

# Ulinastatin Inhibits the Proliferation, Invasion and Phenotypic Switching of PDGF-BB-Induced VSMCs via Akt/eNOS/NO/cGMP Signaling Pathway

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**Background:** Atherosclerosis is a chronic inflammatory disease responsible for thrombosis, blood supply disorders, myocardial infarction and strokes, eventually leading to increased deaths and reduced quality of life. As inflammation plays a vital role in the development of this disease, the present study aims to investigate whether urinary trypsin inhibitor (UTI) with anti-inflammatory property can inhibit the proliferation, invasion and phenotypic switching of PDGF-BB-induced vascular smooth muscle cells (VSMCs) and probe its potential mechanism.

**Methods:** Western blot was used to detect the expressions of the proteins related to the Akt/eNOS/NO/cGMP signaling pathway, phenotypic switching and proliferation. CCK-8 assay and EdU staining were used to detect cell proliferation of VSMCs. Transwell and wound healing assays were respectively conducted to measure the invasion and migration of VSMCs. The concentration of NO was evaluated by NO detection kit. ELISA assay analyzed the expression of cyclic GMP (cGMP).

**Results:** The expressions of p-Akt and p-eNOS were elevated by UTI treatment. Furthermore, UTI inhibited the proliferation, migration and invasion of VSMCs. UTI also increased the expressions of proteins related to phenotypic switching. The amount of NO and expression of cGMP were both elevated under UTI treatment.

**Conclusion:** UTI inhibits the proliferation, invasion and phenotypic switching of PDGF-BB-induced VSMCs via Akt/eNOS/NO/cGMP signaling pathway, which might provide a theoretical basis for the UTI treatment of atherosclerosis.

**Keywords:** ulinastatin, atherosclerosis, proliferation, invasion, phenotypic switching, Akt/eNOS/NO/cGMP signaling pathway

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## Introduction

As a chronic inflammatory disease, atherosclerosis is accountable for other cardiovascular diseases such as thrombosis, blood supply disorders, myocardial infarction and strokes.<sup>1,2</sup> Characterized by lipid accumulation in the vessel wall, atherosclerosis brings about high morbidity and lethality, leading to a large amount of deaths and loss of life quality among patients and their families.<sup>3,4</sup> Despite great expenditure and decades of research, the underlying mechanisms triggering the occurrence, progression and the clinical events at the end stage, such as plaque rupture and myocardial infarction, still puzzle numerous researchers. Thus, it is urgent to take measures to understand the complex mechanism in this disease.<sup>5</sup>

Urinary trypsin inhibitor (UTI) is also called as "ulinastatin", a kind of glycoprotein and a nonspecific wide-spectrum protease inhibitor.<sup>6,7</sup> It could reduce pro-inflammatory

cytokines and increase anti-inflammatory cytokines to protect the organs of the patients and alleviate tissue injury.<sup>8-10</sup> What is more, existing evidence has illustrated that the inhibitory impacts of UTI on inflammation and oxidative stress could attenuate ischemia-reperfusion injury and lower the generation of oxygen free radicals. UTI has been extensively used to treat various diseases, such as pancreatitis, sepsis, and hemorrhagic shock.<sup>11</sup> Considering that the contribution of inflammatory cells to the occurrence and development of atherosclerosis have been emphasized by numerous studies, drugs with anti-inflammatory property may be an excellent option for its treatment.<sup>12</sup>

In mature and normal blood vessels, vascular smooth muscle cells (VSMCs) present contractile phenotype, with elevated levels of contractile markers such as  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and smooth muscle 22 alpha (SM22 $\alpha$ ).<sup>13</sup> However, when VSMCs are stimulated by cytokines like platelet-derived growth factor BB (PDGF-BB), they will convert to synthetic phenotype, along with enhanced dedifferentiation, proliferation and migration, evoking the occurrence of atherosclerosis.<sup>14</sup> Since proliferation and migration of vascular smooth muscle cells (VSMCs) are the crucial factors for atherosclerosis, and phenotypic switching is believed to be a common vascular pathological condition in atherosclerosis, we intended to test the influences of UTI on the proliferation, invasion and phenotypic switching of VSMCs.<sup>15</sup> PDGF family is composed of five proteins, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD contained.<sup>16</sup> Among them, PDGF-BB, one of the most effective stimulants for VSMC proliferation and migration, is chosen to induce VSMCs proliferation and migration as per a previous report.<sup>17</sup>

In the present study, we aimed to investigate whether UTI could suppress the proliferation, invasion and phenotypic switching of PDGF-BB-induced VSMCs and its potential mechanism.

## Materials and Methods

### Cell Culture

VSMC cell line T/G HA-VSMC cells (RRID: CVCL\_4009) from human were obtained from ATCC (Manassas, USA). These cells were cultured with the Dulbecco's modified Eagle's medium (DMEM; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA). These cells were placed in a humid atmosphere at 37°C with 5% CO<sub>2</sub>. PDGF-BB (20 ng/mL; TL644I; Shanghai Xitang Biotechnology Co., LTD., China) was applied to treat the cells for 24 h.

