YY1 affects the levels and function of fibulin-5 in ox-LDL-treated vascular smooth muscle cells

LEIJING WANG, CUIYAN LI, CHUNLI FENG and YUANBO ZANG

Department of Clinical Laboratory, Laixi Municipal Hospital, Laixi, Shandong 266600, P.R. China

Abstract. Fibulin-5 is reportedly involved in the pathological process of atherosclerosis (AS) where low expression has been frequently observed in ruptured atherosclerotic plaques. The aim of the present study was to determine the effects of fibulin-5 on the responses of vascular smooth muscle cells (VSMC) to oxidized low-density lipoprotein (ox-LDL). The expression of fibulin-5 was studied in human aortic-VSMCs (HA-VSMCs) treated with ox-LDL. Fibulin-5 was first overexpressed by the transfection of Ov-Fibulin-5 plasmids in HA-VSMCs challenged with ox-LDL to investigate its influence on cell proliferation, migration and invasion using Cell Counting Kit-8, wound healing and Transwell assays. Yin Yang-1 (YY1) was bioinformatically predicted to bind to the promoter sites of fibulin-5, which was subsequently confirmed by dual-luciferase reporter gene assay. Fibulin-5 overexpression was able to suppress cell proliferation, invasion and migration, which was effectively reversed by YY1 silencing by the transfection of siRNA-Fibulin-5 plasmids which could induced fibulin-5 silencing. YY1 binding sites in the promoter region of fibulin-5 were identified and confirmed *in vitro* by chromatin immunoprecipitation assay and dual-luciferase reporter gene assay. The present results suggested that as a modulator of fibulin-5, YY1 alleviated ox-LDL-induced proliferation, invasion, migration and phenotypic transition from differentiated contractile phenotype to dedifferentiated phenotype in VSMCs. However, the mechanism underlying the YY1-mediated regulation of fibulin-5 expression needs to be confirmed further *in vivo*. Nevertheless, targeting fibulin-5 and YY1 could be further developed for AS therapy.

Introduction

Atherosclerosis (AS) is the common pathological cause of cardiovascular and cerebrovascular diseases (1). Activation

Correspondence to: Dr Yuanbo Zang, Department of Clinical Laboratory, Laixi Municipal Hospital, 8 Weihai West Road, Laixi, Shandong 266600, P.R. China
E-mail: yuanbo09@126.com

and injury of vascular endothelial cells, sub-endothelial deposition of oxidized low-density lipoprotein (ox-LDL), formation of foam cells under the vascular intima and proliferation and migration of vascular smooth muscle cells (VSMCs) are the main events in the pathogenesis of AS (2). Aberrant proliferation and migration of VSMCs are common pathological features of AS, restenosis after stenting and hypertension (3,4).

Fibulin-5 is a glycoprotein that consists of 448 amino acids and is involved in regulating extracellular matrix (5). Recent data showed that fibulin-5 expression was decreased in atherosclerotic plaque tissues in human aneurysmatic aortas compared with healthy vessels (6). Another previous study suggested that fibulin-5 overexpression reduces MMP levels by inhibiting the Wnt/β-catenin signaling pathway (7). Yin Yang-1 (YY1) is a widely expressed transcription factor belonging to the Gli-Kruppel class of zinc finger proteins that can bind transcriptional initiation repeats to alter the activities of target promoters (8,9). Its overexpression can cause the abnormal transcription of downstream genes, participating in disease occurrence, such as diabetes and B lymphoma (8,10,11). YY1 was reported to serve an important role in AS by mediating the proliferation and migration of VSMCs (12). In addition, a previous study indicated that YY1 was involved in the formation of foam cells in response to oxLDL (13). The present study was therefore designed to investigate the potential interaction between YY1 and the promoter region of fibulin-5, and the role of fibulin-5 in ox-LDL-treated VSMCs.

Materials and methods

Collection of clinical samples. In the present study, five patients with coronary heart disease (CHD; male, n=2; female, n=3), with an average age of 65.1±2.4 years, were diagnosed by coronary angiography in The Laixi Municipal Hospital (Laixi, China) between June 2019 and August 2020. The control group was also composed of five individuals (male, n=2; female, n=3) with an average age of 62.3±3.1 years, who were present in the Laixi Municipal Hospital for routine physical examination. The inclusion criterion for coronary heart disease cases was the diameter stenosis of at least one major coronary artery of patients with stable angina (>80%). The exclusion criteria were as follows: i) Unstable angina pectoris or myocardial infarction; ii) comorbidity with other organic heart diseases, such as rheumatic heart disease and myocardiopathy; iii) comorbidity with severe liver and kidney disease; iv) comorbidity with

familial hypercholesterolemia; v) comorbidity with malignancies; and vi) comorbidity with inflammatory diseases. A total of 3 ml venous blood was collected from each participant. Oral informed consent was obtained from all participants prior to enrollment in the present study. The present study was approved by the Ethics Committee of Laixi Municipal Hospital (approval no. ky-2020-041-03).

Human aortic (HA)-VSMC culture. HA-VSMCs were purchased from the American Type Culture Collection (cat. no. CRL-1999) and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. Different concentrations of ox-LDL (Yeasen Biotechnology (Shanghai) Co., Ltd., Shanghai, China) dissolved into DMEM medium (0, 50, 100 and 150 μg/ml) were added to induce cell calcification at 37˚C for 48 or 72 h.

Reverse transcription-quantitative PCR (RT-qPCR) assay. Total RNA from VSMCs was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the instructions of the supplier. A total of 1 μg mRNA per sample was reverse transcribed into cDNA using PrimeScript™ RT Reagent kit (Takara Bio, Inc.) at 37˚C for 15 min and 85˚C for 5 sec. Subsequently, SYBR Green kit (cat. no. 208054; Qiagen GmbH) was used to prepare a 20 μl qPCR reaction mix and qPCR was performed on the StepOne Real Time PCR instrument (Thermo Fisher Scientific, Inc.). The primer sequences were: Fibulin-5 Forward: 5'-CTCACTGTTACC ATTCTGGCTC-3', Reverse: 5'-GACTGGCGATCCAGG TCAAAG-3'. YY1 Forward: 5'-ACGGCTTCGAGGATCAGA TTC-3', Reverse: 5'-TGACCAGCGTTTGTTCAATGT-3'. Smooth muscle α-actin (α-SMA) Forward: 5'-AAAAGACAG CTACGTGGGTGA-3', Reverse: 5'-GCCATGTTCTATCGG GTACTTC-3'. GAPDH: 5'-GGAGCGAGATCCCTCCAA AAT-3', Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'. PCR was performed at 95˚C for 30 sec, followed by 45 cycles of 95˚C for 5 sec and 60˚C for 30 sec. GAPDH was used as an internal reference. The relative levels of mRNA were calculated with the $2^{-\Delta\Delta Cq}$ method (14).

Cell Counting Kit-8 (CCK-8) assay. VSMCs (1×10^4 cells/well) were seeded into 96-well plates and then incubated for 24 h at 37˚C. Following ox-LDL treatment or transfection of Ov-Fibulin-5 plasmids and ox-LDL treatment, the medium was replaced with serum-free DMEM medium. A total of 10 μl CCK-8 solution (Beyotime Institute of Biotechnology) was added to each well for incubation at 37˚C for 1 h. The absorbance at 450 nm was subsequently detected using a microplate reader (Thermo Fisher Scientific, Inc.).

