

Regulation of TGF- β 1 on PI3KC3 and its role in hypertension-induced vascular injuries

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Abstract. The aim of the present study was to investigate the expression and role of transforming growth factor (TGF)- β 1/phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3) in the peripheral blood in patients with hypertension. A total of 28 patients with primary hypertension and 20 healthy control subjects were included. Peripheral blood samples were collected. The mRNA and protein expression levels were detected by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. Cell counting kit-8 assay, Transwell chamber assay and flow cytometry were performed to detect the cell proliferation, migration ability and cellular apoptosis, respectively. Laser scanning confocal microscopy was used to detect the intracellular autophagosomes. The expression of TGF- β 1 was significantly elevated, whereas the expression of PI3KC3 was significantly downregulated in the patients with hypertension compared with controls. There was negative correlation between the TGF- β 1 and PI3KC3 expression. Following treatment with TGF- β 1, the protein expression of PI3KC3 was significantly decreased in human umbilical vein endothelial cells (HUVECs), and the autophagic activity was significantly decreased. Furthermore, following the treatment of TGF- β 1 the proliferation of HUVECs was significantly reduced in the HUVECs, the hypoxia-induced apoptosis rates were significantly elevated and the number of penetrating cells were significantly declined (indicating declined migration ability). However, the overexpression of PI3KC3 significantly ameliorated the proliferation, migration ability and hypoxia tolerance of TGF- β 1-treated HUVECs. In conclusion, the present results indicated that TGF- β 1 expression was elevated in the peripheral blood in hypertensive patients and negatively correlated with the PI3KC3 expression; and that TGF- β 1 regulates the PI3KC3

signaling pathway to inhibit the autophagic activity of vascular endothelial cells, and regulate the cell proliferation, migration and anti-apoptosis ability, thus aggregating the endothelial cell injuries in hypertension. The results of the current study revealed a novel mechanism of TGF- β 1 in the regulation of endothelial cell injury in hypertension, which may provide a potential target for disease therapy.

Introduction

Primary hypertension is a common chronic disease of the circulatory system, with an incidence rate of 25.2% in China 2017 (1), which is also a key factor in the mortality of patients with cardiovascular and cerebrovascular diseases. If not properly controlled, primary hypertension may induce stroke, myocardial infarction, heart failure and chronic kidney diseases, leading to high mortality rates and seriously endangering public health (2,3). It has been demonstrated previously that, due to population aging and living standard improving in China, the incidence of primary hypertension continues to rise, and the proportion of young patients has also been significantly increasing, which has attracted great attention from Scientists (4). Continued elevation of blood pressure is a major feature of primary hypertension, and vascular injury is the underlying mechanism for the dysregulation of blood pressure (5). It has been identified that vascular injuries mainly induce the pathological processes including endothelial cell injury, smooth muscle cell proliferation and vascular remodeling (2,6). Vascular endothelial cells serve important roles in the regulation of blood pressure and the maintenance of blood vessel walls, and vascular endothelial cell injuries represent the important cause of hypertension (7). Hypertension-induced injuries of vascular endothelial cells may lead to the imbalance of the secretion of NO and endothelin, enhance vasoconstriction, increase the peripheral circulation resistance and promote the development of hypertension (8). It has previously been demonstrated that vascular injuries may cause massive apoptosis of microvascular endothelial cells, induce damage in the blood parallel pathways and elevate the peripheral circulation resistance, resulting in elevated blood pressure (9). Vascular endothelial cell injuries serve important roles in the development of hypertension, which is also a focus in medical research (10). However, the associated molecular mechanism has not yet been fully elucidated.

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The transforming growth factor (TGF)- β family of cytokines was identified in the 1980s and comprises >30 members (11,12). At present, four subtypes of TGF- β have been observed in mammals, including TGF- β 1, TGF- β 2, TGF- β 3 and TGF- β 1 β 2 (13). TGF- β 1 is currently the most well-studied member of the TGF family, which serves important regulatory roles in various pathophysiological processes, such as cell proliferation, differentiation, migration and apoptosis (14,15). Activated TGF- β 1 exerts biological functions through cell surface receptors. The classical TGF- β 1 signaling pathway is mainly mediated by the downstream mothers against decapentaplegic homolog (SMAD) family (16). In addition, TGF- β 1 can exert biological functions through a variety of non-classical pathways, such as the c-Jun N-terminal kinase, extracellular signal-regulated kinase, and p38/mitogen-activated protein kinase pathways (17-19). A previous study has demonstrated that TGF- β 1 can regulate the synthesis of collagen and the expression of extracellular matrix-related protein, thus promoting myocardial fibrosis (20,21). Furthermore, in hypertension, TGF- β 1 can induce the proliferation of vascular smooth muscle (22). It has also been reported that the expression of TGF- β 1 is positively correlated with disease development, as detected in the peripheral blood and urine of patients with myocardial infarction and hypertensive nephropathy (23). Furthermore, peripheral blood tests for hypertensive patients have demonstrated that the concentration of TGF- β 1 is significantly increased compared with the healthy control group (24). These findings suggest that TGF- β 1 may be closely associated with vascular injuries in hypertensive patients.

VPS34 was initially identified in yeast, which is analogous to phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3) in mammals (25). PI3KC3 has been demonstrated to serve an important regulatory role in autophagic processes (26). Autophagy is an indispensable physiological process through which normal cells maintain their homeostasis and activities, the main function of which comprises the recycling of aging organelles and the degradation of abnormal proteins (27). Abnormal autophagic processes may lead to mitochondrial damages, thus resulting in cell death (28). Aberrant activity of autophagy has been demonstrated to serve important roles in vascular injuries. In diabetic patients, persistent hyperglycemia induces increased reactive oxygen species in endothelial cells, which in turn activates the endothelial cell autophagy, resulting in endothelial cell death and vascular injuries (29).

In recent years, a number of studies have confirmed that TGF- β 1 can regulate the cell autophagy activity, with differential roles in different disease processes (30,31). Whether TGF- β 1 participates in the vascular injury in hypertensive patients via autophagy remains unclear. In the present study, the regulating effect of TGF- β 1 on PI3KC3 and its role in vascular injuries were investigated.

Materials and methods

Study patients. A total of 28 patients with primary hypertension, 17 males and 11 females, with a mean age of 56.3 ± 4.3 years, who were admitted to the Department of Cardiology at Zaozhuang Municipal Hospital, (Zaozhuang, China) from January 2016-January 2017, were included in the

present study. According to the Guidelines for Hypertension Diagnosis and Treatment in China (32), these patients were divided into three groups as follows: Grade I hypertension ($n=10$), grade II hypertension ($n=9$) and grade III hypertension ($n=9$). All patients were first diagnosis cases with no long-term medication history, combined tumors or other chronic diseases, autoimmune diseases or family genetic diseases. Furthermore, 20 healthy subjects were recruited as the control group. From each patient and healthy subject, 5 ml peripheral blood was collected. Prior written and informed consent was obtained from all subjects and the present study protocol was approved by the ethics review board of Zaozhuang Municipal Hospital (Zaozhuang, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Blood samples were collected and total RNA was extracted with TRIzol (life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) within 30 min. The cDNA template was obtained via the Ipsogen RT kit (Qiagen GmbH, Hilden, Germany). qPCR was performed using a Fast Fire qPCR PreMix kit (Tiangen Biotech Co., Ltd., Beijing, China) with a Step one Plus PCR machine (Thermo Fisher Scientific, Inc.). Primer sequences were as follows: PI3KC3, forward 5'-CTCGCTTCGGCAGCAC-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH forward, 5'-CGGAGTCAACGGATTTGGTTCGTAT-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. The 20- μ l PCR reaction mixture consisted of 10 μ l RT-qPCR-Mix, 0.5 μ l primers each, 2 μ l cDNA and 7 μ l ddH₂O. The PCR conditions were set as follows: 95°C for 5 min and 40 cycles of 95°C for 30 sec, 60°C for 45 sec and 72°C for 30 sec; followed by 72°C for 20 sec. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta C_q}$ method (33). GAPDH was used as the internal control.

