**Signaling Mechanisms That Regulate Smooth Muscle Cell Differentiation**

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**Abstract**—Extensive studies over the last 30 years have demonstrated that vascular smooth muscle cell (SMC) differentiation and phenotypic modulation is controlled by a dynamic array of environmental cues. The identification of the signaling mechanisms by which these environmental cues regulate SMC phenotype has been more difficult because of our incomplete knowledge of the transcription mechanisms that regulate SMC-specific gene expression. However, recent advances in this area have provided significant insight, and the goal of this review is to summarize the signaling mechanisms by which extrinsic cues control SMC differentiation. (Arterioscler Thromb Vasc Biol. 2011;31:1495-1505.)

**Key Words:** gene expression • signal transduction • vascular biology

Smooth muscle cells (SMC) provide structural support to the vasculature and control blood pressure and blood flow through highly regulated contractile mechanisms. Differentiated SMC express a variety of SMC-specific contractile and contractile-associated proteins that contribute to these functions, including smooth muscle (SM) myosin heavy chain, SM22, calponin, and SM α-actin. Importantly, unlike cardiac and skeletal muscle cells, SMC retain significant plasticity even in adult animals. Thus, in response to vessel injury, SMC undergo phenotypic modulation, a process characterized by decreased SMC differentiation marker gene expression and increased proliferation, migration, and matrix synthesis. The search for the transcription regulatory mechanisms that ultimately govern the process of SMC differentiation has been complicated by the plasticity of this cell type and the fact that SMC derive from multiple precursors throughout the embryo.1 The MCM1, agamous, deficiens, serum response factor (SRF) box transcription factor, serum response factor, regulates most SMC differentiation marker genes by binding to highly conserved CArG cis-elements (CC(A/T)_nGG) that are present within nearly all of the SMC-specific promoters (see 2 for review). However, SRF cannot be considered a master regulator of SMC differentiation because it is a ubiquitously expressed protein that also regulates cardiac- and skeletal-muscle–specific gene expression and the expression of a variety of early response and structural genes.3

SRF-dependent transcription is controlled to a large extent by SRF’s interaction with additional transcription factors and coactivators. The first SRF cofactors described were the ternary complex factors, such as Elk-1, that regulate early response gene expression and that interact with SRF when phosphorylated by mitogen-activated protein kinase (see 4 for review). Some Nkx and Gata family members are expressed in certain SMC subtypes (Nkx3.1, Nkx3.2, and Gata-6) and have been shown to interact with SRF to regulate SMC differentiation marker gene expression.5,6

The discovery of the cardiac/SMC-selective SRF cofactor myocardin was a major advance in our understanding of the transcription mechanisms that regulate SMC-specific gene expression.7 Unlike the Nkx and Gata factors, myocardin does not contain a DNA binding domain but powerfully transactivates cardiac and SMC-specific gene expression by physically interacting with SRF.7 Importantly, expression of myocardin activated SMC differentiation marker gene expression in a variety of non-SMC cells, whereas deletion of myocardin in the mouse resulted in embryonic lethality at embryonic day 10.5 due, at least in part, to failure of SMC differentiation within the developing dorsal aorta.8,9 Interestingly, cardiac development did not seem to be affected by deletion of myocardin. Additional analyses of embryonic stem cells and chimeric mice indicated that myocardin-null cells could differentiate into SMC, indicating that other transcription mechanisms could compensate for its loss at least under some conditions.10 Two myocardin-related transcription factors (MRTFs), MRTF-A and MRTF-B, have been identified that have transcriptional properties similar to those of myocardin.11 Although the MRTFs are expressed more ubiquitously, both are strongly expressed in SMC and have been shown to be required for SMC-specific gene expression in several SMC culture models.12–14 Importantly, separate groups have shown that deletion of MRTF-B in the mouse resulted in defective SMC differentiation of the cardiac neural crest cells that populate the aortic arch.15,16 In addition, deletion of MRTF-A prevented the upregulation of SMC-specific gene expression that normally occurs in myoepithelial cells of the mammary gland during...
lactation.\textsuperscript{17,18} Taken together, these data indicate that the myocardin transcription factors have unique functions that are required for SMC differentiation marker gene expression and vascular development in vitro and in vivo. Unfortunately, the overlapping expression patterns, functional redundancy, and potential heterodimerization between myocardin factors has made it difficult to determine their precise roles in SMC, especially for those aspects of SMC function that do not directly involve specification, such as the changes in SMC-specific gene expression that are known to occur during environmental stresses.

