Molecular Determinants of Vascular Smooth Muscle Cell Diversity

Tadashi Yoshida, Gary K. Owens

Abstract—Although the primary role of vascular smooth muscle cells (SMCs) is contraction, they exhibit extensive phenotypic diversity and plasticity during normal development, during repair of vascular injury, and in disease states. Results of recent studies indicate that there are unique as well as common transcriptional regulatory mechanisms that control expression of various SMC marker genes in distinct SMC subtypes, and that these mechanisms are complex and dynamic even at the single cell level. This article will review recent progress in our understanding of the transcriptional regulatory mechanisms involved in controlling expression of SMC marker genes with a particular focus on examination of processes that contribute to the phenotypic diversity of SMCs. In addition, because of considerable controversy in the literature regarding the relationship between phenotypically modulated SMCs and myofibroblasts, we will briefly consider both similarities and differences in regulation of gene expression between these cell types. (Circ Res. 2005; 96:280-291.)

Key Words: smooth muscle cells ■ serum response factor ■ myofibroblasts

The primary function of vascular smooth muscle cells (SMCs) in mature animals is contraction. Differentiated SMCs express a unique repertoire of contractile proteins, contractile agonist receptors, and signaling molecules to perform this function and exhibit an extremely low rate of proliferation and low synthetic activity.1 However, SMCs also maintain considerable plasticity throughout life and can exhibit a diverse range of different phenotypes in response to changes in local environmental cues.1 This plasticity and the diversity of SMC phenotypes presumably evolved to allow SMCs to carry out a broad range of functions that vary considerably in different SMC-containing organs during normal development and maturation (eg, gastrointestinal versus vascular, large versus small arteries, arteries versus veins), as well as to permit repair of injury to SMC tissues resulting from mechanical trauma or inflammation. For example, during early embryonic development, SMCs exhibit a high rate of proliferation and migration.2-3 During late embryogenesis, production of extracellular matrix (ECM) proteins is increased in SMCs,4 while expression of SMC marker genes is robustly induced.5 In contrast, these processes are much reduced in SMCs within mature organisms that are in steady state with respect to expression of the repertoire of proteins required for their specialized contractile function. Indeed, the relative synthesis rates of virtually all SMC marker genes and ECM proteins are extremely low in normal adult SMCs as compared with levels during periods of rapid growth because of the long half-lives of these proteins. After disruption of the normal steady state of

Original received November 22, 2004; revision received December 22, 2004; accepted January 4, 2005.
From the Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville.
Correspondence to Gary K. Owens, PhD, Department of Molecular Physiology and Biological Physics, University of Virginia, MR5 Rm 1220, 415 Lane Rd, PO box 801394, Charlottesville, VA 22908. E-mail gko@virginia.edu
© 2005 American Heart Association, Inc.
Circulation Research is available at http://www.circresaha.org DOI: 10.1161/01.RES.0000155951.62152.2e

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mature SMCs, as occurs in association with vascular injury or disease, SMCs undergo a process often referred to as phenotypic modulation/switching (see a detailed discussion of this topic in Owens et al9) characterized by dramatic increases in the rates of proliferation, migration, and synthesis of ECM proteins, and decreased expression of SMC-specific/selective marker genes such as smooth muscle (SM) α-actin, SM-myosin heavy chain (MHC), SM22α, h1-calponin, smoothelin, caldesmon, and telokin.1 Within normal adult organisms, there is also clear evidence of major functional differences in SMCs in different types of blood vessels and vascular beds including differences in (1) myogenic tone between large versus small arteries,7 (2) calcium regulation in phasic versus tonic blood vessels,7 and (3) signaling pathways resulting in variable sensitivities to drugs such as sildenafil that selectively target blood vessels within the male reproductive system.8 It is thus clear that vascular SMCs exhibit extensive phenotypic diversity at different developmental stages, under different pathophysiological conditions, and even under normal conditions depending on the anatomic location of the vessels.

There is also evidence for SMC heterogeneity within a given SMC tissue under normal conditions. Several investigators have reported the isolation of clonal populations of SMCs or SMC-like cells, under conditions of severe vascular injury, inflammation, or tissue/organ transplantation/rejection.6 Most of these studies have focused on distinguishing two phenotypes: the spindle-shaped “contractile” SMCs and the epithelioid SMCs with a “synthetic” phenotype. These observations are quite interesting although the precise origins and functional significance of these subpopulations in vivo are unclear.

There is also clear evidence that vascular SMCs have diverse embryological origins.10 Most vascular SMCs are derived from local mesodermal populations. However, the major blood vessels in the head and neck contain a significant fraction of cells derived from the neural crest that appear to play a key instructive role in the complex morphogenesis of brachial arch–derived vessels. Similarly, SMCs of coronary arteries are derived from the proepicardial organ. Although highly controversial, there is also recent evidence suggesting that circulating stem cells derived from bone marrow give rise to SMCs or SMC-like cells, under conditions of severe vascular injury, inflammation, or tissue/organ transplantation/rejection.6

The preceding studies provide clear evidence illustrating the remarkable diversity of phenotypic states and origins of vascular SMCs. However, the precise molecular mechanisms that regulate these diverse phenotypic states are relatively poorly understood. Complete consideration of the mechanisms that control SMC plasticity is clearly beyond the scope of this review. Moreover, this area has recently been reviewed elsewhere in considerable depth.8,11 A major focus of this review will be to summarize evidence indicating that there are common as well as unique transcriptional regulatory programs that control expression of SMC marker genes, and to consider how these mechanisms contribute to the diversity of SMC phenotypes.

