Histol Histopathol (2024) 39: 603-610

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

Up-regulation of Trim28 in pregnancy-induced hypertension is involved in the injury of human umbilical vein endothelial cells through the p38 signaling pathway

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Summary. Aims. The present study is to analyze the regulation and potential molecular mechanism of Trim28 on vascular endothelial injury induced by pregnancy-induced hypertension (PIH).

Methods. Trim28 mRNA in placental tissues and peripheral blood from PIH patients were determined by quantitative real-time polymerase chain reaction. The serum from PIH was used to stimulate human umbilical vein endothelial cells (HUVECs). After silencing Trim28 in HUVECs, we used CCK-8 assay, Transwell assay and flow cytometry to investigate proliferation, migration and apoptosis. Western blotting was used to measure Trim28 protein level and p38 phosphorylation level. After addition of p38 inhibitor, the proliferation, migration and apoptosis of HUVECs with silenced Trim28 were studied again.

Results. Trim28 expression in placental tissues and peripheral blood from PIH patients is elevated, and serum from these patients can up-regulate the expression of Trim28 in HUVECs *in vitro*. Trim28 silencing significantly inhibits the proliferation and migration of HUVECs by affecting the cell cycle. Down-regulation of Trim28 expression promotes the apoptosis of HUVECs. Trim28 regulates the biological function of HUVECs by affecting the activity of the p38 signaling pathway.

Conclusions. The present study demonstrates that Trim28 is up-regulated in peripheral blood of patients with PIH and participates in HUVECs injury through the p38 signaling pathway.

Key words: Trim28, Pregnancy-induced hypertension, Human umbilical vein endothelial cells, p38 signaling pathway

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www.hh.um.es. DOI: 10.14670/HH-18-651

Introduction

Gestational hypertension is one of the most common complications during pregnancy (Socha et al., 2020). Epidemiological statistics show that the incidence rate of pregnancy-induced hypertension (PIH) in the world is as high as 10%, seriously threatening the health of pregnant women and perinatal infants (Liu et al., 2016; Magalhães et al., 2020). With the increase of the elderly and obese parturient women in recent years, the incidence rate of gestational hypertension has gradually increased (Younes and Ryan, 2019). If PIH is not controlled, it will develop into pre-eclampsia, or even moderate and severe eclampsia which is a kind of life-threatening multisystem disease with complications such as eclampsia, renal impairment, pulmonary edema, placental abruption or intrauterine fetal death (Berhe et al., 2019; Birukov et al., 2020). Therefore, an in-depth study of the pathophysiological process of gestational hypertension and improving the diagnosis and treatment strategy are of great significance for clinical early intervention and improving the outcome of mother and baby. At present, the pathogenesis of PIH is still unclear and needs further research.

More and more evidence has shown that the basic clinicopathological changes of gestational hypertension are vasoconstriction and damage to uteroplacental perfusion, indicating that abnormal vascular changes play an important role in this process (Püschl et al., 2020). As an important part of the vascular wall, endothelial cells form a natural physical barrier of the vascular wall and play important roles in the stability of vascular tension, blood coagulation and the regulation of inflammatory responses (Cha et al., 2020; Liu et al., 2020b). The activation and dysfunction of endothelial cells can directly regulate vasoconstriction and blood pressure stability, and promote thrombosis and their interaction with immune cells *in vivo* (Li et al., 2009). For example, peripheral CT4+ cytotoxic T cells in

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patients with lupus erythematosus can induce apoptosis of vascular endothelial cells, indicating that the blood environment has a direct impact on the function of vascular endothelial cells (Maehara et al., 2020). Therefore, studies on the molecular mechanism of abnormal function of vascular endothelial cells in PIH is of great significance for understanding the pathway of vascular injury induced by PIH and screening clinical therapeutic targets.

