this regard, Baghavati [68] showed that the co-culture of mESCs together with primary muscle cells suffice for myogenic differentiation, since donor-derived myofibers generated by co-culturing mouse EBs on top of primary muscle fibers could be occasionally found on the surface of the host muscle.

3. Strategies to induce *in vitro* differentiation of mouse and human PSCs towards myogenic cells

3.1. Generation of myogenic precursors and/or terminally differentiated multinucleated myogenic cells by exogenous expression of transcription factors in PSCs

During the last 30 years successful generation of myogenic precursors from mouse and human PSCs has been achieved by exogenous expression of transcription factors crucial for myogenic differentiation. PSCs are especially amenable for genome editing because they can undergo extensive tissue culture manipulations, such as drug selection and clonal expansion, while still maintaining their pluripotency signature and genome stability. In his regard, different authors have explored the possibility to generate PSCs stable cell lines that express the myogenic transcription factor of interest under the control of specific drugs (i.e., antibiotics). The myogenic transcription factor of interest is normally subcloned into a viral vector, which possesses high infection efficiency when transducing PSCs. Other methods involve the use of non-integrative vectors such as transposons or excisable lentiviral vectors. Following these strategies different authors have shown that PSCs monolayers or PSCs-derived EBs can be converted into myogenic cells (see below).

3.1.1. Early studies of myogenic differentiation from mESCs by exogenous expression of transcription factors

Already in 1992 Dekel and colleagues showed that, when mESCs were electroporated with MyoD1 cDNA driven by the Beta-actin promoter, some cells could be converted to skeletal muscle cells [69]. Moreover, authors showed that contracting skeletal muscle fibers could be generated when the transfected cells were allowed to differentiate *in vitro*, via EBs, in low-mitogen-containing medium. Although those studies failed to develop efficient protocols for the generation of high yields of myogenic cells, they helped to understand that environmental factors should control MyoD expression and its myogenic differentiation function, and more importantly, that MyoD was required for the establishment of the myogenic program but not for its maintenance.

Thus, those first observations served as a starting point for the definition of enriched cell culture media for mESCs differentiation towards myogenic cells, and more importantly, for the generation of fine-tuned systems in order to control the expression of the desired myogenic factor at a precise moment during the onset of differentiation. Alongside this line, Ozasa and

colleagues [70] established a mESC line by introducing a MyoD transgene controlled by a Tet-Off system (ZHTc6-MyoD). The possibility to induce MyoD expression during the time course of differentiation, allowed mESCs to differentiate almost exclusively into the myogenic lineage in the absence of doxycycline, and without pre-differentiation into EBs. To start the differentiation process, Ozasa and colleagues removed doxycycline and used a differentiation medium containing 4% fetal bovine serum (FBS). Under those conditions and only after 7 days, primed cells started to fuse into myotubes, and occasionally light muscle contractions were observed. In that study the potential of the generated cells to differentiate into myofibers *in vivo* was also investigated by intramuscular injections into *mdx* mice and clusters of dystrophin-positive myofibers were detected in the injected area.

3.1.2. Myogenic differentiation from human PSCs by exogenous expression of transcription factors

In the same manner, within the last years several studies have demonstrated the possibility to generate myocytes, and even multinuclear myotubes from both hESCs and patient hiPSCs by means of different systems in which the expression of MyoD is driven under the control of soluble factors during the time course of differentiation. In this regard, early in 2012 two different reports indicated that mesodermal [71] or mesenchymal cells [72] could be generated from iPSCs, demonstrating a high potential for myogenic differentiation in response to *MyoD* over-expression.

Also Rao and colleagues (2012) generated a transgenic Tet-inducible MyoD cassette in which all the transgenic elements were inserted in hESCs making use of lentiviral vectors. In that particular study, authors were able to generate multinucleated myotubes with 90% of efficiency in a period that lasted only 10 days. Later on, Yasuno and colleagues [37] improved a previous protocol [36] for the generation of terminal multinucleated cells from iPSCs derived from patients affected with Carnitine palmitoyltransferase II (CPT II). Their protocol consisted in the transduction of a self-contained Tet-inducible MyoD1 expressing *piggyBac* vector (Tet-MyoD1 vector) and transposase into hiPSCs by lipofection. This system allowed the indirect monitoring of induced *MyoD* expression in response to doxycycline by co-expression of a red fluorescent protein (mCherry). Moreover, in that particular setting authors increased the purity of the generated myocytes by culturing the cells in low glucose conditions, a condition that was also reported to increase differentiated cardiomyocytes out of undifferentiated iPSCs based on the substantial biochemical differences in glucose and lactate metabolism between differentiated cells and undifferentiated iPSCs [73].

Very recently, Abujarour and colleagues [41] found that it is possible to derive myotubes from control iPSC and iPSC lines from patients with either Duchenne or Becker muscular dystrophies. In particular, by using a lentiviral system expressing MyoD under the control of a Tet-inducible promoter, and under-optimized culture conditions, the authors achieved an efficient myogenic differentiation setting the bases for the production of scalable sources of normal and dystrophic myoblasts for further use in disease modelling and drug discovery.

MyoD1 has not been the sole transcription factor of choice when differentiating human PSCs towards myogenic cells. Iacovino and colleagues [74] generated an unprecedented system in which it was possible to integrate the gene of interest into the desired cells (mESCs, kidney

murine cells and hESCs) by means of a system that authors called inducible cassette exchange (ICE). In that particular setting, authors were able to integrate one single copy of Myf5 into mESCs and hESCs. Overall, Iacovino and colleagues showed that Myf5 expression is sufficient to promote the myogenic commitment of nascent mesoderm thereby establishing a novel and rapid method of differentiating mESCs and hESCs into skeletal muscle tissue.

Taking advantage of Iacovino´s system [74], Darabi and colleagues generated an improved version of ICE system in order to generate mESCs in which Pax7 expression was controlled under the control of doxycycline, and they succeeded in inducing the myogenic program in mouse cultures [75,76]. Later on, the same authors generated inducible Pax7 hESCs and hiPSCs with a doxycycline-inducible lentiviral vector encoding Pax7 (iPax7 and the expression of the Pax 7 transgene was detected by incorporating an IRES-GFP reporter downstream of the Pax7 gene. Next, iPax7 hESCs and hiPSCs were induced to generate EBs and after three days doxycycline was added into the media in order to induce Pax7 expression. Following 4 days of induction, Pax7+GFP+ cells were purified by FACS and expanded in a secondary monolayer culture in a medium containing doxycycline and bFGF. Under those conditions iPax7 hESCs and hiPSCs expressed markers of early muscle differentiation (Pax7 and Pax3), and terminally differentiated when iPax7 hESCs and iPSCs were subjected to differentiation-inducing conditions (culture media with 5% horse serum and withdrawal of doxycycline and bFGF). Finally, Darabi and colleagues demonstrated that transplantation of Pax7-derived myogenic progenitors into dystrophin-deficient mice (mdx) promotes extensive and long-term muscle regeneration accompanied by functional improvement [77].

3.2. Generation of myogenic precursors and/or terminally differentiated multinucleated myogenic cells by soluble factors

Although in the last years different authors have shown the possibility to generate myogenic cells from human PSCs by means of the ectopic expression of specific transcription factors, these methods do not reflect normal development, and most importantly, are not suitable for therapeutic purposes or *in vitro* disease modelling. For this reason, in the last years different groups have investigated the possibility to expose EBs or monolayers of mouse and human PSCs to different culture media mimicking muscle development. In order to monitor and control the myogenic signature of the produced cells, authors have isolated the different potential populations based on the acquisition of surface markers related to myogenic fate (i.e., paraxial mesoderm) by means of FACS. In the same manner, the majority of these studies have relayed in the analysis of the expression of myogenic-related markers by common techniques such as the expression of myogenic-related factors by polymerase chain reaction or immunohistochemistry. In that way, the different protocols evaluate the efficiency of their method quantifying the percentage of cells that are differentiated towards myogenic cells.

3.2.1. Early studies in myogenic differentiation from mESCs by soluble factors

Although EBs exposed to undefined differentiation cell culture media spontaneously develop skeletal muscle cells and other cells *in vitro*, transplantation of EBs without any induction to direct development along a specific pathway leads to a failure of integration into recipient

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tissues and often forms teratomas in transplanted tissues. Thus, the definition of the specific conditions able to instruct PSCs towards myogenic cells requires establishment of robust conditions able to guide cells through the different stages of muscle differentiation.

In the first moment authors thought that the co-culture of EBs on top of freshly isolated muscle cells could serve as a novel method for myogenic differentiation. Although authors showed that differentiated cells generated by this method developed vascularized and muscle tissue when transplanted in dystrophic mice (mdx mice), still the number of engrafted cells was too low for potential applications in a clinical setting [68]. Later, Zheng and colleagues [78] showed that human EBs (hEBs) from two different hESCs lines cultured in the presence of differentiation media with different percentages of animal serum with or without Epidermal growth factor and 5-azacytidine could give rise to myogenic precursors. Interestingly, in that same work authors demonstrated that when those hESC-derived myogenic precursors were transplanted in NOD-SCID mice they could incorporate into the host muscle efficiently and become part of regenerating muscle fibers; giving rise to myocytes, myotubes, and myofibers, as well as satellite cells.

3.2.2. Generation of myogenic cells from human PSCs by soluble factors

In the quest for protocols suitable for regenerative purposes, Barberi and colleagues [79,80] developed simple feeder-free-monolayer culture systems in order to generate mesenchymal precursors that could be further differentiated towards myogenic cells from hESCs. In those studies multipotent mesenchymal precursors (MMPs) were purified for the acquisition of CD73 surface marker using FACS technology. First, MMPs were maintained in inactivated foetal serum and in the presence of the mouse skeletal myoblast line C2C12 [79]. Later, Barberi and colleagues could avoid the use of C2C12 cells by using serum-free N2 medium. Moreover, in that work authors further purified skeletal muscle myoblasts by means of a second FACS analysis for the neural cell adhesion molecule (NCAM), a marker of embryonic skeletal muscle. Those changes allowed for the expansion of hESC-derived myoblasts in a serum-free N2 medium in the presence of insulin [80].

Following a similar strategy Sakurai and colleagues [81] differentiated a murine ESC line towards paraxial mesodermal progenitors. Specifically, authors selected paraxial mesodermal progenitors based on the expression of platelet-derived growth factor receptor α (PDGFR-α) and the absence of Flk-1–a lateral mesodermal marker. Later on, the same authors demonstrated that mESCs could be directed toward the paraxial mesodermal lineage by a combination of bone morphogenetic protein (BMP) and Wnt signaling under chemically-defined conditions [82]. Interestingly, the same group developed a protocol for the generation of paraxial mesoderm progenitors from both miPSCs and hiPSCs. Although some differences in growth factor requirement between mESCs and miPSCs cells were observed, the PDGFR-α+ population derived from miPSCs was almost identical to that of mESCs. Importantly, the work of Sakurai and colleagues showed that, under their specific conditions, two different lines of hiPSCs could be differentiated towards PDGFR-α+/KDR- cells. Those progenitors could be further differentiated into osteocytes, chondrocytes, and skeletal muscle cells, demonstrating the suitability of their procedures for the generation of myogenic cells for regenerative purposes.

Notably, other authors have shown the possibility to generate PDGFR-α+ from hESCs [83]. However, those same authors showed few engraftments of transplanted hESCs-derived myogenic cells into injured skeletal muscle. Interestingly, the same authors have recently demonstrated that, by incorporating Wnt3a in culture medium, myogenic commitment is rapidly achieved from hESCs, and more significantly, that those cells can contribute to finally regenerate cardiotoxin-injured skeletal muscle of NOD/SCID mice [84]. In the same line, other authors have demonstrated that the inhibition of GSK3B and treatment with FGF2 could specifically promote skeletal muscle differentiation. In particular, Xu and colleagues [85] have demonstrated that simultaneous inhibition of GSK3B, activation of adenyl cyclase and stimulation with FGF2 during EBs formation could promote the generation of myogenic precursors that terminally differentiate *in vitro* and act as satellite cells upon transplantation. Also, Borchin and colleagues [86] have shown that human PSCs can be differentiated towards Pax3/Pax7 double positive cells after GSK3B and FGF2 exposure.

4. Induced pluripotent stem cells from patients: how to model muscle disease in the Petri dish

The possibility to direct cell differentiation from human PSCs opens the door for the development of massive platforms for the study of muscle differentiation and disease progression. Moreover, the possibility to combine gene-editing strategies allowing for the correction of the genetic disorder leading to muscle disease, together with the generation of myogenic cells from patients' cells, represents an unprecedented opportunity for the establishment of *in vitro* systems for the study of MDs.

So far, different groups have demonstrated the suitability of patient iPSCs approaches in order to model MDs. Abujarour and colleagues [41] have derived myotubes from Duchenne Muscular Distrophy (DMD) and Becker Muscular Distrophy (BMD) hiPSCs. In particular, authors showed that myotubes derived from MDM and BMD iPSCs could respond to insulin-like growth factor 1(IGF-1) and wingless-type MMTV integration site family member 7A (Wnt7a) in a similar manner to primary myotubes. These results point out that iPSC derived from MDM and BMD patients have no intrinsic barriers preventing from myogenesis, and thus represent a scalable source of normal and dystrophic myoblast for further use in disease modelling and drug discovery.

Recently, Tedesco and colleagues [71] generated iPSCs from fibroblasts and myoblasts from limb-girdle muscular dystrophy 2D (LGMD2D) patients, developing the first protocol for the derivation of mesoangioblast-like cells from these iPSCs. Moreover, authors expanded and genetically corrected patient iPSC-derived mesoangioblasts *in vitro* by means of a lentiviral vector for the expression of human α-sarcoglycan in striated muscle cells. When LGMD2D disease free iPSC-derived mesoangioblasts were transplanted into α-sarcoglycan-null immunodeficient mice authors showed that they were capable to generate muscle fibers expressing α-sarcoglycan. Interestingly, when the same experiments were conducted using mouse-derived mesoangioblasts authors showed a functional amelioration of the dystrophic pheno-

type and restoration of the depleted progenitors in α-sarcoglycan-null immunodeficient mice. Overall, Tedesco and colleagues showed that transplantation of genetically corrected mesoangioblast-like cells derived from iPSCs from LGMD2D patients could represent a novel therapeutic approach for these patients.

In the same line, other authors [36] have generated iPSCs from patients affected by Miyoshi myopathy (MM), a congenital distal myopathy caused by mutations in dysferlin. Specifically, authors demonstrated that the expression of full-length dysferlin could restore the MM associated phenotype in myotubes differentiated from MM-iPSCs. In the same line, Yasuno and colleagues [87] have shown the possibility to generate iPSCs from patients affected by Carnitine palmitoyltransferase II (CPT II) deficiency, an inherited disorder involving B-oxidation of long-chain fatty acids (FAO).

Very recently, Li and colleagues [88] have demonstrated the possibility to correct iPSCs derived from DMD patients by means of three different strategies: exon skipping, frameshifting, and exon knock-in. In their hands, exon knock-in was the most effective approach. The work of Li reveals the suitability of iPSC technology for the generation of iPSC-based approaches for MDs modelling and therapy.

Overall, these recent advances set the bases for the generation of a previously nonexisting tool for the study of MDs. The possibility to generate human models for the study of MDs by means of iPSC technology opens the door for the development of novel therapeutic compounds for MD treatment, and more importantly, to increase our understanding of MDs and muscle development.

5. Protocols for the generation of patient-specific iPSCs

Within the last years, our group has participated in the development of protocols for the derivation of patient iPSCs for disease modelling and compound screening. Taking advantage of different basic techniques that are commonly used on a daily basis in any laboratory worldwide, we have generated simple methodologies that allow the generation of patient-specific iPSCs in a period that lasts only 50 days from the moment we get the primary samples from patients (i.e., skin biopsy, lipoaspirates, etc.). In this section, we provide two concise protocols for the derivation of patient iPSCs taking advantage of retroviruses and episomal vectors.

5.1. Protocol for the generation of hiPSCs from keratinocytes derived from plucked hair samples

The development of simple methods for the generation of hiPSCs from keratinocytes from plucked hair samples offers an unprecedented scenario for the production of patient-specific iPSCs, making use of a non-invasive procedure when collecting patient samples.

Our protocol is divided into three consecutive steps, which involve: A) Isolation of keratinocytes from plucked hair samples, B) Production of retrovirus, and C) Infection of keratinocytes.

The steps are detailed below. As described elsewhere, the same protocol can be applied when reprogramming cord blood stem cells, kidney tubular epithelial cells, and dermal fibroblasts [11,12,30].

a. Isolation of keratinocytes from plucked hair

A.1 The day before hair isolation coat the required number of 35-mm culture dishes with Matrigel® (Becton Dickinson, S.A. cat. no. 356234) by adding 1 ml of Matrigel® and incubate overnight at 4 ºC.

A.2 The same day of sample recovery, prepare a non-coated 100-mm bacterial plate containing HBSS (Invitrogen cat. no. 14170-088) with 1% (vol/vol) Penicillin/Streptomycin (Invitrogen cat. no. 15140-122).

A.3 After the patient reads and signs the informed consent use tweezers to gently pull the hair out and place it on plates filled with HBSS medium. As recommended by Aasen and colleagues [89] use hair from the occipital part of the head.

A.4 Making use of forceps submerge the hair in HBSS medium. Next, cut off the external part of the hair leaving the bulb and outer root sheath.

A.5 As described by Aasen [89], at this stage two optional methodologies for growing keratinocytes from plucked hair are described: direct outgrowth and enzymatic digestion. In this section, we are going to detail how to get direct outgrowths of keratinocytes from plucked hair. For enzymatic digestion procedure follow Aasen recommendations [89].

A.6 Remove the plate from 4 ºC, aspirate the Matrigel® coating and rinse the plate with 2 ml of hESCs medium: KO-DMEM (Invitrogen, cat. no. 10829-018), 20% KOSR (Invitrogen, cat. no. 10828-028), 10 ng ml-1 bFGF (Peprotech cat. No. 100-18B), 1 mM Glutamax (Invitrogen, cat. no. 35050-038), 100 μM nonessential amino acids (Invitrogen, cat. no. 11140-050), 100 μM 2-mercaptoethanol (Sigma, cat. no. M7522), and 50 U/ml (penicillin and 50 mg/ml streptomicin).

A.7 Place gently the hair obtained from the coated culture plates.

A.8 Add few drops of hESCs medium (0.3 mL) on top of the hair sample in order to keep the hair humid. In the next 3–4 hours, add gently fresh hESCs medium (0.3 mL). The next day carefully check under the microscope that the hair sample is still attached at the bottom of the plate. Refill the plate with more hESCs medium on top of the hair, if necessary.

A.9 Add 1 ml of hESCs medium every following day. After 4 days, outgrowths of typical epithelial keratinocytes are visible.

A.10 After 10–14 days, large colonies of keratinocytes (up to 1 cm of diameter) are visible. At this stage, it is advisable to split the cells for infection or subculture to avoid cells to initiate contact-dependent differentiation.

b. Production of retrovirus

B.1 Seed out $4.3x10^6$ Phoenix Amphotropic 293 cells in 10 ml of DMEM complete medium which consists in DMEM (Invitrogen, cat. no. 11965-092), 10% FBS (Invitrogen, cat. no.

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10270-106), Glutamax 2 mM, Penicillin/Streptomycin (100 U/ml, 100 μg/ml) in 100-mm culture dishes and place in a 37 ºC 5% CO2 incubator.

B.2 Next day, prepare FuGENE:DNA complex according to the manufacturer´s instructions (Roche Applied Science, cat. no. 1181509001). We recommend a ratio of 27 μl FuGENE to 9 μg plasmid DNA for every 10 cm dish. For virus production, we will make use of pMSCV-based plasmids. pMSCV-based retroviral vectors are commercially available for OCT4, SOX2, KLF4, and c-Myc in Addgene (reference numbers are: 20072, 20073, 20074, and 20075 respectively). If the infection efficiency wants to be monitored pMSCV-based retroviral vectors expressing GFP can be used (Addgene plasmid 20672).

B.3 Add the FuGENE:DNA complex solution dropwise onto media (gently). Move the plate carefully in order to distribute the transfection reaction homogenously.

B.4 Place the transfected cells at 37 ºC, with 5% CO2 overnight.

B.5 Next day, change DMEM complete medium gently (10 ml/plate) and incubate the plates overnight at 32 ºC in a 5% CO2 incubator.

B.6 Collect viral supernatants and add fresh complete DMEM medium to the different plates. Take care to avoid cells detaching from the tissue culture plates.

B.7 Every following day, for 2 days, repeat steps B.5 and B.6 in order to collect more viral supernatants.

B.8 Filter the viral supernatant through a 0.45 μm PVDF filter (Millipore® SLHV033NK) to remove any contaminant cells.

B.9 Add 1 μl polybrene (10 mg/ml; Chemicon cat. no. TR-1003-6) for each ml viral supernatant needed (final polybrene concentration of 10 μg/ml).

c. Infection of keratinocytes derived from plucked hair samples

C.1 Wash obtained keratinocyte colonies growing from a hair in hESCs medium as described in section (A) with PBS (Invitrogen, cat. no. 10010-056) and trypsinize them using 1 ml 0.25% Trypsin/EDTA (Invitrogen, cat. no. 25200-056).

C.2 After 5–8 minutes, when cells are released from the plastic surface resuspend them with 10 ml hESCs medium.

C.3 Centrifuge at 200g for 5 min.

C.4 Resuspend the pellets in 4 ml of hESCs medium.

C.5 Plate cell suspensions in the desired wells of six-well plates (Corning, cat. no. 153516) previously pre-coated with Matrigel® as explained in step A1. Seed 80.000 keratinocytes/well.

C.6 Next day add 1 ml of every single viral suspension obtained as described in steps B6-B9 for OCT4, SOX2, KLF4, and cMYC. Perform viral transduction in the same manner for GFP in order to monitor the efficiency of viral infection.

C.7 Centrifuge plates at 650g for 45 min.

C.8 Replace with 2 ml fresh hESCs medium (within 4–5 hours).

C.9 Next day, repeat steps **C.7**-**C.8** to infect cells a second time.

C.10 Change media daily with hESCs medium.

C.11 After 1–2 weeks large colonies are visible and can be picked mechanically and transferred onto irradiated human fibroblasts feeder layer (iHFF) and cultured as normal iPSCs following specifications detailed before by others [12,29,30].

5.2. Protocol for the generation of hiPSCs from mesenchymal stem cells (MSCs) derived from adipose tissue

The possibility to generate iPSCs by means of non-integrative strategies paves the way for the development of clinical grade iPSCs from patients. Here, we detail a specific protocol for the derivation of hiPSCs from mesenchymal stem cells from adipose tissue.

Our protocol makes use of commercial episomal plasmids generated by Okita and colleagues [16]. Our method offers the possibility to generate patient-specific iPSCs in a period that last only 20 to 22 days from the moment the reprogramming experiment starts.

a. Before nucleofection

A.1 Following the Human MSC Nucleofector® Kit (DPE-1001, Amaxa) recommendations the solution for nucleofection is prepared by adding 0.5 ml of Supplement to 2.25 ml Nucleofector Solution. Human MSC Nucleofector Solution is now ready to use and is stable for 3 months at 4°C.

A.2 Under the culture hood prepare plasmid mixture by mixing 1 μg of each pCLXE episomal based plasmid (i.e., if we want to reprogram three different samples 3 μg of each pCXLE plasmid will be added to the final mixture). Plasmids are commercially available in Addgene: pCXLE-hOCT3/4-shp53-F (Plasmid #27077), pCXLE-hSK (Plasmid #27078), pCXLE-hUL (Plasmid #27080).

A.3 Pre-warm the complete Human MSC Nucleofector Solution to room temperature.

A.4 Pre-warm an aliquot mesenchymal stem cells culture medium [DMEM (Invitrogen, cat. no. 11965-092), 10% FBS (Invitrogen, cat. no. 10270-106), Glutamax 2 mM, Penicillin/Streptomycin (100 U/ml, 100 μg/ml)] at 37 °C in a 50 ml tube (500 μl per sample; 1.5 ml for 3 samples).

A.5 Prepare 6-well plates by filling the appropriate number of wells with 1 ml of culture medium containing mesenchymal stem cells culture medium and pre-incubate plates in a humidified 37 °C/5% CO2 incubator. Prepare 2 wells/sample (i.e., 6 wells for 3 samples)

b. Nucleofection and iPSCs generation

B.1 Follow Human MSC Nucleofector® Kit recommendations (http://bio.lonza.com/fileadmin/groups/marketing/Downloads/Protocols/Generated/Optimized_Protocol_90.pd).

B.2 After nucleofection transfer the nucleofected cells from the cuvettes using the plastic pipettes provided by the kit to prevent damage and loss of cells distributing the total amount

of cell suspensions into 2 wells containing the pre-warmed mesenchymal stem cells culture medium. Incubate the cells in a humidified 37 °C/5% CO2 incubator.

B.3 After 4 days transfer, wash nucleofected samples with PBS (Invitrogen, cat. no.10010-056) and trypsinize them using 1 ml 0.25% Trypsin/EDTA (Invitrogen, cat. no. 25200-056).

B.4 Transfer the nucleofected cells into six-well plates (Corning, cat. no. 153516) containing irradiated murine fibroblasts (iMEF;C57BL/6 MEF 4M IRR; Global Stem) in hESCs medium: KO-DMEM (Invitrogen, cat. no. 10829-018), 20% KOSR (Invitrogen, cat. no. 10828-028), 10 ng ml-1 bFGF (Peprotech cat. No. 100-18B), 1 mM Glutamax (Invitrogen, cat. no. 35050-038), 100 μM nonessential amino acids (Invitrogen, cat. no. 11140-050), 100 μM 2-mercaptoethanol (Sigma, cat. no. M7522), 50 U/ml (penicillin and 50 mg/ml streptomicin). Change hES media every other day.

B.5 After 20 days, large colonies are visible and can be picked mechanically and transferred onto iMEF and cultured as normal iPSCs following specifications detailed before by others [12,29,30].

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Chapter 13

Smooth Muscle and Extracellular Matrix Interactions in Health and Disease

Michael Schuliga

Additional information is available at the end of the chapter

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Abstract

Alterations in smooth muscle cell function and phenotype contribute to tissue remodeling in various pathologies including obstructive lung (*e.g.*, asthma) and vascular (*e.g.*, atherosclerosis) diseases. The extracellular matrix (ECM) is a major influence on the biology of smooth muscle cells, being an important support structure that provides signaling cues through its biochemical and biophysical properties. ECM factors activate biochemical and mechano-transduction signaling pathways, which modulate smooth muscle cell contraction, stiffness, survival, growth, cytokine production and migration (*i.e.*, cellular processes which contribute to changes in tissue architecture). The interaction of the ECM with smooth muscle cells is a dynamic multi-directional process, as smooth muscle cells also produce ECM protein, as well as proteases and cross-linking enzymes which regulate ECM form and structure. Understanding the molecular basis of ECM modifications and their impact on smooth muscle cell function in disease may lead to the development of novel therapies. This chapter reviews interactions between the ECM and smooth muscle cell and how they become altered in disease, using obstructive lung and vascular diseases as examples. From a pharmacological and therapeutic perspective, strategies that alter the phenotype of the smooth muscle cell in disease will be discussed. Emphasis will be given to approaches that target the proteases and mediators of ECM-smooth muscle cell signaling as potential treatments for pulmonary and vascular disease. Proteases of the coagulation and plasminogen activation systems have been given particular attention as they not only have a role in forming and modifying ECM, but also can directly stimulate changes in smooth muscle cell function and phenotype via activating receptors such as the protease-activated receptor-1 (PAR-1) and integrins.

Keywords: Extracellular matrix (ECM), Coagulation, Collagen, Fibrinolysis, Integrins, Proteases

1. Introduction

Smooth muscle cells function by contracting following activation of actin and myosin filaments, in a process involving myosin light chain phosphorylation, mediated by Ca^{2+}-dependent pathways [1]. In many diseases, alterations in smooth muscle cell function including contractile responses, growth and phenotype, contribute to tissue remodeling. In obstructive lung diseases such as severe asthma and chronic obstructive pulmonary disease (COPD), increases in the stiffness and mass of the airway smooth muscle (ASM) bundle contribute to fixed airway obstruction and hyper-responsiveness [2, 3]. In vascular injury and diseases such as atherosclerosis and pulmonary arterial hypertension (PAH), the migration, stiffening, proliferation and growth of vascular smooth muscle (VSM) cells contribute to the enlargement of the blood vessel wall, which in effect reduces lumen size, thus an increase in vascular resistance [4, 5]. Alterations in the microenvironment of the smooth muscle cell, particularly in the composition and structure of the ECM, accompany changes in smooth muscle biology in disease. Smooth muscle cells have a large role in modifying their microenvironment in disease by producing ECM protein (*e.g.*, collagen, fibrin, fibronectin and proteoglycans [6]) and factors which regulate ECM formation (*e.g.*, tissue factor in fibrin formation [7]). Furthermore, smooth muscle cells secrete proteases (*e.g.*, urokinase plasminogen activator or uPA [8]) and crosslinking enzymes (*e.g.*, lysyl oxidases [9]) which modulate ECM structure and form. The ECM in turn regulates smooth muscle function by the provision of both biochemical and biomechanical cues, in a process involving complexes formed between integrins, focal adhesion (FA) proteins and the actin-cytoskeleton. Both biochemical and mechano-transduction signaling in smooth muscle cells are mutually interdependent. In disease, the altered ECM may perpetuate tissue remodeling by augmenting smooth muscle growth, migration, cytokine production, cell stiffness and proliferation in a detrimental feed forward mechanism. Aside from important biomechanical contributions in tissue remodelling, smooth muscle cells are also potent producers of an array of inflammatory mediators, including cytokines, chemokines and cell adhesion molecules (CAMs) [10-13]. These inflammatory mediators, as well as the ECM produced by smooth muscle cells, influence the type and quantity of inflammatory cells that infiltrate damaged tissue in disease [14, 15].

