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Vascular Smooth Muscle Progenitor Cells: Building and Repairing Blood Vessels

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Abstract

Molecular pathways that control the specification, migration, and number of available smooth muscle progenitor cells play key roles in determining blood vessel size and structure, capacity for tissue repair and remodeling, and progression of age-related disorders. Defects in these pathways will produce malformations of developing blood vessels, depletion of SMC progenitor pools for vessel wall maintenance and repair, and aberrant activation of alternative differentiation pathways in vascular disease. A better understanding of the molecular mechanisms that uniquely specify and maintain vascular SMC precursors is essential if we are to utilize advances in stem and progenitor cell biology and somatic cell reprogramming for applications directed to the vessel wall.

Keywords

specification; stem cell; embryo; adventitia; differentiation

Introduction

Advances in reprogramming of differentiated adult somatic cells have focused renewed attention on molecular pathways that specify lineage- and cell type-specific progenitor cells [1,2,3]. Multiple sources of vascular SMC progenitor cells in embryos have been identified by fate mapping studies thereby allowing for experimental analysis of the mechanisms that uniquely specify these progenitors for a smooth muscle fate [4,5]. Moreover, recognition that resident SMC progenitor cells are maintained in adult vessel wall raises important new questions about the roles they play in vascular repair, remodeling and disease [6,7,8,9].

Elucidation of molecular pathways that control cell fate decisions within SMC lineages is of fundamental importance at a basic level. It is also important as a basis for development of progenitor cell therapies applied to the vessel wall. Vascular SMC differentiation in embryonic development is recognized by the appearance of cytoskeletal and contractile protein isoforms, including SMα-actin (*Acta2*), SM-calponin (*Cnn1*), and SM-myosin heavy

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chain (*Myh11*), that confer a functional smooth muscle contractile phenotype on these cells [10,11,12]. No such well-characterized markers are available for identification of SMC progenitors *per se* that, by definition, are specified for a smooth muscle fate, but do not express differentiated SMC marker proteins. In the absence of established markers that would selectively identify SMC progenitor cells, we recognize these cells by their ability to differentiate to SMCs *in vitro* or *in vivo*. However, as discussed further below, misuse of SMC markers and particularly the reliance on SMα-actin (*Acta2*) expression as a sole criterion for differentiation of a progenitor cell into a SMC is problematic and can lead to the false conclusion that the progenitor cell type being studied has the capacity to produce functional SMCs.

Phenotype Plasticity of Vascular Smooth Muscle

Since the earliest ultrastructural studies of smooth muscle tissue, it has been apparent that SMCs exhibit a wide range of phenotypic variation [13,14,15,16,17]. In fact, this diversity was considered for many years to be evidence of not one but two different cell types in the tunica media of artery walls, one for contraction and another for synthesis of extracellular matrix (ECM) proteins [18]. In 1979, Chamley-Campbell et al [19] proposed that these two principle functions of arterial medial cells *in vivo*, ECM synthesis and agonist-induced contraction, were embodied in different but interconvertible “synthetic” and “contractile” SMC phenotypes. SMCs adapted to growth in cell culture, as well as SMCs in developing embryonic vessels and proliferating at sites of vascular injury, exhibit a synthetic phenotype, whereas fully differentiated SMCs in mature adult vessels display a contractile phenotype [16,19]. This variation was initially referred to as SMC phenotypic modulation [19,20]. More recently the term SMC phenotypic switching has come into common use to describe this reversible transition [11]. It is important to realize that the two SMC phenotypes share considerable overlap and that contractile cells can replicate and synthetic cells can possess contractile filaments.

Transcriptional Control of Smooth Muscle Differentiation

Analysis of the requirements for transcription of SMC marker genes, including SMα-actin (*Acta2*), SM22α (*Tagln*), SM-calponin (*Cnn1*), and SM-myosin heavy chain (*Myh11*), in differentiated SMCs revealed the importance of interactions of serum response factor (SRF; *srf*), a MADS box-containing DNA binding protein, with a DNA sequence ($CC(AT)_6GG$) known as a CArG box [21,22,23,24]. The central MADS domain of SRF (*Srf*) provides a highly conserved molecular platform for protein-protein interactions and signal-responsive transcriptional regulation that is essential for cytoskeletal organization and SMC differentiation [25,26,27,28]. The discovery of the myocardin (*Myocd*) family of SRF-dependent transcriptional coactivators was a critically important step toward understanding how SMC-selective gene transcription is achieved by combinatorial interactions with the more widely expressed DNA binding protein SRF (*Srf)* [29,30,31,32,33,34,35,36,37]. The identification of transcriptional corepressors and chromatin-associated silencers of SRF/myocardin-dependent gene expression is particularly important for characterization of mechanisms by which SMC progenitors are formed and maintained *in vivo* [38,12,39]. The combinatorial nature of SMC selective transcription provides for rapid and versatile control of SMC phenotype in response to a multitude of environmental cues [29,40]. An additional component of SMC identity that is not well characterized is cell type-specific alternative splicing. For example, myocardin is alternatively spliced into two forms; a long form (935 amino acids) that is selectively expressed in cardiac muscle and contains a Mef2-interacting domain and a short form (856 amino acids) that lacks a Mef2 binding motif and is SMC-specific [41]. Further work shows that the SMC isoform of myocardin synergistically interacts with Nkx3.1 (Nkx3-1, bagpipe) whereas the cardiac isoform of myocardin does not [42]. Vascular SMC-selective alternative splicing has also been reported for α-tropomyosin,

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metavinculin, smoothelin, and Ca(v)1.2 calcium channel α-subunit. SMC progenitors may also be maintained as such by covalent modifications of either SRF or myocardin. For example, phosphorylation of Ser162 in the MADS domain inhibits DNA binding and transcriptional activity of SRF [28]. Moreover, sumoylation of myocardin at Arg445 in a PIAS1-dependent manner was shown to be an activating modification for SRF-mediated myogenesis [43]. In addition, myocardin proteins contain a B-box domain similar to that found in Elk1. Competitive binding to SRF via these related B-box domains shifts in favor of Elk1 when growth factors stimulate the MAP kinase pathway and toward myocardin as growth factor stimulation diminishes [44].

