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**Expression of microRNAs is essential for arterial myogenic tone and pressure-induced  
activation of the PI3-kinase/Akt pathway**

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## **Abstract**

**Aim:** The myogenic response is the intrinsic ability of small arteries to constrict in response to increased intraluminal pressure. Although microRNAs have been shown to play a role in vascular smooth muscle function, their importance in the regulation of the myogenic response is not known. In this study, we investigate the role of microRNAs in the regulation of myogenic tone by using smooth muscle-specific and tamoxifen-inducible deletion of the endonuclease Dicer in mice.

**Methods and results:** In order to avoid effects of Dicer deletion on smooth muscle differentiation and growth, we used an early time point (five weeks) after the tamoxifen-induction of Dicer knockout (KO). At this time point, we found that myogenic tone was completely absent in the mesenteric arteries of Dicer KO mice. This was associated with a reduced pressure-induced Akt-phosphorylation, possibly via increased phosphatase and tensin homolog (PTEN) expression, which was found to be a target of miR-26a. Furthermore, loss of myogenic tone was associated with a decreased depolarization-induced calcium influx, which was restored by the L-type channel agonist Bay K 8644 or by transient stimulation with angiotensin II (Ang II). The effect of Ang II was dependent on AT<sub>1</sub>-receptors and activation of the PI3-kinase/Akt pathway.

**Conclusions:** In this study we have identified novel mechanisms that regulate myogenic tone in resistance arteries, which involves microRNA-dependent control of PI3-kinase/Akt signaling and L-type calcium influx. Furthermore we have demonstrated that transient stimulation by Ang II can have long-lasting effects by potentiating myogenic tone.

## Introduction

Despite substantial variations in perfusion pressure, organs are able to maintain local control of blood flow and capillary blood pressure as a result of precise regulation of the diameter of small resistance arteries. The diameter of these arteries is regulated by perfusion pressure and shear stress as well as by multiple local factors such as metabolites and gases. The contractile response of resistance arteries following an increase in intraluminal pressure is referred to as the Bayliss effect or myogenic response and the resulting myogenic tone contributes significantly to the regulation of arterial diameter in many vascular beds, including the mesenteric, cerebral, coronary, skeletal muscle, and renal circulation<sup>1</sup>. This is an important mechanism to avoid pressure increases in capillaries, which may result in fluid leakage and organ damage. Dysregulation of the myogenic response can cause local ischemia or vasogenic edema while a general increase in myogenic activity results in increased peripheral resistance which could contribute to an elevated systemic blood pressure<sup>2</sup>.

The signaling mechanisms underlying the myogenic response are quite complex and involve stretch-sensitive integrins, receptors and ion channels<sup>1,2</sup>. Stretch-induced activation of these mechanosensors leads to a depolarization and calcium influx via voltage-gated calcium channels. Furthermore, when intracellular calcium levels decline, the myogenic response is maintained by calcium sensitization and actin polymerization via the Rho and protein kinase C signaling pathways<sup>3,4</sup>. In addition to these mechanisms, the PI3-kinase/Akt signaling pathway was recently demonstrated to be involved in regulation of blood pressure and myogenic tone via membrane translocation and activation of L-type calcium channels<sup>5</sup>.

In recent years, microRNAs (miRNAs) have been identified as important regulators of vascular contractility and essential components of stretch-induced contractile smooth muscle cell differentiation in the vascular wall<sup>6-8</sup>. However, the role of miRNAs in the myogenic response in pressurized arteries has not been investigated previously. MicroRNAs are known to be dysregulated in several cardiovascular disease states and may thus represent novel targets for therapeutic intervention<sup>9</sup>. It is therefore of considerable importance to identify their role in physiological processes such as the regulation of myogenic tone and vascular resistance.

In the present study, we have used small mesenteric arteries from a smooth muscle specific and tamoxifen-inducible knockout (KO) of the miRNA-processing endonuclease *Dicer* in order to identify the global role of miRNAs in the myogenic response. We found that miRNAs are essential for the myogenic response and that loss of this response in *Dicer* KO mesenteric arteries is mediated, at least in part, by a reduction of pressure-induced activation of the PI3K/Akt pathway and reduced L-type calcium channel activity.

## Materials and methods

A detailed description of all methods can be found in Supplementary material online, Methods.

### *Animals*

Intraperitoneal tamoxifen and vehicle injections of SMMHC-CreER<sup>T2</sup>/*Dicer*<sup>fl/fl</sup> <sup>10, 11</sup> were performed for five consecutive days at the age of four weeks as described previously <sup>6</sup>. Tamoxifen-treated and vehicle-treated mice are herein referred to as *Dicer* KO and WT, respectively. Unless stated otherwise, all experiments were performed at five weeks post-tamoxifen treatment. At this time point the mice were euthanized by cervical dislocation. In agreement with previous reports <sup>12</sup>, we did not find any significant effect of tamoxifen treatment alone on the contractile function of Cre-negative mesenteric arteries (data not shown). All experiments were approved by the Malmö/Lund animal ethics committee (M167-09 and M213-12). This investigation conforms to Directive 2010/63/EU of the European Parliament.

### *Pressure myography*

Pressure myograph experiments were performed on second order mesenteric arteries as described previously <sup>13</sup>. Distensibility was analyzed by comparing the passive vessel diameter at 45-120 mmHg to the passive vessel diameter at 20 mmHg.

### *Pressurization of small mesenteric arteries*

Mesenteric arterial trees from WT and *Dicer* KO mice were divided in two equal halves and mounted on glass cannulas in a pressure myograph chamber (Living Systems Instrumentation). All the branches on the pressurized half were ligated and the vessels were equilibrated for 3 hours in HEPES buffered saline solution in 0 mmHg. Pressure was then either increased to 95mmHg for 10 minutes or maintained at 0 mmHg. Vessels were snap-frozen in liquid nitrogen or, for determination of myosin LC<sub>20</sub> phosphorylation, immersed in acetone-TCA (10%)-DTT (10 mM) on dry ice.

#### *Wire myography*

Wire myograph experiments for the evaluation of contractile force in mesenteric arteries were performed as previously described<sup>13</sup>.

#### *Calcium measurement*

Second order mesenteric arteries were mounted in a heated myograph chamber (Living Systems Instrumentation) and incubated with the calcium indicator Fluo-4 AM (10 μM; Invitrogen) at room temperature for 40 min at 45 mmHg. The chamber was then placed on the stage of an inverted Zeiss Axiovert 200M microscope, heated to 37°C, and allowed to accommodate for another 40 min at 45mmHg to allow hydrolysis of the Fluo-4 AM. The Fluo-4 fluorescence signal in response to 60mM KCl was monitored by a Zeiss Pascal LSM 5 confocal system with a Zeiss Plan Neofluar 40X (N.A. 1.3) oil immersion lens and normalized to basal fluorescence.

#### *Cell culture and transfection*

Vascular smooth muscle cells were isolated from mouse aorta by enzymatic digestion as described previously <sup>7</sup>. Cells were transfected with commercially available synthetic microRNA mimics for miR-26a or negative control (MISSION<sup>®</sup> microRNA, Sigma-Aldrich) using Oligofectamine transfection reagent (Invitrogen) <sup>7</sup>.

#### *Quantitative real-time PCR (qRT-PCR)*

Total RNA isolation and qPCR analysis was performed using Qiagen miRNeasy mini kit and Qiagen primers as described previously <sup>14</sup>. Array analysis of miRNAs was performed using mouse cardiovascular disease miRNA PCR array (Qiagen).

#### *Protein extraction and Western Blotting*

Protein extraction of preparations frozen in liquid nitrogen and western blotting was performed as described previously <sup>14</sup>. For analysis of LC<sub>20</sub> phosphorylation, preparations frozen in acetone-dry ice were thawed to room temperature, repeatedly washed in acetone-DTT (10 mM), freeze-dried and extracted in SDS-sample buffer overnight at room temperature.

