COL6A1 knockdown suppresses cell proliferation and migration in human aortic vascular smooth muscle cells

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Abstract. Vascular smooth muscle cell (VSMC) migration is an important pathophysiological signature of neointimal hyperplasia. The aim of the present study was to investigate the effects of collagen type VI α1 chain (COL6A1) on VSMC migration. COL6A1 expression was silenced in platelet-derived growth factor (PDGF-BB)-stimulated VSMCs. Cell counting kit-8, wound healing and Transwell assays were used to measure cell viability, migration and invasion, respectively. Reverse transcription-quantitative PCR and western blot analysis were performed to analyze the expression of factors associated with metastasis. COL6A1 silencing attenuated PDGF-BB-induced increases in cell viability and invasive abilities of VSMCs, in addition to partially reversing the increased expression of fibronectin (FN), matrix metalloproteinase (MMP)-2 and MMP-9 induced by PDGF-BB stimulation. The silencing of COL6A also overturned PDGF-BB-induced reduction in tissue inhibitor of metalloproteinase 2 expression in VSMCs. PDGF-BB activated the AKT/mTOR pathway, which was also inhibited by COL6A1 knockdown. Taken together, these findings suggest that COL6A1 silencing inhibited VSMC viability and migration by inhibiting AKT/mTOR activation.

Introduction

The incidence of cardiovascular diseases has been on the rise annually with the onset of aging. In particular, coronary heart disease is becoming increasingly common (1,2). Although percutaneous coronary intervention (PCI) is a commonly used method to treat atherosclerotic coronary heart disease, postoperative restenosis occurs in a considerable proportion of angioplasty patients, leading to a reduction in the success rate of PCI treatment whilst increasing the risk of new cardiovascular complications (3). Indeed, restenosis is an important factor in the long-term outcome of post-angioplasty surgery (4), with the incidence of restenosis potentially reaching between 30 and 50% within 6 months post-surgery without preventive measures (5). Despite developments in novel technology in combatting restenosis such as drug-eluting angioplasty balloons, the occurrence of restenosis remains high (6) due to the mechanism of restenosis being complex and not fully understood. Therefore, understanding the molecular mechanisms underlying vascular remodeling following injury is pivotal to the prevention of restenosis after PCI.

There is substantial evidence that aberrant proliferation of the vascular neointima is central to the pathophysiology of vascular lumen restenosis (7,8); in which the phenotypic transformation of vascular smooth muscle cells (VSMCs) is the main cause. Following transformation, VSMCs display enhanced proliferative and migratory capabilities (9), but may exhibit different characteristics depending on the status of their microenvironment (10). In normal blood vessels, VSMCs are highly differentiated cells that exhibit a strong contractile phenotype, and serve to maintain and regulate vascular tone to stabilize blood pressure (11). However, under pathological conditions, including atherosclerosis and in-stent restenosis, VSMCs begin transforming into a more synthetic phenotype, characterized by high levels of proliferation and migration with a concomitant reduction in contractility (12,13). This transition results in intima thickening of the arteries, which is a common pathogenesis in the formation of multiple vascular lesions *in vivo* (14). Therefore, understanding the changes in the proliferative and migratory capabilities of VSMCs has important implications for the prevention and treatment of these diseases.

Platelet-derived growth factors (PDGFs) are strong mitogens for VSMCs in blood vessels (15). PDGFs function in many vascular pathophysiological processes, such as atherosclerosis, restenosis and angiogenesis (16). PDGFs regulate cell proliferation, cell migration and the production of inflammatory mediators, to maintain tissue permeability and hemodynamics, through modulation of several transcription factors and key molecular signaling pathways (17). PDGF receptor signaling can activate cell proliferation and migration, protein production or secretion, and phenotypic modulation of VSMCs (18). Therefore, in the present study, PDGF-BB, the main isoform of PDGFs, was selected to induce VSMC dedifferentiation.

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Collagen type VI α1 chain (COL6A1) assists in the synthesis of collagen VI (COL6), which is a component of the extracellular matrix and forms distinct microfibrillar networks in the connective tissues of blood vessels and muscles (19,20). High COL6A1 has been previously associated with hypertension, which is a main risk factor for cardiovascular diseases (21), whilst another study showed upregulated COL6A1 expression in the vascular tissues of patients with atherosclerosis (22). In addition, COL6A1 was identified as a metastasis-associated protein using quantitative secretome analysis (23) In light of these reports, it was therefore hypothesized that COL6A1 may serve important roles in the synthetic phenotype of VSMCs and the pathogenesis of vascular lumen restenosis. Therefore, in the present study, a COL6A1 silencing vector was constructed and transfected into aortic VSMCs to study the effects of COL6A1 on VSMC proliferation and invasion.

