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# Upregulation of Protease-Activated Receptor 2 Promotes Proliferation and Migration of Human Vascular Smooth Muscle Cells (VSMCs)

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Statistical Analysis C  
Data Interpretation D  
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Literature Search F  
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**Background:** Protease-Activated Receptor 2 (PAR2), a G-protein-coupled receptor, has been proved to be enhanced in human coronary atherosclerosis lesions. We aimed to investigate whether PAR2 actively participates in the atherosclerosis process.


**Material/Methods:** PAR2 expression was assessed in blood samples by RT-qPCR from healthy controls and patients with atherosclerosis. Human vascular smooth muscle cells (VSMCs) were treated with oxidative low-density lipoprotein (ox-LDL). After PAR2 overexpression by transfection, cell proliferation was determined by CCK-8, and cell migration was evaluated by Transwell assay. The protein expressions associated with cell growth and migration were measured by Western blot. The distribution of  $\alpha$ -SMA in VSMCs was evaluated by immunofluorescence.

**Results:** Expression of PAR2 was higher in patients with atherosclerosis compared with normal controls. PAR2 mRNA and protein expression was increased in ox-LDL-treated VSMCs compared with control cells. Induced overexpression of PAR2 in VSMCs led to a reduction in  $\alpha$ -SMA expression compared to controls. In addition, PAR2 overexpression caused increased migration compared to normal controls, and upregulated MMP9 and MMP14 expression. PAR-2 overexpression promoted cell proliferation compared to control cells, and increased expression levels of CDK2, and CyclinE1, but reduced levels of p27. We preliminarily explored the potential mechanism of PAR2, and results showed that overexpression of PAR2 increased expression levels of VEGFA and Angiotensin 2 compared to controls. Moreover, overexpression of PAR2 enhanced production of tissue factor and IL-8 compared to normal controls.

**Conclusions:** PAR2 promotes cell proliferation and disrupts the quiescent condition of VSMCs, which may be a potential therapeutic target for atherosclerosis.

**MeSH Keywords:** **Atherosclerosis • Cell Dedifferentiation • Cell Migration Assays • Cell Proliferation • Receptor, PAR-2**

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## Background

Atherosclerotic lesion advancement mainly depends on neo-vascularization, lipid-rich macrophages, cell death, calcification, and vascular remodeling. Among various risk factors, the cholesterol level is a sufficient and critical factor driving atherosclerosis. As a chronic inflammatory process, it always shows endothelium dysfunction, uptake of oxidized low-density lipoprotein (LDL), and intimal hyperplasia. The initiation of intimal hyperplasia is mainly led by proliferation and migration of vascular smooth muscle cells (VSMCs) [1,2]. Research indicates that ox-LDL must be present in atherosclerotic lesions to activate various angiogenic signaling pathways such as the sphingomyelinase-2/sphingosine kinase-1 pathway, and reactive oxygen species [3]. Therefore, better understanding of the mechanisms of angiogenesis in VSMCs is an important strategy for the prevention of atherosclerosis.

Proteinase-activated receptor 2 (PAR2), a 7-transmembrane G-protein-coupled receptor, was first found in endothelial cells and later in smooth muscle cells [4,5]. Protease-activated receptors (PARs) include PAR1, PAR2, PAR3, and PAR4. All PARs can be activated by Thrombin, except for PAR2. Recent investigations found that PAR2 can be activated by Cathepsin S, Tissue factor-VIIa protease complex, and trypsin [6–8]. Atherosclerosis lesions are usually induced by various cardiovascular diseases [9]. PAR2 has been reported to be upregulated in human coronary atherosclerotic lesions [10]. A PAR2 antagonist, FSLLRY-NH<sub>2</sub>, is used in type 2 diabetic mice, and the results show that PAR2 is downregulated in coronary arterioles and regulates endothelial dysfunction mediated by promoting TNF $\alpha$  production and NADPH oxidase activation [11]. Transient receptor potential vanilloid receptors may contribute to PAR2-induced protection for cardiac ischemia/reperfusion (I/R) injury, expressed mainly on the epicardial surface and blood vessels of I/R model mice [12]. Trypsin-induced cardiac fibroblasts were shown to be mediated *in vitro* by the PAR2/cyclooxygenase-2 (COX-2) signaling pathway [13]. Also, PAR2<sup>-/-</sup>ApoE<sup>-/-</sup> mice showed fewer atherosclerotic lesions involving lipid deposition, collagen formation, and inflammatory molecule production [14]. PAR2 deficiency reduced atherosclerosis in the aortic sinus and aortic root and further reduced CCL2 and CXCL1 expression, resulting in less monocyte migration [15]. However, little is known about the mechanism by which PAR2 in human vascular smooth muscle cells are related to atherosclerosis.

