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Published in:
Communicative & Integrative Biology

DOI:
[10.4161/cib.22278](https://doi.org/10.4161/cib.22278)

2013

[Link to publication](#)

Citation for published version (APA):
Turczynska, K., Hellstrand, P., Swärd, K., & Albinsson, S. (2013). Regulation of vascular smooth muscle mechanotransduction by microRNAs and L-type calcium channels. *Communicative & Integrative Biology*, 6(1), e22278. <https://doi.org/10.4161/cib.22278>

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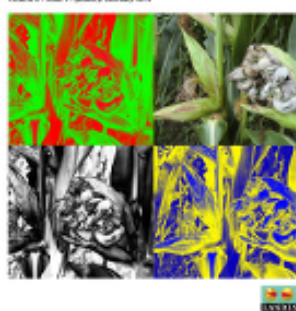
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Communicative & Integrative Biology

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/kcib20>

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Published online: 01 Jan 2013.

To cite this article: Karolina M. Turczyńska, Per Hellstrand, Karl Swärd & Sebastian Albinsson (2013) Regulation of vascular smooth muscle mechanotransduction by microRNAs and L-type calcium channels, *Communicative & Integrative Biology*, 6:1, e22278, DOI: [10.4161/cib.22278](https://doi.org/10.4161/cib.22278)

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Regulation of vascular smooth muscle mechanotransduction by microRNAs and L-type calcium channels

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Keywords: smooth muscle, microRNA, vascular, stretch, differentiation, phenotype

The phenotype of smooth muscle cells is regulated by multiple environmental factors including mechanical forces. Mechanical stretch of mouse portal veins *ex vivo* has been shown to promote contractile differentiation by activation of the Rho-pathway, an effect that is dependent on the influx of calcium via L-type calcium channels. MicroRNAs have recently been demonstrated to play a significant role in the control of smooth muscle phenotype and in a recent report we investigated their role in vascular mechanosensing. By smooth muscle specific deletion of *Dicer*, we found that microRNAs are essential for smooth muscle differentiation in response to stretch by regulating CamKII δ and L-type calcium channel expression. Furthermore, we suggest that loss of L-type calcium channels in *Dicer* KO is due to reduced expression of the smooth muscle-enriched microRNA, miR-145, which targets CamKII δ . These results unveil a novel mechanism for miR-145 dependent regulation of smooth muscle phenotype.

Smooth muscle cells surrounding hollow organs such as the blood vessels, the urinary bladder or the gastrointestinal tract are continuously subjected to mechanical forces. It is known that mechanical stretch can regulate smooth muscle function by stimulating intracellular signaling events, which control smooth muscle cell differentiation and growth.^{1,2} However, studies of this phenomenon in cultured cells must be interpreted with caution since cellular mechanosensing is highly dependent on the surrounding environment including cell-cell and cell-matrix interactions. In *vivo*, on the other hand, the effects of mechanical stretch in the vasculature can be difficult to separate from compensatory mechanisms regulating blood pressure and blood flow in the body. We have in several studies used the murine portal vein in organ culture as a model for examining stretch dependent effects in vascular smooth muscle.³⁻⁷ Similar to certain small arteries, the portal vein smooth muscle exhibits myogenic tone and phasic activity, which may be important factors for stretch-induced effects. Since the portal vein consists of mostly longitudinal smooth muscle, stretch is applied by attaching a weight, which corresponds to the optimal load for force development at one end of the vessel (Fig. 1). The vessels are then incubated in an organ culture environment for up to 5 days. Mechanical stretch of the portal vein results in an early activation of the MAPK/ERK pathway and smooth muscle growth.^{4,6,7} This is followed by a delayed activation of the Rho/Rho-kinase/cofilin pathway, which promotes actin polymerization.⁵ When actin is polymerized, it releases the transcription factor myocardin related transcription factor (MRTF) for