Origins of Vascular Smooth Muscle Progenitors—Over the last three decades, fate-mapping studies have identified at least 8 independent origins for vascular SMC progenitors [4,5,45,46]. Each of these progenitors has a distinctly different lineage history, yet each produces a similar cell type that transcribes a common set of SMC marker genes (SMαActin (*Acta2*), SM22α (*Tagln*), SM-calponin (*Cnn1*), SM-MHC (*Myh11*) upon differentiation. How progenitor cells from such different developmental origins become specified for a common smooth muscle fate, and how they are maintained as smooth muscle myoblasts during the migration, proliferation and heterotypic cell-cell interactions required to position them for differentiation around nascent blood vessels is not known (Figure 1). Moreover, in perinatal and adult vessels recent evidence suggests that SMC progenitor cells reside in a privileged signaling domain or niche environment within the tunica adventitia [6,8,9] (Figure 2). Their persistence in this perivascular location throughout adult life suggests that cells and matrix components of the adventitial niche provide important signals that maintain the progenitor phenotype and prevent premature SMC differentiation [9,47]. Maintenance of SMC progenitors requires transcriptional silencing of SRF-dependent SMC differentiation marker genes within these different environments. Current evidence suggests two types of pathways function coordinately to prevent premature SMC differentiation and thereby allow for the cell migration and cell division events required to position and expand SMC progenitor cell pools during vessel wall growth and repair. One pathway acts at the level of regional chromatin structure to limit access of SRF and its coactivators to critical paired CArG box elements found in most SMC marker genes [12,48,49,50]. A second pathway acts at the level of SRF bound to CArG sequences in SMC target genes to silence gene transcription that would otherwise be activated by the occupancy of SRF and one or more SRF coactivators on SMC gene targets [39,44,51,52].

Epigenetic Controls of Smooth Muscle Differentiation—Genomic DNA is organized into repeating units called nucleosomes consisting of histone octamers wrapped twice by stretches of DNA each an average of 146 bp in length. This compacts genomic DNA about sevenfold [53,54]. Eukaryotic gene transcription, however, takes place in the context of nucleosomal DNA that is further compacted into higher-ordered structures collectively known as chromatin. Chromatin is highly dynamic and organized into chromosomal domains that adopt specific conformations and positions within the overall three dimensional structure of the nucleus [55]. Reversible covalent modifications of histone tail residues by acetylation, methylation, phosphorylation and ubiquitination or histone tail proteolytic cleavage play critical roles in the control of eukaryotic gene expression and cell fate by modifying accessibility of the chromatin-associated DNA template to sequence-specific DNA binding proteins [56,57,58]. Collectively, covalent histone tail modifications together with ATP-dependent DNA conformation-modifying enzymes produce an epigenetic landscape that, to a large extent, determines what genes are available to the general transcriptional machinery for gene expression [54]. Essentially all genes, including SRF-dependent SMC differentiation marker genes, operate within this complex three-dimensional network of higher-ordered chromatin structure.

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With respect to SRF-DNA interactions in SMCs and their progenitors, current evidence suggests that CArG box elements are more accessible for SRF binding in SMCs than the same DNA sequences are in non-SMCs [59]. This would help to explain why SMC marker genes are silent in non-SMCs that nonetheless express SRF together with one or more myocardin-related transcription factors (MRTFs) [30] or other known SRF coactivators [60,61,62,63,64]. The covalent modifications of specific histone residues (called histone marks) in SMC compared to non-SMCs are complex (reviewed in [49]). In principle, histone acetylation on lysine residues is rapid and reversible and correlates with ongoing rates of gene transcription. The addition of negatively charged acetyl groups is thought to disrupt histone protein-DNA interactions effectively relaxing chromatin structure and thereby enabling increased transcription factor access to binding sites on DNA. Counteracting this activating histone modification is a family of histone deacetylases (HDACs) that catalyze the removal of histone acetyl groups and inhibit gene transcription [65]. Recruitment of SRF to CArG elements in SMC promoter/enhancer regions closely correlates with acetylation of lysine residues on histones H3 and H4 [59,66,67]. Histone acetylation at these loci may be catalyzed by the ability of myocardin to bind and recruit histone acetyltransferases, such as P300 (*Ep300*) [38,68]. Further evidence that transcription of SMC marker genes is enhanced by histone acetylation is the finding that treatment of SMCs with trichostatin A, an HDAC inhibitor, increased endogenous SM22α (*Tagln*) gene expression *in vitro* [67]. Histone lysine residues can also be methylated [69]. A well-characterized methylation of histone H3 at lysine 9 (H3K9) is associated with gene silencing. Like histone acetylation, histone methylation is also reversible. A family of jumonji domain-containing histone demethylases that catalyze the removal of methyl groups from specific histone lysine residues has been identified [70,71]. Indeed, Lockman et al reported that the jumonji domain-containing H3K9 histone demethylase, Jmjd1a/JHDM2a, is an MRTF-A (*Mkl1*) interacting protein that stimulates SMC marker gene expression by demethylating H3K9 residues in SMC target genes [72]. Therefore, pathways that control the type and extent of covalent histone modifications on intact chromatin surrounding SMC differentiation target genes are clearly important to identify in future studies to better understand how SMC progenitor cells are formed and maintained *in vivo*.

