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Downregulation of miRNA-1-3p modulates cyclic stretch-mediated proliferation of vascular smooth muscle cells through regulation of ETS-1

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Abstract: Mechanical stretch modulates the proliferation of vascular smooth muscle cells (VSMCs) and plays an important role in the pathogenesis of hypertension, but the underlying mechanisms are unclear. We investigated the role of microRNA-1-3p (miRNA-1-3p) on the proliferation of VSMCs induced by mechanical cyclic stretch. Our data show that miRNA-1-3p is downregulated in the aorta of the spontaneous hypertension rat (SHR). Pathological mechanical stretch at 15% suppressed the expression of miRNA-1-3p, calponin and SM22, but enhanced the proliferation of VSMCs as well as the expression of the V-ets erythroblastosis virus E26 oncogene homolog 1 (ETS-1), collagen type I alpha (Col-1a), collagen type III alpha (Col-3a) and elastin. Overexpression of miRNA-1-3p inhibited cell proliferation and induced the expression of calponin and SM22, but decreased the expression of ETS-1, Col-1a, Col-3a and elastin. Mechanical stretch at 15% combined with losartan treatment increased the expression of miRNA-1-3p, calponin and SM22, and decreased the expression of ETS-1, Col-1a and Col-3a. Dual luciferase reporter assays revealed ETS-1 as a direct target of miRNA-1-3p. These findings suggest that miRNA-1-3p regulates VSMC function through ETS-1 regulation during hypertension-induced vascular remodeling. MiRNA-1-3p may be a viable therapeutic target for hypertension.

Keywords: miRNA-1-3p; cell proliferation; phenotypic switch; cyclic stretch; vascular smooth muscle cells

Abbreviations: spontaneously hypertensive rat (SHR); Wistar-Kyoto (WKY) rat; vascular smooth muscle cells (VSMCs); microRNAs (miRNAs); angiotensin II (Ang II); angiotensin II type 1 receptor (AT1R); V-ets erythroblastosis virus E26 oncogene homolog 1 (ETS-1); collagen type I alpha (Col-1a); collagen type III alpha (Col-3a); extracellular matrix (ECM); cell counting kit-8 (CCK-8); 5-ethynyl-2'-deoxyuridine (EdU); glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

INTRODUCTION

Vascular cells are subjected to continuous mechanical force due to the pulsatile nature of blood flow [1]. Shear stress primarily affects endothelial cells and cyclic stretch influences a range of cells in the vascular wall, in particular VSMCs. Mature VSMCs are fully differentiated cells that express a variety of VSMC-specific genes including calponin, α-actin, SM22, SM myosin heavy chain and smoothelin, which are identified as markers of mature VSMCs. In response to environmental cues, VSMCs switch from a differentiated (contractile) phenotype to a dedifferentiated (synthetic) phenotype accompanying decreased expression of VSMC-specific genes [1-3]. During hypertension, the exacerbated stretch leads to VSMC dysfunction and pathological vascular remodeling. This is characterized by increased VSMC proliferation, migration, excessive extracellular matrix deposition and a cell phenotypic switch [2,3]. Although the role of mechanical stretch on VSMC functions has been studied, its underlying mechanism(s) have not been fully defined.

MicroRNAs (miRNAs) are a class of small non-coding single-stranded RNA molecules, ~18-24 nucleotides in length, which are important regulators

of gene expression [4]. There is increasing evidence that miRNAs are involved in cardiovascular biology, physiology and pathology. For example, both mir-143 and mir-145 are overexpressed in VSMCs and are considered as the key regulators of restenosis, which decreases during vascular injury and arteriosclerosis [5,6]. Mir-125a-5p and mir-128-3p are regarded as novel modulators of the phenotypic transformation of VSMCs during vascular disease [7,8]. Recent reports have shown that a variety of miRNAs also regulate the mechanical stretch-induced differentiation of VSMCs. It was shown that 16% cyclic stretch could inhibit the key phenotypic molecules of VSMCs by inhibiting mir-145 that regulates the transformation of VSMCs [9]. Also, it was found that mir-144/451 plays an important regulatory role in the phenotypic transformation of VSMCs induced by cyclic stretch [10].

Studies have shown that miRNA-1-3p is associated with a variety of cell functions, including the regulation of cell proliferation, migration and apoptosis [11,12]. It was reported that the expression of miRNA-1-3p was significantly inhibited in hepatocellular carcinoma cell lines, and overexpression of miRNA-1-3p markedly restrained the proliferation of hepatocellular carcinoma cell [13]. Downregulation of miRNA-1-3p was found in a variety of cancers, such as gastric cancer [14], colorectal primary tumors [15] and oral squamous cell carcinoma [16]. A recent study showed that the expression of miRNA-1-3p was decreased in hypertrophic cardiomyopathy and was closely associated with left ventricular dysfunction [17].

MiRNA-1-3p has been reported to regulate growth, migration and phenotypic modulation of VSMCs [18,19]. The expression of miRNA-1-3p was reduced by hypoxia in pulmonary VSMCs from rat as well as in pulmonary VSMCs isolated from pulmonary arterial hypertension patients. Overexpression of miRNA-1-3p protects from the development of pulmonary arterial hypertension [18]. In addition, insulin-like growth factor 1 (IGF1) was identified as a target of miRNA-1-3p in VSMCs, and downregulation of IGF1 by the introduction of miRNA-1 mitigates the proliferation of the VSMCs [19]. Furthermore, upregulation of miRNA-1-3p by myocardin resulted in the inhibition of VSMC proliferation and induction of contractile proteins [20]. Although miRNA-1-3p has been shown to be involved in the regulation of VSMC function, whether it affects the proliferation of VSMC in response to cyclic stretch remains unclear.

In this study, we explored the role of miRNA-1-3p and its pathway in VSMCs during cyclic stretch. We observed that ETS-1 was the direct target of miRNA-1-3p. MiRNA-1-3p modulated the proliferation of VSMC at least in part through the regulation of ETS-1.

MATERIALS AND METHODS

Ethics statement

Animal care and experimental protocols were performed in accordance with the Animal Management Rules of China (55, 2002, Ministry of Health, China). Animal procedures were performed according to protocols approved by the Committee for Animal Care and Use (Ethics code SCXK Shanghai 2018-0004).

Rat models of SHR and WKY

Spontaneously hypertensive rats (SHR) and rats with normal blood pressure (WKY) aged 18 weeks were purchased from the Charles River Laboratories Beijing Co., LTD (Shanghai, China). Blood pressure measurements were performed prior to the harvesting of thoracic aortas. Tissues were stored at -80°C prior to analysis.

Cell culture

Sprague-Dawley (SD) rats weighing 125 g were purchased from Shanghai Jiesijie Experimental Animals Co., LTD. (Shanghai, China). Primary VSMCs were cultured as previously described [21]. VSMC purity was confirmed by immunofluorescent detection of alpha smooth muscle actin (Beyotime Biotechnology, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% pen/strep (Thermo Fisher Scientific, USA) at 37°C and 5% CO_2 in a humidified incubator (Thermo Fisher Scientific, USA).

Cell transfection

VSMCs of the rat thoracic aorta were seeded onto 6-well plates (2.0×10^5 cells/well) and cultured overnight to

~70% confluence. VSMCs were then transfected with miRNA-1-3p agomir or negative controls at a concentration of 20 nmol/L using siRNA-Mate reagent as per the manufacturer's recommendations (Genepharma, Shanghai, China). MiRNA-1-3p agomir sequences were as follows: sense (5'-3') UGG AAU GUA AAG AAG UGU GUA U; antisense: (5'-3') ACA CAC UUC UUU ACAU UCC AUU. Negative control sequences were as follows: sense (5'-3') UUC UCC GAA CGU GUC ACG UTT; antisense (5'-3'): ACG UGA CAC GUU CGG AGA ATT. VSMCs were replaced with fresh media 6 h post-transfection and cultured for subsequent experiments.