#### *Statistics*

Values are presented as mean  $\pm$  S.E.M. P-values were calculated by Student's *t*-test for single comparisons, by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc testing for multiple comparisons or by two-way ANOVA for comparison of pressure-diameter relationships. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.). *p* <0.05 was considered statistically significant. \*, *p*<0.05; \*\*, *p*<0.01; \*\*\*, *p*<0.001.



## Results

*Loss of myogenic tone represents an early phenotype in the inducible and smooth muscle specific Dicer KO mice.*

Intraperitoneal tamoxifen injections of inducible *Dicer* KO mice resulted in a time dependent decrease of *Dicer* and miR-145 expression in vascular tissues (Supplementary figure 1A-C). To further evaluate the effect of *Dicer* KO in smooth muscle, we performed an array analysis of 48 different miRNAs in *Dicer* KO aorta at two to four weeks post tamoxifen treatment. This analysis revealed that, on average, approximately 40% of the miRNA expression was still present in the smooth muscle at two and three weeks post tamoxifen treatment (Fig 1 A). At four weeks post tamoxifen treatment the average miRNA expression was reduced to 14%, suggesting that it is primarily during the last week of the five-week period following *Dicer* deletion that loss of miRNAs affect smooth muscle protein expression and function. We thus consider the five week time point to represent early effects of miRNA down regulation in smooth muscle.

In earlier studies we have shown that smooth muscle specific deletion of *Dicer* results in significant reduction of miRNAs in the aorta<sup>6</sup>, portal vein<sup>8</sup> and urinary bladder<sup>15</sup>. To confirm the effect of *Dicer* KO at five weeks post tamoxifen treatment in small mesenteric arteries, we performed qPCR analysis of selected, highly expressed, miRNAs. As shown in Figure 1B, deletion of *Dicer* in smooth muscle caused a significant reduction of these miRNAs. While the miR-143/145 cluster is relatively specific for smooth muscle, miR-26a may also be expressed in other cell types present in the mesenteric artery.

The myogenic response was studied in *Dicer* KO and WT mesenteric arteries using pressure myographs. In this setup, mesenteric arteries were cannulated on glass pipettes and subjected to stepwise increases in intraluminal pressure. In WT vessels, this resulted in a myogenic response and reduced diameter at pressure levels above 45 mmHg (Fig 1C and D). However, *Dicer* KO arteries were unresponsive to increases in pressure and myogenic tone was completely abolished in these mice. The passive diameter was not significantly altered in *Dicer* KO mesenteric arteries compared to WT vessels (Fig 1E) but the distensibility of the *Dicer* KO vessels was significantly reduced suggesting that the elastic properties of the vascular wall are affected by reduced levels of miRNAs.

*The loss of myogenic tone in Dicer KO arteries is not due to vascular remodeling or reduced expression of contractile proteins*

To evaluate if deletion of *Dicer* results in vascular remodeling at five weeks post tamoxifen, mesenteric arteries were perfusion fixed under 95 mmHg pressure in calcium free conditions and then sectioned and stained with hematoxylin and eosin (Fig 2A). Analysis of the vessel morphometry revealed no significant difference in lumen diameter (Fig 2B) or wall thickness (Fig 2C) between WT and *Dicer* KO vessels suggesting that altered smooth muscle mass is not a contributing factor to the lack of myogenic tone in *Dicer* KO arteries.

Over time, loss of *Dicer*-dependent miRNAs has a negative effect on the expression of smooth muscle differentiation markers<sup>6, 8</sup>. However, the effect of *Dicer* KO on smooth muscle differentiation may vary between tissue types and time after tamoxifen treatment. Quantitative PCR (qPCR) of mesenteric arteries at five weeks post tamoxifen treatment showed that some contractile markers were reduced at the mRNA level (Fig 2D). However, by western blot analysis, we only found a modest reduction of one contractile marker

(tropomyosin-1) at the protein level (Fig 2E). Other markers such as  $\alpha$ -actin, desmin, calponin and SM22 $\alpha$  were not affected by loss of miRNAs. Thus, reduced contractile differentiation is likely not the cause of the abolished myogenic tone in mesenteric arteries within five weeks of *Dicer* deletion.

*The L-type calcium channel activator Bay K 8644 rescues the loss of myogenic tone in Dicer KO mesenteric arteries*

We have previously demonstrated that long term deletion of *Dicer* can affect the polymerization of actin filaments and subsequently the contractile machinery of the smooth muscle cells<sup>7</sup>. In order to test the integrity of the contractile filament system, we analyzed calcium-independent contractile function in WT and *Dicer* KO mesenteric arteries using Calyculin A to inhibit myosin light chain phosphatase activity in nominally calcium-free conditions. Calyculin A-induced contractility was unchanged in *Dicer* KO mesenteric arteries, suggesting that loss of myogenic tone is not due to altered function of the contractile machinery in *Dicer* KO vessels at this time point (Fig 3A). However, the contractile response to depolarization by 60mM KCl was significantly reduced in *Dicer* KO arteries (Fig 3B) indicating that voltage gated calcium influx may be affected. To test this possibility, we loaded WT and *Dicer* KO mesenteric arteries with the calcium indicator fluo-4 and evaluated the relative calcium increase in response to KCl in pressurized arteries. In accordance with the reduced contractile response to KCl, we found a significant decrease in fluo-4 signal intensity after KCl stimulation in *Dicer* KO arteries (Fig 3C). Furthermore, pressure-induced phosphorylation of myosin light chains, which is known to be calcium dependent<sup>16</sup>, was completely abolished in *Dicer* KO mesenteric arteries (Fig 3D).

To investigate if a decreased expression of L-type calcium channels is involved in the reduced calcium influx and contractile response to KCl, we performed qPCR and western blot analysis of calcium channel expression in WT and *Dicer* KO mesenteric arteries. In contrast to the effect in portal vein at later time points<sup>8</sup>, the expression of the L-type calcium channel pore-forming subunit (*Cacna1c*/*Ca<sub>v</sub>1.2*) was unchanged at five weeks post tamoxifen in *Dicer* KO mesenteric arteries (Fig 3E and F). Furthermore, the expression of the auxiliary  $\alpha_2\delta_1$ -subunit of the L-type calcium channel, which is known to play a role in the myogenic response<sup>17</sup>, was unchanged (Fig 3G). These results suggest that it may be the activity rather than the expression level of L-type calcium channels that is perturbed in *Dicer* KO mesenteric arteries at this time point. We thus treated the *Dicer* KO mesenteric arteries with an L-type calcium channel activator (Bay K 8644) and found that this restored pressure-induced myogenic tone in *Dicer* KO vessels without affecting contractile tone at lower pressure levels (20-45mmHg) (Fig 3H). This result suggests that a reduced pressure-induced L-type calcium channel activity is the main cause of the abolished myogenic tone in *Dicer* KO arteries.

*Pressure-induced activation of the PI3K/Akt pathway is abolished in Dicer KO mesenteric arteries and essential for myogenic tone*

In vascular smooth muscle, inhibition or deletion of PI3K has been shown to result in loss of myogenic tone and reduced blood pressure via inhibition of L-type calcium influx<sup>5, 18</sup>. To investigate if the reduced calcium channel activity in *Dicer* KO mice is associated with a decrease in Akt activation, we performed western blot analysis on pressurized mesenteric arteries using a phospho-specific (Ser473) Akt antibody. As shown in Figure 4A, pressurization caused a significant increase in Akt-phosphorylation in WT but not in *Dicer* KO

arteries. Furthermore, an essential role of the PI3K/Akt pathway for myogenic tone was confirmed using the PI3K inhibitor LY-294002 (Fig 4B).

Earlier studies have shown that miRNAs are potent regulators of Akt by targeting PTEN, which antagonizes the PI3K-mediated phosphorylation of Akt<sup>19-21</sup>. We thus tested the expression of PTEN in control and *Dicer* KO mesenteric arteries and found that deletion of miRNAs significantly increased the level of PTEN (Fig 4C). By using the miRNA target prediction database, TargetScan, we found that multiple miRNAs are predicted to target PTEN. However, miR-26a is one of few with as many as three preferentially conserved target sites in the PTEN 3'-UTR. Furthermore, miR-26a is highly expressed in WT smooth muscle and rapidly down-regulated in *Dicer* KO by about 70% already at two weeks post tamoxifen treatment (data not shown). Using a miR-26a mimic, we overexpressed this miRNA in cultured vascular smooth muscle cells isolated from *Dicer* KO mouse aorta. In accordance with the bioinformatic prediction, we found that the protein expression of PTEN was significantly reduced by miR-26a overexpression (Fig 4D).

