Am J Transl Res 2019;11(1):370-378
www.ajtr.org /ISSN:1943-8141/AJTR0083022

Original Article

Downregulation of miR143/145 gene cluster expression promotes the aortic media degeneration process via the TGF-β1 signaling pathway

Min Zhang, Zhiwei Wang

Department of Cardiovascular Surgery, Renmin Hospital of Wuhan University, Wuhan 430000, Hubei Province, People's Republic of China

Received July 23, 2018; Accepted December 23, 2018; Epub January 15, 2019; Published January 30, 2019

Abstract: Aortic dissection (AD) is a serious threat to human health; however, the cause of this condition has not yet been fully elucidated. In this study, we found significantly increased expression of phospho-Smad2/3 and phospho-ERK in AD tissues and downregulated expression of miR143 and miR145 in AD tissues. Knockdown of the miR143/145 gene cluster induced phenotypic switching of vascular smooth muscle cells (VSMCs) and activation of the TGF-β1 signaling pathway. When the TFG-β1 signaling pathway was blocked by pretreatment with an LY364947 inhibitor, expression of miR143 and miR145, and VSMC phenotypic markers were not affected by knockdown of the miR143/145 gene cluster. Immunohistochemical staining of aortic tissues donated by AD patients and organ donors showed decrease alpha-smooth muscle actin (α-SMA) expression in pathological tissue, while osteopontin (OPN) expression increased and the arrangement of smooth muscle cells in the tunica media was dysregulated. In conclusion, our study suggests that downregulated expression of the miR143/145 gene cluster promotes phenotypic switching of VSMCs via the TGF-β1 signaling pathway. This may play an important role in the pathogenesis of AD.

Keywords: MicroRNA, TGF-β1, vascular smooth muscle cell, aortic dissection

Introduction

Aortic dissection (AD) occurs when blood breaches the tunica intima of the aorta via a tear, rendering the vascular stratum vulnerable to separation and degeneration [1]. Despite advances in treatments, AD remains a medical emergency wherein rapid intervention is required to avoid mortality.

Many studies have suggested that smooth muscle cells (SMCs) play a pivotal role in the development of arterial diseases owing to their function in vascular wall remodeling. Other studies have demonstrated that aortic tunica media SMCs transition from a contractile to a synthetic phenotype during AD/aneurysm in a process known as "phenotypic switching" [2, 3]. Currently, however, the exact mechanisms underlying AD remain elusive.

MicroRNAs (miRNAs) represent a class of small (20-25 nucleotides) noncoding RNAs that are essential post-transcriptional modulators of gene expression. These molecules coordinate and integrate multiple regulatory pathways involved in cellular differentiation, proliferation, homeostasis, and organ development [4]. Several lines of evidence implicate miRNAs as essential regulators of vascular smooth muscle cell (VSMC) development, differentiation, and contractile function [5-7]. To date, evidence suggests that the miRNA-143/145 gene cluster can direct VSMC fate and regulate differentiation in favor of the contractile versus the synthetic and proliferative phenotype [7-10]. MiR145 is the most highly expressed miRNA in arteries and its expression is attenuated in experimental models of vascular injury and in human atherosclerosis and aneurysms [11-13]. Transient local overexpression of miR145 has been demonstrated to limit formation of the neointima in response to vascular injury [12]. It has been suggested that miR145 regulates VSMC differentiation via several mechanisms, including, but not limited to, KLF4, myocardin, calmodulin kinase IIδ, angiotensin-converting

Table 1. Characteristics of the aortic dissection patients and organ donors

	Aortic dissection group	Organ donor group
Age (year)	50.24 ± 6.58	46.36 ± 5.74
Male/female (n)	6/4	8/2
Weight (kg)	66.42 ± 9.55	58.57 ± 10.06
Type of aortic disease (n)	10	0
Type A aortic dissection	10	0
Type B aortic dissection	0	0
Marfan Syndrome	0	0
Thoracic aortic aneurysm	0	0
Comorbidities (n)	10	0
Hypertensive disease	10	0
Diabetes mellitus	2	0
Heart disease	0	0
COPD	0	0
Renal insufficiency	0	0

enzyme, and, more recently, actin polymerization [5].

The TGF-β superfamily of secreted growth factors comprises 33 ligands that, despite exhibiting pronounced structural similarities, function as regulators of a variety of divergent processes both during embryogenesis and later on in adult homeostasis [14]. Dysregulation of TGF-β1 signaling has been identified as the basis for cancer, cardiovascular, fibrotic, and skeletal diseases [15-18]. In addition, developmental processes are recapitulated during tissue homeostasis regulation and become aberrant during disease development. In this study, we demonstrate that VSMC-specific downregulation of the miR143/145 gene cluster promotes phenotypic switching of VSMCs via the TGF-β1 signaling pathway. These data strengthen the translational basis for novel miRNA-based gene therapy in AD and related disorders.

