

## Original Article

# Regulation of Kruppel-like factor 2 (KLF2) in the pathogenesis of intracranial aneurysm induced by hemodynamics

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**Abstract:** Kruppel-like factor 2 (KLF2) has been found to regulate the reconstruction of vascular wall tissue and participate in the pathogenic mechanism of intracranial aneurysms. However, there is a paucity of research in this area. The present study aimed to investigate the regulatory effect of KLF2 on intracranial aneurysm (IA) and explore novel therapeutic strategies for treating IA. Experimental animal models were established with SPF New Zealand rabbits by bilateral carotid artery ligation (BCAL). Morphology of basilar artery bifurcation was detected using HE, EVG, Masson and immunohistochemical (IHC) staining. Vascular smooth muscle cells were harvested from basilar artery and cultured to establish KLF2 up-regulated and down-regulated cell models. The mRNA expression of KLF2, eNOS, ICAM-1 and MMP-9 were detected using real-time quantitative PCR (RT-qPCR). Protein expression of KLF2 and MAPKs pathway were measured using western blot. IA models were successfully established by bilateral carotid artery ligation. KLF2 expression was inconsistent with the variation of hemodynamics. In the KLF2 overexpression group, the mRNA expression of eNOS was increased, while that of ICAM-1 and MMP-9 was decreased. When KLF2 was up-regulated, the phosphorylation activity of p38 pathway was increased. In conclusion, results reveal that KLF2 is up-regulated in the vascular wall of basilar artery, and its overexpression regulates the pathogenesis of IA, which may be a self-protection mechanism of the arterial wall, providing a novel insight for therapy of IA.

**Keywords:** Kruppel-like factor 2, intracranial aneurysm, hemodynamics, p38 pathway, ICAM-1, MMP-9

## Introduction

Intracranial aneurysm (IA) is a major cause of subarachnoid haemorrhage (SAH) and the pathogenesis is not well understood. Research has found that hemodynamics play an important role in the pathogenesis of aneurysms. Hemodynamic aberrance causes a vascular inflammatory response and cell apoptosis, which influences the remodeling of intracranial vessel walls and reduces the ability to resist tension [1]. Additional studies have confirmed IA might be triggered by the imbalance of local blood pressure and the integrity of the vascular wall. Endotheliocyte is the first physical barrier affected by haemodynamics, and therefore it is extremely important in the pathogenesis of IA.

Kruppel-like factor (KLF), a family of transcription factors found in drosophila, has 17 members in mammals and mediates various impor-

tant physiological and pathological processes [2, 3]. KLF2 is typically expressed by endotheliocyte, which regulates the morphology and cell cycle, which itself is regulated by wall shear stress (WSS) [4]. KLF2 is widely references in atherosclerosis research, and it regulates numerous gene expression of endotheliocyte, including endothelin, endothelial nitric oxide synthase (eNOS), and matrix metalloproteinases (MMPs) regulation pathways [5].

In aneurysmal models induced by blood flow dynamics, it is assumed that hemodynamics result in KLF2 expression variation in endotheliocyte, and mediate the inflammation and reconstruction of vascular wall. Therefore, the present study was designed to investigate the interaction between hemodynamic change and KLF2 expression of the basilar artery and inflammatory factor expression of downstream eNOS, ICAM-1 and MMP-9 in rabbit IA models.

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Further, KLF2 acts as an intervention to affect the expression of MAPK signaling molecule. This study aims to investigate the mechanism of KLF2 in the pathogenesis of IA and provide novel theoretical basis for prevention and therapy.

### Materials and methods

#### *Animals and models*

All experimental protocols were approved by the Ethics Committee of Changhai Hospital and were performed in accordance with the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Experimental animal models were established with 36 male SPF New Zealand rabbits, weighing 2.5 kg-3.5 kg (Laboratory Animal Center, Institute of Naval Medicine, Second Military Medical University, Shanghai, China), by bilateral common carotid artery ligation (BCAL). Rabbits were randomly assigned into three experimental groups: sham (n=12), unilateral common carotid artery ligation (UCAL) (n=12), and bilateral common carotid artery ligation (BCAL) (n=12). Rabbits were anesthetized with xylazine hydrochloride (0.2 ml/kg) and sodium pentobarbital (1%, 20 mg/kg, intraperitoneally). After carotid artery ligation, the incision was sutured. Rabbits had access to food and water ad libitum in a humidity- and temperature-controlled room. Light was controlled on a 12 hour light/dark cycle.

