Atorvastatin regulates vascular smooth muscle cell phenotypic transformation by epigenetically modulating contractile proteins and mediating Akt/FOXO4 axis

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Abstract. Atherosclerosis (AS) is a prevalent cardiovascular disease with severe morbidity and high mortality. Phenotypic regulation of vascular smooth muscle cells (VSMCs) from the contractile and quiescent phenotype to the synthetic type is a critical step for the vascular remodeling of AS. Atorvastatin, as a 3-hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitor, presents an anti-inflammatory effect to improve vascular endothelial functions. The aim of the present study was to examine the effect of atorvastatin on VSMCs phenotypic transformation and the underlying mechanism. The rat primary VSMCs were isolated and identified. The protein expression of contractile proteins, such as α-SMA, SM-MHC, and SM22α, was reduced by angiotensin II (AngII) and enhanced by atorvastatin, in which atorvastatin could reverse the effect of AngII in the VSMCs. The treatment of HDAC inhibitor trichostatin A was able to enhance AngII-inhibited expression of α-SMA and SM-MHC. Atorvastatin regulated AngII-associated VSMCs phenotypic transformation by epigenetically regulating contractile proteins. Moreover, atorvastatin modulated platelet-derived growth factor-BB (PDGF-BB)-induced VSMC phenotypic transformation by modulating the Akt/forkhead Box O4 (FOXO4) axis. Immunofluorescence analysis revealed that PDGF-BB enhanced the accumulation of FOXO4 in the VSMCs, while the treatment of atorvastatin was able to attenuate this effect and the co-treatment of Akt inhibitor LY294002 could further inhibit the phenotype. The treatment of PDGF-BB enhanced the interaction of SRF with FOXO4 and myocardin in the VSMCs, in which the co-treatment of atorvastatin and LY294002 could reverse the effect of PDGF-BB in the system. Thus, atorvastatin regulates VSMCs phenotypic transformation by epigenetically modulating contractile proteins and mediating the Akt/FOXO4 axis. Findings of the present study provide new insights into the mechanism by which atorvastatin modulates VSMCs, providing valuable evidence for the application of atorvastatin in the treatment of AS.

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Introduction

Cardiovascular disease may lead to death and affects approximately 17.9 million individuals annually (1-3). Atherosclerosis (AS) is a prevalent cardiovascular disease and a leading cause of vascular-related death (4,5). The critical function of vascular smooth muscle cells (VSMCs) phenotypic transformation during AS progression has been previously identified (6,7). VSMC phenotypic transformation from a contractile to a synthetic status in vessel walls, stimulated by adverse microenvironmental provocations, accompanied by VSMC proliferation and migration, is crucial to AS development (8,9). Phenotypic regulation of VSMCs from the contractile and quiescent phenotype to the synthetic type is a critical step for the vascular remodeling of AS (10). In addition, it has been identified that VSMCs also experience notable phenotypic transformation stimulated by some signals, such as platelet-derived growth factor-BB (PDGF-BB) and angiotensin II (AngII) (11). Accordingly, the exploration of the potential candidate that is able to regulate VSMCs phenotypic transformation may benefit the treatment of AS.

Atorvastatin is a 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitor and is commonly applied for the treatment of AS patients (12). Atorvastatin can enhance plaque instability and relieve the coronary artery inflammation response, decreasing the morbidity and mortality incidences of cardiovascular disorder (13). Besides, atorvastatin has several other functions, such as vascular endothelial function improvement, anti-oxidation, and anti-inflammation (14). Moreover, it has been reported that histone deacetylases (HDACs) are involved in the modulation of VSMCs (15). In addition, forkhead box O4 (FOXO4) and Akt signaling play a critical role in VSMCs phenotypic transformation during AS (16,17).

In addition, FOXO4 activity is regulated by the PI3K/Akt signaling-mediated phosphorylation (18). Moreover, it has been reported that serum response factor (SRF)/myocardin signaling participates in the regulation of VSMCs shiftiness (19). However, the effect of atorvastatin on these factors in the development of VSMCs phenotypic transformation remains obscure.

In this study, the role and underlying mechanism of atorvastatin in the modulation of VSMCs phenotypic transformation was investigated. A novel function of atorvastatin in regulating VSMCs phenotypic transformation by epigenetically modulating contractile proteins and mediating Akt/FOXO4 axis.

