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MicroRNAs are essential for stretch-induced vascular smooth muscle contractile differentiation via miR-145-dependent expression of L-type calcium channels*

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*Running title: Stretch-induced VSMC differentiation is dependent on miRNAs

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Keywords: microRNA, vascular smooth muscle, differentiation, mechanosensing, calcium

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 phenotype modulation in vascular disease

SUMMARY

Stretch of the vascular wall is an important stimulus to maintain smooth muscle contractile differentiation, 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 and Rho-dependent Cofilin-2 phosphorylation was dramatically reduced in Dicer KO vessels. On the other hand, acute stretch-sensitive growth signaling, which is independent of L-type calcium influx, 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 expression 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 CaMKII inhibitor KN93, suggesting that CamKII δ , 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 (1). 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 (1).

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 (MRTF)², 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). Since 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. It is well 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. revealed that calcium influx via L-type calcium channels promotes Rho-activation, myocardin expression and smooth muscle cell differentiation (15). 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 where 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) or 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. Recent studies have however 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 have 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 arteries and isolated smooth muscle cells, mechanical stretch of the portal vein promotes contractile differentiation via Rho activation and cell growth via activation of PI3-kinase and MAP-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 (SMMHC-CreERT2/Dicer^{fl/fl}) (29,30) were injected intraperitoneally with 0.1 ml 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 previously shown 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/Hams F12 with 2% dialyzed FCS and 10nM insulin as described (10). Vessels were stretched by attaching a 0.3g 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 media containing [³⁵S]-methionine during the last 24h 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 moi Ad.Myocd or Ad.CMV-Null virus for 96 hours in normal growth media.

Force measurement - Portal veins were mounted in a myograph (610M, Danish Myo Technology) in HEPES buffer [composed of (in mM) 135.5 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 11.6 glucose, and 11.6 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 miRNeasy kit (Qiagen) according to the manufacturer's instructions. mRNA and miRNA expression was determined using commercially

available primers (Qiagen) and Sybr-green reagents (Qiagen). The RT/qPCR reaction was performed using a StepOnePlus qPCR machine (ABI).

Western Blotting - Standard protocols for western blotting was used as described previously (3). Briefly, equal amounts of protein were loaded in each lane of Biorad TGX Criterion gels. Proteins were then transferred using either wet transfer over-night or semi-dry transfer for 10 min using the Trans-Blot Turbo system (Biorad). Proteins were detected using commercially available primary antibodies: Desmin (#4024), Tropomyosin (#3910), total- and phospho-Akt (#9272, #9271) total- and phospho-ERK (#9102, #9101), total- and phospho-P70S6K (#9202, #9234) and total-focal adhesion kinase (FAK) (#3285) (Cell Signaling, 1:1000 dilution for all); Calponin (#ab46794, 1:1000), SM22 (#ab14106, 1:2000) and Myocardin (#ab22621, 1:500) (Abcam), α-actin (#A5228, 1:2000) (Sigma), DREAM (#26762, 1:500) and total- and phospho-Cofilin-2 (#07-300, #07-326, 1:500) (Upstate), phospho-FAK (#44624G, 1:500, Invitrogen), HSP90 (#610418, 1:1000, BD Transduction labs.), Ca²⁺/calmodulin-dependent protein kinase CamKIIδ (#MAB4176, 1:1000, R&D Systems), and Cav1.2 (#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 (#7074, #7076 1:5000, Cell Signaling) or fluorescently labeled DyLight800 and DyLight680 secondary antibodies (#5257, #5366, #5470, #5151, 1:5000 Cell Signaling) 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 24h at -80 °C. The film was developed, scanned and analyzed using Quantity One software (Biorad).

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 smooth muscle myosin heavy chain (SMMHC)-CreERT2/Dicer^{fl/fl} mouse (16). However, since 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-1, 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).