The tripartite motif-containing protein (TRIM) family has a wide range of members with complex functions. Existing studies have shown that TRIM family proteins are closely related to biological functions such as cell proliferation, DNA damage, genome stability, and cell differentiation (Yang et al., 2020; Kato et al., 2021). Abnormalities of TRIM family proteins are related to the occurrence and development of a variety of diseases, including tumors, immune inflammation, developmental disorders and chromatin abnormalities (Connacher and Goldstrohm, 2021). Trim28 is a TRIM family protein widely concerned by researchers in recent years, and it has important functions in the process of tumorigenesis and stem cell differentiation (Fong et al., 2018; Liu et al., 2020a). However, the role and potential molecular mechanism of Trim28 in vascular injury in patients with hypertension are not clear. This study intends to analyze the regulation and potential molecular mechanism of Trim28 on vascular injury induced by PIH, so as to provide an experimental basis for further understanding the pathological process of vascular injury induced by PIH.

Materials and methods

Subjects

Thirty parturient women with normal delivery and 30 parturient women with PIH undergoing cesarean section at our hospital between January 2020 and May 2020 were included into control group and PIH group, respectively. Placental tissues and 6 ml peripheral blood were collected from all subjects. PIH group included 11 cases with only PIH (increased blood pressure after 20 weeks of pregnancy; no proteinuria), 12 cases with mild pre-eclampsia (mPE; systolic pressure ≥140 mmHg or diastolic pressure ≥90 mmHg; 24 h urinary protein ≥300 mg), and 7 cases with severe pre-eclampsia (sPE; systolic pressure ≥160 mmHg or diastolic pressure ≥100 mmHg; 24 h urinary protein ≥2 g). The age range of experimental group was 24 to 39 years old, with a mean age of 31.6 years old and a median age of 35 years old; the age range of control group was 22 to 36 years old, with a mean age of 29.5 years old and a median age of 29 years old. The pregnant women in both groups had singleton pregnancy. None of the subjects had diseases of heart, liver, kidney and blood system, diabetes, rheumatism, or autoimmune diseases. None had history of infection and medication within 4 weeks before operation. All procedures performed in the current study were approved by the Ethics Committee of Taian City Central Hospital Affiliated to Qingdao University, China. Written informed consent was obtained from all patients or their families.

Cell culture

Human umbilical vein endothelial cells (HUVECs; Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) under 37°C and 5% CO_2. When the cells reached a density of 70-90%, the cells were trypsinized and passaged. The cells in the 3rd to 5th passages were utilized for subsequent experiments.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Peripheral blood was collected from control group and PIH group and used for the extraction of total RNA by RNAeasy Viral RNA Extraction Kit (Beyotime, Shanghai, China). Then, extracted RNA was reverse-transcribed into cDNA, which was stored under -80˚C. The reverse transcription system contained RNA template (5 µl), 2×Buffer Mix (10 µl), 0.1% BSA (2 µl), RT Enzyme Mix (2 µl) and H_2O (1 µl). The reaction protocol was 37°C incubation for 50 min. The resulting cDNA was diluted to 20 µl with RNAase-free H_2O, and 5 µl was used for qRT-PCR.

Expression of trim28 mRNA was determined by qRT-PCR (miScript SYBR Green PCR Kit, Qiagen, Hilden, Germany), using GAPDH as internal reference. The reaction system (30 µl) contained 5 µl cDNA, 10 µl Mix, 1 µl upstream primer, 1 µl downstream primer and 13 µl ddH_2O. The reaction condition was as follows: initial denaturation at 95°C for 5 min; denaturation at 95°C for 30 sec and annealing at 60°C for 30 sec (40 cycles). The primers for trim28 were 5'-GAGGAAGAGGGACGGATTGC-3' (forward) and 5'-GAGGAAGAGGGACGGATTGC-3' (reverse); the primers for GAPDH were 5'- GGTGA AGGTC GGAGT CAACG -3' (forward) and 5'- CAAAG TTGTC ATGGA TGHACC -3' (reverse). Each sample was tested three times and average values were calculated. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of trim28 mRNA against GAPDH.

CCK-8 assay

HUVECs were seeded into 96-well plates at a density of 2,000/well. Each condition had three replicate wells. At 24h, 48h and 72h, CCK-8 reaction reagent was added onto the cells. After incubation at 37°C and 5% CO_2 for 30 min, the absorbance of each well was measured at 490 nm. Then, cell proliferation curves were plotted.