Materials and methods

Cell transfection. T/G Human aortic vascular smooth muscle cells (T/G HA-VSMC; HA-VSMC thereafter; cat. no. CL-0452; Procell Life Science & Technology Co., Ltd.) were cultured in Medium 231 (Life Technologies; Thermo Fisher Scientific, Inc.) supplemented with 5% smooth muscle growth supplement (SMGS; Thermo Fisher Scientific, Inc.) at 37˚C under 5% CO_2. Fresh culture medium was changed every two days. HA-VSMCs ($1x10^5$ cells) were then transfected with 0.25 µg siRNA specific for COL6A1 (si-COL6A1) or siRNA-control (EV; both purchased from Shanghai GenePharma Co., Ltd.) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol, the cells were named si-COL6A1 and EV cells thereafter. The sequences of si-COL6A1 were: Forward, 5'-CCCACCUGAAGGAGAAUAAUU-3' and reverse, 5'-UUAUUCUCCUUCAGGUGGGUU-3'. The sequences of EV were: Forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. Cells without transfection were designated as the control group (Cntl). Transfection efficiency was determined by measuring COL6A1 mRNA and protein levels after 48 h of transfection.

Cell treatment. Each experiment was designed such that one group of cells received PDGF-BB and the other group did not. In PDGF-BB-treated groups, Cntl, EV and si-COL6A1 cells ($5x10^5$) were seeded into a six-well plate for 24 h, then stimulated with 20 ng/ml platelet-derived growth factor (PDGF-BB; PeproTech, Inc.) diluted in Medium 231 supplemented 5% SMGS for 12, 24 and 48 h at 37˚C.

Cell viability assay. Cell viability was determined using Cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). Cntl, EV and si-COL6A1 cells were seeded into a 96-well plate at $5x10^3$ cells/well and cultured for 12, 24 or 48 h at 37˚C with or without 20 ng/ml PDGF-BB. Following the addition of 20 µl CCK-8 reagent/well and a further 1 h incubation, cell viability was measured by obtaining the optical density values at 450 nm for each well using a microplate reader (Thermo Fisher Scientific, Inc.).

Wound healing assay. HA-VSMC migration was measured using wound healing assay. Cntl, EV and si-COL6A1 cells treated or untreated with 20 ng/ml PDGF-BB were inoculated into a 12-well plate at $1x10^5$ cells/well in Medium 231 supplemented with SMGS, and gently scratched to form a cell-free area. The cells were then incubated for 24 h at 37˚C. The width of each wound was measured using an Olympus DSX100 light microscope (Olympus Coporation; magnification, x200).

Cell invasion assay. The invasive abilities of HA-VSMCs were measured using 24-well Transwell® chambers with 8-µm pore filters (Corning Inc). Cntl, EV and si-COL6A1 cells ($5x10^4$ cells) treated or untreated with 20 ng/ml PDGF-BB, diluted in Medium 231 supplemented with SMGS, were seeded into the Matrigel® GFR (BD Biosciences)-coated Transwell® upper chambers, with the coating process at 37˚C for 30 min. The lower chambers were filled with Medium 231 supplemented with SMGS. Following 24 h incubation, the Transwell membranes were stained using 0.1% crystal violet for 30 min at 37˚C. The number of invasive cells in random 5 fields was then calculated from images captured using Olympus DSX100 optical microscope (Olympus Corporation; magnification, x200).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from Cntl, EV and si-COL6A1 cells ($5x10^5$ cells) treated or untreated with 20 ng/ml PDGF-BB using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. cDNA was obtained using High-capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. The mRNA expression levels of factors associated with metastasis, including fibronectin (FN), matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of metalloproteinases 2 (TIMP-2) were then measured using Fast SYBR® Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol, in an Applied Biosystems 7300 thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: Initial denaturation at 94˚C for 25 sec; 35 cycles of 94˚C for 25 sec, 60˚C for 25 sec and 72˚C for 30 sec; and final extension at 72˚C for 5 min. The quantification was performed using the $2^{-\Delta\Delta Cq}$ method (24). B-actin was used as internal control and the primer sequences were listed in Table I.

