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*Published in:*  
Acta Physiologica

*DOI:*  
[10.1111/apha.12536](https://doi.org/10.1111/apha.12536)

2015

[Link to publication](#)

### *Citation for published version (APA):*

Bhattachariya, A., Dahan, D., Ekman, M., Boettger, T., Braun, T., Swärd, K., Hellstrand, P., & Albinsson, S. (2015). Spontaneous activity and stretch-induced contractile differentiation are reduced in vascular smooth muscle of miR-143/145 knockout mice. *Acta Physiologica*, 215(3), 133-143. <https://doi.org/10.1111/apha.12536>

*Total number of authors:*  
8

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**Spontaneous activity and stretch-induced contractile differentiation are reduced in vascular smooth muscle of miR-143/145 knockout mice**

Journal:	<i>Acta Physiologica</i>
Manuscript ID:	APH-2014-07-0300.R3
Manuscript Type:	Regular Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Bhattachariya, Anirban; Lund University, Experimental Medical Science Dahan, Diana; Lund University, Experimental Medical Science Ekman, Mari; Lund University, Department of Biology Boettger, Thomas; Max Planck Institute for Heart and Lung Research, Max Planck Institute for Heart and Lung Research Braun, Thomas ; Max Planck Institute for Heart and Lung Research, Max Planck Institute for Heart and Lung Research Swärd, Karl; Lund University, Experimental Medical Science Hellstrand, Per; Lund University, Experimental Medical Science Albinsson, Sebastian; Lund University, Experimental Medical Science
Key Words:	miR-145, vascular smooth muscle, mechanotransduction, microRNA, portal vein, L-type calcium channel, spontaneous activity, contractility

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# Spontaneous activity and stretch-induced contractile differentiation are reduced in vascular smooth muscle of miR-143/145 knockout mice

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Short title: Effects of miR-143/145 KO in portal vein

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## Abstract

**Aim:** Stretch is essential for maintaining the contractile phenotype of vascular smooth muscle cells and small non-coding microRNAs are known to be important in this process. By using a *Dicer* knockout model we have previously reported that miRNAs are essential for stretch-induced differentiation and regulation of L-type calcium channel expression. The aim of the present study was to investigate the importance of the smooth muscle enriched miR-143/145 microRNA cluster for stretch-induced differentiation of the portal vein.

**Methods:** Contractile force and depolarization-induced calcium influx were determined in portal veins from wild type and miR-143/145 knockout mice. Stretch-induced contractile differentiation was investigated by determination of mRNA expression following organ culture for 24 hours under longitudinal load by a hanging weight.

**Results and Discussion:** In the absence of miR-143/145, stretch-induced mRNA expression of contractile markers in the portal vein was reduced. This was associated with decreased amplitude of spontaneous activity and of depolarization-induced contractile and intracellular calcium responses, while contractile responses to 5-HT were largely maintained. We found that these effects correlated with a reduced basal expression of the pore-forming subunit of L-type calcium channels and an increased expression of CaMKII $\delta$  and the transcriptional repressor DREAM. In conclusion, our results suggest that the microRNA-143/145 cluster plays a role in maintaining stretch-induced contractile differentiation and calcium signaling in the portal vein. This may have important implications for the use of these microRNAs as therapeutic targets in vascular disease.

1  
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3 **Keywords:** contractility, L-type calcium channel, mechanotransduction, microRNA, miR-145,  
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6 spontaneous activity, vascular smooth muscle, portal vein.  
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11 **List of Abbreviations:**

12 miRNA: microRNA

13 DREAM: Downstream regulatory element antagonistic modulator

14 CaMKII $\delta$ : Ca<sup>2+</sup> /calmodulin-dependent protein kinase II  $\delta$  subunit

15 MLCK: Myosin light chain kinase

16 ACE-1: Angiotensin-converting-enzyme-1

17 5-HT: Serotonin

18 SRF: Serum response factor

19 MRTF: myocardin related transcription factor  
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## Introduction

The vascular smooth muscle cell is normally in a contractile and quiescent state but can under certain conditions revert into a more proliferative and less contractile phenotype. This process, known as phenotypic modulation, is stimulated by changes in the local environment and often observed in vascular disease states such as neointimal hyperplasia (reviewed in (Owens et al., 2004). However, increased contractility of blood vessels may also be detrimental and has been associated with hypertension and hyperglycemia (Aalkjaer et al., 1987, Holloway and Bohr, 1973, Xie et al., 2006).

One of the environmental factors that can influence smooth muscle phenotype is mechanical tension. The vascular wall is constantly subjected to mechanical forces exerted by the blood pressure and flow and it is well known that altered mechanical forces in the vasculature, such as turbulent flow and increased pressure, can result in remodeling of vessels and vascular disease (Intengan and Schiffrin, 2001, Mulvany, 2002). For example, an increased pressure in the rat portal vein caused by partial ligation *in vivo* results in hypertrophic growth of the smooth muscle (Malmqvist and Arner, 1990). The rodent portal vein consists of a dominating longitudinal and a thinner circular smooth muscle layer, and elevated transmural pressure results in hypertrophy and increased force-generating ability of the longitudinal muscle layer. Hence it is relevant to consider longitudinal stretch when evaluating responses of the portal vein to intraluminal pressure (Albinsson et al., 2014). By loading the rat or mouse portal vein longitudinally by a hanging weight in organ culture we have demonstrated that mechanical stress is an important factor for maintaining the contractile phenotype in the intact blood vessel