Regional Heterogeneity in Molecular Control of SMC Marker Gene Expression

Expression of most of the SMC marker genes characterized to date, including SM α-actin,12 SM-MHC,13 SM22α,14 and h1-calponin,15 has been shown to be dependent on multiple CArG elements located within their promoter-enhancer regions. The CArG element has the general sequence motif, CC(A/T-rich)GG, and its binding factor is the ubiquitously expressed transcription factor, serum response factor (SRF).11 The steady-state expression levels of these SMC marker genes appear to be relatively similar between different SMC subtypes.1 However, despite this fact, several recent studies from our laboratory13,16 and others17–20 clearly indicate that SMCs within different vascular beds can be distinguished at the molecular level by virtue of their utilizing distinct cis-elements and modular control regions to regulate expression of these genes. That is, expression of at least some SMC differentiation markers is controlled by different intracellular mechanisms among distinct SMC subtypes.

SMC Marker Gene Expression in SMC Subtypes Exhibit Differential Cis-Element Requirement

One of the most striking examples is illustrated by results of studies in our laboratory showing that expression of the SM-MHC gene requires different modular control regions in different SMC subtypes.13 The SM-MHC gene is one of the most definitive markers for SMC differentiation in that its expression is highly restricted to SMCs throughout development and maturation.21 Studies using transgenic mice harboring SM-MHC promoter-enhancer-LacZ reporter construct (SM-MHC-LacZ mice) demonstrated that SMC-specific expression of the SM-MHC gene required 4.2 kb of the 5′-flanking region, the entire first exon, and 11.5 kb of the first intronic sequence.22 Although wild-type SM-MHC-LacZ mice exhibited expression of the transgene in all SMC tissues including both large and small arteries, mutation of each of the conserved three CArG elements within the SM-MHC promoter-enhancer showed very different effects in distinct SMC subtypes.13 CArG1, which is located at −1112 bp within the 5′-flanking region of the promoter, was found to be required for expression in all SMC subtypes. In contrast, CArG2 at −1231 bp was dispensable for large blood vessels, but had a minor role in controlling expression within the gastrointestinal and urinary tracts. Of major interest, results of mutation of the intronic CArG element located at +1599 bp showed that the intronic CArG element was absolutely required for expression within large elastic arteries such as the abdominal aorta but was completely dispensable for expression in most other SMC tissues (Figure 1). Indeed, extremely strong expression of the intronic CArG mutant LacZ transgene was seen in the renal artery at the point where it branches from the abdominal aorta. That is, over a distance of only a fraction of a millimeter along the vascular tree, expression of SM-MHC gene transitions from being absolutely dependent on the intronic CArG element to being completely independent of that cis-element. Further studies in our laboratory also provided evidence showing that different SMC subtypes utilized unique combinations of regulatory modules contained within the −4.2 to +11.6 kb SM-MHC
promoter-enhancer region to drive expression in vivo. For example, deletion of the region from +9 to +11.6 kb significantly attenuated expression in vascular SMCs but had little or no impact on expression in nonvascular SMCs. These findings support the hypothesis that, although different SMC subtypes all express SM-MHC protein at relatively high levels and in a manner that is highly specific for SMCs, the molecular mechanisms that control its expression show some major differences.

This property of distinct transcriptional control within SMC subtypes is not unique to the SM-MHC gene, in that similar findings have been reported for expression of SM22α and cysteine-rich protein-1 (CRP-1) genes. SM22α is expressed in smooth, cardiac, and skeletal muscle lineages during early embryogenesis and becomes restricted to SM lineages at late stages and adulthood. Several laboratories investigated regulatory mechanisms for cell-specific expression of the SM22α gene. Of interest, each of these groups found that a region of the SM22α promoter containing just over 400 bps of 5'-flanking sequence was sufficient to direct expression in arterial SMCs as well as skeletal and cardiac muscle within mouse embryos, but was virtually inactive in SMCs within small arterioles, veins, venules, the bladder, gastrointestinal tract, and other SMC tissues. Similarly, CRP-1 is also expressed in all muscle cell types during embryogenesis and predominates in vascular and visceral SMCs in the adult. Lilly et al analyzed the 5-kb enhancer within the CRP-1 gene using transgenic mice and found that this region was sufficient to drive expression in arterial but not venous and visceral SMCs in vivo.

Taken together, results from the preceding studies are consistent with our studies of the SM-MHC gene, and indicate that distinct transcriptional regulatory mechanisms exist for the control of SM-MHC, SM22α, and CRP-1 genes within different SMC subtypes.

