

Original Article

Tongxinluo inhibits neointimal formation by regulating the expression and post-translational modification of KLF5 in macrophages

Wen Jiang, Bin Zheng, Xin-Hua Zhang, Ling-Yan Yue, Chan Liu, Dong Ma, Zhan Yang, Jin-Kun Wen

Department of Biochemistry and Molecular Biology, Key Laboratory of Neural and Vascular Biology, Ministry of Education, Hebei Medical University, Shijiazhuang 050017, PR China

Received July 1, 2016; Accepted October 7, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Neointimal hyperplasia is a common pathological characteristic in diverse vascular remodeling diseases. The inflammatory response that follows vascular injury plays an important role in intimal hyperplasia. Tongxinluo (TXL), a traditional Chinese medicine, can ameliorate neointimal formation via suppressing vascular inflammatory response induced by vascular injury. However, the mechanisms underlying anti-inflammatory and anti-intimal hyperplasia of TXL are still not fully understood. The aim of present study was to examine whether the expression and post-translational modification of KLF5 were involved in the vasoprotective effects of TXL. In vivo, TXL inhibited neointimal formation induced by carotid artery injury. In vitro, TNF- α treatment of macrophages resulted in the increased proliferation and migration, but the effects of TNF- α on macrophages were blocked by TXL treatment. Next, KLF5 expression was up-regulated by carotid artery injury in vivo, as well as by exposure of macrophages to TNF- α in vitro, whereas TXL treatment abrogated the up-regulation of KLF5 by TNF- α or vascular injury. Intimal hyperplasia was strongly reduced in macrophage-specific KLF5 knockout (KLF5^{ly-/-}) mice, indicating that TXL inhibits intimal hyperplasia by suppression of KLF5 expression. Furthermore, besides down-regulating KLF5 expression in macrophages, TXL also regulated KLF5 stability by ubiquitination and sumoylation of KLF5. Finally, TNF- α induced KLF5 sumoylation via PI3K/Akt signaling, whereas TXL inhibited Akt phosphorylation induced by TNF- α . We conclude that the multiple ingredients in TXL may act on different targets, which in turn generates a range of actions that manifest as a comprehensively vasoprotective effect.

Keywords: Tongxinluo, intimal hyperplasia, inflammation, KLF5, ubiquitination, sumoylation

Introduction

Inflammatory response plays an important role in the development and progression of vascular remodeling diseases, such as atherosclerosis and restenosis after percutaneous coronary interventions [1]. Vascular injury, including denuding endothelial injury or non-denuding endothelial functional injury in response to low shear stress induced by carotid artery ligation, represents a critical initiating step in vascular remodeling diseases. In response to vascular injury, the injured endothelium produces the multiple cytokines and chemokines, which in turn promotes the adhesion and activation of monocytes/macrophages in the injured endothelium, and triggers the migration and infiltration of macrophages into the vasculature [2, 3], subsequently leading to exacerbated inflammatory response.

The inflammatory response requires the activation of a complex transcriptional program whereby the expression of hundreds of genes are dynamically regulated in a cell-type- and stimulus-specific manner [4]. Several lines of data have suggested that KLF5 is an important transcription factor that regulates the expression of multiple genes and participates in vascular remodeling [5]. Besides its pro-proliferative role, KLF5 is involved in vascular response to injury, as well as in the activation of inflammatory processes after exposure to inflammatory stimuli [6, 7]. It has been known that KLF5 activities are regulated by its posttranslational modifications, including ubiquitination, sumoylation, acetylation, and phosphorylation, thus affecting its downstream cellular functions [8]. For example, the sumoylation of KLF5 regulates its nuclear localization [9]. Phosphorylation of KLF5 by PKC enhances the transcriptional

activities of KLF5 by promoting its interaction with CREB binding protein [10]. In addition, E3 ubiquitin ligase FBW7, a tumor suppressor, interacts with KLF5 for ubiquitination and degradation [11, 12]. Despite understanding of KLF5's role and its activity regulation in cardiovascular remodeling, the function of KLF5 in macrophage infiltration during neointimal formation remains largely unknown.

Tongxinluo (TXL), a traditional Chinese medicine, is extracted and standardized from a mixture of 12 herbal medicines, such as *Radix ginseng*, *Buthus martensi*, *Hirudo*, *Eupolyphaga seu steleophaga*, *Scolopendra subspinipes*, *Periostracum cicadae*, *Radix paeoniae rubra*, *Semen ziziphi spinosae*, *Lignum dalbergiae odoriferae*, *Lignum santali albi*, and *Borneolum syntheticum*. It was registered in the State Food and Drug Administration of China in 1996 and has been used clinically for 20 years to treat cardiovascular and cerebrovascular diseases [13, 14]. The previous studies have demonstrated that TXL can protect the vascular endothelial function [15], lower serum lipid levels [16], inhibit inflammation and apoptosis [17, 18], suppress neointimal hyperplasia [17], protect against blood-brain barrier (BBB) disruption [14], and promote angiogenesis [19]. Despite the therapeutic efficacy of TXL in the treatment of cardiovascular and cerebrovascular diseases and the obvious upregulation of KLF5 in vascular remodeling diseases, it remains unclear whether TXL-suppressed neointimal hyperplasia is responsible for its inhibitory effects on the endothelial injury-induced KLF5 expression and infiltration of macrophages into the vasculature.

In this study, we investigated the effects of TXL on KLF5 expression and macrophage infiltration, and explored the molecular mechanisms of action of TXL both in vivo and in vitro.

Material and methods

Components and preparation of TXL

TXL ultrafine powder was provided by Shijiazhuang Yiling Pharmaceutical Co., Ltd., (Hebei, China). TXL contains 12 medicinal components, its formulation and components have been reported in detail [20]. These herbal medicines were ground to a superfine powder (<10 µm)

using micronization technology after they were authenticated and standardized to marker compounds according to the 2010 Chinese Pharmacopoeia (National Pharmacopoeia Committee, 2010). The TXL ultrafine powder was dissolved in normal saline and detailed preparation methods were described elsewhere [13].

For cell experiments, TXL powder was weighed and dissolved in the phosphate-buffered saline. The ultrasound technology was used to promote the melting of TXL. The drug was then centrifuged at 1000×g for 10 min, and the supernatant was put into the microfilter (0.22 µm) to eliminate bacteria, which was then aliquoted and stored at -20°C before use.

Animals

Macrophage-specific KLF5 knockout (KLF5^{ly-/-}) mice (C57BL/6J background) and wild-type mice (WT, C57BL/6J), 8-10 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mouse model of carotid intimal hyperplasia was established by left common carotid artery ligation near the carotid bifurcation, as described previously [21]. TXL power was administered by intragastric administration at a dose of 0.75 g·kg⁻¹·day⁻¹ (six mice in each group) beginning 3 days before ligation injury and continuing for 14 days thereafter. Ligated animal without TXL treatment received vehicle (water) at an equivalent amount. All animals were anesthetized with 2% isoflurane gas for surgery and harvesting carotid arteries to perform morphology and histological analysis.

All the mice were housed in a specific pathogen free animal laboratory and maintained on a controlled light cycle schedule of 12:12 hour (h, light/dark) at 25°C with food and water ad libitum. Before experiments, animal health was monitored in experimental animal Laboratory according to the NIH guide for care and use of laboratory animals. After operation, the animals were kept warm and observed every half an hour, until they woke up and received water. Euthanize was used at the end of experimental process.

All animal studies were approved by the Ethics Committee of Hebei Medical University. Animal care and procedures conformed with the NIH Guide for the Care and Use of Laboratory

Animals, and all efforts were made to minimize suffering.

