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Shear Stress-mediated Angiogenesis Through Id1 Relevant to Atherosclerosis

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Abstract: Abnormal shear stress in the blood vessel is an important stimulating factor for the formation of angiogenesis and vulnerable plaques. This paper intended to explore the role of shear stress-regulated Id1 in angiogenesis. First, we applied a carotid artery ring ligation to create local stenosis in $ApoE^{-/-}$ mice. Then, 3D geometry of the vessel network was reconstructed based on MRI, and our analysis of computational fluid dynamics revealed that wall shear stress of the proximal region was much higher than that of the distal region. In addition, results from histological staining of the proximal region found more vulnerable-probe plaques with new capillary formation, the presence of macrophages and collagen fibers degradation. Our in *vitro* and in *vivo* experiments further indicated high shear stress can induce endothelial cell-mediated angiogenesis and high expression of Id1. Id1-overexpression promoted endothelial cells migration and angiogenesis through collagen degradation mediated by MT-MMPs. Together, our results support a biomechanical role for Id1 in angiogenesis, suggesting manipulation of the Id1 activity may offer a novel anti-angiogenic therapeutic strategy in vulnerable plaques.

Keywords: Angiogenesis, vulnerable plaques, shear stress, inhibitor of differentiation 1 (Id1), membrane-type matrix metalloproteinase (MT-MMP).

1 Introduction

Destabilization of atherosclerotic plaque is the main cause of acute coronary events [Dempsey et al. (2017), van Hinsbergh, Eringa and Daemen (2015)]. Vulnerable atherosclerotic plaques are often accompanied by pathological angiogenesis [van Hinsbergh, Eringa, and Daemen (2015)]. Angiogenesis contributes to the progression and vulnerability of atherosclerotic plaques by supplying them with lipoproteins, inflammatory cells, matrix proteases and reactive oxygen species (ROS) [Moreno et al. (2004)]. It has been suggested that angiogenesis might be a key

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factor causing plaque rupture, especially when the new blood vessels are still being formed and lacking a complete middle and outer membrane [Lee et al. (2013)]. Angiogenesis is a complex and well-organized event, which requires endothelial cells (ECs) to coordinate multiple aspects, including reduction of intercellular adhesion, degradation of sub-endothelial matrix, migration, proliferation and formation of new capillary tubes.

In this paper, we focus on an important transcription factor, inhibitor of DNA binding 1 or inhibitor of differentiation 1 (Id1), which is a member of helix-loop-helix (HLH) transcription factors [Lasorella, Benezra and Iavarone (2014)]. We choosed to focus on Id1because it can increase the expression of many angiogenic factors in tumor, like matrix metalloproteases (MMPs) [Ruzinova et al. (2003)]. MMPs are needed for angiogenesis to provide enough space for cell growth [Abbas et al. (2014)]. The membrane-type matrix metalloproteinases (MT-MMPs), an important member of extracellular matrix degradation protease families, destroys the cell-cell connection by degrading extracellular matrix, increasing ECs migration and regulating the expression of its downstream genes involved in angiogenesis [Sounni, Paye, Host, and Noel (2011)]. However, the relationship between Id1 and MT-MMPs is still unclear. Other studies have shown that Id1 protein is closely related to tumor angiogenesis [van Hinsbergh, Eringa, and Daemen (2015), Sounni, Paye, Host and Noel (2011)], but there are few publications in atherosclerosis.

During the process of angiogenesis in atherosclerosis, ECs respond to a variety of biochemical factors as well as shear stress from blood flow [Sounni, Paye, Host and Noel (2011), Wei et al. (2012), Walpola, Gotlieb and Langille (1993)]. Although shear stress is known to regulate the expression of genes [Dai et al. (2004), Wragg et al. (2014)], the mechanisms that how to control angiogenesis are not completely understood [Azrad et al. (2015), Zhou et al. (2012)]. Previous studies have discovered a lot of mechanosensitive genes, including Id1[Qiu et al. (2011), Ghaffari, Leask and Jones (2015)]. Therefore, we hypothesize that changes of shear stress could induce the formation of vulnerable plaques in atherosclerosis through angiogenesis and Id1 could play a key role in the process. The identification of biomechanical roles of shear stress in regulating Id1 on angiogenesis may provide insights into the development of new strategies to treat and prevent the acute symptoms of cardiovascular disease.

2 Materials and methods

2.1 Experimental animal model

$ApoE^{-/-}$ mice were anesthetized using 8% (wt/vol) chloral hydrate by intraperitoneal injection. The left carotid artery (LCA) was separated by sterile surgical instruments. Then a silicone ring ligation was made at the LCA to build a narrow blood vessel model according to the previous study [Wei et al. (2012)]. Sham operation was performed by using an identical procedure without a silicone ring ligation at the carotid artery, the control was the normal mice without operation. After surgery, all mice were fed under specific pathogen-free conditions. All procedures were in compliance with the China Council on Animal Care and Chongqing University Animal Use protocol, and all the ethical guidelines for experimental animals were followed.

2.2 Ultrasound measurements

Forty-eight hours after the operation, Vevo 2100 (Canada, VisualSonics company), a high-resolution small animal ultrasound instrument (probe transmit frequency is 24-30 MHz) was used to obtain blood flow data of proximal and distal blood vessels at ring ligation areas on the LCA, as well as the control and sham groups. After being anesthetized, the mice were placed in a supine position. The necks of mice were shaved to facilitate detection, 2D and color doppler mode were used to identify the diameter of carotid arteries, and pulse wave doppler mode was used to detect the velocity of blood flow.

The blood flow velocity and vessels diameter were measured by Vevo 2100 high-resolution small animal ultrasound instrument and then were used to calculate shear stress using a Poiseiulle flow approximation:

$\tau=4\ \mu\nu\ /r$

Where $\mu=0.035$ (the viscosity of blood), ν is the blood flow velocity, and r is the radius of the blood vessel [Walpola, Gotlieb and Langille (1993)].

2.3 3D reconstruction and blood flow simulation

To better understand the distribution of shear stress and blood flow velocity of LCA with local stenosis, a three-dimensional model of the LCA with ring ligation from inlet to outlet was reconstructed and geometrically optimized with the software Mimics (Mimis Medical 19.0, Materialize, Inc) and Studio (Geomagic Studio 12.0, Geomagic, Inc) based on magnetic resonance imaging (MRI) DICOM. MRI were acquired on a 1.5T MR scanner (Magnetom® Avanto, Siemens AG, Germany). The geometric surfaces were then imported into ICEM (ICEM-CFD 15.0, Ansys, Inc.) to generate a volume mesh with approximately 600,000 tetrahedral elements. The blood flow was defined as laminar and incompressible Newtonian fluid with a dynamic viscosity of 0.0035 Pa s and a density of 1060 kg/m^3, and the no-slip condition assuming rigid wall was applied. The inlet boundary condition was set at a flow rate of 380 mm/s measured by doppler ultrasound and zero pressure was set as the outlet boundary condition. Results were analyzed by using CFD-Post (CFD-Post 15.0, Ansys, Inc.)