Materials and methods

Immunohistochemistry

Pathological aortic tissues were obtained from patients (n = 10) who underwent ascending aorta replacement, while normal tissues were obtained from donors who had no aortic disease (n = 10). The baseline characteristics of these AD patients and organ donors are presented in **Table 1**. Tissue samples were fixed in 4% paraformaldehyde/0.1 M phosphate buffered saline (PBS) (pH 7.4) for 24 h. Next, the fixed tissue was dehydrated using a gradient alcohol series, embedded in paraffin and sectioned (4 μm). Sections were then dewaxed, hydrated with gradient alcohol, and antigen-retrieval was performed using the microwave oven method before hydration with 3% hydrogen peroxide to remove endogenous oxidase activity. Prepared slides were incubated overnight at 4°C with approximately 50 mL (1:100 dilution) of the following antihuman primary antibodies: phospho-Smad2/3 (Clone: ab63399; Abcam, UK), phospho-ERK (Clone: ab20-1015; Abcam), α-SMA (Clone: ab5694; Abcam), and OPN (Clone: ab91655; Abcam). Stained slides were then incubated for 1 h at 37°C with approximately 50 mL HRP-conjugated goat antirabbit immunoglobulin G antibody and developed with diaminobenzidine substrate. Finally, slides were stained with hematoxylin and dehydrated with a graded alcohol series and xylene. Immunohistochemical analysis was conducted as previously described [19].

Human aortic smooth muscle cell culture and identification

Human aortic smooth muscle cells (HASMC) were purchased from ATCC (PCS-100-012). HASMCs were first identified by staining tissues with an immunofluorescent monoclonal antibody targeting the alpha-smooth muscle actin (α-SMA) marker (Clone: ab5694; Abcam, England) as previously described [20] (Supplementary Figure 1). HASMCs were then incubated in DMEM containing 10% FBS, 1% penicillin streptomycin mixture, 2 mM glutamine, 50 mg/mL gentamycin, and 50 mg/mL amphotericin-B at 37°C in a 5% CO_2 atmosphere.

Lentivirus-mediated knockdown of miR143/145 gene cluster exression and TGF-β1 blockade

A recombinant lentivirus designed to transfer puromycin resistance was generated using a three-plasmid cotransfection system in human embryonic kidney 293T (HEK 293T) cells, as

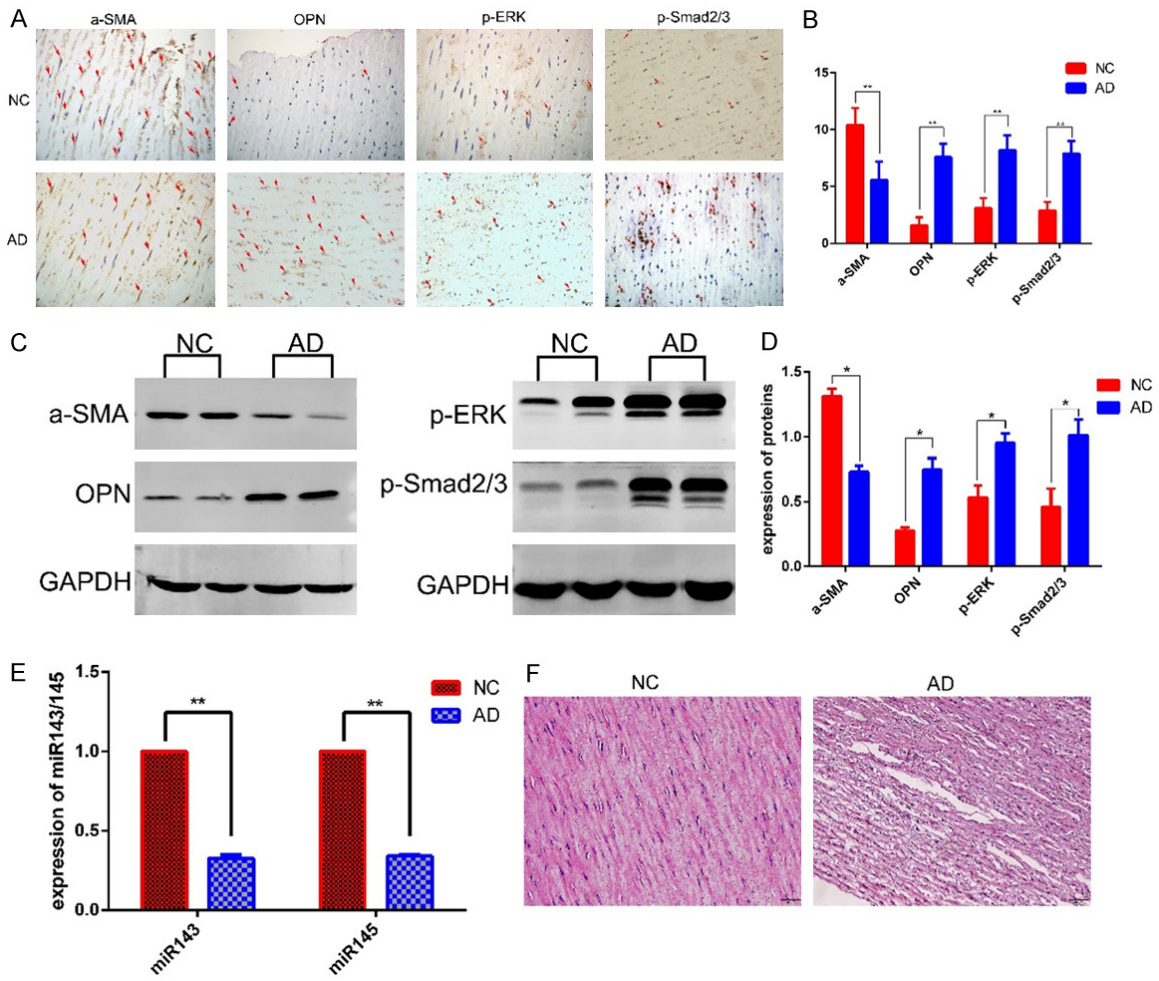


Figure 1. Immunohistochemical staining showing decreased α-SMA expression in the pathological tissues, while the OPN, phospho-Smad2/3 and phospho-ERK expression increased significantly (A and B); Western blot showing the same trend in proteins expression (C and D); RT-PCR analysis suggesting that downregulated expression of miR143 and miR145 in the AD tissues (E); HE staining showing the aortic tissue are disintegrated and fractured and disordered (F). (Data represent means ± SD (n = 10); **P* < 0.05, ***P* < 0.01).

