Programming Smooth Muscle Plasticity With Chromatin Dynamics

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Abstract—Smooth muscle cells (SMCs) possess remarkable phenotypic plasticity that allows rapid adaptation to fluctuating environmental cues. For example, vascular SMCs undergo profound changes in their phenotype during neointimal formation in response to vessel injury or within atherosclerotic plaques. Recent studies have shown that interaction of serum response factor (SRF) and its numerous accessory cofactors with CArG box DNA sequences within promoter chromatin of SMC genes is a nexus for integrating signals that influence SMC differentiation in development and disease. During development, SMC-restricted sets of posttranslational histone modifications are acquired within the CArG box chromatin of SMC genes. These modifications in turn control the chromatin-binding properties of SRF. The histone modifications appear to encode a SMC-specific epigenetic program that is used by extracellular cues to influence SMC differentiation, by regulating binding of SRF and its partners to the chromatin template. Thus, SMC differentiation is dynamically regulated by the interplay between SRF accessory cofactors, the SRF–CArG interaction, and the underlying histone modification program. As such, the inherent plasticity of the SMC lineage offers unique glimpses into how cellular differentiation is dynamically controlled at the level of chromatin within the context of changing microenvironments. Further elucidation of how chromatin regulates SMC differentiation will undoubtedly yield valuable insights into both normal developmental processes and the pathogenesis of several vascular diseases that display detrimental SMC phenotypic behavior. (Circ Res. 2007;100:1428-1441.)

Key Words: smooth muscle cells (SMCs) · serum response factor (SRF) · myocardin · chromatin · histone modifications

Differentiation of vascular smooth muscle cells (SMCs) from embryonic stem cells (ESCs) during development is characterized by the appearance of proteins (eg, smooth muscle [SM] α-actin, SM–myosin heavy chain [MHC]) whose expression is restricted to the SMC lineage and required for SMC contraction and regulation of blood pressure under adult physiological conditions. However, unlike skeletal and cardiac myocytes, which are terminally differentiated, SMCs within adult animals readily switch phenotypes in response to changes in local environmental cues.1 For example, vascular SMCs express high levels of SMC-specific contractile proteins and do not generally proliferate, migrate, or secrete significant amounts of extracellular matrix. However, in response to extracellular cues released at sites of vascular injury or within atherosclerotic lesions, SMCs exhibit decreased expression of SMC-specific contractile proteins and increased migration, proliferation, and production of extracellular matrix components as well as matrix metalloproteases. These changes presumably evolved as an important survival mechanism to repair vascular damage, and the process appears to be fully reversible if the pathological stimuli dissipate.2 Arteries are especially predisposed to this process, often with fatal consequences resulting from arterioocclusive disease, in which SMC pathophysiology plays a prominent role. Many other parenchymal cell lineages (including endothelial cells, various epithelial cell types, fibroblasts, hepatocytes, chondrocytes, and glial cells) display similar reactive changes in their phenotype under pathophysiological conditions, suggesting that some if not many molecular principles underlying SMC physiology might apply to these cell lineages as well.

Transcriptional Control of SMC Differentiation by Serum Response Factor

Transcriptional control of SMC gene expression plays a central role in the maintenance of SMC differentiation and control of SMC phenotypic plasticity. For example, studies in transgenic mice have demonstrated that SMC-specific contractile gene promoters are transcriptionally active within vascular SMCs under physiological conditions, whereas they are transcriptionally repressed in response to vascular injury3 and atherosclerotic plaque formation.4 After resolution of the injury, SMCs resume transcription of these genes and regain their fully differentiated phenotype.3,5 Thus, there is great interest in identifying molecular mechanisms that regulate SMC gene transcription, so that vascular pathologies might
be better understood and potentially controlled. Strikingly, nearly all SMC-restricted contractile protein genes examined to date contain evolutionarily conserved CArG box DNA sequences within their promoters, and these sequences are required for SMC gene transcription in vivo. Paradoxically, many other genes important for induction of SMC phenotypic switching within vascular lesions, including genes important for migration, proliferation, and extracellular matrix production, also contain CArG boxes within their promoters. Thus, it appears that CArG box DNA sequences may have evolved within the context of SMCs for coordinate regulation of the multiple transcriptional programs that underlie differentiation and plasticity of the SMC phenotype.

CArG boxes serve as binding sites for serum response factor (SRF). The original biological function of SRF appears to be that of a generic DNA-binding transcriptional activator important for expansion of cell populations, as the yeast SRF homolog MCM1 regulates DNA synthesis via binding DNA sequences near replication origins and by transcriptional activation of several cell-cycle control genes important for proliferative growth of this organism. Indeed, this function has been evolutionarily conserved as SRF was so named because it was first found to regulate transcription of immediate early genes important for initiation of mitogenesis in response to the addition of serum into the media of various cultured mammalian cell lines. It has been appreciated for quite some time that SRF homodimers perform this function via binding to CArG box DNA sequences within the promoters of immediate early genes (eg, c-fos) and forming a ternary complex with various serum-activated Ets-domain proteins of immediate early genes important for induction of mitogenesis in cultured mammalian cell lines. It has been appreciated for quite some time that SRF homodimers perform this function via binding to CArG box DNA sequences within the promoters of immediate early genes (eg, c-fos) and forming a ternary complex with various serum-activated Ets-domain proteins (ie, the ternary factors [TCFs] Elk-1, Sap-1, Fli-1) that assist SRF in stimulating transcription of these genes. However, during evolution from unicellular to complex multicellular organisms, SRF acquired multiple other functions in addition to induction of cellular proliferation, including regulation of myogenesis, cell migration, cell–cell adhesion, cytoskeletal assembly and organization, and extracellular matrix production. This is consistent with the coevolution of CArG boxes in the promoters of numerous genes important for these diverse cellular operations. For a comprehensive consideration of these and related topics, see several seminal reviews by Miano and colleagues. In addition, a plethora of environmental cues have been found to regulate SRF transcriptional activity in mammals, perhaps because the SRF–CArG interaction was selected by nature to control such a large and varied repertoire of cellular behaviors. It is no surprise then that SRF activity has emerged as a paradigm for how diverse cellular processes are coordinated at the level of transcription for adaptation to changing microenvironments, especially within the context of SMCs.