Materials and methods

Cell culture and treatment. Primary VSMCs were isolated from normal rat aortas (N=5, male, 8 weeks) by applying the explant method as previously reported (20). Briefly, the aortas were isolated and the endothelial cells (ECs) were removed, the smooth muscle layer was stripped and chopped into small fragments (~1 mm^3) in 0.5 ml of fetal bovine serum. The fragments, together with the fetal bovine serum, were transferred to a 25 cm^2 flask and maintained upside down in an incubator at 37˚C for 4 h. The flask was turned over gently and incubated for 4-7 days after the addition of 2 ml of DMEM. VSMCs migrated out of the explants 4-7 days later, and passage was performed 10-14 days after isolation. The VSMCs were cultured in the medium of DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 0.1 mg/ml streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) and 100 U/ml penicillin (Beijing Solarbio Science & Technology Co., Ltd.) at a condition of 37˚C with 5% CO_2. The primary VSMCs were identified using α-SMA. For all the analyses, VSMCs (2-5 generations) were applied followed by quiescence for 12 h. The VSMCs were treated with AngII (10 nM; MilliporeSigma), trichostatin A (TSA, 0.01 ng/ml; MilliporeSigma), atorvastatin (30 μmol/l; MilliporeSigma), mevalonic acid (MVA, 500 μM; MilliporeSigma), PDGF-BB (20 ng/ml; MilliporeSigma), and LY294002 (10 μM, Sigma, USA) as indicated. The morphology of VSMCs was analyzed using a Nikon microscope (Tokyo).

The average weight of the rats at the start of the experiment was 187 g. The rats were fed in a condition of 25˚C and 50% relative humidity with a 12-h light/dark cycle and free access to standard chow and tap water. The rats were euthanized with an overdose of pentobarbital sodium (150 mg/kg, iv). Animal care and method procedure were authorized by the Animal Ethics Committee of Tianjin Fifth Central Hospital (approval no. 2019-0619-37). The procedures were conformed to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication, 8th edition, 2011).

Reverse transcription-quantitative PCR (RT-qPCR). The total RNAs from the mice and cells were extracted by TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) from the VSMCs. The first-strand cDNA was manufactured as per the manufacturer's instructions (Thermo Fisher Scientific, Inc.). The RT-qPCR was carried out by applying SYBR Real-time PCR I kit (Takara Bio, Inc.). The standard control for mRNA was GAPDH. Quantitative determination of the RNA levels was conducted in triplicate independent experiments (n=3). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 30 sec; followed by 39 cycles of 95˚C for 5 sec and 60˚C for 30 sec; and a final extension at 72˚C for 5 min. The $2^{-\Delta\Delta Cq}$ method was used (21). The sequences were designed according to the references (Genscript, Nanjing, China). Primer sequences are listed in Table I.

Western blot analysis. Total proteins were extracted from the cells or mice tissues with RIPA buffer (Cell Signaling Technology, Inc.). Protein concentrations were measured by using the BCA Protein Quantification Kit. The same concentration of protein (25 μg) was divided by SDS-PAGE (12% polyacrylamide gels), and transferred to PVDF membranes (MilliporeSigma) in the subsequent step. The membranes were blocked with 5% milk and incubated overnight at 4˚C with the primary antibodies for α-SMA (1:1,000; cat. no. ab7817; Abcam), SM-MHC (1:1,000; cat. no. ab133567; Abcam), SM22α (1:1,000; cat. no. ab14106; Abcam), FOXO4 (1:1,000; cat. no. ab128908; Abcam), p-FOXO4 (1:1,000; cat. no. ab126594; Abcam), Akt (1:1,000; cat. no. ab8805; Abcam), p-Akt (1:1,000; cat. no. ab38449; Abcam), SRF (1:1,000; cat. no. ab252868; Abcam), myocardin (1:1,000; cat. no. ab107301; Abcam), and β-actin (1:1,000; cat. no. ab7817; Abcam), in which β-actin served as the control. Then, the corresponding secondary antibodies (1:1,000; cat. no. ab96899/ab96879; Abcam) were used for incubating the membranes 1 h at room temperature, followed by the visualization by using an Odyssey CLx Infrared Imaging System. The experiments were conducted in triplicate independent experiments (n=3). The densitometry analysis was performed using ImageJ software (NIH, v1.8.0).