Human umbilical vein endothelial cell (HUVEC) treatment and transfection. HUVECs were purchased from the Cell Bank, Chinese Academy of Sciences (Shanghai, China). TGF- β 1 recombinant protein was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). HUVECs were cultured at 37°C in a 5% CO₂ incubator, in RPMI 1640 culture medium (Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). For cell treatment (at 37°C in a 5% CO₂ incubator), 5 μ g/ml TGF- β 1 was added into the culture medium, to incubate the HUVECs at 37°C in a 5% CO₂ incubator for 48 h.

For cell transfection, following the treatment with TGF- β 1 for 48 h, HUVECs were seeded in a 24-well plate, at a density of 1×10^5 cells/well, and cultured, at 37°C in a 5% CO₂ incubator for 12 h, with RPMI-1640 medium containing 10% FBS. Cells were then divided into the empty vector (0.5 μ g) and PI3KC3 groups. When 60% confluence was reached, 0.5 μ g PI3KC3 plasmid (Shanghai GeneChem Co., Ltd., Shanghai, China) and 2 μ l Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) were added into 50 μ l OptiMem culture medium (Thermo Fisher Scientific, Inc.), in Eppendorf tubes, at room temperature for 5 min. The solution in the two separate tubes (lipofectamine and plasmid, respectively) was then mixed together gently, and the mixture was placed at room temperature for 15 min prior to incubation at 37°C for 6 h.

The incubation medium was then replaced with RPMI-1640 medium containing 20 ng/ml TGF- β 1, followed by incubation for 48 h at 37°C in a 5% CO₂ incubator, prior to further investigation.

Cell Counting Kit (CCK)-8 assay. Proliferation of HUVECs was detected with the CCK-8 assay. Briefly, cells were seeded onto a 96-well plate, at a density of 2×10^3 cells/well, and cultured with 200 μ l RPMI-1640 complete medium at 37°C in a 5% CO₂ incubator. According to the manufacturer's protocol, CCK-8 (Beyotime Institute of Biotechnology, Haimen, China) was added into each well to incubate the cells for 30 min, and the optical density values at 490 nm were detected at 24, 48 and 72 h. The experiment was performed in triplicate, and cell proliferation curves were produced.

Transwell chamber assay. The migration ability of HUVECs was assessed using the Transwell chamber assay. A total of 2×10^5 HUVECs were seeded into the upper chamber of a Transwell insert (8 μ m; Corning Incorporated, Corning, NY, USA) and cultured with 200 μ l serum-free RPMI 1640 culture medium, while 500 μ l RPMI 1640 medium containing 20% FBS was added into the lower chamber. Following 24 h, the cells in the upper chamber were fixed at room temperature with 4% formaldehyde for 10 min. Giemsa staining was then performed at room temperature for 2 min, and the cells were observed under a light microscope (magnification, x100). Five fields were randomly selected, and the number of penetrating cells were counted. Cell migration ability was assessed accordingly.

Flow cytometry. Cellular apoptotic processes were assessed with flow cytometry. HUVEC apoptosis was induced by hypoxia, as follows: Following transfection, HUVECs were cultured in RPMI-1640 culture medium containing TGF- β 1 (20 ng/ml) at 37°C for 48 h, and then cultured in a 1% O₂, 5% CO₂, 37°C incubator for 4 h. Following washing, the cells were collected, and the cell density was adjusted to 1×10^6 cells/100 μ l. Flow cytometry was performed with the Annexin V/FITC Apoptosis Detection kit I (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions, using a flow cytometer. Annexin V single-positive cells were undergoing early apoptosis, propidium iodide (PI; provided by the kit) single-positive cells were undergoing necrosis, and PI and Annexin V double-positive cells were undergoing late apoptosis. For the detection of Ki67, cells were incubated with 20 μ l of the PE-labeled Ki67 (BD Biosciences) in the dark at room temperature for 10 min. Analyses was performed with the ModFit LT 5.0 software (BD Biosciences).

Ad-GFP-LC3 adenovirus transfection and detection of autophagosomes. Following treatment with TGF- β 1, HUVECs were seeded onto a culture dish at a density of 1×10^5 cells/well. The mRFP-GFP-LC3 adenovirus (Hanbio Biotechnology Co., Ltd., Shanghai, China) was used to transfect the cells at multiplicity of infection=20 for 48 h. The cells were then washed with pre-chilled PBS twice, and fixed at room temperature with 4% formaldehyde for 15 min. Following washing, the autophagosomes were observed under laser confocal microscopy (magnification, x400; SP8; Leica Microsystems GmbH,

Wetzlar, Germany). Five fields were randomly selected, and the autophagic process was assessed.

Western blot analysis. Cells were lysed with radio immunoprecipitation assay lysis containing 1% PMSF. Total protein concentration was determined using the BCA method. Protein samples (20 μ g) were separated by SDS-PAGE, and then electronically transferred onto a polyvinylidene difluoride membrane. Following blocking with 5% non-fat milk at room temperature for 1 h, the membrane was incubated with the rabbit anti-human anti-LC3B primary antibody (1:1,000; cat. no. AL221; Beyotime Institute of Biotechnology), rabbit anti-human anti-PI3KC3 primary antibody (1:1,000; cat. no. ab124905; Abcam, Cambridge, UK), rabbit anti-human anti-p62 primary antibody (1:1,000; cat. no. ab155686; Abcam) and mouse anti-human anti-GAPDH primary antibody (1:5,000; cat. no. AF0006; Beyotime Institute of Biotechnology) at 4°C overnight. Following washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse (1:4,000; cat. no. A0192; Beyotime Institute of Biotechnology) or goat anti-rabbit (1:4,000; cat. no. A0208; Beyotime Institute of Biotechnology) secondary antibody, at room temperature for 1 h. Following washing with TBST, protein bands were developed using the P0018 enhanced chemiluminescence kit (Beyotime Institute of Biotechnology). Analysis was performed using quantityone software (V4.62; Bio-Rad Laboratories, Hercules, CA, USA).

ELISA. TGF- β 1 levels were detected using the Human TGF-beta 1 Quantikine ELISA kit (cat. no. DB100B; R&D Systems, Inc.), according to the manufacturer's protocol. Peripheral blood (5 ml) was collected and incubated at 4°C overnight. Serum was obtained via centrifugation at 12,000 x g at 4°C for 10 min, to detect TGF- β 1.

lactate dehydrogenase (LDH) detection. LDH levels were detected using the LDH Cytotoxicity Assay kit (cat. no. C0016) from Beyotime Institute of Biotechnology. The culture supernatant was collected and centrifuged at 12,000 x g at 4°C for 10 min. Detection was performed according to the manufacturer's protocol.

Statistical analysis. Data were expressed as the mean \pm standard deviation. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Student's t-test was performed for comparison between two groups, and one-way analysis of variance was performed for multiple group comparisons, with a Student-Newman-Keuls post hoc test. Spearman's rank correlation analysis was used to measure the correlation between PI3KC3 and TGF- β 1. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PI3KC3 is negatively correlated with TGF- β 1 in peripheral blood of hypertensive patients. Previous studies have demonstrated that the TGF- β 1 expression is significantly elevated in the peripheral blood of the hypertensive patients (34), which has diagnostic values for the hypertension-induced heart or kidney injuries. Therefore, the mRNA expression levels of