Morphology analysis

Carotid arteries were perfusion-fixed in situ with 4% formaldehyde solution, and then harvested and embedded in paraffin. Four-micrometer cross-sections were prepared for hematoxylin and eosin staining, covering the area 3 mm proximal to the ligation site. The neointimal area and intima-to-media ratio were calculated using Image-Pro Plus Analyzer (version 5.1) software (Media Cybernetics, Silver Spring, MD) in a blinded manner. For each section, six random noncontiguous microscopic fields were examined.

Cell culture

The murine RAW 264.7 macrophages were purchased from ATCC, cultured in DMEM containing 10% fetal bovine serum (HyClone, Logan, UT, USA) and maintained in 5% CO₂ at 37°C in a humidified atmosphere. The cells used in all studies were from passages 3 to 6. The cells were pretreated for 2 h with 400 µg/ml of TXL, the PI3K/Akt inhibitor LY294002 (Promega, Madison, WI, USA), or the NF-κB inhibitor CAY10512 (Promega, Madison, WI, USA) before application of TNF-α (10 ng/ml), then cells were harvested and lysed in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10% glycerin, 1 mM Na₃VO₄, 1 mM PMSF and 1 mM DTT) for Western blotting analysis and co-immunoprecipitation assays.

Adenovirus infection

Adenoviruses encoding KLF5 (pAd-KLF5) and GFP control (pAd-null) were entrusted to Invitrogen. RAW 264.7 macrophages grown to 70-80% confluence were infected by pAd-KLF5 or pAd-null at a titer of 50 plaque-forming units/cell. For animal experiments, WT and KLF5^{ly/-} mice received a single intravenous (tail) injection (109 plaque-forming units) of pAd-KLF5 or pAd-null every 4 days beginning 1 day before ligation injury until 14 days.

MTS assay

After appropriate treatment, viability of macrophages cultured in 96-well plates was measured using the MTS assay, as previously

described [22]. In brief, the medium of cultured RAW264.7 cells was replaced with 100 µl serum-free DMEM containing 10 µl of CellTiter 96 AQueous One Solution (Promega, G3582), and incubated at 37°C for 4 h. Then, 60 µl of medium from each well was transferred to a new 96-well plate, and the absorbance at 490 nm was measured using a Multiskan spectrum (Thermo).

Wound healing assay

Macrophages were seeded in a 6-well culture plate. After cells were grown to 80-90% confluence, the center of the cell monolayers was scraped with a sterile micropipet tip to create a denuded zone (gap) of constant width. Cellular debris was washed with PBS, and cells were exposed to 400 µg/ml of TXL, TNF-α (10 ng/ml), or TXL plus TNF-α. Wound closure was monitored and photographed at 0 and 24 h. Wound area was measured by tracing the wound margin and calculated using an image analysis program (NIH Image). The percentage of wound closure was calculated as: (area of original wound-area of actual wound)/area of original wound ×100.

Immunofluorescence staining

Immunofluorescence staining was performed with 5 µm paraffin cross-sections from the carotid artery. After deparaffinized with xylene and rehydrated, the slides were preincubated with 10% normal goat serum (710027, KPL, USA) and then incubated with primary antibodies anti-KLF5 (GTX103289, GeneTex), and anti-MAC2 (60207-1, Proteintech). Secondary antibodies were fluorescein-labeled antibody against rabbit IgG (021516, KPL, USA) and rhodamine-labeled antibody against mouse IgG (031806, KPL, USA). In each experiment, DAPI (157574, MB biomedical) was used for nuclear counter staining. Images were captured by confocal microscopy (DM6000 CFS, Leica) and processed by LAS AF software.

Co-immunoprecipitation assay

Co-immunoprecipitation was performed as described previously [23]. In brief, the cell lysates were immunoprecipitated with anti-KLF5 (GTX103289, GeneTex) for 1 h at 4°C, followed by incubation with protein A-agarose

overnight at 4°C. Protein A-agarose-antigen-antibody complexes were collected by centrifugation at 12,000 g for 1 min at 4°C. The pellets were washed 5 times with 1.0 ml immunoprecipitation-HAT buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 0.1 mM PMSF] for 20 min each time at 4°C. Bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting with antibodies against Fbxw7 and KLF5.

Western blot analysis

The cell lysates were separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 5% milk in Tris-HCl tween buffer solution for 2 h at 37°C and incubated overnight at 4°C with specific KLF5 (1:1000, GTX103289, GeneTex), PCNA (1:10000, ab92552, Abcam), Fbxw7 (1:1000, ab192328, Abcam), Akt1 (1:500, Cell Signaling Technology), phospho-Akt (1:500, Cell Signaling Technology), NF-κB p65 (1:500, NBP1-96139, NOVUS Biologicals), phospho-NF-κB (1:400, AF2006, Affinity Biotech) and β-actin (1:1,000, Santa Cruz Biotechnology) antibodies. After incubation with appropriate secondary antibody, the membranes were developed with the Chemiluminescence Plus Western blot analysis kit (Millipore). For ubiquitination and sumoylation analysis of KLF5, KLF5 was immunoprecipitated from the lysates with rabbit anti-KLF5 (GTX103289, GeneTex), followed by Western blotting with mouse anti-SUMO-1 (Abcam, Cambridge, UK) or mouse anti-ubiquitin (Abcam, Cambridge, UK).

Statistical analysis

Data are presented as bar graphs (means ± SD) of at least three independent experiments. For relative gene expression, the mean value of the vehicle control group is defined as 1. Student's *t* test and ANOVAs were used for statistical evaluation of the data. SPSS 17.0 was used for data analysis. As some sample sizes per group were relatively small, the results were further verified by the Wilcoxon rank sum (two samples) or the Kruskal Wallis test (multiple samples). The results were considered statistically significant at *P* < 0.05.