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3 (Zeidan et al., 2000, Zeidan et al., 2003, Albinsson et al., 2004). The mechanism behind this  
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6 effect involves stretch-induced activation of the Rho/Rho-kinase pathway, which is dependent  
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9 on L-type calcium influx and results in increased myocardin expression and increased  
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11 polymerization of actin filaments (Turczynska et al., 2013b, Ren et al., 2010, Albinsson and  
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13 Hellstrand, 2007, Albinsson et al., 2004, Albinsson et al., 2014). This, in turn, promotes the  
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16 expression of serum response factor (SRF) regulated smooth muscle contractile markers such as  
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18  $\alpha$ -actin, SM22 $\alpha$  and desmin, via myocardin as well as via nuclear translocation of the SRF co-  
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21 factor, myocardin related transcription factor (MRTF) (Miralles et al., 2003).  
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25 In recent years, much effort has been spent to identify novel mechanisms by which microRNAs  
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27 (miRNAs) can regulate the phenotype of vascular smooth muscle, which may open up new  
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29 therapeutic opportunities (Albinsson and Swärd, 2013). MiRNAs are small (~22 nucleotides)  
30  
31 non-coding RNAs, which generally inhibit translation of proteins by causing degradation and/or  
32  
33 translational inhibition of target mRNAs (Bartel, 2004). Although several hundred miRNAs are  
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35 expressed in smooth muscle, only a few have so far been demonstrated to be involved in  
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37 phenotypic modulation of vascular smooth muscle cells (Albinsson and Sessa, 2010, Albinsson  
38  
39 and Swärd, 2013). Out of these, miR-143 and mir-145, which are transcribed as a bicistronic  
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41  
42 unit, are highly expressed in smooth muscle and likely to be two of the most important miRNAs  
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44  
45 for vascular smooth muscle function and contractile differentiation (Cordes et al., 2009,  
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47 Boettger et al., 2009, Xin et al., 2009). Altered expression of miR-145 may contribute to or  
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50 protect against vascular disease making it a potentially interesting therapeutic target (Boettger  
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53 et al., 2009, Cheng et al., 2009, Caruso et al., 2012).  
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3 One strategy to investigate the collective role of miRNAs for the function of a specific cell type is  
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5 genetic deletion of the enzyme dicer, which is essential for the maturation of most miRNAs.  
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8 Since smooth muscle specific *Dicer* KO is embryonically lethal (Albinsson et al., 2010), we have  
9  
10 utilized inducible and smooth muscle specific KO of *Dicer* in order to clarify the importance of  
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12 miRNAs for smooth muscle function in various vascular beds as well as in urinary bladder  
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14 (Albinsson et al., 2011, Bhattachariya et al., 2014, Sadegh et al., 2012, Turczynska et al., 2012).  
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17 In the portal vein, we found that deletion of *Dicer* resulted in loss of stretch-dependent  
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19 contractile differentiation. This effect correlated with a decreased expression of L-type calcium  
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21 channels and a decreased stretch-induced activation of the Rho/Rho-kinase pathway. Using  
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23 smooth muscle cells in culture and synthetic miRNA inhibitors we found that the smooth muscle  
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25 enriched miRNA, miR-145, is a possible candidate behind the effects of Dicer in the portal vein  
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27 (Turczynska et al., 2012). However, the importance of miR-145 *in vivo* for the regulation of L-  
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29 type channel expression and stretch sensitivity remains to be determined.  
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37 In the present study, we investigated if miR-143 and miR-145 are essential for stretch-sensitive  
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39 contractile differentiation in mouse portal vein by using miR-143/145 KO mice. Although the  
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41 effects were milder in constitutive miR-143/145 KO mice compared with inducible *Dicer* KO  
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43 mice, we found that deletion of this miRNA cluster results in reduced stretch-induced  
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45 contractile differentiation and that this effect is associated with a decreased expression of L-  
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47 type calcium channels and reduced contractile function of the portal vein.  
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## Methods

### *Animals*

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. The microRNA-143/145 knockout mice were generated at the Max-Planck-Institute for Heart and Lung Research as described previously (Boettger et al., 2009) and bred in-house by heterozygous mating. Littermate wild type mice were used as controls.

### *Chemicals*

The phosphatase inhibitor Calyculin A (#1336), Angiotensin II (#1158), and 5-hydroxytryptamine (5-HT, #3547) were from Tocris Bioscience.

### *Organ culture*

Portal veins were cleaned from fat and surrounding tissue and one end was tied to a hook in a test tube containing DMEM/Ham'sF-12 with 2% dialyzed fetal bovine serum and 10nM insulin as described previously (Zeidan et al., 2003). The vessels were stretched longitudinally by attaching a 0.3-g gold weight to the other end. This corresponds approximately to the optimal load for force development. At the end of the experiment, portal veins were frozen in liquid nitrogen or in 10% acetone-trichloroethanoic acid – 10mM dithiothreitol (DTT) on dry ice.

### *Force measurements*

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3 Intact portal veins were mounted in a four-channel myograph (610M, Danish Myo Technology)  
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5 in HEPES buffered solution (in mM : 135.5 NaCl, 5.9 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 11.6 HEPES; pH  
6  
7 7.4) and maintained at 37°C as described previously (Turczynska et al., 2012). Vessels were  
8  
9 equilibrated for 30 minutes after applying a basal tension of 3 mN, which corresponds to the  
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11 load applied in the organ culture experiments.  
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17 Longitudinal force was measured by contracting the vessels with 60 mM KCl-containing HEPES  
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19 solution. Following each contraction, the vessels were relaxed in normal HEPES buffer. The  
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21 portal veins were then stimulated with a single concentration of 5-HT (10 µM). To induce  
22  
23 maximal contractions by irreversible myosin light chain phosphorylation, calyculin A (1 µM) was  
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25 added in calcium-free HEPES solution at the end of the experimental protocol.  
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### 29 30 *Intracellular calcium measurements* 31

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34 Portal veins were cut open and mounted as sheets on a nylon mesh with silk sutures and placed  
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36 immersed in HEPES solution on the bottom of a glass culture dish (Mat Tek Corporation, MA,  
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38 USA). After that the vessels were incubated with Fluo-4 AM (10 µM; Invitrogen, Carlsbad, CA,  
39  
40 USA) at room temperature for 90 min. Subsequently the portal veins were allowed to  
41  
42 equilibrate for an additional 40 min in HEPES solution to allow hydrolysis of the Fluo-4 AM.  
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44 Fluorescence was recorded at room temperature using a Zeiss Plan Neofluar 10X (N.A. 0.3) lens  
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46 in a Zeiss Pascal LSM 5 confocal system using excitation at 488 nm and emission at 505 nm.  
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### 51 52 *Quantitative real-time PCR (qRT-PCR)* 53 54 55 56 57 58 59 60