Lentiviral transfection

To generate miRNA-1-3p and ETS-1 overexpression plasmids (miRNA-1-3p-OE, ETS-1-OE), miRNA-1-3p and the open reading frame of ETS-1 were cloned into a pLVX-IRES-Zs Green1 lentiviral vector (Genepharma, Shanghai, China). An empty vector was used as a negative control (NC). HEK-293T cells were cotransfected with three helper plasmids and either miRNA-1-3p-OE, ETS-1-OE or empty vector constructs using the GM easy™ Lentiviral packaging kit (Genomeditech, China). Cell supernatants were then collected 72 h post-transfection and added to the VSMCs. Stable cell lines were selected with puromycin (8 ng/mL). Western blotting was performed to confirm miRNA-1-3p or ETS-1 expression.

Cyclic stretch loading of cells

VSMCs were processed using the Flexcell® cyclic stretch loading system (Flexcell International Corporation, USA) [3]. VSMCs were plated onto collagen coated cell culture plates at a density of 2.0 x 10^5 cells per well at 37°C and 5% CO_2. When the cell density reached ~80%, the VSMCs were synchronized in serum-free DMEM for 24 h and subjected to cyclic stretch at 1 Hz for the indicated times. Cells were divided into three treatment groups as follows: (i) 5% normal cyclic stretch; (ii) 15% high cyclic stretch; (iii) 15% high cyclic stretch combined with losartan.

Real time PCR

Thoracic aorta or VSMCs samples were lysed in TRIzol and cDNA was generated using PrimeScript RT reagent kit with the gDNA eraser (TaKaRa, Japan). RT-PCRs were performed using 2×SGExcel UltraSYBR Mixtures with ROX (Sangon Biotech, China) on an Applied Biosystems 7500 Real-time PCR system (Thermo Fisher Scientific, USA). RNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH. MiRNA-1-3p expression was normalized to U6. The primers used in the study are shown in Table S1.

Western blot analysis

VSMCs were harvested in radioimmunoprecipitation assay buffer supplemented with proteinase inhibitor cocktail (MedChem Express, USA) and lysed using an ultrasonic cell disruption system (Shunmatech, China) at 10% intensity on ice. Lysates were centrifuged at 600 ×g for 5 min at 4°C, and proteins were resolved on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels (PAGE). Resolved proteins were transferred to nitrocellulose membranes, blocked in 5% non-fat milk in tris-buffered saline with 0.1 % Tween 20 for 1 h, and probed with primary antibodies at 4°C overnight. Primary antibodies and dilutions were as follows: anti-α-tubulin (1:1000), anti-ETS-1 (1:2000), anti-elastin (1:2000), anti-SM22 (1:2000), anti-calponin (1:2000), anti-GAPDH (1:2000), anti-Col-1a (1:2000), anti-β-actin (1:2000), (Proteintech, USA). Membranes were then washed and labeled with the corresponding HRP-conjugated goat anti-mouse (1:10000) or goat anti-rabbit secondary antibodies (1:10000) (Proteintech, USA) at room temperature for 1 h. Proteins were visualized using the enhanced chemiluminescence system (Absin Bioscience, China) and signals were quantified on a Tanon image analyzer (Tanon Science, Shanghai, China).

CCK-8 assay

VSMCs were seeded in Flexcell plates at a density of 1.0×10^5 cells/well. CCK-8 solution was added at 0 h, 24 h, 48 h and 72 h of culture, and absorbance values for each well were read at 450 nm on a microplate reader (BioTek, USA).

EdU assay

EdU assays were performed according to the manufacturer's recommendations (Beyotime Biotechnology,

Shanghai, China). Briefly, VSMCs were seeded into Flexcell plates at a density of 2×10^5 cells/well and treated with 100 μL of 50 μM EdU in media for 2 h. Cells were then fixed in 4% paraformaldehyde for 30 min and permeabilized in 0.1% Triton-100 phosphate buffered saline for 20 min. Cells were subsequently washed in phosphate-buffered saline (PBS), labeled with EdU working solution for 30 min and counterstained with Hoechst 33342. Cells were imaged on a fluorescence microscope (Olympus Japan).

Cell cycle analysis

Cell cycle analysis was performed using a cell cycle detection kit (Beyotime Biotechnology, Shanghai, China) as per the manufacturer's recommendations. Briefly, VSMCs were collected, fixed in 70% ethanol at 4°C and stained with propidium iodide (50 μg/mL) for 30 min at 37°C. Cell cycle status was analyzed by flow cytometry (BD Biosciences, USA). ModFIT LT 5.0 (Verity Software House, USA) was used to determine relative cell cycle distributions.

Dual luciferase assays

A 3'-UTR sequence of wild-type (WT) ETS-1 containing the seed sequence for miRNA-1-3p or a TTGG mutation in the seed sequence were cloned into the pmirGLO vector (Promega, USA). WT and mutated recombinant plasmids (mut) of ETS-1 3'-UTR were then cotransfected into HEK-293T cells with miRNA-1-3p agomir or negative controls using Lipofectamine™ 3000 (Invitrogen USA). At 48 h post-transfection, luciferase activity was assessed using the dual-luciferase reporter assay system (Promega). Values were normalized to Renilla luciferase activity.

Statistical analysis

Data are the mean ± SD from three independent experiments. Statistical analyses were performed using GraphPad Prism 7. P-values were calculated using Student's *t* test or AVOVA. P≤0.05 was considered as statistically significant.

RESULTS

Decreased expression of miRNA-1-3p in the thoracic aorta of SHRs

We first investigated the effects of hypertension on miRNA-1-3p expression. Fig. 1A shows that miRNA-1-3p expression in the thoracic aorta of SHRs decreased by ~58% compared to WKY rats. These data indicate that hypertension impaired miRNA-1-3p expression.

Mechanical cyclic stretch suppresses miRNA-1-3p expression in rat VSMCs

We next investigated the effects of 5% and 15% cyclic stretches on miRNA-1-3p expression in VSMCs. Compared to 5% cyclic stretch, the expression of miRNA-1-3p at 15% cyclic stretch decreased by 55%, 58%, 64% and 59% at 3 h, 6 h, 12 h and 24 h, respectively (Fig. 1B).

MiRNA-1-3p inhibits VSMC proliferation induced by mechanical stretch

To investigate the effects of miRNA-1-3p on VSMC proliferation, cells were transfected with 50 and 100 pmol/L miRNA-1 agomir. The expression of miRNA-1-3p in the VSMCs increased by ~60% and ~76%, respectively, compared to control cells (Fig. 2A). In the higher mechanical stretch (15%) condition, CCK-8 assays revealed that the OD values of VSMCs overexpressing miRNA-1-3p from VSMCs were significantly lower than NC controls at 24 h, 48 h and 72 h (Fig.

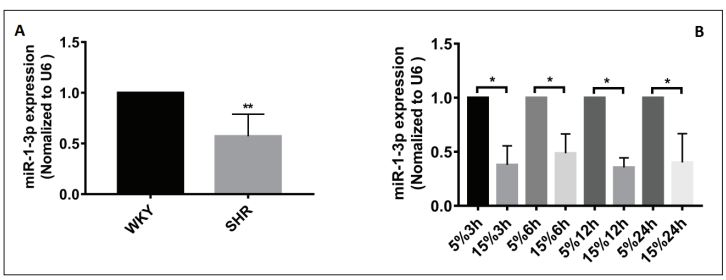

Fig. 1. miRNA-1-3p mRNA expression in the aorta and cyclic stretch-treated VSMCs. **A** – qRT-PCR was used to detect relative mRNA expression levels of miRNA-1-3p in the aorta in SHR and WKY experimental animals; U6 was used as internal normalization. **B** – qRT-PCR was used to detect relative mRNA expression levels of miRNA-1-3p in VSMCs stimulated by cyclic stretch from 3 h to 24 h; U6 was used for internal normalization. All data are expressed as the mean±SD from three independent experiments. *P<0.05; **P<0.01.