Western blot analysis. Cntl, EV and si-COL6A1 cells ($5x10^5$ cells) treated or untreated with 20 ng/ml PDGF-BB were lysed using RIPA lysis buffer (Boster Biological Technology) for 20 min, with the proteins quantified using Bicinchoninic Acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). All proteins were subsequently separated at 10 µg/lane by 15% SDS-PAGE and transferred onto a PVDF membrane (Millipore; Merck KGaA). The membranes were blocked using 5% non-fat dry milk in PBS, at 37˚C for 1 h, before being probed with primary antibodies specific for COL6A1 (cat. no. ab151422; 1:1,000), FN (cat. no. ab23750; 1:1,000), MMP-2 (cat. no. ab37150; 1:1,000), MMP-9 (cat. no. ab73734; 1:1,000), TIMP-2 (cat. no. ab180630; 1:1,000), protein kinase B (PKB/AKT; cat. no. ab8805; 1:500), phosphorylated (p)-AKT (p-AKT; cat. no. ab38449; 1:1,000), mammalian target of rapamycin (mTOR; cat. no. ab2732; 1:2,000), p-mTOR (p-mTOR; cat. no. ab84400; 1:500) and

Table I. The primer sequences used for reverse transcription-quantitative PCR.

Gene	Sequence (5'-3')
β-actin	Forward: GTGGACATCCGCAAAGAC
	Reverse: GAAAGGGTGTAACGCAACT
FN	Forward: ACAACACCGAGGTGACTGAGAC
	Reverse: GGACACAACGATGCTTCCTGAG
MMP-2	Forward: CAGCCCTGCAAGTTTCCATT
	Reverse: GTTGCCCAGGAAAGTGAAGG
MMP-9	Forward: GAGACTCTACACCCAGGACG
	Reverse: GAAAGTGAAGGGGAAGACGC

FN, fibronectin; MMP, matrix metalloproteinase.

β-actin (cat. no. ab8227; 1:2,000) overnight at 4˚C. β-actin was used as loading control. Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (ab6721, 1:5,000) for 2 h at 37˚C. All primary and secondary antibodies were purchased from Abcam. The protein bands were visualized using enhanced chemiluminescence reagents (Millipore; Merck KGaA) and quantified using Bio-Rad ChemiDoc™ XRS+ System with Image Lab™ software (version 4.1; Bio-Rad Laboratories, Inc.,).

Statistical analysis. SPSS 18.0 statistical software (SPSS, Inc.) was used for statistical analyzes. Five repeats were conducted for each experiment. Data were presented as the mean ± standard deviation. One-way ANOVA was followed by Tukey's analysis for further comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

COL6A1 interference inhibits HA-VSMC viability following PDGF-BB stimulation. HA-VSMCs were transfected with the si-COL6A1 recombinant plasmid before transfection efficiency was measured using RT-qPCR and western blotting. The mRNA and protein levels of COL6A1 were significantly downregulated in si-COL6A1 cells compared with those in the negative control EV group (P<0.01; Fig. 1A-C). HA-VSMCs in the Cntl, EV and si-COL6A1 groups were subsequently stimulated with PDGF-BB for 12, 24 and 48 h, before cell viability was assessed using CCK-8 assay. PDGF-BB stimulation significantly increased HA-VSMC viability in the Cntl and EV groups after 24 and 48 h (P<0.05; Fig. 1D). There was no significant difference between the si-COL6A1 group in the absence of PDGF-BB and the si-COL6A1 group in the presence of PDGF-BB, at 24 and 48 h (Fig. 1D). No significant differences were observed in cell viability measured between Cntl, EV and si-COL6A1 groups after 12, 24 or 48 h in the absence of PDGF-BB treatment. These results demonstrate that PDGF-BB stimulation improved HA-VSMC viability, which can be negated by COL6A1 knockdown.

HA-VSMC migration is inhibited by COL6A1 interference following PDGF-BB stimulation. HA-VSMC migration was assessed using wound healing assay. PDGF-BB also appeared to have SIGNIFICANTLY increased HA-VSMC migration si-COL6A1 cell group, albeit not to the same magnitude as the other two conditions (P<0.01; Fig. 2A and B). No significant differences were found between Cntl, EV and si-COL6A1 cell migration in the absence of PDGF-BB stimulation.

COL6A1 interference inhibits HA-VSMC invasion following PDGF-BB stimulation. HA-VSMC invasion was measured using Matrigel-coated Transwell assays. PDGF-BB stimulation significantly increased the invasive capabilities of Cntl and EV cells, but the extent of increase in the si-COL6A1 cell group was significantly lower compared with the corresponding EV group (P<0.01; Fig. 3A and B). In the absence of PDGF-BB, no significant differences were observed between the invasive abilities of Cntl, EV and si-COL6A1 cells.

COL6A1 regulates the expression of factors associated with migration/invasion in HA-VSMCs stimulated by PDGF-BB. RT-qPCR and western blot analysis were used to measure the expression levels of factors associated with migration/invasion in HA-VSMCs following PDGF-BB stimulation. PDGF-BB treatment significantly upregulated the expression levels of FN, MMP-2 and MMP-9 mRNA and protein in Cntl and EV cells, but the scale of this upregulation in si-COL6A1 cells was significantly lower compared with corresponding EV cells (P<0.05; Fig. 4A-C and E-H). By contrast, PDGF-BB treatment significantly reduced TIMP-2 expression in Cntl and EV cells, but the extent of reduction was significantly smaller in si-COL6A1 cells compared with EV cells. (P<0.01; Fig. 4 D, E and I).

