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Alajbegovic, Azra; Turczynska, Karolina; Hien Tran, Thi; Cidad, Pilar; Swärd, Karl; Hellstrand, Per; Corte, Alessandro Della; Forte, Amalia; Albinsson, Sebastian

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Regulation of microRNA expression in smooth muscle by MRTF-A and actin polymerization

Azra Alajbegovic[a], Karolina M. Turczyńska[a], Tran Thi Hien[a], Pilar Cidad[b], Karl Swärd[a], Per Hellstrand[a] Alessandro della Corte[c], Amalia Forte[d] and Sebastian Albinsson[a]

[a] Department of Experimental Medical Science, Lund University, Lund, Sweden.

[b] Departamento de Bioquímica y Biología Molecular y Fisiología and Instituto de Biología y Genética Molecular (IBGM), Universidad de Valladolid and Consejo Superior de Investigaciones Científicas (CSIC), Valladolid, Spain

[c] Departments of Cardiothoracic Sciences, and [d] Experimental Medicine, Second University of Naples, Naples, Italy

Correspondence and proofs to:

Dr. Sebastian Albinsson

Department of Experimental Medical Science

Lund University

BMC D12

SE-221 84 Lund, Sweden

E-mail: sebastian.albinsson@med.lu.se

Tel: +46-46-2227765

Fax: +46-46-2113417

E-mail: sebastian.albinsson@med.lu.se

Abstract

The dynamic properties of the actin cytoskeleton in smooth muscle cells play an important role in a number of cardiovascular disease states. The state of actin does not only mediate mechanical stability and contractile function but can also regulate gene expression via myocardin related transcription factors (MRTFs). These transcriptional co-activators regulate genes encoding contractile and cytoskeletal proteins in smooth muscle. Regulation of small non-coding microRNAs (miRNAs) by actin polymerization may mediate some of these effects. MiRNAs are short non-coding RNAs that modulate gene expression by post-transcriptional regulation of target messenger RNA.

In this study we aimed to determine a profile of miRNAs that were 1) regulated by actin/MRTF-A, 2) associated with the contractile smooth muscle phenotype and 3) enriched in muscle cells. This analysis was performed using cardiovascular disease-focused miRNA arrays in both mouse and human cells. The potential clinical importance of actin polymerization in aortic aneurysm was evaluated using biopsies from mildly dilated human thoracic aorta in patients with stenotic tricuspid or bicuspid aortic valve.

By integrating information from multiple qPCR based miRNA arrays we identified a group of five miRNAs (miR-1, miR-22, miR-143, miR-145 and miR-378a) that were sensitive to actin polymerization and MRTF-A overexpression in both mouse and human smooth muscle. With the exception of miR-22, these miRNAs were also relatively enriched in striated and/or smooth muscle containing tissues. Actin polymerization was found to be dramatically reduced in the aorta from patients with mild aortic dilations. This was associated with a decrease in actin/MRTF-regulated miRNAs.

In conclusion, the transcriptional co-activator MRTF-A and actin polymerization regulated a subset of miRNAs in smooth muscle. Identification of novel miRNAs regulated by actin/MRTF-A may provide further insight into the mechanisms underlying vascular disease states, such as aortic aneurysm, as well as novel ideas regarding therapeutic strategies.

Keywords
Vascular disease, smooth muscle, phenotype, actin polymerization, MRTF-A, microRNA

Abbreviations

miRNA/miR, microRNA; MRTF, myocardin related transcription factor; SMC, smooth muscle cell; HCASMC, human coronary artery smooth muscle cell; mAoSMC, mouse aortic smooth muscle cell; G-actin, globular actin; F-actin, filamentous actin; SRF, serum response factor; TGF-β, transforming growth factor beta; BAV, bicuspid aortic valve; TAV, tricuspid aortic valve; DON, donors; ACTA2, alpha smooth muscle actin; UTR, untranslated region; PGC-1β, peroxisome proliferator-activated receptor gamma coactivator 1-beta; HKG, housekeeping gene; MAPK, mitogen-activated protein kinase; MECP2, methyl-CpG binding protein 2; KChIP3, potassium channel interacting protein 3; CamKIIδ, calmodulin-dependent protein kinase II delta.

1 Introduction

Calcium signaling is an important mechanism for regulation of smooth muscle function as it controls smooth muscle contractility via calcium/calmodulin and activation of myosin light chain kinase [1]. Interestingly, calcium influx via voltage gated, L-type calcium channels can, together with other stimuli, promote the activation of the Rho/Rho-kinase pathway [2-6]. Rho-kinase activation subsequently results in both calcium sensitization and increased polymerization of actin filaments [7]. Polymerization and stabilization of actin filaments by the Rho/Rho-kinase pathway involves inhibition of the actin depolymerizing cofilin as well as activation of the actin binding protein profilin [8]. In certain situations, these effects can cause a hypercontractile smooth muscle phenotype and lead to altered blood flow, increased vascular stiffening and vascular remodeling, which contribute to the development and progression of vascular disease.

Dynamic treadmilling of actin filaments is an essential mechanism in most cell types, and is directly involved in a variety of cellular processes including, cell migration, proliferation, cell adhesion and contractile function of muscle cells. In addition to these functions, actin dynamics can control SRF-dependent gene transcription via myocardin related transcription factors (MRTF aka MAL/MKL) [9, 10]. Unlike myocardin which is primarily expressed in smooth and cardiac muscle, the two MRTF isoforms MRTF-A and -B are ubiquitously expressed and their activity is controlled by a reduction of the monomeric G-actin pool which releases MRTF from binding to G-actin and thereby enables translocation to the nucleus where they act as co-factors for serum response factor (SRF) [9-11]. While MRTF-A is equally potent as myocardin in activating smooth muscle differentiation, MRTF-B appears to be less efficient in this process [12, 13].

The SRF/MRTF complex binds to promoter elements called CArG-boxes with the consensus sequence $CC[AT]_6GG$. These are present in many genes associated with smooth muscle contraction and migration [14]. The regulation of MRTF by actin polymerization allows for a precise titration of contractile/cytoskeletal gene expression in response to cellular demand. This mechanism is involved in growth and/or contractile differentiation of smooth muscle cells in response to a number of stimuli including mechanical stretch, sphingosine-1-phosphate and TGF-beta stimulation [15-18]. Increasing evidence also points towards a role for actin

polymerization and MRTF activity in vascular disease states such as vascular injury [19], neointimal hyperplasia [20], aortic aneurysms [21], retinal disease [22], and stroke [23] [24]. Several of these diseases are known to be associated with a phenotypic shift of smooth muscle cells but the underlying mechanisms are not completely understood. Furthermore, MRTF activation is involved in migration in various cell types, affecting both wound healing and metastasis of cancer cells [25, 26]. These findings emphasize the importance to clarify the role of actin polymerization and MRTF for gene expression in vascular cells.

The regulation of protein-encoding genes by Rho-signaling, actin polymerization and MRTFs has been described previously [27-29]. However, some of the effects of MRTF-mediated transcription may also be due to regulation of non-coding RNAs such as microRNAs (miRNAs) and long-non-coding RNAs. Recent work has identified the first myocardin-sensitive long-non coding RNA called myoslid, which promotes contractile differentiation of smooth muscle cells [30]. Interestingly, the mechanism behind the effects of myoslid involves actin polymerization and nuclear translocation of MRTF-A. MiRNAs are endogenous non-coding RNAs that regulate gene expression post-transcriptionally through interaction with the 3´ untranslated region (UTR) of target messenger RNA (mRNA) resulting in decreased mRNA stability and/or inhibition of protein translation [31]. We have previously demonstrated the importance of Dicer-dependent miRNAs for the regulation of smooth muscle development, differentiation and normal function [32-34]. The role of MRTF-A and myocardin for transcriptional regulation of miRNAs has been reported in cardiomyocytes, where MRTF-A regulates the miR-143/145 cluster, which is known to be enriched in smooth muscle and promote contractile smooth muscle differentiation [35-39]. However, to our knowledge, the transcriptional regulation of miRNAs by MRTF-A and actin dynamics has not been investigated previously in vascular smooth muscle, although multiple lines of evidence points towards a crucial role of actin/MRTF in vascular disease.

Herein, we have identified a number of actin/MRTF-A-regulated miRNAs in mouse and human smooth muscle. Several of these miRNAs are known to be involved in cardiovascular disease states such as hypertension and aortic aneurysms. However, some of the miRNAs regulated by actin/MRTF-A have not yet been ascribed a specific function in smooth muscle.

2. Material and Methods

2.1. Human samples

Aortic biopsies from patients with mild dilation of thoracic ascending aorta (maximal aortic diameter ≤ 4.5 cm) were collected during cardiac valve replacements of either stenotic tricuspid (TAV) or bicuspid aortic valves (BAV). Biopsies from healthy aortas from heart transplant donors with negative personal and familial history of bicuspid valve and aortopathy were used as controls. Human renal arteries belonging to the COLMAH collection (HERACLES network) were collected and cultured as described previously [28]. The study was approved by the involved institutions' ethics committees and performed in accordance with the Declaration of Helsinki. All patients gave their informed consent prior to their inclusion.

