MicroRNAs Are Necessary for Vascular Smooth Muscle Growth, Differentiation, and Function

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Objective—Regulation of vascular smooth muscle (VSM) proliferation and contractile differentiation is an important factor in vascular development and subsequent cardiovascular diseases. Recently, microRNAs (miRNAs) have been shown to regulate fundamental cellular processes in a number of cell types, but the integrated role of miRNAs in VSM in blood vessels is unknown. Here, we investigated the role of miRNAs in VSM by deleting the rate-limiting enzyme in miRNA synthesis, Dicer.

Methods and Results—Deletion of Dicer in VSM results in late embryonic lethality at embryonic day 16 to 17, associated with extensive internal hemorrhage. The loss of VSM Dicer results in dilated, thin-walled blood vessels caused by a reduction in cellular proliferation. In addition, blood vessels from VSM-deleted Dicer mice exhibited impaired contractility because of a loss of contractile protein markers. We found this effect to be associated with a loss of actin stress fibers and partly rescued by overexpression of microRNA (miR)-145 or myocardin.

Conclusion—Dicer-dependent miRNAs are important for VSM development and function by regulating proliferation and contractile differentiation. (Arterioscler Thromb Vasc Biol. 2010;30:1118-1126.)

Key Words: contractile proteins • molecular biology • vascular biology • vascular muscle

MicroRNAs (miRNAs) are short (~22 nucleotides) noncoding RNAs that are involved in the regulation of mRNA expression and protein synthesis, and each miRNA potentially targets multiple transcripts. Immature miRNAs are processed by the 2 RNase III endonucleases, Drosha and Dicer, and then incorporated into the RNA-induced silencing complex. Depending on the complementarity of the miRNA with the 3′-untranslated region of the target mRNA, the RNA-induced silencing complex will mediate either translational repression/activation or degradation of the target mRNA.

One way to decipher the importance of miRNAs in development and cell biology has been by Dicer gene disruption in mice. The global loss of Dicer in knockout (KO) mice results in embryonic lethality associated with a loss of pluripotent stem cells and defective blood vessel formation. However, Dicer is not essential for developmental angiogenesis, because endothelial-specific Dicer hypomorphic mice survive but have impaired postnatal angiogenic responses. Although vascular smooth muscle (VSM)—specific KO of Dicer has not been investigated to date, important functions of miRNAs in VSM are beginning to emerge. MicroRNA (miR)-21 is induced in at least 1 VSM type by the differentiation factors transforming growth factor-β and bone morphogenetic protein. In contrast, platelet-derived growth factor induces miR-221 and the dedifferentiation process in VSM. miR-21 and miR-221 are also upregulated in vivo after vascular injury and may regulate the extent of neointimal growth in vivo. Recently, several groups have found that miR-145 positively regulates VSM-specific gene expression, differentiation, actin cytoskeleton, and contractile function. As expected, the potential mechanisms for these effects are varied, including possible effects on Kruppel-like factors 4 and 5 (KLF4 and KLF5), myocardin activity, and angiotensin converting enzyme. These data suggest the essential role of miR-145 as a critical regulator of VSM differentiation and function. However, despite the role of miR-145 in key aspects of VSM differentiation and stem cell renewal, mice lacking miR-145 develop normally but exhibit hypotension, impaired vascular contractility, reduced injury-evoked neointima, or enhanced aging-dependent neointima consistent with an important, yet nonessential, role of miR-145 in VSM.

Because many miRNAs can regulate the levels of overlapping genes, we wanted to address the global integration of miRNAs in VSM development and function using mice with...
a floxed Dicer allele\textsuperscript{16} bred to VSM-specific SM22α Cre-recombinase. Here we show that inactivation of Dicer in VSM results in late embryonic lethality at embryonic day (E) 16 to 17 due to decreased smooth muscle cell (SMC) proliferation and differentiation resulting in thinner vessel walls, impaired contractility, and hemorrhage. Mechanistically, the loss of Dicer impaired the actin cytoskeleton, and defects in VSM-specific gene expression could be partially rescued by miR-145 or myocardin but not miR-221 or miR-21, suggesting that miR-145 regulates VSM differentiation upstream or at the level of myocardin–serum response factor (SRF). Thus, miRNAs are critical for VSM development independent of miR-145 and regulate blood vessel diameter and contractility.

**Methods**

For expanded Materials and Methods, please see supplemental materials (available online at http://atvb.ahajournals.org).

**Mice**

Constitutive inactivation of Dicer in VSM was achieved by breeding SM22αCre transgenic mice (The Jackson Laboratory) with mice harboring loxP sites flanking an RNaseIII domain (exons 20 and 21) of Dicer (Dicer\textsuperscript{loxP}).\textsuperscript{16} To generate adult mice with SMC-specific deletion of Dicer, Dicer\textsuperscript{loxP} mice were intercrossed with transgenic mice expressing the tamoxifen-dependent Cre recombinase CreERT\textsuperscript{2} under control of the SMC-specific myosin heavy chain (SM-MHC) gene Mvh.\textsuperscript{17} A comprehensive study of these animals is forthcoming. All animal procedures were approved by the Yale Animal Care Committee.

