MicroRNAs Are Essential for Stretch-induced Vascular Smooth Muscle Contractile Differentiation via MicroRNA (miR)-145-dependent Expression of L-type Calcium Channels*1

Karolina M. Turczyńska, Mardjaneh Karbalaei Sadegh, Per Hellstrand, Karl Swärd, and Sebastian Albinsson

From the Department of Experimental Medical Science, Lund University, 221 84 Lund, Sweden

Background: miRNAs regulate smooth muscle phenotype.
Results: Deletion of miRNAs results in impaired stretch induction of contractile differentiation and reduced expression of L-type calcium channels.
Conclusion: miRNAs are crucial for stretch-sensitive smooth muscle differentiation in part via miR-145-dependent expression of L-type calcium channels.
Significance: These findings provide novel insights into the mechanism of smooth muscle phenotypic modulation in vascular disease.

Stretch of the vascular wall is an important stimulus to maintain smooth muscle contractile differentiation that is known to depend on L-type calcium influx, Rho-activation, and actin polymerization. The role of microRNAs in this response was investigated using tamoxifen-inducible and smooth muscle-specific Dicer KO mice. In the absence of Dicer, which is required for microRNA maturation, smooth muscle microRNAs were completely ablated. Stretch-induced contractile differentiation in Dicer KO vessels was dramatically reduced. On the other hand, acute stretch-sensitive growth signaling, which is independent of influx through L-type calcium channels, was not affected by Dicer KO. Contractile differentiation induced by the actin polymerizing agent jasplakinolide was not altered by deletion of Dicer, suggesting an effect upstream of actin polymerization. Basal and stretch-induced L-type calcium channel expressions were both decreased in Dicer KO portal veins, and inhibition of L-type channels in control vessels mimicked the effects of Dicer deletion. Furthermore, inhibition of miR-145, a highly expressed microRNA in smooth muscle, resulted in a similar reduction of L-type calcium channel expression. This was abolished by the Ca2+/calmodulin-dependent protein kinase II inhibitor KN93, suggesting that Ca2+/calmodulin-dependent protein kinase IIδ, a target of miR-145 and up-regulated in Dicer KO, plays a role in the regulation of L-type channel expression.

These results show that microRNAs play a crucial role in stretch-induced contractile differentiation in the vascular wall in part via miR-145-dependent regulation of L-type calcium channels.

Unlike striated muscle cells, vascular smooth muscle cells are not terminally differentiated and therefore retain a remarkable capability of phenotypic modulation. Normally, vascular smooth muscle cells exhibit a quiescent and contractile phenotype characterized by expression of contractile proteins as well as ion channels and signaling molecules involved in contractile function. However, in response to a variety of environmental cues including circulating hormones, autocrine factors, and altered mechanical load, smooth muscle cells may undergo a phenotypic switch characterized by increased proliferation, migration, and synthesis of extracellular matrix proteins. This response plays a crucial role during development of blood vessels and in response to vascular injury but may in some cases be detrimental and promote vascular disease.

Although phenotypic modulation of vascular smooth muscle cells in vivo depends on the integration of multiple environmental cues, we and others have shown that mechanical tension alone is sufficient to promote contractile differentiation (2–9). The mechanisms behind this effect are complex, but stretch-induced Rho/Rho kinase activation and actin polymerization play crucial roles (5, 6, 10). Actin polymerization is known to regulate smooth muscle differentiation by promoting nuclear translocation of myocardin-related transcription factor, a transcriptional co-activator to serum response factor, which then induces transcription of smooth muscle-specific genes (11). Additionally, activation of the Rho signaling pathway may promote myocardin expression and smooth muscle-specific gene transcription via the transcription factor MeF2 (12, 13). Because multiple factors can induce Rho/Rho kinase activation, it has been a challenge to dissect those that play a role in stretch-induced smooth muscle differentiation.
known that depolarization of arterial smooth muscle results in activation of the Rho/Rho kinase pathway and calcium sensitization (14). In addition, work by Wamhoff et al. (15) revealed that calcium influx via L-type calcium channels promotes Rho activation, myocardin expression, and smooth muscle cell differentiation. We later demonstrated that inhibition of L-type calcium channels prevented stretch-induced contractile differentiation in vascular smooth muscle (12).