2.2. Cell culture and treatments

Primary human coronary artery smooth muscle cells (HCASMC) were purchased from Gibco Life Technologies (#C-017-5C) and maintained in Medium 231 (Life Technologies, #M231500) supplemented with 5% smooth muscle growth supplement (Life Technologies, #S-007-25) and 50U/50 ug/ml penicillin/streptomycin (Biochrom, #A2212). Cell culture plates used for experiments were coated with 0.02% collagen (Sigma Aldrich, #G1393) to ensure cell attachment. Mouse aortic SMCs were isolated by enzymatic digestion and maintained in culture as described previously [28, 33]. Animal experiments were approved by the Malmö/Lund Ethical Committee on Animal Research and were carried out in accordance with the EU Directive 2010/63/EU for animal experiments. Human and mouse SMCs were used at passages 2-8 and 2-4 respectively. Media was changed every other day. For actin polymerization, cells were treated with 100 nM jasplakinolide (Tocris Bioscience, #2792) or equivalent volume of DMSO (Sigma Aldrich, # D5879), the last 24 h. Latrunculin B is an actin depolymerizing agent, inactivated by serum. Treating cells with this substance therefore required changing to serum-reduced media, 2% smooth muscle supplement in Medium 231, 24 h after seeding. After another 24 h, 250 nM latrunculin B (Calbiochem, # 76343-94-7), or DMSO as a vehicle control, were added to cells for 24 h.

MRTF-A overexpression was achieved using adenoviral constructs. 24 h after seeding, cells were

transduced with 100 MOI of Ad-MKL1/eGFP (Vector Biolabs, ADV-215499) and maintained in virus-containing media for 96 h. Ad-CMV-null (Vector Biolabs, #1300) was used as a control.

2.3 Quantitative RT-PCR

Homogenized human tissue and cultured cells were lysed in Qiazol (Qiagen). Total RNA was extracted using the Qiagen miRNeasy kit (#217004) in a QIAcube (Qiagen) according to the instructions of the manufacturer. RNA concentration and quality was determined with an ND-1000 spectrophotometer. 250-500 ng of RNA was reverse transcribed to cDNA using the miScript II RT kit (Qiagen, #218161). The relative miRNA expression was determined using the miScript SYBR Green PCR kit (Qiagen, #218076) and miScript Primer Assays (Qiagen): Mm_miR-1_2 (#MS00011004), Hs_miR-7_2 (# MS00032116), Mm_miR-22_1 (# MS00032305), Mm_miR-143_1 (# MS00001617), Mm_miR-145_1 (# MS00009331), Mm_miR-378_2 (#MS00032781), Hs_SNORD95_11 (# MS00033726). The mature miRNA sequences for indicated miRNAs are identical in human and mouse. Amplification was performed by real-time PCR (OneStepPlus qPCR cycler, Applied Biosystems).

2.4 miRNA PCR array

500 ng RNA was reverse transcribed to cDNA using the miScript II RT kit (Qiagen, #218161). The cDNA from several samples in each group was then pooled so that the value in the miRNA PCR array represents a mean of all samples in each experimental group. We then performed several arrays using different conditions to identify the group of miRNAs that were regulated in all conditions.. MiRNA expression profiling was performed using miScript PCR array for cardiovascular diseases (Qiagen, MIHS-113ZC or MIMS-113Z) according to the manufacturer's instructions. Selected miRNAs were then confirmed using individual qPCR-reactions in multiple samples. The miRNA expression was analyzed by PCR using the miScript SYBR Green PCR kit (Qiagen) in an OneStepPlus qPCR cycler (Applied Biosystems).

2.5 F/G-actin ratio measurement

Actin polymerization was evaluated by determining the globular (G-actin) and filamentous (F-actin) fractions using the G-actin/ F-actin In Vivo Assay Biochem kit (Cytoskeleton, Inc, BK037)

according to the manufacturer's instructions. Briefly, cells or homogenized tissue were lysed using the LAS02 lysis buffer, containing F-actin stabilizing reagents, ATP and protease inhibitor cocktail. The lysate was centrifuged at 100,000 x g using the Beckman ultracentrifuge for 1 h at 37 °C. The G-actin was found in the supernatant and was transferred to fresh tubes. F-actin was pelleted and dissolved in actin depolymerizing buffer for 1 h on ice. 5x Laemmli sample buffer was added to each of the pellet and supernatant samples to a final concentration of 1x (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). Equal volumes of F- and G-actin fractions were loaded in each lane on Bio-Rad Criterion TGX 4-15% gels followed by semi-dry transfer to nitrocellulose membranes using the Trans-Blot Turbo system (Bio-Rad). Protein detection was performed by immunoblotting using rabbit smooth muscle alpha-actin primary antibody (Cytoskeleton, # AAN01) and anti-rabbit HRP-conjugated secondary antibody (Cell Signaling, #7076 1: 5,000). Bands were detected by enhanced chemiluminescence (SuperSignal West Femto Maximum Senstitivity Substrate, Thermo Fisher Scientific) and images acquired in a Odyssey Fc Imager (LI-COR Biosciences)

2.6 Statistical analysis

All results are presented as means ±S.E.M. Significance was assessed using the Student's t-test or by one-way analyses of variance (ANOVA) followed by multiple comparisons with Bonferroni post-hoc test. Statistical analysis was performed using GraphPad Prism 5.02 Software. MiRNA expression data from human aortas were log2 transformed prior to statistical analysis. Values two standard deviations away from the mean were considered outliers. $p< 0.05$ was considered statistically significant.

Results

3.1 A number of microRNAs are dysregulated in proliferating SMC versus intact vascular tissue

Phenotypic modulation of smooth muscle cells from a quiescent contractile state to a more proliferative synthetic phenotype is observed in some vascular disease states such as neointimal hyperplasia. By comparing contractile smooth muscle cells in the intact aorta with proliferating aortic smooth muscle cells derived from the same tissue we can get a signature profile of the gene expression in these two phenotypic states of the smooth muscle. We have previously demonstrated that the actin polymerization is significantly decreased in proliferating smooth muscle and that this can inhibit the expression of genes involved in contractile function [28].

To determine miRNA expression in contractile versus proliferating smooth muscle, we used a cardiovascular disease-focused miRNA PCR array and analyzed a pooled sample from cultured mouse aortic smooth muscle cells (mAoSMCs) and compared this to a pooled sample of intact aorta from which the cultured cells were derived. The results reveal dramatic changes in miRNA expression (Fig 1A). In order to filter out the miRNAs with the highest fold changes we set a threshold to 9.0 for upregulated miRNAs presented in the list in Figure 1A.

3.2 Regulation of miRNA expression by actin polymerization in mouse smooth muscle cells

Polymerization of actin filaments in smooth muscle cells can promote the expression of contractile markers via nuclear translocation of the transcription factor MRTF-A. To determine the importance of this mechanism for the regulation of miRNA expression we analyzed cardiovascular disease-associated miRNAs in mouse smooth muscle cells incubated with the actin stabilizer jasplakinolide. As shown in Figure 1B, jasplakinolide promotes the expression of several miRNAs that are associated with the contractile phenotype such as miR-143/145, miR-1, miR-133 and miR-378a. However, the effect of jasplakinolide is not as dramatic as the difference observed in contractile versus proliferating smooth muscle cells. The threshold for miRNAs included in the list in figure 1B and C was therefore set to 1.5 fold.

To test if the expression of MRTF-A is a limiting factor for the effect of jasplakinolide we overexpressed MRTF-A in mAoSMCs and then treated these cells with jasplakinolide for 24 h.

Using the same threshold for fold change as in figure 1B, some additional miRNAs were found to be up-regulated with the combination of MRTF-A and jasplakinolide including miR-22, miR-199 and miR-29a/b (Fig 1C).

3.3 Tissue specificity of microRNAs associated with the contractile phenotype of smooth muscle cells

Many of the genes associated with the contractile phenotype of smooth muscle are also known to be highly enriched in smooth and/or striated muscle cells. To determine the smooth muscle enrichment of actin/MRTF-A-regulated miRNAs, we isolated various tissues from mouse and analyzed the miRNA expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR). As demonstrated previously [36], miR-143/145 is highly enriched in smooth muscle (Fig 2A). Furthermore, miR-1 and miR-133 were enriched in striated muscle. However, these microRNAs are also expressed in significant amounts in contractile smooth muscle as compared to the other non-muscle tissues examined. Interestingly, bladder smooth muscle contained higher amounts of miR-1, miR-133, miR-378a and miR-143/145 compared to aortic smooth muscle (Fig 2A). To confirm the results in figure 1A, we included smooth muscle cells isolated from mouse aorta in the analysis. Similar to the effects in figure 1A, all of the miRNAs except miR-22 were expressed in much higher levels in the contractile smooth muscle of intact aorta compared to proliferating aortic smooth muscle cells (Fig 2A). To further confirm this result, we analyzed expression levels of the miRNA group in human renal arteries compared to cells cultured from each individual artery. In accordance with the results in figure 1A and 2A, we found a significant decrease of the expression of all miRNAs except miR-22 in the cultured cells (Fig 2B).

3.4 Regulation of miRNA expression by actin dynamics in human coronary artery smooth muscle cells

In order to evaluate species differences in the regulation of miRNA expression by actin/MRTF-A in smooth muscle, we used human coronary artery smooth muscle cells (HCASMCs) and analyzed miRNA expression by PCR based miRNA array. This confirmed that miR-1, miR-378a, miR-22 and the miR-143/145 cluster are upregulated following MRTF-A activation in both

mouse and human smooth muscle (Fig 3A). However, we also found an upregulation of additional miRNAs, including miR-7 (Fig 3A).