PAR2 signaling activates macrophages and promotes vascular inflammation in atherogenesis [14]. A previous investigation revealed PAR2 has potential pro-atherogenic effects by activating VEGFR2 in human coronary smooth muscle cells (HCSMCs) overexpressing PAR2 [16]. An *in vivo* study found that PAR2 deficiency alleviates atherosclerosis [15]. However, there are

still no research showing the effects of PAR2 on VSMCs proliferation and migration, which usually results in intima hyperplasia in blood vessels, promoting formation of atherosclerotic plaque. Here, we hypothesize that PAR2 participation in atherosclerosis is partly due to activating VSMC proliferation and migration.

## Material and Methods

### Blood samples

Our study was approved by the Ethics Review Committees of the First Hospital of Hebei Medical University according to the Helsinki Declaration (No. 2015033). All patients provided signed informed consent. Blood samples were collected from 30 patients with atherosclerosis and 30 normal healthy volunteers (Supplementary Table 1). The diagnosis of atherosclerosis in all patients was verified by 3 independent pathologists according to coronary angiography, with at least 1 lesion in a coronary artery or branches. Healthy individuals were randomly recruited among people attending regular health check-ups, with an age range similar to that of the selected atherosclerosis patients. We excluded patients who had received coronary artery bypass graft surgery, or who had congestive heart failure, renal failure, liver disease, malignant cancer, or inflammatory disease.

### Cell lines

Human vascular smooth muscle cells (VSMCs) were purchased from ATCC (USA) and cultured in F12K culture medium supplemented with 10% fetal bovine serum (Gibco, USA). Cells were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere. VSMCs were treated with 100  $\mu$ g/ml ox-LDL for 24 h. Overexpression of PAR2 was performed by transfecting pcDNA3.1 (Invitrogen, USA) encoding PAR2. The transfection of vectors of pcDNA3.1 was performed as negative control (NC). Lipofectamine 2000 (Invitrogen) was used to construct the plasmid according to standard protocol.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

VSMCs were seeded at 7 $\times$ 10<sup>5</sup> cells/well in 6-well plates. Cells were treated with ox-LDL or transfected with PAR2-pcDNA3.1. TRIzol reagent (ThermoFisher Scientific, USA) was used to extract the total RNA of cells and blood samples 24 h later. The RNA concentration was detected and calculated using NanoDrop ND-2000 (Thermo Scientific, USA). The RNA was reversed to cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen, USA), and the cDNA was later used for the qRT-PCR. The primers of PAR2 and 18s were: PAR2 forward primer, 5'-TGGATGAGTTTTCTGCATCTGTCC-3';

and reverse primer, 5'-CGTGATGTTCCAGGGCAGGAATG-3'; 18s forward primer, 5'-CGGCTACCACATCCAAGGAA-3', and reverse primer, 5'-CCTGTATTGTTATTTTCGTCCTACTACCT-3'. The expression of PAR2 was normalized by 18s. mRNA levels were evaluated using the StepOne Plus real-time PCR system (Applied Biosystems, USA).

### Western blot analysis

We seeded  $1 \times 10^6$  cells into each 6-cm culture plate. Treated cells were later isolated using a lysis buffer (Sigma). Subsequently, proteins (30  $\mu$ g) were separated by SDS-PAGE gel (10% and 12%). After proteins were well separated, they were transferred to polyvinylidene fluoride membranes (Millipore, USA) for 2 h and blocked with 5% (v/v TBST) skimmed milk for 1 h. The membranes were incubated with primary antibody at 4°C overnight: rabbit anti-PAR2 (#6976), MMP9 (#13667), MMP14 (#13130), and GAPDH (#5174) were purchased from Cell Signaling Technology (USA), and VEGFA (ab52917) and Angiopoietin 2 (ab8452) were purchased from Abcam (USA). HRP-linked anti-rabbit (#7074; Cell Signaling Technology) secondary antibodies were incubated at room temperature for 2 h. All results were validated through enhanced chemiluminescence detection method.

### Cell proliferation

Cells were transfected with PAR2-pcDNA3.1, and then cultured in 96-well plates at a concentration of  $5 \times 10^3$  cells/well incubated at 37 °C and 5% CO<sub>2</sub> for 24 h after transfection. Cell viability was evaluated using the CCK-8 Kit (Real Times Technology, Beijing, China). Cells were incubated with 10  $\mu$ l CCK-8 solution for 1 h at 37°C. Optical density was assessed at a wavelength of 490 nm.

### Transwell assay to assess cell migration

Cells were overexpressed with PAR2 48 h before cell seeding. Subsequently, cells were planted into the upper chambers (8  $\mu$ m; Millipore, USA) of 24-well plates at a concentration of  $5 \times 10^4$  cells with serum-free medium, and complete medium (F12K supplemented with 10% FBS) was added to the bottom chambers. The cells were fixed and stained 24 h later using methanol and 0.1% crystal violet solution, respectively. Transferred cells were counted by microscope at 200 $\times$  magnification.