Western blotting. Following the x-LDL treatment or transfection of Ov-Fibulin-5 plasmids plus ox-LDL treatment, or the co-treatment of transfection of Ov-YY1 and siRNA-Fibulin-5 plus ox-LDL treatment, VSMCs were collected and lysed by adding RIPA buffer (Beyotime Institute of Biotechnology) on ice for 30 min. The supernatant was collected after centrifugation at 4˚C at 14,000 x g for 20 min. The protein concentration of each group was detected by a BCA kit (Abcam). A total of 20 μg each protein sample was separated by 10% SDS-PAGE

and then transferred into PVDF membrane. The membranes were then blocked with 5% BSA (MilliporeSigma) at room temperature for 2 h and incubated with primary antibodies (Fibulin-5; cat. no. ab109428, 1:1,000; cyclin D1; cat. no. ab16663, 1:200; CDK2; cat. no. ab32147, 1:1,000; MMP2; cat. no. ab92536, 1:1,000; MMP9; cat. no. ab76003, 1:1,000; GAPDH; cat. no. ab8245, 1:5,000. Abcam) at 4˚C overnight. After incubation with a secondary antibody at 37˚C for 2 h (Goat Anti-Rabbit IgG; cat. no. ab7090, 1:10,000. Abcam, England), a ECL color development reagent (cat. no. ab133409; Abcam) was added to the membranes. The density of the protein bands was analyzed using the ImageJ software (version 1.53b; National Institutes of Health).

Wound healing assay. HA-VSMCs in a logarithmic proliferation phase were seeded into six-well plates at 2×10^4 cells per well. After routine overnight culture, the cells received transfection of Ov-Fibulin-5 plasmids and ox-LDL treatment, or the transfection of Ov-YY1 and siRNA- Fibulin-5 and ox-LDL treatment, with three replicates per group. When 80% cell confluence was reached, a scratch was performed using a horizontal line perpendicular to the back with a 20-μl pipette tip. The cells were washed by phosphoric acid buffer three times and cultured in serum-free DMEM medium. PBS was used to rinse the plates and serum-free DMEM medium was added at 37˚C. The cell migration was observed under a light microscope at 0 and 24 h (Olympus Corporation; magnification, x100). The relative migration was calculated using following formula: (Initial width at 0 h-final width at 24 h)/width at 0 h.

Transwell assay. The HA-VSMCs received transfection with Ov-Fibulin-5 plasmids and ox-LDL treatment, or transfection of Ov-YY1 and siRNA-Fibulin-5 and ox-LDL treatment and their concentration was adjusted to 2×10^8 cells/ml. Diluted Matrigel (25 μl; BD Biosciences) with DMEM medium without serum was added to upper chamber at 37˚C. A total of 200 μl cell suspension prepared with serum-free medium was added to the upper chambers of a Transwell insert (cat. no. 3422; 8-μm; Corning). DMEM with 10% FBS was added to the lower chamber. After 24 h of incubation at 37˚C, the cells that have gone through the membrane were fixed using 4% methanol and stained with crystal violet at room temperature for 10 min and images were captured under an inverted microscope (Olympus Corporation, magnification, x100).

Plasmid transfection. Lipofectamine® 3000 transfection reagent (Thermo Fisher Scientific, Inc.) was used according to the manufacturer's instructions to transfect YY1-overexpressing plasmids (0.5 μg; pcDNA-YY1; Ov-YY1; Shanghai GenePharma Co., Ltd.) or its control (0.5 μg; pcDNA), or siRNA targeting fibulin-5 (15 pmol; 5'-GAUUGU UGUGAGGAGUCUAGC-3') or its negative control (15 pmol; siRNA-NC, scrambled RNA, 5'-GUGAUUGCAUGAUUG UGCGGA-3') into HA-VSMCs, which were constructed and purchased from Shanghai GenePharma Co., Ltd. After 24 h at 37˚C, the cells were treated with 100 μg/ml ox-LDL (100 μg/ml ox-LDL significantly promoted cell proliferation) for 24 h at 37˚C for further experiments.

Table I. Clinical characteristics of patients with CHD.

Characteristics	CHD (n=5)	Control (n=5)	P-value
Age, years	65.1±2.4	62.3±3.1	>0.05
Smoking, %	20	20	>0.05
Drinking, %	20	20	>0.05
Hypertension, %	60	60	>0.05
Cholesterol, mmol/l	3.98±0.66	4.01±0.42	>0.05
High-density lipoprotein, mmol/l	0.94±0.12	1.35±0.29	<0.01
Low density lipoprotein, mmol/l	2.12±0.63	2.15±0.64	>0.05

CHD, coronary heart disease.
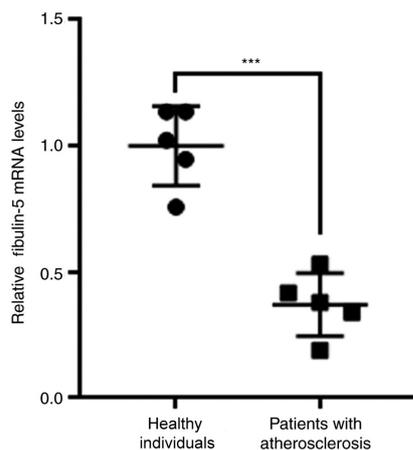
Figure 1. Fibulin-5 mRNA expression levels in blood of healthy individuals and patients with atherosclerosis. Data are presented as the mean ± SD. n=5 per group. ***$P<0.001$.

Figure 2. Ox-LDL treatment decreases the expression of fibulin-5 in HA-VSMC cells. (A) HA-VSMC viability was analyzed by Cell Counting Kit-8 assay after cells were treated with 0, 50, 100 or 150 μg/ml of ox-LDL for 48 or 72 h. (B and C) HA-VSMCs were treated with 100 μg/ml ox-LDL for 48 h. *$P<0.05$, ***$P<0.001$ vs. Ox-LDL 0 μg/ml (B) Endogenous mRNA and (C) protein levels of fibulin-5 were measured by reverse transcription-quantitative PCR and western blotting, respectively. Data are presented as the mean ± SD. ***$P<0.001$. HA-VSMC, human aortic-vascular smooth muscle cells; ox-LDL, oxidized low-density lipoprotein.

Bioinformatics. Using PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3, access date: 10.09.2020) and Transcription Factor DataBase (TFDB; http://bioinfo.life.hust.edu.cn/HumanTFDB#!/, access date: 10.09.2020) tools, YY1 was predicted to bind to the promoter region of fibulin-5.

Chromatin immunoprecipitation (ChIP). After chromatin was subjected to ultrasonication, magnetic beads (60 μl) coated with anti-YY1 or anti-IgG (EMD Millipore) were added to form immune complex. The immune complex was washed with wash buffer (low salt wash buffer, high salt wash buffer, LiCl wash buffer and TE buffer, in sequence) and then was centrifuged at 4°C at 16,000 x g for 2 min. Next, collected immunoprecipitation was eluted with eluent including 10% SDS, NaHCO3 and ddH2O at 65°C for 15 min. 20 μl NaCl was added for incubation at 65°C overnight. After de-crosslink, 1 μl RNaseA was added for incubation for 1 h at 37°C. RT-qPCR was performed to analyze precipitated DNA fragments.