To validate the effects observed in the qPCR-based array we performed individual qPCR-reactions on each of the samples used in the array experiments. In these experiments we also included samples treated with only jasplakinolide or the actin depolymerizing factor latrunculin B. As shown in figure 3B, the effects of MRTF-A in combination with jasplakinolide were confirmed in individual PCR reactions for selected miRNAs. However, in contrast to mouse cells, we did not observe any effect by jasplakinolide alone in human smooth muscle cells. However, depolymerization of actin filaments by latrunculin B vs. control resulted in significant downregulation of all miRNAs except miR-378a, demonstrating that changes in actin polymerization, in a setting of endogenous levels of MRTF-A, is sufficient to regulate most of the selected miRNAs. The combined results from the arrays in figure 1 and 3 are summarized in a Venn-diagram in figure 3C.

3.5 Differences in the F/G-actin ratio in smooth muscle cells of human and mouse origin

To determine the cause of the apparent differences in jasplakinolide-sensitivity between mouse and human cells, we tested the hypothesis that basal levels of F- versus G-actin ratio was higher in human smooth muscle cells compared to mouse cells. This would theoretically sensitize mouse cells to stabilization of actin while human cells would be more sensitive to destabilization of actin. In accordance with this hypothesis we found that the F/G-actin ratio was significantly higher in human cells compared to mouse cells (Fig 4A). Furthermore, miR-145 expression was sensitive to latrunculin B but not jasplakinolide in human smooth muscle cells, while the opposite was observed in mouse smooth muscle cells (Fig 4B).

3.6 *Depolymerization of actin filaments and reduced expression of actin/MRTF-A-regulated miRNAs in human dilated aorta*

Thoracic aortic aneurysms are in some cases associated with mutations in the smooth muscle actin gene, which negatively affects the stability of actin polymerization [40-42]. Recent work has also demonstrated that inducible SMC deletion of myocardin causes spontaneous

aneurysms in multiple arteries [43]. To our knowledge, the polymerized state of actin in early aortic dilations has not been investigated previously. Investigating the early molecular events is crucial in understanding the progression and development of pathologies, such as aneurysm. We used aortic biopsies from donors (DON) without aortic dilation and compared these to patients with mild dilations undergoing valve replacement surgery due to stenotic bicuspid (BAV) or tricuspid (TAV) aortic valves. As shown in figure 5A, the F/G-actin ratio was significantly reduced in both BAV- and TAV-associated aortic dilations. To determine if this loss of filamentous actin also resulted in a reduced expression of actin/MRTF-A-regulated miRNAs, we performed qPCR analysis of miR-1, miR-7, miR-22, miR143/145 and miR-378a. While the differences in miR-22 and miR-378a did not reach statistical significance, all of the other actin/MRTF-A-sensitive miRNAs that were analyzed, exhibited significantly reduced expression levels in dilated aortic tissues.

4.1 Discussion

The expression of specific miRNAs in vascular smooth muscle is essential for vascular function and disease development. Herein we identified a group of miRNAs that are regulated by actin polymerization and the actin-sensitive transcription factor MRTF-A. Increasing evidence points towards a crucial role for actin polymerization in disease development and it is thus important to determine the potential influence of cardiovascular enriched miRNAs in this process. By comparing the regulation of 84 miRNAs by jasplakinolide and MRTF-A in mouse and human smooth muscle cells we identified a group of five miRNAs (miR-1, miR-22, miR-143, miR-145 and miR-378a) that were upregulated in both species. With the exception of miR-22, all of these miRNAs were highly enriched in contractile vs. synthetic smooth muscle in both mouse and human samples. Furthermore, miR-7 was upregulated by MRTF-A specifically in human smooth muscle cells, while miR-133a and miR-133b were specifically upregulated in mouse cells.

In contrast to mouse cells, human smooth muscle cells were relatively resistant to stabilization of actin filaments, while depolymerization of actin caused dramatic effects on miRNA expression. Interestingly, increased basal levels of actin polymerization was observed in human compared to mouse cells, which could explain the differences in sensitivity to reagents that stabilize or disrupts actin filaments. Notably, the difference observed in F/G-actin in human versus mouse smooth muscle cells is in the same range as the effect of 100 nM jasplakinolide in mouse cells [28]. Thus, further stabilization of the actin filaments in human smooth muscle cells may have limited effects on MRTF-translocation and gene transcription.

Several of the actin/MRTF-A-sensitive miRNAs identified in this study have been demonstrated to play important roles in smooth muscle. Most notably, the miR-143/145 cluster is essential for vascular smooth muscle contractile differentiation and vascular function [33, 34, 37-39, 44-46]. Both of these miRNAs are highly expressed in smooth muscle but the effect on contractile differentiation is primarily attributed to miR-145 [36, 45]. Several mechanisms for the effect of miR-145 have been proposed including regulation of actin dynamics, angiotensin signaling and L-type calcium channel expression [33, 34, 36, 37, 47]. The effects of miR-145 on the expression of L-type calcium channels is most likely secondary to inhibition of its direct target

$Ca^{2+/}$calmodulin-dependent protein kinase II δ (CamKIIδ) and reduced activation of the transcriptional repressor DREAM/calsenilin/KChIP3 [34, 38, 48]. Since miR-145 has such prominent effects on smooth muscle differentiation and function, this miRNA has been a particular focus for pharmacological intervention against a number of vascular disease states. In this regard, studies have demonstrated that overexpression of miR-145 can prevent or reduce the development of atherosclerosis [49] and neointimal hyperplasia [44], while inhibition of miR-145 protects against pulmonary arterial hypertension [50].

Some of the actin-sensitive miRNAs identified in this study including miR-1/133a and miR-378a are highly expressed in striated muscle. For example, miR-1 has been demonstrated to account for nearly 40% of all known miRNA reads in cardiac tissues, suggesting extreme enrichment of this miRNA [51]. Despite a relatively lower expression level in vascular tissue, the expression levels of this group of miRNAs are still likely to be physiologically relevant. We also found that the relative expression levels of these miRNAs are higher in bladder smooth muscle compared to vascular smooth muscle suggesting that the miRNA profile can differ substantially among smooth muscle tissues. Information about the relative abundance of a miRNA in specific tissues is likely key in determining the potential for using that miRNA for directed therapeutic intervention. For example, miR-122 is highly enriched in hepatocytes, which results in limited off target effects of the miR-122 inhibitor, Miravirsen [52].

A similar expression pattern of miR-1 and miR-133 is expected considering that these miRNAs are expressed together as a bicistronic cluster [53]. In accordance with our results, this miRNA cluster is regulated by SRF and/or myocardin in striated [53] and smooth muscle [54, 55]. Interestingly, in a negative feedback manner, miR-1 directly targets myocardin, and in striated muscle, where miR-1 expression is relatively high, it suppresses expression of smooth muscle genes by blocking myocardin expression [56, 57]. Similarly, overexpression of miR-1 can inhibit myocardin-induced contractility in human vascular smooth muscle cells [58]. In our screening we found miR-1 to be the most downregulated miRNA in cultured smooth muscle cells versus intact vascular tissue. In mouse aortic cells the expression was reduced over 5000-fold while the downregulation in human renal artery cells was nearly 1000-fold. Thus, miR-1 may act as a

buffer for myocardin expression by acting to maintain the expression at appropriate levels in striated muscle and in proliferating smooth muscle cells.

The importance of miR-133 for vascular smooth muscle function has been investigated both *in vivo* and *in vitro*. Overexpression of miR-133 inhibits smooth muscle proliferation via down regulation of the direct target SP-1 [59]. Accordingly, adenoviral delivery of miR-133 *in vivo* reduces neointimal hyperplasia after balloon injury, while miR-133 inhibitors have the opposite effect. Overexpression of miR-133 can also prevent transdifferentiation of smooth muscle cells to osteoblast-like cells which may play a role in vascular calcification [60].

MiRNA-378a is known to have important functions in both skeletal [61] and cardiac muscle [62], while its role for smooth muscle function remains to be elucidated. Herein, we demonstrate that the expression level of miR-378a in bladder smooth muscle is in a similar range as in striated muscle, while the expression level in vascular smooth muscle is relatively low. Furthermore, we have previously demonstrated, by using a smooth muscle specific dicer KO mouse, that smooth muscle cells are responsible for approximately 98% of miR-378a expression in the bladder [63]. MiR-378a belongs to a group of miRNAs called mirtrons, as they are localized in introns of protein coding genes. As such, miR-378a is localized in the first intron of the *Ppargc1b* gene, encoding the transcriptional co-activator PGC-1β [64]. Mirtrons are known to regulate the same cellular processes as their host gene [65], which, in the case of PGC-1 involves mitochondrial biogenesis, thermogenesis and fatty acid metabolism [66]. By genetic deletion of miR-378a and miR-378a*, Carrer et al. found that mice lacking these miRNAs were protected against diet induced obesity [66]. Interestingly, recent evidence points towards a role of myocardin related transcription factors in regulating lipid homeostasis and adipogenesis but the specific involvement of miRNAs in this process is still unknown [67]. In addition to its role in energy metabolism, miR-378a represses cardiomyocyte hypertrophy by targeting components of the mitogen activated protein kinase (MAPK) pathway [68]. MiR-378a is significantly downregulated in hypertrophic heart and restoration of the disease-associated loss of miR-378a has been suggested as a potential therapeutic strategy against myocardial disease.