In recent work, we have shown that Dicer-dependent microRNAs (miRNAs) are important regulators of smooth muscle development, differentiation, and function (16–18). MicroRNAs are small noncoding RNAs that participate in the regulation of gene and protein expression by interaction with the 3′-UTR of the target mRNA. This interaction results in mRNA degradation and/or inhibition/activation of protein translation (19). Dicer is critical for biogenesis of most miRNAs, and deletion of Dicer in smooth muscle results in embryonic lethality due to widespread hemorrhaging, loss of vascular smooth muscle contractile function, and reduced smooth muscle cell proliferation (18). Reduced contractile differentiation and cell number were similarly evident in mice in which Dicer was postnatally deleted in smooth muscle (16). In these mice, we also demonstrated a severe decrease in blood pressure. Overexpression of a single miRNA, miR-145, could rescue the effect of Dicer KO on smooth muscle cell differentiation, an effect that was mimicked by overexpression of myocardin. Several groups have identified an important role of miR-145 in smooth muscle differentiation and function, and multiple mechanisms have been demonstrated including direct and indirect effects on myocardin expression (20, 21), angiotensin signaling (22), and actin polymerization (23). Our studies showed that actin polymerization could be rescued in Dicer KO smooth muscle cells by overexpression of miR-145 and that inhibition of actin polymerization prevented the effects of miR-145 on smooth muscle differentiation (18). As of yet, no reports have investigated the importance of miRNAs for stretch-induced contractile differentiation of vascular smooth muscle, and the mechanisms of miR-145-dependent actin polymerization are not completely defined. However, recent studies have identified specific miRNAs involved in stretch-dependent effects of skeletal muscle and airway smooth muscle, indicating that miRNAs may play a role in cellular mechanosensing (24, 25).

In this study, we used an inducible and smooth muscle-specific knock-out of Dicer to determine the role of miRNAs for mechanosensing in the vasculature. Stretch-induced effects were investigated in portal veins in organ culture, which enables long term studies of stretch-sensitive protein expression and signaling events in the intact vascular wall. Similar to mechanical stretch of arteries and isolated smooth muscle cells, stretch of the portal vein promotes contractile differentiation via Rho activation and cell growth via activation of phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways (4, 5, 10, 26–28). We found that stretch-induced contractile differentiation was dramatically reduced in Dicer KO portal veins and that this effect is likely due to miR-145-dependent regulation of L-type calcium channel expression.

**Experimental Procedures**

**Animals**—Male inducible and smooth muscle-specific Dicer KO mice (smooth muscle myosin heavy chain (SMMHC)-CreERT2/Dicer<sup>fl/fl</sup>) (29, 30) were injected intraperitoneally with 0.1 ml of tamoxifen (50 mg/kg/day) or vehicle (1:10 EtOH in sunflower oil) for 5 consecutive days at the age of 4 weeks to induce knock-out of Dicer in smooth muscle. All mice were euthanized and used for experiments at 9–10 weeks post-tamoxifen treatment. Vehicle-treated littermate mice were used as controls. We have shown previously that tamoxifen treatment alone does not affect vascular reactivity in Cre-negative mice at 5 or 10 weeks after injection (16). All experiments were approved by the Malmö/Lund animal ethics committee (M167-09).

**Organ Culture**—Portal veins were freed from fat and surrounding tissue and mounted on a hook in a test tube containing DMEM/Ham’s F-12 with 2% dialyzed FCS and 10 nM insulin as described (10). Vessels were stretched by attaching a 0.3-g gold weight at one end of the vessel. This corresponds to the optimal load for force development. The vessels were incubated in cell culture environment for up to 5 days. For protein synthesis measurements, the vessels were incubated in culture medium containing [35S]methionine during the last 24 h of a 5-day incubation.