Immunofluorescence analysis. Cells were solidified at 4% paraformaldehyde for 30 min, treated with Triton X-100 (0.2%) for 10 min and treated with BSA (2%) for 30 min. The slides were hatched with the primary antibody overnight at 4˚C, then hatched with secondary antibodies (Proteintech) for 1 h at 37˚C. The slides were stained with DAPI (Beyotime Institute of Biotechnology) for 10 min at 25˚C. A Nikon microscope (Tokyo) was utilized to analyze the immunofluorescence. The experiments were conducted in triplicate independent experiments (n=3).

ChIP analysis. Chromatin immunoprecipitation (ChIP) was performed using a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, Inc.) according to the manufacturer's instructions. Chromatin prepared from the cells in a 15-cm dish was used to determine total DNA input and was incubated overnight with specific antibodies, H3K9ac (1:500; cat. no. ab32129; Abcam), H4Ac (1:500, 39243, Thermo Fisher, Cambridge, MA, USA), HDAC1 (1:500; cat. no. ab109411; Abcam), HDAC3 (1:500; cat. no. ab32369; Abcam), P300 (1:500; cat. no. ab275378; Abcam), or normal rabbit IgG. Then, the binding DNA was analyzed by qPCR assays and the primer sequences as indicated in Table I. The experiments were conducted as triplicate independent experiments (n=3).

Table I. Primer sequences.

Gene name	Primer	Genebank accession no.	Product sizes (bp)
α-SMA	5'-GAAGAGCTACGAACTGCCTGATG-3' 5'-TAGAAGCATTTGCGGTGGAC-3'	NM_007392.3	417
SM-MHC	5'-TTTGCCATTGAGGCCTTAGG-3' 5'-GTTCACACGGCTGAGAATCCA-3'	NG_009299	427
SM22α	5'-TTGTAATGCAGTGTGGCCCT-3' 5'-CAGGCTGTTCACCAACTTG-3'	NM_003186	369
GAPDH	5'-AAGAAGGTGGTGAAGCAGGC-3' 5'-TCCACCACCCAGTTGCTGTA-3'	XM_036165840.1	203
Calponin	5'-GTCTGGGCATGGAACACTGT-3' 5'-GAGGTACTTACTTGTGAGGGAAT-3'	NM_031747.2	477

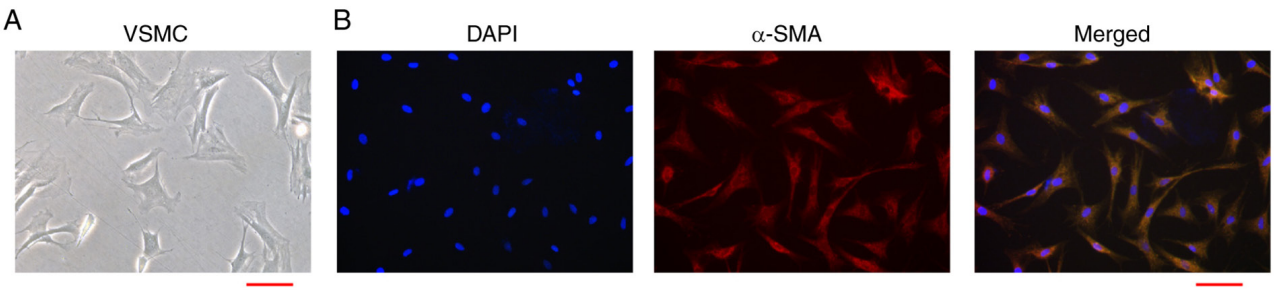

Figure 1. Isolation and identification of rat primary VSMCs. (A) The morphology of VSMCs is shown. Scale bar, 50 μm. (B) Expression of α-SMA was observed by immunofluorescence analysis in the VSMCs. Scale bar, 50 μm. VSMCs, vascular smooth muscle cells.

HDAC activity analysis. The activities of HDAC were analyzed by applying an HDAC Activity Colorimetric Assay Kit (Biovision, Inc.) in the VSMCs according to the manufacturer's protocol. The experiments were conducted as triplicate independent experiments (n=3).

Immunoprecipitation assays. The interaction of the proteins was analyzed by immunoprecipitation in the VSMCs. IP was conducted by Pierce Co-Immunoprecipitation kit (Thermo Fisher Scientific, Inc., Germany) according to the manufacturer's instructions. The experiments were conducted in triplicate independent experiments (n=3).

Statistical analysis. Data are presented as mean ± SD, and the statistical analysis was performed by GraphPad prism 7 (GraphPad Software, Inc.). The unpaired Student's t-test was applied for comparing two groups, and the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test was applied for comparing among multiple groups. $P<0.05$ was considered statistically significant.

