



Regulation of microRNA expression in vascular smooth muscle by MRTF-A and actin polymerization[☆]



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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 vascular 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 vascular 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. This article is part of a Special Issue entitled: ECS Meeting edited by Claus Heizmann, Joachim Krebs and Jacques Haiech.

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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-1 β , 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; CamKII δ , calmodulin-dependent protein kinase II delta; ACE, angiotensin I converting enzyme; KLF4/5, Kruppel-like factor; PKC- ϵ , protein kinase C epsilon; PDGF-R α , platelet derived growth factor receptor alpha.

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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 factor 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 of clarifying 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 [35–38]. 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 point 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 50 U/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 and myocardin overexpression was achieved using adenoviral constructs. 24 h after seeding, cells were transduced with 100 MOI of Ad-hMKL1/eGFP (Vector Biolabs, ADV-215499) or Ad-hMYOCD (Vector Biolabs, ADV-216227) 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). The human small Nuclear RNA, C/D Box 95 (Hs_SNORD95_11; #MS00033726) was used as housekeeping gene. The mature miRNA sequences for indicated miRNAs are identical in human and mouse. Amplification was performed by real-time PCR (OneStepPlus qPCR cyclers, 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 ×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. 5 × Laemmli sample buffer was added to each of the pellet and supernatant samples to a final concentration of 1 × (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 Sensitivity 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.

3. 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 regulation we set a threshold to 9.0 for miRNAs presented in the list in Fig. 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 vascular smooth muscle cells incubated with the actin stabilizer jasplakinolide. As shown in Fig. 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 Fig. 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 Fig. 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 Fig. 1A, we included smooth muscle cells isolated from mouse aorta in the analysis. Similar to the effects in Fig. 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