COL6A1 interference inhibits the AKT-mTOR pathway in HA-VSMCs following PDGF-BB stimulation. AKT and mTOR phosphorylation in Cntl, EV and si-COL6A1 cells following PDGF-BB treatment were next measured using western blotting. p-AKT expression was reduced in the si-COL6A1 group in the absence of PDGF-BB, compared to the other two groups (P<0.05). Relative AKT and mTOR phosphorylation were significantly increased in Cntl and EV cells in response to PDGF-BB stimulation, but not in si-COL6A1 cells (P<0.01; Fig. 5A-C).

Discussion

The phenotypic transformation of VSMCs serves an important pathophysiological role in neointimal hyperplasia after vascular injury and luminal stenosis, which is dependent upon conditions within its microenvironment (25,26). PDGF-BB is an efficient mitogen stimulator of VSMCs that promote the dedifferentiation, proliferation and migration of VSMCs during vascular injury repair (27). Therefore, PDGF-BB was elected as the agonist in the present study to facilitate the phenotypic transformation of VSMCs. HA-VSMC viability, migratory and invasive capabilities were all found to be promoted by PDGF-BB stimulation.

COL6A1 is a protein associated with metastasis of cervical cancer (19) and, by influencing blood pressure, it is also a risk factor for cardiovascular diseases (28). In the present study, COL6A1 expression was knocked down in HA-VSMCs to study

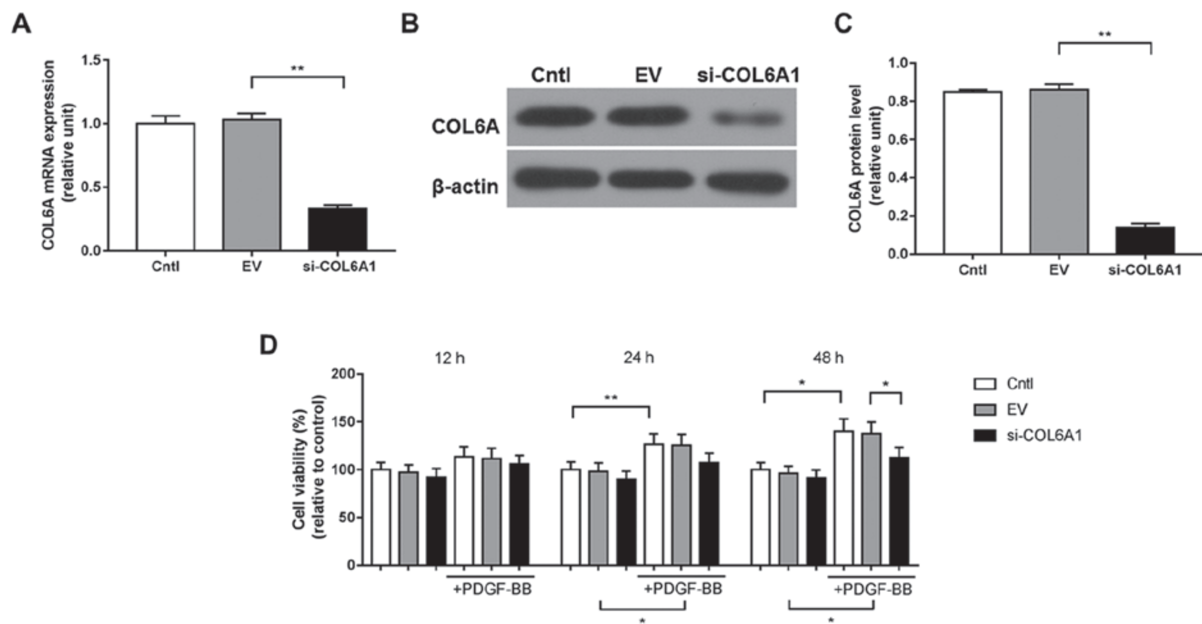

Figure 1. COL6A1 knockdown attenuated cell viability in PDGF-BB-stimulated HA-VSMCs. (A) Expression levels of COL6A1 mRNA were determined using reverse transcription-quantitative PCR following si-COL6A1 transfection. (B) COL6A1 protein levels were determined using western blotting after si-COL6A1 transfection. (C) Quantified densitometry results from the western blot analysis. (D) Cell viability after 12, 24 and 48 h were measured using Cell Counting Kit-8 assay after si-COL6A1 transfection and PDGF-BB stimulation. Each experiment was repeated five times. *P<0.05 and **P<0.01. HA-VSMC, human aortic-vascular smooth muscle cells; COL6A1, collagen type VI α1 chain; PDGF-BB, platelet-derived growth factor; si, short interfering; Cntl, control; EV, siRNA-control.