2.4 Tissue samples collection and histological staining

The mice with silicone ring ligation were sacrificed at 8th week, and the arterial tissues were subjected to hematoxylin and eosin (HE) staining [Azrad et al. (2015)], Masson staining, Van Gieson (VG) staining, Id1 (Abcam, USA) immunostaining, CD31 (Bioss, China) and CD68 (Bioss, China) immunostaining. Pictures were taken by Olympus microscope and analyzed with Image J 6.0 software.

2.5 Cell culture and shear stress Treatment

Human umbilical vascular endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC, Manassas, VA), and cultured in Roswell Park Memorial Institute media (RPMI 1640) with 10% fetal bovine serum (FBS) at 37℃ in 5% CO_2 incubator. HUVECs were transplanted on a 0.5 mm-thick glass slides and were placed on a parallel-plate flow chamber at 37℃ with 5% CO_2 as previously described [Zhou J, et al.

(2012)]. The formula $\tau = 6Q\mu/wh^2$ was used to calculate the fluid shear stress (τ), Q is the flow rate and μ is the dynamic viscosity of the perfusate. HUVECs were exposed to three different shear stress (5 dyn/ cm^2, 15 dyn/cm^2, 25 dyn/cm^2) for 6 hours, respectively.

2.6 Cell migration

To measure the cell migration ability, we scraped cells with a thin 10μl pipette tip across the monolayer to produce lesions with constant diameter. Then the cells were washed three times with phosphate buffer solution (PBS) to remove loose cells, after that the cells were cultured in PRMI -1640 media with 2% FBS. Images were obtained by using a phase contrast microscope at the time of initial wounding and 24 hours post-wounding. Measurements of migration velocity were conducted with image tool software.

2.7 Collagen matrigel invasion assay and analysis of invasion ability

Matrigel (BD Biosciences) was melted at 4℃ overnight and then diluted by 4℃ precooling PBS. 200μl diluted Matrigel was added on the upper surface of 6.5 mm diameter transwell chamber (transwell membrane pore size is 8 μm) and placed for 30 min to solidified. HUVECs were inoculated on the solidified Matrigel. PRMI1640 medium with 10% FBS was added into the lower room of transwell chamber to provide nutrients for cells. After being cultured for 48 hours, invading cells were fixed in 4% paraformaldehyde for 30 min. The cells on the upper surface of the transwell were scraped off, and the cells on the lower surface were stained with 0.1% crystal violet for 10 min. Cells were cleaned with PBS for 3 to 5 times, and pictures were taken by microscopes. The number of cells were counted with Image J 6.0 software.

2.8 In vitro tube formation

Matrigel, kept on ice, was dispensed at 200 μl/well in 24-well culture plates. The plates were then incubated at 37℃ for 30 min to allow matrigel to solidified. HUVECs exposed to shear stress were seeded at 10^4 cells per well on the top of the solidified matrigel, and incubated at 37℃ for 24 hours. Tubes on matrigel were observed through microscopes. The degree of angiogenesis was measured by multiplying the number of branch points between cells.

2.9 Fluorescence quantitative PCR

Total RNA was extracted from cells and reverse-transcribed to cDNA with reverse transcription kit (Takar, Japan). The oligos used for polymerase chain reaction (PCR) were as follows:

MMP14 (MT1-MMP): 5’-TCAACCCAGGACTACCTCCC-3’
5’-CAGCGCTCCTTGAAGACAAAC-3’
MMP17 (MT4-MMP): 5’-TGCACTCCATGTACTACGCCC-3’
5’-ATCGTCAAAGTGGGTGTCCC-3’
Id1: 5’-GATCATCCTTATACCGACGGG-3’
5’-CGGGGGAGCGCTTTTTCCAGG-3’
GAPDH: 5’-TCCCTGAGCTGAACGGGAAG-3’

5’-GGAGGAGTGGGTGTCGCTGT -3’

Each sample had three repeats. The q-PCR program setting was: 95℃, 4 min; 95℃, 30s; 56℃, 20 s; 72℃, 30s; for 30 reaction cycles.

2.10 Western blotting

Total protein was extracted as previously described [Bot et al. (2007)]. Cells were washed three times with PBS and lysated by cell lysis buffer containing 1% phenylmethanesulfonyl fluoride (PMSF) and protease inhibitors. The lysate was centrifuged at 12000 rmp for 5 min and then the supernatant was collected and quantified by using the BCA protein assay kit. Approximately 40 μg of total protein was loaded onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. After transmembrane, PVDF membranes were blocked and then incubated with primary antibodies overnight at 4℃, including Id1 (Abcam, USA), MMP14 (Abcam, USA), MMP17 (Abcam, USA) and GAPDH (Abcam, USA) antibodies, then secondary antibodies (Santa Cruz, USA) was applied to bind to the primary antibodies.

2.11 Lentiviral infections

Plvx-EF1a-EGFP-T2A-Puro vector was used to express Id1. After recombination of Plvx-EF1a-EGFP-T2A-Puro vector with pCMV-dR8.9 and pCMV-VSV-G, the pre-mixed plasmids were transfected into 293T cells, and lentiviruses were amplified. ECs were seeded into 96-well plates at the concentration of 4×10^4 cells/ml. Lentiviruses and polybrene were added into the dishes, the culture medium was replaced 10 hours later by fresh medium that did not contain polybrene and lentiviruses. Three days later, an Olympus fluorescence microscope was used to determine whether the viruses were transfected into cells. Puromycin (1.25 μg/ml) was used to select the cells to make sure all cells were infected by lentiviruses.

2.12 siRNA transfection

Before the transfection experiment, 1x 10^5 cells were planted in a 6-well plate with 2 ml of medium overnight. ECs at 30–50% confluence were transfected with the designated siRNA according to the Lipofectamine™ 2000 Transfection Reagent Protocol. After 48 hours, we detected that the RNAs have entered into cells by fluorescence microscopy.

SiRNA: GGGAUUCCACUCGUGUGUUTT--AACACACGAGUGGAAUCCCT

Negative control:

UUCUUCGAACGUGUCACGUTT—ACGUGACACGUUCGGAGAATT

3 Statistical analysis

All the experiments were carried out for a minimum three times, Data were provided either as mean ± SD or as proportions. In cases of significant differences between the groups, multiple comparisons among groups were made by analysis of variance. Comparisons between two groups were analyzed by independent T test.