In order to determine the role of miRNAs in vascular mechanosensing we incubated portal veins either stretched or unstretched in organ culture for 5 days with [³⁵S]-methionine present during the final 24 hours. 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 1D-gels by 2D-gel electrophoresis, spot excision and mass spectrometry (10). As expected, the synthesis of these proteins was induced by stretch in control vessels (Fig. 1B-G). A previously unidentified, highly stretch-sensitive, 18 kDa band (18kDa) is evaluated in Fig 1H. Deletion of Dicer in smooth muscle significantly reduced or ablated the stretch-induced response (Fig. 1B-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 (Suppl. Fig. 1A-F). The identity of the 18kDa protein is likely to be myosin regulatory light chain as shown by the qPCR results in Suppl. Fig 1B. However, we cannot exclude the possibility that this band represents another ~18kDa stretch-sensitive protein, such as Cofilin-2.

Although the total content of stretch-sensitive proteins is clearly reduced in Dicer KO compared to controls (Fig. 1B, silver stain), we were not able to detect stretch-induced changes in the contents of these proteins using stained gels. In order 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. 2A-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 in part originate from a loss of stretch-dependent myocardin expression.

Reduced L-type calcium channel expression in Dicer KO portal veins - We and others have previously shown 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 MRTFs (10,11). To assess if 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 to 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. We therefore mounted portal veins in a myograph and induced contraction by depolarization with 120 mM KCl and by the contractile agonist 5-HT

(10 μ M). Spontaneous activity, which is readily observed in portal veins, was also analyzed. As shown in Fig. 3B-E, KCl-induced contractions and spontaneous activity were dramatically reduced in Dicer KO vessels while 5-HT-induced contraction was largely maintained. In order 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 μ M) 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).

The effects of Dicer KO on long-term and acute signaling events in portal vein correlate with the effects of L-type calcium channel blockers - We have in earlier reports 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 the transcription factor MRTF (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 phospho-specific antibodies and western blot. In 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-, FAK- and P70S6K-phosphorylation were largely maintained in Dicer KO smooth muscle (Suppl. Fig 2A-C).

Transcriptional regulation downstream of actin polymerization is unaffected in Dicer KO smooth muscle - In order to by-pass 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 hours. Jasplakinolide treatment induced the mRNA expression of smooth muscle specific genes *Cnn1* (Calponin) and *Tagln* (SM22) and *Myh11* (Myosin heavy chain) in control and Dicer KO smooth muscle compared to untreated cells (Fig. 5A-C). The induction of *Cnn1* and *Tagln* was comparable in control and Dicer KO cells while 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 CamKII δ - 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 CamKII δ as a target of miR-145, and a recent study validated CamKII δ experimentally as a direct target for miR-145 (20). Furthermore, in a recent study, Ronkainen et al. demonstrated that CamKII δ negatively regulates the gene (*Cacnal1c*) and protein (Cav1.2) expression of the α 1c-subunit of the L-type Ca²⁺ channel in cardiomyocytes by promoting nuclear translocation of the transcriptional repressor KChIP3/Calsenilin/DREAM (35). We therefore analyzed expression of CamKII δ and DREAM in control and Dicer KO portal veins and found a

significant increase of CamKII δ 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 (B). Moreover, transfection of a miR-145 inhibitor for 96 hours eliminated miR-145 without effects on miR-21 (Fig. 6C), and reduced the expression of *Cacnalc* by approximately 50% in control cells (Fig. 6D). The latter effect was completely absent in the presence of the CamKII inhibitor KN93 (0.5 μ M) (Fig 6E). Since miR-145 is known to promote contractile differentiation by regulating Myocardin expression we also tested if Myocardin was involved in the regulation of *Cacnalc*. Adenoviral-mediated overexpression of Myocardin caused a dramatic increase in the expression of smooth muscle markers (Suppl. Fig. 3). However, no significant effect of Myocardin on the expression of *Cacnalc* (Fig 6F) was observed. Because the reduction in *Cacnalc* expression after inhibition of miR-145 matches the reduction of *Cacnalc* expression in Dicer KO portal veins (compare Fig. 6D 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 development and contractile differentiation. Our goal in this study was to clarify the role of miRNAs for mechanosensing in the vascular wall. To this end, we have demonstrated that Dicer-dependent smooth muscle miRNAs are essential for stretch-dependent myocardin expression and contractile differentiation in the portal vein. However, acute, stretch-sensitive MAPK-signaling, which is important for the growth response in stretched vessels (2), was not affected by depletion of Dicer indicating that stretch-induced growth and differentiation are differentially regulated by miRNAs. We moreover 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 CamKII δ (Figure 6H).