2. Smooth muscle cells

Smooth muscle cells are phenotypically-plastic stromal cells, which are very capable of differentiating in response to injury and inflammation in disease. Whilst myogenic, the structure, mechanical properties, contractility and function of smooth muscle cells are different

to those of striated and cardiac muscle cells. The involuntary non-striated smooth muscle cells are found in many tissues and organs including the gastro-intestinal tract, the respiratory system, reproductive tract, urinary bladder, skin, iris of the eyes, kidneys and blood vessels. Smooth muscle cells contract and relax to regulate the luminal diameter and viscoelasticity of conducting vessels (*e.g.*, the vasculature and bronchioles) [16] and sphincters (*e.g.*, the urethral and pre-capillary sphincters) [17]. Smooth muscle contraction also has a role in the rhythmic peristalsis of tissues and organs of the gastro-intestinal tract and respiratory systems [18]. Whilst the structure and function of smooth muscle cells in different tissues are quite similar, they can contrast in their mode of activation, whether that be spontaneous involving ionic channel dynamics or by physiochemical agents (*e.g.*, hormones and neurotransmitters) or external agents (*e.g.*, CO_2). The actin-myosin contractile apparatus mediates the force generation responsible for smooth muscle contraction, whether that contraction is phasic or tonic (*i.e.*, rapid versus sustained contraction) [19]. Contraction is initiated by calcium-regulated myosin light chain phosphorylation, involving calmodulin rather than the calcium-activated troponin system (as in striated and cardiac muscle), and involves the sliding of actin-myosin filaments [1].

3. Smooth muscle cells in disease

Structural and functional changes to smooth muscle cells and their microenvironment contributes to tissue remodeling in diseases such as lung obstructive and vascular diseases [2-5], which are the focus of this chapter. Tissue remodeling involving smooth muscle cells is likely to be a result of injury and dys-regulated repair processes linked to inflammation and extravascular coagulation and fibrinolysis [20-23].

3.1. Obstructive lung diseases

Obstructive lung diseases, including asthma, COPD, bronchitis and bronchiectasis, are characterized by airway obstruction. The latter is a limitation of airflow, caused by the narrowing of bronchioles. The mechano-contractile properties of the ASM cell make it the primary effecter of bronchospasm, which is an acute contraction of the airways that occurs in asthma and bronchitis. ASM cells are also involved in the array of persistent tissue structural changes of the airway wall that contribute to airway obstruction in asthma and COPD [2, 24, 25]. In airway wall remodeling (AWR), ASM cell hyperplasia, hypertrophy and changes in production of ECM protein contribute to an increase in airway wall thickness and reduction in airway wall distensibility [26]. ASM cells in disease are major contributors to the increases in production of ECM components, including the wound type collagens, I and III, and fibronectin. ASM cells also have an important inflammatory role in airway obstructive diseases, being potent producers of growth factors, cytokines and other pro-inflammatory mediators, including granulocyte macrophage-colony stimulating factor (GM-CSF), intracellular adhesion molecule (ICAM), interleukin-1α (IL-1 α), eotaxin, leukaemia inhibitory (LIF), fractalkine and vascular cell adhesion molecule (VCAM) [10]. Pro-inflammatory mediator expression in ASM cells is stimulated primarily by cytokines and growth factors produced by

inflammatory cells and the epithelium [27]. The ECM in the microenvironment of the ASM cell can modulate cytokine expression by ASM cells [28]. Furthermore, cyclical changes in mechanical strain associated with breathing also modulate cytokine expression by ASM cells [29]. As a consequence of their pivotal role in AWR, therapies that target the ASM cell are continually being explored as possible new treatments for obstructive lung diseases [30]. Although eliminating ASM by bronchial thermoplasty is effective in reducing airway obstruction, less invasive and costly therapies are urgently needed. An effective muscle relaxing, anti-remodeling treatment that specifically targets ASM cells is expected to reduce airway reactivity and symptoms in patients with asthma or COPD [31, 32].

3.2. Vascular diseases

VSM cells reside in the medial layer of blood vessels, mediating changes in vascular tone by contracting or relaxing in response to vasoconstrictors and vasodilators respectively. In vascular injury and disease, VSM cells switch from a quiescent, contractile phenotype to a synthetic phenotype, which produces cytokines and ECM protein (*e.g.,* fibronectin and sulphated proteoglycans), undergoes hyperplasia (*i.e.,* increased proliferation) and/or hypertrophy (*i.e.,* increased cell size). In atherosclerosis, a cause of stenosis and blood vessel occlusion, synthetic VSM cells migrate from the media to the intima where they proliferate and contribute to intimal thickening [22, 23]. Furthermore, within the intima, VSM can alter the composition of the ECM. For example, VSM cells produce increased amounts of LDL-accumulating sulphated proteoglycans, which increase lipid loading, a feature of atherothrombotic disease [33]. In hypertension, VSM cells undergo hypertrophic remodeling in the media of blood vessels, contributing to excessive arterial vascular contractile tone, hence high blood pressure [34]. Hypoxia, inflammation and shear stress contribute to vasoconstriction and pulmonary vascular remodeling in PAH [35, 36]. Medial thickening in proximal pulmonary vessels is caused by the hypertrophy, hyperplasia and ECM production of resident VSM cells, whereas in pre-capillary arterioles, the VSM cells that contribute to medial thickening are derived from intermediate cells in blood vessels or adventitial fibroblasts, which differentiate into VSM. Like ASM cells, VSMs contribute to inflammation in vascular disease by producing pro-inflammatory mediators such as the platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), interferon-γ (IFN- γ), and monocyte chemotactic protein-1 (MCP-1). Furthermore, VSM cells express CAMs (*e.g.,* VCAM-1 and ICAM-1), which have a role in retaining monocytes and macrophages within lesions such as those formed in the intima in atherosclerosis. Aberrant Akt/PI3kinase signaling resulting from a defect in PTEN phosphatase activity is thought to contribute to the pro-inflammatory phenotype of VSM cells in PAH [37]. In a murine model of PAH, the targeting of the PTEN gene specifically in VSM cells augments an inflammatory response and vascular remodeling [38].

4. ECM of smooth muscle

The ECM of the smooth muscle cell microenvironment changes in injury, disease and with ageing. ECM production and modification by smooth muscle cell is regulated by intrinsic

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(*e.g.*, epigenetics) and extrinsic (*e.g.*, oxidative stress, growth factors and cytokines) factors [15]. Abnormalities of the ECM are a key feature of tissue remodeling in obstructive lung [39] and vascular disease [34].

4.1. Collagen

Collagen is the most prominent structural protein of connective tissue. Native collagen I and III form fibrils comprising supra-molecular aggregates of collagen that are stabilized by interactions between their helical domains [40]. These fibrils have a high tensile strength that maintains the structural integrity of tissue by counter-balancing distending forces, such as those evoked by breathing on the airway wall [41]. In the airways of patients with asthma and COPD, the ECM is expanded by fibrils of collagen I and III, including around and within smooth muscle bundles, reducing airway wall distensibility [26]. Increased collagen, as well as ASM hyperplasia and hypertrophy, also contribute to airway wall thickening that is linked to increased airway-reactivity [2]. In the media of the healthy blood vessel wall, collagen I and III are the main components of the ECM, with elastin also being abundant in the media of larger arterioles [42, 43]. Collagen fibrils formed by VSM cells also have an important role in fibrous cap stabilization, a stage of atherogenesis [44]. Whilst the native forms of collagen impose mechanical strain on cells, it is the denatured forms of collagen, which perhaps are more biochemically-reactive. Increases in the activities of plasmin and MMPs, proteases which denature native collagen, occur in both respiratory [45] and vascular [34] disease. The denaturation of collagen fibrils immediately adjacent to and surrounding smooth muscle cells by pericellular proteolysis may have an important role in regulating phenotype and function in disease.

4.2. Fibronectin

Fibronectin is a high molecular weight glycoprotein and constituent of the ECM. Fibronectin binds to the $\alpha 5\beta 1$ integrin on cells to transmit biochemical and mechanical signals, and is a nucleator of fibrillogenesis (*i.e.*, collagen fibril assembly). In asthma, levels of fibronectin, along with collagen I and III, are increased within the smooth muscle bundle of the airway wall [46]. Additionally, ASM cells from asthmatics, when compared to non-asthmatics, secrete more fibronectin, which in turn augments increased ASM cell proliferation and cytokine production [47]. Rhinovirus-infection [48], cigarette smoke extract [49] and the pro-fibrogenic mediator TGF-β [50], all stimulate ASM cells to produce fibronectin. VSM cells in disease also produce increased amounts of fibronectin. During atherogenesis, fibronectin synthesized by VSM cells becomes more abundant in the blood vessel wall, which augments fibrillogenesis [44] and stimulates smooth muscle cell proliferation and cytokine production [42, 43].

4.3. Fibrin

Extravascular accumulation of fibrin, formed by the coagulation cascade, occurs in a number of diseases, including respiratory and vascular diseases [21, 51-55]. Increased vascular permeability allows blood-circulating hemostatic factors such as factor VII (FVII), factor X (FX)

and plasminogen to enter damaged and inflamed tissue to become activated and participate in coagulation and fibrinolysis (Figure 1).

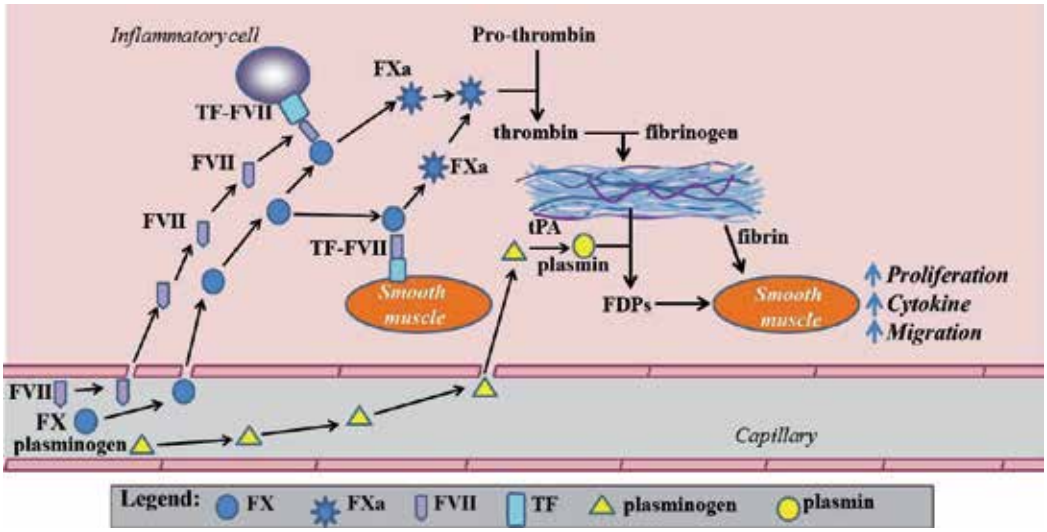

Figure 1. Extravascular coagulation and fibrinolysis. In inflamed tissue such as the airway wall in asthma, plasma containing factor VII (FVII) and FX leak into the extravascular compartment. FVII, combined with tissue factor (TF), formed by epithelial, stromal (e.g., smooth muscle cells) and inflammatory cells, transforms FX into the serine protease, FXa. The latter, combined with FV, activates thrombin, which in turn converts fibrinogen into fibrin. Whilst fibrin has an important role in physiological wound-repair, it also serves as a scaffold for migrating stromal cells, and can be converted into fibrin degradation products (FDPs), which stimulate smooth muscle cell migration and cytokine production. Fibrinolysis (i.e., the break-down of fibrin) is catalysed by plasmin, formed by the activation of plasma-derived plasminogen. The latter is catalysed by tissue-type plasminogen activator (tPA).

Abundant extravascular fibrin is a specific hallmark of lung disease including asthma, and is thought to be a result of hyper-coagulation and suppressed fibrinolysis (*i.e.*, fibrin degradation). The key mediator of fibrinolysis is plasmin, which can be formed by the proteolytic activation of plasminogen by either the tissue-type or urokinase type plasminogen activators (tPA or uPA respectively) [56, 57]. However, only tPA has fibrin-dependent amplification activity, facilitating its role as the primary mediator of fibrinolysis. Levels of tPA are much higher than uPA in the airway lumen [58-60]. The reduced AWR observed in plasminogen activator inhibitor-1 (PAI-1) gene knockout mice challenged with aerosolized allergen is likely to be a consequence of less airway fibrin [61, 62]. PAI-1 inhibits fibrinolysis by blocking the actions tPA in plasmin formation.

The medial layer of blood vessels, where VSM cells are prominent in number, is exposed to plasma exudate, even under normal physiological conditions. Whilst fibrin is present in normal arterial intima, its levels are increased in atherosclerotic lesions, particularly early proliferative, gelatinous-lesions [21]. Again, hypercoaulation and suppressed fibrinolysis are likely to contribute to increased fibrin formation in the vasculature in disease. The coagulant, tissue factor (TF), is highly expressed in VSM cells during atherogenesis [7], being rapidly induced by growth factors and cytokines [7]. Hypoxia, a driver of PAH, induces the up-

regulation of PAI-1 in pulmonary artery VSM cells [63]. In injury and disease, the deposition of fibrin into the ECM serves as a scaffold to support smooth muscle cell migration [64]. Furthermore, fibrin degradation products (FDPs) formed by fibrinolysis, stimulate production of pro-inflammatory mediators (*e.g.*, C-reactive protein in VSM cells [65]).

4.4. Proteolglycans

Proteoglycans are another important component of the ECM synthesized by smooth muscle cells in disease including VSM cells during atherogenesis [42, 43] and ASM cells in asthma [66]. In atherogenesis, sulphated proteoglycans, via ionic interactions with ApoB100 and ApoE, entrap low-density lipoprotein (LDL) within the vessel wall. Bound oxidized-LDL augments macrophage lipid uptake and foam cell formation, and stimulates VSM cells to secrete greater amounts of sulphated proteoglycans [34].

5. ECM remodeling by smooth muscle cells

Asides from producing ECM protein, smooth muscle cells also modulate the structure and form of the ECM. Extracellular modification of ECM protein involves integrins and enzymes which proteolyze and cross-link collagen. The serine protease, plasmin, also has the ability to directly activate smooth muscle cells via receptor based mechanisms. Major modifications of ECM protein by smooth muscle cells are described below:

5.1. Fibrillogenesis

Smooth muscle cells including VSM [67] and ASM [68] cells polymerize collagen fibrils into larger supramolecular collagen assemblies. Collagen fibrils can vary greatly in diameter (20-500 nm) and form different supramolecular structures such as bundles, weaves, and layers to suit differing roles and functions. The ability of smooth muscle cells to increase the diameter of fibrils would increase the tensile strength and resistance of the fibrils to collagenolysis by MMPs. Collagen fibrillogenesis involves integrin binding (*e.g.*, $\alpha 2\beta 1$, $\alpha 11\beta 1$) and is integrated with fibronectin fibril formation [69]. Lipid accumulation by VSM cells inhibits collagen fibrillogenesis [70], contributing to the destabilization of fibrous caps, hence rupture of atherosclerotic plaques [44].

5.2. Proteolysis

Matrix metalloproteases (MMPs) and plasmin are proteases involved in the degradation of ECM proteins such as fibronectin and denatured collagen. Plasmin also cleaves and activates the zymogen forms of MMPs including the MMP collagenases (MMP-1, MMP-13 and MMP-14), which denature collagen fibrils [71]. MMPs including MMP-1, MMP-2, MMP-12 and MMP-14 are expressed by human ASM cells *in vivo* [72] and/or *in vitro* [68] and conversion of plasminogen into plasmin by ASM cells is associated with MMP activation and increases in collageneolytic activity [73]. MMPs and components of the plasminogen activation system are

co-expressed during remodeling in the airways following allergen challenge [74]. VSM cells also produce and secrete MMPs including MMP-2, MMP-3, MMP-7 and MMP-9 [34]. MMPs formed by VSM cells are considered to contribute to the thinning, thus destabilization of the atherosclerotic fibrous cap in atherogenesis [44]. Like ASM cells, VSM cells do not produce plasminogen *de novo*, but express urokinase plasminogen activator (uPA), which readily activates plasminogen convected from plasma through the blood vessel wall [22]. Hypoxia induces uPA expression in VSM cells, in association with increased cell migration and matrix invasion, suggesting a role of plasmin proteolysis in PAH [8].

Interestingly uPA and plasminogen gene deletion (but not tPA gene deletion) also reduces hypoxia-induced PAH and pulmonary vascular remodeling in mice [75]. These effects are likely to be independent of fibrinolysis, as uPA does not bind fibrin with the same high affinity as tPA. Plasmin formed by uPA has pro-inflammatory and -remodeling activities which are not associated with the break-down of fibrin. Furthermore, uPA has plasmin(ogen)-independent effects. The amino-terminal fragment of uPA interacts with its receptor, uPAR to activate other receptors such as the formyl-peptide receptor 2 (FPR2) [76] and the epidermal growth factor receptor (EGFR) [77], to regulate migration, chemotaxis and cytokine production. uPA binding also regulates the affinity of uPAR for the $\alpha 3\beta 1$-integrin [78], to regulate cell adhesion and cell signaling. Furthermore, the kringle domain of uPA interacts with the $\alpha v\beta 1$-integrin in an uPAR-independent manner [79]. Increases in the levels of uPA are associated with a number of pathologies, including asthma, COPD and PAH [59, 60, 80-82]. For smooth muscle cells, plasminogen activation by uPA is accelerated by the annexin A2 hetero-tetramer (AIIt) [83], an extracellular protein complex comprised of annexin A2 and S100A10 (p11). The AIIt also serves as a signal transducer for plasmin in mediating its pro-inflammatory effects on ASM cells [84] and macrophages [85]. The importance of annexin A2 in cancer is becoming increasingly recognised, however, little is still known about the role of annexin A2 in respiratory disease [86-90].

The proteolytic activity of plasmin releases the otherwise latent forms of growth factors such as epidermal growth factor (EGF) and TGF-β [91, 92]. Plasminogen activation by smooth muscle cells is associated with MMP activation [73] and targeting the EGF-receptor (EGFR) or MMPs attenuates plasmin(ogen)-stimulated proliferation [83]. The effects of plasmin(ogen) on EGFR signaling are contributed by heparan sulphate proteoglycan -binding EGF (HB-EGF), an EGFR ligand, which is released from heparin by MMP-mediated proteolysis (Figure 2). Like EGFR transactivation, plasmin-stimulated mobilization of matrix-bound TGF-β contributes to collagen synthesis in smooth muscle cells in a manner involving TGF-β receptor signaling [91]. TGF-β also stimulates ASM cell proliferation [93], in an indirect manner involving autocrine bFGF production [94].

Plasmin also activates the protease-activated receptor-1 (PAR-1), the proto-typical receptor of thrombin (and FXa). PAR-1 is expressed in inflammatory cells including, macrophages, mast cells and eosinophils [96-99], and extravascular structural cells including, smooth muscle cells [100]. Activation of PAR-1 in stromal cells is considered to evoke pro-tissue remodeling activities, which contributes to disease pathology. Levels of PARs are increased in structural cells in tissue remodeling lung diseases [101], and targeting PAR-1 reduces pulmonary

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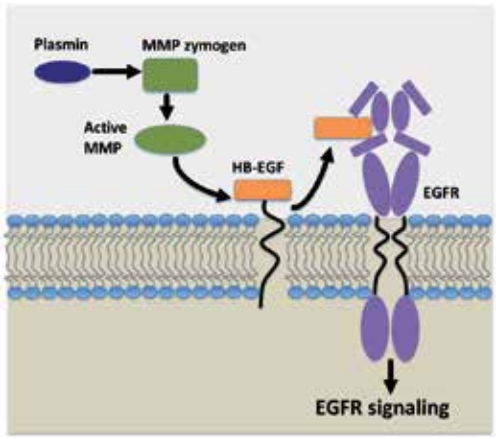

Figure 2. Plasmin-evoked EGFR transactivation. HB-EGF is an important ligand of EGFR, and is released from heparan sulphate proteoglycan by the proteolytic actions of MMPs. HB-EGF is expressed in the airway epithelium and smooth muscle in situ [95].

inflammation and tissue remodeling in mouse models of lung injury and disease, including asthma [98, 102, 103]. Furthermore, PAR-1 activation elicits increased cytokine and collagen expression [103-105] and proliferation [106] of lung stromal cells, including ASM cells [107]. The up-regulation of both PAR-1 and PAR-2 in VSM cells following injury and their subsequent activation by hemostatic proteases is considered to contribute to the pathogenesis of atherosclerosis [5]. Whilst plasmin has ~10 times less affinity for PAR-1 than thrombin [108], integrin co-receptors augment plasmin-evoked PAR-1 activation [109]. Binding to $\alpha_9\beta_1$ integrin localizes plasmin to the cell surface and protects it from α2-antiplasmin inhibition, increasing PAR-1 activation, whilst also activating pathways downstream of $\alpha_9\beta_1$ integrin through integrin-linked kinase (ILK) [109]. Additionally to plasmin, the plasmin-activated MMP-1 and MMP-13 also cleave the N-terminal exodomain of PAR-1, but at sites alternative to those of thrombin, FXa and plasmin, eliciting distinct cellular responses [110].

5.3. Cross-linking

Dysregulated matrix cross-linking and stability contributes to tissue remodeling in vascular disease and ageing. Lysyl oxidases cross-link collagen fibrils by modification of the ε-amino group in the side chain of lysines. Lysyl oxidase activity is increased in the vascular lesions of patients with PAH [9]. The expression of lysyl oxidases in VSM cells is responsive to hypoxia and increases in the lungs of mice in experimental PAH [9]. Glycation of collagens also induces covalent bridges between collagen fibrils, an age-related modification that contributes to arterial stiffness [34]. Similarly, vascular calcification, which involves transglutaminase-2-induced collagen crosslinking and dimerization of osteopontin, is a degenerative and end-stage process of atherosclerosis, which also contributes to the stiffness of ageing arteries [111]. Vascular calcification involves the de-differentiation of the VSM cell to a pro-calcificatory phenotype, where expression of the matrix Gla protein (MGP) and bone morphogenetic protein-2 (BMP-2) are decreased and increased respectively.

6. Smooth muscle cell and ECM interactions

Smooth muscle cells are highly sensitive to the biochemical and mechanical state of the surrounding ECM. Signal transduction, and the transmission and distribution of mechanical forces within the cell are dependent on the actin-cytoskeleton. This deformable polymer network also has important roles in maintaining cell shape (size) and cell migration [112]. The interaction of the actin-cytoskeleton with the contractile apparatus and the ECM involves integrins and focal adhesion (FA) complexes, and is a key determinant of the biochemical and mechano-sensing properties of smooth muscle cells. The highly malleable actin-cytoskeleton constantly rearranges, in a process involving assembly and disassembly, in response to both bio-physical and -chemical stimuli [113]. Alterations in smooth muscle stiffness in health and disease involve changes to actin-cytoskeleton organization, which is influenced by the ECM [114].

6.1. Biochemical transduction

Biochemically, ECM protein signal through integrin receptors and the dystrophin-glycoprotein complex in smooth muscle cells [115]. The native fibrillar and denatured non-fibrillar forms of collagen regulate smooth muscle cell function differentially. The anti-proliferative effect of fibrillar collagen type I on VSM [116] and ASM [68] cells, which arrest cells in the G1 phase of the cell cycle, involves $\alpha 2\beta 1$ integrin binding. Although, the non-fibrillar forms of collagen types I and III do not contribute structurally to tissue integrity, they retain cell signaling activity. The GFOGER motif of the characteristic triple helix of non-fibrillar type I collagen binds with high affinity to the integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, and $\alpha 11\beta 1$ [117]. Furthermore, proteolytic cleavage of the helix by MMPs and plasmin reveals the 'matricryptic' RGD integrin-binding site [118]. Non-fibrillar type I collagen stimulates smooth muscle cell proliferation [119], survival [120], and cytokine release [28] in an integrin-dependent manner. Similarly, fibronectin, which binds $\alpha 5\beta 1$ integrin via its RGD motif, also stimulates smooth muscle cell proliferation [119] and augments cytokine production in response to stimuli such as IL-1β [28]. Fibrin degradation products (FDPs) also regulate smooth muscle cell migration via binding the $\alpha_5\beta_3$ integrin [64], as well as stimulating smooth muscle cell cytokine production and proliferation via binding TLR-4 [121].

6.2. Mechano-transduction

Connections between the ECM and the actin cytoskeleton of smooth muscle cells allows for an efficient transfer of force between the contractile apparatus and the extracellular environment, which is important in myogenic force generation [122]. These same connections, which involve integrins and FA complexes, also regulate smooth muscle cell stiffness and phenotype. Mechanical forces, whether external or internal to tissue in which smooth muscle cells reside, stretch and strain protein-cell surface integrin FA complexes, activating downstream intracellular signaling pathways. This process is termed mechano-transduction, and often elicits mechanically sensitive ion channels to open or close, changing the polarity of the membrane potential, which then activates voltage-gated channels. Mechanical strain in smooth muscle

cells activates RhoA, a pivotal regulator of actin adhesion organization [123]. The β1-integrin is an important mechanoreceptor, regulating cell function and viability via the inositol 3-kinase (PI3K)/Akt signaling pathway, in a process involving FA kinase (FAK) and integrin-linked kinase (ILK) phosphorylation [124, 125].

In arterial blood vessels, integrin-mediated mechano-transduction triggers intracellular Ca^{2+} mobilization in VSM cells to evoke myogenic responses [126]. The integrin-mediated adhesion of VSM cells to the ECM is dynamically regulated in opposing directions by vasoconstrictors (*e.g.*, angiotensin II) and vasodilators (*e.g.*, nitric oxide) to control cell contractility, hence blood vessel tone [127]. VSM cells are in turn subject to varying mechanical strain (*i.e.*, intraluminal pressure) generated by moving blood. In the airways, the ASM cell regulates bronchomotor tone, contracting in response to various contractile agonists including acetylcholine, serotonin, histamine and endothelin-1 [128]. The ASM cell is also subject to mechanical forces resulting from cyclical expansion of airway diameter and cyclical lengthening of the airway wall due to breathing [122]. The ECM modulates the effects of these forces on cell function and phenotype. Cyclical strain (4% elongation) that is equivalent to normal breathing inhibits ASM cell proliferation when grown on laminin, but not collagen type I (denatured) *in* vitro [129]. The ratio of laminin to collagen is higher in the airways of non-asthmatics than asthmatics. When ASM cells grown on type I collagen are subject to increased cyclical strain (17-18% elongation), equivalent to those generated by bronchospasm and hyperinflation, an increase in migration and proliferation occurs in a MMP-dependent manner [130].

In disease, smooth muscle cell biology is affected by changes in the ECM, including increases in substrate stiffness and the generation of isometric forces in tethered collagen lattices, which influence mechano-transduction. In severe asthma, the "strait jacket" effect of ASM cells being embedded in a rigid microenvironment may reduce the amplitude of the oscillatory forces associated with breathing. This is likely to have potential consequences on smooth muscle biology, as has been proposed for lung fibroblasts in fibrotic foci (FF) lesions in idiopathic pulmonary fibrosis (IPF) [131]. Tensile collagen fibrils within FF form a rigid environment that reduces the magnitude of cyclical strain the fibroblasts are normally subjected to in healthy tissue upon breathing. A reduction in the amplitude of force of breathing-associated cyclical strain is thought to increase lung fibroblast fibrogenic activity and stiffness.

6.3. Interconnection of biochemical- and mechanical-evoked signaling

ECM biochemistry and mechanics are very much interconnected in their regulation of smooth muscle behavior. The influence of mechanics on smooth muscle cell biology is modulated by ligand biochemistry. For example, smooth muscle cells grown on fibronectin, as compared to laminin, exhibit increased cell adhesion and cytoskeletal polymerization in response to increasing stiffness of the substrata, *i.e.,* the 'stiffness by ligand' effect [132]. In VSM cells, FAs formed by cell contact with different ECM proteins, exhibit different mechanical characteristics resulting in distinct force-generating reactions [133]. VSM cells attach more strongly to fibronectin than collagen type I, vitronectin and laminin, to evoke greater myogenic force-generation [133]. Integrin co-receptors may also modulate PAR-1 and uPA signaling in response to biomechanical forces. uPA elicits cellular responses via binding its receptor, uPAR,

which lacks a transmembrane or intracellular domain [78]. The interaction of uPA with uPAR to activate co-receptors including integrins, is likely to be influenced by the engagement of integrins with the ECM. In support, fibulin 5 [127] and vitronectin [41] are integrin binding ECM proteins, which modulate uPA-uPAR signaling [134]. uPAR-independent activation of $\alpha v\beta 1$-integrin by uPA in smooth muscle cell may also be similarly affected by the ECM and biomechanical forces [79].

7. Targeting smooth muscle cell biology as therapy

Strategies that target smooth muscle cell biology, including ECM-smooth muscle cell interactions, may be beneficial in the treatment of tissue remodeling diseases. Approaches that target proteases, which modulate the ECM or ECM-signaling of smooth muscle cells in disease, will be of particular focus in this chapter section.

7.1. Contractile, hypertrophic and hyperplasic regulators

Targeting factors which regulate smooth muscle contractility, hyperplasia and/or hypertrophy are currently used in the treatment of airway and vascular diseases. β_2-Adrenergic receptor agonists, which increase the intracellular second messenger, cyclic AMP (cAMP), in ASM cells, are used pharmacologically to dampen bronchoconstriction in asthma and COPD [135]. Blockade of endothelin (ET) receptors are an effective treatment for PAH. Endothelin-1 (ET-1) is both a vasoconstrictor and mitogen, evoking its effects on VSM cells through binding to ET_A or ET_B receptors. Another treatment of PAH is prostacyclin (or its analogs), an eicosanoid, which like the prostaglandins and thromboxane, is vasodilatory [136]. Eicosanoids, which are released by endothelial cells, stimulate increases in the levels of intracellular cAMP in VSM cells via binding prostanoid/G-protein coupled receptors. Increases in cAMP inhibit myosin light chain phosphorylation, causing relaxation, as well as inhibiting proliferation [137]. Similarly, the endogenous vasodilator nitric oxide (NO), which is released by endothelial cells, also inhibits both myosin light chain phosphorylation (via increases in cGMP) and proliferation of VSM cells [36]. Various NO donors such as glycerine trinitrate and sodium nitroprusside (SNP) have been used in the treatment of vascular disease and endothelial dysfunction (*e.g.*, angina pectoris and hypertension) [138].

