Hes-related family BHLH transcription factor with YRPW motif 1-activated proteasome 26S subunit, non-ATPase 14 regulates trophoblast function and endometrial angiogenesis

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Abstract. Proteasome 26S subunit, non-ATPase 14 (PSMD14) expression has been previously reported to be reduced in patients with pre-eclampsia (PE). The present study investigated the interaction network associated with the role of PSMD14 in PE. Reverse transcription-quantitative PCR (RT-qPCR) and western blotting were performed to determine the transfection efficacy following plasmid-based gene transfer of PSMD14 into HTR-8/SVneo cells. Cell proliferation was measured using an MTT assay and 5-ethynyl-2′-deoxyuridine staining. The expression of proliferation-related proteins, including Ki67 and PCNA, was determined using western blotting. Wound healing and Transwell assays were performed to measure cell invasion and migration, whilst the expression of migration-related proteins, including MMP2 and MMP9, was measured using western blotting. The angiogenesis of HUVECs following treatment with the HTR-8/SVneo cell culture supernatant was examined using tube formation assay. Following overexpression of Hes-related family BHLH transcription factor with YRPW motif 1 (HEY1) by transfection of pcDNA3.1 expression vector containing full-length human HEY1 or knockdown by transfection of shRNA plasmids targeting HEY1, the expression of HEY1 and PSMD14 was detected using RT-qPCR and western blotting. The potential interaction between HEY1 and the PSMD14 promoter was examined using dual-luciferase reporter and chromatin immunoprecipitation assays. PSMD14 overexpression was found to promote the proliferation, invasion, migration of HTR-8/SVneo cells and the angiogenesis of HUVECs following treatment with the HTR-8/SVneo cell culture supernatant, accompanied by enhanced expression of proliferation and migration-related proteins. Furthermore, the transcription factor HEY1 activated the expression of PSMD14. Knocking down HEY1 expression partially reversed the promoting effects of PSMD14 overexpression on the proliferation, invasion, migration, angiogenesis, proliferation and migration-related protein expression in trophoblasts. In conclusion, HEY1-activated PSMD14 promoted trophoblast proliferation, invasion and angiogenesis. Therefore, HEY1 and PSMD14 can be potential targets for PE treatment.

Introduction

Pre-eclampsia (PE) is a progressive multisystemic disease that affects pregnancy and poses a serious threat to maternal and fetal health (1,2). It typically manifests as new-onset hypertension and proteinuria after ~week 20 of gestation (1,2). To the best of our knowledge, there is no definitive therapeutic strategy for PE. Current treatment methods, including delivery of the fetus, antihypertensive therapy and excessive fluid administration, are mainly focused on controlling the disease, prolonging the gestational age and maintaining the safety of both the mother and fetus (3). Medication is mainly provided for symptom relief, which is complemented with close monitoring of the maternal and fetal condition (4).

The etiology and specific pathophysiological mechanism of PE remain unclear. However, PE is generally considered to be the result of the joint action of a number of factors, mechanisms and pathways that need to be investigated, with a particular focus on the pregnant woman, placenta and fetus (3). Numerous studies have reported that aberrant utero-placental vascular structural modelling, inflammatory immune overactivation, vascular endothelial cell injury, inadequate trophoblast invasion and increased apoptosis are key processes of PE pathogenesis (5-7). Among these factors, trophoblast dysfunction is considered to serve a key role in the pathogenic process of PE (8,9). In addition, obstructed placental blood flow caused by the reduced angiogenic ability of the placenta is known to be another contributing factor in PE, since uterine spiral arteries of patients with PE tend to be more prone to deficient remodeling (10-12). Therefore,
restoring trophoblast invasion and placental angiogenesis may be an effective treatment strategy for PE.

It has been recently reported that pregnant women with PE have significantly lower expression levels of proteasome 26S subunit, non-ATPase 14 (PSMD14) in the placenta, compared with those with normal blood pressure (13). In view of this finding, PSMD14 was proposed to be a key gene in PE with potential prognostic and therapeutic value (13). Furthermore, PSMD14 expression has been previously found to be increased in liver cancer tissues, which promotes the proliferation, migration and invasion of hepatocellular carcinoma cells to facilitate tumor growth in vivo (14). However, it remains to be investigated if PSMD14 can regulate the proliferation and invasion of trophoblasts and placental angiogenesis. Therefore, the aim of the present study was to investigate the role of PSMD14 in PE and clarify the underlying mechanism.

