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Mesoderm/mesenchyme homeobox gene l promotes vascular smooth muscle cell phenotypic modulation and vascular remodeling

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ABSTRACT

Aims: To investigate the role of mesoderm/mesenchyme homeobox gene l (Meox1) in vascular smooth muscle cells (SMCs) phenotypic modulation during vascular remodeling.
Methods and results: By using immunostaining, Western blot, and histological analyses, we found that Meox1 was up-regulated in PDGF-BB-treated SMCs in vitro and balloon injury-induced arterial SMCs in vivo. Meox1 knockdown by shRNA restored the expression of contractile SMCs phenotype markers including smooth muscle α-actin (α-SMA) and calponin. In contrast, overexpression of Moex1 inhibited α-SMA and calponin expressions while inducing the expressions of synthetic SMCs phenotype markers such as matrix gla protein, osteopontin, and proliferating cell nuclear antigen. Mechanistically, Meox1 mediated the SMCs phenotypic modulation through FAK-ERK1/2 signaling, which appears to induce autophagy in SMCs. In vivo, knockdown of Meox1 attenuated injury-induced neointima formation and promoted SMCs contractile proteins expressions. Meox1 knockdown also reduced the number of proliferating SMCs, suggesting that Meox1 was important for SMCs proliferation in vivo. Moreover, knockdown of Meox1 attenuated ERK1/2 signaling and autophagy markers expressions, suggesting that Meox1 may promote SMCs phenotypic modulation via ERK1/2 signaling-autophagy in vivo.
Conclusion: Our data indicated that Meox1 promotes SMCs phenotypic modulation and injury-induced vascular remodeling by regulating the FAK-ERK1/2-autophagy signaling cascade. Thus, targeting Meox1 may be an attractive approach for treating proliferating vascular diseases.

1. Introduction

The coronary artery disease (CAD) is a common disease seriously endangering the human health. Clinically, percutaneous coronary intervention (PCI) is one of the effective approaches widely used for patients with CAD [1]. Specifically, local application of drug-eluting stent (DES), allowing drug (e.g. anti-proliferative drug) elution into the coronary wall for weeks, has shown the decreased restenosis rate and the subsequent need of vascular re-endothelialization after stent implantation [2,3,4]. However, so far restenosis following PCI is still the main limiting factor affecting the long-term outcome because of repeated revascularization procedures and DES-related thrombosis in patients [1,4]. Therefore, the most effective therapeutic modality needs to be further explored and clearly established.

Data from both clinical and experimental studies provide a classic concept that restenosis is mainly attributed to neointima formation triggered by responses of inflammation, thrombosis, cellular proliferation, and extracellular matrix synthesis to blood vessel injury. In addition, migration and proliferation of vascular smooth muscle cells (SMCs) are considered as the key step during the development of neointima formation [5]. However, the potential molecular mechanisms underlying the SMCs phenotypic modulation still remain elusive [6–10].

Homeobox genes (HOX genes) encoding homeobox protein (Homeoprotein) are essential for cell and organ differentiation [11]. As a subfamily of HOX genes, Meox genes include Meoxl and Meox2. Meoxl is mainly expressed at cardiac neural crest-derived aortic arch and ascending aorta in the neonatal mice [12–14]. Meox2 is mainly expressed in heart, lung, kidney and descending aorta [15]. Meox2 (also known as GAX) has been studied extensively in cardiovascular system [16–18]. In a rabbit artery balloon injury model, exogenous Meox2 expression mediated by adenovirus vector reduced the vascular

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stenosis and intima/media area ratio significantly through down-regulating integrin, which inhibited the migration and apoptosis of SMCs [19,20], suggesting an inhibitory role of Meox2 in neointimal formation [21]. However, the roles of Meoxl, especially in vascular system, are still unclear.

Until recently, Meox1 functions start to be revealed including its roles in hematopoietic stem cell development, endothelial cell senescence, and cancers epithelial to mesenchymal transition have been reported [22–26]. In this study, we found that Meox1 played an important role in SMCs phenotypic modulation and vascular remodeling. Meox1 appeared to modulate SMCs phenotype by activating FAK-ERK1/2 signaling and its related SMCs autophagy.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley (SD) rats (450 ± 500 g) under conventional conditions in the animal care facilities in Experimental Animal Center of Hubei University of Medicine were used to prepare balloon-injury carotid artery model, according to the Guide for the Care and Use of Laboratory Animals recommended by the National Society for Medical Research. All animal surgical procedures were permitted by the Institutional Animal Care and Use Committee of Hubei University of Medicine.

2.2. Cell culture

Rat aortic SMCs from SD rats were isolated and cultured as described in our previous publication [8]. Briefly, SD rats (280–300 g) were anaesthetized using 60 $mg \cdot kg^{-1}$ pentobarbital sodium i.p, and then sterilized with 75% alcohol. Aortic media were removed from the vessels, cut into 1 mm^3 small pieces, and carefully seeded onto the poly-L-lysine coated flask. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 20% (v/v) fetal bovine serum (FBS, Gibco) at 37 °C, 5% CO_2 for about 2 weeks.