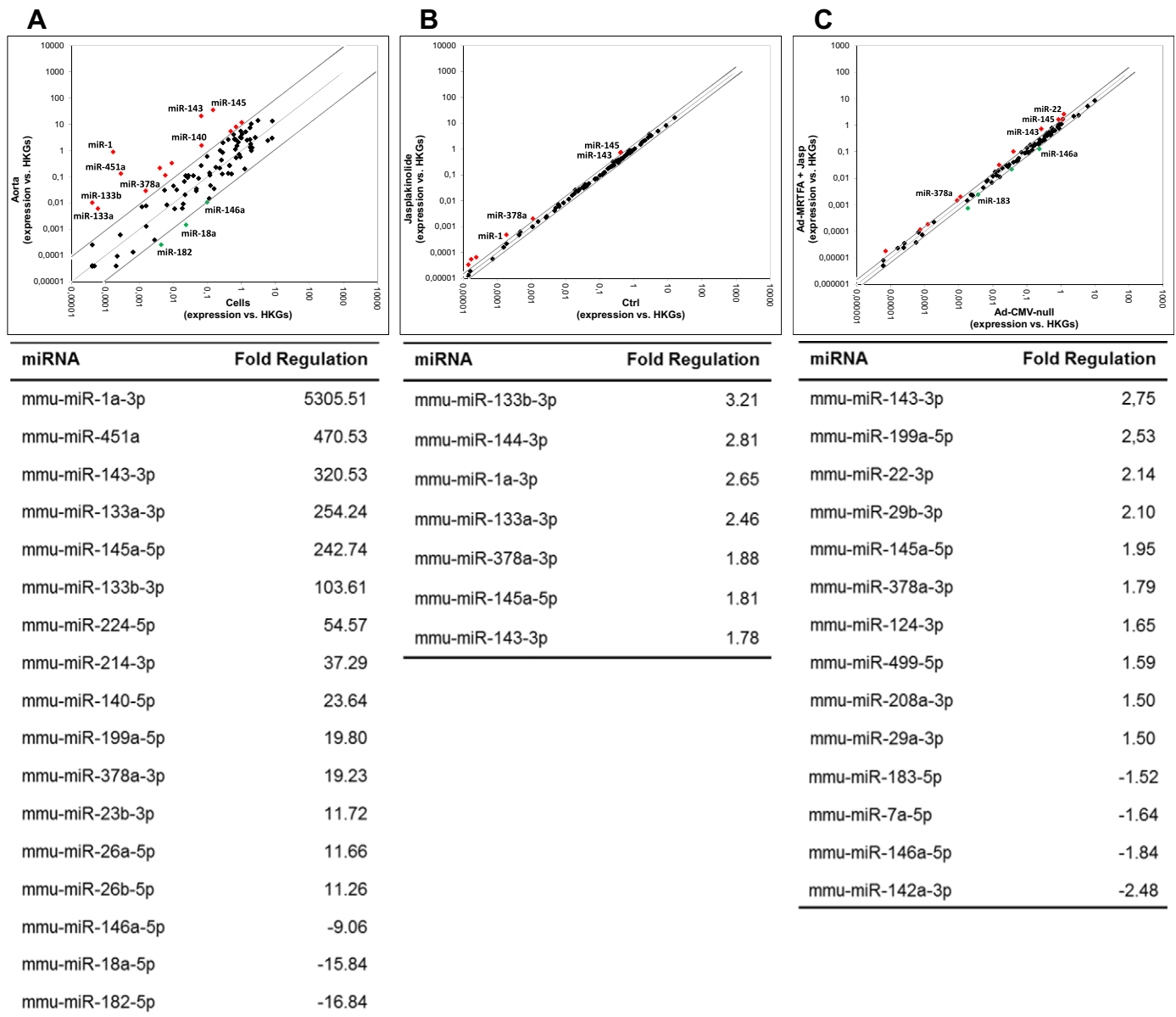


Fig. 1. Differentially expressed miRNAs associated with the contractile phenotype of smooth muscle cells. miRNA expression profiling was performed using a cardiovascular disease-focused miRNA PCR array. Scatter plots show miRNA expression (A) in intact aorta vs. proliferating mouse aortic SMCs (mAoSMC), (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 regulation 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 (A) and 1.5 fold (B and C).

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 Figs. 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). Additional miRNAs such as miR-7 were specifically

upregulated in human cells (Fig. 3A). A number of miRNAs were down-regulated by MRTF and/or jasplakinolide. However, no miRNA was consistently reduced in human and mouse cells and we therefore chose not to proceed with further analysis.

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 Fig. 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