**Regulation of SRF**

A critical step in the activation of SMC-specific gene expression is SRF binding to CArG elements, and several mechanisms regulate this interaction. High SRF expression in all 3 muscle cell types likely promotes SRF binding to the relatively low affinity CArG elements present within the muscle-specific promoters. An increase in SRF expression correlates well with the appearance of the SMC during development\textsuperscript{19} and can be induced by a number of agonists, such as transforming growth factor-$\beta$ (TGF-$\beta$), that are well-known stimulators of SMC differentiation.\textsuperscript{20,21} The transcription mechanisms that regulate SRF expression are not completely clear, but the observation that multiple CArG elements control SRF promoter activity strongly suggests that high SRF expression in muscle cells is facilitated by a positive feedback loop.\textsuperscript{22,23} Phosphorylation of Ser103 by a number of kinases increases SRF affinity for CArG elements,\textsuperscript{24,25} and results from Garat et al suggest that this mechanism is important for arginine vasopressin-mediated induction of the SM $\alpha$-actin promoter.\textsuperscript{26} More recent studies from our laboratory and Iyer et al demonstrated that phosphorylation within SRF's core DNA binding domain (at Ser162 by protein kinase C$\alpha$ and at Thr159 by protein kinase A) decreased SM $\alpha$-actin promoter activity by inhibiting SRF binding.\textsuperscript{27,28} Importantly, neither of these phosphorylation events affected c-fos promoter activity because of the stabilizing effects of Elk-1 within the ternary complex.\textsuperscript{27} SRF interaction with other transcription factors and cofactors influences SRF binding most likely by steric alterations in SRF conformation. The homeodomain proteins Nkx 3.1, Prx-1, and Barx2b and the myocardin factors have been shown to enhance SRF binding,\textsuperscript{5,29–31} whereas the homeodomain-only protein (HOP) and YY1 have been shown to inhibit SRF binding.\textsuperscript{32,33}

It is extremely important to note that the ability of SRF (or any other factor) to regulate SMC differentiation absolutely requires additional mechanisms that control chromatin structure and transcription factor access to the SMC-specific gene promoters. Consistent with the concept of the "histone code," binding of SRF to SMC-specific promoters correlates strongly with positive chromatin marks (ie, H3 acetylation, H3K4 methylation, etc), and a number of laboratories, including our own, have shown that the myocardin factors interact with chromatin modifiers.\textsuperscript{34–39} For a more complete discussion of this important topic, we refer the reader to other reviews,\textsuperscript{40} including the Chen review in this series.

**Regulation of Myocardin**

Clearly, signaling mechanisms that affect myocardin levels or activity play a major role in the regulation of SMC differentiation. Hendrix et al demonstrated that myocardin mRNA expression was significantly reduced in rat carotid arteries following wire injury in a time course that closely paralleled the downregulation of SMC differentiation marker gene expression in this model.\textsuperscript{41} Although the mechanism for this downregulation is not yet clear, several studies have shown that platelet-derived growth factor (PDGF)-BB inhibits myocardin expression,\textsuperscript{42,43} and its release at sites of vascular injury probably contributes to this effect. Interestingly, Doi et al failed to observe a decrease in myocardin protein expression in a rat thoracic aorta injury model perhaps reflecting a differential response to injury governed by SMC origin.\textsuperscript{43} Several environmental cues that enhance SMC-specific gene expression, including angiotensin II and increased calcium influx, have been shown to upregulate myocardin expression.\textsuperscript{31,44,45} Callis et al demonstrated that myocardin expression in cardiomyocytes was upregulated by bone morphogenetic protein-$2$,\textsuperscript{46} but a similar effect was not observed in SMC.\textsuperscript{47} The transcription mechanisms that regulate myocardin expression are starting to be described. Nkx2.5 and nuclear activator of activated T-cells-3 were shown to bind and activate proximal myocardin promoter fragments in cardiomyocytes, and treatment of myocardial cells with aldosterone or the $\beta$-adrenergic agonist isoproterenol enhanced nuclear activator of activated T-cells-3 binding to the myocardin promoter.\textsuperscript{48,49} The relevance of these results is somewhat unclear given the recent demonstration that myocardin expression in vivo is controlled by an enhancer $\approx 25$ kb upstream of myocardin's translational start site.\textsuperscript{50} This enhancer was activated by myocyte enhancer factor-2, and several members of the Foxo and TEAD/TEF-1 transcription factor families, warranting further studies on the expression and control of these factors in SMC.