**SMC Subtypes Exhibit Common as Well as Distinct Transcriptional Regulatory Mechanisms**

Although the precise mechanisms underlying the differential requirement of cis-elements among distinct SMC subtypes remain unknown, there are several possibilities: (1) differential expression and/or function of transcription factors; and/or (2) selective control of binding of transcription factors to these cis-elements. Expression of the SMC marker genes described above, including SM-MHC, SM22α, and CRP-1, has been shown to be dependent on highly conserved CArG elements and their trans-acting factors including SRF and the very potent SRF coactivator, myocardin, which is expressed exclusively in smooth and cardiac muscles. However, it is not at all clear how differences in expression levels and/or
posttranslational modifications of SRF, myocardin, or other known transcription factors including SMC-selective GATA family, MEF2 family, basic Helix-Loop-Helix (bHLH) proteins, homeodomain proteins, and Krippel-like zinc-finger factors (KLFs) might explain differential molecular control of SMC gene expression. For example, why is CArG/SMF-dependent expression of the SM-MHC gene in the aorta dependent on three CArG elements whereas only the two 5′ CArGs are required in other SMC tissues? One possibility is that the intronic CArG is dispensable in SMC tissues other than the aorta because of differences in the local environmental milieu that exists in these tissues as compared with the aorta. That is, environmental cues present in smaller arteries and arterioles may result in activation of alternative regulatory pathways that function independent of the intronic CArG element. However, it is also possible that differences in regulation of the SM-MHC gene in these SMC subtypes are quantitative rather than qualitative. That is, high levels of expression of SRF and/or myocardin may obviate a requirement for three versus two CArG elements. Consistent with this hypothesis, several laboratories including our own have shown that, although multiple CArG elements appear to be required for myocardin-induced gene activation, this requirement is relaxed, at least in cultured cell systems, by marked overexpression of myocardin.25–28 It is also possible, indeed likely, that the differences in alternative splicing, postranslational modifications, or intracellular localization of key SMC transcription factors that vary as a function of the environmental milieu in which the cell exists contribute to differences in the requirements for specific cis-elements among distinct SMC subtypes. Consistent with this possibility, expression of SMC marker genes, at least in cultured cells, has been shown to be influenced by an endogenous dominant-negative form of SRF,29 changes in phosphorylation of SRF,30 and regulation of the translocation of SRF from the nucleus into cytoplasm.31 However, as yet there is no direct evidence that quantitative or functional differences in these regulatory mechanisms contribute to SMC diversity in vivo. In addition, there are no reports whether differential expression and/or function of these regulatory pathways contribute to phenotypic differences between SMCs in arteries versus veins or between muscular versus elastic arteries. Of interest, Adams et al32 performed a systematic analysis of gene expression in the media of aorta and vena cava using a cDNA microarray containing 4048 known human genes to determine differences observed in different SMC subtypes in transgenic mice. However, they found no differences in expression of transcription factors between these two SMCs.

Another possibility is that different SMC subtypes may differ by virtue of the accessibility of transcription factor binding to cis-elements within intact chromatin. Results from our previous studies showed that, in the context of intact chromatin, SRF could bind to CArG elements of the SM-MHC gene in cultured SMCs, whereas SRF could not access CArG elements of this gene in non-SMCs including myoblasts, as determined by chromatin immunoprecipitation assays.13 Moreover, we found evidence for histone modifications consistent with chromatin relaxation within CArG-containing regions of SMC marker genes in SMCs but not other cell types. Qiu et al33 presented evidence implicating involvement of histone acetylation in transcription of the SM22α gene based on studies that administration of inhibitors of histone deacetylases augmented SMC gene expression. It is interesting to speculate that differential control of the accessibility of transcription factors to cis-elements within intact chromatin plays a key role in regulation of SMC plasticity and diversity. Such controls might be “imprinted” during SMC development and persist in adult tissues, and/or may be subject to dynamic regulation by differing environmental cues and signals that exist normally within SMC subtypes or in association with vascular injury or disease.

To What Extent Are the Distinct Transcriptional Regulatory Properties of SMC Subtypes Reversible?

A major unresolved question with respect to regional SMC heterogeneity illuminated by studies in transgenic mice is the extent to which regional diversity of SMCs is patterned during embryonic development and is relatively fixed as opposed to being dynamically regulated by changes in local environmental cues including mechanical forces, cell-matrix interactions, and exposure to vasoactive and neuronal stimuli unique to each respective vascular site. There is a virtual absence of studies in this area, and we know very little about the capacity of SMCs from one tissue or region to undergo reprogramming on transplantation. For example, if one were to transplant SMCs from the renal artery of intronic CArG mutant SM-MHC-LacZ mice into the aorta, would the transgene be silenced? Conversely, would transfer of SMCs from the aorta to the renal artery result in activation of the previously silent transgene? Although intellectually attractive, such experiments are likely to be confounded by the injury associated with the gene transfer process and the resulting effects on SMC phenotype. Indeed, the fact that the transfer process itself is likely to be highly disruptive of the environmental milieu at the recipient site has been a major confounding variable in attempts to carry out SMC transfer experiments.