**Cell Culture, Transfection, and Adenoviral Transduction**—Vascular smooth muscle cells were isolated from mouse aorta by enzymatic digestion as described previously (18). Passages 3–6 were used for experiments. Cells were transfected with commercially available miR-145 inhibitor (Dharmacon) using Oligofectamine transfection reagent (Invitrogen) as described previously (18). Myocardin was overexpressed using adenoviral transduction as described (18, 31). Briefly, cells were transduced using 100 multiplicity of infection Ad.Myocd or Ad.CMV-Null virus for 96 h in normal growth medium.

**Force Measurement**—Portal veins were mounted in a myograph (610M, Danish Myo Technology) in HEPES buffer (composed of 135.5 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 11.6 mM glucose, and 11.6 mM HEPES, pH 7.4) as described (4). Vessels were contracted twice with 120 mM KCl-containing HEPES buffer followed by 10 μM 5-HT. For evaluation, force was integrated over the 5-min stimulation period, and passive force was subtracted.

**qPCR**—Total RNA was isolated using an miRNeasy kit (Qiagen) according to the manufacturer’s instructions. Expression of miRNA and miRNA was determined using commercially available primers (Qiagen) and SYBR Green reagents (Qiagen). The RT-qPCR was performed using a StepOnePlus qPCR machine (Applied Biosystems).

**Western Blotting**—Standard protocols for Western blotting were used as described previously (3). Briefly, equal amounts of protein were loaded in each lane of Bio-Rad TGX Criterion gels. Proteins were then transferred using either wet transfer overnight or semidy transfer for 10 min using the Trans-Blot Turbo system (Bio-Rad). Proteins were detected using the fol-
lowing commercially available primary antibodies: desmin (catalog number 4024), tropomyosin (catalog number 3910), total and phospho-Akt (catalog numbers 9272 and 9271), total and phospho-ERK (catalog numbers 9102 and 9101), total and phospho-p70S6K (catalog numbers 9202 and 9234), and total focal adhesion kinase (catalog number 3285) (Cell Signaling Technology; 1:1000 dilution for all); calponin (catalog number ab46794; 1:1000), SM22 (catalog number ab14106; 1:2000), and myosin (catalog number ab22621; 1:500) (Abcam); α-actin (catalog number A5228; 1:2000) (Sigma); downstream regulatory element against modulator (catalog number 26762; 1:500) and total and phospho-cofilin-2 (catalog numbers 07-300 and 07-326; 1:500) (Upstate); phospho-focal adhesion kinase (catalog number 44624G; 1:500; Invitrogen); HSP90 (catalog number 60418; 1:1000; BD Transduction Laboratories); Ca²⁺/calmodulin-dependent protein kinase (CamK) II δ (catalog number MAB4176; 1:1000; R&D Systems); and Cav1.2 (catalog number ACC-003; 1:500; Alomone Labs). According to the manufacturer, the Cav1.2 antibody recognizes several splice variants of Cav1.2, which explains the occurrence of multiple bands. HRP-conjugated (catalog numbers 7074 and 7076; 1:5000; Cell Signaling Technology) or fluorescently labeled DyLight800 and DyLight680 secondary antibodies (catalog numbers 5257, 5366, 5470, and 5151; 1:5000; Cell Signaling Technology) or fluorescently labeled DyLight800 and DyLight680 secondary antibodies (catalog numbers 5257, 5366, 5470, and 5151; 1:5000; Cell Signaling Technology) were used, and images were acquired using the LI-COR Odyssey Fc instrument (LI-COR Biosciences).

**Autoradiography**—The synthesis rate of stretch-sensitive proteins was determined using autoradiography as described previously (3). Following electrophoresis, gels were silver-stained, dried, and exposed to film for 24 h at −80 °C. The film was developed, scanned, and analyzed using Quantity One software (Bio-Rad).