**Cell Culture**

Aortic SMC were isolated from tamoxifen-inducible SMC-Dicer KO and control mice as described in the supplemental Materials and Methods. For miRNA overexpression, SMC at passages 3 to 5 were transfected with 60 nmol/L miRIDIAN miRNA mimics (Dharmacon) of mmu-miR-145, mmu-miR-21, mmu-miR-221, mmu-miR-143, or negative control mimic for 24 to 96 hours. For actin depolymerization, SMC were treated with 250 nmol/L Latrunculin B 6 hours before transfection with miRNA mimics.

**Force Measurement**

Segments \( \approx 1.7 \) mm long of umbilical arteries from E15.5 to E16.5 embryos were dissected in Ca\textsuperscript{2+}-free HEPES buffer composed of (in mM) 135.5 NaCl, 5.9 KCl, 1.2 MgCl\textsubscript{2}, 11.6 glucose, and 11.6 HEPES (pH 7.4, 37°C) and mounted on a myograph (610 mmol/L, Danish MyoTechnology, Aarhus, Denmark). The vessel segments were then allowed to equilibrate at 37°C at a preload of 0.5 mmN/mm for 1 hour in HEPES buffer with 2.5 mmol/L Ca\textsuperscript{2+} (Ca\textsuperscript{2+}-HEPES). Active and passive circumference-tension relationships were then generated as described in the supplemental Materials and Methods.

**Results**

**SM22α-Targeted Deletion of Dicer Results in Embryonic Lethality**

To address whether SM22α-targeted deletion of Dicer influences vascular development, male SM22αCre/Dicer\textsuperscript{loxP} mice were bred with either female Dicer\textsuperscript{loxP} or SM22αCre/Dicer\textsuperscript{loxP} mice. A total of 97 pups were born, with no live pups of the SM22αCre:Dicer\textsuperscript{loxP} genotype (SMC-Dicer KO; Figure 1A). All other combinations of genotypes were indistinguishable from wild-type (WT) mice. Subsequently, the timing and cause of embryonic lethality were investigated using synchronized breeding of female Dicer\textsuperscript{loxP} mice bred to male SM22αCre/Dicer\textsuperscript{loxP} mice. SMC-Dicer KO embryos die between E16 and E17 because of widespread hemorrhage in the skin and abdomen (Figure 1B through 1D). Surprisingly, SMC-Dicer KO embryos were indistinguishable from WT or any other genotype of embryos at E15.5. No significant difference in growth was observed in live embryos at E16.5 as measured by crown-to-rump length and wet weight (Supplemental Figure IA).

Dicer mRNA and miRNA expression levels were examined in VSM-rich umbilical cords (including the umbilical and vitelline arteries and veins). Dicer mRNA levels were decreased at E14.5 (Supplemental Figure IB) and E16.5 (Figure 1E). The residual Dicer expression is likely due to other cell types present in umbilical cords, such as endothelial cells and fibroblasts. Analysis of miR-21, miR-221, and miR-145 was selected because recent studies have demonstrated that these miRNAs play a role in VSM differentiation and proliferation.\textsuperscript{5–10,11}\textsuperscript{16} whereas miR-133 was selected on the basis of its role in cardiac and skeletal muscle development via regulation of the transcription factor SRF, also important in VSM differentiation.\textsuperscript{18,19} Expression of miR-21, miR-221, and miR-145 were significantly reduced in SMC-Dicer KO umbilical cords at E16.5 (Figure 1E). Because miR-145 and miR-143 are the only miRNAs shown to be relatively specific to SMC in the vasculature, the residual expression of miR-21 and miR-221 in the umbilical cords may represent expression in other cell types. Similarly, expression of miR-133 was unchanged, suggesting that this miRNA is expressed primarily in non-SMC present in the umbilical cord.

**Hemorrhage in SMC-Dicer KO Embryos Originates From the Liver**

Morphometry of E16.5 embryos revealed intraperitoneal bleeding in a majority of the SMC-Dicer KO embryos (Supplemental Figure II). To identify the origin of the intraperitoneal hemorrhage in these embryos, major internal organs were dissected from Dicer\textsuperscript{loxP} and SMC-Dicer KO embryos. In SMC-Dicer KO embryos, distal parts of the liver consistently contained darker red patches, suggesting that intraperitoneal hemorrhage originated from this region (Supplemental Figure IIIA). This correlates with a general increase in red blood cells observed in hematoxylin/eosin–stained sections of distal liver (Supplemental Figure II).