The diverse embryological origins of SMCs are one factor that has been postulated to contribute to the innate diversity of SMC phenotypes. However, it seems unlikely this is the sole explanation for SMC diversity. For example, there appeared to be no obvious correlations between LacZ staining patterns observed in different SMC subtypes in transgenic mice and known difference in SMC origins. Nevertheless, of interest, studies by Topouzis and Majesky34 showed persistence of major differences in morphology, growth, and transforming growth factor-β1 (TGF-β1) responsiveness between cultured avian vascular SMCs isolated from the abdominal aorta that are derived from mesoderm, versus those from the ascending thoracic aorta, which are derived from neural crest. Despite isolation and maintenance of these two SMC subtypes in an identical fashion, these cells exhibited
profound differences in morphology and in their qualitative responses to TGF-β1. That is, the thoracic aorta–derived SMCs were stimulated to grow by TGF-β1, whereas those from the abdominal aorta were growth inhibited. Similarly, the expression of SMC markers appears to be differently regulated by platelet-derived growth factor-BB (PDGF-BB) between SMCs from proepicardial origin and those from other lineages. Although the observation itself implies that cells had some preexisting differences that contributed to these responses, one cannot assume that differences are solely a function of differing embryological origins of cells, and one must always be aware that differences may have evolved due to the particular in vitro growth conditions that may have conferred at least some of the different properties observed.

An experiment performed by Bochaton-Piallat and her colleagues also supports the idea that SMCs have heritable properties that are perhaps much more stable than generally appreciated. Both spindle-shaped and epithelioid SMCs, respectively, were isolated and cultured in vitro, and implanted into a rat carotid artery subjected to endothelial injury in vivo. These investigators found that both of the populations maintained their distinct phenotypic features in vivo. Results seem to indicate that at least certain SMC phenotypes are relatively stable and resistant to reprogramming in response to local environmental cues. However, it must be emphasized that these studies involved growth of the SMC subpopulations in vitro for extended periods of time, and results may not be relevant to what normally occurs in SMCs in vivo. Therefore, it would be interesting to examine whether SMCs derived from small arteries retain their characteristics when they are directly transplanted into an elastic artery and vise versa (or artery to vein and vise versa) to assess the effect of local environmental cues on SMC phenotypes.

Although there is regional heterogeneity in the control of SMC marker gene expression at the molecular level, its pathophysiologically relevant remains unknown. For example, it is recognized that some vascular beds, including the coronary arteries and aorta, are more prone to atherogenesis, whereas certain vascular beds such as the internal thoracic artery are resistant to atherosclerosis. The latter is of particular interest, because this resistance appears to persist even when the vessel is used for coronary bypass surgery, suggesting that this property may be inherent to this particular vessel and not simply a function of varying hemodynamic forces to which the vessels are exposed. The mechanisms responsible for these differences are unknown, but it is of interest to speculate that they may be due at least in part to regional differences in the molecular mechanisms that control SMC phenotypic switching after exposure to atherogenic stimuli. However, it must also be recognized that diseases such as atherosclerosis normally develop over many decades, whereas evidence of the so-called “atherosclerosis resistance” is based on observations over a shorter time period.

**Molecular Regulation of SMC Differentiation: Role of CArG Elements, SRF, and SRF Coactivators**

Expression of most of SMC marker genes has been shown to be dependent on multiple CArG elements and their binding factor, SRF. Landerholm et al have shown that SRF is required for SMC differentiation in an in vitro proepicardial cell model. However, the CArG element was first identified as the core sequence of the serum response element within the growth factor-inducible gene, c-fos, and it is also required for expression of many growth factor-inducible genes. Therefore, a major issue in the field has been to identify mechanisms whereby the CArG-SRF complex can simultaneously contribute to the disparate processes of activation of SMC differentiation marker genes versus activation of growth-regulated genes. The discovery of myocardin by Olson and his colleagues represented a major advance for the SMC field and has provided a significant insight into this question.

Myocardin is a potent coactivator for SRF and is expressed exclusively in SMCs and cardiomyocytes. Myocardin shows marked activation of multiple CArG-containing SMC marker genes including SMα-actin, SM-MHC, SM22α, and h1-calponin. In contrast, it fails to activate the c-fos gene that contains a single CArG element. Either dominant-negative forms of myocardin or short interfering RNA (siRNA)-induced suppression of myocardin in cultured SMCs decreased transcription of SMC marker genes, and knockout of myocardin in mice resulted in embryonic lethality at E10.5 and was associated with failed SMC investment and differentiation. In addition, there are several lines of evidence suggesting that myocardin serves as a point of convergence in mediating effects of environmental cues on expression of SMC marker genes (Figure 2). First, we showed that angiotensin II induced myocardin expression, as well as SMα-actin expression, in rat aortic SMCs, and that suppression of myocardin by either siRNA or dominant-negative myocardin attenuated angiotensin II–induced transcription of the SMα-actin gene. Second, we showed that activation of L-type voltage-gated Ca2+ channels and elevation of intracellular Ca2+ increased the expression of SMC marker genes and these increases were mediated in part by myocardin.

Third, Wang et al showed that growth signals such as PDGF-BB repressed SMC marker expression in A7r5-cultured SMCs by triggering the displacement of myocardin from SRF by one of the ternary complex factor (TCF) members, Elk-1. Taken together, the preceding results indicate that an understanding of myocardin function will not only be critical for understanding normal differentiation of SMCs, but also SMC diversity and/or phenotypic modulation. However, our recent results showed that a subset of SMC marker genes including smoothelin-B, a specific marker for contractile SMCs, is not regulated by myocardin, suggesting that myocardin alone is not sufficient to initiate the complete SMC differentiation program.

