Vascular Smooth Muscle

Structure and Function in Health and Disease

Chi-Ming Hai *editor*

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VASCULAR SMOOTH MUSCLE
Structure and Function in Health and Disease

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This book is dedicated to the many pioneers of smooth muscle research, especially my mentor, colleague and friend, Dr. Richard A. Murphy, to current investigators who have brought unique insight into this complex field of endeavor, and to future investigators who we hope will solve the numerous important questions that remain.

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Contents

Chapter 1

Introduction

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This book covers core concepts in the structure and function of vascular smooth muscle cells in health and disease. Supplemental reading may be drawn from the extensive number of references listed at the end of each chapter. Vascular smooth muscle cell is the major cell type in blood vessels. Dysfunction of vascular smooth muscle cells is an important cause of vascular diseases — for example, atherosclerosis, hypertension, and circulatory shock. Vascular smooth muscle cells are phenotypically plastic, capable of switching between two major phenotypes — contractile/differentiated phenotype and invasive/proliferative phenotype — in response to environmental clues. This book is organized in three sections. Section I (chapters 2 to 4) addresses the structure and function of the contractile/differentiated phenotype of vascular smooth muscle cell. Section II (chapters 5 and 6) addresses the developmental basis of vascular smooth muscle cell phenotype and structure and function of podosomes (invasive organelles) in the invasive/proliferative phenotype of vascular smooth muscle cell. Section III (chapters 7 to 9) addresses the role of vascular smooth muscle cell dysfunction in three vascular diseases — atherosclerosis, hypertension, and circulatory shock.

1. Section I (Chapters 2 to 4)

Structure and Function of Contractile/Differentiated Phenotype of Vascular Smooth Muscle Cell. In Chapter 2, Dr. Thomas Eddinger discusses the structure of blood vessel and contractile phenotype of vascular smooth muscle cell at multiple layers of organization — blood vessel, smooth muscle cell, contractile filaments, cytoskeleton, membrane associated proteins, nucleoskeleton, regulatory proteins, organelles, and extracellular matrix. Contractile filaments include thin and thick filaments, and the associated proteins and isoforms — for example, tropomyosin, myosin heavy chain and light chain isoforms. Cytoskeleton includes actin, intermediate filament (vimentin and desmin), microtubules, and their associated proteins — for example, plectin, filamin, cadherins, and catenins. Regulatory proteins include tropomyosin, caldesmon, calponin, myosin light chain kinase and myosin light chain phosphatase. Organelles include sarcoplasmic reticulum and nucleus. Dr. Eddinger concludes his chapter by posing some unanswered questions on vascular smooth muscle structure and function.

In Chapter 3, Dr. Paul Ratz discusses receptor signaling mechanisms for vascular smooth muscle contraction and relaxation. Dr. Ratz first provides an overview of the classification of smooth muscle cells into fast, phasic and slow, tonic subtypes, and their differential muscle mechanics, intracellular [Ca^{2+}] regulation, and cell signaling. He then discusses extracellular stimuli (neurotransmitters, hormones and local mediators) that regulate smooth muscle contraction and the canonical control of smooth muscle contraction through regulation of myosin light chain phosphorylation. In particular, he discusses the phosphorylation of myosin light chain by Ca^{2+}, calmodulin-dependent myosin light chain kinase and the modulation of Ca^{2+}-sensitivity of myosin light chain phosphorylation by myosin light chain phosphatase. He further details the roles of small GTPases (rac and rhoA), rho-activated kinase (ROCK), calmodulin-dependent kinase II (CaMKII), mitogen activated kinase (Erk), and PKC in the regulation of myosin light chain phosphorylation and contraction. He concludes the chapter by discussing the function of multiple phosphorylation sites of myosin

light chain and non-canonical myosin light chain kinases in the regulation of smooth muscle contraction.

In Chapter 4, Drs. William Cole and Michael Walsh discuss the function of actin filament dynamics during vascular smooth muscle contraction. Drs. Cole and Walsh first discuss the contribution of vascular smooth muscle contraction to the control of blood flow and the concepts of Ca^{2+}-induced vasoconstriction and Ca^{2+}-sensitization of vasoconstriction. Specific Ca^{2+}-sensitization mechanisms include RhoA, Rho-associated coiled-coil kinase (ROCK), myosin targeting subunit of myosin light chain phosphatase (MYPT1) and a 17-kDa cytosolic protein (CPI-17). They then discuss recent findings on the function of actin polymerization in Ca^{2+} sensitization of vasoconstriction and signal transduction pathways mediating stimulus-evoked actin polymerization. Specific signaling mechanisms include Src family kinases (SFK), focal adhesion kinase (FAK), Pyk2, p130CAS and PKC. Specific cytoskeletal proteins include α-actinin, vinculin, talin and paxillin. They conclude the chapter by discussing the potential pathophysiological significance of actin polymerization in vascular dysfunction such as hypertension and cerebral vasospasm following subarachnoid hemorrhage.

2. Section II (Chapters 5 and 6)

Developmental Basis of Vascular Smooth Muscle Cell Phenotype, and Structure and Function of Podosomes (Invasive Organelles) in the Invasive/Proliferative Phenotype of Vascular Smooth Muscle Cell. In Chapter 5, Drs. Christine Cheung and B C Narmada discuss the developmental basis of vascular smooth muscle cell phenotype by first emphasizing the diverse embryonic lineages of vascular smooth muscle cells from different blood vessels and even different regions within the same blood vessel. This observation suggests the hypothesis that lineage differences among vascular smooth muscle cells at different regions of the vasculature can explain region-specific vascular disease development. They then discuss the following specific topics: (a) triggers of phenotypic modulation, (b) influence of embryonic origins on regional differences of vascular smooth muscle cells,

and (c) molecular basis of lineage-specific differences in vascular smooth muscle subtypes — embryonic smooth muscle cells, postnatal smooth muscle cells, and human pluripotent stem cell-derived smooth muscle cells. They conclude the chapter by suggesting that stem cell-derived vascular smooth muscle cells hold great potential for tissue engineering applications and regenerative medicine, high throughput drug screening and pharmacokinetic testing, and targeted therapeutic interventions for restoration of vascular health.

In Chapter 6, Dr. Alan Mak discusses the structure and function of podosomes — invasive organelles that enable vascular smooth muscle cells to degrade and invade the extracellular matrix. He begins the chapter by emphasizing the remarkable plasticity of vascular smooth muscle cells in switching between contractile and synthetic phenotypes and highlighting the importance of acquiring the migratory and invasive phenotype for vascular smooth muscle cells to degrade the extracellular matrix and cross the basement membrane in the process of reaching the intima. He then discusses the following specific topics: (a) podosomes in non-smooth muscle and vascular smooth muscle cells, (b) regulation of podosome formation in vascular smooth muscle cells by the PKC and cSrc-dependent pro-podosome and p53-dependent anti-podosome signaling pathways, and (c) regulators of podosome-mediated extracellular matrix adhesion and degradation. He concludes the chapter by suggesting future directions for research on the structure and function of podosomes in vascular smooth muscle cells in relation to the specific roles of vascular smooth muscle cells in the pathogenesis and progression of atherosclerotic plaques.

3. Section III (Chapters 7 to 9)

Role of Vascular Smooth Muscle Cells in Vascular Diseases — Atherosclerosis, Hypertension, and Circulatory Shock. In Chapter 7, I discuss the role of vascular smooth muscle cell proliferation and invasion in atherosclerosis. I first emphasize the important role of vascular smooth muscle cells in atherosclerosis by highlighting the observation that vascular smooth muscle-rich regions of coronary arteries are more prone to the development of atherosclerosis,

whereas vascular smooth muscle-sparse regions are more resistant to the development of atherosclerosis. I then discuss the multiple stages of atherosclerosis progression and the specific roles of vascular smooth muscle cells in promoting atherosclerosis development and plaque stabilization at each stage of atherosclerosis. I conclude the chapter by suggesting that there is emerging consensus that vascular smooth muscle cells are a central player in all stages of atherosclerosis and re-emphasizing the two opposing roles of vascular smooth muscle cells in atherosclerosis — detrimental role in promoting plaque development during early stage of atherosclerosis but beneficial role in promoting plaque stabilization during later stage of atherosclerosis.

In Chapter 8, Drs. Christopher Nicholson and Kathleen Morgan discuss the role of non-coding RNA in the control of vascular contractility and disease. They begin the chapter by introducing the general structure and function of microRNAs and long non-coding RNAs. In the first section, they discuss the following topics on the molecular biology of non-coding RNA: (a) microRNA biogenesis, (b) RNA-induced silencing complex, (c) microRNA target recognition, and (d) control of microRNA expression. In the second section, they discuss the following topics on the function of microRNA in vascular smooth muscle cells: (a) microRNA-dependent contractile differentiation of vascular smooth muscle cells, (b) role of microRNAs in vascular smooth muscle pathways of contraction, and (c) microRNA dysfunction in hypertension, hyperlipidemia and diabetes, atherosclerosis, and pulmonary vascular disease. They conclude the chapter by discussing the function of microRNA in extracellular communication in vascular cells, function of long non-coding RNAs in vascular smooth muscle, and modulating microRNAs in the treatment of vascular disease.

In Chapter 9, Drs. Liangming Liu, Tao Li, and Chengyang Duan discuss the potential of vascular smooth muscle cells as therapeutic target for the treatment of circulatory shock. They first discuss the clinical significance of circulatory shock, function of vascular smooth muscle cells in vasodilation and vasoconstriction, and the contribution of reduced vascular reactivity to circulatory shock. They then discuss the general concepts of inducing factors of vascular smooth

muscle cell damage and features of vascular dysfunction during circulatory shock. Third, they discuss the following topics on hemorrhagic shock: (a) biphasic change of vascular reactivity, (b) vasculature, gender, and age-differences of vascular reactivity, (c) metabolic diseases suffering from hemorrhagic shock, and (d) similarities and differences between endotoxin/septic shock and hemorrhagic shock. Fourth, they discuss the following topics on shock-induced vascular smooth muscle cell damage and vascular hypo-reactivity: (a) receptor desensitization, (b) membrane hyperpolarization, and (c) calcium desensitization. They conclude the chapter by discussing treatments of circulatory shock based on mechanisms of vascular smooth muscle cell damage and vascular hypo-reactivity.

Chapter 2

Structure of Differentiated/Contractile Vascular Smooth Muscle Cells

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The study of structure and its relationship to function has been, and will continue to be, significant for advancing our understanding of organismal, systems, organ, tissue, cell and sub-cellular physiology. While novel organismal anatomical observations have become rather rare at the gross level, this is far from true at the molecular level where new data on molecular structures continue to expedite advancement of our understanding of their function. William Harvey (1578–1657) is credited with significant advances in our understanding of cardiovascular function through his "exercises" where he applied quantitative reasoning with cardiac and vascular anatomy to derive physiological significance. In so doing he resolved major questions that had no answers, or perhaps worse, had answers but that were incorrect. He is credited with numerous cardiovascular advancements including both ventricles beating simultaneously (not asynchronously), systole forcing blood through the vascular bed (not by vascular contraction), one way circular flow (veins do not carry blood to the tissue), blood recirculation (it is not made in the liver and consumed by the tissue), and valves preventing backflow of blood in the veins (not necessary when the blood is traveling to the tissues via the

veins) to list a few.[1] Significant technological advances since Harvey, especially in the past century, have allowed cellular and subcellular anatomy to continue to add to our understanding of function at these levels. Not least of these advances are a host of new and/or refined crystallization methods and microscopic techniques that allow us to "see" things that were never possible before. Thus while much of what we know about the vascular system in general, and smooth muscle cell structure and function specifically has been known for decades and longer, researchers continue to use old and new knowledge of structure (including protein distribution, localization, and interactions) to facilitate our understanding of function. With this in mind, it is the goal of this chapter to review the major structural organization of the vascular smooth muscle cell (SMC), but also to remind the reader that none of what is in the cell is there in isolation. Everything is connected to something else either physically or through sometimes very complex signaling pathways. This chapter will proceed with a brief overview of the vascular system followed by the major molecules in and around the vascular SMC, and then some observations about where this may lead us with further research.

1. Vascular Structure

Smooth muscle tissue is one of the three contractile muscle tissues in the body (skeletal, cardiac, and smooth). Unlike skeletal and cardiac muscles which are both "striated" (showing a banding pattern with microscopy as a result of the highly organized and repeating pattern of the contractile proteins), smooth muscle is not striated. Smooth muscle tissue is a major component in the wall of hollow organs, which may include, beside the muscle cells, other components such as capillaries, nerves, endothelium, mucosa, secretory cells and so-forth. Smooth muscle cells are present in every major organ system either as a major cell type present (i.e., vascular, digestive, reproductive, urinary, and respiratory systems) or as a critical component (vascular system) for supplying nutrients to and removing waste products from other organ systems (i.e., skeletal, muscle, and nervous systems). In the vascular system the SMC are typically located between the endothelium

(squamous epithelium on the luminal surface of vessels) and the adventitia (loose connective tissue layer with wandering cells, nerves, etc., on the adluminal surface) in all but the smallest of vessels (capillaries)[2] (Fig. 1). In the high pressure arterial side of the vascular system the larger conduit vessels and elastic arteries have both an internal and external elastic lamina (internal between the endothelium and the media and the external between the media and the adventitia), and numerous elastin layers separating the medial SMCs into lamina. These alternating contractile SMC and elastic layers form functional units.[3] With the blood pressure drop along the vascular system the arteries change to "muscular" arteries that do not have elastin layers in the medial layer and eventually lose all of their elastin layers. The capillaries have a single endothelial/pericyte cell surrounding their lumen which minimizes diffusion distances for exchange of gases and nutrients. The venous side of the arterial system is generically similar to the arterial side in containing endothelial, medial and advential layers. However, these layers may not be as well resolved, and include fewer smooth muscle cells with less elastic fibers while generally having more advential tissue.[2] Between the SMCs, there are varying amounts of extracellular matrix including significant elastin (in the larger vessels), collagen, laminin, fibrillin, fibulin and proteoglycans.[4] Primarily in the adventitia, smaller blood vessels and nerves may be observed.

2. Smooth Muscle Cells

Numerous structural studies of smooth muscle including subcellular, cellular, and tissue level organization, going back approximately 50 years, provide the basis for our current understanding of this tissue system and an excellent introduction to and/or review of vascular and smooth muscle cell anatomy and histology.[5–14] Figure 2 shows electron micrographs (longitudinal and transverse to the SMC) at increasing magnifications showing the distribution and density of structural components within the SMC. SMCs develop primarily from the lateral plate mesoderm and the cranial neural crest cells.[15,16] SMCs are spindle shaped with either a single tapered tip at each end or multiple tips at each end.[17] Their size is variable and dependent on being in a

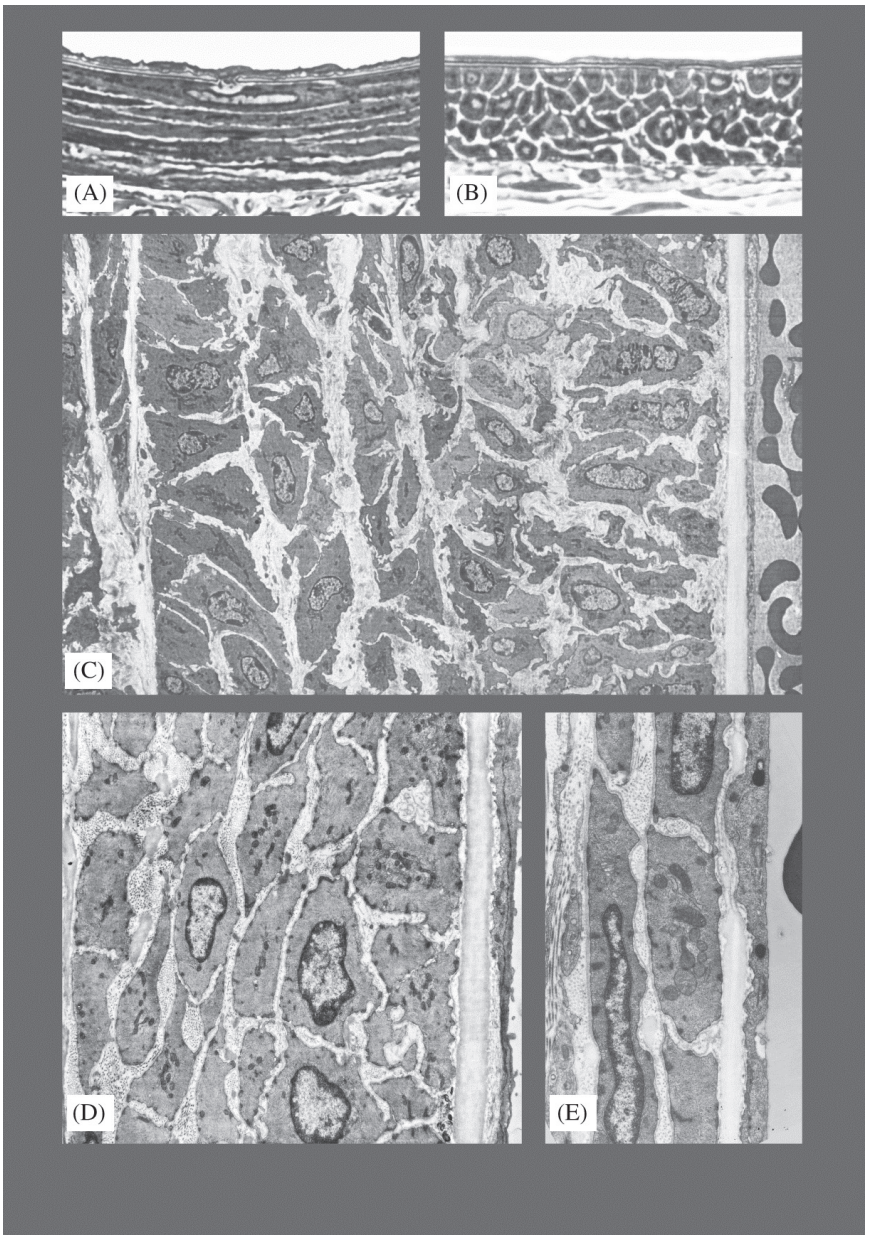

Fig. 1. A and B show a mesenteric artery in transverse section (A) and cut longitudinally right through the middle (B), examined by light microscopy. The lumen, emptied of blood, is at the top and is lined by an endothelium; four or five tiers of

Fig. 1. (*Continued*) muscle cells form the media of this artery, and the adventitia is at the bottom. The thickness of the vessel's wall is about 32 μm. (C), (D) and (E) are electron micrographs of three arteries of decreasing diameter (from the tree of a mesenteric artery), sectioned longitudinally right through the middle of their lumen. In all cases the lumen is to the right, and in (C) and (E) the lumen shows parts of red blood cells, and adjacent to it an endothelium and then an elastic lamella (which appears amorphous and almost unstained). In (C) the total thickness of the artery's wall is about 80 μm, in (D) it is about 22 μm and in (E) it is about 5 μm. All the muscle cells are approximately in transverse section, and some of them show the nucleus; the outlines of the cells are markedly different in the three vessels, in relation to the different mechanical conditions. Courtesy of Dr. Giorgio Gabella, University College London.

relaxed or contracted state. Their length is generally one to two orders of magnitude longer than their width, and varies with species and tissue source. In general, vascular SMCs (50–200 μm) are smaller than digestive (100–600 μm) (personal observation) or urinary (200–1000 μm),[18] and SMCs size also shows a positive correlation with animal size as mouse and rat SMCs are smaller than rabbit which are smaller than dog or pig (personal observation). Freshly isolated arterial SMCs from rabbit carotid or aorta are generally in the 100 μm range for length, which is shorter than those from the stomach (~300 μm).[19–21] This is similar to some values reported using electron microscopy techniques (Gabella, 1984) and shorter than others for visceral and detrusor SMCs which have been reported to be 500–1000 μm in length.[22,23]

Getting an exact size for SMCs is problematic as activation of the cells prior to isolation/fixation may result in cell shortening. Fixation itself can lead to cell shrinkage. Measurements in intact tissue are difficult because of resolution problems (including keeping the entire cell in the tissue section/focal plane), and in isolated cells the isolation proceedure and the fixation can affect the cells. In addition, very long isolated cells are prone to breakage. With activation of smooth muscle tissues, the SMCs may shorten (even if the tissue is held in isometric conditions) which can affect cell length measurements. Cells with radial to length ratios less than ~1:5 are generally believed to be contracted. Unlike striated muscle where shortening is limited by the sarcomere structure and the extent of thin and thick filament

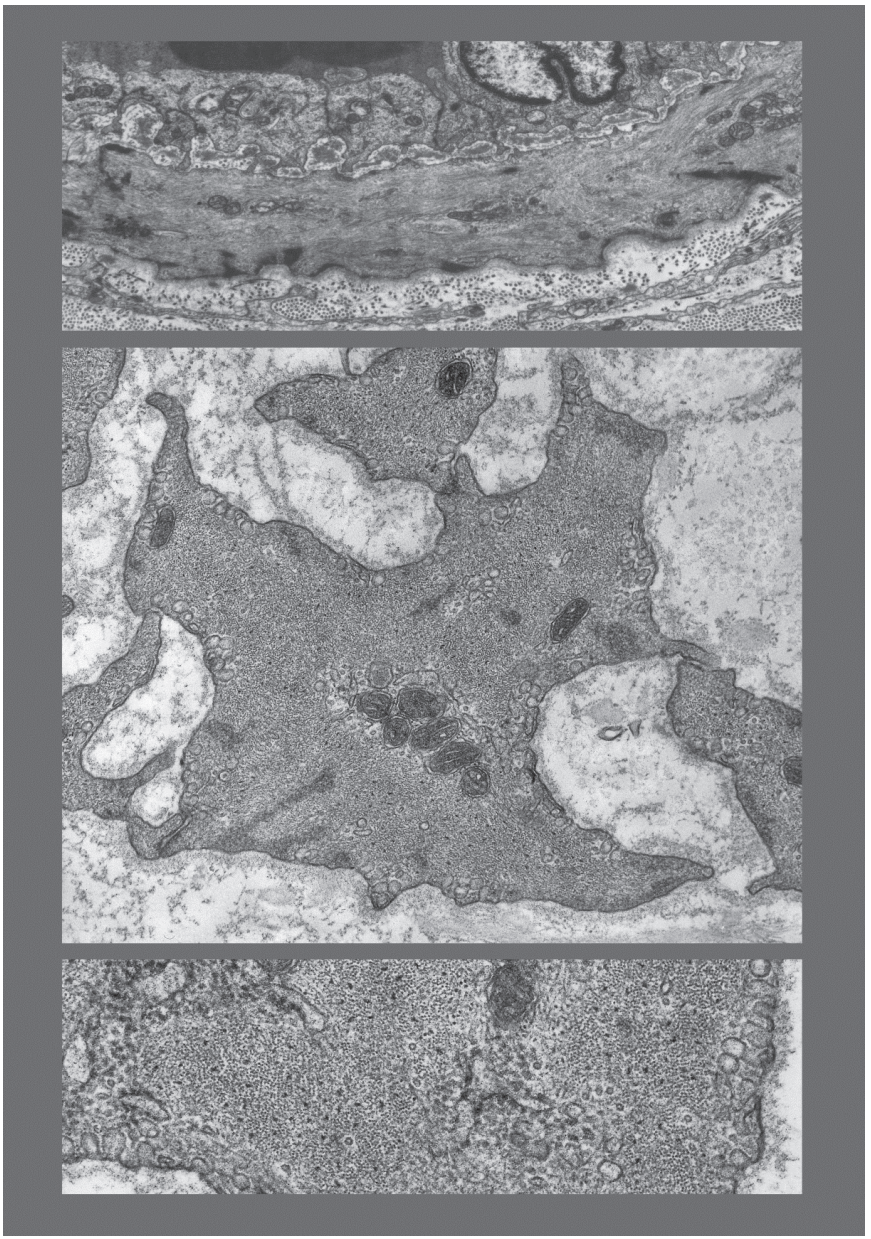

Fig. 2. A. is an electron micrograph of an arteriole cut transversely (this vessel was fixed in a condition of mild contraction); red blood cells and the endothelium are visible at the top, while the media is formed by a single muscle cell which is curved

Fig. 2. (*Continued*) and is sectioned roughly along its length. Prominent dense bands, attachment sites for the contractile apparatus, are well in evidence, especially on the abluminal part of the cell. At the bottom is the adventitia, made of connective tissue; its collagen fibrils run predominantly longitudinally, preventing elongation of the vessel with contraction. The microscopic field is about 18 μm wide. B. shows a transversely sectioned muscle cell from a large artery, embedded in a vascular matrix which consists of collagen fibrils, elastic bands and fibers, amorphous material and microfibrils. The cell profile is mainly occupied by myofilaments (actin and myosin), and it also shows intermediate filaments, microtubules, dense bands that project deeply into the cytoplasm, sarcoplasmic reticulum of smooth and rough type, ribosomes, vesicles and mitochondria. The plasma membrane presents invaginations, known as caveolae, and is lined externally by a thick basal lamina. The microscopic field is about 5 μm wide. C. shows details of a similar cell from the same vessel at higher magnification. The width of the microscopic field is about 3 μm. Courtesy of Dr. Giorgio Gabella, University College London.

overlap, isolated vascular SMCs can shorten to greater than ~50% of their initial length. Isolated visceral and detrusor SMCs have been reported to shorten 70–80% of their initial rest length.[20,22,23] It remains unclear how it is possible for an isolated SMC cell to change its length by 80%, but there must be some significant rearrangment of organelles and filamentous systems for this to occur.

By far the most apparent organelle in the smooth muscle cell is the nucleus, which is generally centrally located in both the longitudinal and cross-sectional axes. Perinuclearly at either end of the nucleus on the long axis of the cell are large numbers of mitochondria, Golgi, and sarcoplasmic reticulum and other smaller organelles. Usually near the plasma membrane but also in other areas in the cytoplasm, regions with few or no filaments that may or may not have organelles are present.[13,21,24] Changes in the shape and distribution of organelles during contraction are consistent with the increasing data that the various filament systems within the cell attach to and push or pull on these organelles during contraction.

3. Contractile Filaments

For this chapter, the term "contractile filaments" will be used in reference to the "thick" (primarily myosin) and "thin" (primarily actin)

filaments that are believed to be the primary source of contractile force generation in SMCs ("thin" filaments are also part of the cell cytoskeleton and are discussed later). The thick filaments are composed primarily of the protein myosin while the thin filaments are composed of actin, tropomyosin and a number of other reported regulatory proteins. Repeated ATP-dependent interactions of the myosin S1 head with actin thin filaments are responsible for force generation and/or shortening of SMCs.

Myosins are a large family of proteins[25] that are expressed in essentially every cell. The contractile muscle protein myosin that most people think of when discussing muscles are part of the type two family of myosin (here simply referred to as "myosin") that include a long α-helical coiled tail that can associate with other myosin to form a thick filament. Multiple reviews and books on myosin have been published[25–28] and so only the basics will be reviewed here. Myosin is a hexamer of two myosin heavy chains (MHC) and four myosin light chains (MLC) (two light chains associate with each of the heavy chains heads (S1 domains). The MHC can be separated into the S1 head (consisting of the motor domain and the lever arm) which is responsible for force generation and/or cell shortening, while the rod (an α-helical coil) is the remainder of the MHC and is responsible for the association of the two MHCs to form the myosin molecule and for thick filament formation.[26] There is a single SM MHC gene in smooth muscle cells which via alternate splicing can generate four different SM MHC isoforms.[29–34] In addition there are three non-muscle (NM) MHC genes with alternate splicing of at least one of these.[35–40] The myosin light chains include two myosin light chain 20 (MLC20 — approximately 20,000 Daltons) isoforms which are the same size but the product of two different genes.[41] This is the MLC that is phosphorylated at serine 19 (and other sites) to regulate myosin ATPase activity required for force generation and or shortening. In addition there are two myosin light chain 17 (MLC_{17} — approximately 17,000 Daltons) isoforms, but these are the product of a single gene with alternate splicing.[42–44] While adult SM tissues express primarily (solely in the case of rabbit) the SM MHC isoforms,[41,45–47] both the SM and NM MLC_{20} and MLC_{17} isoforms are routinely expressed in

adult tissue.[41,48–51] The reported correlations between expression of these various myosin heavy and light chain isoforms with animal development and tissue type appear to correlate with specific function, but this remains controversial and further work is required. Other chapters in this volume will discuss the expression of these SM and NM isoforms with development and disease.

The thin filaments are composed of globular (G) actin monomers (42,000 Daltons, 375 amino acids,[52] which assemble to form filamentous (F) actin. There are six actin isoforms resulting from six different genes, including α- (skeletal, cardiac, and SM), β-non-muscle, and γ- (smooth and non-muscle), which are all the same size (375 AA) with greater than 90% homology (all of the differences include up to 8 AA substitutions located within the first 18 NH_2-termius AA).[52,53] While all SM tissues appear to contain multiple actin isoforms (α-SM, γ-SM, γ-NM, and β-NM), vascular tissues generally express a majority of the α-SM isoform while digestive tissues express larger amounts of the γ (SM and NM) isoform.[54] Biochemical and electron microscopic measurements put the thin (actin) to thick (myosin) filament ratio in smooth muscle at ~15:1[5,55–57] as compared to 2:1 for skeletal muscle. This difference may be relevant for the thin filament's role as part of the cell cytoskeleton in SM in addition to its role as a contractile protein.

Arterial SM is reported to have ~8 mg/g tissue of myosin and 25 mg/g tissue actin which gives both weight and mole ratios of actin: myosin of greater than 3:1 and 30:1 respectively (these numbers are approximately ten times higher than those in skeletal muscle).[55] In veins there is a similar amount of myosin as in arteries, but the actin content is only about half of that expressed in arteries.[58] In spite of only expressing approximately 25% of the myosin that is expressed in skeletal muscle, SM is reported to generate a similar stress per cross-sectional area as skeletal muscle.[55,59]

Associated with the F-actin is tropomyosin (low and high molecular weight forms — ~247 and ~284 amino acids and ~45,000 Daltons), for reviews,[60–64] an α-helical protein that forms dimers which lie along the length of the F-actin filament. A single TM is of a length to span 6–7 actin monomers on the F-actin filament. In

mammals there are four tropomyosin genes that through various regulatory processes can generate over 40 tropomyosin isoforms.[65–67] In striated muscle this protein in association with troponin acts to regulate contractile activity. In smooth muscle there is no troponin, leaving several options for the role of tropomyosin. Because there are several other proteins that can associate with the thin filament, they may function in a similar fashion to troponin in regulating actomyosin interactions via tropomyosin. Alternatively, while possibly difficult to perceive, tropomyosin may not play a regulatory role in SM, but be strictly involved in thin filament stability.[60] Tropomyosin isoforms have been reported to be distributed in discrete subcellular domains on thin filaments, thereby affecting thin filament organization, and myosin interactions with actin.[61,68–70] Proteins that can associate with the thin filament in SM and function to regulate tropomyosin include calponin (a calmodulin-binding troponin like protein), and caldesmon (an actin binding SM protein that may also interact with calmodulin, myosin, and tropomyosin) along with a host of other less prevalent proteins. Specific tropomyosin isoforms have been reported to be involved in a diverse range of functions including formation and stabilization of stress fibers, focal adhesion formation, myosin recruitment, maintaining transverse-tubule structure, filapodia formation and a host of growth and differentiation processes in a wide range of cell types (for review[68]).

An interesting distinction between striated and smooth muscle is the dramatic difference (1) between the expressed levels of actin and myosin, (2) the length and stability of the thick and thin filaments, and (3) the organization of the thick filaments. Unlike skeletal and cardiac muscles, smooth muscles do not have highly organized repeating arrangements of thick and thin filaments resulting in the striated appearance with microscopy. Functionally, there are the equivalent of "sarcomeres" in smooth muscle (dense bodies associated with thin and thick filaments), but their arrangement is significantly less organized with the contractile filaments not all necessarily in the exact same orientation. The thick filaments in SM have been described as being 'side-polar', with all the myosin heads on one side of the filament facing in one direction and all the myosin heads on

the other side of the filament facing in the other direction.[71,72] This arrangement eliminates the "bare-zone" of the bi-polar filament organization of striated muscle, and along with the absence of "Z-lines", allows for an apparent unrestrained thick and thin filament sliding during contraction. This may explain in part the greater amount of cell shortening that is observed in smooth vs. striated muscle.

Thick myosin and thin actin contractile filaments are often reported as being obliquely oriented relative to the long axis of the SMC,[73–75] which can result in the corkscrew shortening that has been reported in isolated SMCs.[74–76] However, there is evidence that the thin and thick filaments run parallel to the long axis of the SMC.[22,77]

4. Cytoskeleton and Membrane Associated Proteins

The cytoskeleton is comprised primarily of thin (actin), intermediate (primarily vimentin and desmin) and larger microtubule filaments. The cytoskeleton is generally considered in its role connecting the various regions and structures of the cell, from the plasma membrane to the nucleus and everything in between. However, it is also involved in organizing and locating intracellular components as well as generating and or transmitting forces throughout the cell. In all these roles the cytoskeleton is dynamic so that where it is and what it is doing may not be the same at any two points in time. There is no way to explain, as mentioned above, how an isolated SMC can shorten by upwards of 80% of its rest length without significant remodeling of these filaments and their association with each other and the rest of the cells components. Each of these filament systems are specific in terms of their mechanical properties, the motors that associate with them to move things within the cell and or generate force, their distribution of monomer to polymer structures and filament stability, the other cell structures they interact with, and their ability to interact with each other.[78,79] Future studies need to take into account not only what is happening in a SMC to thin, intermediate, or microtubule filaments generically with an experiment, but what is happening to the specific isoforms of these proteins in spatial and temporal terms.

It appears that recognizing changes in specific protein isoforms spatially and temporally may be critical to fully understand SMC function and regulation.

As mentioned above, thin filaments are composed primarily of actin which consists of 6 isoforms. Increasing data suggests that there are thin filaments with diverse composition and distribution within the SMC.[80,81] As reviewed,[78] in addition to the typical actin thin filaments, there are also branched networks, filament bundles, cortical networks and stress fibers. There are at least 150 reported actin binding proteins.[82] The nucleus is generally held near the center of the SMC by the cytoskeleton via a host of nuclear membrane proteins,[83,84] including actin stress fibers which may also be involved in positioning and stabilizing intranuclear chromatin.[85] Thus, thin actin filaments near the plasma membrane, near the nuclear membrane, or in the nucleus itself may all have different functional roles depending on these locations, specific proteins they interact with in these locations and/or second messenger pathways working in these regions of the cell.

The major cytoplasmic intermediate filaments (type III) in smooth muscle are composed primarily of vimentin, desmin[86] and synemin (type VI),[87] with vimentin being the major intermediate filament present in vascular SM. These have common α-helical rod domains that form a coiled-coil dimer and then larger associations. Stimulation of SM cells may cause disassembly/reassembly of these intermediate filaments and/or detachment from the dense bodies, which via second messenger pathway activity or re-localization may affect force production, gene regulation, and other cell functions.[86,88,89] Intermediate filaments have also been reported to form connections with the nuclear envelope and mitochondria, possibly affecting function of these organelles and/or allowing their subcellular localization to be maintained or returned to "normal" following extreme cell shortening.[90]

Microtubules are present in smooth muscle cells where the dynamics between the α and β monomers within the microtubules they form has been postulated to be important for, among other things, cell division, cell structure, intracellular transport,

contractility, and nuclear transcription.[91] They can bind to the plasma membrane at caveolae,[92] as well as to most other organelles including the nucleus.[93,94] Being a polarized filament, the molecular motors kinesin and dynein can transport cargo along these filaments toward either the "plus" or the "minus" ends of these filaments, respectively. Determining their roles in SM function has been difficult as there is constant turnover of the tubulin monomers within the microtubule. Microtubules are stiffer than the intermediate or thin filaments, and thus while working well for chromosome sorting in mitosis, they can be a problem for extensive SMC shortening as they may cause an internal load that contraction would have to work against. Microtubule polymerization/depolymerization appears to be regulated in a contraction cycle dependent manner,[95,96] and affects cell alignment in the tissue.[97] Microtubule association with caveolae appears to have a direct effect on increasing polymerization, which would thus also affect intracellular trafficking and cell function.[92]

Plectin is one of numerous plakan isoforms of large MW proteins (~500 KD) that can cross link microtubules, thin, and intermediate filaments to each other, to other proteins, and to proteins in the plasma and nuclear membranes.[79,98,99] The expression and distribution of the various plectin isoforms and their ability to link the various filament systems with each other and the membranes make them critically important in overall SMC mechanics.[100] These molecules along with nucleation and depolymerizing promoting factors all appear instrumental in cell structure and function.

Filamin is a high molecular weight (280 kD) protein that was identified as an actin binding protein.[101,102] Three isoforms have been identified and the protein has been reported to interact with over 90 proteins and is involved in cell signaling, cytoskeletal organization, force transmission, and transcription.[103–106] Its interaction with integrin is important in cell adhesion and migration, while its interaction with small GTPases are important for cytoskeletal remodeling.[107,108] Filamin can be phosphorylated by PKA and PKC,[109,110] which can affect its interaction with actin, integrin and multiple other proteins, and can prevent its cleavage by calpain.[111] Interestingly, its interaction

with actin, caveolae, and PKCα is critical for the linear distribution of caveolae at the plasma membrane.[112] The dynamic inward trafficking of caveolae has been shown to be regulated by hormonal stimulation, PKCα dependent phosphorylation of proteins, and microtubule interactions.[113–115]

Caveolae are flask shaped invaginations of the plasma membrane with caveolin present along with numerous cell signaling molecules, glycosphingolipids and cholesterol. These structures are directly involved in cell regulation via receptors and second messenger pathways, as well as endo- and exo-cytosis.[116] They have been observed to alternate with focal adhesions along the plasma membrane, but are either absent or significantly fewer in number near the tapered ends of the SMCs.[21,117] It remains unclear why these two structures (focal adhesions and caveolae) are so consistent in their close association, but with the increased concentration of receptors and second messenger proteins at the caveolae, interaction and regulation of the extensive network of focal adhesion associated regulatory and cytoskeletal proteins appears to be critically linked.

The cytoskeleton and the contractile component of the SMC need to be attached to the cell membrane (and the extracellular matrix/neighboring SMCs) via cell adhesion molecules (CAMs) including four major classes of proteins (cadherins, immunoglobulin super family, integrins and selectins) in addition to other membrane proteins.[118,119] Cadherins are a large family (greater than 100[120]) of Ca^{2+} dependent cell-cell adhesion molecules that are critical for cell morphology and signaling.[118,121–124] Via catenins, the intracellular domain of cadherin links ultimately to actin thin filaments to transmit force.[125–127] These adherens junctions have also been implicated in signaling microtubule assembly as part of cytoskeletal regulation,[128] interacting with integrin,[129,130] interacting with immunoglobulin cell adhesion molecules[131] and with numerous other signaling events (see[118] for review). Integrins are a large family of single transmembrane-spanning receptors that attach the cell's cytoskeleton to the extracellular matrix[132–134] and ultimately to a vast array of signaling molecules.[135–137] These proteins can be clustered in localized domains or distributed widely throughout the membrane. Adapter proteins in

the cytosol can connect CAMs to the cytoskeleton in order to maintain cell shape and transmit forces, as well as affect gene expression and protein distribution for signalling.

Early evidence for potentially discrete distributions of contractile and cytoskeletal filament domains in SM has been available for 3-4 decades,[138–140] and has more recently found another resurgence[69,141–144] Actin isoforms have also been reported to be uniformly distributed amongst the thin filaments within a given tissue by some groups,[145,146] but to differ in expression between SM tissues.[147] Some more recent studies suggest that there are in fact specific distributions of the actin isoforms that comprise the thin filaments and that these filaments play different roles in the SMCs. Discreet distribution of actin filaments comprised of primarily or exclusively one actin isoform between the sub-plasmalemmal (cortical) vs. cytosolic domains has been described.[142] Fletcher and Mullins[78] review four actin filament organizations (branched networks, bundled filaments, cortical networks, and stress fibers) that are specifically arranged to act on or respond to different cellular forces. It is unclear exactly what determines how these different arrangements are organized, localized and maintained in a given cell, but accumulating data suggests that there is physiological relevance to these specific structures and their distribution within the cell.

5. Nucleoskeleton

The nucleoskeleton, while currently less well understood than the cytoskeleton, is also of major significance in the functioning of the cell. The nucleoskeleton is upwards of an order of magnitude stiffer than the cytoskeleton,[148,149] with the cytoskeleton being critically dependent on it for its own function.[94,150] Lamins (type V intermediate proteins) are present in the nucleus where they contribute to the nuclear skeleton and interact with numerous other proteins in the nucleoplasm and nuclear envelope, and are hypothesized to be involved in nuclear assembly, chromatin organization, and gene expression. Lamins and multiple other nuclear proteins form a nucleoskeleton that is connected to the cytoskeleton via LINC

proteins (linkers of the nucleoskeleton and cytoskeleton) (see[93,151] Meinke *et al.* 2015 for reviews). In addition to the intermediate filament lamins, many of the known cytoskeletal/transport proteins are also present in the nucleus including actin, myosin, titin, spectrin and kinesin, where they are believed to have structural functions similar to their roles in the cytoskeleton. There is also evidence that these proteins are involved with signaling roles for transcription.[152–158] This allows for direct communication between forces at the plasma membrane and genetic information and regulation in the nucleus,[94,159,160] including nuclear and chromatin organization, replication, and transcription.[161–163] LINC complex proteins at the nuclear membrane transmit force to and from the cytoskeleton and the nucleoskeleton (analogous in function to focal adhesions at the plasma membrane transmitting extracellular forces to and from the cytoskeleton).[164] Changes in these LINC complexes affect mechanotransduction (altered gene transcription, replication and repair via second messenger pathways)[165] via lamin and lamin binding proteins,[166–168] cytoskeletal and nucleoskeletal organization, and cell differentiation.[160,169]

6. Regulatory Proteins

Regulatory proteins that are reported to be involved in SM regulation include tropomyosin, desmin, vimentin, caldesmon, calponin, myosin light chain kinase, myosin light chain phosphatase, protein kinases, small GTPases, and a host of other second messengers which have been reviewed extensively. Localization within the SM cell, associations with each other or contractile/intermediate filaments, and localization with cell activation, force generation and or shortening are likely all critical to our full understanding of SM contraction. These proteins are involved in signal transduction for regulation of many cell functions including transcription and translation, contraction and relaxation, cell division and differentiation, and protein translocation.

As mentioned above under contractile proteins, four tropomyosin genes are responsible for over 40 tropomyosin isoforms. These may

specifically interact with particular actin isoforms in thin filaments based on isoform composition and location.[61–63,68–70,81,170] Thus, while there are only four actin isoforms expressed in SMC's, the large number of tropomyosins that can be expressed and associate with them significantly increases their potential specific functional roles in the cell. This includes not only differential regulation of actomyosin interactions throughout the cell, but also localized domains for specific regional control. In addition, specific regulation of metabolic pathways, cell cycling, growth, and probably most other cell activities are affected by the individual expression, distribution and function of these thin filament-tropomyosin pairings (see above references).

Caldesmon in smooth muscle (h-caldesmon, ~88kD; as compared the non-muscle expressed l-caldesmon — ~59kD)[171] appears to co-localize with either thin or thick filaments[172,173] in the cytoskeletal domain.[174] Caldesmon has binding sites for actin and calmodulin,[175] myosin,[176,177] and tropomyosin,[178,179] where it is reported to affect actin-activated myosin ATPase activity.

Calponin was first described as an actin and calmodulin binding protein[180] that was subsequently shown to have multiple isoforms, and bind to numerous other proteins including α-actinin,[181] myosin,[182] PKC,[183] tropomyosin,[184] tubulin,[185] and a host of other proteins. It has been reported to bind to G- and F-actin with similar affinity,[186] thereby affecting polymerization of actin monomers and bundling of F-actin. Calponin has also been reported to inhibit myosin ATPase activity.[187] According to North *et al.*[188] calponin in chicken gizzard can associate with actin in either the contractile (myosin, SM actin and caldesmon) or the cytoskeletal (β-actin, filamin and desmin) domains, and thus is most likely active in regulation of both the cytoskeletal and contractile functions of the SMC.

Myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) are believed to be the primary regulators of smooth muscle contractile function via regulation of myosin light chain phosphorylation levels.[189] These enzymes are regulated in turn via a seemingly endless number of second messenger pathways that continue to perplex our understanding of smooth muscle function.[189–194] Isoforms, expression, distribution, localization and possible translocation of

many or all of these molecules with activation and relaxation of the tissue, appears to be relevant to the function of the SMC.

7. Organelles

The sarcoplasmic reticulum (SR) in SM is directly involved in $[Ca^{2+}]$ regulation. In tonic (aortic) SM there are both peripheral (near the plasma membrane) and central (not near the plasma membrane) SR domains (Fig. 2) with the central SR (often in a perinuclear location) being more prevalent.[195,196] This is the opposite of phasic (vas deferens) SM where the peripheral SR is dominant, with both the peripheral and central pools appearing to be continuous with each other and perhaps not functionally distinct.[195] The SR is in close association with the mitochondria and the caveolae, and may be continuous from the plasma membrane to the nuclear membrane. Mitochondria and vesicles may be observed throughout the cell, but often are present in greatest density near the nucleus.