### Western Blot

Protein extracts were prepared from cells using RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO). Protein concentration was measured using the bicinchoninic acid method. Equal amounts of proteins were separated by 10% SDS-PAGE and next transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for 1 h, followed by incubation with the following primary antibodies: CDK2 (1:1000, Abcam, cat. no. ab206038), PCNA (1:1000, Abcam, cat. no. ab92552), p-Akt Ser473 (1:1000, Abcam, cat. no. ab18206), Akt (1:1000, Abcam, cat. no. ab8805), p-eNOS Ser1177 (1:1000, Cell Signaling Technology, Danvers, MA; cat. no. 9571), eNOS (1:1000, Abcam, cat. no. ab199956), MMP-2 (1:1000, Abcam, cat. no. ab37150), MMP-9 (1:1000, Abcam, cat. no. ab38898),  $\alpha$ -SMA (1:1000, Abcam, cat. no. ab204573), and SM22 $\alpha$  (1:1000, Arigo, cat. no. ARG63416). The membranes were subsequently cultured with horseradish peroxidase-conjugated goat anti-rabbit IgG. The protein was detected using an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL) and the band intensity was quantified with Image-Pro Plus 4.5 software. GAPDH served as the internal control.

### Cell Counting Kit-8 (CCK-8) Assay and EdU Assay

VSMCs were seeded into a 96-well plate at  $3 \times 10^3$  cells/well in DMEM containing 10% FBS. PDGF-BB treatment (20ng/mL) was used for 24 h after starvation of the cells in serum-free medium for 24 h. 10  $\mu$ L of CCK-8 reagent (Dojindo, Japan) was added into per well for 3 h. Absorbance at 450 nm was determined by a spectrophotometer (Model 680; Bio-Rad, USA). For EdU analysis, the nuclear DNA was counterstained using Hoechst 33,342, and the EdU-positive images were captured by a fluorescence microscopy (80i, Nikon, Japan), as previously performed.<sup>18</sup>

### RT-qPCR

Total cellular RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the instruction. SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific) was used to detect mRNA expression level. GAPDH was used as an internal control. The primer sequences are as follows: CDK2: forward 5'-GCGAATTCCCCAGCCCTAATCTC A-3', reverse: 5'-GCCTCGAGAACCCTCTTCAGCAATA

A-3'; PCNA forward: 5'-TCCCAGTTCACCATCCATGTC-3', reverse: 5'-GGTCCCCTAAGGCCCATTCCT-3'; MMP2: forward: 5'-CAACGGTTCGGGAATACAGCAG-3', reverse: 5'-CCAGGAAAGTGAAGGGGAAGA-3'; MMP9: forward: 5'-CGTGTCTGGAGATTCGACTTGA-3', reverse: 5'-TGGAAGATGTCGTGTGAGTTCC-3';, whereas those for the internal control, GAPDH: forward: 5'-ACCACAGTCCATGCCATCAC-3', reverse: 5'-TCCACC ACCCTGTTGCTGTA-3'.

## Wound Healing Assay

Cells of each group were collected and seeded into 24-well plates with a density of  $5 \times 10^3$ /mL. When the cells were completely covered, a vertical line was drawn with a 10 $\mu$ L tip on the well. Cells were washed with PBS thrice and visualized under an inverted microscope. The images were labeled as 0 h as a reference. Then, serum-free DMEM was added and cells were continuously cultured for 24 h. The images were taken under an inverted microscope and labeled as 24 h. The relative cell migration was analyzed by Image J software.

## Transwell Assay

The invasion of cells was evaluated using a Matrigel-based assay in 24-well Transwell chambers.  $2 \times 10^4$  cells were plated in the upper chambers precoated with Matrigel (BD Biosciences) membrane, and DMEM (600  $\mu$ l) containing 10% FBS was added to the lower chamber. Following incubation for 24 h, the cells on the upper surface of the membrane were removed by a sterile cotton swab. The invading cells were stained with hematoxylin and eosin (H&E, Sigma-Aldrich) and then observed under a microscope (Nikon E100; Nikon, Tokyo, Japan). Thereafter, the number of invading cells was counted.

## Immunofluorescence Staining

After being fixed with 4% paraformaldehyde for 30 min, the stimulated VSMCs were permeabilized with 0.1% Triton X-100 in PBS for 15 min. Cells were blocked with 5% BSA for 1 h at room temperature and then incubated with indicated primary antibodies at 4°C overnight. After three times of wash with PBS, cells were detected with goat anti-rabbit IgG H&L Alexa Fluor<sup>®</sup> 488. Finally, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min. Images were photographed by a fluorescence microscope (80i, Nikon, Tokyo, Japan).

