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Invited Review

Multiple repressor pathways contribute to phenotypic switching of vascular smooth muscle cells

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[1]*Department of Molecular Physiology and Biological Physics, University of Virginia School of Medicine, Charlottesville, Virginia; and* [2]*Department of Medicine and Biological Science, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan*

Kawai-Kowase K, Owens GK. Multiple repressor pathways contribute to phenotypic switching of vascular smooth muscle cells. *Am J Physiol Cell Physiol* 292: C59–C69, 2007. First published September 6, 2006; doi:10.1152/ajpcell.00394.2006.—Smooth muscle cell (SMC) differentiation is an essential component of vascular development and these cells perform biosynthetic, proliferative, and contractile roles in the vessel wall. SMCs are not terminally differentiated and possess the ability to modulate their phenotype in response to changing local environmental cues. The focus of this review is to provide an overview of the current state of knowledge of molecular mechanisms involved in controlling phenotypic switching of SMC with particular focus on examination of processes that contribute to the repression of SMC marker genes. We discuss the environmental cues which actively regulate SMC phenotypic switching, such as platelet-derived growth factor-BB, as well as several important regulatory mechanisms required for suppressing expression of SMC-specific/selective marker genes in vivo, including those dependent on conserved G/C-repressive elements, and/or highly conserved degenerate CArG elements found in the promoters of many of these marker genes. Finally, we present evidence indicating that SMC phenotypic switching involves multiple active repressor pathways, including Krüppel-like zinc finger type 4, HERP, and ERK-dependent phosphorylation of Elk-1 that act in a complementary fashion.

serum response factor; platelet-derived growth factor-BB

THERE IS CLEAR EVIDENCE that alterations in the differentiated state of the vascular smooth muscle cell (SMC) play a critical role in the pathogenesis of atherosclerosis, as well as a variety of other major human diseases, including hypertension, asthma, and vascular aneurysms. The precise nature of phenotypic switching is highly variable in these different diseases, with changes in atherosclerosis and vascular aneurysms involving rather profound changes in SMC morphology, function, and gene expression patterns, compared with the much more subtle changes in contractility associated with asthma and hypertension (69). Moreover, the nature and role of SMC phenotypic switching varies greatly at different stages of these diseases. This property is perhaps best illustrated by the pathogenesis of atherosclerosis where accelerated migration, proliferation, and production of extracellular matrix components by phenotypically modulated SMC play a critical role in lesion development. In contrast, the balance in production of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases, and extracellular matrix components by SMC (and macrophages) within the fibrous caps of advanced atheromatous lesions is a critical determinant of plaque stability (51). The fact that vascular SMC are not terminally differentiated and retain the ability to modulate their phenotype to changing environmental cues likely evolved as a key mechanism to allow repair of vascular injury and adaptation of SMC contractile mass to match functional demands. However, an unfortunate consequence of SMC plasticity is that cells are susceptible to various stimuli that can induce changes in phenotype that contribute to the etiology of SMC-related diseases. A major challenge for the field has been to identify environmental cues, signaling pathways, and molecular mechanisms that normally control differentiation of SMC and how these are disrupted in disease states.

Although the term "phenotypic modulation" or "phenotypic switching" was originally based largely on morphological criteria, over the past decade its definition has been expanded by the vascular biology field to encompass the full range of possible alterations in functional and structural properties that can be exhibited by the SMC in response to changing environmental cues, including both profound but also subtle changes in gene expression patterns, signaling mechanisms, contractility. The prevailing theory has been that the majority of intimal SMC are derived from the media of vessel (76), although recent studies have provided evidence suggesting that at least a portion of intimal SMC, particularly in models of graft arteriosclerosis, may be derived from various alternative sources, including circulating bone marrow-derived stem cells (82, 86) and/or resident Sca1+ adventitial stem cells (see Ref. 27 for review; 29). There is also evidence suggesting that SMC or SMC-like cells within an injured blood vessel in animal models, or human atherosclerotic lesion may be derived from a variety of sources, including medial SMC (91), *trans*-differ-

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entiation of endothelial cells (12) and adventitial fibroblasts (46, 83; see Ref. 81 for review). However, irrespective of the source of SMC, the principal of local environmental cues impacting the patterns of gene expression and behavior of these cells apply.

Expression of many SMC marker genes has been shown to be dependent on multiple $CC(A/T)_6GG$ (CArG) elements and their binding factor, serum response factor (SRF) (48, 55, 59, 63). SRF has been reported to activate multiple smooth muscle (SM) genes by recruiting several other cofactors; this topic is elegantly reviewed by Miano et al. (64). Indeed, until now, no master genes have been found to play a role comparable to that of MyoD (15) in skeletal muscle development, although several genes have been found to have some role in this process. One of the most significant advances in this field of SMC differentiation was the identification of myocardin, which was discovered as a co-activator of SRF-dependent transcription exclusively expressed in cardiac and SMC lineages (8, 14, 95, 103). Myocardin activates smooth and cardiac muscle promoters by its interaction with SRF. Of significance, myocardin has been shown to selectively induce expression of all CArG-dependent SMC marker genes tested to date, including smooth muscle α-actin (SM α-actin), SM-myosin heavy chain (SM-MHC), SM22α, calponin, and telokin, but not c-*fos* (102). Studies of telokin (25) are of particular interest in that this gene, unlike all other CArG-containing SMC marker genes characterized to data, has a single rather than multiple CArG elements, as is the case for early response genes, such as c-*fos*. Indeed, a series of highly innovative studies by Herring and co-workers (24) have defined unique mechanisms by which this single CArG-containing SMC promoter confers SMC specificity.

