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ORIGINAL PAPER

RUNX3 is up-regulated in abdominal aortic aneurysm and regulates the function of vascular smooth muscle cells by regulating TGF-β1

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Abstract

Abdominal aortic aneurysm (AAA) has been associated with the dysfunction of vascular smooth muscle cells (VSMCs) and extracellular matrix (ECM) remodelling. Runt-related transcription factor 3 (RUNX3) has been reported to be up-regulated in aneurysmal aorta samples compared with normal aorta. However, its function in VSMCs and the mechanism of function remains unknown. Therefore, our study aimed to investigate the role of RUNX3 in ECM remodelling and VSMC function, and further explore the underlying mechanism. Our results verified that RUNX3 was increased in aortic samples of AAA compared with healthy controls. Overexpression vectors of RUNX3 (ov-RUNX3) and siRNA of RUNX3 (si-RUNX3) were transfected into Human aortic smooth muscle cells (HAoSMCs). The results indicated that ov-RUNX3 promoted cell proliferation, migration, and MMP-2/3/9 secretion, and suppressed TIMP-1, collagen I/III, SM22, MYH11 and CNN1 expression in HAoSMCs. The silencing of RUNX3 has the opposite effect. Furthermore, we found that RUNX3 targets TGF-β1 and suppressed its transcription. The silencing of TGF-β1 increased cell proliferation, migration and MMP-2/3/9 expression, and inhibited TIMP-1, Collagen I/III, SM22, MYH11 and CNN1 expression. In addition, TGF-β1 reversed the effect of RUNX3 overexpression on HAoSMCs. Hence, our study indicated that RUNX3 promotes cell proliferation, migration, and ECM remodelling through suppressing TGF-β1.

Keywords Abdominal aortic aneurysm · RUNX3 · TGF-β1 · Extracellular matrix remodeling

Abbreviations

AAA	Abdominal aortic aneurysm
ECM	Extracellular matrix
VSMCs	Vascular smooth muscle cells
MMPs	Matrix metalloproteinases
TIMP-1	Tissue inhibitor of metalloproteinases 1
RUNX	Runt related transcription factors
HAoSMCs	Human aortic smooth muscle cells
OV-RUNX3	Overexpression vectors of RUNX3
si-RUNX3	RUNX3 siRNA oligo duplex

Introduction

Abdominal aortic aneurysm (AAA) is a degenerative disease with irreversible and progressive dilation of the artery (Chen et al. 2020). Progressive aortic dilation leads to the eventual rupture of the aorta, accompanied by great threat to life (Golledge 2019). In adults older than 50 years, AAA affects approximately 5–7% of men, and 1.3% of women (Guirguis-Blake et al. 2019). It causes 150,000–200,000 deaths every year worldwide. The development of AAA is a complex process, characterised by the depletion and dysfunction of vascular smooth muscle cells (VSMCs), inflammation, and aortic wall extracellular matrix (ECM) remodelling (Klaus et al. 2017; Xu et al. 2019). In healthy aorta, ECM is constantly remodelled to maintain the integrity of the vessel wall, and provides a physical scaffold for the cells that make up the aorta (Kim et al. 2009; Wagenseil and Mecham 2009). In AAA, the ECM homeostasis is imbalanced, characterised by the abnormal deposition of collagen, increased ratio of collagen I/III, and disorganised collagen arrangement (Huang et al. 2016). Previous studies have reported that matrix metalloproteinases (MMPs) were up-regulated in

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the aortic wall of AAA patients, including MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 (Rabkin 2017). It is known that MMPs contribute to the degradation of ECM. However, the pathogenic mechanism of ECM remodelling in VSMCs during AAA progression has not been fully elucidated.

Runt-related transcription factors (RUNX) play crucial roles in multiple signalling pathways and cellular processes through multiple protein-interacting partners (Chuang et al. 2013). Three RUNX genes have been identified in mammals: RUNX1, RUNX2, and RUNX3 (Rahmanian et al. 2017). All of these Runt-related genes were found to be up-regulated in aneurysmal aorta samples, compared with normal aorta (Dubis et al. 2016). RUNX2 was reported to participate in the AAA formation by regulating the microcalcification of VSMCs (Li et al. 2020). Deficient RUNX2 mice exhibited a lower incidence of AAA after stimulation by angiotensin II (Li et al. 2020). RUNX3 exhibits an anti-tumour effect in most tumours (Li et al. 2019; Milner et al. 2017; Sun et al. 2018); nevertheless, it promotes cancer in ovarian cancer and human epithelial cancer cells (Date and Ito 2020; Lee et al. 2011). In addition, RUNX3 overexpression facilitated endothelial-mesenchymal transition and endothelial cell dysfunction (Liu et al. 2017). A previous study of whole genome expression profiling in human AAA showed that RUNX3 was presented as an upregulated gene for immune function in AAA and confirmed the increased expression of RUNX3 by Q-RT-PCR analysis in RNA specimen obtained from AAA tissues (Lenk et al. 2007). However, its role in AAA remains unclear.

In this study, the expression of RUNX3 in AAA tissues was measured, and the effect of the overexpression or inhibition of RUNX3 in human aortic smooth muscle cell (HAoSMC) proliferation, apoptosis, migration, and extracellular matrix remodelling was analysed. In addition, the underlying mechanism of RUNX3 function in HAoSMCs was further explored.

