

## ATVB In Focus Smooth Muscle Cells

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# Combinatorial Control of Smooth Muscle–Specific Gene Expression

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**Abstract**—Alterations in the differentiated state of vascular smooth muscle cells (SMCs) are known to play a key role in vascular diseases, yet the mechanisms controlling SMC differentiation are still poorly understood. In this review, we discuss our present knowledge of control of SMC differentiation at the transcriptional level, pointing out some common themes, important paradigms, and unresolved issues in SMC-specific gene regulation. We focus primarily on the serum response factor–CArG box–dependent pathway, because it has been shown to play a critical role in regulation of multiple SMC marker genes. However, we also highlight several other important regulatory elements, such as a transforming growth factor  $\beta$  control element, E-boxes, and MCAT motifs. We present evidence in support of the notion that SMC-specific gene regulation is not controlled by a few SMC-specific transcription factors but rather by complex combinatorial interactions between multiple general and tissue-specific proteins. Finally, we discuss the implications of chromatin remodeling on SMC differentiation. (*Arterioscler Thromb Vasc Biol.* 2003;23:737-747.)

**Key Words:** smooth muscle cells ■ differentiation ■ transcriptional regulation  
■ serum response factor ■ CArG box

Abnormal growth and proliferation of vascular smooth muscle cells (SMCs) is a key feature of vascular diseases such as atherosclerosis, restenosis, and hypertension.<sup>1,2</sup> Differentiated SMCs of mature animals express a unique repertoire of contractile proteins, ion channels, and signaling molecules that are necessary for their principle function of contraction. However, in response to various injurious stimuli, SMCs can dedifferentiate, converting from a quiescent, contractile phenotype to a highly proliferative, synthetic phenotype.<sup>3</sup> Despite the importance of changes in the differentiated state of SMCs in vascular disease, the molecular mechanisms controlling SMC differentiation are still largely unknown. An understanding of the normal regulation of SMC development and differentiation will not only provide the foundation for elucidating how these pro-

cesses may be disrupted in vascular disease but will also be critical to understanding congenital defects in vascular development and vascular development within solid tumors.

Control of cellular differentiation is ultimately regulated at the level of gene transcription, although cell-specific alternative splicing and posttranslational modifications also play important roles. This review will focus on our present understanding of the transcriptional pathways that normally control SMC differentiation as well as discuss the important, but still poorly understood, role of chromatin modifications on SMC-specific gene expression. But before reviewing transcriptional control in SMCs, we will first briefly discuss the developmental origins of SMCs, SMC differentiation/dedifferentiation markers, and several recently described in vitro SMC differentiation models.

Received December 13, 2002; accepted February 14, 2003.

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*Arterioscler Thromb Vasc Biol.* is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000065197.07635.BA

### Developmental Origin of SMCs

One of the challenges to studying mechanisms of SMC development and differentiation is the fact that unlike cardiac and skeletal muscle (which are derived from specific populations of mesodermal precursors), SMCs are derived from multiple and, in some cases, unknown cell types. For example, SMCs of the ascending aorta and branchial arches are derived from neural crest (ectodermal) cells. SMCs of the coronary vessels arise from epithelial-to-mesenchymal transition of cells in the proepicardial organ. Most of the remaining SMCs, including visceral SMCs, are thought to be derived from condensation of local mesenchyme under the influence of local environmental cues.<sup>4,5</sup> The diverse origins of SMCs likely contribute to their heterogeneity in structure, function, and SMC marker gene expression. Moreover, the signals and molecular pathways leading to SMC differentiation may prove to be somewhat different depending on the cell type of origin.

### Markers of SMC Differentiation and Dedifferentiation

To study cell-specific transcriptional regulation, it is necessary to first identify a set of cell-specific target genes and then determine how these genes may be coordinately regulated at the transcriptional level. This too has proven challenging in the SMC field, because most (if not all) SMC markers, although highly selective for SMCs in adult animals, are expressed, at least transiently, in other cell types. These include the following SM isoforms of contractile apparatus proteins: SM  $\alpha$ -actin, SM myosin heavy chain (SM MHC), calponin, SM 22 $\alpha$ , h-caldesmon, and smoothelin. A detailed description of these proteins and other potentially useful SMC differentiation markers and their expression patterns has been reviewed elsewhere.<sup>3,5,6</sup> Of these factors, the most SMC-restrictive protein seems to be SM MHC.<sup>3</sup> Although we cannot rule out that SM MHC may be expressed, at least transiently or under certain circumstances, in cells other than SMCs *in vivo*, to our knowledge, conclusive evidence of SM MHC expression (by immunostaining with SM MHC-specific antibodies or reverse transcriptase-polymerase chain reaction followed by sequencing) in a non-SMC *in vivo* does not exist. In addition, two proteins with unknown function, aortic preferentially expressed gene 1 (APEG-1)<sup>7</sup> and Crp2/SmLim,<sup>8,9</sup> have been found to be preferentially expressed in arterial SMCs and are likely to be useful markers of this subtype of SMCs. Finally, a secreted protein, aortic carboxypeptidase-like protein (ACLP),<sup>10,11</sup> whose function is also not well-defined, has been shown to be upregulated during SMC differentiation but is by no means SMC-specific. Because no individual marker is exclusive to the entire SMC lineage (with the possible exception of SM MHC), identification of SMCs and investigations into SMC-specific transcriptional regulation requires the use of multiple markers.

When SMCs undergo phenotypic modulation, as occurs in vascular injury, virtually every SMC differentiation marker (with the exception of ACLP<sup>12</sup>) is downregulated.<sup>3,5,6</sup> In addition, certain proteins, which may be used as markers of dedifferentiated VSMCs, are upregulated, including osteopontin (a multifunctional adhesive glycoprotein)<sup>13</sup> and the

### Transcription Factors Known or Likely to be Involved in Regulation of SMC Differentiation/Proliferation

Transcription Factor	Target Gene(s)
<b>MADS Box Proteins</b>	
SRF	↑ numerous SMC differentiation markers <sup>4,6</sup>
MEF2B	↑ SM MHC <sup>101</sup>
MEF2C	SMC specific targets unknown <sup>102</sup>
<b>Homeodomain Proteins</b>	
Phox1/Mhox	↑ SM $\alpha$ -actin <sup>44</sup>
Barx1b	↑ $\beta$ -Tropomyosin <sup>48</sup>
Barx2b	SMC specific targets unknown <sup>45</sup>
Nkx3.1	↑ SM $\gamma$ -actin <sup>46</sup>
Nkx3.2	↑ $\alpha_i$ integrin, SM22 $\alpha$ , and caldesmon <sup>49</sup>
Hox B7	↑ SM22 $\alpha$ and calponin <sup>103</sup>
Hex	↑ SMemb/NMHC-B <sup>104</sup>
Gax (Mox2)	↑ p21; ↓ proliferation <sup>98</sup>
GATA family (GATA 4/5/6)	↑ numerous SMC differentiation markers <sup>49,92,105</sup> ↑ p21; ↓ proliferation <sup>96,106</sup>
Mycardin	↑ numerous SMC differentiation markers <sup>50,51</sup>
<b>Kruppel-like Zinc Finger Proteins</b>	
Sp1/3	↑ ACLP <sup>12</sup> ; ? ↓ SM MHC and SM22 $\alpha$ <sup>95,107</sup>
GKLF/KLF4	↓ SM22 $\alpha$ and SM $\alpha$ -actin <sup>30</sup> ↑ p21; ↓ proliferation <sup>108,109</sup>
BTEB2/KLF5	↑ SMemb/NMHC-B <sup>110</sup> ; ? ↑ SM22 $\alpha$ <sup>30</sup>
<b>Helix-Loop-Helix (HLH) Proteins</b>	
USF	↑ osteopontin <sup>111</sup> , SM $\alpha$ -actin <sup>76</sup> , APEG-1 <sup>74</sup>
CHF/HRT/gridlock	SMC specific targets unknown <sup>112-114</sup>
dHAND/HAND2	SMC specific targets unknown <sup>115</sup>
eHAND/HAND1	SMC specific targets unknown <sup>116</sup>
capsulin/epicardin	? ↑ SM $\alpha$ -actin <sup>117</sup>
Id2/3	↓ p21; ↑ proliferation <sup>99,118,119</sup>
Twist	↓ SM22 $\alpha$ <sup>91</sup>
<b>Single-Stranded DNA Binding Proteins</b>	
Pur $\alpha$ , Pur $\beta$ , and MSY1	? ↓ SM $\alpha$ -actin <sup>83,84</sup>
<b>Others</b>	
Egr-1	↑ proliferation (numerous targets) <sup>100</sup>
TEF-1	? ↑ SM $\alpha$ -actin <sup>83,84</sup>
SSRP1	↑ SM22 $\alpha$ <sup>47</sup>
Mrf2 $\alpha/\beta$	↑ SM $\alpha$ -actin and SM22 $\alpha$ ; ↓ proliferation <sup>28</sup>
AP-1	↑ osteopontin <sup>111</sup>
YY1	↑ SM22 $\alpha$ <sup>37</sup>
p53	↑ SM $\alpha$ -actin <sup>120</sup>
c-myb	↑ SM $\alpha$ -actin <sup>121</sup>
CRP1/2	SMC specific targets unknown <sup>9,122</sup>
NFATc1	↑ SM MHC <sup>123</sup>

embryonic/nonmuscle isoform of myosin heavy chain, SMemb/NMHC-B.<sup>14</sup> Although we will not be discussing the regulation of these SMC dedifferentiation markers in this review, some of the factors that have been shown to upregulate these markers can be found in the Table. Although it is widely accepted that SMCs retain a high degree of plasticity, which allows them to modulate their phenotype when neces-

sary to perform diverse functions, including contraction, extracellular matrix protein synthesis, migration, and replication, an alternate (although not necessarily mutually exclusive) view is that the SMCs that make up the vessel wall are heterogeneous and that the diverse functions of SMCs are carried out by specific subpopulations with different functional capabilities as opposed to modulation of any SMC into functionally different phenotypes. For additional reading on this topic, see reports by Bochaton-Piallat and colleagues,<sup>15,16</sup> Frid et al,<sup>17</sup> and references therein. In addition, an emerging body of evidence now suggests that bone marrow stem cells may be a potential source of neointimal vascular SMCs in conditions such as transplant arteriopathy and atherosclerosis.<sup>18,19</sup>

