Micromanaging Vascular Smooth Muscle Cell Differentiation and Phenotypic Modulation

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Abstract—The phenotype of vascular smooth muscle cells (VSMCs) is dynamically regulated in response to various stimuli. In a cellular process known as phenotype switching, VSMCs alternate between a contractile and synthetic phenotype state. Deregulation of phenotype switching is associated with vascular disorders such as atherosclerosis, restenosis after angioplasty, and pulmonary hypertension. An important role for microRNAs (miRNAs) in VSMC development and phenotype switching has recently been uncovered. Individual miRNAs are involved in promoting both contractile and synthetic VSMC phenotype. In this review, we summarize recent advances in the understanding of miRNA function in the regulation of VSMC phenotype regulation. (Arterioscler Thromb Vasc Biol. 2011;31:2370-2377.)

Key Words: pulmonary hypertension • vascular biology • BMP • TGF-β • microRNA

Vascular smooth muscle cells (VSMCs) are a highly differentiated cell type present within the medial region of arteries and arterioles. VSMCs express proteins that are important for contractility, ion channels, and signaling molecules that allow these cells to regulate systemic blood pressure through the modulation of vascular tone.1 Unlike the majority of differentiated cells, VSMCs maintain phenotypic plasticity. VSMCs can switch between a differentiated (also termed contractile) state and a dedifferentiated (also termed synthetic) phenotype in response to extracellular cues1 (Figure 1A). Differentiated smooth muscle phenotype is characterized by high levels of contractile gene expression and low rates of proliferation, migration, and extracellular matrix synthesis. Conversely, dedifferentiated VSMCs have increased rates of proliferation, migration, and production of extracellular matrix, as well as reduced expression of contractile genes1 (Figure 1A). VSMC phenotype modulation is critically important for the resolution of vascular injury. Immediately after an insult, VSMCs dedifferentiate to promote repair of the vessel; however, once the injury is resolved, healthy VSMCs return to a nonproliferative, contractile phenotype. Although important for normal homeostasis, the plasticity of the VSMC phenotype makes these cells particularly susceptible to both physiological and nonphysiological stimuli. Deregulation of VSMC phenotype switching contributes to the development and progression of vascular pathologies. In particular, abnormal VSMC phenotype is considered to play an important role in the progression of proliferative and obstructive vascular diseases, such as atherosclerosis, postangioplasty restenosis, lymphangioleiomyomatosis, and pulmonary arteriole hypertension (PAH).1,2

VSMC phenotype is determined through the integration of numerous environmental cues, including cytokines, cell-cell contact, cell adhesions, extracellular matrix interactions, injury stimuli, and mechanical force1 (Figure 1A). In particular, growth factor/cytokine signaling can dramatically affect the differentiation status of VSMCs. Platelet-derived growth factor (PDGF) promotes multiple aspects of the synthetic VSMC phenotype, including reduced expression of contractile genes and increased rate of proliferation and migration.3 Conversely, transforming growth factor-β (TGF-β) and its related family member bone morphogenetic protein 4 (BMP4) reduce VSMC proliferation and migration and promote increased expression of VSMC contractile genes.2,4 The importance of TGF-β and BMP in promoting differentiated VSMC phenotype has been confirmed by the identification of mutations in these signaling cascades in patients with the vascular disorders hereditary hemorrhagic telangiectasia and PAH.2

Transcriptional Control of VSMC Contractile Gene Expression

Significant progress has been made in uncovering the basic mechanisms that regulate transcription of VSMC contractile gene expression. The transcriptional regulation of smooth muscle cell markers involves a complex combination of cis-acting elements located within the promoters of VSMC genes and trans-acting factors1 (Figure 1B). In particular, a cis-acting element found in the promoters of contractile genes termed the CArG box (CC(AT)6GG) is critical for the regulation of VSMC gene expression.1 The MADS box...
transcription factor serum response factor (SRF) binds to the CArG box as a homodimer and activates gene transcription. The affinity of SRF for the CArG boxes of VSMC contractile genes is strongly enhanced by the cofactors myocardin and myocardin-related transcription factors A and B (MRTF-A and MRTF-B)\(^1\) (Figure 1B). Moreover, transcription of contractile genes can be negatively regulated by Krüppel-like factor 4 (KLF4). KLF4 is a potent repressor of contractile genes and functions through multiple mechanisms, including remodeling the chromatin of CArG box-containing promoters, sequestering SRF, and reducing myocardin expression\(^1\) (Figure 1B).