#### *Angiotensin II-mediated activation of PI3K/Akt pathway restores myogenic tone in Dicer KO mesenteric arteries*

Previous reports have demonstrated that angiotensin II (Ang II) can promote activation of L-type calcium channels via the PI3K/Akt pathway<sup>18</sup>. To investigate if Ang II-induced contraction was affected by *Dicer* KO in smooth muscle, we stimulated mesenteric arteries mounted in a pressure myograph with 100 nM Ang II. In accordance with a reduced L-type calcium channel activity in *Dicer* KO arteries, Ang II induced contraction was significantly reduced in *Dicer* KO mesenteric arteries (Fig 5A). The remaining Ang II-induced contractile response in *Dicer* KO was further inhibited by LY-294002 suggesting that Ang II (in contrast to

elevated pressure) is able to activate the PI3K/Akt pathway in *Dicer* KO arteries, despite an increased PTEN expression. This was also confirmed by analysis of Ang II-induced Akt phosphorylation in WT and *Dicer* KO mesenteric arteries (Fig 5B).

The effect of PI3K on L-type channel activation is partly mediated via recruitment of the  $\alpha_1$ C-subunit of the channel to the plasma membrane<sup>22</sup>, which suggests that the effect of Ang II-induced PI3K activation should be more sustained than the transient contraction observed upon acute stimulation<sup>5, 23</sup>. To examine if Ang II-induced activation of L-type calcium channels could potentiate myogenic tone in *Dicer* KO mesenteric arteries, we transiently stimulated vessels with 100 nM Ang II under low pressure and then evaluated the myogenic response. Although the vessels were only transiently exposed to Ang II, myogenic tone was completely restored in *Dicer* KO mesenteric arteries after Ang II stimulation (Fig 5C and D). The effect was inhibited by the AT<sub>1</sub>-receptor antagonist candesartan and by transient inhibition of PI3K using LY-294002 during the Ang II stimulation (Fig 5D).

## Discussion

Myogenic tone, which is known to be important for auto-regulation of blood flow and blood pressure, is initiated by stretch-induced depolarization, followed by activation of voltage dependent L-type calcium channels<sup>1,2</sup>. However, despite intense investigation in this field, the mechanisms underlying the regulation of myogenic tone are not fully understood. Several factors including stretch-sensitive activation of ion channels, ion transporters and signaling pathways have been suggested to be involved and it is likely that a combination of these factors regulate myogenic tone.

Herein, we have identified an additional mechanism in the control of myogenic tone involving miRNAs. Over time, deletion of *Dicer*-dependent miRNAs in smooth muscle results in severe effects on smooth muscle differentiation and contractile function, demonstrating the essential role of these small noncoding RNAs. The use of inducible smooth muscle-specific *Dicer* KO has enabled us to decipher processes that are especially sensitive to miRNA regulation by using early time points following tamoxifen treatment. In this work, we show that the myogenic response is abolished at a time point (five weeks) where vessel dimensions and calcium-independent contraction are still unaffected by the loss of miRNAs. The loss of myogenic tone in *Dicer* KO vessels was found to be associated with an increased PTEN expression, abolished stretch-sensitive Akt phosphorylation and reduced calcium influx (Fig 6). Accordingly, the pressure-induced and calcium-dependent phosphorylation of myosin light chains was abolished in *Dicer* KO vessels. Bay K 8644, which is known to promote the opening probability of L-type calcium channels, moreover normalized myogenic tone in *Dicer* KO mesenteric arteries.

L-type calcium channels are known to play an essential role in the regulation of blood pressure and conditional deletion of these channels in smooth muscle results in loss of myogenic tone<sup>24</sup>. We have previously shown that long-term (ten weeks) deletion of miRNAs results in reduced L-type calcium channel expression in the portal vein, likely via up-regulation of CamKII $\delta$ <sup>8</sup>. By analyzing the time-course of miRNA knock-down after *Dicer* deletion we found that most miRNAs were still significantly expressed for up to three weeks after tamoxifen treatment. Thus, the phenotype observed in mesenteric arteries at five weeks after *Dicer* KO represents the initial effects of miRNA knockdown in smooth muscle and cannot be attributed to the long-term effects of *Dicer* KO such as reduced smooth muscle mass<sup>6</sup> or reduced expression of contractile proteins and L-type calcium channels<sup>25</sup>.

Despite the fact that the miRNA levels were reduced only for a short period of time, myogenic tone was completely abolished in *Dicer* KO arteries, suggesting that miRNAs play a crucial role in regulating pressure-induced responses. In agreement with this hypothesis, we found that pressure-induced phosphorylation of Akt in the PI3K signaling pathway was absent in *Dicer* KO vessels. The PI3K/Akt-pathway has been suggested to be activated in models of hypertension and it is known that inhibition of this pathway lowers blood pressure by reducing L-type calcium channel activation and myogenic tone<sup>5, 18</sup>. Furthermore, overexpression of PI3K $\delta$  mediates the increased calcium influx and contractility observed in mouse diabetic vessels<sup>26</sup>. The PI3K-pathway regulates L-type calcium channel activity partly by promoting the trafficking of calcium channels to the plasma membrane<sup>22</sup>. A direct regulation of L-type calcium channels by phosphatidylinositol (3,4,5)-triphosphate (PIP<sub>3</sub>) has also been suggested<sup>27</sup>. Mechanical stretch has been shown to activate Akt in both cultured smooth muscle cells<sup>28</sup> and in portal and jugular veins<sup>8, 29</sup>, and our results herein suggest



that an increase in pressure activates this pathway in small mesenteric arteries. In agreement with our results, pressure-induced Akt phosphorylation was recently demonstrated in myogenically active rat cerebral arteries<sup>30</sup>. In the larger carotid artery, which has limited myogenic tone, pressure-induced activation of Akt is not present<sup>31</sup>. Activation of the PI3K pathway thus appears to be a prerequisite for the myogenic response.

The mechanism behind the loss of pressure-dependent Akt phosphorylation in *Dicer* KO arteries may involve several signaling molecules considering the widespread role of miRNAs in the regulation of protein expression. However, since miRNAs generally down-regulate their target protein, we hypothesized that loss of miRNAs would increase the expression of a negative regulator of the PI3K pathway. PTEN is known to reduce PI3K-mediated Akt-phosphorylation by de-phosphorylating PIP<sub>3</sub> to PIP<sub>2</sub> and thereby inhibiting phosphoinositide-dependent kinase-1 (PDK1)<sup>32</sup>. PTEN is a confirmed target of several miRNAs<sup>19-21</sup> and, accordingly, we found that deletion of *Dicer* caused a significant up-regulation of PTEN protein expression in mesenteric arteries. Furthermore, we demonstrate that overexpression of miR-26a alone reduces the expression of PTEN in *Dicer* KO smooth muscle cells. Although the up-regulation of PTEN in *Dicer* KO arteries is likely to be a combined effect of several miRNAs, the importance of miR-26a is interesting since this miRNA has been shown to be up-regulated by mechanical stretch in airway smooth muscle cells and is suggested to be involved in smooth muscle hypertrophy<sup>33</sup>. Prolonged pressurization of myogenically active arteries could thus potentially result in increased levels of miR-26a, reduced PTEN expression and activation of the Akt-pathway, resulting in augmented myogenic tone and increased growth of the vessel wall. In agreement with this hypothesis,

pharmacological inhibition of PTEN potentiates myogenic responsiveness in rat cerebral arteries<sup>30</sup>.