As the nucleus may take up in excess of 60% of the cross-sectional area of the SMC where the nucleus is located, there needs to be a mechanism to transmit force across this region of the SMC. Data suggests that the contractile filaments attach directly to the nuclear envelope allowing force to be transmitted through the nucleus. Consistent with this, nuclear lengths in relaxed tracheal SMCs are shorter than those in isometrically contracted SMCs as the nucleus gets elongated during SMC contraction as it is pulled on by the force generated by contraction.[77]

8. Extracellular Matrix

The extracellular matrix is a deposition of proteins and glycosaminoglycans that are secreted by the SMC's themselves or by other cell types that are also found in SM tissues. The protein fibers generally consist of collagen, fibrin, elastin, laminins, fibrillins and fibulins.[4] The presence of these fibers and other extracellular components are critical for SMC migration, replication, and differentiation, and thus overall function of SMCs.[197–200]

9. SMC Structure and Function Questions

Smooth muscle cells and a host of other cell types exist in varying ratios within different SM tissue and can consequently have an important impact on how they interact. In the vascular system, the media is generally primarily SMCs, but even then there are always non-muscle type cells closely associated at the luminal and adluminal surfaces. SMCs are either constitutively or transiently generating force, and thus their connections and interactions to each other and or the extracellular matrix is critical for their (patho)- physiological function. In addition, SMC heterogeneity occurs between and within the various regions of the vascular tree. In an attempt to simplify the experimental system of all this variability for studying SMCs, and thereby increase our understanding of SMC function, numerous model systems have been developed. Isolated vessels, vessel rings and strips, isolated cells, cultured cells, and purified filaments or proteins are some of the systems commonly used to good effect. An ongoing concern, however, is the common assumption that much of what is learned from all of these systems is universally relevant to the intact SM tissue system. There is extensive evidence that SMCs removed from their 3-dimentional organization and constraints become at best a modified SMC, or at worst, something other than a SMC all together. There is also an ever increasing data base showing that everything that can potentially have an effect on a SMC in vivo or in vitro does have an effect on it. Thus while we know for example that SMCs and tissues can have a "memory" of previous activity that affects future activity, and that epigenetic changes occur independent of genetic regulation, we do not know enough about either of these things to know how SM tissue or cell preparations for in vitro measurements change these phenomena.

Besides the signals SMCs receive from neural, endocrine, paracrine, and autocrine mechanisms in vivo, their physical interactions and stress/strain relationship with their neighboring cells/matrix is important in determining their shape, function and cellular determination/differentiation.[201,202] The ability of isolated SMCs to shorten by 50–80% of their initial length (not predicted to occur in SMCs in intact vessels), generate peak tension at multiple cell lengths,

and alter organelle distribution and function are all indicative of the distinctive ability of all the filament types to interact with each other, reorganize themselves, and for cell-cell interactions to be reorganized depending on the external conditions leading up to or at the time of measurement (much of this work has been done on respiratory SMCs and tissues.[203–210] Another ongoing unresolved question includes the concentration of specific proteins within the SMC. Myosin light chain kinase for example, is expressed at a 10-fold lower concentration than the protein that it phosphorylates (MLCK ~4 μM and myosin ~40 μM). This raises questions of how full phosphorylation of MLC_{20} occurs in a physiologically relevant time frame. MLCK binding to the thin filament may allow the myosin to be "delivered" to the MLCK on the thin filaments as they slide past the thick filaments.[211] Thus knowing its location and distribution within the SMC may help resolve if there is localized activation of myosin, or if it progresses in a specific pattern across the cell.

Smooth muscles contain approximately 10 times the actin and ~1/4th the myosin expressed in skeletal muscle, but still manages to generate a similar force per cross-sectional area as skeletal muscle.[55] The ratios of actin to myosin filaments have been estimated to be as high as 50:1[212] (Fig. 2) which makes one question why there is such an apparent excess of actin in the SMC. As noted above, there is evidence for specific distributions of actin within the cell, and suggestions that it is compartmentalized into contractile and cytoskeletal domains. The gross excess of actin to myosin filaments suggests that it has significant physiological relevance beyond acto-myosin force generation. Actin isoforms can combine to form a range of thin filaments with distinct function across the phylogenetic tree.[81] In addition, the physiological function of specific actin thin filaments and formation of various large scale actin associated structures (thin filaments, stress fibers, podosomes) are inherently linked to the associating proteins in general, and the tropomyosin isoforms specifically.[68,69] Indeed, as reagents and methodologies advance, it appears that most (all) proteins that are expressed as multiple isoforms have some type of specific distribution within the cell that correlates with a particular function.

Additional unique observations in contracting SM include the seemingly sporadic intracellular domains, (primarily near the plasmalemma) that appear to be devoid of myofilaments or other electron dense structures (Fig. 3 — "LOW" — low density area devoid of filaments) and can result in outward blebbing of the membrane at these sites with contraction.[213] Why these would exist and what possible function they serve remains unknown. There are also multiple reports of cells or portions of cells that fail to contract with the remaining cells in a tissue, or another portion of the SMC. This may result in "wavy" cells that are being pulled shorter by surrounding contracting cells,[21,213] contractile zones in isolated SMCs,[214] or regions of freshly isolated SMCs (1/3 – 1/2 of the cell) that do not contract while the other portion of the cell does (unpublished data).

As schematically shown in Fig. 3, there appears to be a direct physical link between all the contractile and cytoskeletal filaments, which in turn make connections from the plasma membrane to the nuclear membrane, and with all the other organelles in between. This also extends to the nuclear material within the nucleus and the extracellular matrix material outside the cell, so that any changes in force acting on or generated within the SMC affects everything else upstream and downstream from that point.[94,119,215–217] As shown in Fig. 2 (and represented in Fig. 3), there appears to be very little space within the cell that is not occupied by an organelle, structural or contractile protein. Further, this does not take into account the hundreds of second messenger molecules and messenger pathways that are also known to be present within the cell to regulate cell function. How all these components affect each other as they interact and reorganize, with and without the significant cell shortening that is possible, remains an area of much needed work. Relevant to this chapter is the concept that actin for example, (but this would apply to any protein with isoforms present) located near the plasmalemma vs. actin in the cytosol, or near or in the nucleus etc., has specific temporal and spatial function.

A final observation is that there appears to be a consensus in the literature that SMCs need to de-differentiate in order to undergo cell division and that this de-differentiation to a "synthetic" phenotype

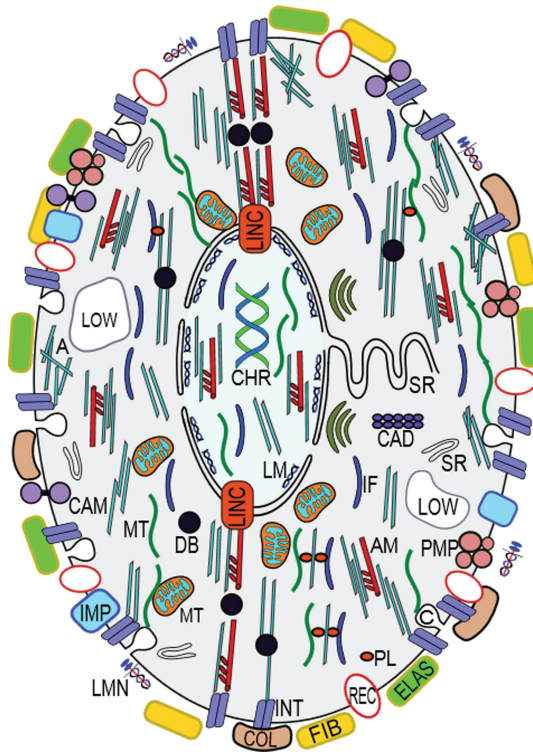

Fig. 3. Schematic of smooth muscle cell showing representative locations, distributions and associations of major contractile, cytoskeletal and membrane associated proteins in the SMC. The complexity of the protein number, type and distribution in the figure is representative of a real SMC (see figure two), although grossly oversimplified. Actin thin filaments for example, may be comprised of different actin isoforms, in different locations (near plasma membrane receptors, caveolae or cell adhesion molecules, near (or not) myosin filaments, near nucleus or other organelles, or in the nucleus) and have different filament stabilities and functions in each of these locations. Abbreviations: AM = Actin-Myosin; C = Calveolae; CAD = Cadherin; CAM = Cell Adhesion Molecules; CHR = Chromosomes; COL = Collagen; DB = Dense Bodies; ELAS = Elastin; FIB = Fibronectin; IF = Intermediate Filaments; IMP = Integral Membrane Proteins; INT = Integrin and Associated Proteins; LAM = Lamins; LINC = Linker of Nucleoskeleton and Cytoskeleton; LMN = Laminin; LOW = Low Density Areas Devoid of Filaments; MICRO = Microtubules; MT = Mitochondria; PL = Plectin; PMP = Peripheral Membrane Proteins; REC = Receptors; SR = Sarcoplasmic Reticulum.

and re-differentiation to a "contractile" phenotype is a routine occurrence in SMCs dependent on external conditions (for reviews[198,218–220]). In fact, there are multiple reports of SMC division without de-differentiation from a "contractile" phenotype.[221–224] This difference in observations is significant as it potentially leads to very different conclusions for relevance of de-differentiation in SMC replication. If SMC de-differentiation for SMC replication is a physiological process for growth and repair, then pathological conditions like intimal wall thickening in atherosclerosis may be a failure to regulate this process correctly. However, if the physiological process of SMC replication for growth and repair does not require de-differentiation of SMCs, then the intimal wall thickening in atherosclerosis may be due to factors causing SMC de-differentiation itself. De-differentiation and re-differentiation of SMCs for vascular growth and repair may not be a physiological process, but a pathological process. Finding treatments and cures for smooth muscle diseases will look very different if the solution is to find a way to alter the mis-regulation of a physiological process *vs.* finding a way to prevent a pathological process.

In conclusion, while we have known a good deal about the anatomy of the SMC for quite some time now, new methods and technologies are allowing us to further clarify when proteins and their isoforms are expressed in SMCs. In addition, the organization, distribution, localization, interaction and potential temporal translocation of these proteins/isoforms continues to be revealed. This new information is allowing us to go beyond our understanding for example, that actin and myosin are the major contractile proteins in the SMC. Expression of their respective isoforms (and possibly also for all the protein isoforms present) are developmentally and (patho-) physiologically regulated. Isoforms appear to be distinctively localized throughout the cell where they can associate with different proteins in different structural organizations at different times that are specifically regulated for particular functions via hundreds of regulatory proteins and pathways. Thus, specific actin and myosin isoforms in specific filament organization and association with specific other proteins in defined locations of the SMC are in fact "contractile filaments". In addition, however, by changing any of these variables (specific actin or myosin

isoform, structural organization, specific associating proteins or their isoforms, location in the SMC, etc.) may result in a functional "cytoskeletal filament" that maintains stress, organizes intracellular distribution and location of other cellular structures, or redistributes cellular components in the cell. Or also possible, when specific isoforms of these proteins associate with each other, change from individual proteins to filaments, or redistribute within the SMC they may now be a part of one or more regulatory pathways signaling contraction, cellular reorganization, changes in protein expression, or cell replication. We know SMC function is much more complex than we previously believed. If we, like William Harvey many centuries ago, will continue to use quantitative reasoning with anatomical information, we will also be able to continue to derive physiological significance from our new observations. In so doing we should be able to resolve just how complex the SMC is in its structure and function.

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Chapter 3

Vascular Structure and Function

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Vascular smooth muscle motor protein regulation acutely adjusts vascular diameters and determines the stress-strain characteristics of arteries and veins. Net muscle contraction occurs when the tension generated by motor proteins is greater than that generated by blood pressure, and net relaxation occurs when pressure- and radius-dependent wall tension exceeds muscle tension. Numerous stimuli generated locally and distantly acting through multiple cell signaling systems modify the level of motor protein activation. Unlike skeletal muscle that is inactive until "called on" to contract, vascular smooth muscle is always "on" to some degree, even when no apparent stimuli are present. Smooth muscle down the vascular tree, and similar vasculatures across species, display some similarities, but also a significant degree of heterogeneity. The canonical vascular smooth muscle contraction control system involves regulation of 20 kDa myosin light chain phosphorylation by Ca^{2+} calmodulin-dependent myosin light chain kinase, rhoA kinase and protein kinase C. The latter two kinases act, in part, by inhibiting the activity of myosin phosphatase, and thus, by increasing the degree of force for a given increase in Ca^{2+}. Several novel kinases also appear to participate in myosin light chain regulation, including Ca^{2+}-calmodulin-dependent kinase II, mitogen activated protein kinases, zipper-interacting kinase, integrin-linked kinase,

kinase inhibitor of NF-κB 2, and AMP-dependent protein kinase. Regulation of vascular smooth muscle contraction is not limited to modulation of myosin light chain phosphorylation. Non-canonical regulation includes control of actin and myosin polymerization, thin filament regulation, latch bridge formation and regulation of force transmission. Stimuli that increase nitric oxide, cyclic nucleotides and their kinases inhibit the contraction control system. The effect of such complex regulation is to maintain blood pressure at levels necessary for perfusion of essential organs, and to ensure appropriate nutrient supply to, and waste removal from, all cells of the body.

1. Introduction

The vasculature is a network of heterogenous tubes through which blood is propelled by a pulsatile pump to perfuse every organ of the body. As multicellular organisms grew in size, the heart and vasculature arose from the need to convectively supply O_2 and substrates to and remove waste products from, cells too distant for diffusion to adequately exchange these metabolites.[303] The vascular system also provides the conduit through which immune cells and hormones reach target tissues. In essence, the development of a vascular system gave life to large animals. Failure of this system leads to disorders such as hypertension, atherosclerosis, heart attacks, stroke, heart failure, thrombosis, and vasodilatory shock due to the traumas of hemorrhage and sepsis. Moreover, diseases such as diabetes and obesity lead to vascular complications. Together, these disorders represent the majority of human mortalities (see http://www.cdc.gov/injury/wisqars/leadingcauses.html).

In mammals, the heart's two muscular ventricles pump blood into the systemic and pulmonary vasculatures, separate circuits connected in-series.[429] In a ~70 kg human at rest, ~5 L of blood is pumped per min (~70 ml/beat × ~72 beats/min). Thus, the entire blood volume circulates through the closed vascular system each minute. At any one moment, the majority of blood volume (~75%) is contained within the systemic veins at low pressures. Venous smooth muscle contraction constricts this reservoir, decreasing venous capacitance rather

than compliance, making more blood available to the heart to increase cardiac output.[425,500] The heart pumps blood delivered from the large veins directly into the aorta and pulmonary artery at high pressure. Muscular arteries of reduced size (both luminal radius and wall thickness) branch off these conduit (elastic) arteries to perfuse each organ system. Muscular arteries that enter (feed) organs branch further and become reduced in size forming the microcirculation, consisting of vascular bed feed arteries, precapillary arterioles, capillaries and postcapillary venules ranging in size from ~5–400 μm.[319,413] The surface area of each capillary bed is about 1,000-fold that of conduit arteries.[54] This, along with the extensive diffusive interactions among microvessels, dynamic VSM vasomotor regulation, and adaptive architectural remodeling, ensure that O_2, nutrient, and fluid exchange occurs between the vascular compartment and the extracellular space bathing the cells of each organ according to local demand.[17,370,381] Most blood returns through many small veins into fewer larger veins in a reverse branching order until blood fills the large vena cava and pulmonary veins, the final reservoir before the heart. Some filtered plasma returns from the extracellular space through the lymphatics, a very low pressure, open, vascular system.[326]

In general, the tubular vasculature is a laminar structure, with an inner intima comprised of an endothelial cell layer, a middle media and outer adventitia.[137,319,413] In the media of large and small arteries, more than a single layer of vascular smooth muscle (VSM) cells and extracellular matrix (ECM) surround the intima. The number of lamina is reduced as the vessel diameter decreases.[319] This is true when moving down the vascular tree, and also when comparing analogous arteries from different species of different sizes. For example, femoral arteries from rabbit and mouse develop, respectively, ~12[155] and ~3 lamina. At a diameter of ~50 μm in the microcirculation, medial lamina give way to a VSM monolayer and then to pericytes, which appear also to exist in the intima of larger blood vessels.[20,352] VSM cells are elongated fusiform bio-engines. Those isolated from pressurized swine carotid artery are ~10 × 240 μm when fully relaxed and ~120 μm long when fully contracted[88] (Fig. 1A). In arteries, the long axis of each VSM cell is oriented perpendicular to the long-axis of the

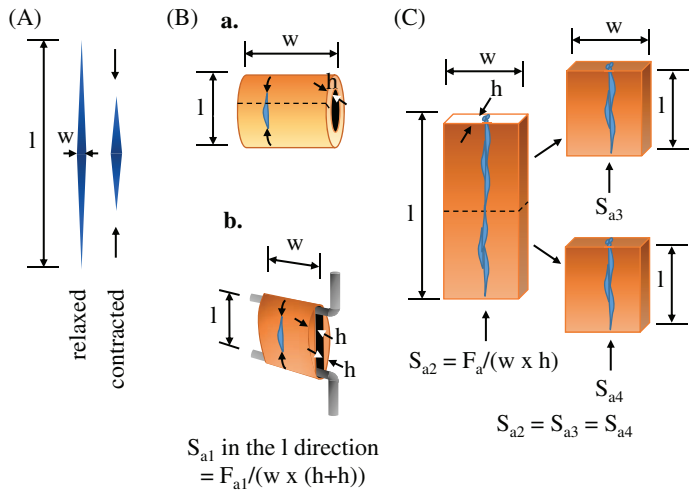

Fig. 1. Vascular smooth muscle (VSM) cells are long and thin (fusiform). The ratio of length, l, to width, w, for a VSM cell at rest is ~24:1. Single VSM cells can be stimulated to contract to ~half the rest-length (A). The long-axis of VSM cells (B.a., l) are oriented perpendicular to the long-axis of the vascular tube (B.a., w). Thus tissue stress, S, should be measured circumferentially, which can be accomplished using a vascular ring (B.b.) or strip (C) preparation. The degree of S is dependent on strain (normalized length, l). The strain at which S_a is a maximum is referred to as a reference length, l_{ref}, or as l_0. The values w and h are width and thickness, respectively. An artery ring hung on tissue pegs (B.b.) is twice as thick and half as long as the same ring cut into a muscle strip, C. The S_a of a tissue strip made by cutting a ring open (B.a., dotted line), S_{a2}, would be equal to the S_a of that strip cut in half (C, dotted line, S_{a3} or S_{a4}).

vascular tube[319,521,522] (Fig. 1B), and in many veins, a fraction of VSM cells are also longitudinally-oriented.[413] The role of VSM is to contract, and thereby increase blood vessel wall stress (Fig. 1C, force per unit wall width × unit wall thickness; i.e., cross-sectional area; tension, the force per unit wall width, is also used for normalizing force of thin-walled blood vessels and to approximate stress). VSM contraction leads to constriction when wall stress is greater than the opposing dilating stress dependent on luminal blood pressure and tube radius (see Fig. 3B). Around the outermost medial lamina of arteries is an adventitial layer comprised of ECM and some cells such as fibroblasts, mast cells, dendritic cells, macrophages, progenitor cells and some

Schwan cells. The percent of medial ECM is largest in elastic, conduit arteries (e.g., aorta, pulmonary artery, carotid artery and common iliac artery) and is reduced as the arterial tree becomes smaller in diameter (e.g., external iliac artery) and branches to perfuse separate organ systems (e.g., renal, femoral and mesenteric arteries and their branches). Arteries downstream from conduit arteries are called large and small muscular arteries. Perivascular adipose tissue encircles the adventitia of conduit and muscular arteries feeding organs. Larger arteries have a vasculature of their own, the vasa vasorum.[318]

VSM is sandwiched between sources of vasoactive stimuli that can be rapidly mobilized to control contractile activity.[538] Endothelial cells release both contractile and relaxant stimuli[31,95,101,256,293] and permit blood-borne hormones access to surrounding VSM.[441] Sympathetic nerve varicosities[35,46] and peptidergic sensory neurons[183,230] invade the adventitia and adventitial-medial junction of certain vascular beds, releasing neurotransmitters and co-transmitters.[538] Recent studies have revealed that perivascular adipose tissue releases stimuli that affect VSM contractile activity, including prostanoids and angiotensin II.[295,356] The cells of each vascular bed produce stimuli that modulate the contractile state of the microcirculation according to local metabolic demand.[450] In addition, regulated gap junctions mediate direct intercellular communication between endothelial cells and between endothelial and VSM cells, permitting very rapid and efficient propagated contraction and relaxation.[17,138,449] Moreover, contractile and relaxant autacoids can be produced directly by VSM cells.

2. Contraction (Shortening, Force Development and "Catch-slip Holding" Tension)

VSM cells are the engines of the vascular wall.[390] The force-generating units, or motors, within smooth muscle cells are actomyosin (AM) crossbridges (XBs) arranged in poorly defined sarcomeric units[6,172] that, nevertheless, behave mechanically in a fashion similar to the well-characterized fast skeletal muscle sarcomere.[323,390,545] In particular, when VSM *in vitro* is clamped at different muscle lengths (isometric) and stimulated to contract at each length to generate length-tension

curves, the maximum active isometric tension generated displays a parabolic dependency on muscle length. That is, muscle motor force-generation is length-constrained (but see below). The maximum concentration of a contractile stimulus will engage the greatest number of motors when the tissue is at its optimum sarcomere length. Fewer motors are engaged at shorter and longer lengths, despite employing a maximum stimulus concentration. Thus, comparisons of the ability of stimuli to contract VSM must take muscle length into account. Both muscle length and tension can modulate the effectiveness of stimulus-contraction coupling.[379,380,512,514]

Myosin is an ATPase with three domains; a head domain that "walks" on actin cables by forming a strong ionic bond with an actin monomer followed by an oar-like power stroke and then release from the cable and reversal of the stroke, a tail domain that is embedded in an assembly of myosins forming a thick filament, and a regulatory domain.[79] Each myosin molecule is a heterohexamer consisting of two heavy chains, each with head and tail domains, and two pairs of myosin light chains (MLCs; 20 kDa and 17 kDa) that wrap around the head's neck region. The 20 kDa MLC, also termed the regulatory light chain, undergoes reversible phosphorylation, thereby regulating the motor's activity (turning it "on" and "off"). Notably, actin-based thin filaments, myosin thick filaments and structures involved in force transmission of smooth muscles all appear to be acutely dynamic,[30,65,277,288,409,451,492,553] so the active length-tension curve is also acutely dynamic and the parabolic relationship can shift along the length-axis.[7,24,452,497,526] Such acute dynamic plasticity enlarges the repertoire of smooth muscle contractile behaviors compared to striated muscles.

Arterial VSM is a composite material consisting of ECM that exerts passive mechanical properties, and VSM cells that appear to act as series-coupled force-transmitting engines.[323,390] That is, isometric stress at any given muscle length is independent of the length of the *tissue* studied (i.e., cutting a tissue in half transversely to retain the original cross-sectional area will not affect the stress-level, Fig. 1C), and mechanical changes in tissue length will result in proportional changes in VSM cell length (and visa versa). Total stress consists of at

least two additive stresses, one from the motors (active stress, S_a) and one from the ECM (passive stress, S_p), because ECM and AM XBs act in-parallel. Other elements may contribute additional stress in-parallel. An example is the giant intracellular protein titin found in striated muscle.[274] Although the stress borne by titin is often considered to represent an additional passive stress, the titin stress-strain (strain = normalized length) curve is acutely regulated by cell signaling systems,[174] indicating that titin's contribution to total stress is not passive. Titin is also expressed by smooth muscle and may serve a mechanical role that is possibly regulated, but the precise function of smooth muscle titin remains to be determined.[68,133] Throughout the functional length-range of bladder, a considerable amount of the stress borne when detrusor smooth muscle is at rest that was originally considered to be passive appears instead to be due to very slowly cycling AM XBs that undergo strain softening.[405,471,473] To a lesser degree, this appears also to be true for VSM, contributing ~5–10% of total stress at the optimum length for contraction. These AM XBs act in parallel with those responsible for shortening more rapidly and developing stress, so their stresses are additive. The ability of AM XBs to "hold" stress for some time is characteristic of non-muscle, smooth muscle and slow striated muscle myosin II isotypes in which the power stroke is completed in 2-steps.[204] "Holding" occurs when the XB power stroke stalls for some time at a position between the first and second steps due to mechanical strain and MLC dephosphorylation.[339,469] Notably, in contrast to most non-covalent bonds that act as slip bonds where bond lifetimes decrease with load, the AM XB can act as a biological catch bond where, up to a point, bond lifetime increases (i.e., detachment rate decreases) with load.[75,145]

The ECM stress-strain curve of VSM is J-shaped such that when the VSM of an artery is fully relaxed, the artery will readily dilate at low pressures (Fig. 2A). The steep stress-strain slope of the curve at long strains provides a stress that resists further tissue strain, limiting the extent of acute increases in arterial lumen diameter with increasing pressures. Failure of this system can result in aneurysm. Adding the parabolic active stress-strain curve to the J-shaped passive curve reveals an N-shaped total stress, S_t, curve (generally called an S-curve

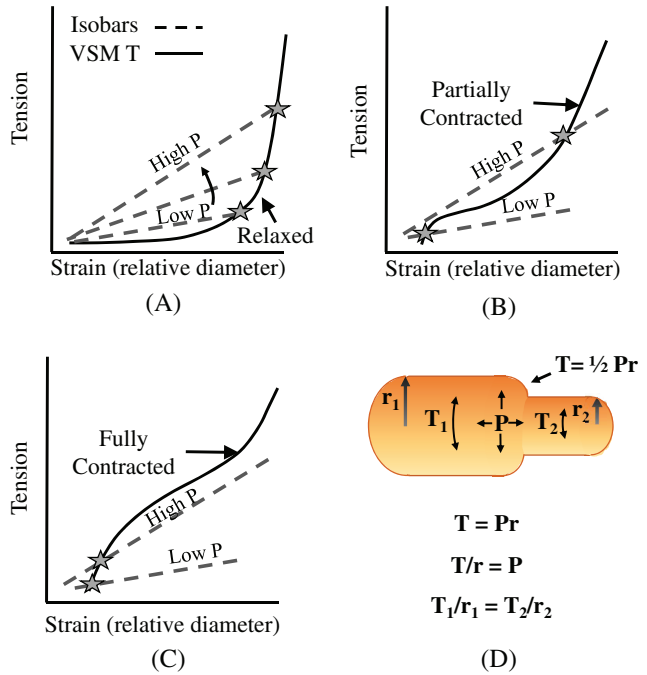

Fig. 2. The preload tension-strain curve (artery segment not exposed to a contractile stimulus) is J-shaped (A), and the total tension-strain curve (artery ring stimulated to contract) includes a parabolic active tension relationship plus the J-shaped preload tension relationship, forming complex shapes dependent on the degree of vascular smooth muscle (VSM) activation (B–C). The intersections (stars) of isobaric curves (dashed lines) and tension curves (solid lines) reveal the level of VSM tension at given pressures in an artery tube not contracted (A), partially contracted (B) and fully contracted (C) where the relationship between wall tension, T, and luminal pressure, P, is according the Laplace relationship (D, T = Pr), assuming a very thin wall.

because of the shape when the axes are inverted; Figs. 2B and 2C). What is apparent is that VSM motor-generated (active) stress dominates at shorter muscle strains (smaller lumen diameters), and ECM stress dominates at longer strains (larger lumen diameters).

Whereas the ECM is acutely static, slowly cycling AM XBs and possibly titin that operate in-parallel with rapidly cycling AM XBs within the smooth muscle compartment are subject to acute adjustments via cell signaling-dependent control. Thus, the stress (or tension) held by

smooth muscle "at rest" prior to activation by a contractile stimulus can be termed adjustable preload tension (T_{ap}). The sum of ECM-dependent passive tension, T_p, and regulated T_{ap} can be termed the preload tension ($T_{preload}$). When stimulated to contract, rapidly cycling AM XBs are activated and the muscle develops T_a. Thus, total tension, T_t, equals $T_a + T_{preload}$, or, $T_t = T_a + T_{ap} + T_p$.

The Laplace relationship reveals that the degree of wall tension, T, is a function of both lumen radius, r, and pressure, P; for a thin-walled tube, T = Pr. In a cylindrically-shaped balloon partially blown up there exists simultaneously wider and narrower segments (Fig. 2D; note that T at the spherical interface between large and small cylinders is less by half than T_1). Pascal's principle is that P is equivalent throughout the lumen of the static, closed system, so $T_1 > T_2$ because $r_1 > r_2$. Compared to the amount of VSM T that must be developed in the narrower segment to just balance the Pr_2-dependent T_2, the VSM T developed in the wider segment needed to offset the Pr_1-dependent T_1 must be greater. Thus, contraction of an artery segment that causes a reduction in r when starting from a value equal to r_1 (i.e., causes constriction) would need to initially be strong, but after causing constriction and a decrease in r (say to r_2), could be weaker to maintain the new, smaller r. Notably, muscle shortening during vessel constriction moves the muscle down the ascending limb of its stress-strain curve, weakening the contractile strength. As will be discussed, mechanical strain also acts as a contractile stimulus in most small (generally <300 μm) arteries, and reduced strain reduces the intensity of this myogenic stimulus to cause contraction. Moreover, the degree of strain can affect stimulus-contraction coupling. The P gradient down the vascular tree falls from an average value of ~100 mm Hg in larger arteries to ~30 mm Hg in smaller arteries. In general, the motor proteins of large arteries are active to some degree so lumen diameters are between full dilation and full constriction. In the anesthetized mouse, the muscular femoral artery is constricted to ~½ its maximally dilated diameter, and most of this "basal tone" is dependent on basal sympathetic nerve activity.[564] The VSM in these arterial segments bear high stresses indefinitely to prevent the luminal Pr product from causing artery expansion and

movement into the strain region of a stress-strain curve where recruitment of ECM proteins are required to prevent further dilation (Fig. 2B). Contraction of smaller muscular arteries and arterioles generally cause more extensive vessel constriction (Fig. 2C), increasing resistance to blood flow through that segment. This action regulates not only organ perfusion, but also contributes to the control of total peripheral vascular resistance, and therefore, blood pressure.

3. Classification of Contraction as Fast/Phasic and Slow/Tonic

Classification of smooth muscles into discrete categories has been a challenge that soon should be met by investigations combining molecular, developmental and functional analyses.[106,406] In general, smooth muscles are classified as fast/phasic and slow/tonic muscles, based on the manner by which they contract.[128] Stomach antrum and fundus provide classic examples because contractions of antrum are spike-like (phasic) and those of fundus are monotonic and sustain high levels of tension for as long as the stimulus is present.[40,574] Moreover, the maximum rate of cell shortening of isolated antrum smooth muscle cells is nearly 3-fold faster than fundus cells.[92] Urinary bladder smooth muscle (detrusor) is relatively fast (maximum speed of shortening ~0.2–0.4 muscle lengths/s),[211,506,507] and can display contractions that are entirely phasic.[77,400] Thus, detrusor is generally classified as a fast/phasic muscle. However, detrusor contractions also can be entirely tonic.[209] Notably, detrusor contractions often exhibit both behaviors because rhythmic contractions develop "on top of" a certain degree of sustained tone.[77] During a tonic contraction, the strength of the sustained phase may be high (e.g., stimulation with endothelin)[217] or moderate and weaker than that produced initially upon muscle activation (e.g., stimulation with KCl or a muscarinic receptor agonist).[209,454] With a maximum rate of muscle shortening of ~0.5 muscle lengths/sec[211,507] and spike-like spontaneous contractions, portal vein fits the category of a fast/phasic VSM, whereas large conduit and muscular arteries generally are classified as slow/tonic VSMs. Large arteries often respond to contractile stimuli in a graded,

monotonic fashion, and stimulus concentration-response curves (CRCs) can be readily constructed. However, electrical oscillations do occur in these vessel segments.[89,167,470] Moreover, many large and most small arteries display rhythmic contractions "on top of" tonic contractions when activated by contractile stimuli and, in some, rhythmicity occurs spontaneously.[18,93,149,334,348–351,443,475] Rhythmic arterial contractile activity is often termed vasomotion,[334] although this term is reserved by some for rhythmic diameter oscillations observed in small arteries *in vivo*. Arteries less than ~300 μm, and some arteries larger than this, display sustained contraction when rapidly stretched (myogenic tone). In summary, whereas large conduit arteries and portal vein generally fit the classifications of, respectively, slow/tonic and fast/phasic smooth muscles, muscular arteries and arterioles fit less well. Regardless, for VSMs stimulated to contract, the initial rapid contractile phase is termed the phasic component, and the sustained phase the tonic component.[44] The strength of the sustained phase relative to the phasic phase often depends on the type and strength of the stimulus used to cause contraction.[383]

4. Ca^{2+}

Multiple highly dynamic Ca^{2+} compartments exist that, despite a high basal Ca^{2+} "leak (spontaneous releases of packets of stored calcium and Ca^{2+} entry through multiple channel types), act to maintain a ~10,000-fold Ca^{2+} gradient between extracellular space and bulk cytosol in resting VSM.[14,43,223,330,367,373,509] In general, application of moderate-to-high concentrations of a contractile stimulus to an isolated artery *in vitro* induces a rapid increase in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) caused by release of intracellular Ca^{2+} stores and Ca^{2+} entry followed by a decline in $[Ca^{2+}]_i$ that is sustained by Ca^{2+} entry.[41,61,198,224,309,396,403,508] The threshold for VSM contraction is ~100 nM, and full contraction occurs at ~300–1000 nM.[83,115,387] Thus, the relationship between steady-state active stress and $[Ca^{2+}]_i$ is steep.

Whether the contractile response of a vascular segment is a direct reflection of the contractile response of individual VSM cells has been a question of interest for some time.[199] When ionotropic P2X receptors

are activated by ATP released initially by periarterial adrenergic nerve stimulation *in vitro*, VSM cells display spatially discrete, asynchronous, junctional Ca^{2+} transients at a relatively high frequency associated with a weak transient contraction.[259] Subsequent (delayed) activation of α_1-adrenergic receptors causes a slow monotonic contraction associated with asynchronous propagating Ca^{2+} waves within individual VSM cells. Addition of exogenous stimuli to a tissue bath induces only a modestly different response. For example, a maximally effective concentration of α_1-adrenergic receptor stimulus in veins[428] and arteries[566] induces an initial synchronous release of Ca^{2+} from sarcoplasmic reticulum stores, followed by asynchronous propagating Ca^{2+} waves. In all cases, Ca^{2+} waves begin in discrete subcellular domains and transient Ca^{2+} waves propagate through the cell.[332] In short, contractile stimuli cause VSM tissue contraction by recruiting VSM cells, and by increasing the frequency of asynchronous Ca^{2+} oscillations once the cells have been recruited.[428] One attractive general model is that, at low stimulus concentrations, weak tissue contractions are sustained by recruitment of cells that generate asynchronous Ca^{2+} waves, and at moderate and high stimulus concentrations, intermediate and strong tissue contractions are sustained by asynchronous Ca^{2+} waves that increase, respectively, in frequency and velocity.[263] Asynchronous Ca^{2+} waves reflect regenerative Ca^{2+} release from the sarcoplasmic reticulum.[34,96,199,440]

Rhythmic contractions appear to reflect entrainment of Ca^{2+} and contractile oscillations through the formation of interconnections.[334] That is, to entrain and produce rhythmic tissue contractions, cell-to-cell coupling and coordination of Ca^{2+} oscillators would be required. Supporting this hypothesis, gap junction inhibition has been shown to abolish rhythmic contractions in small irideal arteries[175] and myogenic contraction of cerebral arteries.[258] Notably, rhythmic contractions originating from Ca^{2+} oscillations in VSM cells can be altered by Ca^{2+} oscillation-induced release of relaxants from endothelial cells.[226] The extensive sarcoplasmic reticulum of smooth muscle that is closely associated with the plasma membrane, caveolae, and mitochondria, acts as a superficial Ca^{2+} buffer barrier that regulates membrane potential, internal Ca^{2+} stores, mitochondrial Ca^{2+} (and ATP levels) and $[Ca^{2+}]_i$,[263,508,509] so subdomain Ca^{2+} signaling does not necessarily

correlate with contraction.[298] In short, Ca^{2+} causes contraction when Ca^{2+} is delivered locally to contractile filaments. Like two circles of a Venn diagram, the set representing global $[Ca^{2+}]_i$ and that representing AM-XBs responsible for contraction may not entirely ovelap.

These descriptions of Ca^{2+}-induced contractions do not account for the likelihood that the tonic phase represents a dynamic process involving Ca^{2+} sensitization,[222,355,465] a transition from rapidly cycling to slowly cycling AM XBs (formation of latch bridges),[150–153,397,407] changes in actin polymerization and myosin filament formation, and sarcomere rearrangements that proceed slowly, perhaps continually.[105,108,288,485,488,496,556] The lack of tight coupling between stimulus-induced $[Ca^{2+}]_i$ and tension in tonic smooth muscle can be seen as a temporal counterclockwise hysteresis between these parameters.[115,177,465] For example, upon stimulation of rabbit muscular arteries with a high [KCl], $[Ca^{2+}]_i$ and tension change in two phases.[402] $[Ca^{2+}]_i$ initially rises rapidly (~22%/sec) within 3 sec to ~65% of the maximum value, then more slowly (3%/sec), achieving the maximum value by 15 sec. During these 15 sec, tension rises at ~3–5%/sec to ~73% of its maximum level. From 15 sec to 3 min, tension continues to rise very slowly (~10%/*min*) to a maximum value that is sustained for at least 10 min, while $[Ca^{2+}]_i$ falls back to ~65% of the maximum value by 10 min. The tension increase corresponding with the increases in $[Ca^{2+}]_i$ corresponds to the phasic phase of contraction, and the delayed, very slow increase in tension that occurs independently of a further increase in $[Ca^{2+}]_i$ (in this case, as $[Ca^{2+}]_i$ falls) corresponds with the tonic phase of contraction.[44] Both the sensitivity and strength of VSM stimulus-contraction coupling is a highly regulated parameter that can adjust up or down (stimuli can become more or less potent) relatively rapidly.[125,131,191,300,377,379,380,387,391,395,399,404,445,446,512,514,566]

5. Cell Signaling

Cells may be thought of as computers that process numerous inputs to produce specific outputs.[49] Rather than hardware and software, cells use wetware to interpret and act on the information derived from extracellular stimuli. Thus, biological information processing involves cell signaling pathways, and the general scheme is stimuli → cell

signaling → response(s). The first step in signaling involves binding of a stimulus to a receptor that generally is located in the plasma membrane but may also be found in other membrane and cytosolic compartments. For clarity, signaling pathways were initially divided into discrete entities (modules), starting from a particular stimulus and ending with the response of interest. For example, the classical epinephrine (Epi)-to-glucose output module for hepatocytes may be written as: Epi → [β-adrenoceptor → adenylyl cyclase (AC) → cyclic-AMP (cAMP) → cAMP-dependent protein kinase (PKA)] → glycogenolysis → glucose, where cell signaling is identified in brackets. However, it is now clear that a single stimulus that produces a certain response often activates multiple cell signaling modules to varying degrees, and that a cell signaling cascade can lead to amplification such that the K_D for a stimulus and its receptor may reside at a much higher stimulus concentration than the EC_{50} for the stimulus and its response.[69] Moreover, temporal feedback mechanisms may "turn off" or "tune down" (i.e., desensitize) certain coupling mechanisms.[573] Most cell signaling pathways do not operated independently because elements within certain signaling modules often interact with elements of other modules. Molecular scaffolds and anchoring proteins such as proteins of the AKAP, annexin, RACK, 14-3-3, paxillin and PDZ families participate not only in spatial organization of signaling, but also in trans-modular crosstalk.[52,136,188,423,460,567] In short, signaling pathways form highly complex networks that include feedback signaling leading to ultrasensitivity, oscillations, bistability, and chaotic patterns,[23] and modest differences in certain protein isoforms can have profound differences in signaling outcome.[99] It is most likely that *in vivo*, multiple stimuli simultaneously activate VSM cells, so threshold modulation, additivity and synergism of signaling systems probably occur.[212,268,477,558] The extreme complexity of signaling systems was the driving force for developing new journals (Science Signaling, for example) and new computational tools used by modelers and systems biologists.[462] The remainder of this chapter will focus on aspects of the arterial VSM signaling network involved in regulating the degree of 20 kDa regulatory myosin light chain (MLC) phosphorylation (MLCp) responsible for controlling the degree of contraction.

The cell signaling pathways that regulate arterial VSM MLCp represent a highly heterogenous system. To gain insight into differences displayed by different arterial segments in VSM cell signaling, comparative studies are required. This area is poorly funded, so progress has been slow.

6. Stimuli that Regulate the VSM Contraction Control System

Inputs that acutely regulate the degree of VSM contractile tension include contractile and relaxant stimuli, often termed vasoconstrictors and vasodilators, respectively (Fig. 3A). VSM cells actively shorten, and passively elongate. That is, the motor moves one-way only to cause VSM shortening. Thus, a relaxant stimulus does not actively cause vasodilation, but instead, reduces the degree of active tension generated by the motor proteins. VSM cells elongate when the motor's tension is less than the opposing tension exerted by luminal pressure, causing vasodilation (Fig. 3B).