2.3. Construction of adenovirus

Meox1 short hairpin RNA (shRNA) (shMeox1) was designed using a dedicated program provided by OriGene. The shRNA sequences were as follows: 5′- CGC GTC GGA CAG CTT TCA CCA AGG AGC AGC TAC GGT TCA AGA GAC CGT AGC TGC TCC TTG GTG AAA GCT GTC CTT TTT TCC AAA-3′ (sense) and 5′- AGC TTT TGG AAA AAA GGA CAG CTT TCA CCA AGG AGC AGC TAC GGT CTC TTG AAC CGT AGC TGC TCC TTG GTG AAA GCT GTC CGA-3′ (antisense). Adenoviral vector expressing shMeox1 was constructed as described previously [27] and packaged in AD-293 cells (Invitrogen) following the manufacture's instruction. Ad-shMeox1 was purified with gradient density ultracentrifugation of cesium chloride.

2.4. Rat carotid artery injury model

According to our previous protocols for generating the model of balloon-injury carotid artery of rat [27], the mixed gases including isoflurane (1.5%–2.5%) and oxygen were used for anesthetizing rats. After introducing a 2F Fogarty arterial embolectomy balloon catheter through the external carotid artery in left, pre-filled saline in the catheter were injected with 0.02 ml to inflate the balloon, and then rotatably withdrawn through the common carotid artery from proximal to the distal end of the heart. The above procedures were repeated for 3 times to achieve the perfect balloon-injury in carotid artery with the traits of utter endothelial denudation. 100 μl of saline, Ad-shCtrl, or Ad-shMeox1 were injected into the balloon-injured carotid arteries through a specific type of syringe (WISP SYR, Hamilton) and incubated for 20 min. The sham-operated or balloon-injured mice were anaesthetized at the designated times with a lethal dose of 60 $mg \cdot kg^{-1}$ pentobarbital sodium, i.p. The common carotid arteries in all groups were removed for Western blot, immunohistochemistry, or histological analyses.

2.5. Histomorphometric analysis and immunohistochemistry staining

The common carotid arteries were fixed in 4% paraformaldehyde and embedded in paraffin. The sections with 5 μm thickness were stained with modified hematoxylin and eosin (H&E), Masson's, and Verhoeff's elastic stain (VEG) Kit, respectively. For immunohistochemistry (IHC), sections with 5 μm thickness were rehydrated, blocked with 5% horse serum and incubated with a series of primary antibodies: goat anti-Meox1 (1:100, SC-10185, Santa Cruz Biotechnology), goat anti-Meox1 (1:200, ab105349, ABCAM), rabbit anti-calponin (CNN1, 1:200, ab46794, ABCAM), mouse anti-smooth muscle α-actin (α-SMA, 1:100, sc-130616, Santa Cruz Biotechnology), or proliferating cell nuclear antigen (PCNA) (1:200, ab29, Abcam) overnight at 4 °C followed by incubation with horseradish peroxidase. For immunoflurescent staining, FITC or TRITC-conjugated secondary antibody was used. The sections were counterstained with hematoxylin or DAPI.

2.6. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from artery tissues or cultured SMCs were extracted using TRIZOL Reagent (Roche). qRT-PCR was performed using FastStart Universal SYBR Green Master (Roche). The primer sequences were: Meox1: 5′-CGA ATC TTC AAT GAG CAG CA -3′ (forward), 5′-AGT CCT CAC CCA ATC CTC CT-3′ (reverse);α-SMA: 5′-ACC CTC TTC CAG CCA TCT TTC A-3′ (forward), 5′-GCG TTC TGG AGG AGC AAT AAT C-3′ (reverse); SM22α: 5′-CGG CAG ATC ATC AGT TAG AAA G-3′ (forward), 5′-GGG CTG AGG CTG AGG ATA GGT-3′ (reverse); Smoothelin: 5′-AAG GCC ATG ATT GAG AAA CTA-3′ (forward), 5′- GCA CCA GGG CAC AGA AAG-3′ (reverse); β-actin (internal control): 5′-GTC CAC CGC AAA TGC TTC TA-3′ (forward), 5′-TGC TGT CAC CTT CAC CGT TC-3′ (reverse). qPCR was performed on a Real-time PCR Detection System (ABI-7000).

2.7. Western blot analysis

The artery segments for Western blotting were grinded in liquid nitrogen and homogenized on ice in RIPA buffer containing protease inhibitors. Cell or tissue debris were removed by centrifugation. 30 μg of proteins were separated in SDS-PAGE and then transferred to polyvinylidene difluoride membrane (Millipore). After being blocked with 5% fat free milk, the membrane was incubated with a series of primary antibodies: Meox1 (1:1000, ab105349, ABCAM), FAK (1:500, 12636-1-AP, Proteintech), p-ERK1/2 (1:500, 4370,Cell Signaling Technology), α-SMA (1:500, SC-130616, Santa Cruz), LC3-I/II (1:500, 12741S, Cell Signaling Technology), CNN1 (1:1000, ab46974. Abcam), Osteopontin (OPN, 25715-1-AP, 1:500, Ptgcn), PCNA (1:1000, ab29, Abcam), Matrix Gla protein (MPG, 1:1000, 10734-1-AP, Ptgcn), or β-actin (1:5000, A5441, Sigma) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:10000, Santa Cruz). The immunoblots exhibited by enhanced chemiluminescence reaction (Amersham Pharmacia Biotech) were measured with densitometry by Image J software (NIH) [28].

2.8. Cell migration assay

CytoSelect™ 24-well Wound Healing Assay Kit was used to evaluate SMCs migration and presented as the percentage of closure by following the protocol described previously [27].

2.9. Cell proliferation assay

Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) Kit was used to assess SMCs proliferation. The optical density at 450 nm was measured as described previously [29].