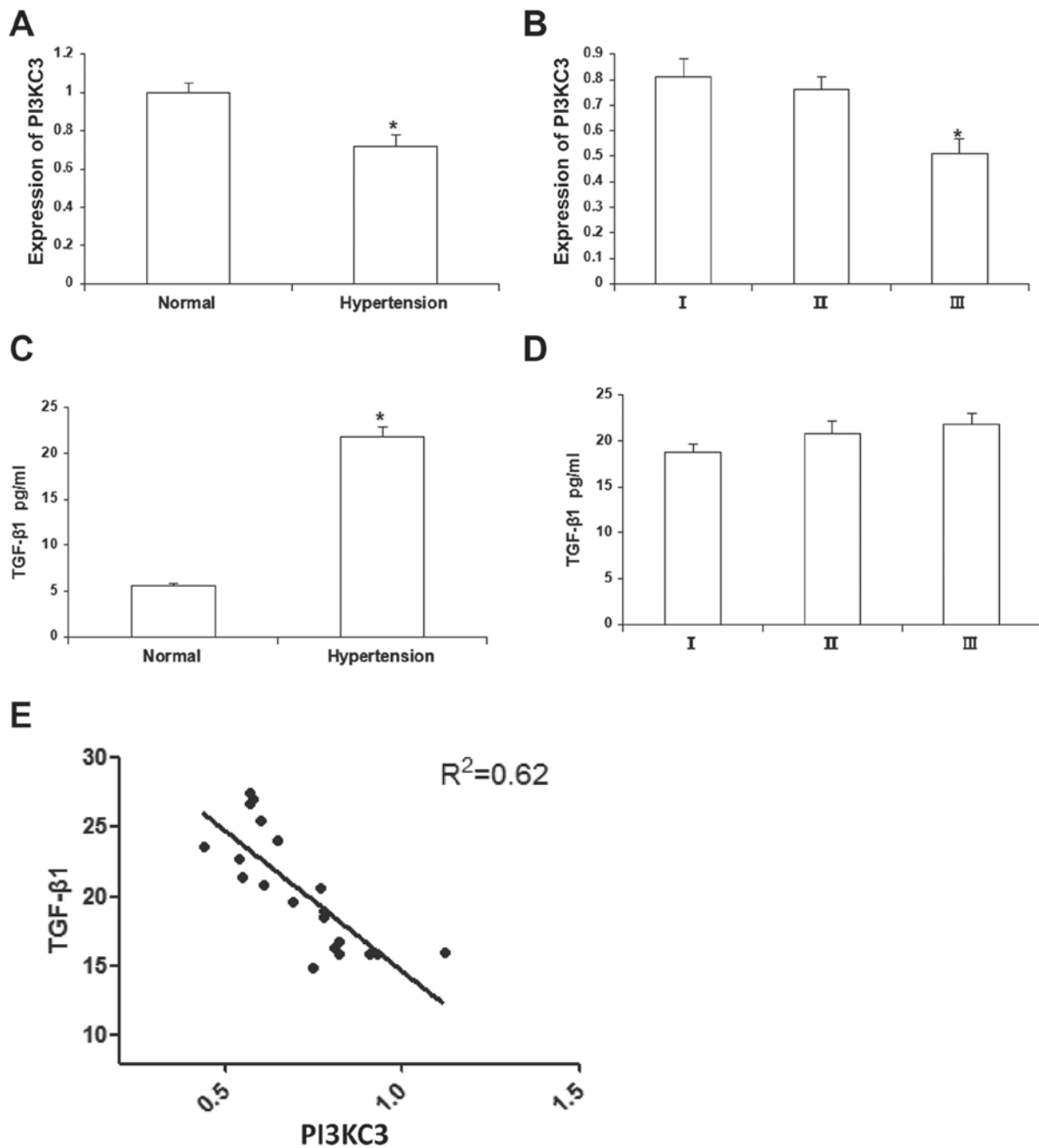


Figure 1. Expression and correlation of PI3KC3 and TGF- β 1 in peripheral blood of hypertensive patients. (A) The mRNA expression levels of PI3KC3 in the peripheral blood in the normal control subjects and patients with hypertension were detected via RT-qPCR. * $P < 0.05$ vs. normal. (B) The mRNA expression levels of PI3KC3 in the peripheral blood in patients with hypertension of grades I, II and III were detected with RT-qPCR. * $P < 0.05$ vs. grades I and II. (C) TGF- β 1 contents in the peripheral blood in the normal control subjects and patients with hypertension were detected with ELISA. * $P < 0.05$ vs. normal. (D) TGF- β 1 contents in the peripheral blood in the patients with hypertension of grades I, II and III were detected with ELISA. (E) Correlation analysis of PI3KC3 and TGF- β 1 in peripheral blood in patients with hypertension. PI3KC3, phosphatidylinositol 3-kinase catalytic subunit type 3; TGF, transforming growth factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

PI3KC3 in the peripheral blood samples of the patients with hypertension were initially investigated via RT-qPCR. The results demonstrated that, compared with the normal control subjects, the mRNA expression levels of PI3KC3 in the peripheral blood of the hypertensive patients were significantly declined (0.72 ± 0.06 ; $P < 0.05$; Fig. 1A). For the sub-group analysis, the results demonstrated that the mRNA expression levels of PI3KC3 in the grades I and II hypertensive patients were 0.81 ± 0.07 and 0.76 ± 0.05 , respectively, which were both

significantly higher than the grade III hypertensive patients (0.51 ± 0.08 ; $P < 0.05$; Fig. 1B). Furthermore, the TGF- β 1 contents in the peripheral blood of these patients were detected with ELISA. The results demonstrated that, the TGF- β 1 contents in the peripheral blood of the hypertensive patients were significantly elevated (21.70 ± 0.34 pg/ml) compared with normal controls ($P < 0.05$; Fig. 1C), whereas no significant differences were observed in the peripheral TGF- β 1 content between patients with different grades of hypertension (Fig. 1D). The

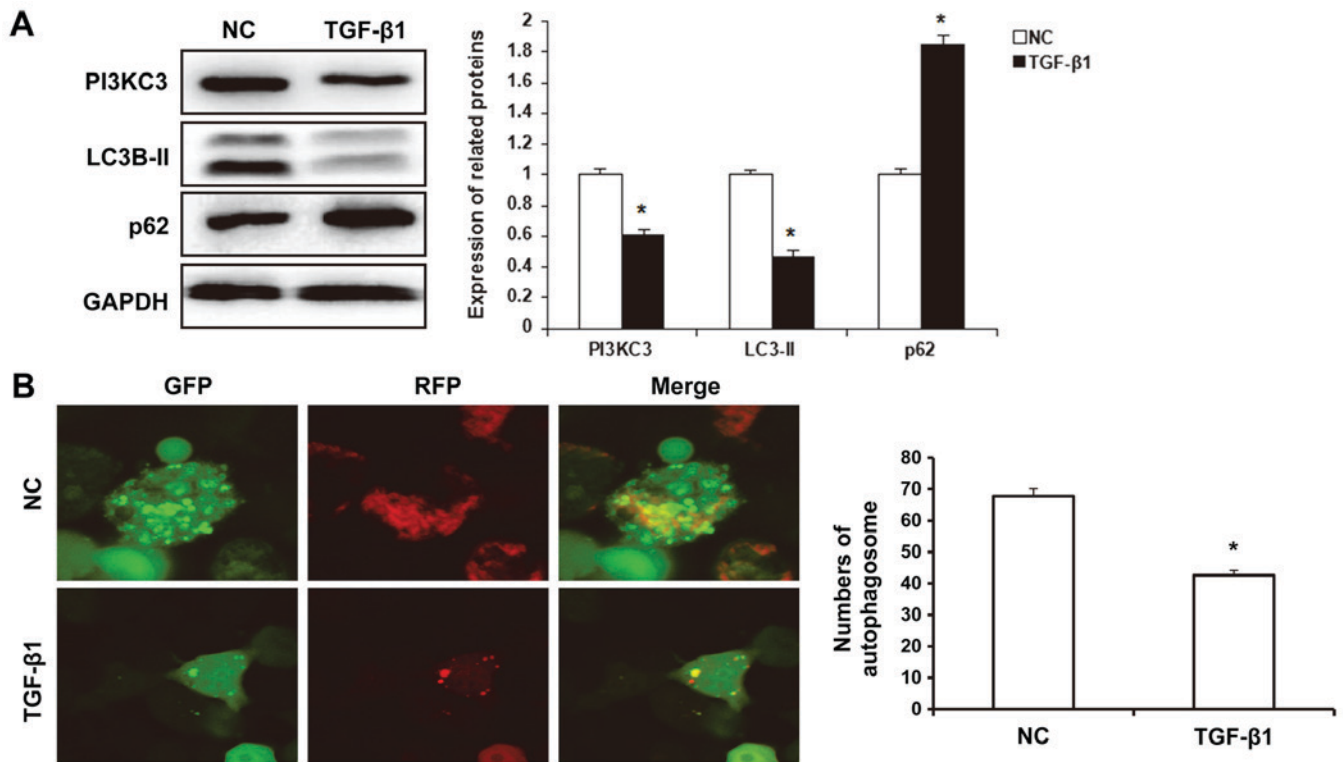


Figure 2. Effects of TGF- β 1 on the PI3KC3 expression and autophagic activities in HUVECs. (A) Following treatment with TGF- β 1, the protein expression levels of PI3KC3 and LC3B-II in the HUVECs were detected with western blot analysis. (B) Following treatment with TGF- β 1, the autophagosomes were detected via laser scanning confocal microcopy and the autophagosome numbers were counted. Magnification, $\times 400$. * $P < 0.05$ vs. NC. TGF, transforming growth factor; PI3KC3, phosphatidylinositol 3-kinase catalytic subunit type 3; HUVECs, human umbilical vein endothelial cells; NC, negative control; GFP, green fluorescent protein; RFP, red fluorescent protein.