There is insufficient cardiac output to fully perfuse all organs simultaneously. In general, whole organism homeostatic regulation of VSM involves systemic production of neural and hormonal contractile stimuli to limit cardiac output distribution and maintain blood pressure at levels sufficient to adequately perfuse the brain and heart, although adrenal medullary production of epinephrine participates with local control in the maintenance of skeletal muscle blood flow at lower exercise intensities.[213,542] The systemic outflow of contractile stimuli is made especially apparent during the trauma of hypovolemic shock.[254] Opposing the demands of essential organs are those of individual cells and tissues that autoregulate blood flow locally by releasing relaxant and contractile stimuli, activating cell-cell coupling mechanisms, and acutely modulating cell signaling systems.[131,170,377,426,448,511] Thus, VSM and adjacent cells such as the endothelium are exposed to numerous stimuli in a dynamic spatiotemporal manner to control the biomechanical activities of various blood vessel segments in all organs.[58,447] A recent model of local blood flow regulation dispenses with hypoxia-induced release of

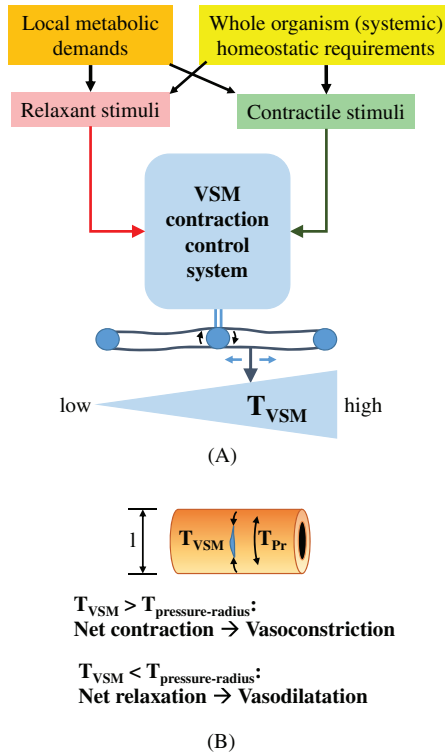

Fig. 3. General model of the vascular smooth muscle (VSM) contraction control system that determines VSM tension (T_{VSM}) based on the degree of activation by contractile and relaxant stimuli (A). Whether an artery constricts or dilates depends also on the degree of counteracting wall tension, which is dependent on luminal pressure and radius (B).

metabolites as controlling agents and incorporates recent information on the opposing actions of reactive oxygen (contractile) and nitrogen (relaxant) species.[129]

Major contractile stimuli released by peri-arterial sympathetic post-ganglionic nerves[71] include ATP and its metabolites (e.g., adenosine, Ado),[53,234] norepinephrine (NE)[564] and neuropeptide Y (NPY).[229,541] Other contractile stimuli include angiotensin II (Ang II) produced by peptidase cleavage of Ang I in blood and tissues, including the vascular wall and periarterial fat,[295,490,539] arachidonic acid generated by phospholipases,[130,304] endothelial-dependent contracting

factors (EDCFs), histamine released from mast cells and adrenergic nerves in some species and blood vessels,[66] serotonin (5-HT) released from platelets and possibly adrenergic nerves,[71,535] thromboxane A_2 (TXA_2) from platelets, urotensin II,[516] and vasopressin (VP) from the posterior pituitary. EDCFs[256] include endothelin-1 (ET1)[489,490] arachidonic acid[227] and its metabolites including 5-lipoxygenase products (leukotrienes), cyclooxygenase products such as TXA_2 as well as other prostaglandins which subsequently diffuse to and activate TXA_2 prostanoid receptors on VSM[212,256,353,547] and the cytochrome p450 metabolite 20-hydroxyeicosatetraenoic acid (20-HETE).[402,421] Notably in some cases, the relaxant agent prostacyclin (PGI_2) generated in this way activates VSM TXA_2 receptors to cause contraction.[102] Coronary artery spasm can be induced by acetylcholine (ACh) infusion in patients with variant angina,[194] exogenous ACh releases endothelium-dependent relaxant factor (EDRFs) in most vasculatures[107,116,117] and EDCFs in the spontaneously hypertensive rat,[546] and cholinergic nerves releasing ACh have been identified in certain vascular beds.[486] However, this neurotransmitter does not appear to act as a physiological VSM stimulus in most species and vascular beds. Mechanical and electrical signals and cell-cell coupling also play important roles as VSM stimuli. In particular, the myogenic response and vascular conducted responses, both vasodilation and vasoconstriction, represent major stimuli in small arteries ensuring that local O_2 delivery balances O_2 demand.[17,176,378,416,448] In arterioles but not arteries of the rat,[338] NADPH oxidase and subsequent generation of reactive oxygen species is a necessary step for stretch-activated myogenic contraction.[338]

There is considerable heterogeneity in vascular responsiveness to contractile stimuli within a particular vascular segment, down the vascular tree (compare Fig. 4A with 4B, 4C and 4E), and across species (compare Fig. 4C and 4D). Mechanisms responsible for transduction of the external signal to internal signals include receptors and associated coupled signaling systems (http://www.cellsignallingbiology.org/csb/). Both receptors and receptor-coupled signaling systems are highly complex, but in general, all of the contractile signaling systems converge on discrete intracellular signaling modules that control the myosin-based motor control system, and non-canonical

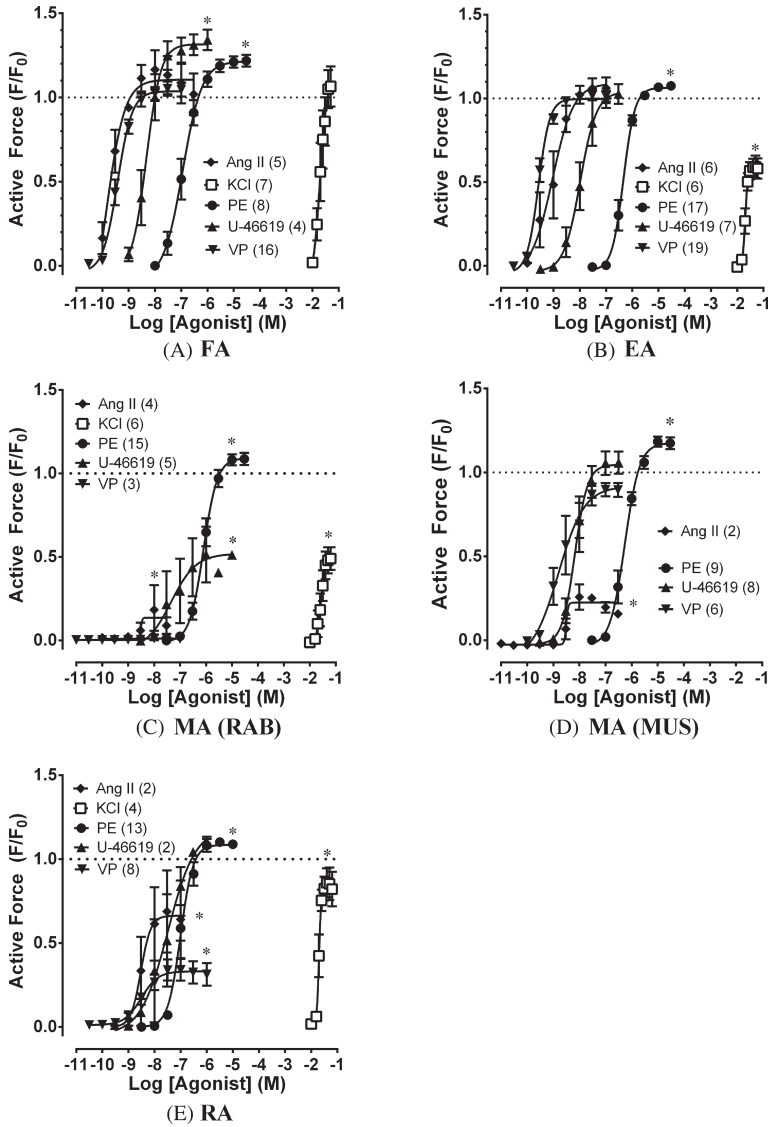

Fig. 4. Examples of heterogeneity of responses to contractile stimuli within a particular arterial segment (A rabbit femoral artery, B rabbit epigastric artery, C rabbit mesenteric branch artery, D mouse mesenteric branch artery, E rabbit renal artery), comparing different arteries (A, B, C & D, E), and comparing the same artery across species (C & D). Data are active force normalized to the maximum force generated by KCl at the optimum length for contraction, and are reported as means ±SE, n values (number of animals) are in parentheses. * indicates maximum stimulus-dependent force is >1 or <1.

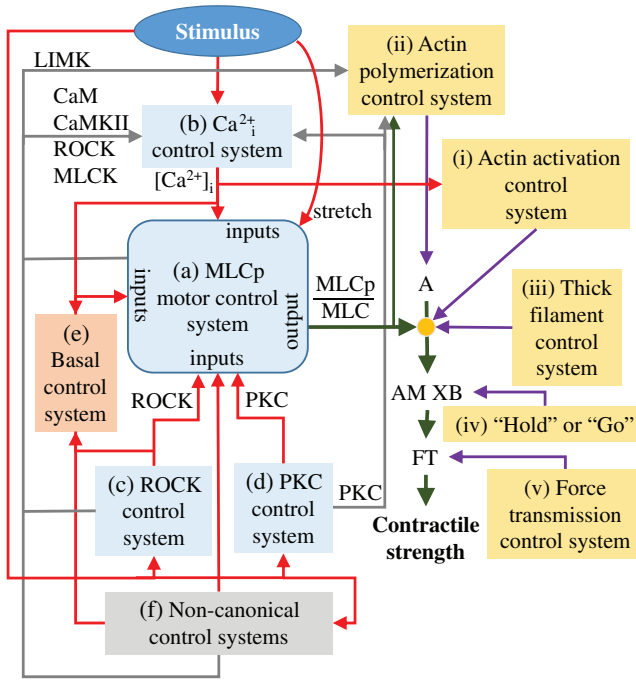

Fig. 5. The canonical vascular smooth muscle (VSM) contraction control system (light blue) activated by a contractile stimulus (dark blue) is comprised of 4 cell signaling control systems; a myosin light chain phosphorylation (MLCp) control system (a) consisting of myosin light chain kinase (MLCK) and protein phosphates 1 (PP1M; see Fig 6B), a Ca^{2+}_i control system (b) that determines the level of Ca^{2+} input to (a), and rhoA kinase (ROCK, c) and protein kinase C (PKC, d) control systems that determine the level of active ROCK and PKC inputs to (a). Additional (non-canonical) control systems that feed into (a) are bundled into (f), and a basal control system (e) regulates (a) when VSM is "at rest". Also shown are non-canonical control systems downstream from the MLCp control system that determines contractile strength (i–v). A, actin; AM XB, actomyosin crossbridge; Ca^{2+}_i, intracellular free Ca^{2+}; FT, force transmission; CaM, calmodulin; CaMKII, calmodulin-dependent protein kinase II; LIMK, lim kinase.

AM XB activating control systems (Fig. 5). For example, the stimulus phenylephrine activates α-adrenergic receptors to elevate the activities of certain trimeric G proteins that, acting as inputs to the Ca^{2+}, ROCK and PKC control systems, alter inputs to the MLCp motor control system to cause a temporal change in the MLCp/MLC ratio. In very

general terms, most VSM contractile stimuli activate G protein-coupled receptors that transduce information delivered by the extracellular signals into specific intracellular signaling via trimeric G proteins belonging to the G_q, $G_{12/13}$ and G_i families.[9,344] Also, in very general terms, the α subunit of G_q, $G_{12/13}$, and G_i, respectively, activates phospholipase C to generate diacylglycerol and inositol-trisphosphate, activates rhoA-GEFs to generate active rhoA, and inhibits adenylyl cyclase. The $G\beta\gamma$ subunits released by activation of G protein-coupled receptors may activate several signaling modules, including those that alter membrane potential. Although not highlighted above, certain growth factors activating receptor tyrosine kinases, and mechanotransduction mechanisms, also can provide inputs to the control systems regulating the MLCp motor control system of VSM. Thus, the red arrows in Fig. 5 linking the contractile stimulus with discrete control systems represent the cell signaling processes activated by each stimulus that provide inputs to Ca^{2+}, ROCK, PKC, and other (non-canonical) control systems that, in turn, send inputs to the MLCp motor control system.

Major relaxant stimuli include Ado,[53] adrenomedullin,[48] ATP,[53] bradykinin (BK),[490] calcitonin gene-related polypeptide (CGRP),[48,165,230,538] cannabinoids,[275,474] epinephrine (Epi),[213,542] EDRFs,[101] natriuretic peptides,[287,548] vasoactive intestinal polypeptide (VIP),[171,538] and urocortin.[216,279,366] EDRFs include nitric oxide (NO) and its metabolites,[107,116,117,555] H_2S,[31,527] and arachidonic acid metabolites including cyclooxygenase products (e.g., PGI_2), cytochrome P450 products (e.g., epoxyeicosatrienoic acids (EETs) and glycerate EETs (GEETs)) and 12- and 15-lipoxygenase products.[2,57,64,95,364] Endothelium-dependent relaxation also involves a phenomenon termed endothelium-dependent hyperpolarization, in which the participation of gap junctions may be obligatory.[82] Relaxant stimuli such as Ado, ATP, BK, and CGRP act largely, but not exclusively, by causing release of EDRFs.[53,170,234,490] Many vasoactive stimuli affect numerous cell signaling systems in VSM and other tissues, and stimuli that generally are considered potent and strong contractile stimuli often also can cause relaxation. For example, the contractile stimulus Ang II and its metabolites can cause VSM relaxation.[26] The mechanical stimulus of

shear stress acting on endothelial cells also is a strong stimulus for release of EDRFs.[58] Many stimuli that act directly on VSM cells to cause contraction can stimulate endothelial cells to release EDCFs and EDRFs, and certain contractile stimuli can act indirectly to alter release of relaxant stimuli. For example, NPY can attenuate release of CGRP in mesenteric arteries,[229] and both urotensin II and ET1, potentially potent contractile stimuli, often cause VSM relaxation by acting on endothelial cells.[516] ACh release from sympathetic cholinergic fibers in skeletal muscle vascular beds of some species,[214] and histamine taken up and released by adrenergic nerves[190] can cause VSM relaxation by stimulating release of vasodilators from endothelial cells.[66,164] ET1 and Ang II stimulate VSM directly, causing contraction, and stimulate endothelial cells to release NO and PGI_2 and cause relaxation.[489,490] NADPH oxidases and reactive oxygen species also participate in the regulation of contraction.[19,156] For example, hydrogen peroxide can act both as a contractile and relaxant stimulus.[121,280]

Most vasodilators reduce the degree of VSM motor activation by generating the cyclic nucleotides, cAMP and cGMP. Cyclic nucleotide signaling is represented by a network that involves significant crosstalk in which these messengers modulate each other's synthesis, degradation, and effectors.[33,110,362] The primary effectors are PKA and PKG, and despite their names, these kinases are not wholly selective for the cyclic nucleotides for which they are named. Notably, cAMP can activate PKG, and the ability of the AC activator forskolin to cause reductions in VSM Ca^{2+} entry is due to activation by cAMP of PKG, not PKA.[210,272,273] In addition to kinase activation, cyclic nucleotides regulate cyclic nucleotide phosphodiesterase activities, and can activate cyclic nucleotide-gated (CNG) Ca^{2+}-permeable nonselective cation channels that, when expressed in endothelial cells cause relaxation and, when expressed in VSM, may enhance contraction.[110,271] Moreover, cAMP binds the exchange protein activated by cAMP (Epac) to elevate activity of ras family members, rap GTPases.[418] In VSM, the cAMP-Epac-rap signaling system causes relaxation by activation of rho-GAP.[576] In cutaneous vasculature, the cAMP-Epac-rap module activates rhoA-ROCK to cause increased translocation along microfilaments of internal α_{2c} adrenergic receptors to the plasma

membrane, with a consequent increase in adrenergic-dependent contraction.[208,314] Relaxant stimuli that couple to cAMP do so via activation of one or more of the 9 adenylyl cyclase (AC) isotypes,[139,158] and relaxant stimuli that couple to cGMP do so via activation of the NO receptor (soluble) guanylate cyclase and the peptide-sensitive particulate guanylyl cyclase.[113,548] Relaxant stimuli that bind G protein-coupled receptors may cause relaxation by activating the α subunit of G_s, which activates all 9 AC isotypes, or by releasing $G\beta\gamma$ subunits that stimulate AC2, AC4 and AC7. However, $G\beta\gamma$ subunits also can inhibit AC1, AC5, and AC6, and increases in $[Ca^{2+}]_i$ inhibit activation of AC5 and AC6. Interestingly, increases in $[Ca^{2+}]_i$ activate AC1, AC3, and AC8. Thus, isoform expression plays an important role in cyclic nucleotide signaling. In VSM cells, G_q-dependent increases in $[Ca^{2+}]_i$ cause reductions in AC5 and AC6 activities,[520] and intimal but not medial VSM cells populating atherosclerotic lesions express AC8.[142] Whereas cyclic nucleotides represent the primary relaxant signaling system, relaxant agents can act by other mechanisms. For example, NO not only elevates cGMP levels, but also can cause nitrosylation reactions that affect contractile activity,[72] and relaxant stimuli can, by elevating microdomain Ca^{2+} (Ca^{2+} sparks), activate Ca^{2+}-dependent K^+ channels to cause VSM hyperpolarization.[261] Interestingly, Ca^{2+} can act as a VSM relaxing signal. Ca^{2+}-activation of a Ca^{2+}-sensing receptor expressed by adventitial perivascular sensory nerves induces release of EETs, GEETs, and anadamide, causing mesenteric artery relaxation.[22] EETs and GEETs cause relaxation by activating VSM K^+ channels, and anadamide acts on VSM cannabinoid receptors.

Contractile and relaxant stimuli control the number of AM XB motors that are "on" and able to transmit force in a fashion that is somewhat distinct from the way that a potentiometer controls a DC motor. A potentiometer "dials in" the amount of energy to supply a motor. Whereas energy availability represents one of several control systems for VSM contraction (see below and[129]), the level of energy (ATP) for each AM XB motor (and for the multiple phosphorylation events involved in motor-control) is not the primary physiological motor controller. The central control system of the canonical VSM

contraction control system is the MLCp *motor* control system (Fig. 5(a)) comprised of calmodulin (CaM), CaM-dependent myosin light chain kinase (MLCK) and the protein phosphatase 1 catalytic subunit[161] (PP1M) that together regulate the degree of MLC phosphorylation. Thus, the output of the VSM contraction control system, via the motor control system, is a certain ratio of phosphorylated-to-total MLC (MLCp/MLC). The rate of force development is limited by the rate of MLC phosphorylation rather than by intrinsic properties of the AM XBs.[468] The three primary canonical inputs to the motor control system include outputs from the Ca^{2+} control system (Fig. 5(b)), the rhoA-dependent coiled coil kinase (ROCK) control system (Fig. 5(c)), and the protein kinase C (PCK) control system (Fig. 5(d)).[382,466] The outputs represent certain levels of Ca^{2+}, and ROCK and PKC activities, determined by the effect of stimulus-induced receptor activation (Fig. 5, red arrows from "Stimulus" to control systems). In VSM, mechanical stretch activates Ca^{2+} and other control systems,[176,262,306,515,537,571] or may bypass these to directly activate MLCK[27] within the motor control system (Fig. 5, curved arrow labeled "stretch"). This latter possibility seems plausible given that MLCK is a member of the family of mechano-sensing cytoskeletal giant proteins that include titin,[122,247] and that MLCK could readily sense sarcomeric strains because opposite ends of this elongated molecule may simultaneously bind myosin and actin.[184,187] The VSM MLCp motor control system is not completely quiescent in the absence of external stimuli. Thus, a basal control system is also included in this model (Fig. 5(e)). Whether this system represents constitutive activities of the Ca^{2+}, ROCK and PKC control systems, other control systems (Fig. 5(f)), or the action of a basal level of stimulation, remains to be determined.

The output of the VSM contraction control system (the MLCp/MLC ratio), in conjunction with non-canonical control systems (Fig. 5(f) and Fig. 5i–v) determines the number of active AM XBs and force transmission to the extracellular matrix, adjacent VSM cells and ultimately the whole tissue. Non-canonical systems include (i) actin activation permitting myosin engagement (tight-binding) with actin, and actin inhibition permitting rapid relaxation,[4,16,146,150,238,366,408,525]

(ii) actin polymerization,[30,180,488,553] (iii) thick filament assembly,[414,461] (iv) latch bridge formation[56,150,152,155,324] and/or sarcomere structural rearrangements,[108] and (v) regulation of force transmission from motor proteins to the ECM and other VSM cells.[135,238,488,525]

Dynamic control of the microfilament-based cytoskeleton is regulated by over 100 actin-binding proteins.[376] The rho GTPase, PKC and other (e.g., p21-activated kinase, PAK) control systems participate in this process by regulating dual specificity LIM kinases (LIMKs), cofilin and heat shock protein 27.[284,306,307,575] In rat cerebral arteries, rhoA knockdown leads to reduced stimulus-mediated contraction not by reduced MLCp, but by attenuated actin polymerization.[78] However, actin polymerization is also regulated in part by MLCp, such that increases in MLCp increase the degree of actin polymerization[65] (Fig. 5, dark green arrow from MCLp motor control system to actin polymerization control system). The myosin head (S1 fragment) can bind two adjacent actin monomers,[45,231] so elevated MLCp may serve as an actin polymerization nucleating factor.[105] In addition to directly altering force transmission, changes in actin polymerization and crosslinking modifies cell viscosity, indirectly affecting contractile biomechanics.[240,255,390] Lastly, non-canonical control systems feed indirectly into regulation of contraction by modulating other control systems and also by feeding directly into the MLCp control system (Fig. 5).[179] Examples of non-canonical control systems include AMP-dependent protein kinase (AMPK), Ca^{2+}-calmodulin-dependent kinase II (CaMKII), extracellular signal-regulated kinase 1/2 (ERK1/2) of the mitogen-activated protein kinase (MAPK) family, kinase inhibitor of NF-κB 2 (IKK2), integrin-linked kinase (ILK), myotonic dystrophy protein kinase (DMPK) and the related myotonic dystrophy-related Cdc42-binding kinases (MRCK), PAK, MAPK-activated protein kinase 1/p90 kDa ribosome S6 kinase 2 (RSK2, a.k.a. p90RSK and MAPKAP-K1), MAPK-activated protein kinase 2 (MK2, a.k.a., MAPKAP-K2), and a member of the ubiquitously expressed death-associated protein kinase (DAPK) subfamily, zipper-interacting kinase (ZIPK). Thus, the degree of pseudo steady-state contractile force is determined by molecular algorithms that use, as inputs, data from the multiple control systems that may be activated

to varying degrees. Relaxant stimuli activate cell signaling control systems that interact with one or more of these contractile control systems to attenuate their ability to increase contractile strength.

7. Motor Control via Regulation of MLCp

7.1. *Canonical Control*

Research on control of the VSM contractile state has focused primary on regulation of MLCp. This is because early work on non-muscle cells, platelets, AM filaments, permeabilized smooth muscle and muscle tissues revealed that a Ca^{2+}-dependent increase in MLCp is necessary for contraction.[1,28,60,62,87,162,463] That is, contraction of smooth muscle and non-muscle cells is primarily thick filament-regulated, which is unlike Ca^{2+}-dependent contraction of striated muscle that is thin-filament-regulated (although MLCp modulates striated muscle contraction long-term).[112,220] While it is generally accepted that MLCp is necessary for *in vitro* assembly of smooth muscle myosin into filaments and for actin-activation of myosin ATPase activity,[186,493,494] whether these steps are sufficient to explain the full-range of *in vivo* contractility is more controversial. As mentioned, other forms of regulation play critical roles (Fig. 5, i–v). In arterial VSM the relationship between active stress and MLCp/MLC is quite steep and saturating.[397] The most rigorous analysis to-date indicates that basal MLCp/MLC is ~0.15 and maximum stress occurs at ~0.25–0.3 (Fig. 6A),[410] supporting the notion of AM XB cooperativity.[468] The function and regulation of high basal levels of MLCp in VSM has not been resolved. However, in detrusor smooth muscle basal MLCp supports T_{ap}, the "tensional platform" upon which active contraction can rapidly develop,[405,471,472] and a similar mechanism may exist in VSM.

Major elements of the canonical MLCp control system that determine the MLCp/MLC ratio are CaM, MLCK, and PP1M (Fig. 6B). CaM is a universal cell Ca^{2+} sensor[70] and regulatory subunit of MLCK.[513] There is insufficient cellular CaM to activate all CaM-dependent systems.[363] However, CaM does not appear to be limiting for smooth muscle activation because it remains tightly bound to myofilaments at low resting Ca^{2+}_i levels, and even in the absence of

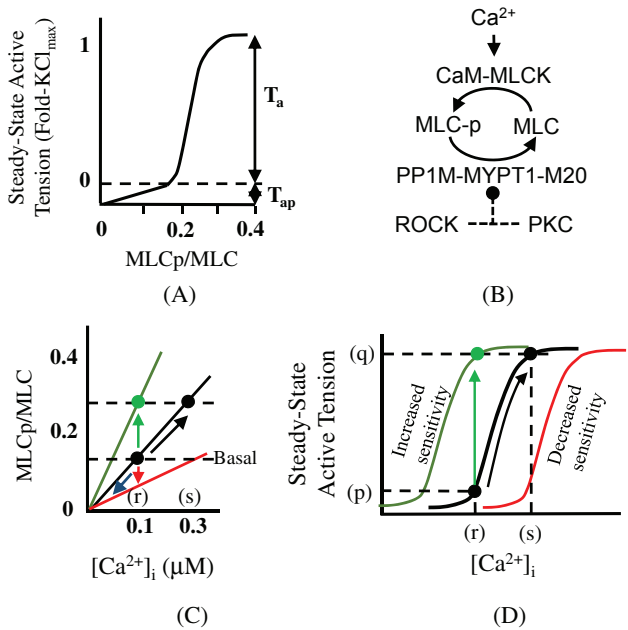

Fig. 6. The relationship between the ratio of myosin light chain phosphorylation to total myosin light chain (MLCp/MLC) and steady-state active tension is steep, and the threshold level for active tension occurs at a relatively high basal MLCp/MLC value of ~0.15 in tonic artery (A). The MLCp control system is comprised of calmodulin-dependent myosin light chain kinase (CaM-MLCK) and myosin light chain phosphatase consisting of the catalytic subunit of protein phosphatase 1 (PP1M), a large myosin phosphatase targeting regulatory subunit (MYPT1) and small subunit (M20). Ca^{2+} and active rhoA kinase (ROCK) and protein kinase C (PKC) serve as inputs to the MLCp control system (B). $[Ca^{2+}]_i$ determines the level of MLCp/MLC (C) and tension (D). These relationships can be shifted leftward (green lines) by ROCK and PKC, and rightward by relaxants (red lines).

Ca^{2+}.[544] As with most proteins, CaM is subject to regulation by phosphorylation,[32] and in VSM, the level of free CaM may be elevated by PKC,[193] increasing the CaM "input" to other control systems.

Early studies identified MLCK as the major cell signaling system responsible for causing increases in MLCp[111,369] leading to VSM contraction.[60] Ca^{2+}_i levels are highly regulated by multiple ion channels and pumps, by unique spatial arrangements and

interactions of subcellular compartments, and by feedback mechanisms.[14,34,91,143,203,294,373,398,509,529,571,572] Thus, activation of MLCK is controlled upstream by complex Ca^{2+} signaling systems that determine the spatio-temporal level of $[Ca^{2+}]_i$ that "feeds" the MLCp control system. Of the many signaling systems involved in $[Ca^{2+}]_i$ control, the L-type voltage-operated Ca^{2+} channel (VOCC) plays a prominent role.[181,331,537] Ablation of the VOCC isotype specific to smooth muscle, $Ca_v1.2b$, decreases mean arterial blood pressure by ~30 mm Hg, and *in vitro*, greatly diminishes contractions induced by α-adrenergic receptor activation and stretch.[305] However, other Ca^{2+} entry pathways, such as receptor-operated Ca^{2+} channels, store-operated Ca^{2+} channels,[100,143,260,270] reverse-phase Na^+–Ca^{2+} exchange),[571,572] and Ca^{2+} release from intracellular pools[34,509] also play major roles. Interestingly, the other primary inputs into the MLCp control system, PKC[337,401,411] and ROCK,[124] and elements of the MLCp control system, MLCK[291] and CaM,[100,154,368] have been reported to participate in control of Ca^{2+}_i and membrane potential (Fig. 5, gray arrows). The actions of ROCK and MLCK on $[Ca^{2+}]_i$ signaling may be indirect, involving the regulation of cytoskeletal proteins and subcellular protein trafficking.[290,291]

7.2. *MLCK*

MLCK, a member of the CaM kinase family of serine-threonine kinases,[285] is highly selective for the regulatory MLC of non-muscle and smooth muscle cell.[186] In fact, MLC appears to be the sole substrate for MLCK.[219] Specific deletion experiments reveal that MLCK is essential for smooth muscle contraction.[168,169,531] MLCK binds tightly to both actin and myosin, and the current view is that MLCK localization to AM is via high affinity actin-binding.[186,187] Notably, although the concentration of smooth muscle myosin is greater than MLCK, one MLCK molecule rapidly phosphorylates many MLC subunits.[185] To accomplish this, MLCK moves along actin filaments, permitting phosphorylation of unphosphorylated MLCs.[184] As with many other ser-thr kinases, an intrasteric autoregulatory sequence, termed a pseudosubstrate domain because of its sequence similarity to

the substrate,[109,233] binds the MLCK catalytic active site, retaining the kinase in an inactive state until activated by Ca^{2+}–CaM.[248]

When bound to the Ca^{2+}-sensor CaM, ~1 μM Ca^{2+} maximally activates MLCK.[481] MLCK activation occurs when the pseudosubstrate domain is sequestered by allosteric binding of Ca^{2+}–CaM to MLCK, permitting MLC access to the active site.[219,247] The [MLC] in smooth muscle is estimated to be similar to the apparent K_m for MLCK, ~30–50 μM, and the estimated molecular activity of MLCK for MLC is ~3/sec.[12,103,481] In maximally stimulated carotid artery, MLCp rises with a half-time of ~1 second.[459] Alternative splicing of the *mylk1* gene produces large (220 kDa) and small (130 kDa) MLCK isotypes.[173,219] Moreover, an Ig domain C-terminal to the protein kinase domain can be transcribed from *mylk1* independently of MLCK and expressed exclusively by smooth muscle tissues as a 17 kDa protein termed telokin (a.k.a., kinase-related protein, KRP).[119,458]

7.3. *CaMKII, ERK and Regulation of MLCK-dependent Ca^{2+} Sensitivity*

A non-canonical VSM contraction control system, CaMKII, can catalyze phosphorylation of MLC *in vitro* with K_m and V_{max} values ~10-fold higher and lower, respectively, than MLCK when MLC is used at about the same substrate concentration.[94] The affinity of CaM for MLCK is over 100-fold greater than for CaMKII, and MLCK is bound to AM even when VSM is "at rest". Thus, CaMKII is generally not considered a physiological activator of MLCp. However, $[Ca^{2+}]_i$ may be elevated to high levels initially upon VSM stimulation which could cause CaMKII activation, and a Ca^{2+}-independent partial CaMKII activity may be retained for some time after Ca^{2+} has declined towards pre-stimulus levels at the steady-state of a stimulus-induced contraction (i.e., CaMKII has "molecular memory").[536] This autonomous CaMKII activity has been associated with VSM tension-maintenance,[320] but not because of CaMKII-dependent MLCp. A more likely form of MLCp regulation by CaMKII is to cause a reduction in Ca^{2+} sensitivity (Fig. 6D, red sigmoidal curve, and Fig. 7). In short,

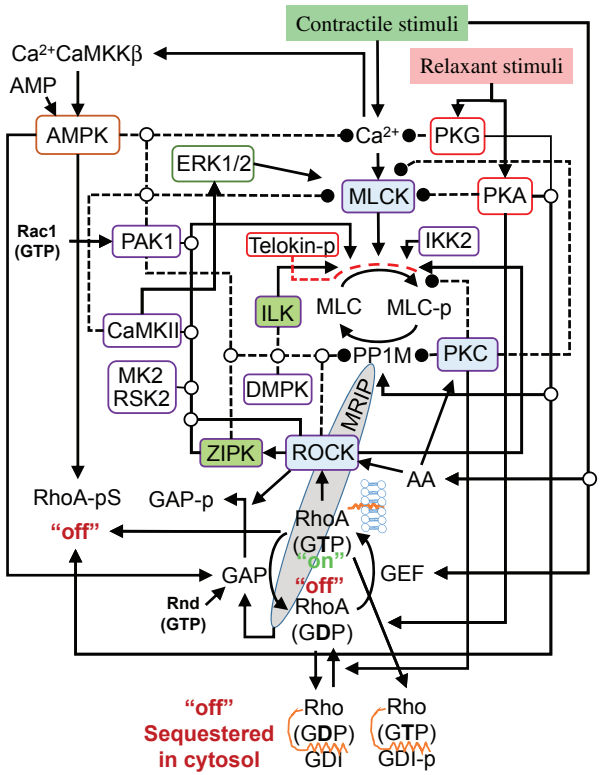

Fig. 7. Vascular smooth muscle contraction/relaxation control system signaling network.

the degree of activation of MLCK by Ca^{2+}-CaM may be limited at high Ca^{2+}-CaM concentrations because CaMKII can phosphorylate MLCK, reducing the affinity of CaM for MLCK.[219,464,487,552] Similar phosphorylation-dependent inhibition of CaM affinity for MLCK is induced by PKC, PKA and PAK[126,219,434] (Fig. 7). Because CaM is tightly bound to MLCK even at low $[Ca^{2+}]_i$, the extent to which this system operates to reduce the sensitivity of MLCK to Ca^{2+} in VSM remains to be fully elucidated. Like CaM, MLCK, PKC and ROCK, CaMKII serves as an input into the $[Ca^{2+}]_i$ control system[232,267] (Fig. 5, gray lines).

Another non-canonical VSM contraction control system, ERK, can enhance the ability of CaM-dependent MLCK to phosphorylate

MLCp.[219,239,244,464,540] That is, ERK can theoretically elevate the degree of force produced for a given $[Ca^{2+}]_i$ (i.e., cause Ca^{2+} sensitization; Figs. 6C and 6D, green lines). ERK activity is associated with VSM contraction,[238] and a CaMKII-γ G2 variant antisense knockdown study revealed that the ERK-activated increase in MLCp in ferret aorta is downstream from CaMKII (286). CaMKII and ERK may also play a role in thin filament and cytoskeletal control systems.[238] In other VSM tissues, ERK does not appear to play a role in regulation of thick or thin filament-regulated contraction.[132,336,392] In rabbit femoral artery in particular, ERK appears to play a role in transmission of information to the cell about the degree of α_1-adrenergic receptor-induced contraction, rather than in a contraction control system.[392] Interestingly, ERK is partially phosphorylated in resting, differentiated rabbit artery. Thus, MEK is constitutively "on" in VSM. However, an intact endothelium reduces the degree of ERK phosphorylation by a mechanism other than PKG activation, which tends to elevate ERK phosphorylation.[251,392]

7.4. *Smooth Muscle PP1cδ (PP1M) and its Regulatory Subunits*

Whereas the human kinome encodes over 400 ser-thr kinases, only 5 catalytic subunit genes encode the two most abundant protein phosphatases, PP1c and PP2Ac.[519] These protein phosphatase catalytic subunits acquire selectivity for particular substrates by binding different regulatory subunits. PP1c alone binds to over 50 regulatory subunits.[76] Many of the regulatory subunits target PP1c to different cellular locations, enhancing dephosphorylation selectivity as well as regulating phosphatase activity. The PP1c isotype expressed in VSM, thought to primarily be PP1cδ, is termed MLCP, SMPP-1M and PP1M. Although inhibitor-1 is the canonical inhibitor of PP1c activity, this regulatory subunit does not appear to play a major role in regulation of PP1M and VSM contraction.[59] However, several other proteins regulate PP1M, including the large regulatory subunit, myosin phosphatase targeting subunit (MYPT1),[161] PKC-dependent protein phosphatase 1 inhibitor 17 (CPI-17) and related

members,[97] telokin, prostate-apoptosis response-4 protein (Par-4),[282,518] smoothelin-like protein (SMTNL1),[47,499,510] and the AMP-dependent protein kinase (AMPK)-related kinase, ARK5 (a.k.a., NUAK1).[565] Binding of VSM PP1M with MYPT1[134] confers selectivity towards myosin,[5] enhances phosphatase activity,[196,457] and provides a mechanism for regulation.[197,206] However, in MYPT1-deleted (smooth muscle conditional knock-out) mesenteric arteries in which ~50% of PP1M was retained, PP1M still recognizes and dephosphorylates MLCp. In addition to MYPT1, a small regulatory subunit, M20, also is associated with PP1cδ in the holoenzyme (PP1M-MYPT1-M20). A highly homologous gene to that expressing MYPT1 transcribes MYPT2 in striated muscle, and a second promoter activated in smooth muscle on the MYPT2 gene generates M20. The function of M20 in smooth muscle remains obscure, but may involve regulation of microtubule assembly.[483] MYPT1 acts as a scaffold for several proteins in addition to PP1M, M20 and MLCp, including 14-3-3β, Par-4, HSP27, and myosin phosphatase-rho interacting protein (MRIP, a.k.a. $p116^{RIP}$).[134]

7.5. *Regulation of Ca^{2+} Sensitivity by PP1M Inhibition*

The relationship between steady-state $[Ca^{2+}]_i$ and tension is not fixed, as might be expected if the sensitivity of MLCK to Ca^{2+} was fixed, and if PP1M activity was constant and unregulated (Fig. 6D, black line). However, MLCK activity for a given Ca^{2+} level can be modulated, and activation of ROCK and PKC, the canonical control systems regulating the MLCp/MLC ratio via inhibition of PP1M activity, can dramatically increase Ca^{2+} sensitivity[222,365,465] (Figs. 6C and 6D). Notably, ROCK, novel and atypical PKC isotypes, and non-canonical MLCp regulators such as ILK and ZIPK, can function independently of global increases in Ca^{2+}. Moreover, relaxant stimuli that activate PKA and PKG can alter the level of steady-state tension independently of alterations in the level of $[Ca^{2+}]_i$ to cause Ca^{2+} desensitization. Thus, the current model is that VSM tension is controlled by (1) changes in $[Ca^{2+}]_i$, and (2) regulation of PP1M activity independently of changes in $[Ca^{2+}]_i$. For example, VSM can be contracted from point (p) to point

(q) in Fig. 6D by increasing $[Ca^{2+}]_i$ from (r) to (s) (Figs. 6C and 6D, black arrows) because elevated $[Ca^{2+}]_i$ would activate MLCK. Alternatively or additionally, VSM can be contracted by increasing the sensitivity of the MLCp control system to Ca^{2+} (increase in the slope of the $[Ca^{2+}]_i$-MLCp relationship) by inhibiting PP1M, which would cause a leftward shift in the $[Ca^{2+}]_i$,-tension curve (Fig. 6D, green arrow and sigmoidal curve). That is, the relationship between $[Ca^{2+}]_i$ and tension is not fixed, but is represented by a series of curves, the nature of which depends on the levels of concomitant regulation of MLCK and PP1M activities. This scenario divides activation mechanisms into Ca^{2+}-dependent and Ca^{2+}-independent, where the former involves Ca^{2+}-dependent activation of MLCK, and the latter involves changes in the ability of Ca^{2+} to activate MLCK and in the regulation of PP1M activity, both of which can be completely or relatively Ca^{2+}-independent.

Unregulated PP1M is "on", and dephosphorylation of MLCp by PP1M turns contraction "off" (Fig. 8Ba). MYPT1 phosphorylation at T472, T696 and T853 inhibit PP1M activity, and phosphorylation at T695 by PKA and PKG prevents T696 phosphorylation, thereby enhancing net T696 dephosphorylation[134,206,327,551] (Fig. 8A). In one model, the mechanisms by which MYPT1-pT696 and MYPT1-pT853 inhibit PP1M activity are distinct.[206] Phosphorylation at pT696 inhibits PP1M activity (Fig. 8Bci), whereas phosphorylation at pT853 displaces PP1M from myosin so that the catalytic site has poorer access to its substrate, MLCp (Fig. 8Bcii).[517] A more recent model of the PP1M-MYPT1-M20 holo-enzyme supports the hypothesis that phosphorylation of MYPT1 at either T696 or T853 converts the phospho-MYPT region into an autoinhibitory domain, where the phosph-MYPT domain docks with and "covers" the PP1M active site, causing complete inhibition of phosphatase activity[236] (Fig. 8Bciii). Because the pT696 site is a more potent autoinhibitor than the pT853 site, this model suggests that the two phospho-MYPTs provide different degrees of PP1M inhibition. MYPT1-pT472 is recognized by 14-3-3β resulting in dissociation of PP1M from myosin and reduction of PP1M activity towards MLCp[249] (Fig. 8Bd).

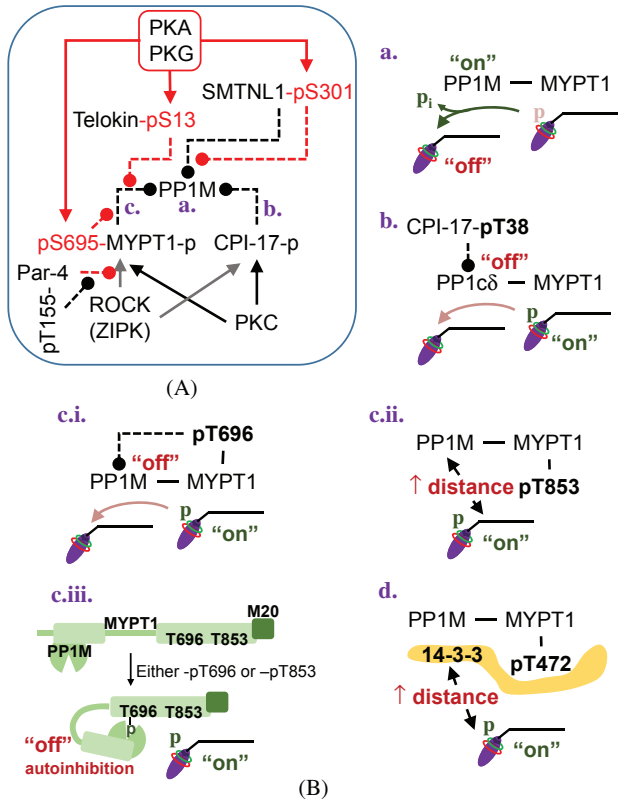

Fig. 8. Vascular smooth muscle protein phosphatase 1 (PP1M) activity is inhibited by multiple mechanisms, including MYPT1 (abbreviations as in other figure legends and text) when phosphorylated at T696, T853 and T472, and by CPI-17 when phosphorylated at T38 (A). MYPT1 and CPI-17 are phosphorylated by ROCK, ZIPK and PKC. PKA and PKG prevent this inhibition by phosphorylating SMTNL1 at S301, telokin at S13 and MYPT1 at S695. Par-4 can inhibit ROCK-induced phosphorylation of MYPT1, and Par-4 phosphorylation at T155 prevents this action. When PP1M is not regulated, then it is "on" and free to catalyze the hydrolysis of MLCp, which turns the actomyosin crossbridge motor "off" (B.a.). Proposed mechanisms by which PP1M is "turned off" (B.b.–B.d.)

Two alternative splice variants of MYPT1, leucine-zipper positive (MYPT1-LZ$^+$) and negative (MYPT1-LZ$^-$) isoforms, are differentially expressed by smooth muscles.[86,192,235,358] Fast, phasic smooth muscles express primarily MYPT1-LZ$^-$ that lacks the C-terminal LZ motif to

which PKG1α can bind, and slow, tonic smooth muscles express MYPT1-LZ$^+$. With regard to VSM, this information supports the hypothesis that the relaxant response of large conduit, not smaller muscular, arteries to stimuli that activate PKG1α may operate via reversal of MYPT1 inhibition of PP1M activity.[86,359] In rabbit femoral artery where KCl causes contraction correlating with a steady-state increase in MYPT1-pT853, stimuli that activate PKA and PKG cause strong Ca^{2+}-independent reductions in Ca^{2+} sensitivity resulting in relaxation, but only PKA activation abolishes the increase in MYPT1-pT853.[377] Notably, activation of PKG causes strong relaxation of mouse mesenteric artery in which MYPT1 was deleted, suggesting that MYPT1 regulation is not essential for PKG-dependent relaxation of this small, muscular, phasic artery.[383]

Several studies indicate that contractile stimuli, including KCl, cause increases in the level of MYPT1-pT853, not MYPT1-pT696.[242,377,543] ROCK can phosphorylate both sites, but MYPT1-pT853 is the preferred site, and is used as an indicator of ROCK activity in smooth muscle and other cell types.[228,321] Members of the non-canonical MLCp regulators that phosphorylate MYPT1, such as ZIPK,[281] ILK,[157,241] and DMPK[322] can phosphorylate MYPT1 at T696 but not T853.[466] Notably, MYPT1-pT853 and MYPT1-p696 levels are elevated at rest in rabbit muscular arteries[10,377,401] and rabbit[375] and mouse[495] bladder. Pharmacological blockade of ROCK and activation of PKA reduce the level of MYPT1-pT853 but not MYPT1-pT696, whereas hypoxia,[533] and the potent non-selective kinase inhibitor staurosporine[225,568] when used at 1 μM,[10] reduce the basal level of both MYPT1-pT853 and MYPT1-pT696.

CPI-17-pT38 inhibits PP1M complexed with MYPT1 (Fig. 8Bb) with an IC_{50} value of ~1 nM.[97] CPI-17 is expressed in greater abundance in VSM compared to visceral smooth muscles[549] (interestingly, CPI-17 is not expressed by chicken arterial VSM).[243] For example, rabbit portal vein and femoral artery express ~4–5-fold more CPI-17 than urinary bladder. MYPT1 expression also is not identical in all smooth muscles, although the degree of difference is less. For example, rabbit portal vein expresses ~2-fold more MYPT1 than femoral artery, and urinary bladder also may express somewhat more MYPT1

than femoral artery.[549] In VSM, CPI-17 is a substrate for PKC, ROCK, ILK, ZIPK and PAK,[97,157] and thus, stimuli that activate these control systems may cause increases in CPI-17-pT38.[242] Of note is the finding that ROCK, not PKC, causes an increase in CPI-17-pT38 induced by U-46619 in VSM cells.[357] As with phosphorylation of MYPT1 at, for example, T696, the net effect of increases in CPI-17-pT38 on PP1M activity is inhibition, which leads to an increase in the MLCp/MLC ratio when a myosin kinase is active.

7.6. *Rac, rhoA and ROCK*

PAK and ROCK are activated by the rho family of small GTPases, rac and cdc42, and rhoA, respectively[42] and play critical roles in signaling systems controlling the cytoskeleton and AM filaments.[11,13,283,382,417,467] Inactive rhoA (rhoA-GDP) translocates from a cytosolic location bound to a guanine nucleotide dissociation inhibitor (GDI) to the plasma membrane where it is activated to rhoA-GTP. Two primary downstream effectors of rhoA include ROCK and the actin polymerization nucleator mDia, the function of which is to establish and maintain bundles of AM filaments.[39,329,424] The Kd values of active rhoA (rhoA-GTP) for mDia and ROCK are, respectively, 6 and 130 nM. Thus, low levels of rhoA activation favor linear actin polymerization, whereas higher rhoA-GTP levels activate ROCK. In rabbit aorta, U-46619, NE, 5-HT, histamine and ET1 cause increases in active rhoA, whereas Ang II and phorbol dibutyrate do not,[431] suggesting that most contractile agonists can potentially activate mDia and ROCK in VSM. Independently of its action to increase linear microfilament formation, mDia stabilizes microtubules.[29] Notably, microtubule disruption enhances the strength of VSM contraction[269,371,455,569] by enhancing GEF-H1 activity, and thus, rhoA and ROCK activities.[38,63,372,570] RhoA-GTP becomes inactivated by intrinsic hydrolysis of GTP, the rate of which is enhanced by rhoGAPs.

ROCK1 (ROKβ) and ROCK2 (ROKα) are AGC-family ser-thr kinases related to DMPK and myotonic dystrophy kinase related-Cdc42 related kinases (MRCK).[13,215,285,360,417,502] ROCK1 and ROCK2

are expressed by different genes and share high sequence homology (92%) within the kinase domains, and low homology within the coiled-coil domains (55%). These isotypes are generally co-expressed and, although some overlap is apparent, they do not appear to be functionally redundant.[562] Recent evidence suggests that ROCK2 plays the primary role in regulation of VSM contraction.[532] ROCK appears to cause smooth muscle contraction indirectly by increasing phosphorylation of MYPT1 and reducing PP1M activity,[382] and directly by acting as a MLC kinase.[257] There are data supporting the model that ROCK can feed back to prolong its duration of activation by phosphorylating p190A-rhoGAP, which inhibits the ability of the GTPase rnd to bind and enhance rhoGAP activity, thus sustaining rhoA activation[312] (Fig. 7). In addition to increasing MYPT1-pT853, ROCK can phosphorylate MYPT1 at S472, a site also phosphorylated by NUAK1.[565] It is interesting that MLCK and ROCK differentially regulate MLCp in different cell compartments within fibroblasts to differentially control cell movement and adhesion.[491] Whether such compartmentation plays a role in the regulation of VSM contraction remains to be determined.