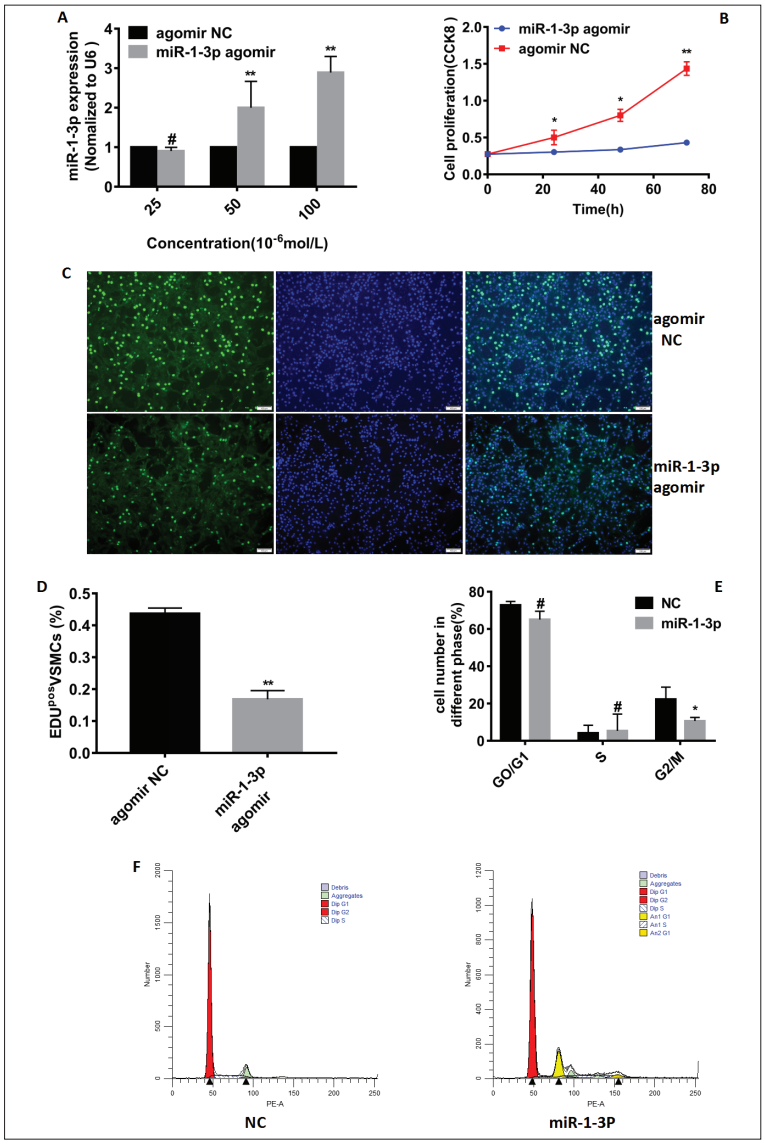

Fig. 2. MiRNA-1-3p inhibits VSMC proliferation induced by mechanical stretch. **A** – Increased expression of miRNA-1-3p was detected by qRT-PCR in VSMCs overexpressing miRNA-1-3p; U6 was used for internal normalization. **B** – Effect of miRNA-1-3p overexpression on VSMCs proliferation analyzed by CCK-8. **C, D** – Effect of miRNA-1-3p overexpression on VSMCs proliferation analyzed by EdU. **E, F** – miRNA-1-3p overexpression decreased the number of VSMCs in the G2/M phase of the cell cycle as demonstrated by flow cytometry. All data are expressed as the mean±SD from three independent experiments. #P>0.05; *P<0.05; **P<0.01.

2B). EdU assays further showed that overexpression of miRNA-1-3p significantly reduced cell growth compared to control cells in VSMCs stimulated by a 15% mechanical stretch for 24 h (Fig. 2C-D). Similarly, flow cytometry analysis indicated that the proportion of VSMCs overexpressing miRNA-1-3p in the G2/M phase were significantly lower than for control cells (Fig. 2E-F). Taken together, these data suggest that the overexpression of miRNA-1-3p inhibits VSMC proliferation stimulated by 15% mechanical stretch.

MiRNA-1-3p increases the expression of cell phenotype molecules

We next examined the effects of miRNA-1-3p on the expression of known contractile phenotype in VSMCs. Compared to the 5% cyclic stretch, the 15% cyclic stretch decreased calponin mRNA (Fig. 3A) and protein expression (Fig. 3C). Similarly, the expression of SM22 mRNA (Fig. 3B) and protein (Fig. 3D) in response to the 15% cyclic stretch were reduced as compared to the 5% cyclic stretch, from 3 h to 24 h. In contrast, the overexpression of miRNA-1-3p increased mRNA and protein (Fig. 3E-H) expression of both calponin and SM22. These data indicate that miRNA-1-3p influences the contractile phenotype of VSMCs.

MiRNA-1-3p decreases the expression of ECM-associated proteins

We investigated the expression of Col-1a, Col-3a and elastin in response to miRNA-1-3p overexpression. As shown in Fig. 4A, the expression of Col-1a mRNA after the 15% cyclic stretch increased when compared to the response to the 5% cyclic stretch from 3 h to 24 h. Consistent with this finding, the expression of Col-1a protein increased in VSMCs in response to the 15% cyclic stretch when compared to the 5% cyclic stretch (Fig. 4C). The expression of Col-3a mRNA following the 15% cyclic stretch similarly increased as compared to the 5% cyclic stretch from 3 h to 24 h (Fig. 4B). Similar data were observed for elastin at 15% cyclic stretch (Fig. 4D). However, the expression of Col-1a and Col-3a in VSMCs overexpressing miRNA-1-3p decreased compared to control cells (Fig. 4E-G). These data indicate that miRNA-1-3p regulates ECM formation in VSMCs.