### In Vitro SMC Differentiation Models

Although cultured SMCs have proven invaluable for addressing many questions related to control of SMC-specific gene expression, inducible SMC lineage systems are likely to prove more powerful for identifying nodal regulatory factors and pathways leading to SMC differentiation. Such a system for the skeletal muscle lineage (based on treatment of 10T1/2 cells with the DNA hypomethylating agent 5-azacytidine) has led to the elucidation of critical regulatory factors and mechanisms governing skeletal muscle differentiation.<sup>20,21</sup> However, until recently, such systems were lacking for SMCs. Fortunately, there are now several SMC-inducible systems, each with its own advantages and disadvantages.

One of the first systems, based on treatment of P19 embryonal carcinoma cells with retinoic acid (RA), was described by our laboratory in 1995.<sup>22</sup> We recently improved on this system by generating stable lines of P19 cells carrying a SM  $\alpha$ -actin promoter/puromycin cassette to enrich for cells that have differentiated into the SMC lineage using puromycin selection.<sup>23</sup> One such stable line, designated as A404, behaves like a SMC precursor/progenitor line in that it shows a high propensity for differentiation into SMC-like cells with RA treatment (even before puromycin selection). As described below, we have already begun to use this system to elucidate SMC-specific chromatin modifications and their effect on transcription factor accessibility and gene expression.<sup>23</sup> For a more extensive review on the effects of retinoids on vascular SMC differentiation and growth, see the study by Miano and Berk.<sup>24</sup>

A second system is an embryoid body model described by Drab et al in 1997<sup>25</sup> in which totipotent mouse embryonic stem (ES) cells are cultured in such a way that they form embryo-like aggregates containing numerous differentiated cell types, including SMCs. This is potentially a very powerful system, and because ES cells with various candidate genes knocked out can be used, it provides a rapid and high throughput method of screening effects of knockouts of various proteins on SMC differentiation. However, to our knowledge, such a study has not yet been performed.

A third system, described by Jain et al in 1998,<sup>26</sup> involves culturing Monc-1 cells, pluripotent neural crest cells first generated and characterized by Rao and Anderson,<sup>27</sup> in a medium that promotes differentiation into the SMC lineage. Using screens to identify factors upregulated in SMC differ-

entiated versus undifferentiated Monc-1 cells, Jain and colleagues<sup>28</sup> have identified several factors that may play important roles in SMC differentiation, including Mrf2 $\alpha/\beta$  (see Table 1) and a Kruppel-like zinc finger protein (M. Jain, personal communication, 2002).

A fourth system, described by Hirschi et al in 1998,<sup>29</sup> involves coculture of 10T1/2 cells with endothelial cells or treatment of 10T1/2 cells with transforming growth factor  $\beta$  (TGF $\beta$ ). A limitation of this system is that, unlike the previous 3 systems in which induction of SM MHC (the most definitive marker for SMCs) has been well documented, it is controversial as to whether SM MHC is induced in the 10T1/2 system. For example, we have been unable to detect SM MHC expression in TGF $\beta$ -treated 10T1/2 cells by either reverse transcriptase-polymerase chain reaction or Western analysis (Manabe and Owens, unpublished observations, 2000). Although Hirschi et al<sup>29</sup> attempted to document its induction in this system using a SM MHC antibody, it is unclear whether the antibody they used specifically recognizes the SM isoform of MHC. We would like to emphasize here the importance of carefully evaluating the specificity of SM MHC antibodies when interpreting previous studies or designing future studies, because we have found that at least one of the principal antibodies used in many studies cross-reacts with a nonmuscle form of MHC (Thompson and Owens, unpublished observations, 1992). This issue is discussed more fully in the study by Owens.<sup>3</sup> Regardless, the TGF $\beta$ -10T1/2 system may still be a useful model of, at least, the early stages of SMC differentiation (ie, before the induction of SM MHC, a relatively late-stage SMC marker) and has been used to investigate mechanisms of TGF $\beta$ -dependent regulation of SMC marker genes.<sup>30,31</sup>

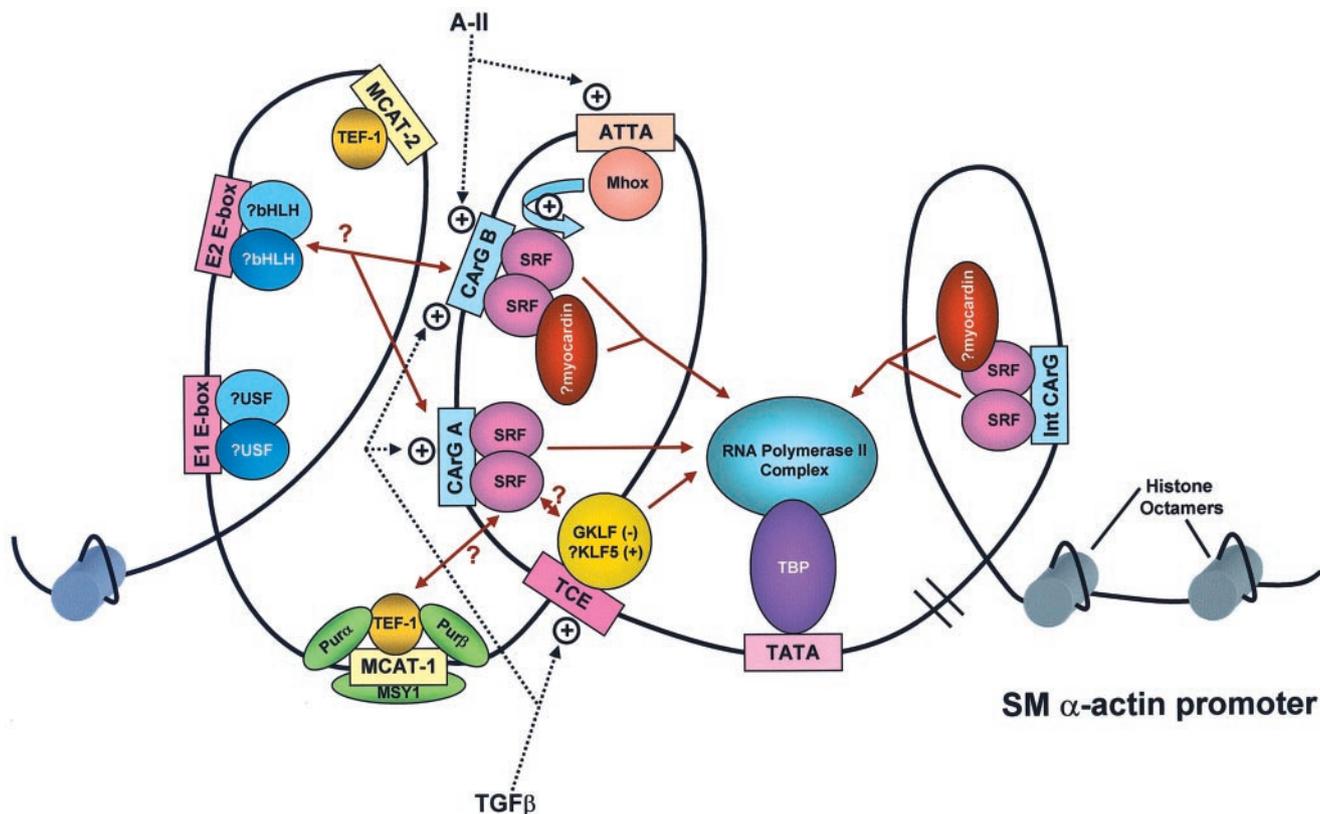
To the extent that these in vitro models mimic SMC differentiation in vivo, they may prove to be very powerful tools for dissecting mechanisms of SMC differentiation. However, because SMC differentiation seems to be highly dependent on the integration of numerous complex local environmental cues<sup>3</sup> that cannot be adequately recapitulated in culture, it is imperative that findings from these systems be verified in vivo using transgenic or knockout/knockin approaches.

### Transcriptional Regulation of SMC Differentiation Markers

Tremendous progress has been made in the last decade in identifying transcriptional regulators of SMC marker genes, most of which are summarized in the Table. Because of space limitations, we will not be able to discuss each of these factors in detail in this review. Rather, we will focus primarily on the serum response factor (SRF)-CArG-dependent pathway, because it seems to play a central and critical role in SMC differentiation, and will draw on several additional factors and regulatory elements as examples to illustrate some common themes, important paradigms, and unresolved issues in SMC-specific gene transcription.