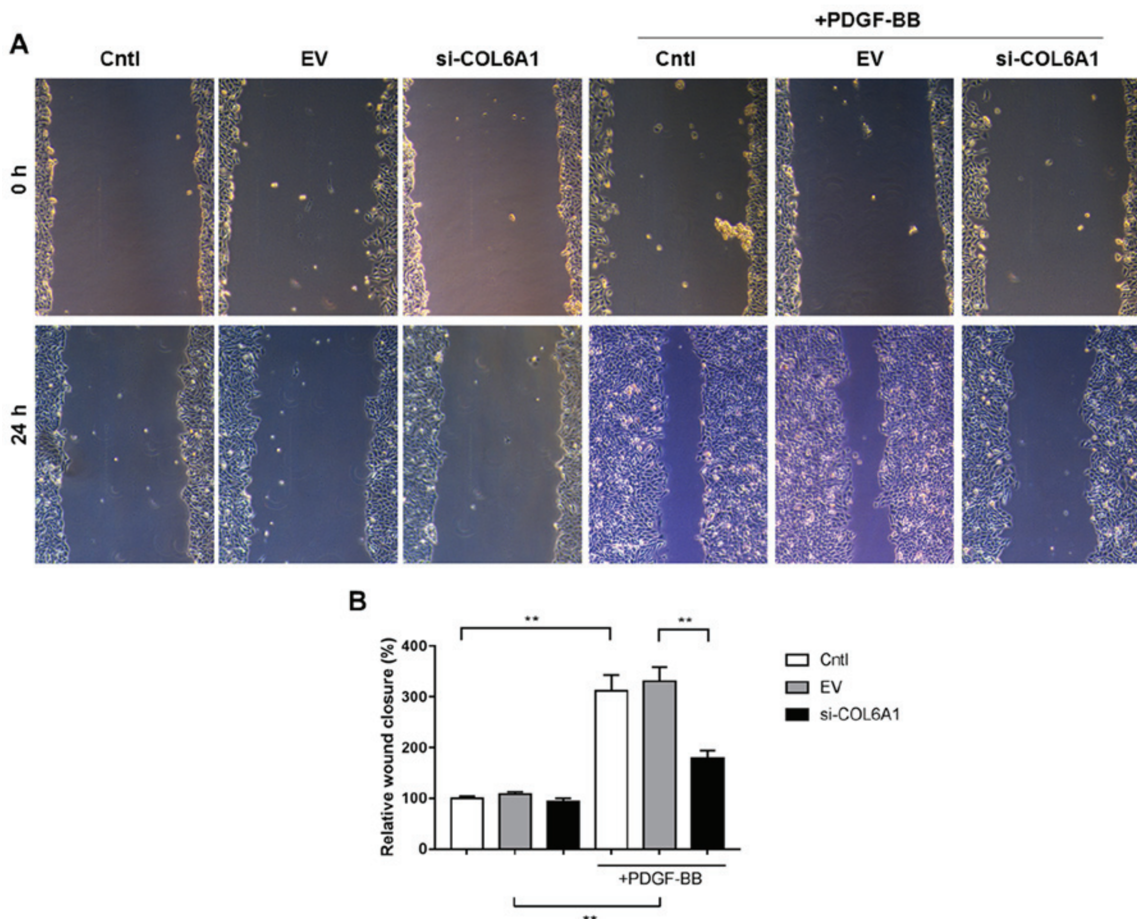

Figure 2. COL6A1 interference inhibited PDGF-BB-stimulated HA-VSMC migration. (A) Wound closure was measured in HA-VSMCs transfected with si-COL6A1 following PDGF-BB stimulation using wound healing assay. (B) Relative wound closure for each condition was quantified. Each experiment was repeated five times. **P<0.01. HA-VSMC, human aortic-vascular smooth muscle cells; COL6A1, collagen type VI α1 chain; PDGF-BB, platelet-derived growth factor; si, short interfering; Cntl, control; EV, siRNA-control.