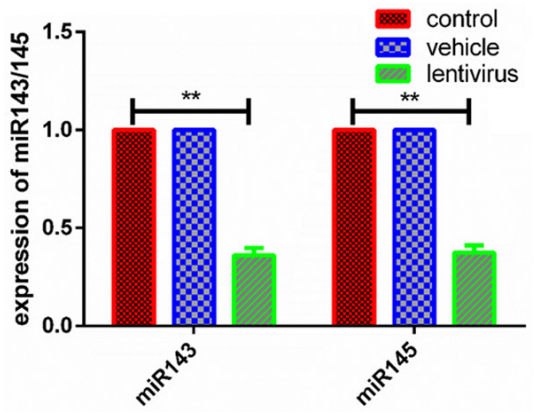


Figure 2. RT-PCR analysis after lentivirus transduction showing significant downregulation of miR143 and miR145, the vehicle had no effect on the expression of miR143 and miR145. (Data represent means ± SD (n = 3); ***P* < 0.01).

previously described [21]. Virus titers were determined based in p24 levels using a Quick-Titer Lentivirus Titer Kit (Cell Biolabs, San Diego, CA, USA). The virus titers were approximately $7 \times 10^4 \pm 2.5 \times 10^4$ transfection units/mL (mean ± SD) in 16 preparations. Transduction efficiency was determined by reverse transcription polymerase chain reaction (RT-PCR) (**Figure 2**).

To study the impact of TGF-β1 signaling pathway inhibition, cells were pretreated with 10 mM LY364947 (Selleck) 30 min before lentivirus treatment.

RNA extraction and RT-PCR

Total RNA was extracted from treated/untreated HASMCs using RNAiso Plus reagent

Table 2. The primers used for different target miRNAs

Gene	Primer sequences
miR143	RT-primer 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCAGAGAT-3'
	Forward-primer 5'-ACACTCCAGCTGGGGGTGCAGTGCTGCAT-3'
	Reverse-primer 5'-TGGTGTCGTGGAGTCG-3'
miR145	RT-primer 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGGGATTC-3'
	Forward-primer 5'-ACACTCCAGCTGGGGTCCAGTTTTCCCAGGA-3'
	Reverse-primer 5'-TGGTGTCGTGGAGTCG-3'
U6	RT-primer 5'-AACGCTTCACGAATTTGCGT-3'
	Forward-primer 5'-CTCGCTTCGGCAGCACA-3'
	Reverse-primer 5'-AACGCTTCACGAATTTGCGT-3'

(Takara, Japan) and used as a template from which complementary DNA was reverse transcribed using a First Strand complementary DNA Synthesis Kit (Takara). MiR levels were subsequently determined by quantitative, SYBR Green-based real-time polymerase chain reaction (PCR; ABI 7500). The U6 gene was used as the control housekeeping gene. Data were normalized against the U6 gene and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method as previously described [22]. The primers and their sequences are listed in **Table 2**.

Western blotting

Aortic tissues/cells were rinsed twice with PBS, and protein was extracted with ice-cold lysis buffer containing 1% protease inhibitor mixture (Sigma, USA) as previously described [23]. Protein concentration was determined with a BCA Protein Assay Kit (Takara) compatible with a detergent-protein assay (Perkin Elmer). Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes, and blocked with 5% nonfat milk for 1 h. Membranes were then incubated overnight at 4°C, with primary antibodies for the detection of Smad2/3 (Clone: #8685S; Cell Signaling Technology), phospho-Smad2/3 (Clone: ab63399; Abcam), ERK (Clone: ab54230; Abcam), phospho-ERK (Clone: ab201015; Abcam), α-SMA (Clone: ab5694; Abcam), and osteopontin (OPN, Clone: ab91-655; Abcam). Subsequently, membranes were incubated with a secondary antibody (LI-COR, USA) for 1 h and blot signals were revealed by addition of an enhanced chemiluminescence reagent (Odyssey).

Statistical analyses

Data were expressed as mean ± standard deviation (SD). Paired and/or unpaired Student's *t*-tests were used to evaluate the significance of differences between the means of two groups, while analysis of variance (ANOVA) was performed to determine significance across multiple groups. A *P*-value < 0.05 was considered to indicate statistical significance. All graphs were fitted with Sigma Plot Version 10.0 software.

Results

The TGF-β1 pathway is activated during AD

Immunohistochemistry and western blotting analyses showed a significant increase in the expression of phospho-Smad2/3 and phospho-ERK (**Figure 1A-D**), thereby indicating that the TGF-β1 pathway is activated during AD.