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3 MicroRNAs and mRNAs were isolated using Qiagen miRNeasy mini kit (Qiagen, #217004) and  
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5 reverse transcribed using miScript II RT kit (Qiagen, #218161) as previously described  
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8 (Turczynska et al., 2013a). Relative expression of miRNAs was analyzed by quantitative real-time  
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10 PCR (StepOnePlus™ Real-Time PCR System, Applied Biosystems) using miScript Primer assays  
11  
12 (Mm\_miR-145\_1, # MS00001631; Mm\_miR-143\_1, # MS00001617; Hs\_SNORD95\_11 #  
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14 MS00033726) and miScript SYBR Green PCR Kit (Qiagen #218076). Relative expression of mRNAs  
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16 was analyzed using QuantiTect Primer Assays (Mm\_Myh11\_1, # QT01060843;  
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18 Mm\_Tpm1\_1\_SG, # QT00137354; Mm\_Des\_1\_SG, # QT00102333, Mm\_Cnn1\_1\_SG, #  
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20 QT00105420) and Quanti-Fast SYBR green RT PCR kit (Qiagen # 204156). Primer sequences are  
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22 proprietary of Qiagen.  
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### 29 *Protein extraction and western blotting*

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32 Portal veins frozen in acetone-TCA were thawed and washed thoroughly in acetone-DTT (10  
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34 mM) to remove any trace of TCA. Samples were then lyophilized for at least 6h and proteins  
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36 were extracted in 2% SDS buffer overnight at room temperature with gentle shaking. Equal  
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38 amounts of protein were loaded in each lane of Bio-Rad TGX Criterion gels. Proteins were then  
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40 transferred either overnight or using semi-dry transfer for 10 min in the Trans-Blot Turbo  
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42 system (Bio-Rad). Membranes were cut horizontally and strips were then incubated with  
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44 primary antibodies. The following primary antibodies were used: desmin (#4024, Cell Signaling  
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46 Technology; 1:1000), calponin (#ab46794, Abcam; 1:1000), SM22 (#ab14106, Abcam; 1:2000),  
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48  $\alpha$ -actin (#A5228, Sigma; 1:2000) ; Ca<sub>v</sub>1.2 (# ACC-003, Alomone Labs; 1:500),  $\beta$ -actin (#A5441,  
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50 Sigma; 1:5000), myocardin (#ab22073, Abcam; 1:1000 ), DREAM (#05-756, Upstate; 1:1000).  
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3 HRP-conjugated (#7074 and 7076, Cell Signaling Technology; 1:5000) or fluorescently labeled  
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5 DyLight800 and DyLight680 secondary antibodies (# 5257, 5366, 5470, and 5151, Cell Signaling  
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7 Technology; 1:5000) were used, and images were acquired using the LI-COR Odyssey Fc  
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9 instrument (LI-COR Biosciences) and analyzed using Image Studio software (LI- COR  
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11 Biosciences). Molecular weight markers were used to identify protein bands and specificity for  
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13 the Ca<sub>v</sub>1.2 antibody was further validated using a blocking peptide (Alomone Labs).  
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### 17 18 19 *Morphometry analysis*

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22 Portal veins were fixed in 4% paraformaldehyde in calcium-free PBS for 1h at room  
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24 temperature, then embedded in O.C.T compound (Tissue-Tek, Sakura Finetek) and sectioned  
25  
26 using a cryostat (10 µm sections). Sections were first washed twice with PBS, permeabilized for  
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28 15 min with 0.2% Triton X-100 in PBS, blocked in 2% bovine serum albumin (BSA) with 0.01%  
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30 Triton X-100 for 1 h, and then stained for G-actin (20 µg/ml and Alexa Fluor 488-DNase I,  
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32 Invitrogen, #D12371) and F-actin (5 µg/ml Alexa Fluor 633-phalloidin; Invitrogen, #A22284) in  
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34 blocking buffer for 1 h. Tissue sections were then washed 6 times for 5 minutes and mounted  
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36 with Aqua poly mount (Polysciences, Inc.). Slides were examined using a Zeiss LSM Pascal  
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38 confocal microscope as described previously (Albinsson et al., 2004). Images were analyzed  
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40 using the Zeiss LSM 510 Pascal analysis software. Media thickness was analyzed using Zeiss LSM  
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42 510 Pascal analysis software. For each vessel the mean of five measurements was used.  
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### 50 51 *Statistics*

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54 Values are presented as mean ± S.E.M. WT values were computed from the mean of each set of  
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56 experiments. Except as noted, p-values were generated by two-tailed Student's t-test for single  
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3 comparisons, and by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc  
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5 testing for multiple comparisons. For comparison of loaded vs. unloaded WT and KO vessels,  
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7 two-way ANOVA with Bonferroni post-hoc testing was used. Statistical analysis was performed  
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9 using GraphPad Prism 5 (GraphPad Software Inc.).  $p < 0.05$  was considered statistically  
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11 significant. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; non significant (n.s.),  $p > 0.05$ .  
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For Peer Review

## Results

### Deletion of microRNA-143/145 results in reduced stretch-dependent contractile differentiation

To confirm loss of miR-143/145 in portal vein, we measured miRNAs using quantitative real time PCR (qRT-PCR). As expected, we found a 50% loss of miR-143/145 in heterozygous mice while the homozygous miR-143/145 KO mice demonstrated a complete lack of these miRNAs (Figure 1A-B).

Dicer-dependent miRNAs are crucial for contractile differentiation induced by mechanical stretch in the portal vein (Turczynska et al., 2012). In order to clarify the specific importance of miRNA-143/145 in this response, we stretched the portal vein from WT and miR-143/145 KO mice using gold weights and incubated the vessels for 24 hours in organ culture. There was a 30 % lower miR-145 expression in WT mice following culture under load for 24 h relative to fresh tissue (Fig. 1C). Previous studies in rat portal vein have indicated that 24 h of culture has no significant effect on the expression of the smooth muscle marker SM22 at the protein level (Zeidan et al., 2003). All comparisons between WT and miR-143/145 KO mice reported in the present study are based on either freshly dissected or cultured vessels under identical conditions for both genotypes.