ROCK is activated not only by rhoA-GTP (active rhoA), but also by arachidonic acid and other lipids including phosphatidylinositol.[103,141,382,466] Inactive rhoA-GDP is retained in its inactive conformation in the cytosol because of enhanced binding of rhoA's prenyl group with guanine nucleotide dissociation inhibitor (GDI). Activation of rhoA involves displacement from GDI, insertion of rhoA's prenyl group into the plasma membrane, and nucleotide exchange catalyzed by a guanine nucleotide exchange factor selective for rhoA (a rhoA-GEF). Activation of ROCK by rhoA is therefore also generally considered to occur at the plasma membrane, and work by Urban *et al.*[503] revealed that KCl induces a transient, Ca^{2+}-dependent translocation of ROCK to plasma membrane caveolae. To activate AM XBs, ROCK or downstream proteins such as ZIPK must translocate to AM XBs in the cytosolic compartment to inhibit PP1M, inducing Ca^{2+} sensitization and tension-maintenance.[466] One proposal is that ROCK activates ZIPK which, in turn, translocates to PP1M at the AM XBs. Alternatively or additionally, rhoA and ROCK may become activated

at the AM XBs by binding to the scaffold protein, MRIP, that colocalizes rhoA, PP1M and actin[315,317,415] (Fig. 7). However, MRIP likely facilitates relaxation rather than contraction, because MRIP overexpression inactivates rhoA.[315–317] MRIP binds both inactive rhoA (rhoA-GDP) and active rhoA (rhoA-GTP),[479] and because MRIP acts as a GTPase activating protein (GAP), will "turn off" rhoA[250] (Fig. 7). Notably, MRIP binds the LZ domain of MYPT1,[479] and thus, will not act in tissues that express MYPT1-LZ$^-$. Of clinical interest is the finding that insulin stimulates MRIP-MYPT1 binding in VSM cells and enhances relaxation,[264] and rac1 knockout causes hypertension by altering the MRIP-rhoA inactivation system.[315–317]

RhoA and the rho-family GTPase rac often act in a counter-regulatory manner.[417] In VSM, rhoA activates ROCK to phosphorylate MYPT1, inhibiting PP1M and causing Ca^{2+} sensitization, and rac1 activates PAK1 to inhibit MLCK activity causing Ca^{2+} desensitization[575] (Fig. 7). Rac1-dependent activation of PAK1 also inhibits the cGMP-specific phosphodiesterase, PDE5,[439] elevating cGMP levels which also would cause Ca^{2+} desensitization, in this case, via PKG-dependent inhibition of rhoA and activation of PP1M. Upstream rac1 signaling in VSM involves NO-induced activation of the tyrosine kinase src via nitrosylation[385] which, in turn, activates the rho-family GEF vav2.[276,439] In addition, both rhoA and rac1 bind MRIP, and the NO pathway promotes dissociation of MRIP-rac1 and promotes association of MRIP-rhoA. Notably, the MRIP-rhoA complex also promotes rhoA phosphorylation and inactivation (Fig. 7). This model is consistent with the finding that inducible inactivation of mouse smooth muscle rac1 causes an ~15 mmHg increase in blood pressure.[15] Thus, rac1 appears to exert a VSM relaxant action in two ways.[15] One is via NO-dependent rac-mediated activation of PAK which inhibits PDE5, reducing cGMP degradation, and another is an increase in MRIP-rhoA association permitting PKG-dependent rhoA phosphorylation and inactivation.

However, rac1 may not act in VSM solely to cause relaxation. In rat aorta, rac1 is activated by receptor-dependent contractile stimuli, but not by KCl, and appears to enhance PKC translocation from the plasma membrane to facilitate PKC-dependent phosphorylation

of MYPT1 and CPI-17, inhibiting PP1M and promoting contraction.[456] In mouse mesenteric and saphenous arteries and aorta, pharmacological inhibition of rac1 attenuates contractions induced by several stimuli, including KCl and PDB, due to alterations in regulation of the Ca^{2+} control system.[384] Rac1-PAK3 may participate in caldesmon phosphorylation and thin filament activation. Rac1 is one component necessary for activation of NADPH oxidase,[301,559] and rac1 overexpression can elevate blood pressure by activation of NADPH oxidase causing generation of reactive oxygen species that mediate VSM contraction.[166,338]

7.7. *Basal MLC Phosphorylation*

A general hypothesis is that the MLC kinase responsible for increasing MLCp upon VSM stimulation is "off" when VSM is "at rest" (i.e., when a stimulus is not present). Moreover, PP1M is assumed to be "on" to ensure a very low level of MLCp. To activate contraction, MLCK is "turned on" by an increase in $[Ca^{2+}]_i$. In this case, simultaneously "turning off" PP1M would increase the degree of MLCp for a given increase in $[Ca^{2+}]_i$ (Ca^{2+}-sensitization). Thus, there is a general notion that the MLCp/MLC ratio is ~zero at rest, and that a stimulus causes dual regulation to "turn on" MLCK by elevating $[Ca^{2+}]_i$ and "turn off" PP1M by elevating ROCK and PKC activities. However, in tonic VSM "at rest", basal MLCp is not zero, but ~0.15.[410] This level can be reduced at least 50% by ROCK blockade, and nearly abolished by 1 μM staurosporine.[10,401] The MLCK inhibitors wortmannin and trifluoperazine do not reduce the basal level of MLCp. Notably, strong reductions in basal MYPT1-pT853 are induced by ROCK blockade,[377,543] adenylyl cyclase activation by forskolin,[377] and bromoenol lactone-induced inhibition of "Ca^{2+}-independent" phospholipase A_2 ($iPLA_2$),[402] an enzyme responsible for basal production of arachidonic acid in VSM.[147] Moreover, reductions in basal MYPT1-pT696 and -pT853 are induced by staurosporine.[10] As these sites are substrates for several kinases, including ROCK, ILK and ZIPK, and reductions in MYPT1 phosphorylation at these sites would be expected to permit elevations in PP1M activity, it is likely that

PP1M in tonic VSM "at rest" is regulated (inhibited), and a kinase other than MLCK is active. *In vitro*, ~50 μM arachidonic acid activates ROCK by ~5.5-fold, whereas active rhoA induces an ~2-fold increase.[103] Thus, basal $iPLA_2$ activity may be responsible for generating sufficient arachidonic acid to activate ROCK and elevate MYPT1-pT853, causing a certain degree of PP1M inhibition that, in conjunction with basal MLC kinase activity, ensures that MLCp is basally "set" at ~0.15 in tonic VSM. Although the CaM-dependent smooth muscle MLC kinase (i.e., MLCK) may be "off" at rest, the notion that all MLC kinases are "off", and that PP1M is "on" and unregulated, is an oversimplification. Rather, PP1M and one or more of the several MLC kinases are most likely at some intermediate value within their range of activities between fully "on" and "off". MLC is a good substrate for ROCK (103). Thus, basal ROCK activity may act both as a MLC kinase and inhibitor of PP1M. Recent evidence supports the contention that IKK2 also is a basal MLC kinase.[389,560] In summary, VSM appears to be "idling" when at rest because MLCp is ~0.15 due in part to the constitutive activities of phospholipases and one or more kinases that, in turn, regulate the activities of MLC kinases and PP1M. Precisely which kinase(s) participate in this regulation remains to be fully elucidated.

These results support the hypothesis that PP1M cannot be considered fully "on" in resting VSM because basal phosphorylation of MYPT inhibits PP1M activity. Moreover, non-myogenic VSM has tone at rest that can be reduced by ROCK inhibition and by strain softening (loading the tissue by stretching from a rest-length to a longer length, then unloading by releasing back to the original rest-length). Inhibition of resting tension by a ROCK inhibitor and strain softening appear synergistic rather than additive, suggesting that they work on separate systems.

7.8. *KCl-induced ROCK-dependent Contraction*

Membrane depolarization using high concentrations of K^+ (KCl or K_2SO_4) in the presence of receptor antagonists to block activation by release of paracrine agents is often used as a tool to bypasses receptor

activation and cause smooth muscle contraction primarily by changing the K^+ equilibrium potential, clamping membrane potential at some value above the resting level, and increasing Ca^{2+} entry through VOCCs.[44] Thus, for many years, KCl was thought to represent a much "simpler" stimulus that could be used for comparison with the more "complex" stimulus-response coupling systems activated by receptor-mediated stimuli.[51] However, KCl can cause release of Ca^{2+} from the sarcoplasmic reticulum[246,504] and can activate channels in addition to VOCCs.[393] Moreover, the Ca^{2+} sensitivity of KCl-induced contractions can change.[399,557] Notably, VOCCs appear to exert not only an ionotropic effect but also a metabotropic effect.[104,181,505] The metabotropic effect of KCl-induced VOCC activation involves downstream G protein activation independently of the ion-conducting property of the $Ca_V1.2$ channel, and the ionotropic effect (Ca^{2+} entry) can activate more proteins than MLCK. For example, Ca^{2+} can activate the tyrosine kinase pyk2 (a.k.a, FAK2)[296,297,561] and PI3K-C2α.[530,563] In many, but not all, experiments assessing KCl-induced contractions, receptor antagonists are duly employed to block spurious activation of G protein-coupled receptors. However, an artery is a collection of several cell-types, and membrane depolarization may release not only paracrine agents from non-smooth muscle cells, but also may cause release of autacoids from VSM itself that potentially could contribute to smooth muscle stimulation. Moreover, receptor tyrosine kinase activation may occur, and inhibitors of this class of receptors are not routinely employed during *in vitro* studies when KCl is used as a stimulus.

With this caveat in mind, Ca^{2+} sensitization appears to play nearly as important a role in KCl-induced tension maintenance as it does in receptor stimulus-induced contractions in tonic VSM.[387,394] Signaling systems that appear to participate in KCl-induced Ca^{2+} sensitization include those that activate ROCK and PKCζ. In the presence of adrenergic, histamine, Ang II and thromboxane A_2 receptor antagonists, KCl causes a strong and sustained activation of rhoA in endothelium-denuded rabbit aorta,[432] and rhoA inhibition reduces KCl-stimulated phasic contractions of guinea pig intestine and rabbit portal vein.[114,354] A different technique employed to "strip away" the complexity of

receptor-dependent signaling and "clamp" Ca^{2+} at known levels is chemical permeabilization of artery segments using Triton X-100, β-escin (and the related detergent, saponin) and α-toxin.[511] Interestingly, in β-escin-permeabilized smooth muscle, rhoA inhibition reduces the maximum efficacy but not the potency of Ca^{2+}-induced contraction of guinea-pig intestinal smooth muscle,[354] and the ROCK inhibitor H-1152 when used at 1 μM produces a similar effect in rabbit and mouse femoral arteries.[74]

ROCK inhibition reduces the tonic phase of KCl-induced contraction in several artery-types.[21,299,430,432,437,503] In rabbit artery, KCl-induced increases in Ca^{2+} appear to cause ROCK translocation to punctate plasma membrane regions rich in caveolae.[503] Potential mechanisms by which KCl can cause an increase in ROCK include activation of a rhoA-GEF,[104,296,297,412,505,561] PI3K-C2α,[530,563] 20-HETE, PKCζ, and $iPLA_2$.[402] KCl can activate PKC and ERK,[392,401] and these signaling systems can elevate the level of 20-HETE. Moreover, PKC is an upstream activator of rhoA and ROCK.[182] KCl, by causing a strong transient increase in $[Ca^{2+}]_i$ can activate CaMKII,[419,476] which along with ERK can activate $cPLA_2$[325] to generate arachidonic acid.

In rat aortic VSM cells, knockdown of pyk2 or the rhoA GEF, PDZ-RhoGEF, reduced rhoA activation by the Ca^{2+} ionophore A-23187, suggesting that pyk2-PDZ-RhoGEF is activated downstream of Ca^{2+} to cause rhoA-ROCK-dependent Ca^{2+} sensitization.[561] In VSM cells stimulated with Ang II, the $[Ca^{2+}]_i$ rise activates a Jak2-dependent mechanism to induce Arhgef1/RhoA signaling and Ca^{2+} sensitization.[144] If Ca^{2+} alone is sufficient to activate ROCK and cause Ca^{2+} sensitization, then any receptor stimulus that elevates Ca^{2+} and membrane depolarization would also be expected to activate ROCK. However, in rabbit tonic arteries, Bay K 8644, a dihydropyridine that promotes Ca^{2+} channel clusters to operate in a persistent Ca^{2+} influx mode, causes a strong increase in $[Ca^{2+}]_i$, MLCp and contraction, and induces a transient increase in rhoA, but fails to elevate the ROCK substrate MYPT1-pT853, suggesting that an increase in $[Ca^{2+}]_i$ alone is insufficient to activate ROCK to cause Ca^{2+}-sensitization. Despite the lack of Bay k 8644-induced activation of ROCK, the ROCK inhibitor H-1152 nearly abolishes a Bay k 8644-induced contraction.

This is due not to inhibition of a stimulus-induced increase in ROCK activity, but to the strong inhibition of basal ROCK activity, as evidenced by the very low basal MYPT1-pT853 in the presence of H-1152, and the inability of Bay k 8644 to elevate the depressed basal MYPT1-pT853.

7.9. *PKC*

PKC, a member of the AGC subfamily of ser-thr kinases,[360] is expressed as 10 isotypes categorized as convention (α, βI, βII, γ), novel (δ, ε, η, θ) and atypical (ζ and ι/λ).[266,302,433] Although most PKC isozymes are ubiquitously expressed,[302] and α, β, γ, δ, ε and ζ isoforms have been shown to be expressed in various arteries,[221,311] there is evidence that PKCβ is not expressed by splanchnic VSM, and that PKCγ expression in arteries is of neuronal origin.[345] Conventional PKCs (cPKCs) are Ca^{2+}-dependent; novel (nPKC) and atypical (aPKC) are not. In the presence of membrane phosphatidylserine, diacylglycerol (DAG), a product of phospholipase D-phosphatidate phosphohydrolase activities, and of hydrolysis of phosphatidylinositol and phosphatidylcholine by phospholipases C (PLC), bind and activate cPKCs and nPKCs.[120] PKC, and DAG acting independently of PKC, also play roles in Ca^{2+} signaling and in membrane potential changes by regulating the activities of several ion channel types, including TRP, VOCC and potassium.[3,245,433,435] Importantly, constitutively active PKCα is necessary for maintaining basal VOCC-dependent Ca^{2+} entry in VSM,[330,331,374] which appears to produce a small but significant degree of basal tone in muscular arteries.[401] PKCα-dependent regulation of VOCCs also are responsible for Ca^{2+} entry during myogenic contraction.[330] Many contractile stimuli activate receptors coupled to PLC, and DAG levels in smooth muscle increase in response to norepinephrine, Ang II and VP.[266] Moreover, VSM contraction is induced by phorbol esters, a DAG-mimetic that induces a sustained and irreversible activation of PKC followed by PKC downregulation.[302] Thus, PKC is considered a canonical activator of VSM.[266,345] Phorbol esters can cause contraction in permeabilized muscle at very low Ca^{2+} levels, and can cause contraction in

intact VSM without causing increases in $[Ca^{2+}]_i$. These findings, along with work identifying the PP1M inhibitor CPI-17 as a PKC substrate, supports the hypothesis that PKC converges with ROCK to participate in PP1M inhibition causing Ca^{2+} sensitization.[97,524] Notably, one action of PKCδ in coronary artery is to activate ROCK. Thus, ROCK can be a downstream effector of PKC.

The precise roles played by PKC isotypes in the regulation of VSM contraction remains an active area of investigation.[433] In addition to regulating multiple ion channels and ATPase-dependent pumps, PKC activates the ras-raf-MEK-ERK1/2 pathway in several cell types[36] by stimulating the production of growth factor autacoids.[501] In smooth muscle, calponin may act as a scaffold to facilitate PKC-ERK1/2 activation.[310] Such activity has been linked to caldesmon phosphorylation and thin filament regulation.[433] In pig coronary arteries, both PKC and ROCK appear to be involved in U-46619- and 5-HT-induced contractions,[221,337] whereas PKC plays no role in the contraction of the rat caudal artery induced by this stimulus.[543] PKC activation appears to potentiate contractile strength independently of an increase in MLCp in pig coronary artery activated by ET1 and 5-HT.[340] In rabbit renal artery, KCl does not cause an increase in inositol-trisphosphate production,[388] suggesting that this stimulus does not activate PLC nor elevate DAG levels. Supporting this notion, KCl does not induce CPI-17 phosphorylation of rabbit femoral artery,[85] and the relatively selective inhibitor of cPKC isotypes, Go 6976,[140,292] does not inhibit a KCl-induced contraction. Interestingly, the green tea catechin, epigallocatechin-3-gallate, enhances the strength of a contraction induced by K^+-depolarization (KCl) via a PKCδ-dependent increase in CPI-17 phosphorylation in pig coronary artery.[343] Based on a study in which long-term application of phorbol ester was used in rat mesenteric artery to cause PKCα and PKCδ degradation, these PKC isotypes were found to be necessary for phorbol ester-induced contraction, and not for contractions induced by norepinephrine and VP.[345]

The general consensus at this time is that, although ROCK and PKCs converge on the Ca^{2+} sensitization signaling system in VSM, ROCK appears to play a more dominant role. Arteries isolated from

the spontaneously hypertensive rat display greater myogenic tone than arteries from Wistar Kyoto rats, and the increase is attributed to ROCK-dependent rather than PKC-dependent Ca^{2+} sensitization.[207] The PKC inhibitor GF-109203X (a.k.a, Bisindolylmaleimide 1 and Go 6850), when used at 1 μM, is a relatively selective strong inhibitor of cPKCs and nPKCs.[80,98,118,357] The rank order of potency of GF-109203X for inhibition of PKC isotype activation in an *in vitro* enzyme assay is $\alpha > \beta I > \varepsilon > \delta > \zeta$, with IC_{50} values ranging from ~0.01 μM for PKCα to ~0.2 μM for PKCδ, and ~6 μM for PKCζ.[292] In rabbit epigastric artery, a small (~400 μm), muscular, phasic artery feeding a musculocutaneous vascular bed, 1 μM GF-109203X reduces the potency of a PE-induced CRC, and reduces the average value of the maximum contraction induced by U-46619, but has no inhibitory effect on Ang II- and VP-induced CRCs (Fig. 9A, 9C–F). In this artery, the MLCK (and PI3K)[554] inhibitor wortmannin (1 μM)[25,80] and ROCK inhibitor H-1152 (1 μM)[436] each produce a relatively strong inhibition of contractions (Fig. 9A, 9C–F). A similar trend is produced in the larger and more tonic rabbit renal artery by these kinase inhibitors (Fig. 9B). These data support the conclusion that cPKC and nPKC isotypes play a minimal role in Ca^{2+} sensitization of VSM.[118,467] However, in other arteries activated by different stimuli, PKC appears to play a more significant role. For example, PKC participates via phosphorylation of HSP-72 in the cytoskeletal reorganization of cerebral arteries required for a strong myogenic contraction.[307] There is evidence that ROCK and PKC act at different times during a stimulus-induced contraction. PE-induced contraction of rabbit femoral artery is biphasic, with a fast/phasic phase followed by a slow/tonic phase. During the phasic phase $[Ca^{2+}]_i$, increases rapidly, then falls slowly to achieve a lower tonic level.[402] During both phases, Ca^{2+} sensitization is activated, but during the phasic phase, increases in CPI-17-T38 dependent on cPKC cause inhibition of PP1M, whereas during the tonic phase, cPKC, nPKC and ROCK maintain CPI-17-pT38, and ROCK increases MYPT1-pT853 to maintain PP1M inhibition.[85]

There are significant differences in the relative effectiveness of protein kinase inhibitors to diminish contractile strength when

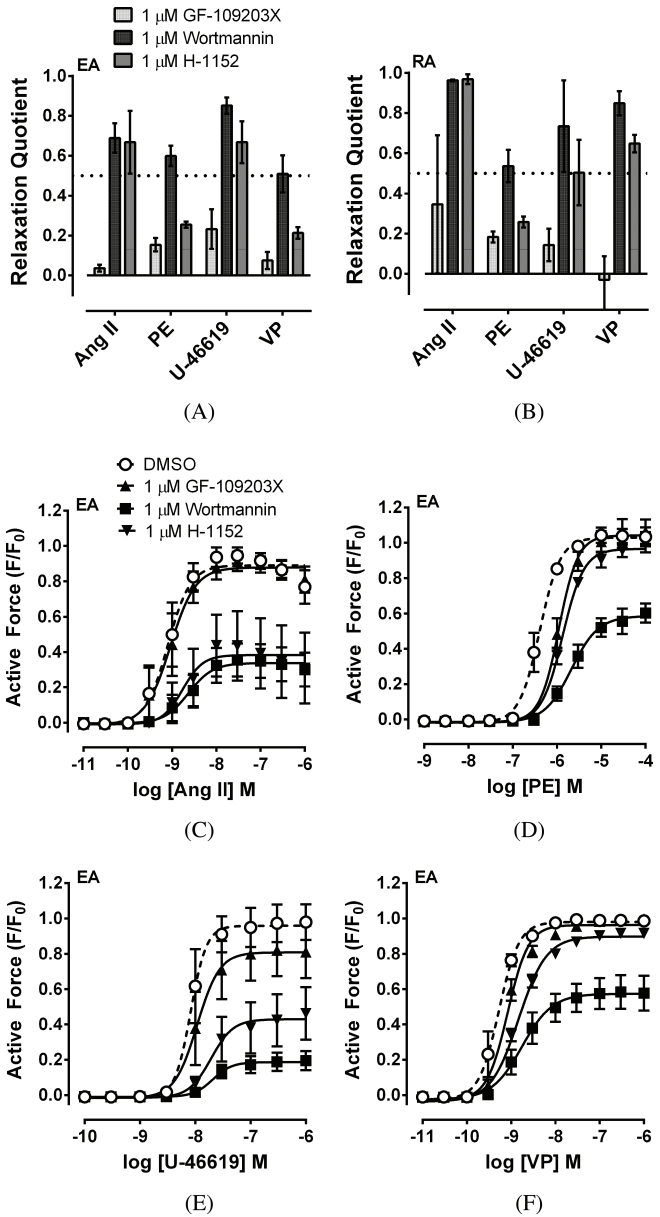

Fig. 9. Relaxation quotient for rabbit epigastric (A) and renal (B) arteries, and concentration-response curves for epigastric artery (C–F) comparing the relative abilities

Fig. 9. (*Continued*) of PKC (abbreviations as in other figure legends and text), MLCK and ROCK inhibitors (1 μM GF-109203X, 1 μM wortmannin and 1 μM H-1152, respectively) to affect contractions produced by Ang II (C), PE (D), U-46619 (E) and VP (F). Data in A and C–F are means ±SE, n = 4. Data in B is for 2–3 arteries. Relaxation quotient = 1-(area under curve with drug/area under control curve). Thus, a value of 0 means no relaxation, and a value of 1 means complete relaxation (inhibition of contraction).

comparing arteries and stimuli (Fig. 9). For example, wortmannin and H-1152 produce a stronger inhibition of contraction induced by VP in renal artery compared to epigastric artery (Fig. 9A–B). These data invite criticism because each drug has some off-target effect. Indeed, using the PKC C1 regulatory domain inhibitor calphostin C (100 nM) in rat small mesenteric artery, PKC was identified as contributing to Ca^{2+} sensitization of norepinephrine-induced contraction.[55] However, subsequent work revealed that off-target effects of calphostin C that do not involve PKC inhibition but likely involve the phorbol ester/DAG (C1)-binding domain of other proteins include the very potent inhibition of VOCC activity,[163] potent inhibition of phospholipase D (IC_{50} ~100 nM),[444] inhibition of rhoA-membrane association,[90] and inhibition of Golgi-associated transport.[8] With this caveat in mind, the comparative assessment of kinase inhibitory activities in Fig. 9 supports the hypothesis that different vascular segments express unique levels and combinations of contractile protein regulatory systems. In short, the VSM of the vascular tree, and likely also vascular trees of different species, cannot be treated as a single homogenous organ.

Atypical PKCs (aPKCs) are not activated by DAG or phorbol esters, but are activated by lipids such as arachidonic acid, phosphatidic acid, ceramide and phosphatidylinositols such as PIP_3.[178] Moreover, certain proteins can bind to and inhibit aPKC activation. For example, Par-4 binds the aPKC C1 motif preventing activation.[313] In several cell-types, PKCζ functions as a MEK kinase to activate ERK independently of raf1. In rabbit VSM, activation of PKCζ by arachidonic acid causes Ca^{2+} sensitization.[118] There is evidence that, like PKCα,[331] ERK,[392] ROCK and $iPLA_2$,[402] PKCζ is active in rabbit arterial VSM at rest, and that basal PKCζ activity

inhibits Ca^{2+} entry through non-VOCC channels that appear to supply intracellular stores rather than AM XBs.[401] Notably, PKCζ is found not only in a peri-nuclear location, but also diffusely throughout the cytosol and in a punctate fashion at the membrane in regions distinct from those housing focal contacts.[401] Moreover, a stimulus-induced increase in $[Ca^{2+}]_i$ further activates PKCζ to cause a feed-back "braking" effect on the degree of increase in $[Ca^{2+}]_i$, through VOCCs, and a feed-forward increase in MYPT1-pT853. Thus, PKCζ and ROCK appear to converge on the MLCp motor control system to inhibit PP1M activity, inducing strong Ca^{2+} sensitization. Interestingly, in certain cell-types, PKCζ activates IKK.[313] As mentioned, IKK2 is thought to act as a MLC kinase in resting VSM, and PKCζ is also active basally, but whether constitutive PKCζ activity participates in basal IKK2 activation in VSM remains to be determined.

8. MLCp Sites and Non-Canonical MLC Kinases

The primary phosphoprotein site involved in covalent regulation of proteins is phospho-serine, accounting for ~86% of 6600 phosphorylations on 2244 human proteins. However, phospho-threonine also participates in regulation, accounting for ~12%.[347] The sequence of the first 19 amino acids at the amino terminal end of MLC reveals 6 potential phospho-serine and phospho-threonine sites. Five of these sites are phosphorylated in tissues and play roles in the regulation of contraction:

S(1)S(2)KRAKAKT(9)TKKRPQRAT(18)S(19).[361,484,534] (1)

8.1. *Mono-phosphorylation*

MLCK phosphorylates S19 and is generally considered to cause MLC mono-phosphorylation, although T18 can also become phosphorylated, but to a lesser degree, with a slower time course, and at a non-physiologically high [MLCK].[94,179,200,201,342] *In vitro* phosphorylation of either S19 or T18 permits myosin filament formation. Interestingly, when MLC is phosphorylated only on T18 *in vitro*,

AM-ATPase activity is lower than when MLC is phosphorylated only on S19, and yet, each moves actin at equivalent velocities in a motility assay.[50]

It is unsurprising that several other kinases have been identified that phosphorylate MLC, because MLC phosphorylation plays critical roles in regulation of cell motility and contraction of non-muscle cells, and in cytokinesis following mitosis. The precise roles kinases other than MLCK play in the regulation of VSM contraction is an ongoing subject of investigation. Other kinases that can cause monophosphorylation of MLC exclusively at S19 include CaMKII,[94] MK2,[252] PAK,[67,386,568] and ROCK.[12] Although RSK2 was shown to monophosphorylate MLC,[478] neither MLC nor MYPT1 are listed among the ~40 substrates of the RSK family in a recent review,[422] suggesting that RSK2 has not been identified as a significant contributor to the regulation of contraction. Whether PAK causes an increase or, through MLCK phosphorylation, decrease in MLC phosphorylation in smooth muscle remains to be fully elucidated.[42] Although ROCK phosphorylates MLC at about half the rate of MLCK, the K_m of MLC for ROCK is about 10-fold lower (~2 μM) than for MLCK, suggesting that ROCK can phosphorylate MLC when this substrate is available at low cellular levels.[12,103] DMPK and MRCK, kinases closely related to ROCK, can phosphorylate MLC and MYPT *in vitro*, but whether they do so in VSM *in vivo* remains to be determined. There is evidence that these kinases may participate in regulation of MYPT in certain cell-types.[502]

MK2 and IKK2 play roles in the cellular response to stress. In particular, MK2 is a primary substrate of the p38 MAPK stress response, and IKK is activated by pro-inflammatory stimuli that generate nuclear factor-κB.[328] Using an MK2 knockout mouse, Martinka *et al.*[289] showed that adenosine, acting not through plasma membrane adenosine receptors but via an intracellular mechanism, enhanced the ability of Ang II to cause mesenteric artery contraction by a p38-MK2-dependent increase in MLCp. MK2 also acts on the actin polymerization control system by elevating phosphorylation of HSP-27, resulting in a loss of HSP-27's actin capping property and increased actin polymerization.[148]

8.2. *Di-phosphorylation*

In vitro S19 phosphorylation of MLC alone is sufficient to increase AM ATPase activity,[218,484] and the Ca^{2+}-MLCK-MLC mono-phosphorylation signaling system, enhanced by ROCK-dependent Ca^{2+} sensitization, is sufficient to cause and maintain maximum arterial contractions induced by such stimuli as KCl[87,394] and U-46619.[543] However, di-phosphoylation of MLC at S19 and T18 can enhance actin-activated myosin Mg^{2+}-ATPase activity *in vitro*, and although physiologically a rare-event, MLC di-phosphorylation has been shown to occur during stimulation with ET1, and to increase the rate of arterial contraction induced by $PGF_{2\alpha}$.[523] Kinases that phosphorylate smooth muscle MLC at both S19 and T18 include ILK and ZIPK.[37,84,333] DAPK primary causes MLC mono-phosphorylation, but can cause di-phosphorylation. DAPK is regulated by CaM in a manner analogous to MLCK, but ZIPK lacks the CaM regulatory domain, and ZIPK and ILK cause MLC di-phosphorylation by an entirely Ca^{2+}-independent mechanism. Par-4, an actin-binding protein that participates in PP1c regulation, contains a death domain-motif and can bind ZIPK. Thus, the ZIPK-Par-4 complex represents a DAPK-mimic.[37] As its name implies, a primary function of DAPK is to regulate cell death in response to stress, resulting in the characteristic death morphology of peripheral super-contraction resulting in cell-rounding and membrane blebbing. Whether ZIPK participates in cell death is not clear. Notably, MLC di-phosphorylation is revealed under conditions of the hyper-contractile states of coronary, cerebral and peripheral artery vasospasm.[482] In motile fibroblasts, ZIPK causes MLC di-phosphorylation primarily of stress fibers in the tail region opposite the leading edge where cell retraction occurs, mono-phosphorylated MLC is located at the leading edge, and ROCK inhibits PP1M.[253] Whether ZIPK and ROCK, as well as MLCK, PKC and ILK, play similar location-dependent roles in the regulation of VSM contraction remains to be determined.

8.3. *Tri-phosphorylation*

The strength and duration of a response can be regulated by multiple phosphorylation and dephosphorylation events. *In vitro*, PKC can

phosphorylate MLC at S1, S2, and T9, with T9 being phosphorylated the most rapidly, followed more slowly by phosphorylation of either S1 or S2, and a third phosphorylation only very slowly.[202,335] Phosphorylation of T9 alone by PKC inhibits subsequent phosphorylation on S19 by MLCK due to a 6-fold increase in the K_m.[498] Myosin phosphorylated by both PKC at T9 and MLCK at S19 has a lower actin-activated Mg^{2+}-ATPase activity than myosin phosphorylated by MLCK alone, due to a lower affinity of myosin for actin. Thus, PKC activation *in vivo* might be expected to exert a negative regulatory influence on contraction both prior to and during activation by a stimulus that increases S19 phosphorylation. However, threonine phosphorylation of MLC was found not to occur in intact pig carotid artery stimulated with phorbol ester, and in permeabilized artery activated by direct addition of PKC, suggesting that, PKC does not cause inhibition of contraction.[480] By contrast, PKC appears to participate in tension decline in dog basilar artery.[342] In this tissue, the increase in $[Ca^{2+}]_i$ and myogenic contraction induced by a slow (over 1 min) step-stretch reaches a peak within another min, and by ~10 min, $[Ca^{2+}]_i$ declines to the basal level and tension fades to ~10% of the peak value. The decline in tension from ~1 to ~5 min correlates with a decline in MLCp, but from 5 to 15 min, MLCp increases. Notably, total MLCp was found to reflect temporal changes in site-specific phosphorylation of MLC not seen when tissues were stimulated instead with KCl. In particular, the early contraction was due to increases in MLCp at S19 induced by MLCK activation, and the subsequent increase in phosphorylation reflected di- then triphosphorylation of MLC. The di-phosphorylation was at S19 and T18 sites, and the third phosphorylation site was thought to reflect PKC activity that served as a "braking" mechanism to prevent a sustained myogenic contraction.[342] Interestingly, the relaxant effect of okadaic acid on canine basilar artery involves activation of PKCα and phosphorylation of MLC at T9.[341]

9. AMPK and Summary of Relaxation Mechanisms

Smooth muscle is dependent on an immediate supply of ATP because, unlike striated muscles that maintain high levels of phosphocreatine as

an energy reserve to resupply ATP during contraction, the level of phosphocreatine in smooth muscle is about equal to the level of ATP.[205] AMPK, a ubiquitous sensor of metabolic stress, is activated by liver kinase B1 (LKB1), a constitutively active enzyme, and by Ca^{2+}-calmodulin-dependent kinase kinase β (CaMKKβ), both of which cause AMPK phosphorylation at T172.[160,195] An increase in the cellular AMP/ATP ratio regulates AMPK by (a) promoting further phosphorylation at T172, (b) inhibiting AMPK-pT172 dephosphorylation, and (c) allosteric activation of AMPK. Increases in $[Ca^{2+}]_i$ and AMP can act synergistically to activate AMPK.[159] AMPK has been shown to potentially interact with multiple smooth muscle contractile protein regulatory systems to decrease stimulus-response coupling, effectively putting a "brake" on contraction to reduce and conserve ATP consumption for use by ion pumps to maintain ion homeostasis and cell viability. AMPK activation inhibits VSM contraction,[127,427,442] and evidence has been presented suggesting that, to cause relaxation, AMPK may inhibit the activities of MLCK,[189,265] PKC[81] and ROCK[528] (Fig. 7). In mesenteric resistance artery, activation of AMPK by A-769662 does not appear to inhibit any of these systems, but instead, reduces $[Ca^{2+}]_i$.[442] Thus, the precise mechanism by which AMPK inhibits VSM contraction under different conditions remains to be determined.

Inhibition of rhoA-GEFs or activation of rhoA-GAPs can reduce the level of active rhoA (rhoA-GTP), and thus, reduce ROCK activity. An alternate mechanism by which rhoA-GTP "turns off" involves rhoA-GTP phosphorylation at S188, which also protects rhoA from ubiquitin/proteasome-mediated degradation in VSM.[123,278,420,438,439] PKA, PKG and AMPK can inactivate rhoA-GTP by this mechanism. PKA inactivates rhoA by a second mechanism involving rhoA-GDI, phosphorylation at S174 that increases rhoA-GTP affinity for binding to rhoA-GDI, resulting in rhoA-GTP sequestration in the cytosol.[346] There is also evidence that a cAMP-activated, PKA- and PKG-independent, Epac-Rap1 GTPase signaling pathway contributes to cAMP-induced inhibition of smooth muscle Ca^{2+} sensitization.[576] In addition to reducing the levels of active rhoA, PKA and PKG can activate PP1M by inducing phosphorylation of SMTNL1 on S301,[47,499,550] MYPT1 on S695, and telokin on S13 (Fig. 8A).

Telokin-pS13 elevates PP1M activity to cause smooth muscle relaxation not by initiating MYPT1-pT853 or –T696 dephosphorylation, but by permitting activation of the full-length, inactive, phospho-MYPT1-PP1M.[237] Another mechanism by which telokin-pS13 inhibits contraction is by shielding the activating phosphorylation sites on MLC (Fig. 7).[453] Telokin does not exert a strong relaxation of VSM compared to visceral smooth muscle (e.g., 30% relaxation of femoral artery compared to 90% for ileum), and this is consistent with the differential telokin expression levels in these tissues (6 and 27 μM, respectively).[73] An early study using the Ca^{2+} indicator aequorin revealed that stimuli that relax contracted VSM by elevating PKA do not concomitantly reduce $[Ca^{2+}]_i$, and in some cases, increase $[Ca^{2+}]_i$, and that although stimuli that activate PKG reduce $[Ca^{2+}]_i$, the degree of relaxation is greater than can be accounted for by the decrease in $[Ca^{2+}]_i$.[308] Mechanisms by which PKG decreases $[Ca^{2+}]_i$ include inhibition of PLC and inositol 1,4,5-trisphosphate formation, activation of Ca^{2+} sequestration, and activation of the large conductance Ca^{2+}-activated K channel.[86,383]

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Chapter 4

Actin Filament Dynamics During Vascular Smooth Muscle Contraction

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Regulation of arterial diameter by control of vascular smooth muscle contraction plays a key role in regulation of blood flow and thereby the supply of O_2 and nutrients to tissues and organs and removal of CO_2 and waste products. A host of contractile stimuli, including agonists such as angiotensin II, serotonin, norepinephrine and endothelin-1, and increases in intravascular pressure (via the myogenic response), elicit vasoconstriction via an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) leading to activation of calmodulin-dependent myosin light chain kinase, phosphorylation of the regulatory light chains of myosin II (LC_{20}) and cross-bridge cycling. Many contractile stimuli also elicit Ca^{2+} sensitization of contraction via activation of the RhoA/Rho-associated kinase (ROCK) pathway leading to phosphorylation of MYPT1 (the myosin targeting subunit of myosin light chain phosphatase), a decrease in phosphatase activity and increased LC_{20} phosphorylation without a change in $[Ca^{2+}]_i$. A third signaling pathway has more recently been implicated in the regulation of vasoconstriction, which involves stimulus-induced polymerization of a pool of actin localized to the sub-plasmalemmal region of the cell and which involves formation of adhesion complexes that transmit force from the myofilaments to the extracellular matrix via transmembrane

integrins. Both ROCK and protein kinase C (PKC) have been implicated in stimulus-evoked actin polymerization, ROCK leading to phosphorylation of cofilin (most likely via activation of LIM kinase) and PKC leading directly to phosphorylation of HSP27. Defects in ROCK and PKC signaling and actin polymerization have been implicated in various cardiovascular pathologies.

1. Vascular Smooth Muscle Contraction and the Control of Blood Flow

Arterial diameter, particularly of so-called resistance vessels (<200 μm internal diameter), controls blood flow and thereby the supply of O_2 and nutrients to downstream tissues and organs, and the removal of CO_2 and metabolic waste products.[1] Arterial blood flow is governed by Poiseuille's Law[2]:

$$\text{Blood flow rate}\,(Q) = \frac{\pi \Delta P r^4}{8\eta L}$$

where ΔP is the pressure difference between the two ends of the artery, r the radius of the vessel, η the blood viscosity and L the length of the vessel. Since flow rate is proportional to the 4th power of the radius, a small change in arterial diameter has a profound effect on blood flow. It is of critical importance, therefore, that arterial diameter be precisely controlled to meet physiological demands. Furthermore, loss of such fine control is associated with severe pathological conditions such as hypertension, diabetes, ischemic stroke and sub-arachnoid hemorrhage.[3–7]

Arterial diameter is regulated by the contraction and relaxation of vascular smooth muscle cells of the medial layer. Numerous physiological stimuli elicit vasoconstrictor responses in arterial smooth muscle, including a variety of circulating or locally-released vasoactive molecules such as angiotensin II, serotonin, norepinephrine and endothelin-1, as well as increased intravascular pressure, which reduce arterial diameter and thereby blood flow.[8–12] Vasoconstriction/vasodilation induced by increased/decreased intraluminal pressure is referred to as the myogenic response and is an inherent property of the vascular smooth

muscle cells[9]. The actions of circulating and locally-released vasoactive molecules are superimposed on the myogenic response to achieve the desired arterial diameter and blood flow for the prevailing physiological conditions.