The miR143/145 gene cluster is downregulated during phenotypic switching of VSMCs in AD

The expression of the miR143/145 gene cluster and phenotypic switching of VSMCs during AD was verified by immunostaining of α-SMA and OPN contractile and synthetic phenotypic markers. Our data showed decreased expression of α-SMA in the tunica media of the pathological aortic tissues, while OPN expression was increased significantly compared with the normal group (**Figure 1A** and **1B**). Furthermore, HE staining and EVG staining showed that the elastic fibers in the middle layer of the aortic tissue were disintegrated, fractured and disordered (**Figure 1F** and Supplementary Figure 2). We also performed quantitative-PCR (q-PCR) analysis of the expression of miR143 and miR145 in AD samples. These data showed significant downregulation of miR143 and miR145 in the pathological specimens (**Figure 1E**).

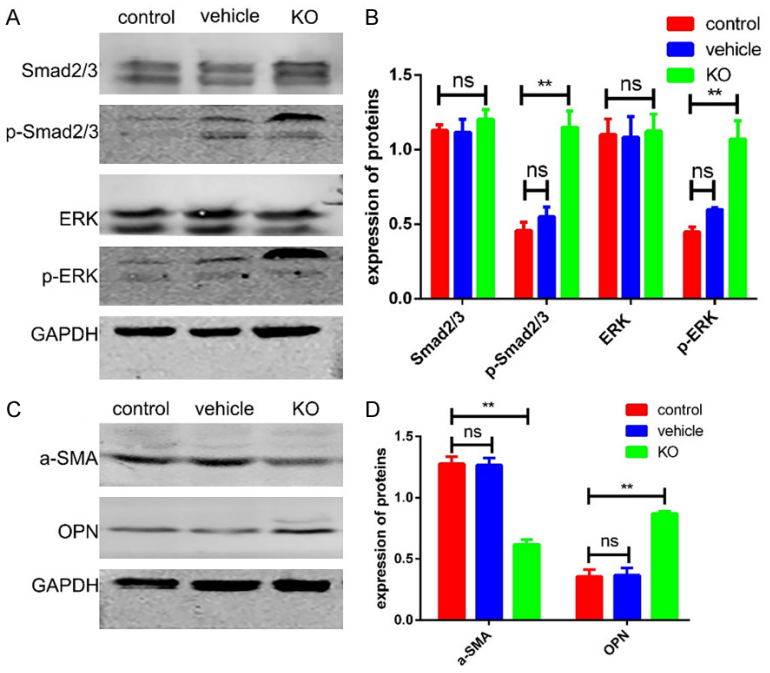


Figure 3. Downregulation of the miR143/145 gene cluster caused a decrease in the activity of the TGF-β1 signaling pathway and phenotypic switching of the VSMC. After knockdown of the miR143/145 gene cluster, the expression of phospho-Smad2/3 and phospho-ERK was significantly decreased (A and B); expression of the contractile phenotype marker of VSMC, α-SMA, decreased, while expression of the synthetic phenotype marker OPN increased (C and D). (Data represent means ± SD (n = 3); $^{**}P < 0.01$).

Knockdown of the miR143/145 gene cluster in VSMCs activates the TGF-β pathway and induced phenotypic switching in vitro

To determine the ability of miR143/145 gene cluster downregulation to activate the TGF-β1 pathway and trigger downstream phenotypic switching, we treated HASMCs in vitro, with lentivirus. Thereafter, we performed western blot analysis of the expression of Smad2/3, phospho-Smad2/3, ERK, phospho-ERK, α-SMA, and OPN. Expression of phospho-Smad2/3 and phospho-ERK was significantly increased (**Figure 3A** and **3B**), while α-SMA was downregulated post-treatment with lentivirus. In contrast, OPN expression was markedly increased (**Figure 3C** and **3D**).

The TGF-β1 pathway mediates phenotypic switching of VSMCs downstream of miR143/145 gene cluster

To verify the impact of the TGF-β1 pathway on phenotypic switching of VSMCs during downregulation of miR143/145 gene cluster, we pre-treated HASMCs with a specific inhibitor of the TGF-β1 pathway immediately before the addition of lentivirus. Our western blotting data showed that TGF-β1 expression was almost completely inhibited (**Figure 4A** and **4B**). Furthermore, the TGF-β1 inhibitor did not affect the expression of miR143/145 gene cluster (**Figure 5**). Western blotting revealed increased expression of α-SMA, although this effect did not reach the level of statistical significance (**Figure 4C** and **4D**), while OPN expression decreased (**Figure 4C** and **4D**).

Discussion

In this study, we discovered a possible mechanism underlying AD. We showed that the miR143/145 gene cluster, an important regulator of VSMC differentiation, can induce the phenotypic switching of VSMCs during the formation of AD, in a process mediated by the TGF-β1 signaling pathway.

Cells respond to changes in the microenvironment by altering many cellular programs, including cell survival, proliferation, differentiation, metabolism, interactions with other cells, and numerous homeostatic loops. VSMCs switch from a contractile to a synthetic phenotype in response to cues in the local environment, in a process known as phenotypic modulation or switching. We demonstrated greater expression of OPN, and reduced α-SMA expression, in the media of specimens taken from tissues undergoing AD compared with the levels in non-pathological controls, thereby indicating that a phenotypic switch had taken place. This result is consistent with that reported by Lesauskaite et al., [2] who also observed phenotypic switching of VSMCs in AD/aneurysm tissues. Detection of the expression of miR143/145 gene cluster in aortic tissues showed significantly reduced expression of miR143 and miR145 in the pathological samples. Following lentivirus-mediated knockdown