Similar to miR-378a, miR-22 regulates cardiac hypertrophy and remodeling in response to stress. However, in contrast to miR-378a, miR-22 is upregulated during cardiac hypertrophy and genetic deletion of miR-22 protects against cardiac hypertrophy and remodeling in mice [69, 70]. Herein we found that, in contrast to other actin/MRTF-A-regulated miRNAs, miR-22 is not reduced in smooth muscle cells cultured from intact mouse aorta or human renal arteries. However, in smooth muscle, miR-22 promotes contractile differentiation by down-regulating methyl CpG-binding protein 2 (MECP2) [71] suggesting that miR-22 may play a role in both hypertrophic growth and contractile differentiation. Previous studies from our group have demonstrated that actin/MRTF signaling is involved in simultaneous contractile differentiation and hypertrophic growth following mechanical stretch of the vascular wall [15, 16, 72]. However, the potential involvement of miR-22 in stretch-induced effects in smooth muscle has not yet been tested.

As discussed herein, several of the miRNAs that are regulated by actin polymerization and MRTF-A have important roles in smooth muscle cells and are dysregulated in cardiovascular disease states. Actin exists in a monomeric and a filamentous pool and the balance between these two pools can shift depending on extracellular cues and intracellular signaling events. By analyzing the F- and G-actin pools in the thoracic ascending aorta of healthy donors and patients with mild aortic dilations we found a dramatic decrease in polymerized actin in the aortic dilations, particularly in patients with tricuspid aortic valves. This is in accordance with previous findings, demonstrating loss of contractile smooth muscle differentiation markers in the concavity of mild aortic dilation from TAV and BAV patients [73]. The contractile unit of smooth muscle cells is essential to maintain structural integrity of the vascular wall and several mutations in actin and other contractile genes have been associated with arterial aneurysms and dissections [74]. More than 40 mutations in the ACTA2 gene have been identified to date but for many of them, the effects on cytoskeletal dynamics are unknown. However, three mutations, R179H R256H and R258C, which are associated with poor prognosis of aortic aneurysms, have been shown to cause destabilization of actin filaments and an increased G-actin pool [40-42]. Although specific mutations were not tested in the patients included in the present study, our data supports impaired actin polymerization as an early event in aortic

aneurysms. Interestingly, a concomitant decrease in actin/MRTF-A-regulated miRNAs was detected in most cases for both TAV and BAV patients. This finding, together with other results presented herein, suggests that the destabilization of actin filaments results in altered transcriptional regulation of miRNAs in smooth muscle, and that this mechanism may be involved in the development and progression of aortic aneurysms. However, it is not clear if the changes in miRNA expression and actin polymerization in the aortic wall depend on a different cellular composition in the obtained biopsies. Even so, transdifferentiation of smooth muscle cells to a macrophage-like phenotype can occur as a consequence of altered miR-143/145 signaling [75]. It is thus possible that altered cellular composition of the media occurs as a consequence of changes in actin polymerization in smooth muscle cells. Further studies are warranted to confirm these effects in a larger cohort of patients and to determine the pathological impact of the observed changes in miRNA expression in aneurysm development. In addition to the limited patient material, this study is limited by the fact that not all miRNAs were tested in the analysis. Thus, we cannot exclude that additional miRNAs are regulated by actin/MRTF in smooth muscle.

In conclusion, the results presented herein suggest that actin polymerization and the actin sensitive transcriptional co-activator MRTF-A regulate a subset of miRNAs that are known to play an important role in smooth and/or cardiac muscle. Furthermore, we demonstrate that several of these miRNAs are enriched in muscle tissues and associated with the contractile phenotype of smooth muscle cells. Finally, the data presented herein suggest a potential role for actin polymerization and actin/MRTF-A-regulated miRNAs in early development of thoracic aortic aneurysms. These findings may be of importance for future therapeutic intervention of aortic aneurysms where signaling pathways involved in actin polymerization, or actin regulated miRNAs can be targeted.

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Figure legends

Fig 1. Differentially expressed miRNAs associated with the contractile phenotype of smooth muscle cells. miRNA expression profiling was performed using a cardiovascular disease-focused miRNA PCR array. Scatter plots show miRNA expression (A) in intact aorta vs. proliferating mouse aortic SMCs (mAoSMC), (B) in mAoSMCs treated with actin stabilizing agent jasplakinolide vs. control and (C) in mAoSMCs transduced with Ad-MRTF-A and treated with jasplakinolide vs. control. Pooled samples from 3-6 replicates were used. Changes in miRNA expression are presented in lists as fold change among groups. HKG refers to housekeeping genes used for data normalization. Grey lines signify upregulated (red circles) and downregulated (green circles) miRNAs with an expression level greater than 9 fold (fig. A) and 1.5 fold (fig. B and C).

Fig 2. Most MRTF/Actin-regulated miRNAs are enriched in muscle containing tissues and highly expressed in contractile vs proliferative smooth muscle. (A) miRNA expression pattern for selected miRNAs was characterized by real-time RT-PCR in indicated adult mouse tissues and cultured mAoSMCs. (B) Same set of miRNAs were analyzed in human renal arteries and proliferating SMCs cultured from the same tissue. Data were normalized to the intact aorta or renal artery of the respective mouse/patient, and are presented as mean± SEM (n=3).

Fig 3. Identification of Actin/MRTF-A-regulated miRNAs in human VSMCs. Human coronary artery smooth muscle cells (HCASMCs) were transduced with Ad-MRTF-A and then incubated with jasplakinolide for 24 h. (A) MiRNA expression was assessed using miRNA PCR array for cardiovascular diseases. Scatter plot shows differentially expressed miRNAs in HCASMCs transduced with Ad-MRTF-A and treated with jasplakinolide vs. control. Pooled samples from six replicates were used. Grey solid lines represent up- and downregulated miRNAs with a threshold set to ≥ 1.5 fold change. (B) Selected miRNAs that were differentially expressed in the PCR arrays were validated using real-time RT-PCR. HCASMCs treated with actin stabilizing agent jasplakinolide (Jasp) or actin destabilizing agent latrunculin B (LatB) were also included in the validation.

(C) Venn diagram depicting common and distinct miRNAs upregulated by the defined thresholds

in the four miRNA arrays. All data are presented as mean± SEM (n=3-6).*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Fig 4. Analysis of actin polymerization in human and mouse smooth muscle cells.
(A) Actin polymerization in human (HCASMCs) and mouse (mAoSMCs) smooth muscle cells was assessed by quantitative immunoblot analysis of F- and G-actin expression levels. (B) Relative miR-145 expression in vascular smooth muscle cells from mouse and human origin treated with either actin stabilizing agent jasplakinolide (Jasp) or actin destabilizing agent latrunculin B (LatB) for 24 h. Expression data was obtained with real-time RT-PCR. Data are presented as mean± SEM (n=3-6).*$P < 0.05$.

Fig 5. Distinct downregulation of actin/MRTF-A-regulated miRNAs in patients with mild aortic dilations. (A) Actin polymerization was assessed by evaluating the F/G-actin ratio in indicated patient groups. Biopsies from mildly dilated thoracic aorta in patients with stenotic tricuspid (TAV) or bicuspid aortic valve (BAV) show a significantly reduced F/G-actin ratio compared to control. Healthy aortas from heart transplant donors (DON) were used as control. A representative immunoblot of F- and G-actin expression is presented below the graph. The F- and G-actin bands were analyzed on the same blot for all samples (n=3-9). (B) Real-time PCR analysis of actin/MRTF-A-regulated miRNAs in indicated patient groups. Data are presented as mean± SEM (n=6-12).*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

References

[1] A.P. Somlyo, A.V. Somlyo, Signal transduction and regulation in smooth muscle, Nature, 372 (1994) 231-236.

[2] M. Fernandez-Tenorio, C. Porras-Gonzalez, A. Castellano, A. Del Valle-Rodriguez, J. Lopez-Barneo, J. Urena, Metabotropic regulation of RhoA/Rho-associated kinase by L-type Ca2+ channels: new mechanism for depolarization-evoked mammalian arterial contraction, Circ Res, 108 (2011) 1348-1357.

[3] J. Urena, J. Lopez-Barneo, Metabotropic regulation of RhoA/Rho-associated kinase by L-type Ca2+ channels, Trends Cardiovasc Med, 22 (2012) 155-160.

[4] J. Ren, S. Albinsson, P. Hellstrand, Distinct effects of voltage- and store-dependent calcium influx on stretch-induced differentiation and growth in vascular smooth muscle, J Biol Chem, 285 (2010) 31829-31839.

[5] B.R. Wamhoff, D.K. Bowles, O.G. McDonald, S. Sinha, A.P. Somlyo, A.V. Somlyo, G.K. Owens, L-type voltage-gated Ca2+ channels modulate expression of smooth muscle differentiation marker genes via a rho kinase/myocardin/SRF-dependent mechanism, Circ Res, 95 (2004) 406-414.

[6] Z. Ying, F.R. Giachini, R.C. Tostes, R.C. Webb, PYK2/PDZ-RhoGEF links Ca2+ signaling to RhoA, Arterioscler Thromb Vasc Biol, 29 (2009) 1657-1663.

[7] Y. Fukata, M. Amano, K. Kaibuchi, Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells, Trends Pharmacol Sci, 22 (2001) 32-39.

[8] T.D. Pollard, G.G. Borisy, Cellular motility driven by assembly and disassembly of actin filaments, Cell, 112 (2003) 453-465.

[9] F. Miralles, G. Posern, A.I. Zaromytidou, R. Treisman, Actin dynamics control SRF activity by regulation of its coactivator MAL, Cell, 113 (2003) 329-342.

[10] A. Sotiropoulos, D. Gineitis, J. Copeland, R. Treisman, Signal-regulated activation of serum response factor is mediated by changes in actin dynamics, Cell, 98 (1999) 159-169.

