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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.

Introduction

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 SM myosin heavy chain (SM MHC), 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 proliferatiion, 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 MADS box transcription factor, serum response factor (SRF), regulates most SMC differentiation marker genes by binding to highly conserved CArG cis elements ($CC(A/T)_6GG$) 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 genes 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 co-activators. The first SRF co-factors described were the ternary complex factors (TCFs) like Elk-1 that regulate early response gene expression and that interact with SRF when phosphorylated by MAP-kinase (see [4] for review). Some Nkx and Gata family members are expressed in certain SMC sub-types (Nkx3.1, Nkx3.2, and Gata-6) and have been shown interact with SRF to regulate SMC differentiation marker gene expression [5, 6].

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The discovery of the cardiac/SMC-selective SRF co-factor, 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 while deletion of myocardin in the mouse resulted in embryonic lethality at E10.5 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 ES 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, MRTF-A and MRTF-B, have been identified that have similar transcriptional properties to 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-Bin 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 up-regulation of SMC-specific gene expression that normally occurs in myoepithelial cells of the mammary gland during lactation [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 three 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 [19] and can be induced by a number of agonists such as TGF-β that are well-known stimulators of SMC differentiation [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 [22, 23]. Phosphorylation of Serine103 by a number of kinases increases SRF affinity for CArG elements [24, 25], and results from Garat et al suggested that this mechanism was important for arginine vasopressin-mediated induction of the SM α-actin promoter [26]. More recent studies from our lab and Iyer et al demonstrated that phosphorylation within SRF's core DNA binding domain (at S162 by PKCα and at T159 by PKA) decreased SM α-actin promoter activity by inhibiting SRF binding [27, 28]. Importantly, neither of these phosphorylation events affected c-fos promoter activity due to the stabilizing effects of Elk-1 within the ternary complex [27]. SRF's interaction with other transcription factors and co-factors influences SRF binding most likely by steric alterations in SRF conformation. The homeodomain proteins, Nkx3.1, Prx-1, and Barx2b, and the myocardin factors have been shown to enhance SRF binding [5, 29–31] while the homeodomain-only protein (HOP)and YY1 have been shown to inhibit SRF binding [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 (i.e. H3 acetylation, H3K4 methylation, etc)and a number of labs including our own have shown that the myocardin factors interact with chromatin modifiers [34–39]. For a more complete discussion of this important topic we refer the reader to other reviews [40] including the Chen review in this series.

Regulation of myocardin

Clearly, signaling mechanisms that affect myocardin levels and/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 down-regulation of SMC differentiation marker gene expression in this model [41]. Although the mechanism for this down regulation is not yet clear, several studies have shown that PDGF-BB inhibits myocardin expression [14, 42] 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 [43]. Several environmental cues that enhance SMC-specific gene expression including angiotensin II and increased calcium influx have been shown to up-regulate myocardin expression [31, 44, 45]. Callis et al demonstrated that myocardin expression in cardiomyocytes was up-regulated by BMP-2 [46], but a similar effect was not observed in SMC [47]. The transcription mechanisms that regulate myocardin expression are starting to be described. Nkx2.5 and NFATc3were shown to bind and activate proximal myocardin promoter fragments in cardiomyocytes, and treatment of myocardial cells with aldosterone or the β-adrenergic agonist, isoproterenol, enhanced NFATc3 binding to the myocardin promoter [48, 49]. The relevance of these results is some what unclear given the recent demonstration that myocardin expression in vivo is controlled by an enhancer ~25kb upstream of myocardin's translational start site [50]. This enhancer was activated by MEF2, and several members of the Foxo and TEAD transcription factor families, warranting further studies on the expression and control of these factors in SMC.

The myocardin transactivation domain can be phosphorylated by GSKβ and ERK resulting in decreased SMC differentiation marker gene expression [51–53], and these modifications probably interfere with the transactivation domain's ability to interact with other cell-selective and/or general transcription factors. Of interest, myocardin has been shown to interact with the estrogen receptor co-activator, SRC3, to enhance SMC-specific expression in human aortic SMC [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 involves additional signals [55]. Tang et al demonstrated that NFkB physically interacts with myocardin to inhibit its activity [56]. This important observation may explain the down regulation 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 Foxo4and this mechanism has potential implications on the regulation of SMC phenotype by agonists that stimulate AKT [57]. These authors demonstrated that IGF-1/AKT-dependent phosphorylation of Foxo4 induced its translocation from the nucleus to the cytoplasm, thus relieving Foxo4-dependent inhibition of SMC differentiation marker gene expression. The bHLH transcription factors, Msx1 and Msx2, interact with myocardin to inhibit SMC-specific transcription, and the up-regulation of these factors by BMPs may be critical for the modulation of SMC phenotype under conditions that promote vessel calcification [47]. Myocardin activity is also inhibited by

the bHLH factor, HERP1/HEY2, and the HMG protein, HMG2L1 [43, 58]. Both of these factors are up-regulated in phenotypically modulated SMC and inhibit myocardin's interaction with SRF.