2.10. Cell autophagy assay

SMCs seeded on a chamber slide were cultured to 50–60% confluence. Cells were then transduced with adenoviral vector expressing mRFP-GFP-LC3-I/II (Ad-mRFP-GFP-LC3-I/II, MOI: 100, Hanbio, Co, China) for 24 h followed by transduction with adenoviral vector expressing Meox1 (Ad-Meox1, purchased from Vigene Biosciences, Jinan, China) (MOI: 100) for 48 h. The autophagosome formations as shown by fluorescent puncta were captured using a fluorescent microscope (Nikon) [30].

2.11. Transmission electron microscopy

SMCs were grown on cover slips, and treated with control adenoviral vector or Ad-Meox1 (MOI: 100) for 24 h followed by serum-starvation for 48 h. The cells were then fixed and imaged as described previously [31].

2.12. Statistical analysis

All values are presented as means ± SD. Comparisons of parameters among groups were made by one-way ANOVA, and comparisons of different parameters between each group were made by a post hoc analysis using a Bonferroni test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Meox1 promoted SMCs phenotypic modulation

PDGF-BB is known to modulate SMCs phenotype [31]. Thus, we detected if PDGF-BB induced Meox1 expressions in cultured rat aortic SMCs. As shown in Fig. 1A, PDGF-BB significantly induced Meox1 expression as early as 4 h of treatment. To test if Meox1 could play a role in PDGF-BB-induced SMCs phenotype alteration, we knocked down Meox1 using its specific shRNA expressed through adenoviral vector transduction (Fig. 1B–C). PDGF-BB modulated SMCs phenotype as shown by the reductions of SMCs markers including α-SMA and calponin 1 (CNN1) (Fig. 1D–E). Knockdown of Meox1, however, restored the expressions of SMCs markers (Fig. 1D–E), indicating that Meox1 was essential for PDGF-BB-induced SMCs phenotypic