[11] C.P. Mack, A.V. Somlyo, M. Hautmann, A.P. Somlyo, G.K. Owens, Smooth muscle differentiation marker gene expression is regulated by RhoA-mediated actin polymerization, J Biol Chem, 276 (2001) 341-347.

[12] Z. Wang, D.Z. Wang, G.C. Pipes, E.N. Olson, Myocardin is a master regulator of smooth muscle gene expression, Proc Natl Acad Sci U S A, 100 (2003) 7129-7134.

[13] D.Z. Wang, S. Li, D. Hockemeyer, L. Sutherland, Z. Wang, G. Schratt, J.A. Richardson, A. Nordheim, E.N. Olson, Potentiation of serum response factor activity by a family of myocardin-related transcription factors, Proc Natl Acad Sci U S A, 99 (2002) 14855-14860.

[14] J.M. Miano, Serum response factor: toggling between disparate programs of gene expression, J Mol Cell Cardiol, 35 (2003) 577-593.

[15] S. Albinsson, P. Hellstrand, Integration of signal pathways for stretch-dependent growth and differentiation in vascular smooth muscle, Am J Physiol Cell Physiol, 293 (2007) C772-782.

[16] S. Albinsson, I. Nordstrom, P. Hellstrand, Stretch of the vascular wall induces smooth muscle differentiation by promoting actin polymerization, J Biol Chem, 279 (2004) 34849-34855.

[17] K. Lockman, J.S. Hinson, M.D. Medlin, D. Morris, J.M. Taylor, C.P. Mack, Sphingosine 1-phosphate stimulates smooth muscle cell differentiation and proliferation by activating separate serum response factor co-factors, J Biol Chem, 279 (2004) 42422-42430.

[18] J.S. Hinson, M.D. Medlin, K. Lockman, J.M. Taylor, C.P. Mack, Smooth muscle cell-specific transcription is regulated by nuclear localization of the myocardin-related transcription factors, Am J Physiol Heart Circ Physiol, 292 (2007) H1170-1180.

[19] T. Minami, K. Kuwahara, Y. Nakagawa, M. Takaoka, H. Kinoshita, K. Nakao, Y. Kuwabara, Y. Yamada, C. Yamada, J. Shibata, S. Usami, S. Yasuno, T. Nishikimi, K. Ueshima, M. Sata, H. Nakano, T.

Seno, Y. Kawahito, K. Sobue, A. Kimura, R. Nagai, Reciprocal expression of MRTF-A and myocardin is crucial for pathological vascular remodelling in mice, EMBO J, 31 (2012) 4428-4440.
[20] L. Weise-Cross, J.M. Taylor, C.P. Mack, Inhibition of Diaphanous Formin Signaling In Vivo Impairs Cardiovascular Development and Alters Smooth Muscle Cell Phenotype, Arterioscler Thromb Vasc Biol, 35 (2015) 2374-2383.
[21] D. Shen, J. Li, J.J. Lepore, T.J. Anderson, S. Sinha, A.Y. Lin, L. Cheng, E.D. Cohen, J.D. Roberts, Jr., S. Dedhar, M.S. Parmacek, R.E. Gerszten, Aortic aneurysm generation in mice with targeted deletion of integrin-linked kinase in vascular smooth muscle cells, Circ Res, 109 (2011) 616-628.
[22] C. Weinl, H. Riehle, D. Park, C. Stritt, S. Beck, G. Huber, H. Wolburg, E.N. Olson, M.W. Seeliger, R.H. Adams, A. Nordheim, Endothelial SRF/MRTF ablation causes vascular disease phenotypes in murine retinae, J Clin Invest, 123 (2013) 2193-2206.
[23] C. Weinl, S. Castaneda Vega, H. Riehle, C. Stritt, C. Calaminus, H. Wolburg, S. Mauel, A. Breithaupt, A.D. Gruber, B. Wasylyk, E.N. Olson, R.H. Adams, B.J. Pichler, A. Nordheim, Endothelial depletion of murine SRF/MRTF provokes intracerebral hemorrhagic stroke, Proc Natl Acad Sci U S A, 112 (2015) 9914-9919.
[24] D.C. Guo, C.L. Papke, V. Tran-Fadulu, E.S. Regalado, N. Avidan, R.J. Johnson, D.H. Kim, H. Pannu, M.C. Willing, E. Sparks, R.E. Pyeritz, M.N. Singh, R.L. Dalman, J.C. Grotta, A.J. Marian, E.A. Boerwinkle, L.Q. Frazier, S.A. LeMaire, J.S. Coselli, A.L. Estrera, H.J. Safi, S. Veeraraghavan, D.M. Muzny, D.A. Wheeler, J.T. Willerson, R.K. Yu, S.S. Shete, S.E. Scherer, C.S. Raman, L.M. Buja, D.M. Milewicz, Mutations in smooth muscle alpha-actin (ACTA2) cause coronary artery disease, stroke, and Moyamoya disease, along with thoracic aortic disease, American journal of human genetics, 84 (2009) 617-627.
[25] L. Leitner, D. Shaposhnikov, A. Mengel, A. Descot, S. Julien, R. Hoffmann, G. Posern, MAL/MRTF-A controls migration of non-invasive cells by upregulation of cytoskeleton-associated proteins, Journal of cell science, 124 (2011) 4318-4331.
[26] S. Medjkane, C. Perez-Sanchez, C. Gaggioli, E. Sahai, R. Treisman, Myocardin-related transcription factors and SRF are required for cytoskeletal dynamics and experimental metastasis, Nat Cell Biol, 11 (2009) 257-268.
[27] A. Selvaraj, R. Prywes, Expression profiling of serum inducible genes identifies a subset of SRF target genes that are MKL dependent, BMC Mol Biol, 5 (2004) 13.
[28] K.M. Turczynska, K. Sward, T.T. Hien, J. Wohlfahrt, I.Y. Mattisson, M. Ekman, J. Nilsson, J. Sjogren, V. Murugesan, A. Hultgardh-Nilsson, P. Cidad, P. Hellstrand, M.T. Perez-Garcia, S. Albinsson, Regulation of smooth muscle dystrophin and synaptopodin 2 expression by actin polymerization and vascular injury, Arterioscler Thromb Vasc Biol, 35 (2015) 1489-1497.
[29] Q. Sun, G. Chen, J.W. Streb, X. Long, Y. Yang, C.J. Stoeckert, Jr., J.M. Miano, Defining the mammalian CArGome, Genome research, 16 (2006) 197-207.
[30] J. Zhao, W. Zhang, M. Lin, W. Wu, P. Jiang, E. Tou, M. Xue, A. Richards, D. Jourd'heuil, A. Asif, D. Zheng, H.A. Singer, J.M. Miano, X. Long, MYOSLID Is a Novel Serum Response Factor-Dependent Long Noncoding RNA That Amplifies the Vascular Smooth Muscle Differentiation Program, Arterioscler Thromb Vasc Biol, (2016).
[31] J. Krol, I. Loedige, W. Filipowicz, The widespread regulation of microRNA biogenesis, function and decay, Nat Rev Genet, 11 (2010) 597-610.
[32] S. Albinsson, A. Skoura, J. Yu, A. Dilorenzo, C. Fernandez-Hernando, S. Offermanns, J.M. Miano, W.C. Sessa, Smooth Muscle miRNAs Are Critical for Post-Natal Regulation of Blood Pressure and Vascular Function, PLoS One, 6 (2011) e18869.
[33] S. Albinsson, Y. Suarez, A. Skoura, S. Offermanns, J.M. Miano, W.C. Sessa, MicroRNAs are necessary for vascular smooth muscle growth, differentiation, and function, Arterioscler Thromb Vasc Biol, 30 (2010) 1118-1126.