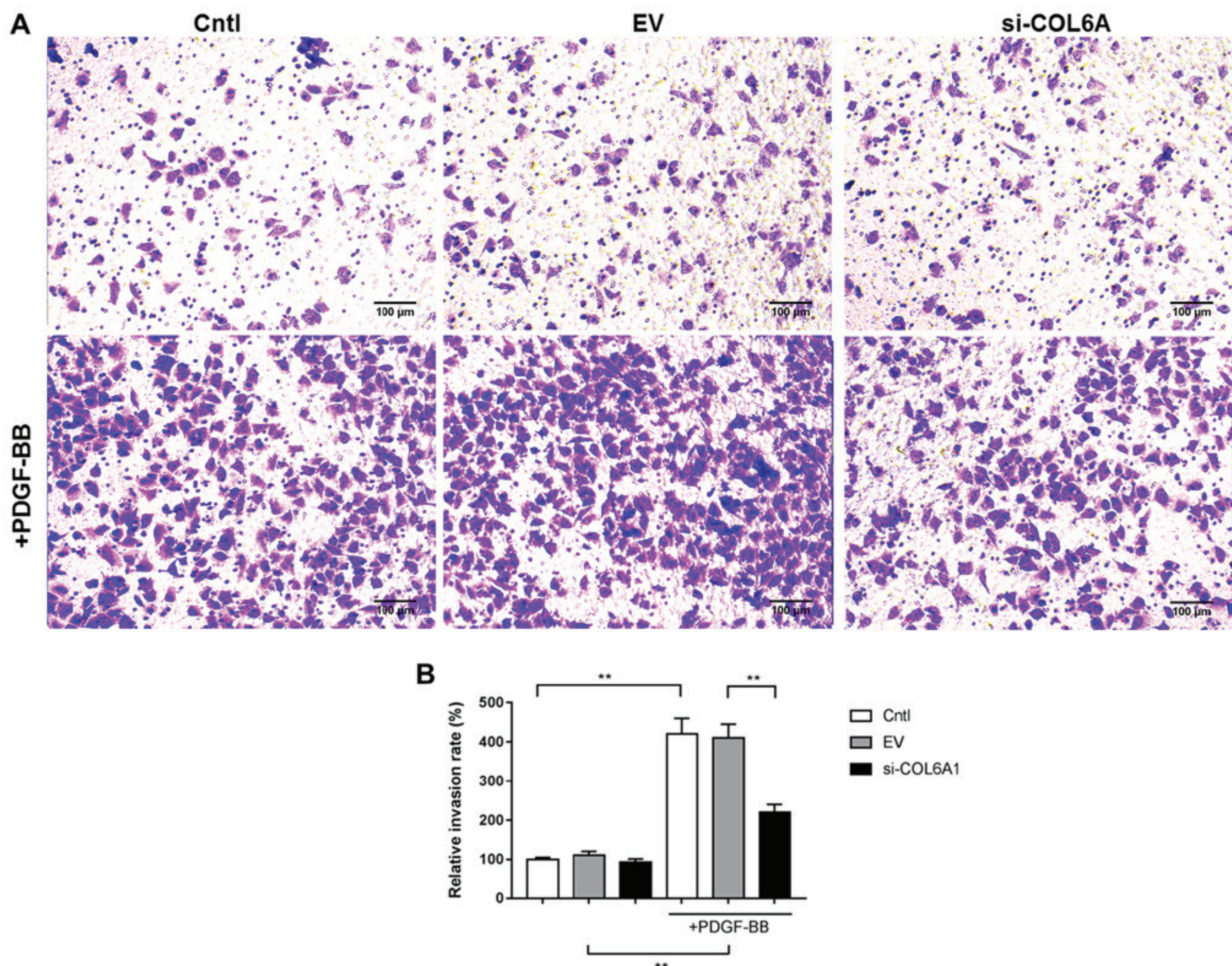

Figure 3. COL6A1 knockdown inhibited HA-VSMC invasion following PDGF-BB stimulation. (A) HA-VSMC invasion was measured using Matrigel-coated Transwell assay after si-COL6A1 transfection and PDGF-BB stimulation. (B) Relative invasion for each condition were quantified. Each experiment was repeated five times. **P<0.01. HA-VSMC, human aortic-vascular smooth muscle cells; COL6A1, collagen type VI α1 chain; PDGF-BB, platelet-derived growth factor; si, short interfering; Cntl, control; EV, siRNA-control.

the function of COL6A1 on HA-VSMCs following PDGF-BB stimulation. It was found that cell viability, migratory and invasive abilities of VSMCs were all significantly potentiated by PDGF-BB treatment, which were partially negated by COL6A1 silencing. RT-qPCR and western blot analysis were subsequently performed to investigate the downstream effects of COL6A1 knockdown on PDGF-BB-stimulated VSMCs, specifically the expression of FN, MMP-2, MMP-9 and TIMP-2, classical factors associated with migration and invasion (29). PDGF-BB promoted FN, MMP-2 and MMP-9 expression whilst downregulating TIMP-2 expression; all of which were partially reversed by COL6A1 knockdown.