2. Ca^{2+}-induced Vasoconstriction

Vasoconstriction typically involves an agonist- or pressure-induced increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), the sources of Ca^{2+} being the extracellular milieu and/or the sarcoplasmic reticulum, depending on the nature of the stimulus. The increase in $[Ca^{2+}]_i$ leads to activation of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK), phosphorylation of the 20-kDa regulatory light chains of myosin II at serine-19, activation of the actomyosin MgATPase activity, cross-bridge cycling and contraction (Fig. 1, left side).[13–16] Following termination of the stimulus, $[Ca^{2+}]_i$ is restored to resting levels, resulting in inactivation of MLCK, and myosin is dephosphorylated by myosin light chain phosphatase (MLCP), leading to dissociation of actin and myosin and relaxation (Fig. 1).[17]

3. Ca^{2+} Sensitization of Vasoconstriction Induced by Inhibition of Myosin Light Chain Phosphatase

It was thought for many years that vasoconstriction could be explained exclusively by MLCK activation, but numerous observations of stimulus-dependent differences in the Ca^{2+} dependence of force generation led to detailed investigations of the regulation of MLCP activity. This resulted in the discovery of the phenomenon of Ca^{2+} sensitization, whereby contractile agonists coupled to the $G_{12/13}$ family of heterotrimeric G proteins activate the small GTPase RhoA via a guanine nucleotide exchange factor (a Rho-GEF) (Fig. 1, right side).[18–20] In the unstimulated vascular smooth muscle cell, RhoA contains bound GDP, is associated with Rho-GDI (a guanine nucleotide dissociation inhibitor protein) and located in the cytosol. Activated Rho-GEF exchanges RhoA-bound GDP for GTP, resulting

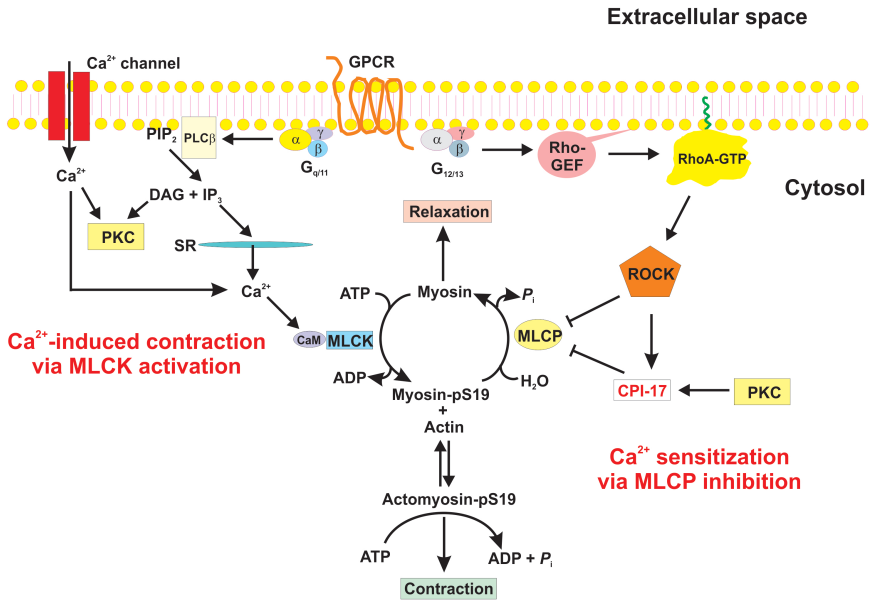

Fig. 1. Ca^{2+}-induced activation and Ca^{2+} sensitization of vascular smooth muscle contraction. Vascular smooth muscle contraction is activated principally by an increase in $[Ca^{2+}]_i$ due to stimulus-induced entry of extracellular Ca^{2+} via sarcolemmal Ca^{2+}-permeant ion channels or G protein-coupled receptor (GPCR)-mediated Ca^{2+} release from the sarcoplasmic reticulum (SR) via inositol 1,4,5-trisphosphate (IP_3) receptors. Ca^{2+} binds to calmodulin (CaM), which activates myosin light chain kinase (MLCK) to phosphorylate serine-19 (pS19) of the two 20-kDa light chain subunits of smooth muscle myosin II. This enables actin interaction, which markedly increases the MgATPase activity of myosin, leading to cross-bridge cycling and contraction driven by the energy derived from the hydrolysis of ATP within the heads of myosin II. Ca^{2+} sensitization of contraction involves GPCR-mediated activation of the RhoA/Rho-associated kinase (ROCK) pathway leading to inhibition of myosin light chain phosphatase (MLCP), either directly by phosphorylation of the myosin targeting subunit of MLCP (MYPT1) or indirectly via phosphorylation of CPI-17 (the 17-kDa phosphoprotein inhibitor of MLCP). Protein kinase C (PKC) can also induce Ca^{2+} sensitization via phosphorylation of CPI-17 in some instances. $G_{q/11}$: heterotrimeric G proteins coupled to phospholipase Cβ (PLCβ), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and IP_3. $G_{12/13}$: heterotrimeric G proteins coupled to guanine nucleotide exchange factors for the small GTPase RhoA (Rho-GEFs), which activate RhoA by GDP-GTP exchange. Activated RhoA-GTP activates ROCK.

in dissociation of the RhoA-GDI complex and translocation of RhoA-GTP to the plasma membrane where it inserts into the membrane via an exposed geranylgeranyl (lipid) moiety, which is buried within the RhoA-GDP/GDI structure under resting conditions. Activated RhoA then activates the serine/threonine kinase Rho-associated coiled-coil kinase (ROCK), which phosphorylates MYPT1 (the myosin targeting subunit of MLCP) at threonine-697 and threonine-855 (rat numbering)[17] and/or CPI-17 (a 17-kDa cytosolic protein) at threonine-38.[21] Phosphorylation of MYPT1 at either site *in vitro* reduces the activity of MLCP, although numerous studies indicate that threonine-855 is the predominant regulatory site *in situ*.[22,23] Phosphorylation of CPI-17 at threonine-38 results in a conformational change, which enables high-affinity binding of the phosphoprotein to the catalytic subunit of MLCP and potent inhibition of its activity.[24–26] CPI-17 can also be phosphorylated at threonine-38 by protein kinase C.[27] The net result of reduced MLCP activity via activation of the RhoA/ROCK pathway is an increase in LC_{20} phosphorylation (due to an increase in the MLCK: MLCP activity ratio) and contraction. Therefore, enhanced force is achieved without a change in $[Ca^{2+}]_i$ since the RhoA/ROCK pathway is not Ca^{2+} dependent. This increased vasoconstrictor response at constant $[Ca^{2+}]_i$ is thus referred to as "Ca^{2+} sensitization".[18] Pressure-induced vasoconstriction also involves Ca^{2+} sensitization via the RhoA/ROCK pathway,[28] but this occurs via phosphorylation of MYPT1 (at threonine-855) and not CPI-17 phosphorylation.[29–31]

4. Ca^{2+} Sensitization of Vasoconstriction Induced by Actin Polymerization

The possibility that vasoconstrictor responses could be explained completely by a combination of Ca^{2+}-induced activation of MLCK and Ca^{2+} sensitization evoked by MLCP inhibition was short-lived. For example, the involvement of an additional mechanism during agonist- and pressure-evoked constriction of cerebral arteries was suggested by several lines of evidence: (i) siRNA-mediated depletion of

RhoA in organ-cultured rat cerebral arteries abolished the vasoconstrictor response to UTP or increased intraluminal pressure without affecting the stimulus-induced increase in LC_{20} phosphorylation[32]; (ii) inhibition of PKC activity abolished the myogenic response of intact cerebral arteries but had no effect on LC_{20}, MYPT1 or CPI-17 phosphorylation[29]; (iii) serotonin-induced vasoconstriction at low (10 mm Hg) and physiological (60 mm Hg) intraluminal pressures was markedly reduced by inhibition of ROCK or PKC activities, but the level of LC_{20} phosphorylation declined only to the level observed in control tissues at 60 mm Hg where a significant myogenic response was observed[29,30]; (iv) serotonin induced an increase in LC_{20} phosphorylation of ~25% at 10 mm Hg and ~10% at 60 mm Hg, but a significantly greater vasoconstrictor response was observed at 60 mm Hg[30]; (v) pressure- or agonist-induced vasoconstriction was observed without an increase in LC_{20} phosphorylation above the pre-existing level of ~0.5 mol P_i/mol LC_{20} under three different experimental conditions: an increase in intraluminal pressure from 80 to 120 mm Hg (the normal physiological pressure range), application of serotonin at 80 mm Hg, or an increase in pressure from 10 to 80 mm Hg in the presence of serotonin[31]; and (vi) spontaneous development of myogenic tone during equilibration of freshly-isolated cerebral arteries at 60 mm Hg was associated with actin polymerization without a change in LC_{20} phosphorylation.[33]

A likely mechanism to explain these results involves dynamic regulation of the actin cytoskeleton, i.e., *de novo* formation of actin filaments is required for force development, actin polymerization and LC_{20} phosphorylation are independent events, and both actin filament formation and LC_{20} phosphorylation are required for smooth muscle contraction.[12,34] Several examples have been described of actin polymerization occurring in vascular smooth muscle tissues exposed to contractile agonists, increased extracellular K^+ concentration (membrane depolarization), tissue stretch, osmotic volume change or increased intravascular pressure.[35–37] An important role for actin polymerization in smooth muscle contraction was originally suggested by the effects of inhibitors of polymerization (latrunculins, which sequester G-actin monomers, and cytochalasins, which bind to

the barbed end of actin filaments): these agents attenuated the contractile responses of various smooth muscles, including vascular smooth muscle.[35,38–46] In contrast, jasplakinolide, which stabilizes actin filaments and opposes depolymerization, induced vasoconstriction.[35] Furthermore, direct measurements of G- and F-actin revealed that smooth muscle contraction is accompanied by a small increase (~10–12%) in F-actin content.[37,47–50] For example, G-actin content progressively decreased as intraluminal pressure was increased in cerebral arteries from 10 to 80 to 120 mm Hg, and G-actin levels were significantly lower in the presence than in the absence of serotonin at 80 mm Hg, and at 80 mm Hg after pre-treatment with serotonin, compared with 10 mm Hg in the presence of serotonin.[33] Latrunculin B dilated vessels pressurized to 80 mm Hg to the passive diameter observed in the absence of extracellular Ca^{2+} without altering the level of LC_{20} phosphorylation. Similar effects of latrunculin B were observed in vessels pre-constricted with serotonin at 80 mm Hg and in vessels constricted by increasing intraluminal pressure from 10 to 80 mm Hg after pre-treatment with serotonin. G-actin content was reduced in every instance in which vasoconstriction occurred in the absence of a change in LC_{20} phosphorylation.[33] In rat cerebral arteries, UTP-induced vasoconstriction was accompanied by actin polymerization and both were prevented by siRNA-mediated RhoA depletion, which did not reduce UTP-evoked LC_{20} phosphorylation.[32]

What has emerged from such studies is the concept of two distinct pools of actin in smooth muscle[51–53]: (i) a contractile actin pool, which is involved in the contractile machinery (mini-sarcomeres), and (ii) a cytoskeletal actin pool, which is localized to the cell cortex (sub-plasmalemmal domain) and subject to dynamic regulation of its polymerization state. The contractile and cytoskeletal actin pools consist of smooth muscle α-actin and non-muscle β- and γ-actin isoforms, respectively.[54,55] Contractile and cytoskeletal actins are associated with different tropomyosin isoforms.[56] Polymerization of cortical actin in response to contractile stimuli is postulated to increase the formation of adhesion complexes, multi-protein complexes that connect the contractile apparatus to integrins (transmembrane proteins that connect to the extracellular matrix).[34] These adhesion complexes

distribute and transmit force generated by cross-bridge cycling at the myofilament level across the plasma membrane and to the extracellular matrix. The fact that latrunculin B induced complete vasodilatation of cerebral arteries at 120 mm Hg, or at 80 mm Hg in the presence of serotonin, to the passive diameter rather than to the pre-existing diameter at 80 mm Hg, or at 10 mm Hg in the presence of serotonin, suggests that pressurization evokes a remodeling process whereby existing connections between the contractile apparatus, plasma membrane and extracellular matrix, required for force distribution and transmission, are broken.[33] The assembly of adhesion complexes is also necessary for the activation of signaling processes that regulate actin polymerization.[34]

There is some debate about whether Ca^{2+} sensitization is the appropriate term to describe the role of actin polymerization in the vasoconstrictor response. Our sense is that, if Ca^{2+} sensitization is defined as an increase in force without a change in $[Ca^{2+}]_i$, then increased actin polymerization via a signaling pathway that does not require Ca^{2+} is indeed a form of Ca^{2+} sensitization. It is important to note that inhibition of MLCP activity or activation of actin polymerization will not evoke contraction in physiologically-relevant conditions unless $[Ca^{2+}]_i$ is sufficiently high to activate MLCK and achieve a threshold level of LC_{20} phosphorylation for cross-bridge cycling to occur.

5. Signal Transduction Pathways Mediating Stimulus-evoked Actin Polymerization

Insights into the signaling pathways that mediate stimulus-induced actin polymerization in smooth muscle have come largely from studies of acetylcholine-induced contraction of canine tracheal smooth muscle.[34,57] There is an increasing body of evidence that early events following agonist-receptor interaction or an increase in intraluminal pressure involve the recruitment of numerous proteins to integrin-containing adhesion complexes at the level of the sarcolemma. These include a variety of protein kinases, including tyrosine kinases such as Src family kinases (SFK), focal adhesion kinase (FAK) and the other FAK family member Pyk2, scaffolding/adaptor

proteins such as Crk-associated substrate (p130CAS),[47,58] cytoskeletal proteins such as α-actinin, vinculin, talin and paxillin,[45,59–63] which link actin filaments to the sarcolemma, proteins that facilitate actin polymerization such as N-WASp (neuronal Wiskott-Aldrich Syndrome protein), cofilin, profilin, HSP20 and HSP27 (heat shock proteins), and VASP (vasodilator-stimulated phosphoprotein),[12,37,46,49,64–68] and the small GTPase RhoA, which regulates adhesome assembly.[50,69] Recent studies of the myogenic response of rat cerebral arteries revealed pressure-dependent increases in phosphorylation of FAK at tyrosine-397 and tyrosine-576/577, SFK at tyrosine-416 (tyrosine-527 phosphorylation decreased), vinculin at tyrosine-1065, paxillin at tyrosine-118 and phospholipase C-γ1 at tyrosine-783[70,71]. Function-blocking antibodies to α_5-integrin, the FAK inhibitor FI-14 or the SFK inhibitor SU6656 attenuated these changes in adhesion complex protein phosphorylation and prevented the pressure-dependent increase in MYPT1 phosphorylation at threonine-855 and of LC_{20} at serine-19, as well as actin polymerization. These observations support a mechanotransduction mechanism involving integrin-containing adhesion complex formation and signaling to enhance Ca^{2+} sensitization via MLCP inhibition and actin polymerization.[70]

PKC has long been implicated in the regulation of smooth muscle contraction,[72,73] but its mechanism, including the identification of key substrates, has been elusive. Recent evidence has implicated PKC in the regulation of actin polymerization in vascular smooth muscle via the phosphorylation of HSP27 at serine-82.[33] HSP27 may suppress actin polymerization by directly binding to G-actin[74,75] or by capping the barbed end of F-actin to prevent filament elongation.[76] Phosphorylation of HSP27 at serine-82[77,78] facilitates actin polymerization by alleviating the inhibition exerted by the unphosphorylated protein or by binding to and stabilizing actin filaments.[79] Indeed, phosphorylation of HSP27 at serine-82 occurs in the same time-frame as agonist-induced vasoconstriction.[37] Inhibition of rat cerebral arterial PKC with GF109203X (which affects both Ca^{2+}-dependent and independent isoforms) or Gö6976 (a selective inhibitor of Ca^{2+}-dependent PKC isoforms) attenuated pressure-induced vasoconstriction without

affecting LC_{20} phosphorylation,[29] whereas GF109203X inhibited serotonin-induced vasoconstriction and LC_{20} phosphorylation.[30] Furthermore, treatment with the PKC-activating phorbol ester, phorbol 12,13-dibutyrate (PDBu), induced significantly greater vasoconstriction than serotonin, but a comparable increase in LC_{20} phosphorylation.[80] PDBu-induced vasoconstriction was largely reversed by sequestration of G-actin monomers by latrunculin B, implicating actin polymerization in the phorbol ester response. In support of this conclusion, direct quantification of G-actin levels revealed that PDBu treatment induced actin polymerization, which was reversed by GF109203X.[80]

ROCK has also been implicated in stimulus-evoked actin polymerization in rat cerebral arteries,[33] most likely via phosphorylation and activation of LIM kinase, which in turn phosphorylates cofilin at serine-3.[81] Similar results were reported for canine pulmonary arterial smooth muscle[82] although a decrease in cofilin phosphorylation was observed upon depolarization of swine carotid arterial rings,[83] which also correlated with actin polymerization,[84] suggesting there may be stimulus-, tissue- or species-dependent variations in the effects of cofilin phosphorylation. Cofilin binds to and severs actin filaments, and this activity is alleviated by phosphorylation at serine-3.[85,86] Phosphorylated cofilin binds to the scaffolding protein 14-3-3, which sequesters cofilin and prevents it from being dephosphorylated.[87] An increase in cerebral arterial pressure from 10 to 120 mm Hg increased the phosphorylation of both HSP27 and cofilin. ROCK inhibition by H1152 prevented the pressure-induced phosphorylation of cofilin and PKC inhibition by GF109203X prevented the pressure-induced phosphorylation of HSP27.[33] Furthermore, enhancement of cofilin phosphorylation by cyclosporin A (an inhibitor of the type 2B protein serine/threonine phosphatase that dephosphorylates cofilin) induced further vasoconstriction and actin polymerization. On the other hand, blockade of PKC-mediated HSP27 phosphorylation by direct binding of the biphenyl isoxazole derivative, KRIBB3, reversed the myogenic response and actin polymerization.[33] The signaling pathways suggested to mediate actin polymerization in vascular smooth muscle are summarized in Fig. 2.

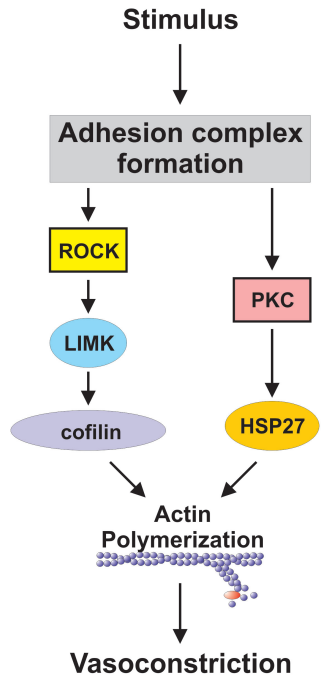

Fig. 2. Signaling pathways leading to actin polymerization in vascular smooth muscle in response to contractile stimuli. Agonists and increased intraluminal pressure trigger the formation of adhesion complexes leading to the activation of ROCK and PKC. One of the substrates of activated ROCK is LIM kinase (LIMK), which phosphorylates cofilin, whereas HSP27 is phosphorylated by PKC. Phosphorylated cofilin and HSP27 enhance actin polymerization in the sub-sarcolemmal domain. This involves branching of cortical actin filaments and the formation of new sites of interaction with the sarcolemma via integrins, thereby enhancing force transmission from the contractile machinery to the extracellular matrix and vasoconstriction.

6. Pathophysiological Considerations

Vascular smooth muscle contractile dysfunction underlies numerous pathologies such as hypertension and cerebral vasospasm following subarachnoid hemorrhage. Defects in signaling mechanisms likely contribute to such pathologies and ROCK and PKC have received particular attention in this regard.[73,88] Given their involvement in regulation of actin filament dynamics, it is conceivable that deficien-

cies in ROCK and/or PKC signaling may be involved in the etiology of cardiovascular defects. For example, inward eutrophic remodeling of resistance arteries, triggered by prolonged vasoconstriction, is detected early in the development of essential hypertension, and is dependent upon actin polymerization.[89] Changes in the actin cytoskeleton of vascular smooth muscle cells also contribute to increases in aortic stiffness during ageing.[90,91] Defective ROCK signaling has been implicated in dysfunctional myogenic regulation of cerebral blood flow in type 2 diabetes.[92–94] In a streptozotocin-induced rat diabetic model, enhanced myofilament Ca^{2+} sensitivity observed in mesenteric and tail arteries was attributed to increased ROCK and, to a lesser extent, PKC activities.[95] Recent studies of the Goto-Kakizaki (GK) rat model of non-obese type 2 diabetes suggested that abnormal ROCK-mediated Ca^{2+} sensitization of rat cerebral arterial constriction contributes to the dysfunctional myogenic control of cerebral blood flow observed in this disease condition.[96] Specifically, basal myogenic tone, LC_{20} phosphorylation and phosphorylation of MYPT1 at threonine-855 were elevated and G-actin content was reduced in cerebral arteries of pre-diabetic GK rats exhibiting normal serum insulin and glucose levels. Furthermore, pressure-dependent myogenic vasoconstriction, LC_{20} and MYPT1 phosphorylation and actin polymerization were suppressed in both pre-diabetic and diabetic GK rats. As more is learned about the signaling pathways responsible for Ca^{2+} sensitization of vascular smooth muscle contraction, and the defects that lead to pathological situations, the potential for development of novel therapeutics will be enhanced.

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Chapter 5

Developmental Basis of Vascular Smooth Muscle Cell Phenotypes

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Vascular smooth muscle cells (SMCs) comprise the predominant cell type in the walls of blood vessels.[1] In adult organisms, SMCs play a role in vascular homeostasis, and their contractile capability helps to sustain vessel tone, as well as mediate hemodynamics. It is intriguing that certain vascular diseases, such as atherosclerosis and aneurysms, tend to afflict specific regions of the larger blood vessels despite most of the identified risk factors being systemic.[2] Interestingly, there is now compelling evidence that SMCs from different blood vessels and even different regions within the same vessel originate from diverse embryonic lineages.[3] This raises the question of whether the intrinsic differences between SMCs underlying different regions of the vasculature may contribute to disease development.

Due to the difficulty of obtaining SMC progenitors from embryos and SMC subtypes from tissues in sufficient quantities, the relation between heterogeneity of SMC embryonic origins and anatomic localization of vascular diseases has not been studied extensively.

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) offer an unparalleled resource to obtain early SMC progenitors and SMCs of defined lineages. The growing importance of SMC lineage diversity in the site-specific manifestation of vascular diseases is beginning to be recognized. Despite a paucity of studies investigating the molecular-genetic differences between SMC subtypes, several notable lineage-specific differences in SMC development, as well as their relevance to disease will be presented.

1. Phenotypic Modulation of Smooth Muscle Cells

Plasticity is a hallmark of SMC phenotypes. In the physiological state, SMCs are highly contractile with low proliferative and migrative capacities. On the other hand, in cases of vascular pathologies such as atherosclerosis, SMCs migrate from media layer into the intima, and transform from contractile state to synthetic phenotype. These two contrasting phenotypes have several demarcating characteristics. Morphologically, contractile SMCs are spindle-shaped whereas synthetic SMCs are epitheloid or form a monolayer with their cobblestone appearance. Functionally, contractile SMCs are important for maintaining the structural integrity of blood vessel walls. Their ability to contract and relax helps to control vessel tone, hence sustaining normal blood pressure. Distinct profiles of secreted proteins also underpin the differences between the two SMC phenotypes. Healthy SMCs express contractile filament proteins such as alpha-smooth muscle actin, smooth muscle myosin heavy chain, smoothelins and cellular retinol binding protein, which are largely involved in their contractile ability and maintenance of vessel wall architecture. However, synthetic SMCs produce a plethora of proteins associated with extracellular matrix, vessel remodeling and repair. Specific proteins such as S1004A and calmodulin have been associated with the synthetic phenotype, whereas smooth muscle myosin heavy chain is only expressed by contractile SMCs.

1.1. *Triggers of Phenotypic Modulation*

Injury to the endothelial lining gradually develops into excessive lipid accumulation, apoptosis, necrosis, and accumulation of plaque within

the vessel wall.[4] Endothelial cells modify circulating low-density lipoproteins (LDLs) into their oxidized form.[5] Exposure to oxidized LDLs induces phenotypic switching of the medial SMCs by down-regulating contractile markers.[6] Oxidized LDLs also cause an increase in nuclear translocation of kruppel-like transcription factor 4 (Klf4).[7] Extensive studies showed that the G/C repressor regulated by Klf4 binding is required for transcriptional supression of SMC marker genes.[8,9] Interestingly, Klf4 and Klf5 are major targets for microRNAs miR-145, miR-146a and miR-25. These microRNAs are known to promote the expression of contractile and cytoskeletal proteins in SMCs. It has also been shown that inflammatory cytokines like TNF-α and IL-1β can increase the expression of lipoprotein scavenger receptors including the lectin-type oxidized LDL receptor 1 in both SMCs and endothelial cells, causing atherogenesis.[10] A recent study demonstrated platelet-derived growth factor–BB-induced dedicator of cytokinesis-2 (DOCK-2) as a novel regulator of the SMC phenotype. High expression of DOCK-2 leads to a suppression of the SMC contractile phenotype. DOCK-2 was shown to work hand in hand with Klf4 in the inhibition of myocardin binding to serum response factor, thereby attenuating myocardin-dependent SMC promoter activity.[11]

Another important contributor of SMC phenotypic switching is mechanical stimuli, both directly on SMCs or indirectly through differences in endothelial-based regulation of vascular tone. Endothelial cells mediate blood flow and pressure through nitric oxide release, as well as direct cell-cell interactions. Mechanical triggers such as shear and tensile stresses, in turn, could induce endothelial cell-based changes in the vasculature and thereby alter SMC phenotypes. Co-culture of SMCs with endothelial cells was able to cause phenotypic switching to a synthetic SMC phenotype.[12] On the other hand, mechanical strain causes a shift towards the contractile phenotype.[13] Changes in the mechanical strain of the matrix substrates can also alter the SMCs phenotype directly as shown by increased expression of caldesmon in SMCs grown on laminin compared to those on collagens.[14] Mechanosensing pathways are postulated to result in an increased expression of smooth muscle myosin heavy chain.[15] Shear stress can vary largely

between a pro-atherogenic and an anti-atherogenic flow. Phenotypic modulation of resident wall SMCs partly contribute plaque development and formation of fibrous cap. Response of SMCs to varying shear stress patterns may determine plaque stability to rupture.[16] Studies in animals have investigated the role of shear stress in plaque formation by exposing the hypercholesterolaemic apolipoprotein E-deficient mice with two different shear stress fields. These studies have found that low non-oscillatory shear stress induces thin-cap fibrous atheromas which are the major cause of coronary heart diseases. Increased expression of adhesion factors, chemokines and macrophage activating factors occur in the presence of low non-oscillatory shear stress. This culminates in elevated uptake and activation of inflammatory cells in the plaques, leading to thin-cap fibrous atheromas found in murine and porcine models, as well as human carotid and coronary arteries.[17–21] On the contrary, low oscillatory shear stress patterns lead to the formation of stable plaques. Thus, phenotypic modulation of SMCs could impact on the regulation of vascular tone, composition and stability of plaque, and pathogenesis of atherosclerosis.

1.2. *Influence of Embryonic Origins on Regional Differences of Vascular Smooth Muscle Cells*

The heterogeneity of vascular SMCs could be attributed to their diverse embrylogical origins. These subtypes localize in particular domains within the vascular tree with almost no intermixing. This leads to an interesting hypothesis that specific SMC subtypes and their location within the systemic vasculature play a critical role in the distinct physiological functions. This heterogeneity might even determine the pathological outcomes that manifest only in certain vessel regions depsite a systemic trigger.[22] One such example would be the proximal aorta with SMCs originating from two diverse embryonic origins: neural crest and somitic mesoderm.[23,24] The SMC subtypes from different embryonic origins in the aorta at such proximity ascertained the existence of distinct phenotypic differences

within the aortic sub-regions, which merges into a single phenotype in the adult aorta.[25] This shows that the specific SMC phenotypic features are determined to a large extent by their embryonic lineages. Congenital cardiovascular disorders also occur at the boundaries of vessel subdomains with SMC subtypes from different lineages. Coarctation of the aorta and aortic arch interruption are two such congenital disorders with regional susceptibility.[26,27] Surgical repair of affected regions does not entirely prevent the patients from recoarctation later in their lives,[28] suggesting that the root of the problem remains unsolved. Further studies focusing on the relationship between SMC embryonic origins and spatial-specific vulnerability would shed light on mechanistic insights and intervention to control these disease conditions. A classic example would be the variant Hox gene expressions that underpin distinct vascular phenotypes.[29,30] Hox6–10 genetic signatures was postulated to preserve the positional identity of vascular tree based upon their embryonic origins. Embryonic origins influence SMC phenotypes and hence may give rise to differential predispositions to atherosclerosis in the thoracic aorta and the atherosclerosis-prone ascending aorta.[31] Such position-specific hox expression profile was also observed during *in vitro* differentiation of human pluripotent stem cells towards neural crest-derived SMCs and somite-derived aortic SMCs. Correspondingly, both basal and TNF-α-stimulated NFκB activation and DNA binding were greater in SMCs of the atherosclerosis-prone ascending aorta compared to that in the atherosclerosis-resistant thoracic aorta.[31,32] Thus embryonic origin of the vascular SMCs indeed is one of the key determinants of their physiological and pathological processes. On the other hand, a corollary hypothesis to investigate would be the study of SMC subtypes of the same lineage but within different vessels to understand importance of regional relevance and the environmental influences. For example, SMCs of the neural crest lineage reside within two different vessels namely the ductus arteriosus and the atherosclerosis-prone ascending aorta. Regional differences in genetic signatures and ion channel expressions are observed despite similar SMC lineage.[33] Further insights into the phenotypic differences from embryonic origins and their positional relevance

could lead to novel therapeutic interventions that address the causal problem of vascular diseases.

2. Molecular Basis of Lineage-specific Differences in Vascular Smooth Muscle Subtypes

2.1. *Embryonic Smooth Muscle Cells*

The molecular mechanisms of how SMC progenitors arising from diverse lineages commit to a common SMC fate during development remain elusive. Nonetheless, a few transcriptional co-activators of serum response factor have been found to be implicated in regulating SMC differentiation in a lineage-specific manner. Myocardin-like 2 protein (MKL2) is one such transcriptional co-activator of serum response factor. Its loss-of-function in mice is embryonic lethal but the main cardiovascular defects are manifested in cardiac outflow tract[34] and branchial arch arteries,[35] both of which are neural crest derivatives. Therefore, there is a unique involvement of MKL2 in neural crest-derived SMCs. On the other hand, TGFβ signaling seems to influence SMC development differently depending on the location of SMCs in the vasculature. Mouse embryos with conditional deletion of the TGFβ type II receptor (TGFBR2) gene in cells expressing the SMC-specific TAGLN gene displayed defective SMC differentiation only in the mesoderm derivatives such as coronary vessels and descending thoracic aorta but not pulmonary trunk, a neuroectoderm derivative.[36] Another SMC-specific TGFBR2 knockout study demonstrated abnormalities of extracellular matrix protein synthesis and SMC differentiation in descending aortas but not cardiac outflow vessels.[37] Impairment of TGFβ signaling exacerbates SMC development primarily in mesoderm-derived vessels. Hence, various embryonic lineages appear to differentiate into SMCs through distinct pathways.

Embryonic SMCs respond in a lineage-dependent way to environmental cues during vascular development and morphogenesis. When cardiac neural crest and nodose placode are ablated in the avian embryo, lateral plate mesoderm-derived SMCs are in turn recruited to make up the walls of aortic arch arteries.[38] Under normal circumstances, lateral plate mesoderm does not contribute SMCs to proximal arterial

structures. Nonetheless, in the absence of neuroectodermal features, replacement by lateral plate mesoderm-derived SMCs has resulted in disorganized and hypoplastic vessel walls of aortic arch, subclavian and pulmonary arteries. This is apparently due to the incapacity of lateral plate mesoderm-derived SMCs to respond correctly to local signals for remodeling of the proximal arteries. Moreover, SMCs isolated from different aortic regions in avian embryos exhibit differential growth and contractile responses to TGFβ1. TGFβ1 promotes cell proliferation of cultured neuroectodermal SMCs but inhibits growth of mesodermal SMCs derived from the same aortic vessels.[39,40] In addition, collagen I and elastin are found to be more abundant in neuroectodermal SMCs upon TGFβ1 stimulation. It is postulated that Myb and Myc function specifically in neuroectodermal SMCs to potentiate TGFβ1-induced mitogenesis and matrix synthesis. Conversely, TGFβ1 elicits a stronger contractile response by mesodermal SMCs than neuroectodermal SMCs in collagen gel contraction assay, possibly mediated through greater expression of α5β1-integrin in mesodermal SMCs.[40] Therefore, SMCs from different embryonic origins respond in lineage-specific ways to common stimuli during growth and remodeling of blood vessels.

2.2. *Postnatal Smooth Muscle Cells*

The phenotypic diversity of SMCs in the mature vascular tree could be attributed to their heterogeneous embryonic origins as well. Interestingly, the wall of aorta is a mosaic of SMC subtypes arising from distinct origins but with well-defined boundaries separating regions of origin-specific SMCs. The frequently observed site-specific pathologic patterns in the aorta have spurred transcriptome profiling of different aortic regions from animal models. A genome-wide microarray compares gene expression of SMCs from aortic arch and thoracic descending aorta in apolipoprotein E-deficient mice which are the standard mouse model for atherogenesis.[41] Genes which are differentially upregulated in aortic arch SMCs of neuroectodermal origin are related to pro-atherogenic processes such as cell proliferation, motility, immune response and apoptosis, consistent with aortic

arch being the more atherosclerosis-prone region. Another microarray analysis of baboon aortas reveals intrinsic expression differences of a panel of HOX genes in the thoracic and abdominal aortas.[42] In particular, HOXA4, a DNA-binding transcription factor, is spatiotemporally regulated during development to regulate morphogenesis and differentiation. HOXA4 is significantly higher in SMCs of human thoracic aortas compared to that of abdominal aortas. Human abdominal aortic aneurysmal tissues demonstrate decreased HOXA4 expression versus non-aneurysmal controls. Furthermore, cultured human SMCs show decreased levels of HOXA4 when treated with interferon-γ, an inflammatory cytokine implicated in the development of abdominal aortic aneurysm. These evidences seem to imply a protective role of HOXA4 and could explain why the thoracic aorta is relatively less susceptible to aneurysms due to higher endogenous level of HOXA4.

SMC subtypes from different origins, and hence different vascular regions may respond variably to systemic disease mediators. Angiotensin II (Ang II) infusion into mice induces medial expansion throughout the aorta but its pathogenesis in ascending aorta proceeds via a different mechanism from the rest of the aorta.[43] Ascending aortic SMCs of neuroectodermal origin undergo hyperplasia when induced by Ang II while SMCs of mesodermal origins from other aortic regions exhibit hypertrophy. Inhibitor of differentiation 3 (Id3), a critical downstream regulator of BMP signaling, is then found to exist in higher abundance in SMCs of ascending aorta and that Id3 deficiency is able to abrogate the effect of Ang II-induced hyperplasia in ascending aortic SMCs. Thus, Id3 may function uniquely in neuroectodermal SMCs to mediate cell proliferation. Aortic arch which is composed of neuroectodermal SMCs is also found to develop medial calcification faster than the other arterial regions in a mouse model deficient in matrix Gla protein, a potent calcification inhibitor. The expression of tissue non-specific alkaline phosphatase, an indicator of SMC osteogenic conversion, is significantly upregulated in aortic arch during mineralization but hardly activated in the descending aorta which is made up of mesodermal SMCs. Therefore, the spatiotemporal development of vascular

diseases may be a consequence of lineage dependent differences in SMC subtypes. Other determinants of differential SMC responses, such as non-uniform hemodynamics and morphogenetic cues should not be neglected nonetheless.

2.3. *Human Pluripotent Stem Cell-derived Smooth Muscle Cells*

A model system which allows us to study the sole contribution of SMC origins to disease susceptibility would be valuable. A myriad of methods to derive smooth muscle progenitors and cells from human ESCs and iPSCs, collectively referred to as human pluripotent stem cells (hPSCs), has been reviewed.[44] Insights from developmental biology are imperative in guiding rationales for hPSC differentiation. Directed differentiation of SMCs from hPSCs induction has leveraged mostly on the developmental paradigm of embryoid body formation, mesoderm induction and further induction to SMCs using specific growth factors.[45–48] It has been found that after an initial mesoderm induction by GSK3 inhibition and BMP4, followed by activin A and platelet-derived growth factor (PDGF)-BB, lead to highly efficient generation of CD140b+ SMCs. CD140b, a receptor for PDGF, marks a highly proliferative subset of SMCs, enabling them to respond to mitogens.[49] Cheung *et al.* developed a methodology to generate lineage-specific vascular SMCs from hPSCs in large numbers using chemically defined conditions.[50,51] This method elegantly allows the derivation of specific SMC subtype progenitors *in vitro* through neuroectoderm- and mesoderm- lineages which would otherwise be difficult to obtain from developing embryos.[52] Such lineage-specific SMCs had comparable characteristics as primary SMCs. Although the morphologies of SMC subtypes are indistinguishable, each SMC subtype maintained its own unique proliferative and secretome profile upon cytokine stimulation. Other recent work demonstrates that faithful recapitulation of early developmental cues *in vitro* enables efficient differentiation of hPSCs into epicardium and its derivatives. Stage-wise activation of BMP and Wnt signaling pathways by Witty *et al.* showed effective derivation of epicardial lineages

from hPSCs.[53] Likewise, Iyer *et al.* reported an *in vitro* model of epicardium, and epicardium-derived smooth muscle cells from hPSCs using a combination of agonists for BMP, Wnt and retinoic acid signaling pathways.[54]

Given a multitude of microenvironment signals that guide differentiation of SMC subtypes in embryos, some aspects of SMC development may be difficult to realize through *in vitro* differentiation. Further morphological maturation could entail incorporation of biomimetic factors. Mechanical stimuli,[55] co-culture with other cell types[56] and three-dimensional cultures[57] have been known to promote vascular differentiation and maturation. It is likely that such dynamic culture situations could enhance refinement of SMC functional phenotypes. Our knowledge of how different embryonic lineages commit to a common SMC fate is still limited. As aforementioned, there are a few studies of lineage-dependent requirement of various serum response factor co-activators in vascular SMC development but epigenetic regulation in origin-specific SMC differentiation remains largely unknown.[58] The hPSC-derived SMC subtype system represents a great opportunity to dissect molecular pathways and to determine which pathways are common SMC pathways and which are lineage-specific.

3. Disease Modeling with Lineage-specific Smooth Muscle Cells

Embryonic origins of SMCs could be crucial in governing blood vessel health and disease. Generation of the lineage-specific SMCs *in vitro* can be an efficient tool to study congenital and vascular disorders with major lineage relevance.[50,51] One of the key findings reveals that SMC subtypes of different embryonic origins displayed differential proteolytic abilities to degrade extracellular matrices in response to interleukin-1β, an inflammatory cytokine commonly involved in vascular diseases. This could suggest that origin-specific SMC subtypes may have different vascular remodeling capabilities during disease settings. Subsequent work showed that specific SMC subtypes have varying levels of Notch 3 gene expression. The differential Notch 3 levels in turn regulate SMC markers in mature neuroectoderm-derived and paraxial mesoderm-lineages but not the lateral mesoderm-derived lineage.[59] For

modeling of brain vasculature in neurodegenerative diseases, neural crest-derived SMCs have been used to recapitulate amyloid beta uptake and clearance mechanism *in vitro*, whereas mesoderm-derived SMCs were not as effective.[60] Such lineage-specific SMC subtypes can create useful platforms to investigate origin-dependent disease susceptibility and downstream therapeutic targets.[61]

There has been increasing recognition of the role of embryonic lineage on the regional distribution of certain congenital and acquired vascular diseases. The regional susceptibility to atherosclerosis in thoracic aneurysm was postulated to be caused by SMC subtypes arising from diverse mesenchymal populations in the embryo.[62] Another example would be Marfan syndrome, caused by the misfolding of fibrillin-1, that results in weakening of vessel walls of the ascending aorta and arch which are populated with SMCs of neuroectodermal origin. Studies have shown that neuroectoderm-derived vascular SMCs respond appropriately to the physiological stimuli of Marfan syndrome.[63]

With the advent of CRISPR/Cas9 technology, genome editing provides an avenue to study genetic disorders affecting specific regions of the vasculatures. Mutations and genetic risk variants could be introduced or genetically corrected to create isogenic sources of SMC subtypes. This allows unbiased comparison between wild type and mutant cell lines for mechanistic interrogation of disease-causing mutations. Genome-wide association studies in patient cohorts have identified polymorphisms that confer susceptibility to coronary artery disease, such as the 9p21 variants.[64] Lineage-specific SMC subtypes could be amenable to genome editing to unravel the causality of genetic risk variants in predisposing individuals to vascular pathologies.

4. Conclusion

Heterogeneity of vascular SMC phenotypes may have been determined early by the diversity of developmental origins (Fig. 1), alongside other factors such as hemodynamics. It is evident from both embryonic and postnatal SMCs that distinct pathways contribute to functional differences among SMC subtypes underlying different sites of the vasculatures. *In vitro* differentiation of SMCs from hPSCs

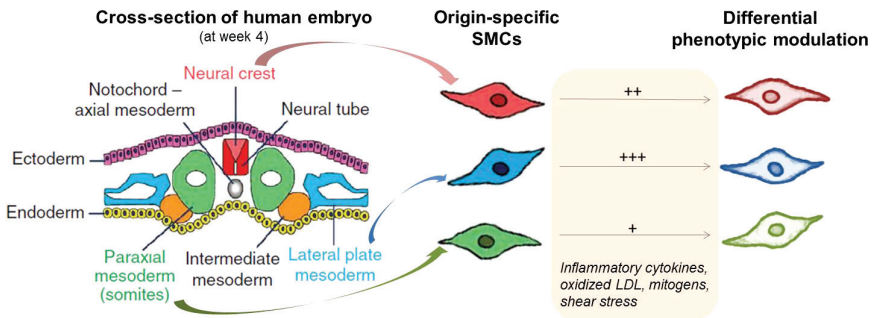

Fig. 1. Diverse embryonic lineages of vascular SMCs influence their responses to disease stimuli, leading to differential phenotypic changes.

has enabled the generation of large numbers of SMCs, which would otherwise be difficult to obtain due to donor unavailability. These stem cell-derived SMCs hold great potential for tissue engineering applications and regenerative medicine.[65] Large populations of pure SMCs can be used to perform high throughput drug screening and pharmacokinetic testing.[63] The robustness of iPSC technology has allowed the derivation of iPSCs from a wide range of tissues.[66] Patient-specific iPSCs could capture genetic risk variants to facilitate investigation of causal disease mechanisms. Such patient iPSC platforms hold promise to predict individual responses to new therapies, moving one step closer to personalized medicine. Taken together, the use of lineage-specific SMCs will underpin the success of modeling genetic disorders affecting certain SMC subtypes. Our knowledge of the developmental basis of SMC could pave the way to devise more targeted therapeutic interventions for restoration of vascular health.

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Chapter 6

Regulation of Podosomes in Vascular Smooth Muscle Cell Invasion of the Extracellular Matrix

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Unlike skeletal and cardiac muscle cells, mature contractile vascular smooth muscle cells (VSMC) are remarkably plastic, capable of switching to the de-differentiated, synthetic/proliferative phenotype, which is motile and invasive. This is achieved in part by remodeling of the actin cytoskeleton triggered by growth factors in response to vascular injury and inflammation. Switching of VSMC from the contractile to the synthetic phenotype is readily demonstrated by passaging VSMC excised from vascular tissues in culture dishes. Expression of contraction-associated markers such as myosin II heavy chain, SM α-actin, calponin, heavy h-caldesmon and SM22α are significantly down-regulated after 3-4 passages of primary VSMC in culture with concomitantly upregulation of migratory markers such as light isoform of caldesmon, l-caldesmon. (Reviewed in).[65,66]

Acquiring the migratory and invasive phenotype is a prerequisite for VSMC to degrade the extracellular matrix (ECM) and cross the internal elastic ileum and basement membrane to reach the intimal layer and injury sites at the endothelium. This innate physiological wound-healing

response, however, can also cause intimal thickening and formation of atherosclerotic plaques. Identification of VSMC and smooth muscle-like cells in the cap and interior of atherosclerotic plaques is indicative of cross-tissue migration of VSMC during atherosclerotic plaque formation. However, investigation of how VSMC contribute to plaque formation and stability have been stymied due to lack of specific VSMC markers and reliable lineage-tracing studies to identify origins of VSMC during atherosclerotic lesion development.[21,35]

The mechanisms of phenotypic switching and possible roles of VSMC in atherosclerosis is reviewed elsewhere in this monograph. In this chapter, I will focus on the structure of actin-based invasive organelles called podosomes, and regulation of their formation in VSMC. It is necessary, however, to provide a brief introduction to podosomes in non-smooth muscle cells such as monocytes, which have been more extensively studied.

1. Podosomes in Non-smooth Muscle Cells

Actin-based structures known as podosomes and invadopodia, collectively named invadosomes, are specialized adhesive and invasive membrane protrusions that degrade ECM proteins allowing cell migration across tissues.[51,83] Though they share many structural and functional characteristics, podosomes and invadopodia differ in life span, size and invasiveness. Invadopodia are large and highly invasive ventral protrusions of several μm in length, found in cancer and transformed cells, with a life time of over an hour. Podosomes are the smaller (< 1 μm), short life (~ 10 min) and less invasive cousins found in normal, untransformed cells of monocytic cell lineage such as macrophages, dendritic cells and osteoclasts, as well as endothelial cells and SMC in the vasculature. Src-transformed cells produce a unique invadosome superstructure called rosette that comprises features characteristic of both invadopodia and podosomes.

Pododomes were first reported in Rous sarcoma virus-transformed BHK cells[78] and have since been identified in other myeloid cell lineage.[9,50,57,82] Podosomes are structurally and functionally tailored to the needs of cell types in which they are produced. They are

required for monocytes in extravasation in inflammation, osteoclasts in bone resorption, endothelial cells in sprouting during angiogenesis, and VSMC in medial-intimal tissue crossing in atherosclerosis. Podosomes are remarkably dynamic organelles. Individual podosomes are assembled and disassembled in a matter of minutes, and capable of turning over core actin a few times within a life span.[20,80] The dynamic nature of podosome structure is necessary for temporal requirement of its multi-faceted functions in adhesion, matrix sensing, recruitment, secretion and degradation of ECM. Dynamics of assembly and disassembly of podosome superstructures such as rosettes, however, is less understood.

A typical podosome comprises a vertical column of actin filaments that ranges from 0.5–1 μm in length, surrounded by a ring of focal adhesion proteins[51] (See Fig. 1). The actin core contains bundles of branched actin filaments and actin-binding proteins such as the Arp2/3 complex, N-WASP, gelsolin, cofilin, cortactin and caldesmon that regulate actin nucleation, polymerization and branching.[13] The ring of focal adhesion proteins include α-actinin, vinculin, talin and paxillin. A cap-like structure has been identified on top of the actin column[52] containing the formin FMNL1[59] and supervillin, a member of the villin family.[5] The function of the cap is not known, but has been suggested to act as a point of attachment for unbranched actomyosin filaments that form a network or 'actin cloud' connecting individual podosomes to form complex superstructures such as sealing zones in osteoclasts, clusters in SMC and rosettes in endothelial cells and Src-transformed cells.[53] Unbranched contractile actomyosin filaments also connect the top of the podosomes to the ventral surface of the plasma membrane thought to be involved in the regulation of vertical growth of actin cores and recruitment of adhesion proteins to the ring.[80]

Recent advances in single molecule activation super-resolution microscopy, such as dSTORM, has provided finer structural details to our standard 'core and ring' model of the podosome structure.[58,81] There is new evidence that vinculin strands form polygonal rings surrounding the actin core and project out of the corners of the polygons, suggesting nucleation sites for new podosomes. Interestingly, talin appears in both the ring and the core.[84] Thus, it now appears that

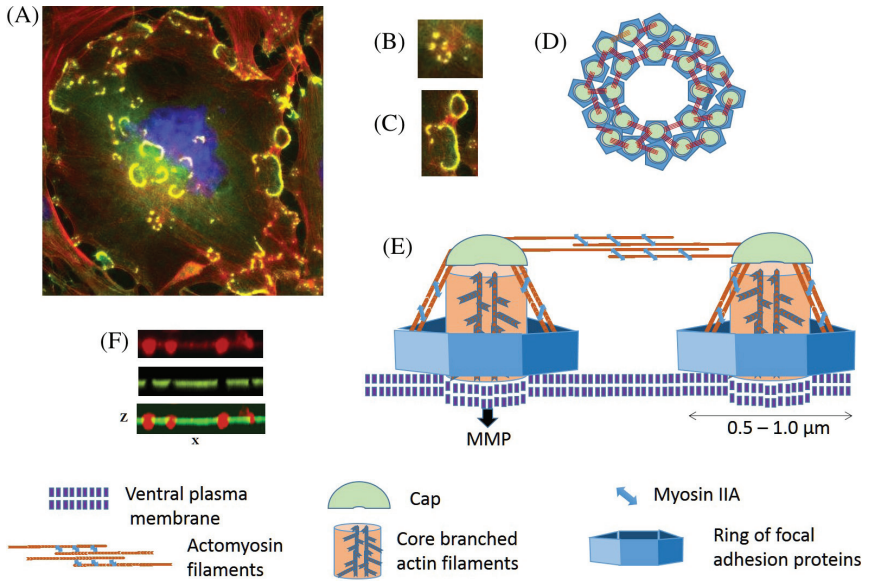

Fig. 1. Podosomes and rosettes in Src(Y527F)-transfected rat aortic smooth muscle cells.