Of particular interest for the focus of this review are epigenetic changes found in SMC progenitors that are not found when compared to differentiated SMCs or to progenitors of non-SMC lineages. One promising epigenetic modification that correlates with smooth muscle identity was reported by McDonald et al [59] using an A404 clonal cell line derived from the multipotential teratocarcinoma cell line P19. In the presence of retinoic acid, A404 cells rapidly adopt a SMC phenotype including expression of SM-MHC (*Myh11*) [66]. Employing CArG box mutants in the SMα-actin (*Acta2*) promoter that cannot bind SRF, McDonald et al showed that failure of SRF binding to DNA led to pronounced decreases in H3K9 acetylation and H3K79 dimethylation most likely because these histone modifications are added to SMC chromatin after SRF binding and transcriptional activation [59]. By contrast, H3K4 dimethylation (H3K4me2) was unaffected by the presence or absence of SRF bound to SMα-actin (*Acta2*) promoter CArG box elements [59]. Moreover, chromatin immunoprecipitation (ChIP) assays showed that SRF-myocardin complexes that activate transcription physically associate with H3K4me2 whereas transcription silencing SRF-Elk1 complexes do not. Finally, in the presence of PDGF-BB, a stimulus known to downregulate mature SMC marker gene expression, reductions in SRF bound to H3K4me2 were found [59]. These results suggest that H3K4me2 is an epigenetic histone mark that may be an important element of a molecular profile that confers SMC identity onto a progenitor cell.

If true, histone methyltransferases responsible for the dimethyl modification of H3K4 would be key targets of one or more pathways that confer SMC specification on a multipotential progenitor cell *in vivo*. There are at least two well-characterized classes of histone

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methyltransferases with this activity, the mixed lineage leukemia (MLL) family and SET1 family [73,74]. But these histone methyltransferase enzymes are widely expressed and are not smooth muscle lineage specific. Moreover, they do not possess intrinsic specificity for histone residues on smooth muscle marker chromatin as opposed to chromatin anywhere else in the genome. This suggests that there must be more specific guidance cues that recruit histone methyltransferase-containing complexes to smooth muscle gene targets in progenitor cells. Preliminary findings raise the interesting possibility that a previously identified DNA binding homeodomain protein that associates with SRF and recruits histone acetyltransferase activity to SMC target genes, known as Pitx2 [75,76], serves to guide MLL-type histone methyltransferases to SMC target genes [77]. While clearly an important objective, identification of these molecular guidance cues for placement of critical histone methyl marks on SMC target gene loci in SMC progenitor cells still leaves open the question of how gene targets so marked are maintained in a transcriptionally silent state until an appropriate time during embryonic development or adult homeostasis for SMC differentiation.

In addition to histone modifications, chromatin structure is also controlled by a family of ATPase-dependent chromatin remodeling enzymes that are key components of the SWI/SNF complex [78]. In vertebrates, the two major ATPase subunits of the SWI/SNF complex are Brahma (*Brm1*) and Brahma-related gene 1 (*Brg1*). Zhang et al reported that the myocardin family member, MRTFA (*Mkl1, Mal*), recruits Brg1 to SRF-dependent SMC marker gene targets to promote gene transcription [50]. The SW13 cell line, deficient in both *Brg1* and *Brm1*, was unable to support MRTFA-mediated increases in SRF-dependent SMC marker gene activity [50]. Chromatin immunoprecipitation (ChIP) assays showed that dominant negative forms of *Brg1* strongly inhibited the MRTFA-enhanced SRF binding to promoter regions of SMC marker genes without affecting SRF binding to CArG elements in immediate early genes such as *c-fos*. Similar results were reported for effects of *Brg1* interaction with myocardin (*Myocd*) on SMC target gene expression [79]. Therefore mechanisms to limit the expression or function of ATPase-dependent chromatin remodeling complexes at SMC differentiation marker genes may be important elements in the maintenance of transcriptional silencing in SMC progenitor cells.

Silencing of SMC Differentiation Marker Genes in SMC Progenitors—When considering how SMC progenitors are formed and maintained, it is important to realize that SRF and CArG box chromatin interacts not only with potent coactivators like myocardin but also with potent transcriptional silencer complexes as well [39,80]. For example, the muscle segment homeobox proteins (*Msx1* and *Msx2*) form ternary complexes with SRF (*Srf*) and myocardin (*Myocd*) that block DNA binding of the SRF-myocardin complex to CArG box motifs in SMC marker genes [80]. Another example is KLF4 (*Klf4),* a zinc finger protein [81] that binds to conserved GC-rich elements located near paired CArG boxes in the SMα-actin (*Acat2*) promoter and other SMC marker genes [82]. *Klf4* physically binds to SRF, recruits HDAC2 (*Hdac2*) and HDAC5 (*Hdac5*) to SMC marker genes, and blocks SRF association with CArG box sequences in intact chromatin [52]. The forkhead transcription factor FoxO4 (*Foxo4*) physically interacts with myocardin and inhibits its SRF-coactivator function thus acting as a repressor for SMC differentiation [51]. In response to signals that activate the PI3-kinase/Akt pathway, *Foxo4* is exported from the nucleus, and SMC differentiation target genes become transcriptionally active. The co-repressors *Msx1, Klf4* and *Foxo4* were found to be coexpressed with SRF and myocardin in SMC progenitor cells obtained from neonatal aortic adventitia [9]. More recently, the HMG box-containing protein HMG2L1 was shown to physically interact with myocardin and abolish the binding of myocardin-SRF complexes to CArG box elements in SMC promoters. Overexpression of HMG2L1 in SMCs repressed SMC marker gene expression whereas depletion of endogenous HMG2L1 increased expression of the same SMC marker genes [83].