Dual-luciferase reporter gene assay. Luciferase activity was detected after co-transfection of fibulin-5 promoter site 1 (TCCCAGCCCCCGCACC) with mutations or wild-type sequence luciferase reporter genes (0.1 μg; pGL3-basic;

Promega Corporation) and YY1 overexpressing plasmids (0.2 μg)or its control plasmids (empty plasmids) into 293T cells (ATCC, USA) with Lipofectamine® 3000 following the manufacturer's protocol. *Renilla* luciferase was used as control reporter gene. After 48 h, luciferase activity was detected using Dual luciferase reporter gene assay kit according to the manufacturer's instructions (cat. no. RG028; Beyotime Institute of Biotechnology).

Statistical analysis. All data were statistically analyzed by GraphPad Prism 8 software (GraphPad Software, Inc.). The
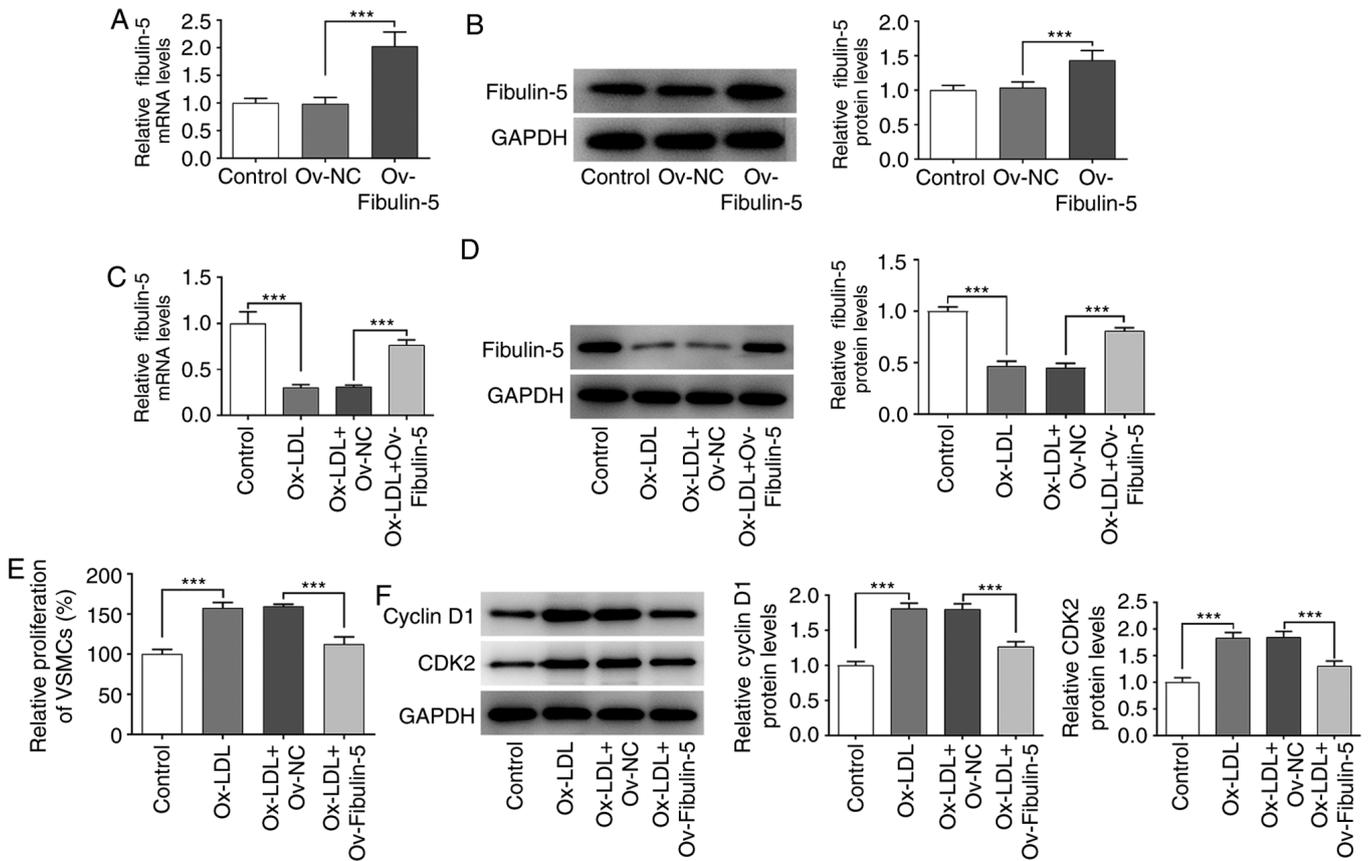
Figure 3. Fibulin-5 overexpression reduces the impacts of ox-LDL on promoting proliferation and the expression of cell cycle proteins, Cyclin D1 and CDK2. (A) RT-qPCR and (B) western blot analyzes were used for assessing the efficiency of fibulin-5 overexpression in HA-VSMCs. Levels of fibulin-5 (C) mRNA and (D) protein detected expression were measured in HA-VSMCs following ox-LDL and/or fibulin-5 overexpression treatment. (E) HA-VSMC viability was measured after ox-LDL treatment and/or fibulin-5 overexpression. (F) Cyclin D1 and CDK2 protein expression was assessed by western blotting in HA-VSMCs following ox-LDL and/or fibulin-5 overexpression treatment. Data are presented as the mean ± SD. ***P<0.001. RT-qPCR, reverse transcription-quantitative PCR; HA-VSMC, human aortic-vascular smooth muscle cells; ox-LDL, oxidized low-density lipoprotein; Ov, Overexpression; NC, negative control.

measurement data were represented by the mean ± SD and one-way ANOVA was used for comparison between multiple groups, followed by Tukey's test. Chi-square test was used to compare the two rates of age, smoking, drinking, or hypertension between CHD and control. Each experiment was repeated at least three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Ox-LDL induces low fibulin-5 expression in HA-VSMCs. The level of high-density lipoprotein in the peripheral blood samples of patients with CHD was significantly lower than that in the control group (Table I). However, there was no statistical significance in the comparison of all other indicators between the two groups (Table I).

To explore the role of fibulin-5 in AS, fibulin-5 mRNA expression was quantified in the peripheral blood of patients with AS. Fibulin-5 mRNA expression was significantly increased in patients with AS compared with that in healthy individuals (P<0.001; Fig. 1). VSMCs have been previously reported to serve an important role in atherogenesis (15). As indicated in Fig. 2A, 100 μg/ml ox-LDL was the concentration that increased VSMC viability the strongest (P<0.001). This concentration was therefore selected for further experiments.

RT-qPCR and western blotting analyses of fibulin-5 expression in VSMCs indicated that the mRNA and protein levels of fibulin-5 were significantly reduced after ox-LDL treatment compared with those in the control group (P<0.001; Fig. 2B and C). To further investigate the role of fibulin-5, HA-VSMCs treated with ox-LDL were transfected with a fibulin-5-overexpressing plasmid.