## Detection of NO

NO production was measured using a Nitric Oxide Colorimetric Assay Kit (BioVision, Mountain View, CA, U.S.), and OD values were measured at 540 nm.

## Enzyme-Linked Immunosorbent Assay (ELISA)

Cell supernatant was collected by centrifugation, and then the concentrations of the inflammatory cytokines were measured as per the instructions of the corresponding ELISA kits (Multi Science, Hangzhou, China). All the experiments were repeated in triplicate.

## Statistical Analysis

All results were defined as mean  $\pm$  standard deviation (SD). Comparisons between two groups were made by Student's *t*-test. For comparisons among multiple groups, statistical analysis was performed by ANOVA followed by Tukey's test. The criterion for statistical significance was set at  $p < 0.05$ .

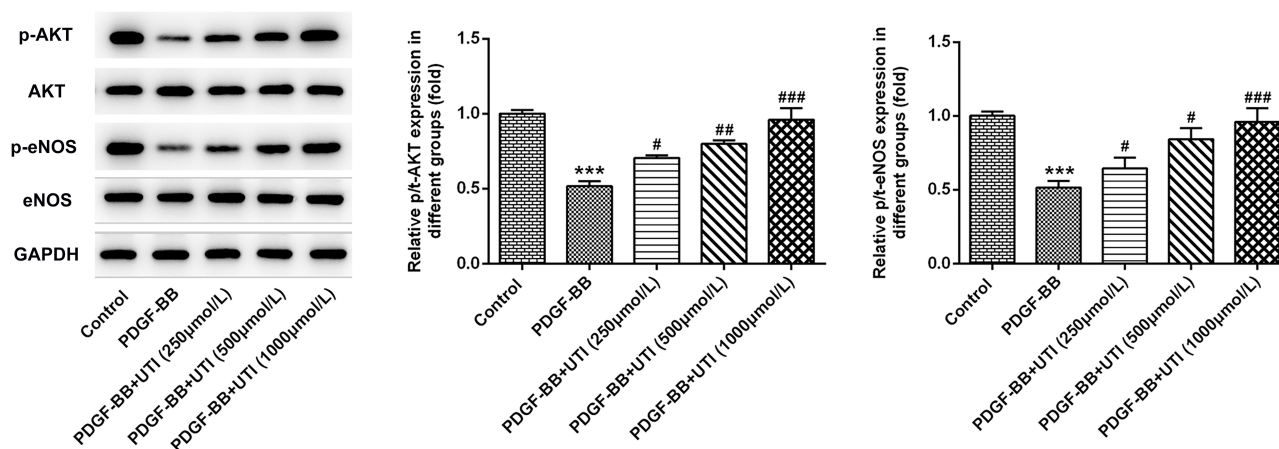
## Results

### UTI Activates the Expression of Akt/eNOS Signal in PDGF-BB-Induced VSMCs

Akt is a crucial signaling pathway regulating eNOS in the endothelium.<sup>19</sup> The expressions of Akt and eNOS were evaluated by Western blot. As shown in Figure 1, compared with the control group, the PDGF-BB group exhibited significantly lower expressions of p-Akt and p-eNOS, while the addition of UTI dose-dependently increased their expressions. Herein, UTI at doses of 500  $\mu$ mol/L and 1000  $\mu$ mol/L was chosen for the next experiments.

### UTI Inhibits the Proliferation of PDGF-BB-Stimulated VSMCs

To further investigate the role of UTI in the proliferation of PDGF-BB-induced VSMCs, CCK-8 assay, EdU staining, RT-qPCR and Western blot were conducted. The results of CCK-8 showed that PDGF-BB dramatically elevated the cell viability of VSMCs; however, the addition of UTI inhibited the cell viability dose-dependently, which could be further reversed by AKT inhibitor or eNOS inhibitor (L-NAME) (Figure 2A). Western blot (Figure 2B) and RT-qPCR (Figure 2C) discovered that the elevated levels of proliferation-related proteins CDK2 and PCNA in VSMCs induced by PDGF-BB were decreased after UTI treatment, and the effects were restored when AKT inhibitor or eNOS inhibitor



**Figure 1** UTI activates the expression of Akt/eNOS signal in PDGF-BB-induced VSMCs. Western blot was used to detect the Akt/eNOS signaling-related proteins. \*\*\* $P < 0.001$  vs Control; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  vs PDGF-BB.

were added. Furthermore, the result of EdU was consistent with that of CCK8 experiment (Figure 2D and E). These results suggest that UTI represses the proliferation of PDGF-BB-treated VSMCs.