7.2. Growth factors

Growth factors such as PDGF, TGF-β and VEGF which regulate smooth muscle proliferation and size, may also be potential targets for the treatment of vascular and lung obstructive diseases [15]. Imatinib, a receptor tyrosine kinase inhibitor which inhibits PDGF signaling, attenuates both VSM cell hyperplasia and hypertrophy in pre-clinical models of vascular disease [139]. However, imatinib was withdrawn from clinical trials for the treatment of advanced PAH, because of serious side effects and increased morbidity [140]. Inhibiting specific aspects of TGF-β signaling may be another growth factor-targeting strategy to treat tissue remodeling in disease. Aberrant TGF-β signaling, important in regulating smooth

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muscle function including cell stiffness and proliferation [141, 142], contributes to tissue remodeling in vascular and respiratory diseases [143-145]. The TGF-β superfamily member, activin A, is linked with the progression of PAH, stimulating VSM cell proliferation [141]. Administration of follistatin, an endogenous inhibitor of activin A, attenuates inflammation and remodeling in experimental models of lung injury and disease [146].

7.3. MMPs

Targeting proteases that modify smooth muscle ECM structure and its biomechanical properties are another strategy to treat vascular and obstructive lung disease. Inhibition of pericellular collagenolysis *in situ* may reduce ASM cell hyperplasia in AWR by maintaining the anti-proliferative effects of fibrillar pericellular collagen. Administration of the MMP inhibitory antibiotic doxycycline reduces airway hyper-responsiveness in allergen-challenged mice [147], supporting the concept that interventions with selective MMP inhibitors could be beneficial in the treatment of diseases such as asthma (see Table 1). In preclinical models of vascular disease and injury, doxycycline has been shown to attenuate acute pulmonary thromboembolism (APT)-evoked pulmonary hypertension and right ventricular dysfunction [148].

The effects of targeting ECM proteases in experimental models of lung and vascular disease

Target	Approach	Model (challenge)	Outcome (as compared to wild type or vehicle control treated mice following challenge or injury)
Annexin A2	Genetic (annexin A2 knockout)	Allergen	Reduced airway inflammation [84].
FXa	Pharmacological (Fondaparinux)	Allergen	Reduced AWR. Administered after AWR was established [149].
Lysyl oxidase	Pharmacological (β-aminopropionitrile)	Arterial injury	Reduced perturbations to right ventricular systolic pressure, right ventricular hypertrophy, and vessel muscularization and normalized collagen cross-linking and vessel matrix architecture
MMPs	Pharmacological (doxycycline)	Allergen	Reduced airway hyper-responsiveness [147].
MMPs	Pharmacological (doxycycline)	APT	Reduced hypertension and right ventricular dysfunction [148].
Plasminogen	Pharmacological	Allergen	Reduced eosinophil and lymphocyte numbers, mucus production, and collagen deposition in the lungs [74].

The effects of targeting ECM proteases in experimental models of lung and vascular disease

Target	Approach	Model (challenge)	Outcome (as compared to wild type or vehicle control treated mice following challenge or injury)
	(tranexamic acid) and genetic (plasminogen knockout)		
Plasminogen	Genetic (plasminogen knockout)	Bleomycin	Reduced alveolar macrophage / Increased lung collagen [150].
Plasminogen	Genetic (plasminogen knockout)	Hypoxia	Reduced pulmonary hypertension and pulmonary vascular remodeling [75].
Plasminogen	Genetic (plasminogen knockout)	Hypoxia	No effect [75].
PAI-1	Pharmacological (IMD-1622)	Arterial injury	Reduced arterial neointimal formation, increases in adhesion molecules, fibrinogen accumulation [151]
PAI-1	Genetic (PAI-1 knockout)	Allergen	Reduced AWR including sub-epithelial fibrosis [61, 62].
PAI-1	Genetic (PAI-1 knockout)	Bleomycin	Reduced lung collagen [152].
PAI-1	Pharmacological (Tiplaxtinin)	Allergen	Reduced AWR [153]
PAI-1	Genetic (PAI-1 knockout)	Endotoxin	Reduced neutrophil recruitment to the lungs [154] and fibrin deposition and AWR in the airways [155].
PAI-1	Genetic (PAI-1 knockout)	Arterial injury	Reduced arterial neointimal formation [156].
PAR-1	Pharmacological (ER-112780-06)	Allergen	Reduced AWR [98].
PAR-1	Genetic (PAR-1 knockout)	Bleomycin	Reduced collagen accumulation in lung and pulmnonary inflammation [102].
PAR-1	Pharmacological (Atopaxar)	APT	Reduced neointimal thickening in arterial blood vessels [157]
PAR-1	Pharmacological (F16618)	Balloon angioplasty	Reduced restenosis [158]
tPA	Therapeutic (nebulized tPA)	Allergen	Reduced airway hyper-responsiveness [52]

The effects of targeting ECM proteases in experimental models of lung and vascular disease

Target	Approach	Model (challenge)	Outcome (as compared to wild type or vehicle control treated mice following challenge or injury)
tPA	Genetic (tPA knockout)	Bleomycin	Increased lung collagen/ alveolar hemorrhage [150].
tPA	Genetic (tPA knockout)	Hypoxia	Had no effect on pulmonary hypertension or pulmonary vascular remodeling [75].
uPA	Therapeutic (anti-uPA antibodies)	Endotoxin	Reduced inflammation and edema [159]
uPA	Genetic (uPA knockout)	Bleomycin	Reduced alveolar macrophages [150].
uPA	Genetic (uPA knockout)	Endotoxin	Reduced lung edema, pulmonary neutrophil accumulation, and pro-inflammatory cytokine levels [160].
uPA	Genetic (uPA knockout)	Hypoxia	Reduced pulmonary hypertension and pulmonary vascular remodeling [75].
uPAR	Genetic (uPAR knockout)	Bleomycin	Reduced alveolar macrophages [150].

Table 1. A summary of findings from studies that have targeted ECM proteases and receptors in experimental models of lung and vascular disease. The animal models used were: (i) Acute pulmonary thromboembolism (APT), Which is induced with autologous blood clots; (ii) Allergen, A model of allergic airway inflammation which has characteristics of asthma pathology. In the model, antigen sensitized animals are repeatedly challenged with aerosolized antigen (e.g., ovalbumin, house dust mite extract); (iii) Balloon angioplasty, A vascular intervention procedure which causes restenosis; (iv) Bleomycin, Intranasal administration of bleomycin induces acute lung injury and subsequent pulmonary inflammation and fibrosis. This model also features extensive remodeling in the upper airways involving ASM cell hypertrophy and hyperplasia; (v) Endotoxin, Intranasal administration of endotoxin (i.e., lipopolysaccharide or LPS) also induces acute lung injury and pulmonary inflammation; and (vi) Hypoxia, A model of pulmonary arterial hypertension (PAH).

7.4. Coagulants

Reducing the accumulation of extravascular fibrin in damaged tissue would be expected to reduce tissue remodeling in disease. Anti-coagulants are already used in the treatment of vascular disease to reduce thrombosis by disrupting hemostasis. Whilst clinical asthma trials with nebulized heparin have provided mixed results [161], systemic administration of *fondaparinux*, a selective inhibitor of FXa, attenuates AWR in experimental asthma [149] (table 1). The success of anticoagulant therapy in treating asthma and other respiratory diseases may depend on the anticoagulant, which differ in potency for coagulation-dependent and -independent targets. Selective small molecule FXa inhibitors (*e.g., apixaban*) are used as treatment for venous thromboembolism and stroke prevention in patients with atrial fibrillation [162], but still have anti-coagulant related bleeding risks, which can be potentially fatal.

The coagulants FXa and thrombin may also contribute to tissue remodeling via their activation of PAR-1 on stromal cells including ASM and VSM cells. PAR-1 antagonists reduce AWR in a murine model of chronic allergic airway inflammation [98] and pulmonary inflammation and fibrosis in mice following bleomycin-induced lung injury [102, 103] (table 1). Similarly PAR-1 antagonists reduce restenosis [158] and intimal thickening [157] in the vascular wall following balloon injury *in vivo* (table 1). Orally administered *Vorapaxar*, a PAR-1 inhibitor, is used as anti-platelet therapy for prevention of secondary thrombotic cardiovascular events in patients with a prior myocardial infarction [163]. However, it is because of the ability to suppress thrombin-stimulated platelet aggregation, thus disrupting hemostasis, that PAR-1 inhibitors such as *Vorapaxar* are associated with potentially fatal bleeding complications [164]. For PAR-1 inhibitors to be used as treatment for tissue remodeling disease, they will need to be modified to selectively target the extravascular cell-mediated actions of coagulant proteases without disrupting hemostasis. Taking advantage of integrin co-receptors and adaptors that differentiate PAR-1 responses in platelet and endothelial cells as compared to inflammatory and extravascular structural cells (*e.g.* smooth muscle cells) may be one approach to achieve selectively in regards to PAR-1-drug targeting.

7.5. Fibrinolytic agents

Potential therapies that reduce fibrin by using fibrinolytic agents, have been considered in the treatment of lung disease for some time [165]. Thrombolytic therapy using tPA-mimetics are already used for stroke and myocardial infarction, despite a higher risk of bleeding complications [166]. A potential strategy to treat asthma is to augment airspace fibrinolysis. Tiplaxtinin, a small molecule inhibitor of PAI-1 [153], or inhaled tPA [52], attenuate AWR or reactivity in allergen-challenged mice (table 1). In animal studies of vascular injury and disease, PAI-1 inhibitors [151] or PAI-1 gene deletion [156] reduced neointima formation.

7.6. uPA and annexin A2

Both uPA and annexin A2 may be potential novel drug targets in the treatment of chronic respiratory disease [167]. Both uPA and annexin A2 gene-deletion reduces pulmonary inflammation in various murine models [84, 150, 160], and uPA antibodies reduce inflammation and edema in a mouse model of acute lung injury [159] (table 1). However, further preclinical characterization of these inhibitors as therapy for tissue remodeling diseases is required. The roles of uPA and annexin A2 in cancer are well established, and their targeting by either pharmacological or antibody-based therapies have been shown to reduce tumour growth and/or metastasis in a number of pre-clinical cancer models [86-90]. Furthermore, clinical trials for cancer have shown uPA inhibitors are well tolerated in humans and have generated promising results [87].

7.7. Cross-linking enzymes

As the expression of lysyl oxidases are dysregulated in PAH, modulation of lung matrix cross-linking may limit pulmonary vascular remodeling associated with PAH. In support, β-

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aminopropionitrile, an inhibitor of lysyl oxidase, attenuates the effect of hypoxia on vascular remodeling in experimental PAH [9] (table 1).

7.8. Actin-cytoskeleton

Cytoskeletal changes in smooth muscle cells occur in association with increases in stiffness (*i.e.*, elastic modulus), size (hypertrophy) and migration [168-170]. A strategy that softens cells by regulating the formation or depolymerisation of the actin-cytoskeleton may be beneficial in the treatment of vascular and lung obstructive diseases. However, there are yet no actin-targeting drugs used clinically [171]. Cytochalasins and latrunculins are cytotoxic as they non-selectively disrupt actin microfilaments. However, there are different populations of actin filaments, spatially organised into distinct cellular compartments with unique functions. Their organization is regulated by associated-actin binding proteins. Therapeutically, actin filament populations need to be targeted specifically, by modulating the binding of specific actin-binding proteins. One approach maybe to target the assembly of FA complexes. Phosphorylation of FAK leads to disassembly of FA complexes-actin filaments [172]. ECM proteins (*e.g.*, collagen and fibronectin) that engage integrins regulate FAK activity and actin-cytoskeleton organization [173]. FAK regulates actin-cytoskeleton organisation by modulating the association of actin with actin-capping proteins such as gelsolin [174]. Complexes between FAK, gelsolin and the actin-cytoskeleton also regulate gene expression of contractile proteins (*e.g.*, α-smooth muscle actin, calponin and transgelin [SM22]) via the actin-filament-dependent transcriptional co-activator, MRTF-A (myocardin-related transcription factor-A) [174].

8. Conclusion

Understanding the molecular mechanisms by which the ECM regulates smooth muscle cell function in disease remains a key challenge to developing effective therapeutics for managing tissue remodeling diseases such as obstructive lung and vascular diseases. Insight into the biology of the smooth muscle cell in these diseases has increased rapidly in the recent years, leading to the concept that successful strategies for managing tissue remodeling may include restoring smooth muscle phenotype. Such approaches could possibly involve modulating the ECM of the smooth muscle microenvironment, or the factors which are involved in the transmission of the biochemical and biomechanical properties of the ECM. Proteases of the coagulation and plasminogen activation systems are of particular interest as therapeutic targets, as they not only have roles in forming and modifying ECM, but also can directly stimulate changes in smooth muscle cell function and phenotype. The challenge is to target these proteases without disrupting their roles in hemostasis, in order to avoid bleeding complications.

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Chapter 14

Novel Therapeutic Approaches for Skeletal Muscle Dystrophies

Emanuele Berardi and Maurilio Sampaolesi

Additional information is available at the end of the chapter

Abstract

Muscular dystrophies (MDs) are inherited diseases that affect skeletal and cardiac muscle tissues. Cases range from mild to very severe, resulting in respiratory or cardiac failures. No cures are available for MDs and corticosteroid treatments, mainly deflazacort and prednisolone, only help to control the inflammatory process and slightly delay the progression of the disease. This is due to the beneficial effect on pulmonary function and scoliosis. Walkers and wheelchairs are used to strengthen patients' independence and walking ability. When respiratory and/or cardiac muscles become weak, mechanical ventilation is mandatory. In addition, hypertension, cataracts, excessive weight gain and vertebral fracture are often serious side effects of deflazacort and prednisolone treatments.

This chapter deals with the advanced therapies used to treat muscle degenerations, ranging from pharmacological to gene and/or cell treatments. We review previous trials that use cell delivery protocols in mice and patients. Here, donor satellite cells and myogenic progenitors are isolated from the bone marrow. We then proceed to describe the recently identified stem/progenitor cells in relation to their ability to exist within a dystrophic muscle and to differentiate into skeletal muscle cells. In this perspective, different known features of various stem cells are compared including mesoangioblasts and mesoderm-derived stem cells, which are associated with the pericyte compartment.

This chapter also provides an outline of the latest techniques used for the isolation/generation and characterization of pluripotent and adult stem cells. We focus on their myogenic differentiation potential and the different strategies used for genetic manipulation including TALEN and CRISPR genome editing. We also explore the use of microRNAs as biological markers or as possible therapeutic targets to improve myogenic commitment of pluripotent and adult stem cells. Finally, based on the rapid advance in stem cell technology, we discuss a prediction of clinical translation for novel cell therapy protocols.

Keywords: Skeletal muscle regeneration, gene and cell therapy, TALEN and CRISPR genome editing, microRNAs

1. Introduction

Muscular dystrophies (MDs) are genetic diseases caused by the continuous degeneration/regeneration cycles of skeletal muscle tissue. Mutations in genes encoding for proteins, either at the plasma membrane or within internal membrane, are responsible for MDs. During contractions, the affected muscle fibres degenerate and the molecular mechanisms are not yet fully understood. Fibre loss is compensated by the regeneration of new fibres, mainly sustained by satellite cells. These are localized underneath the basal lamina of muscle fibres [1]. Damaged dystrophic muscles engage in a remodelling process to generate novel fibres and to produce abundant extracellular matrix (ECM). ECM is necessary for adequate tissue repair. During periods of degeneration/regeneration, myofibroblasts accumulate in dystrophic muscles and are responsible for large amounts of extracellular matrix proteins, generating fibrosis. Additionally, at the final stage, satellite cells become exhausted and are not able to generate new fibres. Cardiac muscle is less efficient in regeneration, compared to skeletal muscle and scar tissues in replacing damaged cardiomyocytes after injuries [2]. However, several research groups have demonstrated the presence of stem/progenitor cells that are able to differentiate into cardiac tissues [3-6], as well as skeletal muscle lineages [7-12]. This paper deals with novel therapeutic approaches for skeletal muscle dystrophies and explores pharmacological treatments. It also provides more recent gene and cell therapeutic protocols. Different sources of myogenic stem cells are discussed, highlighting their advantages and disadvantages, as well as underlining controversies in literature. Finally, we discuss autologous and heterologous cell therapy, considering the viral and non-viral technologies for *ex vivo* cell therapy in the treatment of muscular dystrophies.

2. Epidemiology, diagnosis and clinical management of muscular dystrophies

Muscular dystrophy was described for the first time in 1860s by the neurologist Guillaume-Benjamin-Amand Duchenne (de Boulogne). This followed a study of 13 boys who were affected by the most common type of muscular dystrophy, now carrying his name. Worldwide, Duchenne muscular dystrophy (DMD) affects 1/3,500 born males. Other isoforms of muscular dystrophies include Becker muscular dystrophy (less severe than DMD, with an incidence 3-6: 100,000 male births), limb-girdle muscular dystrophy (mainly affecting hip and shoulder muscles, occurring between 10 and 30 years of age, with an estimated range of incidence between 0.5-4:100,000), congenital muscular dystrophy (present at birth and not affecting the life span, incidence 1:21,500), facioscapulohumeral muscular dystrophy (inherited form of muscular dystrophy, initially affecting skeletal muscles of the face, scapula and upper arms, starting from teenage years. Incidence 4-12:100,000), myotonic dystrophy (an inherited form of muscular dystrophy, normally occurring in patients of any age. European incidence: 3-15:100,000) and finally, oculopharyngeal muscular dystrophy (a type of muscular dystrophy occurring in the middle age and, at the beginning, causes drooping of eyelids, dysphagia and

weakness of the extraocular muscles. This muscular dystrophy has been frequently observed in French Canadian patients, with a prevalence 1:1000). Distal muscular dystrophy is characterized by the onset observed in hands, feet lower arms or lower legs. Its incidence is unknown. Emery–Dreifuss muscular dystrophy affects muscles of the upper arms and lower legs. It causes multiple contractures, as well as heart problems (incidence: 1 for every 100,000).

Muscular dystrophies are typically diagnosed by physical exams, family medical history and tests. These may include muscle biopsies for the histological detection of dystrophin expression and electromyography tests to analyse the electrical activity of muscles at rest and during contraction. Furthermore, nerve conduction tests are conducted to detect possible injuries within the peripheral nervous system and genetic tests - mainly DNA analysis - reveal the presence of different mutated isoforms of dystrophin. Moreover, blood enzyme tests are carried out to detect the presence of creatine kinase, a known marker of fibre muscle damage.

Within the group of muscular diseases affecting the musculoskeletal system, muscular dystrophies represents a serious problem for human health, especially for its clinical management. Muscular dystrophies are characterized by a progressive weakness due to unrestrainable muscle degeneration. Since there are currently no real cures, occupational therapy represents the main tool adopted to ameliorate the patient's quality of life. This therapeutic line aims to assist dystrophic patients with MDs through the engagement of daily activities such as self-care, self-feeding and physical training. Specific instruments have been developed to help patients in their route along the disease including scooters/wheelchairs and some computer interface devices. Occupation therapy also aims to make changes in both the patient's occupational and home environments so as to improve the functionality of the inhabited places. Furthermore, physiological support for patients and relatives is also provided by occupational therapists. The totality of the therapeutic strategies, chosen at an individual level, represents a standard way for the clinical management of MDs in developed countries. Nevertheless, in low-income countries, socioeconomic reasons prevent the adoption of this course. Compared to the rest of the world, this causes a severe worsening of the patient's quality of life.

3. MDs and pharmacological treatments

Pharmacological treatments for MDs aim to stabilize the structural integrity of the muscle fibre membrane by counteracting chronic inflammation. Indeed, lack or genetic mutations of dystrophin causes a chronic influx of calcium into the myofibres. This is largely responsible for cell death and inflammatory response (Figure 1). Accumulation of fibrotic tissues in the replacement of damaged myofibres is another pathophysiological feature of MDs that is responsible for decreasing the contraction force and increasing fatigue. These events characterize dystrophinopathies because dystrophin plays a pivotal role in the anchoring of the dystrophin-associated protein complex, which, in normal conditions, can stabilize the structural integrity of membrane.

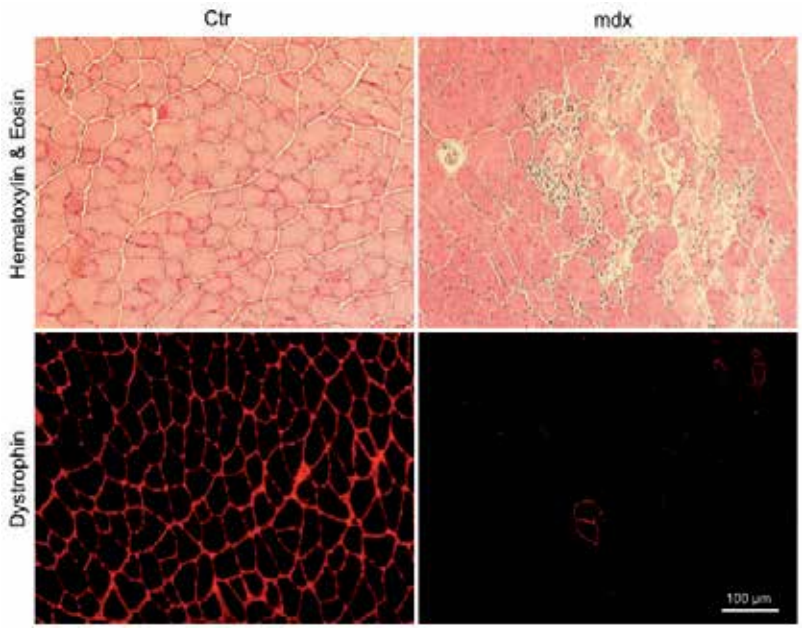

Figure 1. Histological features of muscular dystrophy. Upper panels show the histological architecture of the *tibialis anterior* muscle in a healthy control mouse (left panel) and dystrophic mouse model of human Duchenne muscular dystrophy (mdx, right panel). Haematoxylin and Eosin stain identifies the presence of a huge amount of infiltrating mononuclear cells within the *interstitum* of mdx muscle. Necrotic fibres, as well as centrally located nuclei fibres, are other hallmarks of dystrophic muscle (right panel) compared to healthy control (left panel). Lower panels show the immunofluorescence stain for dystrophin (red). Compared to the healthy control (lower left panel), muscle from mdx shows a strong reduction of dystrophin content. Only a small percentage of revertant fibres are positive for dystrophin expression (lower right panel).

Although there are no proficient cures for dystrophinopathies, several drugs have been used to delay their detrimental effects on muscle tissues, mainly through the attenuation of inflammation. In the treatment of MDs, several drugs are used for their ability to reduce circulating levels of TGF-β, known to play a crucial role in the fibrotic tissue deposition in dystrophic muscles. Among the drugs used to counteract the systemic burden of TGF-β are non-steroidal anti-inflammatory drugs (NSAIDs) such as nabumetone, ibuprofen and isosorbide dinitrate. These drugs have beneficial effects, especially in the treatment of Duchenne, Becker and Limb-Girdle muscular dystrophies [13]. Promising results were recently obtained from phase I studies in healthy volunteers. These studies revealed an optimal tolerability and safety profiles for a combined administration of isosorbide dinitrate, a nitric oxide donor and ibuprofen (NSAIDs) for the treatment of muscular dystrophies [14]. Other established pharmacological approaches aim to hamper the elevated muscle inflammation and necrosis events linked with mitochondrial dysfunction and altered metabolism. In particular, α-methylprednisolone (a synthetic glucocorticoid) stimulates the reduction of cytosolic calcium in dystrophic muscle and prevents apoptosis and/or necrosis events that normally occur during muscle degeneration in dystrophinopathies [15] (Figure 2). Another challenge in the pharmacological treatment of MDs is counteracting the high susceptibility to muscle damage

that characterizes dystrophinopathies. Indeed, among the corticosteroids drugs, prednisone and deflazacort show positive effects in reducing muscle damage and weakness, as well as in counteracting the loss of muscle contraction. Moreover, since the integrity of muscular membrane is a critical determinant of muscle degeneration during the illness progression, many efforts have been made to promote the sarcolemall stability of muscle fibres through the increase of native utrophin as a compensatory mechanism of dystrophin loss. In particular, nabumetone is a small molecule with anti-inflammatory properties, derived from its ability to inhibit cyclooxygenase. In addition, nabumetone can activate the transcription of utrophin. Aminoglycosides antibiotics, such as gentamicin and other small molecules under analysis (e.g., RTC13, RTC14 and ataluren, PTC124) aim to restore full-length dystrophin in patients with stop codon mutations. This promising therapeutic approach arises from the ability of such agents to stimulate the ribosomal read-through. This leads to the suppression of non-sense mutations in Duchenne/Becker muscular dystrophy [13].

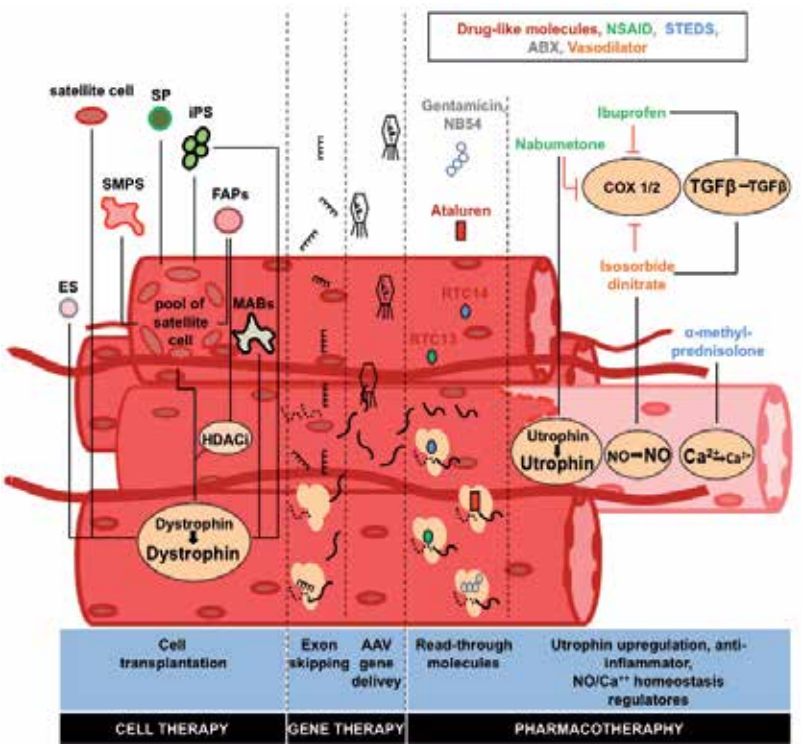

Figure 2. Schematic representation of the strategies adopted in the study/treatment of muscular dystrophy. Pharmaco-therapeutics approaches include drugs that can increase the expression of utrophyn, anti-inflammatory and No/Ca^{2+} homeostasis regulator drugs and small molecules that can promote the read-through of dystrophin. NSAIDs= non-steroidal anti-inflammatory drugs; STEDs= steroid drugs; ABX= antibiotics; COX 1/2= cyclooxygenase type 1 and 2. Gene therapy is based on both exon skipping and Adeno- associated virus (AAV) gene delivery strategies aiming to the increase of dystrophin content. Cell therapy investigates the myogenic potential observed in adult stem cells (such as satellite cells, FAPs, SP, MABs and iPS) and in ES cells, through the increase of both dystrophin content and the pool of resident satellite cells.

4. Inducing muscle hypertrophy as therapeutic strategy for MDs

In adult muscles, satellite cells are quiescence and blocked in the G0 phase of the cell cycle. After activation, these satellite cells move outside of the basal lamina and express Pax7 and MyoD. These cells are now known as myoblasts. They extensively divide and fuse to differentiate and form multinucleated myofibres. During this late differentiation process, myoblasts down-regulate Pax7 and express myogenin. MyoD and Myf5 factors are involved in the early stages of transitioning from undifferentiated myogenic precursors to myoblasts [16]. On the contrary, myogenin and MRF4 regulate the transition from myoblast to mature fibres.

In adulthood, a skeletal muscle can enlarge or reduce its own mass through a complex interplay in which several molecules are involved. During skeletal muscle hypertrophy, the myofibrils increase in number and size to increase muscular strength (Figure 3). While sarcoplasmic hypertrophy is a characteristic of body-builders' muscles, myofibrillar hypertrophy is typically found in weight lifters. In contrast, muscle atrophy, also known as muscle wasting, is the result of muscle protein loss with a reduction in fibres. At a molecular level, signals control both muscle growth and atrophy. These are finely interconnected and the biochemical pathways can be altered by increasing or decreasing specific growth factors [17]. Insulin Growth Factor-1 (IGF-1) is the most reliable muscle growth-promoting factor. IGF-1 is largely produced in the liver. However, skeletal muscle also contributes to the production of two distinct IGF-1 isoforms. Different isoforms of IGF-1 exist due to different RNA spliced variants. Human skeletal muscle has been found to express at least two isoforms [18]. These are IGF-1Ea, which is the liver type or systemic form and IGF-1Ec, also called Mecano Growth Factor (MGF). MGF is an autocrine/paracrine form that is particularly interesting as it is expressed in response to mechanical stimuli and cellular damage.

Increased muscle loading leads to augmented expression of the IGF-1 encoding gene, both in humans and animal models. Several authors have indicated that IGF1Ea induces proliferation and differentiation of satellite cells and muscle hypertrophy [19, 20]. Transgenic mice overexpressing IGF-1Ea display, indeed, skeletal muscle hypertrophy associated with increased muscle strength [21].

IGF-1Ea and other isoforms act via a tyrosin-kinase receptor IGFR-1, enabling AKT1 to be activated by the generation of phosphatidylinositol-3,4,5-triphosphates (PIP3). PIP3 Kinase (PI3Kinase) and the phosphatases PTEN and SHIP2 regulate the formation of PIP3, which recruits AKT1 on the plasma membrane. They can activate the mammalian target of rapamycin (mTOR) or FK506-binding protein 12-rapamycin-associated protein 1 AKT1 (PKBa). Furthermore, mTOR generates two complexes: the Rapamycin-sensitive Ternary complex mTORC1 and Rapamycin-insensitive mTORC2. These complexes control pathways that determine the mass/size (mTORC1) and the shape (mTORC2) of the cells. The general activation of mTOR results in the phosphorylation of several downstream targets in a signalling cascade. In this view, AKT1 is responsible for modulating the muscle growth and protein up-regulation signals in skeletal muscle tissue.