Materials and methods

Subjects

A total of 22 patients with AAA in Weihai Municipal Hospital from 2018 were included. Patient inclusion criteria included: (1) no history of malignancies; (2) no other types of severe disease, for instance, severe mental disorders, and vascular diseases; and (3) newly diagnosed cases which had received no treatment before sampling. The patients included 13 males and 9 females, and ages ranged from 42 to 67 years, with a mean age of 52.3 ± 4.6 years. The control group was consisted of the adjacent non–aneurysmal segment/pieces of aorta from the same patients, according to previous studies (He et al. 2019; Song et al. 2020). Aortic biopsy was performed and aortic samples were collected from each participant. This study was approved by the Ethics Committee of Weihai Municipal Hospital, and all participants signed informed consent.

Cell culture

HAoSMCs (catalog number: PCS-100-012) were provided by the American Type Culture Collection (ATCC, Manassas, VA), and were cultured in medium 231 with smooth muscle growth supplements (Cascade Biologics, Portland, OR) at 37 °C in a classic CO_2 incubator. The cryopreserved cells were at the second passage of culture to guarantee the cell viability and experimental efficiency for the highest level, avoiding effects occurred on our research by adverse cell performance.

Plasmid construction and stable transfection

Overexpression vectors of pCMV6-RUNX3 (OV-RUNX3), RUNX3 siRNA oligo duplex (si-RUNX3) and TGF-β1 siRNA oligo duplex (si-TGF-β1) were purchased from ORIGENE (OriGene, USA). Cell transfection was performed using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. Negative control scramble siRNA (si-NC) or empty overexpression vectors (ov-NC) were transfected into cells as a negative control. The sequences are as follows: si-RUNX3 forward, 5'-TTTGCGGAGTAGTTC TCGTCATACAATGACGAGAACTACTCCGCTTTTT-3'; si-RUNX3 reverse, 5'-CTAGAAAAAGCGGAGTAGTTC. TCGTCATTGTATGACGAGAACTACTCCG-3'.

RNA preparation and quantitative real-time PCR

Total RNA from aortic media specimens and HAoSMCs were extracted using TRIzol reagent (Thermo Fisher Scientific, Inc., Rockford, IL). Complementary DNA (cDNA) was synthesised by reverse transcription using the SuperScript IV Reverse Transcriptase Kit (Thermo Fisher Scientific). Quantitative PCR analysis was performed using SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific) on CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The following primers were used in this study: RUNX3 forward 5'-AGGCAATGACGAGAA CTACTCC-3', and reverse: 5'-CGAAGGTCGTTGAAC CTGG-3'; TGF-β1 forward 5'-TACAGCACGGTATGC AAGCC-3', and reverse: 5'-GCAACCGATCTAGCTCAC

AGAG-3'; and GAPDH forward 5'-TGTGGGCATCAATGG ATTTGG-3', and reverse: 5'-ACACCATGTATTCCGGGT CAAT-3'. The $2^{-\Delta\Delta tC}$ method was used to process all data, and GAPDH was adopted as an endogenous control.

Dual-luciferase reporter assay

Wild-type and mutant of TGF-β1 promoter cDNA fragments were amplified and inserted downstream of the pGL4.1 luciferase reporter plasmid. Luciferase reporter plasmid and the overexpression vectors of RUNX3 or empty overexpression vectors were co-transfected into HAoSMCs cells using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. The luciferase activity was measured through the Dual Luciferase Reporter System Kit (Promega, Madison, WI, USA).

Cell proliferation assay

Cell counting kit-8 (CCK-8, Dojindo, Tokyo, Japan) was adopted in this study to analyse the cell proliferation. HAoSMCs were plated onto 96-well plates and cultured overnight. After being incubated with TGF-β1 for 24 h, CCK-8 reagent (10 μL) was added to each well and cultured for 2 h. The OD values at 450 nm were measured using a microplate reader.

Cell apoptosis assay

Cell apoptosis was measured through Roche's Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics Corporation, Indianapolis, IN) and Caspase-3 activity assay kit (Cell Signalling Technology, Danvers, MA, USA) following the manufacturer's instructions.

Cell migration test

The migration of HAoSMCs was analysed through the transwell chamber migration assay. HAoSMC mixed serum-free culture media were planted into the upper chamber, while culture medium with 10% serum was added to the lower chamber. After incubated for 48 h at 37 °C, cells in the upper surface of chamber were removed by cotton swabs; the migrated cells in the lower chamber were fixed with paraformaldehyde (4%, w/v) for 20 min and then stained with crystal violet for 15 min. Images were obtained in an inverted microscope and the migrated cells were counted.

ELISA test

Culture medium of HAoSMCs was collected and the content of MMP-2, MMP-3, and MMP-9 was measured using the human MMP2 ELISA Kit (ab267813), human MMP3 ELISA Kit (ab269371), and human MMP9 ELISA Kit (ab246539) according to the manufacturer's instructions (Abcam, Cambridge, UK).

Western blot analysis

Western blot was performed as previously described (Hu et al. 2019). Antibodies used in this study include the anti-RUNX3 antibody (ab53066, 1:1000, Abcam), anti-TGF-β1 antibody (ab40278, 1:800, Abcam), anti-caspase 3 antibody, active (cleaved) form (AB3623, 1:200, Sigma-Aldrich), anti-Bax antibody (B3428, 1:500, Sigma-Aldrich), anti-Bcl-2 antibody (B3170, 1:1000, Sigma-Aldrich), anti-TIMP-1 antibody (ab211926, 1:1000, Abcam), anti-Collagen I antibody (ab34710, 1:500, Abcam), anti-Collagen III antibody (ab6310, 1:500, Abcam), goat anti-rat IgG H&L (HRP) (ab7097, 1:2000, Abcam) and goat anti-mouse IgG H&L (HRP) (ab97040, 1:2000, Abcam). Anti-GAPDH antibody (ab9485, 1:1000, Abcam) was used as the loading control.

