Original Research

Fluid shear stress modulates endothelial inflammation by targeting LIMS2

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Impact statement

Whereas we all know that atherosclerosis is the most main common reason of death all over the world, the mechanism of atherosclerosis is still unclear. Recently, more and more evidence indicate that atherosclerosis is a kind of flow-dependent disease, and plenty of mechanosensitive genes have been mentioned in it. Here, we identified for the first time that LIMS2 is a novel flow-sensitive gene and inhibits inflammation by modulating inflammatory factors, VCAM-1, and ICAM-1 in endothelial cells. It will help us to understand the connection between endothelial shear stress and endothelial inflammation deeply; certainly, LIMS2 may also act as a potential target for flow-dependent atherosclerosis.

Abstract

Mechanosensitive genes regulate multiple cardiovascular pathophysiological processes and disorders; however, the role of flow-sensitive genes in atherosclerosis is still unknown. In this study, we identify LIM Zinc Finger Domain Containing 2 (LIMS2) that acts as a mechanosensitive gene downregulated by disturbed flow (d-flow) both in human endothelial cells (ECs) *in vitro* and in mice *in vivo*. Mechanistically, d-flow suppresses LIMS2 expression, which leads to endothelial inflammation by upregulating typical inflammatory factors, VCAM-1, and ICAM-1 in human ECs. The findings indicate that LIMS2, the new flow-sensitive gene, may help us to find a new insight to explain how d-flow caused endothelial inflammation and provide a new therapeutic approach for atherosclerosis in the future.

Keywords: Atherosclerosis, biomarker, endothelial inflammation

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Introduction

Atherosclerosis is the most common reason of death all over the world[1] and thus has been studied extensively; it is a complex chronic disorder and the mechanisms by which it attacks are still being analyzed. So far, what is known is that it usually occurs in the arterial wall and the endothelial layer is attacked primarily in atherosclerosis by inflammation, which induces the endothelium to become more permeable to lipids associated with low-density lipoproteins (LDL). Once the accumulation of these lipids is observed, immune cells such as monocytes migrate across the endothelium and reach the intima, whereupon LDL particles promote the appearance of foam cells to create lesions, which may progressively enlarge with age.[2] Interestingly, it is to be noted that atherosclerotic plaques commonly form at sites experiencing flow separation, like branch, curvature or cross-sectional expansion.[3] At these regions, the flow departs from pulsatile, unidirectional flow to chaotic, disturbed flow (d-flow) characterized by flow-associated zones including reflux, turbulent flow and oscillatory flow.[4–7] d-Flow is known to regulate endothelial cell function via a process known as mechanotransduction. The mechanisms by which d-flow induces pro-atherogenic responses predominantly involve endothelial gene expression, including differential flow-sensitive genes expression.

Recently, many flow-sensitive genes have previously been identified as playing a role in atherosclerosis by their actions in the endothelium, smooth muscle cells, macrophages, or in cholesterol transport.[8–10] Here, we identify that d-flow causes atherosclerosis formation *in vivo* and modulates endothelial inflammation via changing inflammatory factors expression, such as VCAM-1 and ICAM-1. Furthermore, in this study, we demonstrate that LIM Zinc

Finger Domain Containing 2 (LIMS2), a new member of PINCH family, is a novel mechanosensitive gene that is reduced by low endothelial shear stress in endothelial cells (ECs), both *in vitro* and *in vivo*. And then, we demonstrated that the inhibition of LIMS2 expression upregulates VCAM-1 and ICAM-1 level in human ECs. Hence, our findings suggest that LIMS2 is a mechanosensitive and anti-inflammatory gene; it may provide a new possible therapeutic approach to clarify the mechanism of endothelial inflammation and act as a new biomarker of atherosclerosis in the future.

Materials and methods

Partial carotid ligation and endothelial flushing

Two types of mice (C57BL/6 mice and ApoE$^{-/-}$ mice from Jackson Laboratories, eight weeks old, male) were used for all animal experiments. After 2% isoflurane anesthesia, the carotid arteries were ligated partially, and the ineluctable d-flow conditions in the LCA were validated by animal ultrasound (Vevo3100, Visualsonics) with a 30 MHz probe.