Interest in how environmental cues regulate SRF activity in SMCs was intensified by the recognition that there are 3 major paradoxes regarding the transcriptional activity of SRF. First, as noted above, SRF has the ability to simultaneously activate transcription of genes involved in opposing cellular processes, such as differentiation and proliferation. Second, SRF itself is a weak transcriptional activator, probably because its transcriptional activation domain (TAD) does not effectively recruit the basal transcription machinery relative to other transcription factors with strong TADs. Third, SRF is expressed in all cell lineages, yet only activates transcription of SMC-restricted contractile genes in SMC, raising questions as to how this ubiquitously expressed transcription factor can selectively activate SMC-specific gene expression in SMCs and not in non-SMCs. To explain these issues, it was thought that SRF must bind to SMC gene promoters and subsequently recruit other muscle-specific promyogenic accessory factors with strong TADs (ie, similar to the ternary complex factors at growth genes) and that this interaction was amenable to regulation by environmental cues that influence the SMC phenotype.

This idea was validated in dramatic fashion by the discovery of the SRF coactivator myocardin, perhaps the most potent transcriptional coactivator yet identified in nature. Myocardin is exclusively expressed in SMCs and cardiomyocytes, possesses a powerful C-terminal TAD with the capability to selectively activate transcription of cardiac and SMC-specific contractile genes to levels never imagined (eg, several thousand fold by in vitro transient transfection assays), and physically associates with SRF to form a ternary complex on CArG box DNA. Several extracellular cues have since been demonstrated to positively and negatively modulate the expression and transcriptional activity of myocardin in SMCs, and numerous other SRF accessory factors have subsequently been shown to transmit these extracellular signals to myocardin/SRF. Thus, available data suggest a model whereby SRF functions as a protein platform that generically binds CArG box DNA to recruit other downstream accessory factors that either stimulate or repress transcription of antagonistic gene subsets in response to various environmental cues. Although this model will likely prove essentially correct at a simplified level, there is abundant evidence that the story is much more complicated than this model portends, as outlined below.

**SRF Activity Within the Context of SMC Chromatin**

It has become widely appreciated over the past decade that transcription takes place on promoter DNA within the context of chromatin (ie, not a naked linear strand of DNA) and that the structure of this chromatin plays an active and fundamental role in transcriptional control. Ironically, although much of the original work demonstrating that chromatin was an important regulator of transcription was performed by experiments examining the SRF-dependent c-fos promoter nearly 2 decades ago, very little is known regarding how SRF activates transcription within the context of intact chromatin. Because SRF and its accessory factors must operate within this chromatin environment, future revisions to the current model explaining SRF activity will be incomplete without an accurate description of this process.

At its most fundamental level, chromatin is organized into repeating units of nucleosomes, the basic building blocks of chromatin. The nucleosome is composed of 146 base pairs of genomic DNA wrapped around an octamer of histone proteins (2 copies each of histone H2A, H2B, H3, and H4). Nucleosomes are in turn connected to one another by variable lengths of so called linker DNA, forming the characteristic
Chromatin is the form that one would expect SMC genes to influence over silencing of SMC genes in non-SMCs, heterochromatin are depicted in Figure 2. If chromatin holds silent.20,24–26 The opposing properties of heterochromatin and also formed within euchromatic regions of higher eukaryotic genomes; here heterochromatin functions to inhibit transcriptionally. Here, nucleosomes are packed closely together and display restricted mobility. There is little free DNA and access to proteins that bind DNA is severely inhibited. Transcription is a very inefficient and rare event. Much of the gene-poor repetitive DNA sequences originating from past viral integration events are stored here, and deposition of these sequences into condensed heterochromatin is thought to guard against widespread genomic instability. However, recent studies have revealed that foci of heterochromatin are also formed within euchromatic regions of higher eukaryotic genomes; here heterochromatin functions to inhibit transcription of cell-specific genes in cells where they must be kept silent.20,24–26 The opposing properties of heterochromatin and euchromatin are depicted in Figure 2. If chromatin holds influence over silencing of SMC genes in non-SMCs, heterochromatin is the form that one would expect SMC genes to take within non-SMC lineages. Indeed, one could easily envision a scenario whereby CArG box DNA sequences are wrapped into euchromatin or heterochromatin-like structures, thereby dictating the ability of SRF to bind its cognate cis element and activate transcription in SMCs versus non-SMCs. Because of this, investigations into whether chromatin might influence the DNA-binding properties of SRF to SMC gene promoters were initiated.