Myocardin-induced activation of SMC genes appears to depend on the interaction between myocardin and SRF rather than on direct DNA binding, such that an understanding of mechanisms that control SRF binding to CArG elements is critical to fully understanding SMC-selective gene expression. Mouse embryos homozygous for a myocardin loss-of-function mutation died by E box 10.5 and showed failed development of SMC, whereas cardiac development appeared to be normal (50). However, autonomous myocardin is not absolutely required for SMC development, in that we, in collaboration with Olson and co-workers (71), recently showed that homozygous myocardin-null embryonic stem (ES) cells can differentiate into SMC in vitro within an ES cells-embryoid body model of SMC differentiation in vivo as well as in vivo in the context of chimeric knockout mice produced by injection of lineage tagged myocardin null ES cells into wild-type blastocysts. In brief, myocardin-null ES cells populated both vascular and nonvascular SMC tissues, and stained with antibodies to the SMC marker genes such as SM α-actin and SM-MHC. However, it remains to be determined whether myocardin-null ES cells exhibit more subtle defects in SMC differentiation and/or function. In addition, although it is likely that the myocardin-related transcription factors, MRTF-A (MKL1) and/or MRTF-B (MKL2) (96), can compensate for loss of myocardin within chimeric knockout mice, at present there is no direct experimental evidence for this. Indeed, there is emerging evidence that different SMC subtypes may exhibit differential dependence on myocardin and/or MRTFs. For example, in 2005, the laboratories of Parmacek (47) and Olson (68) independently showed that MRTF-B is required for differentiation of SMC in neural crest-derived SMC but dispensable for SMC development in other tissues. As such, there is ambiguity as to the unique vs. overlapping functions of myocardin vs. MRTFs in SMC development. However, myocardin is exclusively expressed in SMC and cardiac myocytes (14), whereas MRTFs are expressed ubiquitously, thus raising key, as yet unresolved, questions as to the mechanisms and role of MRTFs in controlling SMC-selective gene expression. Additional studies (52, 73, 93, 101) have also shown that myocardin plays an important role in mediating inducible expression of CArG-dependent SMC marker genes in response to a variety of environmental cues, including angiotensin II, L-type voltage-gated Ca^{2+} channels/RhoA, transforming growth factor-β (TGFβ), and platelet-derived growth factor (PDGF)-BB. These observations are intriguing in that they suggest that myocardin may play a key role in regulating changes in SMC contractile mass commensurate with functional demands.

Although SMC can undergo quite diverse forms of phenotypic switching, a hallmark feature of SMC phenotypic switching during vascular injury or repair in many SMC-related disease states is downregulation of expression of SMC-specific/selective marker gene, such as SM α-actin, SM-MHC, SM22α, and desmin. As such, there has been interest in identifying molecular mechanisms that repress SMC marker gene expression. As will be summarized in the next section, there is evidence that SMC phenotypic switching results not only from loss of positive differentiation signals and pathways, but also by induction of multiple complementary active repressor pathways that suppress SMC gene expression. This review will summarize recent advances in our understanding of the molecular mechanisms that control SMC phenotypic switching with a particular emphasis on consideration of mechanisms that alter expression of the large repertoire of SMC marker genes whose expression is dependent on CArG elements in their promoter-enhancers and binding of SRF and SRF co-activators, such as myocardin and the MRTFs. For a much more comprehensive review of CArG-SRF transcription, see the elegant recent paper by Miano (62).

PHENOTYPIC SWITCHING IS ACTIVELY REGULATED AND IS NOT SIMPLY DUE TO LOSS OF POSITIVE DIFFERENTIATION FACTORS OR SIGNALS

There are a plethora of local environmental cues that have been postulated to play a key role in normal control of SMC differentiation, including contractile agonists, TGFβ, extracellular matrix proteins (integrins, adhesion molecules, collagen, and elastin), mechanical forces, neuronal influences, and cell-cell interactions (see Ref. 69 for a review). In response to vascular injury, these environmental cues are disrupted and SMC undergo rapid and quite profound changes in their phenotype, including suppression of SMC marker genes, and activation of a host of proliferative and synthetic genes that play a critical role in vascular wound repair.

Although the precise factors that mediate injury-induced phenotypic switching in vivo have not been clearly defined, there is evidence implicating involvement of PDGF-BB. For example, following vascular injury, loss of endothelial integrity results in platelet adhesion, degranulation, release of PDGF-BB and other platelet constituents, as well as upregu-

lation of expression of PDGF receptor-β (PDGFRβ) (2, 77, 99). Conventional knockout of PDGF-A (4), or PDGF-B (44), or their receptors PDGFR-α (89), and PDGFR-β (88) results in early embryonic or perinatal lethality, thus precluding the use of knockout mice to test the role of these growth factors in vivo in SMC phenotypic switching. However, there is evidence that acute injury of the normal artery promotes phenotypic switching of SMC that is significantly dependent on PDGF-B signals. For example, administration of an antibody to PDGF-AB that neutralized the activity of PDGF-AA, -BB, and -AB was found to reduce medial proliferation of SMC (45) as well as SMC migration (32) in a rat carotid artery balloon injury model. Moreover, administration of antisense oligonucleotides to the PDGFR-β (67) and PDGF-B aptamers (43) inhibited SMC accumulation after vascular injury. In contrast, antibodies to PDGFR-α failed to significantly inhibit SMC accumulation following balloon injury in the nonhuman primate (18, 20), suggesting the effects are mediated by PDGF-BB, which can signal only via PDGFRβ rather than PDGFRα. PDGF-BB is also unique among SMC mitogens in its ability to induce profound phenotypic switching of cultured SMC through effects that do not appear to be a function of growth stimulation per se, and which involve active repression of SMC gene expression at both the transcriptional and posttranscriptional levels (3, 10, 11, 92). Indeed, the latter effects are quite remarkable, in that PDGF-BB stimulation is associated with selective degradation of SMC marker gene proteins and mRNAs, suggesting that mechanisms have evolved such that the phenotypic transition process can be extremely rapid, and that are actively mediated rather than simply involving repression of transcription, followed by loss of marker proteins as a function of their normal half lives. There is also compelling evidence that PDGF-BB can actively suppress transcription of SMC marker genes through several different complementary pathways that will be reviewed later in this paper.