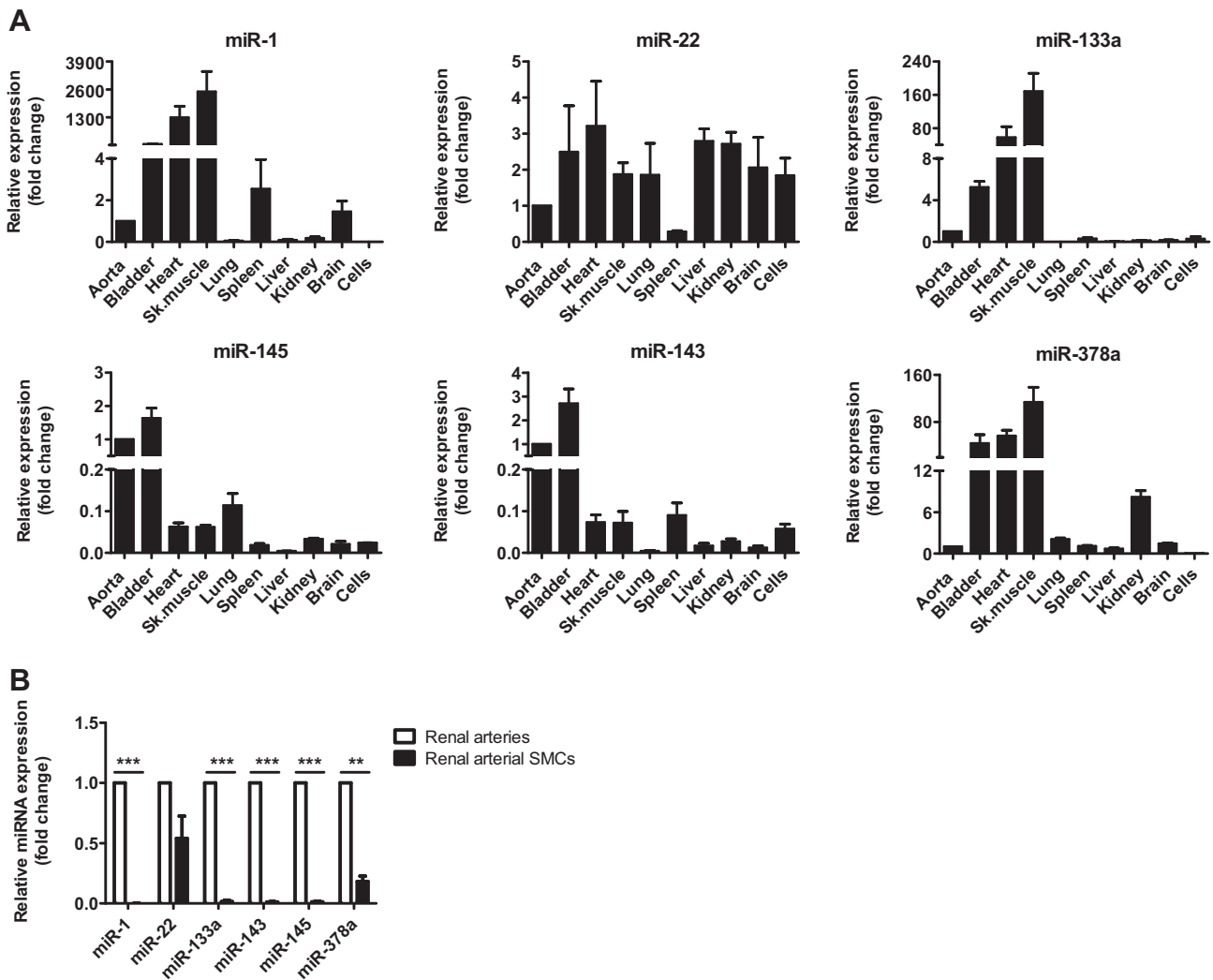


Fig. 2. Most actin/MRTF-A-regulated miRNAs are enriched in muscle containing tissues and highly expressed in contractile vs. proliferative smooth muscle. (A) miRNA expression pattern for selected miRNAs was characterized by real-time RT-PCR in indicated adult mouse tissues and cultured mAoSMCs. (B) Same 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 \pm SEM (n = 3).

miRNAs. The combined results from the arrays in Figs. 1 and 3 are summarized in a Venn-diagram in Fig. 3C. With the exception of miR-7, all of the actin/MRTF-A-regulated miRNAs were also induced by adenoviral-mediated overexpression of myocardin (Supplementary Fig. 1). It should be noted that expression levels of miR-1 in cultured cells is close to the detection limit, which may explain the dramatic increase induced by myocardin overexpression. Low expression levels in control samples also precluded analysis of the effects of latrunculin B on miR-1 expression.