**Chromatin Regulates SRF Binding to CArG Box DNA**

The first piece of evidence that suggested chromatin has the potential to regulate SRF binding was implicitly provided by publications describing of the x-ray crystal structure of SRF bound to CArG box DNA27 and the crystal structure of the nucleosome core particle itself.28 If one compares these structures, it is apparent that SRF makes several critical contacts with DNA minor groove phosphates that would be obscured if this DNA were nucleosomal. This is because acidic minor groove phosphates required for interaction with the SRF MADS box are buried within basic histone amino acids as a functional anchor for DNA as it winds around histone octamers in solution. From thermodynamic considerations alone, the DNA-binding activity of SRF would be greatly inhibited if not impossible if SRF molecules were to encounter CArG box DNA sequences tightly wrapped around histone octamers. The extreme bending of the DNA template as it tracks around the histones would be expected to exert a high degree of steric hindrance as well. The next piece of evidence that suggested chromatin might regulate the ability of SRF to bind CArG box DNA was provided by comparing the ability of SRF to bind SMC gene promoters by electrophoretic mobility assays (EMSAs) and chromatin immunoprecipitation (ChIP) assays.29–31 The EMSAs, which used radiolabeled, naked DNA templates not assembled into chromatin as probes to monitor SRF binding in cell lysates in vitro, showed that SRF promiscuously binds to SMC gene promoter DNA probes with equal affinity in lysates from cultured SMCs and non-SMCs alike. This is consistent with the notion that SRF
is bound to SMC gene promoters in non-SMCs but does not activate transcription because of the absence of cofactors such as myocardin. In stark contrast, results obtained by several independent studies using ChIP assays revealed that SRF binds to endogenous SMC gene promoters much more effectively in SMCs than in non-SMCs within the context of native chromatin.20,31–33 Furthermore, micrococcal nuclease digestion experiments demonstrated that the CArG box chromatin of SMC gene promoters exists in a form that is much more accessible to digestion in SMCs than in non-SMCs, suggesting that SMC genes are euchromatin-like in SMCs and heterochromatin-like in non-SMCs.32 These results revealed that although SRF is expressed in all cell types and has the inherent ability to bind SMC gene CArG box DNA equally in SMCs and non-SMCs, SRF preferentially binds to accessible chromatin of endogenous SMC genes in SMCs as opposed to non-SMCs. The final piece of evidence that implicitly suggested that chromatin might be an important regulator of the ability of SRF to activate SMC gene expression (in the context of the above findings) was the discovery of myocardin-related transcription factors.34 These factors behave in ways that are similar to myocardin in terms of strong activation of SMC gene transcription, but these proteins are expressed in numerous different embryonic and adult cell types (rather than muscle-restricted, like myocardin). Because of this, these proteins are theoretically predicted to ectopically activate SMC gene expression in non-SMCs if SMC gene promoters were constitutively occupied with SRF because of the absence of a heterochromatin conformation, which is not the case under native conditions.18

Collectively, these data argue that the reason SRF does not activate SMC gene expression in non-SMCs is not simply attributable to the absence of myogenic SRF coactivators in these cells. Rather, it appears that there might be some property of the promoter chromatin that inhibits SRF binding to CArG boxes of SMC genes in non-SMCs, which in turn precludes recruitment of SRF coactivators under native conditions. Thus, the process that dictates whether SMC genes exist in a euchromatin or heterochromatin conformation in SMCs versus non-SMCs might represent a fundamental mechanism that underlies SMC identity, via regulation of SMC-restricted SRF binding to SMC genes. Furthermore, if chromatin-based control of SRF binding is important for specifying the SMC lineage, this mechanism might also be used by environmental factors that influence the phenotypic plasticity of adult SMCs, in conjunction with the well-recognized role of SRF cofactors.

Histone Modifications Regulate Chromatin Structure and Function

Recent studies have attempted to characterize components of CArG box chromatin that regulate the SMC-restricted binding activity of SRF. These efforts have thus far primarily focused on identification and functional characterization of posttranslational modifications to histone octamers within the chromatin of SMC genes. This is because a wealth of data obtained from classically studied cell systems (eg, yeast, Drosophila, HeLa cells) have unequivocally demonstrated that chromatin structure and function is profoundly influenced by posttranslational modifications to the histone proteins within the nucleosomes, such as methylation, acetylation, phosphorylation, and ubiquitination of histones by the action of nuclear protein complexes with catalytic activity toward specific histone amino acid residues.20,35 Over the past decade, numerous studies have documented that many of these modifications are found solely within areas of heterochromatin, whereas others are found exclusively in euchromatin. Because studies in SMCs have thus far focused on
acetylation and methylation of histone lysine residues, we have limited our discussion of the vast subject of histone modifications to these. These modifications are thought to serve 2 purposes. First, histone methylation patterns are thought to represent an epigenetic program that preserves information regarding cellular identity when cells are induced to divide or exposed to changes in the native environment.55 Second, histone acetylation is thought to represent a means by which chromatin structure can be rapidly and reversibly adjusted to dynamically regulate transcription factor binding in response to fluctuating environmental cues.36 These concepts are detailed below.

There is a plethora of data supporting the notion that different histone methylation patterns across eukaryotic genomes function as specialized binding surfaces within the chromatin that function to attract protein complexes that contain chromatin remodeling and transcriptional activation/silencing activity. It is also thought that in some circumstances, methylation of histone lysine residues in chromatin represents a means by which information storage regarding basal transcriptional competence is preserved during periods when cells are induced to proliferate or change patterns of gene expression away from baseline.37 Four observations support these concepts. First, bulk turnover of methylated histones is extremely low, indicating that histone methylation is a stable modification that is not often removed once it is deposited into chromatin.29 This implies that once histone methylation patterns are programmed across the genome, they are present for an extended or even indefinite time over a cells lifetime. Second, methylated histones serve as docking sites for protein modules that are members of multisubunit complexes, thereby directly tethering these complexes to the chromatin template.35 Remarkably, some methylated lysine residues recruit complexes that promote assembly of heterochromatin whereas others recruit protein complexes that activate transcription, and these antagonistic modifications are found almost exclusively in areas of heterochromatin and euchromatin, respectively (eg, see the Table). Third, there are data indicating that under some circumstances histone methylation patterns at specific loci remain relatively constant amid fluctuations in transcriptional activity from baseline. In particular, a “methyl/phos” binary switch has been proposed28 whereby reversible phosphorylation of serine/threonine residues located adjacent to methylated lysines can function to transiently displace methyl-docking proteins, thereby potentially antagonizing the action of these protein complexes. However, once the phosphate groups are removed by phosphatases, methyl lysine–binding proteins can relocate to the locus and resume their activities.39–41 Therefore, histone methylation has potential to retain information regarding baseline transcriptional competence of a gene, via directing reactivation or resiliencing after termination of cellular events that trigger histone phosphorylation or other mechanisms that displace methyl-binding modules off the chromatin template.42 Fourth, histone methylation patterns and their functional consequences are faithfully inherited from parent to daughter cell, along with the associated genomic DNA during both mitotic and meiotic cell divisions.24–43 This strongly suggests that histone methylation evolved in part to provide stable units of epigenetic inheritance for information transmission across generations.