For partial carotid ligation of mice, internal carotid, external carotid, and occipital artery of LCA were ligated with 6–0 silk suture except the superior thyroid artery left. Then, mice were sacrificed by CO_2 inhalation. After 48-h ligation, carotids were separated and perfused with heparinized saline for 5 min subsequently. Total intimal RNA of RCA and LCA were isolated and flushed 48 h later with 150 μL QIazol reagent, by previous method that people have demonstrated to be mainly endothelial enriched.[11–13]

Cell culture

Human aortic endothelial cells (HAEC, Lonza CC-2535) were maintained in EGM^{TM}-2 BulletKit (CC-3162), which contains fetal bovine serum (FBS) SDS-attached, multiple growth factors (human fibroblast growth factor basic, h-FGFb; human vascular endothelial growth factor, h-VEGF; analog of human insulin-like growth factor-1, long R3-IGF-1; human epidermal growth factor, hEGF), penicillin/streptomycin (P/S), hydrocortisone solution and Ascorbic Acid Solution (AA). Cells at passages 5–7 were cultured in 10 cm dishes (Falcon 353003) coated with 0.1% gelatin (Sigma Aldrich G9391) at 37°C for following experiments.

Cone and plate viscometer

Briefly, cells were cultured to >90% confluency and then exposed to shear stress in the cone and plate viscometer for 24 h. Cells subjected to laminar shear (LS) stress experienced a one-way shear stress of 20 dyn/cm^2, whereas cells subjected to oscillatory shear (OS) stress experienced one-way shear stress of ±5 dyn/cm^2. Alignment of ECs under LS was visually confirmed by microscopy. Cells were scraped in 600 μL HBSS (Corning 45000–462) and pelleted by centrifugation at 1700 r/min for 3 min. One quarter was resuspended with QIazol (Qiagen 79306) for RNA isolation and the rest was resuspended in phosphate-buffered RIPA with glycerol (Boston Bioproducts BP-421) and protease inhibitor (Sigma Aldrich 11697498001) for protein extraction.

qPCR for target genes

Total RNA was purified using the Qiagen miREasy kit and reverse transcribed using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814). The cDNA was programmed to ABI StepOne Plus qPCR system (10 min at 95°C, followed by 45 cycles of 95°C for 5 s, and 60°C for 30 s) using the target gene primers (LIMS2, KLF2, TSP-1, VCAM-1, ICAM-1) and SYBR Master Mix (ThermoFisher 4472903). 18S was chosen as a housekeeping control. Finally, ΔΔCt method was used to determine the fold changes for all target genes between samples.

BCA and Western blotting

Cell lysate was collected and centrifuged (12,000 × *g*, 10 min, 4°C). The protein-containing supernatant was collected in a separate tube and 2 μL was added to a 96-well plate. Bicinchoninic acid (BCA) was diluted 1:50 in Reagent A (Pierce 23225) and 98 μL of the mixed reagent was added to each protein-containing well. Protein standards were prepared by diluting bovine serum albumin (2 mg/mL, Pierce 23209) to concentrations of 0, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0 and 1.5 mg/mL solutions. After 30 min incubation at 37°C, absorbance was recorded by a microplate reader at 562 nm, and a linear calibration curve was established.

Each protein sample was diluted in 1× RIPA and 6× reducing loading dye (Boston Bioproducts) to achieve a total protein amount of 15 μg, and then subsequently boiled at 95°C for 5 min. Proteins were electrophoresed in 1× running buffer (Tris-Glycine pH 8.8) using a 10% polyacrylamide gel. Then, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad) and blocked in 5% (w/v) non-fat milk in tris-buffered saline with 0.1% Tween (TBST) for 1 h at room temperature. After removing the extra blocking buffer by TBST buffer, the membranes were incubated with the specific primary antibody solution (1:1000) overnight at 4°C cold room (human-LIMS2, Abcam ab272666; mouse-LIMS2, Sigma-Aldrich SAB1302074; VCAM-1, Abcam ab134047; ICAM-1, Abcam ab109361). Following primary antibody exposure, the membrane was incubated with secondary antibody (1:5000) at room temperature (goat anti-mouse or goat anti-rabbit HRP, Santa Cruz SC-2004 or SC-2005) for 1 h. Then, the membrane was filmed by Immobilon Western Chemiluminescent HRP (EMD Millipore, WBKLS0500) and Blue Lite Autorad Film (VWR, 490001–950).