#### SRF-CArG Dependent Pathway

SRF is a MADS (MCM1, Agamous, Deficiens, SRF) box transcription factor that was first identified and named be-



Schematic model of the SM  $\alpha$ -actin promoter depicting many of the protein-protein and protein-DNA interactions discussed in this review. In this model, transcription by the TATA binding protein (TBP)-containing RNA polymerase II complex is dependent on cooperative interactions between multiple *cis* elements and *trans* binding factors. Angiotensin II (A-II) enhances SM  $\alpha$ -actin promoter activity through modulation of protein binding to the CarG B and ATTA elements. TGF $\beta$  regulates SM  $\alpha$ -actin expression, at least in part, through regulation of protein binding to CarGs A and B and the TCE. SRF interacts with multiple cofactors, including homeodomain proteins, such as Mxox, and myocardin. Interactions between SRF and bHLH proteins bound to the E-boxes, TEF-1 family members bound to the MCAT elements, and KLF proteins bound to the TCE are less well defined but likely to play an important role in SM  $\alpha$ -actin regulation. Finally, the loops of DNA and histone octamers are intended to schematically represent the role of chromatin structure in controlling aspects of SMC-specific gene expression, including both transcription factor accessibility to DNA and interactions between transcription factors, to generate unique higher-order complexes.

cause of its ability to confer serum inducibility to the growth-responsive gene *c-fos* through binding to a sequence known as the serum response element or CarG box (CC[A/T]<sub>6</sub>GG). SRF binds CarG boxes as a dimer, with dimerization and DNA binding occurring through the MADS box domain.<sup>32</sup> In addition to regulating growth-responsive genes such as *c-fos*, SRF binding to CarG boxes has been shown to regulate numerous muscle-specific genes.<sup>33,34</sup> In particular, the SRF-CarG-dependent pathway has been demonstrated to play a central and critical role in regulating virtually every SMC-specific gene<sup>4,6,34</sup> with the possible exception of ACLP, whose promoter does not seem to contain a CarG box.<sup>12</sup> (However, because ACLP is also expressed at high levels in nonmuscle tissues such as bone and dermis,<sup>11</sup> it can be argued that ACLP is not a true SMC-specific gene.) Of major significance, transgenic mouse studies have shown that CarG box mutations in the SMC selective promoters SM MHC,<sup>35</sup> SM  $\alpha$ -actin,<sup>36</sup> and SM22 $\alpha$ <sup>37,38</sup> inactivate the respective promoter in one or more SMC subtypes. Because growth and differentiation are generally exclusive processes, a long-standing paradox and unresolved issue in this field is how SRF, a ubiquitously expressed transcription factor, can reg-

ulate both growth-responsive and muscle-specific genes. Although SRF levels seem to be higher in cardiac and skeletal muscle and SM compared with most other tissues,<sup>39,40</sup> this expression pattern alone cannot explain how SRF exerts its selective effects. Several mechanisms have been demonstrated to regulate SRF activity, including association with cofactors, posttranscriptional modifications of SRF, variations in SRF binding affinity among different CarG boxes, and number, position, and spacing of CarG boxes.<sup>41,42</sup> Of course, these mechanisms are not mutually exclusive, and it is likely that a combination of these factors is important in modulating the diverse functions of SRF.

#### SRF Cofactors

The *c-fos* CarG box is adjacent to a binding site for a subfamily of ETS domain transcription factors known as ternary complex factors, which, as their name suggests, form a ternary complex with SRF and the CarG box to activate transcription of the *c-fos* gene.<sup>32,41</sup> However, because muscle-specific CarG boxes are generally not flanked by such ternary complex factor binding sites, other factors are likely to play a role in muscle-specific gene expression. Interestingly, Grueneberg et al<sup>43</sup> demonstrated that the homeodomain

protein, *Phox1*, enhanced SRF binding to the c-fos CarG box without formation of a higher order/ternary complex with SRF-CarG DNA. Hautmann et al<sup>44</sup> subsequently showed that the mouse homologue of *Phox1*, *Mhox*, was able to increase SRF binding to the promoter distal CarG box, CarG B, of the SM  $\alpha$ -actin promoter as well as bind itself to an ATTA element, a homeodomain binding site, 5' to CarG B. Moreover, *Mhox* expression, *Mhox* binding to the ATTA element, and enhanced SRF binding to CarG boxes were all increased by treatment of cultured SMCs with the contractile agonist, angiotensin II (see the Figure). Additional factors, including the homeodomain proteins *Barx2b*<sup>45</sup> and *Nkx3.1*<sup>46</sup> and the high-mobility group protein *SSRP1*,<sup>47</sup> have subsequently been shown to function similarly to *Mhox* in that they enhanced the DNA binding activity of SRF but did not form a detectable ternary complex with SRF-CarG DNA. This suggests that if such a complex exists, either it is transient or unstable or other factors or posttranslational modifications are required to stabilize it. Although interaction of SRF with one or more of the above cofactors may be an important mechanism for regulating SRF activity, it is unlikely on its own to contribute to SMC-specific gene expression, because these cofactors are not SMC selective, nor is there evidence that they can differentiate between differentiation genes and growth-responsive genes (such as c-fos).<sup>43–47</sup>

Interestingly, the homeodomain proteins *Barx1b*<sup>48</sup> and *Nkx3.2*<sup>49</sup> and the zinc finger protein *GATA6*<sup>49</sup> have each been shown to form a stable, detectable ternary complex with SRF-CarG DNA. A recent elegant study from Nishida et al<sup>49</sup> showed that the triad of SRF, *Nkx3.2*, and *GATA6* cooperatively activated several SMC marker genes, including *SM22* and *caldesmon*, but not c-fos. Thus, this combination of factors provides a mechanism by which SMC-specific genes can be regulated selectively and independently of growth-responsive genes. Moreover, although each individual factor was not SMC-specific, this triad of factors was found to be coexpressed only in vascular SMCs, illustrating an important paradigm whereby SMC-specific gene transcription can be achieved in the absence of SMC-specific transcription factors through combinatorial interactions between multiple transcription factors whose presence, at the appropriate levels and stoichiometry, may be SMC-specific.

A major breakthrough in the study of SRF cofactors and cardiac/SMC differentiation came in 2001, when Wang et al<sup>50</sup> reported the identification of a novel SAP (SAF-A/B, Acinus, PIAS) domain containing cardiac and SM-specific SRF cofactor, which they named *myocardin* for its critical role in cardiac-specific gene expression and cardiac development. However, it is becoming increasingly clear that *myocardin* may also play a critical role in SMC differentiation. *Myocardin* has been shown to activate several SMC-specific or -selective promoters, including SM-calponin, SM MHC, SM  $\alpha$ -actin, and *SM22*, while failing to activate the c-fos promoter.<sup>50,51</sup> Thus, like the triad of SRF/*GATA6*/*Nkx3.2*, *myocardin* provides a mechanism by which SRF can discriminate between muscle-specific promoters and growth-responsive promoters. In addition to physically interacting with SRF, additional evidence that *myocardin* acts through the SRF/CarG-dependent pathway comes from the following findings:

(1) *myocardin* forms a stable, ternary complex with SRF/CarG box DNA<sup>50,52</sup>; (2) *myocardin*-mediated transactivation is CarG box-dependent<sup>50–52</sup>; and (3) *myocardin* fails to activate SRF-dependent reporters (including *SM22*) in SRF-null ES cells but can do so when both SRF and *myocardin* are cotransfected into these cells.<sup>52</sup> Interestingly, when overexpressed in L6 skeletal myoblast cells, *myocardin* is sufficient to induce the expression of several endogenous smooth and cardiac muscle genes.<sup>51</sup> Recently, two *myocardin*-related transcription factors, *MRTF-A* and *MRTF-B*, have been identified whose expression is much more widespread than that of *myocardin*.<sup>52</sup> Nevertheless, these factors may be important in modulating the activity of SRF or SRF-dependent genes in SMCs. A key question now is what are the mechanisms by which *myocardin* distinguishes between SM and cardiac muscle genes *in vivo*. Possibilities include additional cell-specific cofactors, posttranslational modifications, or the presence of tissue-specific alternative spliced forms of *myocardin*. Interestingly, the mechanism of *myocardin*-induced activation does seem to be somewhat different between the *SM22* and the cardiac ANF promoter in that mutations in the SAP domain of *myocardin*, although having modest effects on *myocardin*-induced transactivation of the *SM22* promoter, profoundly diminished activation of the cardiac ANF promoter.<sup>50</sup> It remains to be determined whether this observation extends to other smooth versus cardiac selective promoters and how the SAP domain of *myocardin* discriminates between these different target genes.

#### **Posttranscriptional Modifications of SRF**

In addition to interacting with cofactors, SRF has been shown to be regulated by several posttranscriptional mechanisms, including alternative splicing,<sup>40,53,54</sup> phosphorylation,<sup>55,56</sup> and regulated nuclear translocation.<sup>57,58</sup> In fact, several alternatively spliced isoforms of SRF have been reported, including one isoform that seems to function as a dominant-negative inhibitor of SRF.<sup>53</sup> Phosphorylation of SRF by casein kinase II<sup>55</sup> or ribosomal S6 kinase (pp90<sup>sk</sup>)<sup>56</sup> has been shown to increase its DNA-binding activity.

