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ARTICLE

MiR-126-5p promotes contractile switching of aortic smooth muscle cells by targeting VEPH1 and alleviates Ang II-induced abdominal aortic aneurysm in mice

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Abstract

Abdominal aortic aneurysm (AAA) is a potential lethal disease that is defined by an irreversible dilatation (>50%) of the aorta. During AAA expansion, the aortic wall is often remodeled, which is featured by extracellular matrix (ECM) degeneration, medial and adventitial inflammation, depletion and phenotypic switching of vascular smooth muscle cells (SMCs). Recent studies have suggested microRNAs as vital regulators for vascular SMC function. Our earlier work demonstrated an anti-AAA role of miR-126-5p in ApoE$^{-/-}$ mice infused with angiotensin (Ang) II. The present study aimed to further elucidate its role in AAA pathogenesis with a focus on aortic SMC phenotypic switching. Ventricular zone expressed PH domain containing 1 (VEPH1) was identified as a novel negative regulator for vascular SMC differentiation by our group, and its expression was negatively correlated to miR-126-5p in mouse abdominal aortas based on the present microarray data. In vivo, in addition attenuating Ang II infusion-induced aortic dilation and elastin degradation, miR-126-5p agomirs also significantly reduced the expression of VEPH1. In vitro, to induce synthetic transition of human aortic smooth muscle cells (hAoSMCs), cells were stimulated with 1 μM Ang II for 24 h. Ectopic overexpression of miR-126-5p restored the differentiation of hAoSMCs—the expression of contractile/differentiated SMC markers, MYH11, and α-SMA, increased, whilst that of synthetic/dedifferentiated SMC markers, PCNA and Vimentin, decreased. Both *mus* and *homo* VEPH1 genes were validated as direct targets for miR-126-5p. VEPH1 re-expression impaired miR-126-5p-induced differentiation of hAoSMCs. In addition, Ang II-induced upregulation in matrix metalloproteinase (MMP)-9 and MMP2, two key proteases responsible for ECM degradation, in mouse aortas and hAoSMCs was reduced by miR-126-5p overexpression as well. Collectively, these results reveal an important, but previously unexplored, role of miR-126-5p in inhibiting AAA development-associated aortic SMC dedifferentiation.

Introduction

The formation of abdominal aortic aneurysm (AAA) is featured by a 50% or greater dilation in the external diameter of suprarenal aorta [1]. It is a potential lethal vascular disease with an estimated incidence of 6% per decade in men over 65 years old [2]. Currently, no preventive

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therapy is available for AAA patients although the advent of ultrasound, computed tomography, and magnetic resonance imaging makes early diagnosis in clinics possible [1, 3]. Cumulative efforts to understand the cellular mechanisms of AAA have highlighted the pathological alteration of vascular smooth muscle cells (SMCs) in the aortic wall [4].

Although not fully understood, apoptosis, extracellular matrix (ECM) degradation, and phenotypic transition of vascular SMCs often occur in aortic wall, even at the early onset of AAA [5]. Recently, the therapeutic potential of small noncoding RNAs, microRNAs, in limiting AAA formation appears very intriguing [6]. At the post-transcriptional level, microRNAs induce the degradation of gene mRNA and/or prevent gene translation by binding to a 6–8 nt target motif of mRNA transcripts [7]. Data from earlier studies aimed at exploring the pathogenesis of AAA have revealed an intensive involvement of dysregulated microRNAs in AAA formation [8]. For instance, miR-21 limited aneurysm expansion by inhibiting phosphatase and tensin homolog-mediated vascular SMC apoptosis [9]. MiR-181b, miR-712, and miR-29b attenuated or exacerbated AAA symptoms by targeting molecules involved in ECM homeostasis, such as tissue inhibitor of metalloproteinase-3 and Elastin (ELN) [10–12]. Of note, work from our earlier microRNA-based microarray analysis has revealed miR-126-5p as a novel microRNA that decreased in the abdominal aortic tissues of AAA mice [13]. Schober et al. demonstrated that administration of miR-126-5p preserved the function of vascular endothelial cells, and limited atherosclerosis [14]. Although increasing evidence implies that AAA pathogenesis may diverge from atherosclerosis [2], this previous study at least suggests that miR-126-5p plays a role in vascular diseases. Our group has previously demonstrated an anti-AAA role of miR-126-5p in vivo [15]. This study was performed to further explore the mechanisms underlying miR-126-5p's role in AAA development with a focus on SMC phenotype switching.

Unlike other somatic cells, SMCs can switch between two distinct phenotypes: contractile (differentiated) and synthetic (dedifferentiated) phenotypes [16]. It has been reported that maintaining the contractile phenotype of vascular SMCs can limit AAA expansion by conferring vascular homeostasis [17–19]. Activation of transforming growth factor beta (TGF-β)/Smad signaling can promote SMC differentiation by increasing the expression of contractile phenotype-associated proteins, such as Myosin Heavy Chain 11 (MYH11), SM22α, Smooth Muscle Actin Alpha 2 (α-SMA), and calponin [20, 21]. Ventricular zone expressed PH domain containing 1 (VEPH1) has recently been identified as a negative regulator of TGF-β/Smad pathway by Shathasivam's group [22, 23]. In addition to profiling the microRNA expression, we also obtained the messenger RNA (mRNA) expression profiles in mouse AAA tissues. Intriguingly, we found that the expression of VEPH1, a predictive target of miR-126-5p, increased by twofold in AAA tissues as compared with the control. The following Pearson correlation coefficient analysis showed that VEPH1 was negatively correlated to miR-126-5p in the analyzed samples [13]. Moreover, we prior demonstrated VEPH1 as a novel regulator for the phenotypic transition of vascular SMCs [24]. On the basis of such previous evidence, we proposed a hypothesis that miR-126-5p prevents murine AAA formation by targeting VEPH1 in aortic SMCs.