Immunofluorescence staining

Frozen sections of mice carotids were permeabilized in 0.1% Triton-X in PBS for 5–10 min, followed by blocking in either 20% donkey serum or goat serum (Jackson ImmunoResearch, 005–000-121 or 017–000-121) in PBS for 1 h at room temperature. Blocked sides were incubated in the primary antibody (1:100) (mouse-LIMS2, Sigma-Aldrich SAB1302074) overnight at 4°C cold room and followed by next incubation with secondary antibody

(1:250 v/v) (Alexa Fluor 568 conjugated goat anti-rabbit or donkey anti-goat, Life Technologies).

Hematoxylin and eosin staining

Frozen sections were stained using the American MasterTech kit. As per manufacturer's protocol, slides were rinsed in running ddH_2O for 2 min, incubated in Harris hematoxylin for 5 min, rinsed and incubated in differentiating solution for 1 min, rinsed and incubated with Bluing solution for 30 s, rinsed and incubated in 70% ethanol for 1 min, followed by direct incubation in eosin in 1 min. Finally, the slides were dehydrated in three changes of 100% ethanol and xylene. Slides were mounted and imaged on the Hamamatsu Nanozoomer. Arterial diameter was quantified using ImageJ software.

Statistical analysis

Graph-Pad Prism 5 Software was used for statistical analyses in this study. Generally, all error bars measured are SEM unless special instruction. One-way Student's t-tests were used to verify pairwise comparisons. Tukey's multiple comparison tests were used to check multiple comparisons. Differences were considered significant at P-values <0.05.

Results

d-Flow causes atherosclerosis in ApoE$^{-/-}$ mice and active inflammation via upregulating VCAM-1 and ICAM-1 expression both in human and mouse ECs

Initially, we built d-flow model via partial carotid ligation approach in ApoE$^{-/-}$ mice. After 28-day ligation, we found that atherosclerosis was induced, and there were plenty of atherosclerotic plaque formation in left carotid artery (Figure 1(a) and (b)). And then, we tested inflammatory factors, VCAM-1 gene, and ICAM-1 gene expression in endothelial RNA derived from our animal model of acute d-flow-induced atherosclerosis. In ligated C57BL/6 mice, VCAM-1 was increased by six-fold in the flow-disturbed LCAs intima as compared to the RCAs post 48 h ligation. ICAM-1 was increased by more than three-fold in the flow-disturbed LCAs intima as compared to the RCAs post 48 h ligation. Similarly, VCAM-1 was increased by seven-fold in the flow-disturbed LCAs media and adventitia. ICAM-1 was increased by more than four-fold in the flow-disturbed media and adventitia of LCAs (Figure 1(c)). Furthermore, given the well-established link between d-flow-related shear stress and endothelial inflammation, HAECs were subjected to either LS or OS condition for 24 h. VCAM-1 gene level was increased in OS conditions