Materials and methods

Cell culture and treatment. A HTR-8/SVneo trophoblast cell line (15,16) and HUVECs were purchased from Procell Life Science & Technology Co., Ltd. It was generated using freshly isolated extravillous cytotrophoblasts from first trimester (15,16) and HUVECs were purchased from Procell Life Science & Technology Co., Ltd. It was generated using freshly isolated extravillous cytotrophoblasts from first trimester (15,16) and HUVECs were purchased from Procell Life Science & Technology Co., Ltd. It was generated using freshly isolated extravillous cytotrophoblasts from first trimester (15,16) and HUVECs were purchased from Procell Life Science & Technology Co., Ltd. It was generated using freshly isolated extravillous cytotrophoblasts from first trimester (15,16).

Cell transfection. PSMD14-specific pc-DNA3.1 overexpression vector (oe-PSMD14; BC009524) and corresponding negative control (oe-NC), short hairpin RNA (sh) plasmids targeting HEY1 (sh-HEY1-1/2) and the control shRNA (sh-NC) and a pcDNA3.1 expression vector containing full-length human HEY1 (pcDNA3.1-HEY1; BC001873) and corresponding negative control (pcDNA3.1-NC; V790-20) were constructed by Shanghai GenePharma Co., Ltd. Briefly, after annealing, shRNA fragments were integrated into a lentiviral GV493 vector by Shanghai GenePharma Co., Ltd. Briefly, after annealing, shRNA fragments were integrated into a lentiviral GV493 vector (hU6-MCS-CBh-GFP-IRES-puromycin). The sequence of the negative control shRNA fragment was 5'-CCG GCA ATC TGC TAA GCT A-3'. The negative control shRNA sequence was 5'-CCG GCA ATC TGC TAA GCT A-3'. The sequence of the negative control shRNA fragment was 5'-CCG GCA ATC TGC TAA GCT A-3'. The negative control shRNA sequence was 5'-CCG GCA ATC TGC TAA GCT A-3'.

Reverse transcription-quantitative PCR (RT-qPCR). Following total RNA extraction, 2 µg RNA was converted to cDNA using PrimeScript™ RT Master Mix (Takara Bio, Inc.) following the manufacturer's protocol as follows: 25˚C for 5 min, 42˚C for 60 min and 70˚C for 5 min. qPCR was performed in an ABI PRISM™ 7000 Sequence Detection Systems Spectral Calibration Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: Pre-denaturation for 30 sec at 95˚C, followed by 40 cycles of denaturation for 5 sec at 95˚C and annealing for 30 sec at 60˚C. The primer sequences for PCR are presented as follows: PSMD14 forward, 5'-GTCAGGAAACAGGTGTCAGTG-3' and reverse, 5'-AACCAAAACACCATCCTCCGC-3'; HEY1 forward, 5'-CGGCTCTAGTTCCATGTC-3' and reverse, 5'-GCTTAGCAGATCCGTTCTCT-3' and GAPDH forward, 5'-GGGAACACTGTCGGTGAT-3' and 5'-GAAGGTCGTCGTTGTA-3'. The relative mRNA level was normalized with GAPDH using the 2-△△Cq method (17).

Western blotting. Protein samples were isolated from HTR-8/SVneo cells using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.), quantified by an Enhanced Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology) and separated using 10% SDS-PAGE (30 µg per lane) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk powder in Tris-buffered saline containing 0.1% Tween-20 for 2 h at room temperature, followed by incubation with the following primary antibodies overnight at 4˚C: Anti-PSMD14 (1:1,000; cat. no. ab182762; Abcam), anti-nuclear protein Ki67 (1:1,000; cat. no. ab15580; Abcam), anti-proliferating cell nuclear antigen (PCNA; 1:1,000; cat. no. ab29; Abcam), anti-MMP2 (1:1,000; cat. no. ab92536; Abcam), anti-MMP9 (1:1,000; cat. no. ab76003; Abcam) and anti-HEY1 (1:1,000; cat. no. ab154077; Abcam). The secondary antibodies, goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (1:3,000; cat. no. ab6721; Abcam) and goat anti-mouse horseradish peroxidase-conjugated IgG secondary antibody (1:3,000; cat. no. ab6728; Abcam), were incubated at room temperature for 2 h. Super ECL Detection Reagent (Shanghai Yeasen Biotechnology Co., Ltd.) and ImageJ software v1.8.0 (National Institutes of Health) were used to observe and analyze the blots.