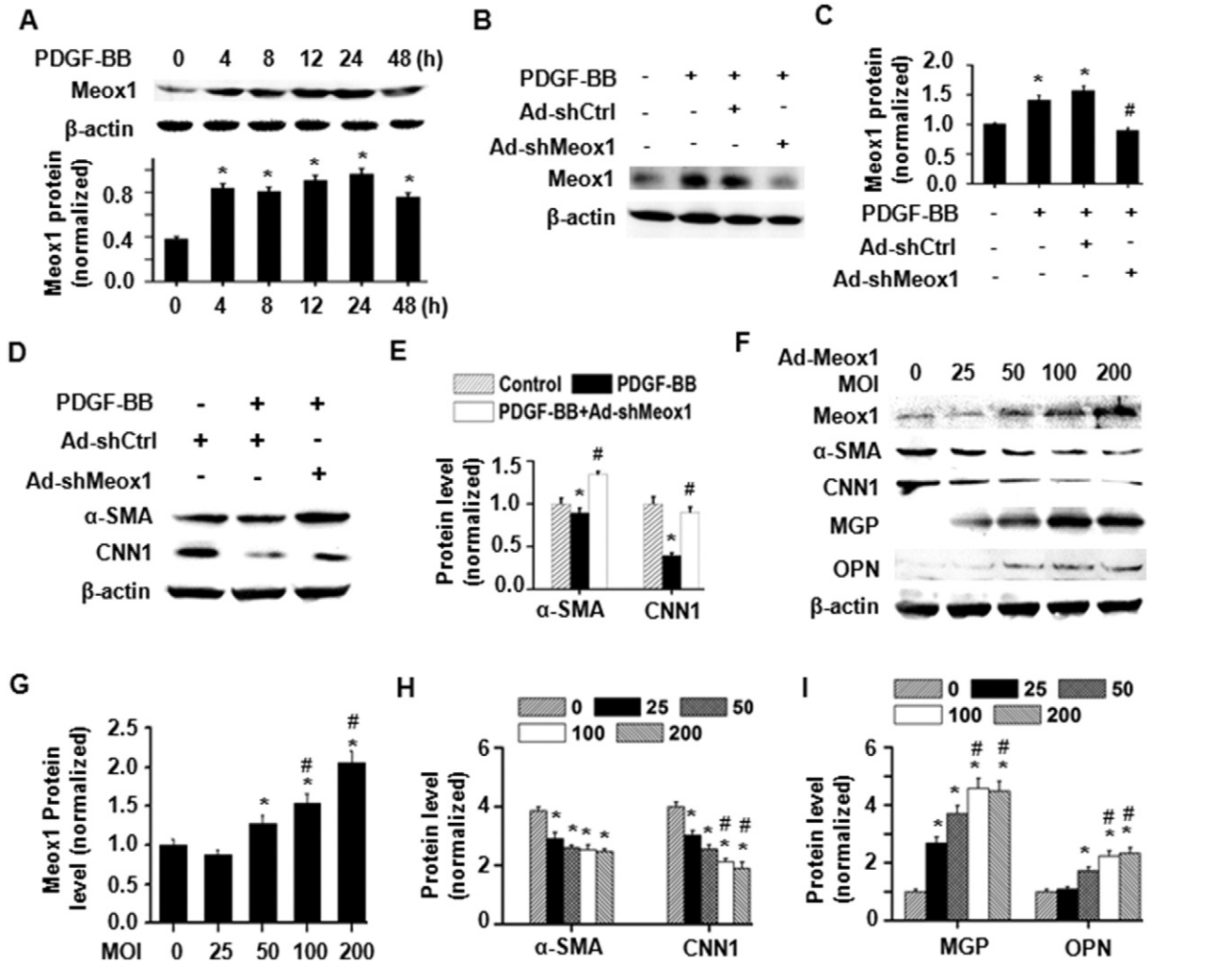

Fig. 1. Meox1 modulated SMCs phenotype. A. PDGF-BB induced Meox1 expression as early as 4 h after the treatment. $^{*}P < 0.05$ compared with vehicle group (0). B. Meox1 shRNA (Ad-shMeox1) effectively knocked down its protein expression. C. Quantification of Mox1 level shown in B by normalized to β-actin. $^{*}P < 0.05$ vs. vehicle-treated cells (-); $^{\#}P < 0.05$ vs. control adenoviral vector-transduced cells (Ad-shCtrl), n = 3. D. Knockdown of Meox1 by its shRNA (Ad-shMeox1) restored PDGF-BB-attenuated α-SMA and CNN1 expression. E. Quantification of protein levels shown in D by normalized to β-actin for each individual protein. $^{*}P < 0.05$ vs vehicle-treated cells (-, Control); $^{\#}P < 0.05$ compared to PDGF-BB with Ad-shCtrl, n = 3. F. Meox1 dose-dependently down-regulated contractile SMCs markers while inducing the synthetic phenotype markers MGP and OPN as indicated. MOI: multiplicity of infection. G–I. Quantification of the protein levels showing in F by normalizing to β-actin. $^{*}P < 0.05$ vs. control vector-transduced cells (0); $^{\#}P < 0.05$ compared with 50 MOI of Ad-Meox1-transduced cells, n = 3.

modulation. In order to determine if Meox1 alone could modulate SMCs phenotype, we overexpressed Meox1 by transducing SMCs with different dosages of Meox1-expressing adenovirus. As shown in Fig. 1F–I, Meox1 dose-dependently inhibited the expressions of contractile SMCs phenotype markers α-SMA and CNN1 while inducing synthetic SMCs phenotype markers MGP and OPN, demonstrating that Meox1 should be a novel factor modulating SMCs phenotype.

3.2. Meox1 promoted SMCs phenotypic modulation through ERK1/2 and FAK signaling pathways

Previous studies have shown that FAK and ERK1/2 play important roles in SMCs phenotypic modulation [32], so we tested if Meox1 could affect ERK1/2 activation and FAK expression. As shown in Fig. 2A–C, forced expression of Meox1 increased while knockdown of Meox1 by shRNA inhibited ERK1/2 phosphorylation and FAK expression, suggesting that ERK1/2 and FAK could be involved in Meox1-mediated SMCs phenotypic modulation. Indeed, when ERK1/2 or FAK signaling were blocked by their pathway-specific inhibitors, the effects of Meox1 on the reductions of contractile phenotype markers were markedly reversed. However, the inductions of synthetic phenotype markers following overexpression of Meox1 in SMCs were obviously weakened by ERK1/2, not FAK blocker (Fig. 2D–F). Since SMCs proliferation and migration have often been taken as characteristics of synthetic SMCs phenotype, we sought to determine if Meox1 promoted SMCs proliferation and migration. As shown in Fig. 2G–H, forced expression of Meox1 stimulated SMCs proliferation and migration. However, ERK1/2 and FAK inhibitors suppressed Meox1-mediated SMCs proliferation (Fig. 2G) and migration (Fig. 2H). These results demonstrated that Meox1 promoted SMCs phenotypic modulation through ERK1/2 and FAK signaling pathways.

3.3. Meox1 induced autophagy through ERK1/2 pathway

Recent studies have shown that ERK1/2 signaling mediates autophagy-regulated SMCs phenotype [31]. Since Meox1 promoted SMCs phenotypic modulation via ERK1/2 signaling, we sought to determine if Meox1 regulated SMCs autophagy. As shown in Fig. 3A–B, forced expression of Meox1 dose-dependently promoted the expression of autophagy marker LC3, and caused an increase of LC3 II/LC3 I ratio, the indicator of autophagy. To further examine this phenomenon, we observed the autophagosome formation using transmission electron