Statistical analysis

Data are presented as the mean of three independent biological replicates. Data were processed by GraphPad software; differences between 2 groups were explored using the unpaired t-test, and more than two groups were analysed by one-way ANOVA combined with the Tukey test. $P < 0.05$ was statistically significant.

Results

RUNX3 is up-regulated in AAA patients

The expression of RUNX3 in aortic media from AAA patients and healthy controls was detected through quantitative PCR analysis. The results show that RUNX3 was significantly increased in AAA patients (n = 22), compared with normal controls (n = 15, $P < 0.001$, Fig. 1A). Meanwhile, three aortic tissue samples were randomly selected from AAA patients and healthy controls; the protein expression of RUNX3 in these six samples was measured. As shown in Fig. 1 B and C, RUNX3 protein expression was markedly higher in AAA patients than in normal controls ($P < 0.001$).

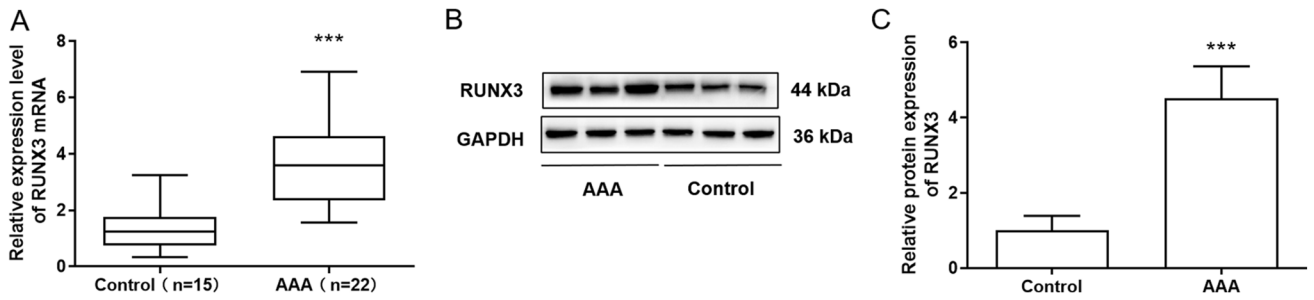

Fig. 1 RUNX3 is upregulated in AAA patients. **A** RUNX3 mRNA expression levels in aortic specimens were measured through quantitative PCR analysis. **B** and **C** RUNX3 protein expression in aortic specimens were tested by Western blotting (n = 3). $^{**}P < 0.001$, compared with healthy control group

Effect of RUNX3 overexpression and inhibition on cell proliferation, apoptosis and phenotype marker expression

To explore the role of RUNX3 in AAA progression, the function of RUNX3 in HAoSMCs was explored. Overexpression vectors of RUNX3 (ov-RUNX3) and siRNA of RUNX3 (si-RUNX3) were transfected into HAoSMCs to promote or suppress RUNX3 expression (Fig. 2A and B. As shown in Fig. 2C, RUNX3 overexpression promoted the cell proliferation of HAoSMCs, while RUNX3 inhibition suppressed HAoSMC proliferation. Also, the silencing of RUNX3 promoted HAoSMC apoptosis (Fig. 2D and E). The protein expression of cleaved-caspase 3 and Bax were promoted by RUNX3 inhibition, while Bcl-2 was suppressed by RUNX3 inhibition in HAoSMCs (Fig. 2F). In addition, overexpression of RUNX3 markedly inhibited SM22, MYH11 and CNN1 expression (Fig. 2G). Conversely, the silence of RUNX3 elicited the opposite effect of SM22, MYH11 and CNN1 expression (Fig. 2G).

Effect of RUNX3 overexpression and inhibition on cell migration and extracellular matrix remodelling

Next, we found that RUNX3 overexpression promoted the migration of HAoSMCs, while the silencing of RUNX3 has an opposite effect (Fig. 3A). The levels of MMP-2, MMP-3, and MMP-9 were markedly increased in the culture medium of HAoSMCs after the overexpression of RUNX3, and decreased after the silencing of RUNX3 (Fig. 3B–D). Meanwhile, we found that the protein expression of TIMP-1 (tissue inhibitor of metalloproteinases 1), Collagen I and Collagen III was decreased in the ov-RUNX3 transfected group, and increased in the si-RUNX3 transfected group (Fig. 3E and F).

RUNX3 targets TGF-β1 and suppressed its transcription

A previous study reported that RUNX3 binds with the promoter of TGF-β1 at base pairs 1154-1296 in SW480 cells (Zhang et al. 2020). The luciferase reporter assay results verified that RUNX3 overexpression inhibited the promoter luciferase activity of TGF-β1 in HAoSMCs (Fig. 4A). Furthermore, we found that transfection with ov-RUNX3 into HAoSMCs significantly suppressed the mRNA and protein expression of TGF-β1; meanwhile, TGF-β1 was up-regulated in HAoSMCs after the silencing of RUNX3 (Fig. 4B and C). Also, the effect of TGF-β1 on the expression of RUNX3 in HAoSMCs was explored. The results indicated that TGF-β1 at a dose of 0–40 ng/mL made no significant changes on the expression of RUNX3 in HAoSMCs (Fig. 4D–F).