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PRISM (PR domain in smooth muscle) (*Prdm6*) is a PR and SET domain-containing protein with transcriptional repressor activity that is selectively expressed in smooth muscle tissues [84]. It is expressed as early as E11.5 in the aortic wall corresponding to initial events in tunica media formation [84]. It is also expressed in developing airway, tracheal and bladder smooth muscle. PRISM is capable of recruiting histone methyltransferases and other chromatin remodeling enzymes including class I HDACs through interactions of these factors with its PR domain and thereby mediate transcriptional repression in SMC progenitors. Among the gene targets whose expression in SMCs is repressed by PRISM are *Gata6* and myocardin (*Myocd*). Indeed, siRNA-mediated knockdown of PRISM produced a concomitant induction of SMC differentiation marker genes, including SM-MHC, suggesting some level of constitutive repressor activity is maintained by PRISM in differentiated SMCs. Other members of the PR/SET domain family are reported to play important roles in the specification of neural crest progenitors [85] and slow-twitch skeletal muscle fibers [86].

Control of cell differentiation by transcriptional corepressors plays important roles in progenitor cell maintenance in skeletal muscle and other progenitor cell types [87]. For example, skeletal muscle myoblasts are maintained, in part, by a MEF2-dependent corepressor complex that normally silences muscle-specific gene expression [88]. This complex consists of MEF2 (*Mef2*), MITR (Mef2-interacting transcriptional repressor), HP1 (heterochromatin interacting protein-1), *Hdac4* and *Hdac5* [89]. Activation of calcium/calmodulin-dependent protein kinase leads to phosphorylation of *Hdac4* and *Hdac5* resulting in nuclear export of these transcriptional repressors [90]. The *Mef2* released from phosphorylated HDACs associates with MyoD (*Myod1*) and E-protein heterodimers resulting in transcriptional activation of skeletal muscle structural genes. *Mef2*, like *Srf*, is a MADS box-containing DNA binding protein that provides a platform for protein-protein interactions in skeletal myoblasts. Another important class of negative transcriptional regulators is the Groucho/TLE gene family, which consists of four unlinked genes named *TLE1-4* in humans and *Grg1-4* in mouse [91]. Grg/TLE proteins interact with engrailed homology-1 (EH1) domain-containing DNA binding proteins and recruit class II HDACs to chromatin via a glycine and proline-rich domain in the N-terminal half of Grg/TLE proteins [92]. EH1 domain proteins important in vascular development include the forkhead box transcription factors of the FoxA, FoxC and FoxD families, T-box proteins, and muscle-segment homeobox proteins of the Msx family [93].

Roles for Noncoding RNA in Formation and Fate of SMC Progenitors—A growing number of studies now indicate the important roles played by noncoding RNAs [94,95] in development and differentiation of vascular smooth muscle [96]. For example, maintenance of fully differentiated phenotypes of vascular SMCs is dependent on expression of microRNA-143 (miR-143) and miR-145 [97,98,99,100,101]. miRNAs are a class of ~ 22-nucleotide noncoding small RNAs that play essential roles in regulating gene expression by posttranscriptional mechanisms including arrest of translation and degradation of mRNAs [95]. Loss of function for miR-143/145 results in arteries with thinner walls and SMCs with a noticeable lack of differentiated features. Systemic blood pressure and smooth muscle contractile activity are reduced in miR-143/145-deficient mice [100], as is the ability to migrate due to disarray of actin stress fibers [99]. Expression of miR-145 alone was sufficient to convert multipotent neural crest cells into vascular SMCs suggesting that miR-145 targets are important for maintaining the SMC progenitor phenotype in the neural crest lineage [97]. Likewise, overexpression of miR-145 was able to substantially rescue the vascular SMC defects resulting from the loss of dicer, an endonuclease required for miRNA synthesis [95], in SMCs [102]. Identified targets of miR-143/145 include two gene products that are known repressors of SRF-myocardin-induced transcription, namely *Elk1* and *Klf4*, as well as the potent SRF coactivator MRTF-B (*Mkl2*) [97,99]. In addition, miR-145 has been shown to

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downregulate expression of Klf5, a factor associated with repression of the mature differentiated SMC phenotype [99,101]. Conversely, miR-221 and miR-222 down-regulate factors that promote expression of the differentiated SMC phenotype thus leading to dedifferentiation of SMCs [103,104]. These two miRNAs are induced by PDGF-BB and target myocardin (*Myocd*) transcripts for down-regulation [104].

Recent studies suggest the paradoxical idea that gene silencing requires transcription initiation [105]. Thus, current evidence suggests that long noncoding RNA molecules (lncRNAs) recruit transcriptional repressors to specific sites in the genome to silence gene expression. For example, mammalian X chromosome-inactivation requires noncoding Xist RNA that forms a hairpin structure that recruits polycomb group repressor complexes (PRC2) to the X inactivation center to silence gene transcription [106,107]. Likewise, a noncoding RNA transcript from the INK4b/ARF locus directs repression of that locus by recruiting PRC2 silencer complexes [108]. Most promoters are now known to produce short transcripts in both 5′ and 3′ orientations that encompass CpG islands frequently found in promoter regions [109]. Short hairpin structures produced by these non-coding transcripts recruit PRC2 repressor complexes that spread across a local region of DNA, catalyze trimethylation of histone H3K27 (H3K27me3) and silence gene expression. Activation of transcription from this repressed locus requires demethylation of H3K27me3 by histone demethylase activity [110]. As discussed above, expression of a jumonji domain-containing H3K9 histone demethylase, Jmjd1a/JHDM2a, in SMCs is associated with transcriptional upregulation of smooth muscle differentiation marker gene expression [72]. More recently a positive role for lncRNAs acting as transcriptional enhancers was reported suggesting that regulatory functions for this important class of noncoding RNAs will likely turn out to be complex and interesting [111]. A role for lncRNAs in control of cell fate in general, and SMC fate in particular, is an important area for future work.