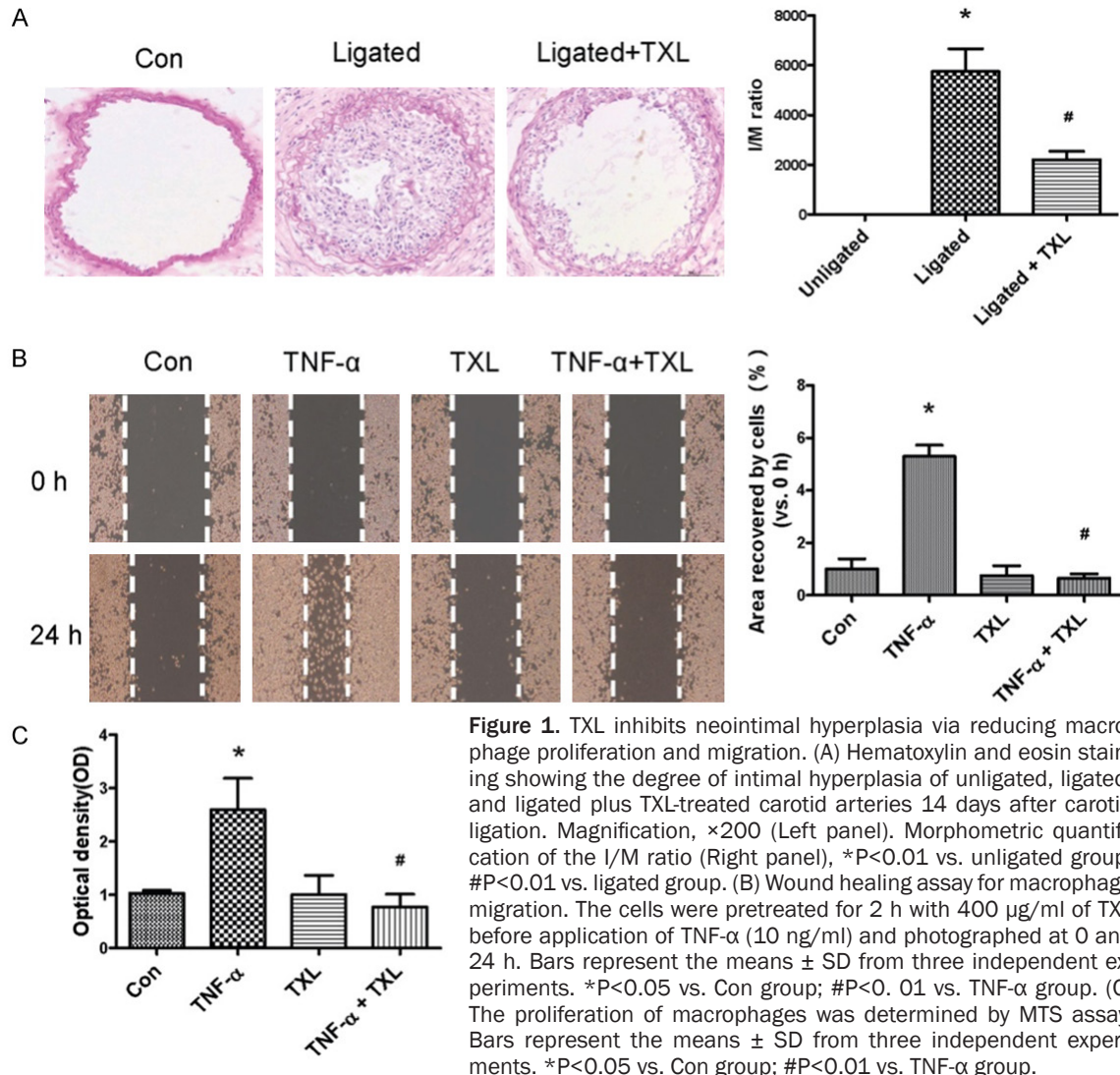
Results

TXL inhibits neointimal hyperplasia induced by carotid artery ligation via reducing macrophage proliferation and migration

At 14 days after carotid artery ligation, the ligated animals showed abundant neointimal hyperplasia, the neointimal area of the ligated group accounted for 70% of the carotid arterial wall thickness (**Figure 1A**). Compared with the ligated group, carotid arterial wall thickness was significantly decreased in the TXL-treated group, and the intima-to-media ratio (I/M ratio) was lower than in the ligated group. The uninjured arteries revealed no significant neointimal hyperplasia. Because it is well known that low shear stress induced by carotid artery ligation promoted TNF-α and IL-1β expression and neointimal hyperplasia [24], and that macrophage proliferation and migration are important for inflammation and neointimal hyperplasia, we investigated whether TNF-α could affect proliferation and migration of macrophages. As shown in **Figure 1B**, migration activities were significantly increased when macrophages were stimulated by TNF-α, the wounded area recovered by cells at TNF-α-treated groups was 5-fold greater than that of the control group. TXL treatment significantly suppressed macrophage migration induced by TNF-α, with the wounded area recovered by cells returning to control level. MTS assay showed that TNF-α increased macrophage proliferation 4-fold over that of control group, whereas cell proliferation decreased to control levels after TXL treatment (**Figure 1C**). These results suggest that TXL inhibits neointimal formation induced by carotid artery ligation, partly through suppressing macrophage proliferation and migration.

TXL inhibits macrophage infiltration to the arterial wall through suppressing KLF5 expression

Because KLF5 can be induced by proinflammatory factors and is an essential regulator of cardiovascular remodeling, we tested the relationship between KLF5 and macrophage infiltration during neointimal formation. As shown by immunofluorescence staining with anti-Mac2 antibody of macrophages, macrophages infiltrated into the neointima of ligated arteries were readily detectable at 14 days after carotid artery ligation (Note: Elastic fibers and collagen



fibers in the arterial wall produce autofluorescence in direct immunofluorescence analysis), whereas they were barely observed in the neointima in TXL-treated group, similar to unligated carotid arteries (control) (**Figure 2A**). Similarly, KLF5 expression was significantly increased in the neointima compared with unligated arteries, but TXL treatment inhibited carotid artery ligation-induced upregulation of KLF5 (**Figure 2A**). Co-immunostaining with anti-KLF5 and anti-Mac2 antibodies showed that besides smooth muscle cells (SMCs), macrophages also expressed KLF5. To further investigate the effect of TXL on the expression of KLF5 induced by TNF- α in vitro, the cultured RAW264.7 cells were treated with TNF- α alone or in conjunction with TXL. Western blotting showed that TXL treatment significantly suppressed TNF- α -

induced KLF5 expression and cell proliferation, as evidenced by the decreased PCNA expression (**Figure 2B**). These results indicate that KLF5 is implicated in macrophage proliferation and migration induced by carotid ligation and TNF- α stimulation.

TXL inhibits neointimal hyperplasia by down-regulating KLF5 expression

To further examine whether the inhibitory effect of TXL on neointimal hyperplasia and macrophage infiltration is related to its regulation of KLF5, in vivo experiments on the gain and loss of KLF5 were carried out. We overexpressed KLF5 using adenovirus (Ad)-mediated gene transfer; pAd-KLF5 was administered intraluminally after carotid artery ligation. As shown in