Flow cytometry

Cells (1×10^6) were first washed with cold PBS

twice, and then subjected to flow cytometry using ANXN V FITC APOPTOSIS DTEC KIT I (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's manual for the detection of apoptosis. Cells with ANNEXIN V-positive values were early apoptotic cells, those with PI-positive values were necrotic cells, and those with double positive values were late apoptotic cells.

Transwell assay

To test migration ability, cells (1×10^5) from each group were seeded into the upper chamber containing 200 μl serum-free DMEM medium. In addition, 500 μl DMEM medium supplemented with 10% fetal bovine serum was added into the lower chamber. After 24h, the chamber was removed and the cells in the upper chamber were wiped off. After being fixed with 4% formaldehyde for 10 min, the membrane was stained using Giemsa method for microscopic observation of 5 random fields (200×). The number of Transwell cells was calculated for the evaluation of cell invasion and migration ability. All procedures were carried out on ice with pipetting tips being cooled at 4°C.

Western blotting

Cells (1×10^6) were trypsinized and collected, and then lysed with precooled Radio-Immunoprecipitation Assay (RIPA) lysis buffer (600 μl; Beyotime, Shanghai, China) for 30 min on ice. The mixture was centrifuged at 12,000 rpm and 4°C for 10 min. The supernatant was used to determine protein concentration by bicinchoninic acid (BCA) protein concentration determination kit (RTP7102, Real-Times Biotechnology Co., Ltd., Beijing, China). The samples were then mixed with 5× sodium dodecyl sulfate loading buffer before denaturation in boiling water bath for 10 min. Afterwards, the samples (5 μl) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (250 mA, 1h) and blocked with 5% skimmed milk at room temperature for 1 h. Then, the membranes were incubated with rabbit anti-human TRIM28 polyclonal primary antibody (1:1000; ab10483; Abcam, Cambridge, UK) or mouse anti-human GAPDH monoclonal primary antibody (1:4000; Beyotime, Shanghai, China) at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween-20 3 times for 15 min, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:4,000; Abcam, Cambridge, UK) for 1h at room temperature before washing with phosphate-buffered saline with Tween-20 3 times for 15 min. Then, the membrane was developed with enhanced chemiluminescence detection kit (Beyotime, Shanghai, China) for imaging. Image lab v3.0 software (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative content of TRIM28 was expressed against GAPDH.

Statistical analysis

The results were analyzed using SPSS 20.0 statistical software (IBM, Armonk, NY, USA). The data were expressed as means ± standard deviations. Comparison between two groups was carried out using paired Student's t-test. Comparison of more than two groups was performed by one-way analysis of variance followed by a Student-Newman-Keuls post-hoc test. $P<0.05$ indicated statistically significant differences.

Results

Trim28 expression in placental tissues and peripheral blood from PIH patients is elevated, and serum from these patients can up-regulate the expression of Trim28 in HUVECs in vitro

To examine the expression of Trim28, qRT-PCR was first performed. The data showed that Trim28 mRNA levels in placental tissues and peripheral blood from PIH group were significantly higher than those from normal control group ($P<0.05$) (Fig. 1A-C). At cellular level, we stimulated HUVECs using the serum obtained from PIH group. qRT-PCR showed that Trim28 mRNA expression in the cells treated with PIH serum was significantly higher than that in untreated cells ($P<0.05$) (Fig. 1D). Western blotting showed that Trim28 protein level in the cells treated with PIH serum was also significantly higher than that in untreated cells ($P<0.05$) (Fig. 1E). The results suggest that Trim28 expression in placental tissues and peripheral blood from PIH patients is elevated, and serum from these patients can up-regulate the expression of Trim28 in HUVECs *in vitro*.