In light of these amazing observations, histone methylation has emerged in the literature as an attractive candidate for a bona fide “epigenetic memory” of long-term transcriptional competence and therefore a primary determinant of cellular identity. Although there is a wealth of data supporting this notion, it must be noted that this idea has been challenged by the recent discovery of several histone demethylase enzymes, although it is still far from clear as to how and to what degree these enzymes operate in vivo.37 Therefore it is currently very controversial as to whether histone lysine methylation is just another dynamic entity similar phosphorylation/acetylation or if it really does represent a genomic indexing system that resists erasure. The answer probably lies somewhere in between, in that there may be some developmental contexts, genomic loci, or histone residues that are resistant to demethylation, whereas others are amenable to it.37 To further complicate matters, histone lysines can be mono-, di-, or trimethylated (abbreviated hereafter as H#K#Me#; eg, H3 Lys4 dimethylation is H3K4Me2, H3 Lys27 trimethylation is H3K27Me3, and so on), and different histone methyltransferases and demethylases appear to possess varying levels of enzymatic activity toward these different states in addition to their different specificities toward histone residues. In light of these findings, other components of the chromatin fiber have been proposed to carry out the functions classically ascribed to histone methylation,44 although it is still believed that histone methylation in some form holds these properties even amid the existence of demethylases.37

Whereas methylated histones appear to function primarily as targeting platforms within chromatin, histone acetylation has been shown to directly relieve structural chromatin compaction, possibly through disruption of interactions between adjacent nucleosomes and through loosening of contacts between histones and DNA.28,45–47 The presence of acetylated histones within promoter chromatin is thought to promote local unfolding of the otherwise condensed chromatin fiber coupled with partial or complete unwrapping of DNA from histones, and the degree to which these adjustments to chromatin structure occur are a function of the quantitative levels of acetylation.36 There is also evidence that acetylation events may also facilitate the action of ATP-dependent chromatin remodeling proteins that mobilize DNA from histone octamers48 as well as directly recruit members

### SMC-Specific Histone Modifications Distinguish SMCs from Non-SMCs

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<th>SMC Gene Modifications</th>
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<td>H3 Lys4 methylation</td>
<td>SMCs/Euchromatin</td>
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<td>H3 Lys9 acetylation</td>
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<td>H3 Lys14 acetylation</td>
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of the basal transcription apparatus. All of these effects are thought to synergize to expose the DNA template to transcription factors that require physical contact with DNA to activate transcription (eg, SRF). Acetyl groups added to histones by histone acetyltransferases (HATs) are readily and often removed by the action of other enzymes with histone deacetylase (HDAC) activity in vivo. Therefore, histone acetylation by HATs actively facilitates chromatin unfolding and presentation of DNA to transcription factors, whereas the removal of acetyl groups from histones by HDACs reverses these effects. The result is 2-fold. First, euchromatin is enriched with acetylated histones, whereas heterochromatin is relatively devoid of acetylated histones. Acetylation therefore provides 1 explanation as to why these 2 forms of chromatin take on opposite properties in terms of chromatin condensation and transcription factor accessibility. In effect, the presence of acetylated histones primes euchromatin for transcriptional activation. Second, quantitative regulation of acetylation levels within euchromatin provides a means whereby chromatin compaction and transcription factor binding can be rapidly and reversibly adjusted in a graded fashion in response to fluctuating extracellular cues that signal to HATs and HDACs. This provides an efficient mechanism for fine titration of transcription of genes within euchromatin, so that cells may effectively adapt to fluxes in the extracellular microenvironment.

A Histone Modification Program Specifies the SMC Lineage

Although there are numerous molecular features that underlie cell lineage specification, there are certain features about histone modifications that make them particularly strong candidates to participate in this function. All histone modifications are in intimate association with DNA, and histone methylation patterns are inherited through cell division, along with the genetic information contained in the DNA sequence. It is therefore conceivable that different histone modification patterns that code for the assembly of euchromatin or heterochromatin are present at the same genetic loci in different cell lineages, thereby giving rise to the cell-specific gene expression potential characteristic of the multitude of diverse cell types found in higher eukaryotes. Indeed, several recent observations support this concept, including studies examining histone modification patterns in SMCs. SMC-specific sets of histone modifications are acquired at the promoter chromatin of SMC genes during development from multipotential stem cells to SMCs. Several studies from our laboratory and others have shown that acetylation to histone H3 and H4 residues are enriched at SMC gene CArG boxes in cultured SMCs, whereas stem cell lines such as cultured ECs, P19 cells, and A404 SMC progenitors possess low levels of acetylation at these promoters. In addition, these genes display high levels of H3 Lys4 and Lys79 dimethylation in SMCs relative to non-SMC lineages, including stem cells and somatic cell lineages. In contrast to SMCs, SMC gene promoters are enriched for H3 Lys27 trimethylated histones in ESCs, a well-studied modification that promotes assembly of heterochromatin via recruitment of polycomb group proteins. Similar to ESCs, SMC gene chromatin in non-SMC somatic cells (eg, endothelial cells, skeletal muscle myotubes, leukocytes) also contains high levels of H3 Lys27 trimethylation, in addition to H4 Lys20 dimethylation (elsewhere and O.G.M. and G.K.O., unpublished observations, 2005). These data closely fit the micrococcal nuclease and SMC-restricted SRF-binding observations. Collectively, these findings suggest that SMC gene promoter DNA is initially wrapped into chromatin harboring modified histones that direct heterochromatin assembly in ESCs (H3K27Me3). At some point during the developmental transition from undifferentiated stem cells to differentiated SMCs, H3K27Me3 is replaced with euchromatic modifications, which are expected to encode chromatin that is permissive to SRF binding. Other non-SMC lineages retain H3K27Me3 (and/or gain H4K20Me2) during their developmental histories, thereby effectively maintaining SMC gene chromatin in a heterochromatic state refractory to SRF binding. Figure 3A and 3B and the Table summarize this data for histones H3 and H4. Clearly, there is an abundance of exciting work that lies ahead to further characterize how histone modification patterns are programmed into SMC gene chromatin during development and how these contribute to SMC fate determination.