Fibulin-5 overexpression suppresses HA-VSMC proliferation in response to ox-LDL. Since ox-LDL decreased fibulin-5 expression, it was hypothesized that fibulin-5 serve a role in HA-VSMCs in response to ox-LDL. As a transfection control, a vector overexpressing (Ov)-fibulin-5 significantly increased the mRNA and protein expression levels of fibulin-5 compared with that in the Ov-negative control (NC) group (P<0.001; Fig. 3A and B). Analyzes of fibulin-5 expression in ox-LDL-treated HA-VSMCs demonstrated that the reduced fibulin-5 expression was significantly reversed by fibulin-5 overexpression (P<0.001; Fig. 3C and D). In VSMCs cycle progression, cyclin D1 and CDK2 are involved in promoting Rb phosphorylation, the inhibition of which can lead to cycle arrest (16). VSMCs were subsequently subjected to CCK-8 analysis to measure cell viability (P<0.001; Fig. 3E) whereas western blotting was used to quantify protein levels of cell cycle proteins
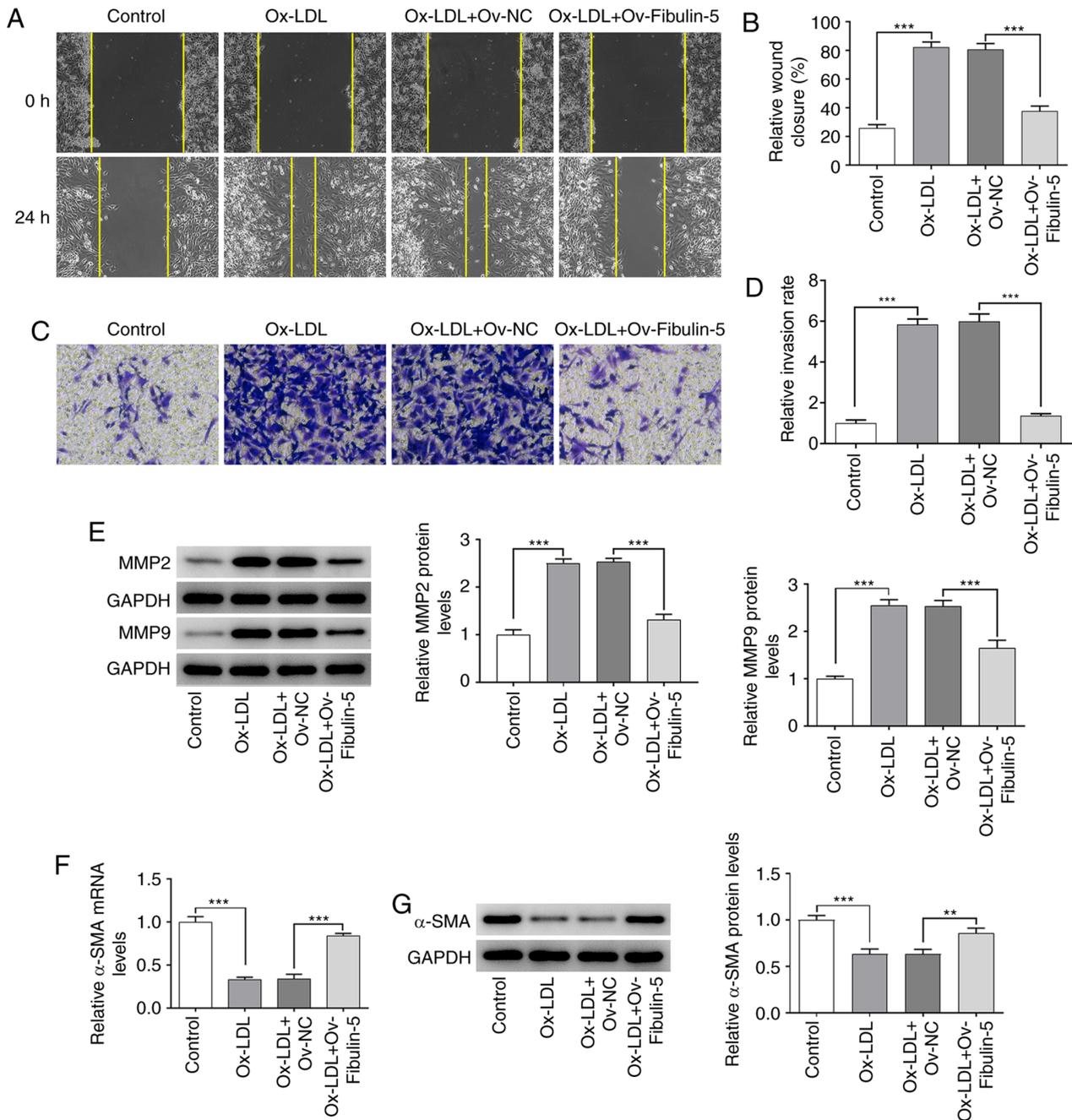
Figure 4. Fibulin-5 overexpression decreases migration and invasion in ox-LDL-induced HA-VSMCs. HA-VSMCs following ox-LDL treatment and/or fibulin-5 overexpression were subjected to (A) wound healing assays to measure (B) cell migration. (C) Transwell assays were performed to compare (D) invasion. Magnification, x100. (E) HA-VSMCs following ox-LDL treatment and/or fibulin-5 overexpression were subjected to western blot analyses to determine the expression levels of MMP2, MMP9. (F) Reverse transcription-quantitative PCR and (G) western blotting were used to measure α-SMA expression following ox-LDL treatment and/or fibulin-5 overexpression in HA-VSMCs. Data are presented as the mean ± SD. **P<0.01 and ***P<0.001. HA-VSMC, human aortic-vascular smooth muscle cell; ox-LDL, oxidized low-density lipoprotein; Ov, vector overexpressing; NC, negative control; α-SMA, smooth muscle α-actin.

cyclin D1 and CDK2 (P<0.001; Fig. 3F). The present results demonstrated that the overexpression of fibulin-5 in HA-VSMCs reversed the increase in cell viability induced by ox-LDL treatment, in addition to reversing the increased protein expression of cyclin D1 and CDK2 induced by ox-LDL treatment (P<0.001; Fig. 3E and F).

Cell migration and invasion were studied by performing wound healing and Transwell assays, respectively. The present results demonstrated that HA-VSMCs exhibited significantly

increased migration and invasion after ox-LDL treatment, which were significantly reversed following fibulin-5 overexpression (all P<0.001; Fig. 4A-D). MMP-2 and MMP-9 expressed in VSMCs, modulated the collagen degradation of the extracellular matrix and served a vital role in AS progression (17).