#### **Variations in SRF-Binding Affinity Among Different CarG Boxes**

Interestingly, CarG boxes from muscle-specific promoters, in general, have a reduced binding affinity for SRF compared with CarG boxes from growth-responsive genes such as c-fos and *egr1*.<sup>59–61</sup> The determinants of SRF binding affinity seem to lie both within the central A/T-rich region of the CarG box and flanking sequences.<sup>61,62</sup> Because muscle tissues contain higher levels of SRF than most other cell types, it has been proposed that the decreased affinity of muscle CarG boxes serves to restrict SRF binding and therefore expression of muscle-specific genes to muscle cells.<sup>60,61,63</sup> In support of this hypothesis, Chang et al<sup>61</sup> demonstrated that in 11.5-day-old mouse embryos, a transgene containing multimerized c-fos CarG boxes upstream of a minimal promoter showed widespread activity whereas a transgene containing multimerized *SM22* CarG-near boxes upstream of a minimal promoter was active primarily in muscle cells. However, they reported that at later developmental time points and postnatally, the *SM22* CarG-near box

reporter was also active throughout the embryo, suggesting that SRF levels may only be rate limiting very early in embryonic development and that other mechanisms subsequently take over to restrict muscle gene expression. Although this study used artificial multimerized CARG box reporters, several studies have actually replaced one or more muscle CARG boxes with *c-fos* CARG boxes and studied the effect of this substitution in the context of the intact muscle promoter. The results of these types of studies have shown either no effect on muscle specificity,<sup>64</sup> increased basal expression with reduced cell specificity,<sup>60</sup> or lack of expression.<sup>65</sup> Possible reasons for these discrepancies include the model system used (ie, cell culture versus *in vivo* systems), the promoter context, and whether CARG flanking sequences were included in the substitution. Of particular note, many investigators failed to document whether the resultant chimeric CARG region created by the substitution truly increased SRF binding affinity as intended. For example, substitution of *c-fos* CARG boxes for the two SM22 CARG boxes abolished activity of the SM22 promoter in transgenic mouse embryos.<sup>65</sup> It was subsequently shown that such a substitution (at least for one of the SM22 CARG boxes [CARG-near]) actually does not increase, and may even decrease, SRF binding affinity.<sup>61</sup> Another consideration is that SRF binding affinity *in vitro* may be very different from SRF binding affinity *in vivo*. Nevertheless, it is intriguing that such weak SRF binding CARG boxes have been conserved throughout evolution in muscle-specific genes, and clearly additional work is needed in this area to elucidate the reasons and implications of this finding.

#### **Number, Position, and Spacing of CARG Boxes**

In contrast to the *c-fos* promoter, which contains a single CARG box, many muscle-specific promoters contain 2 or more CARG elements.<sup>4,35,36</sup> Although the significance of this is unclear, it is thought that this may be one mechanism by which myocardin distinguishes between muscle promoters and the *c-fos* promoter.<sup>50</sup> Position and spacing of CARG boxes also seem to be important determinants of promoter activity. Studies using the SM  $\alpha$ -actin promoter have revealed that the position of the 2 CARG boxes in the 5' promoter region are not interchangeable<sup>60</sup> and that changes in the spacing/phasing of these 2 CARG boxes profoundly affects the activity of this promoter in cultured SMCs.<sup>42</sup>

#### **Other *cis* Elements and *trans* Binding Factors**

Although the SRF-CARG-dependent pathway has been shown to be critically important in the regulation of multiple SMC marker genes, CARG elements are clearly not sufficient for SMC-specific gene regulation. Strong evidence that other *cis* elements and *trans* binding factors are important comes from numerous transgenic studies showing that deletions or mutations of various SMC marker gene promoters, despite having intact CARG boxes, are not properly expressed *in vivo*<sup>30,36,66,67</sup> (and Kumar et al, unpublished observations, 2002). In this section, we will highlight 3 additional *cis* elements, a transforming growth factor  $\beta$  (TGF $\beta$ ) control element (TCE), E-boxes, and MCAT elements, and discuss what is known about their binding factors and their role in

SMC marker gene expression. Although we will focus on the SM  $\alpha$ -actin promoter, these elements are highly conserved in multiple SMC marker genes and are likely to contribute to coordinate regulation of SMC differentiation.

#### **TGF $\beta$ and the TCE**

TGF $\beta$  has been shown to increase the expression of several SMC differentiation marker genes, including SM  $\alpha$ -actin, SM MHC,  $h_1$  calponin, SM22 $\alpha$ , and SM  $\gamma$ -actin, in cultured SMCs or the 10T1/2 model.<sup>29,31,68</sup> Studies on the SM  $\alpha$ -actin promoter revealed that 3 *cis* elements, the 2 5' CARG boxes and a novel TGF $\beta$  control element (TCE), were required for TGF $\beta$  inducibility<sup>68</sup> (see the Figure). TGF $\beta$  enhanced SRF binding to the CARG boxes, and this effect was at least partly attributable to increased SRF expression.<sup>68</sup> The TCE element, G(A/C)GT(T/G)GG(T/G)GA, is found in several SMC-selective promoters,<sup>68</sup> and mutation of this element in the SM22 $\alpha$  promoter inactivated the promoter *in vivo* in transgenic mice.<sup>30</sup> TGF $\beta$  induced the binding of a factor to the TCE element whose identity is unknown but which appears to belong to the Kruppel-like factor (KLF) family of zinc finger proteins that includes GKLf, BTEB2/KLF5, and Sp1 (See Table).<sup>30,68</sup> Interestingly, although GKLf repressed the SM  $\alpha$ -actin and SM22 $\alpha$  promoters, it appears that another related KLF member, possibly KLF5, may activate transcription through the TCE, suggesting a reciprocal role for KLF factors in control of SMC differentiation through the TCE.<sup>30</sup> Additional work is needed to identify the key KLF members that activate or repress SMC marker gene expression *in vivo*.

#### **E-boxes**

E-boxes (CAnnTG motifs) bind to homodimers or heterodimers of basic helix-loop-helix (bHLH) proteins<sup>69</sup> and have been found in many cell-specific promoters, including the SM-specific or -selective promoters SM MHC,<sup>70</sup> SM22 $\alpha$ ,<sup>71</sup> Crp2/SmLim,<sup>72</sup> APEG-1,<sup>73,74</sup> and SM  $\alpha$ -actin.<sup>75,76</sup> The discovery in 1989 that MyoD, a bHLH protein, functions as a master regulatory gene for skeletal myogenesis, sufficient to activate the skeletal muscle differentiation program in a variety of nonmuscle cell types,<sup>77</sup> led to intense interest in identifying similar master regulatory bHLH proteins in other cells. Although this search has resulted in the identification of bHLH proteins critical to the differentiation of numerous cell types, including neurons, pancreatic  $\beta$ -cells, hematopoietic cells, and cardiac muscle,<sup>69</sup> no SMC-specific master regulatory bHLH protein has yet been identified. Although several bHLH proteins have now been found in SMCs (see Table), their role in SMC differentiation is still largely undefined.

We previously showed that 2 E-boxes in SM  $\alpha$ -actin promoter (designated E1 and E2) functioned as positive regulatory elements in cultured SMCs and that members of the upstream stimulatory factor (USF) family of bHLH proteins could bind the E1 E-box and regulate SM  $\alpha$ -actin transcription in an E1-dependent manner.<sup>76</sup> Whether USF proteins are the relevant E1 binding factors *in vivo* and what the nature of the E2 binding factors is remain to be determined. Interestingly, Chen et al<sup>74</sup> recently demonstrated that USF factors can bind to an E-box in exon 1 of the APEG-1 gene and transactivate the APEG-1 promoter. More work is needed to determine the role of E-boxes on the expression of

SMC marker genes *in vivo*, to additionally define the nature of E-box binding proteins in SMCs, and to determine the role of the known SMC-expressed bHLH proteins (see Table) on SMC differentiation. Because myogenic bHLH proteins have been shown to interact with SRF,<sup>78</sup> it is tempting to speculate that interactions between E-box bound bHLH proteins and SRF will prove to be important in the regulation of SMC marker genes (see the Figure).

### MCAT Elements

The SM  $\alpha$ -actin promoter contains 2 MCAT elements (designated MCAT-1 and MCAT-2) with the sequence AG-GAATG. MCAT elements bind the transcription enhancer factor 1 (TEF-1) family of transcription factors and have been implicated in the control of several cardiac, skeletal, and nonmuscle genes.<sup>79–81</sup> However, relatively little is known about their role in SMC marker gene expression. Swartz et al<sup>82</sup> found that the SM  $\alpha$ -actin MCAT elements were capable of binding TEF-1 (or TEF-1-related proteins) in SMC nuclear extract and, in general, functioned as repressors in cultured SMCs but as activators in L6 skeletal myotubes and endothelial cells. Interestingly, studies on the murine SM  $\alpha$ -actin promoter suggest that the MCAT-1 element functions as a cryptic enhancer in both fibroblasts and SMCs, with MCAT-1 flanking sequences conferring repression to the MCAT-1 element.<sup>83</sup> Studies conducted primarily in fibroblasts, but to some extent in SMCs, have demonstrated that the MCAT-1-containing region can be recognized by the single-stranded DNA-binding proteins Pur $\alpha$ , Pur $\beta$ , and MSY1 and have provided evidence in support of at least 2 potential models of cryptic MCAT enhancer regulation.<sup>83–86</sup> One model is a competition model in which binding by Pur $\alpha$ , Pur $\beta$ , and MSY1 maintains the MCAT-containing region in a single-stranded conformation and prevents binding by TEF-1.<sup>83,84</sup> The second, and perhaps more likely, model is that a macromolecular complex of Pur $\alpha$ , Pur $\beta$ , MSY1, and TEF-1 prevents TEF-1-mediated transactivation, which can then occur if the single-stranded binding proteins dissociated from the promoter<sup>83</sup> (see the Figure). Because TEF-1 and SRF have been shown to physically interact and synergistically activate the skeletal  $\alpha$ -actin promoter,<sup>87</sup> it is interesting that Carlini et al<sup>83</sup> found that not only do Pur $\alpha$ , Pur $\beta$ , MSY1, and TEF-1 interact with each other but that all 4 proteins also interact with SRF. Clearly, regulation by MCAT elements seems to be very complex, and additional work is needed to define the role of the SM  $\alpha$ -actin MCAT elements *in vivo* and to determine the mechanisms by which MCAT elements activate or repress SMC marker genes.