Diverse mechanisms have been identified to regulate contractile

Figure 1. Regulation of vascular smooth muscle cell (VSMC) phenotype. A, In response to a variety of stimuli, VSMCs switch between contractile phenotype and synthetic phenotype. Although not mutually exclusive, contractile phenotype is characterized by high expression of contractile genes and low rates of proliferation and migration. Conversely, synthetic VSMCs express low levels of contractile genes and have increased rates of proliferation and migration. MicroRNAs (miRs) reported to promote contractile and synthetic phenotype are indicated. B, Transcriptional regulation of VSMC contractile gene expression involves binding of serum response factor (SRF) and either myocardin or myocardin-related transcription factor (MRTF)-A/B to CArG box elements located within the promoter of contractile genes. Krüppel-like factor 4 (KLF4) is a potent repressor of myocardin/MRTF-A/B activity.
gene expression and other facets of VSMC phenotype. A more complete understanding of the mechanisms used by cytokines and other stimuli to alter VSMC phenotype may allow the development of novel therapeutic approaches for vascular proliferative disorders. The recent identification of microRNAs (miRNAs) as critical regulators of gene expression in a variety of tissues suggested that miRNAs could be involved in the regulation of VSMC phenotype.

miRNA

miRNAs are a class of evolutionarily conserved, ~22-nucleotide noncoding RNAs that control diverse biological functions through the repression of target genes during normal development, as well as during pathological responses. To date, more than 1400 miRNA transcripts have been identified in the human genome, according to miRBase annotation. The mechanism of miRNA biosynthesis is evolutionarily conserved and is initiated by the transcription of a long (several kb) capped and poly-adenylated primary miRNA (pri-miRNA) transcript. The RNase III enzyme Drosha cleaves the pri-miRNA into a ~60- to 100-nucleotide hairpin structure termed the precursor-miRNA (pre-miRNA). Following cleavage, the pre-miRNA is transported out of the nucleus through interaction with exportin-5 and Ran-GTP. The pre-miRNA then undergoes a second round of processing catalyzed by Dicer. This cleavage event gives rise to a double-stranded ~22-nucleotide product composed of the mature miRNA guide strand and the miRNA* passenger strand. The final step of miRNA biogenesis involves loading of the mature miRNA into a large protein complex termed the RNA-induced silencing complex while the passenger strand is degraded. The mature miRNA is then active to promote the association of the RNA-induced silencing complex with specific regions in the 3’ untranslated region of target genes.

Selection of miRNA targets is mediated by imperfect base pairing between the miRNA and miRNA binding sites present in the 3’ untranslated region of the target mRNA. The imperfect nature of the miRNA-mRNA interaction means that a single miRNA can target tens to hundreds of miRNAs. Association of the miRNA–RNA-induced silencing complex results in the repression of the target gene by promoting mRNA degradation, translational inhibition, or both. Through the repression of targets, miRNAs elicit important changes in gene expression programs that contribute to both normal development and disease.

miRNA expression is often tissue specific and developmentally regulated; aberrant expression of miRNAs has been linked to developmental abnormalities and human diseases, including cancer and cardiovascular disorders. Pri-miRNAs are located in diverse regions of the genome and can be transcribed from an independent promoter or, when the miRNA is located in the intron of a protein-coding mRNA, under the control of the host gene promoter. Nucleosome positioning and chromatin immunoprecipitation–on–genomic DNA microarray chip analysis suggests that the promoter structure of miRNA genes is nearly identical to that of mRNAs. Therefore, the DNA binding factors that regulate miRNA transcription largely overlap with those that regulate mRNA expression.