Four smooth muscle markers were tested using qRT-PCR; myosin heavy chain (*Myh11*; Figure 1D), tropomyosin (*Tpm1*; Figure 1E), desmin (*Des*, Figure 1F) and calponin (*Cnn1*; Figure 1G). As expected we found that stretch promotes the expression of these genes in the WT portal veins.

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3 In miR 143/145 KO portal veins, smooth muscle marker expression was markedly reduced and  
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5 no significant differences in mRNA expression levels between loaded and unloaded vessels were  
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7 found, contrasting with the WT results. These results suggest that stretch-induced potentiation  
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9 of smooth muscle marker expression is attenuated following deletion of miR 143/145.  
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### 14 **L-type calcium channel expression is reduced in miR-143/145 KO portal veins**

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18 Stretch-induced contractile differentiation of the portal vein is dependent on calcium influx via  
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20 L-type calcium channels (Ren et al., 2010). Moreover, loss of stretch-induced contractile  
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22 differentiation in *Dicer* KO portal veins is associated with a reduced expression of L-type calcium  
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24 channels, and transient transfection with a miR-145 inhibitor in cultured vascular smooth  
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26 muscle cells mimicked this effect (Turczynska et al., 2012).  
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31 In order to investigate the long term effects of miR-143/145 deficiency on L-type calcium  
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33 channels, we analyzed their expression levels in portal veins. At both mRNA (Figure 2A;  
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35 *Cacna1c*) and protein (Figure 2B; Ca<sub>v</sub>1.2) levels the pore-forming subunit of L-type calcium  
36  
37 channels was significantly less expressed in miR-143/145 KO than in WT portal veins. The effect  
38  
39 of the altered channel expression on intracellular calcium regulation was investigated by  
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41 determination of the increase in Fluo-4 fluorescence upon membrane depolarization by 60 mM  
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43 KCl. The peak fluorescence response relative to baseline was reduced by about 40% in miR-  
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45 143/145 KO as compared with WT portal veins (Figure 2 C).  
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51 This reduced L-type calcium channel expression was associated with increased expression of a  
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53 transcriptional repressor of *Cacna1c* called downstream regulatory element antagonistic  
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55 modulator (DREAM, aka. calsenilin/KChIP3, Figure 2 D). We also investigated the expression of a  
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3 direct target of miR-145 that is known to be associated with expression of L-type calcium  
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5 channels, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II  $\delta$  (CaMKII $\delta$ ), which has been suggested  
6  
7 to activate nuclear translocation of DREAM and thereby repress L-type calcium channel  
8  
9 expression (Ronkainen et al., 2011). CaMKII $\delta$  expression was found to be increased in miR-  
10  
11 143/145 KO vs. WT portal veins (Figure 2 E).  
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### 16 17 **The amplitude of spontaneous contractions is reduced in miR-143/145 KO mice**

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20 Similar to small arteries, a myogenic response is stimulated in the portal vein following an  
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22 increase in wall stress (Johansson and Mellander, 1975). The portal vein exhibits spontaneous  
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24 contractile activity, which is dependent on a fluctuating membrane potential and triggered by  
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26 activation of voltage-dependent L-type calcium channels in the cell membrane (Spencer and  
27  
28 Greenwood, 2003). An oscillating contractile activity, called vasomotion, is also observed in  
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30 pressurized arteries under certain conditions (Osol and Halpern, 1988, Gustafsson et al., 1994),  
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32 which could be an important factor for stretch-sensitive contractile differentiation and  
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34 remodeling in the vascular wall. Vasomotion and spontaneous activity may not be directly  
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36 regulated by L-type calcium channel activity but calcium influx through voltage-gated channels  
37  
38 is essential for this phenomenon to occur (Gustafsson et al., 1994, Spencer and Greenwood,  
39  
40 2003). To investigate if the loss of L-type calcium channel expression seen in miR-143/145 KO  
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42 mice is sufficient to affect smooth muscle contractility we analyzed longitudinal force  
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44 development in the portal vein.  
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54 Portal veins from WT and miR-143/145 KO mice were mounted in a myograph. Force generated  
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56 during 5 minutes of spontaneous activity was reduced in miR-143/145 KO vessels although the  
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3 frequency of contractions was unchanged (figure 3A-D). Similar to spontaneous contractions,  
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5 the contractile response to depolarization by 60 mM KCl was dramatically reduced in miR-  
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8 143/145 KO portal veins (Figure 3E) while calcium-independent contractions elicited by the  
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10 phosphatase inhibitor Calyculin A were somewhat less affected (Figure 3F). Furthermore, the  
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12 peak contractile response to 5-HT, which is largely dependent on calcium release from  
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14 intracellular stores and only weakly inhibited by nifedipine (Turczynska et al., 2012) was not  
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16 significantly reduced in miR-143/145 KO mice (Figure 3G). Together with the reduced  
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18 intracellular calcium response (Figure 2C), these results indicate that the effect of miR-143/145  
19  
20 deletion depends both on reduced calcium influx via voltage-gated channels and on decreased  
21  
22 ability to generate force, via altered function of the contractile machinery and/or via decreased  
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24 smooth muscle mass.  
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### 32 **Reduced smooth muscle mass and expression of contractile markers in miR-143/145 KO portal** 33 34 **vein** 35