The myocardin transactivation domain can be phosphorylated by glycogen synthase kinase-3-$\beta$ and extracellular signal–regulated kinase (ERK), resulting in decreased SMC differentiation marker gene expression,\textsuperscript{51–53} and these modifications probably interfere with the transactivation domain's ability to interact with other cell-selective or general transcription factors. Of interest, myocardin has been shown to interact with the estrogen receptor coactivator steroid receptor co-activator-3 to enhance SMC-specific expression in human aortic SMC.\textsuperscript{54} Somewhat surprisingly, treatment of SMC with estrogen had little effect on this mechanism, suggesting that the protective effects of estrogen on SMC phenotypic modulation involve additional signals.\textsuperscript{55} Tang et al demonstrated that NF$\kappa$B physically interacts with myocardin to inhibit its activity.\textsuperscript{56} This important observation may explain the downregulation of SMC differentiation marker gene expression observed under inflammatory conditions such as those found in atherosclerosis. Liu et al demonstrated that myocardin activity is also inhibited by its interaction with Foxo4, and this mechanism has potential implications on the regulation of SMC phenotype by agonists that stimulate AKT.\textsuperscript{57} These authors demonstrated that insulin-like growth factor-1/AKT-dependent phosphorylation of Foxo4 induced...
protein high mobility group-2L1.43,58 Both of these factors are

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small ubiquitin-like modifier (SUMO), but because sumolya-

tion.64,65 Based on these results, we were the first to demonstrate that myocardin factor stability in SMC was enhanced by proteasome inhibition and that MRTF-A was ubiquitylated.69 Xie et al have now shown that the E3 ligase CHIP (C-terminus of HSC70-interacting protein) ubiquitylates myocardin in SMC.60 Importantly, these authors demonstrated that small interfering RNA-mediated knockdown of CHIP significantly increased the expression of several SMC differentiation marker genes, whereas overexpression of CHIP in isolated aortic rings decreased SMC contractility. Hu et al identified the E3 ligase UBR5 as a myocardin interacting protein using a yeast-2-hybrid screen, but surprisingly, UBR5 increased myocardin stability by a mechanism independent of its E3 ligase function.61 Myocardin and MRTF-A can also be modified by the small ubiquitin-like modifier (SUMO), but because sumoylation activated myocardin but inhibited MRTF-A,62,63 the functional significance of this modification in regard to SMC-specific gene expression remains unknown.

Regulation of SMC Differentiation by RhoA
Early studies from the Treisman laboratory demonstrated that SRF-dependent transcription was regulated by the small GTPase RhoA.64,65 Based on these results, we were the first to show that RhoA activity was an important determinant of SMC differentiation marker gene expression,66 but the mechanisms involved were still unknown. A seminal study by Miralles et al demonstrated that RhoA-dependent actin polymerization promoted the nuclear localization of MRTF-A and that G-actin binding to MRTF-A’s N-terminal RPEL domains inhibited this translocation.67 MRTF-B nuclear localization is also controlled by this mechanism, but because the myocardin RPEL domains do not bind G-actin very strongly, myocardin is constitutively nuclear.68 Many groups have now shown that RhoA activity is required for SMC-specific gene expression in a variety of SMC differentiation models69–74 and for the upregulation of SMC-specific transcription in response to many environmental cues, including thrombin, sphingosine 1-phosphate (SIP), TGF-β, calcium, bone morphogenetic protein-2, and cell tension.44,72,74–80 In several of these models, the activation of SMC-specific gene expression was accompanied by MRTF nuclear accumulation as measured by nuclear fractionation, immunohistochemistry, or localization of green fluorescent protein–tagged MRTF variants.75,78–80 In addition, we have shown that RhoA-dependent SMC-specific promoter activity was inhibited by expression of a nuclear localization–deficient variant of MRTF-A that traps endogenous MRTFs in the cytoplasm.12 Because this intervention does not affect myocardin activity or MRTF expression, these data provided direct evidence for MRTF nuclear translocation as an important signaling mechanism in the control of SMC-specific gene expression.