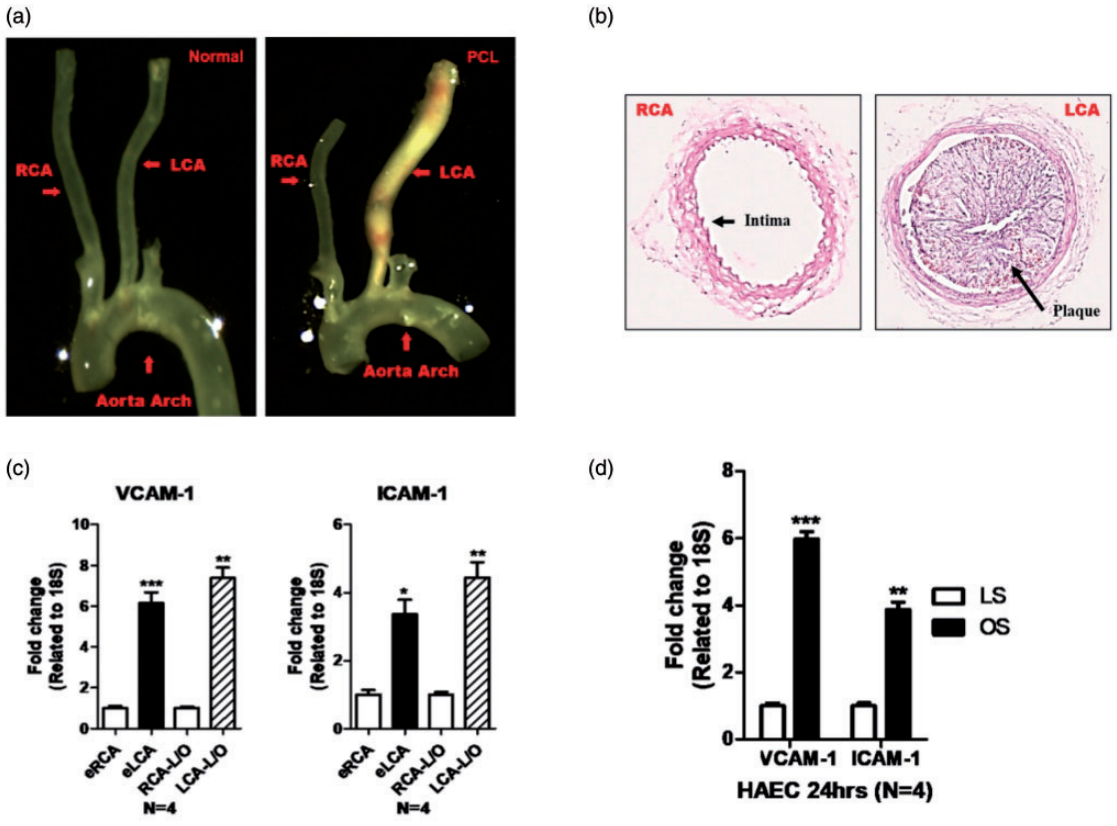

Figure 1. D-flow induces atherosclerotic plaque formation in ApoE$^{-/-}$ mice and causes inflammation in human and ECs. (a) Left carotid artery (LCA) showed atherosclerotic plaque formation compared with right carotid artery (RCA) after 28-day partial carotid ligation (PCL) in ApoE$^{-/-}$ mice. (b) HE staining showed unbroken RCA and obvious atherosclerotic plaque accumulation in LCA after 28-day PCL surgery for ApoE$^{-/-}$ mice (N = 4). (c) C57BL/6 mice were partially ligated for 48 h and then sacrificed. Endothelial-enriched total RNA (eRCA, eLCA) and total RNA of media and adventitia (RCA left over, RCA-L/O; LCA left over, LCA-L/O) were obtained from the LCA and RCA. VCAM-1 and ICAM-1 gene expression were measured and normalized to 18S (N = 4, *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$). (d) HAEC were exposed to LS or OS for 24 h. Following shear, RNA was collected for qPCR and VCAM-1 and ICAM-1 gene level were measured and normalized to 18S. (N = 4, **=$p<0.01$, ***=$p<0.001$). (A color version of this figure is available in the online journal.)

by nearly six-fold as compared to LS condition; ICAM-1 gene level was increased in OS conditions by nearly four-fold (Figure 1(d)). Thus, these results indicated that d-flow was related to atherosclerosis and endothelial inflammation both in human and mouse ECs.

LIMS2 is a novel mechanosensitive gene and a conserved target in human and mice

To figure out how d-flow could affect endothelial inflammation, we found a new mechanosensitive gene, LIMS2, modulated by d-flow both in human and mice. Firstly, we tested LIMS2 levels in endothelial RNA derived from our animal model of acute d-flow-induced atherosclerosis (PCL) and in the chronically flow-disturbed LCA (Figure 2 (a)). Two classic flow-sensitive genes, KLF2 and TSP-1, were treated as positive controls, and their expressions in LCA after 48-h ligation were similar as previous studies (Figure 2(b)). It is represented that d-flow were induced in LCA. Then, in acute d-flow model, LIMS2 was suppressed by 80% in the flow-disturbed intima and more than 70% in the flow-disturbed media and adventitia of LCAs as compared to the RCAs 48 h post-ligation. Moreover, in the chronically flow-disturbed LCAs, LIMS2 was suppressed by more than 70% both in the flow-disturbed intima and media and adventitia (Figure 2(c)). In addition, we also found that LIMS2 protein signal was disappeared in flow-disturbed LCA compared to the RCAs (Figure 2(d)). These *in vivo* results indicated that LIMS2 is a flow-sensitive gene. For further validation, we tested LIMS2 in HAECs and subjected to either OS or LS by cone-and-plate device for 24 h. We noticed that the HAECs pointed in the same direction and were arranged linearly and tightly under LS condition, whereas OS induced chaotic arrangement and abnormal cell morphology (Figure 3(a)). Then, we validated that KLF2 were reduced by OS but TSP-1 were upregulated by OS (Figure 3(b)). Moreover, we identified that LIMS2 gene expression was inhibited in OS conditions by 70% as compared to LS condition (Figure 3(c)). LIMS2 protein level was also decreased in OS-induced HAECs by more than 60% (Figure 3(d) and (e)). These results, taken together, show that LIMS2 is mechanosensitive in both human and mouse endothelium and that LIMS2 may decrease over longer periods of exposure to d-flow.