### UTI Hinders the Invasion and Migration of PDGF-BB-Treated VSMCs

The invasion and migration of PDGF-BB-treated VSMCs were evaluated by transwell and wound healing assays, respectively. Compared with control cells, PDGF-BB induced VSMCs presented enhanced migration (Figure 3A and C) and invasion (Figure 3B and D). Moreover, this effect was inhibited by UTI treatment. In addition, AKT inhibitor and eNOS inhibitor recovered the UTI-inhibited invasion and migration of PDGF-BB-activated VSMCs. Also, compared with control cells, the expressions of MMP-2 and MMP-9 in PDGF-BB-activated VSMCs were upregulated, which was markedly downregulated upon UTI treatment (Figure 3E and F). Furthermore, AKT inhibitor and eNOS inhibitor reversed the suppressive effects of UTI on MMP-2 and MMP-9 in PDGF-BB-induced VSMCs. The expression of MMP-2 and MMP-9 promoted by RT-qPCR was consistent with that of Western blot (Figure 3G). Thus, these results demonstrate that UTI hampers the invasion and migration of PDGF-BB-induced VSMCs.

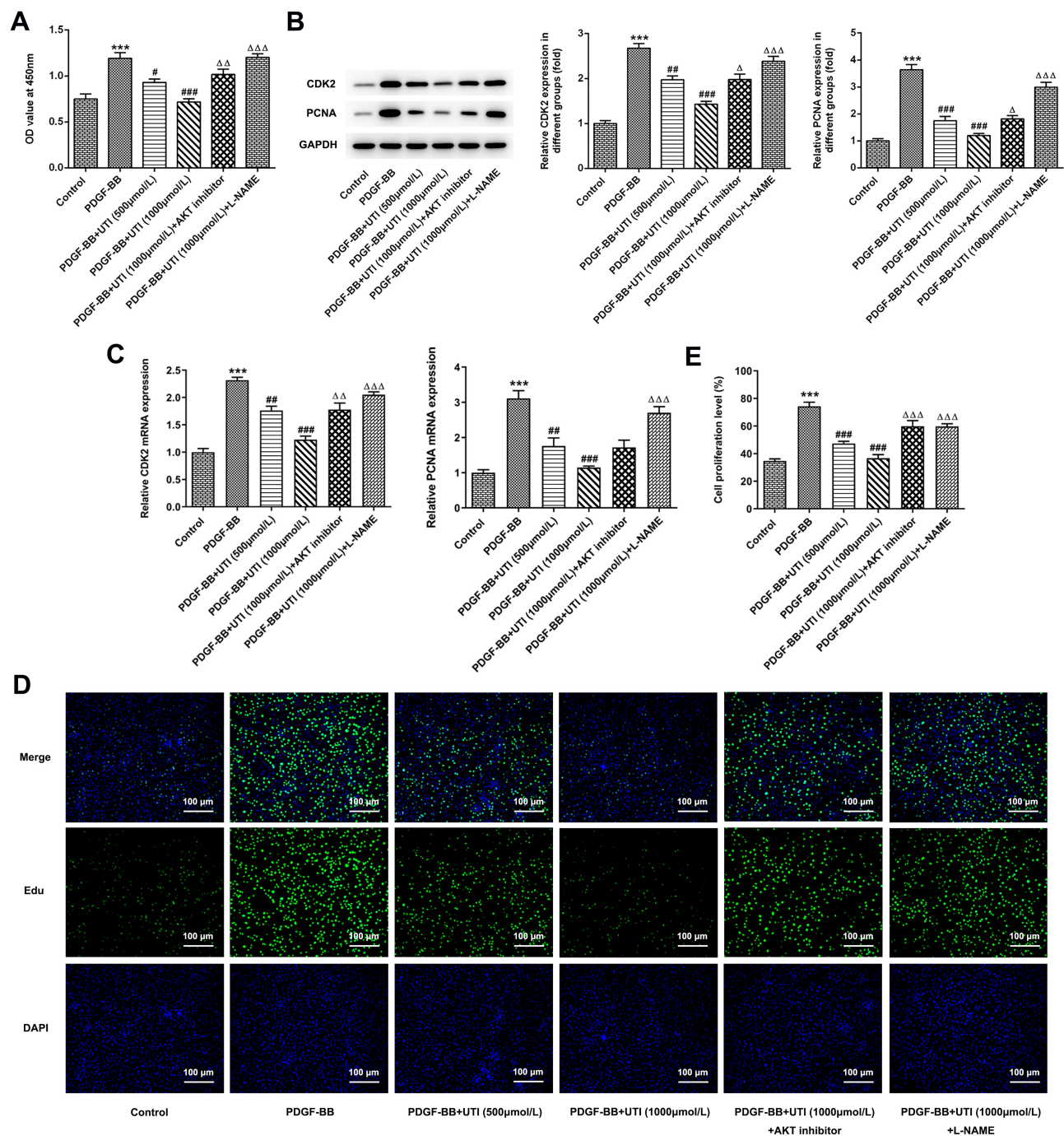
### UTI Resists the Phenotypic Switching of PDGF-BB-Stimulated VSMCs

To further clarify the role of UTI in phenotypic switching of PDGF-BB-stimulated VSMCs, we examined the protein expressions of contraction phenotype makers,

$\alpha$ -SMA and SM22 $\alpha$ . Compared with control, VSMCs treated with PDGF-BB exhibited lower expressions of  $\alpha$ -SMA and SM22 $\alpha$ , which were elevated by the treatment of UTI in a dose-dependent manner (Figure 4A). However, addition of AKT inhibitor and eNOS inhibitor downregulated their expressions. Similar results are found in Figure 4B and C, which confirmed the changes of  $\alpha$ -SMA expression. Thus, the above results hint that UTI resists the phenotypic switching of PDGF-BB-induced VSMCs.