Trim28 silencing significantly inhibits the proliferation of HUVECs by affecting cell cycle

Then, we used the small-interfering RNA of Trim28 to inhibit its expression in HUVECs, and studied the biological functions of the cells. CCK-9 assay showed that silencing of Trim28 expression in HUVECs inhibited the proliferation of the cells, and the absorbance values of the cells at 24h, 48h and 72h were lower than that of control group ($P<0.05$) (Fig. 2A,B). Flow cytometric analysis showed that silencing of Trim28 expression blocked the transition of G1/S phases in HUVEC cells ($P<0.05$) (Fig. 2C). The results indicate that Trim28 silencing significantly inhibits the proliferation of HUVECs by affecting the cell cycle.

Silencing of Trim28 expression inhibits the migration ability of HUVECs

Migration ability is an important feature of HUVECs and plays an important role in vascular endothelial repair and barrier maintenance. Using Transwell assay, we further analyzed the effect of Trim28 on the migration of HUVECs. The data showed that silencing of Trim28 significantly inhibited the migration ability of HUVECs,

and the number of transwell cells in Trim28-silencing group was significantly lower than that in control group (P<0.05) (Fig. 2D). The results indicate that silencing of Trim28 expression inhibits the migration ability of HUVECs.

Down-regulation of Trim28 expression promotes the apoptosis of HUVECs

There are a few inflammatory factors in the peripheral blood of PIH patients, which directly lead to the damage of vascular endothelial cells and the dysfunction of endothelial cells. If the damage cannot be repaired, they will induce the apoptosis of vascular endothelial cells. After treating HUVECs with PIH serum or siRNA of Trim28, flow cytometry was performed to examine the apoptotic rates of the cells. The data showed that PIH serum or siRNA of Trim28 significantly elevates the apoptotic rate of HUVECs compared with control group (P<0.05) (Fig. 2E). The results suggest that down-regulation of Trim28 expression promotes the apoptosis of HUVECs.

Trim28 regulates the biological function of HUVECs by affecting the activity of p38 signaling pathway

The activation of p38 signaling pathway promotes starvation-mediated apoptosis of HUVEC cells, which is regulated by VEGFR. Trim28 has been proven to regulate angiogenesis through VEGF. Therefore, we speculate whether Trim28 regulates peripheral vascular endothelial injury in patients with PIH through p38