Activation of Ang II receptors is known to be involved in the regulation of myogenic tone and it has been suggested that Ang II receptors themselves can act as mechanosensors independently of agonist stimulation<sup>34, 35</sup>. Furthermore, Ang II-induced contraction and calcium influx mediated by L-type channels are partly dependent on PI3K<sup>18, 27</sup>. In contrast to the pressure-induced response, we found that Ang II could promote Akt-phosphorylation despite an increase in PTEN expression. This may simply be due to the fact that 100 nM Ang II is a much stronger stimulus than an increase in pressure. However, it is notable that Ang II has recently been demonstrated to inhibit PTEN activity, which would counteract the increased PTEN expression in *Dicer* KO arteries<sup>36</sup>.

Ang II is known to be an important mediator of blood pressure regulation partly by promoting vasoconstriction. However, Ang II stimulation of pressurized resistance arteries *ex vivo* only results in a transient contraction that peaks at one minute and returns to near baseline after five minutes. Earlier reports show that phosphorylation of Akt in cultured smooth muscle cells peaks at about 2-5 minutes after Ang II stimulation, and remains about two-fold activated after 40 minutes<sup>37</sup>. Thus it is likely that PI3K, apart from its role in the acute response to Ang II, is involved in long-term effects on contractile function of smooth muscle. In accordance with this hypothesis, we show that at 15-20 minutes following a transient Ang II stimulation, after which Ang II was carefully washed out of the myograph chamber, the loss of myogenic tone was completely restored in *Dicer* KO vessels. Interestingly, this effect was dependent on Ang II-induced PI3K activation since a temporary inhibition of PI3K, during the Ang II-stimulation, prevented the Ang II-mediated effect on

myogenic tone. These results suggest that Ang II-mediated PI3K/Akt activation has long-lasting effects on smooth muscle contractility by potentiating myogenic tone, possibly via an increased activation of L-type calcium channels. However, Ang II can also activate other signalling pathways such as protein kinase C, MAP-kinases and reactive oxygen species, all of which are known to be involved in the regulation of myogenic tone<sup>38 30, 39</sup>.

*Ex vivo* pressure myography of small resistance arteries is the most common method to assess myogenic tone. Although it can be considered an advantage that this technique provides an opportunity to study the effects of pressure and flow in the absence of confounding factors, it is also an important limitation that circulating hormones and neural inputs are not present during the analysis. *In vivo*, these factors can likely affect myogenic responses and it may therefore be difficult to predict if the effects observed *ex vivo* can be recapitulated *in vivo*. However, in previous studies we have shown that inducible deletion of *Dicer* in smooth muscle results in reduced blood pressure already at six weeks post tamoxifen treatment, suggesting that myogenic tone is also impaired *in vivo* in *Dicer* KO mice<sup>6</sup>. The reduced distensibility of the *Dicer* KO arteries would, at least in theory, protect against a more drastic reduction in systemic blood pressure at the expense of the vessels ability to adjust to alterations in systemic blood pressure.

In conclusion, our data suggest that the myogenic response is specifically sensitive to loss of miRNAs in smooth muscle. The mechanism behind this effect involves a reduced voltage gated calcium influx and loss of pressure-induced Akt-phosphorylation, possibly due to an increased expression of PTEN. Our results further emphasize the importance of miRNAs in vascular smooth muscle and demonstrate a novel mechanism participating in the regulation of myogenic tone. Since regulation of myogenic tone is essential for the control of blood

pressure and blood flow, the results presented herein can provide a better understanding for the mechanisms underlying vascular diseases, such as hypertension and edema, and suggest novel targets for therapeutic intervention.

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**Conflict of Interest:** none declared.

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## Figure legends

**Figure 1: Deletion of smooth muscle miRNAs in *Dicer* KO mesenteric arteries results in complete loss of myogenic tone.** (A) Time course of the effects of *Dicer* KO on the expression of 48 selected miRNAs in aorta 2, 3 and 4 weeks after tamoxifen treatment (n=3-4). (B) Quantitative PCR analysis of miR-26a, miR-143 and miR-145 expression in *Dicer* KO mesenteric arteries five weeks after tamoxifen treatment. All data were normalized to U6 (n=4-7). (C) The active diameter of WT and *Dicer* KO mesenteric arteries analyzed by pressure myography at stepwise increases in intraluminal pressure (D). Myogenic tone in WT and *Dicer* KO mesenteric arteries calculated by the relative difference in active and passive diameter (n=12-13). (E) Passive diameter analyzed in calcium free conditions (n=12-13). (F) Distensibility of the WT and *Dicer* KO mesenteric arteries calculated by the relative increase in passive diameter compared to the passive diameter at 20 mmHg. For figure 1B, statistical significance was determined by unpaired two tailed t-tests (\*\*P<0.01, \*\*\*P<0.001). For figure 1C-F, p-values were generated by two-way repeated measures ANOVA and multiple comparisons were corrected by Bonferroni's t-test. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

**Figure 2: Vascular morphometry and smooth muscle differentiation are largely maintained in *Dicer* KO mesenteric arteries at five weeks post tamoxifen treatment.**

(A) Mesenteric arteries from WT and *Dicer* KO mice were perfusion fixed and stained with hematoxylin and eosin. Lumen diameter (B) and wall thickness (C) were evaluated using image analysis software (n=6). The effect of *Dicer* KO on the expression of smooth muscle differentiation markers was analyzed using qPCR (D) and western blot (E) (n=5-6 and 6-8 respectively). Abbreviations: calponin (*Cnn1*), SM22 (*Tagln*), myocardin (*Myocd*) and



tropomyosin 1 (*Tpm1*). For all the figures, statistical significance was determined by two-tailed student's *t*-test. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001.

**Figure 3: Reduced calcium influx in *Dicer* KO mesenteric arteries and rescue of myogenic tone by the L-type calcium channel activator Bay K 8644** (A) Contraction in response to the phosphatase inhibitor Calyculin A in calcium-free conditions analyzed by wire myography in WT and *Dicer* KO mesenteric arteries (n=7). (B) Diameter change in pressurized arteries in response to depolarization by 60 mM KCl (n=9). (C) Relative calcium influx in pressurized mesenteric arteries in response to depolarization by 60 mM KCl analyzed using the calcium indicator Fluo-4 (n=8). (D) Mesenteric arteries from WT and *Dicer* KO mice were pressurized at 95 mmHg for 10 minutes and then analyzed for myosin light chain (MLC<sub>20</sub>) phosphorylation using western blot (n=7). The expression of mRNA (E) and protein (F) of the pore forming subunit of L-type calcium channels was analyzed by qPCR and western blot, respectively (n=7 & 5 respectively). (G) Western blot analysis was also performed for the detection of the auxiliary subunit, Ca<sub>v</sub>α<sub>2</sub>δ1 (n=6). (H) Myogenic tone was evaluated by pressure myography in *Dicer* KO mesenteric arteries pre-treated with the L-type calcium channel opener Bay K 8644 (Bay K) (n=5). For figure 3A, 3B, 3E, 3F and 3G, statistical significance was obtained by two-tailed student's *t*-test. For figure 3C, standard error of mean (SEM) is shown as dotted lines. *p* values were generated, for figure 3D, using one-way ANOVA and for figure 3H, using repeated measures two-way ANOVA. Multiple comparisons were corrected by Bonferroni's *t*-test. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001.