Smooth muscle contractile differentiation and myocardin expression in the portal vein is

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 previously been demonstrated. In this study we find that long term stretch promotes the expression of L-type calcium channels in vascular smooth muscle 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. suggests that calcium-induced Rho-activation is dependent on Pyk2/PDZ-RhoGEF in vascular smooth muscle cells (37). 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 (SRF) (38), we have demonstrated herein that myocardin gene expression is insensitive to agents that stabilize actin filaments. Moreover, in addition to SRF, transcriptional regulation of myocardin has been suggested to rely on transcription factors such as Mef2, Tead and Foxo (13) and we have previously reported that KCl-induced myocardin expression requires Mef2 in portal veins (12). It is thus possible that smooth muscle differentiation via L-type calcium channels is regulated both via actin polymerization and MRTF 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 by-passing 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 CamKII δ in cardiomyocytes (35,41). CamKII δ 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 CamKII δ dependent regulation exists in smooth muscle. Moreover, CamKII δ 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 CamKII δ . 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 upregulation of CamKII δ 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 CamKII δ 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 CamKII γ isoform, but a switch to the CamKII δ isoform occurs in proliferating smooth muscle upon vascular injury or in cell culture (44,45). Importantly, silencing or knockout of CamKII δ 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 if this is the triggering factor resulting in phenotypic modulation of smooth muscle cells in vascular disease. 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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FOOTNOTES

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²The abbreviations used are: MRTF, myocardin related transcription factors, CamKII δ , Ca²⁺/calmodulin-dependent protein kinase; SRF, serum response factor; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase, MHC, myosin heavy chain

FIGURE LEGENDS

FIGURE 1. Stretch-induced synthesis of contractile proteins is reduced in Dicer KO portal veins. (A) miRNAs that are known to be highly expressed and relatively specific for smooth muscle were analyzed in Ctrl and Dicer KO portal veins using qPCR. (B) Portal veins from Ctrl and Dicer KO mice were incubated in organ culture with or without stretch for 5 days, with ³⁵S-methionine present during the last 24h. Proteins were separated by SDS-PAGE and the total amount of proteins visualized with silver staining, (left panel). ³⁵S-methionine incorporation was visualized by autoradiography (right panel). Stretch-sensitive contractile proteins are indicated. Summarized data of relative synthesis rates are shown in C-H. MHC: Myosin heavy chain, Tropomyo: Tropomyosin. (n=3)

FIGURE 2. Blunted response to stretch at the protein level in Dicer KO portal veins. Portal veins from Ctrl and Dicer KO mice were incubated in organ culture with or without stretch for 5 days. Protein expression was then analyzed by western blotting using antibodies against SM22, Tropomyosin (Tropo) Calponin, Cofilin-2 (T-Cof) and Myocardin. Expression levels were normalized to the loading control HSP90. A-G shows summarized data and representative blots (n=4-6).

FIGURE 3. Loss of contractility in Dicer KO portal veins is mimicked by the L-type calcium channel blocker nifedipine. (A) Summarized data of the relative protein expression of Cav1.2 in control and Dicer KO portal veins incubated in organ culture with or without stretch for 5 days is shown together with a representative western blot. (B) Isometric contractile force was evaluated in control and Dicer KO portal veins mounted in a myograph. Representative recordings of KCl-induced contraction (left panels), Spontaneous activity (middle panels) and 5-HT-induced force (right panels) are shown. C-E show summarized data of contractile force in response to KCl, spontaneous activity and 5-HT in control and Dicer KO portal veins. In separate experiments, the effects of nifedipine on spontaneous activity and the contractile responses to KCl and 5-HT were evaluated (F-H) (n=3-5).