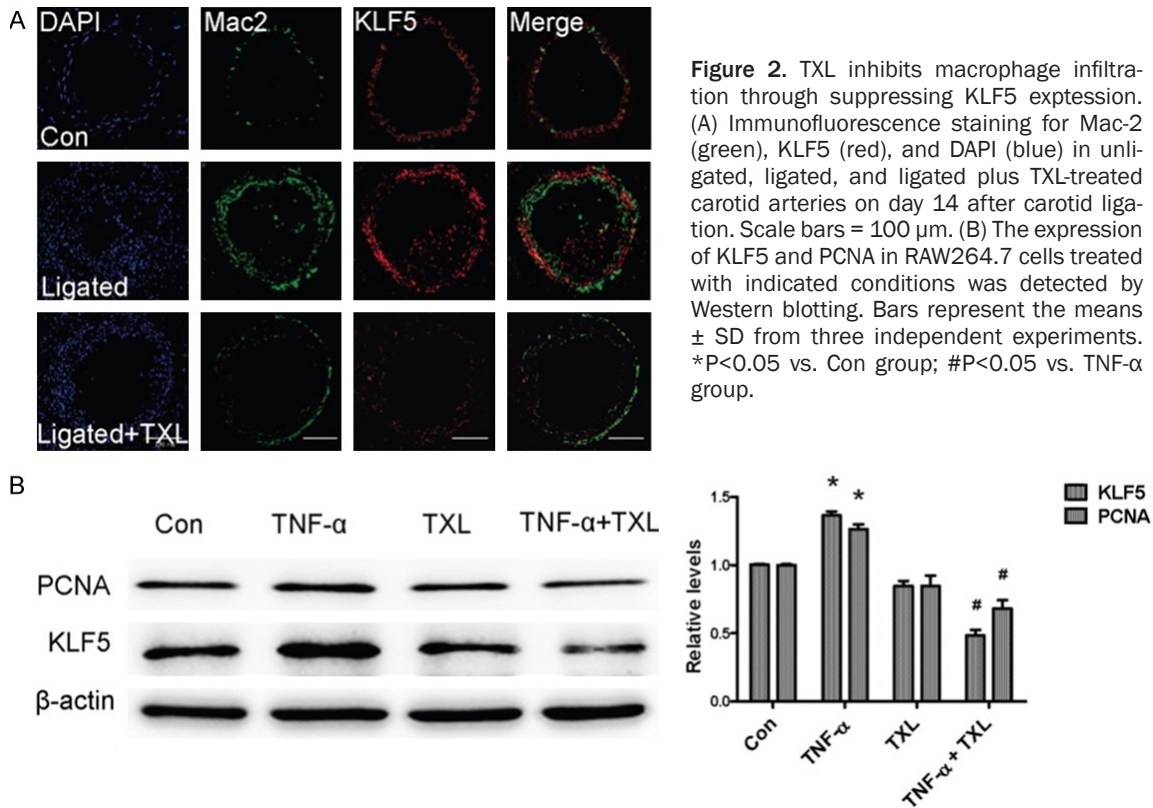


Figure 3A, marked neointimal hyperplasia was observed in ligated carotid arteries of pAd-KLF5-transduced mice compared with those of pAd-transduced mice. As expected, TXL treatment markedly reduced neointimal formation and I/M ratio in ligated carotid arteries of both pAd-KLF5- and pAd-infected mice. Immunofluorescence staining showed that overexpression of KLF5 obviously increased Mac2-positive macrophages in the neointima after carotid artery ligation compared with pAd-transduced mice, indicating an increased macrophage infiltration into the neointima. TXL treatment markedly decreased macrophage infiltration and neointimal thickness induced by carotid artery ligation through down-regulating KLF5 expression, regardless of KLF5 overexpression (**Figure 3B**). Further studies were performed in macrophage-specific KLF5 knockout (KLF5^{ly-/-}) mice and WT (KLF5^{WT}) mice. The results showed that neointimal hyperplasia induced by carotid artery ligation and I/M ratio were strongly reduced in KLF5^{ly-/-} mice compared with KLF5^{WT} mice (**Figure 3C**). Immunofluorescence analysis for KLF5 and Mac2 revealed that Mac2-positive macrophages and neointimal thickness were

obviously reduced in the ligated carotid arteries of KLF5^{ly-/-} mice compared with KLF5^{WT} mice (**Figure 3D**), suggesting that deletion of KLF5 prevents carotid ligation-induced intimal hyperplasia in KLF5^{ly-/-} mice.

TXL inhibits macrophage proliferation and migration in vitro induced by KLF5 overexpression

To further investigate the effect of KLF5 on macrophage proliferation and migration, the cultured RAW264.7 cells were infected or not with pAd-KLF5. As shown in **Figure 4A**, overexpression of KLF5 in macrophages significantly increased PCNA expression, whereas TXL treatment abrogated the up-regulation of PCNA by KLF5 overexpression. Wound healing assay for macrophage migration in vitro showed that KLF5 overexpression promoted, while TXL treatment reduced the migration of the cultured macrophages induced by KLF5 overexpression, with KLF5-promoted migration being abrogated by TXL treatment (**Figure 4B**). Furthermore, using MTS assay, we detected the effect of KLF5 overexpression and TXL

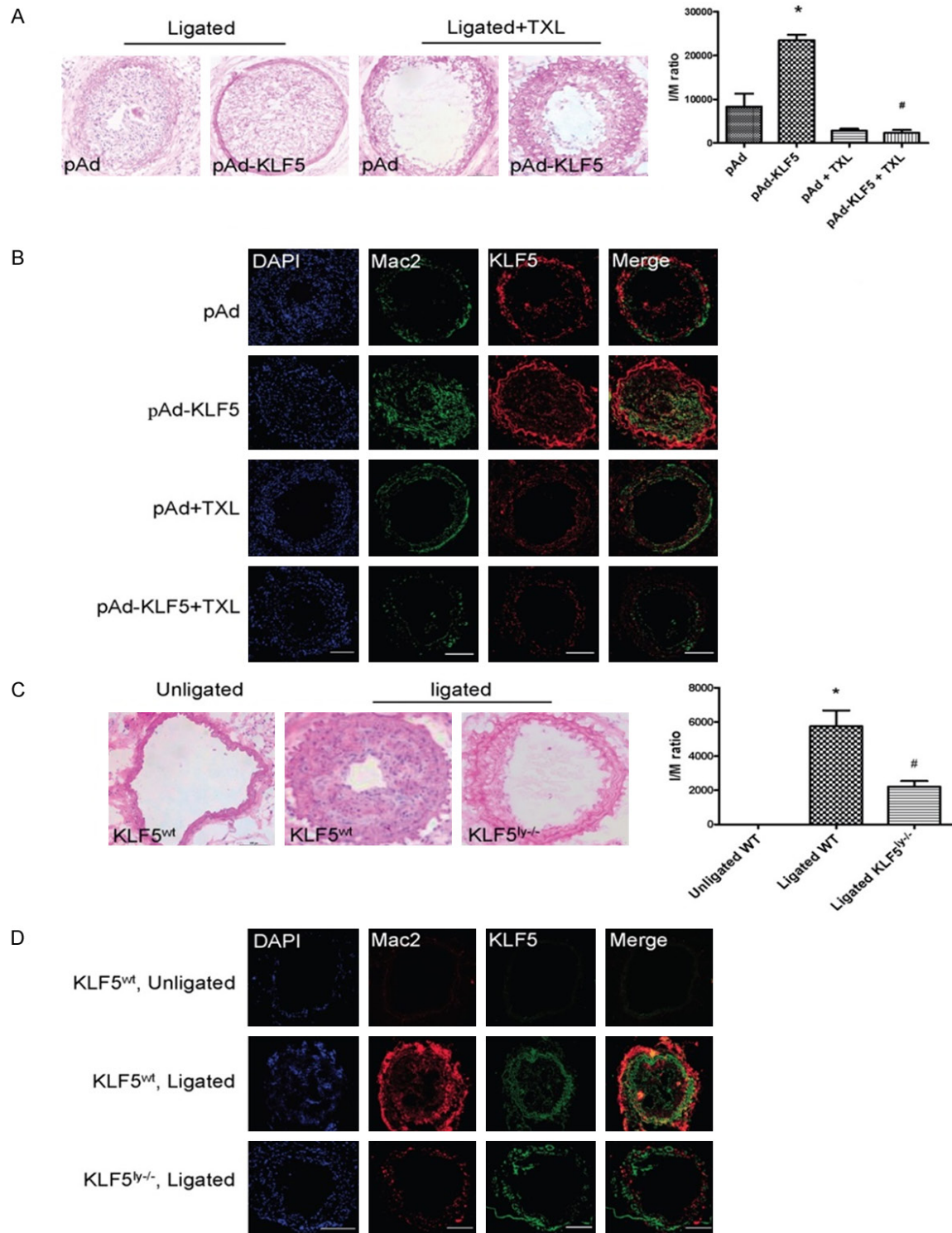


Figure 3. TXL inhibits intimal hyperplasia by down-regulating KLF5 expression. (A) Hematoxylin and eosin staining showing the degree of intimal hyperplasia of ligated and ligated plus TXL-treated carotid arteries infected with pAd or pAd-KLF5. Magnification, $\times 200$ (Left panel). Morphometric quantification of the I/M ratio (Right panel), $*P < 0.05$ vs. pAd group; $\#P < 0.01$ vs. pAd-KLF5 group. (B) Immunofluorescence staining for Mac-2 (green), KLF5 (red), and DAPI (blue) in the carotid arteries treated as in (A). Scale bars = 100 μ m. (C) Hematoxylin and eosin staining showing the degree of intimal hyperplasia of unligated and ligated carotid arteries from KLF5^{WT} and KLF5^{ly-/-} mice. Magnification, $\times 200$ (Left panel). Morphometric quantification of the I/M ratio (Right panel), $*P < 0.01$ vs. unligated KLF5^{WT} group; $\#P < 0.05$ vs. ligated KLF5^{WT} group. (D) Immunofluorescence staining for Mac-2 (red), KLF5 (green), and DAPI (blue) in unligated and ligated carotid arteries from KLF5^{WT} and KLF5^{ly-/-} mice. Scale bars = 100 μ m.