Signal-Responsive Differentiation of SMC Progenitors—As discussed above, SMC progenitor cells are poised to differentiate to SMCs but are restrained from doing so by redundantly acting repressor/silencer mechanisms. Reductions in the activity of SRF-dependent corepressor complexes or increases in the activity of SRF-dependent coactivator complexes are sufficient to trigger SMC differentiation in these cells. A principle pathway for formation of SRF-coactivator complexes involves extracellular signal-dependent activation of RhoA-GTPase (*Rhoa*) and its downstream effectors Rho kinase (*Rock1*), LIM kinase (*Limk1*), protein kinase N (*Pkn1*), and mammalian diaphanous (*mDia*) proteins. Activation of *Rock1* and *Limk1*-dependent signaling leads to actin polymerization, stress fiber formation and cytoskeletal reorganization [112,113,114]. Activated *Rock1* is reported to translocate to the nucleus, phosphorylate P300 and promote SMC gene transcription [115,116,117]. Of particular importance was the finding by Treisman's group that *Rhoa*- GTPase-mediated actin treadmilling, specifically the polymerization of G-actin to F-actin, results in the mobilization of potent SRF coactivators MAL/MRTFs/MKLs from inhibitory binding sites on G actin followed by their translocation to the nucleus [32,118,119]. Accumulation of MRTFs in the nucleus and their partnering with SRF and other coactivators [60,61,62,63,64] leads to transcriptional activation of SMC target genes and SMC differentiation by mechanisms discussed above. It is reasonable to assume that in actively migrating SMC progenitors, repetitive actin filament turnover during extension of lamellipodial and filopodial projections constantly regenerates cytoplasmic G actin and maintains nuclear MRTF activities at low levels. Once these cells reach their destination and take up positions in developing tunica media, F-actin predominates and MRTFs are found in their active nuclear forms. This mechanism would couple morphogenesis of blood vessel walls with differentiation of a major constituent cell type (SMC) present within those walls. For example, conditional deletion of integrin-linked kinase (*Ilk*) produces overactive *Rhoa* and *Rock1*-mediated signaling in SMC progenitors resulting in dermal arterioles with

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smaller diameters and loss of circumferential SMC alignment consistent with premature differentiation, excessive contraction and lack of proliferative expansion of SMC progenitor pools [120]. These findings reinforce the idea that SMC progenitor pool sizes are critical determinants of blood vessel size, length, and function. The relative state of cytoskeletal actin polymerization, and thus levels of active MRTFs, depends upon *Rhoa*-GTPase-mediated signaling pathways that respond to factors present in the local environment thus emphasizing the importance of niche-dependent signaling for the maintenance of a SMC progenitor phenotype [9,47].

Smooth Muscle Progenitors and the Embryonic Vascular System—Genetic approaches to developmental fate mapping reveal the intricate diversity of vascular SMC lineages in development, but they leave us wondering how progenitor cells of such diverse embryonic origins and developmental histories can differentiate into a common cell type [4,5,46]. For example, the dorsal surface of the neural tube contains progenitor cells that migrate into the pharyngeal arch complex between E8.5 and E9.5 and produce SMCs that form the walls of the great arteries [45,121,122,123]. Clonal analysis *ex vivo* shows that individual neural crest progenitor cells are multipotent [124,125]. Thus factors in the environment through which these cells migrate play essential roles in specifying their fate [126,127,128]. SMCs in the descending aorta originate from progenitors in epithelial somites that express Pax3 and FoxC2 [5,129,130]. SMC fate specification occurs when expression levels of FoxC2 exceed those of the promyogenic factor Pax3 [130] and since FoxC2 represses transcription of Pax3, a SMC fate is stabilized over the alternative skeletal muscle fate. The abundance ratio of FoxC2 and Pax3 is controlled by signals in the somite microenvironment [130] and thus resembles SMC fate specification in cardiac neural crest progenitors during delamination and migration from the neural tube. A likely scenario based on current data (Figure 1) is that SMC progenitor cells thus specified make contact with endothelial cells [131], engage notch-dependent signaling pathways [132,133,134,135,136], mobilize MRTF-B (*Mkl2*), and initiate SMC differentiation [137,138]. It is important to point out that the requirement for MRTF-B (*Mkl2*) is restricted to cardiac neural crest-derived SMCs indicating that SMC progenitors originating from other sources in the embryo must have different molecular requirements for SMC differentiation even though the same SMC differentiation marker genes are activated in all cases [137,138]. Indeed, analysis of MRTF-A (*Mkl1, Mal*)-deficient mice revealed that neural crest-derived vascular SMCs differentiated normally whereas myoepithelial cells of mammary gland ductal tissues failed to develop a contractile phenotype [139].

SMCs in the coronary vasculature arise from a separate population of embryonic progenitors. The majority of coronary SMCs (CoSMCs) can be traced back to origins in the proepicardium (PE), a transient collection of mesothelial cells that appears at the sinoatrial junction at about E8.5 in the mouse [140,141,142]. Specification of lateral plate mesoderm for a proepicardial fate is mediated by an antagonistic interplay between BMP and FGF signaling [143,144]. In avian embryos, soluble factors produced by the developing liver bud, but not lung bud, direct multipotent mesoderm progenitors to adopt a proepicardial identity [145]. In zebrafish embryos, an early role for *Tbx5* in specifying PE progenitor cells was identified that results in competence to respond to BMP4 (*Bmp4*) released by the developing myocardium [146]. PE cells thus specified reach the heart around E9.5 in the mouse and grow out over the surface of the myocardium to form a single layer of epicardial cells. Then around E13.5 to E14.5 some epicardial cells undergo an epithelial to mesenchymal transition (EMT), loose their epicardial phenotype and adopt a pre-SMC phenotype in the subepicardium *in vivo* [142,147,148,149]. Epicardial cell EMT is associated with a *Rhoa*-GTPase and *Rock1*-mediated cytoskeletal actin reorganization that is required for CoSMC differentiation [115,150]. Epicardial cells from adult hearts appear to retain their specification to differentiate into CoSMCs, at least *in vitro* [151]. Maintenance of a CoSMC

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progenitor phenotype in the epicardium may be due, in part, to high levels of epicardial expression of EH1 domain-containing T-box proteins including *Tbx5* and *Tbx18* [152,153,154].