MTT assay. HTR-8/SVneo cells were inoculated into 96-well plates (5x10^3/well), cultured for 24, 48 and 72 h at 37˚C, following which they were incubated with 10 µl MTT solution (5 mg/ml; Beyotime Institute of Biotechnology) and the mixture was incubated for 4 h at 37˚C. Next, 200 µl DMSO was added to each well to dissolve the formazan.
culture dish from the EP tube and the conditioned medium was added into the cells. Images of tube formation were captured after 12 h of incubation at 37°C using an inverted light microscope (magnification, x40). Quantification of tube formation was performed by using IncuCyte angiogenesis version 2.0 image analysis (Essen Bioscience).

5-Ethynyl-2’-deoxyuridine (EdU) staining. Following the indicated transfection of HTR-8/SVneo cells and centrifugation at 72 x g/min for 3 min at 4°C, the cells (1x10⁵ cells/well) were resuspended in EdU (50 μM per well; Beyotime Institute of Biotechnology)-containing RPMI-1640 medium for 2 h at 37°C. Subsequently, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature, permeabilized with 1% Triton X100 and incubated with click solution (catalog no. C0075L-3; Beyotime Institute of Biotechnology) for 30 min at room temperature for nuclear staining with 0.1 µg/ml DAPI (Beyotime Institute of Biotechnology) for 20 min at room temperature. Following centrifugation at 600 x g for 10 min at room temperature and resuspension in PBS, cytopsin slides were prepared for observation under a fluorescence microscope (Leica DM3000k; Leica Microsystems, Inc.; magnification, x200).

Wound healing assay. Following transfection, HTR-8/SVneo cells were collected and seeded (1x10⁴/well) in 6-well plates for 24 h. A wound on the cell monolayer was made using a sterile toothpick. Unattached cells were removed by washing with PBS, and the rest of the cells were incubated in RPMI-1640 medium containing 10% FBS with 5% CO₂ at 37°C. Images were captured at 0 and 24 h of incubation under a fluorescence microscope (Leica DM3000k; magnification, x100) and migration distance calculated as follows: Migration (%)=[(0 h average scratch distance-24 h average scratch distance)/0 h average scratch distance] x100.

Transwell assay. An invasion assay was conducted to measure cell invasion using Transwell chambers (6.5-mm in diameter; 8-µm pore-size; Corning, Inc.). The Transwell chambers were first coated with 200 µg/ml Matrigel (BD Biosciences) at 37°C for 1 h. The transplanted cells were harvested and suspended to a final concentration of 5x10⁵ cells/chamber in serum-free RPMI-1640 medium. These cell suspensions were loaded into the upper chamber whilst media containing 10% FBS were added into the lower chamber. Following 24 h of incubation at 37°C, the cells were fixed with 4% paraformaldehyde at room temperature for 15 min and stained with 0.5% crystalline violet dye (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 30 min. The number of invasive cells was counted under a light microscope (magnification, x100). In total, five randomly chosen fields were counted for each chamber.

HUVVEC tube formation assay. Transfected HTR-8/SVneo cells were seeded (1x10⁵/well) in 6-well plates at 37°C for 24 h and then the media of the HTR-8/SVneo cells (conditioned medium) was collected, centrifuged at 500 x g for 5 min and stored in aliquots at -80°C. Matrigel (Corning, Inc.) diluted in DMEM was evenly added into 12-well plates at 300 µl per well to incubate HUVECs (1x10⁵ cells/well) for 30 min at 37°C. HUVECs were selected for digestion, centrifugation and counting. Subsequently, 1x10⁵ HUVECs were added to the
Results