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

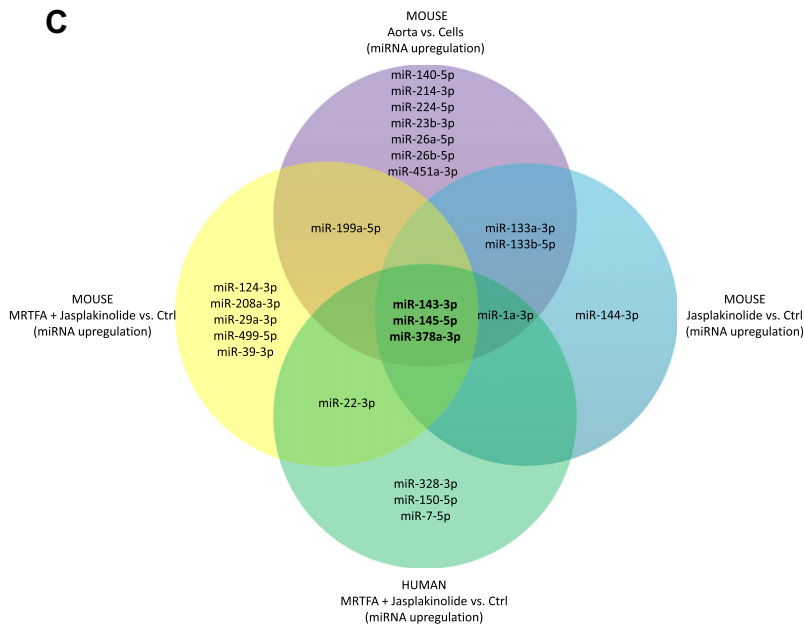
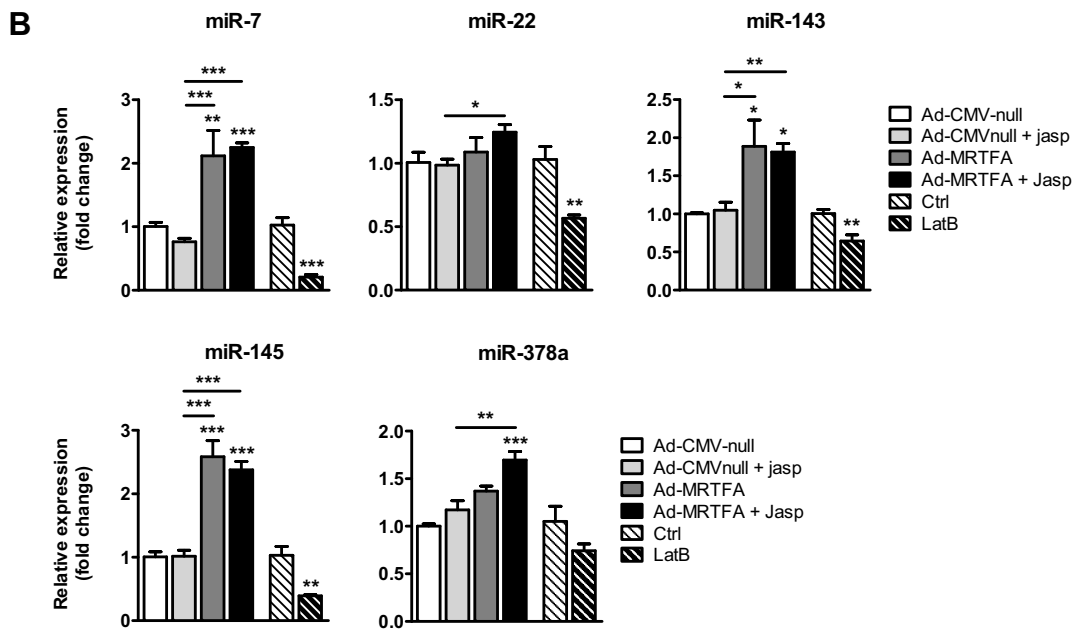
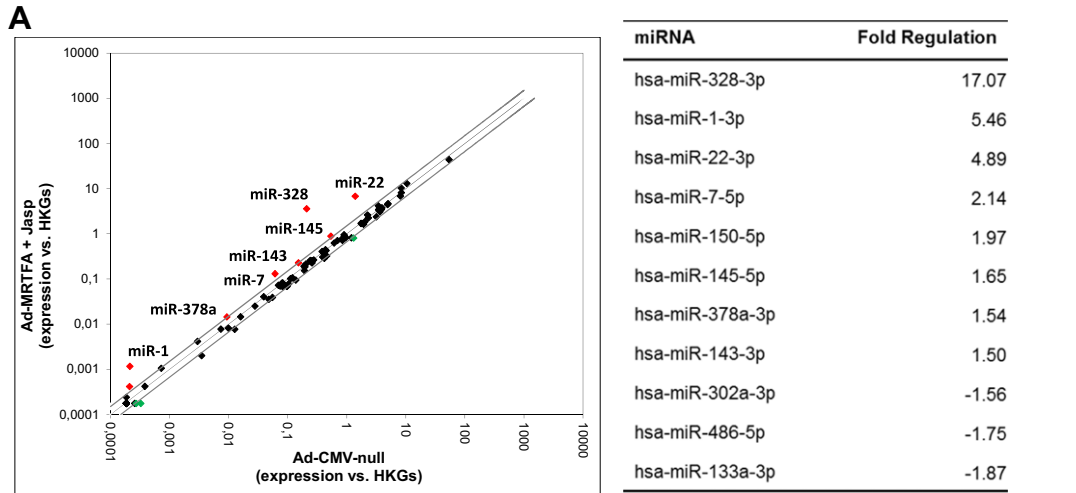
To determine the cause of the apparent differences in jasplakinolide-sensitivity between mouse and human cells, we tested the hypothesis that basal levels of F- versus G-actin ratio was higher in human smooth