3.1 Computational fluid dynamics shows the distribution of flow velocity and shear stress

MRI pictures (shown in Figure.1a) clearly indicated that the lumen of LCA became smaller from anterior to posterior whereas the right carotid artery (RCA) did not. This was consistent with a stenosis in the LCA. Figure1b showed the shape of narrowing LCA caused by the ligated silicone ring in a 3D geometry reconstruction. The streamlines in the vessel showed the flow velocity along the LCA. In the upstream and downstream of the ligated region, a relatively low velocity of flow was observed, and oscillating flow was seen in the downstream of the ligated region, and as expected, the flow velocity increased within the ligated region (Fig.1c). Taken together, these observations meant that increasing wall shear stress was found proximal to the ligated region, decreasing wall shear stress was seen distal to the ligated region, and the stenosis region itself had the highest shear stress (Fig.1d).

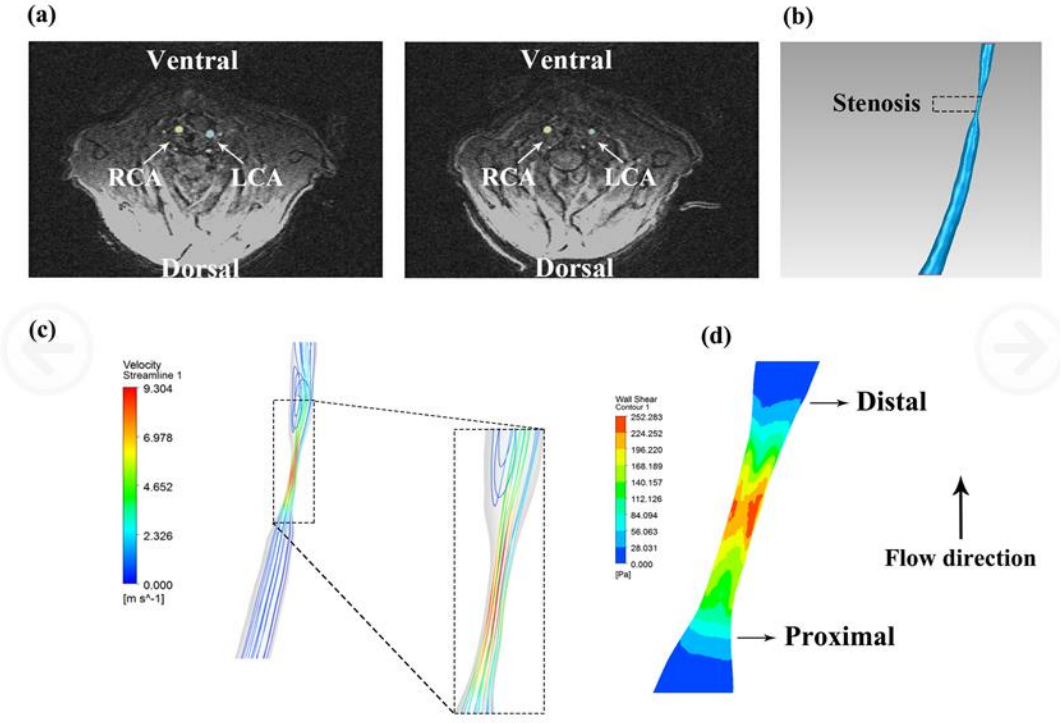

Figure 1: MRI illustrated the stenosis of LCA, computational fluid dynamics showed the distribution of flow velocity and shear stress. (a) MRI showed the lumen of RCA (yellow) and LCA (blue) was similar, but the LCA was smaller than the RCA with the scanning from anterior to posterior. (b) 3D reconstruction of LCA's contour illustrated the stenosis. (c) Streamline representing flow velocity field along the LCA using vessel geometry. (d) Hemodynamic analysis of wall shear stress distribution along the LCA.

3.2 High shear stress promotes angiogenesis in ApoE-/- mice and in Vitro

To study the role of shear stress in the angiogenesis, a silicone ring ligation was constructed on LCA of ApoE$^{-/-}$ mice to control the diameter of the vessel. Then blood flow velocities and diameters were applied to estimate the shear stress. Our results

demonstrated that velocity and wall shear stress in the proximal region of carotid artery were much higher than that in distal region (Fig. 2b, 2d, n=4), but the flow velocities of proximal region and distal region were lower than control and sham group (Fig. s1). HE staining showed that the proximal region with high shear stress had atherosclerotic plaque formation, and the inside of plaque was filled with a large number of newborn capillaries. Meanwhile, the distal region with low shear stress had fewer capillaries (Fig. 2a), same as the blood vessels of the control and sham group (Fig. s2). The density analysis of capillaries indicates that the high shear stress in carotid arteries had a strong correlation with angiogenesis (Fig. 2e, n=3).