It is wildly accepted that clinical AAA formation correlates to renin–angiotensin system (RAS) activation [25–27]. RAS blockers hold the potential to antagonize vascular diseases-associated with angiotensin (Ang) II actions [28, 29]. Therefore, since low dose of Ang II can induce clinical AAA symptoms successfully in rodents [30], we here decided to determine the role of miR-126-5p/VEPH1 axis in Ang II infusion-induced AAA mouse model, and in Ang II-treated human aortic SMCs (hAoSMCs). Our work demonstrated that miR-126-5p directly targeted VEPH1 to regulate the differentiation of AoSMCs.

Materials and methods

Ang II infusion AAA model and miRNA agomir administration

We established murine AAA model based on previously reported methods [31]. In brief, 15-week-old male ApoE$^{-/-}$ mice (C57BL/6J background) were infused with 1 μg/kg/min Ang II for continuous 28 days (GL BioChem., Shanghai, China) through Model 2004 Alzet Osmotic minipumps (Alzet, Germany) implanted subcutaneously. Animals were anesthetized via isoflurane (2–3%) before any surgical procedure.

MiR-126-5p agomirs (MIMAT0000137: 5′cauuauuacuuuugguacgcg3′) and control negative control (NC) agomirs were obtained from GenePharma (Shanghai, China). Agomirs of miR-126-5p were modified by cholesterol, sulfo-skeleton, and methoxylate to stabilize and prevent RNA from degradation. Mice infused with Ang II were given four intravenous injections of miR-126-5p or NC agomirs (20 mg/kg) before and on days 7, 14, and 21 post Osmotic minipump implantation.

For survival experiment, mice were divided into two groups: mice infused with Ang II were administrated with NC ($n = 20$) or miR-126-5p ($n = 14$) agomirs. Mortality rate was recorded during the whole experiment period.

AAA incidence was defined by an increase of suprarenal maximal vascular diameter over 50% compared with basal line [32].

Ultrasound imaging and preparation of mouse aortic tissues

At baseline and 28 days after AAA induction, GE volusonE8 ultrasound imaging was utilized to analyze the aortic diameters. At the end of the experiment, aortas were collected from sacrificed mice, and the fat and connective tissues were removed from the aortas. The tissue samples were embedded into paraffin or frozen immediately.

Elastin analysis

ELN fragments were probed with Elastin van Gieson (EVG) staining. In short, mouse aortas were cut into 5-μm sections, permeabilized with xylene, and hydrated in gradient ethanol. After being washed with distilled water, the tissue samples were stained with EVG for 15 min. Three random areas (200×) per slice per mouse ($n = 3$/group) were quantified with Image-Pro Plus version 6.0.

Immunohistochemical (IHC) staining and fluorescence in situ hybridization (FISH)

For IHC staining of aortic tissues, the slices were boiled in citric acid for 10 min to retrieve the endogenous antigens, and then incubated with H_2O_2 (3%) for 15 min. Primary antibodies utilized in the present work included anti-VEPH1 polyclonal antibody (1:50 dilution for IHC; Novus Biologicals, Littleton, CO, USA). After incubating with primary antibody at 4 °C overnight, samples were incubated with horseradish peroxidase (HRP)-IgG. IHC signals were visualized with DAB (Solarbio, Beijing, China). VEPH1 positive cells were counted in four random areas (400×) per slice per mouse ($n = 3$/group). The in situ expression of miR-126-5p was analyzed with FISH kit containing specific oligonucleotide probe according to the supplier's instructions (GenePharma, Shanghai, China).

In vitro cell culture and lentivirus infection

hAoSMCs obtained from Z.q.x.z Cell Research (Shanghai, China) were maintained in specific SMC culture medium (ScienCell, San Diego, CA, USA). To induce the phenotypic switch, hAoSMCs were treated with 1 μM Ang II or 10 ng/ml Platelet-derived growth factor (PDGF)-BB for 24 h. Knockdown or overexpression of miR-126-5p and VEPH1 in hAoSMCs were mediated by lentivirus vectors. Cells were infected with corresponding lentiviruses (MOI = 10) for 72 h before the Ang II or PDGF-BB stimulation.

RNA quantification

The mRNA expression was determined via real-time quantitative PCR (real-time QPCR). Briefly, to detect the expression of miR-126-5p, RNAs were reversely transcribed into cDNAs via specific adapter primers (5′ gttggctctggtgcagggtccgaggtattcgcaccagagccaaccgcgta3′). For other encoding genes, total RNAs were processed into cDNAs by using random and oligo (dT)15 primers via a Taq PCR Master Mix kit (BioTeke Bio., Beijing, China). Primer information for real-time QPCR for VEPH1 and miR-126-5p were as following: VEPH1 (forwards) 5′ aggc agtagttgaaatctgtatc 3′, VEPH1 (reversed) 5′ ccaaagggtct caggttatgt 3′, miR-126-5p (forwards) 5′ cattattacttttggtacgcg 3′, and miR-126-5p (reversed) 5′ gcagggtccgaggtattc 3′. The relative expression values were expressed as $2^{-\Delta\Delta Ct}$.

Western blot

Mouse aortas and cells were lysed to extract the total proteins. Protein samples were separated on SDS-PAGE, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Following blocking with skim milk (5% M/V) for 1 h, the membranes were treated with anti-VEPH1 (1:1000 dilution), anti-MYH11 (1:500 dilution; ABclonal, Wuhan, China), anti-α-SMA (1:500 dilution), anti-MMP9 (1:1000; WanleiBio, Shenyang, China), anti-MMP2 (1:500 dilution; ProteinTech, Rosemont, IL, USA), anti-Angiotensin II Receptor Type 1 (AT1R; 1:1000 dilution; Affinity, Cincinnati, OH, USA) or anti-Angiotensin II Receptor Type 2 (AT2R; 1:1000 dilution; Abcam, Cambridge, MA, USA) at 4 °C overnight, and with goat anti-rabbit HRP-IgG (1:5000 dilution) at 37 °C for 45 min. BeyoECL Plus (Beyotime, Shanghai, China) was used to visualize the targeted proteins. The relative protein expression was calculated by comparing to β-actin using Gel Pro-analyzer.