### Role of Chromatin Modifications in SMC Differentiation

Up to now, we have discussed the role of interactions between transcriptional regulators and their cognate DNA-binding sites in SMC marker gene expression. However, it must be kept in mind that endogenous genes are not naked DNA sequences but rather components of a complex, higher-order, dynamically changing structure called chromatin whose building block is the nucleosome. Nucleosomes are composed of  $\approx$ 146 base pairs of DNA wrapped around a

histone octamer containing two molecules each of histone H2A, H2B, H3, and H4. Studies from the emerging field of chromatin regulation have revealed that the N-terminal tails of histones are the target of numerous posttranslational modifications, including acetylation, methylation, and phosphorylation, and that these modifications affect numerous biological processes, including transcription.<sup>88</sup> In particular, histone acetylation has been shown to be associated with transcriptional activation.<sup>89</sup> Because transcription factors generally recognize 6 to 10 base pair sequences, their selectivity of action *in vivo* is remarkable. Although combinatorial interactions with other promoter-bound factors (as discussed in this review) are likely to restrict the activity of certain transcription factors, DNA accessibility attributable to chromatin configuration is also likely to play a key role.

Using chromatin immunoprecipitation (ChIP) assays, Manabe et al<sup>85</sup> found that SRF was strongly associated with the SM MHC, but not skeletal actin, promoter in SMCs. In contrast, ChIP assays in skeletal myoblasts and myotubes revealed SRF binding to the skeletal actin, but not SM MHC, promoter in these cells. Additional studies by Manabe et al<sup>23</sup> using the A404 system revealed that SRF accessibility to SMC CarG-containing regions was differentiation state-dependent. Whereas *in vitro* assays showed that SRF-binding activity to CarG box probes was approximately equivalent in nuclear extracts from undifferentiated and differentiated A404 cells, ChIP analyses revealed a preferential association of SRF with the SM  $\alpha$ -actin and SM MHC promoters in differentiated versus undifferentiated A404 cells. Moreover, differentiation resulted in changes in local chromatin structure, with enhanced H3 or H4 acetylation associated with SMC promoters in differentiated A404 cells. Although these studies demonstrate that chromatin remodeling is likely to play an important role in SMC differentiation, the question remains as to what factors are responsible for these changes in chromatin structure.

Interestingly, several transcription factors that we have discussed in this review, including SRF, GATA6, and myocardin, have been implicated either directly or indirectly in chromatin remodeling functions. For example, SRF has been shown to interact with the histone acetyltransferase protein CBP.<sup>90</sup> Qiu et al<sup>91</sup> showed that SRF and CBP were recruited to the CarG containing region of the SM22 promoter in cultured SMCs. GATA6 was shown to interact with the related histone acetyltransferase protein p300, and this combination of factors synergistically activated the SM MHC promoter.<sup>92</sup> Myocardin contains a protein motif called the SAP domain, which has been implicated in diverse aspects of chromatin remodeling.<sup>93</sup> Intriguingly, SRF may also be involved in gene silencing under some circumstances, because it has been shown to interact with SMRT, a protein that recruits histone deacetylases.<sup>94</sup>

Although our knowledge of the complex interplay between transcriptional regulators and chromatin modifying enzymes and their role in SMC differentiation is still clearly in its infancy, this area of research holds great promise for the future in terms of understanding and possibly even manipulating changes in the differentiation state of SMCs.

### Conclusion and Perspectives

Although changes in the differentiated state of SMCs are known to play a key role in vascular diseases such as atherosclerosis and hypertension, the mechanisms controlling SMC differentiation are not fully understood. Studies of SMC development and differentiation are challenging because of the diverse, and sometimes unknown, origin of SMCs in vivo as well as the finding that many (if not all) SMC differentiation markers are not exclusive to the SMC lineage. Nevertheless, the last decade has resulted in tremendous progress and advances in our understanding of the factors controlling SMC differentiation. Although numerous environmental cues, including growth factors, cell-cell contacts, extracellular matrix components, and neuronal input, affect the differentiation state of SMCs,<sup>3</sup> in this review we focused on the final common pathway of most of these influences—the transcriptional regulators and how they interact with each other and with critical *cis* elements in the promoters of SMC marker genes to regulate SMC differentiation.

A common theme that has emerged from these studies is that SRF interacts with multiple proteins, likely forming a macromolecular complex in vivo containing both chromatin-modifying and transcription-activating functions. Although much work has been done investigating mechanisms of SRF-mediated regulation of SMC marker genes, our understanding of control by other *cis* elements (such as the TCE, E-boxes, and MCAT elements) is still in its infancy. Table summarizes most of the transcription factors known or likely to play an important role in SMC differentiation or dedifferentiation. The challenge for the future is to not only add to this list of key players but to continue to draw connections between them, mapping out transcriptional regulatory networks governing SMC-specific gene expression. Hopefully the use of inducible SMC lineage systems will help make this task possible.

Equipped with an understanding of some of the basic molecular pathways that normally control SMC differentiation, the next step is to elucidate how such pathways are disrupted in vascular injury and disease. We now have evidence that decreases in SMC marker gene expression that occur after vascular injury are at least partly transcriptionally mediated.<sup>95</sup> And although we are beginning to elucidate transcriptional regulatory pathways in vascular injury, including downregulation of differentiation promoting/growth inhibitory factors such as GATA-6<sup>96</sup> and Gax<sup>97,98</sup> and upregulation of dedifferentiation/growth promoting factors such as Id2<sup>99</sup> and Egr-1,<sup>100</sup> our understanding of the molecular mechanisms of SMC phenotypic modulation in more complex atherosclerotic lesions is much farther behind. Hopefully, with the combination of mouse models of atherosclerosis, SMC-specific promoter constructs, and conditional/inducible gene expression systems, we will soon be able to not only address these issues but also deliver therapeutic genes in a controlled and precise manner to sites of vascular injury and disease.

In summary, what makes SMCs unique is not the presence of one specific marker protein but a whole family of factors expressed at the appropriate levels and stoichiometry necessary to carry out the differentiated functions of SMCs.

Similarly, it seems that what controls SMC differentiation is likely not one unique SMC-specific transcription factor but rather a combination of ubiquitous and tissue-restricted proteins expressed at the appropriate levels and stoichiometry necessary to initiate, maintain, and, occasionally, modulate the SMC phenotype.

### Acknowledgments

This work was supported by grants NIH R01 HL38854 and NIH PO1 HL19242 (to G.K. Owens) and by fellowship grant 0110071U from the Mid-Atlantic Affiliate of the American Heart Association (to M.S. Kumar).