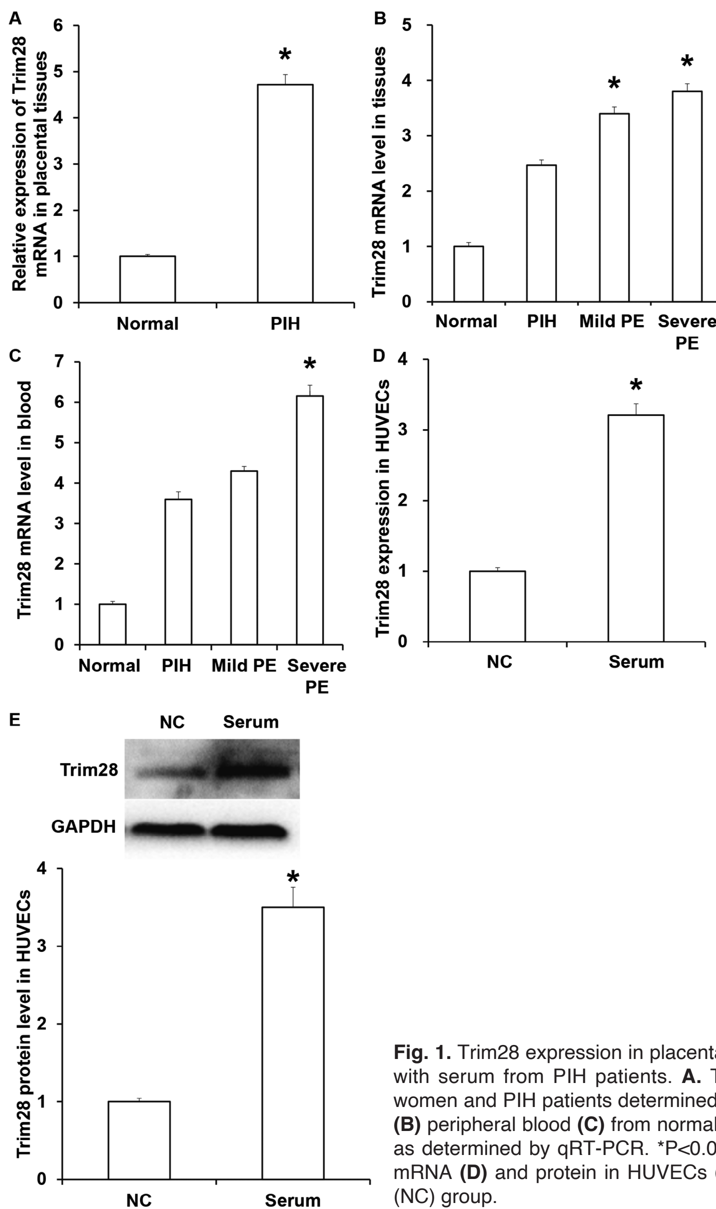

Fig. 1. Trim28 expression in placental tissues and peripheral blood from PIH patients, and HUVECs treated with serum from PIH patients. **A.** Trim28 mRNA expression in placental tissues from normal pregnant women and PIH patients determined by qRT-PCR. (B-C) Trim28 mRNA expression in placental tissues and **(B)** peripheral blood **(C)** from normal pregnant women and PIH patients with or without pre-eclampsia (PE), as determined by qRT-PCR. *P<0.05 compared with normal pregnant women. **D-E.** Expression of Trim28 mRNA **(D)** and protein in HUVECs **(E)** stimulated by PIH serum. *P<0.05 compared with negative control (NC) group.