Results

The isolation and identification of rat primary VSMCs. During AS, VSMCs present a significant phenotypic transformation from the quiescent contractile status to the stimulated synthetic status and are featured by induced migration and proliferation ability. Firstly, we isolated the primary rat VSMCs and the morphology was shown (Fig. 1A). In addition, the purity of VSMCs was validated by the phenotypic transformation markers termed α-SMA in the VSMCs (Fig. 1B).

Atorvastatin regulates AngII-associated VSMCs phenotypic transformation by epigenetically regulating contractile proteins. Next, we explored the effect of atorvastatin on the AngII-associated VSMC phenotypic transformation. To this end, the VSMCs were treated with AngII, atorvastatin, or co-treated with AngII and atorvastatin, AngII and HDAC inhibitor termed trichostatin A (2). Significantly, the protein expression of contractile proteins, including α-SMA, SM-MHC, and SM22α, as the VSMCs-specific genes, was reduced by AngII and enhanced by atorvastatin, in which atorvastatin could reverse the effect of AngII in the VSMCs (Fig. 2A and B). In addition, the treatment of TSA was able to enhance the AngII-inhibited expression of α-SMA and SM-MHC (Fig. 2A and B). Furthermore, ChIP assays showed that the interaction of HDAC, P300, SRF, histone H3 lysine 9 acetylation (H3K9ac), histone H4 acetylation (H4ac) on the promoters of α-SMA and SM-MHC, but not SM22α, in which AngII could regulate the interaction while the co-treatment of atorvastatin or TSA could reverse the effect of AngII in the system (Fig. 2C). Consistently, the treatment of AngII reduced the HDAC activities and atorvastatin presented a reversed effect, in which atorvastatin could attenuate the effect of AngII in the VSMCs (Fig. 2D). Together these suggest that atorvastatin regulates VSMCs phenotypic transformation by modulating HDAC3.