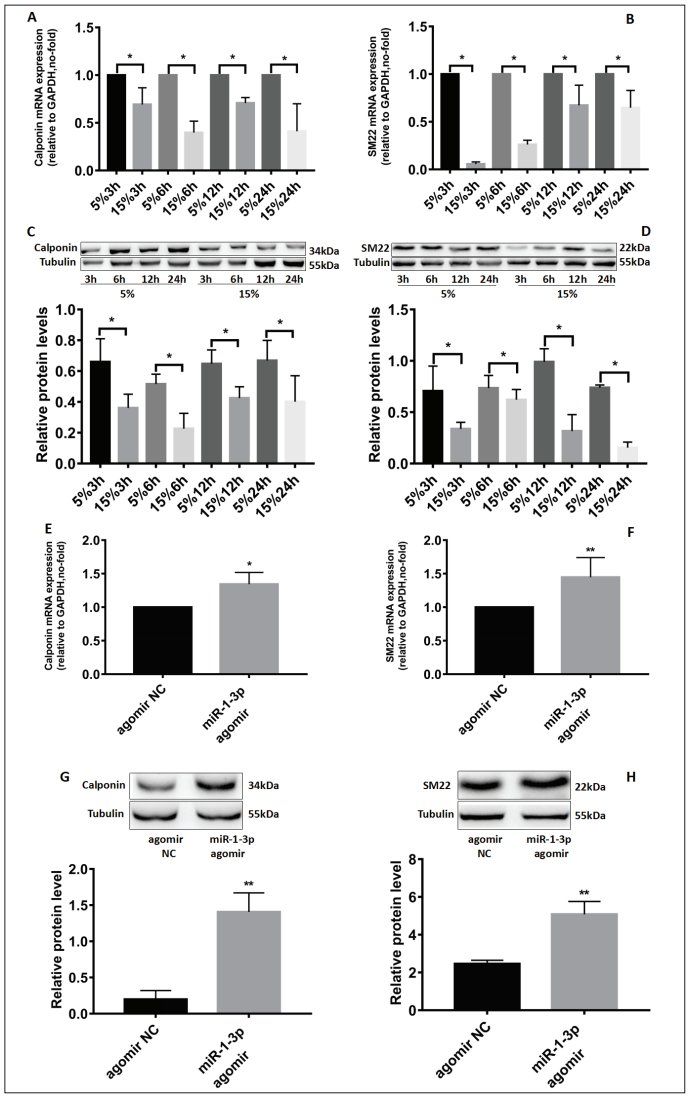

Fig. 3. MiRNA-1-3p increases the expression of cell phenotype molecule. **A, B** – qRT-PCR was used to detect relative mRNA levels for calponin, SM22 gene expression in a 15% cyclic stretch from 3 h to 24 h; GAPDH was used for internal normalization. **C, D** – Western blot analysis showing increased calponin and SM22 protein expression, respectively, in a 15% cyclic stretch from 3 h to 24 h, respectively; α-tubulin was used as the loading control. **E, F** – Decreased expression of calponin and SM22, respectively, was calculated by qRT-PCR in VSMCs overexpressing miRNA-1-3p; GAPDH was used for internal normalization. **G, H** – Decreased expression of calponin and SM22, respectively, was detected by Western blotting in VSMCs overexpressing miRNA-1-3p; α-tubulin was used as the loading control. All data are expressed as the mean±SD from three independent experiments. *P<0.05; **P<0.01.

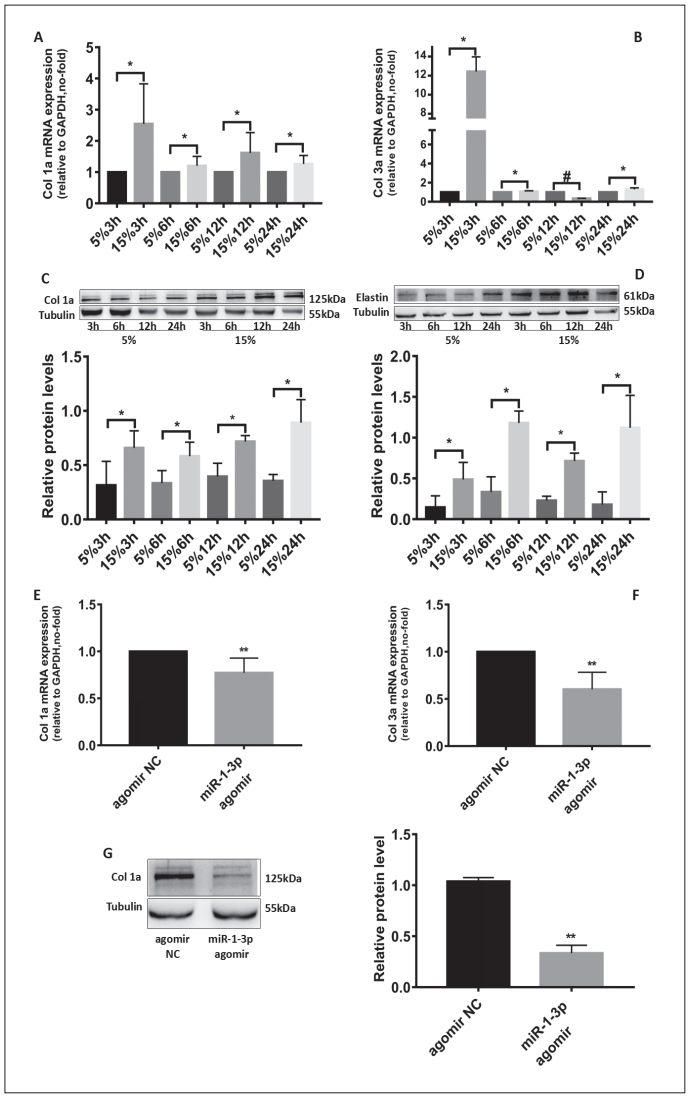

Fig. 4. MiRNA-1-3p decreases the expression of extracellular matrix associated proteins. **A, B** – qRT-PCR was used to detect increased relative Col-1a and Col-3a gene mRNA levels, respectively, after a 15% cyclic stretch from 3 h to 24 h; GAPDH was used for internal normalization. **C, D** – Western blot analysis showing increased Col-1a and elastin protein expression, respectively, in a 15% cyclic stretch from 3 h to 24 h; α-tubulin was used as the loading control. **E, F** – Decreased expression of Col-1a and Col-3a, respectively, was detected by qRT-PCR in VSMCs overexpressing miRNA-1-3p; GAPDH was used for internal normalization. **G** – Decreased expression of Col-1a was detected by Western blotting in VSMCs overexpressing miRNA-1-3p; α-tubulin was used as the loading control. All data are expressed as the mean±SD from three independent experiments. # P>0.05, *P<0.05, **P<0.01.

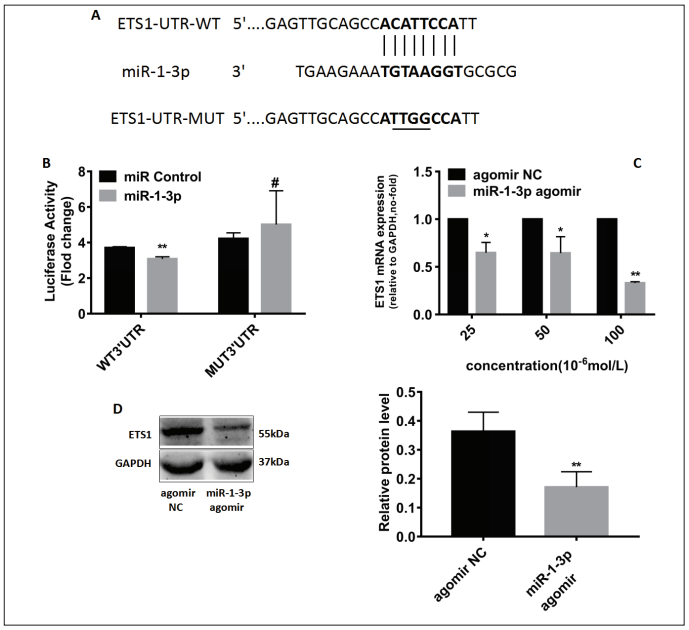

Fig. 5. Regulatory effects of miRNA-1-3p on ETS-1. Schematic of the miRNA-1-3p putative binding site in rat ETS-1 3'-UTR and alignment of wild-type and mutated ETS-1 3'-UTR binding sites of miRNA-1-3p. The four mutated nucleotides are underlined. **B** – Luciferase reporter assay after transfection. **C** – Decreased expression of ETS-1 was detected by qRT-PCR in VSMCs overexpressing miRNA-1-3p; GAPDH was used for internal normalization. **D** – Decreased expression of ETS-1 was detected by Western blotting in VSMCs overexpressing miRNA-1-3p; GAPDH was used as loading control. All data are expressed as the mean±SD from three independent experiments. #P>0.05, *P<0.05, **P<0.01.