### Immunofluorescence

Cells were overexpressed with PAR2 48 h before cell seeding. VSMCs cells were cultured on 24-well chamber slides with  $1 \times 10^4$  cells in 1 ml cultured medium in 24-well plates, and 24 h later, cells were fixed with paraformaldehyde (4%) for 10 min. Subsequently, cells were permeabilized with 0.1% Triton X-100 for 20 min at 25°C. After blocking cells for 30 min with 5%

bovine serum albumin, cells were treated with anti- $\alpha$ -SMA antibody (Cell Signaling Technology, USA) at 4°C overnight, followed by treatment with Anti-rabbit IgG (H+L), F(ab')<sub>2</sub> Fragment (Alexa Fluor® 488 Conjugate; Cell Signaling Technology) at 25°C for 1 h. The nuclei were then stained by 4',6-diamidino-2-phenylindole (DAPI; sigma-Aldrich) for 7 min in the dark. Confocal laser scanning microscopy (Olympus) was performed to valid the immunofluorescence results.

### Tissue factor detection kit

Human Coagulation Factor III/Tissue Factor Quantikine ELISA kits were purchased from R&D systems (USA). PAR2 were overexpressed in VSMCs cells, and the cultured medium was collected after overexpression for 48 h. Tissue factor (TF) from cell supernatant was detected according to the manufacturer's instructions.

### IL-8 ELISA kit

Human IL-8/CXCL8 Quantikine ELISA kits were purchased from R&D Systems (USA). The supernatant from the cultured medium of PAR2-overexpressed VSMCs cells was collected after 48 h. IL-8 from cell supernatant was detected according to the manufacturer's instructions.

### Statistical analysis

All experiments were replicated 3 times (total n=9 from 3 independent experiments). Data are presented as mean $\pm$ SD. Statistical analysis between groups was performed using the *t* test, and multiple comparisons were analyzed using one-way ANOVA. All analyses were performed in GraphPad Prism. Image J (version 1.3.7, USA) software was used for densitometry of Western blotting. *P*<0.05 was considered as statistically significant.

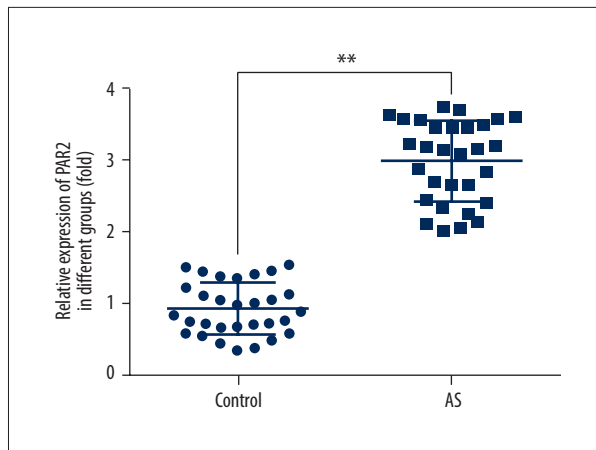
## Results

### PAR2 is associated with atherosclerosis

As shown in Figure 1, PAR2 was highly upregulated in atherosclerosis compared with healthy control. In addition, PAR2 expression was dramatically increased in ox-LDL-induced VSMCs, as shown by immunoblotting and qRT-PCR analysis (Figure 2A, 2B). To investigate whether PAR2 is associated with atherosclerosis progression, PAR2-pcDNA3.1 was transfected into VSMCs cells. The gene and protein expression levels of PAR2 were significantly higher than in untreated cells and pcDNA control (Figure 2C, 2D).

### PAR2 is associated with VSMCs dedifferentiation

Modulation of phenotype in VSMCs, including decreased contractile marker and increased migration, proliferation, and



**Figure 1.** Increased expression of PAR2 in atherosclerosis patients (AS) compared to controls. qRT-PCR was performed to assess the expression levels of PAR2. \*\*  $p < 0.01$  versus controls.

extracellular matrix synthesis, is associated with atherosclerosis progression [17]. The expression of  $\alpha$ -SMA was significantly decreased in PAR2-overexpressed VSMCs (Figure 3A). Consistently, immunofluorescence staining showed that PAR2 overexpression in VSMCs significantly reduced the level of  $\alpha$ -SMA, which is used as a contractile phenotype marker (Figure 3B). There were significantly more migrating cells in PAR2-overexpressed VSMCs compared with control or NC (Figure 4A, 4B), and the expression levels of MMP9 and MMP14 were dramatically