In addition to cardiac neural crest and proepicardium, a third distinct source of SMC progenitor cells in the early embryo is found in an Islet-1 (*Isl1*)-positive cell population that contributes multiple lineages to the developing heart [155]. *Isl1* is a LIM-homeobox transcription factor that marks the second heart field of cardiac progenitors and is required for formation of the atria, right ventricle, and cardiac outflow tract [156]. Genetic fate mapping studies using *Isl1*-cre to mark early cardiac progenitor cells in the mouse showed that SMCs in the walls of the aortic root, pulmonary trunk and coronary stems are produced from *Isl1*-positive progenitors that are distinct from progenitors in the cardiac neural crest. Also labeled by *Isl1*-cre were endothelial cells, endocardial cells, and myocardial cells of the right ventricle suggesting that the progenitor population is multipotential [155]. Clonal analysis of $Isl1^{+}$ progenitor cells verified their unique multilineage potential [155,157]. Similar results were reported by Kattman et al who isolated *VEGF-R2/Flk1*-positive progenitor cells from head-fold stage mouse embryos and showed that single progenitor cells generated colonies that contained cardiac myocytes, endothelial cells and vascular SMCs thus confirming their multilineage potential [158].

Smooth Muscle Progenitors and the Adult Vascular System—Pericytes are smooth muscle-like microvascular mural cells with progenitor-like properties (Figure 2). Proper investment of microvessel walls with pericytes is a required step in vascular development and angiogenesis [159,160]. However, little is known about the developmental origins of pericytes as very few lineage-mapping studies have addressed whether or not pericytes share common origins in the embryo with vascular SMCs. One exception to this is in the brain where pericytes and cerebral vascular SMCs have been shown to originate from cephalic neural crest progenitor cells [161]. Many pericytes exhibit the potential for multilineage mesenchymal cell differentiation [162,163]. Pericytes are classically defined as cells embedded underneath the basal lamina of microvascular endothelial cells. This position is strikingly similar to that of muscle stem cells known as satellite cells that reside under the basal lamina of skeletal muscle myofibers. In both cases, the position of resident pericytes and satellite cells *under a basal lamina* may be important to efficiently receive signals released from endothelial cells or myofibers respectively that maintain their progenitor phenotypes. Cossu and coworkers have shown that pericytes isolated from injured skeletal muscle tissue and injected intra-arterially have the remarkable ability to home to injured skeletal muscle, reconstitute satellite cell pools, form skeletal muscle myofibers, and promote muscle regeneration *in vivo* [164,165,166]. This property may reflect an origin of some pericytes from mesoangioblasts, a population of multipotential mesenchymal progenitor cells that appear early in vascular development as VEGFR2/Flk1-positive cells associated with the abluminal side of the dorsal aorta [167].

The possibility that vascular SMC progenitor cells reside in the outer layer of artery wall, the adventitia and associated perivascular tissues, has been suggested several times over the years (Figure 2) [168,169]. For most investigators, these suggestions were regarded as anecdotal and data consistent with a role for the adventitia as harboring vascular progenitor cells was largely ignored. However, in a survey of adult vessels in $ApoE^{-/-}$ mice, Hu et al reported finding SMC marker-negative, stem cell antigen-1 (Sca1; *Ly6a*)^{pos} progenitor cells in the adventitia of large arteries [6]. When isolated from genetically-marked animals and put back on the adventitial side of vein grafts, adventitia-derived $Sca1^{pos}$ cells were found in the media at 2 weeks and in the neointima at 4 weeks after transplantation where they no longer expressed Sca1 and became immunopositive for SMC differentiation markers [6]. Similarly, a population of $CD34^{pos}/PECAM1^{neg}$ cells was found in human internal thoracic artery with a capacity to form capillary-like microvessels in *ex vivo* aortic ring assays [7]. The

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concentration of these cells in the inner adventitia led Zengin et al to refer to this location as a “vasculogenic zone” in the artery wall [7]. More recently, a novel domain of sonic hedgehog (Shh) signaling was described that is restricted almost entirely to the adventitia of large and medium-sized arteries and veins, and colocalizes with the vasculogenic zone described by Zengin et al [9]. Within this Shh signaling domain a population of $Sca1^{pos}/CD34^{pos}$ progenitor cells was found with the capacity to differentiate into mural cells (pericytes and SMCs) *in vitro* and promote angiogenesis in matrigel implants *in vivo* [9]. Another factor implicated in an adventitial microenvironment for SMC progenitors is stromal cell-derived factor 1-alpha (SDF-1α; *Cxcl12*) [170]. SMC-specific deletion of PTEN (*Pten*), a dual-specificity lipid and protein phosphatase, increased production of SDF-1α by *Pten*-deficient SMCs and resulted in accumulation of CXCR4-positive circulating progenitor cells in a perivascular, adventitial location [170]. These reports are particularly interesting in light of past studies that suggest roles for adventitial cells in artery wall thickening in pulmonary hypertension [8] and in neointimal formation after vascular injury [168]. For example, animals exposed to chronic hypoxia develop markedly thickened pulmonary artery walls (reviewed in [8]). This form of hypertensive remodeling is characterized by additional layers of smooth muscle forming on the adventitial side of the pulmonary artery wall and may well consist of SMCs that originate from local progenitors resident in the adventitia [8]. Likewise, mice that are haploinsufficient for tropoelastin (*Eln*) exhibit additional layers of SMCs and elastin that form on the adventitial side of the aorta during late stages of embryogenesis around E16.5 to postnatal day 3 [171,172]. Whether SMC progenitor cells in the adventitia can detect mechanical stretch of the artery wall, or changes in relative hypoxia, or respond to soluble signals associated with wall remodeling stimuli are important questions for future studies.