**SM22αCre Is Highly Expressed in Vascular but Not Visceral Smooth Muscle**

Although SM22α is considered a marker of smooth muscle, it is, like most other markers for smooth muscle lineage, transiently expressed in the heart during E8 to E12.5.\textsuperscript{20} To investigate the specificity of SM22αCre expression in mouse embryos, SM22αCre mice were crossed to ROSA26 reporter mice. As shown in Supplemental Figure IIIIC, whole mount LacZ staining of E15.5 embryos revealed positive LacZ staining in both venous and arterial vessels in the placenta, umbilical cord, and yolk sac. In paraffin sections, LacZ staining was observed specifically in VSM of the aorta, umbilical artery, and cardiomyocytes, with no or little staining in other tissues, including visceral smooth muscle of the esophagus, trachea, and bladder (Supplemental Figure IIIIC).
Despite the lack of detectable SM22αCre expression or loss of Dicer in visceral and bronchial smooth muscle, a general reduction in the size of the stomach, intestine, urinary bladder, kidney, and lung was observed (Supplemental Figure IIIA). The phenotype of SMC-Dicer KO embryos is clearly distinguishable from a model of cardiac-specific Dicer KO because deletion of Dicer in cardiac progenitor cells by E8.5 results in embryonic lethality due to heart failure caused by pericardial edema and a poorly developed ventricular myocardium.21 SMC-Dicer KO did not exhibit any signs of pericardial edema; however, both ventricles were slightly dilated, and both atria were smaller in size (Supplemental Figure IIIA and IIIB). The absence of a severe cardiac phenotype in the SM22α-mediated SMC-Dicer KO embryos is not clear, but it likely relates to the transient and delayed onset of Cre recombinase expression in cardiomyocytes compared with cardiac-specific promoters such as Nkx2.5 and α-myosin heavy chain.

Transmission Electron Microscopy Revealed Dramatic Changes in Ultrastructure of the Vessel Wall and Medial SMC in Dicer KO Aorta

Transmission electron microscopy was used to clarify how the loss of Dicer affects the ultrastructure of VSM and the surrounding extracellular matrix. Analysis of 4 independent dorsal aortae from either DicerF/wt or SMC-Dicer KO E15.5 embryos revealed dramatic changes in vascular ultrastructure in SMC-Dicer KO aortae. Whereas DicerF/wt embryos showed appropriate elastic lamellae circumscribing layers of medial SMC (Figure 2A and 2B), these structures were discontinuous and highly fragmented in the SMC-Dicer KO embryos (Figure 2C and 2D). Dense plaques and myofilament bundles, which typify the ultrastructure of differentiated SMC in DicerF/wt SMC, were less frequent in the SMC-Dicer KO (Figure 2A through 2D). Moreover, many medial SMC in SMC-Dicer KO embryos were polygonal and poorly organized around the circumference of the dorsal aorta (Supplemental Figure IV). The latter cells also appeared to be overly enriched with rough endoplasmic reticulum (Figure 2D and data not shown). Together, the transmission electron microscopy data illustrate ultrastructural phenotypes in SMC-Dicer KO mice that are consistent with defective vascular structure and altered SMC differentiation.

Altered Vascular Geometry in the Aorta of SMC-Dicer KO Embryos

Physiologically, blood flow and capillary hydrostatic pressure are regulated by vascular contraction of arteries/arterioles that distribute oxygen and nutrients to tissues. Loss of vascular contractility in the arterial tree could potentially increase capillary pressure as systemic blood pressure increases during midgestation, thereby promoting hemorrhage and bleeding. It

Figure 1. Loss of Dicer in smooth muscle is lethal at E16.5 to E17.5. Two different breeding strategies were used to produce SMC-Dicer KO (Dicer+/- + Cre) mice. A, Number of live pups of different genotypes. A total number of 97 pups were genotyped. Timed mating revealed that SM22α-targeted deletion of Dicer results in embryonic lethality at E16.5 to E17.5 (B), and the embryonic lethality was associated with widespread internal hemorrhaging (C). D, No major difference in embryonic development was observed between DicerF/wt (D+/+ ) and SMC-Dicer KO before E16.5. E, Quantitative polymerase chain reaction analysis of smooth muscle–rich umbilical cords demonstrated a significant decrease in Dicer mRNA and miRNAs at E16.5. Data are mean±SEM; *P<0.05.
is well accepted that arterial contractile force is dependent on the differentiated state of VSM and the thickness of the vessel wall. To investigate whether the loss of Dicer in VSM results in altered vascular geometry, quantitative morphometry of the aorta was performed on hematoxylin/eosin–stained sections of E12.5, E14.5, E15.5, and E16.5 embryos. As shown in Figure 3A, decreased medial area (left graphs) and thickness (right graph) was evident in E15.5 and E16.5 SMC-Dicer KO mice. At E15.5, this was also associated with an increase in lumen diameter (Supplemental Figure VA).

Because deletion of Dicer in various cell lineages has been shown to impair cell proliferation,22–24 we tested whether the reduction in smooth muscle medial area in SMC-Dicer KO embryos was due to a decrease in SMC proliferation during
development. As shown in Figure 3B, a significant decrease in Ki67-positive aortic VSM was observed at E14.5 and E15.5 in concordance with a reduced number of aortic VSM cells found in E15.5 to E16.5 embryos. In addition, the level of immunoreactive smooth muscle (SM)-α-actin was reduced in aortae of E14.5 embryos, indicating a decrease in muscle-specific gene expression (Figure 3B). Two important signaling pathways that regulate VSM proliferation are the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways; however, the loss of Dicer in VSM did not affect activation of these pathways in umbilical cords from E16.5 embryos (Supplemental Figure VB).