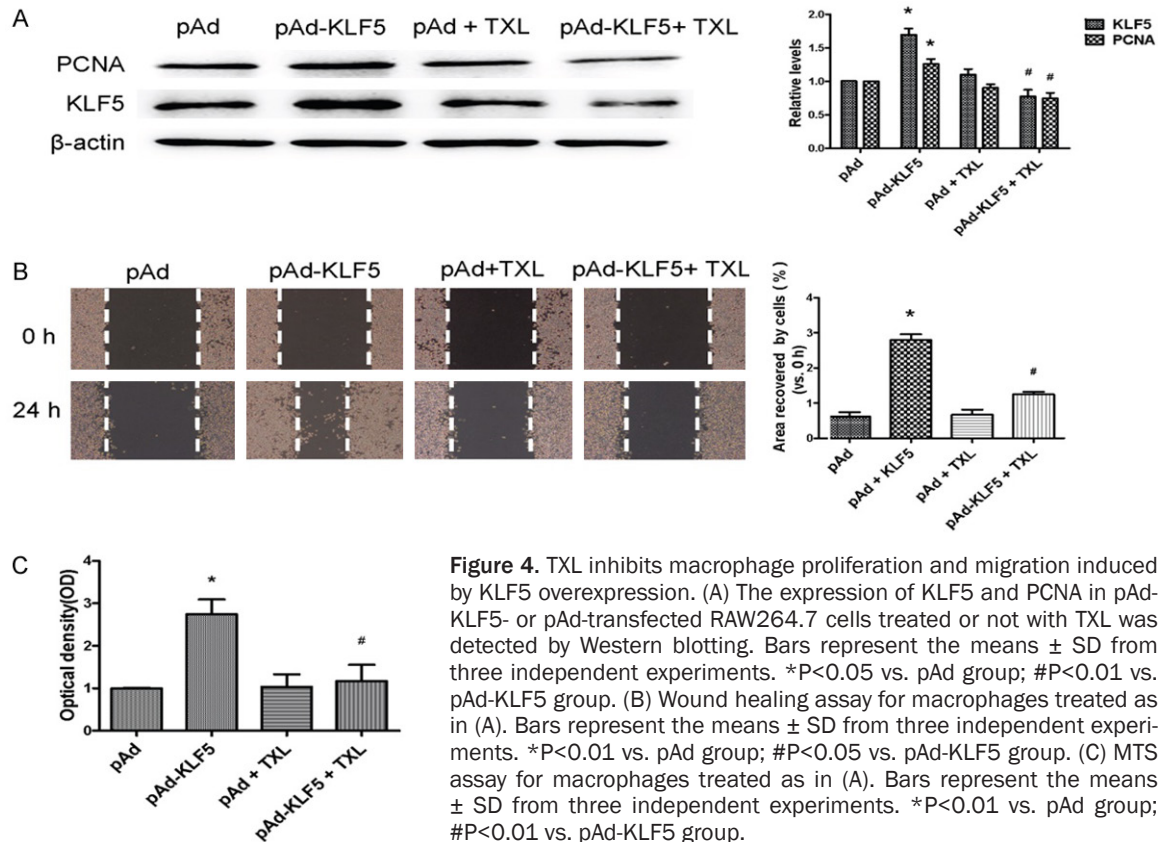


Figure 4. TXL inhibits macrophage proliferation and migration induced by KLF5 overexpression. (A) The expression of KLF5 and PCNA in pAd-KLF5- or pAd-transfected RAW264.7 cells treated or not with TXL was detected by Western blotting. Bars represent the means \pm SD from three independent experiments. * P <0.05 vs. pAd group; # P <0.01 vs. pAd-KLF5 group. (B) Wound healing assay for macrophages treated as in (A). Bars represent the means \pm SD from three independent experiments. * P <0.01 vs. pAd group; # P <0.05 vs. pAd-KLF5 group. (C) MTS assay for macrophages treated as in (A). Bars represent the means \pm SD from three independent experiments. * P <0.01 vs. pAd group; # P <0.01 vs. pAd-KLF5 group.

treatment on macrophage proliferation. Paralleling the above results, TXL treatment abolished macrophage proliferation induced by KLF5 overexpression, as evidenced by MTS assay (**Figure 4C**). These results suggest that KLF5 also mediates macrophage proliferation and migration in vitro.

TNF- α increases, while TXL reduces KLF5 protein level through regulating the ubiquitination and sumoylation of KLF5

Because we have found that TXL treatment reduced KLF5 protein level in macrophages treated with TNF- α (**Figure 2B**), and it is known that KLF5 level can be regulated by ubiquitination and sumoylation [25], we sought to examine whether TXL affected the modification of KLF5 by ubiquitination and sumoylation. As shown in **Figure 5A**, a significant decrease in ubiquitin-conjugated KLF5 was observed in response to TNF- α stimulation (**Figure 5A**, lane 2 versus lane 1). TXL treatment significantly increased the ubiquitin-conjugated KLF5 level compared with TNF- α -treated cells (**Figure 5A**,

lanes 3 and 4 versus lane 2). Next, we determined the effects of TNF- α and TXL treatment on the expression of ubiquitin ligase Fbxw7, which ubiquitinates KLF5, and Fbxw7 interaction with KLF5. The results showed that exposure of macrophages to TNF- α significantly decreased Fbxw7 expression, while TXL treatment abrogated the down-regulation of Fbxw7 expression by TNF- α (**Figure 5B**). Co-immunoprecipitation (Co-IP) assays further showed that the interactions between KLF5 and Fbxw7 were significantly reduced in response to TNF- α (**Figure 5C**, lane 2 versus lane 1). As expected, TXL treatment promoted the interactions of KLF5 with Fbxw7, with Fbxw7 immunoprecipitated with anti-KLF5 antibody returning to control level (**Figure 5C**, lane 4 versus lane 1). These results suggest that TXL enhances KLF5 ubiquitination by Fbxw7, facilitating ubiquitin-mediated proteasomal degradation of KLF5. Interestingly, exposure of macrophages to TNF- α significantly increased KLF5 sumoylation (**Figure 5D**, lane 2 versus lane 1), whereas TXL treatment suppressed the up-regulation of KLF5 sumoylation by TNF- α (**Figure 5D**, lane 4