However, there are several lines of evidence suggesting that fully differentiated vascular SMC are refractive to PDGF-BB stimulation. First, mature SMC appear to express very low levels of PDGFRβ, although it is rapidly induced following mechanical injury of the blood vessel (18, 58, 99). Second, SMC within intact blood vessels in organ culture appear to be unresponsive to PDGF-BB unless first subjected to mechanical injury in vivo or cultured for extended periods in vitro in serum containing medium (65, 75). As such, it is likely that SMC phenotypic switching following injury is quite complex and involve a combination of loss of environmental cues that normally promote and/or maintain the differentiated state of SMC, as well as induction of active repressor and growth activation pathways by factors such as PDGF-BB and other cytokines. Of interest, results of a recent study (6) showed that PDGF-BB and IL-1β were cooperative in repressing SMC differentiation of human aortic SMCs cultured on polymerized collagen through phosphatidylinositol 3-kinase/Akt/P70S6K pathways. Moreover, recent studies have shown that MMP-2 or -9 knockout mice reduced neointimal formation after vascular injury in vivo (34), and combined MMP-2 and ApoE knockout mice showed reduced atherosclerotic plaque formation and intimal SMC mass compared with ApoE knockout mice alone (41). Although the precise mechanisms for these effects are unclear, the results are just one example of many that implicate a key role for extracelluar matrix components in myointimal formation as well as SMC proliferation and phenotypic switching. Taken together, these results suggest that injury-induced phenotypic switching in vivo is complex and is unlikely to be mediated by any single factor alone. Indeed, to date, there are very few direct studies of mechanisms that mediate SMC phenotypic switching in vivo, as will be summarized in the next section.

REPRESSIVE PATHWAYS IMPLICATED IN SMC PHENOTYPIC SWITCHING IN VIVO

To begin to define which of the wide plethora of altered environmental cues associated with vascular injury contribute to phenotypic switching of vascular SMC, we asked the following questions. First, is injury-induced downregulation of SMC marker genes mediated at the transcriptional level? Second, if so, what specific *cis*-elements and *trans*-binding factors are required for this effect? Previously, our laboratory and others have identified sufficient regions of SM α-actin, SM-MHC, and SM22α promoters to drive expression of β-galactosidase in a manner that parallels the expression of the endogenous gene throughout embryonic development and in adult mice (37, 49, 55, 56). We carried out a series of vascular injury experiment in these transgenic mice, and showed nearly complete loss of expression of all three transgenes following vascular injury, thus demonstrating for first time that SMC phenotypic modulation in vivo was mediated at least in part by transcriptional repression (74). We subsequently completed a series of studies using site-directed mutagenesis and transgenic mouse models to define *cis*-elements required for injury-induced repression of SM22α and SM α-actin in vivo (23, 74, 94) (see Fig. 1).

Of major interest, we found that mutation of a conserved G/C repressor element within the SM22α promoter virtually abolished downregulation of this gene in response to wire-induced injury of the carotid artery (74), as well as within phenotypically modulated SMC within myointimal lesions of ApoE knockout mice (94) (see Fig. 1*A*). The G/C-rich repressor element has been shown to be located proximal to the distal CArG element within SM22α and SM-MHC promoters. These promoters share a conserved G/C-rich element, which have been shown to bind with Sp1/Sp3 in electrophoretic mobility shift assays, and possibly other zinc finger transcription factors, including members of the Krüppel-like zinc finger family (KLF) and egr-1, which are normally absent in differentiated SMC in vivo, but rapidly induced in neointima by vascular injury (36, 52, 57, 94, 98). Whereas the precise mechanisms that regulate the G/C repressor are not yet resolved, there is indirect evidence suggesting that repression of SMC marker genes may be regulated by injury-induced increases in expression of these genes that bind to the G/C repressor element. Although chromatin immunoprecipitation assays clearly showed that PDGF-BB greatly decreased SRF enrichment of the CArG-G/C-CArG motif within both the SM22α and SM-MHC promoters, we were not able to detect Sp1 enrichment of this promoter region associated with PDGF-BB treatment within intact chromatin (94), suggesting that Sp1 may not bind directly on G/C repressor. An alternative possibility is that Sp1 may induce (or activate) other transcription factors, which then bind the G/C-rich element. Of interest, we found that overexpression of KLF4, which was originally identified as a binding