Although transcription of the miRNA gene is the first step of miRNA biogenesis and is critically regulated by various stimuli, it has been recently uncovered that posttranscriptional regulation of miRNA processing also plays an important role in the regulation of miRNA expression. In particular, several additional cofactors, such as the RNA helicases p68 (DDX5) and p72 (DDX17), are identified to associate with Drosha and modulate Drosha-dependent miRNA processing. We recently identified that the signal transducers of the TGF-β and BMP4 signaling cascades, the Smads, associate with p68 in the Drosha complex and promote the processing of a subset of miRNAs, including microRNA (miR)-21. Interestingly, although the Smads are bona fide transcription factors that bind to a specific DNA sequence, TGF-β or BMP4 treatment results in induction of the mature levels of several miRNAs in the absence of induction of the pri-miRNA. In addition to the Smads, recent studies have shown that 2 other transcription factors, p53 and estrogen receptor-α, also modulate miRNA processing through association with p68 and Drosha. On activation by DNA damage, p53 facilitates the pri- to pre-miRNA processing of a subset of miRNAs by interacting with p68 and Drosha on the pri-miRNA, similarly to the role of the Smads. Conversely, on estradiol association, estrogen receptor-α attenuates miRNA processing by preventing the association of p68/Drosha with pri-miRNAs. Together, transcriptional and posttranscriptional regulatory mechanisms allow the levels of mature miRNAs to be tightly regulated in response to cellular cues.

miRNAs Are Required for VSMC Differentiation: Implications From Dicer Knockout

One way to interrogate the overall function of miRNAs during development or pathological processes is to examine the effect of genetic deletion of the miRNA processing enzymes, such as Dicer. Targeted disruption of dicer results in a complete ablation of miRNAs. Consistent with the essential role of miRNAs in a wide variety of functions, homozygous disruption of Dicer in mouse results in early embryonic lethality at embryonic day 8.5. Tissue-specific and inducible disruption of Dicer indicates that miRNAs play a critical role in the differentiation and survival of a multitude of cell types, including stem cells, cardiomyocytes, neurons, glia, and skin cells. An important role for miRNAs in VSMCs is supported by the observation that VSMC-specific inactivation of Dicer results in embryonic lethality at embryonic days 16 to 17 due to severe vascular abnormalities and extensive hemorrhage. The vessels in VSMC-specific homozygous Dicer-deficient mice are dilated and thin-walled because of reduced VSMC proliferation. Furthermore, the arteries of these mice exhibit impaired active and passive force contractility and reduced expression of VSMC contractile genes. Inducible disruption of Dicer in the adult also results in dramatic loss of VSMC function, including decreased vessel contractility, VSMC numbers, and VSMC contractile gene expression. Together, these results strongly suggest that miRNAs are essential not only for the differentiation of VSMCs during early development but also for the maintenance of VSMCs in the adult. Although dicer knockout...
mice exhibit a phenotype indicating positive roles of miRNAs in VSMC differentiation or maintenance, it is likely that some miRNAs may also play an inhibitory role in VSMC differentiation or maintenance. Thus, it is important to interrogate the function of individual miRNAs in VSMC phenotype regulation.

**miR-143/145: Critical Regulators of VSMC Differentiation**

miR-143 and miR-145 are very well characterized in the vasculature and are known as critical regulators of VSMC differentiation.

**Function of miR-143/145**

In vitro analysis indicates that miR-143 and miR-145 promote multiple aspects of VSMC contractile phenotype (Figure 1A). In particular, the expression of contractile genes is elevated following overexpression of miR-143 and miR-145. Multiple targets of miR-143 and miR-145 have been identified, including KLF4, KLF5, ELK1, versican, several actin remodeling proteins, and angiotensin-converting enzyme (Figure 2). Interestingly, all these proteins are antagonistic to the VSMC differentiation; thus, repression of these targets by miR-143/145 facilitates VSMC differentiation. In particular, miR-145-mediated repression of KLF4 is required for BMP4- or TGF-β-mediated induction of contractile gene expression. As more targets of miR-143/145 are identified, it will be crucial to examine how downregulation of such targets affects VSMC phenotype.