### UTI Triggers the Activation of Akt/eNOS/NO/cGMP Signaling Pathway

The results from Western blot showed that compared with control, the expression levels of p-Akt and p-eNOS in VSMCs were decreased by PDGF-BB (Figure 5A). What is more, their levels were elevated when PDGF-BB-induced VSMCs were treated with UTI, and the additional treatment of AKT inhibitor and eNOS inhibitor reversed these effects. Next, we detected the concentration of NO, a critical endothelium-derived relaxing factor produced by eNOS.<sup>20</sup> The results elucidated that compared with control, the concentration of NO was decreased in PDGF-BB-induced VSMCs but increased under UTI treatment (Figure 5B). Furthermore, AKT inhibitor and eNOS inhibitor decreased the increase of NO concentration caused by UTI in PDGF-BB-treated VSMCs. The result from ELISA assay illustrated the similar trend of cGMP expression with the changes in NO concentration (Figure 5B). Therefore, the results indicate that UTI triggers the activation of Akt/eNOS/NO/cGMP signaling pathway.

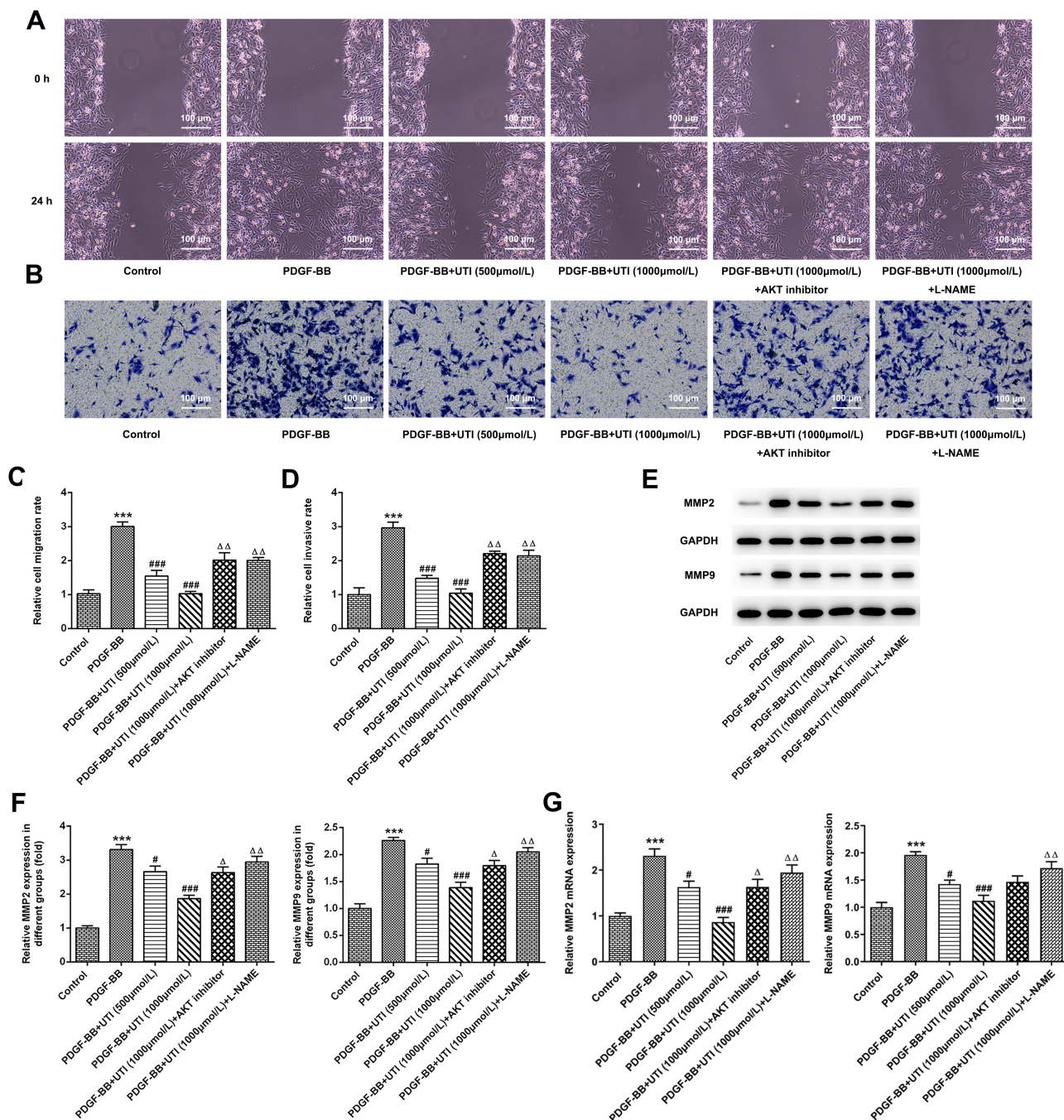