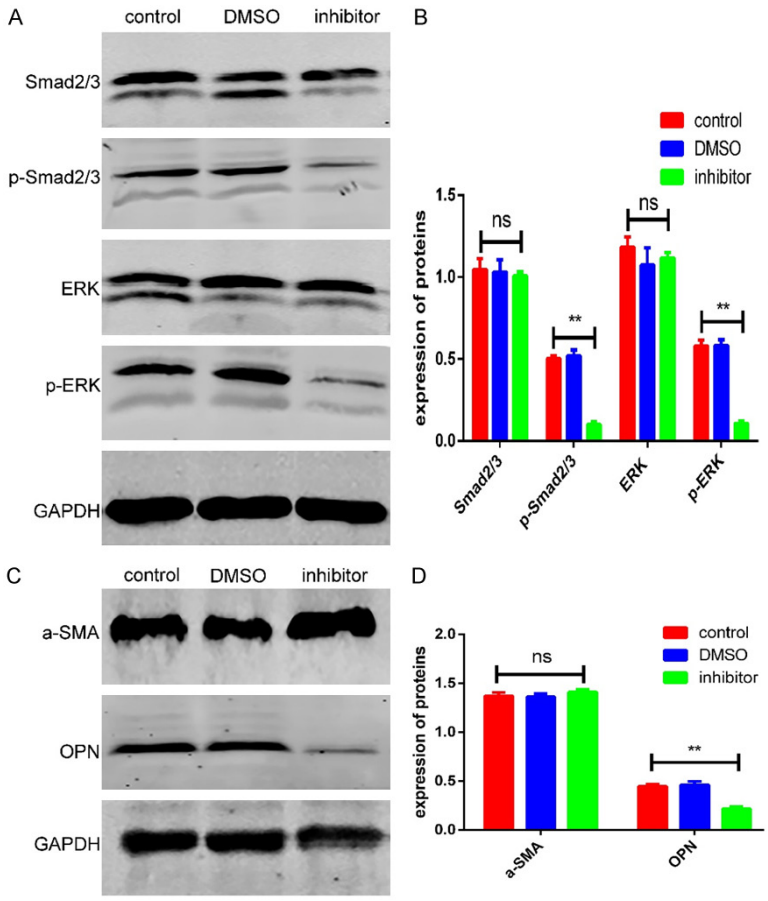


Figure 4. LY364947 maintains the contractile phenotype of VSMC. Activation of the TGF-β1 pathway was blocked by the inhibitor, LY364947 (A and B), and OPN was significantly decreased, while there were no significant changes in α-SMA (C and D). (Data represent means ± SD (n = 3); $^{**}P < 0.01$).

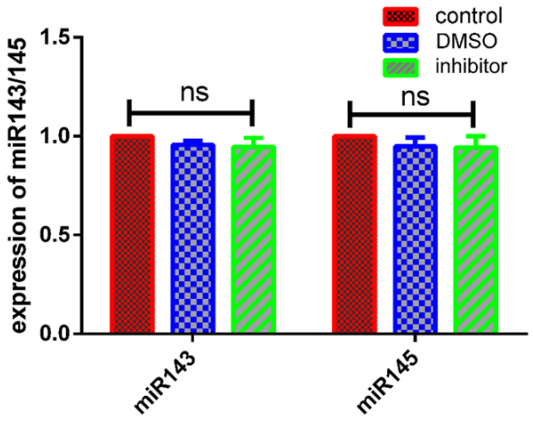


Figure 5. LY364947 does not affect the expression of the miR143/145 gene cluster. Expression of miR143 and miR145 was unaffected by blockade of TGF-β1 pathway activation. (Data represent means ± SD (n = 3); $P > 0.05$).

of the miR143/145 gene cluster in VSMCs in vitro, western blot analysis revealed elevated expression of OPN, and decreased α-SMA expression. These results suggest that downregulation of the miR143/145 gene cluster induced switching of VSMCs from the contractile to the synthetic phenotype.

MiRNAs induce the differentiation of VSMCs in numerous cell culture models [24-29]. Kimberly et al. demonstrated that the miR143/145 gene cluster regulates the fate of VSMCs in regards to processes such as proliferation, differentiation, and migration [9]. However, few studies have examined the pathways underlying the differentiation of VSMCs induced by miRNAs. Therefore, we aimed to characterize the phenotypic response of VSMCs to downregulation of the miR143/145 gene cluster. TGF-β has a wide range of biological functions in vitro and in vivo, including the regulation of cell growth and phenotypes, and inhibition of tumor growth. TFG-β1 pathway dysregulation in humans causes vascular pathologies and cardiovascular diseases, such as arteriovenous malformations, aneurysms, atherosclerosis, cardiac fibrosis, vascular remodeling of the retina, and valvular heart disease [30]. Here, we found that the expression of phospho-Smad2/3 and phospho-ERK was increased in tissues undergoing AD, suggesting that the TGF-β1 pathway was also activated. In addition, downregulation of the miR143/145 gene cluster significantly enhanced the expression of phospho-Smad2/3 and phospho-ERK in HASMCs in vitro. Expression of OPN was also increased under these conditions. Because the expression of OPN tended to increase despite pretreatment with an inhibitor of the TGF-β1 pathway before lentivirus-mediated of the miR143/145 gene cluster, these data indicate the involvement of the TGF-β1 pathway in the phenotypic switching of aortic VSMCs induced by downregulation of the miR143/145 gene cluster.