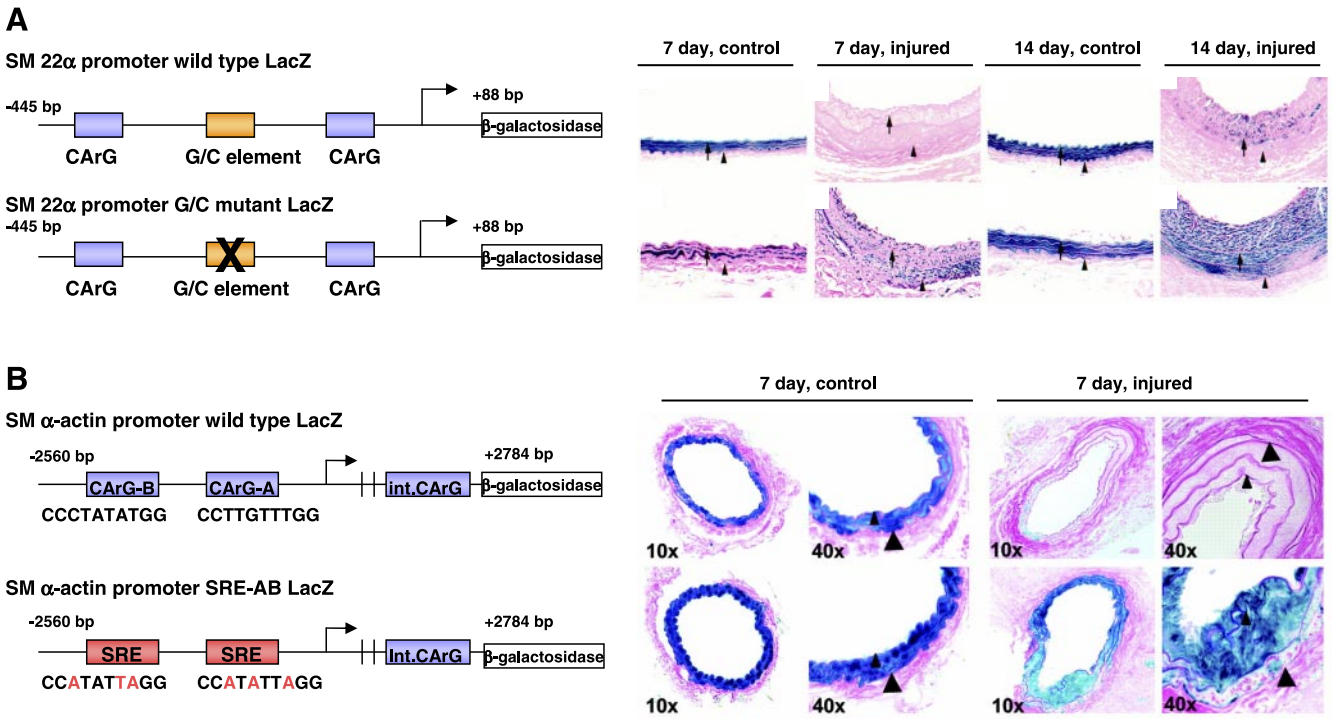

Fig. 1. Injury-induced suppression of smooth muscle cell (SMC) marker gene. *A*: β-galactosidase staining of mouse carotid arteries from SM22α promoter wild-type *Lac*Z and G/C mutant *Lac*Z transgenic mice 7 or 14 days after injury or uninjured controls. Mutation of G/C element within SM22α promoter-enhancer-*Lac*Z transgene significantly attenuated injury-induced suppression of SM22α transactivation (see Ref. 74). *B*: substitution of the c-*fos* serum response element (SRE) consensus CC(A/T)$_6$GG (CArG) for both CArG-A and CArG-B within SM α-actin promoter resulted in a significant attenuation in the transgene response to injury. Unlike the wild-type SM α-actin gene, which was dramatically downregulated in response to injury, the SRE-AB transgene was expressed in the media and developing intima of the injured carotid artery (see Ref. 23).

factor to the G/C-rich TGFβ control element within SM22α and SM α-actin promoter (1, 53): *1*) markedly suppressed expression of myocardin; *2*) nearly abolished myocardin-induced activation of SMC marker genes; *3*) significantly reduced SRF binding to CArG-containing regions of SMC marker genes in the context of intact chromatin, as determined by quantitative chromatin immunoprecipitation assays; and *4*) induced hypoacetylation of histone H4 at SMC CArG regions indicative of transcriptional silencing (52, 61). Moreover, we found evidence for binding of KLF4 to SRF based on immunoprecipitation assays and that overexpression of wild-type SRF, but not a MADS box mutant SRF could partially reverse the effect of KLF4 in suppressing myocardin-induced activation of CArG-dependent SMC marker genes (52). Taken together, results indicate that KLF4 acts through combination of mechanisms, including direct competition for binding of SRF to CArG regions of SMC promoters, marked suppression of myocardin expression, and inducing changes in chromatin structure consistent with formation of heterochromatin and transcriptional silencing.

We have also shown that injury-induced suppression of SM α-actin expression is dependent on degenerate CArG elements found in the 5′-region of this promoter (23) (Fig. 1*B*). Indeed, the promoters of many SMC marker genes contain degenerate CArG element with single G/C substitutions within the internal A/T region of the CArGs that have been conserved across ALL species, and which dramatically lower SRF binding affinity, compared with the consensus CArG sequences found in serum response genes such as c-*fos* (5, 21). Results of our previous studies showed that substitution of SM α-actin 5′ CArGs with the c-*fos* SRE consensus CArG significantly attenuated injury-induced downregulation in transgenic mice, although this substitution had no effect on smooth muscle-specific expression during normal development and maturation in transgenic mice (23). We also demonstrated that expression of myocardin mRNA was significantly decreased within 3 days following injury compared with the uninjured control, but it returned to control level by 7 days after rat carotid artery injury, during which time there was reinduction of SMC gene expression in medial SMC in this injury model (23). We hypothesized that the reduction in myocardin expression resulted in loss of SRF binding to the wild-type degenerate SM α-actin CArG elements (and silencing), whereas the SRE-substituted mutant SM α-actin promoter bound SRF despite reductions in myocardin levels. Consistent with this hypothesis, we showed that the wild-type SM α-actin promoter exhibited a greater myocardin dependency than the SRE substituted promoter. Moreover, myocardin increased SRF association with the CArG-containing region of the SM α-actin promoter but not of the c-*fos* promoter in intact chromatin (23, 61). Taken together, these observations indicate that although CArG degeneracy is not a critical determinant of SMC specific expression of SMC marker genes, it is required for repression of expression of these genes in response to vascular injury.