association between TGF- β 1 content and PI3KC3 mRNA expression in the peripheral blood was analyzed. The results demonstrated that there was a negative correlation between the TGF- β 1 content and PI3KC3 mRNA expression ($R^2 = 0.62$; $P < 0.05$; Fig. 1E). These results suggest that TGF- β 1 and PI3KC3 are negatively correlated in the peripheral blood of the patients with hypertension.

TGF- β 1 reduces PI3KC3 expression and autophagic activities in HUVECs. Continuously increased peripheral blood pressure in patients with hypertension represents a stress factor on endothelial cells, and autophagy, as a physiological process to maintain intracellular stability, is of great importance for the repair of endothelial cell injuries. PI3KC3 is a key regulator for the autophagic process (35). Therefore, the effects of TGF- β 1 on the regulation of PI3KC3 and the autophagic activities were investigated. The results from the western blot analysis demonstrated that, under hypoxia conditions, the expression levels of LC3B-II protein in the TGF- β 1-treated HUVECs (0.46 ± 0.05) were significantly reduced compared with controls ($P < 0.05$), whereas the p62 protein expression levels (1.85 ± 0.06) were significantly elevated compared with the control group ($P < 0.05$; Fig. 2A). Laser scanning confocal microscopy demonstrated that, under hypoxia conditions, the number of autophagosomes in the TGF- β 1-treated HUVECs (42.6 ± 1.5) was significantly declined, compared with the control group (67.8 ± 2.3 ; $P < 0.05$; Fig. 2B). Furthermore, the results from the western blot analysis demonstrated that, the expression levels of PI3KC3 in the TGF- β 1-treated HUVECs (0.61 ± 0.03) were significantly

declined compared with controls ($P < 0.05$; Fig. 2A). These results suggest that TGF- β 1 reduced the expression levels of PI3KC3 and the autophagic activities in the HUVECs.

TGF- β 1 promotes HUVEC injuries via inhibiting PI3KC3-mediated regulation of autophagy. Autophagy helps regulate cellular stress, and factors that inhibit autophagic activities may lead to cellular damages (36). To further investigate whether TGF- β 1 regulates cell viability through PI3KC3-mediated autophagy regulation, the cellular autophagic activities were detected. The results demonstrated that, compared with the TGF- β 1 group, the expression level of LC3B-II protein was markedly elevated in the TGF- β 1+PI3KC3 over-expression group compared with the TGF- β 1 group, which indicated enhanced autophagic activities, and LCSM revealed that the number of autophagosomes in the TGF- β 1-treatment group was significantly lower than the control group, while the number of autophagosomes was significantly higher in the TGF- β 1+PI3KC3 overexpression group compared with the TGF- β 1 group. These results indicate that PI3KC3 restored TGF- β 1-induced autophagy inhibition (Fig. 3A and B). To assess the tolerance of HUVECs against hypoxia-induced stress, the cellular apoptosis rates were detected by flow cytometry. The results demonstrated that for the NC group, the HUVEC apoptosis rate was $18.5 \pm 1.1\%$; for the TGF- β 1 group, the apoptosis rate was $33.8 \pm 2.7\%$; and for the TGF- β 1+PI3KC3 over-expression group, the apoptosis rate was $19.7 \pm 0.9\%$ (Fig. 3C). The LDH release in the cell culture supernatant was also investigated. The results demonstrated that, compared

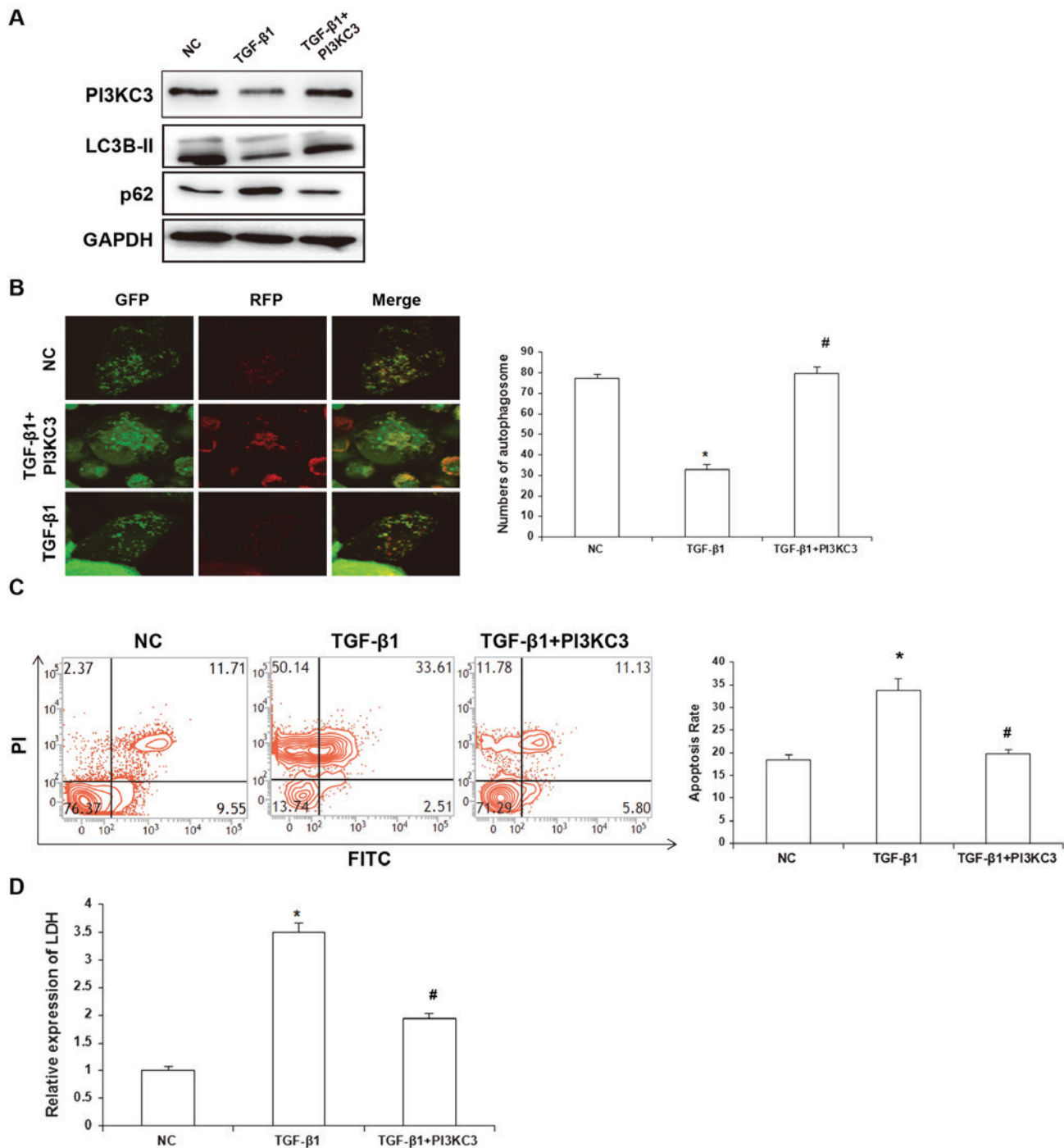


Figure 3. Effects of TGF- β 1 and PI3KC3 overexpression on autophagic activity and apoptotic process of HUVECs. (A) Following the treatment of TGF- β 1, the HUVECs were overexpressed with PI3KC3 and the expression levels of PI3KC3, LC3B-II and p62 were detected via western blot analysis. (B) Following the treatment of TGF- β 1, the HUVECs were overexpressed with PI3KC3 and the autophagosomes were detected with laser scanning confocal microscopy. (C) Following the treatment of TGF- β 1 and overexpression of PI3KC3, under hypoxia conditions the apoptosis of HUVECs was detected with flow cytometry. (D) Following the treatment of TGF- β 1 and overexpression of PI3KC3, under hypoxia conditions, the LDH release in the HUVEC culture supernatant was determined. Magnification, x400. *P<0.05 vs. NC; #P<0.05 vs. TGF- β 1. TGF, transforming growth factor; PI3KC3, phosphatidylinositol 3-kinase catalytic subunit type 3; HUVECs, human umbilical vein endothelial cells; LDH, lactate dehydrogenase; NC, negative control; PI, propidium iodide; FITC, fluorescein isothiocyanate.