#### *Trans cranial doppler sonography (TCD)*

After ligation, the flow velocity of basilar artery was detected using trans cranial doppler sonography (Philips, Holland) at the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup> week. Rabbits were fixed in prone position and the occipitalia was exposed. The occipitalia was scanned using an ultrasonic probe parallel to the basal artery, which was recorded as maximum flow velocity.

#### *HE, EVG, Masson staining*

Anaesthesia method was similar to the aforementioned section. The chest was opened under the xiphoid, and heart was visible. Subsequently, 4% normal saline and 4% paraformaldehyde were perfused for 30 minutes through left ventricular to adequately fix. The

skull was unfolded and basal artery and brainstem were integrally taken out. A part of the top bifurcation area of basal artery was intercepted and further fixed with paraformaldehyde for paraffin-embedded sections. Another part of basal artery was frozen with liquid nitrogen and stored at -80°C for western blot and real-time PCR. 6 µm paraffin sections were performed with HE staining, elastica van Gieson (EVG) staining and Masson staining according to the instruction manual.

#### *Immunohistochemistry*

Sections were blocked in normal goat serum and incubated with primary antibody overnight for 4°C. They were then washed with PBS and incubated with biotin labeled secondary antibody for 30 minutes at 37°C. After being washed with PBS, horseradish peroxidase was added and incubated for 10-30 minutes at 37°C. Finally, sections were stained by DAB (Xinran Biotechnology, Shanghai, China) for 3-5 minutes. Quantification and histogram analysis of immunostained images was performed using the ImageJ software.

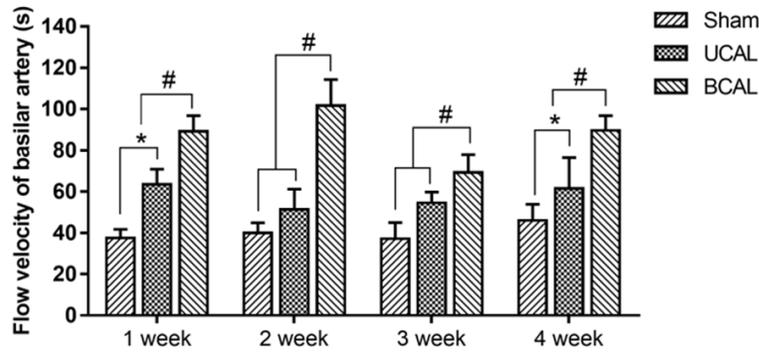
#### *Cell culture*

The basilar artery was harvested from healthy male rabbits. Vascular smooth muscle cells were cultured by explant method. When the cells grew to converge in F-12 medium with 50% FBS, it was digested by 0.25% protease. Three to five passages of cells were inoculated in Transwell lower chamber with  $1 \times 10^5$ . After washed by PBS three times, cells were cultivated in DMEM + 10% FBS medium.

#### *Lipofectamine transfection and siRNA*

In basilar artery smooth muscle cells, Lipofectamine transfection (Invitrogen, Carlsbad, Calif, USA) and siRNA (Santa Cruz, Dallas, TX, USA) were applied to create KLF2 overexpression and under-expression according to the manufacturer's instruction. The sequences were as follows: forward, 5'-AGACCUACACCAAGAGUUCGCAUC-3'; reverse, 5'-CAUGUCCGUUUCAGUGCGC-3'. Lipofectamine 2000 (5 ul) was diluted with 200 ul serum-free RPMI 1640, and incubated for five minutes at room temperature. Plasmids were added into the diluted solution and incubated for 20 minutes to allow the liposome to encase the plasmid sufficiently. 8

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**Figure 1.** Flow velocity of basilar artery was respectively detected by transcranial doppler sonography at 1 week, 2 week, 3 week, 4 week after ligation. Sham represented Sham-operated group. UCAL represented unilateral common carotid artery ligation group. BCAL represented bilateral common carotid artery ligation group. Data was represented as means  $\pm$  standard deviation (SD). \* $P < 0.05$  represented statistical difference between Sham and UCAL group. # $P < 0.05$  represented statistical difference respectively compared with Sham and UCAL group.