Regulatory effects of miRNA-1-3p on ETS-1

A complementary binding region between miRNA-1-3p and the ETS-1 3' untranslated region (3'-UTR) was predicted using TargetScan 7.1 software, indicating ETS-1 as a potential target of miRNA-1-3p (Fig. 5A). Dual luciferase reporter gene assays showed that miRNA-1-3p significantly downregulated the activity of WT ETS-1 (3'UTR). The luciferase activity of the mutant ETS-1 (3'UTR) reporter was not affected. (Fig. 5B). As shown in Fig. 5C, D, the overexpression of miRNA-1-3p also downregulated the expression of EST-1 at both mRNA and protein levels. These data show that ETS-1 is a direct target of miRNA-1-3p through its binding to the 3'UTR.

ETS-1 promotes VSMC proliferation

Following the identification of ETS-1 as a direct target of miRNA-1-3p, we investigated its expression in response to cyclic stretch. As shown in Fig. 6A, ETS-1

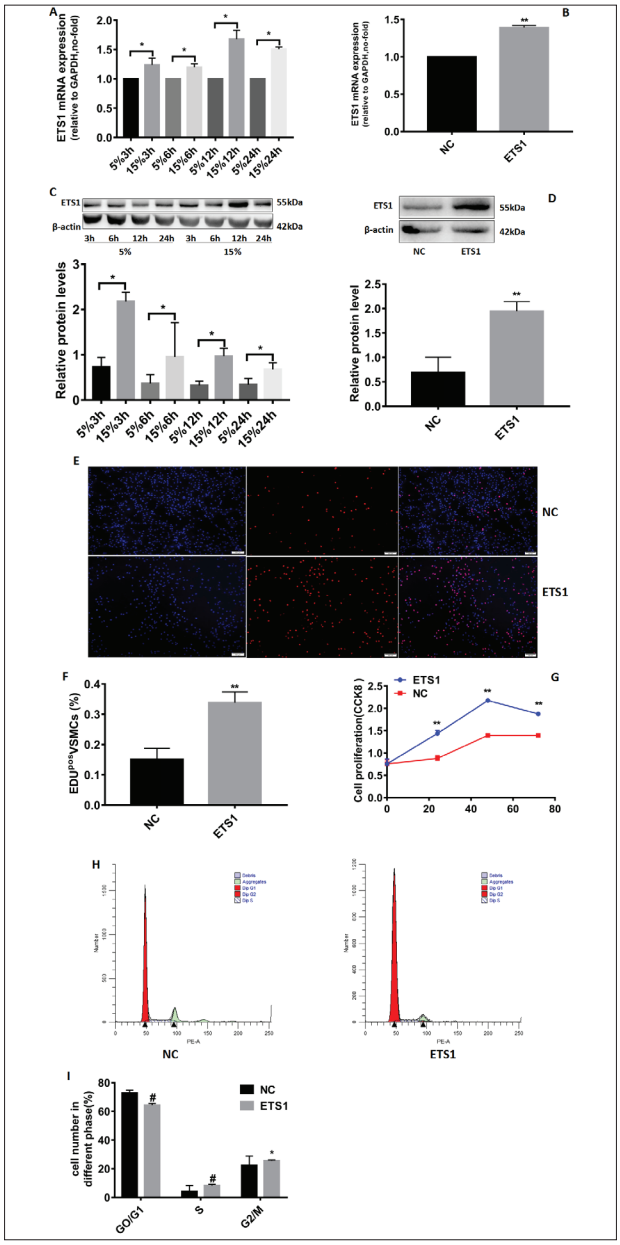

Fig. 6. ETS-1 promotes VSMC proliferation. **A** – qRT-PCR was used to detect the expression of relative ETS-1 gene mRNA levels in a 15% cyclic stretch from 3 h to 24 h; GAPDH was used for internal normalization. **B** – Expression of ETS-1 was detected by qRT-PCR in VSMCs overexpressing ETS-1; GAPDH was used for internal normalization. **C** – Western blot analysis showing increased ETS-1 protein expression in a 15% cyclic-stretched VSMCs from 3 h to 24 h; β-actin was used as the loading control. **D** – Increased expression of ETS-1 was detected by Western blotting in VSMCs overexpressing ETS-1; β-actin was used as loading control. **E, F** – Effect of ETS-1 overexpression on VSMCs proliferation analyzed by EdU. **G** – Effect of ETS-1 overexpression on VSMCs proliferation analyzed by CCK-8. **H, I** – ETS-1 overexpression increased the number of VSMCs in the G2/M phase of cell cycle as demonstrated by flow cytometry. All data are expressed as the mean±SD from three independent experiments. #P>0.05, *P<0.05, **P<0.01.

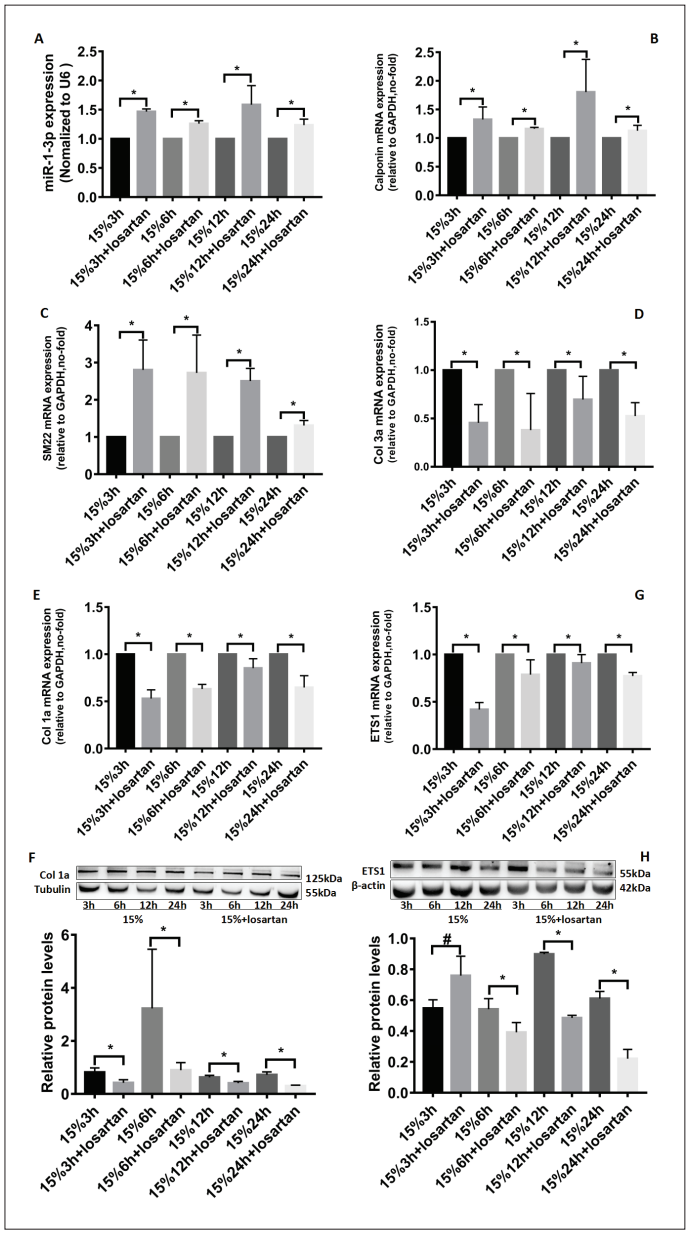

Fig. 7. Ang II-mediated effects of miRNA-1-3p on VSMC function. **A** – qRT-PCR was used to detect the effect of miRNA-1-3p on the relative mRNA expression in a 15% cyclic stretch combined with losartan; U6 was used for internal normalization. **B, C** – qRT-PCR was used to detect relative levels of calponin and SM22 mRNA, respectively, after a 15% cyclic stretch combined with losartan; **D, E** – qRT-PCR was used to detect relative levels of Col-3a and Col-1a mRNA, respectively, after a 15% cyclic stretch combined with losartan; **G** – qRT-PCR was used to detect relative levels of ETS-1 mRNA after a 15% cyclic stretch combined with losartan; GAPDH was used for internal normalization. **F** – Western blot analysis showing decreased Col-1a protein expression in a 15% cyclic stretch combined with losartan; α-tubulin was used as the loading control. **H** – Western blot analysis showing decreased ETS-1 protein expression in a 15% cyclic stretch combined with losartan; β-actin was used as loading control. The time intervals from 3 h to 24 h. #P>0.05, *P<0.05, **P<0.01.