with the control group, the relative LDH content in the culture supernatant in the TGF- β 1 group was 3.5 ± 0.54 -fold greater, and for the TGF- β 1+PI3KC3 over-expression group, the relative LDH content was 1.97 ± 0.36 -fold greater, which was significantly decreased compared with the TGF- β 1 group (Fig. 3D). These results suggest that TGF- β 1 could promote

HUVEC injuries via inhibiting PI3KC3-mediated regulation of autophagy.

TGF- β 1 inhibits HUVEC proliferation via downregulating PI3KC3 expression. Following vascular endothelial injury, endothelial cells may enter a proliferative cycle, which is a key

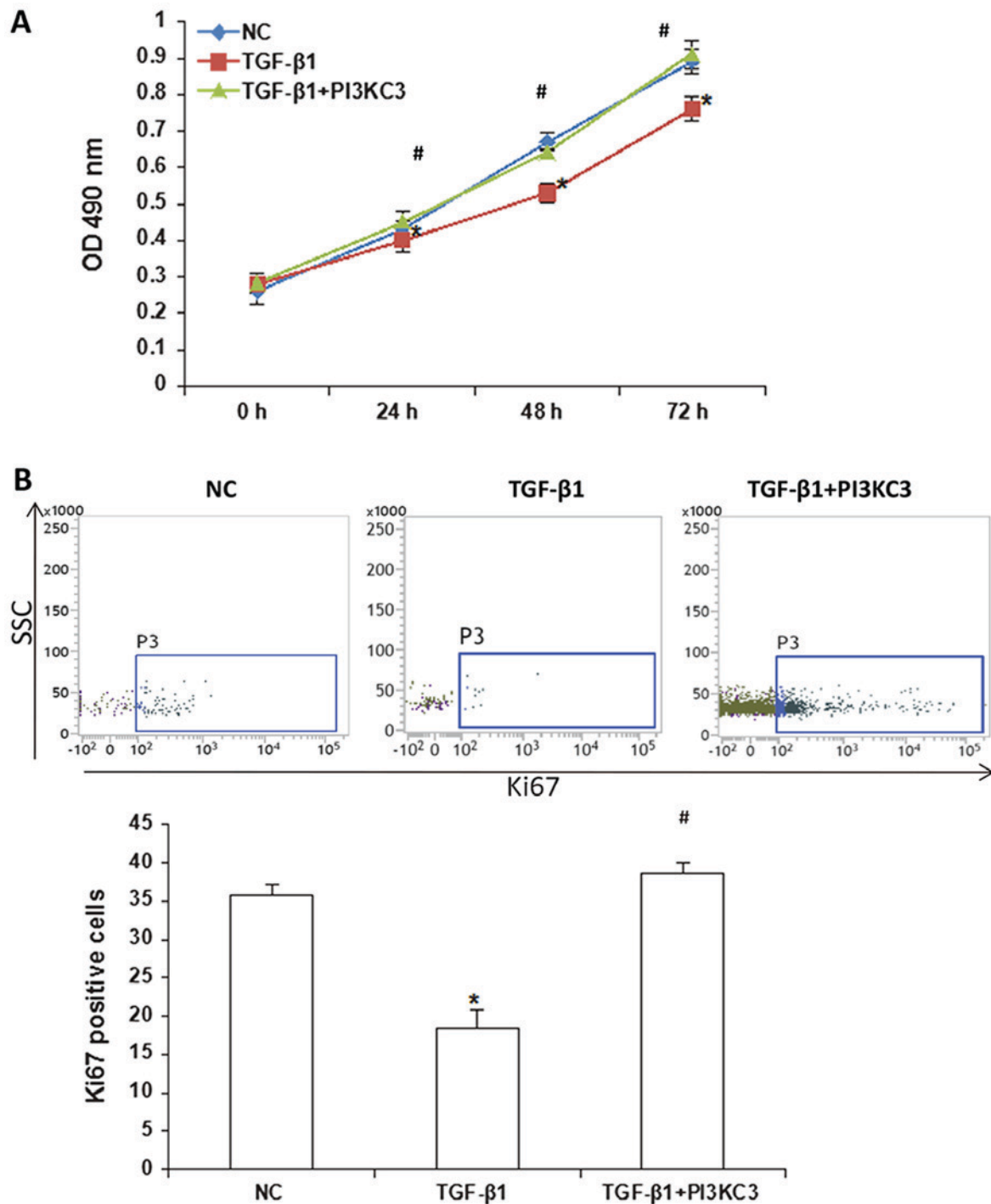


Figure 4. Effects of TGF- β 1 and PI3KC3 overexpression on proliferation of HUVECs. (A) Following the treatment of TGF- β 1, the HUVECs were over-expressed with PI3KC3, and the cell proliferation was detected via a cell counting kit-8 assay. (B) Following the treatment of TGF- β 1, the HUVECs were overexpressed with PI3KC3, and the Ki67 positive-level was detected with flow cytometry. * $P < 0.05$ vs. NC; # $P < 0.05$ vs. TGF- β 1. TGF, transforming growth factor; PI3KC3, phosphatidylinositol 3-kinase catalytic subunit type 3; HUVECs, human umbilical vein endothelial cells; NC, negative control; OD, optical density; SSC, side-scattering.

factors for the vascular repair process (37). In order to study the effect of TGF- β 1-induced downregulation of PI3KC3 expression on the proliferation of HUVECs, a CCK-8 assay was performed. The results demonstrated that, compared with the NC group, HUVEC proliferation ability was significantly declined in the TGF- β 1 group ($P < 0.05$) at 24-72 h. Compared with the TGF- β 1 group, the OD490 values at 24-72 h in the TGF- β 1+PI3KC3 over-expression group were significantly

increased ($P < 0.05$; Fig. 4A). The Ki67 positive cell numbers were further assessed by flow cytometry. The results demonstrated that, the number of Ki67 positive HUVECs in the TGF- β 1 group was significantly lower than the control group and the TGF- β 1+PI3KC3 over-expression group ($P < 0.05$), indicating decreased cell proliferation ability (Fig. 4B). These results suggest that TGF- β 1 may inhibit the HUVEC proliferation ability via downregulation of PI3KC3, and the decreased