A, F-actin filaments were stained with FITC-labelled phalloidin (red) and cortactin with TRITC-labelled anti-cortactin antibodies (green). Three types of podosomes co-stained with actin and cortactin (yellow) are shown in the same cell: individual podosome clusters, incompletely formed open rosettes and closed rings of rosettes.

B, Inset shows a cluster of individual podosomes.

C, Inset shows an open and a closed rosette of podosomes.

D, Illustration of aggregates of individual podosomes in a rosette.

E, Illustration of a vertical section of two podosomes connected by linear actomyosin filaments.

F. Immunofluorescence microscopic images of podosomes (red) invading a FRITC-fibronectin (green) substrate. F-actin was stained with FITC-phalloidin.

key adhesion molecules such as integrins, vinculin and talin are not arranged in a circular ring around the core but rather consists of islets of talin-integrin complexes. There is little doubt that future super-resolution images will further refine our understanding of the podosome structure and function.

There is no known protein that exclusively localizes to podosomes. However, cortactin and its Tyr-phosphorylated counterpart always exist in podosomes, and have been widely used as podosome markers.[87]

Cortactin is a major Src-substrate that stabilizes branch points of actin filament network in lamellipodia and podosomes by binding to the Arp2/3 complex, N-WASP and F-actin. Another Src substrate and adaptor protein, Tks5, has been consistently identified in invadosomes and has also been used as a reliable podosome marker.[16] Considering the functional diversity, structural complexity and dynamic nature of podosomes, it is not surprising that the list of reported podosomal proteins has grown significantly since their discovery. Using Stable Isotope Labelling by Amino acids in Cell culture) (SILAC) / mass spectrometry, a podosome proteomics study performed on human macrophages has identified 203 potential podosomal proteins in podosome-enriched ventral membranes, of which 33 have been previously reported and 170 potentially novel ones.[10] As expected, proteins involved in the regulation of actin cytoskeleton and adhesion are enriched in podosomes. Unexpectedly, ribosomal and RNA-binding proteins such as hnRNP-K and WDR1/AIP-1 have also been identified in the core of macrophage podosomes that echoes previous discovery of RNA-binding proteins in focal adhesions and spreading initiation centers.[19] These studies have certainly provided new targets for future studies on podosome function and structures.

2. Podosomes in Vascular Smooth Muscle Cells

Depending on the pore size of the fibrillary networks of collagen and fibronectin in the ECM, cells can move through the ECM either by non-proteolytic amoeboid movement similar to leukocytes squeezing through inter-fibrillar spaces.[54] or by proteolytic degradation of densely meshed ECM with MMPs secreted by podosomes and invadopodia. To the best of my knowledge, there is no evidence that VSMC are able to move by amoeboid movement through the vascular basement membrane that is densely populated by type IV collagen filaments.[72] On the other hand, podosome formation and degradation of ECM by VSMC have been clearly demonstrated *in vitro* and hinted in *in vivo*.[48,55]

Peripheral SM α actin-containing columns were first reported in the VSMC cell line, A7r5, in response to phorbol-ester treatment.[28] These were recognized as actin- and α-actinin-containing podosomes by Hai *et al* in 2002,[40] who also showed that conventional PKC-α

mediates PDBu-induced podosome formation. Podosomes induced by PDBu exist as individual actin-containing dots under the epifluorescence light microscope. When observed by a confocal microscope, SMC podosomes appear as vertical columns protruding from the ventral plasma membrane of the cell when grow on fibronectin or collagen substrates. Gimona and colleagues have shown that A7r5 cells degrade fibronectin substrate at the podosome sites in a 2-D culture.[7,8] They have also demonstrated that podosomes grow from sites joining the ends of actin stress fibers and focal adhesions, called microdomains. Recruitment of cortactin and the RhoA inhibitor, p190RhoGAP, to the microdomains may cause disassembly of actin stress fibers and local inhibition of contractility signaling the initiation phase of podosome formation in VSMC. This is consistent with the earlier report that *h1* calponin inhibits PDBu-induced podosome formation by stabilizing actin stress fibers.[32] Interestingly, *l*-caldesmon, which is another stress fiber stabilizing protein and actin-branching inhibitor[97] also inhibits podosome formation.[25,63] These early studies suggest that stress fiber-stabilizing proteins such as *h1* calponin and *l*-caldesmon may act as podosome inhibitors in VSMC.[32]

Solid evidence of podosome formation in VSMC *in vivo* is still lacking; however, podosome-like structures are produced by PDBu- stimulated VSMC when embedded in reconstituted basement membrane to simulate the 3-D environment in vascular tissues.[8] Invadopodia-like structures have also been seen in VSMC embedded in a 3-D matrix of type I collagen.[28,29]

3. Regulation of Podosome Formation in Vascular Smooth Muscle Cells

Unlike monocytic cells, primary VSMC and A7r5 SM cell lines do not form podosomes constitutively *in vitro,* but require external stimulants such as phorbol-esters and PDGF. A number of pathways have been identified in the regulation of podosome formation in VSMC that operate under the control of two pro-podosome signaling hubs, PKC and cSrc, and one anti-podosome network involving the tumor suppressor, p53. (Fig. 2).

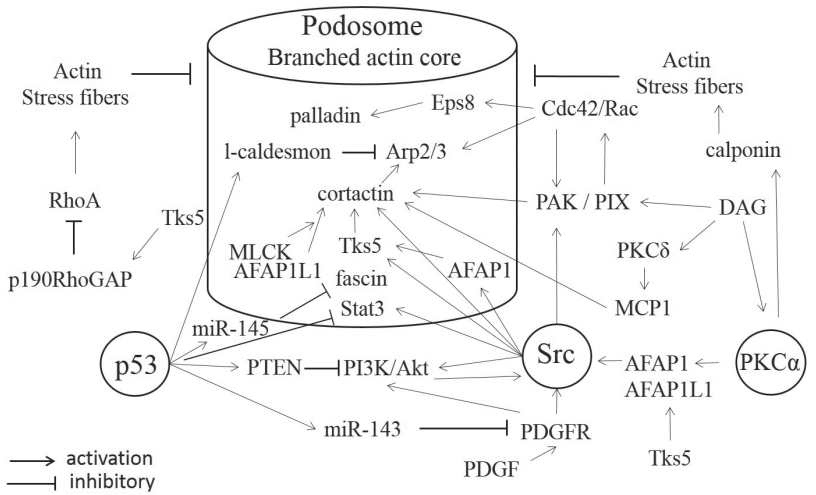

Fig. 2. Pathways known to regulate podosome formation in vascular smooth muscle cells.

The pathways are grouped under the regulation of three major hubs: cSrc, PKC, and p53. The cSrc/PKC- and p53-associated pathways are pro-podosome and anti-podosome, respectively, and are mutually antagonistic.

4. The Pro-Podosome Signaling Hubs: PKC and cSrc

4.1. PKC-associated pathways

Historically, phorbol-esters were first found to induce podosome formation in A7r5 VSMC cell lines.[28] Phorbol-esters such as phorbol 12, 13 dibutyrate (PDBu) and phorbol 12-myristate 13-acetate (PMA) are pharmacological analogues of diacylglycerol (DAG), the physiological activator of conventional PKCs (α, βI, βII, γ), and novel PKCs (δ, ε, η, θ) but not the atypical PKC ζ and λ.[2] Not surprisingly, PKC α was subsequently shown to mediate the effects of phorbol-esters in podosome formation in VSMC.[40] It has also been shown that nicotine and PKC activation synergistically augment podosome formation and ECM degradation in human aortic SMC.[37]

When treated with 1 μM of PDBu for 30 min, over 90% of A7r5 cells develop podosomes, which are capable of degrading fibronectin

substrate.[8,40,46,89] Interestingly, similar dosage of PDBu has no effect on primary rat aorta SMC (RASMC) on either podosome formation or ECM degradation.[29] However, 10-folds increase in PDBu concentration causes 5-10% of RASMC to form podosome-like structures that degrade fibronectin substrate. These findings indicate significant differences in A7r5 cells and primary VSMC in podosome formation in response to PDBu stimulation. This could be attributed to genetic differences in the two cell types that may have altered down- stream effectors of PDBu. A7r5 cells, which are generated from embryonic rat aorta, are tetraploids that display the adult SMC phenotype expressing some differentiated protein markers such as SM-α actin, myosin heavy chain and SM22.[27] RASMC used in invasion and migration studies are usually limited to within 5-6 passages to minimize progressive genetic changes that may alter the primary SMC phenotypes. Nevertheless, A7r5 cells in culture bear resemblance to differentiated VSMC by expressing prominent arrays of stress fibers, thus providing a useful cell model to study dynamics of actin remodeling during the transition from stress fibers to podosome formation.

4.1.1. *AFAP-1 and AFAP1L1*

One of the key signals linking PKCα and podosome formation in A7r5 cells is the family of Actin Filament-binding and Adaptor Proteins (AFAP).[22,31] AFAP-1 (or AFAP-110) is an adaptor protein that links actin filaments and cSrc by virtue of its ability to bind actin filaments and cSrc.[4] Activation of PKCα by PMA leads to phosphorylation of AFAP1 and targets it to form a complex via its SH3-binding domain with cSrc, resulting in cSrc activation and induction of actin cytoskeleton remodeling and podosome formation in A7r5 cells.[31,85]

AFAP1 and its homologue AFAP1L1 have been shown to localize to podosomes in A7r5 cells and overexpression of AFAP1 and AFAP1L1 is able to induce podosome formation without stimulation by phorbolester.[75] AFAP1 alters actin filament integrity and organization by acting as an actin filament cross linker.[4,69] There is evidence that AFAP members can affect podosome formation and dynamics directly by their interaction with the actin core of podosomes. It was shown that PDBu

induces phosphorylation of AFAP1 at Ser277, and a Ser277Ala mutant localizes to podosomes and increase the number of long-lived podosomes, suggesting that phosphorylated AFAP1 promotes disassembly of podosomes.[22] AFAP1 and AFAP1L1 may also play a role in the actin core stability by interacting with cortactin. While AFAP1L1 co-immunoprecipitated with cortactin, AFAP1 does not, suggesting that AFAP1L1 is localized to podosomes via interaction with cortactin, and the two isoforms complement each other in the regulation of podosome formation by interacting with cortactin in A7r5 cells.[75]

AFAP1 and cSrc complex formation and subsequent Src activation has been shown to require activation of phosphatidyl-inositol 3-kinase (PI3K).[85] In addition, PMA directly activates PI3K to produce phosphatidylinositol 3,4,5-trisphosphate (PI(3,4.5)P_3), which recruits AFAP1 by binding to its PH1 domain. This finding provides evidence that the PKCα-PI3K signaling acts upstream of AFAP1-cSrc activation in PMA-stimulated podosome formation. Furthermore, it also suggests a physical link between the PH1 domain of AFAP1 and PI(3,4.5)P_3 at the perinuclear membrane, where inactive cSrc is mainly located and thus activated by AFAP1. Since cSrc is also a known agonist of the PI3K/Akt pathway, which plays a pivotal role in podosome signaling[42] (see below), it suggests the presence of a feed-back loop between PKCα-PI3K and cSrc.

4.1.2. *PAK1-PIX-Rac/Cdc42*

The p21-activated kinases (PAK) have also been shown to play a key role in mediating podosome formation in A7r5 cells induced by phorbol-esters, and by extension, PKC.[88] There are two major families of PAK kinases in mammalian cells, Group 1 (PAK1, 2, 3) and Group 2 (PAK 4, 5, 6).[26] Although all three Group 1 kinases are expressed in VSMC, PAK1 is the most abundant. PAK1 is a key effectors of activated GTP-bound Rac/Cdc42, and its binding partner PIX, which is a guanine exchange factor (GEF) for Cdc42/Rac.

Using kinase active and inactive mutants of PAK1, we have shown that the kinase activity of PAK1 is not required for PDBu-induced formation of podosomes in A7r5 cells, but plays a role in podosome

turnover.[88] Furthermore, interaction between PAK and PIX is required for PAK1 to promote core actin formation in podosomes, and to translocate PIX and the G-protein-coupled receptor kinase interacting protein (GIT) to focal adhesions next to site of podosome growth. These data suggest that podosome formation is initiated by recruitment PAK1, PIX and GIT to focal adhesions at the microdomains, while phosphorylation of podosomal proteins by PAK1 may signal disassembly of the podosomes. This is supported by findings that PAK3, another member of the conventional PAK family, phosphorylates cortactin at Ser113 in the first actin-binding repeat *in vitro*, resulting in a significant reduction of binding of cortactin to actin columns in PDBu-induced podosomes in A7r5 cells.[91] Other podosomal protein substrates of PAK include caldesmon, MLCK and vitmentin. Phosphorylation of caldesmon by PAK has also been shown to downregulate its interaction with actin filament.[23] Expression of wild-type PAK1 in primary RASM cells does not affect podosome formation,[29] but the kinase inactive mutant (PAK1-LL/R), which has lost its PIX-binding site, has also lost most of its ability to induce podosome formation and ECM digestion in primary RASM cells. These data further underscore the importance of the protein-binding domain of PAK1 rather than the kinase activity in podosome formation in VSMC.

The roles of PAK in podosome formation seem to be different in other cell types. Hence, in contrast to VSMC, PAK1 and 2 suppress podosome formation in Rous sarcoma virus-transformed fibroblasts by phosphorylation of caldesmon.[61] PAK4, a member of Group 2 PAK kinases, has also been shown to play a central role in podosome formation in primary human macrophages.[36] siRNA-knockdown of PAK4 and truncation PAK4 mutants cause a reduction of number of podosomes per cell; kinase active mutants increase while inactive mutants decrease podosome sizes.

Evidence for a correlation between PKC and PAK-PIX is strong, however, a causative link between them in podosome formation, especially in VSMC, has not been defined. Nevertheless, recent data from non-VSMC studies have provided some clues. Using PKCγ knock-out mice and a phosphoproteome analysis, it has been shown that βPIX is a direct substrate of PKCγ. βPIX phosphorylation leads

to its translocation to the membrane where it may be involved in Cdc42/Rac activation during dopamine release in the striatum of mice.[73] Another possible link may be provided by direct interaction between PKCγ and fascin, the actin-bundling protein that contributes to cell protrusions.[67] It has been shown that PKCγ/fascin complex formation is regulated by Rac1 in a PAK1-dependent manner in colon carcinoma cells *in vitro*. These data underscore the differences in podosome regulatory mechanisms between VSMC and other cell types.

4.1.3. *Calponin*

h1 calponin inhibits PDBu-induced podosome formation in A7r5 cells by stabilizing actin stress fibers.[32] These studies suggest that local disassembly of actin stress fibers and loss of contractility at the podosome formation sites at the junction of stress fibers and focal adhesions characterize early stages of podosome formation. Stress fiber-stabilizing proteins such as *h1* calponin and *l*-caldesmon may act as podosome inhibitors VSMC.

4.1.4. *Myosin light chain kinase* (*MLCK*)

Using electron microscopic images[77] and FRET analyses,[79] it has been shown that siRNA-knockdown of myosin light chain kinase (MLCK) inhibits PDBu-induced podosome formation in A7r5 cells. It was further demonstrated that the interaction between the actin-binding domain of kinase dead MLCK and the actin core plays a critical role in podosome formation. However, the mechanism by which PKC regulate MLCK and actin interaction and podosome formation is not known.

4.1.5. *Monocyte chemotactic protein 1* (*MCP1*)

Although the roles of phorbol-esters and PKC in podosome formation have been solidly established, data on upstream signaling specific for these events in VSMC involving surface receptors and agonists are surprisingly lacking in the literature. Monocyte Chemotactic

Protein 1 (MCP1), a G protein-coupled receptor agonist, has been shown to stimulate human VSMC migration by upregulating the phosphorylation of cortactin on Ser405 and Ser418, and its interaction with WAVE2.[44] In addition, the MCP1-induced cortactin phosphorylation was dependent on phospholipase C β3 (PLCβ3)-mediated PKCδ activation, both are activated by DAG.

4.2. *The cSrc Signaling Hub*

The PKC-AFAP-cSrc axis is only one of many signaling pathways that converge on the non-receptor tyrosine kinase cSrc, which is a major hub linking membrane receptors such as growth factor receptors and integrins to signaling molecules that regulate cell migration, podosome and invadopodia formation in many cell types including VSMC.

When transformed with the constitutively active cSrc mutant, (SrcY527F), RASMC and fibroblasts undergo major reorganization of the actin cytoskeleton marked by a significant loss of stress-fibers giving way to dot-like podosomes and large ring-like superstructures called rosettes in over 95% of the cells without exogenous stimulation.[29,62] Extensive digestion of ECM containing fibronectin or collagen usually occur within 48 hours. Some of the digested areas co-localize with rosettes, and large imprints of digested areas in cell-free regions are often visible, indicating that these cells are able to exhibit random migration over the ECM substrate.

Many downstream effectors of cSrc contributes to invadosome formation and regulation of their adhesive and invasive functions. These include lipid kinases (PI3K), protein kinases (PAK, Akt) and phosphatases (PTEN), regulators of actin polymerization and branching (RhoGTPases, Arp2/3, NWasp, cofilin), structural podosomal proteins (Tks5, cortactin), and proteases in ECM digestion (MMP1, 2 and 9).

4.2.1. *PI3K-Akt*

One of the key downstream effectors of cSrc, is the PI3K/Akt pathway that plays a pivotal role in podosome signaling.[42] We have shown

that Akt phosphorylation is upregulated in RASMC that stably express Src(Y527F) in a retroviral vector. This is accompanied by podosome formation and subsequent ECM degradation.[68] The mechanisms by which Akt may upregulate podosome formation, however, is not clear. This is complicated by antagonistic roles of Akt isoenzymes in cell migration and invasion. We have recently shown that siRNA-knockdown of Akt1 reduces, while Akt3 knockdown enhances Src(Y527F)-induced podosome and rosette formation and ECM degradation in mouse embryonic fibroblasts. Knockdown of Akt2 has not effect.[24] Interestingly, both Akt1 and Akt3 suppress, while Akt2 enhances, phorbol ester-induced podosome formation. These data show that Akt1, Akt2 and Akt3 play different roles in podosome formation and ECM invasion induced by Src or phorbol ester, thus underscoring the importance of cell context in the roles of Akt isoforms in cell invasion.

Although numerous potential Akt substrates have been identified, few are known to be involved in cell invasion. Akt interaction with Pak1has been reported and may provide a link between Akt and PAK signaling in podosome formation. Phosphorylation of Pak1 by Akt enables Pak1 to bind to the adaptor protein, Nck, and modulates cell migration.[98] Additionally, Pak1 may act as a scaffold for Akt1 and PDK1 allowing for their recruitment to $PI(3,4,5)P_3$ at the plasma membrane resulting in Akt1 activation.[24,41] Whether similar mechanism exist in VSMC requires further study.

4.2.2. *Stat3*

One of the substrates of cSrc and Jak kinases is the Signal Transduction and Activator of Transcription family member Stat3.[30,74] Stat3 plays a role in cancer cell invasion and metastasis via both transcriptional activities, e.g. in the transactivation of MMPs, and non-transcriptional means through protein interactions. We have found that similar mechanisms may be present in VSMC podosome formation. Stat3 and its inactivated mutant, pY705Stat3, are localized to Src(Y527F)-induced podosome formation in primary RASMC.[63] Furthermore, shRNA-knockdown of Stat3 significantly reduces Src-induced podosome for-

mation, indicating that Stat3 is a mediator of Src-induced podosome formation in VSMC. This finding suggests that translocation of Stat3 to podosomes may facilitate its activation by Src. However, the downstream effectors of Stat3 specific for podosome formation remains to be determined.

4.2.3. *RhoGTPases*

The RhoGTPases are key regulators of actin cytoskeleton architecture in cell migration and invasion.[43,64,82] In primary RASM cells, expression of the active Cdc42V12 mutant was almost as effective as Src(Y527F) in inducing podosome formation and ECM invasion, while active Rac1L61 was half as effective. In contrast, expression of the dominant negative Cdc42N17 and Rac1N17 inhibits Src(Y527F) to induce podosome formation indicating that Cdc42 and Rac1 act downstream of cSrc.[29] These findings seem to support the general idea that Cdc42 is the major regulator of invadosome dynamics in a variety of cell types such as endothelial cells[60] and cancer cells, where Cdc42 acts downstream of EGFR and Src to target NWasp to Arp2/3 at sites of actin polymerization.[96] Interestingly, microinjection of the active Cdc42V12 mutant in human macrophages induces podosome disassembly[50] emphasizing cell- and expression level-dependent roles of RhoGTPases.

The role of RhoA and cytoskeletal contractility in podosome formation has been clearly demonstrated in VSMC[7] where activation of cSrc by PKC-AFAP1 induces upregulation of p190RhoGAP resulting in inactivating RhoA and actin stress fiber contractility, which is prerequisite to podosome formation. However, dependent on the cell types, RhoA is required for podosome formation e.g. fibroblasts, osteoclasts and endothelial cells[56] but excess activity appears to inhibit podosome formation in macrophages.

4.2.4. *Tks5*

The adaptor protein, Tks5, is a cSrc substrate that localizes to invadosomes in all cell types studied heretofore, thus providing a

reliable podosome marker. It contains one PX-domain and five protein-interacting SH3 domains that functions as an adaptor protein for recruitment of a number of proteins to the podosomes. Using siRNA knockdown and an approach based on mislocalizing Tks5 to mitochondria, it has been shown that in PDBu-treated A7r5 cells, Tks5 recruits AFAP1, p190RhoGAP and cortactin to sties of podosome formation. Thus, RhoGTPase would downregulate local contractile activity and stress fiber disassembly by inactivating RhoA, AFAP1 would activate cSrc and cortactin may contribute to remodeling actin stress fibers to allow the initiation of podosome formation.[17]

4.2.5. *Cortactin*

Cortactin is an F-actin-binding protein that was originally identified as the major Tyr-phosphorylated protein in Src-transformed cells.[94] Its roles in actin polymerization is emphasized by its localization to cortical actin networks such as invadosomes, lamellipodia and membrane ruffles.[87] Tyr-phosphorylated cortactin is found in all invadosomes and has been used as a podosome marker. Cortactin comprises multiple protein-interacting domains that allow it to bind to actin-branching points, Arp2/3, and NWasp.

We have shown that siRNA-knockdown of cortactin abolishes podosome formation in A7r5 cells and Src-transformed cells indicating that it is required for both PKC- and cSrc-induced podosome formation.[89,90,99] In VSMC, cortactin clusters in the microdomain between focal adhesions and stress fibers in the early phase of podosome formation and localizes in the actin core of mature podosomes.[7,46,89] Using various functional and truncation mutants of cortactin, we have shown that the initial clustering of cortactin requires the C-terminal SH3 domain but not the actin-binding repeat region which, however, is required for subsequent binding to the core actin column.[89] In addition, phosphorylation of cortactin by cSrc at Tyr sites (Y421 and Y466) does not affect its translocation to podosomes in.A7r5 cells. Since Tyr-phosphorylated cortactin and cSrc are present in mature podosomes, phosphorylation by cSrc likely

occurs after cortactin is translocated to podosomes, and plays a critical role in assembly and turnover of the actin core structure in mature podosomes.[99]

These data provide evidence for a model of how cortactin may be involved in early and late phases of podosome formation in VSMC. Many details are still missing, however. For example, the mechanisms for cortactin translocation, the effects of cortactin phosphorylation on podosome dynamics in VSMC and non-smooth muscle cells.

4.2.6. *Palladin*

Palladin is a widely expressed phosphoprotein known to act as a scaffold protein that is involved in polymerization and cross-linking of actin filaments.[34] SiRNA-knockdown of palladin reduce the number of A7r5 cells that produce podosomes by 50% in response to PDBu treatment. Interestingly, knockdown of palladin expression down-regulates Rac activity suggesting that Rac mediates palladin promotion of podosome formation. Using a yeast-two-hybrid screening, palladin has been shown to interact with the Tyr-kinase receptor substrate, Eps8, and both palladin and Eps8 were shown to colocalize in PDBu-stimulated podosomes in A7r5 cells. Since Eps8 contributes to Rac-associated actin remodeling by forming a complex with Abi-1 and Sos-1, it was suggested that palladin interaction with Eps8 may stabilize the Eps8/Abi-1/Sos-1 complex and promote formation of podosome and other cortical actin structures.

5. The p53 Anti-Podosome Signaling

The most widely studied tumor suppressor, p53, better known for its regulatory roles in cell cycle and apoptosis, has emerged in recent literature as a suppressor of cell invasion as well, especially in cancer cell metastasis.[3] Many mutants of p53 in human not only lose the normal capacity to suppress tumor growth and progression, but often acquire new functions that promote cell invasion.[33] Most of these 'gain-of-function' mutations occur within the DNA-binding domain of p53 and account for causing about 50% of all cancers in human. While many of the downstream effectors of p53 and its mutants are

direct p53 transcriptional targets that regulate cell invasion, cell migration and ECM degradation, others are key regulatory proteins in cell invasion and cell division affected indirectly by p53.

In VSMC, we have shown that p53 also functions as an invasion- and podosome-suppressor by a two-pronged mechanism involving the upregulation of PTEN and l-caldesmon on the one hand, and downregulation of cSrc and its effectors, Stat3 and PI3K-AKT on the other.[55,56] It has also been shown that miR-143 and miR-145, are positive p53 targets that may partly mediate p53-suppression of Src-induced podosome formation in VSMC.[70]

5.1. PTEN

The tumor suppressor, PTEN (Phosphatase and Tensin homolog deleted on chromosome Ten), antagonizes the cSrc-PI3K pathway by hydrolyzing $PI(3,4,5)P_3$ to $PI(3,4)P_2$, leading to inhibition of Akt and RhoGTPases.[71] We have shown that PTEN expression in VSMC can be upregulated by p53 and overexpression of PTEN inhibits cSrc and Stat3 activation.[63] This is in agreement with reports that PTEN inhibits the pro-invasion Src-PI3K-Akt pathway in non-SMC.[11,45] Although PTEN is a predominant lipid phosphatase, it also possesses protein phosphatase activities that have been shown to be involved in cell migration and invasion.[18,49] Interestingly, both lipid and protein phosphatase activities of PTEN contribute to the suppression of Src-induced podosome formation in primary RASMC.[68]

These data taken together suggest that PTEN plays a significant role in mediating p53-suppression of podosome formation in VSMC stimulated by the Src-Stat3 and Src-PI3K pathways. The mechanisms appear to require dual lipid and protein phosphatase activities of PTEN; however, *in vivo* protein substrates for PTEN remain to be identified.

5.2. l-Caldesmon

The 'light' isoform of caldesmon (l-caldesmon) is expressed in synthetic VSMC instead of its 'heavy' h-caldesmon counterpart found in differentiated, contractile VSMC.[86] l-caldesmon is a multi-domain

protein containing sites for binding to F-actin, tropomyosin and Ca^{2+}-calmodulin. It stabilizes actin stress fibers by inhibiting actin-severing proteins, enhances contractility by modulating actin-activated myosin ATPase activity,[12] and inhibits Arp2/3-mediated actin nucleation at branching sites by competing with Arp2/3 binding to F-actin.[61,97] Furthermore, PAK phosphorylation of l-caldesmon was shown to augment its inhibition of Arp2/3 and podosome formation.

l-caldesmon was first shown to localize to podosomes and inhibit their formation in Rous sarcoma virus-transformed fibroblasts[61] and later in Src-transformed primary RASMC and PDBu-treated A7r5 cells.[25,62] We have shown that l-caldesmon is translocated from stress fibers to the actin core of podosomes in VSMC that is dependent on its actin-binding domain and, unexpectedly, the Ca^{2+}-calmodulin-binding site at the C-terminal half of the molecule. These data suggest that binding to Ca^{2+}-calmodulin may trigger the release of l-caldesmon from actin stress fibers thus allowing remodeling of actin architecture at the onset of podosome formation. Subsequent binding of l-caldesmon to the actin core may provide stability of the podosome structure.

Using primary RASMC that stably expressing Src(Y527F), we have shown that l-caldesmon plays a role in mediating p53- suppression of podosome/rosette formation and ECM degradation. shRNA-knockdown of p53 expression or chemically inhibition of its activity by pifithrin-α (PFA) reduces caldesmon expression in RASMC and NIH3T3 cells,[62] suggesting that caldesmon may be a transcriptional target of p53. This is consistent with chromatin-immunoprecipitation (ChIP) analyses that caldesmon is a possible transcriptional target of p53.[92]

5.3. miR-143 and miR-145

The microRNAs, miR-143 and miR-145, are known contributors to regulate the switch from contractile to synthetic phenotypes of VSMC. It has been shown that prolong treatment of A7r5 cells with PDGF (>24 hours) downregulates miR-143 and miR-145, and induces podosome formation.[70] Based on a bioinformatics search,

the downstream targets of miR-143 are likely PKC-ε and PDGF receptor α, while fascin was identified as a miR-145 target. Since both PKC-ε and fascin localize to podosomes and are positive regulators of podosome formation, it appears likely that miR-143 and miR-145 suppress podosome formation by downregulating PKC-ε and fascin.

The link between miR-143 and miR145 to p53 was provided by the identification of two possible p53-binding sites at the promoter regions, suggesting that they are potential positive transcriptional targets of p53. In contrast, Src activity can inhibits expression of miR-143 and miR-145 and acts as a p53 antagonist. Taken together, these data suggest that PDGF/PDGF receptor activates cSrc, which in turn upregulates PKC-ε and fascin, by inhibiting p53 and thus downregulating miR143/145, resulting in podosome formation.

6. Regulators of Podosome Functions in Vascular Smooth Muscle Cells: ECM Adhesion and Degradation

ECM degradation and adhesion are hallmarks of podosome functions in the majority of invasive cells.[51] Although podosome formation, ECM degradation and adhesion are intimately linked, they appear to be regulated quite independently depending on the cell type.[83] For example, podosomes of sealing zones of osteoclasts are highly adhesive but have no protease activities; podosomes can be formed in endothelial cells without MT1-MMP recruitment;[51] while conventional PKC-α and PKC-δ are required for podosome formation in A7r5 cells stimulated by phorbol-esters, atypical PKCζ regulates MMP-9 recruitment to podosomes in primary bronchial epithelial cells.[95]

6.1. MMP Recruitment and ECM Degradation

Invadopodia in cancer cells are larger but fewer in number than podosomes, and protrude deeper into the substrate allowing more concentrated ECM degradation. Although podosomes only make

shallow penetrations into the substrate, they are able to degrade ECM extensively due to their large numbers per cell.

It has been well-documented that MMP-2, MMP-9 and MT1-MMP are secreted by podosomes in endothelial cells and Src-transformed fibroblast, and podosomes in A7r5 cells and primary VSMC are capable of causing extensive ECM degradation *in vitro*. Surprisingly little has been reported about MMPs in podosomes of VSMC. Using cultured human saphenous veins it was shown that VSMC migration positively correlated with MMPs-mediated loss of type IV collagen in vascular basement membrane.[1] MT1-MMP colocalizes with Tyr-phosphorylated cortactin in Src-induced podosomes in primary RASMC.[29] Gu *et al*[38] showed that MMP-2 localize to podosomes in PDBu-treated A7r5 cells, and we have shown that siRNA-knockdown of MMP1 reduces cSrc-induced ECM digestion and *in vitro* invasion of Matrigel. In addition, overexpression of p53 suppresses mRNA levels of MMP1 by 35%, suggesting that down-regulation of MMP1 may in part mediate p53-suppression of podosome formation in primary VSMC.[62]

How MMPs are transported to podosomes is not clear. Microtubules may provide an efficient transport system to deliver vesicle-loaded MMPs and perhaps other podosomal proteins to the sites of podosomes,[14,93] whether such mechanisms exist in VSMC remain to be investigated.

6.2. Adhesion and Mechanosensing of ECM

It appears that disassembly of focal adhesions and recruitment of adhesion proteins such as paxillin and integrin is part of the podosome initiation process, perhaps to allow adhesion of budding podosomes at the appropriate spots on the substrate.[46] Mature podosomes must be able to recognize and adhere to the substrate proteins via integrin ECM receptors, and to recruit and secret MMPs. ECM composition and rigidity in turn provides outside-in signals to integrin surface receptors to control podosome numbers and maturation.[42]

Since blood vessel walls are constantly subject to shear stress and static pressure, mechanical stimulation of VSMC migration and

invasion is highly relevant under physiological conditions. Using engineered polyacrylamide gels to mimic microenvironments of blood vessels, and custom-built pressure chambers, Kim *et al*[47] have recently shown that podosome formation can be induced in A7r5 cells by physiological relevant physical cues in a Src- and Cdc42-dependent manner. These include topographical cues presented by microenvironments of arteries, and imposed static pressure that mimics stage II hypertension. Furthermore, they have demonstrated podosome formation occurs in cells at the wound front in a 2-D scratch injury assay. These data demonstrate that physical stimulation by extracellular milieu that simulate arterial walls can induce podosome formation in VSMC *in vitro*. In addition, it suggests that injury to the blood vessels caused by surgical intervention may stimulate podosome formation in VSMC.

7. Conclusion

The involvement of VSMC migration and invasion in atherosclerosis has been recognized and supported by numerous reports. However, our knowledge of mechanisms that regulate actin cytoskeleton remodeling and the formation of podosomes in VSMC trails behind of what is known in endothelial, cancer and monocytic cells. Although podosome formation and regulation in VSMC and other invasive cell types have many similarities in regulatory mechanisms in podosome structure and function, discovery of features specific to VSMC is crucial to our understanding of their roles in initiation and progression of atherosclerosis. In the same vein, in order to understand specific roles of VSMC in the pathogenesis and progression of atherosclerotic plaques, conditional and SMC-specific knockout of individual proteins, rather than global knockout approaches, in animal models are required.

Most of our knowledge about VSMC invasion is based on *in vitro* cell culture studies. Data on VSMC invasion of ECM in a 3-D environment similar to that exists *in vivo* is lacking. With recent developments in super-resolution light microscopy, cryo-electron microscopy and intravital microscopy, we can anticipate exciting and interesting imaging data in the coming years.

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Chapter 7

Vascular Smooth Muscle Cell Proliferation and Invasion in Atherosclerosis

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Atherosclerosis, an inflammatory disease of arteries, is a leading cause of death worldwide.[7] Atherosclerosis is characterized by the formation of lipid-laden plaque in the vessel wall, causing narrowing of the vessel lumen and reduction in blood flow. In advanced stages of atherosclerosis, the rupture of atherosclerotic plaque can cause the catastrophic event of luminal thrombosis, complete vascular occlusion, cessation of blood flow, and organ damage. The three layers of arterial wall — intima, media, and adventitia — play different roles in the development of atherosclerosis.[97] Intima is the inner layer between endothelium and internal elastic lamina; media is the middle layer between internal elastic lamina and external elastic lamina; adventitia is the outer layer. Vascular smooth muscle cells are present in both intima and media. The importance of vascular smooth muscle cells in the development of atherosclerosis is indicated by the observation that vascular smooth muscle-rich regions of coronary arteries are more prone to the development of atherosclerosis, whereas vascular smooth muscle-sparse

regions are more resistant to the development of atherosclerosis.[25] Recent findings suggest that vascular smooth muscle cells play a central role in both the development of atherosclerosis and plaque rupture. During the early stage of atherosclerosis development, vascular smooth muscle cells play a detrimental role in promoting plaque formation. Invasion of medial vascular smooth muscle cells from media to intima and proliferation of vascular smooth muscle cells in the intima together contribute to plaque formation. During the advanced stage of atherosclerosis, vascular smooth muscle cells play a beneficial role in plaque stabilization. Specifically, vascular smooth muscle cells at the fibrous cap of fibroatheroma provide mechanical stability to the structure. Apoptosis of vascular smooth muscle cells at the fibrous cap of atheroma is a major cause of plaque rupture, luminal thrombosis and fatal acute coronary syndrome.[10]

Stary *et al.*[89–91] defined the multiple stages of atherosclerosis progression as adaptive intimal thickening (type I), fatty streak formation (type II), preatheroma formation/pathologic intimal thickening (type III), atheroma formation (type IV), fibroatheroma formation (type V), and complicated lesion with surface defect and thrombus formation (type VI). This review discusses the specific roles of vascular smooth muscle cells in each one of these stages of atherosclerosis.

1. Adaptive Intimal Thickening (Type I)

Vascular smooth muscle cell proliferation is a major mechanism of adaptive intimal thickening. Intimal thickening is a life-long process, beginning early in the peri/post-partum period and continuing throughout life.[36,61] Adaptive intimal thickening appears to be a physiological response to hemodynamic stress/strain and normally does not significantly narrow the vascular lumen.[89] Arteries exhibiting significant intimal thickening — for example, coronary arteries, renal arteries, and carotid arteries — are typically the first to develop atherosclerosis, suggesting that intimal thickening is an initial step of atherosclerosis.[65] The observation of differential spatial distribution of intimal thickening has led to the hypothesis that vascular cells in the atherosclerosis-prone and atherosclerosis-resistant regions of the arterial system are fundamentally different before the development

of atherosclerotic lesions. Van Assche *et al.*[100] tested this hypothesis by comparing transcriptomes of vascular smooth muscle cells in atherosclerosis-prone and atherosclerosis-resistant regions in ApoE$^{-/-}$ mice (animal model of atherosclerosis) before plaque development and in C57Bl/6 mice using whole-genome mouse microarrays. Consistent with the hypothesis, they observed differential expression of 70 and 244 genes between atherosclerosis-prone and atherosclerosis-resistant regions in C57Bl/6 and ApoE$^{-/-}$ mice, respectively. Furthermore, 201 genes related to atherosclerotic processes were expressed exclusively in ApoE$^{-/-}$ mice.

Intimal thickening is caused by the proliferation of vascular smooth muscle cells residing in the intima. Orekhov *et al.*,[72] using light and electron microscopy, showed that majority of the cells in normal intima of human aorta are differentiated smooth muscle cells, characterized by an elongated cell shape and presence of microfilaments and dense bodies. Aikawa *et al.*,[2] using immunolabeling against smooth muscle-specific myosin heavy chain and actin, also identified vascular smooth muscle cells as the major cell type in the intima of human coronary arteries. To determine the origin of intimal vascular smooth muscle cells, Murry *et al.*[63] investigated clonality of intimal vascular smooth muscle cells by examining X chromosome inactivation patterns in the intima of human arteries. They showed that the intima is populated by a monoclonal population of vascular smooth muscle cells. Schwartz and Murry[84] proposed several hypotheses to explain the origin of clonality of vascular smooth muscle cell population in the intima, among which the migration/trapping hypothesis appears to be most consistent with the observed vascular smooth muscle proliferation in early development. The migration/trapping hypothesis suggests that clonality of intimal smooth muscle cells in human coronary arteries arises from a sequence of events during development: a) trapping of rare cells during early embryogenesis, b) migration of rare cells across the internal elastic lamina at later times to become intimal smooth muscle cells, and c) rapid rate of cell proliferation during early development. Chung *et al.*[20] investigated whether clonal expansion occurs only with plaque formation or also with normal development by mapping X chromosome inactivation patterns in micro-dissected samples of normal and atherosclerotic

aortic smooth muscle. They observed monoclonal X-activation patterns in tissue samples of both media and intima from normal and atherosclerotic aorta. Their observation suggests that clonal expansion of vascular smooth muscle cells is part of normal growth and development in both media and intima. The observed relatively large patch size of monoclonal vascular smooth muscle cell population in normal arteries led Chung *et al.*[20] to propose that human arteries grow by expanding coherent vascular smooth muscle clones, with little mixing of adjacent clones. This observation has also led Chung *et al.*[20] to conclude that X-inactivation analysis cannot discriminate between monoclonal and polyclonal origin of plaque vascular smooth muscle cells.

An important mechanism by which intimal thickening leads to the development of atherosclerosis is the retention of lipoproteins in the extracellular matrix secreted by intimal vascular smooth muscle cells.[17,66,67] The response-to-retention hypothesis proposes that retention of lipoprotein by extracellular matrix in the arterial wall is necessary and sufficient for initiating atherogenesis.[28] Consistent with this hypothesis, biglycan, a proteoglycan, has been shown to colocalize with lipoproteins at the site of atherosclerosis.[48] Furthermore, biglycan overexpression in transgenic mice, under control of the smooth muscle α-actin promoter, has been shown to result in a greater degree of atherosclerosis.[99] Intimal thickening appears to be a unique characteristic of human and primate arteries, as it is not observed in arteries in mice and rats.[88] For this reason, research in adaptive intimal thickening has been challenging and underdeveloped.

2. Fatty Streak Formation (Type II)

Fatty streaks are macroscopically visible yellow-colored streaks, patches, or spots on the luminal surface of arteries.[91] Fatty streaks consist primarily of vascular smooth muscle and macrophage foam cells, with the ratio of vascular smooth muscle/macrophage foam cell population changing with the advancement of fatty streaks to fibrous plaques. Katsuda *et al.*,[41] using antibodies against vascular smooth

muscle and macrophage-specific proteins, showed that vascular smooth muscle cells represent the majority of lipid-loaded foam cells in late stage of fatty streak development in human aorta. Similarly, Allahverdian *et al.*,[4] by costaining for oil red O and smooth muscle α-actin, reported that vascular smooth muscle cells accounted for 50% of total foam cells in fatty streaks of human coronary arteries. The process of vascular smooth muscle foam cell formation consists of several steps: a) secretion of extracellular matrix by intimal vascular smooth muscle cells, b) retention of lipoproteins by extracellular matrix in the intima, c) modification of lipoproteins, and d) endocytosis of modified lipoproteins by vascular smooth muscle cells via cell surface scavenger receptors.[40]

Lipid content in a vascular smooth muscle cell is determined by the relative magnitude of cellular endocytosis via scavenger receptors and cellular export via membrane transporters.[3] Scavenger receptors are cell membrane receptors that bind and internalize modified low-density lipoproteins (LDLs) and pathogens.[109] The most important scavenger receptors for binding and uptake of oxidized LDL by vascular smooth muscle cells are the SR class A and SR-B2 (CD36) receptors.[107] Ishikawa *et al.*,[38] by immunostaining for SR-B1/2 and smooth muscle α-actin, demonstrated the presence of SR-B1/2-positive vascular smooth muscle foam cells in the intima around fatty streak in atherosclerotic human aorta. In comparison, SR-B1/2 receptors-positive vascular smooth muscle cells were not detected in normal intima. This observation suggests the presence of a vicious cycle, in which uptake of oxidized LDL and cholesterol by vascular smooth muscle cells induces up-regulation of scavenger receptors for further uptake of lipid, resulting in high level of cellular lipid accumulation and vascular smooth muscle foam cell formation.[51,60,79,105] Other classes of scavenger receptors — for example, SR-D1 (CD68), SR-E1 (LOX-1), SR-G, and SRJ receptors — are also expressed in vascular smooth muscle cells.[106] It is noteworthy that the scavenger receptor, SR-D1 (CD68), is often considered a marker of macrophages. By costaining cells in atherosclerotic human coronary arteries for CD68 and smooth muscle α-actin, Allahverdian *et al.*[4] recently showed that 18% and 40% of CD68-positive cells express

smooth muscle α-actin in early (type I and II) and advanced (type III and IV) atherosclerotic lesions, respectively. Their finding suggests that many CD68-positive intimal cells, previously considered macrophages, are actually vascular smooth muscle cells exhibiting the macrophage-like phenotype. There is emerging consensus that vascular smooth muscle cells may play a more important role in foam cell formation than previously recognized.[16]

Export of cellular lipids by vascular smooth muscle cells is mediated mostly by ATP-binding cassette transporter A1 (ABCA1). It is noteworthy that ABCA1 expression is down-regulated in intimal vascular smooth muscle cells in atherosclerotic lesions in human coronary arteries.[19] In vitro experiments indicate that oxidized LDL and cholesterol induce down-regulation of ABCA1 in vascular smooth muscle cells.[79,105] Thus, oxidized LDL-induced up-regulation of scavenger receptors and down-regulation of ABCA1 expression together enhance cellular lipid accumulation and vascular smooth muscle foam cell formation.