The mechanisms that regulate RhoA in SMC have not been completely described and are likely to be complex. Like all small GTPases, RhoA activation is tightly regulated by GTPase activating proteins that facilitate RhoA’s intrinsic GTPase activity (activating RhoA) and guanine exchange factors (GEFs) that facilitate exchange of GDP for GTP (activating RhoA) (see 81 for review). In SMC, RhoA activity is mainly regulated by agonists, such as angiotensin II, thrombin, and SIP, that signal through G-protein coupled receptors (GPCRs). GPCR-dependent activation of RhoA is mediated, at least in part, by Gα12/13-dependent activation of the regulator of G-protein signaling family of Rho GEFs (LARG, p115RhoGEF, and PDZRhoGEF)82 or Gα11-dependent activation of the Trio family of Rho GEFs (Trio, Duet, and p63RhoGEF).83,84 SMC typically express multiple receptors for the same agonist, and because these receptors have different G-protein coupling properties it has been difficult to predict or determine the relative importance of these proteins to SMC-specific transcription. Using a combination of receptor subtype-specific agonists for the 5 SIP receptors (S1PR1 to S1PR5), we and others have demonstrated that S1PR2 is a major activator of RhoA in SMC and is required for S1P-dependent upregulation of SMC-specific gene expression.85,86 Our data also suggest that the S1PR2 signals mainly through LARG to activate RhoA in SMC.86 Interestingly, S1PR2-deficient mice do not show an overt SMC phenotype, but Shimizu et al have recently demonstrated that they are more susceptible to injury-induced restenosis,87 suggesting that SIP signaling through this pathway helps maintain SMC differentiation in vivo. Angiotensin II–dependent activation of RhoA in SMC is much more complex and has been shown to be mediated by JAK2-dependent activation of p115RhoGEF,88 inhibition of p190Rho GTPase activating protein,89 upregulation of LARG,90 and calcium-activated phosphorylation of PDZ-RhoGEF by the tyrosine kinase PYK2.91

The signaling mechanisms downstream of RhoA that control MRTF nuclear localization involve multiple RhoA effectors that enhance actin polymerization. Rho-kinase stimulates actin polymerization by inhibiting the disassembly of actin polymers through LIM-kinase–dependent inhibition of cofillin.92 Pharmacological inhibition of Rho-kinase with Y-27632 significantly attenuates SMC-specific gene expression but does not completely inhibit it.12,44,75 The diaphanous-related formins mDia1 and mDia2 are RhoA effectors that directly catalyze actin polymerization through a mechanism that involves profilin.93,94 and we have shown that both are highly expressed in SMC and are required for SMC differentiation marker gene expression.95 Another diaphanous-related formin, formin homology domain containing protein-1, is also highly expressed in SMC.96 Although formin homology domain containing protein-1 binds Rac and not RhoA, its activity is regulated by ROCK-dependent
phosphorylation, and we have shown that this signaling mechanism contributes to SMC-specific transcription. The RhoA effector, protein kinase N, was shown to upregulate SMC-specific gene expression by a mechanism yet to be described.

Taken together, these data strongly suggest that RhoA-dependent regulation of MRTF nuclear localization is an important mechanism for the regulation of SMC differentiation marker gene expression and is facilitated in SMC by a combination of parameters, including high SRF and myocardin factor expression levels, strong GPCR-dependent activation of RhoA, and relatively high expression of downstream RhoA effectors. Finally, it is important to note that like SRF, RhoA also regulates cell proliferation, and several studies have demonstrated that RhoA signaling is required for the stimulation of SMC growth by angiotensin II, thrombin, mechanical stretch, and vessel injury. However, it is also clear that SMC growth and differentiation are not mutually exclusive and that activation of RhoA by itself is not sufficient to stimulate SMC proliferation.