Contractile Function and Differentiation Is Decreased in Umbilical Arteries of Dicer KO Embryos

In the adult heart, inducible deletion of Dicer results in a decreased cardiac function and activation of the fetal gene program.25 To assess the contractile function of SMC-Dicer KO vessels, umbilical arterial vessels were used for functional studies. To our knowledge, analysis of contractile function in embryonic mouse umbilical arteries has not been attempted previously, likely because of the small size of these vessels. We examined both active force (due to calcium activation of actin-myosin cross bridges) in response to depolarization by 80 mmol/L potassium (high-K) and passive force (an index of vascular compliance or structure) in response to nominally calcium-free buffer at increasing luminal circumferences of the embryonic vessels. As quantified in Figure 4A, the loss of Dicer in VSM markedly impairs contractile function in arteries. Reduced contractile responses to depolarization in SMC-Dicer KO vessels could be caused by altered expression or function of ion channels, such as L-type voltage gated calcium channels in the cell membrane. To test this hypothesis, we also measured contractile force in response to the protein phosphatase (PP1, PP2A) inhibitor Calyculin A, which causes calcium-independent contraction of smooth muscle by increasing myosin light chain phosphorylation.26 Similar to high KCl, the contractile force to Calyculin A (0.2 μmol/L) was reduced in SMC-Dicer KO vessels, suggesting that the cause of contractile dysfunction in the absence of miRNAs is mainly an effect on the contractile machinery per se and not signaling events upstream of myosin phosphorylation (Figure 4B). Part of the decrease in contractile response is clearly due to a decrease in VSM mass in SMC-Dicer KO umbilical arteries (Supplemental Figure VC and VD). However, although VSM mass in SMC-Dicer KO umbilical arteries was decreased by ≈33%, maximal contractile force was decreased by ≈75%, indicating that contractile properties of the SMCs are disturbed in SMC-Dicer KO vessels. This could be a result of a decrease in actin filament content as suggested by transmission electron microscopy. A rightward shift in the passive length-tension relationship in SMC-Dicer KO together with a right shift in the calculated Lmax (optimal length for maximal force; lumen circumference: WT, 968 μm, KO, 1039 μm) suggests that loss of Dicer increases vascular compliance.

To directly examine the state of differentiation of VSM, the levels of SMC-specific proteins SM-MHC, calponin, SM22α,
and α-actin were examined in Dicer<sup>fl/wt</sup> and SMC-Dicer KO cords by Western blotting. The levels of SM-MHC, calponin, and SM22α were significantly reduced in SMC-Dicer KO vessels, suggesting that miRNAs play an important role in SMC differentiation (Figure 4C). A minor part of this effect may be due to the reduction in VSM in SMC-Dicer KO umbilical cords. However, the expression of SM-α-actin was not significantly decreased in SMC-Dicer KO umbilical cords, suggesting that comparable amounts of SMC from Dicer<sup>fl/wt</sup> and SMC-Dicer KO were loaded on the gel. The expression of SMC-specific genes is regulated by the transcription factor SRF together with the coactivators myocardin and myocardin-related transcription factors-A/-B (MRTF-A and -B).<sup>27,28</sup> In addition, the transcription factors KLF4 and KLF5 repress SMC-specific gene expression by both downregulating myocardin expression and preventing SRF/myocardin-dependent gene expression.<sup>15,29</sup> miR-145 appears in some studies to enhance myocardin expression<sup>10</sup> while repressing KLF4 and KLF5<sup>15</sup> to reduce cell proliferation and promote VSM-specific gene expression. However, in E16.5 umbilical cords, neither the protein levels of SRF, KLF4, or KLF5 (Figure 4C) nor the mRNA levels of myocardin or KLF4 (Supplemental Figure VE) were altered in SMC-Dicer KO cords, suggesting alternative mechanisms are operational in these mice.

**miR-145 Rescues SMC-Specific mRNA and Protein Expression in Dicer KO SMC**

To clarify the roles of specific miRNAs for SMC differentiation, SMC-Dicer KO and control SMCs were isolated from adult, inducible SMC-Dicer KO and Cre-negative (control) mice and then transfected with miRNA mimics (see Methods). Dicer mRNA levels were reduced by approximately 75% in Dicer KO SMC, whereas miR-145 levels were reduced by approximately 85% compared with control. Interestingly, transfection of miR-145 completely rescued the expression of SMC-specific genes in Dicer KO SMCs (Acta2, SM-α-actin; Myh11, SM-myosin heavy chain; Cnn1, calponin; Figure 5A). miR-143 did not significantly alter the effect of miR-145 when added in combination, and the fold increase in SMC gene expression was similar in Dicer KO and control SMC transfected with miR-145 (data not shown), suggesting that miR-145 functions independently of other miRNAs. No significant difference in SMC-specific gene transcription was observed with transfection of miR-21, miR-221, or miR-143 alone in control or Dicer KO SMCs (Figure 5A and data not shown). As shown in Supplemental Figure VIA, mRNA levels of myocardin, KLF4, and KLF5 were not affected by transfection of miR-145, miR-21, or miR-221 mimics.

