Molecular Determinants of Vascular Smooth Muscle Cell Diversity

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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. 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. 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. During late embryogenesis, production of extracellular matrix (ECM) proteins is increased in SMCs, while expression of SMC marker genes is robustly induced. 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
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 within large blood vessels that exhibit morphologically and functionally distinct properties when grown in culture.⁹ Several investigations are quite interesting although the precise origins and mechanisms of this diversity have been viewed elsewhere in considerable depth.⁹,¹¹ 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 in 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 cis-regulatory elements.
promoter-enhancer region to drive expression in vivo.\textsuperscript{16} 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 SM22\textsuperscript{α} and cysteine-rich protein-1 (CRP-1) genes.\textsuperscript{17–20} SM22\textsuperscript{α} is expressed in smooth, cardiac, and skeletal muscle lineages during early embryogenesis and becomes restricted to SM lineages at late stages and adulthood.\textsuperscript{23} Several laboratories investigated regulatory mechanisms for cell-specific expression of the SM22\textsuperscript{α} gene.\textsuperscript{17–19} Of interest, each of these groups found that a region of the SM22\textsuperscript{α} 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.\textsuperscript{24} Lilly et al\textsuperscript{20} 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,\textsuperscript{13} and indicate that distinct transcriptional regulatory mechanisms exist for the control of SM-MHC, SM22\textsuperscript{α}, 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, SM22\textsuperscript{α}, 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.\textsuperscript{25–28} However, it is not at all clear how differences in expression levels and/or

**Figure 1.** Mutation of only 5 bps within the intronic CarG element of the ~16 kb SM-MHC promoter-enhancer-LacZ transgene resulted in selective silencing of this gene in large conduit arteries (B), whereas it retained activity in all other SMC tissues including small arteries, arterioles, and the urinary tract. In contrast, the wild-type promoter was active in all SMC subtypes (A). Results provide evidence for diversity of transcriptional control within different SMC subtypes in that the intronic CarG element was absolutely required for expression in SMCs within large conduit arteries, but was completely dispensable for expression in other SMCs. Adapted from Manabe I, Owens GK. CarG elements control smooth muscle subtype-specific expression of smooth muscle myosin in vivo. J Clin Invest. 2001;107:823–834, by permission of The American Society for Clinical Investigation.
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 Krüppel-like zinc-finger factors (KLFs) might explain differential molecular control of SMC gene expression. For example, why is CArG/ SRF-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, posttranslational 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 in expression pattern between arterial and venous SMCs. They found that 68 genes, including regulator of G-protein signaling 5 (RGS-5), embryonic/non-muscle isoform of myosin heavy chain-B (SMemb/NMHC-B), vimentin, and aortic preferentially expressed gene-1, were elevated in the aorta compared with vena cava. 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 al13 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 Majesky14 showed persistence of major differences in morphology, growth, and transforming growth factor-β (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
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.

Although there is regional heterogeneity in the control of SMC marker gene expression at the molecular level, its pathophysiological relevance remains unknown. For example, it is recognized that some vascular beds, including the coronary arteries and aorta, are more prone to atherosclerosis, 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.

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 Not Expressed in Vascular SMCs?** Indeed, in vitro it has been shown that overexpression of myocardin activates these genes as well as an artificial 4× 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.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,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 its ability to potently repress expression of virtually all SMC marker genes in cultured cell systems.36,49 Of major interest, its ability to potently repress expression of virtually all SMC marker genes and SMC phenotype.

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)56–59 and MKL2 (also referred to as MRTF-B)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 al58 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 multiple SMC marker genes in cultured SMCs, whereas the inhibition of RhoA pathway reduced transcription.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.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 al55 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,
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 \( \approx 16 \) 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.\(^6\) 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.\(^2\) 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 \( \beta\)-galactosidase gene within the ubiquitously expressed ROsa26 locus.\(^6\) 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\(^2\) may be caused by SMC heterogeneity within focal vessel areas.\(^6\) For example, Frid et al\(^6\) 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 \( \alpha\)-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. Metavinucin, 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 metavinucin in subpopulation of SMCs. As such, it is possible that the heterogeneous staining in SM-MHC-LacZ mice is resultant from the SMC heterogeneity 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 transiently 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 fluorescent proteins, that will be a useful tool to further analyze the dynamic regulation of SMC-specific genes at the transcriptional 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. It has been postulated, 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 SM α-actin, SM22α, desmin, and vimentin, but not SM-MHC. Myofibroblasts also express Smemb, os teopontin, and cellular retinol-binding protein-1, 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 (see a detailed discussion of this topic in Sartore et al). The first evidence for adventitial cell investment within the intima, they do not provide direct evidence that normal adventitial fibroblasts function in this manner. Finally, long-term outcome of adventitial fibroblasts investing the neointima is still unclear whether they transdifferentiate into stable differentiated SMCs or they simply provide an early wound-healing response followed by apoptosis as occurs in dermal wounds.

Thus, a major uncertainty in the field has arisen as to how one can distinguish activated myofibroblasts from phenotypically modulated SMCs. In the final analysis, these differences may be very subtle or nonexistent. However, of major significance, results of in vitro studies by several groups suggest that the transcriptional regulatory mechanisms that control activation of some of SMC marker genes including SM α-actin may be quite different between SMCs and myofibroblasts. Such differences may be required because SM α-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 SM α-actin in SMCs versus myofibroblasts.

Results of transient transfection experiments in cultured SMCs versus mouse AKR-2B embryonic fibroblasts suggest that MCAT elements (AGGAATG) located at −320 bp (MCAT2) and −184 bp (MCAT1) in rat SM α-actin promoter may play a role in mediating these opposite effects. In SMCs, mutation of MCAT2, MCAT1, or both resulted in significant increases in SM α-actin promoter activity, indicating that these cis-elements acted to repress transcription in SMCs. In contrast, these same mutations reduced activity in AKR-2B fibroblasts, rat L6 skeletal myoblasts, and bovine aortic endothelial cells. Strauch and coworkers have extensively characterized the underlying mechanisms that regulate SM α-actin expression in AKR-2B cells. They found that transcriptional activation and repression of mouse SM α-actin promoter in these cells was dependent on MCAT1 element spanning −182 to −176 bp that bound Transcription Enhancer Factor-1 (TEF-1). Moreover, inspection of the sequence spanning −195 to −165 bp revealed that MCAT1 element was centered within a region of high polypurine/polypyrimidine (Pu/Py) asymmetry, and that mutation of these flanking regions increased the transcriptional activity. In addition, three single-stranded DNA-binding proteins, Purα, Purβ, and MSY1, were identified as binding factors for these flanking regions. Currently, it has been postulated that the balance between TEF-1, as a positive regulator, and these single-stranded DNA-binding proteins, as negative regulators, determines the activity of the SM α-actin transcription. However, there is no direct evidence showing that these factors are able to alter transcription of the SM α-actin gene by either gain or loss of function studies. Moreover, it is unclear whether the results seen in AKR-2B fibroblasts really reflect what happens in myofibroblasts in vivo. Further studies are required to define these questions.

The features of SMCs at the early embryonic stages are also similar to both phenotypically modulated SMCs and myofibroblasts. They are characterized by higher proliferation rate, migratory activity, and lack of some SMC marker gene expression. Indeed, SM α-actin and SM22α genes are expressed as early as E9.5, whereas SM-MHC and 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\alpha-actin and SM22α 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.

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