Effect of TGF-β1 silence on cell proliferation, migration, extracellular matrix remodelling and phenotype marker expression

As shown in Fig. 5A and B, TGF-β1 was inhibited by transfection with si-TGF-β1. TGF-β1 inhibition promoted cell proliferation and the migration of HAoSMCs (Fig. 5C and D). At the same time, the levels of MMP-2, MMP-3, and MMP-9 were notably increased in HAoSMCs after the silencing of TGF-β1 (Fig. 5E). The expression of TIMP-1, Collagen I and Collagen III was decreased in the si-TGF-β1 transfected group, compared with the si-NC transfected group (Fig. 5F and G).

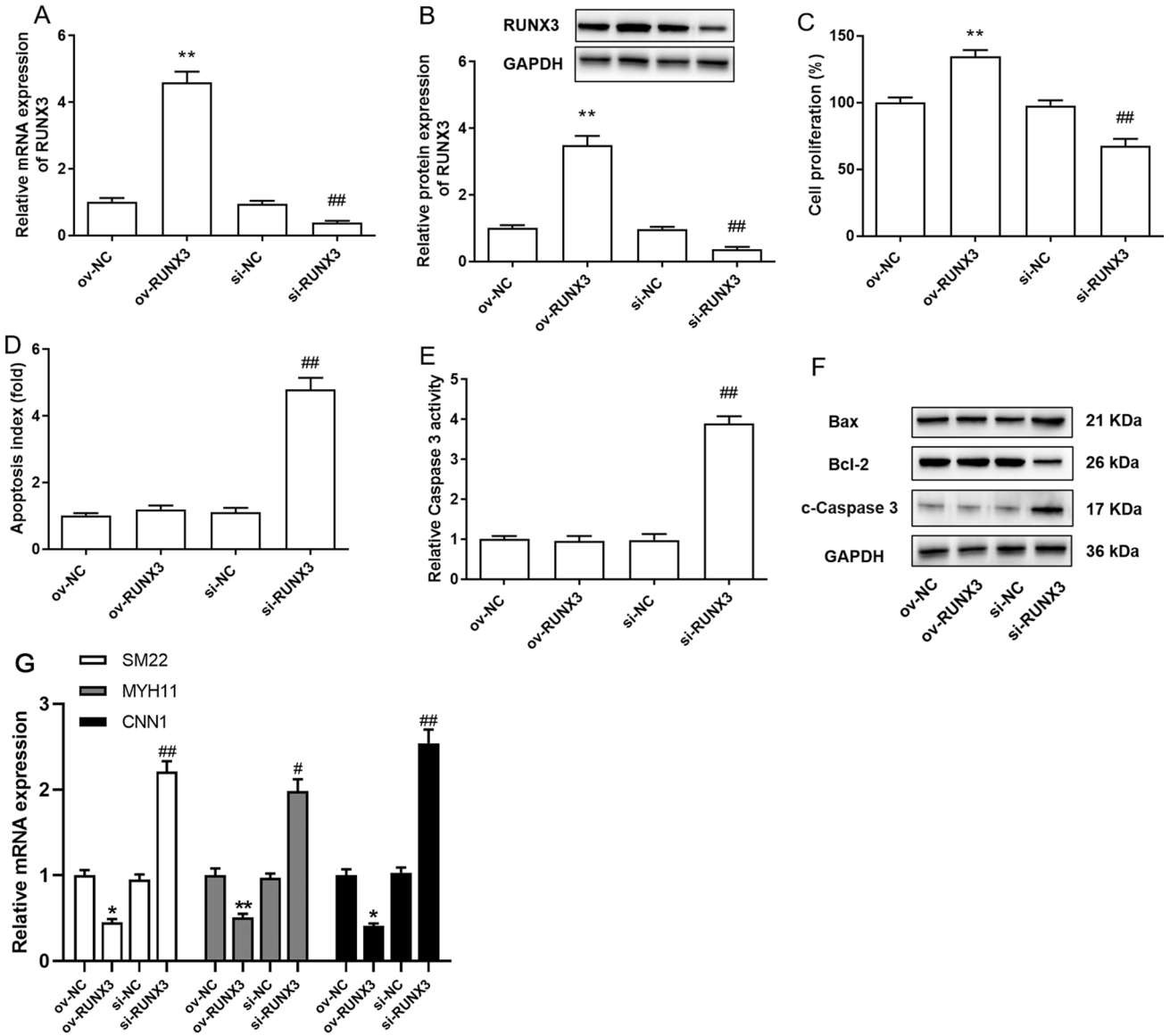

Fig. 2 Effect of RUNX3 overexpression and inhibition on cell proliferation and apoptosis. **A** and **B** Transfection efficiency of overexpression vectors of RUNX3 (ov- RUNX3) and siRNA of RUNX3 (si-RUNX3) in HAoSMC cells. **C** Cell proliferation was measured by CCK-8 assay kit. **D** and **E** Cell apoptosis was analyzed through Roche's Cell Death Detection ELISA^{PLUS} Kit and Caspase 3 activity kit. **F** The expression of cleaved-Caspase 3, BCL-2 and Bax were detected through Western blotting. G. The expression of SM22, MYH11 and CNN1 were detected using qPCR. $^{**}P < 0.01$, compared with ov-NC group. $^{\#\#}P < 0.01$, compared with si-NC group