Recently, it was demonstrated that the gene encoding for the β1-subunit of the large conductance calcium-activated potassium (MaxiK) channels channel, Kcnmb1, is transcriptionally regulated by myocardin-SRF.<sup>30</sup> We found that expression of Kcnmb1 was nearly abolished in Dicer KO SMC and that this effect was partly rescued by transfection of miR-145, confirming that Kcnmb1 is regulated by similar mechanisms as SMC-specific contractile genes.

To test whether miRNAs are required for myocardin activity, human myocardin was overexpressed in Dicer KO SMCs using adenoviral-mediated gene transfer. As shown in Supplemental Figure VIB, overexpression of myocardin significantly increased the expression of VSM-specific genes in Dicer KO SMCs, demonstrating that myocardin is functional in a setting of reduced miRNA levels.

Because miRNAs often target translation of mRNA to protein, the effects of some miRNAs are only observed at the protein level. We thus quantified the effect of miR-145 on SMC protein markers and transcription factors by Western blotting. As shown in Figure 5B, SM22 and calponin protein levels were significantly reduced in Dicer KO SMC versus control, and all SM markers were upregulated in response to miR-145. In addition, we found an increased expression of KLF4 in Dicer KO SMC, which was reduced by addition of miR-145. Surprisingly, SMC proliferation rate, in vitro, was increased in Dicer KO versus control, and this effect was not sensitive to miR-145 transfection (Supplemental Figure VIC). The cause of the discrepancies in Dicer KO SMC proliferation rates, as well as KLF4 expression, in vivo and in vitro are not clear but may depend on differences in environmental cues.

**miR-145 Induces SMC-Specific Gene Transcription by Promoting Actin Polymerization**

A possible mechanism for the reduced vascular contractility and differentiation of SMC-Dicer KO may be perturbed actin dynamics. Naturally, an increased number of actin filaments will promote smooth muscle contractile function, but it is also well known that increased actin polymerization promotes SMC-specific gene transcription and protein synthesis in vitro and ex vivo.<sup>31,32</sup> Globular (monomeric) G-actin binds to the SRF cofactor MRTF-A, or MAL, thereby preventing its translocation to the nucleus.<sup>33</sup> An increase in actin filament polymerization results in a reduction of the G-actin pool, nuclear translocation of MAL/MRTF-A, and activation of SMC-specific gene transcription. It was recently suggested that miR-143/145 regulates the expression of several factors involved in smooth muscle actin dynamics.<sup>13</sup> To analyze actin filament structure, Dicer KO and control SMC were isolated and stained with fluorescent phalloidin (F-actin) and DNase1 (G-actin). Initial experiments were performed using nontransfected SMC and showed that loss of Dicer results in decreased stress fiber formation compared with control cells (Supplemental Figure VII). In subsequent experiments, control and Dicer KO SMC were transfected with either negative control or miR-145 mimic (Figure 6A). Surprisingly, addition of miR-145 to Dicer KO SMC rescued the loss of actin filaments, indicating that this miRNA plays an important role in actin dynamics. Moreover, we found that depolymerization of actin filaments using Latrunculin B prevents the increase in SMC-specific gene expression induced by miR-145 (Figure 6B). This suggests that miR-145 promotes SMC differentiation via an increased actin polymerization.

**Discussion**

The present study demonstrates that miRNAs are essential for VSM development and function by regulating SMC differentiation and proliferation. The loss of miRNAs in VSM during early development results in hemorrhage and embry-
onic lethality. The phenotype of the SMC-Dicer KO mouse is clearly distinguishable from Dicer KO in cardiomyocytes.\textsuperscript{21,34} We also found a decreased medial thickness (E15.5 to 16.5) in the aorta of SMC-Dicer KO embryos caused by a decrease proliferation of SMC at E14.5. In addition, unique measurements of vascular function in E16.5 embryonic arteries revealed a nearly abolished contractile force in SMC-Dicer KO, likely caused by a combination of a decreased SMC mass, decreased expression of contractile proteins, and decreased formation of actin filaments. We suggest that the loss of SMC proliferation and arterial contractile function results in hemorrhage and embryonic lethality because of dysregulation of blood flow into the microcirculation. Although overexpression of miR-145 and myocardin restored SMC-specific gene expression in Dicer KO SMC, our results indicate that additional miRNA-dependent mechanisms must be operational during VSM development, because the loss of Dicer in SMC is lethal, whereas the global loss of miR-145 is not.