expression at the mRNA level in response to a 15% cyclic stretch increased by 24%, 27%, 82% and 51% compared to the 5% cyclic stretch at 3 h, 6 h, 12 h and 24 h, respectively. A similar increase in ETS-1 expression was observed at the protein level (Fig. 6C). We further confirmed that ETS-1 expression in VSMC-EST-1-overexpression cell lines increased when compared to control cells (Fig. 6B-D). EdU analysis demonstrated that EST-1-overexpression enhanced the proliferation of VSMCs compared to control cells under the 15% mechanical stretch (Fig. 6E-F). Similarly, the CCK-8 assay revealed that the OD values of VSMCs overexpressing ETS-1 were significantly higher than NC controls (Fig. 6G). Cell cycle assessment indicated that the proportion of VMSC in the G2/M phase post ETS-1-overexpression VSMCs was markedly higher than in control cells (Fig. 6H-I). These data suggested that the overexpression of ETS-1 increased VSMC proliferation.

Ang II mediated effects of miRNA-1-3p on VSMC function

Angiotensin II is an important regulator of hypertension. To assess its contribution to the effect of miRNA-1-3p on VSMCs, the cells were exposed to a 15% cyclic stretch in the presence or absence of losartan, an angiotensin II receptor antagonist that competes with the binding of angiotensin II to AT1R. Compared to a 15% cyclic stretch, losartan treatment increased miRNA-1-3p expression (Fig. 7A). However, losartan treatment led to a loss of ETS-1 mRNA and protein expression (Fig. 7B-C). Losartan treatment also increased calponin and SM22 mRNA and protein expression (Fig. 7D-E). However, the expression of Col-1a and Col-3a mRNAs in the 15% cyclic stretch group when combined with losartan decreased when compared to the 15% cyclic stretch alone (Fig. 7F-G). Consistent with this finding, Col-1a protein expression in VSMCs exposed to a 15% cyclic stretch in combination with losartan decreased (Fig. 7F).

DISCUSSION

In this study, we show that (i) miRNA-1-3p expression is downregulated in the aorta of SHR; (ii) in response to mechanical stretch, miRNA-1-3p and ETS-1 are downregulated; (iii) miRNA-1-3p and ETS-1 inhibit VSMC proliferation and regulate the expression of contractile markers and markers of the extracellular matrix *in vitro*; (iv) ETS-1 is a direct target of miRNA-1-3p in VSMCs; (v) AT1 receptors regulate the mechanical stretch-mediated expression of miRNA-1-3p and ETS-1. Accumulating evidence implicates miRNAs in the regulation of VSMC functioning [22,23]. Thus, mir-221 is upregulated in proliferating VSMCs following vascular injury, and the loss of mir-221 suppresses VSMC proliferation and migration [24]. Mir-145 is highly expressed in VSMCs. The levels of mir-145 decrease in atherosclerotic vessels and its overexpression suppresses VSMC proliferation [25]. Several miRNAs have been shown to modulate the mechanical stretch-mediated functions of VSMCs, including the upregulation of mir-21 in response to mechanical stretch-induced VSMC proliferation [26]. In contrast, increased mechanical stretch inhibits the expression of miRNA-1-3p, which contributes to the increased proliferation of VSMCs [3]. In the present study we report that miRNA-1-3p is downregulated in the aorta of SHR. The expression of miRNA-1-3p also decreased when VSMCs were subjected to a 15% cyclic stretch, reducing VSMC proliferation; these results are consistent with those reported previously [20]. MiRNA-1-3p has been shown to play an important role in the growth, migration and apoptosis of cancer cells [27]. MiRNA-1-3p modulates skeletal and cardiac muscle proliferation and is considered a muscle-specific miRNA [28]. It was shown that miRNA-1-3p is highly expressed in VSMCs and the inhibitory effect of miRNA-1-3p on VSMC is myocardin-dependent [20]. Hypoxia suppresses miRNA-1-3p expression in pulmonary VSMCs, whereas the overexpression of miRNA-1-3p inhibits pulmonary VSMC (PVSMC) proliferation through the targeting of sphingosine kinase 1 [18]. The downregulation of miRNA-1 was observed in VSMCs from SHR, and overexpression of miRNA-1-3p inhibited VSMC proliferation and migration by targeting IGF 1 [19]. Taken together, these results implicate miRNA-1-3p in mechanical stretch-induced VSMC proliferation.

Our data highlight ETS-1 as a direct target of miRNA-1-3p. ETS-1 belongs to the ETS family, functioning as a transcription factor that modulates a variety of cellular processes during cardiovascular disease [29]. Recent studies indicate that angiotensin II-induced ETS-1 expression promotes cardiac fibrosis; the deletion of ETS-1 alleviates angiotensin II-mediated cardiac hypertrophy and fibrosis [30]. ETS-1 is highly expressed in VSMCs and has been implicated in the regulation of cell apoptosis, migration and proliferation. *In vivo* studies demonstrated that infusion of angiotensin II increases ETS-1 expression in VSMCs of the mouse thoracic aorta [31]. ETS-1 inhibits the apoptosis and proliferation of VSMCs through activation of the cyclin-dependent kinase inhibitor p21 [32]. ETS-1 accelerates the progression of cerebral aneurysms through its ability to upregulate MCP-1 in VSMCs [33]. Moreover, ETS-1 blockade also prevents neointima formation in the rat carotid arteries of balloon injury models [34]. Based on these findings, we hypothesized that ETS-1 mediates the inhibitory effects of miRNA-1-3p on VSMCs. We found that high cyclic stretch stimulation enhanced ETS-1 expression in VSMCs, while the overexpression of miRNA-1-3p suppressed EST-1 levels. The dual luciferase reporter assays confirmed ETS-1 as a direct target of miRNA-1-3p. Furthermore, the overexpression of EST-1 enhanced the proliferation of VSMCs. These results suggest that miRNA-1-3p modulates the cyclic stretch-induced proliferation of VSMCs through the upregulation of ETS-1.