**Regulation of SMC Differentiation by TGF-β**

TGF-β is a multifunctional cytokine that regulates vascular development and maintenance by controlling the growth, differentiation, and matrix synthesizing properties of endothelial cell, SMC, and lymphocytes. In most cells including SMC, TGF-β signals mainly through a heteromeric complex of 2 serine/threonine kinase receptors, the type II TGF-β receptor and the type I receptor ALK5 (see 105 for review). Within the ligand-induced receptor complex, constitutively active type II TGF-β receptor phosphorylates ALK5, resulting in the recruitment and phosphorylation-dependent activation of Smads 2 and 3. The activated Smads complex with Smad4 and then translocate to the nucleus, where they stimulate gene expression. The Smads contain DNA binding motifs and have been shown to interact with DNA at a consensus Smad binding element (GTGTC). However, because Smads bind these elements with very low affinity, it is thought that they regulate transcription in combination with additional transcription factors and cofactors.

Extensive studies have shown that TGF-β strongly stimulates SMC differentiation marker gene expression in a number of cell types, including 10T1/2, Monc-1, mesenchymal and embryonic stem cells, lung fibroblasts, and aortic SMC. This effect requires activation of Smad2, Smad3, or both, and several studies have demonstrated that these Smads interact with the SMC-specific promoters at putative Smad binding elements. The effects of TGF-β are also Cux/SRF-dependent, and studies by Qiu et al have shown that Smad3 physically interacts with SRF to facilitate SMC-specific gene expression. Interestingly, this group demonstrated that Smad3 can recruit myocardin to the SM22 promoter by a direct interaction between these transcription factors. Nishimura et al have shown that Smad3 also interacts with δEF1, a zinc finger- and homeodomain-containing protein expressed in SMC. δEF1 was upregulated by TGF-β, formed a complex with SRF and Smad3 on the SM α-actin promoter, and was required for the full effects of TGF-β on SMC-specific gene expression. TGF-β also upregulates the expression of many proteins that have secondary effects on SMC phenotype, including many matrix and matrix remodeling proteins (see 116 for review). The discovery that the effects of TGF-β on SMC-specific gene expression were mediated at least in part by the NADPH oxidase Nox4 was particularly intriguing and supported previous reports that reactive oxygen species regulate SMC-specific transcription. Interestingly, reactive oxygen species have been shown to regulate a number of critical SMC processes, but the precise mechanisms involved are currently unknown.

By poorly understood mechanisms, TGF-β directly activates (within minutes) several signaling pathways that affect SMC phenotype (see 124 for review). Activation of p38, most-likely by TGF-β-activated kinase, was shown to required for TGF-β-induced SM α-actin expression in fibroblasts and Pac-1 SMC. Activation of p38 also inhibits cell cycle progression and is required for TGF-β’s ability to inhibit SMC proliferation. Lien et al have demonstrated that TGF-β activates AKT in 10T1/2 cells and that this signaling pathway was required for TGF-β’s effects on SMC-specific transcription. TGF-β also activates RhoA in SMC and SMC precursors, and inhibition of RhoA signaling prevented TGF-β-dependent SMC differentiation marker gene expression in these models. In our hands, TGF-β is a relatively weak and slow activator of RhoA and did not stimulate MRTF nuclear localization in SMC at early time points (<2 hours), suggesting a different mechanism for its requirement. Interestingly, inhibition of RhoA prevented Smad2 and Smad3 nuclear localization, but this potentially important mechanism will require further investigation. Recent studies have shown that TGF-β upregulates the expression of multiple RhoA signaling proteins, including the Rho GEFs, Net-1, and GEF-H1, and RhoB, suggesting that TGF-β’s ability to promote long-term activation of RhoA signaling may contribute to its effects on SMC differentiation.

**Notch Signaling Plays a Dual Role in the Regulation of SMC Differentiation**

Recent studies have demonstrated that Notch signaling plays an important role in vascular development and maintenance (see 132 for reviews). Four single-pass transmembrane Notch receptors (Notch1 to Notch4) have been described in mammals, with Notch3 most strongly expressed in SMC. Importantly, the Notch receptors interact with ligands that are also transmembrane proteins (Jagged1 and 2 and Delta-like1, 3, and 5) thus limiting Notch signaling to adjacent cells. Activation of Notch by ligand binding results in proteolytic cleavage of the receptor by γ-secretase, release of the Notch intracellular domain (NICD), and translocation of the NICD to the nucleus, where it interacts with the multifunctional transcription regulator RBPJ. In the absence of NICD, RBPJ/CBF-1 binds and represses target genes by recruiting histone deacteylases and other transcriptional repressors. Displacement of the repressive factors on NICD binding and the eventual recruitment of transcriptional activators such as mastermind-like and histone acetyltransferases result in activation of Notch target genes. Major gene targets of Notch are the Hes and Hey/HERP transcription regulators.