nuclear translocation.⁸ Like myocardin, MRTF is a co-factor for the transcription factor serum response factor (SRF), which promotes the transcription of smooth muscle specific genes. The concerted action of MRTF and SRF thereby increases contractile differentiation of smooth muscle cells. The identity of the sensor that activates signaling pathways in response to stretch is still unclear but it is likely that stretch sensitive components in the plasma membrane such as stretch-sensitive ion channels, G-protein coupled receptors or integrins coupled to focal adhesion kinase play an important role.⁹⁻¹¹ Interestingly, activation of focal adhesion kinase is biphasic in response to stretch in the portal vein with an early peak that correlates with MAPK activation and a delayed peak, which correlates with Rho activation.⁴ It is thus possible that integrins and focal adhesions are involved in stretch-induced activation of both pathways. Earlier studies have shown that L-type calcium channel influx is an important mediator for activation of the Rho pathway.^{12,13} Using pharmacological calcium channel inhibitors we found that stretch-induced MAPK pathway activation in the portal vein depends on store-operated calcium influx while Rho pathway activation requires L-type calcium channel activation.¹⁴ This suggests that these signaling pathways may be selectively activated depending on the mode of influx and/or intracellular release of calcium.

A novel mechanism involved in the regulation of protein expression and cell function was revealed by the discovery of microRNAs (miRNAs).¹⁵ These small noncoding RNAs bind the 3'UTR of their target mRNA, which in most cases results in inhibition of protein translation or degradation of

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Submitted: 08/08/12; Revised: 09/17/12; Accepted: 09/18/12
<http://dx.doi.org/10.4161/cib.22278>

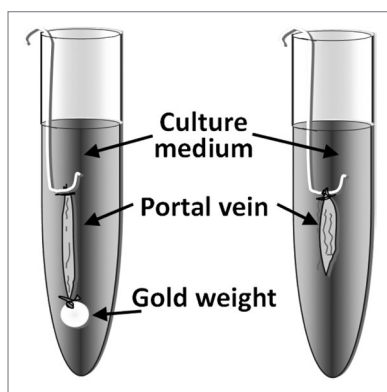


Figure 1. Mouse portal veins are stretched by attaching a gold weight at one end of the vessel. The portal veins are then placed in a cell culture incubator for up to 5 days.