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 [39–41]. Recent work has also demonstrated that inducible SMC deletion of myocardin causes

Fig. 3. Identification of Actin/MRTF-A-regulated miRNAs in human VSMCs. Human coronary artery smooth muscle cells (HCASMCs) were transfected 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 transfected 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 \pm SEM (n = 3–6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



spontaneous aneurysms in multiple arteries [42]. To our knowledge, the polymerized state of actin in early aortic dilations has not been investigated previously. Determining 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 Fig. 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, miR-143/145 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 (Fig. 5B). To verify that our results were not due to a general downregulation of all miRNAs, we determined the expression level of miR-146a, which was unchanged in dilated aorta.

4. 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 transcriptional co-activator MRTF-A (Fig. 6). 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 vascular 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 by targeting specific mRNA sequences (Table 1). Most notably, the miR-143/145 cluster is essential for smooth muscle contractile differentiation and vascular function [33,34,37,38,43–47]. Both of these miRNAs are highly expressed in smooth muscle but the effect on contractile differentiation is primarily attributed to miR-145 [36,44]. Several mechanisms for the effect of miR-145 have been proposed including regulation of actin dynamics, angiotensin signaling and L-type calcium channel expression [33,34,36,37,48]. 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 (CamKII δ) and reduced activation of the transcriptional repressor DREAM/calsenilin/KChIP3 [34,38,49]. 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 [50] and neointimal

hyperplasia [43], while inhibition of miR-145 protects against pulmonary arterial hypertension [51].

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 [52]. 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, Miravirsin [53].

A similar expression pattern of miR-1 and miR-133 is expected considering that these miRNAs are expressed together as a bicistronic cluster [54]. In accordance with our results, this miRNA cluster is regulated by SRF and/or myocardin in striated [54] and smooth muscle [55,56]. 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 [57,58]. Similarly, overexpression of miR-1 can inhibit myocardin-induced contractility in human vascular smooth muscle cells [59]. However, miR-1 has also been demonstrated to play a critical role in the determination of SMC fate during embryonic stem cell differentiation by targeting Krüppel-like factor 4 (KLF4) [60]. Furthermore, miR-1 targets the serine/threonine kinase Pim-1, leading to decreased proliferation of smooth muscle cells [56]. 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 [61]. 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 [62]. Both miR-1 and miR-133 can influence calcium signaling in cardiomyocytes by regulating protein phosphatase 2A activity resulting in increased phosphorylation and activation of the ryanodine receptor 2 (RYR2) [63,64]. These miRNAs are upregulated in heart failure and they are associated with an increased arrhythmogenic potential in heart failure myocytes.

MiRNA-378 is known to have important functions in both skeletal [65] and cardiac muscle [66,67], 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 [68]. MiR-378 belongs to a group of miRNAs called mirtrons, as they are localized in introns of protein coding genes. As such, miR-378 is localized in the first intron of the *Ppargc1b* gene, encoding the transcriptional co-activator PGC-1 β [69]. Mirtrons are known to regulate the same cellular processes as their host gene [70], which, in the case of PGC-1 involves mitochondrial biogenesis, thermogenesis and