The role of miR-143/145 in VSMC phenotype is further supported by in vivo analysis. To date, several mouse models of miR-143/145 deletion have been generated. Despite different transgenic strategies, all studies have found that loss of miR-143 and miR-145 significantly compromises VSMC contractile phenotype. The blood vessels of miR-143/145 knockout animals are thinner and contain fewer VSMCs compared with control animals. Furthermore, knockout animals exhibited reduced systolic and diastolic blood pressures, supporting an important role for miR-143/145 in the maintenance of vascular tone.

**Regulation of miR-143/145 Expression**

miR-143 and miR-145 are encoded in close proximity on chromosome 5 and are transcribed as a bicistronic transcript from a common promoter (Figure 2). In vivo analysis in mouse indicates that the region ∼0.9 kb upstream of miR-143 is sufficient for regulated expression during development; however, additional regulatory regions may also play a role in the expression of miR-143/145 in response to various stimuli. The miR-143/145 promoter contains several highly conserved cis-acting elements that represent potential binding sites for Nkx2.5 and SRF. Indeed, the SRF cofactors myocardin and MRTF-A/B strongly activate the miR-143/145 promoter both in vivo and in vitro.

The expression of miR-143/145 is regulated by cytokines that control VSMC differentiation. For example, pri-miR-143/145 is dramatically elevated by BMP4 and TGF-β. Whereas MRTF-A is required for BMP-4-mediated induction of miR-143/145, myocardin is required for induction by TGF-β (Figure 2). BMP4 enhances MRTF-A nuclear localization by activating Rho signaling and actin polymerization and promotes transcription of pri-miR-143/145. Conversely, TGF-β elicits the rapid transcriptional induction of myocardin (Figure 2). Interestingly, time-course analysis showed that TGF-β induction of miR-143/145 is more rapid than that for MRTF-A. The miR-143/145 promoter additionally contains a Smad response element that can be directly activated in response to TGF-β signal. Thus, at early time points following TGF-β, Smads induce both miR-143/145 and myocardin, whereas sustained elevation of miR-143/145 requires myocardin induction. Pri-miR-143/145 transcription is also regulated through activation of Notch by Jagged-1 (Jag-1) (Figure 2). Similarly to BMP and TGF-β, Jag-1 promotes differentiation of VSMCs through activation of Notch receptors. Activation of Notch results in C promoter binding factor 1 binding to the miR-143/145 promoter and transcriptional activation of miR-143/145 (Figure 2). Importantly, induction of miR-143 and miR-145 is required for the prodifferentiation activities of Jag-1.

In addition to positive regulation by prodifferentiation factors, miR-143/145 are negatively regulated in response to prosynthetic stimuli, such as PDGF. PDGF-BB promotes activation of Src, which in turn inhibits p53. As p53 promotes miR-143/145 expression through both transcriptional and posttranscriptional mechanisms, reduced p53 activity leads to loss of miR-143/145 expression (Figure 2). Together, these results position miR-143/145 as a central target of multiple growth factor signaling pathways that regulate VSMC phenotype.

**Clinical Relevance of miR-143/145**

The expression of miR-143/145 is dramatically reduced in several models of vascular disease, including carotid artery ligation injury in mouse, carotid balloon injury in rat, and ApoE knockout mice. In each of these studies, miR-143/145 was dramatically downregulated in the experimental condition compared with controls. Importantly, overexpression of miR-145 reduced neointimal formation following balloon injury, suggesting that modulation of miRNA expression may represent a viable therapeutic option for VSMC proliferative disorders. The significance of miR-143/145 in human vascular diseases is suggested by the observation that miR-143/145 levels are decreased in aorta from patients with aortic aneurism. Moreover, lower levels of circulating miR-145 are detected in the serum of patients with coronary artery disease. This finding suggests the exciting possibility that the level of miR-145 in circulation may serve as a biomarker for coronary artery disease and potentially other vascular disorders. Unlike VSMC-specific dicer knockout mice, miR-143/145 knockout animals are viable through adulthood. Therefore, although miR-143/145 may be critical for the homeostasis of VSMCs, additional miRNAs are required to provide robust control of VSMC differentiation during development and in response to cellular stimuli.

**Additional miRNAs Promote VSMC Differentiation**

miR-1

Similarly to miR-143/145, myocardin has also been reported to regulate the expression of miR-1 in VSMCs.