MMP activity detection

The activities of MMP9 and MMP2 in aortic tissues and cell supernatants were determined by zymography. In brief, equal amounts of proteins were run on gelatin (Sigma-Aldrich) SDS-PAGE. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250 (Solarbio, Beijing, China) for 3 h, and then washed with de-staining solution. The fluorescence intensity was analyzed with gel imaging system.

Luciferase assay

We used miR-126-5p to represent hsa-miR-126-5p (MIMAT0000444) and mmu-miR-126a-5p (MIMAT0000137) because of their identical sequence information (miRBase).

Direct targeting of *mus* and *homo VEPH1* 3′ UTR by miR-126-5p was validated by transfecting HEK293 cells with pmirGLO Dual-Luciferase constructs (Promega Corporation, Madison, WI, USA). Wild or mutant type of 3′ UTR of *VEPH1* gene was inserted into pmirGLO plasmid, and co-transfected with NC or miR-126-5p agomirs into HEK293 cells. Luciferase activity was determined 48 h post the transfection as per protocol ($n = 5$).

Statistics

Values were expressed as means ± SD. The statistic comparison was performed by using GraphPad Prism software version 8.0 (GraphPad software, San Diego, CA, USA). Student's t test was used to analyze data between two groups, while one-way ANOVA followed by Tukey's multiple comparison test was utilized to compare data among multiple groups. Survival rate was analyzed with Kaplan–Meier curve followed by log-rank (Mantel–Cox) test. Vascular diameters were analyzed with repeated measure of ANOVA followed by Sidak's multiple comparison test. A p less than 0.05 was considered significant.

Results

MiR-126-5p agomirs limit murine AAA formation and reduce VEPH1 expression

AAA formation was induced in ApoE$^{-/-}$ mice by a 28 days infusion of Ang II. By performing FISH assay, we observed that miR-126-5p was detectable in the normal aortic wall, and mainly expressed in tunica media (Fig. s1). After AAA induction, its expression decreased in aortic wall. Further, sporadic staining of miR-126-5p was present with in arterial aneurysm (Fig. s1). Overexpression of miR-126-5p in mouse aortic tissues was validated with real-time QPCR (Fig. s2). Treatment of miR-126-5p agomirs reduced Ang II-induced dilation of abdominal aortas, suppressed AAA incidence, and improved mouse survival (Fig. 1a–e). MiR-126-5p agomirs prevented ELN deposition (Fig. 1f, g). Immunoblotting results revealed that administration of miR-126-5p decreased VEPH1 expression in the aortas of AAA animals (Fig. 1h–j). The activities of MMP9 and MMP2, two key ECM proteases, were analyzed with gelatin zymography. MiR-126-5p administration was found to inhibit their enzymic activities (Fig. 1k). Results from Western blot showed that the expression of AT1R and AT2R decreased following the administration of miR-126-5p agomirs (Fig. s3). Taken together, these data reveal an anti-AAA action of miR-126-5p. VEPH1 downregulation is an concurrent event occurred to miR-126-5p's anti-AAA action.

Overexpression of miR-126-5p inhibits MMP activation induced by Ang II in hAoSMCs

In vitro, miR-126-5p expression significantly decreased in primary hAoSMCs stimulated with Ang II (Figs. 2a and s4a). Lentiviruses overexpressing miR-126-5p, but not the NC viruses, markedly upregulated the expression of miR-126-5p in hAoSMCs (Figs. 2a and s4b). Like in vivo, the enzymatic activities of MMP9 and MMP2 were repressed by miR-126-5p overexpression (Fig. 2b). The protein levels of these two MMPs were analyzed with Western blot. We noted that miR-126-5p overexpression attenuated Ang II-triggered cleavage of MMPs (Fig. 2c, d). These data suggest that the role of miR-126-5p in protecting ELN integrity may be associated with its inhibitory effects on MMPs.

Overexpression of miR-126-5p suppresses Ang II-induced synthetic switching of hAoSMCs