FN is an important component of the extracellular matrix which is upregulated in renal cell carcinoma cells (30). Non-enzymatic glycation interferes with FN-integrin interactions in VSMC and glycation of FN shifts the nature of cellular adhesion from integrin- to receptor for advanced glycation end products-dependent mechanisms (31). MMPs are proteolytic enzymes that require calcium, zinc and other metal ions as cofactors (32,33). MMP-9 was reported to be associated with cell proliferation and migration of VSMCs (34). In particular, MMP-2 and MMP-9 serve important roles in the proliferation and migration of VSMCs (35). During VSMC proliferation and migration, the outer membrane of VSMCs interacts with the extracellular matrix to release MMPs for subsequent degradation (36). In contrast, TIMP-2 is a key inhibitor of MMPs and suppresses cell migration/invasion by inhibiting the function of MMPs (37). In the present study, the reduction in FN, MMP-2 and MMP-9 expression, in conjunction with the increase in TIMP-2 expression were caused by COL6A1 knockdown in VSMCs.

The PI3K/AKT signaling pathway commonly serves roles in a number of physiological processes, including cell proliferation, migration, invasion and angiogenesis (38,39). Importantly, it is also the central signaling component of PDGF-BB induced invasion in VSMCs. PDGF-BB-induced cell growth and migration of human airway smooth muscle (40), retinal pigment epithelial (41) and endothelial progenitor cells (42) were all closely associated with the PI3K/AKT pathway. The activation of mTOR has also been reported in the PDGF-BB-induced proliferation and migration of VSMCs (43-45). In the present study, the phosphorylation levels of AKT and mTOR were significantly increased by PDGF-BB treatment in VSMCs, consistent with data from the