Overexpres-
The expression of myocardin in human aortic smooth muscle cells (SMCs) strongly induces the expression of miR-1 and inhibits SMC proliferation. Myocardin-induced inhibition of SMC proliferation is partially reduced when miR-1 is repressed by a miRNA inhibitor against miR-1, indicating that myocardin-induced inhibition of SMC proliferation is in part mediated by miR-1. Moreover, Pim-1, a serine/threonine kinase shown to promote SMC proliferation, has been found to be a direct target of miR-1. Interestingly, compared with uninjured controls, the expression of myocardin and miR-1 is significantly reduced in neointimal lesions induced by carotid artery ligation in mice, whereas the expression of Pim-1 is...
reduce apoptosis. Moreover, knockdown of miR-21 using antisense oligonucleotides in the rat reduces vascular remodeling following balloon injury in carotid arteries. Although differentiation processes are typically coupled to a decrease in proliferation, this is not necessarily the case in VSMCs. The dual action of miR-21 in promoting both differentiation and proliferation may reflect the interesting possibility that miR-21 could target a diverse set of target genes and mediate differential biological outcomes depending on the cellular context.

miRNAs Promote VSMC Dedifferentiation and Proliferation

miR-221
In response to PDGF-BB, VSMCs switch from a contractile to a synthetic phenotype. One aspect of PDGF-mediated VSMC phenotype switch is mediated by induction of miR-221. In response to PDGF treatment, the transcription of pri-miR-221 is rapidly elevated, leading to increased mature miR-221 levels within 3 hours. Mature miR-221 exerts multiple effects on the phenotype of VSMCs, including increased proliferation and migration, as well as reduced expression of contractile genes. miR-221 promotes VSMC proliferation through the repression of the cyclin-dependent kinase inhibitor p27Kip1. Inhibition of miR-221 prevents PDGF-mediated reduction of p27Kip1, as well as VSMC proliferation. Elevation of miR-221 levels is also required for PDGF-mediated repression of contractile genes. However, this effect is independent of the regulation of p27Kip1 as knockdown of p27Kip1 by small inhibitory RNA had no effect on contractile gene expression. These results indicate that the expression of contractile genes and cell growth are not coupled but instead can be regulated by distinct mechanisms. Overexpression of miR-221 is accompanied by dramatic reduction of myocardin expression. Interestingly, miR-221 does not target myocardin directly. This is due to the downregulation of the tyrosine kinase c-Kit, which positively regulates myocardin expression. Similarly to the effect of PDGF-BB, miR-221 overexpression also increased VSMC migration; however, small inhibitory RNA–mediated knockdown of p27Kip1 or c-Kit does not recapitulate the PDGF or miR-221-like promigratory effect, suggesting that a yet-unknown target of miR-221 is responsible for this aspect of VSMC phenotype switch. Together, these findings provide an example of the potency of a single miRNA in mediating diverse cellular outcomes by regulating multiple targets.

It is of note that miR-221 and miR-222 are clustered on the X chromosome and share a common seed sequence, and some reports indicate that they are transcribed from a common promoter. The importance of these miRNAs in regulating VSMC switching is further emphasized by the finding that miR-221 and miR-222 are strongly elevated in vivo in VSMCs following balloon injury of the vessel. Importantly, targeted knockdown of miR-221 and miR-222 in the vessel reduced VSMC proliferation and intimal thickening in response to vascular injury. This response is due to elevated expression of p27Kip1, c-Kit, and p57Kip2 in response to miR-221/222 knockdown, which results in inhibition of cyclin-dependent kinases. These findings suggest the exciting possibility that modulation of miR-221 and miR-222 may provide an effective treatment in vascular proliferative disorders, such as restenosis following angioplasty.

miR-146a
Similarly to miR-221, miRNA microarray analysis indicates that the expression of miR-146a is elevated in rat balloon-injured arteries compared with uninjured controls. In addition, the expression of miR-146a was elevated in proliferative VSMCs. Overexpression of miR-146a increases VSMC proliferation, whereas knockdown of miR-146a attenuates PDGF-mediated increase of VSMC growth. The relevance of miR-146a in vivo is supported by the observation that treatment of balloon-injured rat carotid arteries with antisense oligonucleotides (antagomir) against miR-146a results in reduced neointima formation and VSMC proliferation.
terestingly, a validated target of miR-146a is KLF4.40 This is not consistent with the observation that KLF4 promotes synthetic phenotype and is targeted by miRNAs with procontractile activity, such as miR-14522,24 and miR-1.32 It is possible that additional targets of miR-146a may be responsible for miR-146a-mediated VSMC proliferation.