$\mu$ l siRNA duplexes and 6  $\mu$ l siRNA transfection reagent were added into 100  $\mu$ l siRNA transfection medium and incubated for 30 minutes at room temperature. The mixture of transfection reagent and basilar artery smooth muscle cells were incubated at 37°C for six hours. Finally, the medium was changed to conventional medium and cultured sequentially for 48 h.

### Real-time polymerase chain reaction (PCR)

Cerebrum tissue was frozen and thawed. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, Calif, USA) according to the manufacturer's instructions. The RNA concentration was detected by ultraviolet spectrophotometer at 260-280 nm and the appropriate OD value was 1.8-2.0. The total reverse transcription reaction system was 20  $\mu$ l (RNA 1  $\mu$ l) to synthesize cDNA with SuperScript First-Strand Synthesis system (Invitrogen, Carlsbad, Calif, USA). The PCR primers were as following: eNOS, forward: 5'-TGG TACATGAG CACTGAGA TCG-3', reverse: 5'-CCACGTTGATTCCACTGCTG-3'; ICAM-1, forward: 5'-GAATGGAAGGCTGGA-TGAAA-3', reverse: 5'-TTCTGGTG CAGCATAGTT-GG-3'; MMP-9, forward: 5'-ACGCACGACGTCTT-CCAGTA-3', reverse: 5'-CCACCTGGTTCAACTCA-CTCC-3'; GAPDH, forward: 5'-GCACCGTCAAGG-CTGAGAAC-3', reverse: 5'-TGGTGAAGACGCCAG-TGGA-3'. PCR amplification was carried out by ABI PRISM 7900 thermocycler using SYBR Premix Taq (Applied Biosystems, Foster, Calif, USA). The reaction conditions contains: initial

denaturation at 94°C for 30 s, 94°C for 20 s for 40 cycles, 61°C for 30 s, 72°C for 30 s, final elongation step at 72°C for one min. The results were calculated compared to GAPDH using the  $2^{-\Delta\Delta Ct}$  method.