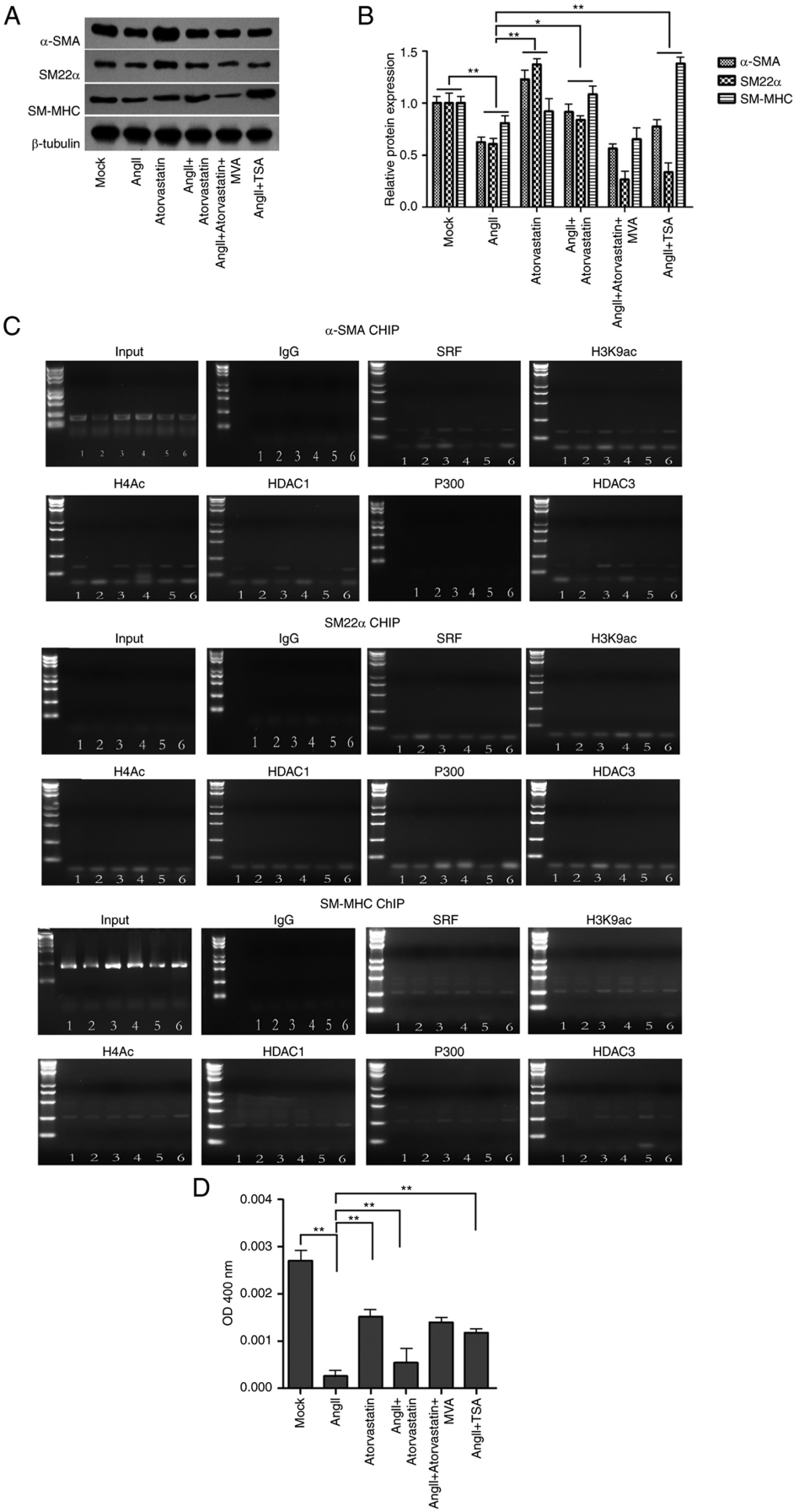

Figure 2. Atorvastatin regulates AngII-associated VSMCs phenotypic transformation by epigenetically regulating contractile proteins. (A-D) VSMCs were treated as described in Materials and methods. (A and B) The expression of α-SMA, SM-MHC, and SM22α was measured by western blot analysis in the VSMCs and the results were quantified by ImageJ. (C) IP analysis was performed in the VSMCs. (D) The enzyme activities of HDAC were analyzed. n=3, mean ± SD. *P<0.05, **P<0.01. VSMCs, vascular smooth muscle cells.

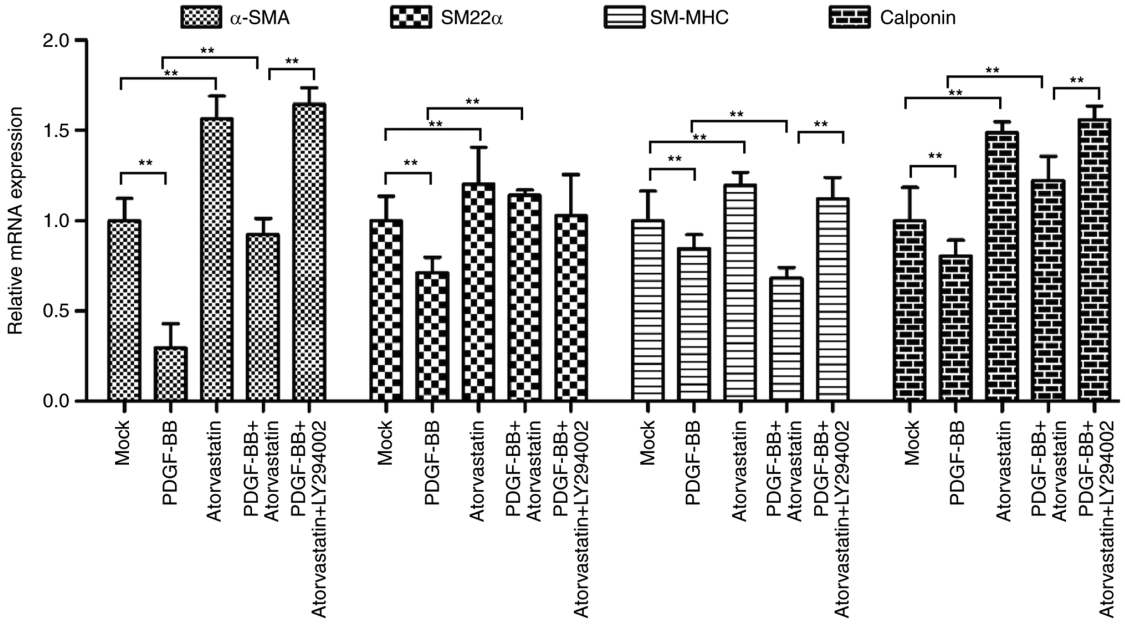

Figure 3. Atorvastatin regulates PDGF-BB-induced VSMC phenotypic transformation by modulating PI3K signaling. VSMCs were treated as the described in Materials and methods. The expression of α-SMA, SM-MHC, SM22α, and calponin was analyzed by RT-qPCR in the VSMCs. n=3, mean ± SD. **P<0.01. PDGF-BB, platelet-derived growth factor-BB; VSMCs, vascular smooth muscle cells.