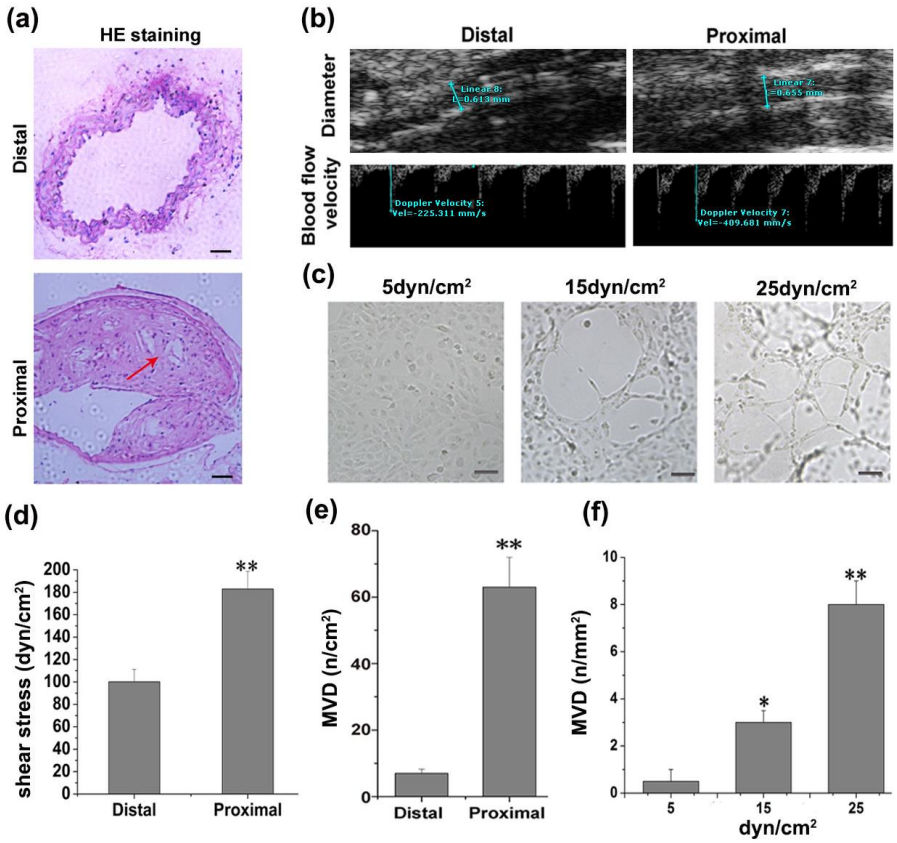

Figure 2: High shear stress promotes angiogenesis in ApoE$^{-/-}$mice and in vitro. (a) HE staining of proximal and distal blood vessel, scale bar, 100μm. (b) Small animal ultrasonic method measured blood flow velocity and the vascular diameter. (c) Exposing cells to different shear stress (5, 15, 25 dyn/cm^2) for 6h, then culture the cells on martrigel, scale bar, 100μm. (d) Statistic analysis of the shear stress of the proximal region and distal region, n=4, ***P*<0.01. (e) Quantitative analysis of angiogenesis, MVD represents vessel numbers/area, n=3, ***P*<0.01. (f) Quantitative analysis of cell angiogenesis, n=3, **P*<0.05, ***P* < 0.01.

Next, we confirmed the influence of shear stress on angiogenesis in vitro. Tube formation results showed that high shear stress induced angiogenesis, and the amount of

angiogenesis was remarkably increased compared to that in low shear stress (Fig. 2c, 2f, n=3).

3.3 High shear stress activates the expression of Id1 in Vivo and in Vitro

Id1 is one of the most important transcription factors regulating angiogenesis, especially in tumor angiogenesis, but there were few publications reporting the relationship between Id1 and shear stress. We next examined whether Id1 expression levels were changed by shear stress. Immunohistochemical staining of proximal and distal blood vessel with Id1 antibody showed that the Id1 expression of proximal region with high shear stress was significantly increased (Fig. 3a, 3b). Then we used a shear stress apparatus to apply shear stress to HUVECs with 5, 15, 25 dyn/cm^2 for 6 hours. RT-PCR (Fig. 3c, n=3) and Western blot (Fig. 3d, 3e, n=3) were used to detect the expression of Id1. Our results showed that both mRNA and protein expression of Id1 were upregulated when shear stress became higher.

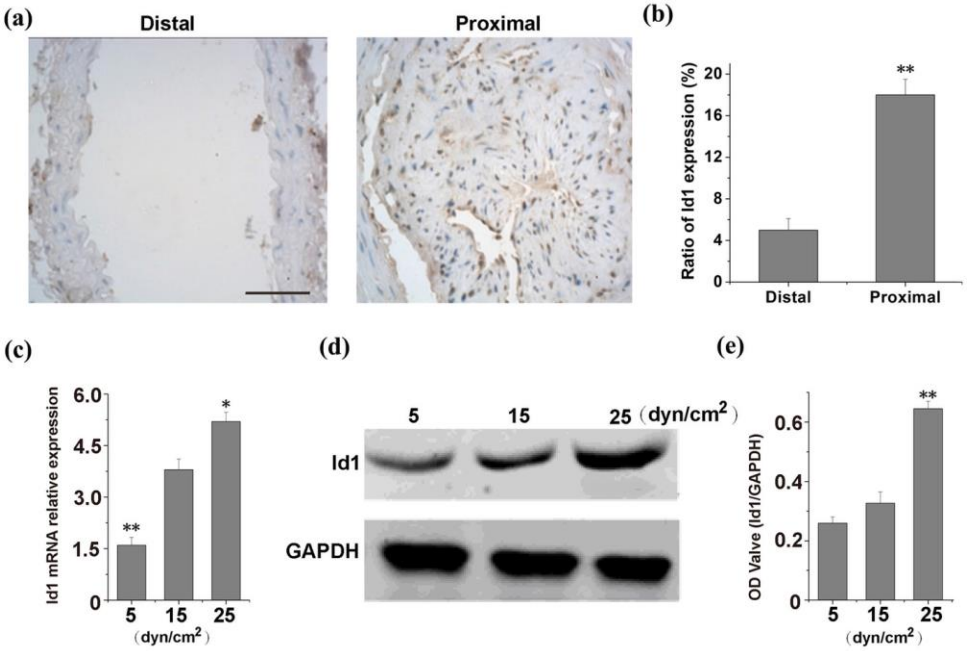

Figure 3: High shear stress activates the expression of Id1 in vivo and in vitro. (a) Immunohistochemical staining of proximal and distal blood vessel with Id1 antibody. scale bar, 100μm. (b) Quantitative analysis of Id1 expression, *p<0.05, **p<0.01, n=3. (c) Quantitative analysis of Id1 mRNA expression after cells were exposed to various shear stress (5, 15, 25 dyn/cm^2), n=3, *p<0.05, **p<0.01 versus 15dyn/cm^2 group. (d) Western blotting analysis and (e) Quantitative analysis of Id1 protein expression. The expression was normalized to that of GAPDH, n=3, *p<0.05, **p<0.01 versus 15dyn/cm^2 group.

3.4 Id1 faciliates ECs migration and angiogensis through collagen degradation

To investigate whether Id1 was involved in regulating EC functions, we established two transfectant clones: Id1 overexpression (Id1-t) and knockdown (Id1-si) ECs to examine whether ectopic Id1 expression in ECs could regulate cell migration and angiogensis. Cell migration experiments showed that Id1 overexpressing (Id1-t) cells had a 50% increase in wound healing rate than the control cells (con-t), migration of the Id1 knockdown (Id1-si) cells was slower than that of the control group (con-si) (Fig. 4a). Thus, Id1-t could significantly increase migration speed compared with Id1-si (Fig. 4b, n=3).

We next cultured cells on the three-dimensional matrix gel with 20% FBS to observe vascular lumen formation. As shown in figure 4c, Id1-t group cells elongated, gradually forming the end-to-end annular tube structure. The vascular tube branch number were analyzed (Fig. 4d, n=3), and the statistical results showed that the angiogenic ability of Id1-t was three times higher than the control group. Meanwhile, it was difficult to connect Id1-si group cells, and they rarely formed a sample tube structure. The angiogenesis ability of Id1-si group also decreased by 45% compared with con-si group. These results showed that high expression of Id1 improved endothelial cells angiogenesis in vitro.