Enhanced TGF-β1 reversed the effect of RUNX3 overexpression on HAoSMCs

To explore the role of TGF-β1 in RUNX3-mediated cell proliferation, migration, extracellular matrix remodeling and phenotype marker expression in HAoSMCs. TGF-β1 (20 ng/mL) was added into the culture medium of HAoSMCs and incubated for 24 h. The results suggested that TGF-β1 suppressed cell proliferation and migration in HAoSMCs (Fig. 6A–B). Meanwhile, TGF-β1 reversed the promoting effect ov-RUNX3 on cell proliferation and migration in HAoSMCs (Fig. 6A–B). Also, TGF-β1 treatment suppressed the expression of MMP-2, MMP-3, and MMP-9 in HAoSMCs, and also partially reversed the promoting effect

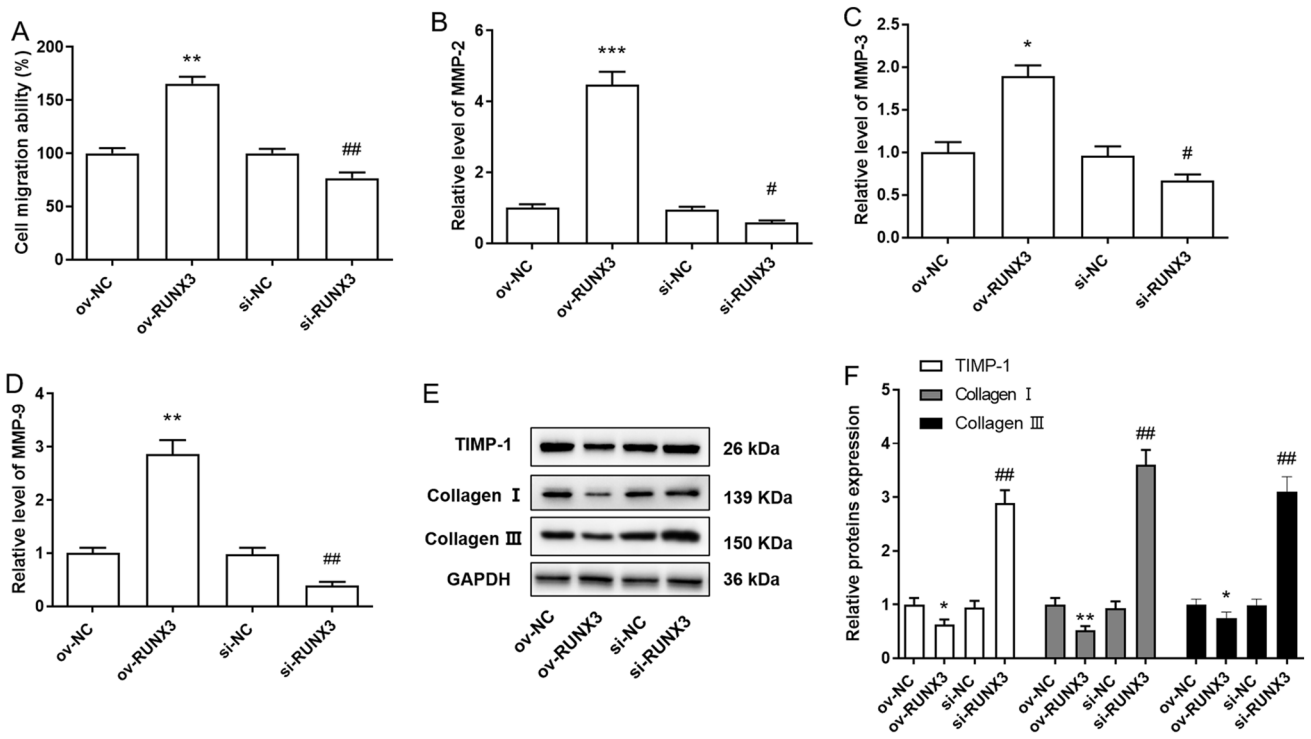

Fig. 3 Effect of RUNX3 overexpression and inhibition on cell migration and extracellular matrix remodeling. **A** Cell migration was measured through the transwell assay. **B**–**D** Relative expression of MMP-2, MMP-3 and MMP-9 in the culture medium of HAoSMC cells. **E** and **F** Protein expression of TIMP-1, Collagen I and Collagen III in HAoSMC cells were detected through Western blotting. $^{*}P<0.05$, $^{**}P<0.01$, compared with ov-NC group. $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with si-NC group

of ov-RUNX3 on MMP-2, MMP-3, and MMP-9 production (Fig. 6C–E). In addition, TGF-β1 stimulation promoted TIMP-1, Collagen I and Collagen III expression, and suppressed the expression of TIMP-1, Collagen I and Collagen III induced by RUNX3 overexpression in HAoSMCs (Fig. 6F and G).

Discussion

In this study, we found that RUNX3 was up-regulated in AAA patients and its up-regulation promoted HAoSMC proliferation and migration. Meanwhile, the silencing of RUNX3 suppressed the proliferation and migration of HAoSMCs, and induced cell apoptosis. SMCs are the main intrinsic cells in the aortic wall; their dysfunction contributes to the gradual dilatation and eventual rupture of the aorta (Wang et al. 2018). They perform their functions through proliferation, migration, and the secretion of various cytokines (Xue et al. 2019), while VSMC hyperplasia was regarded as critical for AAA (Li et al. 2018). Hence, it is predicted that RUNX3 is involved in the initiation and progression of AAA.