**Figure 4: Loss of pressure-induced activation of Akt in *Dicer* KO arteries is associated with an increased expression of PTEN, which is inhibited by miR-26a.** (A) Akt phosphorylation was analyzed by western blot in WT and *Dicer* KO mesenteric arteries

following 10 minutes pressurization at 95 mmHg (n=3-9). (B) The role of PI3K/Akt signaling for the development of myogenic tone was evaluated by pressure myography using WT mesenteric arteries treated with the PI3K inhibitor LY-294002 (10 $\mu$ M) (n=4). The regulation of PTEN by miRNAs was analyzed by western blot in WT and *Dicer* KO mesenteric arteries (C) and in isolated *Dicer* KO smooth muscle cells transfected with either negative control or miR-26a mimic (n=8 and 6 respectively). For figure 4A, statistical significance was determined by one-way ANOVA and for figure 4B using repeated measures two-way ANOVA. Multiple comparisons were corrected by Bonferroni's t-test. Statistical significance of figure 4C and 4D were determined using two-tailed Student's *t*-test.\*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

**Figure 5: Angiotensin II mediated activation of PI3K/Akt pathway can restore myogenic tone in *Dicer* KO mesenteric arteries.** (A) Contractile responses of mesenteric arteries to angiotensin II (Ang II; 100 nM) were evaluated by pressure myography in WT and *Dicer* KO vessels with or without the PI3K-inhibitor LY-294002 (LY; 10  $\mu$ M) (n=6-8). (B) Western blot analysis of Akt phosphorylation in mesenteric arteries following 5 minutes stimulation with Ang II (100 nM) (n=5). (C) Original recordings from pressure myography experiments showing the effect of *Dicer* KO and Ang II pre-treatment on the myogenic response. (D) Summarized data from pressure myography experiments showing the effect of Ang II pre-treatment on myogenic tone in *Dicer* KO mesenteric arteries. In some experiments the vessels were incubated with either the PI3K inhibitor LY-294002 (LY; 10  $\mu$ M) or the Ang II receptor blocker, candesartan (1  $\mu$ M), during the temporary Ang II stimulation (n=8). For figure 5A and 5B, statistical significance was determined using one-way ANOVA and multiple comparisons were corrected by Bonferroni's t-test.\*P<0.05, \*\*P<0.01.

**Figure 6: Schematic model of how a reduction of microRNA expression can affect myogenic tone via up-regulation of PTEN and inactivation of L-type calcium channels.** This model does not exclude any alternative mechanisms of microRNAs in smooth muscle or alternative signaling pathways involved in the development of myogenic tone. The mechanism for calcium-induced Rho-activation is not well understood but may involve PYK2, JAK2 and/or Rho-GEF activation<sup>40,41</sup>. Abbreviations: PTEN; Phosphatase and tensin homolog, PI3K; Phosphoinositide 3-kinase, AT<sub>1</sub>; Angiotensin II receptor type 1, LTCC; L-type calcium channel, BayK; Bay K8644, CaM; Calmodulin, GEF; Guanine nucleotide exchange factor and MLC; Myosin light chain.

**Supplementary figure 1: Time-course of *Dicer* and miR-145 down-regulation after tamoxifen treatment.** (A) The protein expression of *Dicer* was analyzed in mesenteric arteries from inducible *Dicer* KO mice at 0-5 weeks (w) following tamoxifen treatment. Quantitative PCR analysis was performed to evaluate *Dicer* (B) and miR-145 (C) expression in aorta at 0-4 weeks after tamoxifen treatment. Statistical significance was determined using one-way ANOVA and multiple comparisons were corrected by Bonferroni's t-test. n=3-4, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