Lipid-loading of vascular smooth muscle cells can lead to stimulation of inflammatory gene expression and cell proliferation by the release of autocrines — for example, platelet-derived growth factor and fibroblast growth factor-β.[43] The effects of these growth factors on phenotypic modulation and proliferation of vascular smooth muscle cells have been reviewed recently.[18]

3. Preatheroma Formation/Pathologic Intimal Thickening (Type III)

Type III and more advanced atherosclerotic lesions are often described as "plaque". Preatheroma/pathologic intimal thickening (type III) is characterized by the presence of extracellular lipid pools among the layers of smooth muscle cells. Lipid pools typically contain microvesicular lipid without vascular smooth muscle cells. However, the presence of "caged basal lamina" — thick basement membrane that previously surrounded vascular smooth muscle cells — suggests death of vascular smooth muscles within lipid pools.[44,82] Pathologic intimal thickening can be detrimental. One study showed

that pathologic intimal thickening constitutes higher than 10% sudden coronary deaths.[95] Pathologic intimal thickening, followed by subsequent infiltration of macrophages, is an important step in the progression of atherosclerosis toward atheroma and fibroatheroma formation.[73]

4. Atheroma (Type IV) and Fibroatheroma Formation (Type V)

Atheroma and fibroatheroma are characterized by the presence of a core of extracellular lipid. The lipid core is also known as necrotic core, because it consists of lipids and cell debris. The necrotic core is surrounded by a fibrous cap consisting of vascular smooth muscle cells and extracellular matrix. Stary *et al.*[90] classified atheroma (type IV) lesion histologically as the first advanced atherosclerotic lesion having extensive accumulation of extracellular lipid in the form of a lipid core, and classified fibrotheroma (type V) lesion as having a fibrous cap layer in addition to the lipid core. Virmani *et al.*[103] have proposed an alternative classification of atherosclerotic lesions, which categorizes types IV and V lesion together as fibroatheroma having a lipid core and a fibrous cap. Virmani *et al.*[102,103] defined the fibrous cap as a distinct layer of connective tissue completely covering the lipid core. Comparison and illustration of these two classifications of human atherosclerosis have been presented by Plasschaert *et al.*[75] The fibrous cap consists of vascular smooth muscle cells in a collagen-proteoglycan matrix, with varying degrees of infiltration by macrophages and lymphocytes. Vascular smooth muscle cells are the major producers of collagen, elastin, and proteoglycans in the fibrous cap. The fibrous cap is critical for stabilizing the lesion.

Growth of atheroma/fibroatheroma is driven by invasion of vascular smooth muscles from the media to intima and proliferation of vascular smooth muscle cells in the intima.[10,18] Invasion of vascular smooth muscle cells from media to intima is a well-recognized mechanism of atheroma formation,[32,81] but data in support of this mechanism is relatively scanty. Thomas *et al.*[98] using radioactive labeling of arterial cells with tritiated thymidine prior to feeding swines with

hypercholesterolemic diets, following by radioautography of intima and media in atherosclerotic arteries, showed that cells in atherosclerotic lesions are polyclonal in origin. Their finding is significant in suggesting that non-intimal cells, presumably medial cells, contribute to the development of atherosclerotic lesions. Feil *et al.*,[26] using a genetic approach to investigate the contribution of medial vascular smooth muscle cells to atherosclerotic plaque formation, pulse-labeled vascular smooth muscle cells using the tamoxifen-dependent Cre recombinase, CreERT2, expressed from the endogenous SM22α locus combined with Cre-activatable reporter genes that were integrated into the ROSA26 locus. The fate of medial vascular smooth muscle cells during atherogenesis was then tracked by tamoxifen treatment of ApoE–/– mice before the development of atherosclerosis. They found that, during atherogenesis, medial vascular smooth muscle cells underwent clonal expansion, lost expression of the smooth muscle marker (smooth muscle α-actin), and converted to macrophage-like cells. Their data analysis indicated that vascular smooth muscle-derived macrophage-like cells constituted majority of the cellular component of advanced atherosclerotic lesions.

Transdifferentiation of vascular smooth muscle cells to macrophage-like cells has been confirmed by cell culture studies. Rong *et al.*[79] loaded mouse aortic smooth muscle cells with cholesterol using cholesterol: methyl-β-cyclodextrin complexes and demonstrated vascular smooth muscle foam-cell formation by staining lipid droplets. Using immunostaining against smooth muscle and macrophage marker proteins, they demonstrated down-regulation of protein expression of smooth muscle cell-related genes — for example, smooth muscle α-actin and myosin heavy chain — and up-regulation of protein expression of macrophage-related genes — for example, CD68 — in cholesterol-loaded vascular smooth muscle cells. Furthermore, these changes in protein expression were accompanied by macrophage-like phagocytotic activity. Recently, Vengrenyuk *et al.*[101] reported that down-regulation of the miR-143/145-myocardin axis induced by cholesterol loading is an important mechanism underlying the conversion of vascular smooth muscle cells to macrophage-like cells. Findings from these and other studies have led to the recognition that transdifferentiation of medial

vascular smooth muscle cells to macrophage-like cells contributes substantially to plaque formation.[49]

Several alternative hypotheses have been proposed to address the origin of intimal vascular smooth muscle cells during the development of atherosclerotic lesions. Schwartz and Murry *et al.*[84] asserted that the conventional paradigam — that invasion of vascular smooth muscle cells from media to intima is a major mechanism of plaque formation — has been developed mostly based on animal models of balloon injury to normal blood vessels. They proposed the alternative hypothesis that intimal vascular smooth muscle cells in atherosclerosis-prone arteries spontaneously form atherosclerotic lesions in response to hyperlipidemia. Other investigators have proposed the alternative hypothesis that circulating hematopoietic and multipotent vascular stem cells are the major contributors to plaque formation,[83,94] but findings from several laboratories appeared to contradict this hypothesis.[9,11,12] In a recent review, Tabas *et al.*[93] emphasized the current controversy regarding the origin of vascular smooth muscle cells in atherosclerotic lesions, and stated that the concept that medial vascular smooth muscle cells contribute to atheroma formation in atherosclerosis remained unproven in humans. Despite the controversy regarding the origin of vascular smooth muscle cells in atherosclerosis plaques, there is emerging consensus that interconversion of vascular smooth muscle cells among three phenotypes — contractile phenotype, synthetic phenotype, and macrophage-like phenotype — is an important mechanism underlying plaque formation and stabilization. In particular, differentiated vascular smooth muscle cells play a critical role in plaque stabilization by forming the fibrous cap of atheroma, but relatively little has been published on the origin of differentiated vascular smooth muscle cells in the fibrous cap of atheroma. One possibility is that a population of differentiated vascular smooth muscle cells is pushed from intima and/or media toward the luminal surface to form the fibrous cap. Another possibility is that synthetic or macrophage-like vascular smooth muscle cells near the luminal surface convert to the contractile phenotype during fibrous cap formation.

Degradation of basement membrane and extracellular matrix by matrix metalloproteinases is necessary for invasion of medial vascular

smooth muscle cells from media to intima during atheroma formation.[68] Matrix matalloproteinases are released by multiple cell types, including vascular smooth muscle cells. Galis *et al.*,[29] using in situ zymography, demonstrated matrix metalloproteinase activity in frozen sections of atherosclerotic but not normal human arterial tissues. Segers *et al.*[85] demonstrated the colocalization of matrix metalloproteinase activity with vascular smooth muscle cells in histological studies of aortic segments dissected from hypercholesterolemic rabbits. Furthermore, results from cell culture studies indicate that vascular smooth muscle cells of the synthetic phenotype are capable of releasing matrix metalloproteinases in response to inflammatory cytokines and growth factors.[23,68] The release of matrix metalloproteinases by invasive cells, for example, macrophages, is regulated by podosomes — cellular organelles consisting of columns of filamentous actin and actin-binding proteins, surrounded by a ring of adhesion and signaling proteins.[52,53,57,62] Vascular smooth muscle cells form podosomes in response to PKC activation.[35,50,55] Using immunoelectron microscopy of podosome markers, Quintavalle *et al.*[77] demonstrated the formation of podosomes in vascular smooth muscle cells in the aorta of microRNA-143 knockout mice, but not in the aortas of wildtype mice. Their finding suggests that down-regulation of microRNA-143 and -145 promotes the formation of podosomes in vascular smooth muscle cells. It is noteworthy that down-regulation of the miR-143/145-myocardin axis promotes the conversion of vascular smooth muscle cells to macrophage-like cells.[101] Findings from these two studies together suggest the intriguing concept that podosome formation may represents a step in the process of transdifferentiation of vascular smooth muscle cells to macrophage-like cells. Nicotine, an addictive substance in cigarette smoke, induces the formation of podosome rosettes in vascular smooth muscle cells.[33] Podosome rosettes are highly invasive cellular organelles found in highly invasive cells. This observation suggests that enhancement of vascular smooth muscle cell invasion via formation of podosomes and podosome rosettes is an important mechanism by which cigarette smoking and nicotine consumption increase the risk of developing atherosclerosis.

5. Complicated Lesion (Type VI)

Complicated lesion is characterized by plaque rupture and thrombus formation. Rupture of atherosclerotic plaque can potentially cause the catastrophic event of luminal thrombosis, complete vascular occlusion, cessation of blood flow, and organ damage. Plaques vulnerable to rupture are characterized by the presence of thin fibrous caps, large number of macrophages, small number of vascular smooth muscle cells, small amount of extracellular matrix proteins, and large lipid cores. A major cause of plaque rupture is the progressive thinning of fibrous cap as a result of vascular smooth muscle cell apoptosis and extracellular matrix degradation by macrophage-derived matrix metalloproteinases.[69,70,87] Apoptosis and necrosis of vascular smooth muscle cells are also important contributors to necrotic core formation. The vast majority (95%) of the fibrous caps of ruptured plaques is less than 64 μm in thickness. Based on this observation, Kolodgie *et al.*[47] introduced the term "thin-cap fibroatheroma" to describe fibroatheroma having a fibrous cap less than 65 μm in thickness. A major difference between the thick fibrous cap in early fibroatheroma and the thin fibrous cap in late fibroatheroma is the different extent of vascular smooth muscle cell death. Because fibrous cap thinning can potentially lead to plaque rupture and thrombosis, thin-cap fibroatheroma is also known as vulnerable plaque or high-risk atherosclerotic plaque.

Apoptosis of vascular smooth cells is recognized as a major cause of fibrous cap thinning and plaque rupture. Bennett *et al.*,[8] using time-lapse videomicroscopy, electron microscopy, and DNA fragmentation, studied apoptosis of vascular smooth muscle cells isolated from normal human coronary arteries and human coronary plaques. They found that apoptosis was a major cause of cell death in both normal and plaque-derived vascular smooth muscle cells. However, normal vascular smooth muscle cells died only upon removal of serum growth factors, whereas plaque-derived vascular smooth muscle cells died even in high serum conditions. Their finding suggests that vascular smooth muscle cells at atherosclerotic plaques are highly susceptible to apoptosis. To study the effect of vascular smooth muscle cell apoptosis on atherosclerotic plaque stability in vivo, Clarke *et al.*[21] generated

transgenic ApoE$^{-/-}$ mice expressing the human diphtheria toxin receptor for inducing apoptosis. They showed that high level of vascular smooth muscle cell apoptosis induced significant thinning of the fibrous cap in atherosclerotic plaques, whereas high level of vascular smooth muscle cell apoptosis in normal arteries did not induce vascular remodeling or aneurysm formation. Using the same mouse model of inducible vascular smooth muscle-specific apoptosis in ApoE$^{-/-}$ mice, Clarke *et al.*[22] showed that chronic low level of vascular smooth muscle apoptosis accelerated growth of fibroatheroma. Based on the recognition that vascular smooth muscle cell apoptosis is a major cause of plaque destabilization, pharmaceuticals are being developed to target apoptotic processes in the treatment of atherosclerosis.[58]

Pro-inflammatory cytokines, for example, IFN-γ, FasL, and TNF-α, promote vascular smooth muscle proliferation and/or apoptosis during atherosclerosis.[31] IFN-γ, a member of T-cell-derived pro-inflammatory cytokines, is present at high levels in atherosclerotic plaques and has been shown to promote apoptosis of vascular smooth muscle cells and atherosclerosis. Gupta *et al.*,[34] using IFN-γ$^{-/-}$/ApoE$^{-/-}$ double-knockout mice fed with Western-style diet, showed that IFN-γ deficiency substantially reduced atherosclerotic lesion size. Taking a different approach, Koga *et al.*[45] investigated the effect of blocking IFN-γ function by overexpressing a soluble function-blocking mutant of IFN-γ receptor in ApoE$^{-/-}$ mice fed with Western-style diet. They found that blockade of IFN-γ function increased vascular smooth muscle cell number, increased fibrotic area, and improved plaque stability. Koga *et al.*[46] then investigated the effect of blocking postnatal IFN-γ function by repeated gene transfers of the soluble mutant of IFN-γ receptor into the thigh muscle of ApoE$^{-/-}$ mice fed with high-fat diet. They found that blockade of postnatal IFN-γ function decreased plaque progression and stabilized advanced plaques by increasing the size of fibrotic area and number of vascular smooth muscle cells. Altogether, these findings suggest that IFN-γ promotes atherosclerosis and plaque vulnerability in part by modulating proliferation and apoptosis of vascular smooth muscle cells.

Fas-mediated vascular smooth muscle cell apoptosis has been hypothesized as a mechanism of atheroma cap thinning.[31] The death

receptor Fas and its ligand FasL have been identified in atherosclerotic human carotid and coronary arteries. Majority of the Fas-positive vascular smooth muscle cells are found in atherosclerotic plaques in the intima.[14,30] Double staining of atherosclerotic plaques indicated colocalization of Fas and DNA fragmentation in vascular smooth muscle cells in areas containing T cells and macrophages, suggesting that activated T cells induce vascular smooth muscle cell apoptosis by activating Fas.[30] In cell culture experiments, treatment with IFN-γ, TNF-α, and IL-1β together has been shown to induce Fas expression on the cell surface of vascular smooth muscle cells. Furthermore, incubation of cytokine-primed vascular smooth muscle cells with an activating anti-Fas antibody triggered apoptosis.[30] This finding supports the hypothesis that induction of Fas expression is an important mechanism by which inflammatory cytokines promote vascular smooth muscle cell apoptosis. In support of this hypothesis, Geng *et al.*[30] showed that activated monocytes and supernatant collected from activated monocytes induced apoptosis of vascular smooth muscle cells. They further identified FasL as the causative agent in the supernatant, and showed that anti-Fas IgG1 blocked the ability of supernatant to induce apoptotis. These findings together suggest the involvement of Fas/FasL pathway in monocyte-induced apoptosis of vascular smooth muscle cells. Fas can translocate dynamically between cell surface and intracellular compartments.[96] Rosner *et al.*[80] showed that IFN-γ primes vascular smooth muscle cells to FasL-induced apoptosis by stimulating the translocation of Fas from intracellular compartment to the cell surface. Taking a genetic approach to investigate FasL-mediated vascular smooth muscle cell apoptosis, Zadelaar *et al.*[108] induced FasL overexpression in the fibrous cap of plaque in ApoE$^{-/-}$ mice using adenovirus-mediated FasL gene transfection. They showed that FasL overexpression significantly increased vascular smooth muscle cell apoptosis in the fibrous cap, accompanied by enhanced plaque vulnerability. Altogether, these findings implicate the involvement of Fas and FasL in apoptosis of vascular smooth muscle cells and atheroma cap thinning.

TNF-α is a member of the TNF superfamily molecules. TNF-α is present at high concentration in the extracellular matrix within

atherosclerotic human arteries. Persistently elevated plasma concentrations of TNF-α is a characteristic of post-myocardial infarction patients at increased risk for recurrent coronary events.[78] To investigate the role of TNF-α in atherogenesis, Ohta *et al.*[71] generated TNF-$\alpha^{-/-}$/$ApoE^{-/-}$ double knockout mice and showed that TNF-α gene disruption diminished development of atherosclerosis in $ApoE^{-/-}$ mice. Consistently, cell culture studies indicated the ability of TNF-α to induce vascular smooth muscle cell apoptosis. Jia *et al.*[39] studied the effect of TNF-α on vascular smooth muscle cells isolated from human carotid plaques, and showed that TNF-α induced apoptosis of vascular smooth muscle cells by stimulating caspase-3 activity and activating FoxO1 transcription factor. Altogether, these and other human and animal studies indicate that TNF-related molecules, including TNF-α, regulate cell survival and promote atherosclerosis progression and plaque rupture.[6]

Autophagy, or "self-eating", is an important mechanism for regulating death and survival of vascular smooth muscle cells in atherosclerosis. Autophagy is a cellular process that degrades cellular components for recycling. Basal autophagy promotes plaque stabilization by enhancing survival of vascular smooth muscle cells in the fibrous cap of atheroma. However, excessive autophagy can cause plaque destabilization by inducing autophagic death of vascular smooth muscle cells.[86] Xu *et al.*,[104] using fluorescence microscopy and transmission electron microscopy for detecting autophagic vacuoles, showed that loading of vascular smooth muscle cells with excess free cholesterol induced autophagy. Furthermore, they showed that inhibition of autophagy by 3-methyladenine enhanced apoptosis of free-cholesterol-loaded vascular smooth muscle cells, whereas stimulation of autophagy by rapamycin attenuated cell death. Their finding suggests that autophagy promotes vascular smooth muscle cell survival. Swaminathan *et al.*[92] compared the gene expression of 59 selected proteins, including proteins involved in autophagy, between carotid plaques excised from symptomatic patients (unstable plaques) and asymptomatic patients (stable plaques). They observed differential gene expression of several proteins associated with autophagy pathways between the two patient groups. In particular, mRNA and protein expression of MAP1LC3B, a marker of autophagy, exhibited a five-fold decrease in

symptomatic samples, suggesting that autophagy enhances vascular smooth muscle cell survival and promotes plaque stability. Based on these findings, Swaminathan *et al.*[92] concluded that carotid atherosclerotic plaques exhibiting under-expression of MAP1LC3B would have low levels of autophagy, high levels of dead cell accumulation, and plaque destabilization.

Autophagy has also been shown to promote plaque vulnerability. ATG16L1 is an essential protein for early stages of autophagy. Magne *et al.*,[54] using histological analysis, demonstrated expression of ATG16L1 in areas surrounding the necrotic core and shoulder regions of plaques excised from human atherosclerotic carotid arteries. Using double immunofluorescence labeling and immunogold labeling, Magne *et al.*[54] demonstrated abundant expression of ATG16L1 in phagocytic cells, endothelial cells, vascular smooth muscle foam cells, and mast cells in advanced plaques. Furthermore, they correlated protein expression of ATG16L1 with plaque content of proinflammatory cytokines and matrix metalloproteinases. By analyzing ATG16L1 expression during atherogenesis induced by incomplete ligation and cuff placement in carotid arteries of $ApoE^{-/-}$ mice, Magne *et al.*[54] showed that colocalization of ATG16L1 and vascular smooth muscle cells occurred only in early atherosclerotic lesions. Altogether, the findings of Magne *et al.*[54] indicated that early stage of autophagy as measured by ATG16L1 protein expression is associated with events at earlier stages of atherosclerosis — foam cell formation, development of inflamed plaque phenotype, and plaque vulnerability.

Thrombotic occlusion of a coronary plaque can result in myocardial infarction, cardiac death, or stroke. Based on this recognition, detection of highly vulnerable plaque by invasive and noninvasive imaging modalities has been proposed as an approach to identifying high-risk patients and improving prognosis.[27] However, recent findings suggest that plaque rupture does not always result in catastrophic thrombotic occlusion. Instead, plaque rupture and its healing are often asymptomatic, but could lead to progressive lumen obstruction.[1,5,13] The healing process consists of thrombolysis by the fibrinolytic system, followed by proliferation of vascular smooth muscle cells, secretion of extracellular matrix by vascular smooth muscle cells at the rupture site, and resurfacing of the rupture site by

endothelial cells.[13,56] As a result, healing of plaque rupture increases the number of vascular smooth muscle cells and amount of extracellular matrix in the plaque, thereby contributing to plaque enlargement and further narrowing of the vessel lumen.

In addition to promoting atheroma formation and plaque stabilization, vascular smooth muscle cells also mediate vascular calcification, an unfavorable event in the progression of atherosclerosis that strongly predicts cardiovascular morbidity and mortality.[24,59] Vascular smooth muscle cells contribute to plaque calcification by two mechanisms — apoptosis and osteogenic transdifferentiation.[40,74,76] Vascular smooth muscle cells promote two types of vascular calcification — micro-calcification (0.5 to 15 μm in diameter) and sheet-like macro-calcification (>5 mm segment of continuous calcium), with different impacts on plaque vulnerability. In micro-calcification, also known as spotty calcification, apoptotic bodies and matrix vesicles released by vascular smooth muscle cells and macrophages serve as spotty nuclei of calcification. Micro-calcification induces viscous cycles of further inflammation and calcification, progressive thinning of the fibrous cap and plaque rupture. Clarke *et al.*,[22] using a mouse model of inducible vascular smooth muscle cell-specific apoptosis, showed that chronic apoptosis of vascular smooth muscle cells caused development of calcified plaques in young animals and promoted calcification within established plaques. Biomechanical modeling predicts that the presence of small micro-calcifications within the fibrous cap of the plaque can increase the local stress on the cap to the level sufficient for plaque rupture.[15,37] Kelly-Arnold *et al.*[42] tested this model prediction by examining the spatial distribution, clustering, and shape of almost 35,000 micro-calcifications in the fibrous caps of 22 non-ruptured human atherosclerotic plaques using high-resolution microcomputed tomography. Their analysis indicated that only a small subset of micro-calcifications had the potential for rupture, suggesting that micro-calcification does not necessarily increase the risk of plaque rupture.

Macro-calcification, also known as dense calcification, is characterized by large plates of organized calcium deposits resembling bone formation. Macro-calcification is considered beneficial for plaque stabilization. Vascular smooth muscle cells promote macro-calcification

by transdifferentiating to osteoblast-like cells. Naik *et al.*,[64] using genetic fate mapping to trace cells of smooth muscle origin via SM22α-Cre recombinase and Rosa26-LacZ Cre reporter alleles, showed that vascular smooth muscle cells accounted for 80% of osteochondrogenic cells and all of chondrocytic cells in atherosclerotic arteries of $ApoE^{-/-}$ mice. By tracing cells derived from vascular smooth muscle cells during vascular calcification in matrix Gla protein deficient mice using the SM22-Cre recombinase and Rosa26-LacZ alleles, they found that vascular smooth muscle cells transdifferentiate to osteochondrogenic precursor- and chondrocyte-like cells by down-regulating the expression of smooth muscle lineage markers (α-smooth muscle actin, SM22α) and up-regulating the expression of the osteochondrogenic transcription factor Runx2/Cbfa.

6. Conclusion

There is emerging consensus that vascular smooth muscle cells are a central player in all stages of atherosclerosis. Paradoxically, vascular smooth muscle cells play two opposing roles in atherosclerosis — a detrimental role in promoting plaque development during early stage of atherosclerosis but a beneficial role in promoting plaque stabilization during later stage of atherosclerosis. Proliferation, invasion, and transdifferentiation of vascular smooth muscle cells promote the development of atherosclerotic lesions. Apoptosis of vascular smooth muscle cells promotes thinning of fibrous cap of atheroma and plaque rupture. Given the two opposing roles of vascular smooth muscle cells in promoting the development of atherosclerosis and plaque rupture, it is challenging but necessary to find new approaches to controlling these two processes in the treatment and prevention of atherosclerosis.

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Chapter 8

The Role of Non-coding RNA in the Control of Vascular Contractility and Disease

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Over the last decade, the cellular impact of non-coding RNAs, defined as RNA molecules not translated into protein, have emerged as an area of great interest. Once thought to only regulate generic functions of cells, namely transcription, translation and splicing, several diverse functions of non-coding RNAs, such as described below for microRNAs and long non-coding RNAs, are now well established.[1]

1. MicroRNAs

By far the most studied of these molecules are microRNAs (miRNAs), a family of short (21–25 nucleotide) RNAs, which normally post-transcriptionally downregulate gene expression through interacting with the 3′ untranslated (3′ UTR) region of mRNA targets. The first miRNA, lin-4, was discovered more than 20 years ago to be important in C. Elegans larval development.[2] The current release of the miRNA database (miRBase) has documented 2661 human miRNAs, which could collectively regulate the expression of thousands of protein-encoding genes with many cellular and developmental processes. However, it should be noted that the true functional importance of these miRNAs has yet to be fully determined.[3,4] Each miRNA has only

a moderate effect on gene expression of their targets, and hence provides a 'fine-tuning' role on gene expression.[5] It is common, however, for one miRNA to influence multiple targets in the same biological pathway, and consequently miRNAs can have profound influences on gene expression.[6] Moreover, miRNAs can become aberrantly expressed in pathophysiological conditions, such as cardiovascular disease and cancer, leading to an important role in disease development.

1.1. *MicroRNA Biogenesis*

In humans, miRNAs are primarily encoded by introns of non-coding or coding transcripts, however, some are encoded by exonic regions.[4] It is also common for several miRNA loci to be clustered together in a polycistronic unit, and therefore co-transcribed.[7] Some miRNA loci are located in the introns of protein coding genes, and could therefore be under the influence of the protein-encoding promoter and, thus, transcription factors.[4,7] In the nucleus, RNA polymerase II transcribes miRNAs into long primary transcripts (Pri-miRNA)[7] (Fig. 1A). Following transcription, pri-miRNAs are modified by the RNAse III enzyme Drosha[8] into a 70 nucleotide hairpin structure termed preliminary miRNAs (pre-miRNA) and exported from the nucleus by a nuclear pore complex, formed by the protein exportin 5.[9–11] In the cytoplasm pre-miRNAs undergo further processing into a 21–25 nucleotide duplex, by the distinct RNAse III endonuclease enzyme Dicer (Fig. 1B).[12] Dicer binds to pre-miRNAs at the PAZ (PIWI-AGO-ZWILLE) domain and cleavage is initiated by the c-terminal RNAse III domain.[13] The proximity of these domains has been proposed to act as a 'molecular ruler' to determine the 21–25 nucleotide size of the miRNA duplex.[14]

1.2. *RNA-induced Silencing Complex*

The mature miRNA strand is incorporated onto Argonaute (AGO) protein to form the RNA-induced silencing complex (RISC) (Fig. 1C).[12,15] The miRNA sequence acts as a guide to target mRNA, whereas the AGO proteins recruit cofactors that ultimately lead to the translational repression and/or decay of target mRNA. In humans,

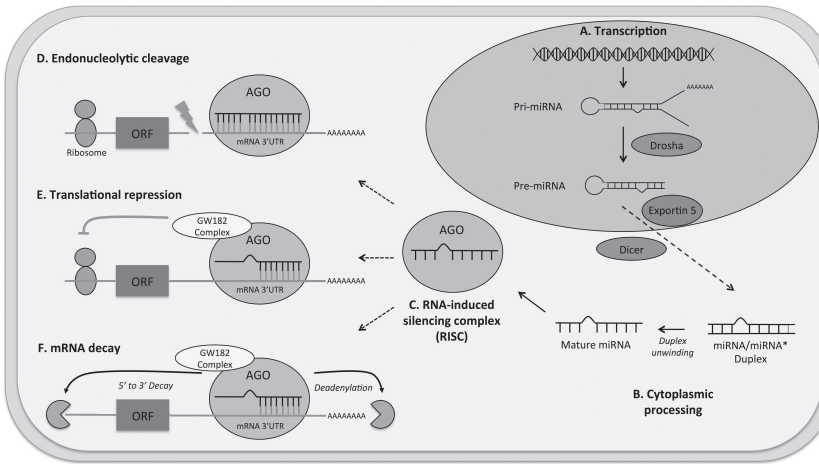

Fig. 1. Summary of the main mechanisms of miRNA biogenesis and function. (A) MiRNAs are primarily encoded by introns of non-coding or coding transcripts, into long primary transcripts termed pri-miRNAs. Following transcription, pri-miRNAs are modified by Drosha into a 70 nucleotide hairpin structure termed preliminary miRNAs (pre-miRNA). (B) Following export into the cytoplasm through the Exportin 5 containing pore complex, pre-miRNAs undergo further processing, by Dicer, into a 21–25 nucleotide duplex. (C) The mature 'guide' miRNA strand is incorporated onto AGO protein to form the RNA-induced silencing complex (RISC), whilst the 'passenger' strand is unwound and degraded in the cytoplasm. The miRNA sequence acts as a guide to target mRNA, whereas the AGO proteins recruit cofactors, such as GW182, that ultimately lead to the translational repression and/or decay of target mRNA. (D) When miRNAs form near perfect complimentary base pairs with their targets, mRNAs are endonucleolytically cleaved by RISC (often observed in plants). Mammalian miRNAs associate with target mRNA through binding of a 6–8 nucleotide 'seed' sequence at the 5′ end of the miRNA. This has been proposed to; (E) result in translational repression through the recruitment of translational repressors or dissociation of the poly (A)-binding protein, and/or (F) recruit deadenylases onto target mRNAs, leading to mRNA decay.[122]

there are four AGO proteins (AGO 1-4) that associate with distinct sets of miRNAs.[4,16–18] The guide strand is preferentially loaded into the RISC by virtue of a relatively unstable 5′ end terminus and, additionally, a U at nucleotide position 1.[19–23] The passenger strand is often degraded immediately upon release. The selection is not entirely rigid, however, and the passenger strand in some cases has cellular function, albeit far less pronounced than the predominant guide strand.[4]

In plants, miRNAs form near perfect complimentary base pairs with their targets, and this results in miRNA endonucleolytic cleavage by RISC (Fig. 1D).[24] This degree of complementarity is rarely observed in mammals. Alternatively, mammal miRNAs associate with target mRNA through binding of a 6-8 nucleotide sequence at the 5′ end of the miRNA.[6,25] This 'seed' sequence functions as a guide for RISC, which subsequently recruits the effector protein GW182, which influences mRNA translation by recruiting (i) deadenylases onto target mRNAs, leading to mRNA decay (Fig. 1F), and/or (ii) translational repressors (Fig. 1E).[26] The association of AGO1 with GW182 is essential for this process since gene silencing is abolished in the absence of GW182.[27]

1.3. *MicroRNA Target Recognition*

The influence of miRNAs on target mRNA translation is in large part determined by the degree of complementarity of the seed sequence to the 3′ UTR of target mRNA. Indeed, even a change of one nucleotide in this region of a miRNA results in a vastly different set of mRNA targets.[28] Furthermore, this region is the most evolutionarily conserved site across species, suggesting great importance to miRNA function.[25] Although less important, complementarity at the 3′ end of miRNAs may enhance target recognition or compensate for mis-matches in the seed sequence.[25] The position of interaction on the target mRNA is also important in determining the potency of miRNA effects. For example, the miRNA is most likely to influence mRNA translation if the seed sequence interacts with the 3′ UTR of mRNA, at least 15 nucleotides away from a stop codon, away from the center of long UTRs and close to target sites of co-expressed miRNAs.[25] Binding to the 5′ UTR is ineffective due to the RISC complex becoming displaced by the transcriptional machinery.[29]

1.4. *Control of miRNA Expression*

The importance of miRNA biogenesis has been elucidated in Knockout (KO) studies, in which KOs of Drosha, Dicer, and AGO are embryonically lethal.[17,30,31] It is also important to note that each stage

of miRNA biogenesis can be regulated, which may have profound effects on miRNA abundance. For example, the activity of transcription factors or changes in the methylation status of CpG regions associated with miRNA genomic loci may influence miRNA transcription.[32–34] In addition, the expression levels of the downstream effector molecules Drosha, Dicer, Exportin and AGO can all affect miRNA biogenesis and eventual loading into RISC.[4] Furthermore, cell signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, can phosphorylate and stabilize Drosha, Dicer and AGO, therefore regulating miRNA abundance.[4,35–37] It is possible that defects in these pathways lead to aberrant miRNA expression that have been implicated in human disease.

2. Long Non-coding RNAs

In addition to miRNAs, LncRNAs (long non-coding RNAs of more than 200 nucleotides), which were once thought to be merely 'transcriptional noise' or 'genetic junk', have emerged as critical regulators of gene expression (reviewed in[38–44]). LncRNAs have a much more widespread mode of action than miRNAs through DNA, RNA and protein interactions, making the establishment of LncRNA functions and mechanisms a challenging task.[40] Perhaps the most well characterized function of LncRNAs is in the recruitment of histone-modifying complexes, through which they play an important role in X chromosome inactivation.[45,46] However, they have also been implicated in other epigenetic processes, such as DNA methylation[47] and chromosome looping.[48,49] LncRNAs are sometimes transcribed as natural antisense transcripts (NATs) to protein-coding transcripts (i.e. on the opposite strand of DNA), and can influence how their sense counterparts are processed by interacting with transcription factors or altering splicing patterns.[50–53] Interestingly, LncRNAs also modulate protein activity, location and structure, thereby influencing the post-transcriptional processes that they regulate.[41,51,54,55]

Adding to the complexity of LncRNAs, their function is intertwined with that of miRNAs. For instance, they can post-transcriptionally silence the expression of genes by way of binding complimentary

sequences in target mRNA 3′ UTR.[56] Furthermore, they can bind to and sequester the activity of miRNAs, thus attenuating miRNA regulation of gene expression.[57,58] Recently, it has been suggested that certain LncRNAs are also precursors to small non-coding RNAs like miRNAs. Although the exact function of LncRNAs has yet to be fully elucidated, they are emerging as important regulators of cardiovascular development and disease.[41]

3. MicroRNAs in Vascular Smooth Muscle

The expression of microRNAs in vascular smooth muscle is now known to play a vital role in (i) the differentiation of VSMCs into the mature, contractile phenotype in development, and (ii) the maintenance of VSMC contractile protein expression. Mismanagement of miRNA expression in VSMCs plays a role in the development of cardiovascular diseases, such as hypertension, atherosclerosis and aortic aneurysms. As noted above, Dicer KO mice are embryonically lethal, but a great deal of knowledge has been revealed from the phenotype of the smooth muscle-specific Dicer KO mouse. Interestingly, smooth muscle miRNAs are vital to the entire body since SM22α-targeted deletion of Dicer also induced embryonic lethality due to widespread hemorrhaging.[59] The authors further demonstrated that the SM22α-targeted deletion of Dicer was restricted to the aorta, umbilical artery and cardiomyocytes, with no effect on smooth muscle from the esophagus, trachea or bladder. Taken together, this study suggests that miRNA expression in cardiovascular smooth muscle is essential for development. Furthermore, KO of dicer was associated with a dramatic reduction in contractility of ex vivo umbilical arteries from embryonic mice, partially explained by a reduction in the expression of myocardin, smooth muscle myosin heavy chain (SM-MHC), calponin and SM22α.[60] Further studies using a tamoxifen-inducible SMC-specific knockout of Dicer, which allows knockout of Dicer after development, suggested miRNAs are required for blood pressure regulation and VSMC contractile function.[60] It was suggested that the VSMC Dicer KO results in a decrease in contractile protein expression, leading to impairment of both receptor- and calcium-mediated

contractions of small mesenteric arteries, which consequently resulted in reduced blood pressure.[60] Furthermore, the stretch-dependent contractile differentiation of mouse portal veins is dependent on Dicer expression.[61] Myogenic tone, an important response in resistance arteries to increased blood pressure, was abolished from Dicer KO mice, which was associated with a loss of calcium influx through the L-type calcium channel.[62] Since myogenic tone is crucial in the maintenance of peripheral arterial resistance, aberrant miRNA expression may be involved in the development of hypertension. These studies, when taken together, suggest that miRNAs play a role in VSMC differentiation into the contractile phenotype.

3.1. *MicroRNA-dependent Contractile Differentiation of Vascular Smooth Muscle Cells*

The miR-143/145 cluster has emerged as a master regulator of vascular smooth muscle differentiation. Indeed, knockout of this cluster induces a phenotype that resembles the one observed for Dicer KO, whilst overexpressing miR-145 rescues the effect of Dicer KO on vascular smooth muscle cell differentiation.[59,60,63–68] The miR-143/145 family can influence ion channel (see below), actin cytoskeletal and contractile gene expression,[65] to support the promotion of the vascular smooth muscle contractile phenotype. In smooth muscle cells (SMCs), the serum response factor (SRF) and myocardin transcriptional complex drive contractile gene expression via CArG boxes in promoter regions of SMC differentiation markers, such as SM-MHC, calponin and SM22α. This mechanism is counteracted by KLF4-mediated inhibition of SRF binding to the CArG-containing genes. The primary pro-contractile property of the miR-143/145 cluster is through the targeted downregulation of KLF4 and 5, which results in the upregulation of contractile proteins (Fig. 2).[69–72] The contractility to AngII and phenylephrine was almost abolished in the femoral artery and aorta from miR-143/145 KO mice, confirming an important pro-contractile function for this cluster.[59,63,67–69,72,73] Interestingly, the same impairment was observed in airway, but not bladder smooth muscle, suggesting tissue-specific effects of miR-143/145 in smooth

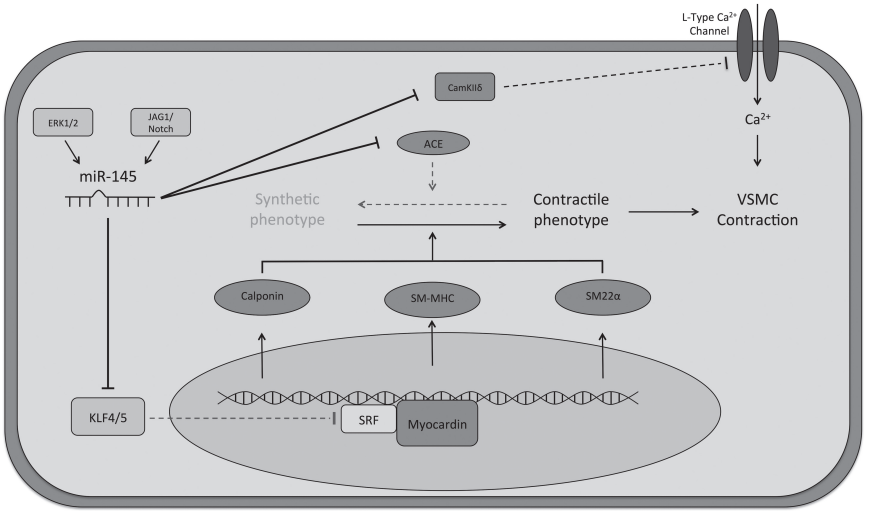

Fig. 2. The pro-contractile functions of miR-145 in vascular smooth muscle cells. miR-145 primarily promotes the contractile phenotype through the targeted down-regulation of KLF4/5, which relieves its suppression on SRF/myocardin-induced expression of contractile genes, including calponin, SM-MHC and SM22α. It also negatively regulates the expression of ACE, which upregulates AngII signaling and reversion to the synthetic, proliferative smooth muscle phenotype. Finally, miR-145 targets CamKIIδ, relieving its suppression of L-Type calcium channel expression, therefore promoting VSMC contraction. The expression of miR-145 can itself be regulated by signaling pathways, such as ERK1/2 and JAG1/Notch.

muscle.[67] The impaired contractile response in these mice is partially due to an accumulation of synthetic SMCs, but also specific defects in receptor-mediated signaling pathways (see below).[63] An alternative mechanism involving aberrant angiotensin signaling is also involved in the regulation of VSMC function by miR-143/145.[63] Angiotensin converting enzyme (ACE) is a verified target of miR-145, and its cleavage of angiotensin I to, angiotensin II (AngII), plays an important role in VSMC contraction and phenotype regulation.[63] Loss of miR-145 leads to enhanced angiotensin stimulation through the upregulation of ACE. The subsequent chronic promotion of the synthetic phenotype leads to impaired contractility and stimulation of atherosclerotic pathways. In addition, this aberrant chronic signaling

may desensitize VSMCs to angiotensin, explaining the reduced AngII-induced contractility of vascular strips from miR-143/145 KO mice.[63]

The expression of miR-24 is similarly induced by bone morphogenic protein (BMP) signaling, which leads to contractile differentiation by targeting Tribbles-like protein-3.[74] The expression of certain miRNAs can also act in negative feedback loops, perhaps as mechanisms to fine-tune the expression of contractile proteins. For example, myocardin ordinarily promotes contractile differentiation, but it also enhances miR-1 expression, which blocks the expression of contractile proteins α-SMA and SM22.[75] Additionally, miR-21 promotes the contractile phenotype by targeting Programmed Cell Death 4 in response to BMP signaling.[76] However, it also negatively regulates the expression of myosin phosphatase, Rho-interacting protein and cofilin-2, which are involved in smooth muscle relaxation.[77]

3.2. *Smooth Muscle Pathways of Contraction: A Role for MicroRNAs?*

Further to having a role in phenotypic regulation of VSMCs, miRNAs may directly regulate both classical and novel pathways of VSMC contraction, such as those affecting actomyosin activity or focal adhesion remodeling, respectively. For example, miR-1, -133 and -155 downregulate myosin light chain kinase (MLCK) expression, thereby influencing myosin activation or smooth muscle contraction.[78–82] In addition to targeting MLCK, miR-1 also downregulates calmodulin expression, and could therefore play an anti-contractile function in smooth muscle. The microRNAs of the miR-200 family target numerous members of cytoskeletal effectors that control actin polymerization and actomyosin contractility.[83]

The focal adhesion, which had previously been assumed to be largely static in differentiated smooth muscle, remodels in response to vasoconstrictor stimulation, facilitating the transmission of force developed by actomoyosin bridges. This has been suggested to occur through activation of the non-receptor tyrosine kinase Src, which promotes focal adhesion kinase-dependent tyrosine phosphorylation

of the focal adhesion proteins Crk Associated Substrate (CAS) and Paxillin.[84–86] This regulatory pathway is impaired in aged mouse aorta, which was associated with defects in aortic stiffness and contractility maintenance.[84,87] Interestingly, the expression levels of miRNAs that target Src tyrosine kinase were elevated with aging in mouse aorta. Therefore, abnormal miRNA expression could play a role in the reprogramming of focal adhesion in VSMCs, leading to impairment of vascular contractile and stiffness regulation in aged aorta.[87]

3.3. *MicroRNA Expression Gone Awry: Implications for Vascular Disease*

3.3.1. *Hypertension*

The aberrant expression of miRNAs involved in controlling the phenotypic switch of VSMCs from the synthetic to the contractile state could contribute to the development of vascular disease. A diminished or enhanced capacity of an artery to respond to agonist-mediated contraction will display aberrant regulation of blood pressure. Clinical studies have highlighted miRNA expression changes that favor the contractile phenotype, and hence could increase the risk of hypertension. For example, the expression of miR-145, -143, and -133 were lower in peripheral blood mononuclear cells from hypertensive patients, and miR-1 and -21 were higher.[88] The finding that miR-145 was lower in hypertensive patients is contradictory to the known procontractile function of miR-145. It also remains to be determined if the same pattern is seen in VSMCs, which are less accessible than blood cells. Moreover, the expression of miR-145 is induced by Jag1/Notch signaling,[69] and repressed by ERK1/2 signaling (Fig. 2).[89] Therefore, the expression levels of miR-145 are altered in different physiological situations, such as stress and mechanical stretch, in-turn altering the phenotype of VSMCs and risk of hypertension.

A clinical study of a Chinese population demonstrated a single nucleotide polymorphism in the prostaglandin $F_2\alpha$ receptor gene to be more likely in hypertensive individuals.[90] This was associated with a defect in miR-590-3p binding, thereby upregulating prostaglandin

$F_2\alpha$ receptor expression and augmenting prostaglandin-mediated contractility.[90]

3.3.2. *Hyperlipidemia and diabetes*

In mouse models for diabetes and hyperlipidemia, in which contractility of the renal artery is increased, the expression of miR-10a, -139b, -206 and -222 are drastically reduced.[91] This was associated with increases in the expression of Rho kinase and Cx43. The increase in contractility was attenuated by overexpression of miR-10a, -139b, -206 and -222 in VSMCs.[91] In a separate study, miR-145 was upregulated in VSMCs from type 2 diabetic rats[92] and humans,[93] which consequently heightened the expression of contractile proteins. In addition, glucose-induced contractile differentiation of mouse SMCs was recently found to be dependent on miR-143/145, further supporting this view.[94] In the diabetic rat model, the increase in miR-145 expression was associated with increased aortic contractility and calponin levels.[92] Furthermore, high glucose treatment to human coronary SMCs, which increases AngII signaling, suppressed miR-145, promoting a switch to the synthetic phenotype.[95] Taken together, these studies suggest that miRNAs could play a role in the increased cardiovascular disease risk associated with type 2 diabetes.