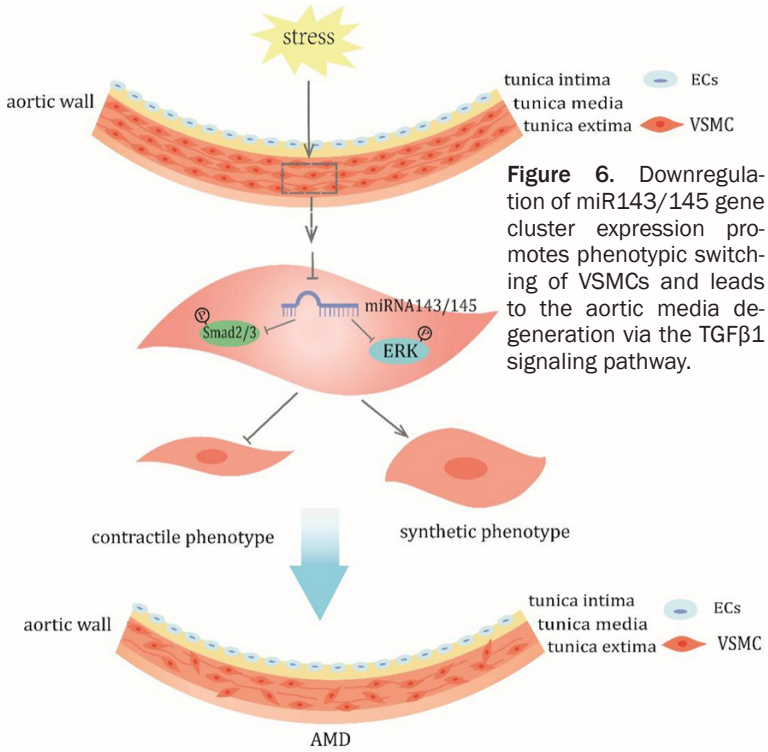


Figure 6. Downregulation of miR143/145 gene cluster expression promotes phenotypic switching of VSMCs and leads to the aortic media degeneration via the TGFβ1 signaling pathway.

VSMC dysfunction leads to a variety of arterial diseases in humans, including atherosclerosis, AD/aneurysm, cancer, and Alzheimer's disease [6, 31-34]. AD is an acute and catastrophic condition, and a serious threat to human health. The normal aorta contains an elastic layer (mainly composed of SMCs), and a layer of elastic fibers that links the continuous frame structure. This arrangement, however, along with the morphology of the fibrous layer was irregular in AD samples. The formation of AD and the characteristic degeneration of the aortic media involve damage and loss of the elastic fibers, phenotypic switching and loss of SMCs, and accumulation of proteoglycan material. However, the pathogenesis of AD is likely to be multifaceted, with other researchers reporting activation of inflammation genes, degradation of extracellular matrix proteins, and upregulated transformation and transcription, while genes encoding the extracellular matrix, cell adhesion proteins, and cytoskeleton proteins are downregulated, thereby indicating that the development of AD is promoted by inflammatory responses [35-37]. In this study, we aimed to demonstrate the mechanism underlying the phenotypic switching of VSMCs during the development of AD. For this, we knocked down the miR143/145 gene cluster by lentivirus, subsequently linking the TGF-β1 pathway to the miR143/145 gene cluster during the phenotypic switching of human aortic VSMCs. Our preliminary experiments demonstrated first that the miR143/145 gene cluster is a positive factor in the differentiation of VSMCs and second, that the TGF-β1 pathway is involved in the phenotypic switching of VSMCs induced by downregulation of the miR143/145 gene cluster (**Figure 6**).

We acknowledge that our investigations are limited by the nature of the retrospective study design. Accordingly, using our data, it is impossible to determine if the downregulation of the miR143/145 gene cluster following activation of the TGF-β1 signaling pathway occurred before or after onset of the pathogenesis of AD. Experimentation using a miR143/145 gene cluster-knockdown animal model will be critical in verifying our findings.

In summary, we have shown that downregulation of the miR143/145 gene cluster affects the expression of TGF-β1 signaling pathway proteins in human aortic VSMCs and promotes phenotypic switching of VSMCs. These outcomes may represent another mechanism by which downregulation of miR143/145 gene cluster induces VSMC phenotypic modulation during the pathogenesis of AD. Our study implicates the miR143/145 gene cluster as an attractive target for strategies aiming to prevent the formation of AD.

Acknowledgements

The authors would like to thank all the patients and donors and the Ethics Committee of Renmin Hospital of WuHan University (Judgement reference number is WDRY2015-K021) for supporting this study. This study was supported by grants from the National Natural Science Foundation of China (no. 81570428) and the Natural Science Foundation of Hubei Provincial (no. 2017CFB340).

Disclosure of conflict of interest

None.

Address correspondence to: Zhiwei Wang, Department of Cardiovascular Surgery, Renmin Hospital of Wuhan University, 238# Jiefang Road, Wuhan 430000, Hubei Province, People's Republic of China. Tel: +86-027-88041911; E-mail: wangzhiwei@whu.edu.cn

References

[1] Hiratzka LF, Bakris GL, Beckman JA, Bersin RM, Carr VF, Casey DE Jr, Eagle KA, Hermann LK, Isselbacher EM, Kazerooni EA, Kouchoukos NT, Lytle BW, Milewicz DM, Reich DL, Sen S, Shinn JA, Svensson LG and Williams DM. 2010 ACCF/AHA/AATS/ACR/ASA/SCA/SCAI/SIR/STS/SVM guidelines for the diagnosis and management of patients with thoracic aortic disease: executive summary. A report of the American college of cardiology foundation/American heart association task force on practice guidelines, American association for thoracic surgery, American college of radiology, American stroke association, society of cardiovascular Anesthesiologists, society for cardiovascular angiography and interventions, society of interventional radiology, society of thoracic surgeons, and society for vascular medicine. Catheter Cardiovasc Interv 2010; 76: E43-86.