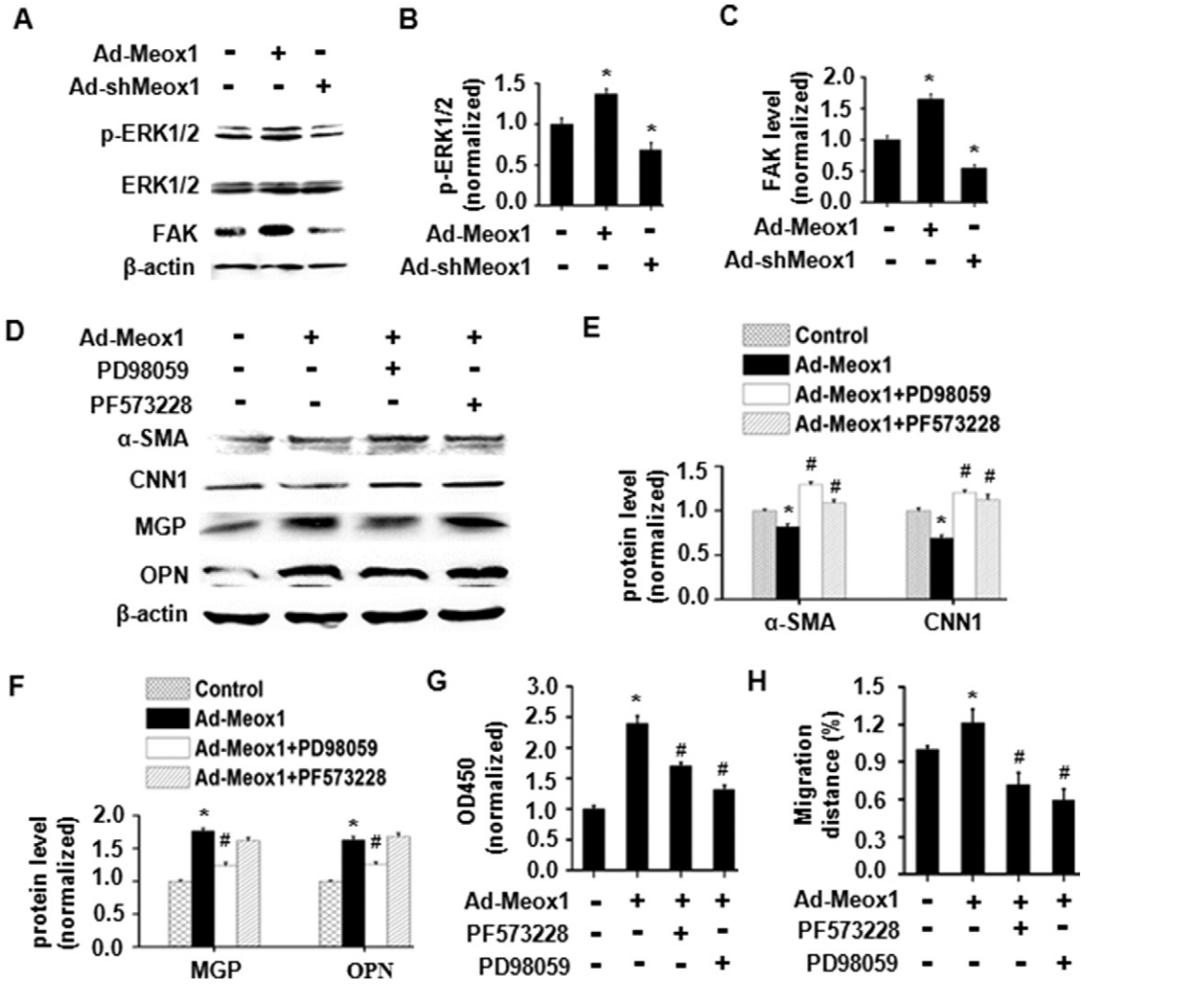

Fig. 2. Meox1 modulated SMCs phenotype via ERK1/2 and FAK signaling. A. Overexpression of Meox1 (Ad-shMeox1) attenuated while knockdown of Meox1 (Ad-Meox1) increased phospho-ERK1/2 (p-ERK1/2) and FAK levels as detected by Western blot. B–C. Quantification of p-ERK1/2 and FAK levels shown in A by normalized to β-actin. $^{*}P < 0.05$ compared to control adenoviral vector-transduced cells (Ctrl), n = 3. D. Blockade of ERK1/2 (PD98059) or FAK (PF573228) signaling by their specific inhibitors attenuated Meox1 effects in regulating SMCs contractile and synthetic phenotype markers. E–F. Quantification of protein levels shown in D by normalized to β-actin. $^{*}P < 0.05$ compared to control adenoviral vector-transduced and vehicle-treated cells (-, Control); $^{\#}P < 0.05$ compared to Ad-Meox1 group, n = 3. G–H. Blockade of ERK1/2 (PD98059) or FAK (PF573228) signaling inhibited Meox1-mediated SMCs proliferation (G, measured by CCK8 assay) and migration (H, measured by wound healing assay). $^{*}P < 0.05$ compared to control adenoviral vector-transduced and vehicle-treated cells (Control, -); $^{\#}P < 0.05$ compared to Ad-Meox1 group in each measurement, n = 3.

microscopy. The ultrastructure displaying the formation of autophagosome is considered as the 'gold-standard' for cell autophagy [31]. Indeed, Meox1 caused >3-fold increase in single-membrane autophagic vacuoles as well as early double-membrane vacuoles (Fig. 3C–D), comparable to the vacuoles in SMCs shown previously [31]. In addition, Meox1 also induced the formation of singular large double-membrane phagosomes, which could serve as a designated compartment for the digestion of intracellular materials.

To acquire additional evidence for the Meox1-induced autophagy, we overexpressed mRFP-GFP-LC3 by adenoviral delivery in SMCs, an established method for detecting the autophagosome formation by the appearance of fluorescent puncta [30]. As Fig. 3E shows, after incubated with Ad-mRFP-GFP-LC3, SMCs were successfully infected with this adenovirus, and RFP, GFP double-positive puncta were observed. In addition to accumulation of LC3, there was a 1.5-fold more fluorescent puncta formed in Meox1-overexpressed SMCs. Furthermore, autophagy activator Brefeldin A further enhanced the autophagy induction (Fig. 3E and F). Conversely, autophagy inhibitor 3-Methyladenine (3-MA) and ERK1/2 inhibitor PD98059 inhibited Meox1-mediated formation of puncta. These results further confirmed the induction of autolysosome formations in Meox1-overexpressed SMCs, indicating that Meox1 mediated a pro-autophagy flux via ERK1/2 signaling in SMCs. In fact, blockade of ERK1/2 signaling by its pathway inhibitor attenuated the LC3 expression and decreased the LC3 II/I ratio that was increased by Meox1 (Fig. 3G–H). These data indicated that Meox1 induced SMCs autophagy via ERK1/2 signaling.