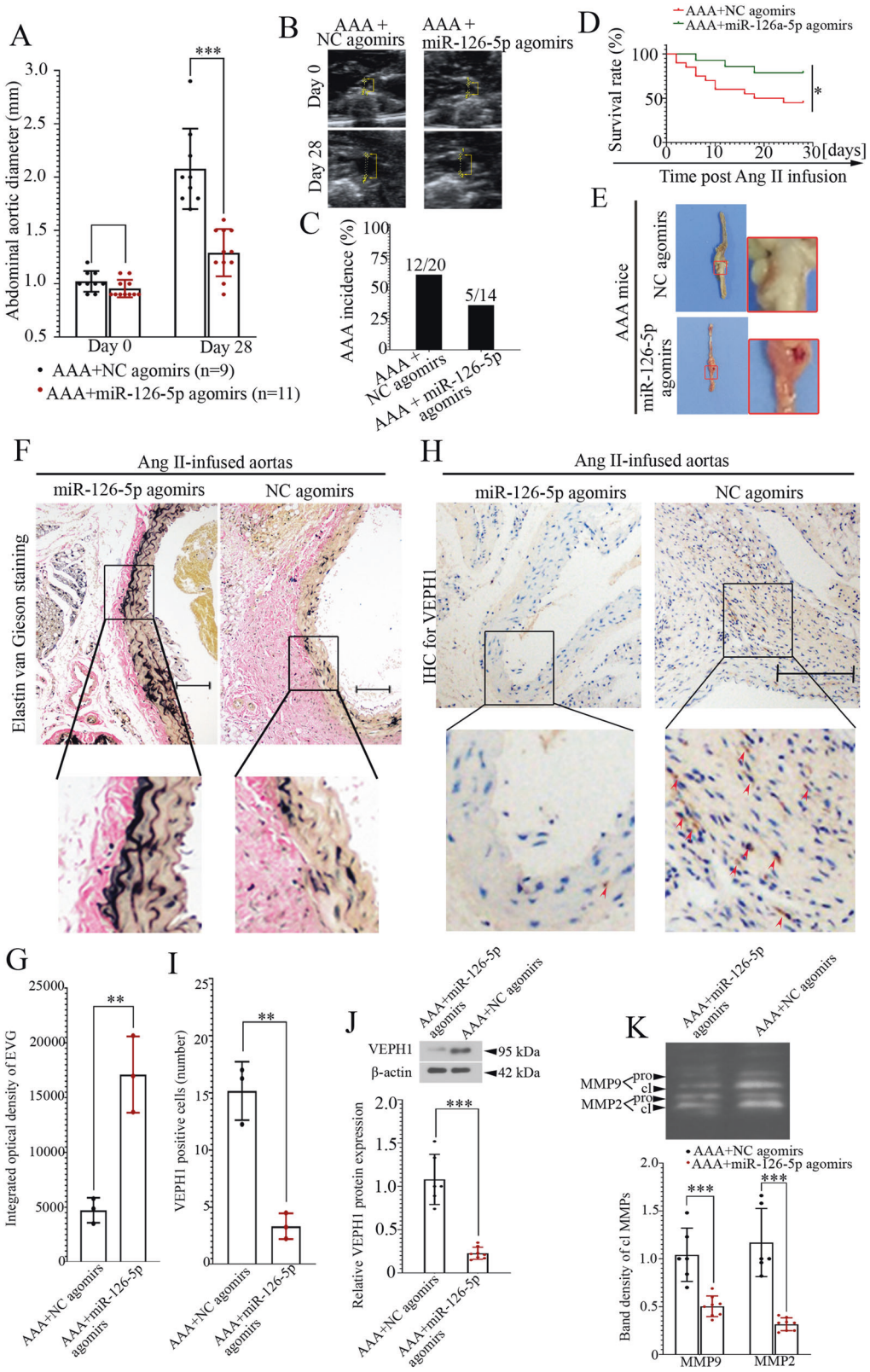
As miR-126-5p was markedly downregulated by Ang II and was anticipated to regulate hAoSMC differentiation, we then explored the physiological properties of this miRNA by performing gain-of-function experiments. After exposing to 1 μM Ang II for 24 h, hAoSMCs switched to the synthetic phenotype—cell proliferation ability was enhanced (Fig. 3a), and the expression of contractile SMC markers, MYH11, and α-SMA were downregulated (Fig. 3b, c). Interestingly, in presence of Ang II, α-SMA, and MYH11 expression partly restored in hAoSMCs post miR-126-5p overexpression (Fig. 3b, c). These data suggested that miR-126-5p prevented hAoSMCs against Ang II-induced pathological dedifferentiation.

RAS activation is not the only trigger that is responsible for pathological aortic SMC dedifferentiation. PDGF-BB, a vital mitogen for mesenchymal cells, is able to reduce the expression of contractile SMC markers [33]. We then treated hAoSMCs with PDGF-BB, and found that PDGF-BB-induced vascular SMC phenotypic switching was also suppressed by miR-126-5p overexpression (Fig. s5).

MiR-126-5p directly targets VEPH1 to regulate hAoSMC phenotypic switching

In keeping with our in vivo findings, the expression of VEPH1 was upregulated by Ang II in hAoSMCs (Fig. 3d, e). This elevation was attenuated when miR-126-5p was overexpressed (Fig. 3d, e). These results demonstrated that VEPH1 was sensitive to Ang II, and may be negatively regulated by miR-126-5p.

To confirm that VEPH1 is directly targeted by miR-126-5p, both gain- and loss-of-function assays of miR-126-5p



were carried out in hAoSMCs. As indicated in Fig. 4, miR-126-5p was proved to negatively regulate VEPH1 expression. Furthermore, luciferase data showed that miR-126-5p could bind to and induce the degradation of wild-type VEPH1 mRNA (Fig. 5). Both *mus* and *homo VEPH1* genes were targeted by miR-126-5p (Fig. 5).

◀ **Fig. 1 MiR-126-5p agomirs limit murine AAA formation and reduced VEPH1 expression.** A 28-day infusion of Ang II was utilized to induce AAA formation in ApoE$^{-/-}$ mice. AAA mice were administrated with NC or miR-126-5p agomirs (20 mg/kg/week, intravenous injection), and were sacrificed at the end of the experiment. **a** Maximal abdominal aortic diameters. **b** Representative ultrasound images of suprarenal aortas. **c** AAA incidence. **d** Survival rate. **e** Representative images of whole aortas. **f**, **g** Abdominal aortic tissue slices were stained via Elastin van Gieson (bars = 100 μm). Elastin was stained in ash black. **f–i** VEPH1 expression was determined with IHC staining (bars = 200 μm; red arrowheads indicated VEPH1 positive cells) and **j** Western blot ($n = 6$–8/group). **k** The activities of MMP9 and MMP2 in aortic tissues were determined with gelatin zymography. Blot density of the first repetition of indicated molecules in AAA+NC agomirs was normalized as 1 (**j**, **k**). Ang II angiotensin II, IHC staining immunohistochemical staining, NC negative control, cl cleaved. *, <0.05; **, <0.01; ***, <0.001.

To demonstrate VEPH1 indeed participates in miR-126-5p-orchatrated hAoSMC differentiation, cells were co-infected with lentiviruses overexpressing miR-126-5p and VEPH1. As expected, the pro-differentiation effects of miR-126-5p on hAoSMCs were markedly weaken if VEPH1 was re-expressed in these cells (Fig. 6).