VSMCs reside in the medial layer of blood vessels and are subject to mechanical stretch as a result of pulsating blood flow [1,2]. Studies have revealed that physiological stretch is essential for the maintenance of normal vascular structure. However, pathological and mechanically activated intracellular signal cascades lead to adverse vascular remodeling, characterized by abnormal proliferation, migration and excessive deposition of the extracellular matrix [3]. Previous studies showed that an 18% mechanical stretch led to a loss of contractile marker protein expression, including α-SMA, SM-HMC and calponin in human umbilical arterial smooth muscle cells following the activation of Yes-associated protein/transcriptional coactivator with PDZ-binding motif (WWTR1, also known as TAZ (YAP/TAZ) signaling [35]. Recent studies found that 18% cyclic stretch enhanced the expression of collagen I and collagen III in human aortic smooth muscle cells [36], and in hypertensive rat aortas [37]; mechanical stretch also increased elastin synthesis [38]. It is known that miRNA-1-3p and ETS-1

regulate the expression of VSMC phenotype molecule [20,39]. Loss of miRNA-1-3p significantly enhances collagen I expression in bone marrow mesenchymal stem cells [40]. Similarly, increased expression of collagen I, collagen III, matrix metalloproteinase (MMP) 1 and MMP-9 were observed in ETS-1-overexpressing VSMCs [41]. In response to a 15% cyclic stretch when compared to the 5% cyclic stretch group, we observed a decrease in SM22 and calponin expression and an increase in extracellular matrix collagen I, collagen III and elastin. The overexpression of miRNA-1-3p increased the expression of SM22 and calponin, but reduced the expression of collagen I, collagen III and elastin. As miRNA-1-3p modulates ETS-1 expression, our results suggest that miRNA-1-3p controls the expression of the cell phenotype molecule and the extracellular matrix, thereby controlling vascular remodeling.

The renin-angiotensin system (RAS) plays a key role in the regulation of vascular function and structure [42]. As the main effector of RAS, angiotensin II exerts a variety of adverse cardiovascular effects through binding to Ang II type I receptor (AT1R) [41]. Previous reports have implicated the RAS in cyclic stretch-mediated cellular functions. Ang II expression was induced in rat VSMCs stimulated by a 20% mechanical stretch [43]. It was found that stretch led to an increase in Ang II secretion in VSMCs [44]. Mechanical stretch significantly augments the expression of AT1R in SHR VSMCs, which contributes to effects on cell proliferation [45]. Given that Ang II regulated the expression of miRNA-1-3p and ETS-1 [30], we hypothesized that RAS modulates mechanical stretch-mediated miRNA-1-3p and ETS-1 expression. Indeed, we found that treatment with the AT1 receptor antagonist losartan increased the expression of miRNA-1-3p, SM22 and calponin, but decreased the expression of ETS-1 and the extracellular matrix. These data imply that under situations of high cyclic stretch, the expression of miRNA-1-3p and ETS-1 are regulated by Ang-II paracrine mechanisms.

Several limitations of this study should be considered. First, we only examined the effects of miRNA-1-3p on the functions of VSMCs in overexpression studies, so the effects of miRNA-1-3p were not precisely defined; second, the role of miRNA-1-3p on vascular remodeling was not investigated *in vivo*. Both warrant further investigations.

CONCLUSION

MiRNA-1-3p was shown to regulate cell proliferation in rat VSMCs in response to mechanical stretch through direct targeting of ETS-1. Ang II was shown to regulate the stretch-induced expression of miRNA-1-3p and ETS-1. These findings provide new insight into the pathogenesis of vascular remodeling. The results reveal novel targets for the treatment of vascular diseases, including hypertension and atherosclerosis.

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REFERENCES

1. Yamashiro Y, Yanagisawa H. The molecular mechanism of mechanotransduction in vascular homeostasis and disease. Clin Sci (Lond). 2020;134(17):2399-418.
2. Rodríguez AI, Csányi G, Ranayhossaini DJ, Feck DM, Blose KJ, Assatourian L, Vorp DA, Pagano PJ. MEF2B-Nox1 signaling is critical for stretch-induced phenotypic modulation of vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2015;35(2):430-8.
3. Wang WB, Li HP, Yan J, Zhuang F, Bao M, Liu JT, Qi YX, Han Y. CTGF regulates cyclic stretch-induced vascular smooth muscle cell. proliferation via microRNA-19b-3p. Exp Cell Res. 2019;376(1):77-85.
4. Orang AV, Safaralizadeh R, Kazemzadeh-Bavili M. Mechanisms of miRNA-mediated gene regulation from common downregulation to miRNA-Specific upregulation. Int J Genomics. 2014;2014:970607.
5. Shi L, Tian C, Sun L, Cao F, Meng Z. The incRNA TUG1/miR-145-5P/FGF10 regulates proliferation and migration in vsmcs of hypertension. Biochem Biophys Res Commun. 2018;501(3):688-95.
6. Gareri C, De Rosa S, Indolfi C. Micro RNAs for Restenosis and Thrombosis After Vascular injury. Circ Res. 2016;118(7):1170-84.
7. Zheng XT, Wu ZH, Xu K, Qiu YH, Su X, Zhang Z, Zhou MT. Interfering histone deacetylase 4 inhibits the proliferation of vascular smooth muscle cells via regulating MEG3/miR-125a-5p/IRF1. Cell Adh Migr. 2019;13(1):41-9.
8. Farina FM, Hall IF, Serio S, Zani S, Climent M, Salvarani N, Carullo P, Civilini E, Condorelli G, Elia L, Quintavalle M. miR-128-3p Is a Novel Regulator of Vascular Smooth Muscle Cell Phenotypic Switch and Vascular Diseases. Circ Res. 2020;126(12):e120-35.