**Statistics**—Values are presented as mean ± S.E. unless otherwise stated. p values were calculated by Student’s t test or one-way analysis of variance followed by Bonferroni post hoc testing using GraphPad Prism 5 (GraphPad Software Inc.). p < 0.05 was considered statistically significant.

**RESULTS**

Reduced Stretch-induced Contractile Differentiation in Dicer KO Portal Veins—Tamoxifen treatment significantly reduces Dicer expression in vessels of the SMMHC-CreERT2/DicerKO mouse (16). However, because blood vessels also contain cell types other than smooth muscle, Dicer expression per se is not a reliable indicator of the knockdown efficiency in smooth muscle-specific Dicer KO mice. Therefore, we selected three miRNAs that are relatively specific for smooth muscle, miR-143, and miR-145, and compared the expression of these miRNAs in control and Dicer KO portal veins. The expression of these miRNAs was reduced by 98–99% in Dicer KO portal veins 10 weeks following tamoxifen treatment (Fig. 1A).

To determine the role of miRNAs in vascular mechanosensing, we incubated portal veins either stretched or unstrained in organ culture for 5 days with [35S]methionine present during the last 24 h. Stretch-sensitive protein synthesis in control and Dicer KO portal vein was then analyzed by autoradiography (Fig. 1B, right panel). Equal loading was confirmed by silver staining of the gel (Fig. 1B, left panel). We have in previous work identified several of the stretch-sensitive proteins in the portal vein that are seen on the one-dimensional gels by two-dimensional gel electrophoresis, spot excision, and mass spectrometry (10). As expected, the synthesis of these proteins was induced by stretch in control vessels (Fig. 1, B–G). A previously unidentified, highly stretch-sensitive, 18-kDa band is evaluated in Fig. 1H. Deletion of Dicer in smooth muscle significantly reduced or ablated the stretch-induced response (Fig. 1, B–H), indicating that miRNAs are essential for smooth muscle contractile differentiation in response to stretch. This result was confirmed by qPCR analysis of smooth muscle-specific genes (supplemental Fig. 1, A–F). The identity of the 18-kDa protein is likely to be myosin regulatory light chain as shown by the qPCR results in supplemental Fig. 1B. However, we cannot exclude the possibility that this band represents another ~18-kDa stretch-sensitive protein such as cofilin-2.

Although the total content of stretch-sensitive proteins is clearly reduced in Dicer KO compared with controls (Fig. 1B, Silver stain), we were not able to detect stretch-induced changes in the contents of these proteins using stained gels. To demonstrate that the increased synthesis rate also has an effect
on total protein content, we analyzed the expression of specific proteins by Western blotting. Although the effects were quite modest due to the slow turnover of contractile and cytoskeletal proteins, we were able to detect a significant stretch-dependent increase in the expression of several smooth muscle marker proteins in the control portal veins (Fig. 2, A–D). In accordance with the effects on protein synthesis, the stretch-sensitive protein expression was reduced or abolished in Dicer KO portal veins. Interestingly, expression of the transcription factor myocardin, which is essential for smooth muscle differentiation (32), was stretch-sensitive in control but not Dicer KO portal vein (Fig. 2E). Thus, reduced stretch-sensitive smooth muscle differentiation in Dicer KO mice may originate in part from a loss of stretch-dependent myocardin expression.

**Reduced L-type Calcium Channel Expression in Dicer KO Portal Veins**—We and others have shown previously that myocardin expression and smooth muscle cell differentiation in vascular smooth muscle depend on L-type calcium channels and downstream activation of the Rho/Rho kinase pathway (12, 15). In addition to activating myocardin expression, Rho kinase may also promote smooth muscle differentiation independently of myocardin via cofilin-2 phosphorylation, actin polymerization, and nuclear translocation of myocardin-related transcription factors (10, 11). To assess whether loss of stretch sensitivity in Dicer KO portal veins could be due to a decreased expression of L-type calcium channels, we analyzed Cav1.2 expression in control and Dicer KO portal veins following 5 days of stretch. Interestingly, basal and stretch-sensitive expressions of Cav1.2 were both reduced in Dicer KO portal veins compared with control vessels (Fig. 3A). Reduced expression was also confirmed at the mRNA level (see below).