To further confirm whether Id1 can promote matrix degradation, we performed collagen martrigel invasion experiments [Wei D, et al. (2012)]. Crystal violet staining showed that the average invasion cell numbers of Id1-t group and con-t group were $430 \pm 21.5/mm^2$ and $269 \pm 33.5/mm^2$, respectively. Compared to con-t group, Id1-t group were significantly increased (Fig. 4e, 4f, n=4). Consistently, the average invasion number of Id1-si group ($146 \pm 16.5/mm^2$) was significantly decreased compared with that of Id1-si group (Fig. 4e, 4f, n=4).

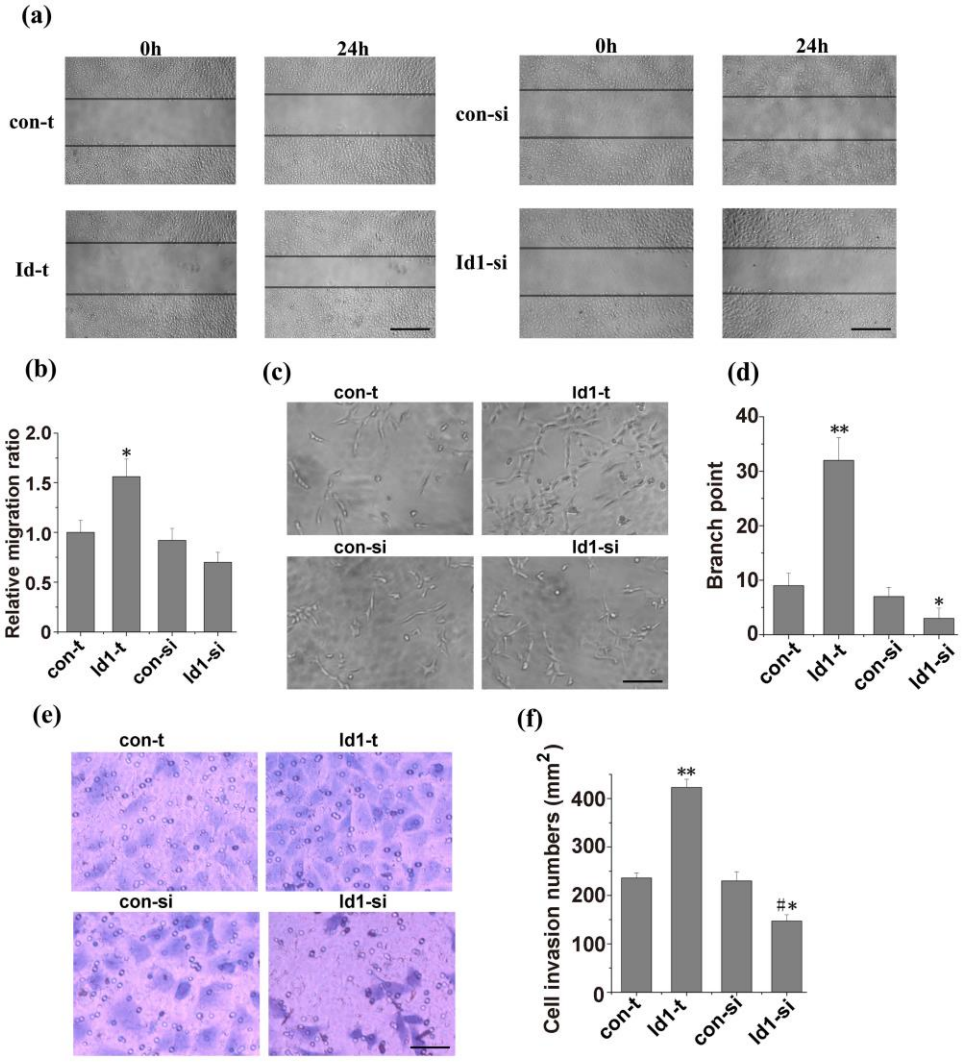

Figure 4: Id1 faciliates ECs migration and angiogensis through collagen degradation. (a) Cells migration after 24 hours, scale bar, 200μm. (b) Cells migration ability analysis, migration velocity of Id1-t group cells was greater than the interference group cells. Id1-t compared with Id1-si, n=3, **P*<0.05. (c) Cells angiogenesis after culturing cells for 2 days, scale bar, 50μm. (d) Statistics analysis the number of branch points between cells, n=3, **P*<0.05, ***P*<0.01 versus con-t group. (e) Martrigel invasion experiments after culturing cells for 48 hours, scale bar, 30μm. (f) Quantitative analysis cell invasion ability, n=4, **P*<0.05, ***P*<0.01 versus con-t group, $^{\#}P$<0.05 versus con-si group.

3.5 Id1 regulates MMP14 and MMP17 expression

To investigate whether Id1 regulates the expression of MMP14 (TM1-MMP) and MMP17

(TM4-MMP), RT-PCR was used to analyze MMP14 and MMP17 expression in Id1-t, con-t, Id1-si and con-si groups, respectively. Results showed that the expression of MMP14 and MMP17 in Id1-t group was increased by nearly 150% and 100%, respectively, compared with con-t group. In Id1-si group, the expression of MMP14, MMP17 was decreased by 50% and 30%, respectively (Fig. 5a, n=5). Likewise, western blot was used to detect the expression of protein levels, Id1-t cells could promote MMP14 and MMP17 protein expression, in contrast, the protein levels of MMP14 and MMP17 were suppressed when Id1 expression was knocked down (Fig. 5b, 5c, n=3).