[2] Lesauskaite V, Tanganelli P, Sassi C, Neri E, Diciolla F, Ivanoviene L, Epistolato MC, Lalinga AV, Alessandrini C and Spina D. Smooth muscle cells of the media in the dilatative pathology of ascending thoracic aorta: morphology, immunoreactivity for osteopontin, matrix metalloproteinases, and their inhibitors. Hum Pathol 2001; 32: 1003-1011.

[3] Zhang J, Wang L, Fu W, Wang C, Guo D, Jiang J and Wang Y. Smooth muscle cell phenotypic diversity between dissected and unaffected thoracic aortic media. J Cardiovasc Surg (Torino) 2013; 54: 511-521.

[4] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[5] Albinsson S and Sessa WC. Can microRNAs control vascular smooth muscle phenotypic modulation and the response to injury? Physiol Genomics 2011; 43: 529-533.

[6] Albinsson S, Suarez Y, Skoura A, Offermanns S, Miano JM and Sessa WC. MicroRNAs are necessary for vascular smooth muscle growth, differentiation, and function. Arterioscler Thromb Vasc Biol 2010; 30: 1118-1126.

[7] Rangrez AY, Massy ZA, Metzinger-Le Meuth V and Metzinger L. miR-143 and miR-145: molecular keys to switch the phenotype of vascular smooth muscle cells. Circ Cardiovasc Genet 2011; 4: 197-205.

[8] Boettger T, Beetz N, Kostin S, Schneider J, Kruger M, Hein L and Braun T. Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster. J Clin Invest 2009; 119: 2634-2647.

[9] Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, Lee TH, Miano JM, Ivey KN and Srivastava D. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 2009; 460: 705-710.

[10] Zhang C. MicroRNA-145 in vascular smooth muscle cell biology: a new therapeutic target for vascular disease. Cell Cycle 2009; 8: 3469-3473.

[11] Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, Dean DB and Zhang C. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. Circ Res 2007; 100: 1579-1588.

[12] Cheng Y, Liu X, Yang J, Lin Y, Xu DZ, Lu Q, Deitch EA, Huo Y, Delphin ES and Zhang C. MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. Circ Res 2009; 105: 158-166.

[13] Elia L, Quintavalle M, Zhang J, Contu R, Cossu L, Latronico MV, Peterson KL, Indolfi C, Catalucci D, Chen J, Courtneidge SA and Condorelli G. The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. Cell Death Differ 2009; 16: 1590-1598.

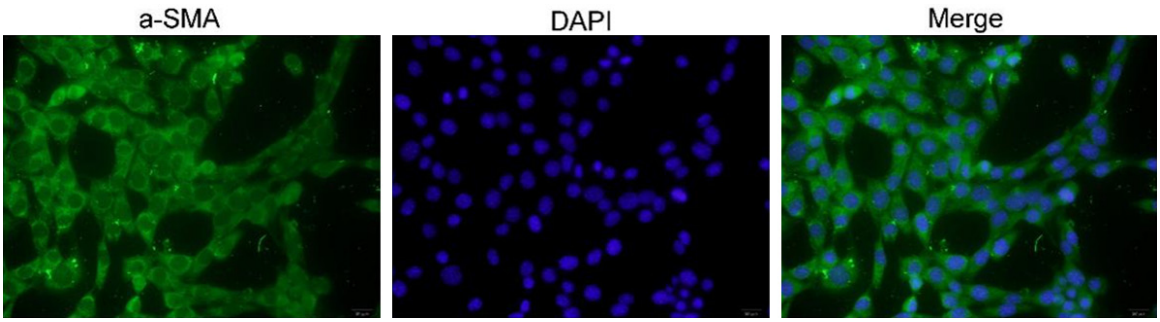
[14] Horbelt D, Denkis A and Knaus P. A portrait of transforming growth factor beta superfamily signalling: background matters. Int J Biochem Cell Biol 2012; 44: 469-474.

[15] Miles FL, Kurtoglu S, Ahmer C, Soori M, Favate JS and Sikes RA. Transforming growth factor-beta signaling induced during prostate cancer cell death and neuroendocrine differentiation is mediated by bone marrow stromal cells. Prostate 2015; 75: 1802-1813.

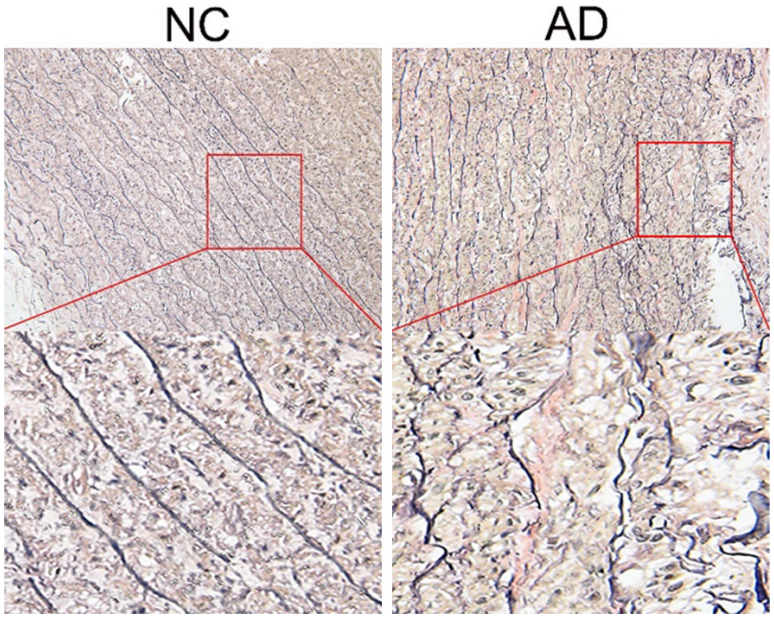
[16] Barnes RM and Black BL. Nodal signaling and congenital heart defects. In: Nakanishi T, Markwald RR, Baldwin HS, Keller BB, Srivastava D, Yamagishi H, editors. Etiology and morphogenesis of congenital heart disease: from gene function and cellular interaction to morphology. Tokyo: Springer; 2016. pp.183-192.