Reduced voltage-gated calcium entry is expected to affect smooth muscle contractility. Therefore, we mounted portal veins in a myograph and induced contraction by depolarization with 120 mM KCl and by the contractile agonist 5-HT (10 μM). Spontaneous activity, which is readily observed in portal veins, was also analyzed. As shown in Fig. 3, B–E, KCl-induced contractions and spontaneous activity were dramatically reduced in Dicer KO vessels, whereas 5-HT-induced contraction was largely maintained. To assess the role of L-type calcium channels in the separate contractile responses, we investigated their sensitivity to the L-type calcium channel blocker nifedipine. In wild-type portal veins, nifedipine (0.3 μM) effectively inhibited KCl-induced contractions and spontaneous activity but only weakly inhibited 5-HT-induced contraction, indicating that the 5-HT response is relatively independent of voltage-gated calcium influx in the portal vein (Fig. 3, F–H).

**Effects of Dicer KO on Long Term and Acute Signaling Events in Portal Vein Correlate with Effects of L-type Calcium Channel Blockers**—In earlier reports, we have demonstrated that long term stretch-induced Rho/Rho kinase activation results in phosphorylation and inhibition of the downstream actin-depolymerizing factor cofilin-2 (3, 4, 10). This effect is highly sensitive to inhibition of L-type calcium channels by verapamil (12). An increase in cofilin-2 phosphorylation results in stabilization of actin filaments, which promotes smooth muscle differentiation by releasing G-actin binding and enabling nuclear translocation of myocardin-related transcription factor (11). Conversely, acute signaling events, which include ERK1/2 in the MAPK pathway, are insensitive to verapamil (12). Thus, if the effects of Dicer KO are mainly a result of reduced L-type calcium channel expression, only long term and not acute signaling events would be sensitive to Dicer KO in the portal vein. To test this hypothesis, we incubated portal veins for 5 days with and without stretch and determined the phosphorylation of cofilin-2 using phosphospecific antibodies and Western blot.
accordance with the effects of verapamil, deletion of Dicer prevented stretch-induced cofilin-2 phosphorylation (Fig. 4A) when the total cofilin level was used for normalization. As shown in Figs. 2D and 4A, total cofilin-2 expression is highly stretch-sensitive in WT, but not Dicer KO, vessels. Comparison with the stable loading control HSP90 confirmed reduced cofilin-2 phosphorylation in KO vessels (Fig. 4B). On the other hand, acute (5 min) stretch-induced activation of ERK1/2 was unaffected by Dicer KO (Fig. 4C), consistent with the idea that loss of stretch sensitivity in Dicer KO smooth muscle is due to reduced expression of L-type calcium channels. In addition, we found that other acute signaling events including Akt, focal adhesion kinase, and p70S6K phosphorylation were largely maintained in Dicer KO smooth muscle (supplemental Fig. 2, A–C).

Transcriptional Regulation Downstream of Actin Polymerization Is Unaffected in Dicer KO Smooth Muscle—To bypass the Rho/cofilin-2 pathway and directly promote stabilization of actin filaments, we incubated control and Dicer KO smooth muscle cells from the aorta with jasplakinolide for 24 h. Jasplakinolide treatment induced the mRNA expression of smooth muscle-specific genes Cnn1 (calponin), Tagln (SM22), and Myh11 (myosin heavy chain) in control and Dicer KO smooth muscle compared with untreated cells (Fig. 5, A–C). The induction of Cnn1 and Tagln was comparable in control and Dicer KO cells, whereas the induction of Myh11 was augmented in Dicer KO. This result indicates that the effects of Dicer KO reside upstream of actin polymerization and that transcriptional regulation downstream of actin polymerization is unaffected. Of note, the expression of myocardin was not affected by jasplakinolide in either control or Dicer KO cells, arguing that the transcriptional regulation of myocardin is independent of actin polymerization (Fig. 5D).