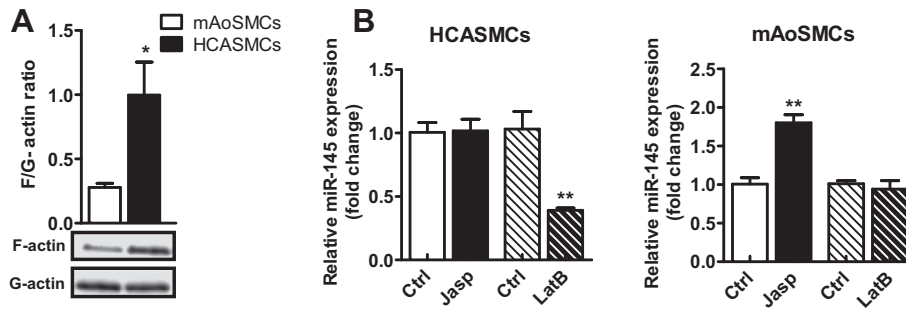


Fig. 4. Analysis of actin polymerization in human and mouse smooth muscle cells. (A) Actin polymerization in human (HCASMCs) and mouse (mAoSMCs) smooth muscle cells was assessed by quantitative immunoblot analysis of F- and G-actin expression levels. (B) Relative miR-145 expression in vascular smooth 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 \pm SEM (n = 3–6). *P < 0.05.

fatty acid metabolism [71]. By genetic deletion of miR-378 and miR-378*, Carrer et al. found that mice lacking these miRNAs were protected against diet induced obesity [71]. 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 [72]. In addition to its role in energy metabolism, miR-378 represses cardiomyocyte hypertrophy by targeting multiple components of the RAS/mitogen activated protein kinase (MAPK) pathway [66,73]. MiR-378 is significantly downregulated in hypertrophic heart and restoration of the disease-associated loss of miR-378 has been suggested as a potential therapeutic strategy against myocardial disease. The targets of miR-378a in the MAP-kinase pathway are also involved in regulation of smooth muscle hypertrophy and proliferation in vascular disease. It is thus possible that elevated expression of this miRNA in differentiated smooth muscle cells can assist in maintaining a quiescent phenotype.

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 [75,76]. 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) [76] suggesting that miR-22 may play a role in promoting 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 [15,16,77]. 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