Additionally, the protein expression of MMP2 and MMP9, which participate in phenotype transition (18,19), was quantified by western blotting, whilst the mRNA and protein
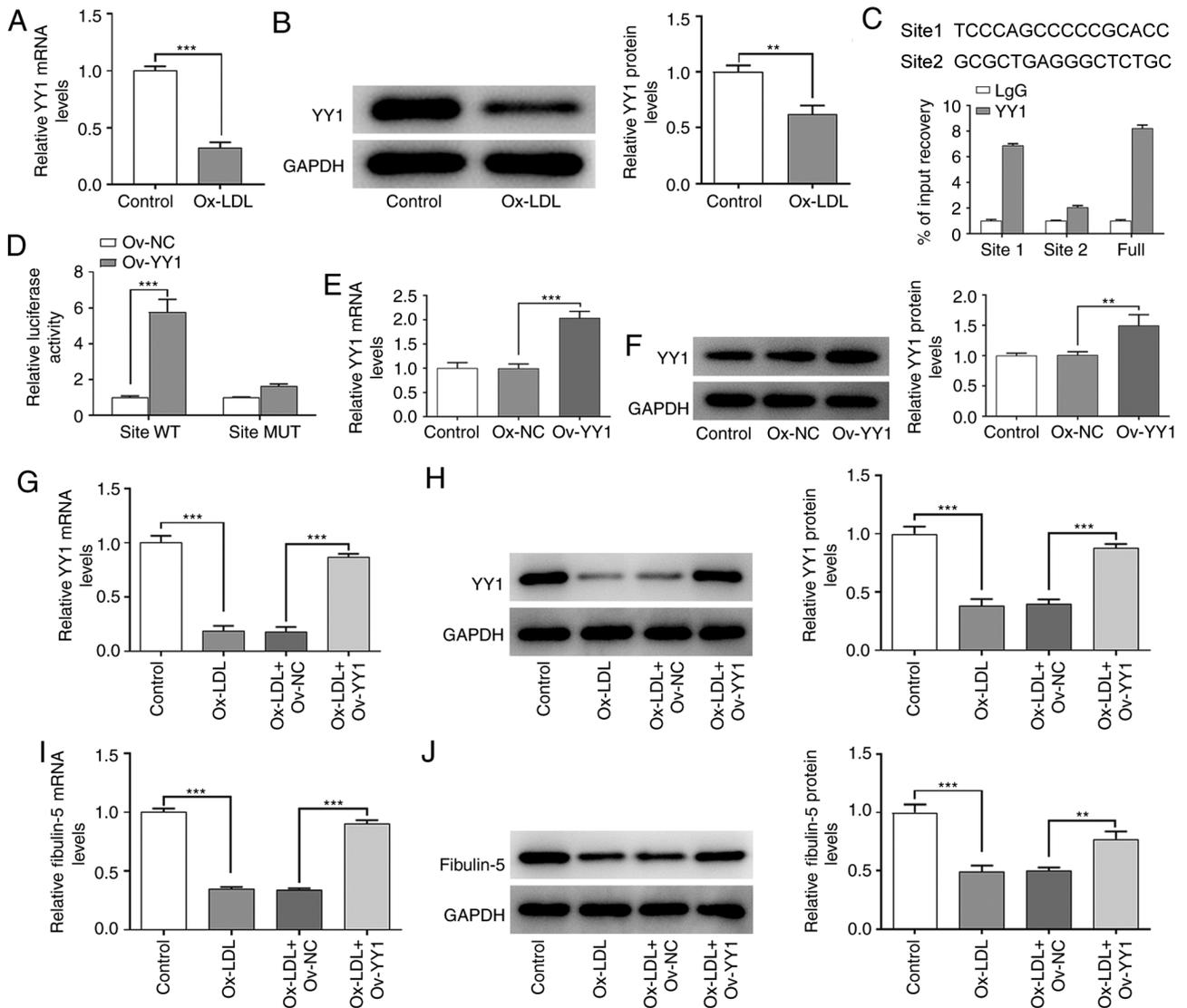
Figure 5. YY1 overexpression upregulates the expression of fibulin-5. YY1 (A) mRNA and (B) protein expression was assessed in control and ox-LDL-treated HA-VSMCs by RT-qPCR and western blotting assays, respectively. (C) Following ChIP assay, the immune complex pulled down by YY1 antibody or IgG (negative control) was used to analyze the PCR amplification level of the two promoter sites and the total promoter sites (site 1 and site 2) using RT-qPCR. (D) Luciferase activity as detected by dual-luciferase reporter gene assays. Ov-YY1 transfection induced YY1 overexpression on both (E) mRNA and (F) protein levels. RT-qPCR and western blot analyzes were performed in HA-VSMCs treated with ox-LDL and/or overexpressing YY1 to determine the levels of YY1 (G) mRNA and (H) protein expression. RT-qPCR and western blot analyses were performed in HA-VSMCs treated with ox-LDL and/or overexpressing YY1 to determine the levels of fibulin-5 (I) mRNA and (J) protein expression. Data are presented as the mean ± SD. **P<0.01 and ***P<0.001. HA-VSMC, human aortic-vascular smooth muscle cell; ox-LDL, oxidized low-density lipoprotein; RT-qPCR, reverse transcription-quantitative PCR; YY1, Yin Yang-1; Ov, vector overexpressing; NC, negative control.

expression of α-SMA was measured by RT-qPCR and western blotting, respectively. MMP2 and MMP9 protein expression was significantly increased after ox-LDL treatment, an effect that was significantly reversed by fibulin-5 overexpression (all P<0.001; Fig. 4E). Similarly, α-SMA mRNA and protein expression were significantly decreased after ox-LDL treatment (P<0.001), which was significantly reversed by fibulin-5 overexpression (P<0.01 and P<0.001; Fig. 4E-G).

YY1 binds to the promoter of fibulin-5 to induce its upregulation in HA-VSMCs. To further explore the role of fibulin-5 in AS, PROMO and TFDB databases were used to predict whether YY1 can bind to the promoter region of fibulin-5 to regulate fibulin-5 expression in ox-LDL-treated HA-VSMCs.