Indeed, the preceding studies of G/C-repressive element and CArG degeneracy provide a potential mechanism to explain a long-term paradox in the field as to how CArG-SRF dependent SMC marker genes could be repressed, yet CArG-SRF-depen-

dent growth regulatory genes like *egr*-1 and c-*fos* activated in the context of vascular injury, in which SRF expression is dramatically induced. That is, although SRF levels are high and contribute to activation of growth regulatory genes, SMC marker genes are repressed due to: *1*) loss of myocardin and/or other pathways required for enhancing SRF binding to degenerate CArG elements within the promoters of SMC marker genes; and *2*) active suppression of SMC marker gene expression as a result of induction of G/C-element binding factors including KLF4 which in turn suppress SRF binding to CArG regions of SMC marker genes within intact chromatin and by suppressing myocardin expression. Of interest, upregulation of KLF4 expression would simultaneously modulate SMC growth since it can both activate and repress genes that are involved in cell-cycle regulation and differentiation, such as p53 and p21, respectively, in cancer cells (78, 100, 104). The preceding model is undoubtedly overly simplistic. Indeed, results thus far clearly suggest that SMC phenotypic switching in vivo is extremely complex and likely dependent on a multitude of complementary repressor mechanisms, which may play differential roles depending on the nature of the inducing signals and other environmental cues present.

PDGF-BB REPRESSES SMC GENE EXPRESSION THROUGH MULTIPLE COMPLEMENTARY REPRESSOR PATHWAYS

Although there is no direct evidence that PDGF-BB is required for phenotypic switching of vascular SMC in vivo, there is certainly circumstantial evidence for its involvement (see *Repressive Pathways Implicated in SMC Phenotypic Switching in vivo*). Indeed, PDGF-BB is the only factor described to date that can induce rather profound suppression of SMC marker genes (3, 10, 11, 92). The aim of this section will be to summarize studies that have examined molecular mechanisms by which PDGF-BB-induces phenotypic switching of SMC both for purposes of highlighting potential mechanisms and experimental approaches for further testing PDGF-BB involvement in vivo, as well as for defining important down stream effectors of PDGF-BB induced phenotypic switching that may also be involved in mediating responses to other growth factors or cytokines that may contribute to SMC phenotypic switching during vascular injury as well as experimental atherosclerosis. Key transcriptional repressors and pathways that will be considered include: KLF4, phosphorylated Elk-1, HERP1, FOXO4, YY1, FHL2, and several homeobox proteins. These mechanisms are not mutually exclusive, and it is likely that combinations of these factors are important in modulating SMC differentiation through both myocardin/MRTF-A/B-dependent and -independent pathways (Fig. 2).

As summarized in *PDGF-BB represses SMC gene expression through multiple complementary repressor pathways*, we have previously shown that the G/C repressor element located between the 5′-CArG elements of the SM22α promoter is required for downregulation of this gene in vivo following vascular injury or experimental atherogenesis (74, 94). Moreover, we have shown that KLF4, which can bind to the G/C-repressive element, can potently repress expression of multiple SMC marker genes through a combination of effects including suppression of myocardin expression, inhibiting SRF binding to intact chromatin, and suppressing myocardin-induced gene activation (52, 61). Of interest, PDGF-BB treatment of cultured SMC is associated with marked induction of KLF4 expression in a manner similar to vascular injury in vivo. Moreover, we have shown that suppression of KLF4 expression using either siRNAs or antisense oligonucleotides markedly inhibited PDGF-BB induced repression of multiple SMC marker genes including SM α-actin, SM-MHC, and SM22α (Ref. 52 and R. A. Deaton and G. K. Owens, unpublished data). In addition, suppression of KLF4 expression in control cultures also resulted in increased SMC marker gene expression suggesting that KLF4 plays some role in suppressing SMC gene expression, even under basal condition in cultured SMC. Moreover, we demonstrated that myocardin and KLF4 exert opposing influences over SRF-CArG binding to SMC genes in cultured SMC (61). That is, myocardin overexpression increased SRF binding and H4 acetylation of histones at SMC CArG promoter regions within intact chromatin, and markedly activated SMC marker gene expression. In contrast, KLF4 had the opposite effects of decreasing SRF binding and H4 acetylation in cultured SMC. Moreover, we showed that KLF4 could completely suppress myocardin-induced activation of SM-MHC *Lac*Z transgene in vivo (61). Finally, we presented evidence that the effects of KLF4 on chromatin structure were dependent on recruitment of HDAC2 to SMC genes, resulting in deacetylation of histone H4, chromatin compaction, and loss of myocardin/SRF binding. However, the precise mechanisms that regulate both normal and abnormal differentiation of SMC in vivo are presently very poorly understood, since conventional knockout of KLF4 (84), PDGFs and PDGF receptors (4, 44, 88, 89), which are implicated in SMC phenotypic switching, results in early embryonic or prenatal lethality. Key unsolved questions include the following. *1*) Is KLF4 required for phenotypic switching of SMC in vivo? *2*) Does KLF4 also contribute to alternation in CArG independent SMC marker genes and if so by what mechanisms? *3*) Is KLF4 a common downstream effector of SMC phenotypic switching in response to other environmental cues or cytokines involved in vascular disease? Of interest, KLF5, which is a member of KLF family, induces embryonic type MHC (SMemb/NMHC-B) and PDGF-A genes, and the heterozygotes knockout mice of KLF5 revealed diminished levels of SMC and adventitial cell activation in response to external stress (85). Moreover, oral administration of Am80, which inhibits the activity of KLF5, significantly inhibited in-stent neointima formation in rabbit stent-placement models (17). Taken together, these results provide evidence that KLF family members play an important role in phenotypic modulation of SMC following vascular injury.