The recent demonstration that the myocardin factors are targeted for proteasome-mediated degradation could have significant implication on the regulation of SMC differentiation. We were the first to demonstrate that myocardin factor stability in SMC was enhanced by proteasome inhibition and that MRTF-A was ubiquitylated [59]. Xie et al have now shown that the E3 ligase, CHIP (C-terminus of HSC70-intreacting protein), ubiquitylates myocardin in SMC [60]. Importantly, these authors demonstrated that siRNA-mediated knockdown of CHIP significantly increased the expression of several SMC differentiation marker genes while 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-two-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 Ubiqutin-like MOdifier (SUMO), but because sumolyation 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 lab demonstrated that SRF-dependent transcription was regulated by the small GTPase, RhoA [64, 65]. Based upon 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 since 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 models [69–74] and for the up-regulation of SMC-specific transcription in response to many environmental cues including thrombin, sphingosine 1-phosphate (S1P), TGF-β, calcium, BMP-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 GFP-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]. Since 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 (GAPs) that facilitate RhoA's intrinsic GTPase activity (inhibiting 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 (AII), thrombin, and S1P that signal through G-protein coupled receptors (GPCRs). GPCR-dependent activation of RhoA is mediated, at least in part, by $G\alpha_{12/13}$-dependent activation of the RGS family of RhoGEFs (LARG, p115RhoGEF, and PDZRhoGEF) [82] and/or $G\alpha_{q/11}$-dependent activation of the Trio family of RhoGEFS (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 sub-type-specific agonists for the fiveS1P receptors (S1PR1-5), we and others have demonstrated that S1PR2 is a major activator of RhoA in SMC and is required for S1P-dependent up-regulation of SMC-specific gene expression [85, 86]. Our data also suggest that theS1PR2 signals mainly through LARG to activate RhoA in SMC [86]. Interestingly, S1PR2deficient 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 S1P 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 byJAK2-dependent activation of p115RhoGEF [88], inhibition of p190RhoGAP [89], up-regulation 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 cofilin [92]. Pharmacologic inhibition of Rho-kinase withY-27632 significantly attenuates SMC-specific gene expression, but does not completely inhibit it [12, 44, 75]. The diaphanous-related formins (DRFs), 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 DRF, FHOD1 (formin homology domain containing protein-1), is also highly expressed in SMC [96]. Although FHOD1 binds Rac and not RhoA, its activity is regulated by ROCK-dependent phosphorylation [97], and we have shown that this signaling mechanism contributes to SMC-specific transcription [96]. The RhoA effector, protein kinase-N, was shown to up-regulate SMC-specific gene expression by a mechanism yet to be described [77].

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 demonstrate that RhoA signaling was required for the stimulation of SMC growth by AII, thrombin, mechanical stretch, and vessel injury [98–102]. 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 [75, 95, 103, 104].

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 EC, SMC, and lymphocytes. In most cells including SMC, TGF-β signals mainly through a heteromeric complex of two serine/threonine kinase receptors, the type IITGF-β receptor (TβRII)and the type I receptor, ALK5 (see [105] for review). Within the ligand-induced receptor complex, constitutively active TβRII 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 (GTCT). However, because Smads bind these elements with very

low affinity it is thought that they regulate transcription in combination with additional transcription factors and co-factors.