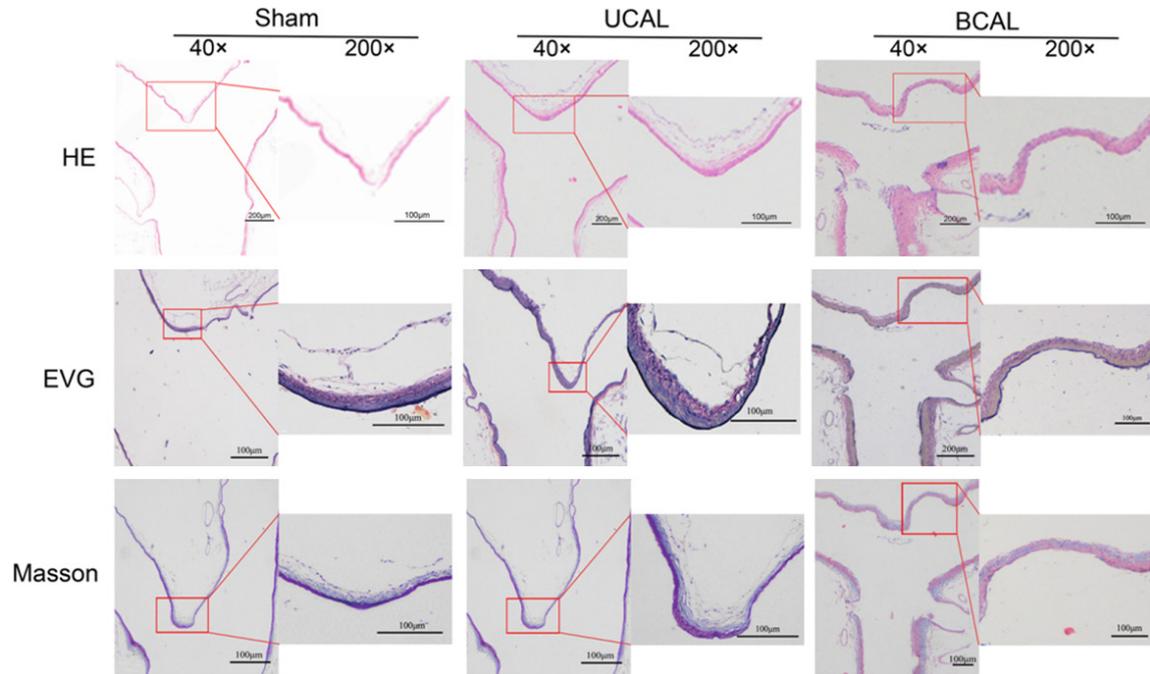
### Western blot

Total protein of basilar artery bifurcation were dissected and homogenized on ice in RIPA buffer (Invitrogen, Carlsbad, Calif, USA) containing a protease inhibitor cocktail PMSF (Roche, Mannheim, Germany). The lysate was centrifuged at 20,000 g for ten minutes, and the supernatant was sub-packaged into EP tube for subsequent experiments. Protein concentrations were measured by comparison with a known concentration of coomassie brilliant blue (Beyotime, Jiangsu, China). A mixture of protein and sample buffer with 10% 2-mercaptoethanol was subjected to 15% SDS-PAGE. After electrophoresis, the separated protein was transferred onto a PVDF membrane. After blocking, the membrane was probed with the primary antibodies and incubated at 4°C overnight. Primary antibodies used for immunoblotting were as follows: KLF2 (1:200, mouse anti-KLF2, HZ47054, Yuanmu, China), p38, p-p38, ERK1/2, p-ERK1/2, JNK, p-JNK (all from Cell Signaling, Danvers, MA, USA). GAPDH (1:200, sc-51907, Santa Cruz, USA). Then, the PVDF membrane was incubated with secondary antibody (1:200, goat anti-mouse, A0216, Beyotime, China) at room temperature for one hour. Finally, the PVDF membrane was washed again with TBST three times for 10 minutes. Quantification of the protein bands was performed using the software Quantity One (Bio-Rad Laboratories, Hercules, CA). Data were normalized against GAPDH.

### Statistical analysis

All quantitative values are presented as the mean  $\pm$  SD. Data were statistically analyzed using one-way ANOVA followed by Fisher's LSD test and student's t-test. All the Statistical analyses were carried out by using SPSS 19.0 (Chicago, IL, USA) and Graphpad prism soft-

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**Figure 2.** The HE, EVG and Masson staining of basilar artery endotheliocyte. HE staining revealed the coronal section of basilar artery bifurcation vessel. EVG staining revealed the internal elastic membrane of basilar artery bifurcation vessel. Masson staining revealed the medial smooth muscle of basilar artery bifurcation vessel. The left part of each image was the bifurcation vessel of basilar artery (40×), and the right part was the partial enlarged view (200×). Scale was in the lower right corner. Sham represented Sham-operated group. UCAL represented unilateral common carotid artery ligation group. BCAL represented bilateral common carotid artery ligation group.

ware. The level of significance was set at  $P < 0.05$ .

### Results

#### *Flow velocity of basilar artery*

Flow velocity of basilar artery was detected by trans cranial doppler sonography at the 1<sup>st</sup> week, 2<sup>nd</sup> week, 3<sup>rd</sup> week, 4<sup>th</sup> week after ligation (**Figure 1**). There was no obvious change in Sham group from week 1 to week 4. Then, the value in UCAL group was slightly increased, while it was significantly elevated in BCAL group. The results revealed that the flow velocity of BCAL group was observably faster than that of Sham and UCAL group.