### References

- Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med.* 1999; 340:115–126.
- Schwartz SM, Campbell GR, Campbell JH. Replication of smooth muscle cells in vascular disease. *Circ Res.* 1986;58:427–444.
- Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev.* 1995;75:487–517.
- Solway J, Forsythe SM, Halayko AJ, Vieira JE, Hershenson MB, Camoretti-Mercado B. Transcriptional regulation of smooth muscle contractile apparatus expression. *Am J Respir Crit Care Med.* 1998;158: S100–S108.
- Hungerford JE, Little CD. Developmental biology of the vascular smooth muscle cell: building a multilayered vessel wall. *J Vasc Res.* 1999;36:2–27.
- Sobue K, Hayashi K, Nishida W. Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. *Mol Cell Biochem.* 1999;190:105–118.
- Hsieh CM, Yoshizumi M, Endege WO, Kho CJ, Jain MK, Kashiki S, de los SR, Lee WS, Perrella MA, Lee ME. APEG-1, a novel gene preferentially expressed in aortic smooth muscle cells, is down-regulated by vascular injury. *J Biol Chem.* 1996;271:17354–17359.
- Jain MK, Fujita KP, Hsieh CM, Endege WO, Sibinga NE, Yet SF, Kashiki S, Lee WS, Perrella MA, Haber E, Lee ME. Molecular cloning and characterization of SmLIM, a developmentally regulated LIM protein preferentially expressed in aortic smooth muscle cells. *J Biol Chem.* 1996;271:10194–10199.
- Jain MK, Kashiki S, Hsieh CM, Layne MD, Yet SF, Sibinga NE, Chin MT, Feinberg MW, Woo I, Maas RL, Haber E, Lee ME. Embryonic expression suggests an important role for CRP2/SmLIM in the developing cardiovascular system. *Circ Res.* 1998;83:980–985.
- Layne MD, Endege WO, Jain MK, Yet SF, Hsieh CM, Chin MT, Perrella MA, Blonar MA, Haber E, Lee ME. Aortic carboxypeptidase-like protein, a novel protein with discoidin and carboxypeptidase-like domains, is up-regulated during vascular smooth muscle cell differentiation. *J Biol Chem.* 1998;273:15654–15660.
- Layne MD, Yet SF, Maemura K, Hsieh CM, Bernfield M, Perrella MA, Lee ME. Impaired abdominal wall development and deficient wound healing in mice lacking aortic carboxypeptidase-like protein. *Mol Cell Biol.* 2001;21:5256–5261.
- Layne MD, Yet SF, Maemura K, Hsieh CM, Liu X, Ith B, Lee ME, Perrella MA. Characterization of the mouse aortic carboxypeptidase-like protein promoter reveals activity in differentiated and dedifferentiated vascular smooth muscle cells. *Circ Res.* 2002;90:728–736.
- Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM. Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. *J Clin Invest.* 1993;92:1686–1696.
- Kuro-o M, Nagai R, Nakahara K, Katoh H, Tsai RC, Tsuchimochi H, Yazaki Y, Ohkubo A, Takaku F. cDNA cloning of a myosin heavy chain isoform in embryonic smooth muscle and its expression during vascular development and in arteriosclerosis. *J Biol Chem.* 1991;266:3768–3773.
- Bochaton-Piallat ML, Ropraz P, Gabbiani F, Gabbiani G. Phenotypic heterogeneity of rat arterial smooth muscle cell clones: implications for the development of experimental intimal thickening. *Arterioscler Thromb Vasc Biol.* 1996;16:815–820.
- Bochaton-Piallat ML, Clowes AW, Clowes MM, Fischer JW, Redard M, Gabbiani F, Gabbiani G. Cultured arterial smooth muscle cells maintain distinct phenotypes when implanted into carotid artery. *Arterioscler Thromb Vasc Biol.* 2001;21:949–954.

17. Frid MG, Aldashev AA, Dempsey EC, Stenmark KR. Smooth muscle cells isolated from discrete compartments of the mature vascular media exhibit unique phenotypes and distinct growth capabilities. *Circ Res.* 1997;81:940–952.
18. Shimizu K, Sugiyama S, Aikawa M, Fukumoto Y, Rabkin E, Libby P, Mitchell RN. Host bone-marrow cells are a source of donor intimal smooth-muscle-like cells in murine aortic transplant arteriopathy. *Nat Med.* 2001;7:738–741.
19. Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med.* 2002;8:403–409.
20. Lassar AB, Paterson BM, Weintraub H. Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. *Cell.* 1986;47:649–656.
21. Lassar AB, Davis RL, Wright WE, Kadesch T, Murre C, Voronova A, Baltimore D, Weintraub H. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell.* 1991;66:305–315.
22. Blank RS, Swartz EA, Thompson MM, Olson EN, Owens GK. A retinoic acid-induced clonal cell line derived from multipotential P19 embryonal carcinoma cells expresses smooth muscle characteristics. *Circ Res.* 1995;76:742–749.
23. Manabe I, Owens GK. Recruitment of serum response factor and hyperacetylation of histones at smooth muscle-specific regulatory regions during differentiation of a novel P19-derived in vitro smooth muscle differentiation system. *Circ Res.* 2001;88:1127–1134.
24. Miano JM, Berk BC. Retinoids: versatile biological response modifiers of vascular smooth muscle phenotype. *Circ Res.* 2000;87:355–362.
25. Drab M, Haller H, Bychkov R, Erdmann B, Lindschau C, Haase H, Morano I, Luft FC, Wobus AM. From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: a retinoic acid and db-cAMP in vitro differentiation model. *FASEB J.* 1997;11:905–915.
26. Jain MK, Layne MD, Watanabe M, Chin MT, Feinberg MW, Sibinga NE, Hsieh CM, Yet SF, Stemple DL, Lee ME. In vitro system for differentiating pluripotent neural crest cells into smooth muscle cells. *J Biol Chem.* 1998;273:5993–5996.
27. Rao MS, Anderson DJ. Immortalization and controlled in vitro differentiation of murine multipotent neural crest stem cells. *J Neurobiol.* 1997;32:722–746.
28. Watanabe M, Layne MD, Hsieh CM, Maemura K, Gray S, Lee ME, Jain MK. Regulation of smooth muscle cell differentiation by AT-rich interaction domain transcription factors Mrf2alpha and Mrf2beta. *Circ Res.* 2002;91:382–389.
29. Hirschi KK, Rohovsky SA, D'Amore PA. PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate [published erratum appears in *J Cell Biol* 1998 Jun 1;141(5):1287]. *J Cell Biol.* 1998;141:805–814.
30. Adam PJ, Regan CP, Hautmann MB, Owens GK. Positive- and negative-acting Kruppel-like transcription factors bind a transforming growth factor beta control element required for expression of the smooth muscle cell differentiation marker SM22alpha in vivo. *J Biol Chem.* 2000;275:37798–37806.
31. Hirschi KK, Lai L, Belaguli NS, Dean DA, Schwartz RJ, Zimmer WE. Transforming growth factor-beta induction of smooth muscle cell phenotype requires transcriptional and post-transcriptional control of serum response factor. *J Biol Chem.* 2002;277:6287–6295.
32. Shore P, Sharrocks AD. The MADS-box family of transcription factors. *Eur J Biochem.* 1995;229:1–13.
33. Sartorelli V, Kurabayashi M, Kedes L. Muscle-specific gene expression: a comparison of cardiac and skeletal muscle transcription strategies. *Circ Res.* 1993;72:925–931.
34. Owens GK. Molecular control of vascular smooth muscle cell differentiation. *Acta Physiol Scand.* 1998;164:623–635.
35. 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.
36. Mack CP, Owens GK. Regulation of smooth muscle alpha-actin expression in vivo is dependent on CARg elements within the 5' and first intron promoter regions. *Circ Res.* 1999;84:852–861.
37. Kim S, Ip HS, Lu MM, Clendenin C, Parmacek MS. A serum response factor-dependent transcriptional regulatory program identifies distinct smooth muscle cell sublineages. *Mol Cell Biol.* 1997;17:2266–2278.
38. Li L, Liu Z, Mercer B, Overbeek P, Olson EN. Evidence for serum response factor-mediated regulatory networks governing SM22alpha transcription in smooth, skeletal, and cardiac muscle cells. *Dev Biol.* 1997;187:311–321.
39. Croissant JD, Kim JH, Eichele G, Goering L, Lough J, Prywes R, Schwartz RJ. Avian serum response factor expression restricted primarily to muscle cell lineages is required for alpha-actin gene transcription. *Dev Biol.* 1996;177:250–264.
40. Belaguli NS, Schildmeyer LA, Schwartz RJ. Organization and myogenic restricted expression of the murine serum response factor gene: a role for autoregulation. *J Biol Chem.* 1997;272:18222–18231.
41. Treisman R. Ternary complex factors: growth factor regulated transcriptional activators. *Curr Opin Genet Dev.* 1994;4:96–101.
42. Mack CP, Thompson MM, Lawrenz-Smith S, Owens GK. Smooth muscle alpha-actin CARg elements coordinate formation of a smooth muscle cell-selective, serum response factor-containing activation complex. *Circ Res.* 2000;86:221–232.
43. Grueneberg DA, Natesan S, Alexandre C, Gilman MZ. Human and Drosophila homeodomain proteins that enhance the DNA-binding activity of serum response factor. *Science.* 1992;257:1089–1095.
44. Hautmann MB, Thompson MM, Swartz EA, Olson EN, Owens GK. Angiotensin II-induced stimulation of smooth muscle alpha-actin expression by serum response factor and the homeodomain transcription factor MHOx. *Circ Res.* 1997;81:600–610.
45. Herring BP, Kriegel AM, Hoggatt AM. Identification of Barx2b, a serum response factor-associated homeodomain protein. *J Biol Chem.* 2001;276:14482–14489.
46. Carson JA, Fillmore RA, Schwartz RJ, Zimmer WE. The smooth muscle gamma-actin gene promoter is a molecular target for the mouse bagpipe homologue, mNkx3-1, and serum response factor. *J Biol Chem.* 2000;275:39061–39072.
47. Spencer JA, Baron MH, Olson EN. Cooperative transcriptional activation by serum response factor and the high mobility group protein SSRP1. *J Biol Chem.* 1999;274:15686–15693.
48. Nakamura M, Nishida W, Mori S, Hiwada K, Hayashi K, Sobue K. Transcriptional activation of beta-tropomyosin mediated by serum response factor and a novel Barx homologue, Barx1b, in smooth muscle cells. *J Biol Chem.* 2001;276:18313–18320.
49. Nishida W, Nakamura M, Mori S, Takahashi M, Ohkawa Y, Tadokoro S, Yoshida K, Hiwada K, Hayashi K, Sobue K. A triad of serum response factor and the GATA and NK families governs the transcription of smooth and cardiac muscle genes. *J Biol Chem.* 2002;277:7308–7317.
50. Wang D, Chang PS, Wang Z, Sutherland L, Richardson JA, Small E, Krieg PA, Olson EN. Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell.* 2001;105:851–862.
51. Chen J, Kitchen CM, Streb JW, Miano JM. Myocardin: a component of a molecular switch for smooth muscle differentiation. *J Mol Cell Cardiol.* 2002;34:1345–1356.
52. Wang DZ, Li S, Hockemeyer D, Sutherland L, Wang Z, Schmitt G, Richardson JA, Nordheim A, Olson EN. Potentiation of serum response factor activity by a family of myocardin-related transcription factors. *Proc Natl Acad Sci U S A.* 2002;99:14855–14860.
53. Belaguli NS, Zhou W, Trinh TH, Majesky MW, Schwartz RJ. Dominant negative murine serum response factor: alternative splicing within the activation domain inhibits transactivation of serum response factor binding targets. *Mol Cell Biol.* 1999;19:4582–4591.
54. Kemp PR, Metcalfe JC. Four isoforms of serum response factor that increase or inhibit smooth-muscle-specific promoter activity. *Biochem J.* 2000;345(pt 3):445–451.
55. Manak JR, Prywes R. Mutation of serum response factor phosphorylation sites and the mechanism by which its DNA-binding activity is increased by casein kinase II. *Mol Cell Biol.* 1991;11:3652–3659.
56. Rivera VM, Miranti CK, Misra RP, Ginty DD, Chen RH, Blenis J, Greenberg ME. A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA-binding activity. *Mol Cell Biol.* 1993;13:6260–6273.
57. Camoretti-Mercado B, Liu HW, Halayko AJ, Forsythe SM, Kyle JW, Li B, Fu Y, McConville J, Kogut P, Vieira JE, Patel NM, Hershenson MB, Fuchs E, Sinha S, Miano JM, Parmacek MS, Burkhardt JK, Solway J. Physiological control of smooth muscle-specific gene expression through regulated nuclear translocation of serum response factor. *J Biol Chem.* 2000;275:30387–30393.