Experiments to determine if circulating progenitor cells of bone marrow origin contribute intimal SMCs to atherosclerotic plaques were initially interpreted as supporting such an origin [173,174]. In those early studies, up to 50% of intimal SMCs within mouse atherosclerotic plaques were reported to be derived from progenitors of bone marrow origin [174]. However, as work progressed on this possibility, an origin for intimal SMCs from marrow-derived progenitors seemed less likely [175,176]. A large influx of marrow-derived inflammatory cells, mostly monocytes and macrophages, occurs early in lesion formation. High-resolution confocal imaging is required to determine if the marker used to detect cells of bone marrow origin is actually coexpressed with markers used to identify intimal SMCs [175]. This is not trivial as intimal SMCs and inflammatory cells are often in very close proximity, if not actual physical contact, during these early time points. In addition, common methods for marrow-derived mononuclear cell isolation lead to their contamination with platelet membrane fragments (microparticles, MPs) carrying markers used to identify other cell types (e.g, CD31, endothelial cells)[177]. By extrapolation, inflammatory cell MPs may be shed and incorporated into nearby SMCs leading to false conclusions about their origins. Upon extending the time course of experiments out to 16 weeks after wire injury to femoral artery, Daniel et al clearly showed that bone marrow-derived cells of any type were dramatically reduced in numbers while intimal cells expressing smooth muscle markers continued to increase [176]. By 16 weeks after injury there were very few, if any, cells that coexpressed the bone marrow lineage marker and SMC specific proteins. Taken together, the current data suggests that inflammatory cells contribute an early paracrine activity that diminishes greatly with time, and that there is little, if any, long-term contribution of marrow-derived progenitor cells to the vascular SMC population in these vessels.

Myofibroblasts are derived from resident tissue fibroblasts and are found in abundance in a number of different reactive and pathogenic conditions [178]. Myofibroblasts are present in granulation tissue during wound healing, in connective tissue stroma surrounding solid tumors, and are abundant in fibrotic tissue. Because myofibroblasts express some of the commonly used SMC markers, such as SMα-actin (*Acta2*) and SM22α (*Tagln*) it is

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frequently assumed that these cells are SMC progenitors that can go on to complete a differentiation sequence and become SMCs. In fact, although SMCs and myofibroblasts both express the SMα-actin (*Acta2*) gene, they use distinct molecular mechanisms to do so [77]. For example, analysis of MCAT element mutations in the SMα-actin promoter/enhancer in transgenic mice showed that myofibroblasts within granulation tissue of skin wounds required intact MCAT elements for reporter gene expression whereas vascular SMCs as well as SMCs of the stomach, bladder and intestine did not [77]. Moreover, different TEF-1 (*Tead1*) family members associate with MCAT elements in myofibroblasts (RTEF-1; *Tead4*) compared to SMCs (TEF-1; *Tead1*) [77]. In addition, myofibroblasts differ from SMCs in an absence of expression of smoothelins, relatively late stage differentiation markers for SMCs [178,179]. Thus activation of SMC markers in myofibroblasts occurs by distinct molecular pathways compared to SMCs, arguing that resident tissue fibroblasts or myofibroblasts are not SMC progenitors under most conditions.

Summary

The number of different smooth muscle types far exceeds that of skeletal or cardiac muscle. This diversity reflects a multiplicity of different kinds of SMC progenitors found in embryonic and adult tissues. It also reflects the versatility of a common molecular mediator for SMC differentiation, a complex of SRF and a myocardin family member occupying one or more CArG box *cis*-elements. Through interactions with a large number of co-activators and co-repressors that recruit epigenetic regulators of regional chromatin structure, this core transcription module is made responsive to a wide variety of extracellular signaling cues that specify and direct cell fate. Maintenance of SMC progenitor pools requires signals that allow for proliferative expansion during tissue growth or repair together with activation of redundant transcriptional silencing mechanisms to maintain the SMC progenitor phenotype and prevent SMC differentiation. A more complete understanding of the identity of these signals and the molecular mechanisms that are utilized to form and maintain vascular SMC progenitor cells will enable advances in somatic cell reprogramming and stem cell biology to be more effectively applied to disorders of the vessel wall.

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Non-standard Abbreviations and Acronyms

ACLP	aortic carboxypeptidase-like protein
APEG	aorta preferentially-expressed gene
BMP	bone morphogenic protein

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CArG	$(CC(AT)_6GG)$
ChIP	chromatin immunoprecipitation
CoSMC	coronary smooth muscle cell
E*n*	embryonic day *n*
ECM	extracellular matrix
Flk1	fetal liver kinase-1
H3K4me2	histone H3 lysine-4 dimethyl modified
HAT	histone acetyltransferase
HDAC	histone deacetylase
HP1	heterochromatin interacting protein-1
MADS	mcm-agamous-deficiens-srf
miRNA	micro-RNA
MKL	megakaryoblastic leukemia
MLL	mixed lineage leukemia
MRTF	myocardin-related transcription factor
PIAS1	protein inhibitor of activated STAT-1
PRC2	polycomb group repressor complex 2
Rock1	rho kinase-1
SCA1	stem cell antigen-1
siRNA	small interfering RNA
SMC	smooth muscle cell
SRF	serum response factor
VEGF-R2	vascular endothelial cell growth factor receptor-2