#### *HE, EVG, Masson staining*

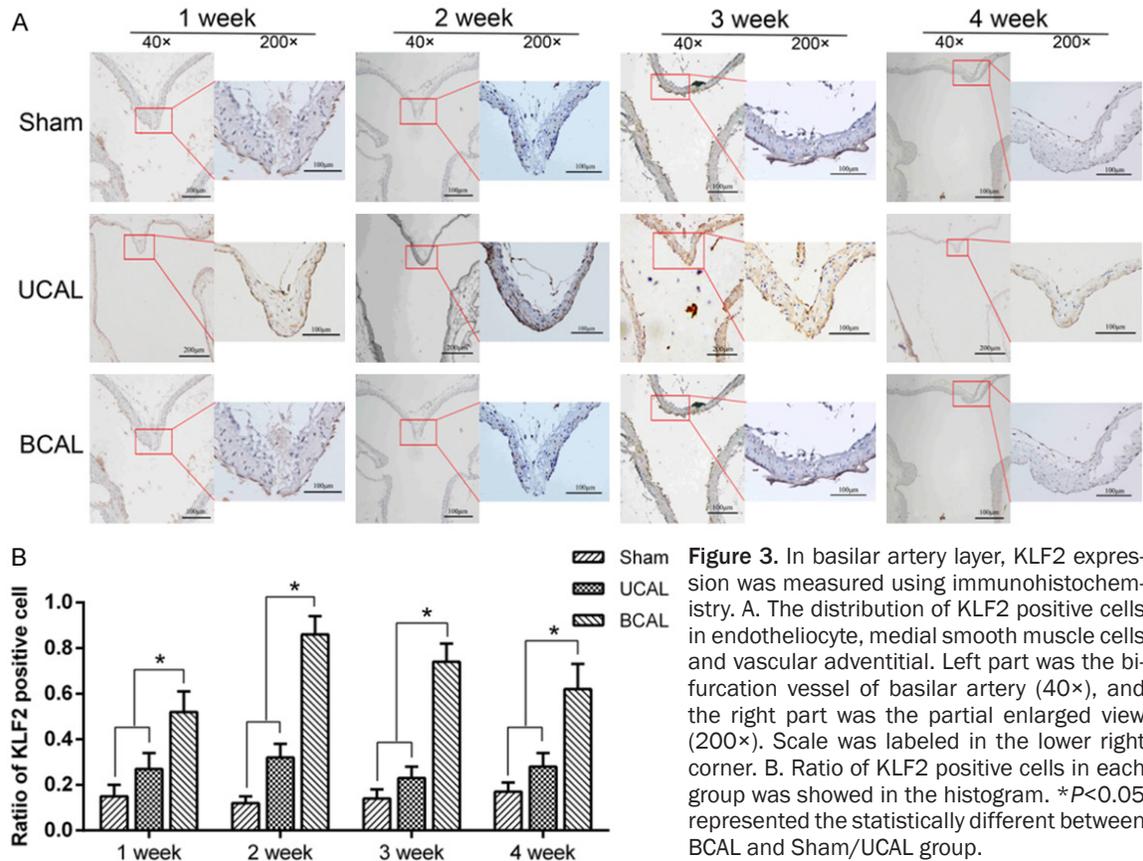
The morphological changes of basilar artery endotheliocyte were tested using HE staining, EVG staining, and Masson staining (**Figure 2**). HE staining showed the top of the basilar artery bulged outwards in UCAL group (upper row). EVG staining showed the elastic membrane

was partial unsubstantial and deleted as a result of high blood-fluid pressures in UCAL group (middle row). Analogously, Masson staining showed the thickness of medial smooth muscle became thin with extrorse embossment (lower row). The staining results revealed bilateral carotid artery ligation observably altered the construction and morphology of the basilar artery. Results demonstrated that the animal model of intracranial aneurysm was successfully established.

#### *Immunohistochemistry showed that KLF2 was motivated by haemodynamics in IA*

The expression of KLF2 in various layers of the basilar artery was measured using immunohistochemistry. KLF2 was distributed in the endotheliocyte, medial smooth muscle cell and vascular adventitial. With increased exposure time, KLF2 expression was significantly increased in each layer, specifically in UCAL group (**Figure 3A**). The ratio of KLF2 positive cells indicated the KLF2 expression in UCAL group was higher than the other two groups (**Figure 3B**). The above results indicate KLF2 is influenced by

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haemodynamics in IA models and varies over time.

*KLF2 protein expression was increased in IA induced by bilateral carotid arteries ligation*

Expression of KLF2 protein in basilar artery was detected using western blot (**Figure 4**). There was no obviously fluctuation in the expression of KLF2 protein in the Sham group (**Figure 4A**). However, the expression of KLF2 protein was significantly increased in the other two groups, particularly in the BCAL group. From the 1<sup>st</sup> week to the 4<sup>th</sup> week, the KLF2 protein expression increased significantly and then maintained a stable level (**Figure 4B**). Results showed that KLF2 protein significantly increased in intracranial aneurysm induced by bilateral carotid artery ligation.