Atorvastatin regulates PDGF-BB-induced VSMCs phenotypic transformation by modulating PI3K signaling. PDGF-BB is able to induce the phenotypic transformation of VSMCs and plays a critical role in the regulation of VSMCs phenotypes. Accordingly, we further evaluated the effect of atorvastatin on the PDGF-BB-induced VSMCs phenotypic transformation. For this purpose, the VSMCs were treated with PDGF-BB, atorvastatin, or co-treated with PDGF-BB and atorvastatin, PDGF-BB, atorvastatin, and PI3K inhibitor LY294002. The results showed that the mRNA expression of contractile proteins, such as α-SMA, SM-MHC, SM22α, and calponin, was inhibited by PDGF-BB but enhanced by atorvastatin, in which co-treatment of atorvastatin with PDGF-BB could rescue the PDGF-BB-reduced phenotypes in the VSMCs (Fig. 3). Meanwhile, LY294002 was able to enhance α-SMA, SM-MHC, SM22α, and calponin expression in the PDGF-BB and atorvastatin co-treated VSMCs (Fig. 3).

Atorvastatin modulates PDGF-BB-induced VSMC phenotypic transformation by modulating PI3K/FOXO4 axis. Moreover, the protein levels of α-SMA, SM-MHC, SM22α, and calponin, were reduced by PDGF-BB but upregulated by atorvastatin, in which the co-treatment of atorvastatin with PDGF-BB could rescue the PDGF-BB-inhibited phenotypes in the VSMCs (Fig. 4A and B). Furthermore, LY294002 induced α-SMA, SM-MHC, SM22α, and calponin expression in the PDGF-BB and atorvastatin co-treated VSMCs (Fig. 4A and B). In addition, the expression and phosphorylation of Akt were increased by PDGF-BB, in which atorvastatin could attenuate the effect of atorvastatin and LY294002 was able to further inhibit the phenotype (Fig. 4A and C). Significantly, the expression and phosphorylation of FOXO4, especially the serine 262 phosphorylation, was induced by PDGF-BB, while atorvastatin reduced the effect in the system and LY294002 could further reinforce the inhibitor phenotype in the VSMCs (Fig. 4A and D), suggesting that atorvastatin modulates PDGF-BB-induced VSMC phenotypic transformation by modulating the PI3K/FOXO4 axis.

Atorvastatin is able to regulate FOXO4/SRF/myocardin axis. The immunofluorescence analysis revealed that PDGF-BB enhanced the accumulation of FOXO4 in the VSMCs, while the treatment of atorvastatin was able to attenuate this effect and the co-treatment of LY294002 could further inhibit the phenotype (Fig. 5A). Subsequently, the treatment of PDGF-BB enhanced the interaction of SRF with FOXO4 and myocardin in the VSMCs, in which the co-treatment of atorvastatin and LY294002 could reverse the effect of PDGF-BB in the system (Fig. 5B), suggesting that atorvastatin is able to regulate FOXO4/SRF/myocardin axis.

Discussion

AS is a predominant type of cardiovascular disease with severe morbidity and high mortality (4). Atorvastatin has presented anti-inflammatory effects and improvements in vascular endothelial function. Nevertheless, the role and the potential mechanism of atorvastatin in the regulation of VSMCs phenotypic transformation is still unclear. In this study, we discovered a novel function of atorvastatin in modulating VSMCs phenotypic transformation by epigenetically modulating contractile proteins and mediating Akt/FOXO4 axis.

Previous findings identified the function of atorvastatin in AS and VSMCs regulation. It has been reported that atorvastatin upregulates ACE2 expression by epigenetic histone modifications in rabbit AS mode (22). Atorvastatin reduces pyroptosis via NEXN-AS1/NEXN signaling in vascular endothelial cells during AS (23). Atorvastatin represses the PDGF-ββ-stimulated migration and proliferation of VSMCs by G0/G1 cell cycle suppression and the arrest of PDGFRβ/PI3K/Akt signaling (24).