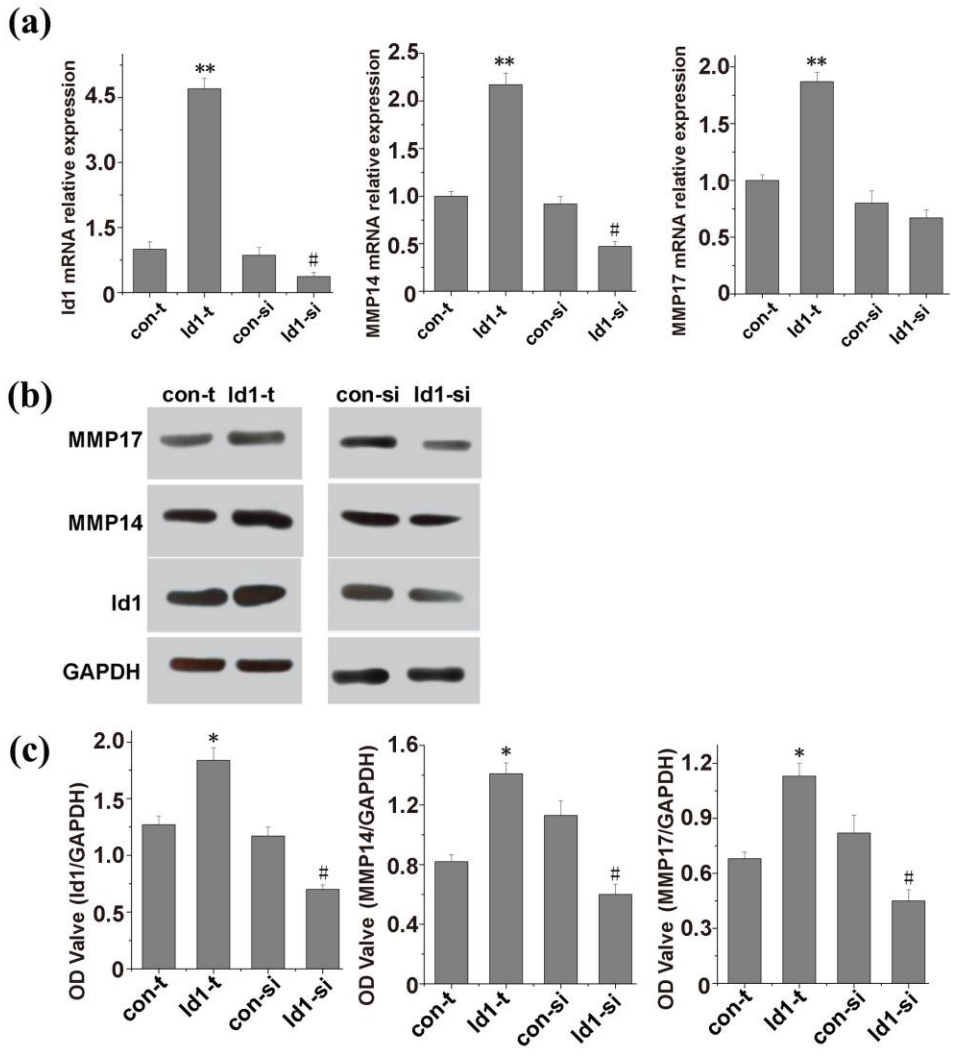

Figure 5: Id1 regulates MMP14 and MMP17 expression. (a) Quantitative PCR detected MMP17, MMP14, Id1 expression, n=5 (b) Western blot detected protein levels of Id1, MMP14, MMP17 and (c) Statistical analysis the protein level of Id1, MMP14 and MMP17, n=3, **P*<0.05 versus con-t group, ***P*<0.01 versus con-t group, $^{\#}P$<0.01 versus

con-si group.

3.6 Vulnerable-probe plaques are localized in high shear stress region in ApoE$^{-/-}$ mice

To further study the role of shear stress in the angiogenesis during vulnerable plaque formation in blood vessels, we performed a silicone ring ligation at the carotid artery of ApoE$^{-/-}$ mice. HE staining (Fig. 2a) and CD31 immunostaining (Fig. 6a) results showed that the proximal region with high flow velocity had plaque formations, and the inside of plaque had a large number of newborn capillaries. Consistently, the distal region with low flow velocity had less capillary formation. CD68 immunostaining results indicated that there were a lot of macrophages in the proximal plaque, however, there were few macrophages in the vessels of the distal region (Fig. 6b).

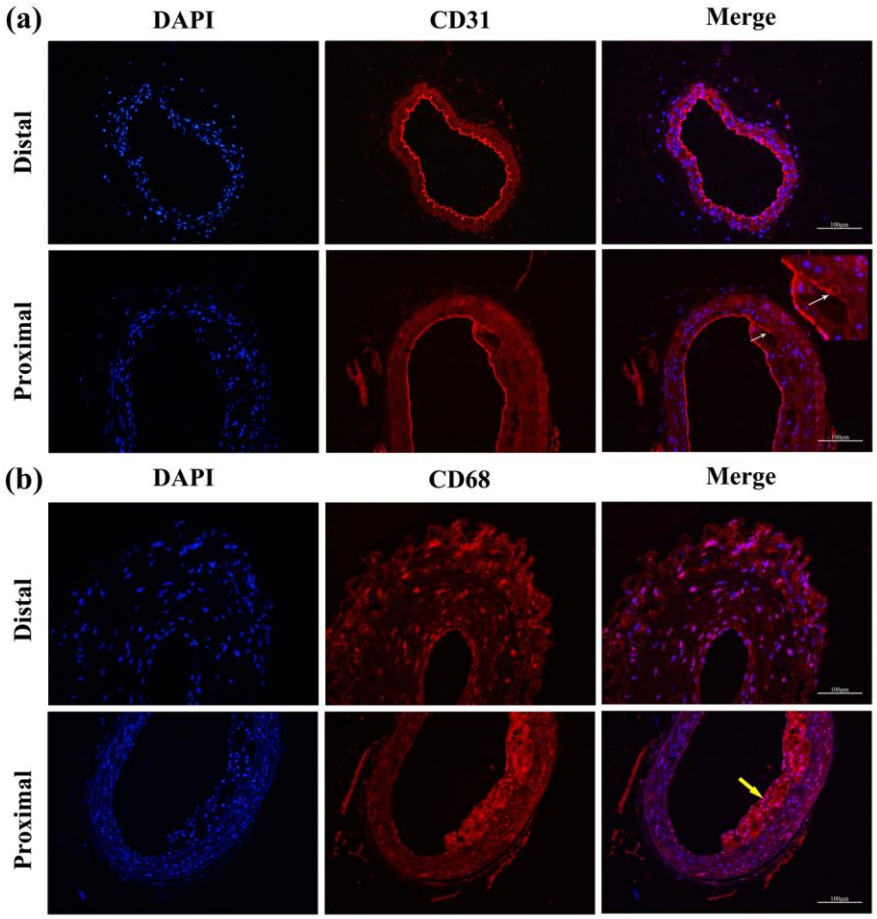

Figure 6: Vulnerable-probe plaque localized in high shear stress with many newborn blood vessels and macrophages. (a) CD31 immunostaining of the proximal and distal blood vessels. The white arrow showed the representatively newborn vessels, (b) CD68 immunostaining of the proximal and distal blood vessels. The yellow arrow displayed the

macrophages recruitment, scale bar, 100 μm.

It is well known that angiogenesis needs protease to degrade extracellular matrix. Masson and VG staining observed various collagen fibers and muscle fibers. Figure 7a illustrated that the total collagens of blood vessels significantly decreased in high shear stress region, especially in the middle and inner layer; whereas, the collagen fiber structure was intact at the distal region with low shear stress, in control and sham group (Fig. 7a, Fig. s2). Moreover, VG staining (Fig. 7a) showed that muscle fibers and elastic plate were degraded and broken at the proximal region with high shear stress. However, in the distal region with low shear stress, muscle fiber layer structure was relatively intact, without fractures. We further quantified the degradation percentage of collagen and muscle fibers. The results showed that high shear stress regions had more serious degradation than low shear stress regions (Fig. 7b, n=3).