Collectively, the above mentioned findings demonstrated that miR-126-5p can prevent hAoSMCs from Ang II-triggered dedifferentiation by targeting VEPH1.

Discussion

Two mature strands, miR-126-5p and miR-126-3p, are generated from the precursor mir-126 (pre-mir-126). Depletion of the gene encoding pre-mir-126 leads to impaired vascular integrity and hemorrhage during embryonic development [34], suggesting an involvement of the two mature miRNAs generated from pre-mir-126 in vascular homeostasis. The profiles of genes targeted by miR-126-5p and miR-126-3p are distinctly different due to their discrepant binding patterns to mRNAs. Although the role of miR-126-5p in AAA formation remains poorly studied, miR-126-3p has been demonstrated to arrest AAA growth in a mouse model [35]. Our work here demonstrated that miR-126-5p limited Ang II-induced AAA expansion in ApoE$^{-/-}$ mice—aortic diameter was

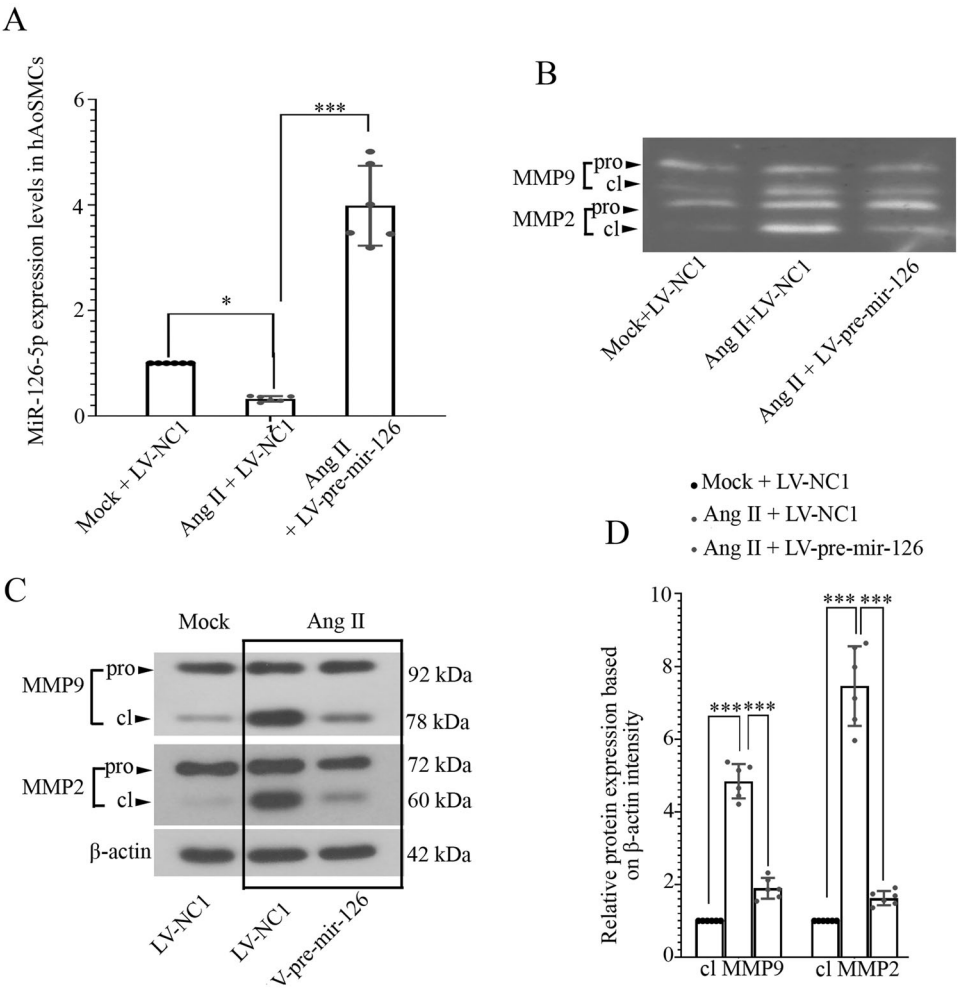


Fig. 2 Overexpression of miR-126-5p inhibits MMP activation induced by Ang II in hAoSMCs. hAoSMCs were infected with control or lentiviruses expressing miR-126-5p, and 72 h later, they were treated with 1 μM Ang II for 24 h. **a** MiR-126-5p expression was first determined with real-time QPCR. The protein levels and activities of MMP9 and MMP2 were assessed with (**b**) zymography, and (**c**, **d**) western blot analysis. Values of indicated molecules in cells infected with LV-NC1 (Mock + LV-NC1) were normalized as 1 (**a**, **d**). Mock cells treated with Ang II vehicle, cl cleaved, MMP matrix metallopeptidase, NC negative control, LV lentiviruses. *, <0.05; **, <0.01; ***, <0.001 ($n = 6$).