Met-Activating Genetically-Improved Chimeric Factor 1 (Magic-F1) is a recombinant protein that also triggers AKT pathway [22]. Magic-F1 is constructed as a bivalent ligand from HGF,

Figure 3. Muscle remodelling is a complex process and is among many key factors that modulate both satellite cells activation and protein synthesis. As a final result, fibre size and nuclear content can be increased (hypertrophy, right panel). In the cartoon, molecules can induce muscle growth and the key players in the hypertrophy-signalling pathway are indicated.

containing the signal peptide, the N-domain and the first two kringle-domains K1 and K2 of HGF. However, the kringles repeat in tandem and are joined by a linker. Magic-F1 binds c-Met and the HGF receptor activates Akt but not the Erk signalling pathway. Therefore, this recombinant molecule, which enhances the myogenic differentiation process, is a safe molecule. It does not have the potential risk of stimulating uncontrolled proliferation observed in several growth factors including IGF1. Previous studies in transgenic mice expressing Magi-F1 under muscle specific promoter showed that the recombinant protein cooperates with Pax3 signal pathway in early embryogenesis. This generates a more active skeletal muscle progenitors in early embryogenesis [23]. This results in a constitutive muscular hypertrophy in the adulthood of transgenic mice, since Magic-F1can down-regulate myostatin, a potent muscle mass regulator. Furthermore, it can directly activate MyoD, Myf-5 and several anti-apoptotic pathways.

The therapeutic potential of Magic-F1 was evaluated on α-sarcoglycan null mice (Scga-/-), an established model of limb-girdle muscular dystrophy type 2D. The Scga-/-/Magic-F1 transgenic mice showed a stronger muscle phenotype than their Scga-/- counterparts. Furthermore, the physiological benefit of muscular hypertrophy partially recovered the dystrophic phenotype [22].

Mutations occurring in the myostatin gene, also named growth and differentiation factor 8 (GDF8), are responsible for a hypertrophic phenotype. There is a great increase in muscle mass in a breed of beef cattle known as Belgium Blue [24]. Mdx mice that do not express myostatin are stronger and more muscular than their mdx counterparts [25]. Fibrosis and fatty remodelling are less evident in the diaphragm of those transgenic mice, suggesting improved muscle regeneration. In 2004, the first mutation of the myostatin gene in humans was reported and correlated with an enlargement of the skeletal muscle apparatus [26].

In summary, inducing muscular hypertrophy is relevant in clinical applications as a potential treatment of muscle diseases, including muscular dystrophy and cachexia, which cause

wasting in muscle mass and force. Insulin-like Growth Factor-1 (IGF-1), MAGIC-F1 and myostatin regulate the key steps during muscle regeneration (Figure 3). In animal models for Duchenne muscular dystrophy [22, 25, 27], these molecules have demonstrated a therapeutic value, without redressing the primary cause of the lesion and, in principle, could be adopted in patients suffering from muscular dystrophies. The delivery strategies of these molecules and potential side effects require more investigation. So far, their translational potential has been hindered in clinical trials.

5. Gene therapy and gene editing for MDs

In previous decades, significant advancements in direct gene replacement approaches in genetic muscle diseases have been achieved. Two strategies are currently being tested in the dystrophic animal model and have already entered - or are ready to enter - clinical experimentation. These are exon skipping and the expression of dystrophin variants of reduced size (Figure 2).

For the exon-skipping experiments, adeno associated viral vectors (AAV) were engineered to produce small nuclear U7 RNA targeting exons [28, 29]. These excluded the mutation of dystrophin from an in-frame transcript that is translated in a 'quasi' normal dystrophin protein. High-pressure intravenous delivery was adopted to guarantee an efficient systemic delivery. Phase I and II clinical protocol in patients was designed by scaling up AAV production for total body delivery and transient immune suppression to enable reinfusions.

As an alternative strategy, dystrophin variants of reduced size were considered, since the large size of the transcripts (14 kb) is an impediment to generate viral vectors. Indeed, a mild phenotype was observed in Becker muscular dystrophy patients, characterized by a huge deletion of dystrophin gene. This resulted in the expression of truncated, yet partially functional, dystrophin. This observation led to the idea of using truncated dystrophins for therapeutic use in mdx mice via rAAV vector-mediated gene transfer (for a detailed review see [30]). Recombinant adeno-associated viral (rAAV) vector-mediated gene transfer represents a promising approach for genetic diseases of muscles. Despite the limited DNA packaging capacity (~4.8 kb), its transduction efficiency is very impressive. Recently, Chamberlain group showed that functional dystrophin transgenes could be reconstituted *in vivo* by homologous recombination (HR), following intravascular co-delivery of two independent rAAV6 vectors [31]. Systemic delivery of dystrophin variants of reduced size has also been effective in pigs (Pichavant et al., 2010). Unfortunately, a phase 1/2 clinical trial using intramuscular injections of AAV2 into DMD patients did not result in the restoration of dystrophin expression. This is likely due to T cell immunity to dystrophin proteins. An alternative strategy is the delivery of utrophin, a dystrophin related protein normally present at neuromuscular junctions, which should not elicit an immune response. Utrophin has 3,433 amino acids, with a predicted molecular mass of 395 kDa. It is slightly smaller than dystrophin (427 kDa) and ubiquitously expressed. In addition, while dystrophin is expressed throughout the sarcolemma of skeletal muscle fibres, utrophin is restricted at the neuromuscular and myotendinous junction.

A significant inverse correlation between utrophin expression and disease severity in DMD has been observed [32]. Davies group developed a range of strategies to up-regulate utrophin for therapeutic approaches in muscular dystrophies [33]. A number of drugs were tested in a stable H2K mdx myoblast cell line. Here, a luciferase reporter gene was under control of the mouse utrophin promoter to identify an effect on utrophin expression [34]. This long screening study allowed the identification of SMT C1100, which also showed therapeutic potential in the mdx mouse [35]. Since the drug also demonstrated a synergistic effect when administered with prednisolone [35], the gold standard in treatment of DMD patients in the clinic, SMT C1100 was tested in phase I trials by BioMarin Pharmaceuticals (as BMN-195; Novato, CA, USA). Unfortunately, the plasma levels of the drug were not high enough for the trials to continue. Although there were no safety issues, new formulations are necessary.

In conclusion, the systemic delivery of AAV, plasmids and molecules to counteract muscle muscular dystrophy still face significant technical hurdles and alternative strategies are necessary.

6. Multipotent and pluripotent stem cells for the treatment of MDs

Satellite cells are quiescent mononucleated myogenic cells, located between the sarcolemma and basement membrane of terminally-differentiated muscle fibres [36]. For a long time, adult muscle was considered as a static tissue. Furthermore, due to its histological nature, it was considered formed by spatially-oriented post-mitotic multinucleated muscle fibres. Since 1961, the discovery of satellite cells by Katz and Mauro, together with vast studies about their biological role, revealed the existence of plasticity potential in adult skeletal muscle tissue. Their name derived from the distinctive location, wedged between the basal lamina and sarcolemma of myofibres, in a separate place from the fibre. With an estimated number between 1×10^{10} to 2×10^{10} satellite cells per person, these cells represent the main source of muscle progenitors within the adult skeletal muscle tissue [36]. Indeed, they are unipotent stem cells that, in the case of acute muscle damage and during muscle regeneration/degeneration, are able to re-enter the cell cycle and contribute to muscle repair by offering new myogenic progenitors capable to fuse and form new fibres. Pax7 (a paired box transcription factor) was the first identified marker required for myogenic specification of satellite cells. However, in the last decade, several surface markers were identified within the pool of satellite cells. These include CD34, M-cadherin, syndecan-3/4, c-met and the chemokine receptor CXCR4, as well as $\alpha7\beta1$-integrin (a transmembrane domain protein), Pax3, barx2, myocyte nuclear factor (MNF) (the latter three are known transcription factors) and caveolin-1 (a scaffolding protein within caveolar membrane) (Table 1). Since these markers are all expressed by satellite cells and not by post-mitotic myonuclei of fibres, their identification within the muscle microenvironment outside the fibres has been relatively easy. These markers were investigated both *in vitro* and *in vivo* for the marked regenerative ability potential of satellite cells [13]. Pre-clinical studies of DMD in the mdx mouse model, which recapitulates the pathophysiological features of human DMD such as the absence of dystrophin (Figure 1), contributed to the increase in knowledge of the therapeutic potential of satellite cells. It was

demonstrated that the satellite cells derived from a single fibre of a healthy donor and transplanted into a muscle of mdx mouse can actively contribute to the repopulation of the satellite cell pool of dystrophic muscle, as well as to the regeneration of new dystrophin expressed fibres. Several combinations of markers have been used to identify donor satellite cells and their myogenic contribution in dystrophic mice, including Pax7/CD34 [37] and CD34/integrin-α [38]. In 2004, a distinct subpopulation of satellite cells, named skeletal myogenic precursors (SMPS), positive for Cxcr4 and β1 integrin and negative for CD45 and Sca1 (a known maker of hematopoietic progenitor cells), were discovered, highlighting the heterogenic nature of satellite cells [39] (Table 1). SMPS showed a robust regenerating ability, as well as a strong capability of repopulating the satellite cell poll when injected in the muscles of mdx mice. Additionally, their potential role in counteracting muscle wasting was confirmed by a strong improvement of muscular contractile properties such as contractile force, observed in treated dystrophic muscles compared to the healthy controls [7].

Cell type	Localizaton	Surface markers expression (+ or -)	Transcription factors	Cytoplasmatic markers
satellite cells	between the sarcolemma and basal lamina of muscle fiber	$CD34^+$, M-cadherin$^+$, syndecan-3/4$^+$, c-met$^+$, $Cxcr4^+$, $\alpha7\beta1$-integrin$^+$	Pax7, Pax3, Barx2, myocyte nuclear factor (MNF)	caveolin-1
skeletal myogenic precursors (SMPS)		$Cxcr4^+$, $\beta1$-integrin$^+$, $CD45^-$, Sca-1^-		
muscle side population (SP)	Interstitial	Sca1+/Cd45+/Abcg2+ Syndecan4+	Pax7	
fibro-adipogenic progenitors (FAPs)	Interstitial	$CD45^-/CD31^-$ $CD34^+$, Sca-1^+ PDGFRα^+		
mesoangioblasts (MABs)	Interstitial	SMA$^+$, PDGFR$^+$, PDGFRβ^+, Ng2$^+$		alkaline phosphatase

Table 1. Cell markers identifying adult stem cells currently used in cell therapy.

Although satellite cells are the main source of myogenic renewal in adult skeletal muscle tissue, in recent years, other adult stem cells have been discovered. A subpopulation of muscle precursors associated with skeletal muscle tissue, named muscle side population (SP) cells, is a rare source of multipotent stem cells that contribute to muscle regeneration, upon transplantation. SP cells are characterized by a complete permeability to the Hoechst 33342 dyes, derived from their high expression level of Abcg2 transporter (Table 1). The myogenic potential ability of SP cells has been tested by *in vitro* co-culture with myoblast cells. In these conditions, SP cells were able to fuse with myoblast to form mature myotubes. At the same time, *in vivo* experiments confirmed their involvement in myogenic differentiation (see below). Interestingly, as observed in satellite cells, SP cells also show certain heterogeneity inside their population. Analysis of the expression pattern of specific markers revealed that 80% of SPs are positive for the vascular endothelial marker CD31, while 2-10% of total muscle SPs are blood-

derived and positive for the immune marker CD45. In the case of muscle damage and during the followed early phase of regeneration, a fraction of SP cells have been identified as highly positive for CD45, Abcg2 and CD31 (the latter two suggested a possible intervention in modulation of both vascularization and immune system) [40]. Furthermore, a third fraction of SP cells, representing 5% of total population, were recently identified. These cells are characterized by the absence of both CD45 and CD31 expression, while they may express Pax7, Sca1 and Syndecan4 [40-42] (Table 1). Interestingly, although in physiological conditions this subpopulation of muscle resident SP cells represents the smallest fraction within the rest of population, if engrafted in a regenerating muscle (pre-treated with cardiotoxin to induce acute tissue damage), they showed the highest myogenic differentiation potential [13].

In 2010, a new population of muscle interstitial stem cells were identified. These are characterized by their ability to undertake both fibrogenic and adipogenic differentiation [43, 44]. These mesenchymal fibro-adipogenic precursors (FAPs) show an intriguingly functional crosstalk with satellite cells. The nature of this relationship is mutually exclusive within the homeostatic processes of skeletal muscle and, indeed, the presence of FAPs during muscle regeneration enhances the myogenic potential of satellite cells. At the same time, the presence of satellite cells derived from new myofibres inhibits the adipogenic differentiation of FAPs [45]. FAPs were also investigated in the pre-clinical models of MDs. In particular, the treatment of FAPs with histone deacetylase inhibitors (HDACi) increased their *in vitro* myogenic differentiation (Figure 2), while, if transplanted in advanced dystrophic muscle (old mdx mouse), FAP cells can enhance the regenerative potential of resident satellite cells [46].

Another important source of muscle progenitors associated with vasculature, named mesoangioblasts (MABs), were investigated for their therapeutic potential in the treatment of muscular dystrophies (Figure 2). Mesoangioblasts were originally isolated from embryonic aorta of a quail and mouse and, later on, in the adult skeletal muscle of a mouse, dog and human. MABs are multipotent stem cells positive for CD34, SMA, Pdgfrα, Pdgfrβ, Ng2, AP and, accordingly with such expression pattern, can undertake several differentiation fates (Table 1). These include myogenic, osteogenic, chondrogenic and adipogenic. Studies of *Scga*-null mice (a limb-girdle muscular dystrophy mouse model) and GRMD (golden retriever muscular dystrophy) dogs showed that intra-vein injection of MABs can restore both the histological structure and function of large areas of dystrophic muscles [11, 12]. These promising results provided important knowledge regarding the therapeutic use of MABs for the treatment of muscular dystrophies. Recently, these efforts were finalized in a phase I/II clinical trial of donor mesoangioblasts transplantation from HLA-identical donors in five DMD patients, nearing completion (EudraCT Number: 2011- 000176-33). This clinical trial will provide useful information regarding the safety for the systemic delivery of stem cells in dystrophic patients, as well as the assessment of the ability of MABs to increase the dystrophin expression (Figure 2).

The scaling up of the research on pluripotent stem cells of the last decades has offered the interesting prospective to adopt this new precious source of stem cell in the treatment of MDs.

Embryonic stem (ES) cells are pluripotent stem cells, originally isolated from the inner cell mass of the blastocyst in a mouse in 1981 and from human in 1998. Their efficient pluripotency arises from the ability of ES cells to differentiate in all three germ layers - meso-

derm, ectoderm and endoderm. It was demonstrated in the early 1990s that, if cultured *in vitro*, murine ES cells can develop aggregates of cells (embryoid bodies) and differentiate in skeletal muscle cells expressing myogenic markers in the same muscle-specific determination genes order observed during embrional development: myf5, myogenin, myoD and myf6 [47]. Later on, both *in vitro* and *in vivo* studies confirmed their myogenic differentiation potential [13]. Nonetheless, the possibility of a therapeutic adoption of ES cells met the criticisms of both civil and scientific communities (see below). In 2006, Yamanaka published a revolutionary, paradigm-shift study. For the first time, a fate conversion of somatic cells (fibroblasts) into pluripotency was demonstrated [48]. As a result of this study, Yamanaka won the Nobel Prize in 2012 and began a new era for pluripotent stem cells-based therapeutic approach of chronic illness. So far, the myogenic potential of the induced pluripotent stem (iPS) cells, either from mouse or human origin, have been provided to counteract muscle degeneration in MDs [13] (Figure 2). In particular, *in vitro* and *in vivo* analyses showed that myogenic precursors generated from iPS cells could produce chimeric myotubes if co-cultured with C2C12 myogenic cell line. Furthermore, if transplanted in dystrophic muscles, their contractile properties could also be improved [49].

7. Muscle progenitor cell transplantation: Causes of failure and new perspectives

So far, in pre-clinical models, the exploration of cell-based therapy for muscle degeneration in MDs showed promising results. However, both technical and ethical issues are still the main determinants that hinder this therapeutic approach in clinics. The low abundance of satellite cells in human muscle, their heterogeneity and the reduced myogenic potential when expanded *in vitro* are the reasons why this source of stem cell is not ideal for the treatment of muscular dystrophies. Previously, satellite cells showed a general limited cell migration from the transplantation site and were not able to cross the endothelial barrier when injected systemically [12]. To overcome this important issue, MABs were proposed as an alternative source of cells. This is due to their ability to cross the vessel walls after intra-arterial delivery in dystrophic mice [11, 12]. Nevertheless, a large number of cell deaths can still occur if the immune response occurs within a few hours of the treatment. Thus, immune suppression is needed in the heterologous transplantation, as planned in the ongoing phase I/II clinical trial where Duchenne patients were transplanted intra-arterially with HLA-identical allogeneic MABs (EudraCTno. 2011-000176-33). This clinical trial faced three problems: the age of enrolled patients (frequently in advanced stage of diseases), the low dose of cells transplanted (from 1/5 to 1/10 of that administered to the GRMD dogs) and the intra femoral arteries delivery (limiting the treatment only for the muscle located downstream of femoral artery). For the treatment of muscular dystrophies, pluripotent stem cells have been recently explored. In such pre-clinical studies, the major problem encountered was controlling their myogenic differentiation and avoiding tumour formation. In addition, the therapeutic use of ES cells has been strongly contested within the scientific community and from public opinion. This is because

of ethical concerns (due to the embryonic origin of ES cells) and it does not seem that the employment of iPS cells will help this.

In conclusion, many scientific issues can be solved by further investigations in the cell biology of myogenic stem cells. With this in mind, further studies on mechanisms regulating skeletal muscle regeneration in basic and applied research are needed in order to solve several practical problems, as described above.

8. New frontiers for the treatment of MDs: Exosomes, MicroRNAs and gene editing

MicroRNAs (miRs) are non-coding RNA transcripts, ~22 nucleotides long that promote mRNA degradation by annealing to complementary sequences in the 3'untranslated regions (UTR) of specific target mRNA. Furthermore, miRs can target several transcripts and system individual mRNAs can be targeted by multiple miRs.

The biogenesis of miRNAs starts with the generation of pri-miRs by RNA polymerase II. These pri-miRs are transformed in pre-miRs by the microprocessor complex. They then transport them in the cytosol, where Dicer cleaves pre-miRs in ~22nt-long double-stranded molecules [50]. The guide strand responsible for the recognition of target mRNAs is loaded on the RNA-induced silencing complex (RISC), which contains multiple proteins including a ribonuclease enzyme.

Several biological processes, including muscle growth and differentiation, are mediated by a collection of specific miRs. These miRs can be released from the cells in the surrounding areas or in the circulation and circulating miRs (circ-miRs) appear resistant to harsh conditions [51]. Circ-miRs are protected by carriers, making them stable and valuable biological markers. Among the different carriers identified, exosomes are small vesicles (50-100nm diameter) that act as important regulators of long-range miR shuttling [52]. After the unknown processes of maturation, exosomes are released from the plasma membrane and are identified by specific markers, as Hsp-60/70 in the lumen and CD9/63/81 and tissue-specific membrane proteins on the surface [53]. Despite the lack of detail concerning receptors and intracellular processing generate debate and controversies, it is largely accepted that pre- or mature miRs are delivered to other cells, eliciting their regulation in target non-miR-originating cells.

The importance of miRNAs in the muscle development was established in a study involving conditional transgenic mice lacking Dicer in myogenic progenitors. This study resulted in aberrant muscle differentiation, accompanied by hyperplasia [54].

Furthermore, miR-206 is the most abundant miRNA in adult vertebrate skeletal muscle and promotes muscle skeletal muscle development and differentiation [55].

Interestingly, a mutation in myostatin gene that causes a dramatic muscle increase in textil sheep creates a target site for miR-206 and miRNA1. In these sheep, myostatin down-regulation determines a phenocopy of the double muscling Belgiun Blue cattle previously described (see

Myostatin section). Our knowledge of miRNA biology is still in its infancy and future investigation needs to be carried out in order to clarify the molecular mechanism and the precise involvement of these miRNAs in muscle development and regeneration.

Recently, several groups tested gene editing to correct point mutation using TALEN and CRISPR genome editing. TALENs are endonucleases that possess two domains: a TAL effector DNA binding domain and a DNA cleavage domain. Left and right TALENs can induce a double strand break (DSB) in the DNA that allows homologous recombination of the target DNA.

Also, CRISPR (clustered regularly interspaced short palindromic repeats) is a RNA-guided gene-editing system that can introduce a double strand break at any desired location by delivering the Cas9 protein and appropriate guide RNAs. Olson group used CRISPR/Cas9-mediated genome editing to correct the dystrophin gene mutation in the germ line of mdx mice [56]. This procedure produced genetically mosaic animals, containing 2-100% correction of the dystrophin gene. In principle, this technology could facilitate the genome editing of post-natal somatic cells, avoiding the use of viral vectors. Li et al. recently performed three correction methods (exon skipping, frame-shifting and exon knockin) in DMD-patient-derived iPSCs to restore the dystrophin protein. In their study, exon knockin was the most effective approach and identified clones with a minimal mutation load. We further investigated the genomic integrity by karyotyping, copy number variation array and exome sequencing to identify. TALEN and CRISPR-Cas9 corrected iPSCs were able to differentiate into skeletal muscle cells and express full-length dystrophin protein [57]. This is innovative technology that will be further investigated. However, no field trials have been planned.

9. Concluding remarks

To date, many efforts have been made to increase the understanding of genetic and molecular mechanisms of different types of muscular dystrophy. Historically, the flux of knowledge achieved from patients to laboratories of research, or from bed to bench, allowed the design, test, production and administration of new molecules that only perform a partial restoration of dystrophin expression. Nonetheless, the use of common drugs, such as non-steroidal anti-inflammatory drugs, corticosteroids and aminoglycosides antibiotics administered alone or combined with new drugs, showed poor beneficial effects regarding the restoration of dystrophin or the up-regulation of utrophin as compensatory mechanisms.

Thus, despite the limited results achieved by the pharmacological approaches tested so far, pharmacotherapy is still considered to be a useful tool in delaying the process of muscular degeneration and palliate the symptoms of the late stage of diseases.

An alternative approach for the treatment of dystrophies derives from the specific strategies adopted in gene therapy. Adeno-associated virus and lentiviral vector technologies have been studied in pre-clinical models to mediate the delivery of micro-dystrophin or mini-utrophin. Furthermore, an exon skipping strategy was proposed to restore the endogenous expression of dystrophin. Unfortunately, this also produced poor results in terms of dystrophin restoration and safety.

However, in recent decades, the stem cell therapy for muscular dystrophies has represented a new field of interest. In particular, it has sparked an increase in the understanding of biology in both multipotent (*i.e.* SP, MABs and FAPs) and pluripotent stem cells (*i.e.* ES and iPS cells), leading to the discovery and identification of new muscle progenitor cells. The exploitation of their myogenic potential has been investigated in several animal models including the mouse and dog. Extraordinary results were obtained in terms of dystrophin expression, decrease in inflammatory burden and increase in muscular function. More studies are likely to be conducted on myogenic derivatives from pluripotent stem cells and, possibly, in combination with miRNA and gene editing (CRISPR and TALEN) technologies for the treatment of muscle diseases. However, stem cell-based protocols still rely on adult stem cells. Many practical issues negatively interfere with their potential use in clinics. Low cell motility after transplantation, as well as the high immune rejection observed in pre-clinical models, are the main problems for obtaining a systemic treatment for cell-based therapy of dystrophies. A combined approach between pharmacotherapy and cell-based therapy can increase the beneficial effects of cell transplantation and give hope for the treatment of muscle degenerations. This could be achieved by increasing the myogenic differentiation potential of both multipotent and pluripotent stem cells by exosome, as well as miRNAs and gene editing technologies associated with pharmacological anti-inflammatory effects.

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Section 7

Vascular Smooth Muscle Cells

Chapter 15

Use of Biomaterials and Biomolecules for the Prevention of Restenosis

Nelisa Türkoğlu Laçin, Kadriye Kızılbey and Banu Mansuroğlu

Additional information is available at the end of the chapter

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Abstract

Coronary balloon angioplasty and coronary stenting are the procedures used in healing coronary artery disease. However, injury of arteries during angioplasty and stenting causes cell stimulations in tissue. Cell movement and thrombosis lead to re-narrowing of widened vessel called restenosis. Several new types of carriers and technology have been developed to suppress and/or prevent restenosis via prevention of migration/proliferation of smooth muscle cells (SMCs). The conventional approaches are not fully effective for inhibiting restenosis. In order to eliminate such problems, stent-based delivery methods are developed to replace traditional vascular approaches. A series of materials have been improved for controlled delivery/release of genes, miRNAs, peptide structures, siRNAs, miRNAs, and antisense molecules to the target tissue. Agents to be delivered are either attached to the materials or entrapped in polymeric structure. In particular, biodegradable polymers have held great interests in drug delivery for targeting or prolonging implantable drug release. This chapter summarizes the molecular mechanisms of in-stent restenosis, the role of SMCs and endothelial cells in restenosis, and recent researches about the polymeric materials featured in drug/gene carrier systems, nanovehicles, and stent coating materials to prevent restenosis.

Keywords: Restenosis, smooth muscle cells, biomolecules, drug delivery

1. Introduction

Atherosclerosis is a disease in which a plaque builds up inside your arteries. The plaque builds up of fat, cholesterol, calcium, and other substances found in the blood. Over time, plaque hardens and narrows your arteries [1]. Arteries are blood vessels that carry oxygen-rich blood to organs in the body, and this plaque limits the flow of oxygen-rich blood to organs and other parts of body. Thus, atherosclerosis can lead to serious problems, including heart attack, stroke, or even death. Percutaneous transluminal coronary angioplasty (PTCA) is a technique used to widen the narrowing in a coronary artery without surgery.

2. What happens during angioplasty?

At the beginning, the doctor moves a guiding catheter into the artery with the blockage. Once the guiding catheter is in right place, a guide wire is moved across the blockage site and then a balloon catheter is moved to the blockage site. The balloon is inflated for a few seconds to compress the blockage against the artery wall and then the balloon is deflated. This proceeding can be repeated for a few times. Each time the balloon is pumped, the plaque widens a little more and enables the blood to flow through. If it is needed, a stent is placed within the coronary artery to keep the vessel open. Following this, the catheter is removed and the procedure is completed, as seen in Figure 1. As a result, the narrowed artery is enlarged by PTCA. PTCA is sometimes called coronary angioplasty. Coronary angioplasty has become increasingly popular as a result of its low morbidity and mortality and reduced hospital stay in comparison with surgery. Coronary angioplasty is generally effective and safe, but restenosis is frequent, occurring in about 30-40% of cases [2]. Restenosis limits the long-term beneficial effects of PTCA and related procedures. PTCA may be defined as the initial gain in artery lumen size, and restenosis can be defined as the loss of gain. Prevention of restenosis after successful PTCA remains one of the most challenging issues in the obstructive treatment of coronary artery disease [3].

3. What causes restenosis?

As we mentioned above, stent placement is another option that is applied during angioplasty. Stent is a metallic scaffold that keeps the narrowed coronary artery portion open. Besides, as a metallic scaffold, the body may also perceive as alien. In fact, the trauma created by angioplasty and stenting in tissue is more effective on restenosis. The trauma created by angioplasty and stenting leads cell stimulations in tissue, triggers cells in that region, and causes cell proliferation, migration, and thrombosis. Finally, cell movement and thrombosis lead to the renarrowing of the vessel.

Restenosis following balloon dilation of the vascular endothelium is thought to occur in three steps:

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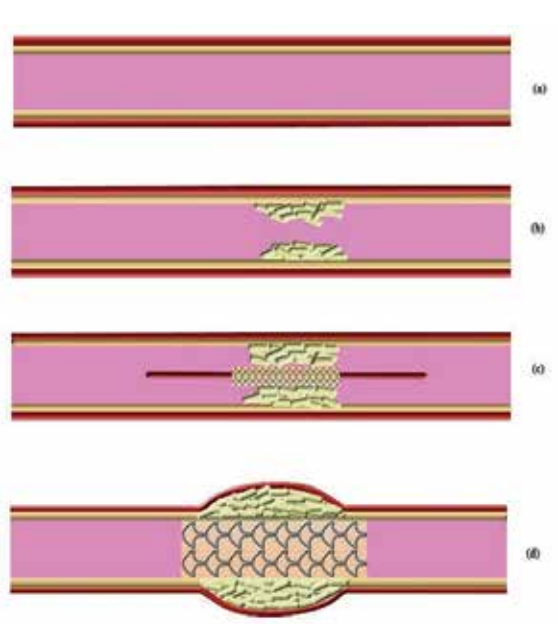

Figure 1. Balloon angioplasty and stent placement: (a) artery, (b) plaque formation, (c) balloon catheter at the blockage site, (d) balloon inflation and stent placement.

1. Elastic recoil, which tends to occur within the first 24 h of the procedure
2. Thrombus formation, which occurs within the first 2 weeks
3. Neointimal hyperplasia involving smooth muscle cell (SMC) activation and synthesis of extracellular matrix, which occurs over the course of the first 3 months [4]

Over 1.5 million percutaneous coronary revascularization procedures are performed annually worldwide, most being intracoronary stenting. Clinically significant restenosis continues to occur in ◎14% of elderly patients within the first year undergoing PTCA. Therefore, there are great efforts for the prevention of restenosis [3]. For the prevention of restenosis, infusing a drug in solution at the site of injured artery is a simple approach and is not successful because of the rapid washout of infused drug from the arterial tissue. Almost 90% of infused drug is lost within 30 min with almost complete loss occurs in less than 24 h [5].

4. Drug-Eluting Stent (DES)

Early difficulties with coronary stents included a risk of early thrombosis (clotting) resulting in occlusion of the stent. Coating stainless steel stents with some other materials such as platinum or gold were evaluated. However, this approach by itself did not eliminate the problem. Then researchers coated stent surface with biocompatible polymers; moreover, the idea of using these polymers as drug reservoir is generated. Scientists developed drug-eluting stents and used the devices themselves as a tool for delivering medication directly to the

arterial wall. The medication is entrapped in polymer layers or loaded into polymeric nanoparticles, and nanoparticles are embedded in polymeric layers. A drug-eluting stent consists of three main parts.

The first part is the metallic scaffold. The metallic scaffold may be constituted by using different types of metallic materials such as stainless steel, nitinol, cobalt, chromium, platinum, gold, magnesium alloy, etc.