At the very least, these distinct histone modification patterns can be used to distinguish SMCs from non-SMCs and vice versa. Even more fundamental, SMC-specific patterns of histone modifications might represent an epigenetic program that defines the SMC lineage through selective control of SRF binding to SMC gene chromatin. All of the results described thus far reflect ChIP data around the 5′-CArG box regions of these genes. Interestingly, we mapped the distribution of these modifications across the SMC α-actin locus for ∼3 kb on either side of the CArG box in cultured SMCs, which captures regions outside the promoter on the 5′ end and extends to regions inside the coding region on the 3′ end (Figure 3C). We found that all of the euchromatic modifications examined were essentially absent from chromatin 5′ of the promoter, whereas the heterochromatic modifications were highly enriched. At a point approximately 1 kb 5′ of the CArG boxes, these distributions abruptly reversed (elsewhere and O.G.M. and G.K.O., unpublished observations). These results suggest that these modification patterns are specifically programmed into this locus around the CArG box promoter region (by yet unknown factors), presumably to serve some functional role in activating transcription. Drawing from our earlier discussions regarding histone acetylation and methylation, it can be hypothesized that some of the histone modifications present around the CArG boxes in SMCs function to decompartment the chromatin and expose CArG box DNA to SRF (eg, acetylation), whereas others directly recruit and/or stabilize SRF binding to the chromatin via a tethering function (eg, methylation). In contrast, the absence of these modifications combined with the presence of heterochromatic modifications observed in the non-SMC lineages may direct the assembly of heterochromatin at SMC loci, thereby precluding efficient SRF binding (Figures 2 and 3). In this way, SMC-specific gene expression is accomplished. Much work is needed to test these possibilities and demonstrate causality, both in SMCs and in non-
Recent evidence has demonstrated a role for histone modifications in control of chromatin-binding activity of SRF, as would be expected if these modifications played a role in SMC specification. From these studies a sequence of step-wise molecular events within chromatin that direct activation of SMC gene chromatin contains skeletal muscle genes effectively precluded SRF binding to these promoters, thereby blocking myocyte differentiation. Similar functional studies addressing silencing of SMC genes non-SMCs are needed.

**SMC Histone Modifications Regulate the Chromatin-Binding Properties of SRF**

Recent evidence has demonstrated a role for histone modifications in control of chromatin-binding activity of SRF, as would be expected if these modifications played a role in SMC specification. From these studies a sequence of step-wise molecular events within chromatin that direct activation of SMC gene transcription can be derived as depicted in Figure 4 and the text below. In ESCs, SMC gene chromatin contains H3 histones methylated at Lys27, which maintains these genes in a heterochromatin conformation that is refractory to SRF binding (Figure 4A). ESCs that are destined for non-SMC lineages retain H3K27Me3 (and/or acquire H4K20Me2). However, in ESC subsets that commit to the SMC lineage, H3K27Me3 is replaced with the permissive modifications characteristic of differentiated SMCs. A key challenge to understanding this process is deciphering the temporal sequence in which these different modifications are programmed into the SMC chromatin during development, as well as what (if any) roles they play in promoting SRF binding and/or other events during transcriptional activation. Assuming that activation of transcription is a linear step-wise event, any histone modifications that regulate the chromatin-binding properties of SRF must be added to the chromatin before SRF can bind. That is, these modifications are predicted to lie upstream of SRF binding during transcriptional activation within chromatin. Under this premise, we have generally observed only H3K9Me3 at SM α-actin in ESCs, whereas we have observed the others within CArG box chromatin of multiple SMC-specific contractile genes in several SMC and non-SMC lines (and from rat tissues in vivo). One exception is that H4K20Me2 is not found at SMC genes in ESCs, although it is found in non-SMC somatic cells. Distribution of heterochromatic (red) and euchromatic (green) histone modification enrichments across the SM α-actin locus. SRF, TATA-binding protein/TFIID, and RNA polymerase II distributions are also included, as are areas with the highest probability of nucleosome occupancy (gray cylinders) as predicted by micrococcal nucleosome digestion experiments. Data in this figure represent previous ChIP-mapping experiments and unpublished observations by O.G.M.

and G.K.O., 2005. Note: modifications to different histone residues are color coded in all figures according to Figure 3. Ac indicates acetylation; Me, methylation.

SMCs. In this regard, experiments in undifferentiated myoblasts demonstrated that the absence of acetylated histones and the presence of H3K27Me3 within CArG box chromatin of skeletal muscle genes effectively precluded SRF binding to these promoters, thereby blocking myocyte differentiation. Similar functional studies addressing silencing of SMC genes non-SMCs are needed.
In this regard, multiple SMC contractile genes anchor these protein complexes within the chromatin. It is thought that this extra stability assists to provide rewarding insights into the nature of both SMC physiology and chromatin biology.