Figure 1

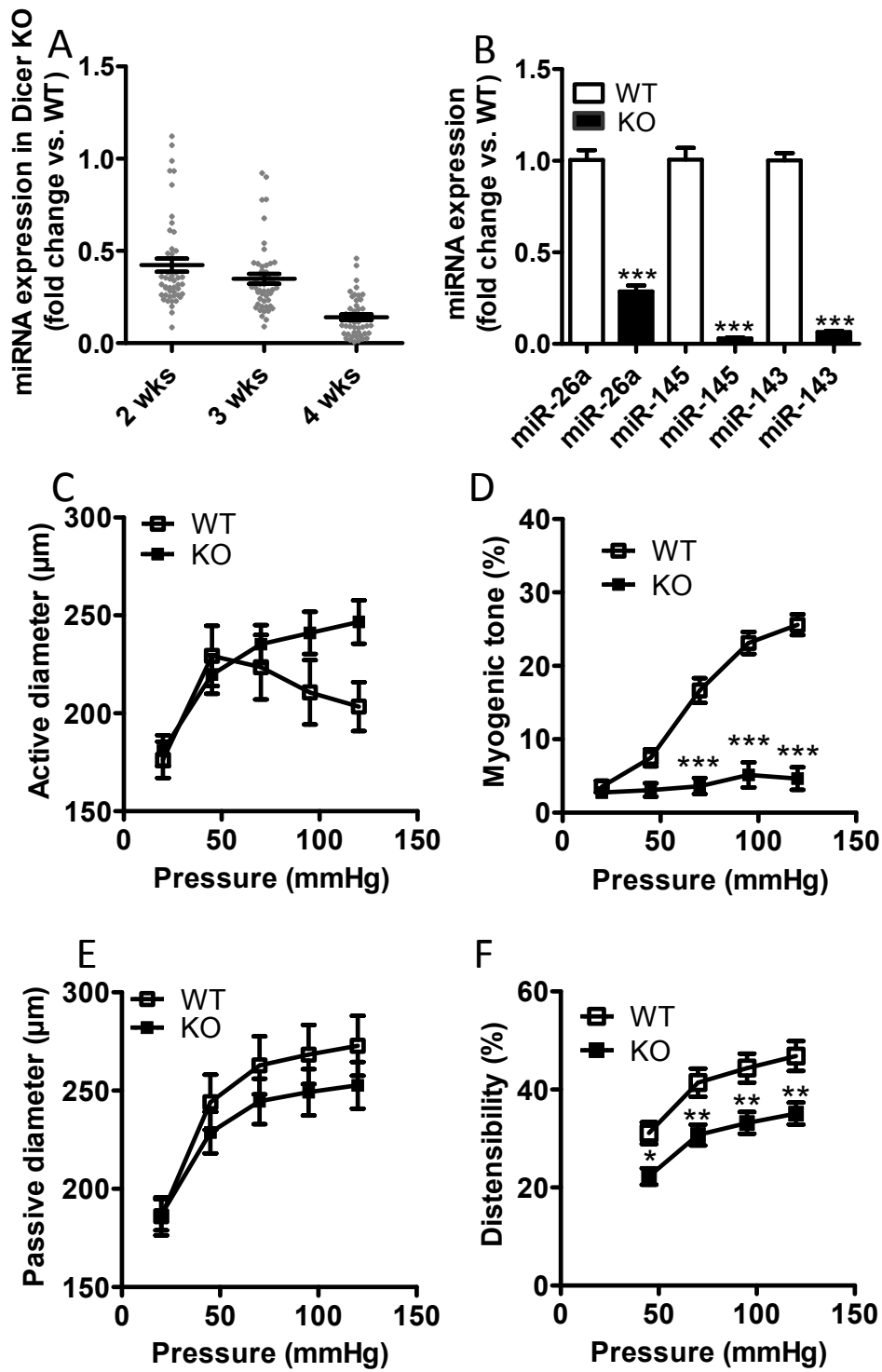


Figure 2

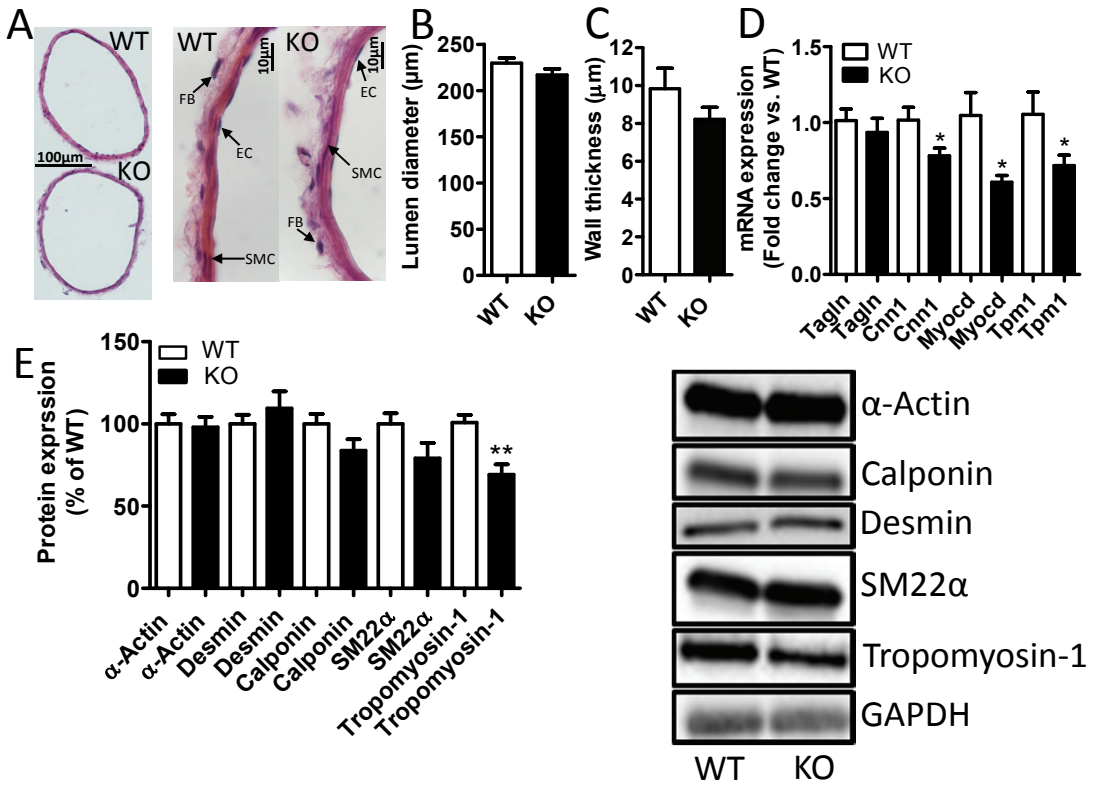


Figure 3

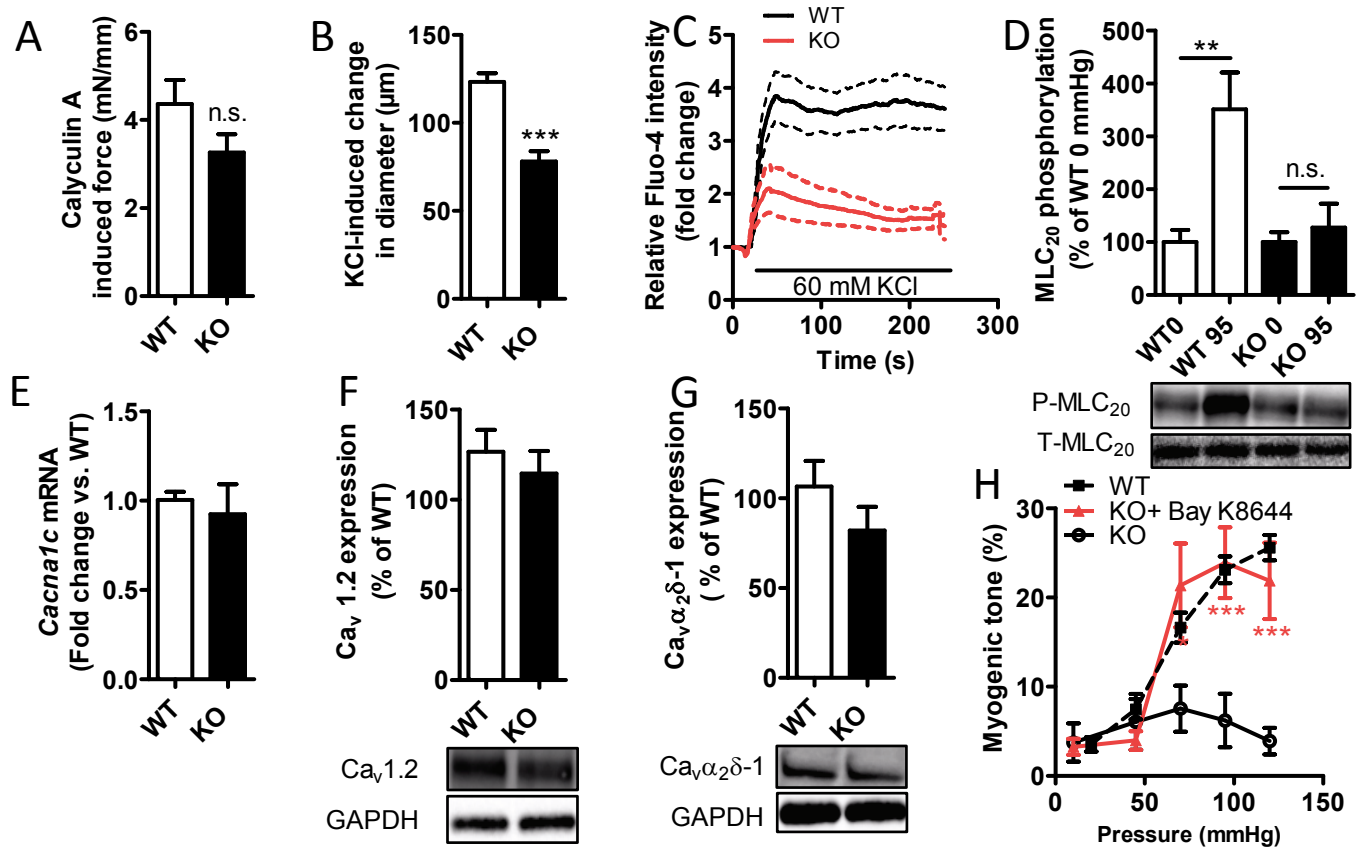


Figure 4

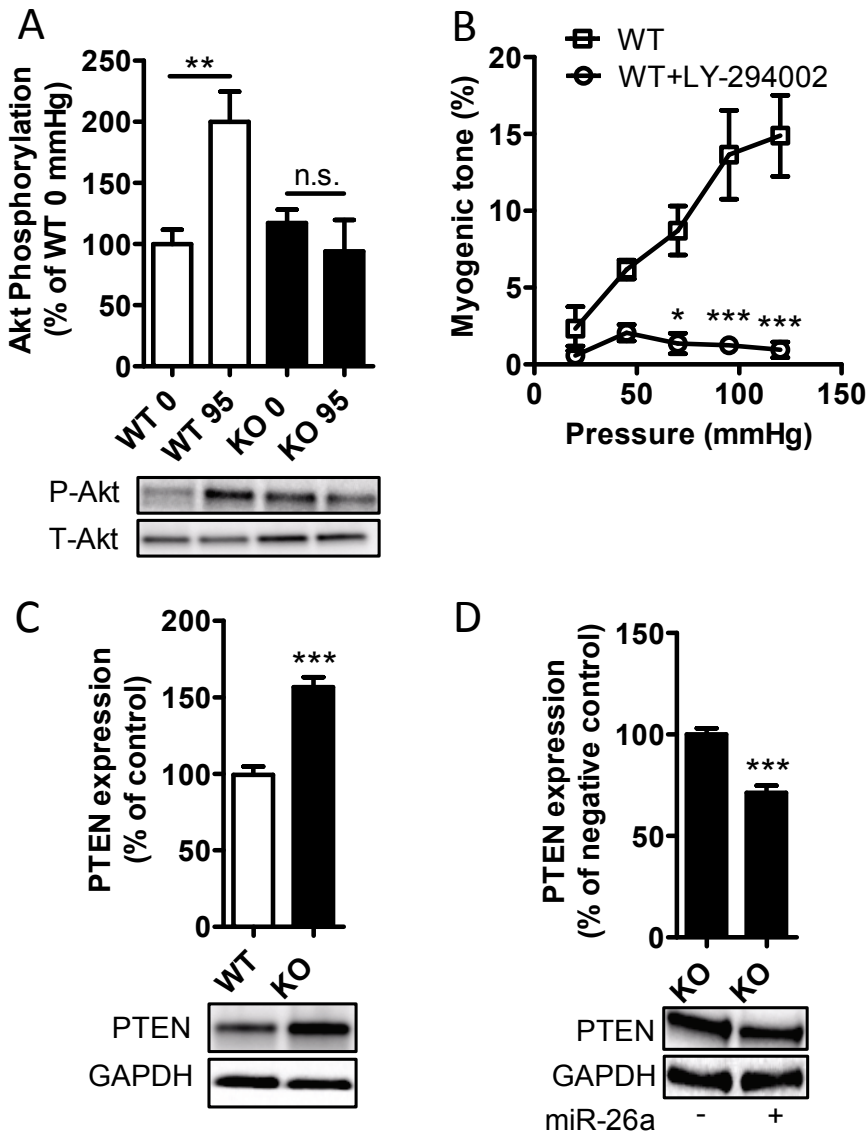


Figure 5

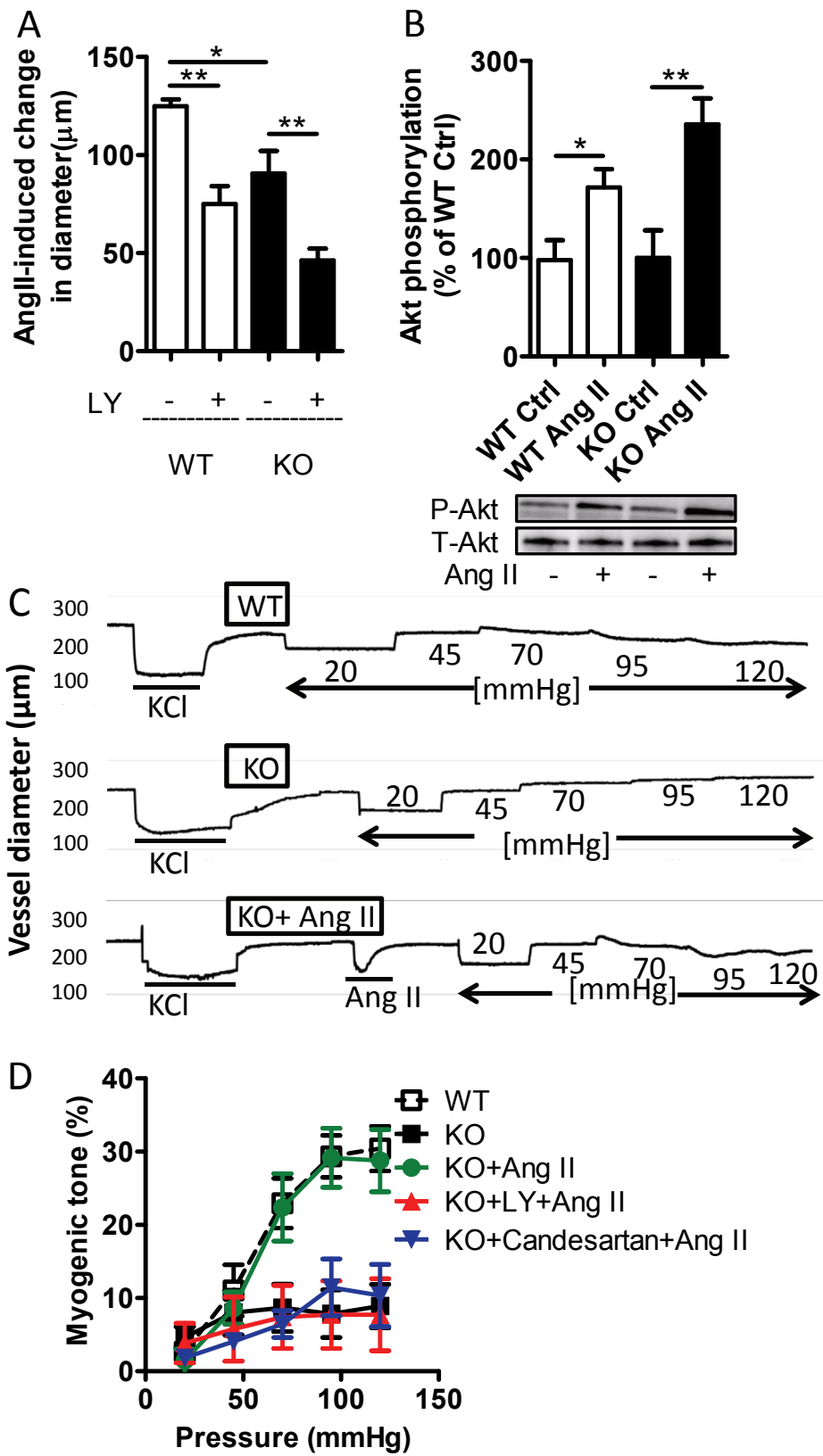
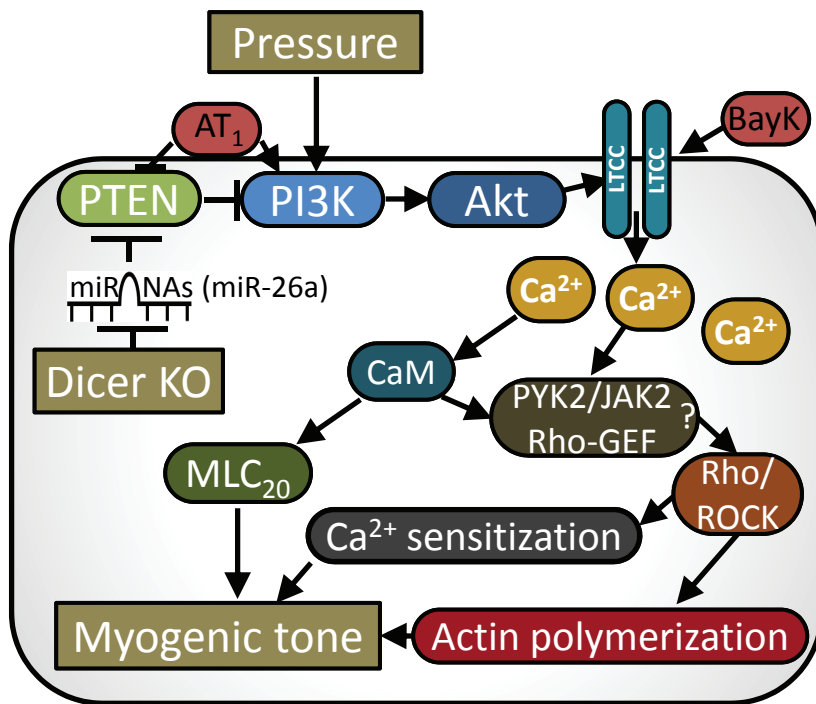
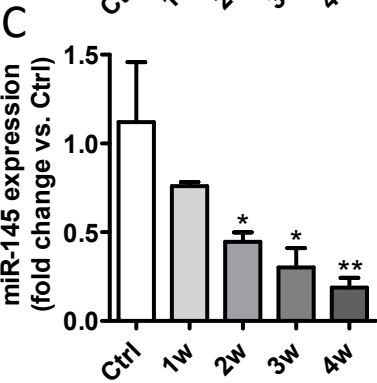
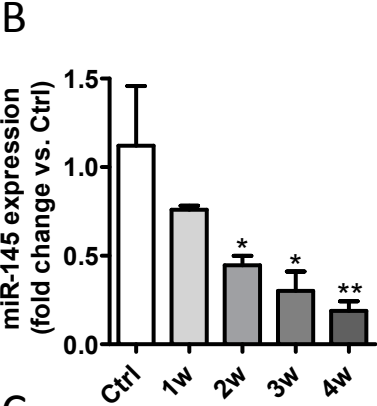
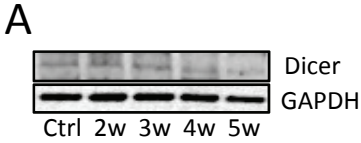




Figure 6



# Supplementary Figure 1



## Supplementary materials and methods

### *Animals*

Male inducible and smooth muscle-specific Dicer KO mice (SMMHC-CreERT2/Dicer<sup>fl/fl</sup>)<sup>1, 2</sup> 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. Unless stated otherwise, all experiments were performed at 5 weeks post-tamoxifen treatment. Vehicle-treated littermate mice were used as controls. All experiments were approved by the Malmö/Lund animal ethics committee (M167-09 and M213-12).