The mRNA and protein expression levels of YY1 were significantly decreased in ox-LDL-treated HA-VSMCs compared with those in the control group (P<0.01 and P<0.001; Fig. 5A and B). Next, the two promoter sites and the overall promoter region level of fibulin-5, were detected by qPCR in an immunoprecipitation complex formed by using YY1 antibody. Promoter site 1 was shown to be markedly enriched (Fig. 5C). The luciferase activity of the pGL4 luciferase reporter vectors containing the promoter region of fibulin-5 with binding site (site 1) for YY1 was significantly increased when YY1-overexpressing plasmids were co-transfected into HA-VSMCs, compared with that in cells co-transfected with the Ov-NC plasmid (P<0.001, Fig. 5D). When the pGL4 luciferase reporter vectors with muted sites and Ov-NC or Ov-YY1
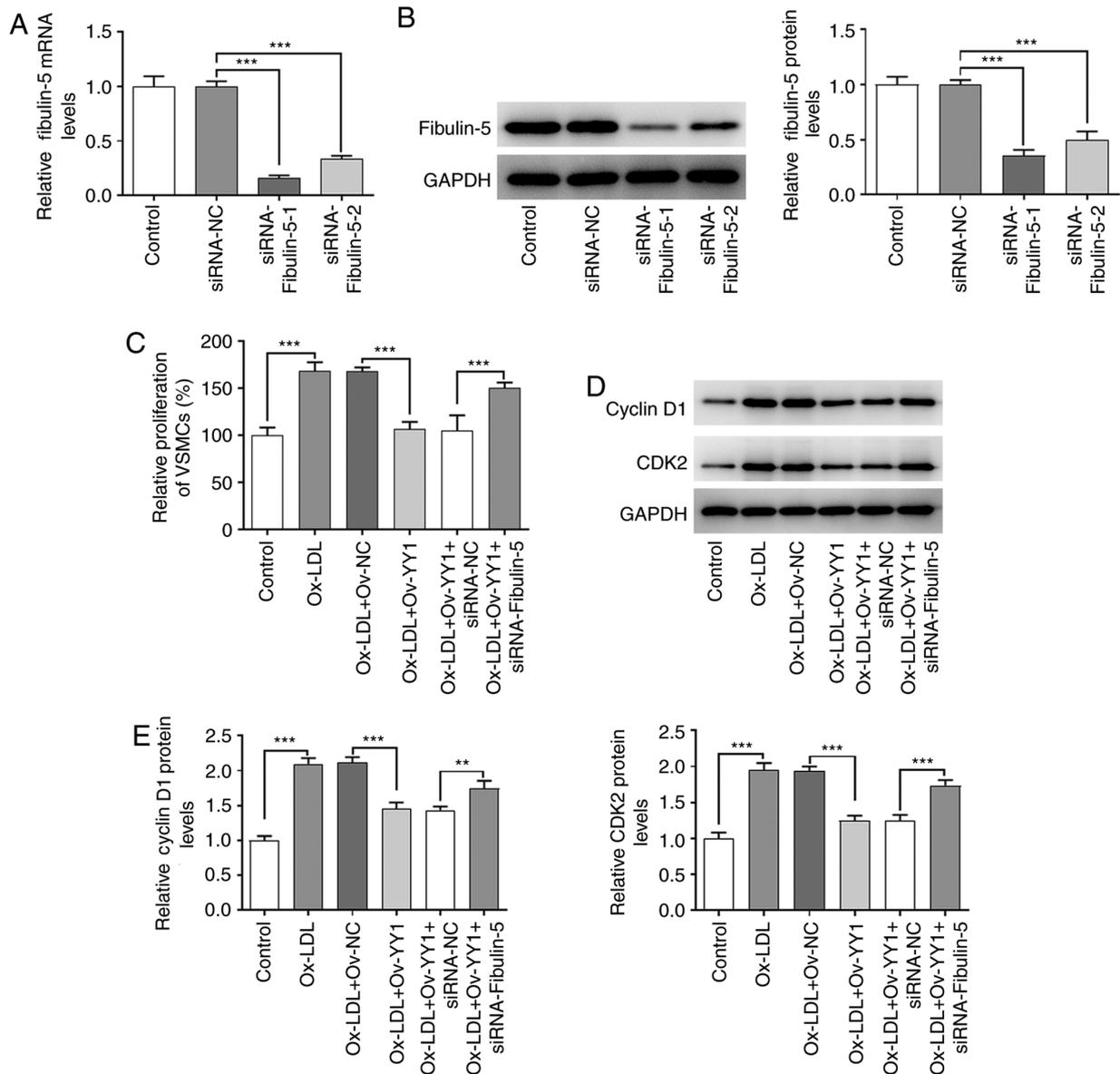
Figure 6. The effects of YY1 overexpression on proliferation and expression of cell cycle proteins are reversed by fibulin-5 silencing. (A and B) HA-VSMCs were transfected with either siRNA-NC or siRNA-fibulin-5. Reverse transcription-quantitative PCR and western blot analyzes were performed to determine the levels of fibulin-5 (A) mRNA and (B) protein expression. (C) Cell Counting Kit-8 assay was performed to measure cell viability in HA-VSMCs after ox-LDL treatment, YY1 overexpression and/or fibulin-5 knockdown. (D) Western blot analysis was performed after ox-LDL treatment, YY1 overexpression and/or fibulin-5 knockdown to detect the levels of (E) cyclin D1 and CDK2 expression. Data are presented as the mean ± SD. **P<0.01 and ***P<0.001. Ov, vector overexpressing; siRNA, small interfering RNA; NC, negative control; HA-VSMC, human aortic-vascular smooth muscle cells; ox-LDL, oxidized low-density lipoprotein; YY1, Yin Yang-1.

were co-transfected into 293T cells, there was no significant difference in luciferase activities between Ov-NC and Ov-YY1 (Fig. 5D). These results suggest that YY1 can bind to site 1 of the fibulin-5 promoter. The transfection efficiency of the YY1-overexpressing plasmid was tested in Fig. 5E and F. As shown in Fig. 5E and F, cells transfected with Ov-YY1 showed significantly higher YY1 expression levels compared with those in cells transfected with Ov-NC (P<0.01 and P<0.001). As indicated in Fig. 5G and H, overexpression of YY1 in the HA-VSMCs significantly reversed the decreased mRNA and protein expression of YY1 induced by ox-LDL treatment (P<0.001). Similarly, YY1 overexpression also significantly restored the expression of fibulin-5, which was decreased after ox-LDL treatment (P<0.01 and P<0.001; Fig. 5I and J).

YY1 mediates the effects of fibulin-5 on proliferation, migration and invasion in HA-VSMCs stimulated with ox-LDL. The present study next aimed to determine whether fibulin-5 mediated the effects of YY1 in cultured HA-VSMCs stimulated with ox-LDL. First, the transfection efficiency of small interfering (si)RNA-fibulin-5 was tested in the HA-VSMCs. Analyzes of fibulin-5 expression in HA-VSMCs demonstrated that it was effectively knocked down by siRNA-Fibulin-5 transfection compared with cell transfected with siRNA-NC (P<0.001; Fig. 6A and B). Since it was the most efficient in knocking down fibuin-5 expression, si-fibulin-1 was selected for further experiments. Based on the present results, it was hypothesized that YY1 interacted with the promoter sites of fibulin-5 to regulate cell proliferation, migration and invasion
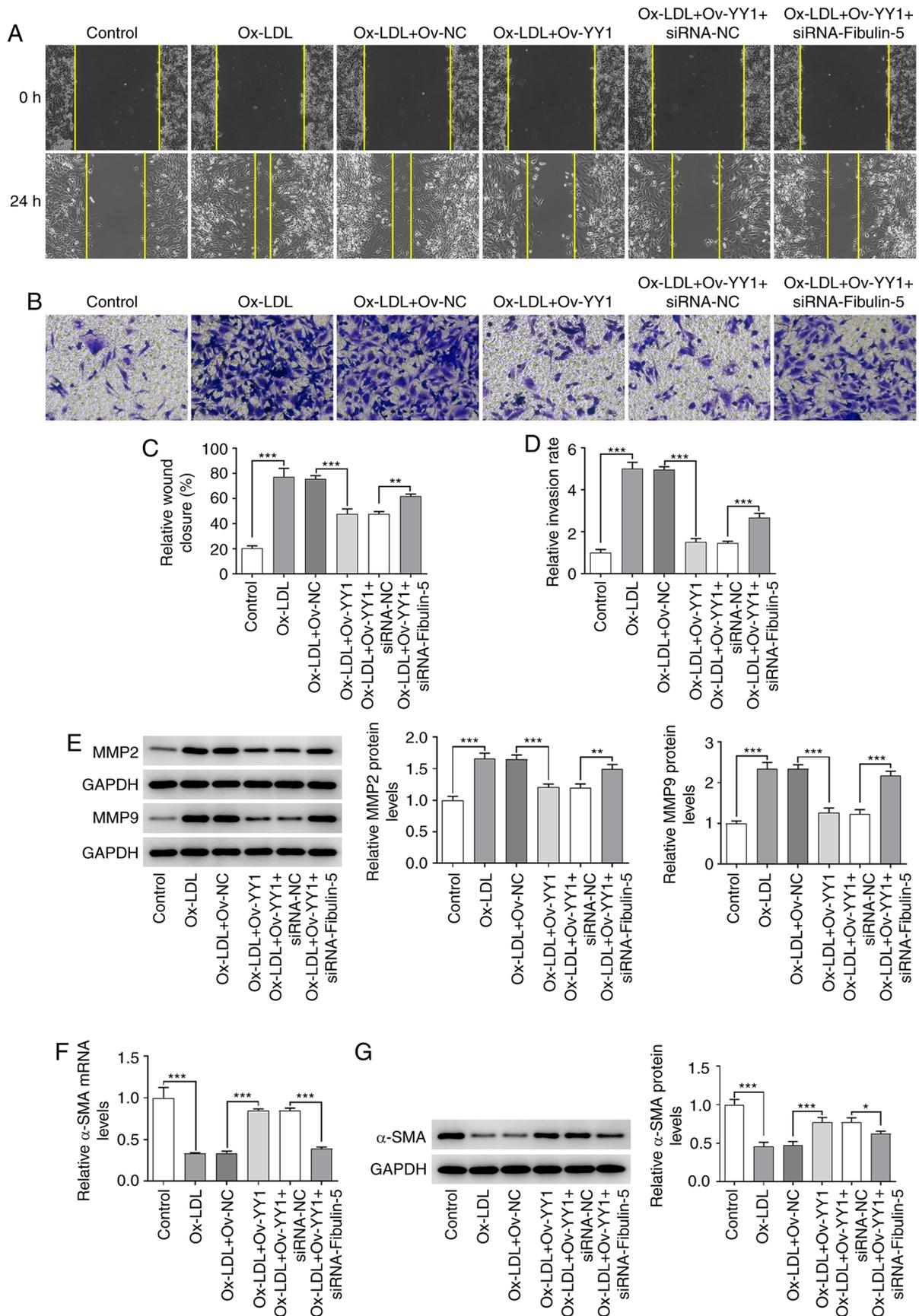
Figure 7. The effects of YY1 overexpression migration and invasion were reversed by fibulin-5 silencing. HA-VSMCs after ox-LDL treatment, YY1 over-expression and/or fibulin-5 knockdown were subjected to (A) wound healing and (B) Transwell assays to compare (C) cell migration and (D) invasion. Magnification, x100. (E) Western blot analyses were performed after ox-LDL treatment, YY1 overexpression and/or fibulin-5 knockdown to measure the protein expression of MMP2 and MMP9. (F) Reverse transcription-quantitative PCR and (G) western blotting were performed to determine α-SMA expression levels. Data are presented as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001. HA-VSMC, human aortic-vascular smooth muscle cell; ox-LDL, oxidized low-density lipoprotein; YY1, Yin Yang-1; siRNA, small interfering RNA; NC, negative control; α-SMA, smooth muscle α-actin.