The second part is the drug-eluting polymer-coated inner surface of the scaffold. The polymer-coated inner surface of the scaffold is generally used as a drug carrier, which holds and elutes the drug in a controlled manner. The polymers used for DES is generally biodegradable polymers like polylactic acid (PLA), polyglycolic acid (PLGA), and polycaprolactone (PCL). Besides, nonbiodegradable polymers like polybutylmethacrylate (PBMA), polymethylmethacrylate (PMMA), phosphorylcholine, and polyethylene terphthalate (PET) are also evaluated. The third part is the medication that is released from stent directly to arterial wall. Drugs used in DES are immunosuppressive and antiproliferative drugs like sirolimus, everolimus, zotarolimus, paclitaxel, etc., to inhibit neointimal growth, which would cause restenosis [2].

Although the drug-eluting stents significantly reduced the rate of restenosis, it did not completely eliminate restenosis, especially in complex lesions. Additionally, delayed endothelialization after drug-eluting stent implantation is considered to be the cause of late thrombosis. Therefore, scientists have suggested that gene transfer can be an option to address these problems by inhibiting proliferation of vascular smooth muscle cells (VSMCs) and by promoting endothelialization with some genes [6]. Then scientists used stents as a tool to deliver growth factors, plasmids, and antisense oligonucleotides directly to arterial wall. Several studies have been carried out for the delivery and controlled release of genes encoding antiproliferative proteins, miRNAs, peptide structures, and siRNAs to the target tissues through different polymeric materials.

5. Gene therapy for the prevention of restenosis

Gene therapy is the use of genes as a means to achieve high levels of the therapeutic gene product to treat acquired cardiovascular diseases. It can be used as a gene replacement strategy to enhance normal protein function to correct genetic defects. Also, it can be used for local gene transfer to provide a means of delivering a high concentration of therapeutic proteins at the targeted tissue. The vectors used for gene delivery can be classified into two categories, nonviral and recombinant viral vectors. Given the focus of this chapter on gene delivery approaches, we will just briefly discuss the nonviral (polycationic) vector choices. A delivery vehicle of either viral or nonviral origin is essential to carry the foreign gene into a cell. The each of the vector choices has unique advantages and disadvantages.

Viral vectors take advantage of the easy integration of the target gene into the host and long-term expression of gene. Immunogenicity is the major problem of using viral vectors in clinical studies. Attention has turned therefore to nonviral vectors, which possess many advantages

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over viruses in terms of safety and ease of use, and many clinical studies have now been performed using nonviral technology [7]. Although nonviral vectors are less efficient at introducing and maintaining foreign gene expression compared to viral vectors, they have the profound advantage of being nonpathogenic and nonimmunogenic [8]. Plasmid DNA is the simplest gene delivery vector. In cell transfection, the minimum amount of negatively charged naked plasmid can go by the cell membrane. Therefore, it is necessary to carry genetic materials to target cell by a vector, which are commonly liposomes or polycationic materials.

In nonviral gene therapy, the negatively charged DNA is conjugated with a positively charged cationic polymer. Nevertheless, the conjugate prepared has to be positively charged. By this way, pDNA is wrapped in a protective envelope to be delivered. Once the conjugate is inside the cell, pDNA expresses the targeted proteins to cure the target disease as seen in Figure 2.

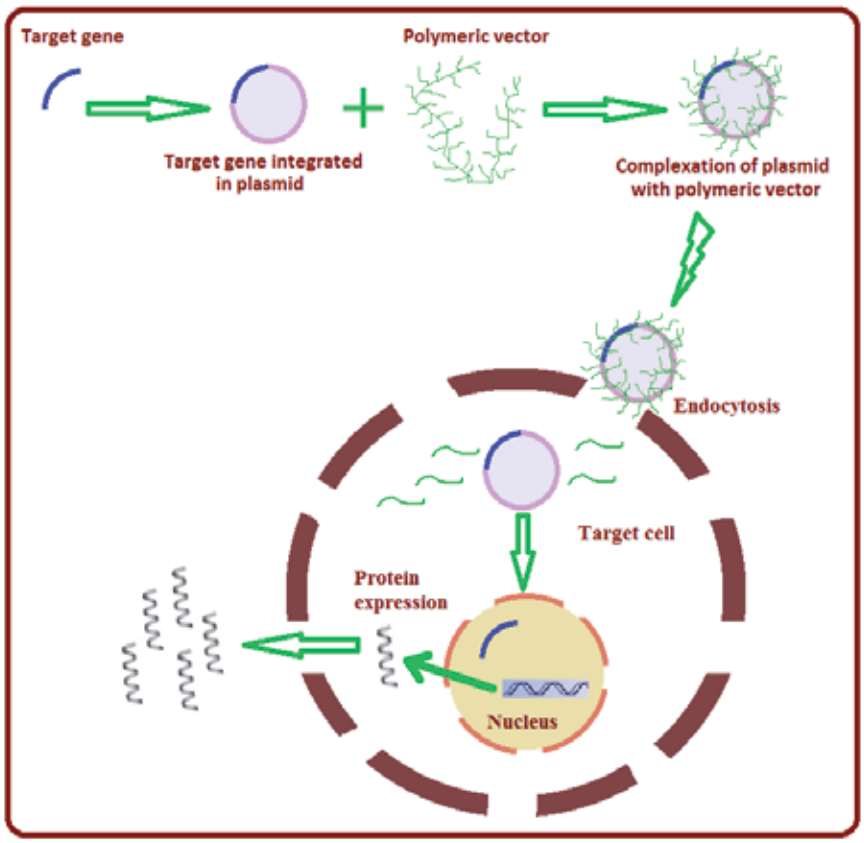

Figure 2. Polycationic gene transfer.

Human gene therapy for the prevention of restenosis is expected to provide important advances in therapeutic restenosis management. If applied in humans, it will be possible to provide long-term beneficial therapeutic effects. However, some key issues, including vector

safety and delivery mechanisms, still have to be resolved before percutaneous gene therapy can be widely applied in clinic. With the aim of inhibition of restenosis, several new types of carriers and technology have been developed, and a great number of gene therapy methods have been studied.

Vascular gene transfer is used to overexpress therapeutically important proteins and correct genetic defects. Promising therapeutic effects have been obtained in animal models of restenosis via transfer of genes, such as encoding vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), nitric-oxide synthase, thymidine kinase, tissue inhibitor of metalloproteinases, etc. [9]. In vascular gene therapy, it is required to combine a therapeutic gene or a therapeutic gene product with an appropriate vector. These complexes are delivered to target cells from a device.

6. Catheter-mediated local gene transfer

Catheter delivery system is one of the devices used in vascular gene therapy. Several balloon catheters (porous and microporous catheters, hydrogel catheters, dispatch catheters, and infiltrator catheters) have been used for gene-based delivery. Rolland et al. [10] have investigated hydrogel-coated catheters for the delivery of interested drug and gene. In recent years, Saurer et al. [11] have designed ultrathin multilayered polyelectrolyte films fabricated on embolectomy catheter balloons by alternately adsorbing layers of a hydrolytically degradable poly (β-amino ester) for the localized delivery of plasmid DNA to vascular tissue. Although catheters seem to be a simple tool for gene delivery, several factors limit its efficiency. Most of the catheters cause localized vascular injury with increased inflammatory response and neointimal proliferation. Additionally, in direct injection method and catheter-based gene delivery, transgene expression is limited within the injection site and homogenous expression is not achieved. In point of fact, in stent-based gene delivery approach, a homogenous transgene expression is achieved in comparison to catheter-based gene delivery methods.

7. Gene eluting stents

Stents represent an attractive alternative for targeted gene delivery, thanks to their permanent scaffolding structure. Polymer-coated stents are used as delivery devices for the elongated time release of small molecules. The greatest challenge with this delivery system lies in achieving a compatible relationship between the stent, coating matrix, and vessel wall. As a result of the long residence times of coatings on the stents, attention has been focused on using them as reservoirs for prolonged local drug administration. While there is much known about stent coatings for drug elution, less is known about the use of these substances for gene elution [12]. The polylactic-polyglycolic acid copolymer (PLGA) is an FDA-approved, biodegradable, and biocompatible polymer and is widely used in various drug release applications, as graft materials in tissue engineering studies.

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Although the emergence of drug-eluting stents significantly reduced the rate of restenosis after the interventions, it is not completely eliminated especially in complex lesions. Beside, delayed endothelialization after drug-eluting stent implantation is reported and considered to be the cause of late thrombosis, which is a critical complication. Gene transfer can be an option to address these problems by inhibiting VSMCs proliferation, and with some genes, promoting endothelialization [6].

Klugherz et al. [13] have developed stents coated with polylactic-polyglycolic acid copolymer (PLGA), and they incorporated green fluorescent protein (GFP) plasmid DNA via emulsion coating. They reported the first successful in vivo transfection using a DNA controlled-release stent. GFP-encoding plasmid was efficiently expressed in rat aortic SMCs with 1% percent efficiency. In later years, the same group developed an intravascular stent with a denatured collagen-polylactic-polyglycolic acid for controlled release of GFP-encoding plasmid. Target protein expression was determined with 10.8% efficiency. The level of expression was significantly higher than previous study. They have concluded that denatured collagen incorporated into plasmid DNA-stent coating formulation increased the target protein expression via integrin-related mechanisms and associated changes in the arterial smooth muscle cell actin cytoskeleton [14]. In another study, Takahashi et al. have developed metallic stent-coated polyurethane emulsion containing plasmid DNA. They have evaluated in vivo transgene expression levels, and they have reported that transgene expression has occurred only in vessel segments in contact with the stent. Moreover, analysis of the GFP expression pattern revealed a high frequency of marker protein-positive cells occurring at or near the luminal surface. They have concluded that polymer-coated stents provide a new capability for transgene delivery to immune cells that are believed to contribute to the development of in-stent restenosis [15].

Walter et al. [16] have evaluated the delivery of human vascular endothelial growth factor (hVEGF-2)-encoding plasmid delivery from a gene-eluting stent. They did not use a vehicle to encapsulate the plasmid DNA, encoding for could achieve similar reductions in neointima formation while accelerating, rather than inhibiting, re-endothelialization. They have found that the lumen cross-sectional area (4.2 ± 0.4 versus 2.27 ± 0.3 mm2, $P < 0.001$) was significantly greater and the percentage of cross-sectional narrowing was significantly lower (23.4 ± 6 versus 51.2 ± 10, $P < 0.001$) in VEGF stents compared with control stents implanted in hypercholesterolemic rabbits [16]. In another study, Walsh et al. developed metallic stents coated with a polyurethane emulsion containing plasmid DNA (plasmid-encoded marker genes, b-galactosidase, luciferase, and GFP), which were implanted by Takahashi et al. [15] in rabbit iliac arteries to evaluate transgene delivery. They have observed transgene expressions only in vessel segments in contact with the stent, and they have also emphasized that the extent of transgene expression was dependent upon the quantity of DNA loaded onto the stent [15].

Nitric oxide (NO) is an important regulator of vascular cellular proliferation. NO promotes EC growth and inhibits proliferation of SMCs in the vessel. Additionally, NO reduces platelet adhesion and aggregation. ECs produce NO via nitric oxide synthase (NOS). Accordingly, Bohl Masters et al. [17] have evaluated the effects of localized delivery of NO from hydrogels covalently modified with S-nitrosocysteine (Cys-NO) on neoinitma formation in a rat balloon

injury model. They have reported that localized the delivery of NO from hydrogels inhibited neointima formation by approximately 75% at 14 days. Recently, Sharif et al. [18] have studied therapeutic gene delivery from a stent. They have developed lipoplexes composed of lipofectin and therapeutic eNOS gene. They have coated lipoplexes directly onto the surface of stents and have demonstrated efficient gene delivery for 28 days via liposome-mediated gene delivery.

In another study, Zhu et al. [19] have developed stent-coated dodecylated chitosan-plasmid DNA nanoparticles (DCDNPs) and used them as scaffolds for localized and prolonged delivery of reporter genes into the diseased blood vessel wall. As prepared DCDNPs were spray coated on stents, and a thin layer of dense DCDNPs was successfully distributed onto the metal struts of the endovascular stents. Both in vitro and in vivo expression levels of plasmid DNA-encoding GFP were evaluated. In cell culture, DCDNP stents containing plasmid EGFP-C1 exhibited high level of GFP expression in cells grown on the stent surface and along the adjacent area. In animal studies, reporter gene activity was observed in the region of the artery in contact with the DCDNP stents, but not in adjacent arterial segments or distal organs. Thus, they have concluded that the DCDNP stent provides a very promising strategy for cardiovascular gene therapy [19].

In recent years, Paul et al. [20] have developed a really functional nanobiohybrid hydrogel-based endovascular stent device. The hydrogel was comprised of fibrin matrices, assembled layer by layer on stent surface with alternate layers carrying endosomolytic Tat peptide/DNA nanoparticles or nanoparticles hybridized to polyacrylic acid wrapped single-walled carbon nanotubes. In vitro studies have demonstrated that CNTs incorporated in the hydrogel layers play a major role in tuning the bioactivity of the stent. In addition, the developed stent formulation can significantly reduce the loss of therapeutics while traversing through the vessel and during deployment. In addition to all these, they have demonstrated that the hydrogel-based scaffold carrying therapeutic gene significantly enhances the re-endothelialization of injured artery via in vivo experiments compared to controls. In conclusion, they have declared that this new technology is going to be very useful for controlled delivery of multiple biotherapeutics from stent and other biomedical devices [21].

Since the long-term clinical studies of DES have reported high incidence of late thrombosis, Yang et al. [22] have developed a drug and a gene containing system. They have coated the stent with bilayered PLGA nanoparticles containing VEGF plasmid in the outer layer and paclitaxel in the inner core. They have suggested that while re-endothelialization is going to promote by early release of VEGF gene, slow release of Paclitaxel is going to suppress smooth muscle cell proliferation. They have demonstrated that VEGF/Paclitaxel containing NP-coated stents showed complete re-endothelialization and significantly suppressed in-stent restenosis after 1 month compared to commercial DES [22].

Evidence about restenosis suggests that vascular injury during stent placement and angioplasty procedure activates medial VSMCs, changing them from a quiescent to a proliferative phenotype, and leads them to migrate from the media into the intima. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases. They digest extracellular matrix components, and they play a major role in the formation of restenosis. It is well known that following

angioplasty, MMPs are secreted increasingly. MMPs are secreted as zymogens. In normal physiological vascular remodeling, the activity of MMPs is tightly regulated at the transcription level, the activation of their pro-form or zymogens, the interaction with specific ECM components, and the inhibition by endogenous inhibitors. Tissue inhibitor of matrix metalloproteinases (TIMPs) are the inhibitors of MMPs. Many MMPs and TIMPs are regulated at the level of transcription by a variety of growth factors, cytokines, and chemokines [23]. The interruption of MMPs activity by tissue inhibitor metalloproteinase infection has been shown to limit SMC proliferation and migration through various models by researchers [24]. Local gene transfer of tissue inhibitor of metalloproteinase-2 (TIMP-2) has been studied on a mouse model. TIMP-2 recombinant adenoviruses overexpressing human TIMP-2 gene have been transferred to SMCs, and the findings demonstrated significant decrease in vein graft diameter [25]. Thus, VSMCs seem to be the most promising cell type to be targeted for inhibition of restenosis. Recently, Laçin et al. [26] have used PEGylated nanoparticles poly(St/PEG-EEM/DMAPM) monosized nanoparticles with significantly high cationic charge for the transfection of TIMP-2-encoding plasmid to SMCs. Increased TIMP-2 protein expression in SMCs according to nontransfected SMCs confirmed the successful delivery and expression of the tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) gene via a nonviral transfection gene therapy approach. This PEG-lated monosized, nontoxic, and highly positively charged nanoparticle poly(St/PEG-EEM/DMAPM) was successfully used in SMCs transfection studies.

Polyethylene glycol (PEG) is the polymer of choice for nonviral vector systems because it possesses several favorable properties such as the lack of immunogenicity, antigenicity, and toxicity and a high solubility in water and in many organic solvents. The cytotoxicity of the polycationic carriers used in gene therapy is an important consideration, especially when polycations with high positive charge were used. Thus, to overcome the cationic polymeric vector-induced toxicity, many researchers encapsulate genetic materials or drugs into a PEG shielded cationic liposomal bilayer [27]. PEG is also approved by the FDA for human use. PEGylation of a drug or a material helps to reduce its excretion by the kidneys and avoids its degradation by proteolytic enzyme. Additionally, PEGylation prevents molecule from reticuloendothelial (RES) clearance by enhancing the water solubility of the molecule and to reduce its immunogenicity and antigenicity [28-30].

Cardiovascular gene therapy is the third most popular application for gene therapy. Although preclinical studies of gene therapy studies for restenosis have shown promising results for the potential application of the gene delivery methods in cardiovascular disease, numerous cardiovascular gene therapy clinical trials have not demonstrated substantially positive results for effective gene transfer. A major disappointing feature of the trials is that while preclinical and uncontrolled phase-I gene therapy trials have been continued in a positive matter, none of the randomized controlled phase-II/III cardiovascular gene therapy trials have shown clinically relevant positive effects [31]. Low gene transfer efficiencies were observed with most of trials. A sophisticated efficient delivery method for cardiovascular applications is still not existing, and only low gene expression levels could be detected in target tissues [31]. Recently, several delivery approaches have been designed for the treatment of restenosis, but a number of challenging obstacles must be solved. For example, for different types of biomolecules

(miRNA, siRNA, plasmid, peptide, etc.), different types of materials and different types of vector systems are used. Therefore, it is important to develop unique gene delivery systems that have enhanced transgene efficacy, are safe, and are clinically reliable.

8. Prevention of in-stent restenosis via other biomolecules and peptides

After coronary artery angioplasty (PCI, heart stent surgery), several biomolecules participate in formation of cellular response. Leucocytes and thrombosites discharge cytokines and growth factors inside the blood vessel, adventitia, and encompassing tissue after blood vessel damage. It is well known that tumor necrosis factor α (TNF-α), platelet-derived growth factor (PDGF), and transforming growth factor β (TGF-β) modulate cellular behaviors. Following the activation and proliferation of smooth muscle cell by fibroblasts, significant cumulation and response of extracellular matrix (ECM) in the vessel wall occur. Due to the responsibilities in cellular interactions, ECM, the active component of the vessel wall, is known as a considerable player in vascular diseases. The ECM consists of a diversity of molecules, including collagen, elastin, glycoproteins, and proteoglycans.

Type III collagen is the most abundant matrix protein in a muscular coronary artery. MMPs move through and interact with the C-terminus of the collagen molecule. Several MMPs attend in the collagen degradation mechanism. Interstitial collagenases (MMP1, MMP8, and MMP13) are the most prevalent MMPs that cleave fibrillar collagens, while gelatinases are active against nonfibrillar collagen components of the ECM.

While some of the cytokines and growth factors such as uPA, MT-MMPs, IL-1, PDGF, and TNF-α arrange MMP activation, TGF-β, heparin, steroids, and tissue inhibitor of metalloproteinases (TIMP 1-4) inhibit MMP activity.

Besides, the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) are also another biomolecule group related with atherosclerosis and possibly restenosis. Protease family ADAMTS enzymes regulate ECM transformation by reducing versican (VCAN—a large extracellular matrix proteoglycan) and procollagen-type matrix components. The degradation of versican by ADAMTS-1 catalyses the migration of SMCs and intimal hyperplasia. Also, ADAMTS-7 enables SMC migration and intimal thickening by degradation of cartilage oligomeric matrix protein (COMP). ADAMTS [2, 3, and 14] involve in the removal of N-terminal peptides from procollagen to form mature collagen. Due to their substrate specificity, ADAMTS enzymes are considered as attractive pharmaceutical targets.

Devices having biomimetic surfaces coated with sequences of extracellular matrix proteins, peptides, and enzymes could accelerate endothelial regeneration and prevent from both the thrombic and proliferative effect after stent implantation. In around 25% of patients, th e development of scar tissue underneath the covering of the course may be thick to the point that it can block the bloodstream and produce a vital blockage. At the point when a stent is set in a vein, new tissue becomes inside the stent, covering the struts of the stent. At first, this new tissue comprises healthy cells to cover the blood vessel endothelium. This is a great impact in

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light of the fact that the improvement of typical covering over the stent permits blood to stream easily over the stented territory without coagulating. Later, scar tissue may structure underneath the new healthy coating [32].

Due to the mechanisms of restenosis after angioplasty operation, it is known that TGF-β increases. Yamamoto et al. [33] studied on ribozymes to inhibit TGF-β by cleaving the targeted gene. TGF-β gene demonstrates 100% homology among the human, rodent, and mouse species. They built ribozyme oligonucleotides targeted to the sequence of the TGF-β gene and used it in a rat balloon injury model. Ribozyme inhibits TGF-β mRNA in cultured VSMCs, and using ribozyme oligonucleotides, TGF-β was inhibited, resulting in a significant reduction in neointimal formation in a rat balloon injury model. They also modified ribozyme oligonucleotides containing phosphorothioate DNA and RNA targeted to the TGF-β gene. TGF-β expression was decreased with modified ribozyme oligonucleotides. It was shown that the selective blockade of TGF-β resulted in the inhibition of neointimal formation and reduction in collagen synthesis. It was assumed that the modification of ribozyme oligonucleotide pharmacokinetics would create potential therapeutic strategy for the treatment of cardiovascular disease related to high TGF-β.

Merrilees and colleagues [34] mentioned the importance of viscoelastic properties of vessel wall. They concentrated on arterial matrix proteoglycans, which are related to increasing tissue volume and atherogenicity. One of the basic stimulants of proteoglycans is transforming growth factor β1 (TGF-β1). The aim of the researchers was to investigate the effects of diminishing TGF-β_1 and proteoglycan synthesis in vivo. They used rabbit with balloon catheter damage treated with a TGF-β1 antisense phosphorothioate oligonucleotide connected in a pluronic gel to the adventitia. Statistical information showed that intimal thickening and proteoglycan synthesis were inhibited with the inhibition of TGF-$\beta_1$antisense. These data affirm a part for TGF-β1 in creating neointima and exhibit a particular impact on the combination, appropriation, and gathering of proteoglycan matrix.

There has been extraordinary enthusiasm for the way of stents themselves and the methods used to embed them as boosts for in-stent restenosis. Stent design, arrangement, length, and measures of coronary stream have gotten significant consideration. Additionally, there has been incredible enthusiasm for the stents coated with gradually eluting antirestenotic specialists. The use of drug-eluting stents limits neointima hyperplasia. Pyrrole-imidazole (PI) polyamide targeting TGF-β1 is one of the candidate agents for the drug-eluting stents. In one study, the effects of PI polyamide targeting the TGF-β1 promoter in rat after balloon injury were studied. PI polyamide was designed to connect with the TGF-β1 promoter and carried out for 10 min after inducing balloon injury. Neointimal thickening and re-endothelialization were analyzed [35]. TGF-β1 was significantly decreased with PI polyamide, targeting the expression of TGF-β1 mRNA. Fibronectin and collagen were also affected after targeting. It was understood from the research that synthetic PI polyamide has potential to extinguish neointimal hyperplasia after arterial injury. It was assumed from the article that PI polyamide targeting TGF-β1 could be coated on the stent for the prevention of in-stent restenosis as next-generation drug-eluting stents [35]. Besides, the long-term benefit and safety of coated active stents are crucial research field and should be examined extensively in further studies. The

application of prohealing substances and antirestenosis drugs together as coated on stent represents a diversified approach to reduce restenosis without an increased risk for stent thrombosis [36].

The prevention of new techniques of in-stent restenosis such as peptide-loaded stents to inhibit the biological reactions of the vessel wall gains greater importance in novel studies.

According to the previous studies in the 90s, it was found that fibrin-coated stents lessened thrombogenicity. Baker et al. [37] loaded RGD peptide into fibrin-coated stents due to the inhibition effect of RGD peptide on interaction between fibrinogen with platelets. They have used those stents in an atherosclerotic rabbit model. Four weeks after stent implantation, myointimal hyperplasia in coated and uncoated stent groups were measured and it was seen from the analysis that the extension of myointimal hyperplasia in coated stent group was lower than in the uncoated stent group. Vessel cross-sectional areas of coated stents also were lesser than the uncoated stents. As a result, it was thought that RGD-loaded fibrin-coated stents have prevented vascular complications after stent implantation.

Hong et al. [38] have estimated the advantage and controlling of angiopeptin in a porcine coronary in-stent restenosis model. They have used forty pigs arranged in four groups in the experiments. Out of the control group, the other three groups were treated respectively with one-time treatment (200 μg angiopeptin) at the site of stent placement, continuous angiopeptin over a 1-week period via a subcutaneous osmotic pump (200 μg/kg total dose), and combination of both locally and systematically. In conclusion, this study has demonstrated that the group applied with continuous subcutaneous treatment with angiopeptin after stent implantation significantly has reduced in-stent restenosis by inhibiting neointimal hyperplasia.

In 1999, a synthetic octapeptide, angiopeptin, was used to inhibit tissue response against growth factor, insulin-like growth factor, and interleukin-1-mediated endothelial cell adhesion.

Wiktor brand stents were coated with polyorganophosphazene. Researchers loaded angiopeptin into that biodegradable polymer and implanted the stent in porcine coronary arteries. The group has indicated that angiopeptin increased lumen diameter and morphometric lumen area in significantly as a percentage [39].

We can observe several studies in stent implantation area about local biomolecule delivery made since 1999. Coating of stent is necessary for carrying, prolongation, and elution of the drug through the targeted area effectively and without any loss arising from catheter. Studies on physical strengths of polymers coated on stents and eliciting inflammatory reactions occurring after operation are still ongoing. A portion of the presently accessible gadgets, coatings, and stents are drawing near to making this point an achievable reality. Stent thrombosis remains an important problem after the implantation of different stent types. Coating of stents impacts thrombogenicity. Simple chemical coating lessens platelet adhesion, fibrinogen binding, and effectual against in-stent restenosis in clinical trials. Fuchs et al. [40] were also interested in solving this problem about thrombosis with vasoactive agents. They studied on in vitro and in vivo effects of C-type natriuretic peptide (CNP) that has dual effects on different cell types in a porcine restenotic model. Although gene transfer of CNP in cultures

of porcine vascular cells had achieved 30% reduction of growth of SMCs, the suppression of endothelial growth using CNP had failed. Usage of the CNP gene could be a solution for compress formation of restenosis while preventing late thrombosis [40].

Recent evidence point out endocrine activities are mediated by growth hormone. Shu and colleagues made studies on Ghrelin, a 28-amino acid peptide, which had been isolated from both human and rat stomach that was mediated by growth hormone secretagogue receptor. Ghrelin is expressed in stomach tissue and has several important physiological effects in secretion of growth hormone, inflammation, cell proliferation, differentiation, and apoptosis. Besides, it has wide role on cardiovascular system, such as increasing myocardial contractility, improving cardiac function, inhibiting ventricular remodeling, and attenuating cardiac ischemia-reperfusion injury. Novel studies indicated inhibition of ghrelin on vascular inflammation and proliferation of VSMCs. It also repairs endothelial cells, promotes vascular endothelial function, inhibits platelet aggregation, and exerts antithrombotic effects. Volante et al. [41] had found its protective effect on vascular endothelial function by increasing endothelial nitric oxide synthase (eNOS) expression and improving endothelial function.

Another research group has also stated that ghrelin has prevention against platelet aggregation, MCP-1 expression, and exerts antithrombotic effects. Consequently, ghrelin is considered as therapeutic candidate for the prevention and treatment of ISR [42].

In the 2000s, subjects on expanded polytetrafluoroethylene-covered stent-graft have been carried out. Hamm et al. [43] have used 15-amino acid peptide (P-15), which had cell adhesion property in supporting the endothelization on inner surface after implantation. The recovery of a utilitarian endothelium over the surfaces of the embedded gadgets may restrain both the thrombotic and proliferative reaction after gadget implantation. It was discovered from studies that matrix proteins such as collagen and laminin could improve and increase endothelial regeneration. Starting from this idea, P-15 synthetic peptide, which had cell binding cell of collagen [44], has been tried in in vitro studies with endothelial cells [45]. It was shown that cell migration and adhesion had increased on P-15-coated surface. According to those experiments, P-15 peptide-coated stents had been used in clinical applications. P-15 peptide-coated stents had demonstrated that similar healing with uncoated stents had provided high luminal support and protected from distal emboli. Based on the results from this preparatory, it was figured out that a peptide-treated stent is an alluring methodology for the treatment of stenosed saphenous vein grafts [43].

A different approach with angiotensin-[1-7], an endogenous, biologically active peptide, has come from Langeveld et al. [46]. Angiotensin-[1-7] is a part of the renin-angiotensin system, which has vasodilatory, antithrombotic, and antiproliferative properties. The effects of angiotensin-[1-7] infusion on neointimal formation after stent placement in male Wistar rats have been investigated in this study. Other than the control group, angiotensin-[1-7] [24 g/kg per hour) had been given to rats that underwent stent implantation in the abdominal aorta or sham surgery by placing an osmotic minipump. The endothelial function has been measured in isolated thoracic aortic rings after 4 weeks by histomorphometric and histological analyses. Researchers have found out that angiotensin-[1-7]-treated group has exhibited a significant decrease in neointimal thickness, neointimal area, and percentage stenosis compared with the

control group [46]. Results have showed that angiotensin-[1-7] treatment has reduced neointimal formation after stent implantation in rats. This consequence has supported the idea of Ang-[1-7] could be an alternative to the presently available aggressive antiproliferative drug-loaded stents [46].

Yu et al. [47] were interested in calcineurin/nuclear factor of activated T cells (NFAT) axis. It plays an important role in VSMCs that inhibits NFAT. In earlier studies, the main epitope site on NFAT for calcineurin was discovered. The optimization of this site had induced to the exploration of synthetic peptide VIVIT. Yu et al. [47] have used VIVIT to examine the inhibition NFAT activation and NFAT-mediated proliferation and inflammation in RAW 264.7 macrophages, Ea.Hy.926 endothelial cells and VSMCs, and blocked ionomycin-elicited nuclear import of NFAT. It was also found that VIVIT suppressed platelet-derived growth factor-BB (PDGF-BB) and thrombin induced VSMC proliferation. According to the data, it was reported that NFAT is a regulator of PDGF-BB induced vSMC proliferation. This study stents coated with VIVIT could be a candidate to more specific approaches in the antirestenosis therapy.