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 [20, 106–113]. This effect requires activation of Smad2 and/or Smad3, and several studies have demonstrated that these Smads interact with the SMC-specific promoters at putative Smad binding elements [107, 108, 114, 115]. The effects of TGF-β are also CArG/SRF-dependent [20], and studies by Qui et al have shown that Smad3 physically interacts with SRF to facilitate SMC-specific gene expression [114]. Interestingly, this group demonstrated that Smad3 can recruit myocardin to the SM22 promoter by a direct interaction between these transcription factors [115]. Nishimura, et. al. have shown that Smad3 also interacts with δEF1, a zinc finger- and homeodomain-containing protein expressed in SMC [112]. δEF1 was up-regulated 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 up-regulates 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, [117–120] was particularly intriguing and supported previous reports that reactive oxygen species regulate SMC-specific transcription [121, 122]. Interestingly, reactive oxygen species have been shown to regulate a number of critical SMC processes [123] 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 [77, 125, 126]. Activation of p38 also inhibits cell cycle progression and is required for TGF-β's ability to inhibit SMC proliferation [127]. 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 [128]. 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 [74, 77]. 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 (< 2h) [12] suggesting a different mechanism for its requirement. Interestingly, inhibition of RhoA prevented Smad2 and Smad3 nuclear localization [74], but this potentially important mechanism will require further investigation. Recent studies have shown that TGF-β up-regulates the expression of multiple RhoA signaling proteins including the Rho GEFs, Net-1 and GEF-H1, and RhoB [129–131] 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 (Notch1–4) 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 binds and represses target genes by recruiting HDACs

and other transcriptional repressors. Displacement of the repressive factors upon NICD binding and the eventual recruitment of transcriptional activators such as MAML and histone acetyltransferases results 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 upon cell context. Over-expression 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 ChIP assays [133, 134]. Endothelial-specific deletion of Jagged1 resulted in embryonic lethality and severe defects in SMC investment of vessels [135]. In addition, neural crest cell-specific expression of a dominant negative MAML that inhibits all Notch family members disrupted aortic arch development and reduced SMC differentiation [136]. The decrease in artery maturation observed in Notch3 deficient mice [137] and the requirement for Notch3 in EC-dependent mural cell differentiation [138] 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 [139].

A number of studies have demonstrated that expression of the NICD inhibited SMC differentiation marker gene expression significantly complicating our understanding of this pathway [140, 141]. These inhibitory effects are due, at least in part, to up regulation 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 [43, 142]. HERP1/HEY2 is up-regulated following vascular injury and HERP1/HEY2 knockout mice have reduced neointimas suggesting that that this mechanism plays a role during SMC phenotypic modulation [43, 143]. 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 [144]. Thus, in regard to the regulation of SMC differentiation, the eventual outcome of Notch activation seems to depend upon the efficacy and timing of these competing transcriptional events. Notch receptor and ligand expression levels are known to change during development and following injury [145] 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 EC and is required for the initial recruitment and subsequent proliferation of pericytes and SMC within the maturing vasculature [146]. However, treatment of differentiated SMC with PDGF-BB strongly stimulates SMC phenotypic modulation by enhancing SMC proliferation and migration and down-regulating SMC differentiation marker gene expression (see [147] for review). The observations that PDGF-BB is released upon 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 PDGFRβ triggers the Ras/Raf/MEK/ERK kinase cascade leading to Elk-1 phosphorylation and the SRF-dependent up-regulation 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, KLF4. These authors demonstrated that KLF4was strongly induced by PDGF-BB and vascular injury, and 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 down-regulation upon injury or treatment with PDGF-BB [155, 156], it is somewhat unclear whether the inhibitory effects of KLF4 are mediated by this 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 re-expression 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 α and β 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 non-receptor tyrosine kinases, FAK and c-src. Tyrosine phosphorylation of FAK, Shc, and other focal adhesion scaffolding proteins such paxillin a p130Cas, leads to activation of the Raf/Ras/MEK/ERK kinase cascade through Grb2/Sos-dependent mechanisms. As discussed above, up- or down-regulation 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, although the mechanism for this effect was not clear [162]. 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 [76, 80, 164, 165], the signaling pathways by which integrins, FAK, and mechanical forces regulate RhoA in cells are only partially understood [166]. Interestingly, Kogata et al have shown that integrin-linked kinase (ILK), a very weak serine/threonine kinase that interacts with the β1 integrin receptor, negatively regulates RhoA activity in SMC and that deletion of ILK in PDGFRβ expressing cells in vivo resulted in defects in SMC investment and hypercontractility [167]. Although Wu, et al have demonstrated that ILK negatively regulates SMC differentiation marker gene expression [168], additional studies will be required to confirm that these effects were due to alterations in RhoA signaling.