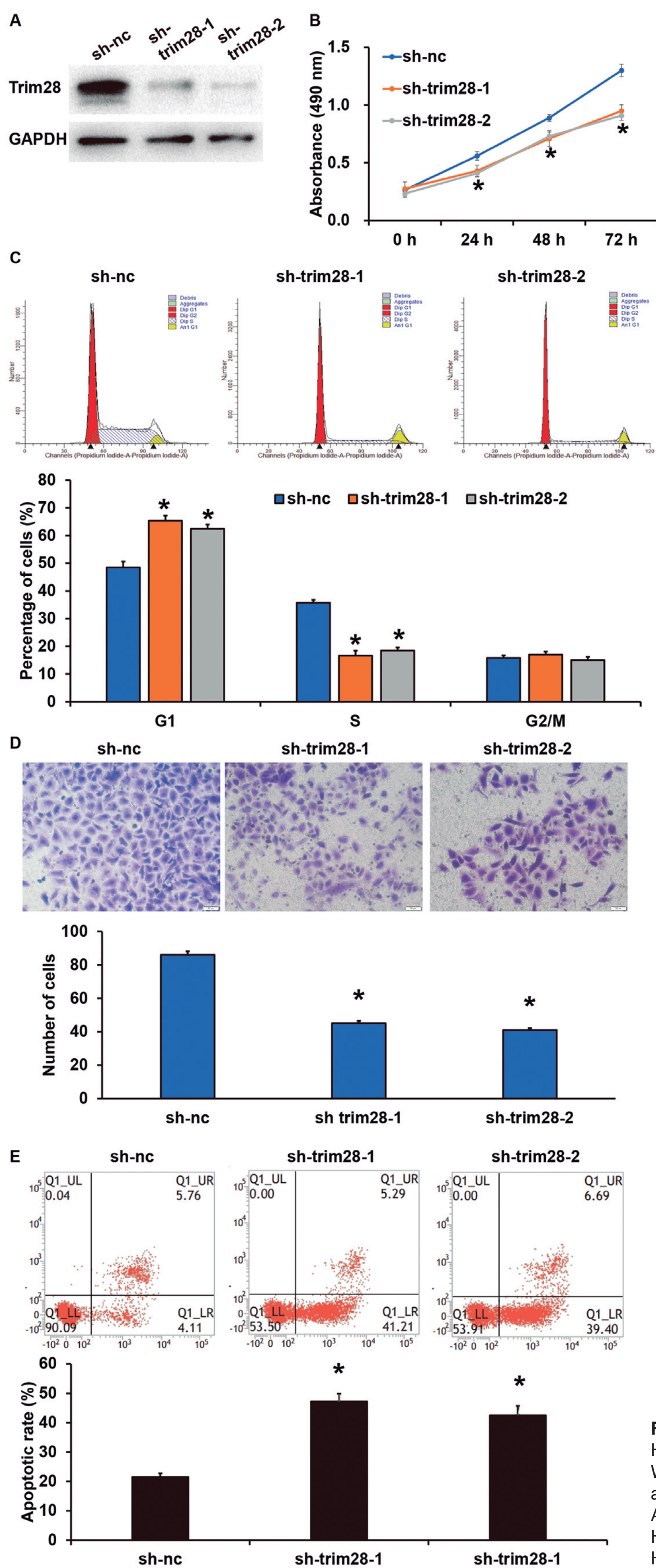

Fig. 2. Effect of silencing of Trim28 on the biological function of HUVECs. **A.** Expression of Trim28 in HUVECs determined by Western blotting. **B.** Proliferation of HUVECs determined by CCK-8 assay. **C.** Migration of HUVECs examined by Transwell assay. **D.** Apoptosis of HUVECs studied by flow cytometry. sh-nc, untreated HUVECs; sh-trim28-1, HUVECs treated with PIH serum; sh-trim28-2, HUVECs with silenced Trim28. *P<0.05 compared with sh-nc group.

signaling pathway. Western blotting showed that p38 phosphorylation level was elevated in Trim28 silencing group after stimulation by PIH serum, compared with control group (Fig. 3A). CCK-8 assay showed that p38 inhibitor elevated the proliferation of HUVECs stimulated with PIH serum at 24h, 48h and 72h (P<0.05 for all three time points) (Fig. 3B). Transwell assay showed that p38 inhibitor elevated the migration ability of HUVECs treated with PIH serum (Fig. 3C). Flow cytometry showed that p38 inhibitor reduced the apoptosis of HUVECs stimulated by PIH serum (P<0.05) (Fig. 3D). These results indicate that Trim28 regulates the biological function of HUVECs by affecting the activity of p38 signaling pathway.