**Figure 2** UTI inhibits the proliferation of PDGF-BB-induced VSMCs. **(A)** CCK-8 assay was used to detect the cell viability of VSMCs. **(B)** Western blot detected the expressions of proliferation-related proteins. **(C)** RT-qPCR detected the expressions of proliferation-related proteins. **(D)** EdU staining was employed to detect the proliferation of VSMCs. **(E)** Statistical graph of D. \*\*\* $P < 0.001$  vs Control; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  vs PDGF-BB;  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$ ,  $\Delta\Delta\Delta P < 0.001$  vs PDGF-BB+UTI (1000μmol/L).

## Discussion

UTI is broadly known as a serine protease inhibitor that can suppress several pro-inflammatory proteases, inhibit neutrophil elastase, and decrease the release of inflammatory cytokines.<sup>21,22</sup> Based on its physiological characteristics, UTI has been extensively applied in the treatment of many

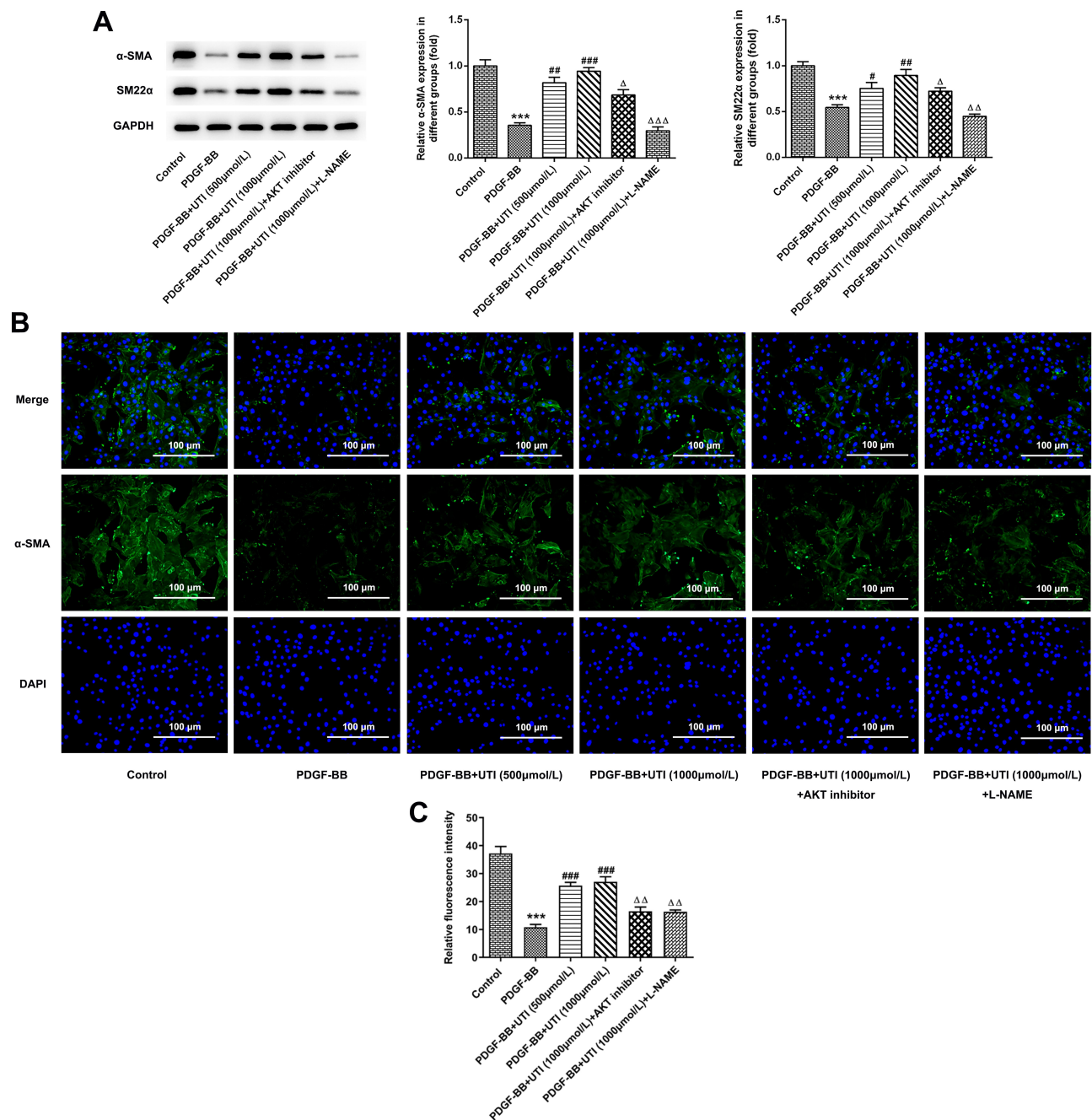
acute inflammatory disorders.<sup>23,24</sup> Studies have demonstrated that the serum levels of UTI are decreased in patients with sepsis, and genetically modified mice without genes for synthesis of UTI possess relatively higher risk of sepsis.<sup>25,26</sup> Some researchers also found the vital role of UTI in preserving the functions of myocardial mitochondria to prevent