FIGURE 4. Stretch-induced Cofilin-2 phosphorylation is abolished in Dicer KO portal veins. Portal veins from Ctrl and Dicer KO mice were stretched in organ culture for 5 days. Phosphorylation of Cofilin-2 (Ser3) was used as a marker of activation of the Rho-signaling pathway. Cofilin-2 was determined using phospho-specific antibodies and western blotting. In A, the summarized ratio of phosphorylated versus total Cofilin-2 is shown together with a representative western blot. Since the total level of Cofilin-2 is highly stretch-sensitive, phospho-Cofilin-2 was also normalized to the loading HSP90 (B). Erk1/2 phosphorylation was analyzed in portal veins from Ctrl and Dicer KO mice in control conditions and after 5min of stretch (C) (n=4-6)

FIGURE 5. Stabilization of actin filaments induces expression of contractile proteins but not myocardin in both control and Dicer KO smooth muscle cells. Aortic smooth muscle cells were incubated with 0-100nM jasplakinolide for 24h. mRNA expression of *Tagln* (SM22), *Cnn1* (Calponin), *Myh11* (Myosin heavy chain) and *Myocd* (Myocardin) was then analyzed by qPCR and normalized to the respective untreated sample (A-D) (n=4).

FIGURE 6. miR-145 regulates the expression of L-type calcium channels Summarized data and representative western blots of CamKII δ and DREAM/Calsenilin expression in control and Dicer KO portal veins are shown in A and B respectively. In C-E, aortic smooth muscle cells were transfected with negative control (NC) or miR-145 inhibitor (miR-145i) for 96 hours. The expression of miR-145 and miR-21 was analyzed by qPCR in C, while D and E shows relative mRNA expression of the L-type calcium channel α_{1c} -subunit (*Cacnalc*) after miR-145 inhibition in the absence or presence of 0.5 μ M KN93, respectively. *Cacnalc* expression was also analyzed in isolated smooth muscle cells following overexpression of Myocardin (Myocd) by adenoviral transduction (F) and in control and Dicer KO portal veins (G) (n=4-9). (H) Tentative model of how stretch-induced contractile differentiation may be regulated via miRNAs and L-type calcium channels (LTCC) in portal vein. This model represents our findings in portal vein and does not exclude any alternative mechanisms of miR-145 in smooth muscle.