Discussion

With the changes of lifestyle and the increase of

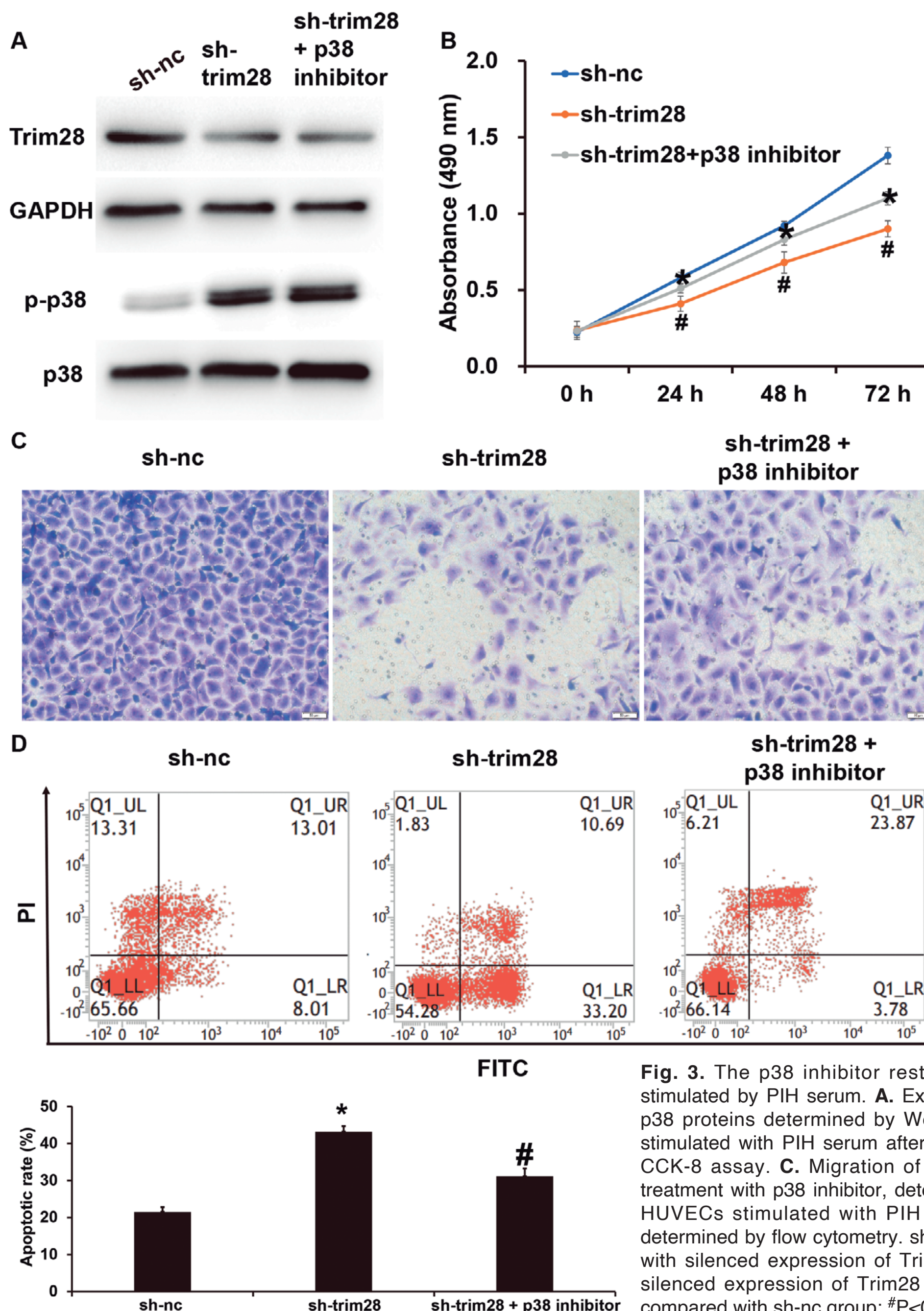

Fig. 3. The p38 inhibitor restores the biological functions of HUVECs stimulated by PIH serum. **A.** Expression of Trim28, p38 and phosphorylated p38 proteins determined by Western blotting. **B.** Proliferation of HUVECs stimulated with PIH serum after treatment with p38 inhibitor, determined by CCK-8 assay. **C.** Migration of HUVECs stimulated with PIH serum after treatment with p38 inhibitor, determined by Transwell assay. **D.** Apoptosis of HUVECs stimulated with PIH serum after treatment with p38 inhibitor, determined by flow cytometry. sh-nc, untreated HUVECs; sh-trim28, HUVECs with silenced expression of Trim28; sh-trim28-p38 inhibitor, HUVECs with silenced expression of Trim28 after treatment with p38 inhibitor. *P<0.05 compared with sh-nc group; #P<0.05 compared with sh-trim28 group.

elderly pregnant women in recent years, PIH has increased year by year. If it is not effectively controlled, its development will lead to systemic organ injury and endanger the safety of mothers and infants (Liu et al., 2020a). Vascular endothelial cells are the natural barrier of the inner wall of blood vessels. They maintain the stability of the internal environment by regulating the transport of substances inside and outside the cells and maintaining vascular pressure (Potente and Mäkinen, 2017; Lu et al., 2020). It is of great significance to maintain the normal function of vascular endothelial cells (Potente and Mäkinen, 2017; Lu et al., 2020). In the present study, we first found that Trim28 was up-regulated in peripheral blood from PIH patients. *In vitro* experiments further demonstrated that peripheral serum of PIH patients stimulated the expression of Trim28 in HUVECs, inhibited the proliferation and migration of HUVECs and promoted the apoptosis of these cells. In terms of molecular mechanism, we found that Trim28 might exert its biological function by inhibiting the p38 signaling pathway of HUVECs.