During vascular development, VSM proliferate and mature into the differentiated, contractile state to maintain pressure and flow throughout the growing embryo. Postnatally, VSM is in its differentiated state expressing markers such as myocardin, SM-MHC, calponin and SM22\textsubscript{α} and remains quiescent until injury or disease processes such as atherosclerosis promote a less differentiated and proliferative VSM phenotype. Recent data suggests that miRNAs may differentially modulate the proliferative versus the differentiated state of VSM. Growth factor upregulation of miR-221 promotes VSM proliferation via targeting the negative regulators of the cell cycle, p27 and p57.\textsuperscript{7,8} On the other hand, both miR-21 and miR-145 promote VSM differentiation. miR-21 negatively regulates programmed cell death 4, promoting VSM differentiation, whereas miR-145 enhances myocardin ex-

Figure 5. miR-145 rescues SMC-specific gene and protein expression in Dicer KO SMC. A, Control (Ctrl) and Dicer KO SMCs were transfected for 96 hours with 60 nmol/L negative control (Neg Ctrl), miR-145, miR-21, miR-221, or miR-143 mimics. The expression of SMC-specific genes (Acta-2, SM-α-actin; Cnn1, calponin; Myh11, myosin heavy chain; Kcnmb1, B1-subunit of MaxiK channel), Dicer, and miR-145 was analyzed by quantitative polymerase chain reaction and normalized to control VSM cells. 18S was used as internal control for SMC-specific genes and Dicer, and 5S was used as internal control for miR-145. B, SMC protein markers and transcription factors were analyzed by Western blotting in Ctrl and Dicer KO SMC transfected with Neg Ctrl or miR-145 mimics. Original data are shown from different parts of a single nitrocellulose membrane, and the signal intensity was quantified and normalized to HSP90. Data are mean±SEM; *P<0.05, #P<0.05 versus KO (Neg Ctrl).
expression/activity, possibly via inhibition of the myocardin negative regulators KLF4 and KLF5. In support of previous findings, we show that addition of miR-145 in Dicer KO SMC is sufficient to partly or completely restore SMC-specific gene transcription (Figure 5A and 5B). Thus, miR-145 seems to play an important role in SMC differentiation. However, recent studies of miR-143/145 KO mice show that these miRNAs are not necessary for VSM development in vivo. In contrast, in our experiments on miR-143/145 KO SMCs transfected with negative control or miR-145 mimic for 96 hours, actin filaments were depolymerized by Latrunculin B (Latr B, 250 nmol/L) to depolymerize actin filaments. The cells were then transfected with negative control or miR-145 mimic for 24 hours. The expression of SMC-specific genes (Acta-2, Sm-α-actin; Myh11, myosin heavy chain) and Dicer was analyzed by quantitative polymerase chain reaction and normalized to Ctrl SMC. 18S was used as internal control. Data are mean±SEM; *P < 0.05.

Figure 6. Decreased stress fiber formation in Dicer KO SMC results in loss of SMC-specific gene expression. A, Ctrl and Dicer KO (KO) SMC were transfected with negative control (Neg Ctrl) or miR-145 mimic for 96 hours. Actin filaments (F-actin) and monomeric actin (G-actin) were visualized using rhodamine phalloidin (red) and Alexa 488 DNase1 (green), respectively. The staining intensity of F- and G-actin in 12 to 16 fields from 4 separate slides was quantified and shown as the F/G-actin ratio. B, Ctrl and Dicer KO SMC were pretreated with vehicle or Latrunculin B (Latr B, 250 nmol/L) to depolymerize actin filaments. The expression of SMC-specific genes accompanying an apparent defect in VSM cell migration in the miR-143/145 KO. Future experiments elucidating the repertoire of miRNAs that negatively regulate VSM-specific contractile/regulatory gene function (including the VSM master regulatory factor, myocardin) will enhance our understanding of miRNA regulation of vascular function in health and disease. For these studies, the SMC-Dicer KO model may be of great value: in this model, individual miRNAs can be reintroduced without the confounding effect of endogenous miRNAs.

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Disclosures
None.

References
9. Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C. MicroRNA expression signature and antisense-mediated depletion reveal involved in miRNA-dependent SMC-specific gene transcription in vivo. Recently, it was shown that miR-143/145 regulates the expression of several factors that control actin polymerization, which has a profound effect on SRF-dependent gene transcription via control of nuclear translocation of the SRF cofactor MAL/MRTF-A. Herein, we show that stress fiber formation in isolated Dicer KO SMCs is severely impaired in accordance with a loss of actin filaments in vivo in aortas of E15.5 SMC-Dicer KO embryos. Interestingly, the loss of actin filaments was rescued by transfection of miR-145. In addition, miR-145 was not able to promote SMC gene expression in the presence of Latrunculin B, an inhibitor of actin polymerization. These results suggest that miR-145 is involved in the control of actin dynamics, and these data are congruent with those of Xin et al, who showed impaired actin cytoarchitecture and reduced cytoskeletal genes accompanying an apparent defect in VSM cell migration in the miR-143/145 KO. Future experiments elucidating the repertoire of miRNAs that negatively regulate VSM-specific contractile/regulatory gene function (including the VSM master regulatory factor, myocardin) will enhance our understanding of miRNA regulation of vascular function in health and disease. For these studies, the SMC-Dicer KO model may be of great value: in this model, individual miRNAs can be reintroduced without the confounding effect of endogenous miRNAs.