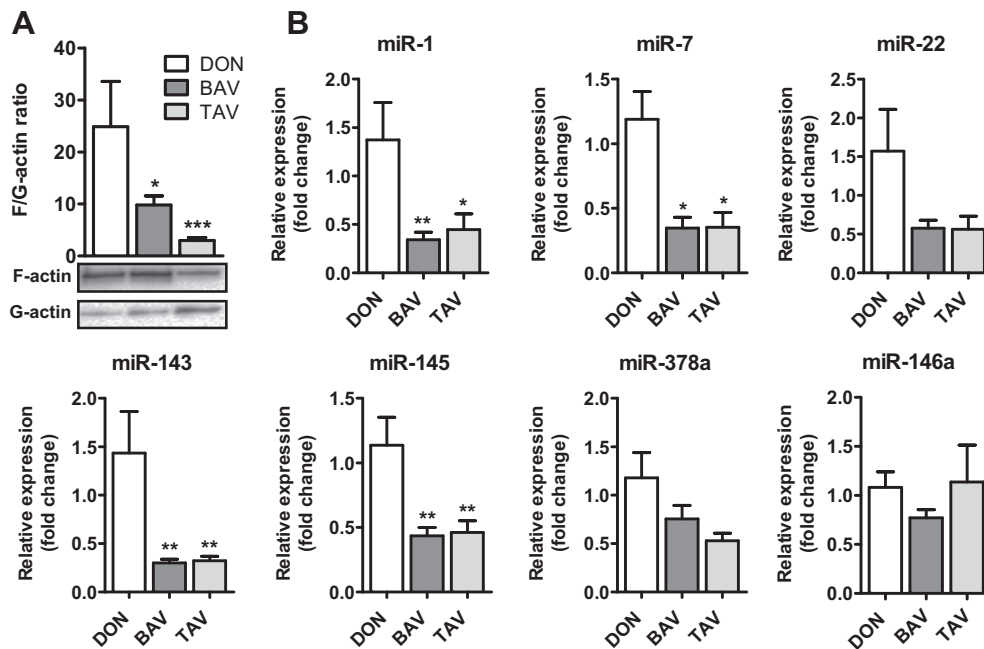


Fig. 5. Distinct downregulation of actin/MRTF-A-regulated miRNAs in patients with mild aortic dilations. (A) Actin polymerization was assessed by evaluating the F/G-actin ratio in indicated patient groups. Biopsies from mildly dilated thoracic aorta in patients with stenotic tricuspid (TAV) or bicuspid 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 and miR-146a in indicated patient groups. Data are presented as mean \pm SEM (n = 6–12). *P < 0.05, **P < 0.01, ***P < 0.001.

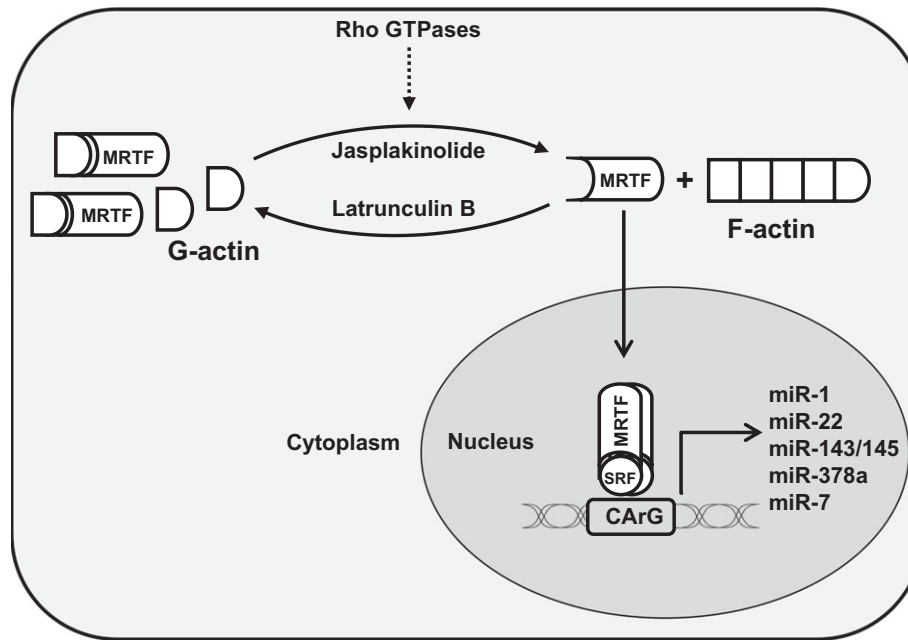


Fig. 6. Regulation of miRNA expression by MRTF in smooth muscle cells. Schematic figure illustrating the influence of actin polymerization on miRNA expression in smooth muscle. Increased stabilization of actin filament results in a decreased G-actin pool and increased nuclear translocation of MRTF, where it can associate with SRF. Actin dynamics can be experimentally modulated using agents that stabilize (jasplakinolide) or disrupt (latrunculin B) actin filaments. Abbreviations: MRTF: myocardin related transcription factor, SRF: serum response factor.

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 [78]. 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 [79]. 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 [39–41]. 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. The decrease in miR-22 and miR-378a in dilated aorta did not reach statistical significance, which may be related to their relatively lower sensitivity to actin/MRTF modulation. Taken together, our findings in dilated aorta, together with other results presented herein, suggest 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 [80]. 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 striated 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.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamcr.2016.12.005>.

Table 1
Selected validated targets of actin-regulated miRNAs in smooth muscle.

miRNA	Target genes
miR-1	Pim1 [56], KLF4 [60]
miR-22	MECP2 [76]
miR-143	ELK-1 [44], PKC- ϵ [46], PDGF-R α [46], Versican [47]
miR-145	KLF4 [37,44], KLF5 [37,43], Myocardin [44], ACE [36], CamkII δ [44], Fascin [46]
miR-378a	–
miR-7	–

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Transparency Document

The Transparency document associated with this article can be found in online version.

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