in VSMCs treated with ox-LDL. First, cell viability was compared between VSMCs transfected with siRNA-NC and with siRNA-fibulin-5. Fibulin-5 silencing induced a significant reversal of the potentiating effects due to YY1 overexpression on cell viability, cyclin D1 and CDK2 expression (P<0.01 and P<0.001; Fig. 6C-E). Significant differences were also observed in the cell migration and invasion of HA-VSMCs, and the expression of MMP2, MMP9 and α-SMA between Ov-YY1 vs. Ov-NC and between Ov-YY1+siRNA-NC and with Ov-YY1+siRNA-fibulin-5 (P<0.05, P<0.01 and P<0.001; Fig. 7A-G). This suggests that YY1 participated in mediating the effects of fibulin-5 on cell viability, migration and invasion induced by ox-LDL.

Discussion

The present study demonstrated that fibulin-5 participated in ox-LDL-induced cell viability, invasion and migration in VSMCs. A previous study on the fibulin-5 mechanism revealed that fibulin-5 was involved in arterial stiffening in patients with AS (20). The present study indicated that YY1 regulated the promoter activity and expression levels of fibulin-5 in VSMCs under ox-LDL treatment, which provided a new insight in the understanding of the role of fibulin-5 in AS.

The role of fibulin-5 in AS was found to involve the binding of extracellular superoxide dismutase to the vascular tissue to modulate vascular superoxide production, which was identified to serve a vital role in AS (21). As for the regulatory mechanism of fibulin-5 promoter activity, a previous report indicated that SOX9 could regulate the fibulin-5 promoter activity in VSMCs under the stimulus of TNF-α (22). In the present study, a YY1 binding sequence on the fibulin-5 promoter was identified and predicted, where it was verified further that YY1 could directly bind to the fibulin-5 promoter and regulate its expression. This translated downstream to the regulation of the biological processes of VSMCs in response to ox-LDL. However, the molecular mechanism by which YY1 binding activates fibulin-5 transcription was not uncovered in the present study, which was predicted to be associated with YY1 involvement in transcriptional activation by forming complexes with some proteins, such as INO80 or Sp1 (23) in the promoter of fibulin-5. YY1 has been reported to be a transcriptional activator of some genes, such as Xist in ES cells and CDC6 in HEK293 cells, (24,25). For instance, YY1 initiated the activity of the X-inactive specific transcript (Xist) promoter by directly binding to the Xist 5' region in ES cells (24). Downstream, YY1 was also revealed to be involved in the pathophysiology of AS by regulating cell proliferation and apoptosis (12,26,27). For example, a previous study reported that YY1 was involved in the promoting effects of miR-544 on the maturation and antioxidative effects of stem cell-derived endothelial-like cells (28). A previous mechanistic analysis of YY1 in ox-LDL-treated macrophages of AS demonstrated that YY1 can translocate into the nucleus and increase the expression of miR-29a by binding to its transcriptional promoter region (27).

In addition to its regulatory role in cell viability, invasion and migration, fibulin-5 overexpression markedly reduced the expression levels of cyclin D1 and CDK2, which regulate G_1-phase cell cycle progression and subsequently proliferation

in VSMCs (29,30). A previous study found that the expression levels of YY1, which is a negative regulator of cyclin D1, could be regulated by miR-29a (31). The present results suggest that cyclin D1 and CDK2 can participate in the regulation of YY1/fibulin-5 during cell proliferation. Although the present study revealed that fibulin-5/YY1 affected the viability, invasion and migration of HA-VSMCs stimulated with ox-LDL, whether this axis is involved in the pathogenesis of AS requires further study, which is a limitation of the present study.