Wang et al. (97) have presented evidence showing that PDGF-BB-induced suppression of CArG-SRF-myocardin dependent transcription is mediated by phosphorylation of Elk-1, a ternary complex factor family of Ets domain proteins. In brief, they showed that PDGF-BB induced phosphorylation of Elk-1 through MEK1/2 and that phosphorylated Elk-1 but not unphosphorylated Elk-1 competed for binding of SRF to CArG elements in the promoters of SMC marker genes, including SM22α, and SM α-actin. These studies were subsequently extended by Herring and co-workers (105, 107), who presented evidence that ERK-dependent phosphorylation of the SRF co-activator Elk-1 is likely to modulate not only selective silencing of CArG-dependent SMC genes but also activation of growth regulatory gene. That is, it is well established that phosphorylation of Elk-1 increases its binding to the c-*fos*

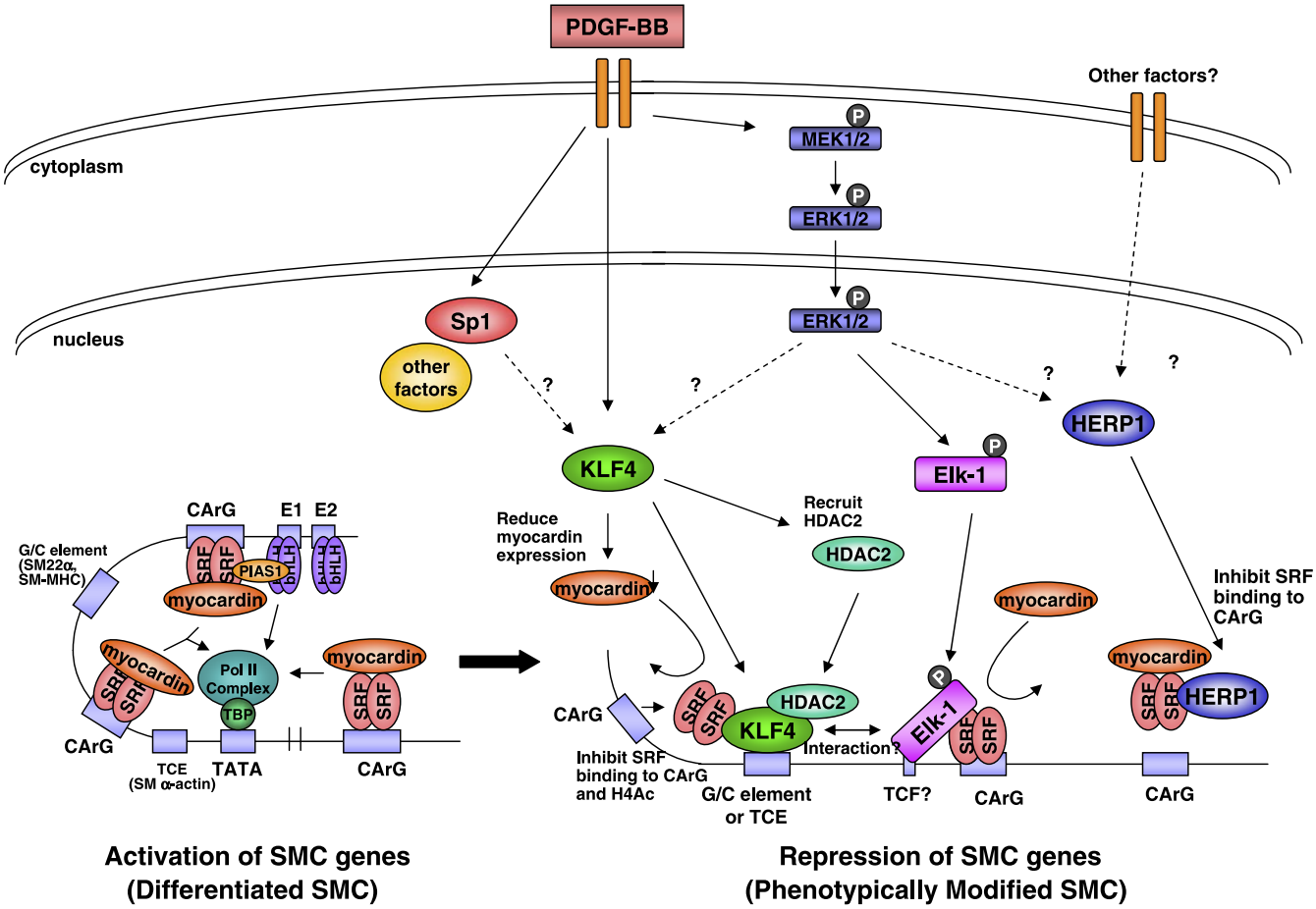

Fig. 2. Model for the regulation of phenotypic switching of SMC selective/specific gene. This figure presents a schematic model illustrating some of the complex protein-protein and DNA-protein interactions thought to be important in determining PDGF-BB-induced repression of multiple SMC selective/specific marker genes. In the differentiated state, transcription by the TATA binding protein (TBP)-containing RNA polymerase II complex is dependent on cooperative interactions between multiple *cis*-elements and transcription factors, such as CArG-serum response factor (SRF)-myocardin complexes (8, 14, 95, 103) and E box-bHLH protein-PIAS1 complex (35, 40) in SM α-actin promoter, as shown at *left*. In this model, PDGF-BB-induced phenotypic switching of vascular SMC has been shown to be mediated by multiple mechanisms, including expression of Krüppel-like zinc finger factor type 4 (KLF4) (52, 61), Sp1 (94), HERP1 (13, 72, 79), and/or phosphorylation of Elk-1 (97). One simple model is that PDGF-BB simultaneously induces phosphorylation of Elk-1, as well as increased KLF4 expression. KLF4 suppresses myocardin expression and recruits HDAC2, which in turn causes histone hypoacetylation and reduces SRF binding. There is also evidence that KLF4 binds to G/C repressive element within SM22α and SM-MHC promoter, and the TGFβ control element within SM α-actin promoter, and inhibits cooperative interaction of paired CArG elements located in the 5′-region of virtually all SMC marker genes. PDGF-BB induced MEK1/2-ERK1/2-dependent phosphorylation Elk-1, which inhibits myocardin-SRF interactions. There is also evidence that HERP1 may further augment these effects by inhibiting binding of SRF/myocardin binding to CArG elements. Finally, injury-induced suppression of SMC marker genes may also involve loss of positive differentiation signaling pathways and mechanisms, including downregulation of the homeodomain factor Prx1, which at least in culture is required for binding of SRF to degenerate CArG elements, found the majority of SMC marker genes characterized to date (22, 101).