3.3.3. *Atherosclerosis*

In contrast to the above, an uncharacteristic miRNA expression that promotes a switch to the synthetic VSMC phenotype could enhance the development of atherosclerosis. Vascular injury, such as endothelial dysfunction, mechanical stress or inflammation, induces VSMCs to switch from the contractile to the synthetic phenotype. This involves a suppression of genes that promote contraction, and an enrichment of those that regulate proliferation, inflammation, and migration, which precedes the development of atherosclerosis.[96,97] The downregulation of miR-145 would lead to an increase in KLF4/5 expression, subsequently decreasing the expression of contractile markers, such as calponin and MHC. Indeed, the expression

of miR-145 is suppressed in both animal models of vascular injury and human atherosclerosis.[70,98]

3.3.4. *Pulmonary vascular disease*

There is accumulating evidence that miRNAs play an important role in the development and progression of pulmonary hypertension, which is marked by increased pulmonary artery smooth muscle cell (PASMC) proliferation and vasoconstriction. Several studies have suggested a role for the miR-130/301 family in the promotion of pulmonary artery remodeling, through the control of peroxisome proliferator activated receptor (PPAR-ϒ).[99–101] Furthermore, miR-21,[102] -124,[103] -145,[104] and -204,[105,106] have all been suggested to modulate proliferation pathways in PASMCs. In addition to its pro-proliferative role, the miR-130/301 family also indirectly increases the expression of endothelin-1, which enhances the contractility of PASMCs.[107] Abnormal vasoconstriction is also caused by the aberrant expression of ion channels in the VSMC membrane, which are influenced by miRNAs. For example, the expression of α_{1C} subunit of the L-type calcium channel is downregulated by miR-328.[108] Conversely, miR-145 promotes the targeted down-regulation of CamKIIδ, resulting in increased expression of the α_{1C} subunit of the L-type calcium channel, which causes increased VSMC contractility (Fig. 2).[64] In a rat model of pulmonary arterial hypertension, miR-190 expression was increased in PASMCs. This miRNA increases the contractility of pulmonary arteries, through the targeted downregulation of the potassium channel Kv7.5, which, when activated, promotes VSMC relaxation.[109] Indeed, overexpression of miR-190 in ex vivo PA rings increased both KCl- and PE-induced contractility.[109] Similarly, miR-9a-3p downregulates the expression of the SUR2B domain of the K_{ATP} channel, which causes dysregulation of vascular contractility.[110]

3.4. *Extracellular Communication in Vascular Cells*

Importantly, miRNAs expressed in one cell type are able to transport to other cell types. This cell-to-cell communication has been proposed

to be crucial in vascular biology, especially with regard to the expression of miR-143/145. For example, endothelial cells subjected to physiological shear stress or statin treatment were induced to transport miR-143/145 in extracellular vesicles to SMCs, promoting an atheroprotective phenotype.[111] On the other hand, miR-143/145 transfer from aortic SMCs to endothelial cells (ECs) inhibits EC proliferation, through targeted downregulation of hexokinase II and integrin beta 8. This transfer occurs through tunneling nanotubules, which are small plasma membrane protrusions, and is promoted by TGFβ signaling. Therefore, this transfer arises under vascular stress, and hence may be critical for the response.[112] Similarly, TGFβ can promote the transfer of miR-143-3p from PASMCs to pulmonary artery endothelial cells (PAECs) via exosomes, which are small (50–90 nm) membrane vesicles. Interestingly, and in contrast to the former study in aortic SMCs, not only was this specific to miR-143 (i.e. miR-145 was not enriched in these exosomes), but it promoted the migration and angiogenesis of PAECs. Moreover, miR-143 enriched exosomes were discovered in both calf models of pulmonary hypertension (PAH) and PAH patients. The use of *in vivo* anti-miR-143 therapy reversed experimental PAH in mice. This highlights both the potential of detecting miRNA-enriched exosomes as biomarkers of disease and the ability to subsequently treat such disorders.[113]

4. Long Non-coding RNAs in Vascular Smooth Muscle

LncRNAs are both expressed in VSMCs and play a role in phenotype regulation. Deep sequencing studies identified smooth muscle and endothelial cell-enriched migration/differentiation-associated long non-coding RNA (SENCR), which acts in the phenotype regulatory pathway of SMCs. Depletion of SENCR acts in similar manner to miR-145 KO, decreasing the expression of myocardin, which drives the switch from the contractile to the synthetic phenotype.[114] Similarly, knock down of the p53-induced LincRNA-p21, led to increased VSMC proliferation through interfering with p53 transcriptional activity. Significantly, this LncRNA was reduced in carotid

arteries and peripheral blood mononuclear cells from atherosclerotic patients.[115] In contrast, Lnc-Ang362 is induced by AngII, which may promote the risk of hypertension by encouraging the proliferation of VSMCs.[116] It is not currently known how LncRNAs influence the expression or activity of VSMC contractile proteins, but this is a rapidly developing field and we expect many further advances in the coming years.

5. Modulating MicroRNAs in the Treatment of Vascular Disease

Two main techniques have been utilized to study the functions of miRNAs *in vitro* and *in vivo*; (i) increasing the expression of a downregulated miRNA by use of a miR mimic, which is chemically designed to mimic the endogenous sequence, and (ii) blocking the effects of an abnormally upregulated miRNA by using an anti-miRNA, which is designed to be complimentary to the endogenous miRNA and hence block its function. The cellular entry of a miR mimic is enhanced by the addition of a passenger strand, which is chemically modified to aid membrane uptake (e.g. by the addition of a phosphate group). The passenger strand is also designed to contain many mismatches so that it quickly dissociates from the guide strand after entry into the cell. The *in vivo* stability of anti-miRNAs has been improved by several modifications, such as locked nucleic acid and phosphodiester additions.

The aberrant expression of miRNAs in disease states, and the relative ease at which to manipulate them, has given great promise to pharmacologically target them to counteract disease. There are many benefits to regulating miRNAs such as; (i) their fine-tuning effect on target protein expression, meaning responses would not be severe, (ii) their evolutionary conservation amongst species, enabling preclinical studies, and (iii) their ability to target many members of the same signaling pathway, therefore having a greater combined effect than siRNA treatments (which typically only regulate one target). The latter of these benefits is also a potential weakness since they could have multiple off-target effects. To work around this problem,

miRNA adenovirus constructs have been combined with delivery systems to target the therapy to specific tissues. This also has the considerable advantage of moderately modulating miRNA levels back to physiological levels, rather than using miR mimics, which typically increase miRNA expression to supraphysiological levels. This approach has been demonstrated to treat hypoxia-induced pulmonary hypertensive rats with an anti-miR-145 therapy without significant off-target effects.[117] In addition, several *in vivo* mouse studies have demonstrated the usefulness of miRNA therapy in treating experimental pulmonary hypertension[107,109] and abdominal aortic aneurysm,[118–121] signifying the potential for miRNA therapeutics to treat cardiovascular disease. Furthermore, miRNA therapies, such as anti-miR-33 (atherosclerosis, Regulus Therapeutics), anti-miR-92 (peripheral artery disease, miRagen Therapeutics) and anti-miR-145 (vascular occlusion, miRagen Therapeutics), have already emerged in preclinical trials. However, there is still a great deal to be determined in the field of vascular smooth muscle contractility. Further advancements in the knowledge of miRNAs and LncRNAs in both small and large arteries may aid the development of treatments to prevent systemic hypertension and aortic stiffness, which are major risk factors for age-related morbidity and mortality.

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Chapter 9

Vascular Smooth Muscle Cells as Therapeutic Target for the Treatment of Circulatory Shock

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Vascular smooth muscle cells (VSMCs), the important functional cell in blood vessels, play important roles in vaso dilation and vaso consctriction functions. Circulatory shock-induced ischemia and hypoxia, and internal environment disorder can damage the structure and function of VSMCs, and result in the disorder of vascular contraction and relaxation, which presents as low reactivity to vasoconstrictors and vasodilators is called vascular hypo-reactivity. This reduced vascular reactivity severely interferes with the treatment of circulatory shock, especially interferes with the efficacy of vasoactive agents. Consequently, it is very important to elucidate the mechanisms and search for the effective treatments for VSMC damage and vascular hypo-reactivity. In recent years, many studies focused on the factors inducing VSMC damage, the different features and mechanisms for vascular hyporeactivity, and the treatment approaches following shock. VSMCs are an important target for the treatment of circulatory shock.

Circulatory shock, a common clinical critical illness, includes hemorrhagic, traumatic, endotoxic/septic, cardiogenic and anaphylactic shock. Traumatic hemorrhagic shock is often seen both in civilian and military situations. It is the major cause of early death at the battle field and during disasters, accounting for about 50% of deaths of battle personnel.[1] Sepsis and septic shock are the common and severe complications in intensive care unit. Despite substantial advances in medical science and technology, the mortality of severe sepsis remains between 52%–60%.[2–6] There are about 1,400 deaths because of sepsis everyday globally.[7]

Vascular smooth muscle cells (VSMCs), the important functional cell in blood vessels, play key roles in vaso dilation and vaso consctriction. Shock-induced ischemia and hypoxia, and internal environment disorder can damage the structure and function of VSMCs, and result in abnormal vascular contraction and relaxation, called vascular hyporeactivity. This reduced vascular reactivity severely interferes with the development and treatment of circulatory shock, especially interferes with the application of vasoactive agents. Also, this reduced vascular reactivity severely interferes with the tissue perfusion and organ functions, which is the important reason for the incidence of irreversible shock. In recent years, many studies focused on the mechanisms and treatment of vascular hyporeactivity following shock.[8] VSMCs are thought to be an important target for the treatment of circulatory shock.

1. The Inducing Factors of VSMCs Damage and the Features of Vascular Dysfunction during Circulatory Shock

1.1. *The Inducing Factors for VSMCs Damage*

A large number of factors may induce damage the VSMCs and interfere with the vascular function/reactivity following circulatory shock including cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, inflammatory mediators such as nitric oxide (NO), endothelin (ET) and bradykinin, endogenous opioid peptides (EOP) such as β-endorphin, and so on.[8] These factors can impact the

function of VSMCs by interfering with the ion channels and receptors of VSMC membranes.[9–11] Also, these factors can induce cellular oxidative stress and endoplasmic reticulum stress (ERS), which affect the mitochondrial function and induce necrosis and apoptosis, in VSMCs. finally they damage the vascular function.[12] In addition, circulatory shock induced ischemia and hypoxia, endotoxin released by bacteria and internal environment disorder such as acid-base disorder can directly cause damage to VSMCs.

1.2. *The Features of Vascular Dysfunction following Circulatory Shock*

Studies show that there were obvious changes in vascular function/reactivity after shock. The main focus in recent years is hemorrhagic shock and endotoxic/septic shock. The main features of vascular dysfunction after shock are vascular hyporeactivity. Vascular reactivity has biphasic change, vascular difference and gender/age-differences.

1.3. *Hemorrhagic Shock*

1.3.1. *Biphasic change of vascular reactivity*

Research showed that vascular reactivity appeared as biphasic change after hemorrhagic shock.[13–17] Most studies demonstrated that vascular reactivity is increased at early-stage shock and decreased at late-stage or prolonged shock (2 hours after shock). Available studies showed that save for the neural and humoral factors, the balance of RhoA/Rac and angiopoietin 1/2 (Ang1/Ang2) are present in the occurrence of the hemorrhagic shock-induced biphasic change of vascular reactivity. Our research team found that the activity of RhoA was increased and the activity of Rac1 was decreased in early-stage of shock, while in late-stage of shock, the activity of RhoA was decreased, and the activity of Rac1 was increased,[18] and that the changes of the ratio of RhoA/Rac1 positively correlated with the vascular reactivity. RhoA/Rac1 regulates the vascular reactivity mainly through their downstream molecules, Rho kinase and P21-activated kinase (PAK).

Rho A may activate Rho kinase and inhibit PAK to increase the vascular reactivity, while Rac1 may activate PAK and inhibit Rho kinase to decrease the vascular reactivity.[17–20] Further study found Rho kinase regulates vascular reactivity mainly through inhibition of myosin light chain phosphatase (MLCP) and induction of 20-kDa myosin light chain (MLC20) dephosphorylation. PAK regulates vascular reactivity mainly through inhibition of myosin light chain kinase (MLCK) and MLC20 phosphorylation.[19]

Angiopoietin (Ang) is the important factor promoting angiogenesis and vascular repair. Xu J *et al.* found that Ang participated in the occurrence of biphasic change of vascular reactivity following hemorrhagic shock.[21] They found that Ang-1 was increased at early shock, which positively correlated with the changes of vascular reactivity after hemorrhagic shock, whereas Ang-2 was increased at the late stage of shock, and negatively correlated with the changes of vascular reactivity after hemorrhagic shock.[21] The exogenous application of Ang-1 maintained the vascular hyper-reactivity at early-stage shock and improved hypo-reactivity at late-stage shock while exogenous application of Ang-2 suppressed the vascular hyper-reactivity at early-stage shock and aggravated hypo-reactivity at late-stage shock. Studies showed that Ang-1 and Ang-2 regulate vascular reactivity mainly through the regulation of the endothelial cell-selective receptor tyrosine kinase Tie2.[21,22] Ang-1 increases the vascular reactivity at early-stage shock mainly through the activation of Tie2-Akt- eNOS pathway, resulting in the appropriate amount of nitric oxide release, which brings the protection of vascular endothelial cells, while Ang-2 decreases the vascular reactivity mainly through Tie2-ERK-iNOS pathway at late-stage shock leading to a considerable amount of NO release, which brings the damage to vascular endothelial cells.

1.3.2. *Vasculature difference*

Hemorrhagic shock induced-vascular hypo-reactivity appears as vascular bed diversity, which means blood vessels at different sites appear with different change features. For example, our studies[23–25] found that following hemorrhagic shock, superior mesenteric artery (SMA), renal artery (RA), femoral artery (FA), celiac artery (CA), and middle

cerebral artery (MCA) had different speeds and severity in vascular reactivity loss. SMA, RA and FA decreased more severely and rapidly than that in CA and MCA. This may be the main reason that causes the different blood distribution and different tissue perfusion to different organ following shock.

Further studies showed that vasculature differences in vascular reactivity correlated with the different expressions of cytokines and inflammatory mediators in various vasculatures. Our studies found that the expressions of cytokines and inflammatory mediators (IL-1β, TNF-α and ET-1, *et al.*) in intestinal and kidney tissues were significantly higher than that in brain and liver tissues after shock.[25] These inflammatory mediators have been confirmed as the inducing factors for vascular hypo-reactivity.[10,11,22,25–27]

1.3.3. *Gender- and age-difference of vascular reactivity*

Studies demonstrated that the host responses to trauma and hemorrhagic shock presented age and gender diversity. The research team of Chaudry found that females tolerated trauma stimuli much better than males, and estrogen played a protective role in this process.[28] Angele *et al.* found that male patients under the age of 50 had a higher mortality late than females after severe blunt trauma, while the difference was not evident in patients older than 50, which suggests that postmenopausal women without the protection of estrogen had no advantages against trauma insult.[29–30] In addition, Maranon R *et al.* found that pre-menopausal women had less cardiovascular events as compared with men of the same age. However, after menopause, this advantage was diminished.[31] These results suggest that estrogen has an important contribution to the protection of organ function in females following trauma and hemorrhagic shock.

Studies showed that the gender- and age-differences not only exist in the tolerance and outcome of trauma, but also in vascular function/reactivity. Proctor *et al.* found that 7-week-old rats had higher vascular reactivity than rats of other ages, and as age increased the vascular reactivity was gradually decreased.[32] Our studies found that female rats in reproductive age had higher vascular reactivity and better tolerance to traumatic shock than male rats of the same age or

female rats not at the reproductive age.[33] A clinical study found that in healthy subjects, the vascular reactivity was gradually decreased as the subjects aged. Middle-aged and young healthy women had higher vascular reactivity than men of the same age. Sex-based differences in vascular reactivity were not obvious in an aged population. Similar to the healthy participants, vascular responsiveness in middle-aged and young trauma patients was greater than in the elderly trauma patients. Female patients had stronger responsiveness than males, whereas aged population had no obvious sex-based differences in vascular reactivity.[33] Further studies showed that estrogen and its receptor (GPR 30)-mediated activation of Rho kinase and PKC lead to the protective effects on vascular reactivity.

1.3.4. *Metabolic diseases suffering from hemorrhagic shock*

Hypertension, diabetes, and hyperlipidemia are the common cardiovascular diseases which seriously threaten human health; the morbidity is increasing year-by-year. There are 190 million diabetic patients and two billion hypertensive patients globally as at 2012.[34] In addition, the number of patients with hyperlipidemia is also rapidly increased. Some studies indicate that these cardiovascular diseases can affect the presentation and outcome of trauma and shock. Ahmad *et al.* reported that patients with diabetes exposed to trauma had higher hospital morbidity and mortality, and longer intensive care unit stays, and increased complications.[35] Lusternberger analyzed 1272 patients with traumatic brain injury and showed that patients with traumatic brain injury and diabetes had nearly a 1.5-fold increase in mortality as compared to similar patients without diabetes.[36] Our studies showed that hemorrhagic shock (40% fixed hemorrhage or mean arterial blood pressure at 40 mmHg for 2h) induced more severe damage on vascular reactivity, hemodynamics, tissue perfusion and mitochondrial function of vital organs, and led to a more rapid death in hypertensive, diabetic, and hyperlipidemic rats than in health rats. Our results show that some basic diseases can aggravate the cardiovascular injury when suffering from severe trauma or shock. More attention should be paid to the diagnosis

and treatment for these diseases subjected to severe trauma or shock.[37]

1.4. *Endotoxic/septic Shock*

Studies showed that vascular reactivity after endotoxic shock also appears biphasic change and vasculature-difference as compared with hemorrhagic shock.[38,39] As compared with hemorrhagic shock, vascular hypo-reactivity is more serious than hemorrhagic shock but appears late.[40,41] Our studies found that vascular reactivity was increased within 1 hour after endotoxic shock, while 2 hours after endotoxic shock, the vascular reactivity was rapidly and seriously decreased. The vascular reactivity had only 20% of normal level at 4 hours after endotoxic shock.[11,42]

We found that the vascular reactivity after endotoxic shock also appeared differently in different vasculatures. At the early stage of endotoxic shock, vascular reactivity in superior mesenteric artery was not obviously increased while in the celiac artery and renal artery it was obviously increased, at the late stage of endotoxic shock, the vascular reactivity in superior mesenteric artery was greatly decreased (reduced by 34.8%), next for renal artery (33.7%) and celiac artery (16.7%). As for the vasodilation to Ach, the superior mesenteric artery showed significant hypo-reactivity while celiac artery showed hyper-reactivity and renal artery had no obvious change.[42] Studies showed that males and aged individuals had a higher risk of the development of sepsis and multiple organ failure after severe trauma.[43,44] Bone *et al.* reported that male patients with sepsis had more morbidity and mortality as compared with females.[45] Schroder *et al.* found that women had a significantly higher survival rate (74%) as compared with men (31%) following the onset of sepsis.[46]

2. The Mechanisms for VSMCs Damage and Vascular Hyporeactivity After Shock

As mentioned above there are many factors affecting VSMCs and vascular function. Shock-induced ischemia, hypoxia, cellular ERS and

oxidative stress can directly damage VSMCs. Cytokines and inflammatory mediators can affect the signal transduction of VSMC and which in turn, affect vascular function (vascular reactivity). Available documents show there are three mechanisms responsible for vascular hypo-reactivity after circulatory shock: receptor desensitization mechanism; membrane hyper-polarization mechanism; and calcium desensitization mechanism.

2.1. *Receptor Desensitization Mechanism of Vascular Hypo-reactivity*

It has been reported that the adrenergic receptors (ARs) are desensitized following circulatory shock including hemorrhagic or endotoxic/septic shock.[47] Studies showed that following shock, a high concentration of catecholamines could cause receptor desensitization. In addition, shock-induced ischemia and hypoxia, the release of cytokines as well as endogenous opioid peptide (EOP) may also inhibit the functions of adrenergic receptors and result in receptor desensitization. Receptor desensitization includes receptor amount down-regulation, receptor affinity drop and uncoupling.[47]

2.1.1. *Down-regulation of receptor amount*

Down-regulation of the receptor number is one of the important mechanisms for receptor desensitization. Sandrini *et al.*[48] investigated the changes of ARs with hypovolemic shock rats and found that the amount of α1- and, β-ARs in the heart and α2-ARs in the spleen were significantly reduced. Tait and Onuma *et al.* investigated the changes of ARs with traumatic shock rats, and found the β-ARs were down-regulated in the liver and heart.[49–50]

Studies recognized that down-regulation of the receptor number includes two steps. The first step is the decrease of receptor number on the cell membrane, but the total quantity of receptors in each cell did not change. The second step is the decrease in total quantity of receptors, which means that the real down-regulation of receptors. In the early stage of shock, receptor desensitization may be mainly due

to the decrease of the receptor amount on the cell membrane and this possibly correlated to the internalization of surface receptors.[51] The real down-regulation of receptors is related to the degradation of internalized receptors and the decrease of receptor expression, which appears at 3–5 hours even 1–2 days after the first step. Many factors including high concentration of catecholamine and cytokines during shock may down-regulate the adrenergic receptors. High concentration of catecholamine can result in the internalization of adrenergic receptors.[47] Cytokines such as TNF-a and IL-1β may down-regulate the amount of adrenergic receptors via inhibition of the transcription of the adrenergic receptor. Our studies.[10,11] found that in-vitro incubation with IL-1β (12.5–50ng/ml) could significantly decrease the vascular reactivity of superior mesenteric artery to phenylephrine and down-regulate the mRNA expression of α1-adrenergic receptors (α1-AR). AG490 (10μmol/L), an inhibitor of JAK2 (Janus kinase 2), could partly reverse IL-1β-induced down-regulation of α1-AR mRNA and suppressed the DNA binding ability of STAT3 (Signal transducer and activator of transcription 3). The results indicate that IL-1β down-regulates the expression of α1-AR mainly by activating JAK2-STAT3 pathway.[11]

2.1.2. *The decease of receptor affinity*

A drop in receptor affinity is another important mechanism for AR desensitization and often happens before the decrease of receptor number on the cell membrane. Previous studies showed that the affinity of β-AR was generally declined but the affinity of α-AR remained constant during endotoxic and hemorrhagic shock.[52,53] A decrease in receptor affinity may also lead to receptor uncoupling and thereby decrease the binding ability of adrenergic receptor to agonists. Therefore, the coupling obstacles between adrenergic receptor and adenylyl cyclase (AC) may be the most important factor for receptor desensitization. Save for causing the internalization of adrenergic receptor, a high concentration of catecholamine, EOP and cytokines can also cause the decrease of adrenergic receptor affinity. For e.g., Romano *et al.* found that agonist-induced receptor desensi-

tization was mainly correlated with the phosphorylation of adrenergic receptor in the early stage of shock.[54] Besides, Shepherd *et al.* found that the decrease of adrenergic response was closely related to the inhibition of adenylyl cyclase activity. These findings suggest that the inhibition of adenylyl cyclase activity may be the key point to affect the coupling between adrenergic receptor and adenylyl cyclase. The mechanism for the down-regulation of adenylyl cyclase activity may be correlated with G proteins. For e.g., Wong *et al.* found EOP and TNF-α inhibited adrenergic receptor affinity and the decrease of adenylyl cyclase activity.[55,56] However, the precise mechanisms for the interaction of adrenergic receptor, G protein and adenylyl cyclase are not clear and need further investigation.

2.2. *Membrane Hyperpolarization Mechanism of Vascular Hypo-reactivity*

Membrane hyperpolarization is another crucial mechanism for vascular hypo-reactivity after shock. Membrane hyperpolarization of vascular hypo-reactivity mainly involves in two kinds of potassium channels: ATP-dependent K+ channel (K_{ATP}) and Large conductance Ca2+-activated K+ (BK_{Ca}) channel.

2.2.1. *K_{ATP} channels*

In physiological conditions, cytoplasm ATPs are in mMol level, which is of course enough to completely close the K_{ATP} channels on cell membranes.[57] While in some pathophysiological conditions such as shock, cell oxidative stress or severe decrease of ATP would cause K_{ATP} channels open on cell membranes.[58,59] The over-open of K_{ATP} channels in VSMCs would result in membrane hyperpolarization of VSMC.[60] This event would inhibit potential dependent calcium channels and decrease the Ca2+ inflow, and finally result in vascular hypo-reactivity. The important inducing factors that cause the opening of K_{ATP} channels include intracellular acidosis, nitric oxide (NO), and so on.

Intracellular acidosis induced by ischemia and hypoxia after shock may markedly reduce the inhibitory effect of ATP on K_{ATP} channels.

Liu J *et al.*[61] found that, in the absence of ATP, a decrease of intracellular pH (pHi) significantly reduced the conductance of single K_{ATP} channel. The open time and time constant of the channels were slightly increased. While in the presence of ATP, a decrease of pHi significantly increased the conductance of single channel and the open probability of K_{ATP} channel. These results suggest that intracellular acidosis activates K_{ATP} channels in the presence of ATP. NO has strong activity to dilate the vascular vessels. In physiological conditions, the endothelial NO synthase (eNOS) in vascular endothelial cells (VECs) may catalyze the continuous synthesis of NO, which makes great contribution to vaso-dilation. In the early stage of hemorrhagic shock, NO is slightly increased, which makes the vascular reactivity a little bit increase. In the late stage of or severe hemorrhagic shock, NO is significantly increased and large amount of OONO- is generated with superoxide anion, which induces over-opening of K_{ATP} channels in VSMCs, and finally results in vascular hypo-reactivity.[62]

2.2.2. *BK_{Ca} channels*

Although K_{ATP} channel makes a great contribution to membrane hyperpolarization of VSMC and vascular hypo-reactivity following shock, it has only one channel per 10μm² membranes. However, BK_{Ca} channels broadly distributes on VSMCs (1~4 channels/μm² membrane), which can be activated by voltage and intracellular Ca^{2+} and plays important role in the regulation of vascular reactivity.[63] The BK_{Ca} channel consists of α-subunit and accessory β-subunit, which co-influence the characteristics of its physiology and pathophysiology.[64] Nelson *et al.* reported that Ca^{2+} sparks are the physiological activators of BK_{Ca} channels. A single Ca^{2+} spark may induce the opening of its surrounding BK_{Ca} and K^+ outflow, which forms spontaneous transient outward current (STOC).[65] This process may induce membrane hyperpolarization. In turn, over-opened BK_{Ca} channels decrease the external calcium influx and finally make the VSMCs in the state of hypo-reactivity. Similar to the K_{ATP} channel, many factors such as nitric oxide (NO), endothelin (ET) and endogenous opioid peptide (EOP) can regulate the opening of BK_{Ca} channels and result in vascular hyporeactivity.[65]

Studies showed that NO regulates the opening of BK_{Ca} channels following hemorrhagic shock mainly through the tyrosine phosphorylation of BK_{Ca} α subunit. Zhou R *et al.* found that this tyrosine phosphorylation of BK_{Ca} α subunit was further regulated by protein tyrosine kinase (PTK) and/or protein tyrosine phosphatase (PTP).[9,65,66] Further, Wu L *et al.*[67] reported that after the treatment with dehydrosoyasaponin (DHS, a BK_{Ca} β subunit probe), the open probability of BK_{Ca} channel did not change, while the excitatory effect of sodium nitroprusside (SNP) on BK_{Ca} channel was reduced. This suggests that SNP regulates BK_{Ca} channel mainly through β subunit of BK_{Ca} channel, but the precise mechanisms need further investigation. In addition, although ET is a peptide with vasoconstrictor properties, our research showed that long-time ET stimulation could also induce vascular hypo-reactivity through cAMP-PKA pathway and BK_{Ca} channel activation.[66]

It was reported that opioid receptors play an important role in the pathogenesis of shock.[68,69] Our studies found that naloxone (10 μM), a non-selective opioid receptor antagonist, significantly down-regulates the activity of BK_{Ca} by reducing its open probability and open frequency. Naltrindole (δ-opioid receptor antangonist) and nor-binaltorphimine (κ-opioid receptor antagonist) have the similar effects to naloxone, while no significant effect was found on the activity of channels after β-funaltrexamine (μ-opioid receptor) treatment. These results suggested that δ- and κ-opioid receptors, but not μ-receptors, participate in the regulation of BK_{Ca} channel after hemorrhagic shock.[64]

2.3. *Calcium Desensitization Mechanism of Vascular Hypo-reactivity*

An interesting phenomenon is that restoration of adrenergic receptors, K+ and Ca2+ channels' function cannot return the vascular reactivity to normal level, which suggests that there are other mechanisms to regulate the vascular reactivity following shock. The key event of receptor desensitization and membrane hyperpolarization mechanism responsible for vascular hyporeactivity as mentioned above is the decrease of intracellular [Ca2+]. While at late stage of shock or in severe shock, the intracellular [Ca2+] in VSMCs is often

over loaded. Nevertheless, vascular hyporeactivity still exists.[15] This phenomenon indicates that VSMCs may exist with calcium desensitization after shock and that calcium desensitization may play a critical role in vascular hypo-reactivity.[70] Our studies proved the hypothesis that VSMCs have calcium desensitization after shock and this calcium desensitization contributes a critical role to vascular hypo-reactivity. Further studies showed that Rho kinase and PKC pathways are the two main pathways to regulate the calcium sensibility of VSMCs following shock.

2.3.1. *Rho kinase pathway*

Rho kinase, a Ser/Thr protein kinase and important member of the small G protein family, is identified as a GTP- Rho binding protein. A number of previous studies showed that Rho kinase is present in the regulation of many biological cellular functions, such as proliferation, differentiation and migration of tumor cells, and the migration and invasion of trophoblast cells, etc.[71] Li T and Schmitz *et al.* reported that Rho kinase played an important role in the regulation of vascular reactivity and calcium desensitization following hemorrhagic shock.[13,71] The regulation of calcium sensitivity of VSMC depends on the phosphorylation and dephosphorylation of myosin light chain (MLC), which is respectively regulated by myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP).[72] Studies showed that there are three ways for Rho kinase to regulate the calcium sensitivity of VSMC: (1) Rho kinase phosphorylate MLC20 directly, but the extent of Rho kinase to phosphorylate MLC20 is far less than MLCK does. The strength of Rho kinase phosphorylating MLC20 is about one third of MLCK. So this way is not the main way. (2) Rho kinase phosphorylates myosin-binding subunits (MBS) of MLCP at Thr2695, Thr2850 and Ser2854, and via which inhibits the activity of MLCP and increases the phosphorylation level of MLC20.[73] This way is the main way of Rho kinase regulating calcium sensitivity. (3) Rho kinase activates CPI-17 via phosphorylation of the Thr238 site of CPI-17. The activated CPI-17 enhances the phosphorylation of MLC20 through inhibiting the MLCP.

2.3.2. *PKC pathway*

Protein kinase C (PKC), a Ser/Thr protein kinase, plays a critical role in cell adaptability to extracellular environment. PKC is also involved in varieties of physiologic functions including cell proliferation, differentiation and migration, cytoskeletal structure, and apoptosis.[74,75] PKC is a big family consisting of at least 12 isoforms, and the main isoforms distributed in the vascular system are PKC α, ε, δ and ξ. Basic research shows that PKC isoforms, especially PKCα and PKCε, may be activated by transferring from cytoplasm to membrane, and then trigger a series of cascade reactions that ultimately interacts with the contractile myofilaments and leads to VSMC contraction.[76]

Many studies showed that PKC participated in the regulation of vascular reactivity and calcium sensitivity following shock. Our previous study found that PKC agonist, phorbol-12- myristate-13-acetate (PMA), could improve and stabilize the hemodynamic parameters and play beneficial effect for hemorrhagic shock in rats through improving the vascular reactivity and calcium sensitivity.[18,77] There are several mechanisms by which PKC regulates the vascular reactivity and calcium sensitivity.[76–78] Woodsome T *et al.* found that PKC may phosphorylate CPI-17, and then inhibits the MLCP activity, via which increases the MLC20 phosphorylation and calcium sensitivity of VSMC.[76] Our research team found that the inhibitory effect of PKC on MLCP is not only related to CPI-17 but also related to zipper-interacting protein kinase (ZIPK)[78] and integrin-linked kinase (ILK).[77,78] Our results showed that ZIPK and ILK may be the direct downstream molecules of PKC α and ε, in which CPI-17 may play an indirect modulating role on MLCP. Our very recent study found that Rho kinase is the downstream molecule of ILK and ZIPK, and the upstream molecule of CPI-17[33].

2.3.3. *MAPKs pathway*

Except for Rho kinase and PKC pathways, mitogen-activated protein kinases (MAPKs), adenosine receptors and myoendothelial gap junction (MEGJ) and its connexin proteins were also found participating in the regulation of vascular reactivity and calcium sensitivity of VSMCs. MAPKs belong to a family of serine/threonine

protein kinases, which include extracellular-signal regulated kinase (ERK), jun NH2 -terminal kinase (JNK), and p38 MAPK in mammals. It was reported that they mediated the fundamental biological process to external signals, such as cytokines and inflammatory mediators.[15]

Previous studies showed that MAPK played a critical role in regulating cell differentiation, proliferation and cell death.[79,80] A recent study of Yang[15] investigated the potential role of MAPK in vascular reactivity after hemorrhagic shock and found that the changes of ERK and p38MAPK activity were positively correlated with the changes of vascular reactivity after hemorrhagic shock. In SMAs, ERK and p38MAPK activity was significantly increased at early shock (0.5 hour) and decreased at late shock (2 hour), ERK and p38MAPK inhibitors decreased the vascular reactivity. This suggests that MAPKs pathway participates in the regulation of vascular reactivity and calcium sensitivity following shock.

2.3.4. *Adenosine receptor*

Adenosine is one of the most important endogenous modulators released excessively in tissue after severe trauma, ischemia or hypoxia. It has been demonstrated that adenosine mainly produces the marked effect through adenosine receptor in VSMC. There are four types of adenosine receptors (AR) in VSMCs, including A1AR, A2aAR, A2bAR and A3AR. Adenosines which combine with specific AR may cause vasoconstriction (A1AR) or vasodilatation (A2aAR, A2bAR). Srinivas SP *et al.* reported that exogenous adenosine may reduce the phosphorylation of MLC in bovine cornea epithelial cells.[81] However, the report of Lai EY *et al.* demonstrated that exogenous adenosine may induce MLC phosphorylation on VSMCs and increase its calcium sensitivity.[82] The results suggest that adenosine is closely related to vascular reactivity and calcium sensitivity. Huang J *et al.*[73] demonstrated that A1AR agonist (N6- cyclopentyladenosine, CPA, 10-5mol/L) can induce renal artery constriction, which can be antagonized by Rho kinase inhibitor (Y-27632). This indicates that Rho kinase is correlated with A1AR in the regulation of vascular tone. Tawfik HE *et al.*[83] found that the PKC inhibitor U-73122 could

abolish the vasoconstriction induced by A1AR, and A1AR agonist may enhance the activity of PKCε. This indicates that A1AR regulating vascular calcium sensitivity is also related to PKC pathway.

However, A2aAR and A2bAR can activate adenylate cyclase which causes the increase in cAMP concentration and PKA activation. The activated PKA may inhibit the activity of Rho kinase. This will induce MLC dephosphorylation and vascular smooth muscle dilation. Besides, Gardner AM *et al.* showed that A2aAR may down-regulate the activity of PKC and A2aAR agonist CGS21680 may inactivate PKCε.[84] The study of Zhou R and Abbracchio M *et al.* found that A3AR was also involved in the modulation of vascular reactivity following shock and this regulation is closely related to Rho kinase pathway.[85,86] These studies suggest that A3AR regulating vascular reactivity and calcium sensitivity is related to PKCδ pathway.

2.3.5 *MEGJ*

MEGJ, the important connecting structure between vascular endothelial cell and VSMCs, plays important roles in vascular tone and vascular synchronism motion. Our studies found that MEGJ and its connexin proteins participated in the regulation of vascular reactivity following shock.[87–89] There are over 20 connexins (Cx) distributed in varieties of tissue cells. Studies showed that the cardiovascular system mainly contains Cx37, Cx40, Cx43, Cx45 and Cx46. Our studies showed that Cx43 participated in the regulation of the vascular reactivity following hemorrhagic shock. Further studies found that Cx43 mediated the vasoconstriction effect of platelet derived growth factor (PDGF) and high dosage of bradykinin which mainly related to Rho kianse and PKC pathways.[88,89]

3. Treatment Measures Based on VSMCs Damage and Vascular Hypo-reactivity after Shock

3.1. *Based on VSMC Damage*

As mentioned above, many factors including ischemia-, hypoxia-induced oxidative stress, ERS and mitochondrial dysfunction may

damage VSMCs. Based on these factors, some measures were found protective. Polydatin, an effective monomer extracted from a Chinese medicine polydate, was found to be protective for the mitochondrial function of VSMCs and vascular reactivity in hemorrhagic shock rats by Dr. Zhao KS *et al*.[90] Our lab found that after traumatic hemorrhagic shock in rats, the mitochondrial permeability transition pore (MPTP) in VSMCs was over opened. Over opened MPTP inhibited the vascular constriction function. Cyclosporine A (CsA, 5mg/kg, iv), the inhibitor of MPTP, could inhibit the opening of MPTP of VSMCs and improve the vascular constriction function and overall outcome of shock rats.[12] Some studies showed that activated protein C (APC) and recombinant human erythropoietin (rhEPO) could prevent septic shock-induced VSMC damage and vascular hypo-reactivity.[91–93] Nacira S and Favory R *et al.* found APC increased the pressor response of adrenergic receptors agonists in septic shock rats and patients. The mechanism was related to APC decreasing tissular inflammation and oxidative stress.[91,92] Bianca R *et al.* found rhEPO had important non-erythropoietic effects including inhibition of inflammatory response and apoptosis.[93]

Our recent study found 4-phenylbutyrate (PBA) has beneficial effects on traumatic hemorrhagic shock in rats by attenuating oxidative stress. It could protect vital organ functions and markedly improve the survival outcomes of shock rats. Studies showed PBA could significantly inhibit the production of reactive oxygen species, increase the antioxidant enzyme levels such as superoxide dismutase, catalase, and glutathione, and improve the mitochondrial function in rat artery and VSMCs. Further studies found PBA increased the nuclear levels of Nuclear factor NF-E2-related factor 2 (Nrf2), and decreased the nuclear levels of Nuclear factor kappa B (NF-κB) in hypoxic VSMCs.[94]

3.2. *Based on Receptor Desensitization Mechanism of Vascular Hyporeactivity*

Studies showed that glucocorticoid (GC) may promote the catecholamine biosynthesis and potentiate the vasoconstriction effect of

vasopressin (AVP), Angiotensin II and endothelin (ET) by increasing the sensitivity of their receptors.[95–97] Further, cortisol has significant inhibitory effect on pro-inflammatory mediators, such as TNF-α and IL-1β, which are confirmed to be correlated with adrenergic receptor desensitization.[98] A recent study showed that dexamethasone improved LPS-induced hypo-reactivity of VSMC. Except for increasing the sensitivity of adrenergic receptors, dexamethasone was found to be able to increase the phosphorylation of MLC20 via activation of RhoA-Rho kinase pathways.[99] This finding demonstrated that glucocorticoid increasing the vascular reactivity is not only related to increasing the sensitivity of related receptors, but also related to increasing the calcium sensitivity of VSMC.[99] The survival benefit of glucocorticoid in septic shock patients is controversial. Annane D *et al.* reported that a 7-day treatment with low doses of hydrocortisone and fludrocortisone significantly reduced the risk of death in patients with septic shock and relative adrenal insufficiency without increasing adverse events.[100] However, Sprung CL *et al.* reported that hydrocortisone did not improve survival or reversal of shock in patients with septic shock, either overall or in patients who did not have a response to corticotropin, although hydrocortisone hastened reversal of shock in patients in whom shock was reversed.[101] Thus, the application of glucocorticoid in sepsis or septic shock including the application dosage, opportunity need more clinical trials and investigations.

Recently, two research teams from France and Australia found α2 adrenergic receptor agonists clonidine and dexmedetomidine could decrease the vasopressor requirements and increase the pressor response of sepsis or septic shock animal (rats and sheep) to norepinephrine (NE), phenylephrine and angiotensin II. They found that the possible mechanisms included central and peripheral actions[102–104] Clonidine and dexmedetomidine can activate the centralα-2 adrenergic receptors to reduce the peripheral sympathetic nerve activity. Thus, the decreased α-1 receptors in peripheral blood vessels in septic shock become progressively up-regulated upon α-2 agonist administration.[102–104]

3.3. *Based on K_{ATP} Channels and Membrane Hyperpolarization*

Glybenclamide is a kind of K_{ATP} channel antagonist. Zhao KS *et al.*[105] found that vascular reactivity was significantly increased after use of glybenclamide combined with NaHCO3 in hemorrhagic rats. The blood pressure, arteriolar blood flow as well as the 24-hour survival rate were also markedly increased in shock rats after treatment, which indicates that glybenclamide combined with NaHCO3 is an effective regimen in the treatment of severe hemorrhagic shock with vascular hypo-reactivity.

Nitric oxide (NO) is an important inducing factor for the opening of K_{ATP} channels, OONO- with superoxide anion is the important executive of NO. Kim HW *et al.* found NO scavenger hemoglobin alone or in combination with NO synthase inhibitor N-nitro L-arginine methyl ester (L-NAME) could significantly improve septic shock induced vascular hypo-reactivity.[106] Zhao KS *et al.* found that superoxide anion scavenger Tiron could block the effect of OONO- in hemorrhagic shock rats, inhibited the membrane hyperpolarization of VSMC and improve shock-induced vascular hypo-reactivity.[107] Bianca E *et al.* found dexamethasone, except for increasing receptor sensitivity and inhibiting tissular inflammation, could also modulate the opening of K_{ATP} channels and improve the vascular hypo-reactivity induced by LPS.[108] The recent studies of Liu C and Vellinga NA *et al.* found that Ketanserin, a serotonin receptor antagonist, have protective effect on endotoxic shock by inhibiting the expression of inducible NO synthase (iNOS) and improving micro-circulation.[109,110]

Methylene blue (MB) has been used as an antidote for toxin-induced and hereditary methemoglobinemia, ifosfamide-induced encephalopathy, and ackee fruit and cyanide poisoning. Recent years, MB was found protective in the treatment of shock states including septic shock, anaphylactic shock, and toxin-induced shock.[111–113] The putative mechanism of the action of MB in the treatment of shock is inhibition of endothelial nitric oxide within the microvasculature and improving the responsiveness to endogenous catecholamines such as

norepinephrine (NE).[114,115] Up to date, since no many clinical trials have been done, so the effects of MB on morbidity and mortality of sepsis/septic shock remain unknown. Well-designed, prospective evaluations are needed to define the role of MB in the treatment of septic shock.[116]

3.4. *Based on Rho A-Rho Kinase Pathway*

Based on "calcium desensitization mechanism of vascular hyporeactivity", Rho kinase and PKC pathway are two important potential targets for the treatment of hemorrhagic shock and endotoxic shock. Studies showed arginine vasopressin (AVP) and its analog terlipressin (TP) have important roles in vasodilatory shock animal and patients.[117] Some previous reports showed that the anti-shock effect of AVP was mainly related to its V1a receptor activation and then the increase in intracellular Ca^{2+}.[118–122]

Our recent study found that AVP (0.03 U/kg/h) and TP(2.6μg/kg/h) significantly improved the decreased vascular reactivity in hemorrhagic shock and endotoxic shock rats and rabbits, This effect of AVP and TP is closely related to activation of Rho A-Rho kinase pathway.[39,123,124] In septic shock patients, we further found that a small dose of TP (1.3 μg/kg/h) in combination with NE continuous infusion, except for decreasing the mortality and NE requirement, could better improve and stabilize the hemodynamics, improve the tissue blood flow, increase the blood oxygen saturation and urine volume, and decrease the lactate level and complication rate. These results show that low-dose of TP continuous infusion can help NE achieves good resuscitation effect by improving vascular reactivity, stabilizing hemodynamics, and protecting organ function in septic shock patients.[125]

Further studies showed that ischemic preconditioning may activate Rho A-Rho kinase pathway and improve the vascular reactivity in hemorrhagic shock rats.[126] Hu Y *et al.*[126] observed the effects of ischemic pre-conditioning on vascular reactivity after hemorrhagic shock in rats and found that a 5% hemorrhage for 30 minutes before hemorrhagic shock may prevent the decrease in vascular reactivity and calcium sensitivity after hemorrhage. The study showed that hemorrhagic shock

may attenuate Rho-kinase activity while ischemic preconditioning may reverse this process. The further study of Hu Y *et al.* found BK_{Ca} opener, NS1619 pre-treatment could protect against shock-induced vascular hypo-reactivity through PDZ-Rho GEF-RhoA-Rho kinase pathway in rats.[127]

3.5. *Based on PKC Pathway*

Phorbol-12- myristate-13-acetate (PMA), a non-specific PKC isoform agonist, was found to have good protective effect on shock. Fang YQ *et al.* observed the beneficial effect of PMA in rats that suffered hemorrhagic shock. They found 1μg/kg PMA could significantly enhance the vascular reactivity and calcium sensitivity of hemorrhagic shock rats and improve the hemodynamic indexes as well as hepatorenal function.[77] Although PMA is not used in clinic now, these findings provide a rational ground to develop this kind of drug or search for other approaches to induce or activate PKC to play protective effect on shock in clinic.

Pinacidil, an adenosine triphosphate–sensitive potassium channel (K_{ATP}) opener, is a common agent used to induce preconditioning protection against ischemia insult.[126,129–130] Xu J *et al.* used 25μg/kg of pinacidil administered 30 min before hemorrhagic shock to mimic ischemic pre-conditioning in rats and found that pinacidil pretreatment could activate PKC α and ε and improve the vascular reactivity and calcium sensitivity in rats suffering hemorrhagic shock.[131] This suggests that pre-treatment with pinacidil can improve the vascular reactivity and calcium sensitivity after hemorrhagic shock, the mechanism is closely related to the activation of PKC α and ε.

4. Conclusion

Circulatory shock-induced ischemia, hypoxia, inflammatory response and internal environment disorder can damage VSMCs and vascular function. Shock-induced vascular function disorder (hypo-reactivity) can severely interfere with the pathological process and treatment of circulatory shock, especially interferes with the application of vasoactive

agents. Three mechanisms including receptor desensitization, membrane hyperpolarization, and calcium desensitization mechanisms for shock- induced vascular hypo-reactivity have been raised. Based on the inducing factors and the important signal regulatory molecules, some beneficial treatment measures have been found for VSMCs damage and vascular hypo-reactivity. Of course, there are still some issues that need further investigation such as the more detailed mechanisms and the more beneficial measures for VSMCs damage and vascular reactivity needing further studies. In addition, some available measures also need to be confirmed with large scale of clinical trials.

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