[34] K.M. Turczynska, M.K. Sadegh, P. Hellstrand, K. Sward, S. Albinsson, MicroRNAs are essential for stretch-induced vascular smooth muscle contractile differentiation via microRNA (miR)-145-dependent expression of L-type calcium channels, J Biol Chem, 287 (2012) 19199-19206.
[35] T. Thi Hien, K.M. Turczynska, D. Dahan, M. Ekman, M. Grossi, J. Sjogren, J. Nilsson, T. Braun, T. Boettger, E. Garcia Vaz, K. Stenkula, K. Sward, M.F. Gomez, S. Albinsson, Elevated glucose levels promote contractile and cytoskeletal gene expression in vascular smooth muscle via Rho/protein kinase C and actin polymerization, J Biol Chem, (2015).
[36] T. Boettger, N. Beetz, S. Kostin, J. Schneider, M. Kruger, L. Hein, T. Braun, Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster, J Clin Invest, 119 (2009) 2634-2647.
[37] M. Xin, E.M. Small, L.B. Sutherland, X. Qi, J. McAnally, C.F. Plato, J.A. Richardson, R. Bassel-Duby, E.N. Olson, MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury, Genes Dev, 23 (2009) 2166-2178.
[38] A. Bhattachariya, D. Dahan, M. Ekman, T. Boettger, T. Braun, K. Sward, P. Hellstrand, S. Albinsson, Spontaneous activity and stretch-induced contractile differentiation are reduced in vascular smooth muscle of miR-143/145 knockout mice, Acta Physiol (Oxf), (2015).
[39] D. Dahan, M. Ekman, A.K. Larsson-Callerfelt, K. Turczynska, T. Boettger, T. Braun, K. Sward, S. Albinsson, Induction of angiotensin converting enzyme after miR-143/145 deletion is critical for impaired smooth muscle contractility, Am J Physiol Cell Physiol, (2014).
[40] H. Lu, P.M. Fagnant, C.S. Bookwalter, P. Joel, K.M. Trybus, Vascular disease-causing mutation R258C in ACTA2 disrupts actin dynamics and interaction with myosin, Proc Natl Acad Sci U S A, 112 (2015) E4168-4177.
[41] H. Lu, P.M. Fagnant, E.B. Krementsova, K.M. Trybus, Severe Molecular Defects Exhibited by the R179H Mutation in Human Vascular Smooth Muscle alpha-Actin, J Biol Chem, (2016).
[42] L.E. Malloy, K.K. Wen, A.R. Pierick, E.W. Wedemeyer, S.E. Bergeron, N.D. Vanderpool, M. McKane, P.A. Rubenstein, H.L. Bartlett, Thoracic aortic aneurysm (TAAD)-causing mutation in actin affects formin regulation of polymerization, J Biol Chem, 287 (2012) 28398-28408.
[43] J. Huang, T. Wang, A.C. Wright, J. Yang, S. Zhou, L. Li, A. Small, M.S. Parmacek, Myocardin is required for maintenance of vascular and visceral smooth muscle homeostasis during postnatal development, Proc Natl Acad Sci U S A, 112 (2015) 4447-4452.
[44] Y. Cheng, X. Liu, J. Yang, Y. Lin, D.Z. Xu, Q. Lu, E.A. Deitch, Y. Huo, E.S. Delphin, C. Zhang, MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation, Circ Res, 105 (2009) 158-166.
[45] K.R. Cordes, N.T. Sheehy, M.P. White, E.C. Berry, S.U. Morton, A.N. Muth, T.H. Lee, J.M. Miano, K.N. Ivey, D. Srivastava, miR-145 and miR-143 regulate smooth muscle cell fate and plasticity, Nature, 460 (2009) 705-710.
[46] L. Elia, M. Quintavalle, J. Zhang, R. Contu, L. Cossu, M.V. Latronico, K.L. Peterson, C. Indolfi, D. Catalucci, J. Chen, S.A. Courtneidge, G. Condorelli, The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease, Cell death and differentiation, (2009).
[47] D. Dahan, M. Ekman, A.K. Larsson-Callerfelt, K. Turczynska, T. Boettger, T. Braun, K. Sward, S. Albinsson, Induction of angiotensin-converting enzyme after miR-143/145 deletion is critical for impaired smooth muscle contractility, Am J Physiol Cell Physiol, 307 (2014) C1093-1101.
[48] J.J. Ronkainen, S.L. Hanninen, T. Korhonen, J.T. Koivumaki, R. Skoumal, S. Rautio, V.P. Ronkainen, P. Tavi, Ca2+-calmodulin-dependent protein kinase II represses cardiac transcription of the L-type calcium channel alpha(1C)-subunit gene (Cacna1c) by DREAM translocation, J Physiol, 589 (2011) 2669-2686.

[49] F. Lovren, Y. Pan, A. Quan, K.K. Singh, P.C. Shukla, N. Gupta, B.M. Steer, A.J. Ingram, M. Gupta, M. Al-Omran, H. Teoh, P.A. Marsden, S. Verma, MicroRNA-145 targeted therapy reduces atherosclerosis, Circulation, 126 (2012) S81-90.

[50] P. Caruso, Y. Dempsie, H.C. Stevens, R.A. McDonald, L. Long, R. Lu, K. White, K.M. Mair, J.D. McClure, M. Southwood, P. Upton, M. Xin, E. van Rooij, E.N. Olson, N.W. Morrell, M.R. MacLean, A.H. Baker, A role for miR-145 in pulmonary arterial hypertension: evidence from mouse models and patient samples, Circ Res, 111 (2012) 290-300.

[51] P.K. Rao, Y. Toyama, H.R. Chiang, S. Gupta, M. Bauer, R. Medvid, F. Reinhardt, R. Liao, M. Krieger, R. Jaenisch, H.F. Lodish, R. Blelloch, Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure, Circ Res, 105 (2009) 585-594.

[52] M.H. van der Ree, A.J. van der Meer, J. de Bruijne, R. Maan, A. van Vliet, T.M. Welzel, S. Zeuzem, E.J. Lawitz, M. Rodriguez-Torres, V. Kupcova, A. Wiercinska-Drapalo, M.R. Hodges, H.L. Janssen, H.W. Reesink, Long-term safety and efficacy of microRNA-targeted therapy in chronic hepatitis C patients, Antiviral Res, 111 (2014) 53-59.

[53] Y. Zhao, E. Samal, D. Srivastava, Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis, Nature, 436 (2005) 214-220.

[54] M. Imamura, Y. Sugino, X. Long, O.J. Slivano, N. Nishikawa, N. Yoshimura, J.M. Miano, Myocardin and microRNA-1 modulate bladder activity through connexin 43 expression during post-natal development, J Cell Physiol, 228 (2013) 1819-1826.

[55] J. Chen, H. Yin, Y. Jiang, S.K. Radhakrishnan, Z.P. Huang, J. Li, Z. Shi, E.P. Kilsdonk, Y. Gui, D.Z. Wang, X.L. Zheng, Induction of microRNA-1 by myocardin in smooth muscle cells inhibits cell proliferation, Arterioscler Thromb Vasc Biol, 31 (2011) 368-375.

[56] K. Wystub, J. Besser, A. Bachmann, T. Boettger, T. Braun, miR-1/133a clusters cooperatively specify the cardiomyogenic lineage by adjustment of myocardin levels during embryonic heart development, PLoS Genet, 9 (2013) e1003793.

[57] A. Heidersbach, C. Saxby, K. Carver-Moore, Y. Huang, Y.S. Ang, P.J. de Jong, K.N. Ivey, D. Srivastava, microRNA-1 regulates sarcomere formation and suppresses smooth muscle gene expression in the mammalian heart, Elife, 2 (2013) e01323.

[58] Y. Jiang, H. Yin, X.L. Zheng, MicroRNA-1 inhibits myocardin-induced contractility of human vascular smooth muscle cells, J Cell Physiol, (2010).

[59] D. Torella, C. Iaconetti, D. Catalucci, G.M. Ellison, A. Leone, C.D. Waring, A. Bochicchio, C. Vicinanza, I. Aquila, A. Curcio, G. Condorelli, C. Indolfi, MicroRNA-133 controls vascular smooth muscle cell phenotypic switch in vitro and vascular remodeling in vivo, Circ Res, 109 (2011) 880-893.

[60] X.B. Liao, Z.Y. Zhang, K. Yuan, Y. Liu, X. Feng, R.R. Cui, Y.R. Hu, Z.S. Yuan, L. Gu, S.J. Li, D.A. Mao, Q. Lu, X.M. Zhou, V.A. de Jesus Perez, L.Q. Yuan, MiR-133a modulates osteogenic differentiation of vascular smooth muscle cells, Endocrinology, 154 (2013) 3344-3352.

[61] J. Gagan, B.K. Dey, R. Layer, Z. Yan, A. Dutta, MicroRNA-378 targets the myogenic repressor MyoR during myoblast differentiation, J Biol Chem, 286 (2011) 19431-19438.

[62] R.S. Nagalingam, N.R. Sundaresan, M.P. Gupta, D.L. Geenen, R.J. Solaro, M. Gupta, A cardiac-enriched microRNA, miR-378, blocks cardiac hypertrophy by targeting Ras signaling, J Biol Chem, 288 (2013) 11216-11232.

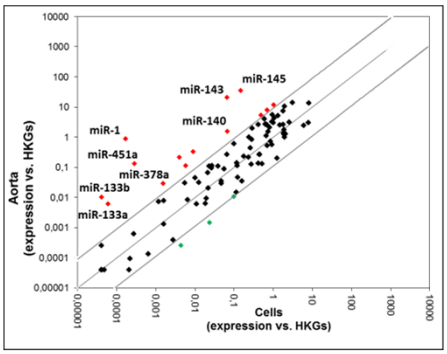
[63] M.K. Sadegh, M. Ekman, C. Rippe, B. Uvelius, K. Sward, S. Albinsson, Deletion of dicer in smooth muscle affects voiding pattern and reduces detrusor contractility and neuroeffector transmission, PLoS One, 7 (2012) e35882.

[64] L.J. Eichner, M.C. Perry, C.R. Dufour, N. Bertos, M. Park, J. St-Pierre, V. Giguere, miR-378(*) mediates metabolic shift in breast cancer cells via the PGC-1beta/ERRgamma transcriptional pathway, Cell Metab, 12 (2010) 352-361.