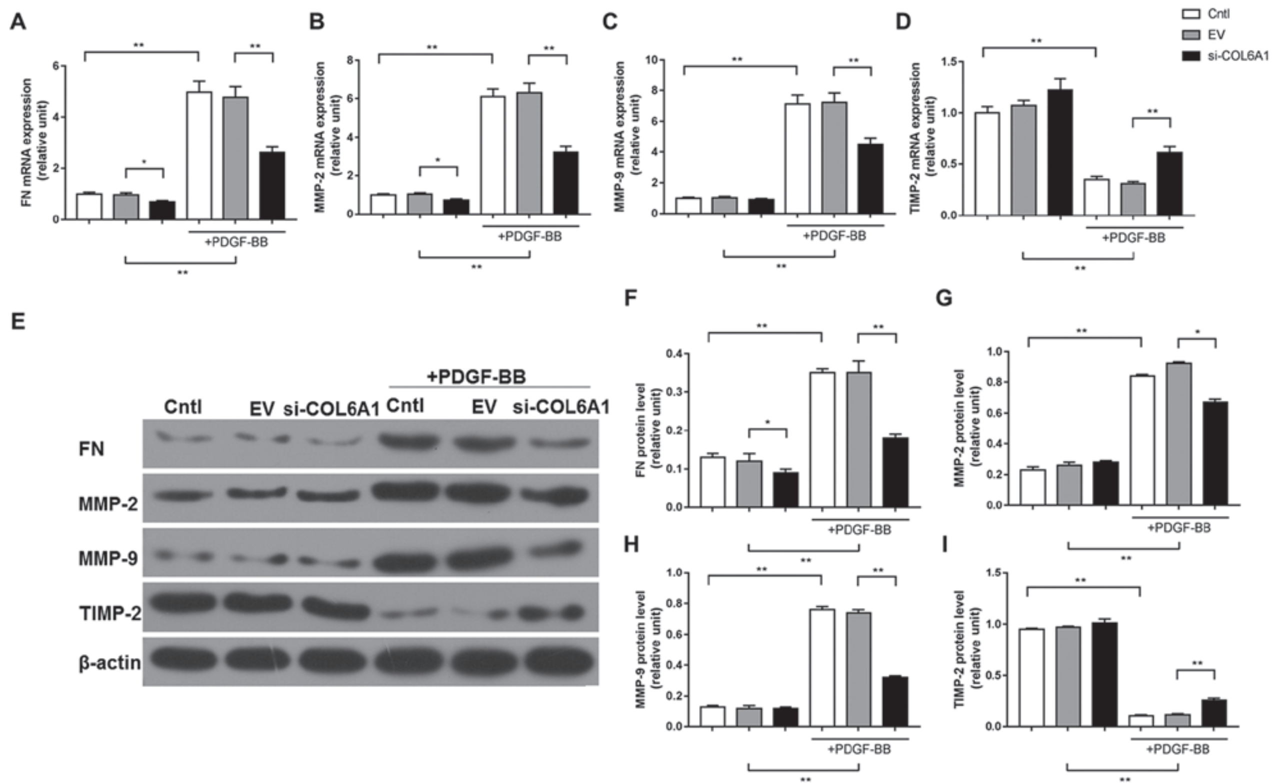

Figure 4. COL6A1 regulate the expression of factors associated with migration and invasion in HA-VSMCs following PDGF-BB stimulation. (A) Reverse transcription-quantitative PCR was performed to determine the levels of mRNA expression of FN, (B) MMP-2, (C) MMP-9 and (D) TIMP-2 after si-COL6A1 transfection and PDGF-BB stimulation. (E-I) Western blot analysis was performed to determine the protein expression levels of (F) FN, (G) MMP-2, (H) MMP-9 and (I) TIMP-2 after si-COL6A1 transfection and PDGF-BB stimulation. Each experiment was repeated five times. *P<0.05, and **P<0.01. HA-VSMC, human aortic-vascular smooth muscle cells; COL6A1, collagen type VI α1 chain; PDGF-BB, platelet-derived growth factor; FN, fibronectin; MMP, matrix metalloproteinase; TIMP-2, tissue inhibitor of metalloproteinase-2; si, short interfering; Cntl, control; EV, siRNA-control.

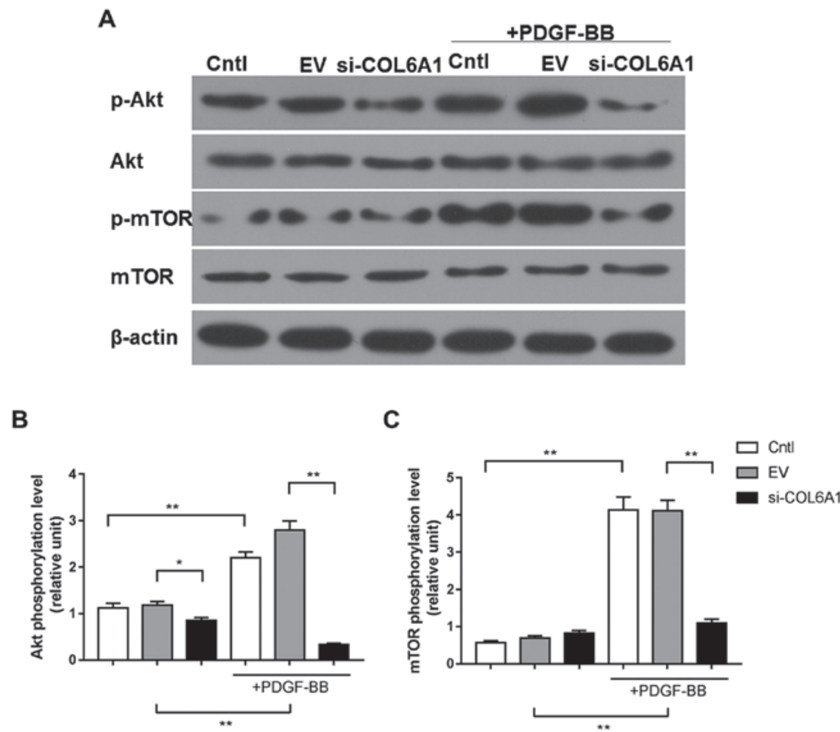

Figure 5. AKT-mTOR pathway was inhibited by COL6A1 knockdown in HA-VSMCs following PDGF-BB stimulation. (A-C) The levels of phosphorylation relative to the total expression of their respective proteins of (B) AKT and (C) mTOR in HA-VSMCs were analyzed and quantified using western blot analysis following si-COL6A1 transfection and PDGF-BB stimulation. Each experiment was repeated five times. *P<0.05 and **P<0.01. HA-VSMC, human aortic-vascular smooth muscle cells; COL6A1, collagen type VI α1 chain; PDGF-BB, platelet-derived growth factor; si, short interfering; Cntl, control; EV, siRNA-control.

previous studies aforementioned. The silencing of COL6A1 potently reversed PDGF-BB-induced activation of AKT and mTOR in VSMCs. These observations suggest that COL6A1 knockdown inhibited VSMC viability, migration and invasion by suppressing Akt/mTOR activation.

In conclusion, the silencing of COL6A1 inhibited cell viability, migration and invasion of PDGF-BB-stimulated VSMCs by suppressing the expression of MMPs and Akt/mTOR activation. This suggest that COL6A1 may be a potential therapeutic target in the treatment of cardiovascular diseases, but this needs to be investigated further.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author upon reasonable request.

Authors' contributions

ZC and QW performed PCR and western blotting assays. CY performed the remaining assays. JD conceived and designed the study.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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