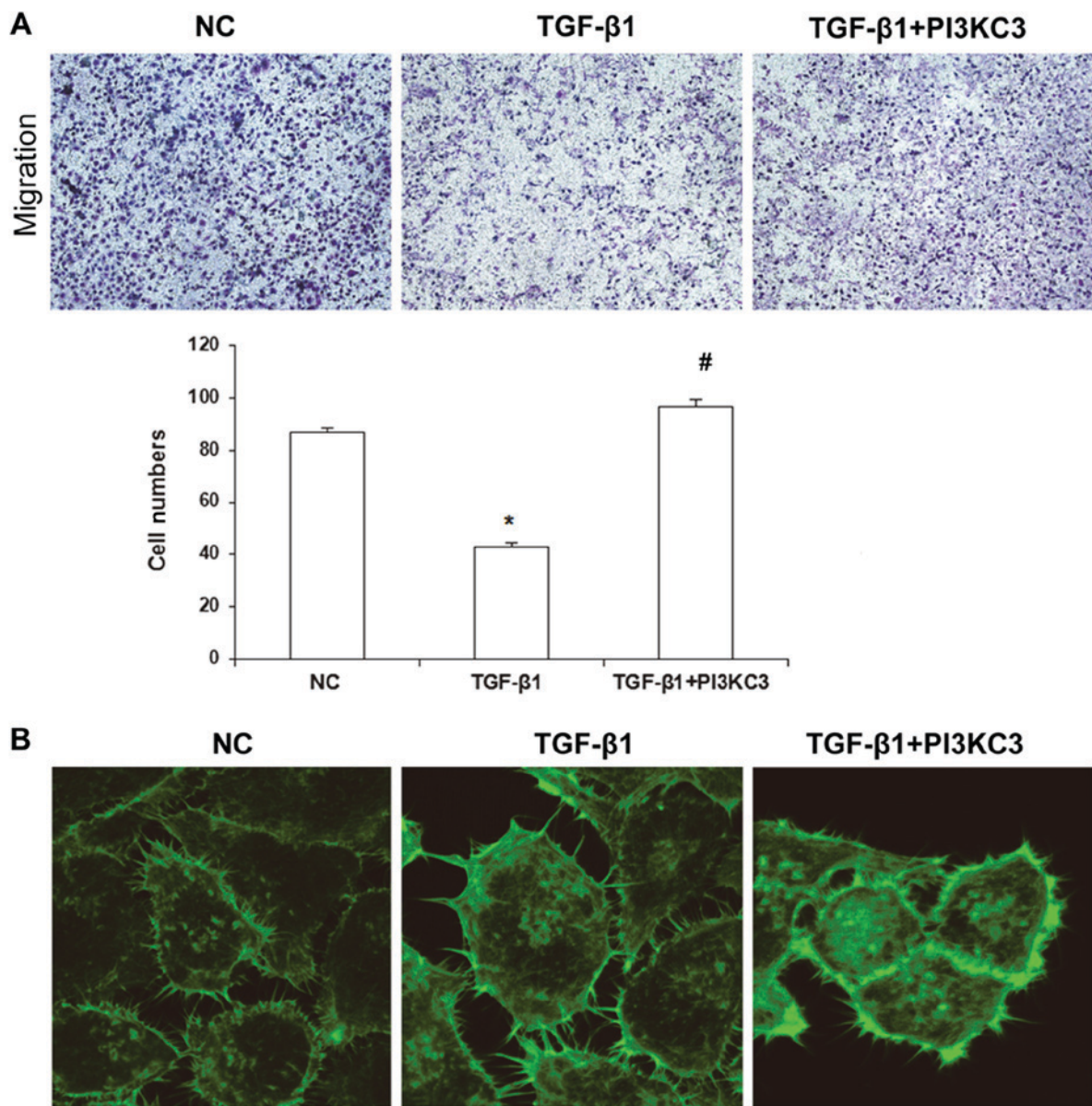


Figure 5. Effects of TGF-β1 and PI3KC3 overexpression on migration ability of HUVECs. (A) Following the treatment of TGF-β1, the HUVECs were overexpressed with PI3KC3 and the cell migration ability was assessed with the Transwell chamber assay. (B) Following the treatment of TGF-β1, the HUVECs were overexpressed with PI3KC3, and the synapses on the cell membrane were detected with laser scanning confocal microscopy. Magnification, x400. *P<0.05 vs. NC; #P<0.05 vs. TGF-β1. TGF, transforming growth factor; PI3KC3, phosphatidylinositol 3-kinase catalytic subunit type 3; HUVECs, human umbilical vein endothelial cells; NC, negative control.

cell proliferation ability may inhibit the vascular endothelial injury repair process.

TGF-β1 inhibits HUVEC migration ability via downregulating PI3KC3 expression. It has been demonstrated that endothelial cells have the ability to migrate. Following vascular injury, the adjacent vascular endothelial cells may begin to proliferate or migrate to the lesion sites under the direction of local chemotactic factors, repairing the vascular wall (38). To investigate the migration ability of HUVECs, a Transwell chamber assay was performed. The results demonstrated that, compared with the NC group, the HUVEC migration ability in the TGF-β1 group was significantly decreased, as indicated by the decreased penetrating cells (P<0.05), whereas the penetrating cells were significantly increased in the

TGF-β1+PI3KC3 over-expression group compared with the TGF-β1 group (85.6±3.5; P<0.05; Fig. 5A). The cytoskeletal changes in these cells were further observed by laser confocal microscopy. The results demonstrated that, compared with the NC and TGF-β1+PI3KC3 groups, the synapses on the HUVEC membrane were significantly decreased in the TGF-β1 group, as indicated by the declined fluorescence intensity and quantity, which also suggests decreased migration ability (Fig. 5B). These results suggest that TGF-β1 may inhibit the migration ability of HUVECs via downregulating PI3KC3 expression.

Discussion

Vascular endothelial injury is one of the most commonly reported pathological changes in patients with hypertension,

which has a marked impact on the occurrence and development of hypertension and associated complications (29). TGF- β 1 is associated with various physiological and pathological biological processes. Due to its diverse functions and complex mechanism of action, TGF- β 1 has notable clinical value in various diseases, including tumor metastasis and tissue fibrosis (13,39), and has thus become a focus in medical research. Previous studies have demonstrated that TGF- β 1 serves an important role in the development of hypertension and its associated complications, particularly in the regulation of various pathological processes (40,41). In the present study, the results demonstrated that the expression of TGF- β 1 in the peripheral blood was significantly elevated in patients with hypertension, which was negatively correlated with the autophagy regulator PI3KC3. The *in vitro* results demonstrated that TGF- β 1 could downregulate the expression of PI3KC3 to inhibit the proliferation, migration and autophagy of HUVECs, while promoting cellular apoptosis and aggregating the vascular endothelial injuries in patients with hypertension.

TGF- β 1, as a multi-functional cytokine, serves an important role in the development of hypertension. A number of studies have demonstrated that the contents of TGF- β 1 in the peripheral blood are significantly increased in patients with hypertension (42,43). In accordance with this, the present results demonstrated that, compared with the healthy control group, the TGF- β 1 contents in the peripheral blood were significantly elevated in hypertensive patients, which was also associated with hypertension grading. In addition, TGF- β 1 is associated with various hypertension-induced complications. Wei *et al* (44) have recently demonstrated that the TGF- β 1/SMAD3 pathway mediates hypertension-induced myocardial fibrosis (45). The TGF- β 1/p53 signaling pathway serves an important role in hypertension-induced renal fibrosis (46). For vascular injuries, it has been reported that, TGF- β 1 can promote the proliferation of vascular smooth muscle, leading to thickened vascular muscle layer and enhanced contraction, further increasing the peripheral circulation resistance and promoting hypertension development (47). For vascular endothelial injuries, the role of TGF- β 1 remains unclear. Li *et al* (48) have recently demonstrated that miRNA-146a-mediated TGF- β 1 silencing can promote angiogenesis. In the present study, the results demonstrated that following treatment with TGF- β 1, the HUVEC proliferation and migration ability were significantly decreased, and under hypoxic conditions, the cellular apoptosis rates were significantly elevated. In addition, the release of LDH in the cell culture supernatant was also investigated. The results demonstrated that TGF- β 1 could promote the release of LDH, indicating the occurrence of endothelial cell injuries. These results suggest that TGF- β 1 may promote the development of vascular endothelial injuries.

Autophagy is an important biological process in vascular injury, and PI3KC3 is a key gene regulating autophagic activity. It has been demonstrated that TGF- β 1 is associated with the regulation of autophagy (49,50). In the present study, the expression levels of PI3KC3 mRNA in the peripheral blood and its correlation with TGF- β 1 were analyzed. The results demonstrated that the PI3KC3 mRNA expression levels were significantly decreased in

patients with hypertension, which was also associated with the disease grading. Correlation analysis demonstrated that the TGF- β 1 and PI3KC3 mRNA expression demonstrated moderate negative correlation. *In vitro* results demonstrated that, TGF- β 1 could inhibit the PI3KC3 expression and the autophagic activity in the HUVECs. In order to further verify whether TGF- β 1 functioned through PI3KC3, following treatment with PI3KC3, the HUVECs were overexpressed with PI3KC3. The overexpression of PI3KC3 significantly ameliorated the proliferation, migration and hypoxia tolerance of the HUVECs. These results suggest that TGF- β 1 was able to inhibit autophagic activity through PI3KC3 and promote the injury of vascular endothelial cells. Conversely, it has previously been reported that TGF- β 1 can promote the EMT of vascular endothelial cells and enhance the migration ability (51). The present authors speculate that this disparity may be attributed to the different downstream pathways of TGF- β 1 and the different models used. Further in-depth studies are required to elucidate the detailed mechanism.