**Regulation of BMP/TGF-β Signal by miRNAs**

**miR-24**

As discussed above, BMP and TGF-β promote and maintain VSMC contractile phenotype. Consistently, inhibition of BMP or TGF-β signaling promotes PDGF-mediated induction of synthetic phenotype. One mechanism of inhibition of BMP signaling is mediated by miR-24. In response to PDGF-BB, the expression of miR-24 is elevated and the miR-24 target, Tribbles-like protein 3, is decreased.41 Tribbles-like protein 3 promotes the degradation of the Smad ubiquitin ligase Smurf1. Reduced expression of Tribbles-like protein 3 in response to miR-24 thus increases Smurf1, leading to decreased Smad1 expression and reduced BMP signal.41,42 Knockdown and overexpression analysis indicate that miR-24-mediated repression of Tribbles-like protein 3 is required for PDGF-mediated induction of synthetic phenotype, including induction of VSMC proliferation and repression of contractile gene expression.41

**miR-26a**

miR-26a promotes VSMC proliferation and attenuates serum starvation–induced VSMC differentiation.43 Interestingly, the signal transducers of the BMP signaling pathway, Smad1 and Smad4, have both been identified as direct targets of miR-26a, and knockdown of miR-26a results in elevation of expression of Smad1 and Smad4.43 Consistent with inhibition of the Smads, miR-26a overexpression reduces transcription of a promoter containing consensus Smad binding sites.43 Together, these studies indicate miRNAs regulate key pathways involved in VSMC differentiation and suggest that disruption of miRNA function may result in disorders associated with altered BMP and TGF-β signaling, such as PAH.

**Clinical Applications of miRNAs**

Modulation of miRNA levels represents an exciting opportunity for the development of novel therapeutics for cardiovascular disorders. It has been just over a decade since the discovery of miRNAs in humans; however, the first miRNA-based therapy is already in phase II clinical trials.30,44 Identification of miRNAs that modulate VSMC phenotype in vitro and in vivo is a critical step forward for the use of this technology for the treatment of cardiovascular diseases. Although modulating miRNA expression levels appears to be promising in animal models, much work still needs to be done before miRNA-related therapies can be brought to the clinic. It will be important to find ways to safely and efficiently deliver miRNA mimics or antagonists to the tissue of interest.45 Additionally, because a single miRNA has the potential to target hundreds of genes, it will be critical to better define the targeting potential of miRNAs to reduce the potential of detrimental effects due to unknown targets.45 Despite these challenges, the prospect of using miRNAs as therapeutics and diagnostics for vascular diseases in the near future is an exciting possibility. Finally, recent findings that altered levels of miRNA(s) are found in the plasma or sera of patients with specific conditions may provide a novel approach for the blood-based detection of cardiovascular disorders.30,46

**Future Prospects**

miRNAs represent a critical layer of regulation in the control of VSMC phenotype, and miRNA expression is often deregulated in vascular disorders, including PAH, postangioplasty restenosis, and atherosclerosis. Through the regulation of diverse targets, miRNAs regulate gene expression and VSMC differentiation processes. The identification of miRNAs that regulate VSMC phenotypes may provide novel therapeutic targets for the treatment of cardiovascular disease. In addition to modulating the expression of microRNAs themselves, therapeutics that directly modulate expression of miRNA target genes may be useful for the development of novel cardiovascular disorders. Although great progress has been made in the understanding of VSMC regulation by miRNAs, much exciting work remains.

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**Disclosures**

None.

**References**


41. Davis-Dusenbery et al Regulation of VSMCs by miRNAs.
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