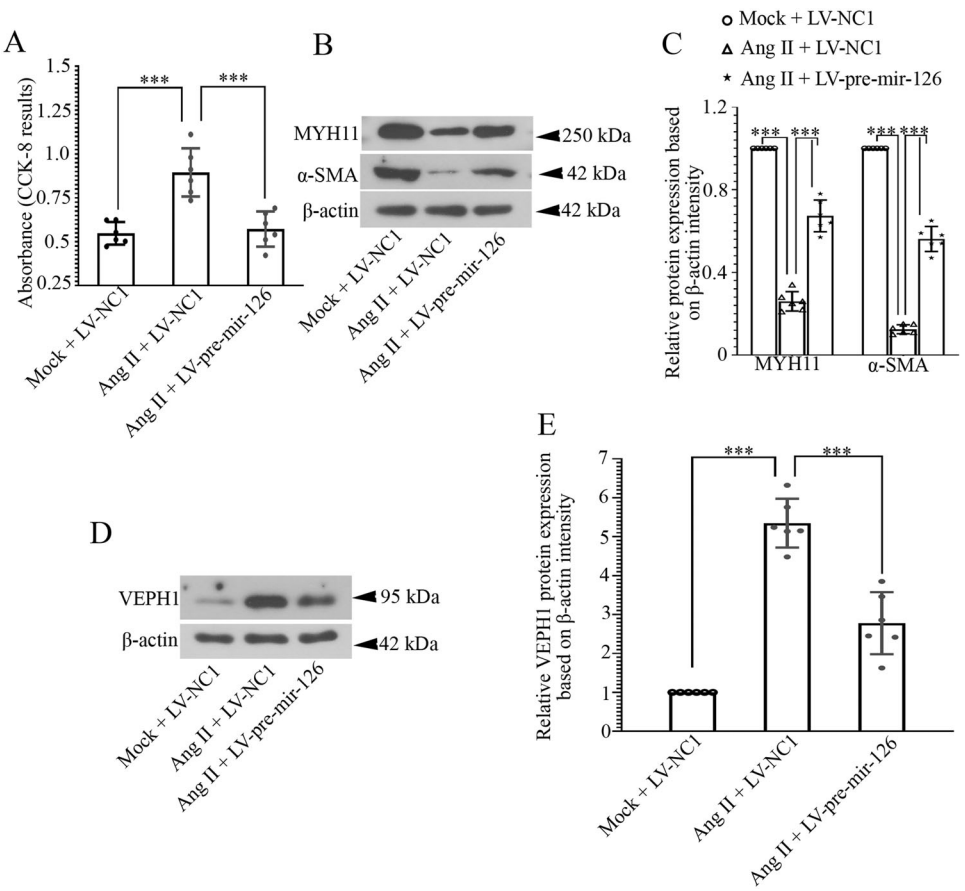


Fig. 3 Overexpression of miR-126-5p suppresses Ang II-induced synthetic phenotype of hAoSMCs. hAoSMCs were infected with NC or lentiviruses expressing miR-126-5p. To induce the dedifferentiation of hAoSMCs, cells were stimulated with Ang II (1 μM) for 24 h at 72 h following the virus infection. **a** Cell proliferation was determined with CCK8 assay. **b**, **c** Western blot was performed to analyze the protein expression of α-SMA and MYH11 in hAoSMCs. **d**, **e** Western blot was performed to analyze the protein expression of VEPH1 in hAoSMCs. Blot densities of indicated molecules in cells infected with LV-NC1 (Mock + LV-NC1) were normalized as 1 (**c**, **e**). Mock cells treated with Ang II vehicle, NC negative control, LV lentiviruses, VEPH1 ventricular zone expressed PH domain containing 1, MYH11 myosin heavy chain 11, α-SMA smooth muscle actin α 2. *, <0.05; **, <0.01; ***, <0.001 ($n = 6$).

reduced and ELN was increased. Further, miR-126-5p overexpression reduced Ang II-stimulated VEPH1 expression in mouse aortic tissues in vivo and hAoSMCs in vitro. Dual-luciferase results validated VEPH1, a molecule that potently suppresses the contractile SMC switching [24], as a novel target gene of miR-126-5p. MiR-126-5p overexpression augmented hAoSMCs to switch into a contractile phenotype in the presence of Ang II. These data add our understanding into the role of mir-126 family miRNAs in AAA development.

An upregulation of MMPs, especially MMP9 and MMP2, has been confirmed to contribute to AAA expansion [36, 37]. Several previous studies on exploring the function of certain miRNA, such as miR-33, miR-516a, and miR-712/miR-205 [11, 38, 39], in aortic aneurysm formation showed that the expression, activity, or cleavage of MMP9 and MMP2 was altered by these miRNAs though they did not directly target MMP9 and MMP2. Several online databases (TargetScan, miRDB, or Starbase) predict *mus MMP10*, *MMP13*, *MMP16*, and *MMP20*, but not *mus MMP9* and *MMP2*, as potential targets for mmu-miR-126a-5p. Nonetheless, we observed that the activities of MMP9 and MMP2 decreased in response to miR-126-5p agomirs. These findings suggest that miR-126-5p may indirectly regulate MMP9 and MMP2 activities.

The pro-inflammatory cytokines over-produced by injured vascular endothelial cells and immune cells infiltrated into aortic wall, such as interleukin (IL)-1β, are well-known risk factors for arterial aneurysm growth [40, 41]. hAoMSCs exposed to IL-1β showed an upregulated expression of MMP9 and MMP2 [42]. Our microarray data revealed a >3-fold increase of IL-1β in aortic tissues of AAA mice relative to the control aortas [13]. Interestingly, two genes important for IL-1β signaling transduction, *mus Interleukin 1 Receptor Type 2* and *mus Interleukin 1 receptor accessory protein*, are predictive targets (TargetScan database) of mmu-miR-126a-5p, suggesting miR-126-5p as a potential negative regulator for IL-1β signaling pathway. A hypothesis that miR-126-5p reduces MMP9 and MMP2 activities by suppressing IL-1β signals is thus proposed by our group, which will be validated in the near future.