In conclusion, the present results demonstrated that TGF- β 1 may inhibit the autophagic activity of vascular endothelial cells via the PI3KC3 pathway, promoting the vascular endothelial injuries. The present study primarily investigated the mechanism through which TGF- β 1 regulated the autophagy, and the results revealed the regulating correlation between TGF- β 1 and PI3KC3. These findings may contribute to the understanding of the role of TGF- β 1 in hypertension-induced vascular injuries.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

QZ, HL and JY designed the current study, performed the experiments, collected the data, performed statistical analysis and prepared the manuscript.

Ethics approval and consent to participate

Prior written informed consent was obtained from all subjects and the present study protocol was approved by the ethics review board of Zaozhuang Municipal Hospital (Zaozhuang, China).

Patient consent for publication

Prior written informed consent was obtained from all subjects.

Competing interests

The authors declare that they have no competing interests.

References

- Yang Y, Zhang E, Zhang J, Chen S, Yu G, Liu X, Peng C, Lavin MF, Du Z and Shao H: Relationship between occupational noise exposure and the risk factors of cardiovascular disease in China: A meta-analysis. *Medicine (Baltimore)* 97: e11720, 2018.
- Wang X, Cai X, Wang W, Jin Y, Chen M, Huang X, Zhu X and Wang L: Effect of asiaticoside on endothelial cells in hypoxia-induced pulmonary hypertension. *Mol Med Rep* 17: 2893-2900, 2018.
- Kuang ZM, Wang Y, Feng SJ, Jiang L and Cheng WL: Association between plasma homocysteine and microalbuminuria in untreated patients with essential hypertension: A case-control study. *Kidney Blood Press Res* 42: 1303-1311, 2017.
- Johns CS, Wild JM, Rajaram S, Swift AJ and Kiely DG: Current and emerging imaging techniques in the diagnosis and assessment of pulmonary hypertension. *Expert Rev Respir Med* 12: 145-160, 2018.
- Yeates K, Campbell N, Maar MA, Perkins N, Liu P, Sleeth J, Smith C, McAllister C, Hua-Stewart D, Wells G and Tobe SW: The effectiveness of text messaging for detection and management of hypertension in indigenous people in Canada: Protocol for a randomized controlled trial. *JMIR Res Protoc* 6: e244, 2017.
- Roy C, Tabiasco J, Caillon A, Delneste Y, Merot J, Favre J, Guihot AL, Martin L, Nascimento DC, Ryffel B, *et al*: Loss of vascular expression of nucleoside triphosphate diphosphohydrolase-1/CD39 in hypertension. *Purinergic Signal* 14: 73-82, 2018.
- Rocha HNM, Garcia VP, Batista GMS, Silva GM, Mattos JD, Campos MO, Nóbrega ACL, Fernandes IA and Rocha NG: Disturbed blood flow induces endothelial apoptosis without mobilizing repair mechanisms in hypertension. *Life Sci* 209: 103-110, 2018.
- Bakrania BA, Spradley FT, Satchell SC, Stec DE, Rimoldi JM, Gadepalli RSV and Granger JP: Heme oxygenase-1 is a potent inhibitor of placental ischemia-mediated endothelin-1 production in cultured human glomerular endothelial cells. *Am J Physiol Regul Integr Comp Physiol* 314: R427-R432, 2018.
- Navia-Pelaez JM, Campos GP, Araujo-Souza JC, Stergiopoulos N and Capettini LSA: Modulation of nNOS^{ser852} phosphorylation and translocation by PKA/PP1 pathway in endothelial cells. *Nitric Oxide* 72: 52-58, 2018.
- Rol N, de Raaf MA, Sun X, Kuiper VP, da Silva Gonçalves Bos D, Happé C, Kurakula K, Dickhoff C, Thuillet R, Tu L, *et al*: Nintedanib improves cardiac fibrosis but leaves pulmonary vascular remodeling unaltered in experimental pulmonary hypertension. *Cardiovasc Res*: Jul 18, 2018 (Epub ahead of print).
- Ungferoren H, Witte D, Fiedler C, Gädeken T, Kaufmann R, Lehnert H, Gieseler F and Rauch BH: The role of PAR2 in TGF- β 1-induced ERK activation and cell motility. *Int J Mol Sci* 18: E2776, 2017.
- Liakouli V, Elies J, El-Sherbiny YM, Scarcia M, Grant G, Abignano G, Derrett-Smith EC, Esteves F, Cipriani P, Emery P, *et al*: Scleroderma fibroblasts suppress angiogenesis via TGF- β /caveolin-1 dependent secretion of pigment epithelium-derived factor. *Ann Rheum Dis* 77: 431-440, 2018.
- Jin X, Aimaiti Y, Chen Z, Wang W and Li D: Hepatic stellate cells promote angiogenesis via the TGF- β 1-Jagged1/VEGFA axis. *Exp Cell Res* 15: 34-43, 2018.
- Wang Z, Wang K, Wang R and Liu X: SUMOylation regulates TGF- β 1/Smad4 signalling in-resistant glioma cells. *Anticancer Drugs*: Dec 18, 2017 (Epub ahead of print).
- Huang CH, Chang YH, Lin CY, Wang WH, Kuan HC, Hsieh YJ, Wang YW, Yang CH, Chiu JY, Tsai SF, *et al*: Shared IgG infection signatures vs. hemorrhage-restricted IgA clusters in human dengue: A phenotype of differential class-switch via TGF β 1. *Front Immunol* 8: 1726, 2017.
- Zheng W, Song J, Zhang Y, Chen S, Ruan H and Fan C: Metformin prevents peritendinous fibrosis by inhibiting transforming growth factor- β signaling. *Oncotarget* 8: 101784-101794, 2017.
- Ohmaru-Nakanishi T, Asanoma K, Fujikawa M, Fujita Y, Yagi H, Onoyama I, Hidaka N, Sonoda K and Kato K: Fibrosis in preeclamptic placentas is associated with stromal fibroblasts activated by the transforming growth factor- β 1 signaling pathway. *Am J Pathol* 188: 683-695, 2018.
- Qu Y, Zhang H, Sun W, Han Y, Li S, Qu Y, Ying G and Ba Y: MiR-155 promotes gastric cancer growth and invasion by negatively regulating transforming growth factor β receptor 2. *Cancer Sci* 109: 618-628, 2018.
- Niu J, Yue W, Le-Le Z, Bin L and Hu X: Mesenchymal stem cells inhibit T cell activation by releasing TGF- β 1 from TGF- β 1/GARP complex. *Oncotarget* 8: 99784-99800, 2017.
- Qiong W, Xiaofeng G and Junfang W: Transforming growth factor- β 1 (TGF- β 1) induces mouse precartilaginous stem cell differentiation through TGFRII-CK1 ϵ - β -catenin signalling. *Int J Exp Pathol* 99: 113-120, 2018.
- Wang P, Luo L, Shen Q, Shi G, Mohammed A, Ni S and Wu X: Rosuvastatin improves myocardial hypertrophy after hemodynamic pressure overload via regulating the crosstalk of Nrf2/ARE and TGF- β /smads pathways in rat heart. *Eur J Pharmacol* 820: 173-182, 2018.
- Zhang N, Dong M, Luo Y, Zhao F and Li Y: Danshensu prevents hypoxic pulmonary hypertension in rats by inhibiting the proliferation of pulmonary artery smooth muscle cells via TGF- β -smad3-associated pathway. *Eur J Pharmacol* 820: 1-7, 2018.
- Beaudoin J, Dal-Bianco JP, Aikawa E, Bischoff J, Guerrero JL, Sullivan S, Bartko PE, Handschumacher MD, Kim DH, Wylie-Sears J, *et al*: Mitral leaflet changes following myocardial infarction: Clinical evidence for maladaptive valvular remodeling. *Circ Cardiovasc Imaging* 10: e006512, 2017.
- Li X, Tan H, Chen M and Zhou S: Transforming growth factor beta 1 related gene polymorphisms in gestational hypertension and preeclampsia: A case-control candidate gene association study. *Pregnancy Hypertens* 12: 155-160, 2018.
- Stjepanovic G, Baskaran S, Lin MG and Hurley JH: Unveiling the role of VPS34 kinase domain dynamics in regulation of the autophagic PI3K complex. *Mol Cell Oncol* 4: e1367873, 2017.
- Thongchot S, Yongvanit P, Loilome W, Seubwai W, Phunichom K, Tassaneeyakul W, Pairojkul C, Promkotra W, Techasen A and Namwat N: High expression of HIF-1 α , BNIP3 and PI3KC3: Hypoxia-induced autophagy predicts cholangiocarcinoma survival and metastasis. *Asian Pac J Cancer Prev* 15: 5873-5878, 2014.
- Zhang Q, Wang D, Zhang H, Wang M, Li P, Fang X and Cai X: Detection of autophagy processes during the development of nonarticulated laticifers in *Euphorbia kansui* Liou. *Planta* 247: 845-861, 2018.
- Yang B, Sun J, Yuan Y and Sun Z: Effects of atorvastatin on autophagy in skeletal muscles of diabetic rats. *J Diabetes Investig* 9: 753-761, 2018.
- Holmberg J, Bhattacharya A, Alajbegovic A, Rippe C, Ekman M, Dahan D, Hien TT, Boettger T, Braun T, Swärd K, *et al*: Loss of vascular myogenic tone in miR-143/145 knockout mice is associated with hypertension-induced vascular lesions in small mesenteric arteries. *Arterioscler Thromb Vasc Biol* 38: 414-424, 2018.
- Gao P, Wu W, Ye J, Lu YW, Adam AP, Singer HA and Long X: Transforming growth factor β 1 suppresses proinflammatory gene program independent of its regulation on vascular smooth muscle differentiation and autophagy. *Cell Signal* 50: 160-170, 2018.
- Cheng JZ, Chen JJ, Wang ZG and Yu D: MicroRNA-185 inhibits cell proliferation while promoting apoptosis and autophagy through negative regulation of TGF- β 1/mTOR axis and HOXC6 in nasopharyngeal carcinoma. *Cancer Biomark* 23: 107-123, 2018.
- Khera R, Lu Y, Lu J, Saxena A, Nasir K, Jiang L and Krumholz HM: Impact of 2017 ACC/AHA guidelines on prevalence of hypertension and eligibility for antihypertensive treatment in United States and China: Nationally representative cross sectional study. *BMJ* 362: k2357, 2018.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Sun M, Ishii R, Okumura K, Krauszman A, Breitling S, Gomez O, Hinek A, Boo S, Hinz B, Connelly KA, *et al*: Experimental right ventricular hypertension induces regional β 1-integrin-mediated transduction of hypertrophic and profibrotic right and left ventricular signaling. *J Am Heart Assoc* 7: e007928, 2018.
- Li L, Huang C, He Y, Sang Z, Liu G and Dai H: Knockdown of long non-coding RNA GAS5 increases miR-23a by targeting ATG3 involved in autophagy and cell viability. *Cell Physiol Biochem* 48: 1723-1734, 2018.