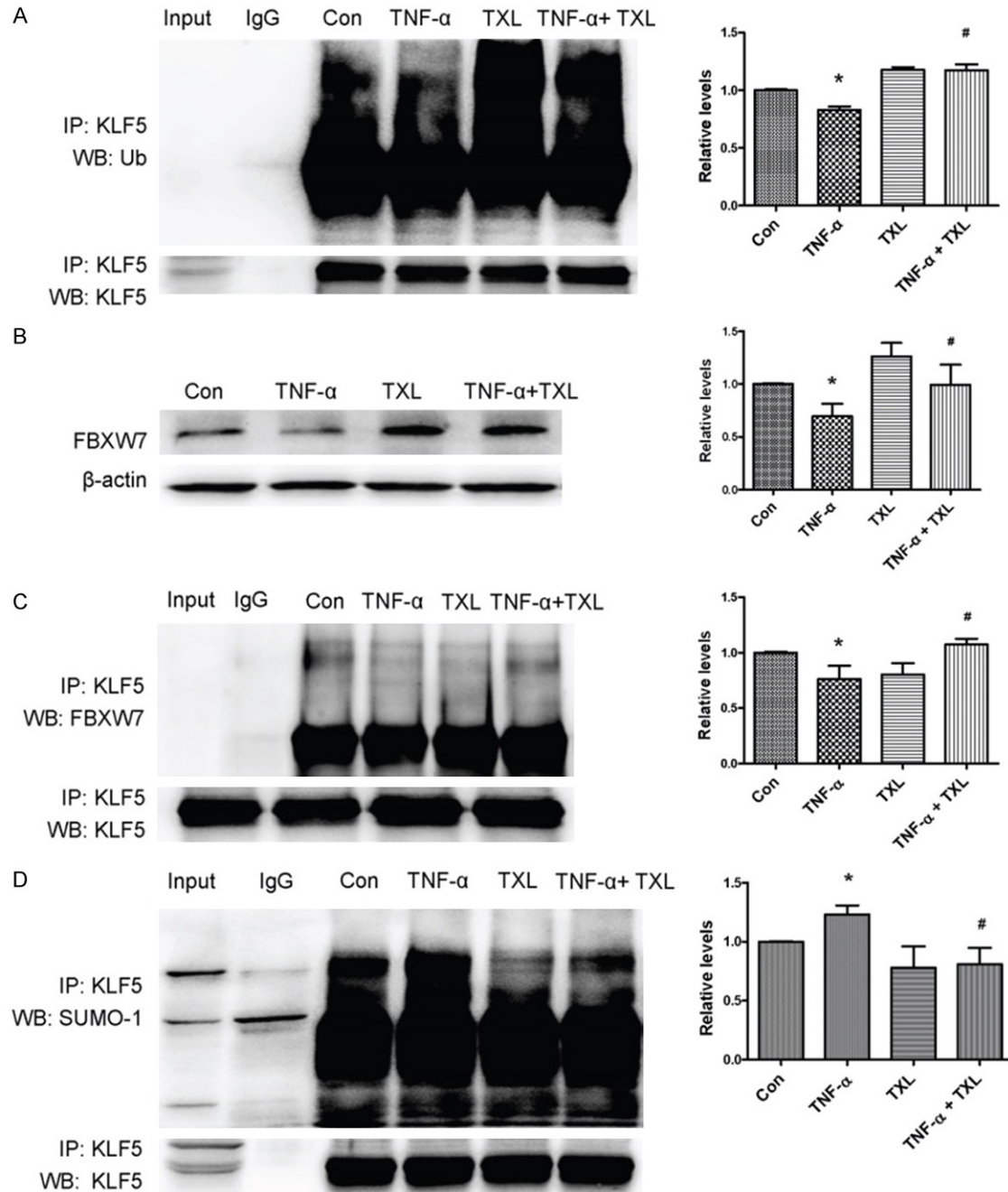


Figure 5. TNF- α increases, while TXL reduces KLF5 level through regulating the ubiquitination and sumoylation of KLF5. (A) The lysates from TNF- α -, TXL-, or TNF- α plus TXL-treated macrophages were immunoprecipitated with anti-KLF5, and then detected by Western blotting with anti-ubiquitin. Bars represent the means \pm SD from three independent experiments. * P <0.05 vs. Con group; # P <0.05 vs. TNF- α group. (B) Western blot analysis for Fbxw7 expression in macrophages treated as in (A). Bars represent the means \pm SD from three independent experiments. * P <0.05 vs. Con group; # P <0.05 vs. TNF- α group. (C) The lysates from macrophages treated as in (A) were immunoprecipitated with anti-KLF5, and then detected by Western blotting with anti-Fbxw7. Bars represent the means \pm SD from three independent experiments. * P <0.05 vs. Con group; # P <0.05 vs. TNF- α group. (D) The lysates from macrophages treated as in (A) were immunoprecipitated with anti-KLF5, and then detected by Western blotting with anti-SUMO-1. Bars represent the means \pm SD from three independent experiments. * P <0.05 vs. Con group; # P <0.05 vs. TNF- α group.

versus lane 2). These findings imply that KLF5 sumoylation induced by TNF- α may impede its

interaction with Fbxw7, subsequently leading to the decreased KLF5 ubiquitination by Fbxw7.

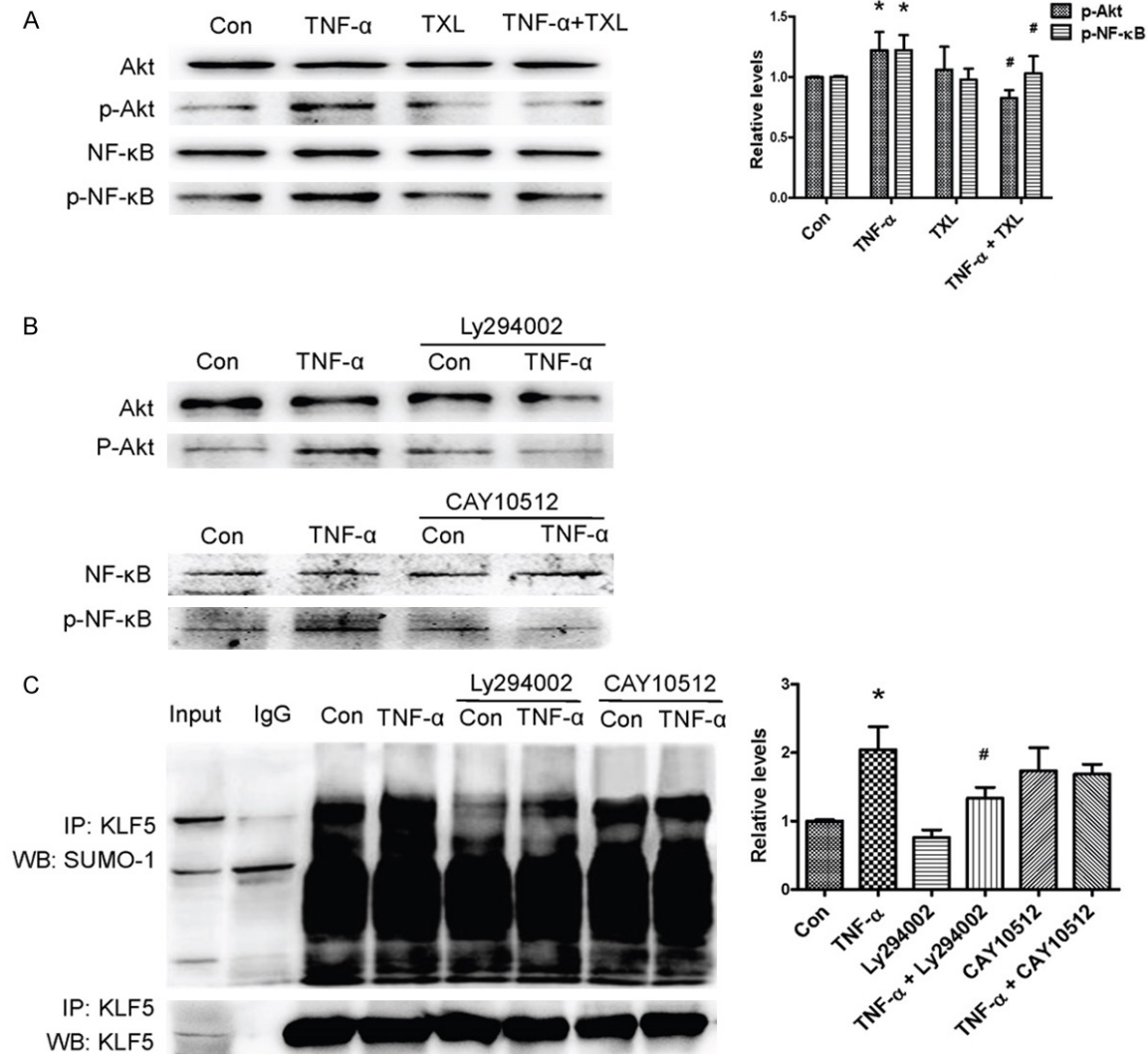


Figure 6. TNF- α induces KLF5 sumoylation via PI3K/Akt signaling, whereas TXL inhibits Akt phosphorylation induced by TNF- α . (A) Western blot analysis for Akt, p-Akt, NF- κ B and p-NF- κ B in macrophages treated with TNF- α , TXL, or TNF- α plus TXL. Bars represent the means \pm SD from three independent experiments. * P <0.05 vs. Con group; # P <0.05 vs. TNF- α group. (B) Western blot detection of p-Akt and p-NF- κ B in macrophages treated or not with PI3K/Akt inhibitor LY294002 or NF- κ B inhibitor CAY10512. (C) The lysates from macrophages treated as in (B) were immunoprecipitated with anti-KLF5, and then detected by Western blotting with anti-SUMO-1. Bars represent the means \pm SD from three independent experiments. * P <0.05 vs. Con group; # P <0.05 vs. TNF- α group.