L-type Calcium Channel Expression Is Regulated by miR-145 and CamKIIβ—We aimed to specify the role of a specific miRNA in smooth muscle mechanosensing to further explain the effects observed in Dicer KO mice. Generally, miRNAs inhibit protein translation, although exceptions to this rule have been reported (33, 34). In silico analysis suggests CamKIIβ as a target of miR-145, and a recent study validated CamKIIβ experimentally as a direct target for miR-145 (20). Furthermore, in a recent study, Ronkainen et al. (35) demonstrated that CamKIIβ negatively regulates the gene (Cacna1c) and protein (Cav1.2) expression of the α1c-subunit of the L-type Ca2+ channel in cardiomyocytes by promoting nuclear translocation of the transcriptional repressor KChIP3/Calsenilin/DREAM. Therefore, we analyzed expression of CamKIIβ and DREAM in control and Dicer KO portal veins and found a significant increase of CamKIIβ protein in Dicer KO vessels (Fig. 6A). The expression of DREAM tended to be increased in Dicer KO vessels, but this difference did not reach statistical significance (Fig. 6B). Moreover, transfection of a miR-145 inhibitor for 96 h eliminated miR-145 without effects on miR-21 (Fig. 6C) and reduced the expression of Cacna1c by ∼50% in control cells (Fig. 6D). The latter effect was completely absent in the presence of the CamKII inhibitor KN93 (0.5 μM) (Fig. 6E). Because miR-145 is known to promote contractile differentiation by regulating myocardin expression, we also tested whether myocardin was involved in the regulation of Cacna1c. Adenovirus-mediated overexpression of myocardin caused a dramatic increase in the expression of smooth muscle markers (supplemental Fig. 3). However, no significant effect of myocardin on the expression of Cacna1c (Fig. 6F) was observed. Because the reduction in Cacna1c expression after inhibition of miR-145 matches the reduction of Cacna1c expression in Dicer KO portal veins (Fig. 6, compare D and G), it seems likely that miR-145 is a key player in the reduced expression of L-type calcium channels and the subsequent loss of smooth muscle mechanosensing in Dicer KO vessels.

DISCUSSION

MicroRNAs have emerged as key players in a number of biological processes including vascular smooth muscle develop-
Induced Rho activation is dependent on Pyk2/PDZ-RhoGEF in but a recent report by Ying...ation by L-type calcium influx is not completely understood, marker expression. The regulation of myocardin and smooth muscle differentiation in the portal vein, indicating that stretch-induced growth and differentiation are differentially regulated by miRNAs. Moreover, we found that the effects in Dicer KO vessels could be explained by a reduction in L-type calcium channels consequent to loss of miR-145 and up-regulation of CamKIIα (Fig. 6H).

Smooth muscle contractile differentiation and myocardin expression in the portal vein are dependent on calcium influx via L-type calcium channels and downstream activation of the Rho/Rho kinase pathway and actin polymerization (4, 6, 10, 12). It is well known that stretch promotes the activity of voltage-dependent calcium channels (36), but to our knowledge, stretch-sensitive regulation of calcium channel expression has not been demonstrated previously. In this study, we found that long term stretch promotes the expression of L-type calcium channels in vascular smooth muscle cells and that this effect is dependent on miRNAs, which is in accordance with the effects of stretch on myocardin and smooth muscle marker expression.