promoter and activation of transcription in response to growth factors. Conversely, it is likely that the inability of myocardin to activate the c-*fos* promoter is due, at least in part, to the binding of Elk-1 to the serum response element of the c-*fos* promoter. Consistent with this idea, they showed that deletion of the Elk-1 binding site within the c-*fos* promoter conferred partial sensitivity of the c-*fos* promoter to myocardin (106). Of interest, these studies showed that Elk-1 suppressed the activity of smooth muscle-restricted promoters, including the telokin promoter that does not contain a consensus Elk-1 binding site, through its ability to block myocardin-induced activation of the promoters (107).

An additional repressor pathway implicated in control of SMC phenotypic switching both in vivo and in response to PDGF-BB in cultured SMC are HERP1 (also known as CHF1/Hey2, Hesr-2, and HRT2) (9, 30, 38, 42, 66, 105) transcription factors. HERP1-deficient vascular SMC proliferated slowly compared with wild-type SMC and also showed decreased migration in response to PDGF-BB and HB-EGF in vitro using cultured HERP1-null SMC (79). Moreover, neointimal formation after arterial wire injury was markedly diminished in HERP1 knockout mice when compared with wild-type mice (79). Of interest, results of recent studies showed that both HERP1 and HERP2 transcripts were abundantly expressed in aorta as well as the heart. HERP1 expression was colocalized with SM α-actin-positive cells in rat neointima in rat balloon injury models and human coronary atherosclerotic lesion (13). HERP1 suppressed myocardin-dependent transactivation of SM-MHC and SM22α promoter, and HERP1 interfered with SRF binding to CArG box within intact chromatin through physical association with SRF (13, 72). In summary, results suggest that HERP1 may act in concert with KLF4, phospho-

Elk-1, to suppress transcription of CArG-SRF-myocardin/MRTF-dependent genes. However, it must be noted that the effects observed on SMC in conventional HERP1 knockout mice may be indirect since HERP1 could be expressed in a wide variety of cell types, such as macrophage, endothelial cells, and other inflammatory cells, which are known to contribute to vascular diseases. In addition, further studies are needed to explore the precise molecular interactions of HERP1 with SRF, myocardin/MRTFs, phospho-Elk-1, and KLF4, as well as if different vascular SMC subtypes differentially utilize one repressor pathway vs. another.

Other possible transcription factors which have been implicated in PDGF-BB-induced suppression of SMC marker genes include Sp1 and ets1. Previously, we demonstrated that PDGF-BB increased the expression of Sp1 and ets1, and that over expression of these factors suppressed SMC marker genes expression in cultured SMC (11, 94). In addition, we found that siRNA or antisense oligonucleotide induced suppression of Sp1 inhibited PDGF-BB-induced suppression of multiple CArG dependent SMC marker genes. As described above, we initially postulated that Sp1 binding to the G/C repressor which lines between the two 5′ CArGs in both the SM22α and SM-MHC promoters may directly impair SRF binding and/or cooperativity between 5′ CArG elements and thereby inhibit transcription. However, we were unable to detect Sp1 binding to the G/C repressor within intact chromatin either in control or PDGF-BB treated SMC. Of interest, since the KLF4 promoter contains four conserved Sp1 binding sites, it is interesting to postulate that the role of Sp1 in inhibiting SMC gene expression is indirect and mediated through induction of KLF4.

Thus far, we have considered evidence that PDGF-BB-induced suppression of SMC genes involves inhibition of myocardin function by phosphorylated Elk-1, as well as increased expression of KLF4, HERP1, Sp1, and/or ets1. Of interest, results of several recent studies have identified several additional factors that may contribute to control of SMC phenotypic switching, but which have not yet been implicated in the effects of PDGF-BB. For example, Liu et al. (54) showed that the forkhead transcription factor FOXO4 repressed SMC differentiation marker gene expression by interacting with and inhibiting the activity of myocardin as well as SRF. In addition, FOXO4 was upregulated in proliferating SMC of the injured neointima. Although mice homozygous for a FOXO4 null allele are viable and do not display overt abnormalities (28), it is important to extend this study to determine whether FOXO4 contributes to vascular remodeling following injury in vivo. Similarly, the small homeodomain-only protein HOP was found to inhibit the ability of myocardin to activate SRF-dependent transcription of ANF and SM22α, and interacted with SRF and modulated its DNA binding. These workers also provided evidence that HOP increased binding of HDAC2 to the SM22α promoter thus promoting deacetylation of histone H4 and transcriptional repression (39). Of interest, the CArG boxes present in the promoters of many genes has been shown to contain binding sites for the transcription factor Yin Yang-1 (YY1) that was induced in SMC in response to vascular injury and human carotid media (31, 33, 37, 80). Although YY1 has been shown to inhibit SRF binding, repress c-*fos*, skeletal α-actin and cardiac α-actin expression (7, 19), and inhibit SRF activity by actin cytoskeleton (16), overexpression of YY1 can transactivate the mouse SM22α promoter in cultured rat aortic SMC (37). Strobeck et al. showed that at least two additional nuclear proteins other than SRF and YY1 are enriched in SMC and bind to CArG elements within SM22α promoter (90). It is thus interesting to speculate that a SM-specific ternary factor, possibly myocardin, may change YY1 function within SMC-specific genes. Another transcription factor implicated in CArG-SRF transcriptional control is FHL2/DRAL/Slim3, a LIM domain containing protein that has been shown to interact with SRF and bind to the promoter of SMC-specific CArG-dependent genes in response to RhoA (70). Of interest, FHL2 was shown to inhibit SRF-dependent transcription of SMC-specific genes by competing with MRTF-A for SRF binding (70). Since FHL2 is highly expressed in the heart and has not been reported to be expressed in vascular SMC, it is conceivable that FHL2 functions as an inhibitor of SMC genes in cardiac muscle. However, the role of FHL2 for SMC phenotypic switching in vivo has been unknown. Thus, it is interesting to postulate that regulation of myocardin/MRTFs by forkhead transcription factors, homeobox proteins, YY1 and/or FHL2 may also play a key role in regulation of SMC phenotypic switching. However, further direct studies are needed examining contributions of these factors in vivo, as well as to factors like PDGF-BB that can induce phenotypic switching of cultured SMC.