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38 To identify which factors other than L-type calcium channel expression that could be involved in  
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40 the loss of contractile force in the portal vein we investigated the expression of contractile  
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42 proteins, basal actin polymerization and wall thickness.  
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47 Previous studies have suggested that genetic deletion of miR-143/145 results in phenotypically  
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49 modified smooth muscle cells (Boettger et al., 2009, Elia et al., 2009). However, conflicting  
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51 results have been reported concerning the expression of contractile smooth muscle markers in  
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53 these mice, and different effects of miRNA KO may be observed in different vascular beds. To  
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55 test if the reduced contractile function could be dependent on loss of contractile marker  
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3 proteins, we performed western blot analysis of WT and miR-143/145 KO mice. The expression  
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5 levels of the actin binding protein SM22 (Figure 4A),  $\alpha$ -actin (Figure 4B) and myosin light chain  
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7 kinase (MLCK; Figure 4C) were slightly but significantly reduced in KO portal veins, whereas  
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9 there was no significant reduction in calponin expression (Figure 4D). The transcriptional co-  
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11 activator myocardin was significantly decreased in miR-143/145 KO portal veins (Figure 4E),  
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13 possibly mediating a general effect of this deletion on the expression of several SRF-dependent  
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15 smooth muscle marker proteins.  
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21 MiR-145 has previously been suggested to regulate actin polymerization in smooth muscle (Xin  
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23 et al., 2009, Albinsson et al., 2010) and actin dynamics is known to affect stretch-sensitivity of  
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25 the portal vein. We thus investigated the effect of miR-143/145 KO on actin polymerization in  
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27 portal vein smooth muscle using fluorescently labeled probes. Neither F- nor G-actin levels were  
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29 significantly decreased in KO portal veins, and there was no difference in F/G-actin ratio (Figure  
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31 4 F-H, K). On the other hand, the thickness of the longitudinal smooth muscle layer was  
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33 significantly smaller in miR-143/145 KO vs. WT portal veins (Fig. 4 I,K)  
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## Discussion

In order to clarify the underlying mechanisms in vascular disease states it is important to understand how mechanical factors can affect smooth muscle phenotype and function. Using *Dicer* KO mice, we have recently identified essential roles for miRNAs in maintaining myogenic reactivity (Bhattachariya et al., 2014) and stretch-induced contractile differentiation (Turczynska et al., 2012) in the vascular wall. Furthermore, earlier studies have suggested that several of the defects in *Dicer* KO smooth muscle are mediated by loss of the highly expressed miRNA, miR-145 (Turczynska et al., 2012, Albinsson et al., 2010, Albinsson and Sessa, 2010), but the importance of this miRNA for stretch-induced effects in smooth muscle has not been directly investigated previously.

In the present study, we investigated the importance of miR-143/145 for stretch-induced smooth muscle differentiation and the specific role of these miRNAs for the regulation of underlying mechanisms that control contractile differentiation in the vascular wall. We found that genetic ablation of the miR-143/145 cluster results in reduced stretch-dependent contractile differentiation, which was associated with a decreased expression of L-type calcium channels and a diminished spontaneous activity. While the miR-143/145 KO mouse is constitutive and global, the *Dicer* KO mouse that we have used previously is inducible and smooth muscle specific. Although this difference must be taken into consideration, it is still clear that deletion of the miR-143/145 cluster results in less severe effects on both basal and stretch-dependent smooth muscle differentiation compared to loss of all smooth muscle miRNAs. This is in accordance with the fact that other smooth muscle miRNAs than miR-143/145 are essential