Figure 1

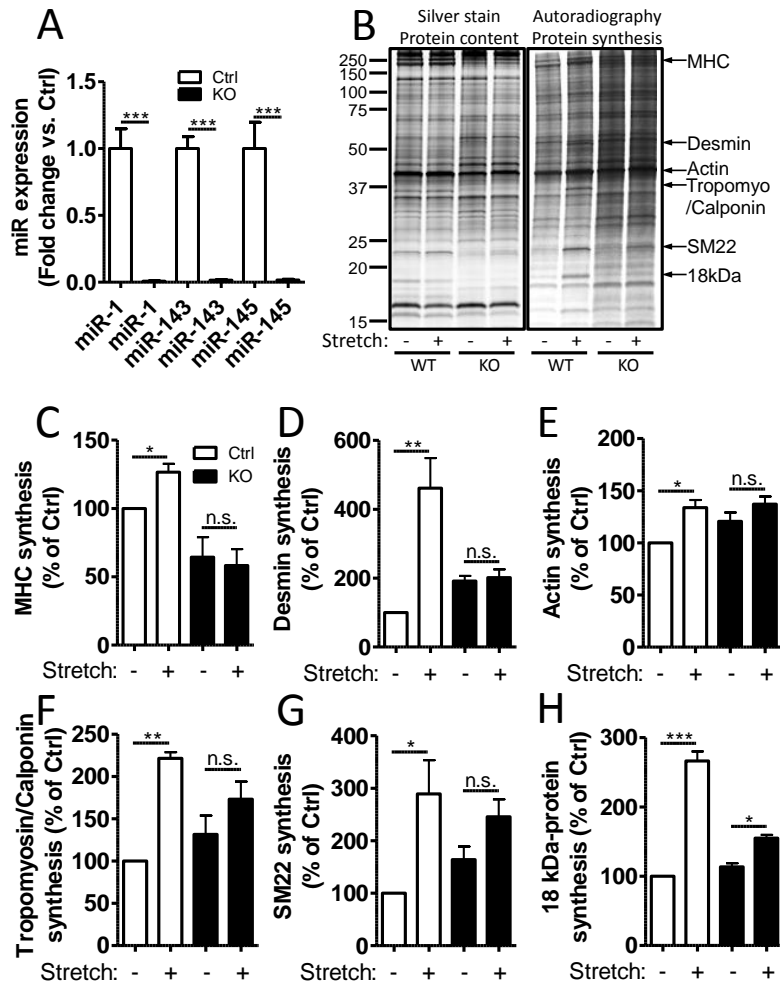


Figure 2

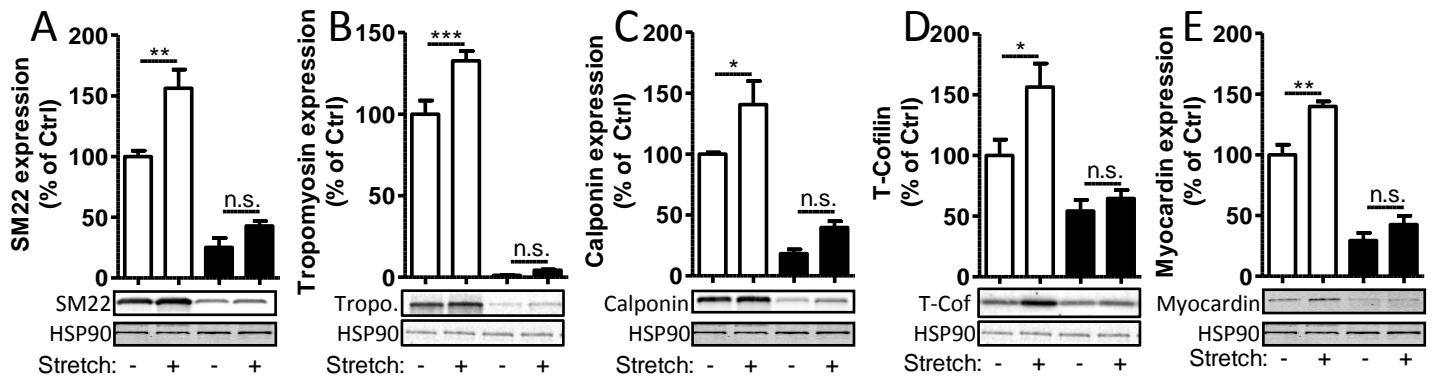