**PSMD14 overexpression improves trophoblast proliferation.** To examine the role of PSMD14 in trophoblast function, a PSMD14-expressing plasmid was transfected into HTR-8/SVneo cells. RT-qPCR and western blotting revealed a significant increase in the expression of PSMD14 following cell transfection compared with that in cells transfected with Oe-NC (Fig. 1A and B). MTT assay results showed a significant increase in the proliferation of PSMD14-overexpressing HTR-8/SVneo cells, compared with that in the Oe-NC group at 48 and 72 h (Fig. 1C). Similarly, the EdU staining results showed that the number of proliferating cells was markedly increased following PSMD14 overexpression compared with that in the Oe-NC group (Fig. 1D). Furthermore, western blotting results revealed that PSMD14 overexpression significantly promoted the expression of proliferation markers Ki67 and PCNA in HTR-8/SVneo cells (Fig. 1E). These results support the findings from MTT assay and EdU staining in that PSMD14 overexpression promoted HTR-8/SVneo cell proliferation.
PSMD14 overexpression enhances trophoblast migration and invasion in addition to promoting HUVEC angiogenesis. As shown in Fig. 2A and B, HTR-8/SVneo cells transfected with oe-PSMD14 possessed significantly higher migratory and invasive capacities compared with those in the oe-NC group. In addition, significant increases in the expression levels of migration-related proteins MMP2 and MMP9 were observed following PSMD14 overexpression in HTR-8/SVneo cells compared with those in cells transfected with Oe-NC (Fig. 2C). Tube formation assay results subsequently revealed a stimulating effect of cell culture supernatant of the HTR-8/SVneo trophoblasts overexpressing PSMD14 on the tube-like structure formation by HUVECs (Fig. 2D). These results suggest that PSMD14 overexpression enhances trophoblast migration and invasion, in addition to HUVEC angiogenesis.

Transcription factor HEY1 activates PSMD14 expression. The Human Transcription Factor Database predicted the binding site of HEY1 in the PSMD14 promoter region (Fig. 3A). The role of HEY1 in the expression mechanism of PSMD14 was therefore examined following HEY1 overexpression or knockdown in HTR-8/SVneo cells. The transfection efficiency of pcDNA3.1-HEY1 or sh-HEY1-1/2 was detected by RT-qPCR and western blotting. The results revealed that HEY1 expression was prominently increased after transfection of pcDNA3.1-HEY1, shRNA transfection decreased the expression levels of HEY1, and sh-HEY1-1 exhibited superior transfection efficacy (Fig. 3B and C). Therefore, sh-HEY1-1 was selected for subsequent experiments. PSMD14 expression was also significantly higher in HTR-8/SVneo cells transfected with pcDNA3.1-HEY1 compared with that in the pcDNA3.1-NC group, whereas HTR-8/SVneo cells transfected with the sh-HEY1 exhibited significantly lower PSMD14 expression levels compared with those in the sh-NC group (Fig. 3D and E).

Dual-luciferase reporter assay results revealed significantly higher luciferase activities in HTR-8/SVneo cells co-transfected with pcDNA3.1-HEY1 and PSMD14-WT compared with those in cells co-transfected with pcDNA3.1-NC and PSMD14-WT, but luciferase activity remain unchanged between the pcDNA3.1-HEY1 and PSMD14-MUT or pcDNA3.1-NC and PSMD14-MUT groups (Fig. 3F). ChiP assay results also revealed significantly higher PSMD14 enrichment in the anti-HEY1 group compared with that in the IgG group (Fig. 3G). Collectively, these results suggest that the transcription factor HEY1 can activate PSMD14 expression in HTR-8/SVneo trophoblast cells.

HEY1 silencing reverses the positive effects of PSMD14 overexpression on trophoblast proliferation. PSMD14 overexpression was found in the present study to promote the proliferation of HTR-8/SVneo trophoblast cells. Following HEY1 knockdown, PSMD14-overexpressing HTR-8/SVneo cells exhibited significantly lower cell proliferation levels (Fig. 4A). Similarly, HEY1 knockdown also decreased the number of proliferating cells following PSMD overexpression, as evidenced by the EdU staining results (Fig. 4B). The expression of Ki67 and PCNA in PSMD14-overexpressing HTR-8/SVneo cells was also significantly downregulated following HEY1 knockdown compared with that in PSMD14-overexpressing cells transfected with sh-NC (Fig. 4C). These results suggest that knocking down HEY1 expression weakens the positive effects of PSMD14 overexpression on trophoblast proliferation.