[65] E.M. Small, E.N. Olson, Pervasive roles of microRNAs in cardiovascular biology, Nature, 469 (2011) 336-342.
[66] M. Carrer, N. Liu, C.E. Grueter, A.H. Williams, M.I. Frisard, M.W. Hulver, R. Bassel-Duby, E.N. Olson, Control of mitochondrial metabolism and systemic energy homeostasis by microRNAs 378 and 378*, Proc Natl Acad Sci U S A, 109 (2012) 15330-15335.
[67] K. Sward, K.G. Stenkula, C. Rippe, A. Alajbegovic, M.F. Gomez, S. Albinsson, Emerging roles of the myocardin family of proteins in lipid and glucose metabolism, J Physiol, (2016).
[68] J. Ganesan, D. Ramanujam, Y. Sassi, A. Ahles, C. Jentzsch, S. Werfel, S. Leierseder, X. Loyer, M. Giacca, L. Zentilin, T. Thum, B. Laggerbauer, S. Engelhardt, MiR-378 controls cardiac hypertrophy by combined repression of mitogen-activated protein kinase pathway factors, Circulation, 127 (2013) 2097-2106.
[69] Z.P. Huang, J. Chen, H.Y. Seok, Z. Zhang, M. Kataoka, X. Hu, D.Z. Wang, MicroRNA-22 regulates cardiac hypertrophy and remodeling in response to stress, Circ Res, 112 (2013) 1234-1243.
[70] X.D. Xu, X.W. Song, Q. Li, G.K. Wang, Q. Jing, Y.W. Qin, Attenuation of microRNA-22 derepressed PTEN to effectively protect rat cardiomyocytes from hypertrophy, J Cell Physiol, 227 (2012) 1391-1398.
[71] H. Zhao, G. Wen, Y. Huang, X. Yu, Q. Chen, T.A. Afzal, A. Luong le, J. Zhu, Y. Shu, L. Zhang, Q. Xiao, MicroRNA-22 regulates smooth muscle cell differentiation from stem cells by targeting methyl CpG-binding protein 2, Arterioscler Thromb Vasc Biol, 35 (2015) 918-929.
[72] A. Zeidan, I. Nordstrom, S. Albinsson, U. Malmqvist, K. Sward, P. Hellstrand, Stretch-induced contractile differentiation of vascular smooth muscle: sensitivity to actin polymerization inhibitors, Am J Physiol Cell Physiol, 284 (2003) C1387-1396.
[73] A. Forte, A. Della Corte, M. Grossi, C. Bancone, R. Provenzano, M. Finicelli, M. De Feo, L.S. De Santo, G. Nappi, M. Cotrufo, U. Galderisi, M. Cipollaro, Early cell changes and TGFbeta pathway alterations in the aortopathy associated with bicuspid aortic valve stenosis, Clin Sci (Lond), 124 (2013) 97-108.
[74] D.C. Guo, H. Pannu, V. Tran-Fadulu, C.L. Papke, R.K. Yu, N. Avidan, S. Bourgeois, A.L. Estrera, H.J. Safi, E. Sparks, D. Amor, L. Ades, V. McConnell, C.E. Willoughby, D. Abuelo, M. Willing, R.A. Lewis, D.H. Kim, S. Scherer, P.P. Tung, C. Ahn, L.M. Buja, C.S. Raman, S.S. Shete, D.M. Milewicz, Mutations in smooth muscle alpha-actin (ACTA2) lead to thoracic aortic aneurysms and dissections, Nat Genet, 39 (2007) 1488-1493.
[75] Y. Vengrenyuk, H. Nishi, X. Long, M. Ouimet, N. Savji, F.O. Martinez, C.P. Cassella, K.J. Moore, S.A. Ramsey, J.M. Miano, E.A. Fisher, Cholesterol loading reprograms the microRNA-143/145-myocardin axis to convert aortic smooth muscle cells to a dysfunctional macrophage-like phenotype, Arterioscler Thromb Vasc Biol, 35 (2015) 535-546.

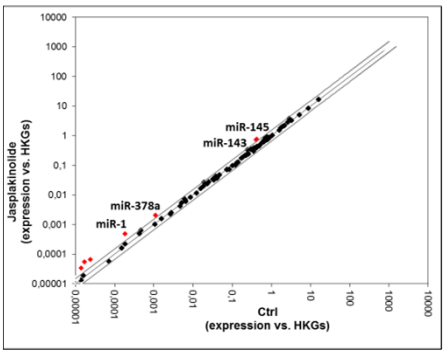
Figure 1.

A



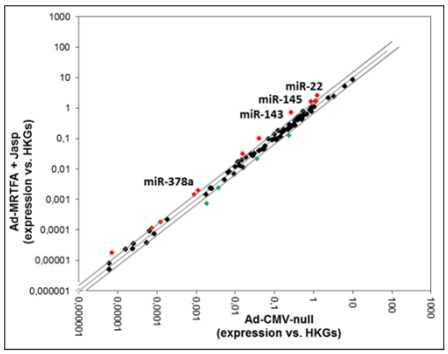
miRNA	Fold Change
mmu-miR-1a-3p	5305.51
mmu-miR-451a	470.53
mmu-miR-143-3p	320.53
mmu-miR-133a-3p	254.24
mmu-miR-145a-5p	242.74
mmu-miR-133b-3p	103.61
mmu-miR-224-5p	54.57
mmu-miR-214-3p	37.29
mmu-miR-140-5p	23.64
mmu-miR-199a-5p	19.80
mmu-miR-378a-3p	19.23
mmu-miR-23b-3p	11.72
mmu-miR-26a-5p	11.66
mmu-miR-26b-5p	11.26

B



miRNA	Fold Change
mmu-miR-133b-3p	3.21
mmu-miR-144-3p	2.81
mmu-miR-1a-3p	2.65
mmu-miR-133a-3p	2.46
mmu-miR-378a-3p	1.88
mmu-miR-145a-5p	1.81
mmu-miR-143-3p	1.78

C



miRNA	Fold Change
mmu-miR-143-3p	2,75
mmu-miR-199a-5p	2,53
mmu-miR-22-3p	2.14
mmu-miR-29b-3p	2.10
mmu-miR-145a-5p	1.95
mmu-miR-378a-3p	1.79
mmu-miR-124-3p	1.65
mmu-miR-499-5p	1.59
mmu-miR-208a-3p	1.50
mmu-miR-29a-3p	1.50

Figure 2.

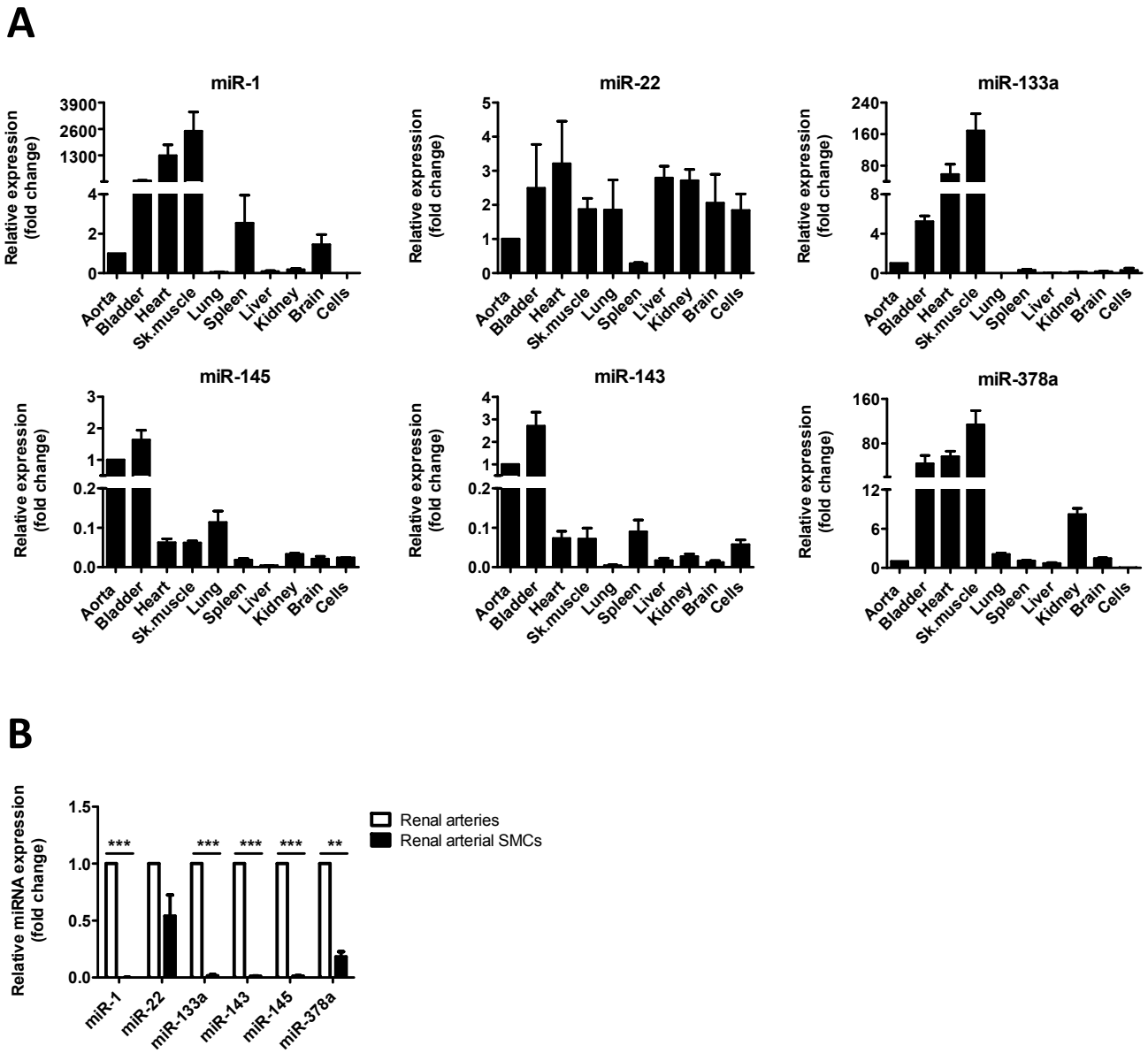
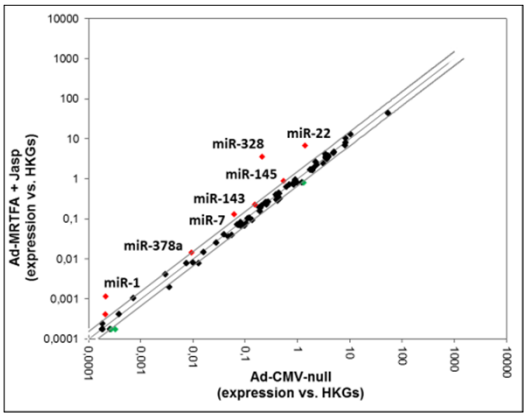