Figure 3

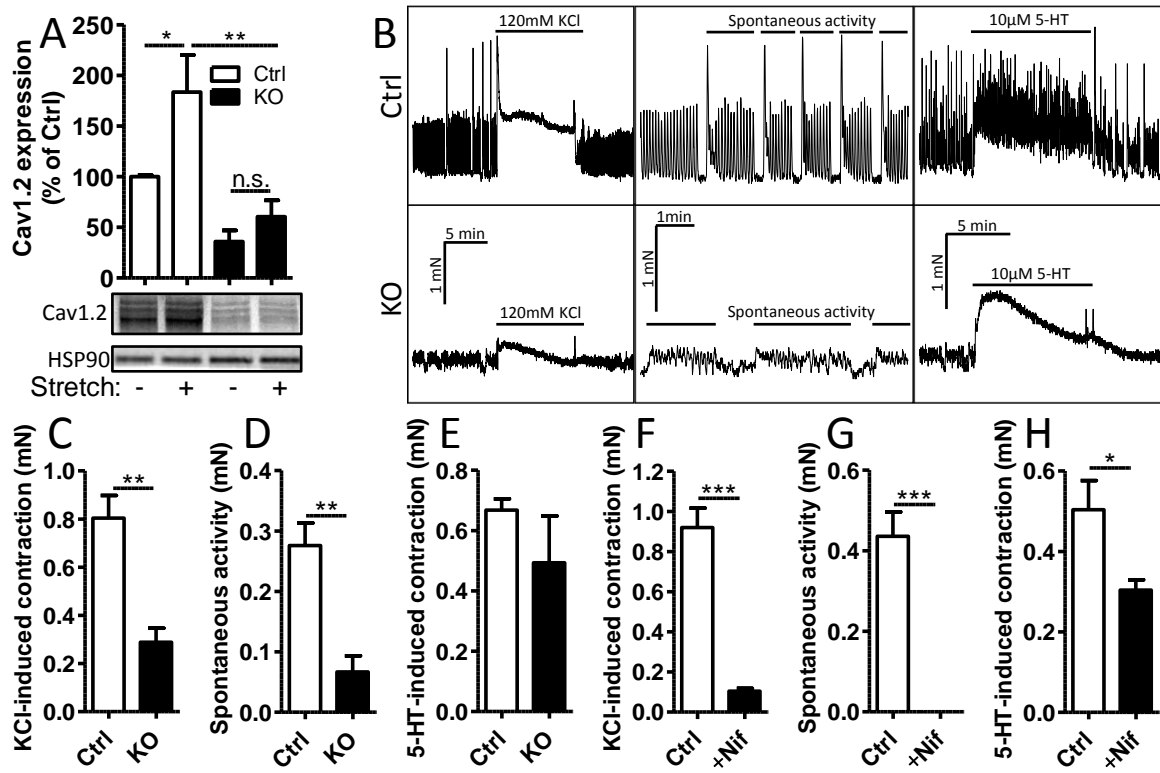


Figure 4

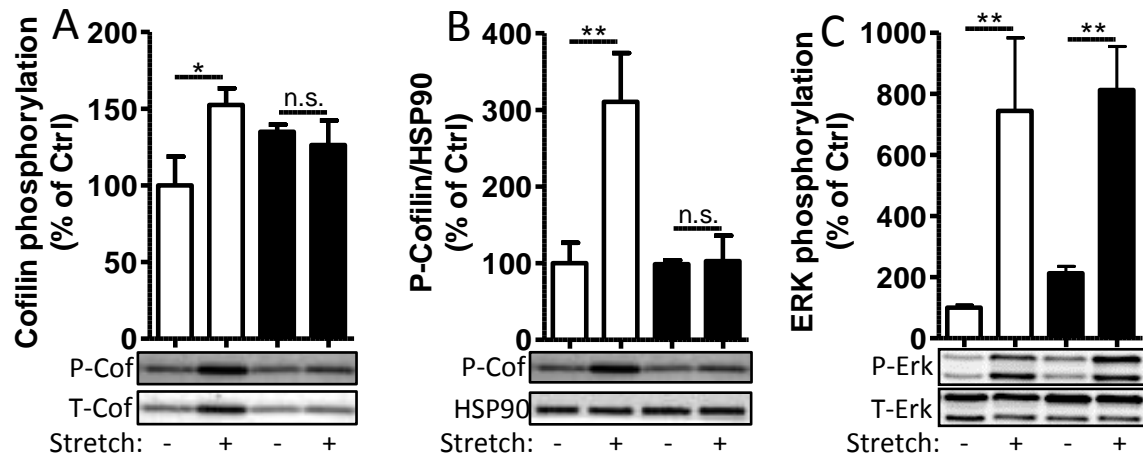