*KLF2 overexpression upregulated eNOS expression and suppressed ICAM-1, MMP-9 expression*

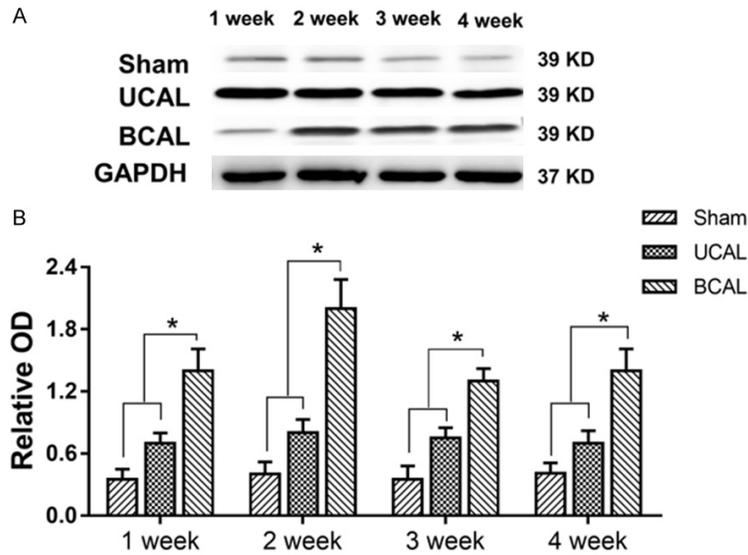
KLF2 acted as a transcription factor and regulated various downstream target factors.

Expression of eNOS, ICAM-1 and MMP-9 mRNA were detected using real time PCR (**Figure 5**). Expression of eNOS was increased in the KLF2 up-regulated group and showed an evident time-varying trend, and was decreased in KLF2 down-regulated group. Conversely, expression of ICAM-1 and MMP-9 mRNA was inhibited in KLF2 up-regulated group and promoted in KLF2 down-regulated group. Results demonstrate that KLF2 overexpression markedly accelerated eNOS expression, and concurrently suppressed ICAM-1 and MMP-9 mRNA expression.

*KLF2 regulated p38, JNK, ERK1/2 pathway*

The phosphorylation activity of p38, JNK, ERK1/2 pathways was detected using western blot (**Figure 6A-D**). The experimental results of up-regulation expression of KLF2 showed that the phosphorylation activity of p38 significantly increased, while JNK and ERK1/2 did not significantly change. Further, the results of down-regulation expression of KLF2 showed that the phosphorylation activity of p38 decreased, however, JNK and ERK1/2 did not

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**Figure 4.** KLF2 expression was measured using western blot. A. Representative western blot bands of KLF2 in each group. B. Quantitative analysis of the band densities for KLF2. \* $P < 0.05$  represented statistical difference compared with UCAL and BCAL group.

significantly change. Above results demonstrate that up-regulated expression of KLF2 activates the p38 pathway. To confirm this relationship, a p38 inhibitor (SB203580) was used (Figure 6E, 6F). Under the SB203580 function, the phosphorylation activity of p38 was significantly decreased, and was significantly different compared to the prior experiment.

### Discussion

There are currently no definite theories regarding the pathogenesis of intracranial aneurysms (IA). As previous investigations demonstrate, the occurrence and development of IA are influenced by multiple risk factors, including heredity risk factors, hypertension and the environment. The present study investigates the expression and role of KLF2 in the pathogenesis of intracranial aneurysm (IA) in an animal model and cell model.