**Key Questions That Remain to be Resolved**

**How Does Myocardin Selectively Activate a Very Distinct Subset of CArG-Containing Genes Including Those That Contain a Single CArG Element?**

It has been demonstrated that most of the SMC marker genes possess multiple CArG elements in their promoter-enhancer regions and are activated by myocardin, whereas a single CArG-containing c-fos gene is not activated by myocardin. Wang et al proposed the “2-CArG model” in which
homodimerization of myocardin, which is mediated by its leucine zipper-like structure, was required for efficient transcriptional activation of SMC marker genes. However, results of several studies have shown that some SMC marker genes, including telokin and CRP-1, contain only a single CArG element in their promoter-enhancer regions, but are activated by myocardin. It is unknown how myocardin distinguishes these single CArG-containing SMC marker genes from the c-fos gene. One plausible mechanism is that the presence of a TCF binding site in the c-fos gene plays a key role in modulating the responsiveness to myocardin. Indeed, Wang et al showed that Elk-1 could compete with myocardin for SRF binding within the SM22α promoter. However, unlike the SM22α promoter, most SMC marker genes contain no conserved TCF binding sites such that it is unclear to what extent this mechanism contributes to overall control of expression of CArG-dependent SMC marker genes. An alternative possibility is that differences in the sequence of CArG element itself and/or the presence of adjacent cis-elements and their trans-acting factors may alter the action of myocardin on SMC-selective single CArG-containing genes. However, as yet, this hypothesis has not been directly tested.

What Are the Mechanisms That Normally Prevent Myocardin-Dependent Activation of Genes Such as Skeletal α-Actin, Cardiac α-Actin, and Atrial Natriuretic Factor That Contain Multiple CArG Elements in Their Promoter but That Are NotExpressed in Vascular SMCs? Indeed, in vitro it has been shown that overexpression of myocardin activates these genes as well as an artificial 4× c-fos CArG elements—containing promoter-reporter gene in cultured cells including SMCs. One potential explanation is that at least some of the results seen in overexpression studies may be an artifact of expression of supraphysiological levels of myocardin. It is highly likely that that precise control of the stoichiometry of myocardin relative to levels of other required transcription factors is critical for its proper function. In addition, we have presented evidence indicating that a key regulatory step in control of CArG-SRF—dependent activation of SMC marker genes is at the level of selective regulation of SRF binding to CArG elements within intact chromatin. Briefly, we found selective enrichment of SRF binding to CArG-containing regions of SMC promoters, including the SM α-actin and SM-MHC genes, within intact chromatin after retinoic acid—induced expression of SMC marker genes in an A404 SMC progenitor line developed in our laboratory. As such, it is interesting to speculate that there may be SMC-lineage specific patterning of chromatin structure that selectively controls SRF binding and subsequent recruitment of myocardin to a very select subset of SMC marker genes but not growth response genes like c-fos or skeletal and cardiac multiple CArG-containing genes.

**Does Knockout of Myocardin Directly Prevent Formation of SMC Lineages?** Li et al demonstrated that myocardin-deficient mice died at E10.5 and failed to form SMC tissues. However, there are several lines of evidence that the failure to form SMC lineages may represent an indirect rather than a direct effect. First, myocardin-null embryos were much smaller than their wild-type counterparts and showed major defects in the development of extraembryonic blood vessels, suggesting that knockout of myocardin had effects other than simply impairing SMC development in the embryo proper. Second, myocardin-null embryos died before mRNA is detectable in SMC tissues such as the aorta (ie, normally E12.5), despite that fact that SM α-actin and SM22α are induced at E9.5 to E10.5. In contrast, myocardin is detected in the primitive
heart at E9.5 by in situ hybridization.\textsuperscript{25,28} Taken together, these results indicate that the failure to form SMC tissues is secondary to defects in formation of the extraembryonic circulation and/or cardiac defects. Moreover, results suggest that initial induction of SMC marker genes at the early developmental stages may not require myocardin. However, given clear evidence that myocardin is required for expression of many SMC marker genes in adult SMCs,\textsuperscript{26–28} it is possible that myocardin is expressed within SMCs at early developmental stages at levels sufficient to drive transcription but below the level of sensitivity of in situ hybridization assays. Alternatively, initial induction of CArG-SRF–dependent SMC marker genes, at least in some SMC subpopulations, may not be dependent on myocardin. As such, further studies are needed in this area to test whether early stages of SMC differentiation are directly dependent on myocardin, and if not, what alternative mechanisms and factors are required.