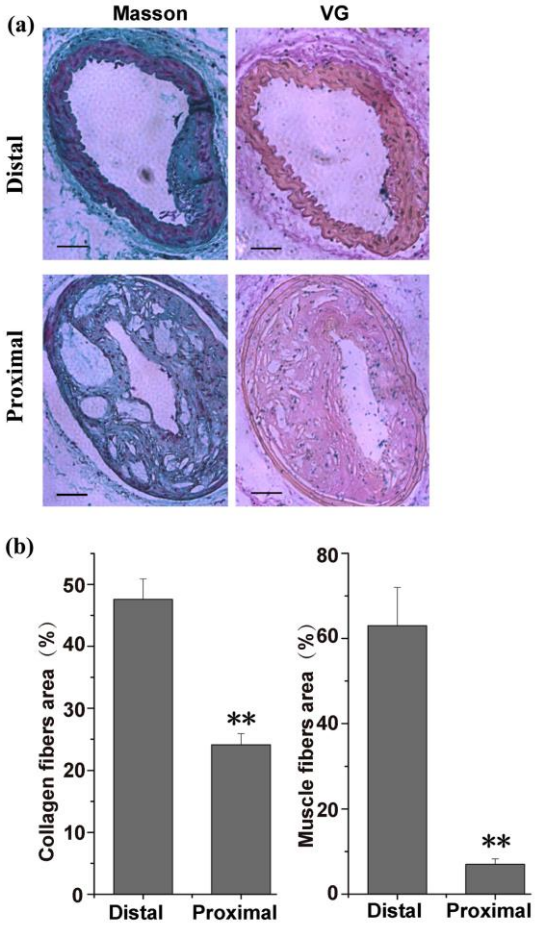

Figure 7: Vulnerable-probe plaque has more collagen and muscle fibers degradation in ApoE$^{-/-}$mice. (a) Masson and VG staining of proximal and distal blood vessel, scale bar, 100μm. (b) Quantitative analysis of intravascular collagen and muscle fibers, n=3, ***P*<0.01 versus distal group.

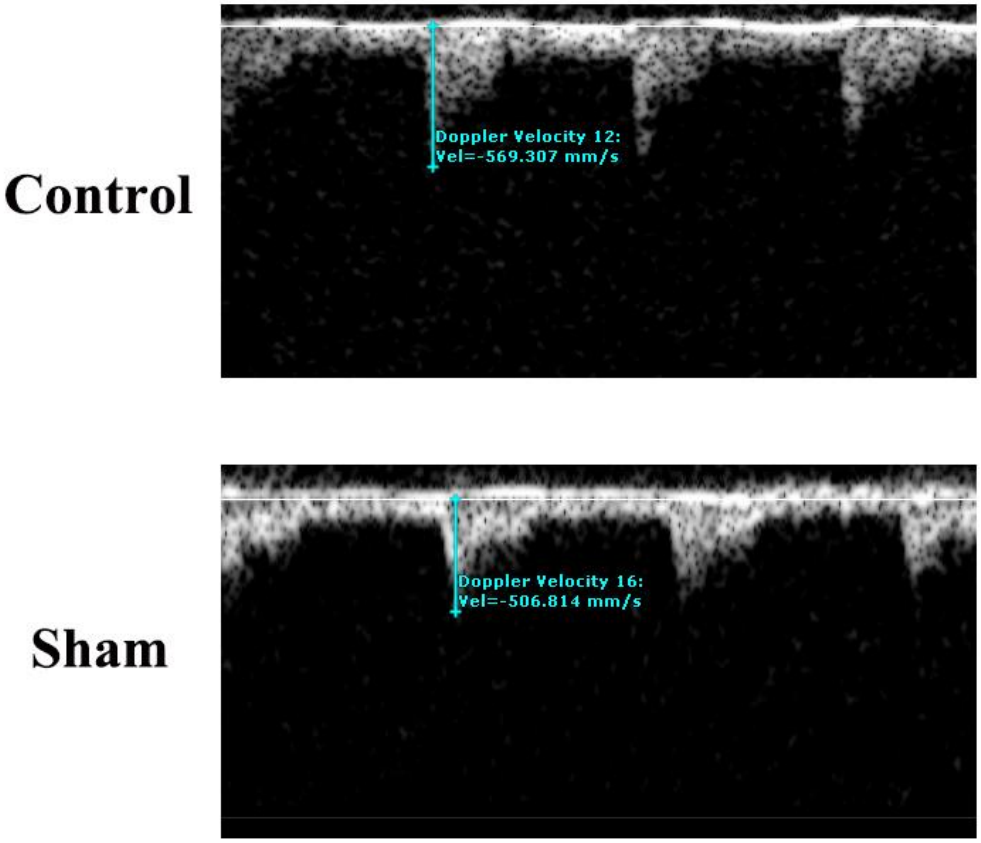

Figure 8: The flow velocity of the carotid artery in control and sham group. It was found that the flow velocity in control and sham group was higher than that in the proximal and distal region of carotid artery with ligation.