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Supplemental Materials

Supplemental Methods

*Generation of SM22-Cre;Dicer^f/f Embryos*

In initial experiments, heterozygous SM22αCre/Dicer^f/wt male mice were either bred with Cre negative Dicer^f/wt female mice or heterozygous SM22-Cre/Dicer^f/wt female mice. In subsequent timed breeding experiments, only the former breeding strategy was used. Females were checked daily for the presence of vaginal plug, which indicated E0.5. SM22αCre negative Dicer^f/wt embryos (D^f/wt) were used as controls for SM22αCre/Dicer^f/f embryos (Dicer KO). Genotyping was performed using PCR as reported previously.

*Cell culture*

At the age of 3 weeks, male MHCCreER^T2/Dicer^f/f mice were treated with intraperitoneal injections of tamoxifen (50g/kg/day) for 5 consecutive days. Excision of the floxed Dicer allele was confirmed by genotyping and aortic smooth muscle cells were then isolated using a standard enzymatic digestion technique. Control cells were isolated simultaneously from age matched, tamoxifen treated, Cre negative Dicer^f/f mice. Briefly, aortas were isolated and incubated for 30 min at 37°C in serum free DMEM cell culture media containing 1 mg/ml collagenase type 2 (Worthington Biochemical Corporation). The adventitia was then pulled of using forceps and the aorta was incubated for 4h at 37°C in serum free DMEM cell culture media containing 2 mg/ml collagenase type 2 (Worthington Biochemical Corporation) and 0.2mg/ml elastase (Sigma).
qRT-PCR

Quantitative real time PCR for Dicer and selected miRNAs was performed as reported previously \(^1,^2\). The qPCR primer sequences used for \textit{Acta2}, \textit{Cnn1}, \textit{Myh11}, \textit{Myocd}, \textit{KLF4} and \textit{KLF5} have been published previously \(^3^5\). Commercially available primer sequences for selected miRNAs were used.

Adenoviral gene transfer

For adenoviral gene transfer of myocardin SMC were incubated overnight in DMEM / 10\%FCS with Ad.MYOCD or Ad.LacZ at moi 100 \(^6\). After removal of the virus, SMCs were cultured for 36h in DMEM / 10\%FCS.

Western blotting

The umbilical and vitelline artery and vein from each embryo was dissected free from surrounding tissue and snap frozen in liquid nitrogen. Standard western blot analysis was conducted using Dicer (Santa Cruz Biotechnology Inc.), HSP90 (BD Biosciences), \(\alpha\)-actin (Sigma), SM-MHC (Biomedical Technologies Inc.), Calponin, SM22, KLF4, KLF5 (Abcam) antibodies.

\(\text{\[^{3}\text{H}]\text{Thymidine incorporation}}\)

Control and Dicer KO SMC were starved for 36h, transfected for 15h with negative control or miR-145 mimic and then stimulated for 36h with 10\% FCS with 1\(\mu\)Ci \(\text{\[^{3}\text{H}]\text{Thymidine}}\) (PerkinElmer). After washing and standard TCA-precipitation, the incorporated \(\text{\[^{3}\text{H}]\text{Thymidine}}\) was quantified by liquid scintillography.
Histological analysis

Embryos generated by timed breeding were isolated, fixed in 4% paraformaldehyde, paraffin embedded and sectioned. The sections were stained with hematoxylin and eosin (H&E) and analyzed by microscopy. Freshly dissected whole embryos and organs were photographed using a dissecting microscope.

Immunofluorescence

Following deparaffinization and rehydration, slides were incubated in Tris-EDTA buffer (10mM Tris, 1mM EDTA, 0.05% Tween 20, pH 9) at 97°C for 15 min then blocked using 10% goat serum and incubated over night with antibodies against Ki67 (Abcam) and smooth muscle alpha actin (Sigma). For F-actin and G-actin staining, isolated smooth muscle cells, passage 4-5, were fixed in 4% paraformaldehyde for 10 min, permeabilized for 5 min in 0.1% triton and incubated for 30 min at room temperature with rhodamine phalloidin (Molecular Probes) for F-actin and alexa fluor 488 conjugated DNAse1 (Invitrogen) for G-actin.

β-Galactosidase staining of SM22Cre;ROSA26 embryos

Heterozygous SM22αCre female mice (The Jackson Laboratory) were timed bred with a homozygous ROSA26 (The Jackson Laboratory) male mouse. At E15.5 the embryos were dissected from the yolk sacs and opened at the thorax and abdomen to allow penetration of the staining solutions. Whole staining of embryos were then performed as previously described 7.
**Electron microscopy**

Embryos (E16.5; n=5) were fixed at 4°C in 0.1M phosphate buffered 2.5% glutaraldehyde, postfixed 60 minutes in phosphate buffered 1.0% osmium tetroxide, dehydrated in a graded series of ethanol to 100% (3 times), transitioned to propylene oxide, infiltrated with EPON/Araldite resin, embedded in fresh resin and polymerized for two days at 70°C. Thin sections of the dorsal aorta (70nm) were placed onto 100 mesh copper grids, stained with uranyl acetate/lead citrate and analyzed in a blind manner with a Hitachi 7650 transmission electron microscope equipped with a GATAN Erlangshen 11 mega pixel digital camera. Final images were processed in Adobe Photoshop.