LIMS2 silencing induces endothelial inflammation in human ECs

The previous results indicated that d-flow induced atherosclerosis and endothelial inflammation. To test whether the loss of LIMS2 may drive d-flow-related endothelial inflammation in atherosclerosis or not, first of all, we exposed HAECs in different doses of siLIMS2 with static condition to ensure an effective dose of siLIMS2. We noticed that the effect of siLIMS2 showed dose-dependent character in

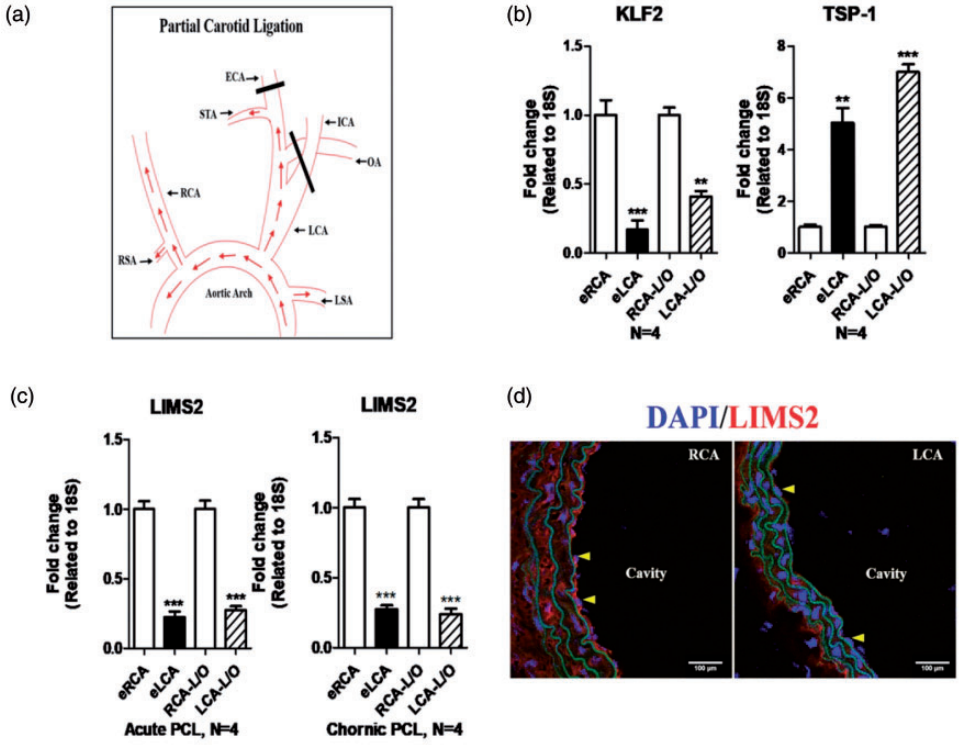

Figure 2. LIMS2 is a novel mechanosensitive gene that is inhibited by d-flow in mice. (a) For building PCL animal model, C57BL/6 mice left carotid artery were partial ligated by suture, included internal carotid artery (ICA), external carotid artery (ECA), occipital artery (OA). (b) C57BL/6 mice were partially ligated for 48 h and then sacrificed. Endothelial-enriched total RNA (eRCA, eLCA) and total RNA of media and adventitia (RCA-L/O, LCA-L/O) were obtained from the LCA and RCA. KLF2 gene and TSP-1 gene level as positive control were measured and normalized to 18S ($N = 4$, **$=p < 0.01$, ***$=p < 0.001$). (c) C57BL/6 mice were partially ligated for 48 h (acute PCL group) or 2 week (chronic PCL group) and then