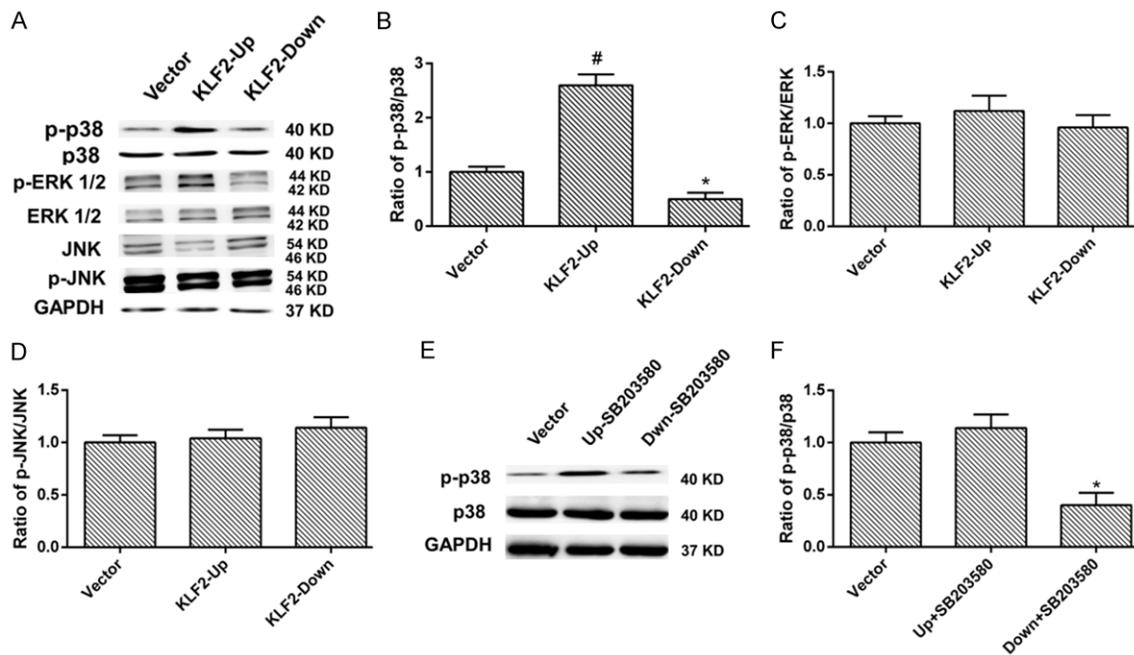
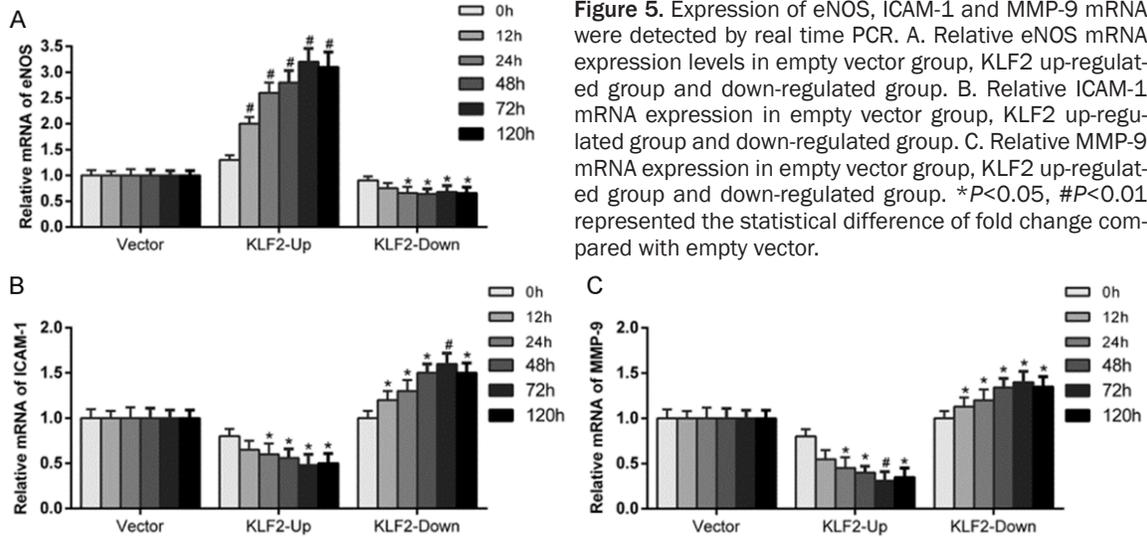
In an animal model, flow velocity was measured using trans cranial doppler sonography in different treatment groups. Results revealed the flow velocity of BCAL group was significantly faster than that of Sham and UCAL group (Figure 1). Research has found that complex blood flow changes appear at basal artery bifurcation in aneurysm models induced by hemodynamics. These are characterized by local high WSS and WSSG, which is considered a

predictive factor for aneurysm [6, 7]. In UCAL group, the top of basilar artery was found to bulge outwards and the thickness of medial smooth muscle and elastic membrane decreased. This was measured using HE, EVG and Masson staining (Figure 2). The construction and morphology transformation of basilar membrane illustrates that blood flow variation may lead to intracranial aneurysm [6, 7]. Existing research has found that endothelial shear stress may up-regulate KLF2 mRNA expression through MEK5/ERK5/MEF2 pathway, mediated by AMPK signaling [8].