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3 for embryonic development of the vasculature since both the constitutive and inducible smooth  
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5 muscle *Dicer* KO is lethal, while the miR-143/145 KO appears to have a normal life span  
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8 (Boettger et al., 2009).  
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11 The portal vein has several important features that are advantageous for its use as a model to  
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13 investigate stretch-induced differentiation, including phasic contractile activity and myogenic  
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15 responsiveness. These characteristics can under certain conditions also be found in small  
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17 resistance arteries and arterioles. However, it is not straightforward to compare the  
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19 longitudinally loaded portal vein to a pressurized myogenically active vessel lacking vasomotion.  
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21 Vasoconstriction in response to pressure tends to counteract increased circumferential wall  
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23 stress, although longitudinal stress will increase with pressure in any vessel that like the portal  
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25 vein is not supported longitudinally by extravascular tissue. The tissue mechanics of  
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27 myogenically active small arteries thus complicates investigations into the molecular basis of  
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29 pressure-induced remodeling, although possible differences at the cellular level relevant for the  
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31 comparison of the portal vein with arterial smooth muscle should be investigated further.  
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33 Furthermore, it is possible the organ culture in itself affects the responsiveness of portal veins in  
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35 our model and it is difficult to predict if this effect may influence the results reported herein.  
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45 As shown in *Dicer* KO, stretch-induced contractile differentiation in the portal vein is nearly  
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47 abolished in the absence of smooth muscle miRNAs (Turczynska et al., 2012). We can now  
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49 report that part of the effect of *Dicer* deletion on stretch-induced contractile differentiation is  
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51 due to loss of the miR-143/145 cluster. Deletion of these miRNAs resulted in a decreased,  
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53 although possibly not abolished, stretch-dependent transcription of several contractile smooth  
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55 muscle markers including myosin heavy chain, tropomyosin, calponin and desmin. While desmin  
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3 is a component of intermediate filaments in smooth muscle, the other proteins are actin binding  
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5 and involved in contractile function of smooth muscle cells. However, all of the genes encoding  
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7 these proteins are known to be regulated by serum response factor and its cofactors myocardin  
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9 and MRTF (Miano et al., 2007). The activity of MRTF is known to be dependent on nuclear  
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11 translocation, which in turn is regulated by actin polymerization (Miralles et al., 2003). The  
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13 effects of stretch on smooth muscle stimulate this pathway by activating the small GTPase Rho  
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15 in the cell membrane and increasing actin polymerization (Albinsson et al., 2004). While there  
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17 was a large and significant difference in expression between loaded and unloaded WT vessels  
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19 for all genes examined, differences in KO vessels were considerably smaller and not significantly  
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21 different from zero. This may partly reflect the lower expression levels in KO mice and we  
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23 cannot rule out that possible stretch dependence of myocardin expression or actin  
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25 polymerization may confer stretch sensitivity to KO vessels. However, any such effect is not  
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27 sufficient to rescue the phenotype from miR 143/145 deletion.  
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36 Mechanical stretch of the portal vein enhances spontaneous activity and thus promotes calcium  
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38 influx via L-type channels (Johansson and Mellander, 1975). Subsequent activation of the Rho  
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40 signaling pathway and contractile differentiation of smooth muscle can be stimulated by  
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42 depolarization and activation of L-type calcium channels (Wamhoff et al., 2004), leading to  
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44 increased myocardin expression and actin polymerization. On the other hand, calcium influx via  
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46 store-operated calcium channels promotes smooth muscle proliferation and the expression of  
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48 growth-related genes (Ren et al., 2010, Golovina et al., 2001). In agreement with this hypothesis,  
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50 stretch-induced contractile differentiation in the portal vein is sensitive to inhibition by L-type  
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3 calcium channel blockers while stretch-sensitive MAP-kinase phosphorylation and global protein  
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5 synthesis is inhibited by blockers of store-operated channels (Ren et al., 2010).  
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9 Since miRNAs generally target multiple mRNAs it is likely that several factors are responsible for  
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11 the reduced stretch-induced differentiation in miR-143/145 KO mice. However, we have  
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13 identified at least one possible mechanism in the regulation of L-type calcium channels by miR-  
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15 145. In a previous study we found that transfection of cultured smooth muscle cells with miR-  
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17 145 inhibitor reduced *Cacna1c* expression similar to *Dicer* KO (Turczynska et al., 2012).  
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19 Naturally, it must be taken under consideration that transient transfection of cultured cells is an  
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21 artificial condition and the effects may be different in the *in vivo* situation. However, we show  
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23 herein that constitutive miR-143/145 KO results in reduced expression of L-type calcium  
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25 channels both at the protein and mRNA level. A functional consequence of this decrease in  
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27 channel expression was demonstrated to be a decreased elevation of intracellular calcium in  
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29 response to membrane depolarization in miR-143/145 KO portal vein. Taken together, the  
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31 results obtained here suggest that deletion of miR-143/145 results in a decrease of voltage-  
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33 dependent calcium influx, which may influence gene expression including its stretch  
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35 dependence, partly by virtue of the effects of stretch on membrane ion channel activity and its  
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37 downstream signaling pathways.  
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48 One possible mechanism for the effect of miR-143/145 deletion on L-type channel expression is  
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50 an increased expression of the transcriptional inhibitor DREAM. In cardiomyocytes, an increase  
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52 in DREAM expression can reduce L-type channel expression (Ronkainen et al., 2011) and in the  
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54 portal vein we found that deletion of miR-143/145 results in an increased expression of this  
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3 protein. The cause of the increased DREAM expression in miR-143/145 KO is yet unknown but  
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5 likely involves an indirect effect since neither miR-143 nor miR-145 are predicted to target  
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7 DREAM mRNA. However, DREAM is known to be activated by CaMKII $\delta$ , a known target for miR-  
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9 145 (Cordes et al., 2009) and in the present study we were also able to detect an increase in  
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11 CaMKII $\delta$  expression. Further evidence is however needed to establish this mechanism and its  
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13 generality in vascular smooth muscle.  
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19 In addition to the effect on mechanosensing, a reduced calcium channel expression is expected  
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21 to result in diminished contractile function. However, contractile function is dependent on  
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23 several factors other than L-type channel expression such as the organization and amount of  
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25 contractile filaments, the activation of calcium sensitization and the cross sectional area of the  
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27 vessel to name a few. In order to differentiate between calcium dependent and -independent  
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29 effects of miR-143/145 KO, we used the phosphatase inhibitor calyculin A in nominally calcium-  
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31 free solution. The effect of miR-143/145 KO on contractile force induced by depolarization was  
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33 more prominent than the effect on calyculin A-induced force, suggesting that part of the effect  
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35 of miR-143/145 deletion is due to altered extracellular calcium influx, which may depend on the  
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37 reduced L-type calcium channel expression. However, although the effect was smaller, calyculin  
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39 A-induced contractile force was still significantly decreased in miR-143/145 KO portal veins,  
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41 which indicates that part of the reduced KCl-induced force and spontaneous activity is due to  
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43 factors not directly related to the role of calcium in activation of contraction, such as reduced  
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45 wall thickness, reduced amount of contractile proteins and altered contractile filament  
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47 organization. In support of this we found a reduced media thickness of the longitudinal smooth  
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3 muscle layer and a slightly decreased protein expression of  $\alpha$ -actin, MLCK and the actin binding  
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5 protein SM22.  
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9 No effect was observed on the basal F/G-actin ratio although we and others have previously  
10 demonstrated that miR-145 can regulate this process in cultured smooth muscle cells (Albinsson  
11 et al., 2010, Xin et al., 2009). In the present study, actin polymerization was determined from  
12 fixed sections of fresh tissue, and hence the degree of actin polymerization seen here may  
13 approximate conditions in the living animal with physiological portal venous pressure. We  
14 cannot however exclude that stimulation of actin polymerization by stretch is affected by miR-  
15 143/145 deletion in the portal vein. It is notable that myocardin expression, which is not  
16 dependent on actin polymerization (Turczynska et al., 2012), was reduced in the KO vessels,  
17 possibly as a result of decreased L-type channel activity (Wamhoff et al., 2004, Ren et al., 2010).  
18 This may be one reason for the lower expression of smooth muscle markers in the KO.  
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35 The reduction in smooth muscle mass (~32%) is comparable with the decrease in calyculin A-  
36 induced force (~35%) and this effect alone can therefore explain the decreased contractile  
37 response to calyculin A in miR-143/145 KO portal veins. Since mechanical stimulation is an  
38 important factor for normal growth and differentiation of vascular smooth muscle, one can  
39 speculate that the reduced wall thickness of miR-143/145 KO mice may be due to a reduced  
40 stretch sensitivity during vascular development. Interestingly, peak contractile responses to 5-  
41 HT, which is largely dependent on calcium release from intracellular stores, were not  
42 significantly decreased in miR-143/145 KO portal vein. This effect is similar to what we have  
43 previously observed in *Dicer* KO portal veins (Turczynska et al., 2012) and may depend on  
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3 compensatory mechanisms in miR-143/145 KO smooth muscle, such as increased intracellular  
4 calcium stores or an increase in store operated calcium influx. In fact, miR-143/145 KO smooth  
5 muscle cells contain more rough endoplasmic reticulum (Boettger et al., 2009, Xin et al., 2009),  
6 which has been suggested to be involved in the activation of store operated calcium entry (Lur  
7 et al., 2009).  
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17 In summary, the results of this study suggest that miR-143 and miR-145 are important  
18 regulators of stretch-induced contractile differentiation and that this effect may be mediated  
19 via decreased L-type calcium channel expression. Furthermore, the effect on L-type calcium  
20 channels likely contributes to the reduced contractile function in response to depolarization and  
21 spontaneous activity in the portal vein. The results of this study may have important  
22 implications for therapeutic targeting of the miR-143/145 cluster in vascular disease.  
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### 43 **Acknowledgements**