sacrificed. Endothelial-enriched total RNA (eRCA, eLCA) and total RNA of media and adventitia (RCA-L/O, LCA-L/O) were obtained from the LCA and RCA. LIMS2 gene level was measured and normalized to 18S ($N = 4$, ***$=p < 0.001$). (d) C57BL/6 mice were partially ligated for 48 h (acute PCL group) and then sacrificed. RCA and LCA were separated and stained for LIMS2. (A color version of this figure is available in the online journal.)

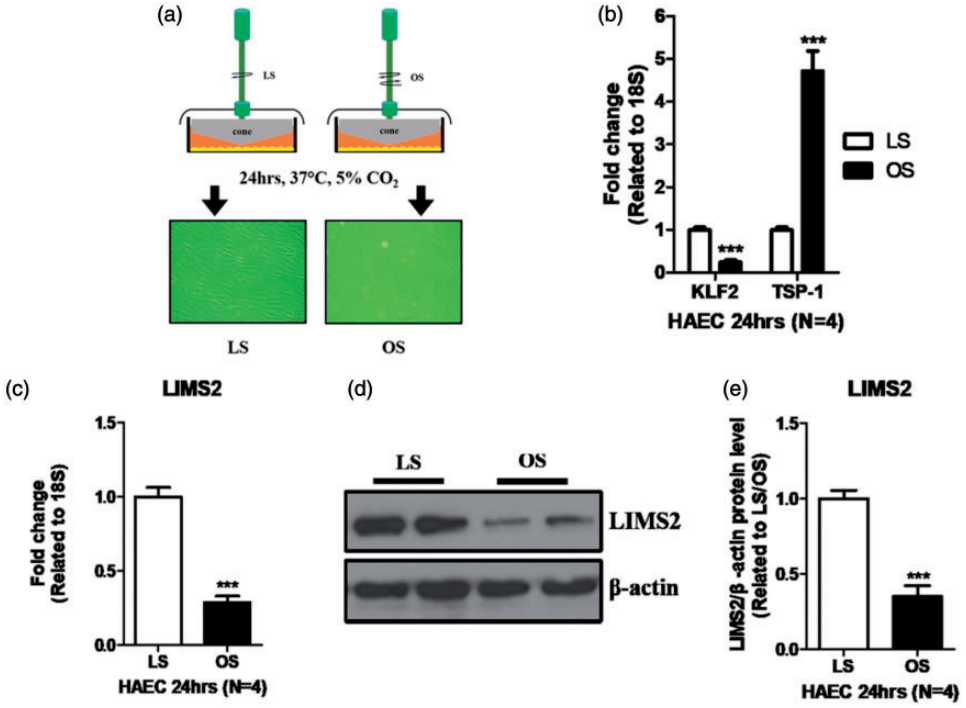

Figure 3. LIMS2 expression are suppressed by oscillatory shear (OS) in human ECs. (a) HAEC were subjected to laminar shear (LS, 20 dyn/cm^2) or oscillatory shear (OS, ±5dyn/cm^2) for 24 h. (b) HAEC were exposed to LS or OS for 24 h. Following shear, RNA was collected for qPCR and KLF2 gene and TSP-1 gene acted as positive control were measured and normalized to 18S. (N = 4, ***=$p < 0.001$). (C) HAEC were exposed to LS or OS for 24 h. Following shear, RNA was collected for qPCR and LIMS2 gene was measured and normalized to 18S. (N = 4, ***=$p < 0.001$). (d and e) HAEC were exposed to LS or OS for 24 h. Following shear, LIMS2 protein level was measured by western blot (N = 4, ***=$p < 0.001$). (A color version of this figure is available in the online journal.)