9. Hu B, Song JT, Qu HY, Bi CL, Huang XZ, Liu XX, Zhang M. Mechanical stretch suppresses microRNA-145 expression by activating extracellular signal-regulated kinase 1/2 and upregulating angiotensin-converting enzyme to alter vascular smooth muscle cell phenotype. PLoS One. 2014;9(5):e96338.
10. Turczynska K M, Bhattachariya A, Säll J, Göransson O, Swärd K, Hellstrand P, Albinsson S. Stretch-sensitive down regulation of the miR - 144/451 cluster in vascular smooth muscle and its role in AMP-activated protein kinase signaling. PLoS One. 2013;8(5):e65135.
11. Kura B, Kalocayova B, Devaux Y, Bartekova M. Potential Clinical Implications of miR-1 and miR-21 in Heart Disease and Cardioprotection. Int J Mol Sci. 2020;21(3):700.
12. Han C, Shen JK, Hornicek FJ, Kan QC, Duan ZF. Regulation of microRNA-1 (miR-1) expression in human cancer. Biochim Biophys Acta Gene Regul Mech. 2017;1860(2):227-32.
13. Zhang H, Zhang ZY, Gao LL, Qiao ZD, Yu MH, Yu B, Yang T. miR-1-3p suppresses proliferation of hepatocellular carcinoma through targeting SOX9. Onco Targets Ther. 2019;12:2149–57.
14. Chen YC, Wu CC, Tu YT, Chen YR, Ming-Cheng Lee MC, Tsai KW. Involvement of the MicroRNA-1-LITAF Axis in Gastric Cancer Cell Growth and Invasion. Anticancer Res. 2020;40(11):6247-56.
15. Moreno EC, Pascual A, Prieto-Cuadra D, Laza VF, Molina-Cerrillo J, Ramos-Muñoz ME, Rodríguez-Serrano EM, Soto JL, Carrato A, García-Bermejo ML, Guillén-Ponce C. Novel molecular characterization of colorectal primary tumors based on miRNAs. Cancers (Basel). 2019;11(3):346.
16. Wang ZS, Wang JL, ChenZH, Wang K, Shi LS. inhibits the proliferation and migration of oral squamous cell carcinoma cells by targeting DKK. Biochem Cell Biol. 2018;96(3):355-64.
17. Li MM, Chen X, Chen L, Chen K, Zhou JY, Song JP. MiR-1-3p that correlates with left ventricular function of HCM can serve as a potential target and differentiate HCM from DCM. J Transl Med. 2018;16(1):161.
18. Sysol JR, Chen JW, Singla S, Zhao SP, Comhair S, Natarajan V, Machado RF. Micro-RNA-1 is decreased by hypoxia and contributes to the development of pulmonary vascular remodeling via regulation of sphingosine kinase 1. Am J Physiol Lung Cell Mol Physiol. 2018;314(3):L461-72.
19. Liu K, Ying Z, Xia QX, Shi Y, Tang Q. MicroRNA-1 regulates the proliferation of vascular smooth muscle cells by targeting insulin-like growth factor 1. Int J Mol Med. 2015;36(3): 817-24.
20. Chen J, Yin H, Jiang YL, Radhakrishnan SK, Huang ZP, Li JJ, Shi Z, Kilsdonk EP, Gui Y, Wang DZ, Zheng XL. Induction of microRNA-1 by myocardin in smooth muscle cells inhibits cell proliferation. Arterioscler Thromb Vasc Biol. 2011;31(2):368-75.
21. Tan JJ, Yang LG, Liu CC, Yan ZQ. MicroRNA-26a targets MAPK6 to inhibit smooth muscle cell proliferation and vein graft neointimal hyperplasia. Sci Rep. 2017;7:46602.
22. Albinsson S, Swärd K. Targeting smooth muscle microRNAs for therapeutic benefit in vascular disease. Pharmacol Res. 2013;75:28-36.
23. Solly EL, Dimasi CG, Bursill CA, Psaltis PJ, Tan JTM. MicroRNAs as therapeutic targets and clinical biomarkers in atherosclerosis. J Clin Med. 2019;8(12):2199.
24. Chistiakov DA, Sobenin IA, Orekhov AN, Bobryshev YV. Human miR-221/222 in Physiological and Atherosclerotic Vascular Remodeling. Biomed Res Int. 2015;2015:354517.
25. Vacante F, Denby L, Sluimer JC, Baker AH. The function of miR-143, miR-145 and the MiR-143 host gene in cardiovascular development and disease. Vascul Pharmacol. 2019;112:24-30.
26. Song JT, Hu B, Qu HY, Bi CL, Huang XZ, Zhang M. Mechanical stretch modulates microRNA 21 expression, participating in proliferation and apoptosis in cultured human aortic smooth muscle cells. PLoS One. 2012;7(10):e47657.
27. Li SM, Wu HL, Yu X, Tang K, Wang SG, Ye ZQ, Hu J. The putative tumour suppressor miR-1-3p modulates prostate cancer cellaggressiveness by repressing E2F5 and PFTK1. J Exp Clin Cancer Res. 2018;37(1):219.
28. Elia L, Contu R, Quintavalle M, Varrone F, Chimenti C, Russo MA, Cimino V, De Marinis L, Frustaci A, Catalucci D, Condorelli G. Reciprocal regulation of microRNA-1 and insulin-like growth factor-1 signal transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. Circulation. 2009;120(23):2377-85.
29. Shiu YT, Jaimes EA. Transcription Factor ETS-1 and Reactive Oxygen Species: Role in Vascular and Renal Injury. Antioxidants (Basel). 2018;7(7):84.
30. Hao GH, Han ZH, Meng Z, Wei J, Gao DF, Zhang H, Wang NP. Ets-1 upregulation mediates angiotensin II-related cardiac fibrosis. Int J Clin Exp Patho. 2015;8(9):10216-27.
31. Zhan YM, Brown C, Maynard EM, Anshelevich A, Ni WH, Ho IC, Oettgen P. Ets-1 is a critical regulator of Ang II-mediated vascular inflammation and remodeling. J Clin Invest. 2005;115(9):2508-16.
32. Zhang C, Kavurma MM, Lai A, Khachigian LM. Ets-1 protects vascular smooth muscle cells from undergoing apoptosis by activating p21WAF1/Cip1: ETS-1 regulates basal and and inducible p21WAF1/Cip: ETS-1 regulates basal and inducible p21WAF1/Cip1 transcription via distinct cis-acting elements in the p21WAF/Cip1 promoter. J Biol Chem. 2003;278(30):27903-9.
33. Aoki T, Kataoka H, Nishimura M, R Ishibashi R, Morishita R, Miyamoto S. Ets-1 promotes the progression of cerebral aneurysm by inducing the expression of MCP-1 in vascular smooth muscle cells. Gene Ther. 2010;17(9):1117-23.
34. Feng WG, Xing DQ, Hua P, Zhang Y, Chen YF, Oparil S, Jaimes EA. The transcription factor ETS-1 mediates proinflammatory responses and neointima formation in carotid artery endoluminal vascular injury. Hypertension. 2010;55(6):1381-8.
35. Wang YS, Cao W, Cui JJ, Yu Y, Zhao YB, Jian Shi J, Wu J, Xia ZY, Yu B, Liu JJ. Arterial Wall Stress Induces Phenotypic Switching of Arterial Smooth Muscle Cells in Vascular Remodeling by Activating the YAP/TAZ Signaling Pathway. Cell Physiol Biochem. 2018;51(2):842-53.
36. Liu XX, Huang XZ, Chen L, Zhang Y, Li MM, Wang L, Ge C Wang H, Zhang M. Mechanical stretch promotes matrix metalloproteinase-2 and prolyl-4-hydroxylase α1 production

in human aortic smooth muscle cells via Akt-p38 MAPK-JNK signaling. Int J Biochem Cell Biol. 2015;62:15-23.
37. Zhou N, Zhang Y, Wang T, He JY, He HZ, He LC. The imperatorin derivative OW1, a new vasoactive compound, inhibits VSMC proliferation and extracellular matrix hyperplasia. Toxicol Appl Pharmacol. 2015;284(2):125–33.
38. Wahart A, Hocine T, Albrecht C, Henry A, Sarazin T, Martiny L, El Btaouri H, Maurice P, Bennasroune A, Romier-Crouzet B, Blaise S, Duca L. Role of elastin peptides and elastin receptor complex in metabolic and cardiovascular diseases. FEBS J. 2019;286(15):2980-93.
39. Dandré F, Owens GK. Platelet-derived growth factor-BB and Ets-1 transcription factor negatively regulate transcription of multiple smooth muscle cell differentiation marker genes. Am J Physiol Heart Circ Physiol. 2004;286(6):H2042-51.
40. Wang JG, Zhao XG, Wang XL, Liu MX, Wan W. Low expression of miR-1 promotes osteogenic repair of bone marrow mesenchymal stem cells by targeting TLR1. Eur Rev Med Pharmacol Sci. 2020;24(7):3492-500.
41. Rao VH, Rai V, Stoupa S, Agrawal DK. Blockade of Ets-1 attenuates epidermal growth factor-dependent collagen loss in human carotid plaque smooth muscle cells. Am J Physiol Heart Circ Physiol. 2015;309(6):H1075-86.
42. Te Riet L, van Esch JH, Roks AJ, van den Meiracker AH, Danser AH. Hypertension: renin-angiotensin-aldosterone system alterations. Circ Res. 2015;116(6):960-75.
43. Chiu CZ, Wang BW, Shyu KG. Effects of cyclic stretch on the molecular regulation of myocardin in rat aortic vascular smooth muscle cells. J Biomed Sci. 2013;20(1):50.
44. Wang BW, Chang H, Shyu KG. Regulation of resistin by cyclic mechanical stretch in cultured rat vascular smooth muscle cells. Clin Sci (Lond). 2009; 118(3):221-30.
45. Gang Liu G, Hitomi H, Hosomi N, Bai Lei B, Pelisch N, Nakano D, Kiyomoto H, Hong Ma H, Nishiyama A. Mechanical stretch potentiates angiotensin II-induced proliferation in spontaneously hypertensive rat vascular smooth muscle cells. Hypertens Res. 2010;33(12):1250-7.

Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Wang%20et%20al_5934_Supplementary%20Material.pdf