HEY1 knockdown inhibits the promoting effects of PSMD14 overexpression on trophoblast migration, invasion and angiogenesis. It was observed from wound healing and Transwell assays that HEY1 knockdown significantly decreased the migratory and invasive capabilities of PSMD14-overexpressing trophoblasts compared with those in PSMD14-overexpressing cells transfected with sh-NC (Fig. 5A-D). In addition, MMP2 and MMP9 expression in PSMD14-overexpressing HTR-8/SVneo cells was also significantly reduced by HEY1 knockdown compared with those in PSMD14-overexpressing cells transfected with sh-NC (Fig. 5E). Furthermore, the PSMD14 overexpression-activated stimulation of cell culture supernatant of the HTR-8/SVneo trophoblasts on the tube-like structure formation ability by HUVECs was significantly reversed by HEY1 knockdown (Fig. 5F). Therefore, although PSMD14 overexpression enhanced trophoblast migration, invasion and HUVEC angiogenesis, HEY1 knockdown was able to at least partially reverse these changes.

Discussion

PE is a hypertensive disorder that typically occurs during pregnancy and can become a serious obstetric complication in addition to being a major cause of maternal and fetal mortality (3). Globally, 4-5% pregnant women are affected by PE, who face higher risks of seizures or falling into coma if the condition deteriorates into eclampsia, which threatens the life of both the mother and the baby (18,19). The past two decades have witnessed promising advances in terms of the accuracy and efficiency of PE diagnosis, in addition to improvements in the clinical management of this disease (20,21). However, the mechanism underlying the pathogenesis of PE remains to be fully elucidated (20,21). Trophoblasts can differentiate into highly proliferative and invasive extravillous trophoblasts with physiology similar to that of cancer cells, which can invade the endometrium and anchor the placenta to the uterus (22). There they promote angiogenesis, which provide nutrients and oxygen supply that is essential for fetal development (22). Defective trophoblast function has been previously shown to be a major cause of PE development (23-25). In addition, insufficient angiogenesis of vascular endothelial cells, which contributes to dysfunctional vascular recast, has also been proposed to be an important cause of PE, which ultimately leads to placental ischemia and hypoxia (23-25). To the best of our knowledge, the molecular mechanism underlying these pathological processes remain unclear.

Recently, a gene expression profiling study revealed that PSMD14 expression was aberrantly reduced in pregnant women with PE in comparison with healthy pregnant women (13), suggesting a role for PSMD14 in PE pathogenesis. PSMD14 has been previously reported to be a potential prognostic biomarker and treatment target in several types of cancer, including breast cancer (26), lung adenocarcinoma (27) and melanoma (28). In hepatocellular carcinoma, PSMD14 was found to serve an oncogenic role, where its upregulation promoted cell proliferation, migration and invasion in vitro.
and tumor growth in vivo (14). Furthermore, elevated PSMD14 expression has been observed in breast cancer tissues compared with that in adjacent non-tumor tissues, such that PSMD14 knockdown was found to inhibit proliferation and migration whilst facilitating apoptosis and G0/G1 arrest (29). It was therefore hypothesized in the present study that PSMD14 expression may also affect the cellular processes of trophoblasts. In the present study, increased proliferation was observed alongside
enhanced expression levels of Ki67 and PCNA following PSMD14 overexpression in HTR-8/SVneo cells. Previous studies have shown that cell viability and invasion of trophoblast cells are closely associated with PE progression (30,31). Extravillous trophoblasts (EVTs) are progeny trophoblasts following epithelial-mesenchymal transition and are highly invasive, which enables them to migrate away from the attached embryo and invade the uterine epithelium and spiral arteries to establish maternal-fetal linkage (32). Dysfunctional EVT migration and invasion frequently result in the failure of establishing maternal-fetal connection, which has been previously associated with PE pathogenesis (33). In the present study, HTR/SVneo cell invasion and migration after PSMD14 upregulation were both promoted according to results from wound healing and Transwell assays, which were coupled with significantly increased levels of MMP2 and MMP9 expression. In addition, the supernatant of PSMD14-overexpressing trophoblasts also improved the tube formation capabilities of HUVECs. Therefore, PSMD14 overexpression was suggested to promote trophoblast function and HUVEC angiogenesis.