Notch signaling has been shown to promote SMC differentiation marker gene expression, but its effects are likely depend on cell context. Overexpression of NICD in 10T1/2 or human SMC cells stimulated SMC differentiation marker gene expression.
and RBPJ was shown to interact with the SM α-actin promoter by chromatin immunoprecipitation assays. Endothelial-specific deletion of Jagged1 resulted in embryonic lethality and severe defects in SMC investment of vessels. In addition, neural crest cell–specific expression of a dominant-negative mastermind-like that inhibits all Notch family members disrupted aortic arch development and reduced SMC differentiation. The decrease in artery maturation observed in Notch3-deficient mice and the requirement for Notch3 in EC-dependent mural cell differentiation provide additional evidence that Notch activation is important for SMC identity. Interestingly, a mutation in Notch3 is causal for CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephaly), a neurovascular disorder associated with SMC abnormalities.

A number of studies have demonstrated that expression of the NICD inhibited SMC differentiation marker gene expression significantly complicating our understanding of this pathway. These inhibitory effects are due, at least in part, to upregulation of the canonical Notch target genes of the HERP/HEY family, which have been shown to inhibit SMC differentiation marker gene expression by interfering with SRF/myocardin binding to CArG elements and by directly inhibiting the function of the NICD/RBPJ complex. HERP1/HEY2 is upregulated following vascular injury and HERP1/HEY2 knockout mice have reduced neo-intimas, suggesting that this mechanism plays a role during SMC phenotypic modulation. Further supporting this idea, Notch3-deficient mice do not develop pulmonary hypertension in response to hypoxia and knockdown of HES-5 in human pulmonary artery SMC decreased proliferation and increased SMC differentiation marker gene expression. Thus, in regard to the regulation of SMC differentiation, the eventual outcome of Notch activation seems to depend on the efficacy and timing of these competing transcriptional events. Notch receptor and ligand expression levels are known to change during development and following injury, and it will be important to determine whether alterations in these parameters have significant effects on the dynamics of Notch activation. It will also be critical to determine how the Notch pathway in SMC is influenced by additional signaling and transcription mechanisms, especially those that regulate chromatin structure.

Inhibition of SMC Differentiation by PDGF-BB

The growth factor PDGF-BB is a critical regulator of early vascular development. It is highly expressed by endothelial cell and is required for the initial recruitment and subsequent proliferation of pericytes and SMC within the maturing vasculature. However, treatment of differentiated SMC with PDGF-BB strongly stimulates SMC phenotypic modu-
oration by enhancing SMC proliferation and migration and downregulating SMC differentiation marker gene expression (see 147 for review). The observations that PDGF-BB is released on vessel injury \[148\] and that inhibition of PDGF-BB signaling inhibits neointimal growth \[149\] strongly suggest that it is a major regulator of SMC phenotype in vivo. It is clear that PDGF-BB regulates SMC growth and differentiation by multiple but partially overlapping mechanisms. Activation of tyrosine kinase receptors such as PDGF receptor- \[\beta\] triggers the Ras/Raf/MEK/ERK kinase cascade, leading to Elk-1 phosphorylation and the SRF-dependent upregulation of multiple early response growth genes. Importantly, myocardin and Elk-1 bind to the same region of SRF and compete for SRF binding. \[150\] Thus, in PDGF-BB-treated SMC, phosphorylated Elk-1 inhibited SMC differentiation marker gene by displacing myocardin (and likely the MRTFs) from the SMC-specific promoters. \[150,151\] ERK-dependent phosphorylation of MRTF-A at Ser454 was also shown to inhibit MRTF-A nuclear accumulation in HeLa cells. \[152\] Results from the Owens laboratory suggest that the negative effects of PDGF-BB are at least partially mediated by the pluripotency transcription factor Krüppel-like factor-4. These authors demonstrated that Krüppel-like factor-4 is strongly induced by PDGF-BB and vascular injury and that it inhibited SMC differentiation marker gene expression by decreasing myocardin expression, interfering with SRF/myocardin factor binding to the SMC-specific promoters, and modulating the chromatin environment near the SMC-specific promoters. \[14,153,154\] Although this group has also identified a G/C repressor within the SM22 promoter that is required for its downregulation on injury or treatment with PDGF-BB, \[155,156\] it is somewhat unclear whether the inhibitory effects of Krüppel-like-factor-4 are mediated by this \textit{cis}-element.