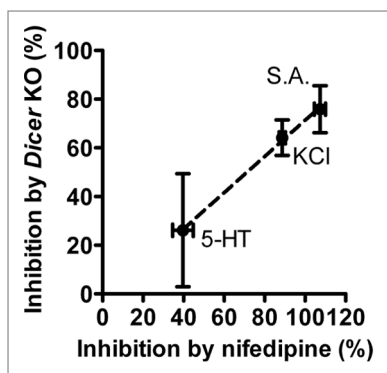


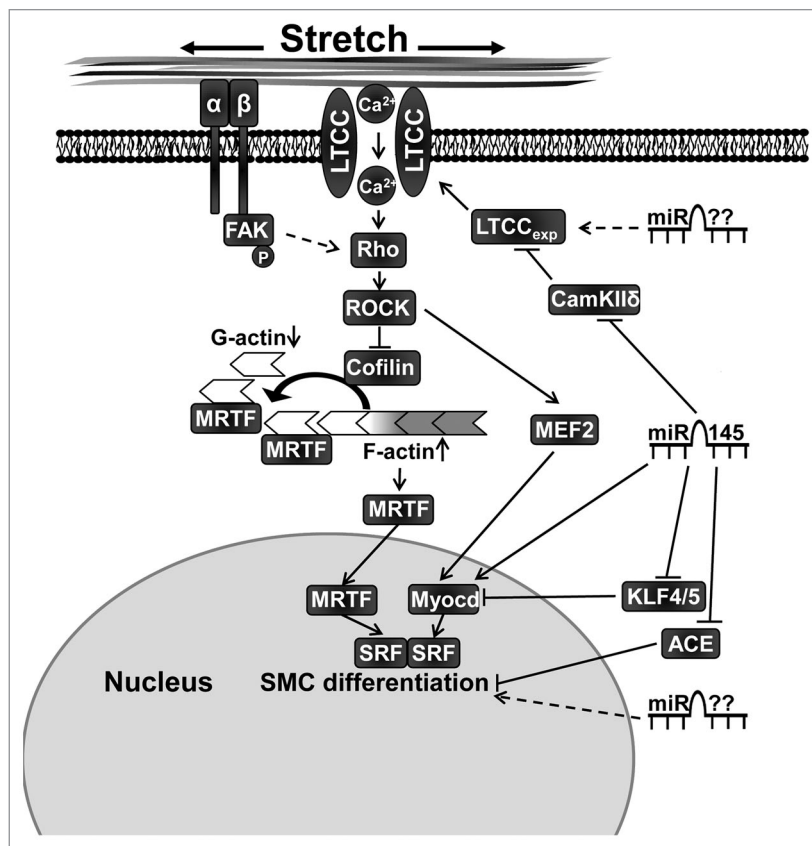
Figure 2. The level of inhibition of various contractile responses in portal vein by *Dicer* KO correlates with force inhibition by the L-type calcium channel blocker Nifedipine. 5-HT: serotonin, KCl: potassium chloride, S.A.: Spontaneous activity. n = 3–4.

the mRNA. MicroRNAs are known to play an important role in smooth muscle development and contractile differentiation and a number of specific miRNAs have been identified to be of particular importance.^{16–24} In a recent study, we examined the role of miRNAs in smooth muscle mechanosensing in portal vein by using tamoxifen-inducible and smooth muscle specific *Dicer* KO mice (*Dicer* KO).²⁵ *Dicer* is an essential enzyme for the biosynthesis of most miRNAs and deletion of *Dicer* therefore results in a general loss of miRNA expression. Similar to other vascular beds, *Dicer* KO portal veins exhibited a reduced basal expression of smooth muscle contractile markers, confirming the important role of miRNAs in smooth muscle differentiation. Interestingly, we found that stretch-induced expression of smooth muscle markers was reduced or ablated in *Dicer* KO portal veins suggesting that miRNA expression is essential for stretch-induced contractile differentiation. However, it is important to note that mechanosensing per se was not affected by loss of miRNAs since acute stretch-induced MAPK activation was maintained in *Dicer* KO vessels. It is thus likely that the effect of *Dicer* KO on stretch-induced

contractile differentiation is downstream of activation of integrins and focal adhesions. The *Dicer* KO portal veins exhibited a reduced expression of the pore forming $\alpha 1C$ subunit of voltage dependent L-type calcium channels at both mRNA (*Cacna1c*) and protein (Cav1.2) levels. As mentioned earlier, inhibition of L-type calcium channels using verapamil or nifedipine is sufficient to prevent stretch-induced contractile differentiation.¹⁴ A key finding of our recent study was a close correlation between effects on force of Ca^{2+} -channel inhibition and *Dicer* deletion, respectively. This suggests that *Dicer* deletion impairs force in smooth muscle in part via effects on L-type Ca^{2+} channels. The observed correlation is illustrated in **Figure 2**, where the effects of *Dicer* KO on responses to contractile agonists are seen to correlate with the effects of nifedipine in wild type vessels. The reduced *Cacna1c* expression in *Dicer* KO portal veins suggests a transcriptional effect on the L-type calcium channels mediated by miRNAs. Since miRNAs generally repress protein translation of their target we hypothesized that L-type calcium channels could be indirectly regulated in *Dicer* KO portal veins by a transcription factor or signaling molecule that inhibits L-type calcium channel expression and is upregulated in the absence of miRNAs. In a recent study by Ronkainen et al. CamKII δ was shown to inhibit L-type calcium channel expression via the transcriptional inhibitor calsenilin/DREAM/KChIP3.²⁶ Furthermore, CamKII KO mice display an increased expression of L-type calcium channels in cardiomyocytes.²⁷ In addition to DREAM translocation, the effect of CamKII KO may depend on decreased nuclear translocation of the NF κ B component p65, which suppresses transcription of *Cacna1c*.²⁷ Since CamKII δ is a confirmed target of miR-145 in vascular smooth muscle cells¹⁷ and is upregulated in *Dicer* KO portal veins,²⁵ we hypothesized that miR-145 could regulate L-type calcium channels via CamKII. To test this hypothesis, we used isolated smooth muscle cells in culture transfected with miR-145 inhibitor. Interestingly, inhibition of miR-145 resulted in a reduction of L-type calcium channel mRNA expression, which closely correlated with the effect observed in *Dicer* KO portal vein. This indicates that the reduced expression of L-type calcium channel in *Dicer* KO smooth muscle is primarily caused by loss of miR-145. Furthermore, the effect of miR-145 on L-type calcium channel expression could be prevented by the CamKII inhibitor, KN93.