3.4. Meox1 modulated SMCs phenotype via ERK1/2-mediated autophagy in vivo

To determine if Meox1 could play a role in SMCs phenotypic modulation in vivo, we firstly used rat carotid artery balloon injury model to induce vascular remodeling. As shown in Data in brief Fig. 1A, injury induced progressive neointima formation in the arteries, Meox1 was initially induced in medial SMCs (1 and 3 days after the injury) and subsequently in neointimal SMCs, showing time-dependently induced expressions along with a cell proliferating marker PCNA in carotid arteries following injury (Data in brief Fig. 1B-1D). By contrast, knockdown of Meox1 (Data in brief Fig. 2) significantly inhibited the neointima formation as well as the intima/media ratio by nearly 40%, and decreased the elastin and collagen production/deposition that were typical indicators of synthetic SMCs producing

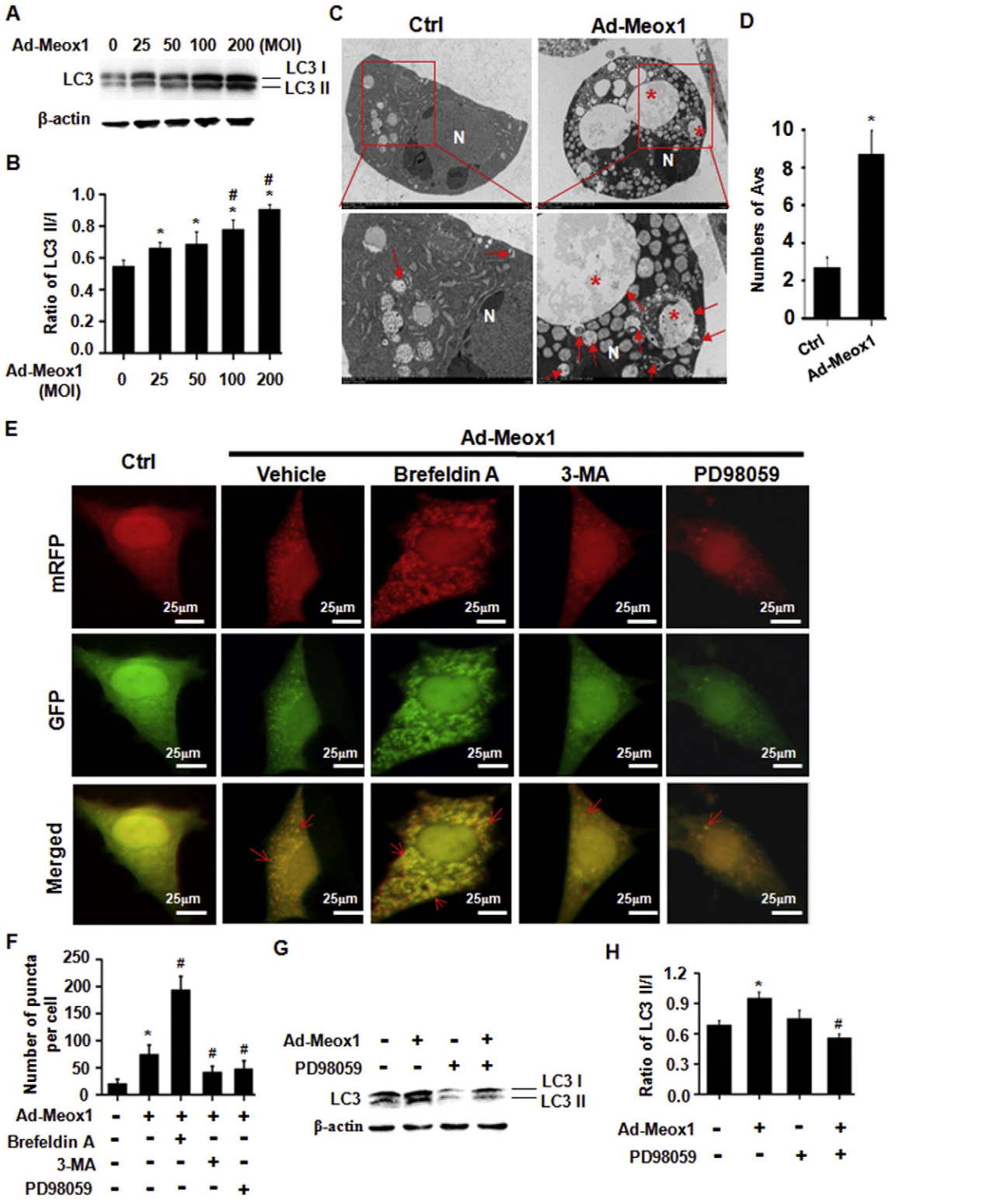

Fig. 3. Meox1 induced autophagy in SMCs. A–B. Meox1 dose-dependently induced the expression of autophagy markers LC3I/II expression (A). Quantification of LC3II/I (B) after their levels were normalized to β-actin, respectively. $^{*}P < 0.05$ vs. control adenovirus-transduced cells (0); $^{\#}P < 0.05$ vs. cells transduced with 25 or 50 MOI (multiplicity of infection) of Meox1-expressing adenovirus (Ad-Meox1). C–D. Meox1 increased the number of autophagosomes in SMCs as observed by transmission electron microscopy (C); the lower panels showed the higher magnification images, N: nucleus. The large heterolysosome/phagosome is indicated by the asterisk. Magnification: ×5000. The autophagic vacuoles (Avs, red arrows) were counted in 8 randomly selected fields (×2500) and averaged (D). $^{*}P < 0.05$ vs. control adenovirus-transduced cells (Ctrl), n = 8. E–F. Brefeldin A promoted while autophagy inhibitor 3-methyladenine (3-MA) and ERK1/2 inhibitor (PD98059) inhibited Meox1-mediated autophagosome formation as indicated by the fluorescent puncta (E) formed by mRFP- and GFP-infused LC3. The fluorescent puncta were quantified by counting and averaging 20 different cells in randomly selected fields (F). $^{*}P < 0.05$ vs. control adenovirus transduced cells (-); $^{\#}P < 0.05$ vs. Ad-Meox1-transduced cells with vehicle treatment, n = 20. G–H. Meox1-mediated autophagy was attenuated by ERK1/2 inhibitor (PD98059) as indicated by the alteration of LC3 II and LC3 I expression (G). The LC3 II/I ratios (H) were compared to the control adenovirus-transduced cells. $^{*}P < 0.05$ vs. control adenovirus-transduced cells (-); $^{\#}P < 0.05$ vs. Ad-Meox1-treated cells; n = 3.

more matrix proteins including collagen. These results suggested that Meox1 could play an important role in SMCs phenotype during vascular remodeling.