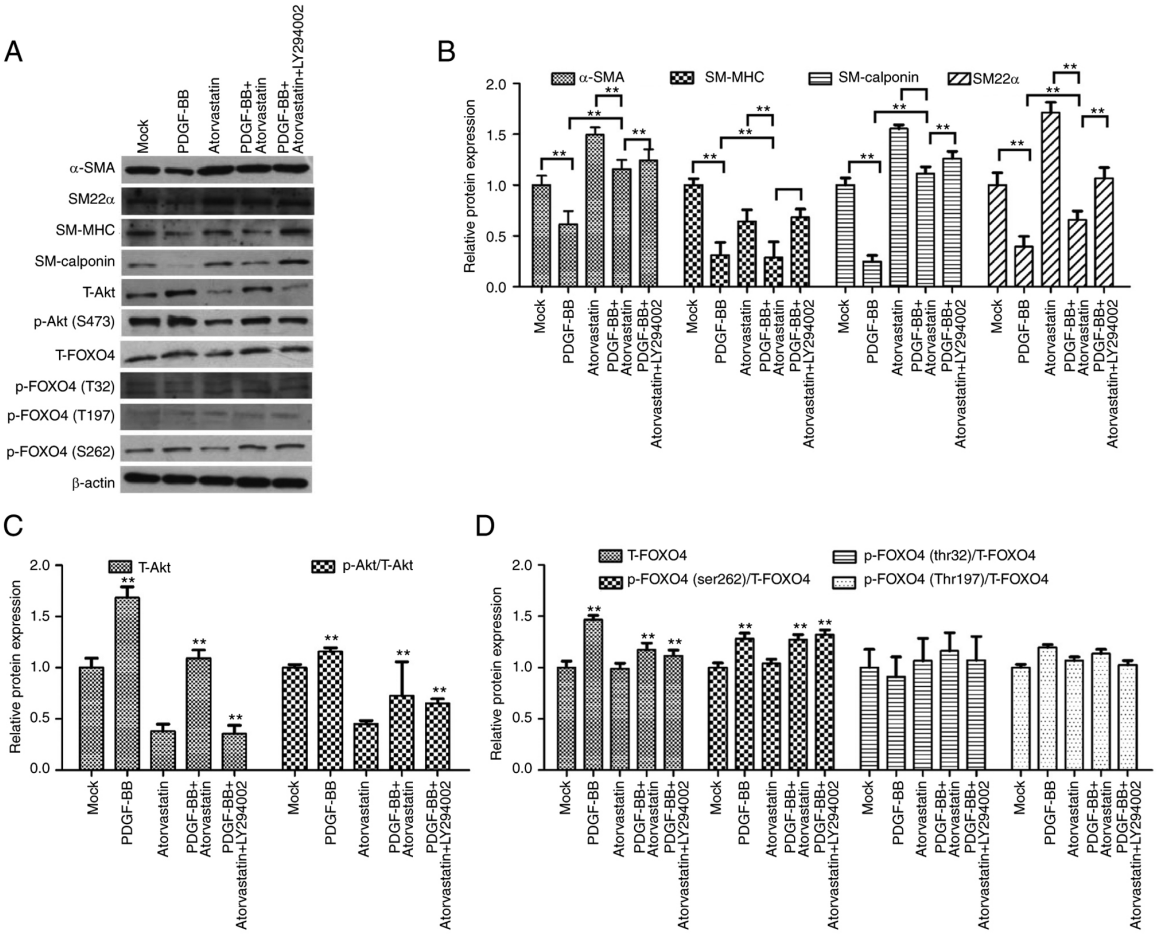

Figure 4. Atorvastatin modulates PDGF-BB-induced VSMC phenotypic transformation by modulating Akt/FOXO4 axis. (A-D) VSMCs were treated as described in Materials and methods. The expression of α-SMA, SM-MHC, SM22α, FOXO4, and Akt and the phosphorylation of FOXO4 and Akt were measured by Western blot analysis and the results were quantified by ImageJ. n=3, mean ± SD. **P<0.01. PDGF-BB, platelet-derived growth factor-BB; VSMCs, vascular smooth muscle cells.