Next, the role of miR-126-5p in phenotypic switching (contractile–synthetic transition) of vascular SMCs was investigated in hAoSMCs exposed to Ang II. The phenotypic switching of vascular SMCs is originally depicted by Chamley et al. [43], and further amplified by Ross [44] and Owens [45]. The expression of SMC markers, such as MYH11, α-SMA, calponin, and SM22α, is normally downregulated in the synthetic SMCs. The capability of

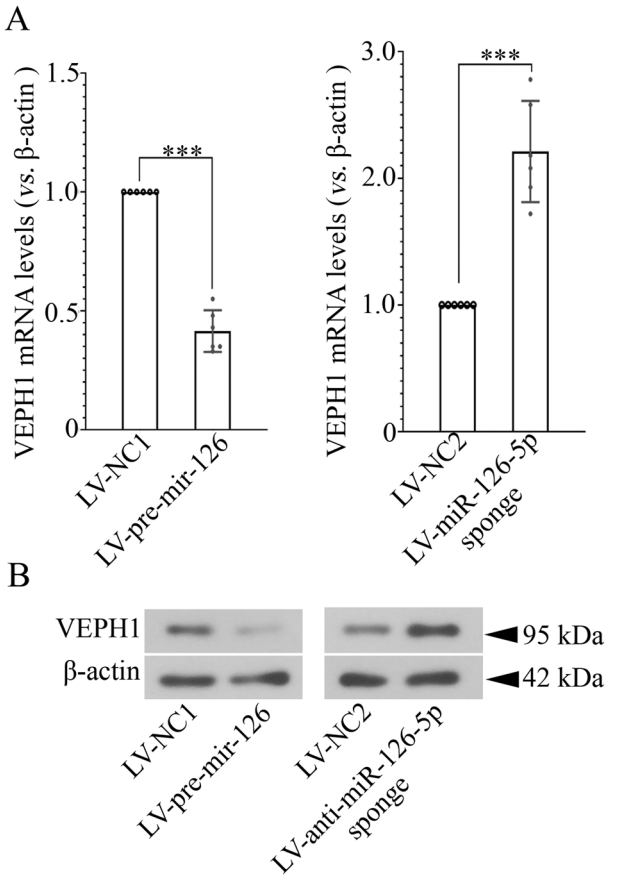


Fig. 4 MiR-126-5p negatively regulates VEPH1 expression in hAoSMCs. hAoSMCs were infected with NC or lentiviruses expressing miR-126-5p, and 72 h later, the total RNAs or proteins were isolated to detect VEPH1 expression at (**a**) transcriptional or (**b**) translational level with real-time QPCR and western blot analysis. Values of indicated molecules in cells infected with LV-NC1 (Mock + LV-NC1) were normalized as 1 (**a**). NC negative control, LV lentiviruses, VEPH1 ventricular zone expressed PH domain containing 1. *, <0.05; **, <0.01; ***, <0.001 ($n = 6$).

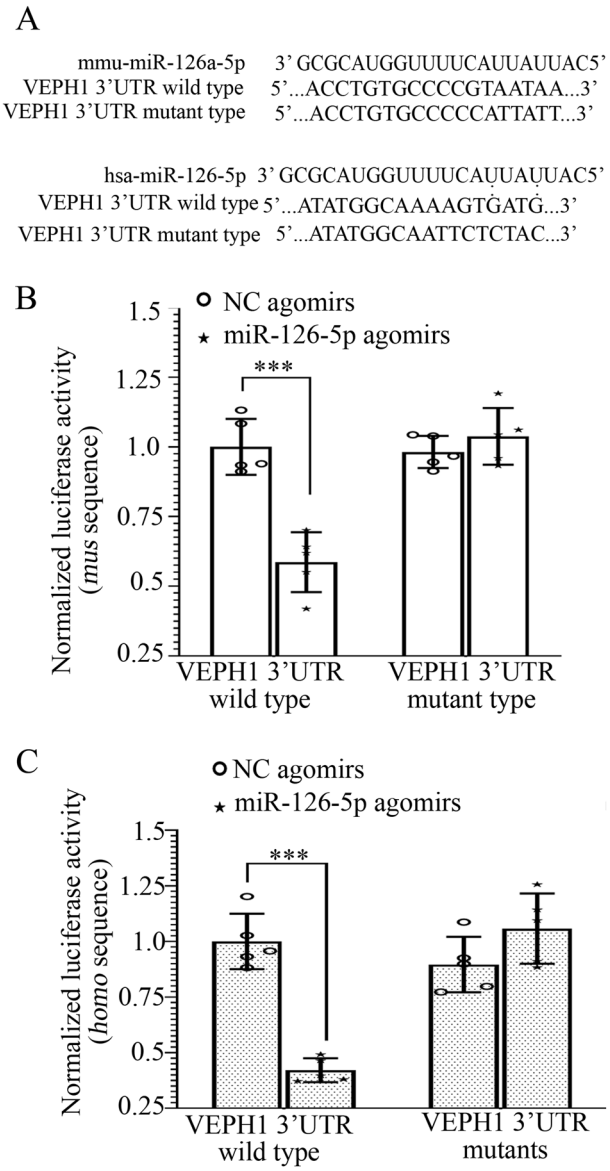


Fig. 5 MiR-126-5p directly targets both *mus* and *homo VEPH1* mRNA. The sequence information of wild and mutant types of *VEPH1* mRNA was shown in **a**. Dual-luciferase reporter assay was performed to analyze the binding between miR-126-5p and *VEPH1* (**b**, **c**). Luciferase ratio of the first repetition of cells transfected with VEPH1 wild-type plasmid and NC agomirs (VEPH1 wild type + NC agomirs) was normalized as 1. VEPH1 ventricular zone expressed PH domain containing 1, NC negative control. *, <0.05; **, <0.01; ***, <0.001 ($n = 5$).

Ang II in inducing dedifferentiation of hAoSMCs has been reported before [46], and was supported by our current data. Furthermore, re-expression miR-126-5p in Ang II-treated hAoSMCs upregulated the expression of MYH11 and α-SMA. In addition, miR-126-5p overexpression also inhibited SMC phenotypical switch induced by PDGF-BB, suggesting that the effect of miR-126-5p on SMC phenotypical switch is not Ang II dependent. Results from our earlier study [24] has demonstrated VEPH1 as a novel negative regulator for the contractile transition of vascular SMCs. Dual-luciferase assays performed in the present study revealed that miR-126-5p bound to the mRNA of both *mus* and *homo VEPH1*. Gain- and loss-of-function experiments validated that miR-126-5p directly targeted and suppressed VEPH1 expression in hAoSMCs. Additional experiments showing that VEPH1 overexpression impaired miR-126-5p-induced hAoSMC differentiation indicated that miR-126-5p regulated vascular SMC polarity by targeting VEPH1. These data collectively uncover a pivotal, but previously unexplored, role of miR-126-5p in regulating SMC polarity.