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47 This work was supported by the Swedish Research Council; the Swedish Heart and Lung  
48 Foundation, the Crafoord Foundation; the Royal Physiographic Society; the Åke Wiberg  
49 Foundation; the Jeansson Foundation; the Tore Nilson Foundation; the Greta and Johan Kock  
50 Foundation; the Magnus Bergvall Foundation and the Lars Hierta Memorial Foundation. A.B  
51 were supported by the European Union FP7 Marie Curie Initial Training Network Small Artery  
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3 Remodeling (SmArt). We thank Dr. Mario Grossi for help with the immunofluorescence and Ina  
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6 Nordström for assistance with western blotting.  
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13 **Conflict of Interest:** none declared.  
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For Peer Review

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## Figure legends

**Figure 1: Deletion of microRNA-143/145 causes reduced stretch-dependent contractile differentiation.** Deletion of miR-143/145 was confirmed using qRT-PCR (A-B). All data were normalized to U6 (WT: n=4, Het: n=3, KO: n=5 ). To evaluate effect of stretch, portal veins were either loaded (lo, 0.3 g weight) or left unloaded (unlo), and organ cultured for 24h. The effect of culture on miR-145 expression was evaluated in stretched vessels and compared to fresh, non-cultured veins (C). Expression of the smooth muscle markers myosin heavy chain (*Myh11*, D), tropomyosin (*Tpm1*, E), desmin (*Des*, F) and calponin-1 (*Cnn1*, G) were determined using qRT-PCR. All data were normalized to 18S (WT: n=3, KO: n=4-5). In panels D-G, two-way ANOVA was used to analyze effects of load and genotype. For all four genes, differences in expression between KO and WT were significant ( $p < 0.05$ ). 95% confidence intervals for the difference between loaded and unloaded vessels were: *Myh11*: WT [0.07, 1.10], KO [-0.50, 0.84], *Tpm1*: WT [0.41, 2.79], KO [-0.83, 1.43], *Des*: WT [3.38, 6.39], KO [-0.26, 2.21], *Cnn1*: WT [0.05, 2.21], KO [-0.36, 1.42]. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; non significant (n.s.),  $p > 0.05$ .

**Figure 2: Expression of pore forming subunit of L-type voltage dependent calcium channel is reduced in miR-143/145 KO portal vein.** Expression of the pore-forming subunit of L-type calcium channels was measured at both mRNA (A) and protein (B) level (WT: n=7, KO: n=8). For qRT-PCR, 18S was used as an internal control. Peak stimulated/basal Fluo-4 fluorescence of intact portal veins stimulated with 60 mM KCl (C; WT, KO: n=4). Expression of the transcriptional repressor DREAM (D; WT, KO: n=4) and CaMKII $\delta$  (E; WT: n=6, KO: n=5) was measured with western blotting. The original blot for  $\beta$ -actin is identical in B and D since the same samples