In parallel with the ongoing experiments, integrin-binding cyclic Arg-Gly-Asp peptide (cRGD)-loaded stents were used to bound coronary neointima formation and to increase endothelialization by attracting endothelial progenitor cells. It has been stated again that stent coating with cRGD may be useful for reducing in-stent restenosis by accelerating endothelialization [48]. Another study was about RGD-modified liposomes targeted to integrin GPIIbeIIIa on activated platelets [49]. RGD-conjugated liposomes have also been tested in vivo in a rat carotid injury model. As seen from the experiments, cyclic RGD liposomes have binded activated platelets significantly higher compared to linear RGD liposomes. Huang et al. [49] have found an approach on optimization of platelet-targeting ability of ligand-modified liposomes. It has been thought to be a solution for sensitive and selective delivery of therapeutic agents in cardiovascular diseases such as atherosclerosis, thrombosis, and restenosis where activated platelets play significant role in disease development, progression, and outcome.

In-stent restenosis is a pathobiologic methodology, histologically different from restenosis after balloon angioplasty and embodied generally of neointima arrangement. Since percutaneous coronary mediation progressively includes the utilization of stents, in-stent restenosis is moreover getting to be correspondingly more regular. Novel applicable and therapeutic approaches in humans for re-endothelialization are about coating of stents with some substances to give acceleration for the formation of endothelial coverage safely. It was indicated in a porcine model study that cRGD-coated stents expedite endothelialization [50].

In a novel study, it has been focused on the binding ratio of integrin receptor to subendothelial matrix proteins. When integrin binds to arginine-glycine-aspartic acid (RGD) peptide, it imitates naturally occurring adherent interactions. The surface modification of stents with RGD peptide also contributes selectivity for integrin alpha V beta 3, which stimulates endothelialization after stent implantation. Joner and colleagues [51] studied on the availability of RGD peptide-loaded titanium-oxide nitinol stents. Functionality of the engrafted RGD peptide has been examined by in vitro endothelial bioassays, and a subsequent 7-day in vivo endothelialization has been studied by using cRGD-coated self-expanding nitinol stents in rabbits.

Significant increase in endothelial coverage with cRGD stent implants has been stated. This study has represented as an innovative strategy to improve endothelialization and to catalyze vascular healing after stent implantation [51].

Besides, Kramer et al. [52] insisted on interventional cardiology was revolutionized by stent implantation. Stents were developed with antiplatelet therapy and new materials. They have defended the importance of oral drug usage with newly developed stents together. Angiotensin II (Ang II) is an important vasoactive peptide associated with in-stent restenosis, which is produced locally from vessel wall. Due to the Ang II AT_1 receptors' effects on relationship of Ang II with growth and inflammatory signals, A T1-receptor blocking drugs are widely used to treat hypertension and heart failure [53]. Experimental clinical trials has estimated the effect of AT_1-receptor blockers on ISR but no significant result was obtained from patients who were treated with drugs [52].

Different treatment about the movement, growth, and adhesion of endothelial cells has been tried to improve the re-endothelization of stents. Yin and colleagues [54] had synthesized muscle adhesive polypeptide mimics including dihydroxyphenylalanine and l-lysine (MAPDL). They had attached MAPDL on ethylene vinyl acetate (EVA)-coated stent with different molecular weight PEG spacers to find out optimum cell bioactivity. According to in vitro analysis, endothelial cells layer formation had significantly increased on the MAPDL-EVA-coated stents in contrast with the control bare stent. In this manner, it was demonstrated that MAPDL-coated EVA surface had decrease platelet adhesion and appeared to be promising solution for re-endothelialization of intravascular stent devices.

As is seen from the experiments, metal-based stents are mostly preferred for coronary artery disease. The recovery of endothelium around the lesion site can be achieved by coating stents with bioactive molecules. Due to restrictions in availability of proper bioactive signals that would selectively stimulate growth of endothelium and immobilization of such signaling molecules on the metal surface, Ceylan et al. had developed self-assembly, pH-dependent, Dopa-conjugated peptide amphiphile and REDV-conjugated peptide amphiphile nanofibers. Those nanofibers had mimicking property of native endothelium extracellular matrix and had been easily immobilized on stainless steel surface. In vitro experiments had showed that peptide nanofiber-coated stainless steel surface had increased adhesion of vascular endothelial cells as against uncoated surface. Besides, it had decreased viability, proliferation of vascular endothelial cells, and platelet attachment to the peptide. It was suggested in this study that this bioactive stent design has provided a futuristic approach for clinical use in prolonged cardiovascular treatments [55].

9. The role of small RNAs in-stent restenosis

Recent progress in molecular biology has resulted in development of numerous effective gene therapy methods via transferring RNA molecules for the treatment of variety diseases. There are also many studies with the purpose of prevention and treatment of vascular neointima proliferation after balloon angioplasty and stent implantation, using RNA molecules [56-58].

Recent studies suggest that microribonucleic acid-based (miRNA) are important gene regulators and seems to be suitable for the treatment of various cardiovascular diseases [56, 59]. Delivery and controlled release of miRNA through different polymeric materials to target tissues is one of the nucleic acid-based therapy approaches.

Discovered little more than decade ago, non-protein-coding RNAs are single-stranded endogenous RNAs, approximately 25 nucleotides long and they are called as miRNAs [60, 61]. They regulate gene expression negatively at the posttranscriptional level by binding to specific mRNA target, leading either to degradation or to translational target protein repression, rarely they can promote gene expression [56, 59, 62-64]. Small interfering RNAs (siRNA) are short, double-stranded RNAs (20-25 nucleotides) that induce the degradation of target mRNA and inhibits the production of the target protein, and the procedure is called RNA silencing. Unfortunately, clinical applications of RNA interference-based therapeutics such as siRNAs and miRNAs have been limited mainly due to low intracellular delivery efficiency in vitro and in vivo. However, RNA molecules promising therapeutic potential, safe, and efficient delivery methods have to be developed for targeted controlled release. Over the last decade, there has been great effort to develop effective nonviral delivery systems for the transfection of siRNA and miRNA [60]. As it is well known, RNAs are short double-stranded molecules. Due to this reason, they have more rigid structures and inappropriate distribution, making them difficult to form stable and compact particles using a wide range of cationic condensing reagents, such as polylipids, polypeptides, and polyamines, via simple electrostatic interactions. Thus, to achieve maximum target gene silencing, improved gene carrier systems have to be prepared. Therefore, attention has become focused on development of nonviral gene delivery vectors to carry small RNA molecules to target cells [65].

Several experimental and clinical data showed that miRNAs are associated with restenosis or renarrowing of the arteries which primarily results from the proliferation and migration of VSMCs into the intima after stent implantation [66, 67]. Recent evidence by several groups has decelerated that miRNAs have an important role in prevention of atherosclerosis and restenosis [62, 63, 67-71]. In fact, knockdown of miR-21, miR-221, and miR-222 and overexpression of miR145 were found to be intimately relevant to neointimal formation after vessel injury [57, 58, 62, 71-73]. miR-21 is encoded by a single gene and autonomously transcribed from a conserved promotor that is located within the intron of the overlapping protein coding gene [73]. The oncogenic activity of miR-21 has been identified by several groups [74-76]. Besides, it has been found to play important role in proliferation of VSMCs, cardiac cell growth, and death and cardiac fibroblast functions [73]. Indeed, both basic and clinical studies have demonstrated that the overexpression of miR-21 in human reduces cardiac fibrosis and prevents vascular neointima proliferation after balloon angioplasty and stent implantation [72, 77].

Similarly, Liu et al. reported that both of miR-221 and miR-222 were recognized in rat carotid arteries after angioplasty, in which their expression was upregulated and localization in VSMCs at the injured regions of vascular walls [78]. Moreover, it was shown that the overexpression of miR-221 and miR-222 decreased VSMC proliferation in vitro. Also, the knockdown of miR-221 and miR-222 in rat carotid arteries suppressed VSMC proliferation in vivo and

neointimal lesion formation after angioplasty [78]. However, among miRNAs, miR-145 is the most abundant type in vascular walls [58]. In addition to these, especially both miR-143 and miR-145 are significantly expressed in vascular endothelial cells (VECs), which is able of controlling vascular neointimal lesion formation [57].

siRNAs mediate specific gene silencing through a highly regulated enzyme-mediated process. Nowadays, siRNAs are established as the most important biological strategy for gene silencing that includes the degradation of target mRNA and block production of the related protein [79, 80]. Yanming et al. [81] have found that siRNAs reduce neointima formation significantly as reflected by a decreased intima/media area ratio in carotid artery sections after surgical mechanical injury of the rat carotid artery. Wang et al. [59] reported that c-myc siRNA, when given immediately after the surgery, is an effective approach for the prevention of vein graft restenosis.

Usage of nanoparticle eluting stent technologies is an important approach. Walter and colleagues [16] have developed nanoparticles containing plasmid DNA-encoding sequence hVEGF-2 and explored the ability of delivery of target sequence by NP through the stent. An alternative and novel treatment strategy, acceleration of re-endothelialization via VEGF-2 gene-eluting stents, is achieved through endothelial cell proliferation by serving to activate endothelial cell proliferation pathways [16, 82].

Strategies for enhancing gene delivery and gene transfer through stents typically involve the complexations of siRNA/miRNA molecule with cationic polymers, which can be loaded on the stent surface [82, 83].

Although there are several in vitro gene therapy studies for the prevention of restenosis, a few studies with the use of miRNA/siRNA-based therapy for the treatment of cardiovascular diseases have been carried out in humans. Although in vivo studies of miRNA-based agents, conjugated to biodegradable polymers or encapsulated in nanoparticles, were promising, to date there have been a few studies consisting of miRNA-vehicle complexes to a polymer-coated stent that allow delivery of the miRNA for achieve endothelial cell proliferation by serving to activate endothelial cell proliferation pathways [62, 82].

Patil and Panyam [84] have developed nanoparticles using the biodegradable polymer, poly(d,l-lactide-co-glycolide) (PLGA), for siRNA delivery. Additionally, they have incorporated in the PLGA matrix, a cationic polymer, polyethylenimine (PEI), to improve siRNA encapsulation in PLGA nanoparticles. The effectiveness of siRNA-loaded PLGA-PEI nanoparticles was investigated in vitro. They have reported that PEI in PLGA nanoparticle matrix has increased siRNA encapsulation by about 2-fold and also improved the siRNA release profile. Moreover, they have observed higher cellular uptake and cytosolic delivery with the encapsulated siRNA.

In order to avoid such blockages, at the site of angioplasty or stent placement, the suppression of SMCs near the implanted stent, etc., has developed a new delivery technique for Akt1 (Akt1 is a protein that plays a key role in cellular proliferation) siRNA nanoparticles to release from a hyaluronic acid (HA)-coated stent surface. For this purpose, they have used disulfide cross-linked low molecular polyethyleneimine (PEI) (ssPEI) as a gene delivery carrier. Disulfide

bonds are stable in an oxidative extracellular environment but degrade rapidly in reductive intracellular environments. They have immobilized Akt1 siRNA/ssPEI nanoparticles (ASNs) on the HA-coated stent surface. They have reported that the Akt1 released from the stent suppressed the growth of the smooth muscle at the peri-stent implantation area in the balloon-injured external iliac artery in rabbits [85].

Encouragingly, the current developments in the understanding of RNAs have reveal both miRNAs and siRNAs as a potential targets for the development of new diagnostic and therapeutic strategies for the prevention of restenosis [56, 62, 63]. Therefore, attention has become focused on the development of chemically modified RNAs to cure or prevent in-stent restenosis.

10. Conclusion

As conclusion, platelets are the main reason for the formation of thrombus. After stent implantation, platelets are activated and stimulate SMCs migration. In-stent restenosis occurs by the proliferation of SMCs to the injury site. In future studies, the blood flow can be improved, and no platelets are aggregated by coating and biomolecule loading instead of loading to the stent surface.

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Chapter 16

Three-Dimensional "Honeycomb" Culture System that Helps to Maintain the Contractile Phenotype of Vascular Smooth Muscle Cells

Itsuko Ishii and Masashi Uchida

Additional information is available at the end of the chapter

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Abstract

Vascular smooth muscle cells (VSMCs) in the normal aorta are described as having a contractile phenotype because they can contract and do not proliferate. VSMCs in pathological conditions such as atherosclerosis and restenosis can proliferate and migrate, but lose their ability to contract, which is referred to as a synthetic phenotype. VSMCs show plasticity by changing their phenotype according to the surrounding environment. When VSMCs are cultured on a plastic plate, which is a normal two-dimensional culture system, they display the synthetic phenotype because they proliferate and migrate without contraction. Recently, we successfully cultured VSMCs that display features similar to the contractile phenotype, using type I collagen three-dimensional matrices, "honeycombs," in the presence of abundant fetal bovine serum albumin. VSMCs cultured in honeycombs stop proliferating and can contract. The honeycomb culture system can maintain VSMCs in the contractile phenotype for a long period of time. In this chapter, we show the method of this new culture system and the characteristics of VSMCs in honeycombs. It is expected that the use of this culture system will generate new information on the characteristics of VSMCs.

Keywords: smooth muscle cell, three-dimensional culture, collagen, proliferation, contraction

1. Introduction

Vascular smooth muscle cells (VSMCs) are the major cell type in the vascular wall and their main role is contraction. VSMCs in the normal aorta are classified as having a contractile phenotype because they contract without proliferation. Conversely, VSMCs in vascular diseases, such as restenosis after percutaneous coronary intervention and the formation of atherosclerotic plaques, are described as having a synthetic phenotype because they can proliferate and migrate, but lose their ability to contract. The phenotypic modulation of VSMCs depends on the surrounding environment [1].

In order to conduct basic studies on the physiological function of VSMCs and to develop novel medical treatments, cultured VSMCs are used. When VSMCs are cultured on a plastic plate in the presence of 10% fetal bovine serum (FBS), which is the normal two-dimensional monolayer culture system, the cells can migrate and proliferate, but lose their contractile ability [2]. Cultured VSMCs are classified as having a synthetic phenotype and are used as a model of atherosclerotic lesion cells. As phenotypic modulation of VSMCs is responsible for restenosis and the progression of atherosclerosis, they could be the main target of medicinal treatment, and VSMCs cultured on plates are useful to evaluate the effects of medicines. However, there is no acceptable cell model that reflects the nature of VSMCs in the normal aorta of a living body. For this reason, information generated from synthetic VSMCs cannot be compared with that from contractile VSMCs in the normal aorta. In this chapter, we describe a new culture system for VSMCs that maintain their contractile phenotype.

2. Method of culture of VSMCs in type I collagen three-dimensional "honeycomb" matrices

We reported a method for culturing contractile VSMCs by using three-dimensional matrices, so-called "honeycombs." Honeycombs are type I collagen sponges that can be obtained from Koken Co., Ltd. (Japan). Honeycombs are prepared from 0.5% type I atelocollagen in an acid solution by neutralization with ammonia gas to separate the collagen fibrils and lyophilization [3]. The structure of the honeycomb is porous and consists of many tubes aligned side by side, similar to a beehive [4, 5]. The pore diameter of the honeycomb is controlled by altering the concentration of the collagen solution and ammonia gas. When a higher concentration of ammonia gas is used, smaller pores are produced, and vice versa [3]. The diameter of each pore of the tubes is 100–500 μm, and we usually use a pore size of 200–300 μm for rabbit and human VSMCs. The honeycombs are cut vertically into cubes of dimensions 5 × 5 × 2 or 3 × 3 × 2 mm for culturing VSMCs.

The initial culture of VSMCs in a honeycomb is as follows (Figure 1): rabbit or mouse VSMCs cultured on plates (synthetic phenotype) in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS are used. The honeycombs are pre-incubated with DMEM containing 10% FBS and the air in each pore of the honeycomb is removed by centrifugation. VSMCs cultured on plates are incubated with trypsin-EDTA, and the released cells are collected by centrifuga-

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tion. The collected cells are suspended in 300–400 μL DMEM containing 10% FBS and are incubated with the honeycombs (approximately 2.0 – 3.0 × 10^6 cells per 30 honeycombs) on a dish (diameter, 6 cm) for 3 h at 37°C. Then, 3–5 mL culture medium is added. The medium is changed every 2–3 days. For human VSMCs, a commercial medium supplemented with epidermal growth factor, fibroblast growth factor-B, insulin, and 2% FBS is used.

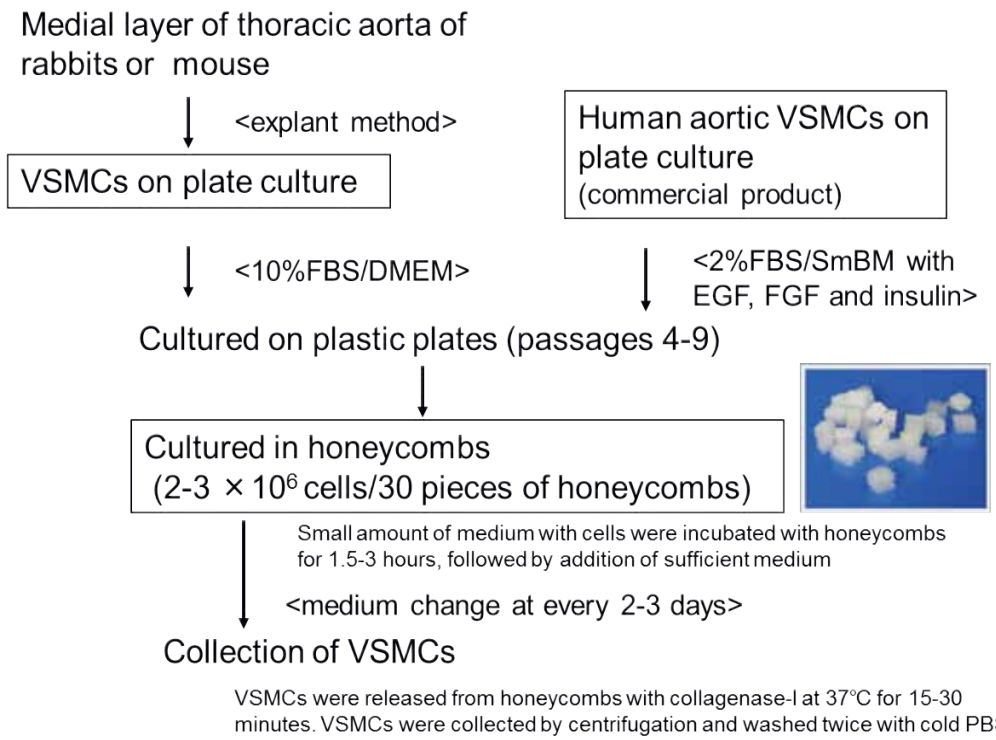

Figure 1. Preparation of VSMCs into honeycombs. Rabbit, mouse, and human VSMCs can be cultured successfully in honeycombs with this chart. The required number of cells are cultured on a plate as synthetic VSMCs and transferred to the honeycombs. To collect VSMCs in honeycombs for analysis, the honeycombs are treated with collagenase-I.

By visualizing the binding process of VSMCs to honeycombs using a real-time cultured cell monitoring system [6], VSMCs are fixed at one attachment point and move little by little using other attachment points, resembling the movement of an inchworm. After 25 h incubation, the positions of the VSMCs are fixed, and they form cross-bridges in the honeycombs. VSMCs in honeycombs form 2–5 attachment points per cell (Figure 2). However, mouse VSMCs (length of long axis, 33–139 [80 ± 29] μm) do not make cross-bridges in honeycombs in which the diameter of the pore is about 500 μm. These results suggest that the relationship between pore size and the length of the long axis of VSMCs is important for the formation of cellular cross-bridges. The inner wall of honeycombs with a larger pore size may function as a "flat floor" for VSMCs, as if it were a plastic plate.

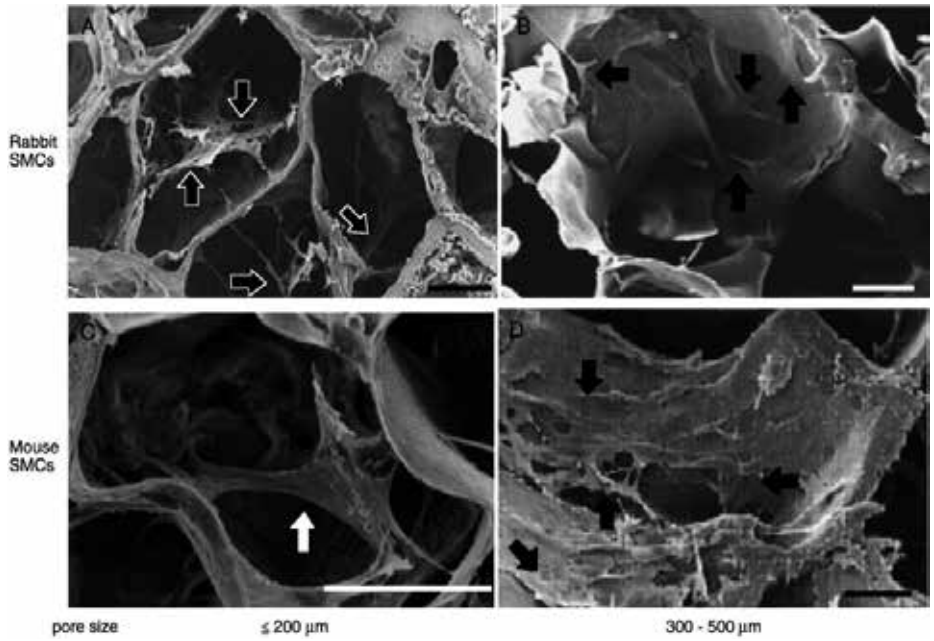

Figure 2. Cross-bridges of VSMCs on honeycombs. These pictures are electron microscopic observations of rabbit and mouse VSMCs cultured in honeycombs. (A) Rabbit VSMCs cultured in honeycombs of pore size ≤200 μm; (B) rabbit VSMCs cultured in honeycombs of pore size 300–500 μm; (C) mouse VSMCs cultured in honeycombs of pore size ≤200 μm; and (D) mouse VSMCs cultured in honeycombs of pore size 300–500 μm. Arrows show VSMCs. Scale bars, 50 μm. Data adapted from reference 6.

3. Proliferative inhibition of VSMCs cultured in honeycombs

Various kinds of cells, such as human fibroblasts, CHO-K1, BHK-21, and bovine endothelial cells [3], can be cultured successfully in honeycombs. These cells grow normally in honeycombs. However, the proliferation of VSMCs can be controlled easily by the pore size of the honeycombs.

When synthetic rabbit VSMCs are used for culture in honeycombs (pore size, about 200 μm), they stop proliferating immediately when they form cross-bridges [4]. [^{3}H]Thymidine is incorporated at a low level into VSMCs cultured in honeycombs, and cell number does not change during culture. VSMCs can be cultured in honeycombs for approximately 3 months with medium change. However, when rabbit VSMCs are cultured in honeycombs with larger pore size (100–500 μm) (Figure 3), they proliferate for the first few days, but then stop proliferating and cell number does not change [6]. The reason why cell number increases only at the beginning of culture is as follows: in honeycombs with a smaller pore (≤200 μm), VSMCs form cross-bridges independently of each other, but a large number of connected cells form cross-bridges together at the wall of honeycombs with larger pores (100–500 μm) after the initial increase in cell number at the beginning of culture. These data suggest that the formation

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of cross-bridges by VSMCs in honeycombs may be a significant step for the cessation of proliferation.

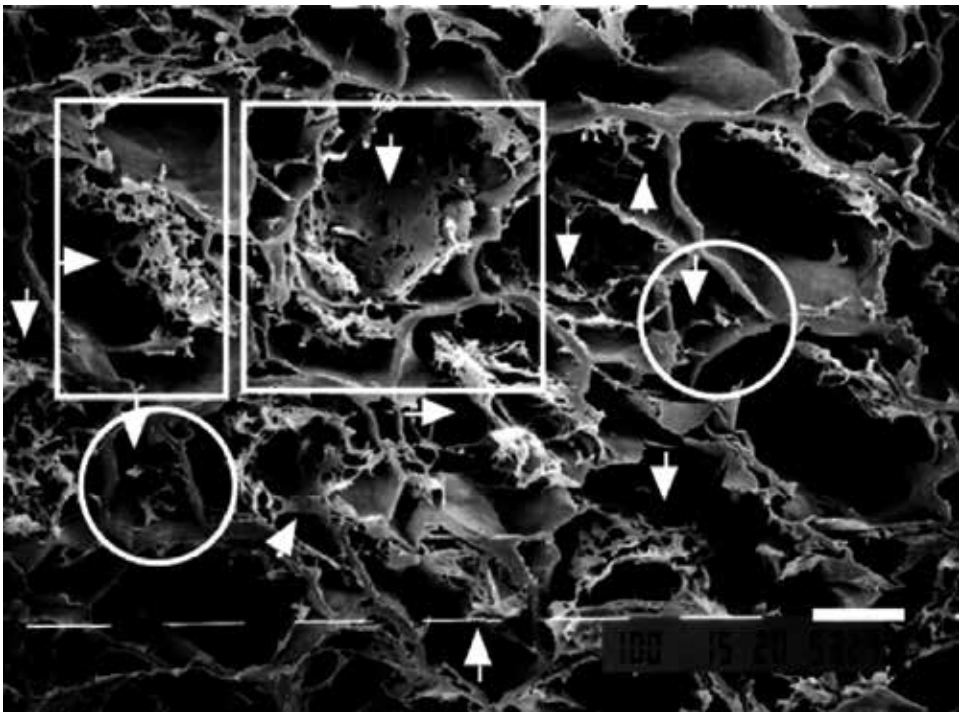

Figure 3. Cross-bridges of rabbit VSMCs cultured in honeycombs for 14 days. Electron microscopic observation of rabbit VSMCs cultured in honeycombs for 14 days. Rabbit VSMCs were cultured in honeycombs of pore size 100–500 μm. The pores enclosed by squares contain a large number of VSMCs, and the pores enclosed by circles contain a small number of VSMCs forming cross-bridges. Arrows show VSMCs. Scale bar, 100 μm. Data adapted from reference 6.

When VSMCs are cultured on collagen-coated plates, their proliferation rate increases. Conversely, when VSMCs are cultured on collagen gels, their proliferation stops via the cdk2 inhibitor $p27^{kip1}$ [7]. However, proliferative inhibition persists for less than 1 week because VSMCs dissolve the collagen gel and start to proliferate again (unpublished data). Therefore, it is expected that both the higher-order structure of collagen and pore size of the honeycombs are considerable factors for the proliferative inhibition of VSMCs.

Although VSMCs in honeycombs stop proliferating immediately, $p27^{kip1}$ expression increases after incubation for 2–3 days (unpublished data). From this observation, it is expected that $p27^{kip1}$ may work to keep VSMCs at a resting state and not as the initiator of proliferative inhibition. Taken together with electron microscopic observations and data for growth in honeycombs, proliferative inhibition occurs in parallel with a decrease in the number of focal adhesions, including reduced levels of focal adhesion kinase (FAK). As a high level of phosphorylated FAK promotes proliferative activity [8], the low level of phosphorylated FAK is one of the reasons for proliferative inhibition of VSMCs in honeycombs.

An additional mechanism for the proliferative inhibition of VSMCs in this three-dimensional culture system is the expression of ornithine decarboxylase antizyme 1 (OAZ1), which is a key regulator of intracellular polyamines. Polyamines (putrescine, spermidine, and spermine), which are multivalent organic cations, are essential for cell growth [9]. The proliferation and transformation of cells induced by oncogenes, carcinogens, and viruses are characterized by increases in the levels of intracellular polyamines due to their increased biosynthesis and uptake [10]. Ornithine decarboxylase (ODC) is the rate-limiting enzyme of polyamine biosynthesis. OAZ1 inhibits the activity of ODC and increases its degradation by forming an OAZ1-ODC complex [11, 12]. OAZ1 also decreases the uptake of polyamines independent of its effects on ODC [13, 14].

When VSMCs proliferate on plates, the intracellular content of polyamines increases. Conversely, polyamine content is maintained at a low level in VSMCs cultured in honeycombs [15]. VSMCs stably transfected with the ODC gene (ODC-VSMCs) and cultured on plates increase their rate of proliferation, which is accompanied by an increase of polyamine content (especially spermidine) and phosphorylated FAK and a decrease in marker proteins of differentiation, α-actin, and myosin heavy chain in comparison to VSMCs cultured on plates (Figure 4). As ODC is an oncogene and induces an excess of polyamines [16], it is assumed that ODC may promote the proliferative activity of VSMCs cultured in honeycombs. However, the proliferation of ODC-VSMCs also ceases in honeycombs, similar to normal VSMCs with low levels of spermidine and phosphorylated FAK (Figure 4). Finally, culture of ODC-VSMCs in honeycombs increases the expression of α-actin and myosin heavy chain.

As shown in Figure 4, OAZ1 levels are higher in both VSMCs and ODC-VSMCs cultured in honeycombs than in both VSMCs and ODC-VSMCs cultured on plates. OAZ1 is degraded in ODC-VSMCs cultured on plates for up to 3 h after treatment with cycloheximide, but OAZ1 degradation in ODC-VSMCs cultured in honeycombs is limited over a 12 h incubation period (Figure 4). This difference in OAZ1 stability may be the major reason for the high levels of OAZ1 observed in VSMCs cultured in honeycombs. These results suggest that OAZ1 in VSMCs cultured in honeycombs might enhance the degradation of ODC and inhibit polyamine uptake. In fact, our data show that polyamine uptake is significantly lower in ODC-VSMCs cultured in honeycombs than in ODC-VSMCs cultured on plates [15]. This could be one of the reasons for the lower polyamine levels in VSMCs cultured in honeycombs than in VSMCs cultured on plates.

When OAZ1 is transiently overexpressed in ODC-VSMCs (Figure 5), OAZ1 decreases the number of ODC-VSMCs cultured on plates at day 3, whereas OAZ1 overexpression does not change the number of ODC-VSMCs cultured in honeycombs. OAZ1 overexpression slightly decreases the expression of ODC and phosphorylated FAK levels in ODC-VSMCs cultured on plates. Surprisingly, OAZ1 overexpression influences the levels of myosin heavy chain in ODC-VSMCs cultured on plates and in honeycombs, and influences the levels of α-actin in ODC-VSMCs cultured in honeycombs. These results suggest that OAZ1 in VSMCs might regulate intracellular polyamine levels and cellular proliferation. Moreover, OAZ1 levels may influence the expression of α-actin and myosin heavy chain, which are the main components of the contractile apparatus.