Figure 4. Step-wise assembly of SMC-specific chromatin at SMC gene promoters. In this model, SMC-specific H3K4Me2 and H4 acetylation (Ac) combine to encode a chromatin structure permissive for binding of myocardin/SRF. Myocardin/SRF then recruit other transcription factors to activate transcription. ESCs and other non-SMC lineages contain heterochromatic histone modifications and compacted chromatin, thereby inhibiting SRF binding (A). Methylation (Me) of H3 Lys4 by unknown histone methyltransferases is programmed into SMCs during development of SMCs from ESCs (B) to provide a docking site for either myocardin or some myocardin-associated factor that binds methylated histones (MBP). H4 acetylation by as of yet unknown HAT(s) is also required to "open" up the chromatin and expose degenerate CArg box DNA accessible to SRF (C). However, SRF binding to these degenerate CArg boxes is weak and transient in the absence of myocardin. The appearance of myocardin and/or putative MBPs that interact with myocardin therefore stabilizes binding of SRF to the degenerate CArg boxes via their interaction with nearby histones harboring H3K4Me2 (D and E). In this way, H4 acetylation and H3K4Me2 synergize to assemble chromatin that is permissive for binding myocardin/SRF complexes. Myocardin/SRF then recruits other transcription factors (eg, CBP/p300) via the TAD of myocardin that subsequently add other histone modifications (eg, H3 acetylation, H3K79Me2) and recruit the basal transcription machinery (TATA-binding protein/TFIID [TBP], RNA polymerase II [Pol II], etc) downstream of SRF to robustly activate transcription (F).
Numerous investigations into the functions of H4 acetylation in traditional cell systems have demonstrated a role for this modification in direct regulation of chromatin compaction. Indeed, one of the first studies to demonstrate H4 acetylation is a prerequisite for activation of transcription in response to physiological stimuli did so in the context of SRF-dependent activation of c-fos.56 This concept appears applicable to SMCs as well. Two proteins, homeodomain-only protein 1 (HOP) and Kruppel-like factor 4 (KLF4) have been found to physically associate with HDAC2 and recruit this protein to SMC gene promoters.32,57 HDAC2 possesses deacetylase activity specific to histone H4 residues,58 and expression of both HOP and KLF4 in cultured cells results in loss of H4 acetylation from SMC gene chromatin. This in turn is accompanied by chromatin compaction, loss of SRF binding, and transcriptional repression consistent with an upstream role for H4 acetylation in control of SRF binding to SMC gene promoter chromatin. Indeed, these effects are blocked by the HDAC inhibitor trichostatin A. Remarkably, both KLF4 and HOP potently antagonize the transcriptional activity that myocardin conveys to SRF, and this action is also dependent on the ability of these proteins to recruit HDAC activity to SMC genes and block SRF association with CArG box chromatin. Furthermore, treatment of SMCs with trichostatin A under native conditions results in H4 hyperacetylation and increased binding of SRF within chromatin of SMC genes.33 These studies reveal that the SMC-specific presence of H4 acetylation likely functions to maintain SMC gene chromatin in a conformation in which CArG box DNA sequences are accessible to SRF (Figure 4C and 4E). The absence of this modification, as in non-SMCs or in SMCs expressing proteins such as HOP or KLF4, results in compaction of this chromatin into a conformation that blocks binding of SRF, most likely by tight packaging of CArG box DNA with histone octamers, which is predicted to strongly inhibit SRF binding based on crystal structure data (Figure 4A and 4B). It is also likely that the quantitative levels H4 acetylation within SMC gene euchromatin are adjusted in response to various environmental cues for reversible, graded regulation of SRF binding to SMC genes. This would allow fine tuning of SMC gene expression levels in adult SMCs for adaptation to fluctuating extracellular signals.

Collectively, available evidence implies that H3K4Me2 serves as a docking site for myocardin/CArG complexes within chromatin, whereas H4 acetylation functions to “open up” the chromatin and provide CArG box DNA suitable for binding SRF. These 2 modifications operate in combination to establish a chromatin structure that is permissive to SRF binding (Figure 4), consistent with the histone code hypothesis.20,59 Several pieces of data support this concept, although a definitive demonstration of causality has yet to be realized. This is attributable to a number of confounding experimental obstacles that are commonly encountered in chromatin biology, most notably the inherent difficulties in minimizing indirect effects caused by loss-of-function strategies targeted toward chromatin-based processes, as these events play universal roles in a variety of cell behaviors besides transcription of gene subsets. Nevertheless, progress on these fronts will require development of effective strategies to test causality, such as in vitro reconstitution of SMC gene promoters wrapped into nucleosomes harboring various histone modifications or identification of SMC-restricted proteins that recruit HATs and histone methyltransferases to SMC gene promoters.

Other Factors Regulating SRF Activity Within SMC Chromatin

There are unequivocal data that the SMC-specific histone modification program is just 1 component of a fascinating multilayered transcriptional regulatory process that has evolved around SRF within SMC chromatin. Indeed, a truly integrated model is emerging in the literature that suggests control of SMC differentiation involves complex interplay among (1) SRF cofactors, (2) the SRF–CArG interaction itself, and (3) histone modifications within CArG box chromatin. Although much work regarding interconnections between these 3 processes is needed, we favor the view that all 3 of these regulatory levels (and others yet discovered) operate simultaneously in synergy and/or provide redundancy with one another to provide tight regulatory control over the SMC phenotype. As such, below, we very briefly review nonhistone based regulatory control of SRF activity within chromatin to provide a comprehensive glimpse into what is known about the chromatin dynamics underlying SMC transcription.

First, numerous SRF accessory proteins have been discovered that either displace promyogenic cofactors off of SRF to repress transcription or facilitate interactions SRF with promyogenic factors to activate transcription. These events occur while SRF is bound to CArG box DNA, consistent with the model that SRF is a platform for recruitment of other accessory modules. For example, Olson and colleagues60 completed very elegant studies showing that activation of the extracellular signal-regulated kinase (ERK) signaling pathway in SMCs results in phosphorylation of the TCF Elk-1, a protein that has the ability to form a ternary complex with SRF on CArG box DNA within promoters of both SMC-specific genes and growth-responsive immediate early genes. Phosphorylation of Elk-1 directly relocalizes of this protein from cytoplasm to the nucleus, where Elk-1 proceeds to compete with myocardin for a common docking surface on SRF. Displacement of myocardin off of SRF by Elk-1 has the resultant effect of blocking the strong transcriptional activation conveyed to SRF by myocardin.60 Whereas SRF-dependent growth-responsive genes (which do not use myocardin) are transcriptionally stimulated by the presence Elk/ SRF ternary complexes, SMC-specific contractile genes are repressed after replacement of myocardin/CArG by Elk/ SRF,60 albeit to different degrees.61 Other SRF-binding partners have been found to operate similar to Elk-1 in response to other signaling pathways.62,63 In contrast, other SRF accessory factors such as cysteine-rich proteins (CRP1/2),64 myocardin-related transcription factors,34 Prx1,19 and GATA factors64 have been found to stimulate the transcriptional activity of myocardin/CArG ternary complexes via their association with SRF on SMC gene promoters. This leads to increased levels of SMC gene transcription. Putting this together, tipping the balance between the activities of positive and negative SRF
cofactors by different signaling pathways offers a dynamic means by which SMC gene expression might be controlled.