Trim28 is a member of the Trim family, and it plays important roles in heterochromatin regulation, DNA damage, signal transduction and apoptosis of cells (Lu et al., 2020; Jin et al., 2021). Trim28 protein is highly conserved in structure, and its sequence from N-terminal to C-terminal is Ring Box, B-Box domain and a coiled coil domain (Peng et al., 2019). It is discovered that Trim28 protein is closely related to cell survival (Peng et al., 2019). For example, silencing of Trim28 expression can up-regulate the expression of E2F1, so as to promote the sensitivity of non-small cell lung cancer to chemotherapeutic drugs (Liu et al., 2017). Trim28 protein inhibits DNA breakage induced by replication pressure through SUMOylated PCNA protein, indicating that Trim28 promotes cell survival by maintaining genomic stability (Li et al., 2020). In the present study, we first found that the expression of Trim28 mRNA was significantly increased in peripheral blood, and then we speculated that Trim28 might be related to vascular endothelial injury in PIH, in view of the important role of Trim28 in the survival of vascular endothelial cells. Therefore, we cultured HUVECs with the serum from PIH patients, and the results showed that the expression of Trim28 in HUVECs was significantly up-regulated after stimulation with PIH serum, suggesting that Trim28 might be related to peripheral vascular injury in hypertension. For HUVECs with silenced expression of Trim28, we stimulated these cells with PIH serum to analyze the effect of Trim28 in endothelial injury. The results suggested that silencing Trim28 expression not only inhibited the proliferation and migration of HUVECs, but also promoted the apoptosis of HUVECs induced by serum derived from PIH patients. Based on these results, we believe that Trim28 silencing can promote the injury of HUVECs and Trim28 may play important roles in vascular endothelial injury induced by PIH.

Studies show that p38-MAPK induces the transcription of many related genes by phosphorylating transcription factors, regulates the expression of active substances, and participates in the process of oxidative stress injury and even apoptosis of vascular endothelial cells (Coulthard et al., 2009; Lee and Kim, 2017). Blocking and regulating the expression and activity of p38-MAPK at the signal pathway level not only reduces endothelial cell injury or apoptosis, but also makes the p38 signaling pathway a therapeutic target for cardiovascular diseases (Kojonazarov et al., 2017; Yokota et al., 2020). It is shown that Trim28 can regulate the angiogenesis of endothelial cells through VEGF-Notch signaling pathway, suggesting that Trim28 may have a regulatory relationship with p38 (Wang et al., 2020). In the present study, Western blotting data showed that the phosphorylation level of p38 protein in HUVECs with Trim28 knockout was increased significantly. We speculate that Trim28 affects HUVEC survival through the p38 signaling pathway. After adding p38 inhibitor, we found that the survival of HUVECs in Trim28 knockout group was significantly restored, and the proliferation and migration ability of HUVECs were also recovered significantly. These observations demonstrated that the expression of Trim28 in HUVECs can regulate and affect the biological function of HUVECs through the p38 signal pathway. However, there are still some problems to be solved in our future work. For example, the regulation of Trim28 elevation in HUVECs is still unclear. We speculate that it may be related to the injury of inflammatory factors in peripheral blood of PIH patients. In addition, Trim28 plays a role in the nucleus, and whether it affects the p38 signal pathway remains to be further studied.

In conclusion, the present study demonstrates that Trim28 is up-regulated in peripheral blood of patients with PIH and participates in HUVECs injury through the p38 signaling pathway.

Acknowledgements. The authors wish to thank their department and research team for their help and dedication.

Funding. N/A.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions. MC, PS and LZ contributed to the design of the study. MC, PS, PW, TZ and JZ performed the experiments. MC and PS analyzed the data. MC, PS and PW interpreted results and prepared the manuscript. The final version of the manuscript has been read and approved by all authors.

Ethical approval and consent to participate. All procedures performed in the current study were approved by the Ethics Committee of Taian City Central Hospital. Written informed consent was obtained from all patients or their families.

Consent for publication. Written informed consents for publication of any associated data and accompanying images were obtained from all patients or their parents, guardians or next of kin.

Conflict of interest. The author declares that there is no financial or any conflict of interests related to this paper.

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Accepted July 21, 2023