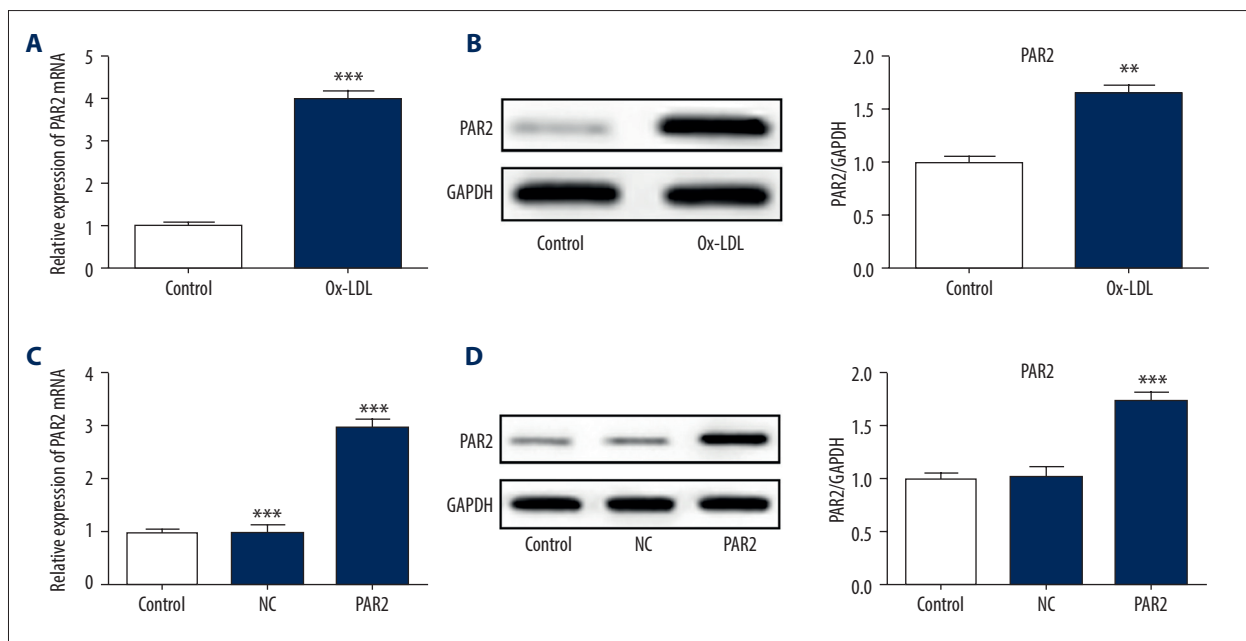
increased compared with control or NC (Figure 4C). In short, PAR2 dedifferentiates the quiescent contractile phenotype of VSMCs and promotes cell migration.

### PAR2 promotes cell proliferation in VSMCs

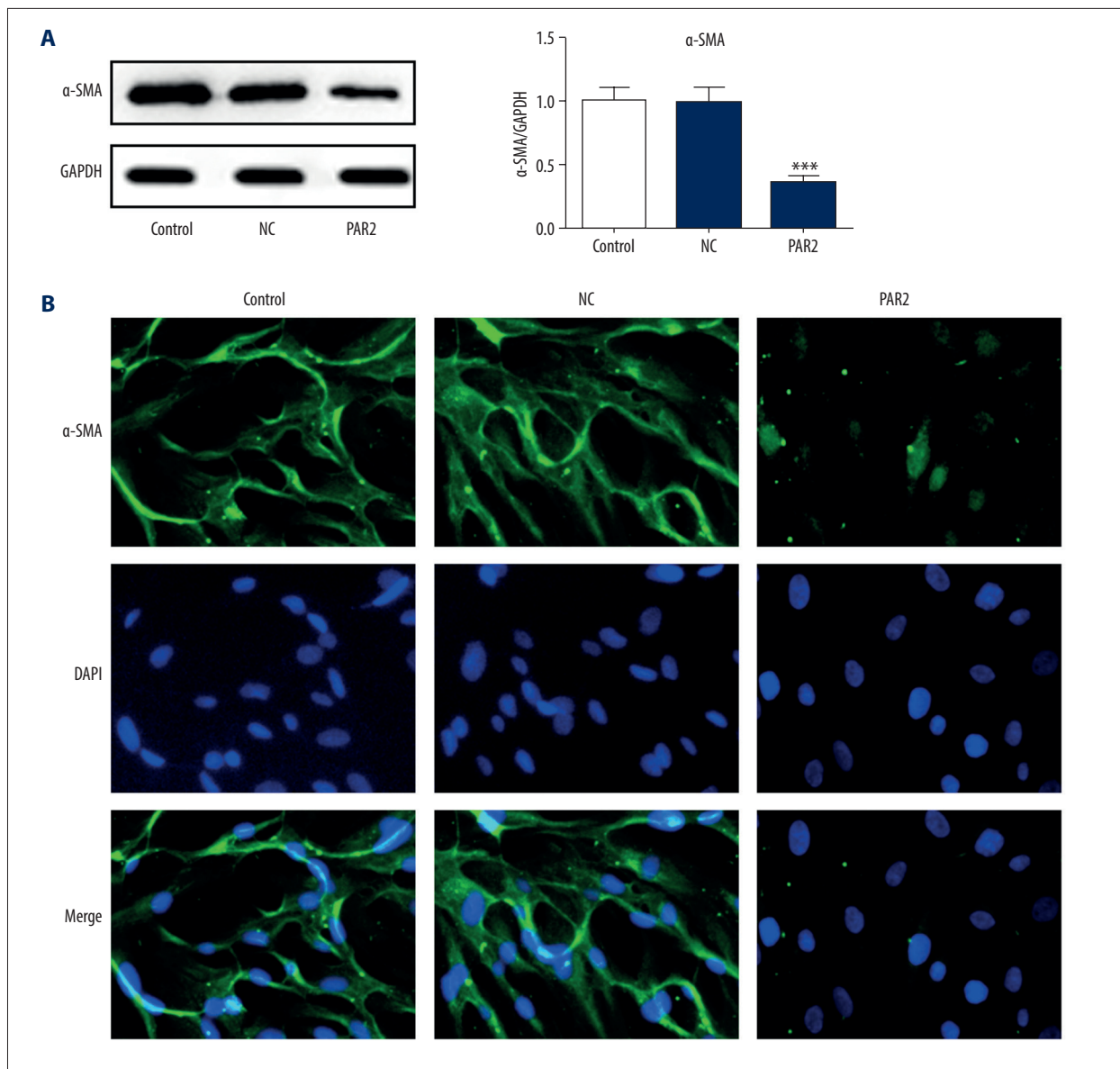
In contrast with control or NC, PAR2 overexpression significantly increased the cell viability of VSMCs (Figure 5A). Activation of CDK2/CyclinE complex is essential for the cell cycle by promoting progression of cells from G1 phase to S phase. Overexpression of PAR2 significantly promoted the upregulation of CDK2 and CyclinE1 in VSMCs (Figure 5B, 5C). Moreover, the CDK2/CyclinE1 complex inhibitor, p27, was significantly inhibited by overexpression of PAR2 (Figure 5B, 5C). These data show that PAR2 promotes proliferation of VSMCs.