### *Chemicals*

The phosphatase inhibitor Calyculin A (#1336), L-type Ca<sup>2+</sup> channel activator (S)-(-)-Bay K 8644 (#1544), Angiotensin II (#1158) and LY 294002 hydrochloride (#1130) were from Tocris Bioscience. The AT<sub>1</sub>-receptor antagonist candesartan (pure bulk drug) was from Astra Zeneca, Sweden.

### *Pressure myography*

Dicer knock-out mice (Dicer KO) were euthanized by cervical dislocation. Small mesenteric arteries (SMAs) were cleaned from surrounding tissues and mounted on glass cannulas in a heated pressure myograph chamber (Living Systems Instrumentation, Burlington, VT) containing 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 @37°C). The myograph chamber was placed on a Nikon Diaphot 200 inverted microscope equipped with a charge-coupled device (CCD) camera.

VediView 1.2 software (Danish MyoTechnology) was used to monitor lumen and vessel diameter. The intraluminal pressure was continuously monitored by two pressure transducers (on the inflow and outflow side), which were connected to a pressure servo (Living Systems Instrumentation, Burlington, VT). The pressure servo adjusted intraluminal pressure via a peristaltic pump (Living Systems Instrumentation, Burlington, VT). The temperature was kept stable at 37,5°C throughout the experiment. Contractile responses to 60 mM KCl and 100nM Ang II were evaluated at 45mmHg to exclude any effect of myogenic tone on these responses.

#### *Pressurization of small mesenteric artery*

Mesenteric trees from Dicer KO mice were cleaned from surrounding tissue and divided into two equal halves. They were mounted on glass cannulas in a pressure myograph chamber (Living Systems Instrumentation, Burlington, VT). All branches on the pressurized half were ligated using silk sutures while the un-pressurized control vessels were left open. The vessels were equilibrated for one hour 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 @37<sup>0</sup>C) in 0 mmHg. After equilibration period, pressure was increased to 95mmHg for 10 minutes. Control samples were left at 0 mmHg during this period. Vessels were then snap-frozen in liquid nitrogen or, for determination of myosin light chain phosphorylation vessels were frozen in acetone-TCA (10%)-DTT (10 mM) on dry ice.

#### *Vessel morphometry*

One specific branch of the mesenteric artery was mounted in a pressure myograph chamber, pressurized to 95 mmHg in calcium-free and then fixed in 4% paraformaldehyde in

calcium-free PBS for one hour in room temperature. The artery was then embedded in O.C.T compound (Tissue-Tek, Sakura Finetek) and sectioned using a cryostat (10  $\mu\text{m}$  sections). The sections were stained with hematoxylin-eosin (#01656, HistoLab, Sweden) following manufacturer's instructions. Sections were visualized with light a microscope (Olympus) and, internal and external circumference was calculated with CellSens software (Vers 1.5, Olympus). Lumen diameter and wall thickness was obtained from circumference calculated by the software.

### *Wire myography*

Intact mesenteric arteries (1.5-2 mm length) were mounted in four-channel Mulvany myographs (610M, Danish Myo Technology) in HEPES buffer (in mM : 135.5 NaCl, 5.9 KCl, 2.5  $\text{CaCl}_2$ , 1.2  $\text{MgCl}_2$ , 11.6 HEPES; pH 7.4) and maintained at 37°C. Vessels were equilibrated for 30 minutes after applying a basal tension of 2.5mN<sup>4</sup>.

Vessels were then contracted twice with 60mM KCl-containing HEPES solution. Following each contraction, they were relaxed in normal HEPES buffer. To induce maximal contractions by irreversible myosin light chain phosphorylation, calyculin A (1  $\mu\text{M}$ ) was added in calcium free HEPES solution at the end of the experimental protocol.

### *Calcium measurement*

After mounting of a vessel segment close to the cover slip at the bottom of a Living Systems myograph chamber it was incubated with Fluo-4 AM (10  $\mu\text{M}$ ; Invitrogen, Carlsbad, CA, USA) at room temperature for 40 min. Then the chamber was placed on the stage of an inverted Zeiss

Axiovert 200M microscope, heated to 37°C and connected to a fluid reservoir for pressurization of the vessel by gravity. The vessel was allowed to accommodate for another 40 min to allow hydrolysis of the Fluo-4 AM and then intracellular calcium levels were monitored by a Zeiss Pascal LSM 5 confocal system using with excitation at 488 nm and emission at 505 nm. Fluorescence was recorded using a Zeiss Plan Neofluar 40X (N.A. 1.3) oil immersion lens, which allowed focusing at the widest part of the vessel so that the outer diameter could be measured simultaneously.

#### *Cell culture and transfection*

Vascular smooth muscle cells were isolated from mouse aorta by enzymatic digestion as described previously <sup>5</sup>. Mouse aortic smooth muscle cells (mAoSMCs) were used for experiments at passages 3-5. Cells were transfected with commercially available synthetic microRNA mimics for miR-26a (MISSION<sup>®</sup> microRNA-26a mimic, Sigma) or negative control (MISSION<sup>®</sup> microRNA negative control, Sigma) using Oligofectamine transfection reagent (Invitrogen) as described previously <sup>5</sup>.

#### *Quantitative real-time PCR (qRT-PCR)*

Total RNA was isolated using miRNeasy mini kit (Qiagen, #217004) according to the manufacturer's instructions. mRNA was detected using QuantiFast SYBR Green RT-PCR Kit (Qiagen) and QuantiTect primer assays (Qiagen). For miRNA measurement, 300ng of template RNA was reverse transcribed to cDNA using miScript II RT Kit (Qiagen, #218161) according to the manufacturer's instructions. The relative expression of miRNAs was analyzed by real-time qPCR (StepOnePlus qPCR cycler, Applied Biosystems) using miScript SYBR Green PCR Kit (Qiagen, #

218073) and miScript Primer Assays (Mm\_miR-145\_1, # MS00001631; Mm\_miR-143\_1 # MS00001617, Mm\_miR-26a\_2 # MS00032613, Hs\_RNU6-2\_1, #MS00033740).

### *Protein extraction and Western Blotting*

Proteins were extracted as described previously<sup>6</sup> or for determination of myosin LC<sub>20</sub> phosphorylation, frozen arteries were thawed and washed thoroughly in acetone-DTT (10 mM) to remove any trace of TCA. Arteries were then lyophilized for at least 5 hours and proteins were extracted in 2% SDS buffer overnight at room temperature with gentle shaking. Equal amounts of protein were loaded in each lane of Bio-Rad TGX Criterion gels. Proteins were then transferred either overnight or using semi-dry transfer for 10 min in the Trans-Blot Turbo system (Bio-Rad). Proteins were detected using the following commercially available primary antibodies: desmin (#4024, 1:1000), tropomyosin (#3910, 1:1000), total and phospho-Akt (# 9272 and 9271, 1:500), PTEN (# 9559, 1:1000), total and phospho-MLC (#3672 and #3675, 1:500) (Cell Signaling Technology); calponin (# ab46794; 1:1000), SM22 (# ab14106; 1:2000);  $\alpha$ -actin (#A5228; 1:2000) (Sigma); Ca<sub>v</sub>1.2 (# ACC-003; 1:500; Alomone Labs), Ca<sub>v</sub> $\alpha$ <sub>2</sub> $\delta$ -1(#OAEB01261, Aviva Systems Biology, 1:500), GAPDH (#MAB374, Millipore, 1:1000-1:5000), Dicer (#NB200-591, Novus Biologicals, 1:500). HRP-conjugated (# 7074 and 7076; 1:5000; Cell Signaling Technology) or fluorescently labeled DyLight800 and DyLight680 secondary antibodies (# 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) and analyzed using Image Studio software (LI-COR Biosciences).

### *Statistics*

Values are presented as mean  $\pm$  S.E.M. unless otherwise stated. P-values were calculated by Student's t-test for single comparisons, one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc testing for multiple comparisons or 2-way ANOVA for comparison of pressure-diameter relationships and myogenic tone. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.).  $p < 0.05$  was considered statistically significant. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

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