A
miR-1
miR-22
miR-133a
miR-145
miR-143
miR-378a
Relative expression (fold change)
Aorta
Bladder
Heart
Sk.muscle
Lung
Spleen
Liver
Kidney
Brain
Cells
B
Relative miRNA expression (fold change)
Renal arteries
Renal arterial SMCs
***
**
miR-1
miR-22
miR-133a
miR-143
miR-145
miR-378a

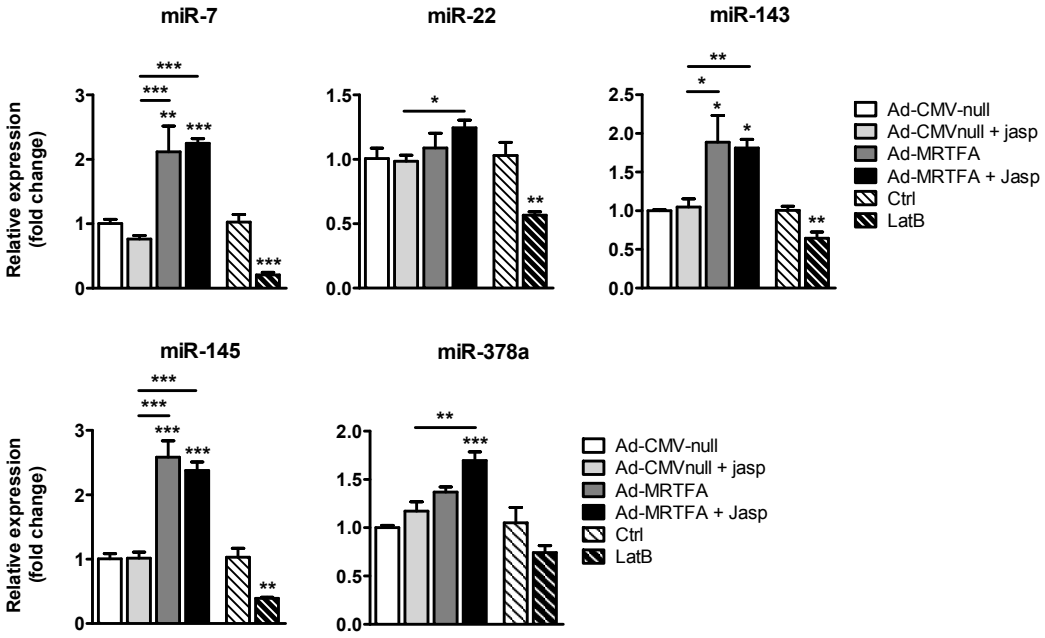
Figure 3.

A



miRNA	Fold Change
hsa-miR-328-3p	17.07
hsa-miR-1-3p	5.46
hsa-miR-22-3p	4.89
hsa-miR-7-5p	2.14
hsa-miR-150-5p	1.97
hsa-miR-145-5p	1.65
hsa-miR-378a-3p	1.54
hsa-miR-143-3p	1.50

B



C

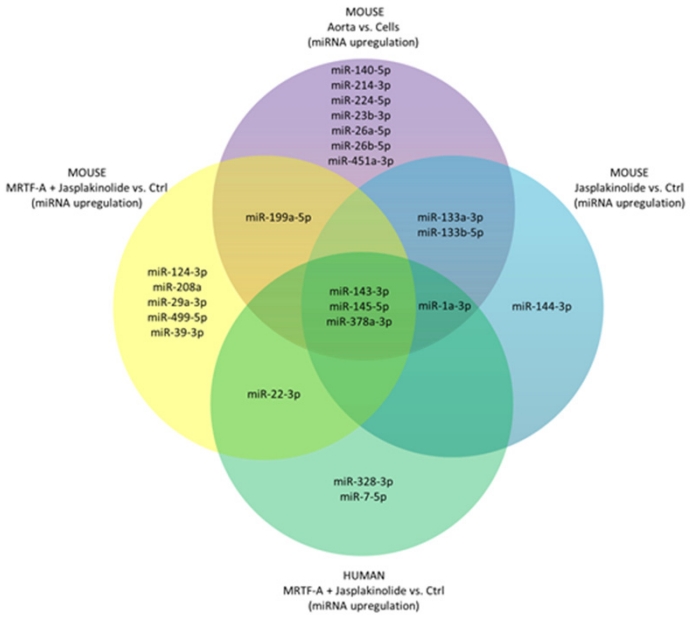
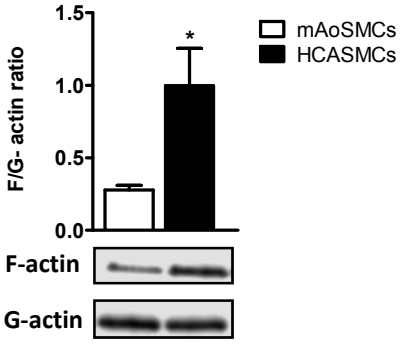


Figure 4.

A


1.5
1.0
0.5
0.0
F/G- actin ratio
*
mAoSMCs
HCASMCs
F-actin
G-actin

B

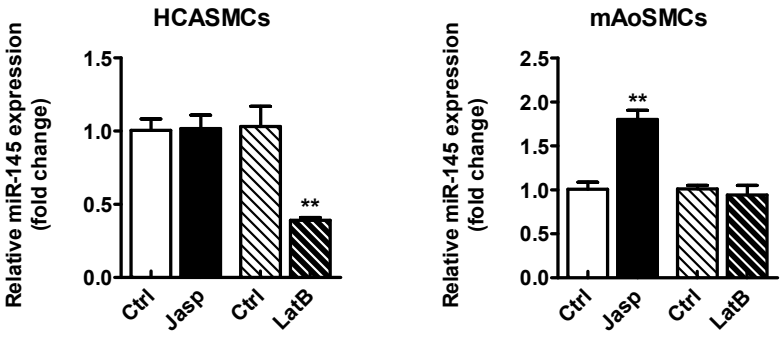
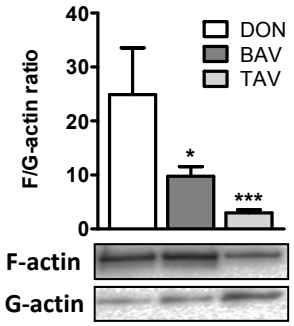
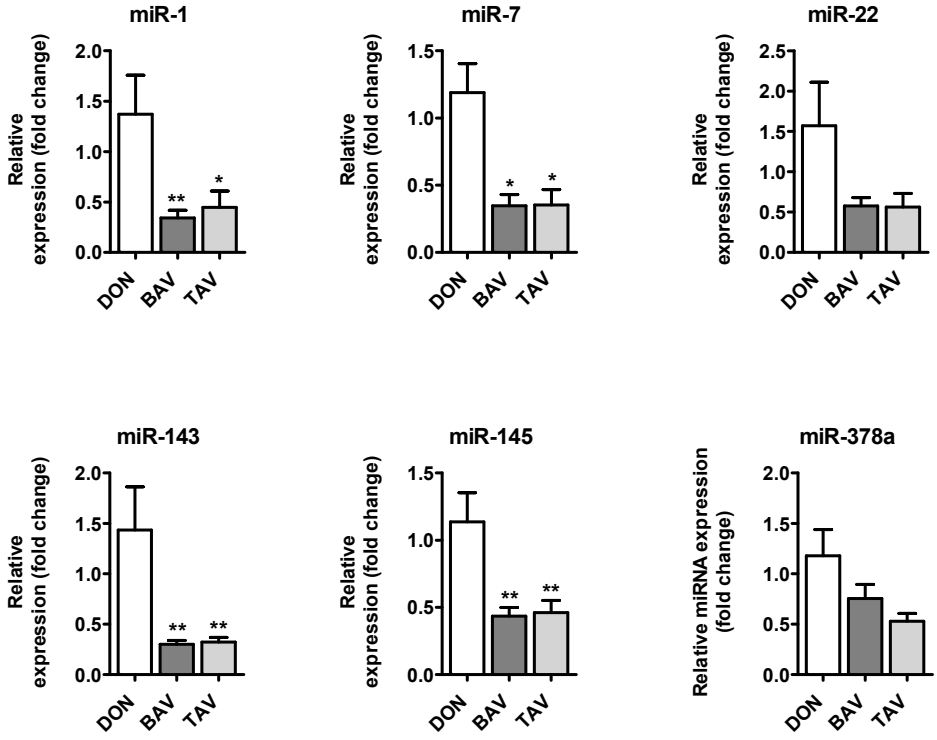

HCASMCs
Relative miR-145 expression (fold change)
1.5
1.0
0.5
0.0
**
Ctrl
Jasp
Ctrl
LatB
mAoSMCs
Relative miR-145 expression (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
**
Ctrl
Jasp
Ctrl
LatB

Figure 5.

A


DON
BAV
TAV
F/G-actin ratio
40
30
20
10
0
*
***
F-actin
G-actin

B


miR-1
miR-7
miR-22
miR-143
miR-145
miR-378a
Relative expression (fold change)
Relative miRNA expression (fold change)
DON
BAV
TAV