The regulation of myocardin and smooth muscle differentiation by L-type calcium influx is not completely understood, but a recent report by Ying et al. (37) suggests that calcium-induced Rho activation is dependent on Pyk2/PDZ-RhoGEF in...vascular smooth muscle cells. Downstream of Rho activation, transcriptional regulation of myocardin may differ from other contractile and cytoskeletal proteins that are considered to be smooth muscle markers. Although myocardin and contractile proteins are dependent on the transcription factor serum response factor (38), we have demonstrated herein that myocardin gene expression is insensitive to agents that stabilize actin filaments. Moreover, in addition to serum response factor, transcriptional regulation of myocardin has been suggested to rely on transcription factors such as Mef2, Tead, and Foxo (13), and we have reported previously that KCl-induced myocardin expression requires Mef2 in portal veins (12). Thus, it is possible that smooth muscle differentiation via L-type calcium channels is regulated both via actin polymerization and myocardin-related transcription factor nuclear translocation and via Rho kinase/Mef2-dependent transcription of myocardin.

The expression of L-type calcium channels is known to correlate with the differentiated state of smooth muscle cells (39), and elevated expression of L-type calcium channels has been suggested to be an important factor for maintaining smooth muscle differentiation via activation of the Rho/Rho kinase pathway (40). Accordingly, we found that the basal expression of L-type calcium channels was decreased in Dicer KO portal veins. Two lines of evidence indicate that loss of L-type channels is responsible for the effects on smooth muscle contractility and the ablated stretch-induced differentiation and cofilin-2 phosphorylation in Dicer KO portal veins. First, stabilization of actin filaments with jasplakinolide, thus bypassing the membrane activation step, led to a similarly increased expression of contractile markers in control and Dicer KO cells. Second,
pharmacological inhibition of L-type channels mimicked the effect of Dicer deletion on vessel contractility (present study) and on differentiation marker expression and cofilin-2 phosphorylation (12). We speculate that decreased expression of L-type calcium channels in Dicer KO smooth muscle prevents stretch-induced smooth muscle differentiation both through inhibition of myocardin expression and through inhibition of actin polymerization via reduced activation of the Rho pathway (Fig. 6H).

Expression of L-type calcium channels has been suggested to be repressed by CamKIIδ in cardiomyocytes (35, 41). CamKIIδ is regulated by calcium/calmodulin, and findings in smooth muscle have demonstrated a calcium dependence in the long term control of calcium current (42). This is consistent with the idea that a similar CamKIIδ-dependent regulation exists in smooth muscle. Moreover, CamKIIδ is a confirmed target of the most highly expressed miRNA in differentiated smooth muscle, miR-145 (20). Accordingly, Dicer KO portal veins exhibited an increased expression of CamKIIδ. We also found that inhibition of miR-145 reduced the expression of L-type calcium channels to the same extent as deletion of Dicer did and that this effect was dependent on CamKII activity. However, L-type calcium channel expression was independent of myocardin. Loss of miR-145 and up-regulation of CamKIIδ may thus be sufficient to explain the reduced L-type calcium channel expression in Dicer KO smooth muscle.

Similar to down-regulation of miR-145 and L-type calcium channels, up-regulation of CamKIIδ is a prominent observation in phenotypically modulated smooth muscle cells in vivo and in vitro (43–45). Quiescent smooth muscle cells in vivo mainly express the CamKIIγ isoform, but a switch to the CamKIIδ isoform occurs in proliferating smooth muscle upon vascular injury or in cell culture (44, 45). Importantly, silencing or knock-out of CamKIIδ decreases smooth muscle cell proliferation in vitro (43, 45) and neointima formation in vivo (43, 44). The mechanisms underlying the CamKII isoform switch are so far not well understood. It is possible that down-regulation of miR-145 may be involved, but it is still not clear whether this is the triggering factor resulting in phenotypic modulation of smooth muscle cells in vascular disease. In this work, we have shown that miRNA expression is essential for stretch-induced contractile differentiation of vascular smooth muscle. However, we have not yet investigated the effects of stretch or pressure on miRNA expression in the vascular wall. Further studies are therefore warranted in this area.

Herein, we have shown that miRNAs are crucial for stretch-sensitive contractile differentiation in the vascular wall via miR-145-dependent expression of L-type calcium channels. These findings may have implications for our understanding of phenotypic modulation of smooth muscle cells in vascular disease.

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