**Does Myocardin Play a Key Role in PDGF-BB–Induced Suppression of SMC Marker Genes in Cultured SMCs?**

PDGF-BB has been shown to be unique among mitogens in suppression of SMC marker genes in postconfluent cultures.\textsuperscript{49} Of major interest, PDGF-BB has been shown to be unique among mitogens in suppression of SMC marker genes in adult SMCs,\textsuperscript{26–28} it is possible that myocardin is expressed within SMCs at early developmental stages at levels sufficient to drive transcription. However, there are several lines of evidence suggesting that this is not the only mechanism whereby PDGF-BB suppresses SMC marker gene expression (Figure 2B). First, as noted earlier, most promoter regions of SMC marker genes have no TCF binding site for Elk-1, which in turn competed with myocardin for SRF binding within CArG elements of the SM22α gene. Because Erk1/2 is known to phosphorylate Elk-1, these findings are consistent with those of Hayashi et al\textsuperscript{50} who found that activation of Erk1/2 by PDGF-BB reduced expression of SMC marker genes. However, there are several lines of evidence suggesting that this is not the only mechanism whereby PDGF-BB suppresses SMC marker gene expression (Figure 2B). First, as noted earlier, most promoter regions of SMC marker genes have no TCF binding site for Elk-1.\textsuperscript{11} Second, many factors including angiotensin II, endothelin-1, and sphingosine 1-phosphate activate Erk1/2, but increase rather than reduce the expression of SMC marker genes.\textsuperscript{43,51–54} Third, although Wang et al\textsuperscript{45} found no change in myocardin expression in A7r5 cells on treatment with PDGF-BB, this has not been the case in several other studies including our own (Y. Liu and G.K. Owens, unpublished data, 2004). Results of studies in our laboratory suggest several alternative mechanisms for PDGF-BB–induced suppression of SMC marker genes, including induction of expression of ets-1\textsuperscript{16} and Sp-1.\textsuperscript{55} As such, much additional work is needed in this area and, of key importance, studies are needed to directly ascertain the potential role of PDGF-BB in suppression of SMC marker gene expression in vivo in response to vascular injury or disease.

**What Is the Role of Myocardin in Control of SMC Phenotypic Switching That Occurs In Vivo in Response to Vascular Injury or in Association With Development of Vascular Disease?**

Although there is clear evidence that myocardin plays a key role in control of SMC marker gene expression in cultured SMCs and its positive and negative modulation by factors such as angiotensin II\textsuperscript{43} and PDGF-BB,\textsuperscript{45} respectively, there is no direct evidence that it plays a similar role in vivo. Surprisingly, as yet, there are no reports describing whether there are differences in expression levels of myocardin in SMCs that have undergone phenotypic switching in vivo. Further studies are needed to determine whether disruption of myocardin expression and/or function contributes to SMC phenotypic switching in vivo in response to vascular injury, or in association with neointima formation in experimental or human atherosclerosis.

**What Is the Role of the Two Myocardin-Related Factors, MKL1 and MKL2, in Control of SMC Diversity?**

Because tissue distribution of both MKL1 (also referred to as MAL, MRTF-A, and BSAC)\textsuperscript{56–59} and MKL2 (also referred to as MRTF-B)\textsuperscript{59,60} is ubiquitous and they are normally localized in the cytoplasm, their roles in the regulation of CArG-dependent SMC marker genes and the heterogeneity of SMC subtypes are unclear. Of interest, Miralles et al\textsuperscript{56} identified MKL1 as a RhoA-signal regulated SRF coactivator. That is, MKL1 is normally sequestered by G-actin in the cytoplasm of serum-starved fibroblasts, but accumulates in the nucleus after the activation of actin polymerization by RhoA. Consistent with this, we previously demonstrated that RhoA increased transcription of SMC marker genes in cultured SMCs, whereas the inhibition of RhoA pathway reduced transcription.\textsuperscript{61} In addition, we showed that treatment with agents that enhance formation of F-actin profoundly increased SMC gene transcription, whereas treatment with agents that increase G-actin markedly inhibited SMC gene expression.\textsuperscript{61} Taken together, these observations indicate that MKL1 may play a role in linking RhoA-actin signaling to SMC marker expression. In support of this, recent data from Lockman et al\textsuperscript{43} showed that sphingosine 1-phosphate increased the expression of multiple SMC marker genes by RhoA-mediated translocation of MKL1 in cultured SMCs. Establishment of the role of myocardin and MKL1/2 may provide important insights into the molecular control of SMC marker genes and SMC phenotype.

In summary, it is clear that the CArG-SRF-myocardin complex plays a critical role in regulation of SMC differentiation marker genes. However, it alone is not sufficient to control SMC marker gene expression or to initiate the SMC lineage. Rather, there is compelling evidence showing that SMC differentiation and the diversity of SMC phenotypes exhibited under various physiological and pathological circumstances are controlled by complex combinatorial interactions of many cis-elements and their trans-acting factors, including GATA family, MEF2 family, bHLH proteins,
homeodomain proteins, and KLFs, in concert with SRF and myocardin.6,11

**Periodicity in the Transcription of SMC Marker Genes**

One of the interesting findings that has emerged from transgenic mouse studies is that regulation of SMC marker genes in SMCs appears to be much more dynamic than was originally appreciated. For example, expression of a SM-MHC-LacZ reporter construct containing ≈16 kb of the SM-MHC genomic region from −4.2 to +11.6 kb coupled to a LacZ reporter gene showed heterogeneous LacZ staining pattern in a wide array of embryonic and adult SMC tissues, including the aorta, coronary vessels, the intestine, stomach, and many smaller blood vessels.22 That is, a fraction of SMCs were strongly LacZ positive, whereas others in close proximity or even immediately adjacent to these cells were negative. In contrast to these observations, analysis of SM-MHC protein expression in individual SMCs by immunostaining with an SM-MHC antibody showed detectable expression in all SMCs within these tissues.22 The striking differences in LacZ staining seen between adjacent SMCs suggests either that heterogeneous populations of SMCs exist that require distinct regulatory modules to activate the gene (ie, negative cells may require control regions lacking from the transgene tested) or that there is periodicity of transgene expression and that the observed staining patterns reflected episodic expression. To distinguish these possibilities, we generated SM-MHC promoter–driven Cre-recombinase transgenic mice (SM-MHC-Cre mice) and crossed them with indicator mice containing a Cre-recombinase activatable β-galactosidase gene within the ubiquitously expressed ROSA26 locus.62 As such, SM-MHC activation of LacZ in these animals represents the integral of activation of the SM-MHC promoter over the entire lifespan of the mouse (ie, any cells that have ever expressed the SM-MHC promoter-enhancer should be positive in LacZ staining even if gene expression is downregulated subsequently). Of major interest, results of these studies showed nearly 100% LacZ-positive SMCs (Figure 3A), suggesting that the heterogeneous expression seen in the SM-MHC-LacZ mice (Figure 3B) was likely due to episodic changes in expression of the transgene within individual SMCs. Although the mechanisms underlying this phenomenon are still unclear, results have several important implications. First, regulation of SMC marker gene expression may be much more dynamic than often appreciated, ie, differentiated SMCs may not show constitutive transcription of their entire repertoire of SMC-specific genes in vivo.