Interestingly, several SMC-selective integrin and/or signaling molecules have been described that regulate SMC differentiation marker gene expression. First, in close collaboration with the Taylor lab, we have shown that FAK activity in SMC is regulated by a dominant negative FAK variant termed FRNK (FAK-related non-kinase) that is selectively expressed in large arteries [169]. FRNK expression is up-regulated during early post-natal 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 [170, 171]. In a second collaborative effort with the Taylor lab, we demonstrated that the SMC-selective LIM protein, leupaxin, interacts with SRF to activate SMC-specific gene expression most likely by scaffolding additional transcription co-activators through its multiple LIM domains [172]. 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, CRP1 and CRP2 enhanced SMC-specific gene expression by facilitating SRFs interaction with Gata–6 [173]. The CRPs have been shown to bind α-actinin and to localize to actin stress fibers within the cytoplasm [174]. 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 [175]. 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 [176] suggesting that FHL2 may act as a feedback inhibitor of RhoA/SRF-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. Figures 1 summarizes many of the signaling mechanisms that stimulate and/or maintain SMC differentiation while 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 still 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 trans-acting factors that mediate Smad interactions with the SMC-specific promoters. What are the mechansims by which integrin-matrix signaling regulate RhoA and the localization of the LIM protein SRF co-factors in SMC? Do signaling pathways have differential effects on SMC phenotype based upon 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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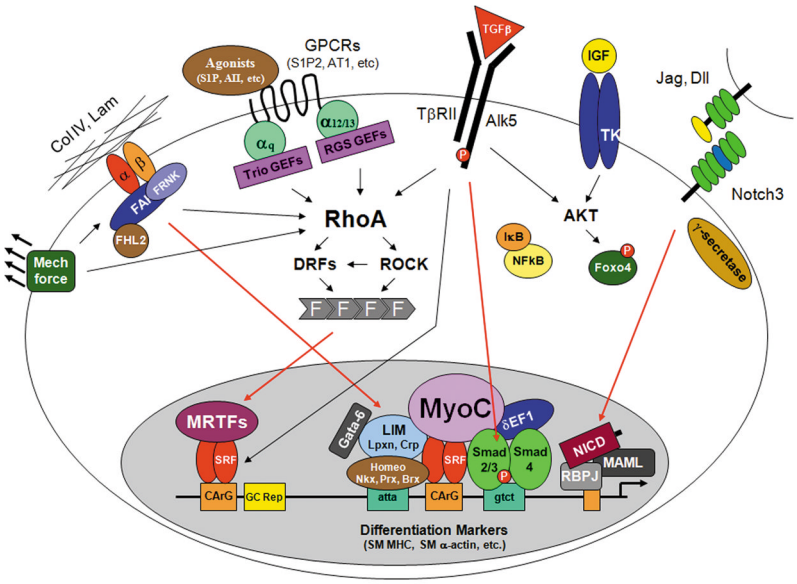

Figure 1. Signaling pathways that stimulate and/or maintain SMC differentiation
The red arrows denote nuclear translocation events.

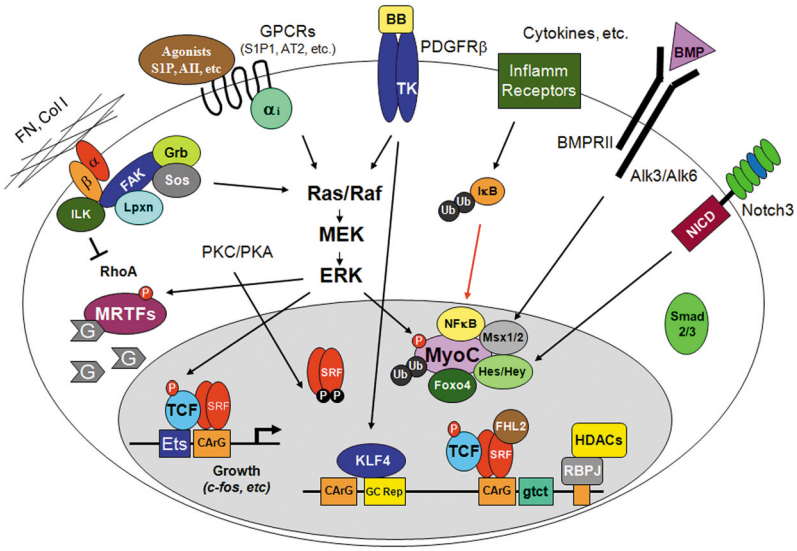

Figure 2. Signaling pathways that promote the phenotypic modulation of SMC

Note that myocardin levels are significantly reduced. The red arrows denote nuclear translocation events.