### Exploration of further mechanisms for PAR2 in VSMCs

Figure 6A shows that overexpression of PAR2 upregulated the expression levels of VEGFA and Angiopoietin 2. Moreover, overexpression of PAR2 promoted the production of tissue factors (Figure 6B). A previous study found that expression of TF and PAR2 is accompanied by the expression of VEGF and IL-8. In the present study, IL-8 levels were significantly increased after VSMCs overexpressed PAR2 (Figure 6C).



**Figure 2.** ox-LDL induction in the PAR2 expression in VSMCs. (A, B) Cells were treated with ox-LDL (100  $\mu$ g/ml) for 24 h. qRT-PCR and Western blotting were performed to estimate the expression of PAR2. \*\*  $p < 0.01$  versus control. (C, D) Cells were transfected with PAR2-pcDNA3.1 or NC for 48 h. Transfection efficacy was detected by qRT-PCR and Western blotting. NC, pcDNA3.1 vector as the negative control. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  versus control or NC.

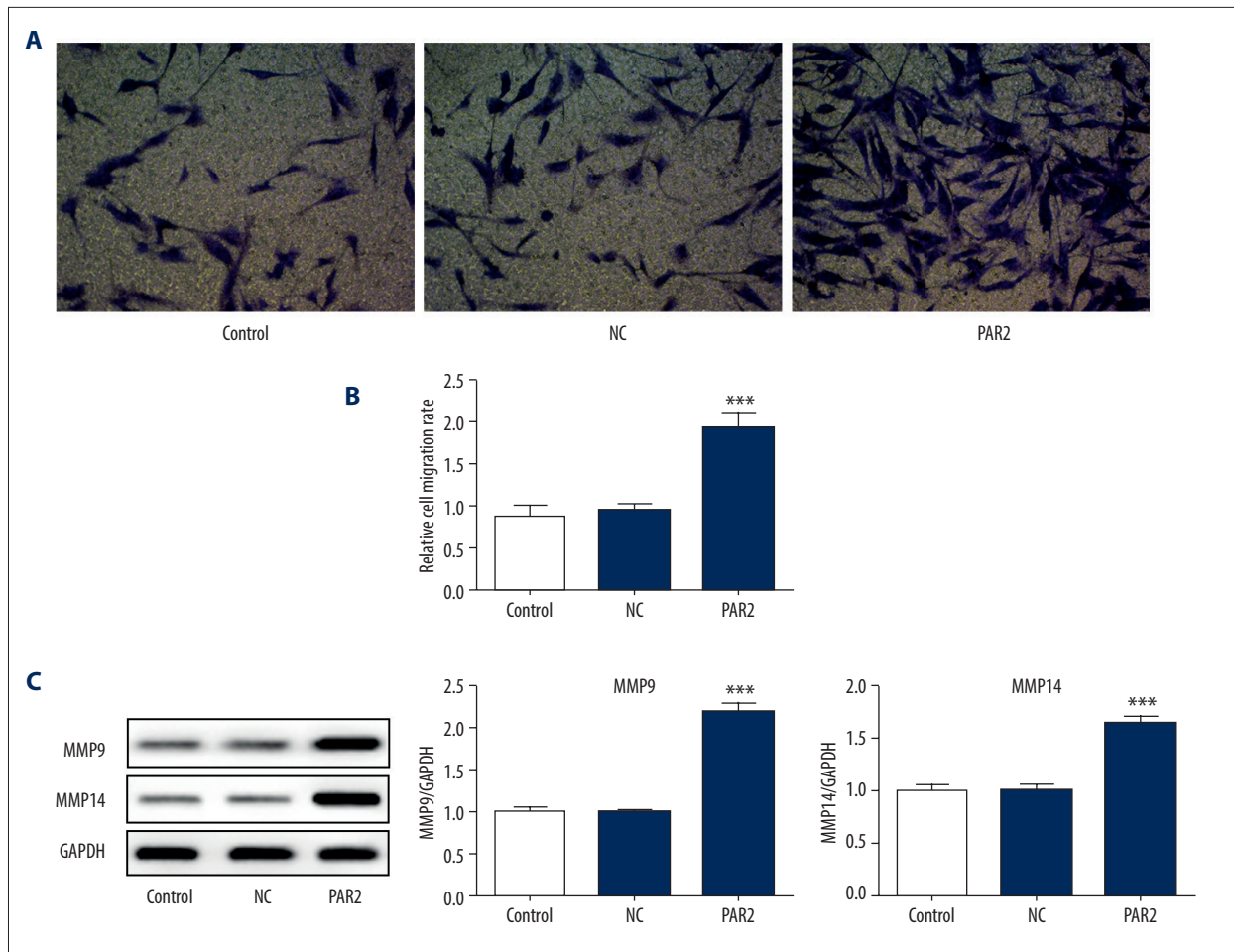


**Figure 3.** Effect of PAR2 on  $\alpha$ -SMA expression in VSMCs. Cells were transfected with PAR2-pcDNA3.1 or NC. **(A)** The expression levels of  $\alpha$ -SMA were validated by Western blotting. \*\*  $p < 0.01$  versus control or NC. **(B)** Immunofluorescence assay was also performed to detect the alteration of  $\alpha$ -SMA.

## Discussion

During atherosclerosis, the phenotype of VSMCs was switched from contractile to proliferative and migrative types [18]. In the present study, we show that upregulation of PAR2 promotes the proliferation and migration of the cells in VSMCs, and ox-LDL induces upregulation of PAR2 in VSMCs. These novel findings show that ox-LDL-induced PAR2 overexpression promotes VSMCs proliferation and migration and elucidate the intercellular mechanism underlying the role of VSMCs in progression of atherosclerosis.

Ox-LDL has been proved to be a critical endogenous ligand that has various pathogenic roles. Ox-LDL is associated with formation of atherosclerotic plaque via induction of macrophage foam cell formation, as well as smooth muscle cell migration, proliferation, and inflammation [19,20]. A previous study found that VSMCs induced by ox-LDL can form foam-like cells, which contributes to the fatty streaks in atherosclerotic plaque [21]. In the present study, ox-LDL was used to stimulate VSMCs, and the expression of PAR2 was significantly upregulated. Furthermore, our results indicate that overexpression of PAR2 promotes proliferation, migration, and upregulation of the pro-inflammatory factor IL-8 in VSMCs.