Another major function of SMCs is the maintenance of ECM homeostasis. The results in this study indicated that RUNX3 promoted the secretion of MMP-2, MMP-3, and MMP-9, and inhibited the expression of TIMP-1, and collagens I/III. Previous studies suggested that RUNX3 up-regulated MMP-2 and MMP-9 expression in colorectal tumour cells (Kim et al. 2016; Xue et al. 2020). Accumulating evidence indicates that MMPs are overexpressed in AAA and promote its progression. MMP-3 deficiency attenuates ECM damage and suppresses the susceptibility of mice to develop AAA (Hadi et al. 2018). Meanwhile, SMC-derived MMP-3 promotes AAA progression (Hadi et al. 2018). Also, MPPs can destruct the orderly collagen and elastin network of the aorta. MMP-2/9 can degrade solubilised monomers of type I and III collagen (Van Doren 2015). TIMP-1 as an inhibitor of MMP-1, -3, and -9 could suppress the activity of MMPs (Rabkin 2014). Collagens I/III are the building blocks for the aortic wall, and play an important role in keeping the strength and stiffness of the aortic wall (Deguchi et al.

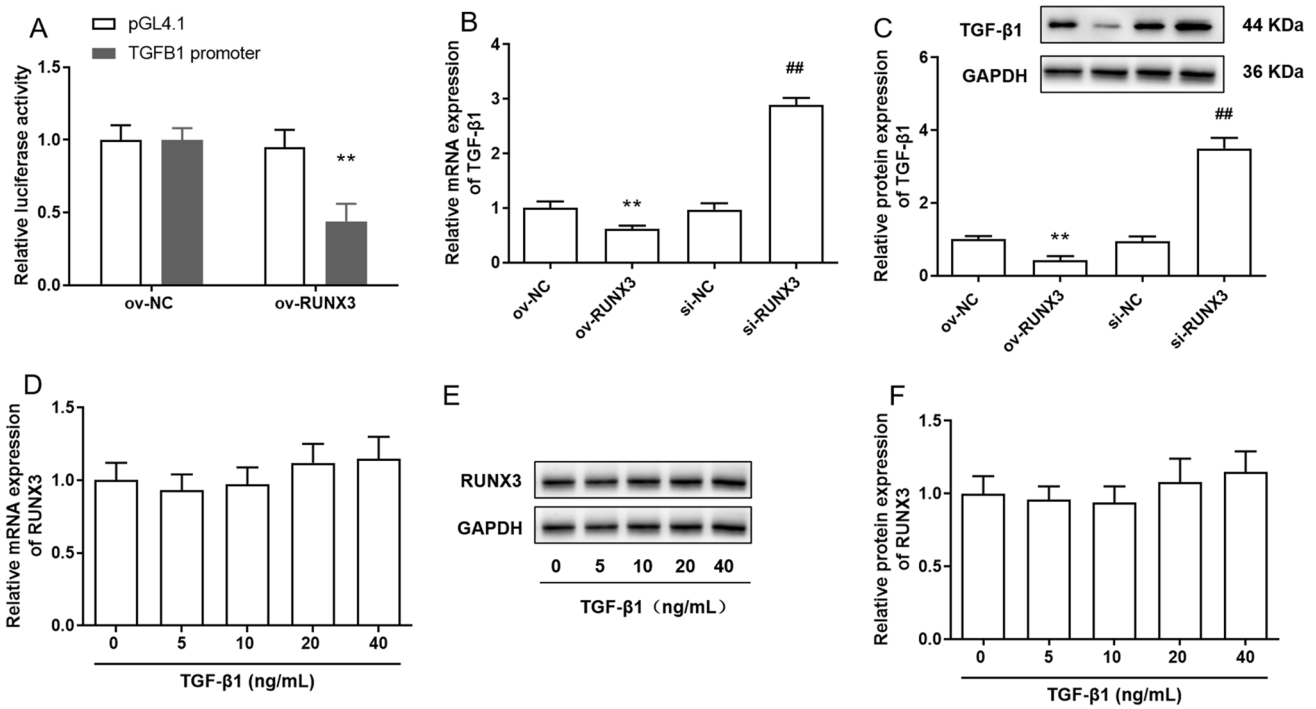

Fig. 4 RUNX3 targets TGF-β1 and suppressed its transcription. **A** Relative luciferase activity of TGFB1 promoter was measured after transfection with ov-RUNX3 or ov-NC. $^{**}P<0.01$. **B** and **C** Expression of TGF-β1 mRNA and protein in HAoSMC cells were measured after transfection with ov-RUNX3 or si-RUNX3. $^{**}P<0.01$, compared with ov-NC group. $^{\#\#}P<0.01$, compared with si-NC group. **D–F** Effect of TGF-β1 on RUNX3 expression

2009). Hence, it is indicated that RUNX3 may be involved in ECM remodelling through promoting MMPs and inhibiting TIMP-1 and collagen I/III accumulation. In addition, inflammation is also a pivotal factor in the development and progression of AAA (Xu et al. 2019). Runx3 is involved in many important immune processes. In Runx3 knockout mice, Runx3 deletion resulted in multiple immunological defects (Lotem et al. 2017). Besides, it has been shown that RUNX3 is associated with immune-related diseases of the gastrointestinal tract and several other organs (Guo et al. 2010; Weersma et al. 2008). These data demonstrate the significance of RUNX3 in inflammation. In this work, we focused only on the role of RUNX3 in VSMC proliferation, migration, and ECM remodelling. In the next study, we will devote to explore the role of RUNX3 in AAA inflammatory process.