### Integrin-Matrix Signaling

Early studies demonstrated that SMC differentiation during development correlated strongly with a change in basement membrane composition from fibronectin, which supports SMC proliferation, to collagen IV and laminin, which promote SMC differentiation. \[157-159\] Matrix degradation and the reexpression of fibronectin and other growth-promoting matrix components, such as collagen I, also occur following vessel injury and likely contribute to SMC phenotypic modulation. \[160\] Matrix components signal through heterodimeric integrin receptors composed of \[\alpha\]/\[\beta\] subunits and the mechanisms by which integrins regulate cell growth are fairly well described (see 161 for review). In brief, integrin activation leads to the formation of multiprotein focal adhesion signaling complexes and the activation of the nonreceptor tyrosine kinases focal adhesion kinase (FAK) and c-src. Tyrosine phosphorylation of FAK, Shc, and other focal adhesion scaffolding proteins, such as paxillin and p130Cas, leads to activation of the Raf/Ras/MEK/ERK kinase cascade through Grb2/Sos-dependent mechanisms. As discussed above, upregulation or downregulation of ERK signaling may explain the effects of integrin-matrix signaling on SMC differentiation marker gene expression, but other mechanisms are likely to be involved. Orr et al recently demonstrated that plating SMC on collagen IV but not on collagen I enhanced myocardin expression by enhancing SMC proliferation and migration and downregulating SMC differentiation marker gene expression (see 147 for review). The observations that PDGF-BB is released on vessel injury \[148\] and that inhibition of PDGF-BB signaling inhibits neointimal growth \[149\] strongly suggest that it is a major regulator of SMC phenotype in vivo. It is clear that PDGF-BB regulates SMC growth and differentiation by multiple but partially overlapping mechanisms. Activation of tyrosine kinase receptors such as PDGF receptor- \[\beta\] triggers the Ras/Raf/MEK/ERK kinase cascade, leading to Elk-1 phosphorylation and the SRF-dependent upregulation of multiple early response growth genes. Importantly, myocardin and Elk-1 bind to the same region of SRF and compete for SRF binding. \[150\] Thus, in PDGF-BB-treated SMC, phosphorylated Elk-1 inhibited SMC differentiation marker gene by displacing myocardin (and likely the MRTFs) from the SMC-specific promoters. \[150,151\] ERK-dependent phosphorylation of MRTF-A at Ser454 was also shown to inhibit MRTF-A nuclear accumulation in HeLa cells. \[152\] Results from the Owens laboratory suggest that the negative effects of PDGF-BB are at least partially mediated by the pluripotency transcription factor Krüppel-like factor-4. These authors demonstrated that Krüppel-like factor-4 is strongly induced by PDGF-BB and vascular injury and that it inhibited SMC differentiation marker gene expression by decreasing myocardin expression, interfering with SRF/myocardin factor binding to the SMC-specific promoters, and modulating the chromatin environment near the SMC-specific promoters. \[14,153,154\] Although this group has also identified a G/C repressor within the SM22 promoter that is required for its downregulation on injury or treatment with PDGF-BB, \[155,156\] it is somewhat unclear whether the inhibitory effects of Krüppel-like-factor-4 are mediated by this \textit{cis}-element.