**Figure 3** UTI impedes the invasion and migration of PDGF-BB-treated VSMCs. (A and C) Transwell and (B and D) wound healing were utilized to analyze the invasion and migration of VSMCs. (E) Western blot was used to detect the expressions of proliferation-related proteins. (F) Statistical graph of E. (G) RT-qPCR was used to detect the expressions of proliferation-related proteins. \*\*\*P<0.001 vs Control; #P<0.05, ###P<0.001 vs PDGF-BB; ΔP<0.05, ΔΔP<0.01 vs PDGF-BB+UTI (1000µmol/L).

against hemorrhagic shock.<sup>27</sup> One animal study indicated that UTI is crucial for the reduction of key inflammatory mediators in the vascular endothelium resulting in atherosclerosis.<sup>28</sup> But there was no evidence exploring its role in the proliferation, invasion and phenotypic switching of VSMCs during the development of atherosclerosis.

Atherosclerosis is mainly triggered by the excessive proliferation and migration of VSMCs, and phenotypic switching is the key element to control the proliferation and migration of VSMCs.<sup>15,29</sup> Phenotypic switching has long been thought as a major force for the atherosclerosis development, generating a pro-atherogenic VSMC

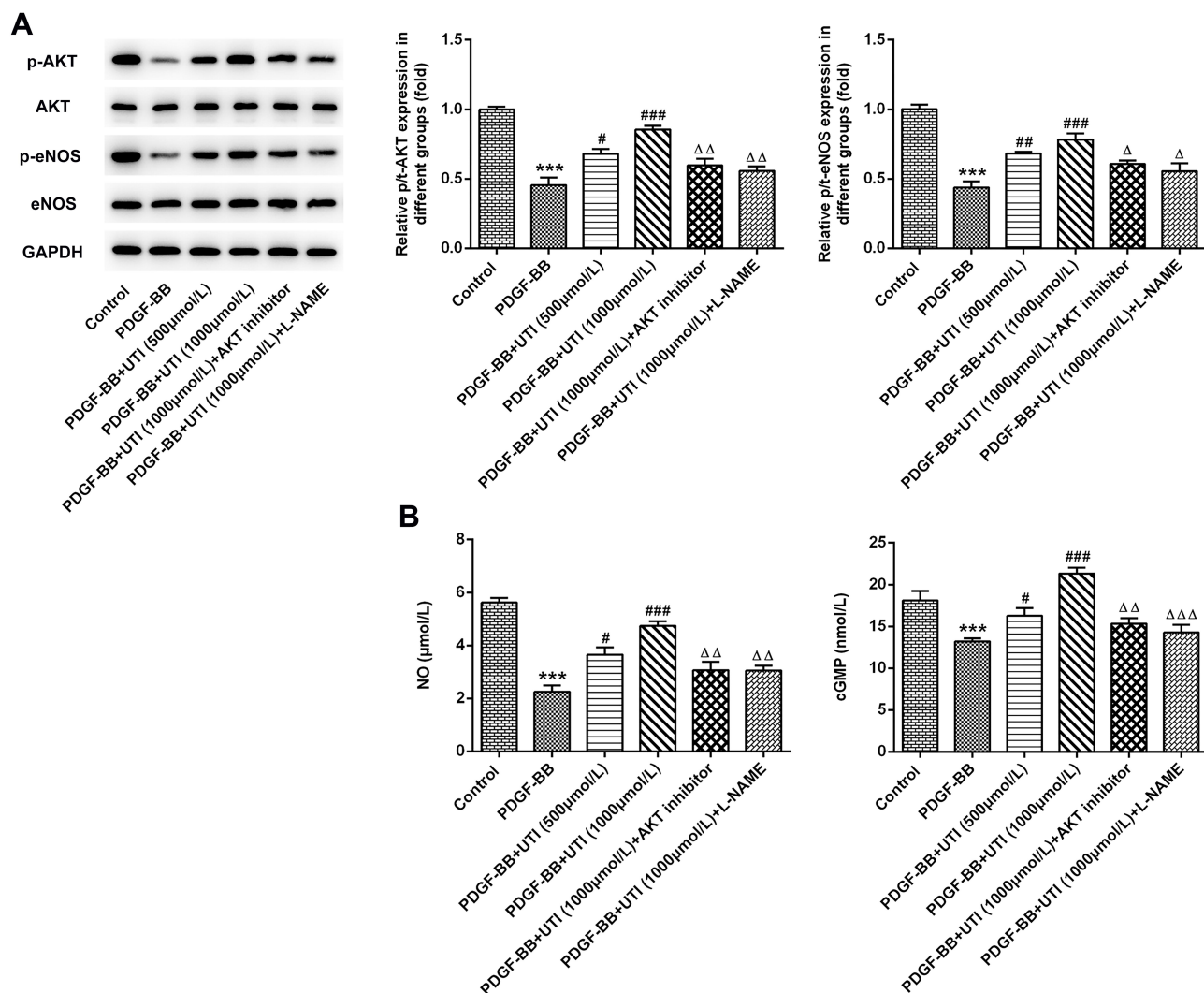


**Figure 4** UTI restrains the phenotypic switching of PDGF-BB-treated VSMCs. **(A)** Western blot was applied to detect the expressions of  $\alpha$ -SMA and SM22 $\alpha$ . **(B)** Immunofluorescence staining analysis of the expression of  $\alpha$ -SMA. **(C)** Statistical graph of B. \*\*\* $P$ <0.001 vs Control; # $P$ <0.05, ## $P$ <0.01, ### $P$ <0.001 vs PDGF-BB;  $\Delta$  $P$ <0.05,  $\Delta\Delta$  $P$ <0.01,  $\Delta\Delta\Delta$  $P$ <0.001 vs PDGF-BB +UTI (1000 $\mu$ mol/L).

phenotype.<sup>15</sup> VSMCs express a series of “SMC markers” in the normal arterial media, including SM22 $\alpha$  and  $\alpha$ -SMA.<sup>15</sup> However, under the pathological conditions of atherosclerosis, the expressions of these markers are reduced in VSMCs, giving rise to enhanced proliferation, migration and secretion of multiple extracellular matrix proteins and cytokines.<sup>30</sup> In this study, PDGF-BB significantly reduced expressions of SM22 $\alpha$  and  $\alpha$ -SMA in

VSMCs, in agreement with the observed phenomena in the previous study.<sup>19</sup> It was also proved that UTI could inhibit the phenotypic switching induced by PDGF-BB in VSMCs, which might be beneficial to the treatment of advanced atherosclerosis.<sup>31</sup>