The potential relationship between the transcription factor HEY1 and the PSMD14 promoter was predicted using bioinformatics analysis. This prediction was meaningful as HEY1 has been previously shown to be expressed at significantly lower levels in patients with PE compared with those in healthy pregnant women (34). In hepatocellular carcinoma, HEY1 upregulation has been identified to be a tumorigenic factor, which correlated negatively with prognosis, overall survival and recurrence-free survival (35). In addition, high HEY1 expression levels have been detected in clinical glioblastoma
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In conclusion, the present study demonstrated that HEY1 can activate PSMD14 expression, thereby promoting trophoblast proliferation and HUVEC angiogenesis. However, the present study only investigated the role of PSMD14 and its transcriptional regulation by HEY1 in vitro. Further in vivo and clinical studies are required to verify the findings in the present study.

Figure 4. HEY1 knockdown reverses the positive effects of PSMD14 overexpression on trophoblast proliferation. (A) The cell proliferation of HTR-8/SVneo cells transfected with oe-PSMD14 in the presence or absence of HEY1 knockdown were detected by Cell Counting Kit-8. *P<0.05 and ***P<0.001 vs. oe-NC; ^P<0.05 vs. oe-PSMD14 + sh-NC. (B) The proliferative capacity of HTR-8/SVneo cells transfected with oe-PSMD14 in the presence or absence of HEY1 knockdown were detected by EdU staining. (C) The protein expression of Ki67 and PCNA in HTR-8/SVneo cells transfected with oe-PSMD14 in the presence or absence of HEY1 knockdown were detected by western blotting. **P<0.001 vs. oe-NC; ^P<0.05 and ##P<0.01 vs. oe-PSMD14 + sh-NC. PSMD14, proteasome 26S subunit, non-ATPase 14; oe, overexpression plasmid; NC, negative control; sh, short interfering; HEY1, Hes-related family BHLH transcription factor with YRPW motif 1; PCNA, proliferating cell nuclear antigen.
Figure 5. HEY1 knockdown reverses the positive effects of PSMD14 overexpression on trophoblast migration and invasion and HUVEC angiogenesis. (A) Migration and (B) invasion of HTR-8/SVneo cells transfected with oe-PSMD14 in the presence or absence of HEY1 knockdown were detected by wound healing and Transwell assays. (C) Migration and (D) invasion assay results were quantified. ***P<0.001 vs. oe-NC; ###P<0.001 vs. oe-PSMD14 + sh-NC. (E) The protein expression of MMP2 and MMP9 in HTR-8/SVneo cells transfected with oe-PSMD14 in the presence or absence of HEY1 knockdown was detected by western blotting. ***P<0.001 vs. oe-NC; #P<0.05 and ###P<0.001 vs. oe-PSMD14 + sh-NC. (F) Formation of tube-like structures by HUVECs treated with HTR-8/SVneo cell supernatants with or without PSMD14 overexpression in the presence or absence of HEY1 knockdown was measured. ***P<0.001 vs. oe-NC; ***P<0.001 vs. oe-PSMD14 + sh-NC. PSMD14, proteasome 26S subunit, non-ATPase 14; HEY1, Hes-related family BHLH transcription factor with YRPW motif 1; oe, overexpression plasmid; NC, negative control; sh, short interfering.
trophoblast proliferation, invasion and migration, in addition to downstream endothelial angiogenesis. PSMD14 and HEY1 are therefore promising therapeutic targets for PE. However, further research on PSMD14 and HEY1 is warranted to obtain an in-depth understanding of their roles in PE pathogenesis.

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Availability of data and materials

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

Authors' contributions

LZ and FC designed the study. LZ, SZ and FC performed the experiments and analyzed the data. All authors read and approved the final manuscript. LZ and SZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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