Rather, expression may be like the thermostat regulating the temperature in your house, turning off and on as needed to maintain an appropriate level of expression of the gene products. Second, it reinforces the fact that one cannot directly extrapolate transcription rates from mRNA or protein levels.

On the other hand, it is possible that at least some of the differences in LacZ staining in SM-MHC-LacZ mice may be caused by SMC heterogeneity within focal vessel areas.63,64 For example, Frid et al64 demonstrated that at least four phenotypically distinct SMC populations can be identified within the normal adult media from bovine pulmonary arteries in vivo. They performed simultaneous analysis of a number of different SMC markers including SM α-actin, SM-MHC, calponin, desmin, and vinculin, in the mature and developing vessels, and found that the mature arterial media...
was a highly complex and heterogeneous structure. Metavin- 
culin, a splicing variant of vinculin, was restricted to a 
specific SMC population throughout all stages of fetal and 
postnatal development and was never identified in other 
population of SMCs. Also, they found that two other proteins, 
h-caldesmon and SM-2, a splicing variant of SM-MHC, were 
coordinately expressed with metavinulin in subpopulation of 
SMCs. As such, it is possible that the heterogeneous staining 
in SM-MHC-LacZ mice is resultant from the SMC heteroge-
nity and that the strong homogeneous staining in SM-MHC- 
Cre/indicator LacZ mice is due to the fact that all SMC 
subpopulations express the SM-MHC promoter at least tran-
siently sometime during development.

It is clear that resolution of some of the preceding issues 
will require live imaging system using shorter turnover 
indicator proteins, such as destabilized enhanced green fluo-
rescent proteins, that will be a useful tool to further analyze 
the dynamic regulation of SMC-specific genes at the tran-
scriptional levels in vivo.

Similarities and Differences Between 
Myofibroblasts and Phenotypically 
Modulated SMCs

Myofibroblasts arise as a consequence of tissue injury and are 
characterized by expression of several SMC differentiation 
markers, proliferation, migration, and production of ECM 
proteins, cytokines, and chemokines.\textsuperscript{65,66} It has been postu-
lated, although not proven, that induction of expression of 
these SMC marker genes is required for myofibroblasts to 
produce sufficient contractile force necessary for wound 
closure. SMC marker genes expressed in myofibroblasts 
include \textit{SM \alpha-actin}, \textit{SM22a}, \textit{desmin}, and \textit{vimentin}, but not \textit{SM-MHC}.\textsuperscript{65–67} Myofibroblasts also express \textit{SMemb},\textsuperscript{68} os-
teoponin,\textsuperscript{69} and cellular retinol-binding protein-1,\textsuperscript{70} which 
are known to be induced in phenotypically modulated SMCs.

It is also worth noting that it has been reported that 
myofibroblasts derived from adventitia can participate in 
neointimal formation after severe vascular injury and in 
transplant vasculopathy\textsuperscript{71–73} (see a detailed discussion of this 
topic in Sartore et al\textsuperscript{65}). The first evidence for adventitial cell 
migration to the subendothelial space was suggested based on 
experiments using bromodeoxyuridine to selectively label 
proliferating cells within the adventitia.\textsuperscript{71–74} However, a 
limitation of these studies was that medial SMCs and partic-
ularly those at the medial-adventitial boundary would also 
have been labeled with bromodeoxyuridine such that the 
lineage tracing method was selective rather than specific for 
adventitial fibroblasts. To attempt to address this limitation, 
investigators used primary cultures of adventitial fibroblasts 
that were stably transfected with LacZ retrovirus and intro-
duced them into the adventititia of rat carotid arteries imme-
diately after balloon injury.\textsuperscript{75} LacZ staining was detected in 
the media at 5 days after injury and in both media and 
neointima at 7, 10, and 14 days after injury, indicating that the 
exogenously administered myofibroblasts had migrated 
through the medial layer and into the neointimal compart-
ment.\textsuperscript{75} Whereas these latter results clearly show that exog-
enously administered adventitial fibroblasts have the capacity 
to invest within the intima, they do not provide direct 
evidence that normal adventitial fibroblasts function in this 
manner. Finally, long-term outcome of adventitial fibroblasts 
investigating the neointima is still unclear whether they transdif-
ferentiate into stable differentiated SMCs or they simply 
provide an early wound-healing response followed by apo-
potis as occurs in dermal wounds.\textsuperscript{55,66}