Studies have indicated that eNOS is one of the downstream substrates of Akt that can induce eNOS phosphorylation.<sup>32</sup> In the present study, UTI promoted



**Figure 5** UTI triggers the activation of Akt/eNOS/NO/cGMP signaling pathway. **(A)** Western blot examined the Akt/eNOS/NO/cGMP signaling-related proteins. **(B)** The amount of NO and the expression of cGMP were measured by corresponding commercial kits. \*\*\* $P < 0.001$  vs Control; # $P < 0.05$ , ### $P < 0.01$ , #### $P < 0.001$  vs PDGF-BB;  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$ ,  $\Delta\Delta\Delta P < 0.001$  vs PDGF-BB + UTI (1000µmol/L).

the phosphorylation of Akt and activated its downstream effector eNOS in PDGF-BB-treated VSMCs. Furthermore, AKT inhibitor and eNOS inhibitor enhanced the proliferation, migration and invasion that were suppressed by UTI in PDGF-BB-induced VSMCs. Decreased concentration of NO, a characteristic of endothelial dysfunction, is a significant mediator in close association with cardiovascular diseases.<sup>33</sup> High amount of NO protects the vasculature through hindering platelet aggregation, monocyte adhesion, and smooth muscle cell proliferation.<sup>34</sup> Herein, UTI treatment abolished the inhibitory effect of PDGF-BB on the concentration of NO, suggesting that the endothelial dysfunction of VSMCs was alleviated by UTI. The activation of eNOS can bring about rapid vasodilatation.

Specifically, NO release can promote endothelial cells to proliferate into VSMCs and activate soluble guanylyl cyclase (sGC), thereby producing cyclic GMP (cGMP).<sup>35,36</sup> In the present study, the activation of p-Akt and p-eNOS by UTI gave rise to higher production of NO and expression of cGMP, demonstrating that UTI could activate the whole Akt/eNOS/NO/cGMP signaling pathway.

## Conclusion

Taken together, this study demonstrates that UTI inhibits the proliferation, invasion and phenotypic switching of PDGF-BB-induced VSMCs via Akt/eNOS/NO/cGMP signaling pathway, which might provide a theoretical basis for the UTI treatment of atherosclerosis.



## Data Sharing Statement

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

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## Disclosure

The authors declare that they have no competing interests.

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