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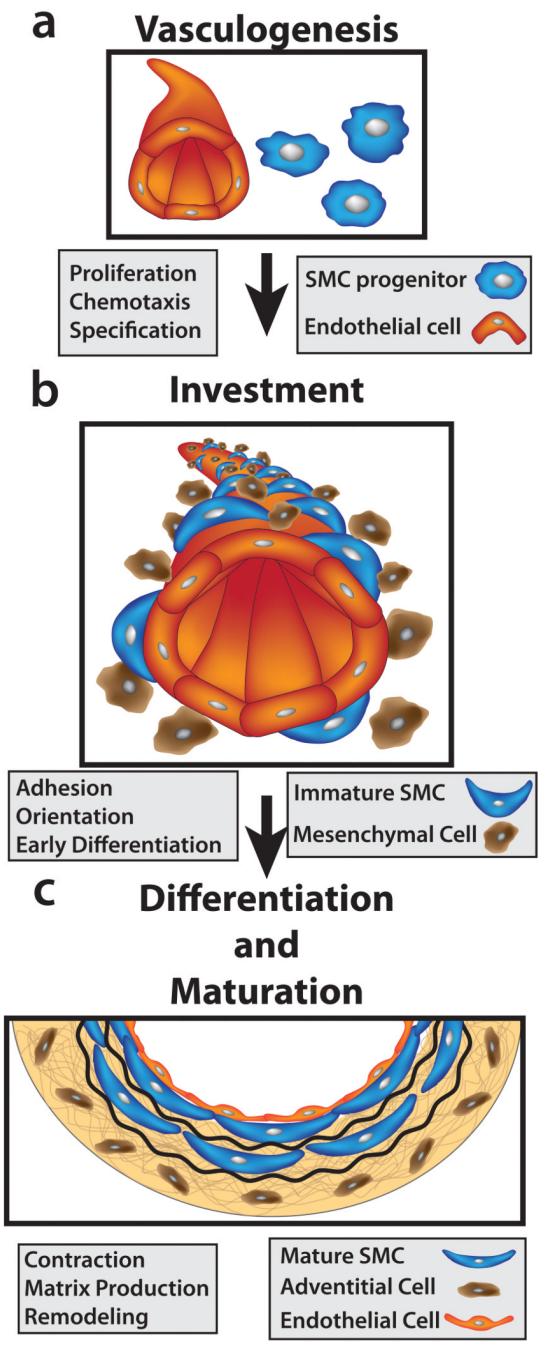

Figure 1. Development of vascular smooth muscle from embryonic progenitors

(a) Vasculogenesis: Vascular development begins when angioblasts differentiate into endothelial cells (red) that self-assemble into a nascent capillary-like vascular network. (b) Investment: Increasing cardiac output from the developing heart stimulates production of mesenchymal cell chemoattractants by endothelial cells. SMC progenitors (brown) begin to invest the vessel wall around E10.5 in the mouse. Close contact with endothelial cells initiates SMC differentiation (blue cells). The position of the vessel within the embryo determines what type of SMC progenitor will be involved in producing the tunica media. Proliferation of SMC progenitor cells is required to supply numbers of SMCs sufficient for continued vascular development. (c) Differentiation and maturation: As layers of SMCs are

added to the developing artery wall, a cross-linked extracellular matrix is formed that defines the structure of the mature tunica media.

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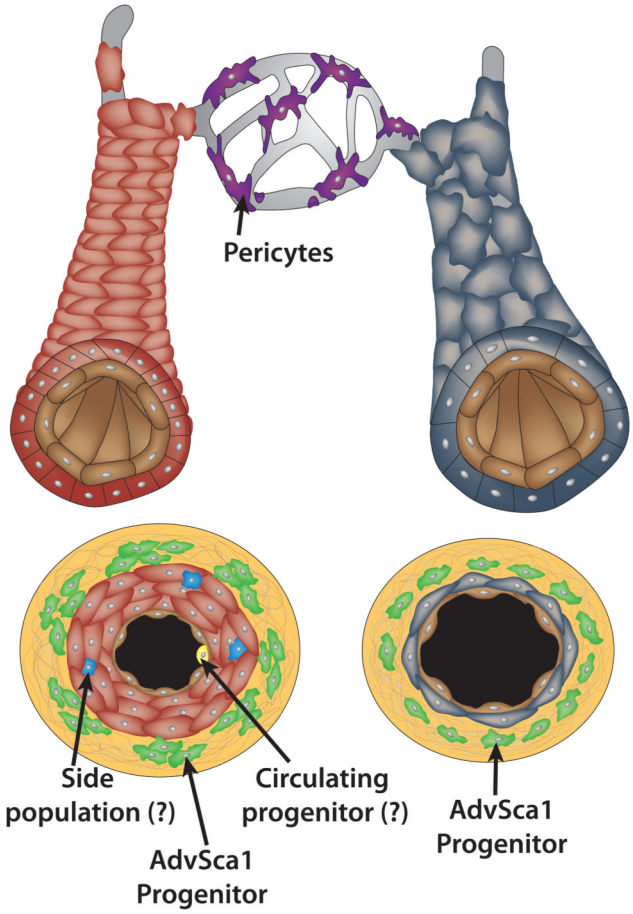

Figure 2. Sources of vascular smooth muscle progenitor cells in adults

In large arteries (red) and veins (grey), resident SMC progenitors have been identified in the adventitial layer (green cell clusters) [6,7,9] and in the medial layer (scattered blue cells) [180]. Some reports suggest that SMCs in atherosclerotic plaques and intimal masses can arise from circulating, bone marrow-derived progenitor cells (yellow) [173,174] while others report finding no evidence to support that possible origin [175,176]. In microvessels, pericytes (purple) have been proposed to have multilineage differentiation potentials [162,166], and can act as SMC progenitor cells [159,163].