And then, we detected the SMCs contractile phenotype markers in balloon-injured arteries. As shown in Fig. 4A–B, knockdown of Meox1 by its shRNA increased α-SMA and CNN expression in neointimal SMCs, suggesting that Meox1 indeed modulated SMCs phenotype in vivo. Since SMCs proliferation is also a vital process in vascular remodeling, and Meox1 promoted SMCs proliferation in vitro, we sought to determine if Meox1 could be important for SMCs proliferation in vivo. Thus, we stained PCNA in both control and Meox1 shRNA-transduced arteries with injury. We found that knockdown of Meox1 significantly reduced the number of PCNA-positive cells in the neointima (Fig. 4C–D), indicating that in addition to the phenotypic modulation, Meox1 also played a role in SMCs proliferation in vivo.

Since ERK1/2 and autophagy mediated Meox1 function in SMCs phenotypic modulation in vitro, we tested if Meox1 regulated ERK1/2 signaling and SMCs autophagy in vivo. As shown in Fig. 4E–F, knockdown of Meox1 inhibited autophagy marker LC3 expression and ERK1/2 phosphorylation while decreasing the PCNA expression in injured arteries, indicating that Meox1 promoted ERK1/2 signaling and SMCs autophagy in vivo, which could contribute to the injury-induced vascular remodeling.

4. Discussion

In the present study, we have identified Meox1 as a novel regulator for SMCs phenotypic modulation and vascular remodeling. The involvement of Meox1 in SMCs phenotype and vascular remodeling is supported by several lines of evidences. Firstly, Meox1 can be induced by PDGF-BB in cultured SMCs in vitro and by balloon injury in rat carotid artery in vivo. Secondly, knockdown of Meox1 restores PDGF-BB-attenuated expressions of contractile SMCs phenotype markers in vitro. Thirdly and most importantly, Meox1 knockdown enhances the contractile proteins expressions in neointimal SMCs while blocking neointima formation in injured arteries. Finally, Meox1 alone down-regulates the contractile phenotype markers while inducing synthetic phenotype markers.

Meox1 appears to mediate SMCs phenotypic modulation by regulating ERK1/2 and FAK signaling, which is consistent with previous finding that FAK-ERK1/2 is involved in the regulation of SMCs phenotype and function [33–35]. In addition, ERK1/2 is known to mediate PDGF-BB-induced SMCs proliferation. Since PDGF-BB induces Meox1 expression, and blockade of ERK1/2 inhibits Meox1-induced SMCs proliferation, Meox1 is likely to act upstream of ERK1/2. Our results and those from others indicate that ERK1/2 signaling is important for both SMCs phenotypic modulation and subsequent proliferation/migration.

Previous studies have shown that autophagy is involved in SMCs phenotype plasticity [36,37], including the PDGF-BB-treated SMCs [31]. PI3K-Akt signaling appears to mediate PDGF-BB-induced autophagy in SMCs. Our results suggest that both PI3K and ERK1/2 signaling are important for Meox1-induced SMCs autophagy because blockade of either PI3K (by 3-MA) or ERK1/2 signaling (by PD98059) attenuates Meox1-mediated formation of autophagosomes as shown by the fluorescent puncta (Fig. 3E). PI3K induces autophagy via the activation of mammalian target of rapamycin (mTOR), a key regulator of autophagy