36. Chen J, Yan L, Wang H, Zhang Z, Yu D, Xing C, Li J, Li H, Li J and Cai Y: ZBTB38, a novel regulator of autophagy initiation targeted by RBICC1/FIP200 in spinal cord injury. *Gene* 678: 8-16, 2018.
37. McDonald AI, Shirali AS, Aragón R, Ma F, Hernandez G, Vaughn DA, Mack JJ, Lim TY, Sunshine H, Zhao P, *et al*: Endothelial regeneration of large vessels is a biphasic process driven by local cells with distinct proliferative capacities. *Cell Stem Cell* 23: 210-225.e6, 2018.
38. Rong X, Ge D, Shen D, Chen X, Wang X, Zhang L, Jia C, Zeng J, He Y, Qiu H, *et al*: miR-27b suppresses endothelial cell proliferation and migration by targeting Smad7 in Kawasaki disease. *Cell Physiol Biochem* 48: 1804-1814, 2018.
39. Grampp S and Goppelt-Strube M: Receptor-independent modulation of TGF- β -induced pro-fibrotic pathways by relaxin-2 in human primary tubular epithelial cells. *Cell Tissue Res*, 2018.
40. Chen K and Sun Z: Activation of DNA demethylases attenuates aging-associated arterial stiffening and hypertension. *Aging Cell*: Apr 16, 2018 (Epub ahead of print).
41. Fu S, Li YL, Wu YT, Yue Y, Qian ZQ and Yang DL: Icariside II attenuates myocardial fibrosis by inhibiting nuclear factor- κ B and the TGF- β 1/Smad2 signalling pathway in spontaneously hypertensive rats. *Biomed Pharmacother* 100: 64-71, 2018.
42. Bellaye PS, Yanagihara T, Granton E, Sato S, Shimbori C, Upagupta C, Imani J, Hambly N, Ask K, Gauldie J, *et al*: Macitentan reduces progression of TGF- β 1-induced pulmonary fibrosis and pulmonary hypertension. *Eur Respir J* 52: 1701857, 2018.
43. Cai Z, Li J, Zhuang Q, Zhang X, Yuan A, Shen L, Kang K, Qu B, Tang Y, Pu J, *et al*: MiR-125a-5p ameliorates monocrotaline-induced pulmonary arterial hypertension by targeting the TGF- β 1 and IL-6/STAT3 signaling pathways. *Exp Mol Med* 50: 45, 2018.
44. Wei W, Rao F, Liu F, Xue Y, Deng C, Wang Z, Zhu J, Yang H, Li X, Zhang M, *et al*: Involvement of Smad3 pathway in atrial fibrosis induced by elevated hydrostatic pressure. *J Cell Physiol* 233: 4981-4989, 2018.
45. Higgins SP, Tang Y, Higgins CE, Mian B, Zhang W, Czekay RP, Samarakoon R, Conti DJ and Higgins PJ: TGF- β 1/p53 signaling in renal fibrogenesis. *Cell Signal* 43: 1-10, 2018.
46. Liu Z, Huang XR and Lan HY: Smad3 mediates ANG II-induced hypertensive kidney disease in mice. *Am J Physiol Renal Physiol* 302: F986-F997, 2012.
47. Shang P, Liu T, Liu W, Li Y, Dou F, Zhang Y, Sun L, Zhang T, Zhu Z, Mu F, *et al*: Telmisartan improves vascular remodeling through ameliorating prooxidant and profibrotic mechanisms in hypertension via the involvement of transforming growth factor- β 1. *Mol Med Rep* 16: 4537-4544, 2017.
48. Li Y, Zhu H, Wei X, Li H, Yu Z, Zhang H and Liu W: LPS induces HUVEC angiogenesis in vitro through miR-146a-mediated TGF- β 1 inhibition. *Am J Transl Res* 9: 591-600, 2017.
49. Ding Y and Choi ME: Regulation of autophagy by TGF- β : Emerging role in kidney fibrosis. *Semin Nephrol* 34: 62-71, 2014.
50. Wang H, Zhang Y, Wu Q, Wang YB and Wang W: miR-16 mimics inhibit TGF- β 1-induced epithelial-to-mesenchymal transition via activation of autophagy in non-small cell lung carcinoma cells. *Oncol Rep* 39: 247-254, 2018.
51. Wang Z, Fei S, Suo C, Han Z, Tao J, Xu Z, Zhao C, Tan R and Gu M: Antifibrotic effects of hepatocyte growth factor on endothelial-to-mesenchymal transition via transforming growth factor-beta1 (TGF- β 1)/Smad and Akt/mTOR/P70S6K signaling pathways. *Ann Transplant* 23: 1-10, 2018.



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