Aside from aortic SMCs, AAA formation involves dysregulation of other types of cells, such as macrophages and vascular endothelial cells. FISH images here showed that miR-126-5p was detectable in the normal aortic wall,

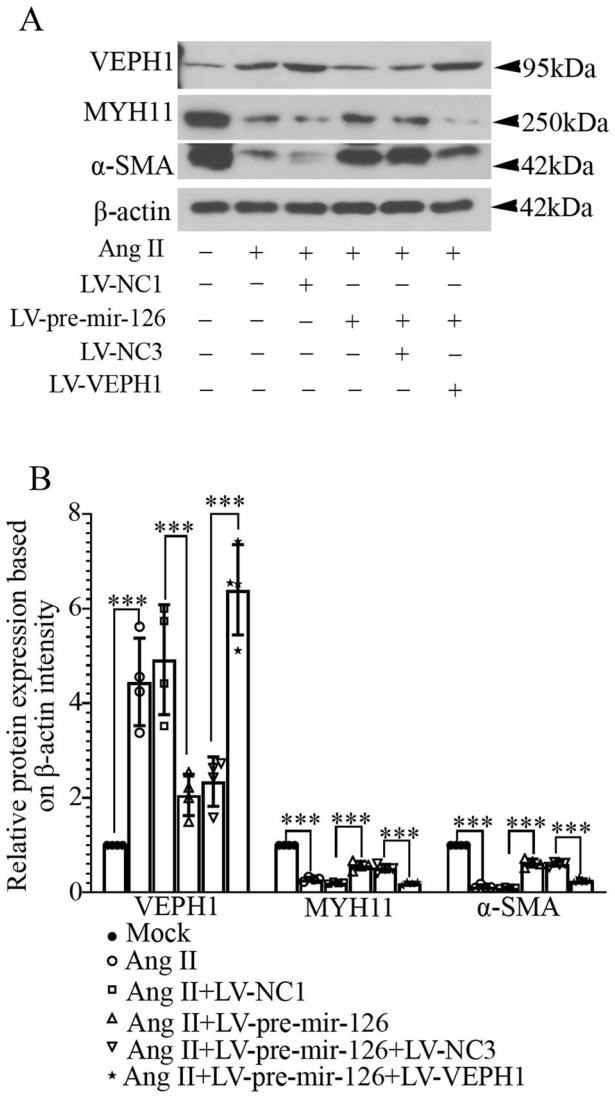


Fig. 6 Re-expression of VEPH1 impaired miR-126-5p-triggered hAoSMC differentiation. hAoSMCs were infected with lentiviruses expressing miR-126-5p and VEPH1 (or NC). To induce the dedifferentiation of hAoSMCs, cells were stimulated with Ang II (1 μM) for 24 h at 72 h following the virus infection. **a**, **b** The expression levels of VEPH1, MYH11, and α-SMA were determined with western blot analysis. Blot densities of indicated molecules of control cells (Mock) were normalized as 1 (**b**). Mock cells treated with Ang II vehicle, NC negative control, LV lentiviruses, VEPH1 ventricular zone expressed PH domain containing 1, MYH11 myosin heavy chain 11, α-SMA smooth muscle actin α 2. *, <0.05; **, <0.01; ***, <0.001 ($n = 4$).

mainly in tunica media. To determine miR-126-5p expression patterns, FISH co-staining, one probe for miR-126-5p, and another for markers of specific cell types, such as α-SMA (vascular SMC marker), CD31 (vascular endothelial cells), and CD68 (macrophage marker), is needed.

The first limitation of our work was that the protective effects of miR-126-5p were only validated in one experimental animal model. Although low-dose infusion of Ang II appropriately simulates clinical AAA symptoms in rodents [30], the anti-AAA role of miR-126-5p should be tested in porcine pancreatic elastase- or calcium chloride-induced AAA models [47, 48]. Second, the contribution of VEPH1 in miR-126-5p-mediated phenotypic switching of AoSMCs was only demonstrated in vitro. To confirm the role of miR-126-5p-VEPH1 axis in AAA-associated SMC phenotypic switching, re-expression VEPH1 in vivo is needed. We analyzed the mRNA sequence information of *AT1R* and *AT2R* genes, and found no potential binding sites for miR-126-5p. Nonetheless, our data illustrated a reduction in AT1R and AT2R expression following miR-126-5p administration in vivo, implying that miR-126-5p may indirectly interfere Ang II signaling transduction. The underly mechanisms require to be further elucidated.

Systemic neutralization of TGF-β activity increased the susceptibility of normocholesterolemic C57BL/6 mice to Ang II-induced AAA formation [49]. Our prior work has demonstrated that VEPH1 suppresses TGF-β signaling pathway [24]. An assumption that miR-126-5p can affect TGF-β signaling transduction through regulating VEPH1 is thus proposed, which needs further validation. Besides TGF-β, dysregulation of other intracellular signaling pathways, such as mitogen-activated protein kinase [50–52] and AKT Serine/Threonine Kinase (AKT) [53], also occurs during Ang II-induced vascular SMC dedifferentiation. There is thus a need to investigate whether miR-126-5p-VEPH1 axis regulates these pathways during AAA formation.

In summary, our work demonstrates that the anti-AAA role of miR-126-5p is associated with its promoting effects on contractile switching of AoSMCs, which involves VEPH1 suppression. Identification of the dysregulated miR-126-5p-VEPH1 axis in experimental AAA formation provides novel insights that help to better understand the underlying mechanisms of this vascular disease.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Ethic Committee of Dalian Medical University. The animal experiments conformed to the NIH Guide for Care and Use of Laboratory Animals.

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