HAECs, 150 nM siLIMS2 could inhibit 80% LIMS2 level, and was treated as an effective dose in following steps (Figure 4(a)). Then, HAECs were treated with siLIMS2 for 48 h, subjected to LS condition, and either lysed for RNA or protein to determine the expression of LIMS2 and inflammatory factors, such as VCAM-1 and ICAM-1. Finally, the treatment with 150 nM siLIMS2 in LS condition significantly decreased LIMS2 gene expression by more than 70% (Figure 4(b)), whereas VCAM-1 and ICAM-1 were upregulated separately by more than four-fold and two-fold (Figure 4(c)). Certainly, we also mentioned that LIMS2 protein level was suppressed by more than 60%, whereas VCAM-1 and ICAM-1 were upregulated separately by more than four-fold and three-fold (Figure 4(d) and (e)). Hence, as LIMS2 is lost by d-flow and induces endothelial inflammation, these results indicate that the loss of LIMS2 can replicate the effects seen by d-flow. Furthermore, it represents that rescuing LIMS2 in d-flow-related area of artery may blunt endothelial inflammation and LIMS2 may act as a possible biomarker for atherosclerosis in the future.

Discussion

It is well accepted that atherosclerosis is an inflammatory, chronic and fibroproliferative disease. Increasingly, evidence demonstrates that d-flow plays a major role in atherosclerotic plaque initiation and aggressive progression and plaque activation.[14,15] Previous studies indicated that the whole arterial network was not implicated in atherosclerosis; the most common site of atherosclerosis is d-flow susceptible area, including branch point, bifurcation of arteries.[13] In addition, endothelial cells attached to blood flow directly and were influenced by d-flow primarily.[16–18] In this study, we validated that d-flow induced atherosclerosis formation in ligated LCA of ApoE$^{-/-}$ mice and endothelial inflammation was activated via upregulating some inflammatory factors, VCAM-1 and ICAM-1 in OS condition both in human and mice ECs.[19,20] These findings proved that flow-dependent endothelial inflammation may be the typical mechanism of atherosclerosis.

Then, to figure out the connection between d-flow and endothelial inflammation, we mentioned that there are many endothelial genes that have mechanosensitive features and these genes were affected by d-flow in atherosclerosis. Some of flow-sensitive genes were reduced by d-flow in ECs, and loss of them would cause endothelial dysfunction and break the normal, healthy vessel microenvironment.[10,21] KLF2, a classic flow-sensitive gene, has already been reported as a main atheroprotective master molecule *in vivo* and *in vitro*. Silencing KLF2 in endothelial cells by d-flow could lead to inflammation via inhibiting activated NF-kB signaling pathway and suppressing anti-inflammatory factors such as eNOS secretion.[10,22,23] For TIMP3, NOS3, KLF4, and other flow-downregulated genes, they also possessed similar beneficial functions to atherosclerosis.[24–26] On the contrary, some other genes like TSP-1, BMP-4, HIF-1α, etc.[27–29] were increased by d-flow in ECs and improved endothelial inflammation via modulating multiple signaling pathways, including NF-kB signaling pathway, PI3K/AKT signaling pathway,