Second, the ability of SRF proteins to directly bind CArG box DNA molecules is regulated through multiple mechanisms. Yin-yang 1 (YY1) is a transcriptional repressor that possesses CArG box DNA-binding activity. YY1 in turn has the ability to compete with SRF for binding to CArG box DNA by EMSAs. Exchange of YY1 for SRF on SMC gene promoters thus has the effect of repressing SMC gene transcription. HERP1 also has the ability to interfere with SRF binding to DNA, but via physical association with the SRF MADS box rather than with CArG DNA. The MADS box of SRF can also be phosphorylated at sites that inhibit SRF binding to DNA specifically at SMC gene promoters but not at promoters of growth-responsive genes. Finally, SRF can translocate from nucleus to cytoplasm in response to various signals, which may also reduce levels of SRF binding to CArG box DNA over time via reductions in the numbers of SRF molecules available to bind SMC gene promoters. In contrast, other proteins, such as CRP2, PIAS1, and various homeodomain proteins (eg, Phox1) enhance binding of SRF to CArG box DNA to stimulate transcription. Importantly, the mechanisms described in this passage were demonstrated to be operational not only via EMSAs (ie, raw SRF DNA-binding activity) but also within the context of CArG box DNA wrapped into euchromatin by ChIP assays.

The Chromatin Dynamics of SMC Plasticity

To truly integrate chromatin into the current model describing the myogenic activity of SRF, investigations into the interplay between SRF cofactors, the SRF–CArG interaction, histone modifications, and environmental cues are required. Currently, there are only a handful studies that have attempted to make connections between these regulatory layers. As such, future work in the field should attempt to address modular, synergistic, and/or redundant functions for these different processes relative to each other. Treatment of cultured SMCs with platelet-derived growth factor BB (PDGF-BB) has shed some light on what might be revealed by studies such as these. PDGF-BB is a molecule released at sites of vascular injury and atherosclerosis that potentiy induces phenotypic switching of cultured SMCs, including transcriptional repression of SMC-specific contractile genes, proliferation, and migration. These effects are completely reversible after removal of PDGF-BB from the media. Treatment of cultured SMCs with PDGF-BB results in rapid (≈30 minutes) activation of the ERK pathway and phosphorylation/nuclear translocation of Elk-1, thereby putting competition between Elk and myocardin for SRF into motion. Expression of KLF4 is also upregulated in response to PDGF-BB and 24 hours after treatment, there are reversible reductions in H4 acetylation and SRF binding from the chromatin of SMC genes. In contrast, growth-responsive genes such as c-fos retain high levels of acetylation, and Elk-1/SRF complexes stimulate transcription within the chromatin of these genes. Finally, there is evidence that SRF partially translocates out of the nucleus into the cytoplasm several hours after treatment of SMCs with PDGF-BB. These processes may operate to infuse redundancy into the actions of PDGF-BB, such that if 1 pathway is inhibited, genes remain partially or fully repressed because of compensation by the other pathways.

Alternatively, the diverse events that are initiated in response to PDGF-BB signaling might operate synergistically within a linked pathway. This is expected given that several studies indicate that inhibition of multiple different PDGF-BB–triggered events can antagonize PDGF-BB–mediated repression of SMC gene expression, although it is difficult to know how efficiently these diverse processes repress SMC transcription relative to each other without comparative studies. This concept is depicted in Figure 5, where physiological SMC chromatin (assembled during development as in Figure 4) is reorganized in response to PDGF-BB into a transcriptionally repressive configuration. This action is accomplished synergistically by the numerous events that are triggered by PDGF-BB signaling (eg, KLF4, Elk-1, Herp, phosphorylation, etc), thereby creating a pathological SMC chromatin environment that inhibits binding of myocardin/CArG complexes. These events are transient and fully reversible after removal of PDGF-BB from the culture medium, which allows reconstruction of physiological SMC chromatin that is permissive to myocardin/CArG. Reconstruction would proceed similar to Figure 4 via reacetylation of histone H4 coupled with reintroduction of myocardin to SRF complexes. This is predicted to make SMC chromatin transcriptionally permissive by both “reopening” chromatin (via acetylation) to SRF coupled with stabilization of SRF binding to CArG box DNA (via interaction of myocardin or a myocardin-associated protein with H3K4Me2). To illustrate, data from our laboratory indicate that the ability of KLF4 to repress SMC gene expression is dependent on activation of the ERK pathway, and we have found a strong physical interaction between Elk-1 and KLF4 in extracts isolated from SMCs where the ERK pathway has been activated (R. Deaton, O.G.M., and G.K.O., unpublished observations, 2006). Although speculative, it is possible that displacement of myocardin off SRF by Elk-1 might be the sole mediator of the PDGF-BB response early (eg, 30 minutes), when myocardin levels are high and KLF4 levels are low. Once KLF4 is upregulated, occupancy of Elk-1 within SMC gene chromatin might also attract KLF4/HDAC2 complexes to SMC promoters leading to deacetylation of histones, chromatin compaction, and ejection of SRF from the chromatin template, further enhancing the repressive effects of PDGF-BB. Any other potential transcriptional activation by residual myocardin/CArG binding attributable to stochastic/transient opening of compacted chromatin would continue to be antagonized by the competitive function of Elk-1. Translocation of SRF out of the nucleus might further reduce any residual SRF binding. Theoretically, the synergistic actions of these processes would generate tight repression of SMC gene expression. Again, removal of PDGF-BB from the culture media terminates these repressive molecular forces and allows reconstruction of physiological SMC chromatin, thereby restoring SMC differentiation. Many of these cell culture observations have been confirmed by experimental observations in rats and mice, including demonstration of reversible loss of SRF binding.
SMC Plasticity: A Model System to Study Chromatin Dynamics

The profound phenotypic plasticity of the SMC lineage has historically represented a confounding obstacle for study of SMC behavior. However, we feel that this property represents a unique advantage for the study of how cell-specific gene expression/transcription is dynamically controlled at the level of chromatin. Differentiated SMCs can be induced to reversibly switch their phenotype virtually on command, representing a potential model system whereby chromatin is reversibly remodeled into pathophysiological configurations and yet still retains information critical for restoration back to its original physiological form.