TGF-β1 was found to be elevated in the AAA tissues of humans and experimental animals (Doyle et al. 2015; Spin et al. 2011). In addition, TGF-β1 regulates vascular remodelling by promoting collagen synthesis and suppressing collagen degradation (Jones et al. 2009). Meanwhile, TGF-β1 participates in regulation of the growth, migration, and differentiation of VSMCs (Pardali et al. 2010). Activated TGF-β1 could enhance the transcription of collagen, and MMPs. Previous studies suggested that TGF-β signalling in VSMCs plays a protective role in abdominal aortic health (Angelov et al. 2017). In our study, we found that the overexpression of RUNX3 suppressed the transcription of TGF-β1, while RUNX3 inhibition promoted TGF-β1 expression. This result is consistent with a previous report that RUNX3 binds to the promoter region of TGF-β1 in colorectal cancer cells and suppresses the transcription of TGF-β1 (Zhang et al. 2020). Besides, knockdown of RUNX3 in hypoxia-induced human cardiac microvascular endothelial cells (HCMECs) increased the level of TGF-β1 isoform TGF-β2 (Liu et al. 2016). Given the important role of TGF-β1 in the AAA process and the few studies on RUNX3 and TGF-β1 isoforms, herein, we mainly investigated whether RUNX3 regulates TGF-β1 expression to influence AAA onset and progression. In addition, our study found that TGF-β1 reversed the promoting effect of RUNX3 on VSMC proliferation and migration, and MMP production, and promoted collagen protein

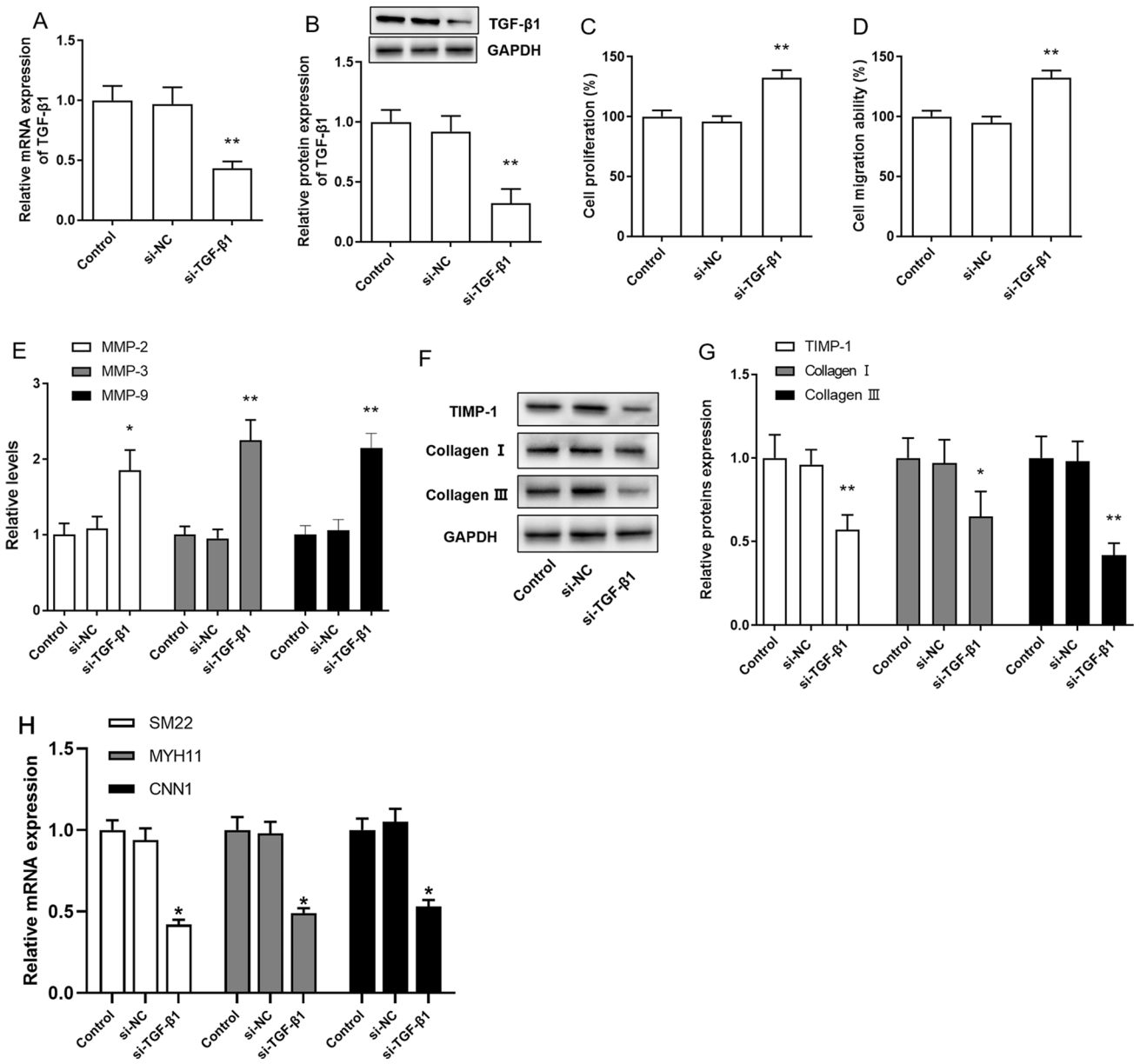

Fig. 5 Effect of si-TGF-β1 on HAoSMC cells function. **A** and **B** Transfection efficiency of TGF-β1 siRNA (si-TGF-β1) in HAoSMC cells. **C**–**G** Cell proliferation (**C**), migration (**D**), contents of MMP-2, MMP-3 and MMP-9 (**E**), protein expression of TIMP-1, Collagen I and Collagen III (**F** and **G**) in HAoSMC cells were detected after transfection with si-TGF-β1. $*P<0.05$, $**P<0.01$, compared with si-NC group

expression. These results suggest that RUNX3 aggravates VSMC proliferation, migration, and ECM remodelling, at least in part by suppressing TGF-β1 transcription.