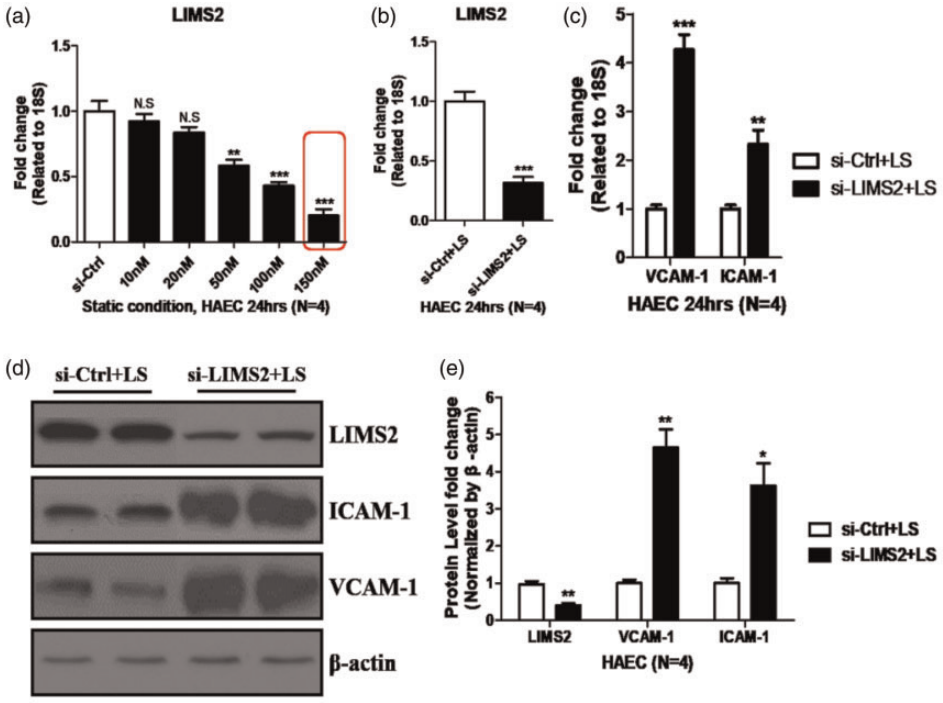

Figure 4. Silencing LIMS2 expression modulates endothelial inflammation via upregulating VCAM-1 and ICAM-1 in human ECs. (a) HAECs were transfected with different dose of siLIMS2 for 48 h. Then, the cells were lysed and RNA was collected for determination of the LIMS2 gene level. ($N = 4$, **=$p < 0.01$, ***=$p < 0.001$). (b) HAECs were transfected with 150 nM of siLIMS2 and subjected to LS 24 h later. 24-h post shear, the cells were lysed and RNA was collected for determination of the LIMS2 gene level. ($N = 4$, ***=$p < 0.001$). (c) Alternatively, the cells were lysed at 24 h and RNA was collected for determination of the inflammatory markers VCAM-1 and ICAM-1. ($N = 4$, **=$p < 0.01$, ***=$p < 0.001$). (d and e) The protein was collected and measured by western blot for determination of the VCAM-1 and ICAM-1 protein expression. ($N = 4$, *=$p < 0.05$, **=$p < 0.01$). (A color version of this figure is available in the online journal.)

Notch signaling pathway, etc.[30–32] In this study, we used KLF2 and TSP-1 as positive controls to ensure that d-flow has been built in animal model and cell experiment. We found that decreasing KLF2 and increasing TSP-1 expression in OS condition were as similar as previous studies. Furthermore, we demonstrated for the first time that LIMS2 was reduced by OS in human ECs as well as acute and chronically flow-disturbed regions in mice. The result represents that LIMS2 is a novel mechanosensitive gene both in human and mouse ECs. We also discovered that silencing LIMS2 expression in ECs by siLIMS2 profoundly induced EC inflammation by upregulating VCAM-1 and ICAM-1. VCAM-1 and ICAM-1 are two classic inflammatory factors related to atherosclerosis and modulated by multiple inflammatory signaling pathways.[33–35] The loss of LIMS2 in normal shear stress condition caused upregulation of VCAM-1 and ICAM-1, suggesting that LIMS2 is an anti-inflammatory target for flow-dependent endothelial inflammation. In addition, the limitation also should be mentioned because the anti-inflammatory function of LIMS2 never been reported before, the specific mechanism of LISM2 in endothelial inflammation should be clarified, and the connection between LIMS2 and atherosclerosis should be confirmed in the next step.

So far, there are a few researches that have been reported about LIMS2; we just known that LIMS2 is a new member of PINCH family and is a cytoskeletal-associated protein localized in focal adhesions.[36] Some reports showed that the loss of LIMS2 could modulate tumor cells migration in gastric cancer and colon cancer[37,38]; silencing LIMS2 could also cause severe cardiomyopathy and heart failure in mice,[39] whereas the mechanism of LIMS2 in these disorders has never been reported. In this study, LIMS2 is firstly mentioned as a novel mechanosensitive gene in endothelial cells and has a potential anti-inflammatory effect in endothelial inflammation. In the following steps, we will try to clarify the mechanism of LIMS2 in flow-dependent endothelial inflammation. We hope that LIMS2 will help us to find a new insight to explain how d-flow caused endothelial inflammation and provide a new therapeutic approach for flow-associated diseases in the future.

Conclusions

In this study, we validate that LIMS2 is a novel mechanosensitive gene and is downregulated by d-flow or OS both in human and mice. The loss of LIMS2 in human ECs activates endothelial inflammation via upregulating inflammatory factors such as VCAM-1 and ICAM-1 expression.

Authors' contributions: JYW was responsible for conception and design. SYJZ measured data analysis and interpretation. All authors wrote the article.

DECLARATION OF CONFLICTING INTERESTS

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