TNF- α induces KLF5 sumoylation via PI3K/Akt signaling pathway, whereas TXL inhibits Akt phosphorylation induced by TNF- α

We next examined which signal pathway mediates KLF5 sumoylation in macrophages in response to TNF- α . As shown in **Figure 6A**, TNF- α significantly increased the level of phospho-Akt and phospho-NF- κ B (**Figure 6A**, lane 2 versus lane 1). TXL treatment effectively reversed the stimulatory effect of TNF- α on the phosphorylation of these two signaling molecules (**Figure 6A**, lane 4 versus lane 2). Under

the same experimental conditions, the expression level of these signal molecules did not change significantly during the course of stimulation with TNF- α (**Figure 6A**). Macrophages were then incubated with the PI3K/Akt inhibitor LY294002, or the NF- κ B inhibitor CAY10512 for 2 h before exposure to TNF- α . The results showed that inhibition of PI3K/Akt or NF- κ B by their respective pharmacological inhibitors blocked the TNF- α -induced phosphorylation of Akt and NF- κ B (**Figure 6B**, lane 4 versus lane 2). Correspondingly, we observed the effect of pharmacological inhibition of the NF- κ B and

PI3K/Akt signaling pathways on KLF5 sumoylation induced by TNF- α . As shown in **Figure 6C**, inhibition of PI3K/Akt by LY294002 reduced sumoylation of KLF5 induced by TNF- α (**Figure 6C**, lane 4 versus lane 2), whereas NF- κ B inhibition by CAY10512 had only a slight impact on KLF5 sumoylation induced by TNF- α (**Figure 6C**, lane 6 versus lane 2). Taken together, these results further suggest that PI3K/Akt pathway mediates TNF- α -induced KLF5 sumoylation in macrophages, and TXL treatment suppresses KLF5 sumoylation through blocking PI3K/Akt signaling.

Discussion

Neointimal formation is a common pathological characteristic in diverse cardiovascular diseases such as atherosclerosis, coronary heart diseases, postangioplasty restenosis, and transplantation arteriopathy. The inflammatory response that follows endothelial injury is recognized to play an important role in neointimal formation. Inflammatory cells adhering to sites of endothelial dysfunction produce cytokines and growth factors, including tumor necrosis factor (TNF)- α and platelet-derived growth factor (PDGF). These induce the migration and proliferation of smooth muscle cells [26]. These cells secrete extracellular matrix components, including elastin and collagen, to form neointimal hyperplasia and restenosis. Our previous study showed that TXL can improve the vascular endothelial function via up-regulating tight junction protein expression [27]. In this study, we found that TXL decreased macrophage migration and infiltration through inhibiting KLF5 expression in macrophage, as well as through regulating the ubiquitination and sumoylation of KLF5. These results suggest that TXL-mediated cardiovascular protection is not only via improvement of endothelial function but also via anti-inflammatory effect on macrophages.

KLF5 exerts growth-promoting functions in a variety of cell types through its activation of key cell-cycle-promoting genes [28, 29], thus participating in vascular remodeling process induced by vascular injury. In endothelial cells (ECs), KLF5 mediates the expression of tight junction proteins, and TXL increases tight junction protein levels by inducing KLF5 expression [27]. In VSMCs, KLF5 drives phenotypic switch-

ing by suppression of smooth muscle cell (SMC) marker genes, and meanwhile KLF5 inhibits p21 expression and G1/S cell cycle progression by promoting interaction of KLF5 with c-Jun, with KLF5 binding to its cis-elements of the p21 promoter [30]. In this study, we found that TXL treatment repressed macrophage proliferation and migration induced by TNF- α in vitro, and reduced macrophage infiltration into the arterial wall induced by vascular endothelial injury. Mechanistic studies suggested that the decreased proliferation and migration of macrophages in TXL-treated mice or cells was attributable to a reduction in KLF5 expression. Importantly, KLF5 overexpression or macrophage-specific knockout of KLF5 significantly increased or reduced macrophage migration, as evidenced by Mac2 immunopositive macrophages infiltrated into the neointima of ligated arteries. These results clearly indicated that KLF5 expression and activity have profound pleiotropic effects on different cell types that participate in vascular pathophysiology.

TXL is a compound prescription formulated according to the meridian theory of traditional Chinese medicine. It has been demonstrated that ginsenoside Rb1, the major ingredient of TXL, could effectively block homocysteine-induced endothelial dysfunction, superoxide anion production and down-regulation of endothelial nitric oxide synthesis [31], and paeonia, another ingredient of TXL, has antioxidative, vasodilatory, antiplatelet, lipid-lowering and anti-inflammatory activities [32]. These findings support the notion that Traditional Chinese Medicine (TCM) has characteristics of multiple ingredients, multiple targets, and multiple pathways, and that multiple ingredients simultaneously act on multiple targets to cause different effects.

Besides down-regulating KLF5 expression in macrophages, TXL also regulated KLF5 stability by ubiquitination and sumoylation of KLF5. It has been known that KLF5 is an unstable protein with a short half-life [25]. KLF5 can be degraded through the ubiquitin-dependent and ubiquitin-independent mechanisms [25, 33]. In this article, our data showed that TNF- α and endothelial injury up-regulated KLF5 expression, whereas TXL treatment promoted the interactions of KLF5 with Fbxw7 and enhanced KLF5 ubiquitination by Fbxw7, facilitating KLF5

degradation. The results of the present study prompted us to hypothesize that multiple ingredients and multiple pathways in TCM may act on different targets simultaneously to generate a range of actions that manifest as a comprehensive overall effect.

TNF- α activated the PI3K/Akt signaling pathway, as shown by the increased level of phospho-Akt, subsequently leading to the increase in KLF5 sumoylation. TXL treatment abrogated TNF- α -induced Akt phosphorylation. In addition, inhibition of PI3K/Akt by its pharmacological inhibitor LY294002 decreased the TNF- α -induced phosphorylation of Akt. Simultaneously, inhibition of PI3K/Akt abrogated the stimulatory effect of TNF- α on the sumoylation of KLF5, suggesting PI3K/Akt signaling plays a key role in TNF- α -induced KLF5 sumoylation, as well as in TXL-suppressed sumoylation of KLF5. Considering the previous reports and the present study, TXL-mediated cardiovascular and cerebrovascular protection may be via multiple different pathways. Modulation of KLF5 expression and stability in macrophages is one of the mechanisms underlying the vasoprotective effects of TXL.

Acknowledgements

This study was supported by the National Basic Research Program of China (No. 2012CB518-601), the National Natural Science Foundation of China (No. 31271396, 31271224, 31301136) and the Hebei Key Scientific and Technological Project (No. 13967607D).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jin-Kun Wen, Department of Biochemistry and Molecular Biology, Hebei Medical University, Shijiazhuang 050017, PR China. Tel: +86-311-86265563; Fax: +86-311-86266180; E-mail: wjk@hebmh.edu.cn

References

- [1] Tellides G and Pober JS. Inflammatory and immune responses in the arterial media. *Circ Res* 2015; 116: 312-322.
- [2] Gimbrone MA Jr and García-Cardena G. Endothelial cell dysfunction and the pathobiology of atherosclerosis. *Circ Res* 2016; 118: 620-636.