Figure 5

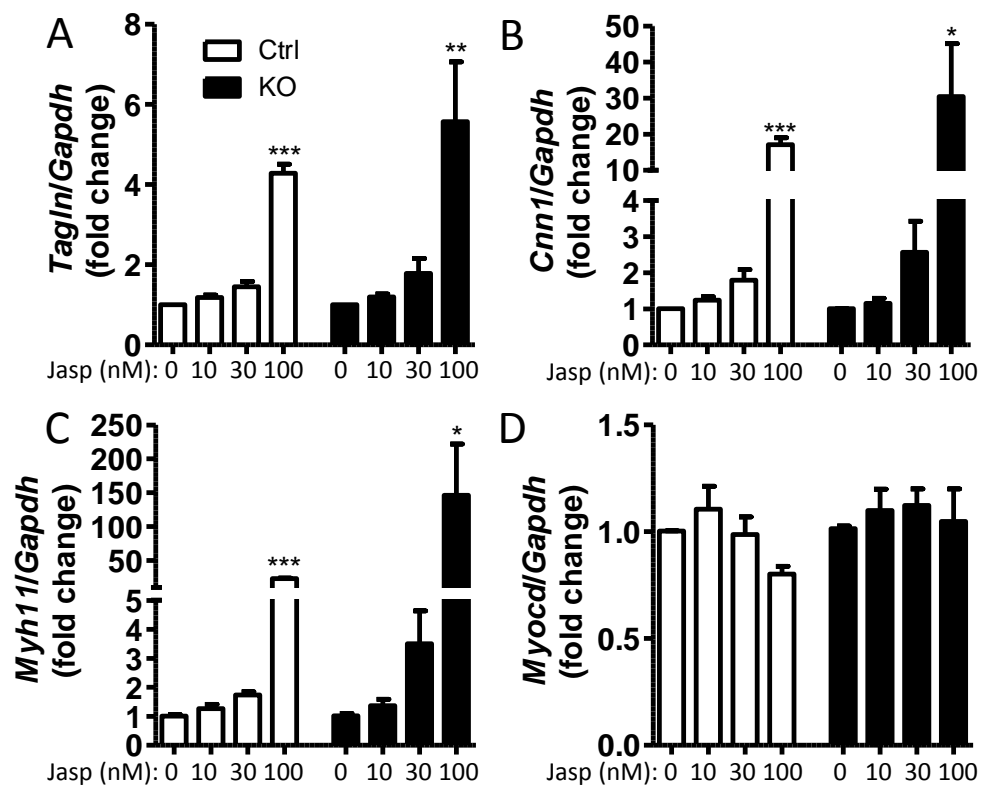
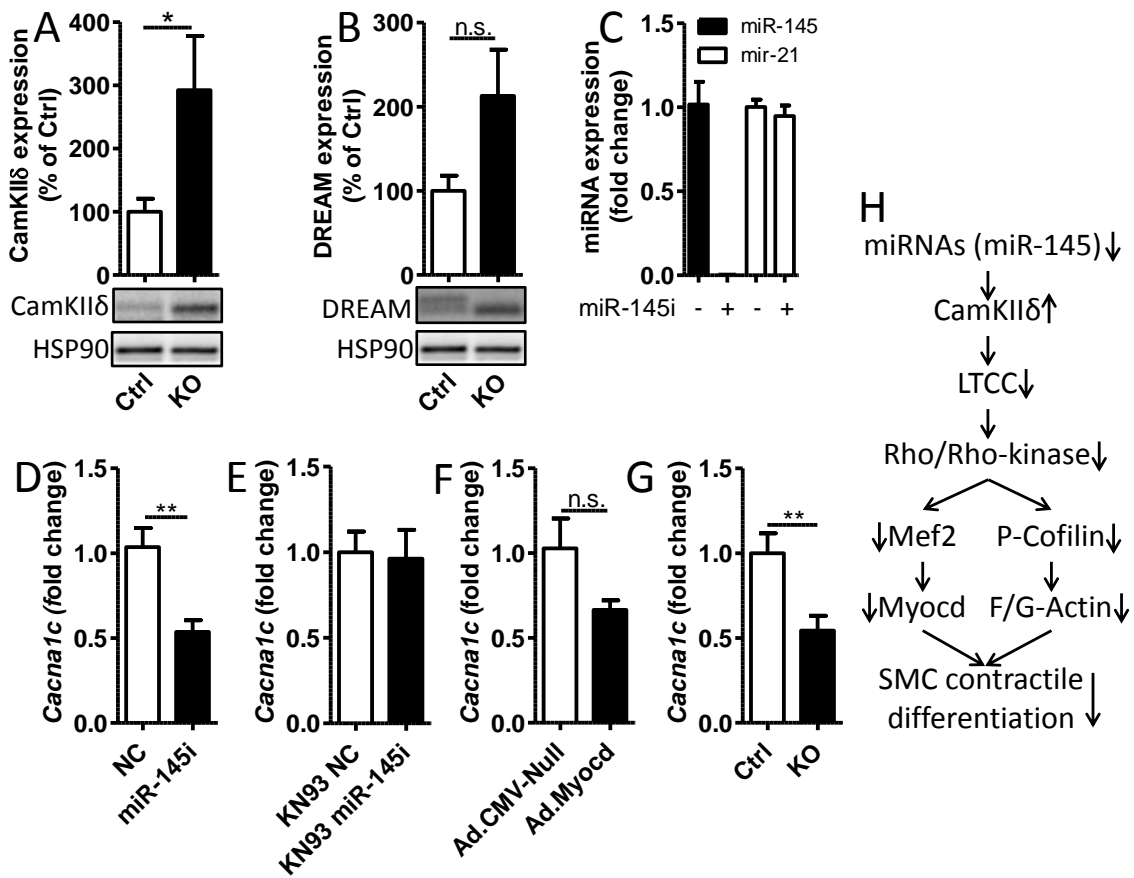