**Force measurement**

Active and passive circumference-tension relationships were then generated as previously described. Briefly, vessels were contracted twice using High-KCl HEPES buffer obtained by exchanging 80 mM NaCl for KCl before circumference-tension relationships were generated by increasing wire distance in a stepwise fashion. At each wire distance, preparations were allowed to equilibrate in Ca^{2+}-HEPES for 5 min, then stimulated with High-KCl buffer for 6 min (active force) and relaxed in nominally Ca^{2+}-free solution for 10 min (passive force). Calcium chelating agents such as EGTA was not used in the Ca^{2+}-free solution since this may negatively affect subsequent contractile responses. However, previous experience shows that smooth muscle myogenic tone in nominally Ca^{2+}-free solution is negligible.
Supplemental Figure Legends

**Supplemental Fig. I** (A) Crown-to-rump length and wet weight of E16.5 embryos. (B) qPCR analysis of Dicer in umbilical cords of E14.5 embryos.

**Supplemental Fig. II** Paraffin embedded, H&E stained section of the abdominal area of Dfl/wt and SMC Dicer KO E16.5 embryos. Four 4X photos were acquired and assembled into one montage. Note intraperitoneal hemorrhage in the SMC-Dicer KO embryo indicated by arrows. Areas of hemorrhage within the liver are indicated by asterisks.

**Supplemental Fig. III** (A) Organs isolated from E16.5 Dfl/wt and SMC-Dicer KO embryos. Bar indicates 5mm. (B) Paraffin embedded, H&E stained section of E16.5 hearts. Bar indicates 500mm . (C) LacZ staining of tissues from SM22-Cre positive and Cre negative embryos carrying the ROSA26 reporter gene (A). Bl; bladder, UA; umbilical artery, Ao; aorta, Eso; esophagus, Tra; trachea.

**Supplemental Fig. IV** TEM showing a cross-section of a Dfl/wt E15.5 dorsal aorta (top) and a similar region from a SMC-Dicer KO embryo (bottom). Note disordered array of medial SMC in the Dicer KO aorta and their less spindled morphology. Scale bars are indicated at bottom. Magnification is 6,000x.

**Supplemental Fig. V** (A) Analysis of outer and inner diameter of the aortic media of E12.5-E16.5 embryos shown in figure 3A. n=4-6 *=p<0.05 (B) ERK1/2 and Akt phosphorylation in umbilical cords of E16.5 embryos. n=3-5 *p<0.05. (C) Vascular
geometry of H&E stained frozen sections of E16.5 umbilical arteries. n=3. (D) H&E stained cross section of the umbilical arteries from E16.5 embryos. m indicates the smooth muscle media. (E) qPCR analysis of myocardin and KLF4 in umbilical cords of E16.5 embryos.

**Supplemental Fig. VI** (A) Analysis of mRNA expression of Myocd, KLF4 and KLF5 in Dicer KO and Ctrl SMC following 96h transfection of Neg Ctrl, miR-145, miR-21 or miR-221. (B) Ctrl and Dicer KO SMC transduced with Ad-lacZ or Ad-myocardin expressing adenoviral vectors for 48h. miR-145 and SMC specific genes (Acta-2: SM-a-actin, Cnn1: calponin, and Myh11: myosin heavy chain) were analyzed by qPCR. (C) Proliferation of Dicer KO and control SMC stimulated with 10% fetal calf serum (FCS) was analyzed using 3H-Thyminine incorporation. Cells were either transfected with Neg Ctrl or miR-145 mimic prior to FCS stimulation. n=3-5, *p<0.05

**Supplemental Fig. VII** Actin filaments (F-actin) and monomeric actin (G-actin) were visualized in Dicer KO (KO) and Ctrl SMC using rhodamine phalloidin (red) and Alexa 488 DNase1 (green), respectively. Upper four panels show representative images of Ctrl and Dicer KO cells using a 10X objective. Lower two panels show representative cells using a 63X objective.
References


Supplemental figure I

A

Length (mm)

Weight (mg)

D^{fl/wt}  KO

D^{fl/wt}  KO

B

E14.5

Dicer mRNA (fold change)

D^{fl/wt}  KO

***
Supplemental figure III

A

Liver

Heart

Lung/heart

B

D_{fl/wt} KO

D_{fl/wt} KO

C

SM22αCre/ROSA26

ROSA26

Stomach/Bladder

Intestine

Yolk sac

Placenta/Umbilical cord

Bladder

Thorax

Ao

Eso

Tra

UA

Bl

Bi
Supplemental figure V

A

Outer diameter (µm) Lumen diameter (µm)

E12.5

E14.5

E15.5

E16.5

B

Dfl/wt KO

Dfl/wt KO

Dfl/wt KO

Dfl/wt KO

P-ERK

T-ERK

P-Akt

T-Akt

C

Outer diameter (µm)

Inner diameter (µm)

Media area (µm²)

Wall thickness (µm)

D

KO

Dfl/wt KO

E

Myocardin (fold change)

KLF4 (fold change)

E16.5

Dfl/wt KO
Supplemental figure VII

F-actin/G-actin

10X

10X

63X