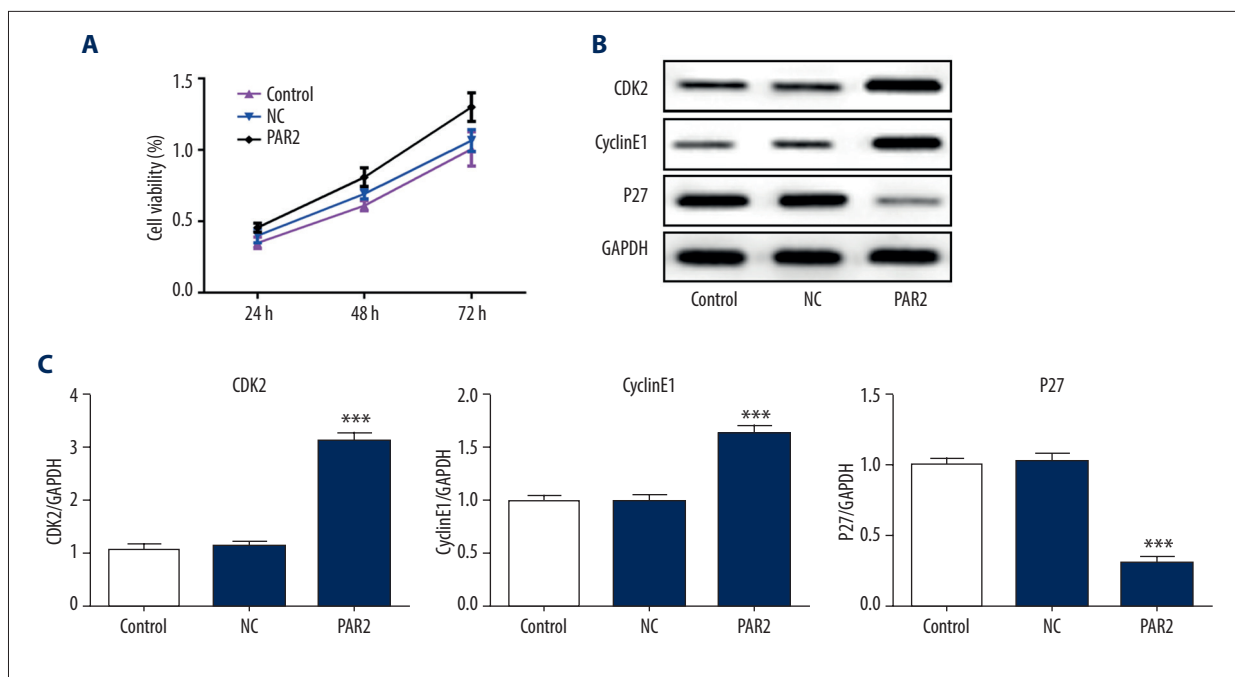


**Figure 4.** VSMCs overexpression of PAR2 results in increased migration and MMP9/14 activity. (A) Transwell assay of cell migration. \*\*  $p < 0.01$  versus control or NC. Representative western blot analysis (B) and quantification (C) of MMP9 and MMP14 in VSMCs transfected with PAR2-pcDNA3.1 or NC. \*\*\*  $p < 0.001$  versus control.

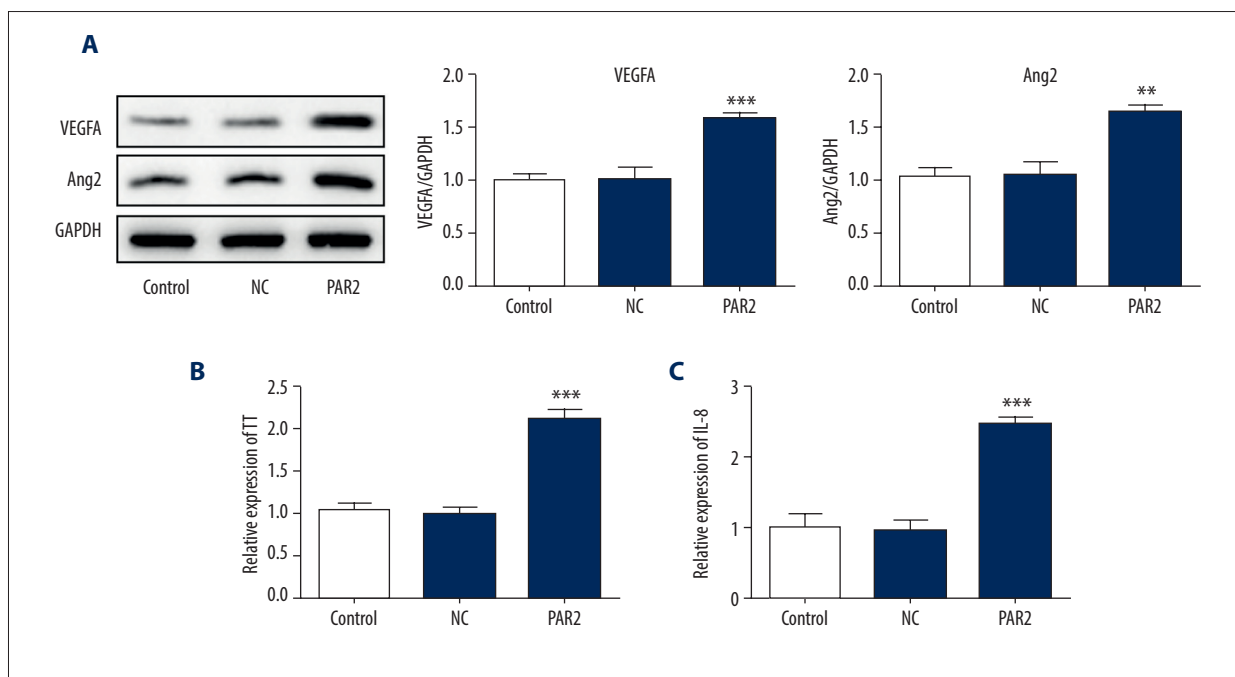
It has been reported that PAR2 deficiency is associated with attenuation of atherosclerosis [15], but the main cellular mechanism has been unknown. VSMCs have a quiescent contractile phenotype under normal conditions. When cells are stimulated, VSMCs switch to a dedifferentiated state by suppressing the expression of VSMC-specific markers, including  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), to increase the ability of cells to cell proliferate and migrate [22]. Except for the VSMCs dedifferentiation, the cell-cell and cell-matrix interactions were also induced by VSMCs proliferation and migration [23,24]. Our data show that  $\alpha$ -SMA was decreased in PAR2-overexpression VSMCs. In addition, the expression levels of MMP9 and MMP14 were increased by overexpression of PAR2, suggesting that overexpression of PAR2 promotes ECM degradation, which is related to migration of VSMCs [25].