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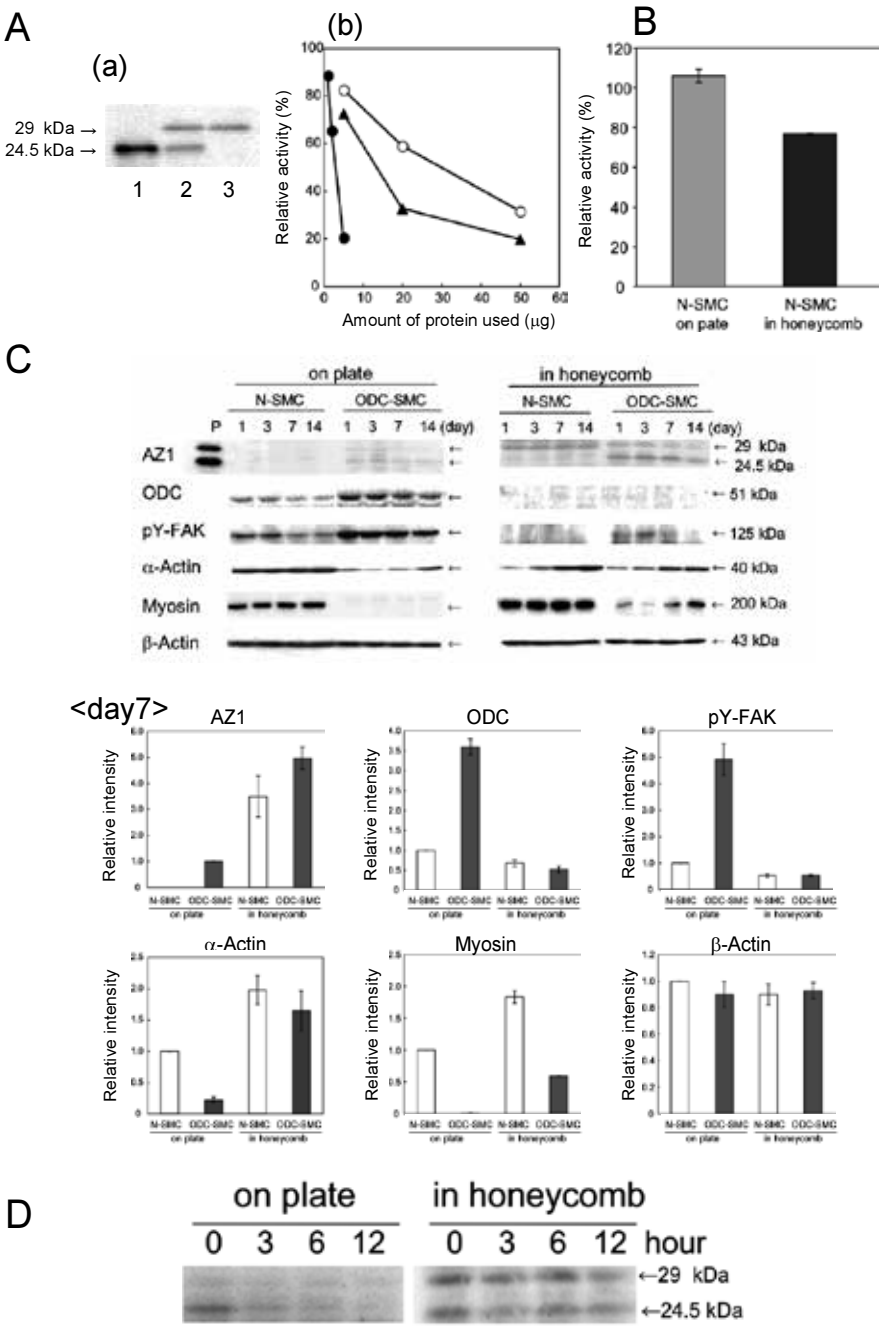

Figure 4. OAZ1 in normal VSMCs and ODC-VSMCs, and the levels of various proteins expressed in VSMCs cultured on plates and in honeycombs. (A) (a) VSMCs were transfected with three kinds of pTracer-CMV containing OAZ1 without T205, which do not need a frameshift for their expression of each type of OAZ1. Lane 1, pTracer-OAZΔT205ΔAUG1, which expresses 24.5-kDa OAZ1; lane 2, pTracer-OAZΔT205, which expresses 24.5- and 29-kDa

OAZ1; lane 3, pTracer-OAZΔT205ΔAUG2, which expresses 29-kDa OAZ1. After transfection, VSMCs were collected at 24 h, a cell extract was prepared, and Western blot analysis was performed. (b) Degree of inhibition of ODC by OAZ1 was measured. The activity of OAZ1 was determined as an inhibitory percentage of ODC activity of the extract from ODC-overproducing FM3A (EXOD-1) cells. ●, extract from VSMCs expressing 24.5-kDa OAZ1; ▲, extract from VSMCs expressing 29- and 24.5-kDa OAZ1; and ○, extract from VSMCs expressing 29-kDa OAZ1. Values are means of duplicate determinations. (B) Inhibition of ODC activity using extracts from VSMCs cultured on plates and in honeycombs. OAZ1 activity was determined as an inhibitory percentage of ODC activity of the extract from EXOD-1 cells. Values are means ± standard deviation (SD) of triplicate determinations. (C) Western blot analysis of OAZ1, ODC, tyrosine-phosphorylated FAK (pY-FAK), α-actin, myosin heavy chain, and β-actin of VSMCs and ODC-VSMCs cultured on plates and in honeycombs. P, EXOD-1 cells as a positive control. Relative intensity on day 7 was quantified. The intensity of OAZ1 was quantified as the sum of the 29- and 24.5-kDa bands. ND, not detectable. Values are means ± SD of triplicate determinations. (D) OAZ1 degradation. ODC-VSMCs cultured on plates and in honeycombs for 3 days were treated with 20 μg/mL cycloheximide for the indicated time. Data adapted from reference 15.

VSMC proliferation is reportedly up-regulated via an increase in the stability of S-phase kinase-associated protein-2, E3 ubiquitin protein ligase (SKP2) by autophosphorylation of FAK-Tyr397 [17]. In ODC-VSMCs cultured on plates, the increased rate of proliferation is accompanied by an increase of phosphorylated FAK. Interestingly, when the effect of spermine on FAK autophosphorylation *in vitro* was investigated, FAK phosphorylation was stimulated by spermine [15]. The low levels of intracellular polyamines in VSMCs cultured in honeycombs could inhibit the autophosphorylation of FAK, and this may contribute to the proliferative inhibition of VSMCs.

4. Differentiation of VSMCs cultured in honeycombs

Caldesmon is an actin-linked regulatory protein, and caldesmon heavy chain (h-CaD) is expressed abundantly and specifically in contractile VSMCs. h-CaD localizes to the actomyosin contractile structure in VSMCs as an integral component of smooth muscle thin filaments [18]. Caldesmon also works as a potent repressor of cancer cell invasion because its ectopic expression reduces the number of podosomes/invadopodia and decreases extracellular matrix degradation. The depletion of caldesmon facilitates the formation of podosomes/invadopodia and cell invasion [19]. As VSMCs cultured in honeycombs for 14 days express h-CaD [4, 6], these cells could be classified to the contractile phenotype.

Some other cells also differentiate in honeycombs. By usage of honeycomb scaffolds, rat axonal regeneration [20], differentiation of pluripotent embryonic stem (ES) cell-derived embryoid bodies (EBs) into hepatocyte-like cells [21], and differentiation of mesenchymal stem cells into osteoblasts [22] have been reported. For example, EBs formed from ES cells become liver-specific gene-positive and albumin-positive cells, and they form cord-like structures in honeycombs that are not present in two-dimensional monolayer culture systems [21]. When a honeycomb scaffold including EB-derived hepatocyte-like cells is transplanted into the median lobes of partially hepatectomized nude mice, cells that are positive for both albumin and cytokeratin 18 appear in the transplant and form clustered aggregates after 7 and 14 days. These data suggest that a honeycomb is a useful scaffold culture system for regulation of cellular differentiation and for advanced tissue engineering.

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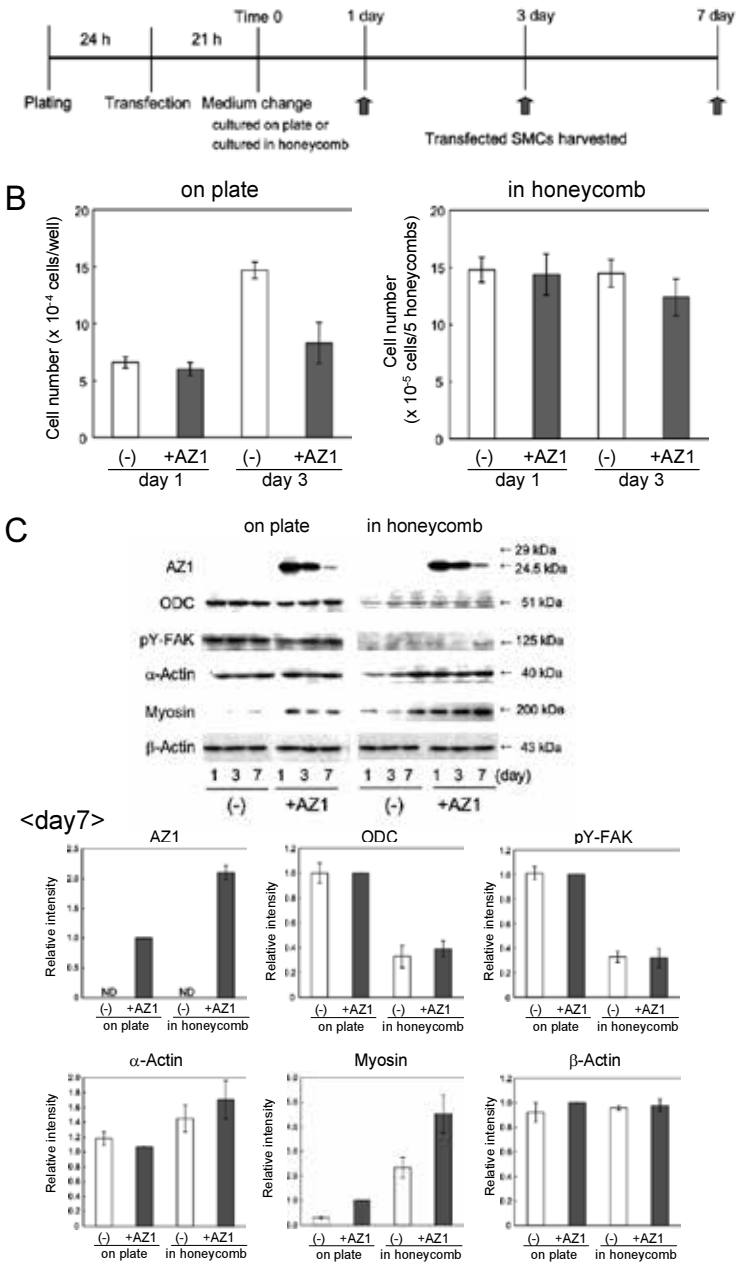

Figure 5. Effect of the transient expression of OAZ1 in ODC-VSMCs cultured on plates and in honeycombs. After ODC-VSMCs were transfected with pTracer-OAZΔT205ΔAUG1 (24.5-kDa OAZ1), the cells were cultured as shown in (A). Transfected ODC-VSMCs were then cultured either on plates or in honeycombs for 1, 3, and 7 days. (B) Cell number on days 1 and 3. (C) Western blot analysis of OAZ1, ODC, pY-FAK, α-actin, myosin heavy chain, and β-actin. Relative intensity of proteins on day 7 was quantified. Values are means ± SD of triplicate determinations. Data adapted from reference 15.

5. Contraction of VSMCs cultured in honeycombs

VSMCs cultured on plates express the main components of the contractile apparatus and cytoskeleton (including β-non-muscle actin filaments), but VSMCs cultured on plates do not contract under normal culture conditions [23]. The size of honeycombs containing VSMCs is significantly reduced over time [5], which could be due to the contraction of the VSMCs. A key protein for the contraction of VSMCs cultured in honeycombs is filamin. Filamin A has a molecular weight of 280 kDa and a molecular length of approximately 160 nm [24]. Filamin dimers link actin filaments into orthogonal networks or parallel bundles, and an *in vitro* study showed that the manner in which actin filaments are organized depends on the ratio of filamin to actin [25]. Filamin mainly co-localizes with the β-non-muscle actin filaments in gizzard SMCs [26, 27]. When ODC-VSMCs are used, the function of filamin in VSMCs becomes clear. Filamin expression is dramatically decreased in ODC-VSMCs compared to VSMCs (Figure 6). β-Non-muscle actin filaments in ODC-VSMCs cultured on plates are thinner than those of VSMCs, although β-actin expression, as determined by Western blot analysis, is almost the same in ODC-VSMCs as in VSMCs (Figure 6). These results suggest that the 280-kDa form of filamin is responsible for the bundling of β-non-muscle actin filaments in VSMCs.

It is well known that filamin is degraded by μ- and m-calpain [28, 29]. When VSMCs are cultured in honeycombs, 280-kDa filamin is degraded to 180-kDa filamin by calpains [5]. It is expected that degraded filamin cannot induce the bundling of actin filaments, resulting in the relative structural weakness of β-non-muscle actin filaments in VSMCs cultured in honeycombs. Observation of the intracellular distribution of filamin and β-actin by immunofluorescence microscopy shows that filamin is situated along fibers and co-localizes with β-actin in subconfluent VSMCs cultured on plates (Figure 7A and B). In honeycombs, filamin staining is detected evenly in the cytoplasm and is not stained as fibers in most cells (Figure 7C and D). It can be assumed that degraded filamin, which does not bind to β-non-muscle actin filaments, is present in the cytoplasm of VSMCs cultured in honeycombs.

The expression levels of proteins involved in both the contractile apparatus (α-actin, myosin heavy chain, and tropomyosin) and cytoskeleton (β-actin and α-actinin) in VSMCs cultured in honeycombs are almost identical to those in VSMCs cultured on plates [5]. However, VSMCs on plates express the 280-kDa form of filamin, whereas VSMCs cultured in honeycombs express both the 280- and 180-kDa forms of filamin. α-Actin and β-actin co-localize in subconfluent VSMCs cultured on plates and in VSMCs cultured in honeycombs, suggesting that the contractile apparatus is aligned with β-non-muscle actin filaments [5].

These data from VSMCs cultured in honeycombs could explain why contraction, which is a result of shortening of the contractile apparatus, is not observed in VSMCs cultured on plates. VSMCs attach to rigid plastic plates via focal adhesions, and the resulting cytoskeletal tension, which is maintained by β-non-muscle actin filaments, inhibits shortening of the contractile apparatus. In honeycombs, VSMCs can contract because filamin degradation reduces cytoskeletal tension and allows shortening of the contractile apparatus [5].

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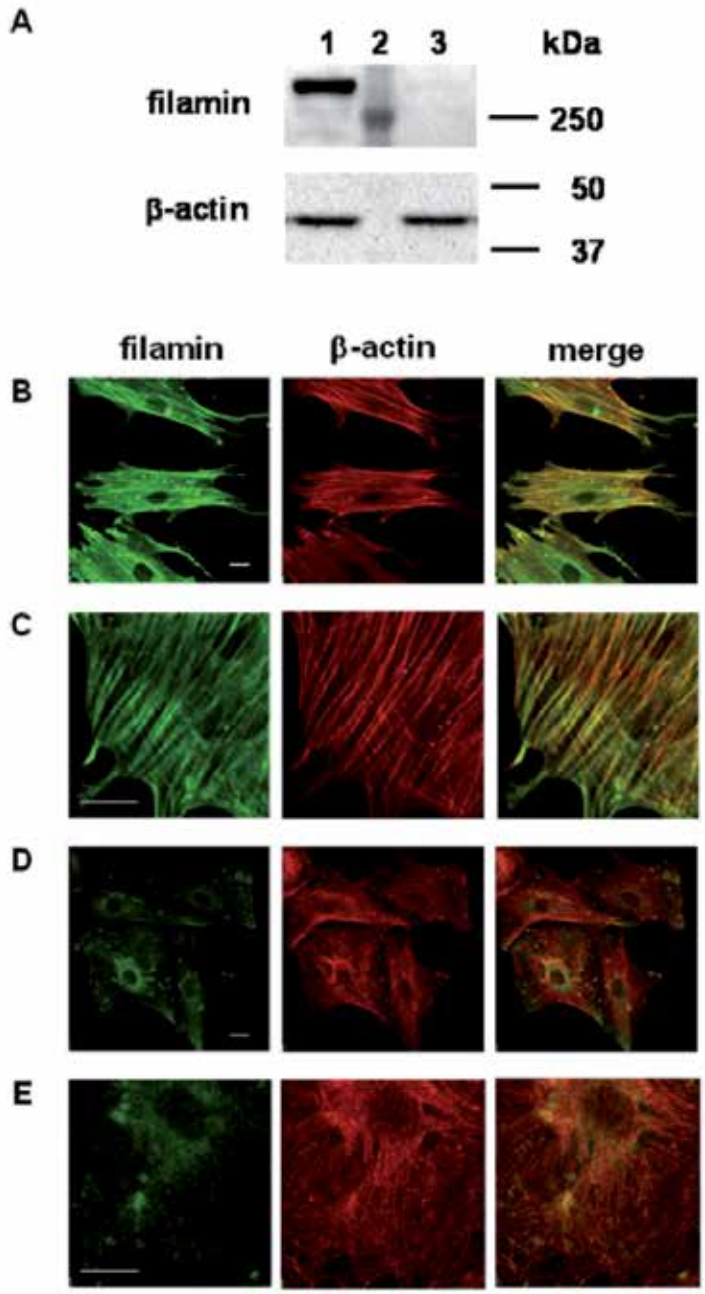

Figure 6. Expression and localization of filamin and β-actin in ODC-VSMCs. (A) Western blot analysis of filamin and β-actin in VSMCs and ODC-VSMCs. VSMCs and ODC-VSMCs were cultured on plates to subconfluence. Lane 1, VSMCs; lane 2, molecular marker; and lane 3, ODC-VSMCs. Localization of filamin and β-actin in VSMCs (B, C) and ODC-VSMCs (D, E). VSMCs and ODC-VSMCs were cultured on plates to subconfluence. Green indicates filamin, and red indicates β-actin. Scale bars, 20 μm (B, D); 5 μm (C, E). Data adapted from reference 5.

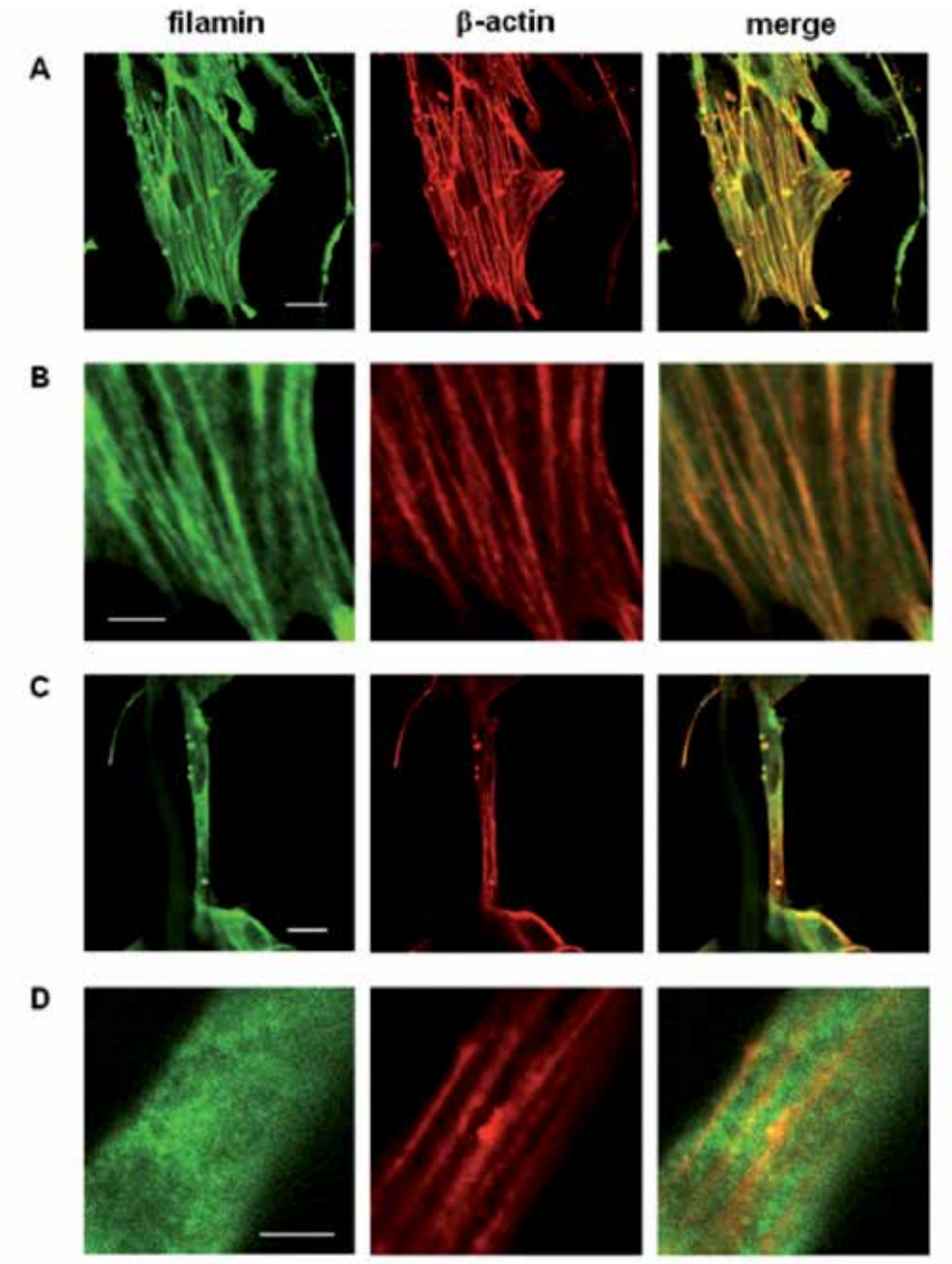

Figure 7. Localization of filamin and β-actin in VSMCs. (A, B) VSMCs were cultured to subconfluence on a plate. (C, D) VSMCs were cultured in honeycombs for 3 days. Green indicates filamin, and red indicates β-actin. Scale bars, 20 μm (A, C); 5 μm (B, D). Data adapted from reference 5.

When myocardial cells derived from newborn rats are cultured in honeycombs, the cells attach to the honeycombs and start beating from the first day. The rate of beating increases gradually,

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and reaches the highest frequency of 162 beats per minute at day 8 [30]. However, the number of myocardial cells increases significantly. From these data, it is shown that the honeycomb scaffold may induce the functional aspects of cells even though it is unclear whether or not cells cultured in honeycombs proliferate.

6. Reversibility of VSMCs between the contractile and synthetic phenotypes

When VSMCs cultured in honeycombs are treated with collagenase-I and the released cells are seeded in plastic plates, they start to proliferate again [5]. This result shows the remarkable plasticity of VSMCs. The relationship between the culture system and phenotypic modulation is shown in Figure 8.

Filamin A mRNA expression is almost identical in VSMCs cultured in honeycombs and on plates, and VSMCs moved to plates mainly express the 280-kDa form of filamin after 12 h [5]. These observations indicate that filamin might contribute to the phenotypic modulation seen in VSMCs.

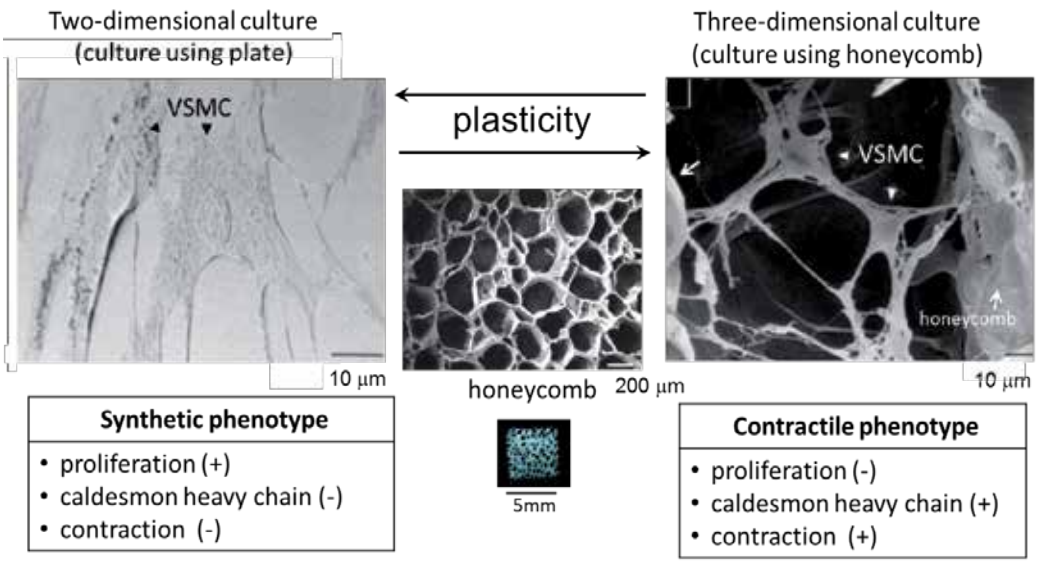

Figure 8. Summary of VSMC phenotypic modulation in culture. These images are scanning electron microscopic observations. Arrowheads show VSMCs and arrows show honeycombs. VSMCs cultured on plates express the synthetic phenotype. This type of VSMC is de-differentiated and can proliferate. VSMCs cultured in honeycombs display similar features to the contractile phenotype. These two phenotypes are reversible depending on the type of culture used. These photographs were taken from reference 4 to make it easy to understand the culture system.

7. Vision and agenda

The honeycomb culture system has been used for tissue engineering. For example, when honeycombs filled with grown human adipose-derived stem cells were transplanted into nude mice, bone formation was observed *in vivo* [31]. Another report showed that new bone induction by KUSA/A1cells was promoted by using honeycomb culture [32]. Furthermore, when human embryonic stem cells (hESCs) were co-cultured with Swiss 3T3 cells in honeycombs, type I collagen synthesis by Swiss 3T3 cells contributed to the hepatic maturation of hESCs [33]. From these data, it can be seen that the honeycomb culture system has the potential to help the regeneration of organs and to facilitate the analysis of intercellular interactions. In the near future, in order to clarify the mechanism of the phenotypic modulation of VSMCs involved in atherosclerosis, we intend to co-culture VSMCs and macrophages. In addition, the reason for the proliferative inhibition of VSMCs cultured in honeycombs is still unclear. This is our agenda that remains to be solved.

8. Conclusion

A summary of VSMC phenotypic modulation in culture is shown in Figure 8. The contractile phenotype of VSMCs can be cultured stably using type I collagen three-dimensional matrices, i.e., honeycombs, in the presence of 10% FBS. VSMCs in honeycombs stop proliferating and can contract. The expression of OAZ1 and reduction of phosphorylated FAK levels contribute partially to the proliferative inhibition of VSMCs in honeycombs. Filamin degradation reduces cytoskeletal tension and allows shortening of the contractile apparatus. However, the detailed mechanism by which VSMCs display features similar to those of aortic VSMCs when cultured in honeycombs is still unclear. In order to clarify the regulation of phenotypic modulation, further studies are needed.

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Chapter 17

Role of Platelet-Activating Factor and Hypoxia in Persistent Pulmonary Hypertension of the Newborn — Studies with Perinatal Pulmonary Vascular Smooth Muscle Cells

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Stephen M. Douglass, Rebecca Davis,
Shaemion McBride and Basil O. Ibe

Additional information is available at the end of the chapter

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Abstract

Platelet-activating factor (PAF) plays an important physiological role of maintaining a high vasomotor tone in fetal pulmonary circulation. At birth, endogenous vasodilators such as nitric oxide and prostacyclin are released and facilitate pulmonary vasodilation via cAMP-dependent protein kinase (cAMP/PKA) and cGMP-dependent protein kinase (cGMP/PKG) pathways. Interaction between the cyclic nucleotides and PAF receptor (PAFR)-mediated responses in pulmonary arterial smooth muscle is not well understood. To further understand the interactions of PAF-PAFR pathway and the cyclic nucleotides in ovine fetal pulmonary arterial smooth muscle cells (FPASMC), effects of cAMP and cGMP on PAFR-mediated responses in pulmonary arterial smooth muscle cells (PASMC) were studied. Ovine FPASMC were incubated with 10μM cAMP or cGMP in normoxia (5% CO_2 in air, pO_2~100 Torr) or hypoxia (2% O_2, 5% CO_2, pO_2~30-40 Torr). Proteins were prepared and subjected to Western blotting. Effect of cell permeable cAMP and cGMP on PAFR binding was also studied and effect of cAMP on cell proliferation was also studied by RNAi to PKA-Cα. cAMP and cGMP significantly decreased PAFR binding and protein expression in normoxia and hypoxia, more so in hypoxia, when PAFR expression was usually high. PKA-Cα siRNA demonstrated that inhibition of PAFR-mediated responses by the cyclic nucleotides occurred through PKA. These data suggest that the normally high levels

of cyclic nucleotides in the normoxic newborn pulmonary circulation assist in the downregulation of postnatal PAFR-mediated responses and that under hypoxic conditions, increasing the levels of cyclic nucleotides will abrogate PAF-mediated vasoconstriction thereby ameliorating PAF-induced persistent pulmonary hypertension of the newborn.

Keywords: Pulmonary artery, PAFR binding, cyclic nucleotides, siRNA

1. Introduction

Platelet-activating factor (PAF) is an endogenous phospholipid which evokes a wide range of biological activities, such as vasoconstriction and systemic hypotension [1], mainly under pathophysiological conditions. The discovery of PAF, its cellular origin, and biological actions were first reported by Benveniste and Associates [2, 3]. Following these reports, investigations of the physiological effects of PAF involved its roles in fetal lung maturation and lung function [4-9], and its role in reproduction where it is involved in implantation of embryos, among other effects [10, 11]. PAF produces a myriad pathological effects in vivo, including platelet aggregation [12-14], mediation of immune response and bronchoconstriction [15, 16], and smooth muscle contraction [17-21], which hinges on its role as an inflammatory mediator and vasoconstrictor [17]. In the fetus, PAF plays an important physiological role in maintaining a high level of vasomotor tone in the pulmonary circulation [22]. Therefore, the high PAF receptor (PAFR) binding in fetal lamb lungs supports the existence of a high level of pulmonary vasomotor tone in utero [23]. On the other hand, in lungs of the newborn lamb, PAFR binding and receptor mRNA expression are low, suggesting a down regulation of PAFR-mediated effects in vivo [22, 23].