58. Phiel CJ, Gabbeta V, Parsons LM, Rothblat D, Harvey RP, McHugh KM. Differential binding of an SRF/NK-2/MEF2 transcription factor complex in normal versus neoplastic smooth muscle tissues. *J Biol Chem.* 2001;276:34637–34650.
59. Tuil D, Clergue N, Montarras D, Pinset C, Kahn A, Phan-Dinh-Tuy F. CC Ar GG boxes, cis-acting elements with a dual specificity: muscle-specific transcriptional activation and serum responsiveness. *J Mol Biol.* 1990;213:677–686.
60. Hautmann MB, Madsen CS, Mack CP, Owens GK. Substitution of the degenerate smooth muscle (SM) alpha-actin CC(A/T-rich)6GG elements with c-fos serum response elements results in increased basal expression but relaxed SM cell specificity and reduced angiotensin II inducibility. *J Biol Chem.* 1998;273:8398–8406.
61. Chang PS, Li L, McAnally J, Olson EN. Muscle specificity encoded by specific serum response factor-binding sites. *J Biol Chem.* 2001;276:17206–17212.
62. Leung S, Miyamoto NG. Point mutational analysis of the human c-fos serum response factor binding site. *Nucleic Acids Res.* 1989;17:1177–1195.
63. Santoro IM, Walsh K. Natural and synthetic DNA elements with the CARG motif differ in expression and protein-binding properties. *Mol Cell Biol.* 1991;11:6296–6305.
64. Taylor M, Treisman R, Garrett N, Mohun T. Muscle-specific (CARG) and serum-responsive (SRE) promoter elements are functionally interchangeable in *Xenopus* embryos and mouse fibroblasts. *Development.* 1989;106:67–78.
65. Strobeck M, Kim S, Zhang JC, Clendenen C, Du KL, Parmacek MS. Binding of serum response factor to CARG box sequences is necessary but not sufficient to restrict gene expression to arterial smooth muscle cells. *J Biol Chem.* 2001;276:16418–16424.
66. Li L, Miano JM, Mercer B, Olson EN. Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J Cell Biol.* 1996;132:849–859.
67. Madsen CS, Regan CP, Hungerford JE, White SL, Manabe I, Owens GK. Smooth muscle-specific expression of the smooth muscle myosin heavy chain gene in transgenic mice requires 5'-flanking and first intronic DNA sequence. *Circ Res.* 1998;82:908–917.
68. Hautmann MB, Madsen CS, Owens GK. A transforming growth factor beta (TGFbeta) control element drives TGFbeta-induced stimulation of smooth muscle alpha-actin gene expression in concert with two CARG elements. *J Biol Chem.* 1997;272:10948–10956.
69. Massari ME, Murre C. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol Cell Biol.* 2000;20:429–440.
70. White SL, Low RB. Identification of promoter elements involved in cell-specific regulation of rat smooth muscle myosin heavy chain gene transcription. *J Biol Chem.* 1996;271:15008–15017.
71. Osbourn JK, Weissberg PL, Shanahan CM. A regulatory element downstream of the rat SM22 alpha gene transcription start point enhances reporter gene expression in vascular smooth muscle cells. *Gene.* 1995;154:249–253.
72. Yet SF, Folta SC, Jain MK, Hsieh CM, Maemura K, Layne MD, Zhang D, Marria PB, Yoshizumi M, Chin MT, Perrella MA, Lee ME. Molecular cloning, characterization, and promoter analysis of the mouse Crp2/SmLim gene: preferential expression of its promoter in the vascular smooth muscle cells of transgenic mice. *J Biol Chem.* 1998;273:10530–10537.
73. Hsieh CM, Yet SF, Layne MD, Watanabe M, Hong AM, Perrella MA, Lee ME. Genomic cloning and promoter analysis of aortic preferentially expressed gene-1: identification of a vascular smooth muscle-specific promoter mediated by an E box motif. *J Biol Chem.* 1999;274:14344–14351.
74. Chen YH, Layne MD, Watanabe M, Yet SF, Perrella MA. Upstream stimulatory factors regulate aortic preferentially expressed gene-1 expression in vascular smooth muscle cells. *J Biol Chem.* 2001;276:47658–47663.
75. Shimizu RT, Blank RS, Jervis R, Lawrenz-Smith SC, Owens GK. The smooth muscle alpha-actin gene promoter is differentially regulated in smooth muscle versus non-smooth muscle cells. *J Biol Chem.* 1995;270:7631–7643.
76. Johnson AD, Owens GK. Differential activation of the SMalphaA promoter in smooth vs skeletal muscle cells by bHLH factors. *Am J Physiol.* 1999;276:C1420–C1431.
77. Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, Lassar AB, Miller AD. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. *Proc Natl Acad Sci U S A.* 1989;86:5434–5438.
78. Groisman R, Masutani H, Leibovitch MP, Robin P, Soudant I, Trouche D, Harel-Bellan A. Physical interaction between the mitogen-responsive serum response factor and myogenic basic-helix-loop-helix proteins. *J Biol Chem.* 1996;271:5258–5264.
79. Mar JH, Ordahl CP. M-CAT binding factor, a novel trans-acting factor governing muscle-specific transcription. *Mol Cell Biol.* 1990;10:4271–4283.
80. Karns LR, Kariya K, Simpson PC. M-CAT, CARG, and Sp1 elements are required for alpha 1-adrenergic induction of the skeletal alpha-actin promoter during cardiac myocyte hypertrophy: transcriptional enhancer factor-1 and protein kinase C as conserved transducers of the fetal program in cardiac growth. *J Biol Chem.* 1995;270:410–417.
81. Davidson I, Xiao JH, Rosales R, Staub A, Chambon P. The HeLa cell protein TEF-1 binds specifically and cooperatively to two SV40 enhancer motifs of unrelated sequence. *Cell.* 1988;54:931–942.
82. Swartz EA, Johnson AD, Owens GK. Two MCAT elements of the SM alpha-actin promoter function differentially in SM vs. non-SM cells. *Am J Physiol.* 1998;275:C608–C618.
83. Carlini LE, Getz MJ, Strauch AR, Kelm RJ Jr. Cryptic MCAT enhancer regulation in fibroblasts and smooth muscle cells: suppression of TEF-1 mediated activation by the single-stranded DNA-binding proteins, Pur alpha, Pur beta, and MSY1. *J Biol Chem.* 2002;277:8682–8692.
84. Sun S, Stoflet ES, Cogan JG, Strauch AR, Getz MJ. Negative regulation of the vascular smooth muscle alpha-actin gene in fibroblasts and myoblasts: disruption of enhancer function by sequence-specific single-stranded-DNA-binding proteins. *Mol Cell Biol.* 1995;15:2429–2436.
85. Kelm RJ Jr, Elder PK, Strauch AR, Getz MJ. Sequence of cDNAs encoding components of vascular actin single-stranded DNA-binding factor 2 establish identity to Puralpha and Purbeta. *J Biol Chem.* 1997;272:26727–26733.
86. Kelm RJ Jr, Cogan JG, Elder PK, Strauch AR, Getz MJ. Molecular interactions between single-stranded DNA-binding proteins associated with an essential MCAT element in the mouse smooth muscle alpha-actin promoter. *J Biol Chem.* 1999;274:14238–14245.
87. Gupta M, Kogut P, Davis FJ, Belaguli NS, Schwartz RJ, Gupta MP. Physical interaction between the MADS box of serum response factor and the TEA/ATTS DNA-binding domain of transcription enhancer factor-1. *J Biol Chem.* 2001;276:10413–10422.
88. Cheung P, Allis CD, Sassone-Corsi P. Signaling to chromatin through histone modifications. *Cell.* 2000;103:263–271.
89. Marmorstein R, Roth SY. Histone acetyltransferases: function, structure, and catalysis. *Curr Opin Genet Dev.* 2001;11:155–161.
90. Ramirez S, Ait-Si-Ali S, Robin P, Trouche D, Harel-Bellan A, Ait Si AS. The CREB-binding protein (CBP) cooperates with the serum response factor for transactivation of the c-fos serum response element. *J Biol Chem.* 1997;272:31016–31021.
91. Qiu P, Li L. Histone acetylation and recruitment of serum responsive factor and CREB-binding protein onto SM22 promoter during SM22 gene expression. *Circ Res.* 2002;90:858–865.
92. Wada H, Hasegawa K, Morimoto T, Kakita T, Yanazume T, Sasayama S. A p300 protein as a coactivator of GATA-6 in the transcription of the smooth muscle-myosin heavy chain gene. *J Biol Chem.* 2000;275:25330–25335.
93. Aravind L, Koonin EV. SAP: a putative DNA-binding motif involved in chromosomal organization. *Trends Biochem Sci.* 2000;25:112–114.
94. Lee SK, Kim JH, Lee YC, Cheong J, Lee JW. Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule of activating protein-1, nuclear factor-kappaB, and serum response factor. *J Biol Chem.* 2000;275:12470–12474.
95. Regan CP, Adam PJ, Madsen CS, Owens GK. Molecular mechanisms of decreased smooth muscle differentiation marker expression after vascular injury. *J Clin Invest.* 2000;106:1139–1147.
96. Mano T, Luo Z, Malendowicz SL, Evans T, Walsh K. Reversal of GATA-6 downregulation promotes smooth muscle differentiation and inhibits intimal hyperplasia in balloon-injured rat carotid artery. *Circ Res.* 1999;84:647–654.
97. Weir L, Chen D, Pastore C, Isner JM, Walsh K. Expression of gax, a growth arrest homeobox gene, is rapidly down-regulated in the rat carotid artery during the proliferative response to balloon injury. *J Biol Chem.* 1995;270:5457–5461.
98. Smith RC, Branell D, Gorski DH, Guo K, Perlman H, Dedieu JF, Pastore C, Mahfoudi A, Deneffe P, Isner JM, Walsh K. p21CIP1-