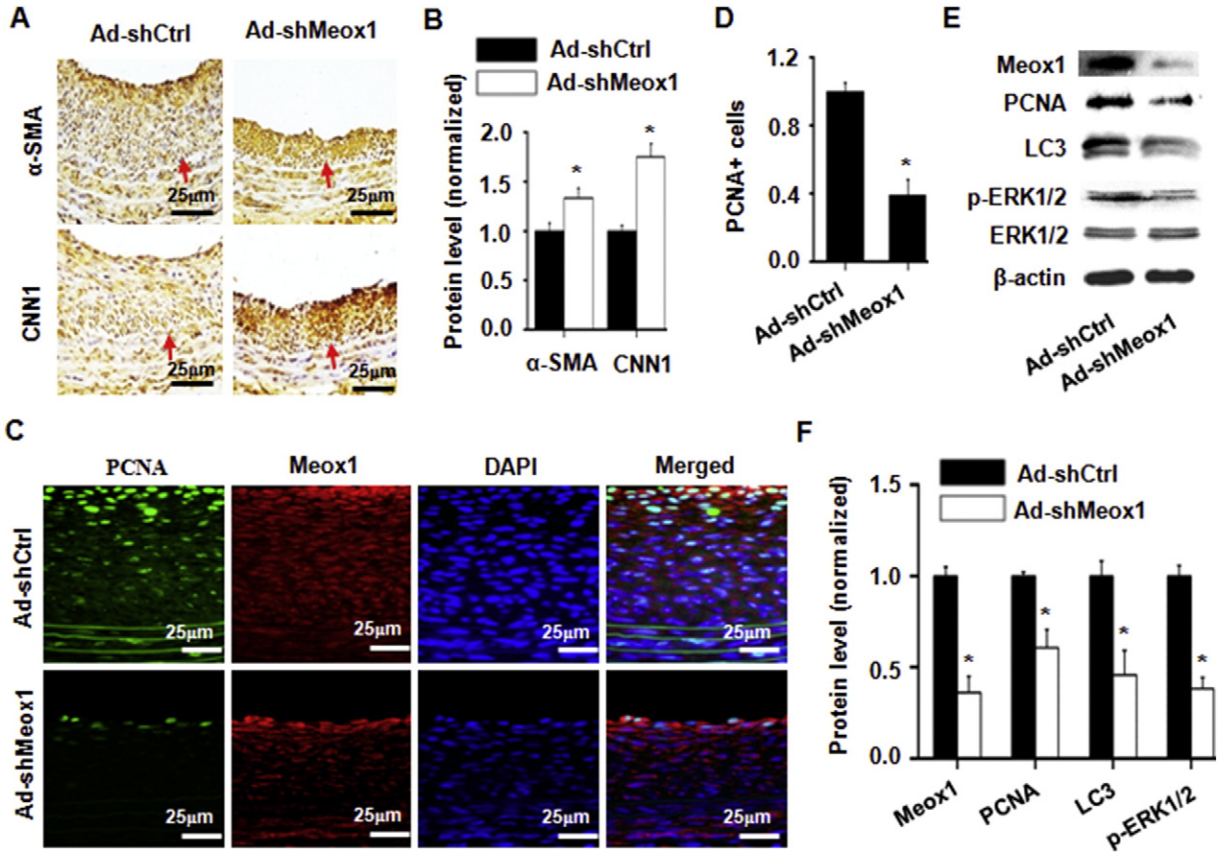

Fig. 4. Knockdown of Meox1 attenuated SMCs phenotypic modulation in vivo. A–B. Knockdown of Meox1 by its shRNA (Ad-shMeox1) increased SMCs contractile protein expression in injured arteries as shown by immunostaining of α-SMA and CNN1. The relative protein levels shown in A were quantified (B) by measuring the optical density of the staining with subtraction of background and comparing to control adenoviral vector-treated arteries (Ad-shCtrl, set as 1). *$P < 0.05$ vs. Ad-shCtrl group for each individual protein. C. Knockdown of Meox1 (Ad-shMeox1) inhibited injury-induced SMCs proliferation in vivo as shown by the reduction of PCNA-expressing cells. PCNA and Meox1 expression were detected by immunostaining. DAPI stains nuclei. D. PCNA positive (PCNA+) cells were counted and averaged from 10 sections of 5 injured rat arteries each group. *$P < 0.05$ vs. Ad-shCtrl group, n = 5. E. Knockdown of Meox1 (Ad-shMeox1) diminished the autophagy and ERK1/2 signaling in injured arteries. Western blot was performed to detect the expression of indicated proteins. F. Quantification of protein levels shown in E by normalized to β-actin. *$P < 0.05$ vs. Ad-shCtrl groups, n = 5.

[38]. Whether or not ERK1/2 mediates PDGF-BB/Meox1-induced autophagy through PI3K-mTOR signaling-dependent or -independent mechanism requires extensive future investigation. In addition to autophagy, PI3K/Akt signaling can directly regulate SMCs proliferation and survival through other different mechanisms. Therefore, inhibition of PI3K signaling by 3-MA may affect SMCs phenotype partially through mechanisms independent of the autophagy, which should also be studied in the future.

Interestingly, although Meox1 and Meox2 have similar function in inducing cell cycle arrest and cell senescence of endothelial cell [29], they show an opposite expression patterns and functions in arterial SMCs in response to vessel injury. Meox2 appears to be down-regulated in SMCs by PDGF-BB in vitro and artery injury in vivo [17,19]. Overexpression of Meox2 promotes SMCs contractile SMCs marker expression while decreasing SMCs proliferation and injury-induced neointima formation [39]. These divergent roles of Meox1 and Meox2 suggest that they do not have redundant activities during SMCs phenotypic modulation and vascular remodeling. Instead, they may counter each other's function to maintain the SMCs homeostasis in normal arteries.

Collectively, we have found for the first time that Meox1 plays a critical role in SMCs phenotypic modulation and vascular remodeling. Our results also suggest a novel mechanistic axis, i.e., PDGF-BB-Meox1-ERK1/2-autophagy, controlling PDGF-BB function in inducing synthetic SMCs phenotype and related proliferation/migration. Although many other links are required to fully understand how this axis regulates SMCs phenotypic modulation, Meox1 may serve as a potential target for treating proliferative vascular disorders.

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Author contributions

B Wu, L Zhang, YH Zhu, YE Zhang, F Zheng, JY Yang, LY Guo, L Wang, and XY Li performed the experiments, L Zhang and JM Tang analyzed the data and wrote the manuscript, SY Chen and JM Tang significantly revised the manuscript, JN Wang conceived and designed the study.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

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