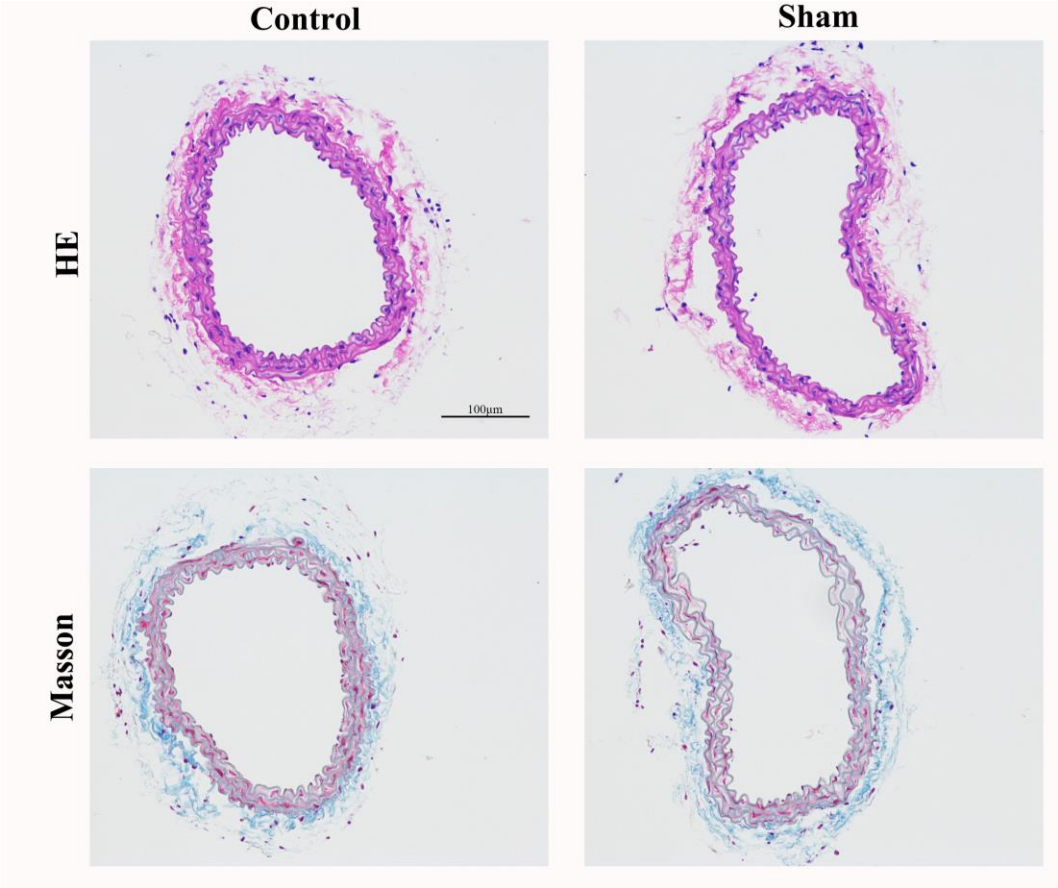

Figure 9: The left carotid artery in control and sham group with HE staining and Masson staining. HE staining showed there were hardly seen intimal hyperplasia and newborn vessels in control and sham group. Masson staining indicated that intravascular collagen was not regraded in control and sham group compared with the proximal, scale bar, 100μm.

4 Discussion

The aim of this study was to investigate the function of high shear stress on angiogenesis. The main findings were that: 1) High shear stress promoted angiogenesis and Id1 expression in vitro and in vivo, 2) Id1 facilitated ECs migration and angiogenesis mediated by collagen degradation, and the expression of MMP14 and MMP17 was needed by collagen degradation and 3) Vulnerable-probe plaques were localized in high shear stress regions in $ApoE^{-/-}$mice.

Some researchers proposed that the biomechanical factors may play an important role in the process of atherosclerosis [Bot et al. (2007), Vucevic, Radak, Milovanovic, Radosavljevic and Mladenovic (2013)]. Chronic changes of blood flow conditions including shear stress may cause the artery wall remodeling, disturb dynamic balance between degradation and

synthesis of extracellular matrix, decrease vascular elasticity modulus, and increase vascular brittleness [Ghaffari, Leask and Jones (2015)].

To test these ideas, we built an animal blood vessel thimble stenosis model to explore the role of shear stress in the process of the plaque formation, and this was a classic model to study plaque formation [Lovett and Rothwell (2003)]. Our results showed that shear stress in the proximal part of carotid artery was much higher than the distal region. These results were consistent with Slager's review that high shear stress localized in the upstream side of the stenosis and high shear stress induced the destabilized cap into a rupture-prone or vulnerable plaque through changing the local biologic effects [Slager et al. (2005)]. Our model was modified from this review. Kuhlmann's paper mentioned an interesting shear stress modifier (cuff) [Kuhlmann et al. (2012)] and we actually found the proximal region in our model was similar to the region in the shear stress modifier (cuff) with high shear stress, but without changing the tensile stress of the proximal region. Based on this model, we demonstrated that the proximal region had a large number of tiny capillaries within the plaque, which almost blocked the whole blood vessel lumen, while the low shear stress in distal region induced serious intimal hyperplasia.

It was reported that the angiogenesis of plaques began at the early stages of atherosclerosis and later gradually increased the density of new capillaries inside plaque [Camare, Pucelle, Negre-Salvayre and Salvayre (2017)]. However, the signaling factors regulating angiogenesis are still not entirely understood, especially under shear stress condition. Our results showed that the high shear stress region had more severe intravascular substrate degradation, with badly damaged layers, outer membrane structures and significantly degraded collagen fibers and muscle fibers. Id1 is a helix-loop-helix transcription factor, which can be expressed by vascular endothelial cells and regulate angiogenesis [Lasorella, Benezra and Iavarone (2014)]. Some recent studies showed that Id1 was also a mechanical response factor [Ni et al. (2010)]. Our analysis of the impact of shear stress on Id1 showed that high shear stress promoted the expression of Id1, and the overexpression of Id1 significantly increased cell migration and angiogenesis through collagen degradation in vitro.

Recent studies also found that MMP expression can increase blood capillary sprout, collagen degradation and ECs invasion, thereby promoting angiogenesis [Bot et al. (2007)]. Therefore, we detected whether Id1 influenced collagen degradation by regulating the expression of MT-MMPs. We used Id1 overexpression and knockdown ECs to demonstrate that Id1 regulated the expression of MMP14 and MMP17. The results showed that Id1 overexpression cells promoted MMP14 and MMP17 expression. These findings suggested that MT-MMPs may be involved in Id1-induced angiogenesis process. Because we did not detect collagen degradation when MMP14 and MMP17 were inhibited in Id1 overexpressing cells, our results only stated that MMP14 and MMP17 were needed in collagen degradation. Another limitation in our present study was that we didn't confirm whether high shear stress mediated angiogenesis via ECs could be abolished by si-Id1 group, which deserved further study in the future. But we had investigated that Id1 facilitated ECs migration and angiogenesis in vitro, and these results can illustrate Id1 participated in the high shear stress-mediated angiogenesis to a certain degree.

Previous studies showed that vulnerable atherosclerotic plaques were often accompanied with pathological angiogenesis [Vucevic, Radak, Milovanovic, Radosavljevic and Mladenovic (2013)]. We then investigated the role of shear stress in the angiogenesis during vulnerable plaque formation in ApoE$^{-/-}$mice. The results showed high shear stress regions had a more serious degradation and more likely to form a vulnerable plaque than low shear stress regions.

In summary, the present study applied a combination of in vitro cell experiments and in vivo animal experiments to demonstrate that high shear stress promote angiogenesis and vulnerable plaque formation through activating Id1. These findings provide a novel mechanism for angiogenesis, and Id1 may be a new therapeutic factor to control angiogenesis, which is related to cardiovascular diseases.

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Conflict of interest：The authors declare that there are no conflicts of interest.

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