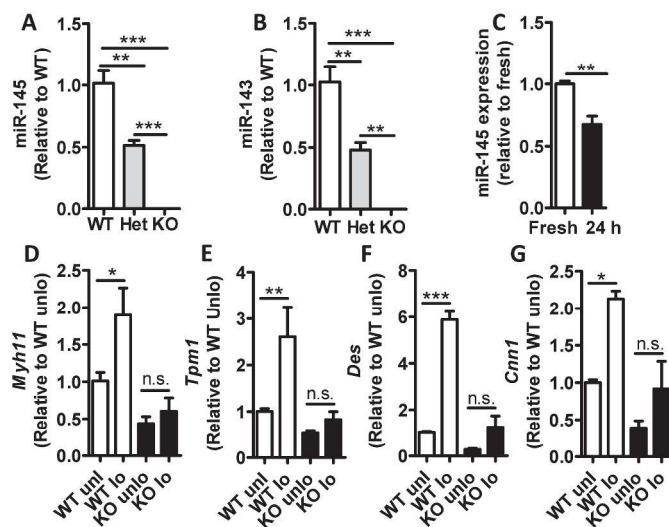


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3 were used for the original blots of Ca<sub>v</sub>1.2 and DREAM. Since CaMKIIδ is a known target of miR-  
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5 145, up-regulation in the KO was tested using one-tailed Student's t-test. \*, p<0.05; \*\*, p<0.01.  
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9 **Figure 3: Amplitude but not frequency of spontaneous activity is reduced in miR-143/145 KO**  
10 **portal vein.** Spontaneous activity was measured using isometric force recording. A  
11 representative original recording is shown for WT (A) and KO (B). The integrated force of  
12 spontaneous activity (C), the frequency spontaneous contractions (D; p=0.794), integrated KCl-  
13 induced force (E), integrated Calyculin A-induced force (F) and peak 5-HT induced force (G;  
14 p=0.135) was evaluated using Lab Chart 7 (WT: n=5, KO n=8). \*, p<0.05; \*\*\*, p<0.001; non  
15 significant (n.s.), p>0.05.  
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28 **Figure 4: Decreased smooth muscle mass and reduced expression of some contractile markers**  
29 **in miR-143/145 KO mice.** Expression of SM22α (A; WT: n=10, KO: n=11), α-actin (B; WT: n=10,  
30 KO: n=11), MLCK (C; WT: n=5, KO: n=4), calponin (D; WT: n=10, KO, n=11; p=0.320) and  
31 myocardin (E; WT, KO: n=5) was measured with western blotting. Filamentous (F-) actin (F;  
32 p=0.174), globular (G-) actin (G; p=0.096), F/G-actin ratio (H; p=0.692) and media thickness of  
33 the longitudinal smooth muscle layer (I) were determined from portal vein sections stained with  
34 Alexa Fluor 633-phalloidin (red) and Alexa Fluor 488-DNase I (green) for (F-) and (G-) actin,  
35 respectively (K). On average 80 images from 4 different animals of both genotypes were  
36 analyzed using the Zeiss LSM 510 Pascal analysis software. \*, p<0.05; \*\*\*, p<0.001; non  
37 significant (n.s.), p>0.05.  
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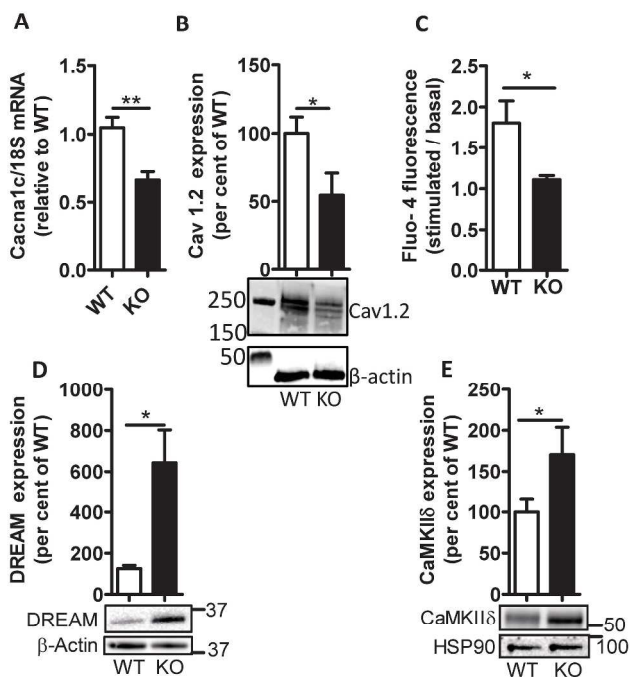
Figure 1



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Figure 2



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Figure 3

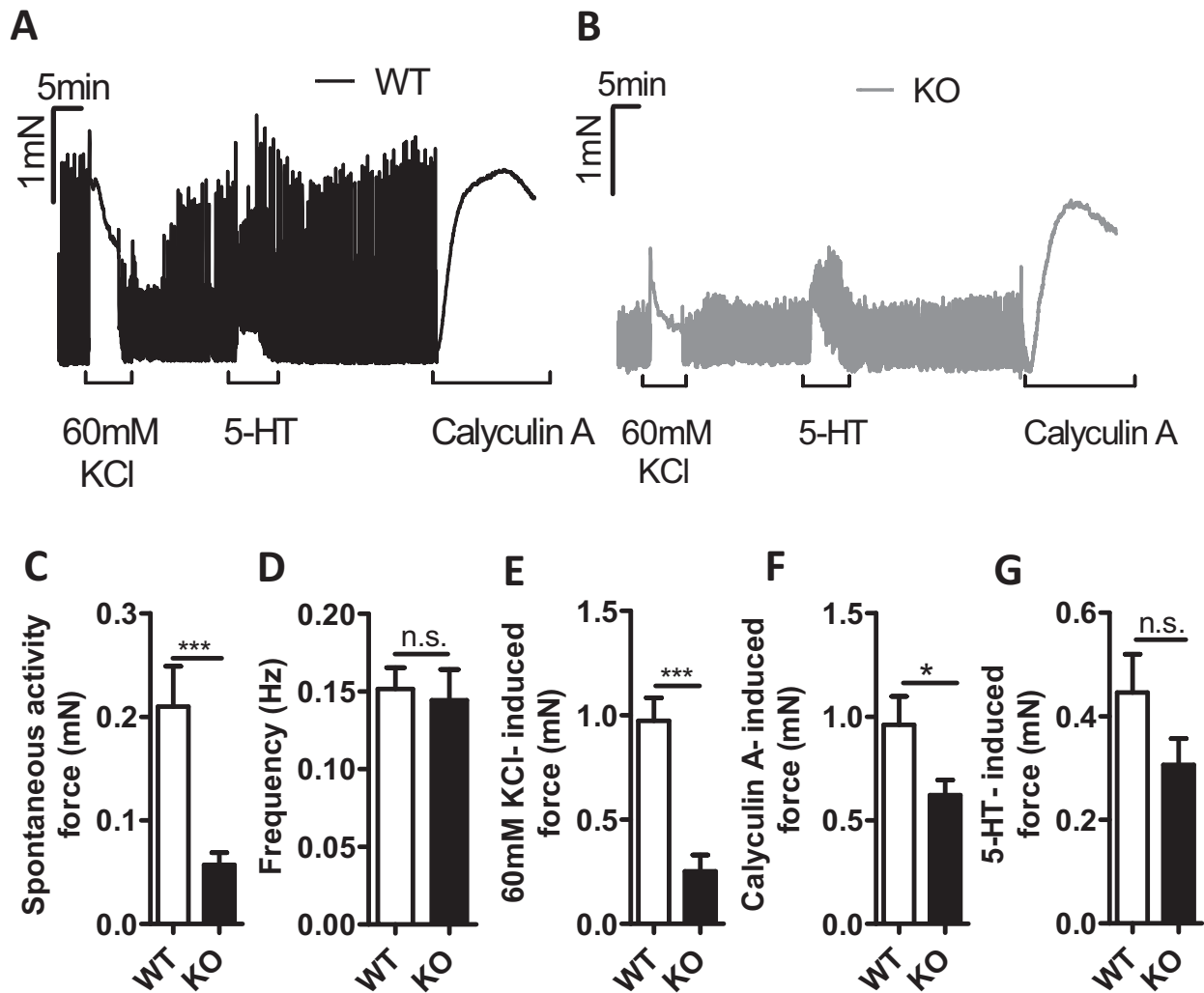


Figure 4

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