[17] Lamora A, Talbot J, Mullard M, Brounais-Le Royer B, Redini F and Verrecchia F. TGF-beta

signaling in bone remodeling and osteosarcoma progression. J Clin Med 2016; 5.
[18] Verrecchia F and Redini F. Transforming growth factor-beta signaling plays a pivotal role in the interplay between osteosarcoma cells and their microenvironment. Front Oncol 2018; 8: 133.
[19] Goldstein NS, Hewitt SM, Taylor CR, Yaziji H and Hicks DG. Recommendations for improved standardization of immunohistochemistry. Appl Immunohistochem Mol Morphol 2007; 15: 124-133.
[20] Li B, Wang Z, Hu Z, Zhang M, Ren Z, Zhou Z, Huang J and Hu X. P38 MAPK signaling pathway mediates angiotensin II-induced miR143/145 gene cluster downregulation during aortic dissection formation. Ann Vasc Surg 2017; 40: 262-273.
[21] Stewart SA, Dykxhoorn DM, Palliser D, Mizuno H, Yu EY, An DS, Sabatini DM, Chen IS, Hahn WC, Sharp PA, Weinberg RA and Novina CD. Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA 2003; 9: 493-501.
[22] Long X, Bell RD, Gerthoffer WT, Zlokovic BV and Miano JM. Myocardin is sufficient for a smooth muscle-like contractile phenotype. Arterioscler Thromb Vasc Biol 2008; 28: 1505-1510.
[23] Long X, Tharp DL, Georger MA, Slivano OJ, Lee MY, Wamhoff BR, Bowles DK and Miano JM. The smooth muscle cell-restricted KCNMB1 ion channel subunit is a direct transcriptional target of serum response factor and myocardin. J Biol Chem 2009; 284: 33671-33682.
[24] Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL and Wang DZ. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 2006; 38: 228-233.
[25] Ivey KN, Muth A, Arnold J, King FW, Yeh RF, Fish JE, Hsiao EC, Schwartz RJ, Conklin BR, Bernstein HS and Srivastava D. MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. Cell Stem Cell 2008; 2: 219-229.
[26] Kwon C, Han Z, Olson EN and Srivastava D. MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. Proc Natl Acad Sci U S A 2005; 102: 18986-18991.
[27] Srivastava D. Making or breaking the heart: from lineage determination to morphogenesis. Cell 2006; 126: 1037-1048.
[28] Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ and Srivastava D. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell 2007; 129: 303-317.
[29] Zhao Y, Samal E and Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature 2005; 436: 214-220.
[30] Goumans MJ and Ten Dijke P. TGF-beta signaling in control of cardiovascular function. Cold Spring Harb Perspect Biol 2018; 10.
[31] Pauletto P, Sarzani R, Rappelli A, Chiavegato A, Pessina AC and Sartore S. Differentiation and growth of vascular smooth muscle cells in experimental hypertension. Am J Hypertens 1994; 7: 661-674.
[32] Owens GK, Kumar MS and Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 2004; 84: 767-801.
[33] Chow N, Bell RD, Deane R, Streb JW, Chen J, Brooks A, Van Nostrand W, Miano JM and Zlokovic BV. Serum response factor and myocardin mediate arterial hypercontractility and cerebral blood flow dysregulation in Alzheimer's phenotype. Proc Natl Acad Sci U S A 2007; 104: 823-828.
[34] Xu Q. Stem cells and transplant arteriosclerosis. Circ Res 2008; 102: 1011-1024.
[35] Muller BT, Modlich O, Prisack HB, Bojar H, Schipke JD, Goecke T, Feindt P, Petzold T, Gams E, Muller W, Hort W and Sandmann W. Gene expression profiles in the acutely dissected human aorta. Eur J Vasc Endovasc Surg 2002; 24: 356-364.
[36] Cifani N, Proietta M, Tritapepe L, Di Gioia C, Ferri L, Taurino M and Del Porto F. Stanford-A acute aortic dissection, inflammation, and metalloproteinases: a review. Ann Med 2015; 47: 441-446.
[37] Baliyan V, Parakh A, Prabhakar AM and Hedgire S. Acute aortic syndromes and aortic emergencies. Cardiovasc Diagn Ther 2018; 8: S82-S96.



Supplementary Figure 1. HASMC were identified by immunofluorescence staining of α-SMA protein.



Supplementary Figure 2. EVG staining showed that the elastic fibers (black) in the aortic dissection specimen collapsed and collagen (red) deposition increased compared with the normal aortic specimen.