In conclusion, the present study presented evidence that fibulin-5, regulated by YY1, can suppress the viability, invasion and migration of HA-VSMCs stimulated with ox-LD. The present results provided a new insight for the further investigation of the mechanism of AS. Targeting fibulin-5/YY1 can be a potential therapeutic option for AS. However, further studies should be performed, mainly on the molecular mechanism by which the binding of YY1 can activate fibulin-5 transcription. In addition, the mechanism of YY1/fibulin-5 would need to be validated *in vivo*.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LW and YZ made substantial contributions to the conception and design of the study. LW collected clinical samples and interpreted the patient data regarding fibulin-5 expression. LW, CL, CF and YZ performed other experiments and collected data. LW and YZ interpreted the data, drafted and revised the manuscript for important intellectual content. LW and YZ confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Laixi Municipal Hospital (approval no. ky-2020-041-03). Oral informed consent was obtained from all participants prior to enrollment in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Cao Q, Guo Z, Du S, Ling H and Song C: Circular RNAs in the pathogenesis of atherosclerosis. Life Sci 255: 117837, 2020.
2. Kattoor AJ, Goel A and Mehta JL: LOX-1: Regulation, signaling and its role in atherosclerosis. Antioxidants (Basel) 8: 218, 2019.
3. Cheng YC, Sheen JM, Hu WL and Hung YC: Polyphenols and oxidative stress in atherosclerosis-related ischemic heart disease and stroke. Oxid Med Cell Longev 2017: 8526438, 2017.
4. Lu QB, Wan MY, Wang PY, Zhang CX, Xu DY, Liao X and Sun HJ: Chicoric acid prevents PDGF-BB-induced VSMC dedifferentiation, proliferation and migration by suppressing ROS/NF-κB/mTOR/P70S6K signaling cascade. Redox Biol 14: 656-668, 2018.
5. Ito S, Yokoyama U, Nakakoji T, Cooley MA, Sasaki T, Hatano S, Kato Y, Saito J, Nicho N, Iwasaki S, *et al*: Fibulin-1 integrates subendothelial extracellular matrices and contributes to anatomical closure of the ductus arteriosus. Arterioscler Thromb Vasc Biol 40: 2212-2226, 2020.
6. Xu BF, Liu R, Huang CX, He BS, Li GY, Sun HS, Feng ZP and Bao MH: Identification of key genes in ruptured atherosclerotic plaques by weighted gene correlation network analysis. Sci Rep 10: 10847, 2020.
7. Gao JB, Lin L, Men XQ, Zhao JB, Zhang MH, Jin LP, Gao SJ, Zhao SQ and Dong JT: Fibulin-5 protects the extracellular matrix of chondrocytes by inhibiting the Wnt/β-catenin signaling pathway and relieves osteoarthritis. Eur Rev Med Pharmacol Sci 24: 5249-5258, 2020.
8. Meliala ITS, Hosea R, Kasim V and Wu S: The biological implications of Yin Yang 1 in the hallmarks of cancer. Theranostics 10: 4183-4200, 2020.
9. Liu H, Schmidt-Supprian M, Shi Y, Hobeika E, Barteneva N, Jumaa H, Pelanda R, Reth M, Skok J, Rajewsky K and Shi Y: Yin Yang 1 is a critical regulator of B-cell development. Genes Dev 21: 1179-1189, 2007.
10. Blättler SM, Cunningham JT, Verdeguer F, Chim H, Haas W, Liu H, Romanino K, Rüegg MA, Gygi SP, Shi Y and Puigserver P: Yin Yang 1 deficiency in skeletal muscle protects against rapamycin-induced diabetic-like symptoms through activation of insulin/IGF signaling. Cell Metab 15: 505-517, 2012.
11. Sui G, Affar el B, Shi Y, Brignone C, Wall NR, Yin P, Donohoe M, Luke MP, Calvo D, Grossman SR and Shi Y: Yin Yang 1 is a negative regulator of p53. Cell 117: 859-872, 2004.
12. Yue Y, Lv W, Zhang L and Kang W: miR-147b influences vascular smooth muscle cell proliferation and migration via targeting YY1 and modulating Wnt/β-catenin activities. Acta Biochim Biophys Sin (Shanghai) 50: 905-913, 2018.
13. Tian FJ, An LN, Wang GK, Zhu JQ, Li Q, Zhang YY, Zeng A, Zou J, Zhu RF, Han XS, *et al*: Elevated microRNA-155 promotes foam cell formation by targeting HBP1 in atherogenesis. Cardiovasc Res 103: 100-110, 2014.
14. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
15. Grootaert MOJ, Moulis M, Roth L, Martinet W, Vindis C, Bennett MR and De Meyer GRY: Vascular smooth muscle cell death, autophagy and senescence in atherosclerosis. Cardiovasc Res 114: 622-634, 2018.
16. Kavurma MM and Khachigian LM: Sp1 inhibits proliferation and induces apoptosis in vascular smooth muscle cells by repressing p21WAF1/Cip1 transcription and cyclin D1-Cdk4-p21WAF1/Cip1 complex formation. J Biol Chem 278: 32537-32543, 2003.
17. Nasiri-Ansari N, Dimitriadis GK, Agrogiannis G, Perrea D, Kostakis ID, Kaltsas G, Papavassiliou AG, Randeva HS and Kassi E: Canagliflozin attenuates the progression of atherosclerosis and inflammation process in APOE knockout mice. Cardiovasc Diabetol 17: 106, 2018.
18. Terzuoli E, Nannelli G, Giachetti A, Morbidelli L, Ziche M and Donnini S: Targeting endothelial-to-mesenchymal transition: The protective role of hydroxytyrosol sulfate metabolite. Eur J Nutr 59: 517-527, 2020.
19. Viedma-Rodríguez R, Martínez-Hernández MG, Martínez-Torres DI and Baiza-Gutman LA: Epithelial mesenchymal transition and progression of breast cancer promoted by diabetes mellitus in mice are associated with increased expression of glycolytic and proteolytic enzymes. Horm Cancer 11: 170-181, 2020.
20. Paapstel K, Zilmer M, Eha J, Tootsi K, Piir A and Kals J: Association between Fibulin-1 and aortic augmentation index in male patients with peripheral arterial disease. Eur J Vasc Endovasc Surg 51: 76-82, 2016.
21. Nguyen AD, Itoh S, Jeney V, Yanagisawa H, Fujimoto M, Ushio-Fukai M and Fukai T: Fibulin-5 is a novel binding protein for extracellular superoxide dismutase. Circ Res 95: 1067-1074, 2004.
22. Orriols M, Varona S, Martí-Pàmies I, Galán M, Guadall A, Escudero JR, Martín-Ventura JL, Camacho M, Vila L, Martínez-González J and Rodríguez C: Down-regulation of Fibulin-5 is associated with aortic dilation: role of inflammation and epigenetics. Cardiovasc Res 110: 431-442, 2016.
23. Sarvagalla S, Kolapalli SP and Vallabhapurapu S: The two sides of YY1 in cancer: A friend and a foe. Front Oncol 9: 1230, 2019.
24. Makhlouf M, Ouimette JF, Oldfield A, Navarro P, Neuillet D and Rougeulle C: A prominent and conserved role for YY1 in Xist transcriptional activation. Nat Commun 5: 4878, 2014.
25. Cai Y, Jin J, Yao T, Gottschalk AJ, Swanson SK, Wu S, Shi Y, Washburn MP, Florens L, Conaway RC and Conaway JW: YY1 functions with INO80 to activate transcription. Nat Struct Mol Biol 14: 872-874, 2007.
26. Wei SY, Shih YT, Wu HY, Wang WL, Lee PL, Lee CI, Lin CY, Chen YJ, Chien S and Chiu JJ: Endothelial Yin Yang 1 phosphorylation at S118 induces atherosclerosis under flow. Circ Res 129: 1158-1174, 2021.
27. Jian D, Dai B, Hu X, Yao Q, Zheng C and Zhu J: ox-LDL increases microRNA-29a transcription through upregulating YY1 and STAT1 in macrophages. Cell Biol Int 41: 1001-1011, 2017.
28. Guo J, Xiang Q, Xin Y, Huang Y, Zou G and Liu T: miR-544 promotes maturity and antioxidation of stem cell-derived endothelial like cells by regulating the YY1/TET2 signalling axis. Cell Commun Signal 18: 35, 2020.
29. Qin L, Yang YB, Yang YX, Gong YZ, Li XL, Li GY, Luo HD, Xie XJ, Zheng XL and Liao DF: Inhibition of smooth muscle cell proliferation by ezetimibe via the cyclin D1-MAPK pathway. J Pharmacol Sci 125: 283-291, 2014.
30. Vadiveloo PK, Filonzi EL, Stanton HR and Hamilton JA: G1 phase arrest of human smooth muscle cells by heparin, IL-4 and cAMP is linked to repression of cyclin D1 and cdk2. Atherosclerosis 133: 61-69, 1997.
31. Zhang Y, Li YH, Liu C, Nie CJ, Zhang XH, Zheng CY, Jiang W, Yin WN, Ren MH, Jin YX, Liu SF, *et al*: miR-29a regulates vascular neointimal hyperplasia by targeting YY1. Cell Prolif 50: e12322, 2017.