MiR-145 has previously been demonstrated to promote smooth muscle differentiation by targeting multiple factors involved in the regulation of smooth muscle phenotype including Krüppel-like factors,^{17,19} myocardin¹⁷ and angiotensin converting enzyme.¹⁶ Regulation of the L-type calcium channel by miR-145 therefore represents an additional mechanism by which miRNAs can control smooth muscle differentiation and contractile function (**Fig. 3**). We and others have shown that miR-145 is involved in smooth muscle actin polymerization but the role of L-type calcium influx in this process is not fully understood.^{18,22} Although miR-145 has been shown to directly target several factors involved in actin dynamics it is tempting to speculate that miR-145 promotes actin polymerization via increased expression of L-type calcium channels.

Figure 3. Contractile differentiation of vascular smooth muscle cells is promoted by mechanical stretch and miR-145. Regulation of L-type calcium expression (LTCC_{exp}) via miR-145 and possibly other miRNAs plays an important role for stretch-induced differentiation. Stretch activates the Rho/Rho-kinase (ROCK), which promotes actin polymerization partly via inhibition of cofilin. Myocardin related transcription factor (MRTF) is then released from monomeric actin (G-actin) and translocates to the nucleus where it, as a co-factor to serum response factor (SRF), promotes smooth muscle differentiation. MicroRNA-145 also regulates contractile differentiation via additional targets such as angiotensin converting enzyme (ACE), Kruppel-like transcription factors (KLF) 4 and 5 and a direct positive regulation of myocardin (Myocd). Furthermore, it is likely that several so far unknown miRNAs are involved in smooth muscle cell (SMC) contractile differentiation. FAK, focal adhesion kinase; MEF2, myocyte enhancer factor-2.



Although we find the portal vein to be a robust model to investigate stretch-induced effects in vascular smooth muscle, the effects of *Dicer* KO may differ between veins and arteries and even between different arterial beds. It is therefore important to investigate the role of miRNAs in pressure-induced effects in small resistance arteries as well. Regulation of L-type calcium channel expression by miRNAs, may play a role in other cell types including cardiomyocytes and skeletal muscle. Indeed, we recently reported that smooth muscle specific deletion of *Dicer* also results in reduced expression of L-type calcium channels in smooth muscle of the urinary bladder, indicating that miRNAs regulate these channels in multiple tissues.²⁸

Disclosure of Potential Conflicts of Interest

There were no potential conflicts of interest to disclose.

Acknowledgments

This work was supported in part by the Swedish Research Council; the Swedish Heart and Lung Foundation; The Crafoord Foundation; The Royal Physiographic Society; The Ake Wiberg Foundation; The Tore Nilson Foundation; The Greta and Johan Kock Foundation; The Magnus Bergvall Foundation and The Lars Hierta Memorial Foundation.

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