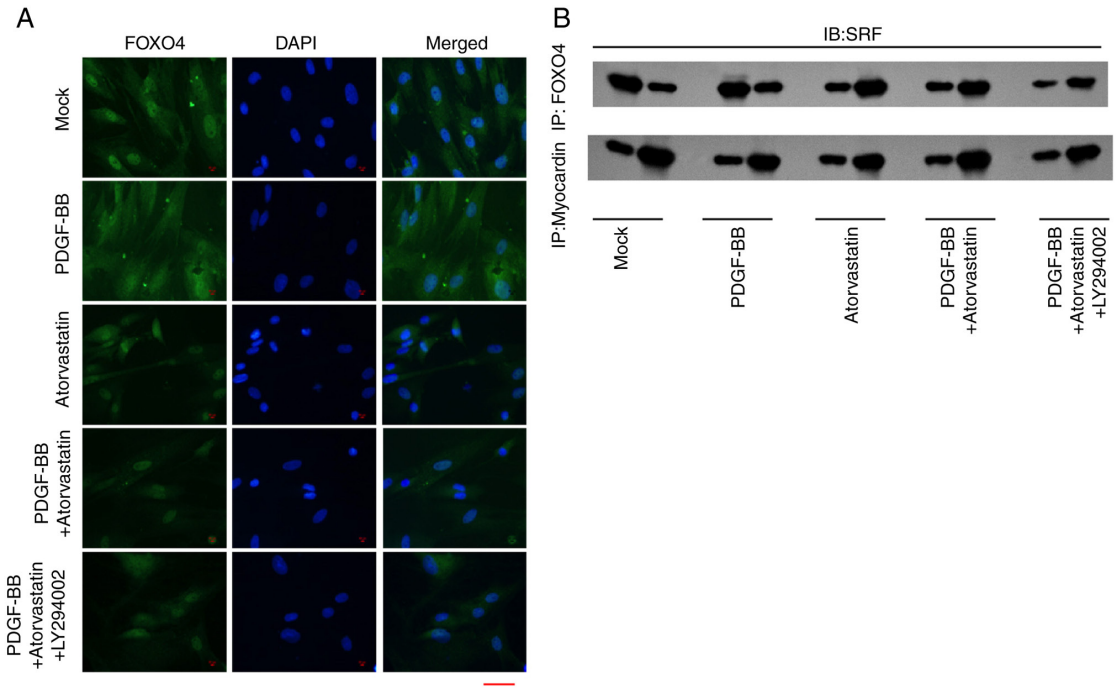

Figure 5. Atorvastatin is able to regulate FOXO4/SRF/myocardin axis. (A and B) VSMCs were treated as described in Materials and methods. (A) The expression of FOXO4 was analyzed by immunofluorescence analysis. Scale bar, 50 μm. (B) The interaction of SRF with FOXO4 and myocardin was tested by IP assay. FOXO4, forkhead Box O4; VSMCs, vascular smooth muscle cells; SRF, serum response factor.

Atorvastatin enhances apoptosis of VSMCs by downregulating Rho A prenylation and Bcl-2 expression (25). Results of the present study showed that, atorvastatin was able to regulate PDGF-BB/AngII-induced VSMCs phenotypic transformation. This is a novel function of atorvastatin in VSMCs regulation, providing valuable evidence for the fundamental role of atorvastatin in the development of AS.

Moreover, HDAC is known to participate in VSMCs and AS development (26,27). Protein kinase B/AKT regulates insulin-like growth factor 1-accociated phosphorylation and nuclear export of HDAC5 by activating NADPH oxidase 4 in VSMCs (26). TSA suppresses VSMCs proliferation by enhancing WAF1 (27). Additionally, FOXO4 plays a critical role in the progression of VSMCs and AS. MiR-23b downregulation enhances phenotypic switching of VSMCs by targeting FOXO4 (28). MiR-128-3p decreases VSMCs migration and proliferation by inhibiting FOXO4 signaling (29). In addition, Akt signaling is involved in the modulation of VSMCs and AS (18,30). In the present study, atorvastatin modulated VSMCs phenotypic transformation potentially by epigenetically regulating contractile proteins and regulating PI3K/FOXO4 signaling. These data identify the unreported correlation of atorvastatin with these critical factors in the modulation of VSMCs during AS development.

In conclusion, findings of the present study showed that atorvastatin regulated VSMCs phenotypic transformation by epigenetically modulating contractile proteins and mediating Akt/FOXO4 axis. This finding provides new insights into the mechanism by which atorvastatin modulates VSMCs, providing valuable evidence for the application of atorvastatin in the treatment of AS.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XD, XL and GZ designed the study; XD performed the experiments in figures 1-4. ZS, ZC, XZ, DW, and KW performed the experiments in figures 4 and 5. XD, XL and GZ wrote the manuscript. XD, XL and GZ confirm the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

Animal care and method procedure were authorized by the Animal Ethics Committee of Tianjin Fifth Central Hospital (approval no.: 2019-0619-37). The procedures were conformed to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication, 8th edition, 2011).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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