Taken together, the results presented in this section indicate that there appear to be multiple repressive pathways that contribute to SMC phenotypic switching. Of course, these mechanisms are not mutually exclusive, and it is likely that a combination of these factors is important in modulating SMC phenotype.

CONCLUSION AND PERSPECTIVES

SMC differentiation is an essential component of vascular development and these cells perform biosynthetic, proliferative, and contractile role in the vessel wall. SMC are not terminally differentiated and possess the ability to modulate their phenotype in response to changing environmental cues. There is also compelling evidence that "phenotypic switching" of SMC is actively regulated, and is not simply a function of loss of normal positive differentiation cues. One of the keys to understanding SMC differentiation and phenotypic switching is to identify the *cis*-regulatory elements and transcription factors that regulate transcription of SMC selective/specific genes. Although numerous environmental cues, including growth factors, cell-cell contacts, and extracellular matrix components, affect the modulation of SMC phenotype in culture systems, the precise mechanisms controlling SMC phenotypic switching in vivo are poorly understood. Indeed, at present, to our knowledge, only three studies have defined specific *cis*-elements required for injury-induced and/or atherosclerosis associated downregulation of SMC marker genes in vivo (23, 74, 94). The results of these studies indicated that: *1*) CArG degeneracy is required for SMC phenotypic switching in vascular injury, and *2*) the G/C repressor element within the SM22α promoter mediates transcriptional repression of this gene within phenotypically modulated SMC in experimental atherosclerosis lesions as well as vascular injury. There are several transcriptional repressor pathways, including PDGF-B, PDGFRβ, KLF4, and Sp1 that have been shown to mediate silencing of SMC marker genes in in vitro model systems but

as yet there is no direct evidence these are functional in vivo. However, direct investigation of their role in SMC phenotypic switching following vascular injury or in experimental atherosclerosis will be dependent on development of conditional knockout and/or chimeric knockout mice, since conventional knockout of each of these genes is associated with early embryonic or prenatal lethality (4, 44, 60, 84, 88, 89). In addition, there is a critical need to *1*) identify humoral factors that mediate SMC phenotypic switching other than PDGF-BB and in particular to investigate the role of inflammatory cytokines and other pro-atherogenic factors found within diseased blood vessels; *2*) elucidate specific molecular mechanisms and factors that confer responsiveness of SMC in intact blood vessels to humoral factors like PDGF-BB which can clearly induce profound changes in gene expression patterns in cultured SMC but which appear to be incapable by themselves of doing this in fully differentiated SMC in vivo; *3*) more fully characterize the nature of changes in gene expression that characterize SMC phenotypic switching in different disease states including examination of genes that play a functional role in the disease pathophysiology; and *4*) better understand differences and similarities in regulation of SMC phenotypic switching between diverse SMC subtypes, including coronary arteries, the outflow tract, large conduit arteries in the body trunk, and peripheral arteries, which have diverse embryological origins (26), as well as functional properties (87).

Finally, the so-called synthetic state SMC presumably evolved as a highly regulated state of the SMC for purpose of vascular injury repair, a process that is undoubtedly critical for survival, and hence these mechanisms have been conserved across very diverse species and hundreds of millions of years of evolution. In contrast, virtually all diseases in which SMC phenotypic switching plays a key role, including atherosclerosis, have their negative survival consequences beyond the reproductive period such that there presumably has been little or no evolutionary selection pressure against these changes. As such, although SMC phenotypic switching in disease may share some common regulatory mechanisms and mediators with those that occur with vascular injury/repair, there may also be key differences that are critical to the pathobiology of SMC-related diseases. It is also important to emphasize the importance of temporal changes in the nature and consequences of SMC phenotypic switching at different stages of atherosclerotic disease development, and/or within different plaque regions. For example, during development of fibrous cap, SMC proliferation and migration are required to stabilize the plaque, although in end-stage disease, these cells can alter gene expression patterns and contribute to destabilization of the fibrous plaque. That is, rather than inducing a phenotype that aids in vascular repair and is beneficial, in some case these factors may induce a form of SMC phenotype that is pathophysiological and exacerbates the disease state through production of MMPs that degrade the extracellular matrix, expression of inflammatory cytokines that further augment recruitment of inflammatory cells to the site of injury, and/or cause SMC apoptosis. If this occurs within an advanced atherosclerotic plaque, the consequence may be devastating and lead to plaque rupture, and a fatal myocardial infarction.

Taken together, it is becoming clear that regulation of phenotypic switching of SMC is a complex trait of this unique cell type that has evolved in higher organisms as means to optimize repair of vascular damage, as well as blood vessel repair and regeneration. This extensive plasticity of SMC has unfortunately also made the cell susceptible to maladaptive changes in phenotype in a number of major disease states. Thus elucidation of mechanisms that control this process are of critical importance not only for understanding normal SMC development, but also the etiology of major human diseases such as atherosclerosis, hypertension, asthma, and cancer that are characterized by abnormal control of SMC differentiation.

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