PAF acts by binding to its Gq protein isoform of G protein-coupled receptors, which is a seven transmembrane receptor [24]. Activation of G protein-coupled receptors by an agonist results in activation of signal transduction pathways [25], which may involve recruitment of intracellular second messengers such as cAMP, cGMP, inositol 1,4,5-triphosphate (IP_3), and calcium [26, 27]. cAMP and cGMP act via their endogenous receptors, cAMP-dependent protein kinase (cAMP/PKA) and cGMP-dependent protein kinase (cGMP/PKG), respectively, to elicit relaxation of smooth muscle, and cAMP and cGMP mediate relaxation of pulmonary vessels, but cGMP has been shown to be more effective than cAMP in producing relaxation of perinatal ovine pulmonary vessels [28, 29]. Acute hypoxia upregulates PAFR-mediated intracellular signaling in fetal ovine pulmonary vascular smooth muscle [30]. Chronic hypoxia in the perinatal period may result in abnormal upregulation of PAFR protein expression, PAFR binding, and PAFR-mediated cell signaling, leading to increased pulmonary vasomotor tone and vascular remodeling, a key event in the onset of clinical disorders such as persistent pulmonary hypertension of the newborn (PPHN) [31].

We are interested in understanding the mechanisms of pulmonary vascular relaxation at birth. Our primary hypothesis is that with oxygenation at birth and the increased production of

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cAMP and cGMP in pulmonary vascular smooth muscle, PAFR protein expression and PAFR-mediated cell signaling may be inhibited via cross-talk between the cyclic nucleotides and PAFR-mediated responses. This hypothesis was investigated in ovine fetal pulmonary vascular smooth muscle cells using cAMP and cGMP on PAFR binding and PAFR-mediated cell signaling in both normoxia and hypoxia. After birth, during normoxia, pulmonary levels of cAMP rise significantly, perhaps contributing to the decrease in PAFR binding, PAFR protein expression and the ensuing fall in pulmonary vascular resistance. We employed siRNA to the catalytic domain of PKA to define the role of PKA in decreased pulmonary PAFR-mediated responses at birth.

2. Materials and methods

2.1. Materials

The study was approved by the Institutional Animal Care and Use Committee of Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center. Pregnant ewes (146-148 d gestation, term being 150 d) were purchased from Nebekar Farms (Santa Monica, CA). Authentic standards of PAF (C_{16}-PAF) as well as 8-Br-cAMP, Rp-cAMPS, 8-Br-cGMP, Rp-8-pCPT-cGMPS were purchased from Biomol, Plymouth Meeting, PA. Radiolabeled PAF standards and substrates: hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, 1-O-[acetyl-^{3}H-(N)]-, (^{3}H-acetyl-C_{16}-PAF), 21.5 Ci/mmol (370 GBq/mmol), and ^{3}H-thymidine were purchased from Perkin Elmer Life Sciences (Boston, MA). siRNA to PKA-Cα and its control were purchased from Cell Signaling Technologies (Carlsbad, CA). Phenylmethysulfonyl fluoride, leupeptin, pepstatin, as well as bovine serum albumin, were purchased from Sigma Chemical Company (St. Louis, MO). Antibody to PKA and PKG were purchased from Cell Signaling, while PAFR antibody was purchased from Cayman Chemical Company (Ann Arbor, MI). Studies were done with freshly made reagents. Ecolite(+) liquid scintillation cocktail was purchased from MP Biochemicals (Irvine, CA).

2.2. Methods

Arterial intrapulmonary vessels were isolated from freshly killed term fetal lambs and then smooth muscle cells were harvested from the excised arteries and veins under sterile conditions as previously reported [20, 32]. Cells were used at the 3rd to 10th passage. Cell phenotype did not change from 1st to 10th passage as determined by the expression of α-smooth muscle actin and myosin light-chain kinase proteins.

2.3. Study conditions

Studies were done with adherent cells in normoxia and in hypoxia.

Normoxia: Cells were studied in humidified incubator at 37 °C aerated with 5% CO_2 in air.

Hypoxia: An incubator set at 37 °C was first equilibrated for at least 1 h with a gas mixture of 2% O_2, 10% CO_2, and balance N_2 to maintain incubator culture media $pO_2 < 40$ Torr, and to

mimic fetal lung environment, and monitored with TED 60T percent oxygen sensor, Teledyne Analytical Instruments (City of Industry, CA). Cells were then placed in this equilibrated incubator and continuously aerated with the hypoxia gas mixture throughout the duration of the study.

2.4. Study of PAFR binding

2.4.1. General protocol

Receptor-binding assays were performed during hypoxia and normoxia as we previously reported [30]. Briefly, after incubation in normoxia or hypoxia, unbound ^{3}H-PAF was washed off with ice-cold phosphate buffer saline, and then incubated on ice for 30 to 45 min in saline/EDTA mixture containing 154 mM saline and 5 mM EDTA. Receptor bound ^{3}H-PAF was extracted on Whatman GF/C membrane filters using inline vacuum system. Then culture flasks or dishes were washed with calcium-free 0.25% bovine serum albumin-containing Tyrodes buffer, pH 6.4. Cell-bound PAF radioactivity was quantified by scintillation spectrometry (Beckman Instruments, Fullerton, CA). In studies probing the interaction of PAF with its receptors in the presence of other agonist or antagonists, cells were pre-incubated with the agent before the addition of ^{3}H-PAF, and then incubated further according to the specific experimental protocol.

2.4.2. Specific protocols

Effect of cAMP/cGMP on PAFR binding: Cells were pre-incubated for 30 min, in normoxia or hypoxia, with buffer for controls or 10 μM each of cell permeable cyclic nucleotide analogs, 8-Br-cAMP or 8-Br-cGMP; the cAMP/PKA receptor inhibitor Rp-cAMPS or the cGMP/PKG receptor inhibitor Rp-8-pCPT-cGMPS. Then 1.0nM ^{3}H-PAF was added and incubated for 30 min more.

Effect of cAMP/cGMP on PAFR protein expression: Cells were pre-incubated for 3 hr min, in hypoxia or normoxia, with buffer for controls or 10 μM each of cell-permeable cyclic nucleotide analogs, 8-Br-cAMP or 8-Br-cGMP. Membrane proteins were isolated from cultured cells and probed for PAFR protein expression by Western blotting.

Proliferation assay: Sub-confluent cells were serum starved by culturing in 0.1% fetal bovine serum (FBS) for 72 hr, then cells were cultured in 10% FBS with or without the test agents in the presence of 5μCi/well of ^{3}H-thymidine and incubated for 24 hr more in the oxygen condition [32]. Cell DNA that were labeled with ^{3}H-thymidine were extracted with 0.5N NaOH and quantified on LKB 6500 scintillation counter (Beckman Coulter, Fullerton, CA).

Transient cell transfection: Cells were seeded in 6-well culture plates at 5×10^{4} cells per well in an antibiotic-free growth media and allowed to stabilize for 24 h. Then they were treated with 1.5μg/ml of each plasmid in lipofectamine transfection reagent with 50nM of PKA-Cα siRNA according to the vendor's protocol (Cell Signaling) and incubated for 48 hr after which the transfection medium was replaced with fresh 10% FBS culture medium, which was also used to study cell proliferation or to study PAFR binding. Transfection efficiency was between 25%

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and 35% within 24 hr of transfection as judged by the pGFP fluorescence [32]. The proliferative phenotype of transfected cells was compared to that of untransfected cells. In studying cell proliferation or PAFR binding, transfected cells were incubated for 24 hr for proliferation study or 30 min for PAFR binding, with and without 10nM PAF in 10% FBS. Control for cell proliferation is 10% FBS alone while cells transfected with scrambled siRNA (sham siRNA) were used as control for PKA-Cα siRNA effect.

2.5. Western blotting

Preparation of proteins for Western analysis: Proteins were prepared from stimulated and unstimulated cells that were studied in normoxia or hypoxia according to our previous reports. Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Western blotting for PAFR quantification following our published methods [20, 30, 32, 33]. After x-ray exposure, band corresponding to PAFR protein was quantified and normalized to β-actin density.

2.6. Data analysis

All numerical data are mean ± SEM. In all instances where radioisotope was used, background radioactivity was subtracted before quantifying radioactivity. Data were analyzed with two-tailed *t*-test followed with ANOVA (GraphPad Prism 6, San Diego, CA). Results were considered significant at $p < 0.05$.

3. Results

Figure 1 shows the effect of 8-Br-cAMP and 8-Br-cGMP on PAF binding to its receptors in pulmonary arterial smooth muscle cells (PASMC) (fmol/10^6 cells). For both 8-Br-cAMP and 8-Br-cGMP, data are mean ± SEM, $n = 6$; $^*p < 0.05$, different from normoxia; $^+p < 0.05$, different from PAF alone in normoxia or hypoxia. Cells treated with 10nM PAF alone, binding in normoxia was 9.7 ± 1.0, which increased to 17.2 ± 1.0 in hypoxia. Pretreatment of cells with 10μM of 8-Br-cAMP decreased PAF by 31% in normoxia and by 29% in hypoxia, respectively. Inhibition of endogenous cAMP effect with 1μM of the cAMP/PKA receptor inhibitor, Rp-cAMPS in normoxia or hypoxia increased PAF binding by 65% compared to effect of PAF alone in normoxia and by 35% compared to effect of PAF alone in hypoxia. Also, pretreatment of cells with 10μM Br-cGMP decreased PAF binding by 38% and 40% in normoxia and hypoxia, respectively, compared to the effect of PAF alone. Inhibition of endogenous cGMP activity with the cGMP/PKG receptor antagonist Rp-8-pCPT-cGMPS (Rp-pCPT) increased PAF binding by 20% compared to PAF alone in normoxia, and by 30% in comparison to binding by PAF alone in hypoxia. In general, hypoxia increased PAFR binding; exogenous cAMP or cGMP attenuated PAFR binding while inhibition of endogenous cAMP or cGMP activity with specific cAMP/PKA or cGMP/PKG receptor antagonists restored PAFR binding to levels higher than effect of PAF alone.

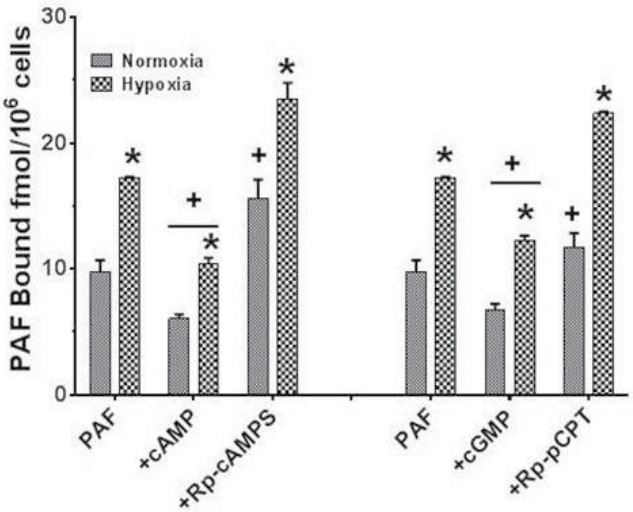

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Figure 1. Effect of cAMP and cGMP on PAF receptor binding FPASMC. Data are means ± SEM, n = 6. Cells were pre-incubated for 30 min in normoxia or hypoxia with buffer alone, or 10μM of 8-Br-cAMP or 10μM of 8-Br-cGMP then 10nM PAF was added and incubated for 30 min more as described in the methods section. Hyoxia increased PAF receptor binding and both 8-Br-cAMP and 8-Br.cGMP inhibited PAF receptor binding in normoxia and hypoxia. The specific inhibitors of PKA and PKG signaling; Rp-cAMPs and Rp-8-pCPT-cGMP reversed inhibitory effects of 8-Br-cAMP and 8-cGMP. *p <0.05, different from PAF normoxia; +p <0.05, different from PAF alone in normoxia or hypoxia.

Effect of cyclic nucleotides cAMP and cGMP on PAF stimulation of fetal pulmonary arterial smooth muscle cells (FPASMC) growth (^{3}H-thymidine DPM × 10^3) is shown in Figure 2. For both 8-Br-cAMP and 8-Br-cGMP, data are mean ± SEM, $n = 6$, $^*p < 0.05$, different from normoxia; $^+p < 0.05$, different from PAF alone in normoxia; $^\#p < 0.05$, different from PAF alone in normoxia or hypoxia. Treatment of cells with 10nM of PAF in normoxia caused a 15% increase in cell proliferation compared to effect of FBS control (55.9 ± 2.2). In hypoxia, PAF increased cell proliferation by 25% compared to effect of FBS control in hypoxia (70.5 ± 8.4). Incubation with 10μM 8-Br-cAMP in normoxia or hypoxia produced no difference in cell proliferation compared to PAF effect in hypoxia (Figure 2a). However, co-incubation of cells with 10nM PAF in the presence of cAMP significantly inhibited cell proliferation below effect of FBS control or 10nM PAF in normoxia and hypoxia. With cGMP (Figure 2b), treatment of cells with 10μM 8-Br-cGMP in normoxia or hypoxia decreased cell proliferation by 40% in normoxia compared to FBS control, and by 48% in compared to effect of 10nM PAF in normoxia. On the other hand, 8-Br-cGMP significantly increased cell proliferation compared to the effect of FBS control and 10nM PAF in hypoxia. Co-incubation of cells with PAF and 8-Br-cGMP in normoxia decreased cell proliferation by 77% in compared to FBS control and by 80% compared to PAF alone. Co-incubation with 8-Br-cGMP and PAF in hypoxia produced no significant difference in cell growth compared to effects of FBS and 10nM PAF in hypoxia. Thus, cAMP and cGMP produced different effects of PAF stimulation of FPASMC growth during normoxia and hypoxia.

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Figure 3 shows representative Western blots of effect of cAMP and cGMP on PAFR protein expression. For both 8-Br-cAMP and 8-Br-cGMP, data are mean ± SEM, $n = 3$. The statistics are as shown in the figures. The figures are not meant to be a cross-comparison of the effects of 8-Br-cAMP and 8-Br-cGMP on PAFR protein expression, but rather to compare effect of each cyclic nucleotide on PAFR protein expression. However, the figures show that hypoxia increased PAFR protein expression and in both cases, cAMP (Figure 3a) and cGMP (Figure 3b) decreased PAFR protein expression compared to control conditions.

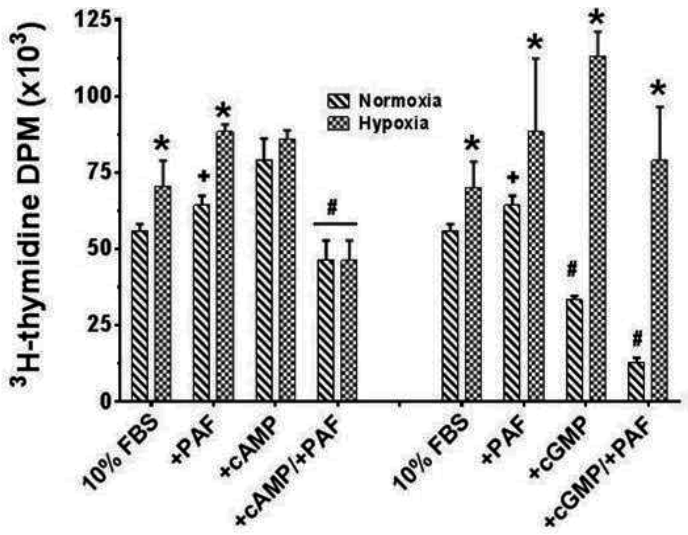

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Figure 2. Effect of cAMP and cAMP on PAF stimulation of FPASMC proliferation. Data are means ± SEM, n = 6. Cells were pre-incubated for 30 min with buffer alone, or with 10 μM 8-Br-cAMP, or 10μM of 8-Br-cGMP, then PAF was added and incubated for 24 hr more as described in methods section. PAF increased cell proliferation in normoxia and hypoxia. Effect of 8-Br-cAMP on cell proliferation in normoxia or hypoxia, co-incubation of 8-Br-cAMP and PAF decreased PAF-induced cell proliferation. 8-Br-cGMP alone decreased cell proliferation in normoxia, and co-incubation with PAF further decreased PAF stimulation of cell proliferation. *p <0.05, different from normoxia; +p <0.05, different from effect of 10% FBS; #p <0.05, different from PAF effect.

We then investigated the effect of PKA siRNA PAFR binding and PAF stimulation of FPASMC proliferation in normoxia only. For both binding and proliferation, data are mean ± SEM, $n = 6$; $^*p < 0.05$, different from 10% FBS control, $^+p < 0.05$, different from PAF alone, $^\#p < 0.05$, different from 10% FBS control, PAF alone, and 8-Br-cAMP (+cAMP). In Figure 4a, 8-Br-cAMP decreased PAFR binding by 26% (PAF alone control, 10.2 fmol/10^6 cells). Pretreatment of cells with 50nM of the PKA-Cα siRNA increased PAFR binding by 47% compared to effect of PAF alone (control) and by 52% compared to effect of cAMP alone. The effect of sham PKA-Cα siRNA (sham) was not different from PAF alone. In Figure 4b, PAF alone stimulated greater cell proliferation (^{3}H-thymidine DPM × 10^3) by 45% compared to 10% FBS control (101.2 ± 5.3). Effect of 8-Br-cAMP on cell proliferation was 120.3 ± 7.8 which is 19% lower than effect of 10% FBS control and 20% lower than effect of 10nM PAF alone. As with PAFR binding, PKA-Cα increased cell proliferation and reversed the effect of cAMP on PAF binding. PAFR binding

produced a 2-fold increase in cell proliferation compared to 10% FBS control and 37% increase compared to effect of 10nM PAF. The effect of the sham siRNA control was not different from effect of 10% FBS control.

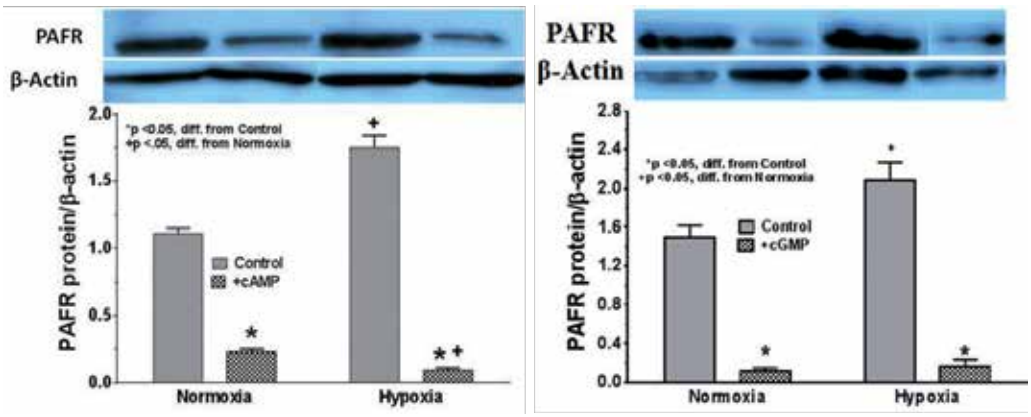

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Figure 3. Effects of cAMP and cGMP on PAF receptor protein expression in normoxia and hypoxia. Data are means ±SEM, n= 3. Cells were incubated in for 24 hr in normoxia or hypoxia with buffer alone, or with 10μM of 8-Br-cAMP, figure 3a, or with 10μM 8-Br-cGMP, figure 3b. PAF receptor (PAFR) expression was measureb by Western blotting and normalized to expression of beta-actin standard. Rp-8-pCPT-cGMPS, then 5nM PAF was added as needed and incubated for 20 min more. Treatment of cell with 8-Br-cAMP or 8-Br-cGMP surpressed PAFR protein expression in normoxia and hypoxia. *p <0.05, different from control; +p <0.05, different from normoxia.

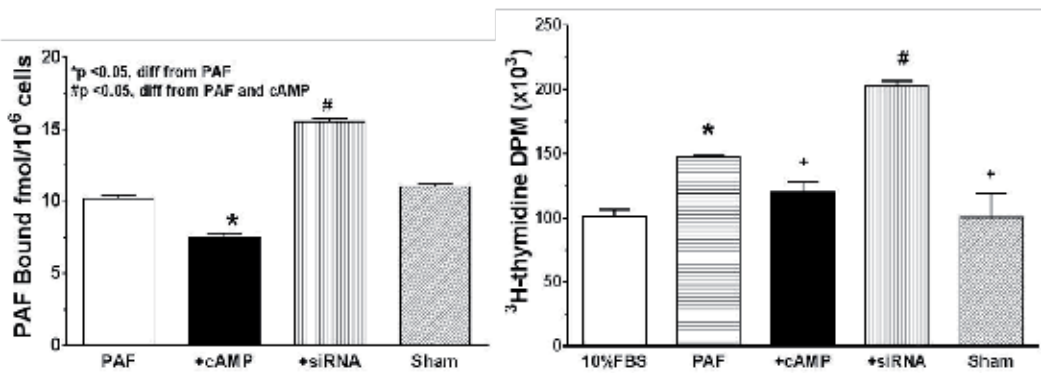

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Figure 4. Effects of cAMP and and siRNA to PKA-cα on PAFR binding and PAF stimulation of cell proliferation. Data are means ± SEM, n= 5. Studies were done as described in methods section. PAFR binding (figure 4a) and cell proliferation were determined. PAFR binding was attenuated by 8-Br-cAMP, but siRNA to PKA-cα increased PAFR binding. Effect of sham siRNA was not different from PAF alone. In figure 4b, 8-Br-cAMP decreased cell proliferation compared to effect of PAF alone. siRNA to PKA cα increased cell proliferation. Effect of sham siRNA was not different from 10% FBS or 8-br-cAMP. *p <0.05, different from PAF alone of 10% FBS control; +p <0.05, different from PAF alone; #p <0.05, different from all other condition.

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4. Discussion

In fetal pulmonary circulation, vasomotor tone is maintained high by multiple mechanisms such as elevated calcium, PAF, and thromboxane A_2 [22, 34, 35]. At birth, endogenous vasodilators induce smooth muscle relaxation via increased intracellular concentrations of the cyclic nucleotides, cAMP and cGMP, acting through their respective receptors PKA and PKG [27]. We have published previously that in ovine fetal pulmonary vascular smooth muscle cells, hypoxia upregulates PAFR binding and PAFR-mediated intracellular IP_3 and calcium release [30], suggesting that the hypoxic environment of the fetus facilitates PAFR binding and PAFR-mediated signaling and the maintenance of a high pulmonary vasomotor tone in utero. The possibility that vasoconstrictors such as PAF may actively downregulate vasodilator pathways in the hypoxic environment of fetal pulmonary circulation is being actively explored. Similarly, the decreased PAFR-mediated activity in the higher oxygen environment of the postnatal lung may involve the downregulation of PAFR-mediated cell signaling by other endogenous mediators such as cAMP and cGMP [36, 37]. The present report investigates the interaction between cyclic nucleotides, cAMP and cGMP, and PAF signaling pathway in FPASMC. We have found that in ovine fetal pulmonary vascular smooth muscle cells, both cAMP and cGMP decrease PAFR binding in normoxia through the actions of their respective kinases, PKA and PKG. Addition of PAF in physiologic concentrations to pulmonary venous smooth muscle cells decreased PKA and PKG protein expression and kinase activities during normoxia and hypoxia, suggesting that in the hypoxic environment of the fetal lungs, PAF may be actively downregulating cAMP- and cGMP-dependent signaling pathways and that postnatally, in the normoxic environment, cAMP and cGMP actively inhibit PAF binding to its receptors and PAFR-mediated signaling. This cross-talk between the two pathways will effectively maintain a high pulmonary vasomotor tone in utero and facilitate vasorelaxation at birth.

4.1. Cyclic nucleotides inhibit PAFR binding and PAFR-mediated cell proliferation

Role of cAMP: cAMP production is linked to β-adrenergic receptor-mediated activation of adenylyl cyclase [38, 39]. Inhibition of cAMP- and cGMP-dependent phosphodiesterases (PDEs) can also result in high cellular levels of cAMP and cGMP [38, 40], but the effect of cAMP and cGMP on PAFR-mediated signaling in PASMC has not been reported. Unlike in pulmonary venous smooth muscle cells, the cell permeable analog of cAMP, 8-Br-cAMP, decreased PAFR binding in normoxia and hypoxia. Inhibition of cAMP-dependent PKA, the endogenous receptor of cAMP, with Rp-cAMPS, reversed the inhibitory effect of endogenous cAMP and significantly increased PAFR binding. Also, when PKA was inhibited with Rp-cAMPS, PAF binding in hypoxia was increased beyond the effect of addition of exogenous PAF alone. This suggests that cAMP interacts with PAFR after activation of its own receptor, PKA.

Effect of cAMP downstream from PAFR in the nucleus of PASMC is not clear. 8-Br-cAMP enhanced cell growth in normoxia, with no change in cell growth in hypoxia compared to PAF effect in hypoxia. However, when cells were pulsed with 8-Br-cAMP and exposed to PAF, cell proliferation was significantly decreased in normoxia and hypoxia, suggesting that cAMP

effect occurs after activation of its receptor. This relationship is relevant physiologically because it suggests that postnatally, cAMP will stimulate growth of PASMC and under this condition, the presence of PAF will be detrimental cell growth and pulmonary vascular development. Thus, we can speculate that cAMP/PKA-mediated inhibition of PAF effects, in vivo, may constitute one mechanism whereby the postnatal vasodilator properties of cAMP are maintained. Our studies with PKA siRNA demonstrate that cAMP acts at its receptor, PKA, to inhibit postnatal adverse PAFR-mediated responses in the pulmonary circulation of the newborn lamb lung.

Role of cGMP: Endothelium-derived nitric oxide produced under basal conditions, or by a stimulus, readily diffuses into the contiguous smooth muscle to activate soluble guanylyl cyclase, resulting in an increase in cGMP synthesis and smooth muscle relaxation [37, 38]. Nitric oxide is a potent vasodilator in the pulmonary circulation and it is important in the transition of the pulmonary circulation from fetal to postnatal life. In this study, 8-Br-cGMP blunted PAFR binding to PASMC in physiologic oxygen level (normoxia) and in hypoxia. This suggests that cGMP produced in vivo will counteract the vasoconstricting properties of PAF. Interestingly, inhibition of endogenous cGMP activity with the inhibitor of cGMP-dependent PKG, the endogenous receptor of cGMP, resulted in abrogation of the ability of cGMP to inhibit PAF binding to its receptors, similar to the effect of PKA receptor antagonist. This shows that cGMP acts via its receptor to inhibit PAFR binding.

cGMP has been shown to inhibit endothelin-stimulated inositol phosphate release in pulmonary artery of fetal lambs studied in organ bath [41]. Inositol phosphate is released downstream from PAFR effect. Both endothelin and PAF are potent endogenous vasoconstrictors in the pulmonary circulation. This shows that increased levels of endogenous PAF under normoxic conditions can inhibit cGMP effect and as a corollary, increased levels of cGMP after birth can inhibit PAF effect in normoxia, leading to increased vasodilation. Our data show that cAMP and cGMP produce different effects on cell proliferation. During normoxia, cGMP inhibited PAF stimulation of cell proliferation, but the effect in hypoxia seemed to be stimulatory. This physiological significance of this effect is not clear, but may indicate a protective role of cGMP against unwarranted cell growth in the presence of PAF.

We can infer that in vivo, activation of PKA will result in inhibition of PAFR-mediated effects such as stimulation of inositol phosphate release, calcium mobilization, and vasoconstriction. As with cGMP effect, cAMP/PKA-mediated inhibition of PAF effects, in vivo, may constitute one mechanism whereby the postnatal vasodilator properties of cAMP are maintained.

4.2. PAF and regulation of PKG and PKA activity and role in perinatal pulmonary adaptation

Previous reports have shown that the activities of PKG and PKA are upregulated by normoxic condition [36, 37, 42]. The physiologic implications of these findings are that upregulation of PKG and PKA activities by normoxia, after birth, is one mechanism by which fetal high pulmonary vasomotor tone is downregulated to facilitate postnatal pulmonary adaptation. It can be deduced that favorable perinatal pulmonary vascular adaptation can be achieved by both downregulation of mediators of pulmonary vascular constriction, such as PAF, and upregulation of mediators of pulmonary vasodilation such as cGMP/PKG and cAMP/PKA.

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Endothelin [43] and protein kinase C (PKC) [44] are two other mediators that have been reported to evoke vasoconstriction in the perinatal pulmonary circulation. In this report, we show that during normoxia, PAF significantly downregulates the activities of both cGMP- and cAMP-dependent protein kinases. We also found that exposure of the smooth muscle cells to 8-Br-cAMP and 8-Br-cGMP for 30 hr in normoxia and hypoxia, resulted in significant downregulation of PAFR in line with attenuation of PAFR binding to the cells. These findings strongly indicate that congenial perinatal pulmonary adaptation entails a combination of downregulation of PAFR-mediated effects by cyclic nucleotide-mediated pathways as we have shown in this report, as well as by upregulation of cGMP- and cAMP-mediated pathways as has been previously reported [31, 36, 37, 42].

PPHN is a pathological condition with different etiologies. High PAF levels have been reported in neonates with PPHN [31, 45], suggesting that persistence of high PAF levels postnatally may lead to abnormal perinatal pulmonary adaptation. In addition, we speculate that inhibition of PKG and PKA activities by high levels of PAF and the inability of the cyclic nucleotides to downregulate PAFR-mediated effects postnatally will also contribute to the development of PPHN.

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In order to complete tissue regeneration, various cells such as neuronal, skeletal, smooth, endothelial, and immune (e.g., macrophage) interact smoothly with each other. This book, Muscle Cells and Tissues, offers a wide range of topics such as stem cells, cell culture, biomaterials, epigenetics, therapeutics, and the creation of tissues and organs. Novel applications for cell and tissue engineering including cell therapy, tissue models, and disease pathology modeling are discussed. The book also deals with the functional role of autophagy in modulating muscle homeostasis and molecular mechanism regulating skeletal muscle mass. The chapters can be interesting for graduate students, postdocs, teachers, physicians, and for executives in biotech and pharmaceutical companies, as well as researchers in the fields of molecular biology and regenerative medicine.

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