Thus, a major uncertainty in the field has arisen as to how 
one can distinguish activated myofibroblasts from phenotyp-
ically modulated SMCs. In the final analysis, these differ-
ences may be very subtle or nonexistent. However, of major 
significance, results of in vitro studies by several groups\textsuperscript{76–81} 
suggest that the transcriptional regulatory mechanisms that 
control activation of some of SMC marker genes including 
\textit{SM \alpha-actin} may be quite different between SMCs and 
myofibroblasts. Such differences may be required because 
\textit{SM \alpha-actin} expression is normally induced in myofibroblasts 
under conditions such as vascular injury when the gene is 
suppressed in SMCs, ie, the same stimulus has completely 
opposite effects on expression of \textit{SM \alpha-actin} in SMCs versus 
myofibroblasts.

Results of transient transfection experiments in cultured 
SMCs versus mouse AKR-2B embryonic fibroblasts suggest 
that MCA\textit{T} elements (AGGAATG) located at \text{-320 bp} (MCA\textit{T}2) and \text{-184 bp} (MCA\textit{T}1) in rat \textit{SM \alpha-actin} pro-

myofibroblasts. They are characterized by higher prolifera-
tion rate, migratory activity, and lack of some SMC marker 
gen expression. Indeed, \textit{SM \alpha-actin} and \textit{SM22a} genes are expressed as early as E9.5, whereas \textit{SM-MHC} and \textit{h1-}
calponin appear to be expressed at later stages.\textsuperscript{1,21,23} Notably, myocardin is undetectable within the mouse developing aorta until E12.5 as determined by in situ hybridization,\textsuperscript{25,28} whereas SM\textalpha-actin and SM22\alpha have already been expressed. Based on these results, it is interesting to speculate that initial induction of early SMC differentiation markers may not require myocardin but rather may utilize mechanisms similar to those involved in activation of these genes in myofibroblasts. Moreover, recent results from our laboratory\textsuperscript{27} and others\textsuperscript{82} showed that myocardin and SM-MHC are not expressed in multipotential 10T1/2 fibroblasts irrespective of treatment with TGF-\beta\textsubscript{1}, strongly suggesting that: (1) myocardin is a cell-selective transcription factor that can distinguish differentiated SMCs from activated fibroblasts; and (2) that TGF-\beta\textsubscript{1}-treated 10T1/2 cells do not represent a valid model for studying later stages of SMC differentiation.

In summary, there are somewhat provocative in vitro results suggesting the presence of fundamental differences in the transcriptional regulatory processes in control of SM \alpha-actin expression between myofibroblasts and SMCs. However, direct validation of these results in vivo is needed.

Conclusions and Perspectives

The model that has emerged is that expression of SMC marker genes involves the complex interplay of multiple positive- and negative-acting regulatory modules and their binding factors. In addition, results indicate that, although there are many common transcriptional regulatory mechanisms used by individual SMC subtypes, there are also clear cases in which at least part of the regulatory scheme is accomplished by utilization of distinct combinations of these control modules and their binding factors. Control of SMC-specific gene expression does not appear to be mediated by a single transcription factor that is completely specific for SMCs, but rather is regulated by unique combinations of transcription factors that are ubiquitous (eg, SRF), widely expressed (eg, MEF2), or selective for SMCs (eg, myocardin and GATA6). Indeed, the multiplicity of promoter control regions and trans-acting factors that regulate expression of SMC genes and modulation of the activity of these pathways by changing environmental cues is paramount to control of SMC diversity and plasticity. However, this being said, there are major unresolved issues with respect to the extent to which single SMCs can undergo phenotypic change, the nature of fixed changes that occur during SMC lineage determination, the molecular mechanisms that may control these fixed lineage changes, and whether regeneration of SMCs in adult tissues, at least under some circumstances, is dependent on recruitment of new SMCs from relatively poorly differentiated SMC precursor populations or stem cells.

A growing body of evidence in all cell types indicates that the binding of transcription factors to cis-elements and subsequent gene transcription are profoundly affected by the chromatin structure. Not surprisingly, these mechanisms also appear to be critical in regulation of SMC differentiation, although studies in this area are truly in their infancy. Indeed, it is clear that the transcriptional activity observed in vivo is not a sum of activities of individual transcription factors, but instead adjusted by chromatin modifying enzymes to make a network of complex interactions among transcriptional regulatory modules. As such, a critical need in the field is to analyze the interactions between cis-elements, transcription factors, and chromatin structures in vivo, and to resolve how these are modified by environmental cues that contribute to the phenotypic diversity of SMCs.

Finally, in the long term, it is hoped that a further understanding of the molecular mechanisms that control the diverse SMC phenotypes will have important clinical interventions for directing expression of therapeutic agents that can modify SMC phenotype and contribute to stabilization of the fibrous cap of atherosclerotic lesions, thereby reducing the risk for heart attacks.

Acknowledgments

This work was supported by NIH grants P01HL192442, R01HL38854, R21HL071976, and R37HL57353 to G.K.O. We thank Sanjay Sinha and Brian Warnhoff for providing helpful comments.

References


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Tadashi Yoshida and Gary K. Owens

doi: 10.1161/01.RES.0000155951.62152.2e
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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