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**Figure 2.** Signaling pathways that promote the phenotypic modulation of SMC. Note that myocardin levels are significantly reduced. The red arrows denote nuclear translocation events. All indicates angiotensin II; PDGFR, PDGF receptor; Inflamm, inflammation; BMP, bone morphogenetic protein; BMPRII, bone morphogenetic protein receptor II; TK, tyrosine kinase; HDAC, histone deacetylase; Ub, ubiquitin; TCF, ternary complex factor.
expression, although the mechanism for this effect was not clear. Integrin signaling is also a major regulator of the actin cytoskeleton and is required for a cell’s ability to sense and respond to mechanical forces (see 163 for review). Although the effects of these cues on SMC-specific gene expression are mediated, at least in part, by RhoA/MRTF-dependent mechanisms, the signaling pathways by which integrins, FAK, and mechanical forces regulate RhoA in cells are only partially understood. Interestingly, Kogata et al have shown that integrin-linked kinase, a very weak serine/threonine kinase that interacts with the β1 integrin receptor, negatively regulates RhoA activity in SMC and that deletion of integrin-linked kinase in PDGF receptor-β expressing cells in vivo resulted in defects in SMC investment and hypercontractility. Although Wu et al have demonstrated that integrin-linked kinase negatively regulates SMC differentiation marker gene expression, additional studies will be required to confirm that these effects were due to alterations in RhoA signaling.

Interestingly, several SMC-selective integrin and signaling molecules have been described that regulate SMC differentiation marker gene expression. First, in close collaboration with the Taylor laboratory, we have shown that FAK activity in SMC is regulated by a dominant-negative FAK variant termed FAK-related nonkinase (FRNK) that is selectively expressed in large arteries. FRNK expression is upregulated during early postnatal development and following vascular injury when SMC are transitioning from the proliferative to contractile phenotype, and FRNK promotes this transition by inhibiting SMC growth and migration and by stimulating SMC differentiation marker gene expression. In a second collaborative effort with the Taylor laboratory, we demonstrated that the SMC-selective LIM protein leupaxin interacts with SRF to activate SMC-specific gene expression, most likely by scaffolding additional transcription coactivators through its multiple LIM domains. Importantly, leupaxin localizes to focal adhesions and the nucleus, and we demonstrated that nuclear translocation of leupaxin was inhibited by plating cells on fibronectin or by expression of an active FAK variant. Chang et al demonstrated that the SMC-specific LIM proteins C/EBPα and C/EBPβ bind to SRF and facilitate SRF interaction with C/EBPα and C/EBPβ, respectively. The CRPs have been shown to bind α-actin and to localize to actin stress fibers within the cytoplasm. Whether CRP nuclear/cytoplasmic shuttling is regulated by integrin-matrix signaling is unclear but seems likely. The four and a half LIM domain–containing protein, FHL2, is a CArG-dependent cardiac/SMC-selective protein that also interacts with SRF. In contrast to the effects of leupaxin and the CRPs, FHL2 was shown to inhibit SMC-specific transcription by competitively interfering with MRTF binding to SRF. Like leupaxin, FHL2 localizes to focal adhesions, and its nuclear accumulation was enhanced by activation of RhoA, suggesting that FHL2 may act as a feedback inhibitor of RhoA/SMC-dependent gene expression.

Summary and Unanswered Questions

The discovery of the myocardin factors has provided a regulatory framework for our understanding of the control of SMC phenotype. Figure 2 summarizes many of the signaling mechanisms that stimulate or maintain SMC differentiation, and Figure 2 summarizes those that promote SMC phenotypic modulation. It is important to emphasize that significant cross-talk exists between these signaling mechanisms and that SMC phenotype likely reflects the integrated sum of these pathways. It is clear that many important questions remain. What are the epigenetic mechanisms that allow transcription factor access to the SMC-specific genes? What are the mechanisms that regulate myocardin factor expression? To what extent does RhoA-dependent MRTF nuclear localization regulate SMC differentiation in vivo? How do the direct and indirect actions of Notch affect SMC differentiation? Can we define the cis-regulatory elements and transacting factors that mediate Smad interactions with the SMC-specific promoters. What are the mechanisms by which integrin-matrix signaling regulates RhoA and the localization of the LIM protein SRF cofactors in SMC? Do signaling pathways have differential effects on SMC phenotype based on developmental origin? Can we identify novel signaling targets for the treatment of cardiovascular diseases that involve SMC phenotypic modulation? The recent development of tools for studying signaling, transcription, and chromatin networks on a genome-wide basis will certainly facilitate these efforts and will hopefully increase our understanding of the complex signaling and transcription mechanisms that regulate SMC differentiation.

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Disclosures

None.

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