Recently, an increasing number of studies have found Kruppel-like factors (KLFs) are the main regulatory molecule in the physiological regulation of endotheliocyte. Specifically, KLF2 is an important regulatory factor in vascular endothelial function [9]. The present study verified that KLF2 is up-regulated when stimulated by hemodynamics in an animal model of IA, which was detected using immunohistochemistry and western blot (Figures 3, 4). These results are in accord with previous studies [10]. KLFs regulate the expression of various genes in endotheliocyte and participate in the inflammatory response, including endothelin, eNOS, MMPs and NF- $\kappa$ B [11]. These downstream gene and transcription products play an important role in the molecular pathways involved in the process of the formation of IA.

In previous animal experiments, results found bilateral carotid artery ligation successfully established an IA model and verified the hemodynamic effects in the pathogenesis of IA. Studies have reported that p38 pathway participates in the pathogenesis of IA [12, 13]. Endotheliocyte cultured in vitro was used to confirm the underlining interaction mechanisms of KLF2 in the MAPK pathway. Vascular smooth muscle cells are harvested from basilar artery and cultured for establishing KLF2 up-regulated and down-regulated cell model by liposome transfection and siRNAs.

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In the present study, KLF2 overexpression promoted eNOS mRNA expression, and inhibited MMP-9 and ICAM-1 mRNA expression (Figure 5). Further, the regulatory effect of KLF2 changes with time. MMP-9 and ICAM-1 are pro-inflammatory factors and play an important role in the progression of inflammation, an important factor in the pathogenesis of IA. Inflammatory cell

infiltration, particularly macrophages, produces a large amount of matrix metalloproteinases, such as MMP-2 and MMP-9, which is observed in human aneurysm wall and aneurysm animal model induced for several months [14-16]. Immunohistochemical assay has found that eNOS expression is decreased in aneurysm walls and ICAM-1 expression is elevated in the

inner membrane [17, 18]. The pathogenesis of cerebral aneurysms is closely associated with endothelial dysfunction and inflammation [19]. Furthermore, gene chip analysis found KLF2 combined with vascular endothelial growth factor (VEGF) receptor 2 suppresses the expression of VEGF and the inflammatory reaction [20]. Conversely, when the KLF2 expression is interrupted using siRNA, it accelerates the expression of endothelin and epinephrine, and inhibits the expression of vasodilator gene eNOS [21]. Further studies have found that KLF2 immediately combines with eNOS promoter [9]. The present results support this conclusion, which is in accordance with previous research.

The present study found KLF2 overexpression activated the phosphorylation activity of p38, except for JNK and ERK1/2. Similarly, lower expression of KLF2 decreased the ratio of p-p38/p38 (**Figure 6A-D**). When p38 inhibitor (SB203580) was added into experimental group, ratio of p-p38/p38 in the two groups was significantly decreased (**Figure 6E, 6F**). It has been found that p38 inhibitor significantly inhibits neutrophil granulocyte adhesion and hyperoxide generation to reduce inflammation and protect the arterial vascular wall [22]. Research has shown that hypoxia stress activates the p38 MAPK signaling molecule and regulates MMPs/TIMPs and uPA/PAI to affect vascular endothelial cells in arterial aneurysm [23]. The interaction mechanism of KLF2 and p38 signaling molecule remain not well understood and require further investigation.

Taken together, the present study investigates the expression of KLF2 in an animal model and its regulatory effect on progression of IA. Furthermore, the potential mechanism of KLF2 and p38 pathway was investigated, and may provide a potential therapeutic target in the non-surgical treatment of IA.

### Acknowledgements

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### Disclosure of conflict of interest

None.

### Abbreviations

KLF2, Kruppel-like factor 2; IA, intracranial aneurysm; WSS, wall shear stresses; eNOS, endothelial nitric oxide synthase; MMP-9, matrix metalloproteinases; ICAM-1, intercellular adhesion molecule-1.

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