In conclusion, we discovered that RUNX3 was increased in AAA patients, and its overexpression induced VSMC proliferation, migration, and ECM remodelling, while the silencing of RUNX3 has the opposite effect. The underlying mechanism might involve binding to the promoter of TGF-β1 and suppressing its transcription. These findings suggest that RUNX3 may be a promising therapeutic target for VSMC hyperplasia and vascular ECM remodelling in AAA patients.

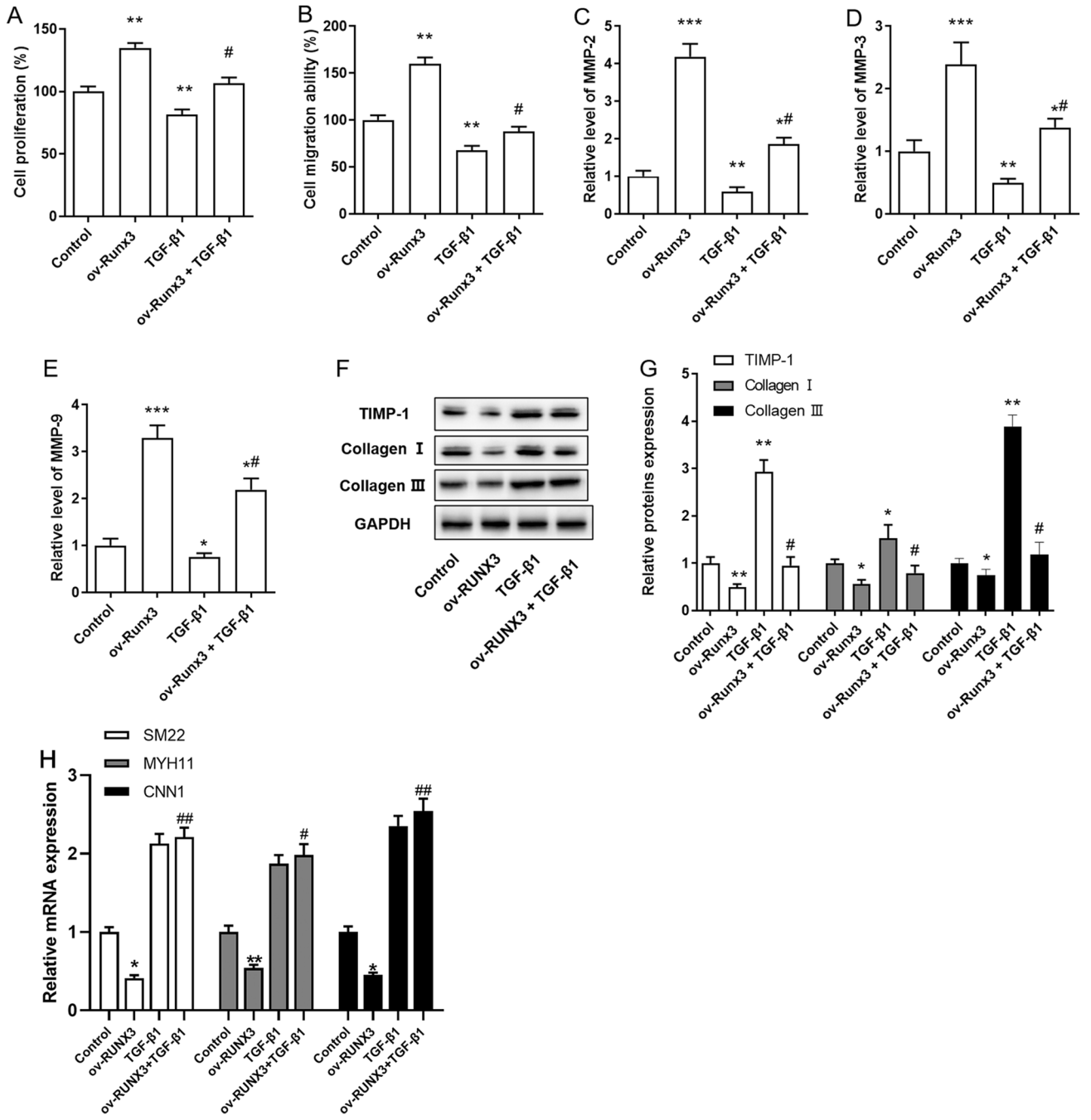

Fig. 6 Enhanced TGF-β1 reversed the effect of RUNX3 overexpression on HAoSMC cells. Cell proliferation (**A**), migration (**C**), content of MMP-2, MMP-3 and MMP-9 (**C**–**E**), protein expression of TIMP-1, Collagen I and Collagen III (**F** and **G**) in HAoSMC cells were detected. $^{*}P<0.05$, $^{**}P<0.01$, compared with the control group, $^{\#}P<0.05$, compared with ov-RUNX3 transfected group

Author contributions ZZ and HZ: conception, design and analysis of data, performed the data analyses, and wrote the manuscript. XZ: contributed to the conception of the study. XW: contributed significantly to analysis and manuscript preparation. All authors contributed to interpretation of date and review of the manuscript, and approved this manuscript for submission.

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

Declarations

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

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