Thrombosis usually forms based on atherosclerosis. Tissue factor is sensitive to initiation of atherosclerotic plaque and induces thrombosis [26,27]. In astrocytoma samples, elevated expression of VEGF and IL-8 was positively correlated with

TF and PAR2 expression [28]. Camerer et al. found that PAR2 can be activated by TF/FVIIa complex [29]. The present study shows that PAR2 overexpression promotes the production of TF. Whether the endogenous TF affects the biological effects of VSMCs in atherosclerosis requires further investigation. VSMCs migration is related to increased expression of chemoattractants, including VEGF. Furthermore, the neovascularization process is tightly associated with the acceleration of atherosclerotic plaque formation. Vascular endothelial growth factor-A (VEGFA) is essential in the formation of angiogenesis [30,31]. The expression of VEGFA was significantly increased in PAR2 overexpressed VSMCs compared with control and NC. VEGF receptor 2 is the primary signaling receptor of VEGFA. A study reported that PAR2-activated VEGFR exerts pro-atherogenic effects [16]. It appears that PAR2-activated VEGFA/VEGFR signaling exert pro-atherosclerosis effects, but this requires further study. Angiotensin-2 levels are clinically relevant to atherosclerosis, and this has been confirmed in mouse models [32]. In the present study, Angiotensin-2 expression was significantly



**Figure 5.** PAR2 increased VSMCs proliferation. (A) CCK-8 demonstrated the effect of PAR2 on cell viability. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  versus control or NC. (B, C) Western blot analysis of PAR2 regulated CDK2, cyclin E1, and p27 expression. The results of western blotting were exhibited as band image (B) or quantitative analysis (C). \*\*\*  $p < 0.001$  versus control or NC.



**Figure 6.** The potential mechanisms for the effects of PAR2. (A) Expression of VEGFA and Angiopoietin 2 (Ang2) were estimated by western blotting. Production of tissue factor (TF) (B) and IL-8 (C) were analyzed by ELISA. \*\*\*  $P < 0.001$  versus control or NC. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

upregulated by overexpression of PAR2. These data show the potential molecular mechanism of PAR2 in VSMCs. Whether PAR2 affects VSMCs in atherosclerosis progression via these signaling processes remains unknown.

of atherosclerosis, showing its potential roles as a diagnostic and therapeutic target. Although PAR2 was significantly up-regulated in ox-LDL-treated VSMCs, the relationship between PAR2 and ox-LDL requires further exploration, and the effects of PAR2 need to be confirmed by *in vivo* experiments.

## Conclusions

We assessed the molecular and cellular mechanisms by which PAR2 affects cell proliferation and migration in development

## Conflict of interests

None.

**Supplementary Table 1.** Baseline characteristics of cardiac atherosclerosis under angiography.

Age (year)	Sex	Smoking history	Family history of atherosclerosis	History of myocardial infarctions	Number of blood vessel lesions	LM*	LAD*	LCX*	RCA*
53	Female	No	No	No	3	No	No	No	No
51	Female	No	No	No	2	No	Yes	Yes	No
53	Female	No	Yes	No	3	No	Yes	Yes	Yes
54	Female	No	Yes	No	3	No	Yes	Yes	No
55	Female	No	No	No	3	No	No	Yes	Yes
46	Female	No	No	No	2	No	Yes	No	No
52	Female	No	No	No	3	No	No	No	No
55	Female	No	No	No	3	No	No	No	No
50	Female	No	No	No	3	Yes	No	No	No
51	Female	No	No	No	2	No	Yes	No	No
52	Female	No	No	No	2	No	No	Yes	No
54	Female	No	No	No	2	No	Yes	No	Yes
44	Female	No	Yes	No	2	No	Yes	Yes	No
50	Female	No	No	No	2	No	No	No	Yes
49	Female	No	Yes	No	3	Yes	No	No	No
42	Male	Yes	No	No	3	No	Yes	Yes	No
43	Male	Yes	Yes	No	2	No	No	No	Yes
43	Male	No	Yes	No	3	No	No	Yes	Yes
41	Male	Yes	No	No	3	No	No	No	No
40	Male	Yes	No	No	3	No	No	No	Yes
45	Male	No	Yes	No	2	No	No	Yes	No
45	Male	Yes	No	No	3	No	No	Yes	No
46	Male	Yes	Yes	No	2	No	No	No	Yes
45	Male	No	No	No	3	Yes	No	No	No
43	Male	Yes	Yes	No	3	No	No	No	No
45	Male	Yes	Yes	Yes	3	No	No	Yes	No
43	Male	Yes	No	No	2	No	No	Yes	No

\* Distribution of the location of lesions in LM, LAD, LCX, or RCA. LM – left main; LAD – left anterior descending; LCX – left circumflex branch; RCA – right coronary artery.



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