Figure 6

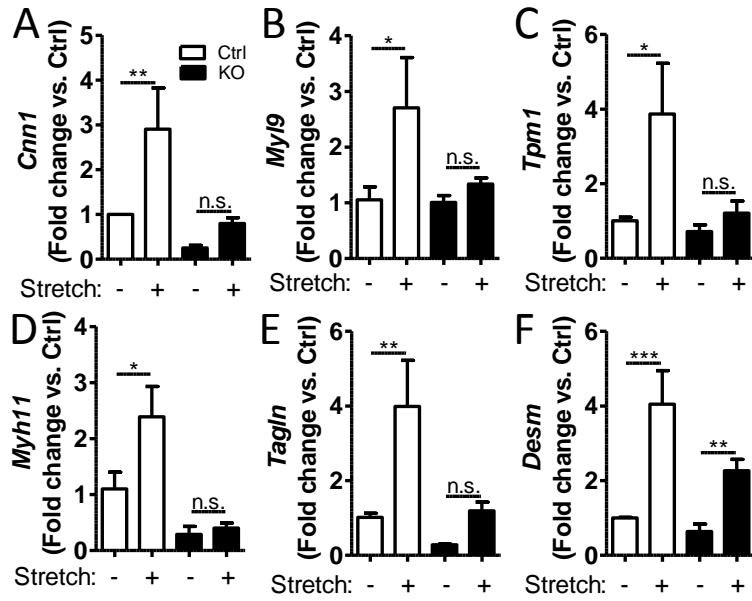


SUPPLEMENTARY FIGURE 1. Stretch-induced expression of SMC specific genes is reduced in Dicer KO portal veins. (A -F) Portal veins from Ctrl and Dicer KO mice were incubated in organ culture with or without stretch for 72h. SMC marker gene expression was analyzed using qPCR. The genes encode the following proteins: *Cnn1*: Calponin, *Myl9*: Myosin regulatory light chain, *Tpm1*: Tropomyosin, *Myh11*: Myosin heavy chain, *Tagln*: SM22, *Desm*: Desmin. (n=3-7)

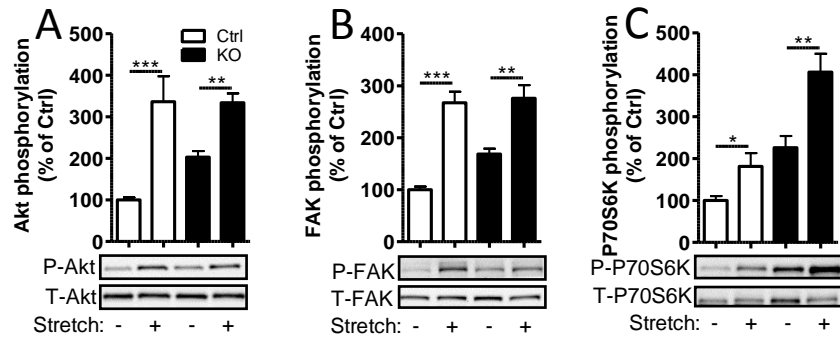
SUPPLEMENTARY FIGURE 2. Acute signaling events in response to stretch are maintained in Dicer KO portal veins. Portal veins from Ctrl and Dicer KO mice were equilibrated unstretched in organ culture for 24h and then stretched for 5min or left unstretched. Activation of pathways involved in stretch-induced growth and contractile differentiation were determined using phospho-specific antibodies. The relative difference in the signal intensity ratio of phospho- vs. total was analyzed and summarized in A-C together with representative blots. FAK: Focal adhesion kinase. n=3-6

SUPPLEMENTARY FIGURE 3. Overexpression of Myocardin promotes contractile differentiation in vascular smooth muscle cells. Wild type aortic smooth muscle cells were transduced with Ad.Myocd or control virus and analyzed by qPCR after 96h. Summarized data of the effect of Myocardin overexpression on the levels of *Cnn1* (Calponin) and *Myh11* (Myosin heavy chain) mRNA are shown in A and B, respectively (n=3).

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