- [3] Tabas I and Bornfeldt KE. Macrophage phenotype and function in different stages of atherosclerosis. *Circ Res* 2016; 118: 653-667.
- [4] Smale ST and Natoli G. Transcriptional control of inflammatory responses. *Cold Spring Harb Perspect Biol* 2014; 6: a016261.
- [5] Aizawa K, Suzuki T, Kada N, Ishihara A, Kawai-Kowase K, Matsumura T, Sasaki K, Munemasa Y, Manabe I, Kurabayashi M, Collins T and Nagai R. Regulation of platelet-derived growth factor-A chain by Krüppel-like factor 5: new pathway of cooperative activation with nuclear factor-kappaB. *J Biol Chem* 2004; 279: 70-76.
- [6] Alaiti MA, Orasanu G, Tugal D, Lu Y and Jain MK. Krüppel-like factors and vascular inflammation: implications for atherosclerosis. *Curr Atheroscler Rep* 2012; 14: 438-449.
- [7] Fujii K, Manabe I and Nagai R. Renal collecting duct epithelial cells regulate inflammation in tubulointerstitial damage in mice. *J Clin Invest* 2011; 121: 3425-3441.
- [8] Luan Y and Wang P. FBW7-mediated ubiquitination and degradation of KLF5. *World J Biol Chem* 2014; 5: 216-223.
- [9] Du JX, Bialkowska AB, McConnell BB and Yang VW. SUMOylation regulates nuclear localization of Krüppel-like factor 5. *J Biol Chem* 2008; 283: 31991-32002.
- [10] Zhang Z and Teng CT. Phosphorylation of Krüppel-like factor 5 (KLF5/IKLF) at the CBP interaction region enhances its transactivation function. *Nucleic Acids Res* 2003; 31: 2196-2208.
- [11] Liu N, Li H, Li S, Shen M, Xiao N, Chen Y, Wang Y, Wang W, Wang R, Wang Q, Sun J and Wang P. The Fbw7/human CDC4 tumor suppressor targets proliferative factor KLF5 for ubiquitination and degradation through multiple phosphodegron motifs. *J Biol Chem* 2010; 285: 18858-18867.
- [12] Zhao D, Zheng HQ, Zhou Z and Chen C. The Fbw7 tumor suppressor targets KLF5 for ubiquitin-mediated degradation and suppresses breast cell proliferation. *Cancer Res* 2010; 70: 4728-4738.
- [13] Zhang L, Liu Y, Lu XT, Wu YL, Zhang C, Ji XP, Wang R, Liu CX, Feng JB, Jiang H, Xu XS, Zhao YX and Zhang Y. Traditional Chinese medication Tongxinluo dose dependently enhances stability of vulnerable plaques. *Am J Physiol Heart Circ Physiol* 2009; 297: H2004-H2014.
- [14] Liu Y, Tang GH, Sun YH, Lin XJ, Wei C, Yang GY and Liu JR. The protective role of Tongxinluo on blood-brain barrier after ischemia-reperfusion brain injury. *J Ethnopharmacol* 2013; 148: 632-639.
- [15] Liang JQ, Wu K, Jia ZH, Liu C, Ding J, Huang SN, Yin PP, Wu XC, Wei C, Wu YL and Wang HY. Chinese medicine Tongxinluo modulates vas-

- cular endothelial function by inducing eNOS expression via the PI-3K/Akt/HIF-dependent signaling pathway. *J Ethnopharmacol* 2011; 133: 517-523.
- [16] Chen WQ, Zhong L, Zhang L, Ji XP, Zhao YX, Zhang C, Jiang H, Wu YL and Zhang Y. Chinese medicine tongxinluo significantly lowers serum lipid levels and stabilizes vulnerable plaques in a rabbit model. *J Ethnopharmacol* 2009; 124: 103-110.
- [17] Zhang RN, Zheng B, Li LM, Zhang J, Zhang XH and Wen JK. Tongxinluo inhibits vascular inflammation and neointimal hyperplasia through blocking positive feedback loop between miR-155 and TNF- α . *Am J Physiol Heart Circ Physiol* 2014; 307: H552-H562.
- [18] Li N, Yang YJ, Cui HH, Zhang Q, Jin C, Qian HY, Dong QT and Zhang H. Tongxinluo decreases apoptosis of mesenchymal stem cells concentration-dependently under hypoxia and serum deprivation conditions through the AMPK/eNOS pathway. *J Cardiovasc Pharmacol* 2014; 63: 265-273.
- [19] Zheng CY, Song LL, Wen JK, Li LM, Guo ZW, Zhou PP, Wang C, Li YH, Ma D and Zheng B. Tongxinluo (TXL), a traditional Chinese medicinal compound, improves endothelial function after chronic hypoxia both in vivo and in vitro. *J Cardiovasc Pharmacol* 2015; 65: 579-586.
- [20] Luo WM, Kong J, Gong Y, Liu XQ, Yang RX and Zhao YX. Tongxinluo protects against hypertensive kidney injury in spontaneously-hypertensive rats by inhibiting oxidative stress and activating Forkhead box O1 signaling. *PLoS One* 2015; 10: e0145130.
- [21] Kumar A and Lindner V. Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscler Thromb Vasc Biol* 1997; 17: 2238-2244.
- [22] Yang Z, Zheng B, Zhang Y, He M, Zhang XH, Ma D, Zhang RN, Wu XL and Wen JK. miR-155-dependent regulation of mammalian sterile 20-like kinase 2 (MST2) coordinates inflammation, oxidative stress and proliferation in vascular smooth muscle cells. *Biochim Biophys Acta* 2015; 1852: 1477-1489.
- [23] Zhang XH, Zheng B, Gu C, Fu JR and Wen JK. TGF β 1 downregulates AT1 receptor expression via PKC-d-mediated Sp1 dissociation from KLF4 and Smad-mediated PPAR-g association with KLF4. *Arterioscler Thromb Vasc Biol* 2012; 32: 1015-1023.
- [24] Rectenwald JE, Moldawer LL, Huber TS, Seeger JM and Ozaki CK. Direct evidence for cytokine involvement in neointimal hyperplasia. *Circulation* 2000; 102: 1697-1702.
- [25] Chen C, Sun X, Ran Q, Wilkinson KD, Murphy TJ, Simons JW and Dong JT. Ubiquitin-proteasome degradation of KLF5 transcription factor in cancer and untransformed epithelial cells. *Oncogene* 2005; 24: 3319-3327.
- [26] Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; 352: 1685-1695.
- [27] Li LM, Zheng B, Zhang RN, Jin LS, Zheng CY, Wang C, Zhou PP, Guo ZW, Ma D and Wen JK. Chinese medicine Tongxinluo increases tight junction protein levels by inducing KLF5 expression in microvascular endothelial cells. *Cell Biochem Funct* 2015; 33: 226-234.
- [28] Nandan MO, Chanchevalap S, Dalton WB and Yang VW. Krüppel like factor 5 promotes mitosis by activating the cyclin B1/Cdc2 complex during oncogenic Ras-mediated transformation. *FEBS Lett* 2005; 579: 4757-4762.
- [29] McConnell BB, Ghaleb AM, Nandan MO and Yang VW. The diverse functions of Krüppel-like factors 4 and 5 in epithelial biology and pathology. *Bioessays* 2007; 29: 549-557.
- [30] He M, Han M, Zheng B, Shu YN and Wen JK. Angiotensin II stimulates KLF5 phosphorylation and its interaction with c-Jun leading to suppression of p21 expression in vascular smooth muscle cells. *J Biochem* 2009; 146: 683-691.
- [31] Zhou W, Chai H, Lin PH, Lumsden AB, Yao Q and Chen C. Ginsenoside Rb1 blocks homocysteine-induced endothelial dysfunction in porcine coronary arteries. *J Vasc Surg* 2005; 41: 861-868.
- [32] Ghayur MN, Gilani AH, Rasheed H, Khan A, Iqbal Z, Ismail M, Saeed SA and Janssen LJ. Cardiovascular and airway relaxant activities of peony root extract. *Can J Physiol Pharmacol* 2008; 86: 793-803.
- [33] Chen C, Zhou Z, Guo P and Dong JT. Proteasomal degradation of the KLF5 transcription factor through a ubiquitin-independent pathway. *FEBS Lett* 2007; 581: 1124-1130.