Figure 5. Histone methylation (Me) provides epigenetic memory of SMC identity during synergistic repression of SMC gene transcription. In this model, various pathways that have been shown to repress SMC gene expression are linked together in synergy. These events are dependent on each other to achieve the full function of inhibiting SRF-dependent transcription via positive feedback. In addition, some or all of these processes may be physically linked in a step-wise manner over time (eg, Elk-1 binds SRF, then recruits KLF4/HDAC2, which subsequently deacetylates H4). During these events, histone H3K4Me2 is preserved within pathological chromatin, thereby providing a means by which the original physiological chromatin configuration can be restored by directing relocalization of myocardin/SLF and/or H4 HATs to SMC gene promoters after termination of repressive environmental cues (ie, the reconstruction phase). The black arrow adjacent to myocardin represents decreased myocardin expression, as the event has been documented in some SMC lines in response to PDGF-BB and during vascular injury/atherosclerotic plaque formation in vivo. This event would be predicted to decrease SRF binding to SMC genes via disruption of the myocardin–H3K4Me2 interaction. Finally, the hypothetical situation of a “methyl/phos” switch to temporarily displace myocardin/SLF from SMC gene promoters during transcriptional repression is also included, which might also antagonize myocardin–H3K4Me2. Ac indicates acetylation; P, phosphorylation.
our opinion, investigation into this phenomenon is very important, as H3K4Me2 (or some other modification not yet tested) patterns across SMC genomes could represent a molecular memory of which genes must retain the ability to become reactivated on resolution of conditions (eg, vascular injury) in which SMCs undergo reversible phenotypic switching (ie, cell lineage memory). This could also provide a practical means by which to follow neointimal SMCs in vessels exposed to various stimuli such as atherosclerotic plaque formation, an exercise that has historically been confounded because of loss of markers such as SMC-specific contractile proteins that are typically used to identify the SMC lineage in vivo.

A simple first step to explore this hypothesis would be to transfect SMCs with histone peptides methylated at H3 Lys4 and determine whether these “squelch” myocardin and/or SRF from SMC promoters. Immunohistochemistry of sections from arteries exposed to vascular injury or atherosclerotic plaque with H3K4Me2 antisera conjugated to fluorescent DNA probes homologous to SMC gene sequences could confirm whether these genes remain methylated under these conditions. Although certainly technically challenging, these experiments are envisioned to proceed in a fashion similar to standard in situ cytogenetic protocols, except that conditions would be such that hybridization would depend on binding of the antisera in close proximity to DNA probe targets, in addition to binding of the DNA probe to the target itself (in this case, the targets/probes are SMC gene promoters). Subsets of H3 Lys4 methylated histones have been found to be phosphorylated at Thr3 in vivo, and the presence of this modification inhibits binding of proteins that dock to H3 Lys4 methylated histones via the “methyl/phos” binary-switch process described earlier (Figure 5). It is also possible that myocardin or a myocardin-associated protein is modified so as to inhibit interaction with H3K4Me2. Antibodies that recognize histones with dual methyl and phosphoryl groups are becoming commercially available (Upstate, Abcam), providing a starting point for investigation into whether a methyl/phos switch might be operational at SMC gene promoters. Eventually, purification of all proteins present in myocardin/SRF complexes, along with identification and functional characterization of any modules that bind methylated histones, will provide the most definitive experiments addressing this issue. Clearly, opportunities are plentiful for investigation into chromatin dynamics underlying SMC phenotypic plasticity.

**Closing Remarks**

The inherent phenotypic plasticity of the SMC lineage has presented remarkable insights and unique challenges regarding our understanding of transcriptional control of cellular differentiation. Indeed, the unfolding story documenting the molecular behavior of SMCs under physiological conditions versus times of pathophysiological stress is emerging as a general paradigm for how fluctuating environmental cues dynamically regulate differentiation and plasticity of most if not all cell types in higher organisms. The model that is emerging from studies of SMCs (and other cell lineages) suggests that cellular differentiation is tightly regulated at the level of chromatin through a complex, synergistic combination of DNA-binding transcription factors (eg, SRF), accessory cofactors for the DNA-binding proteins (eg, myocardin/Elk-1), the direct interaction of DNA and transcription factor complexes (eg, the SRF–CArG interaction), and histone modifications present within promoter chromatin (eg, SMC-specific H3K4Me2 and H4 acetylation at CArG boxes). In SMCs, this multilayered orchestra evolved around SRF to provide multiple avenues by which environmental cues and signaling pathways may signal to chromatin to dynamically control gene expression.

Further investigation into the issues addressed in this review will undoubtedly yield important insights into the SMC component of vascular development and the multitude of disease processes in which SMC pathology is a prominent component (eg, atherosclerosis). In addition to the multiple experimental directions proposed earlier, several clever cell systems that capture the transit from undifferentiated ESCs to differentiated SMCs have recently been developed. These systems still largely await investigation into how histone modifications are programmed into SMC gene chromatin during normal SMC development. These latter experiments are critical as they will provide a wealth of information necessary to engineer healthy SMCs in vitro that may be used for therapeutic purposes in vivo. Future studies regarding this issue should focus on identification of trans factors and cis elements important for programming histone modifications into SMC gene promoters during development. Continued efforts in combining mutation of promoter cis elements with ESC knockout of trans factors identified by yeast 2-hybrid assays or bioinformatics methodology are the most powerful initial approaches to address these issues. In addition, expansion of our knowledge regarding epigenetic regulation of SMC chromatin is a must. Investigations must include the following: how other histone modifications regulate SMC differentiation, identification of new mechanisms whereby histone modifications operate in SMCs, examination of other epigenetic processes such as ATP-dependent chromatin remodeling, micro-RNAs, and DNA methylation in SMCs, nuclear packaging/localization of SMC genes, and assembly of heterochromatin at SMC gene loci in non-SMCs.

Over the past decade, efforts aimed at elucidation of the molecular processes that underlie SMC differentiation have intensified at an exponential rate. These studies have begun painting what promises to be an elegant picture depicting an incredibly complex and finely tuned molecular process that bestows SMCs with their remarkable cellular properties. We anticipate that future studies have the potential to offer particularly instructive insights into how cell differentiation is controlled at the level of chromatin, both within the context of normal SMC development and SMC phenotypic plasticity within dynamic tissue microenvironments.

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Disclosures

None.

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