- mediated inhibition of cell proliferation by overexpression of the gax homeodomain gene. *Genes Dev.* 1997;11:1674–1689.
99. Matsumura ME, Lobe DR, McNamara CA. Contribution of the helix-loop-helix factor Id2 to regulation of vascular smooth muscle cell proliferation. *J Biol Chem.* 2002;277:7293–7297.
  100. Lowe HC, Fahmy RG, Kavurma MM, Baker A, Chesterman CN, Khachigian LM. Catalytic oligodeoxynucleotides define a key regulatory role for early growth response factor-1 in the porcine model of coronary in-stent restenosis. *Circ Res.* 2001;89:670–677.
  101. Katoh Y, Molkentin JD, Dave V, Olson EN, Periasamy M. MEF2B is a component of a smooth muscle-specific complex that binds an A/T-rich element important for smooth muscle myosin heavy chain gene expression. *J Biol Chem.* 1998;273:1511–1518.
  102. Lin Q, Lu J, Yanagisawa H, Webb R, Lyons GE, Richardson JA, Olson EN. Requirement of the MADS-box transcription factor MEF2C for vascular development. *Development.* 1998;125:4565–4574.
  103. Bostrom K, Tintut Y, Kao SC, Stanford WP, Demer LL. HOXB7 overexpression promotes differentiation of C3H10T1/2 cells to smooth muscle cells. *J Cell Biochem.* 2000;78:210–221.
  104. Sekiguchi K, Kurabayashi M, Oyama Y, Aihara Y, Tanaka T, Sakamoto H, Hoshino Y, Kanda T, Yokoyama T, Shimomura Y, Iijima H, Ohyama Y, Nagai R. Homeobox protein Hex induces SMemb/nonmuscle myosin heavy chain-B gene expression through the cAMP-responsive element. *Circ Res.* 2001;88:52–58.
  105. Belaguli NS, Sepulveda JL, Nigam V, Charron F, Nemer M, Schwartz RJ. Cardiac tissue enriched factors serum response factor and GATA-4 are mutual coregulators. *Mol Cell Biol.* 2000;20:7550–7558.
  106. Perlman H, Suzuki E, Simonson M, Smith RC, Walsh K. GATA-6 induces p21(Cip1) expression and G1 cell cycle arrest. *J Biol Chem.* 1998;273:13713–13718.
  107. Madsen CS, Regan CP, Owens GK. Interaction of CARg elements and a GC-rich repressor element in transcriptional regulation of the smooth muscle myosin heavy chain gene in vascular smooth muscle cells. *J Biol Chem.* 1997;272:29842–29851.
  108. Zhang W, Geiman DE, Shields JM, Dang DT, Mahatan CS, Kaestner KH, Biggs JR, Kraft AS, Yang VW. The gut-enriched Kruppel-like factor (Kruppel-like factor 4) mediates the transactivating effect of p53 on the p21WAF1/Cip1 promoter. *J Biol Chem.* 2000;275:18391–18398.
  109. Chen X, Johns DC, Geiman DE, Marban E, Dang DT, Hamlin G, Sun R, Yang VW. Kruppel-like factor 4 (gut-enriched Kruppel-like factor) inhibits cell proliferation by blocking G1/S progression of the cell cycle. *J Biol Chem.* 2001;276:30423–30428.
  110. Watanabe N, Kurabayashi M, Shimomura Y, Kawai-Kowase K, Hoshino Y, Manabe I, Watanabe M, Aikawa M, Kuro-o M, Suzuki T, Yazaki Y, Nagai R. BTEB2, a Kruppel-like transcription factor, regulates expression of the SMemb/Nonmuscle myosin heavy chain B (SMemb/NMHC-B) gene. *Circ Res.* 1999;85:182–191.
  111. Bidder M, Shao JS, Charlton-Kachigian N, Loewy AP, Semenkovich CF, Towler DA. Osteopontin transcription in aortic vascular smooth muscle cells is controlled by glucose-regulated upstream stimulatory factor and activator protein-1 activities. *J Biol Chem.* 2002;277:44485–44496.
  112. Chin MT, Maemura K, Fukumoto S, Jain MK, Layne MD, Watanabe M, Hsieh CM, Lee ME. Cardiovascular basic helix loop helix factor 1, a novel transcriptional repressor expressed preferentially in the developing and adult cardiovascular system. *J Biol Chem.* 2000;275:6381–6387.
  113. Nakagawa O, Nakagawa M, Richardson JA, Olson EN, Srivastava D. HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. *Dev Biol.* 1999;216:72–84.
  114. Zhong TP, Rosenberg M, Mohideen MA, Weinstein B, Fishman MC. Gridlock, an HLH gene required for assembly of the aorta in zebrafish. *Science.* 2000;287:1820–1824.
  115. Yamagishi H, Olson EN, Srivastava D. The basic helix-loop-helix transcription factor, dHAND, is required for vascular development. *J Clin Invest.* 2000;105:261–270.
  116. Hollenberg SM, Sternglanz R, Cheng PF, Weintraub H. Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. *Mol Cell Biol.* 1995;15:3813–3822.
  117. Hidai H, Bardales R, Goodwin R, Quertermous T, Quertermous EE. Cloning of capsulin, a basic helix-loop-helix factor expressed in progenitor cells of the pericardium and the coronary arteries. *Mech Dev.* 1998;73:33–43.
  118. Nickenig G, Baudler S, Muller C, Werner C, Werner N, Welzel H, Strehlow K, Bohm M. Redox-sensitive vascular smooth muscle cell proliferation is mediated by GSKF and Id3 in vitro and in vivo. *FASEB J.* 2002;16:1077–1086.
  119. Mueller C, Baudler S, Welzel H, Bohm M, Nickenig G. Identification of a novel redox-sensitive gene, Id3, which mediates angiotensin II-induced cell growth. *Circulation.* 2002;105:2423–2428.
  120. Comer KA, Dennis PA, Armstrong L, Catino JJ, Kastan MB, Kumar CC. Human smooth muscle alpha-actin gene is a transcriptional target of the p53 tumor suppressor protein. *Oncogene.* 1998;16:1299–1308.
  121. Buck M, Kim DJ, Houghlum K, Hassanein T, Chojkier M. c-Myb modulates transcription of the alpha-smooth muscle actin gene in activated hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol.* 2000;278:G321–G328.
  122. Crawford AW, Pino JD, Beckerle MC. Biochemical and molecular characterization of the chicken cysteine-rich protein, a developmentally regulated LIM-domain protein that is associated with the actin cytoskeleton. *J Cell Biol.* 1994;124:117–127.
  123. Wada H, Hasegawa K, Morimoto T, Kakita T, Yanazume T, Abe M, Sasayama S. Calcineurin-GATA-6 pathway is involved in smooth muscle-specific transcription. *J Cell Biol.* 2002;156:983–991.

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JOURNAL OF THE AMERICAN HEART ASSOCIATION

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*Arterioscler Thromb Vasc Biol.* 2003;23:737-747; originally published online March 6, 2003;  
doi: 10.1161/01.ATV.0000065197.07635.BA

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272  
Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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