

Original Article

STAT3-mediated effects of methyltransferase inhibitor 5-aza-2'-deoxycytidine on preeclampsia

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Abstract: Objective: To investigate the effects of 5-aza-2'-deoxycytidine (5-AZA-DC) on preeclampsia (PE) and functional mechanisms dependent on STAT3. Materials and methods: Trophoblastic cells (HTR8/Svneo, JEG-3, JAR and BeWo) were used to construct STAT3-overexpressing or -silenced cells. qRT-PCR, Western blot, and FISH were used to detect mRNA and protein expression. GST-pull down, ChIP and dual luciferase reporter were used to prove the association of STAT3 and PTEN or TSC2, LC-MS/MS for proteome, and MeDIP-Seq for transcriptome. CCK-8 and flow cytometry were used to examine cell proliferation and apoptosis. C57BL/6J mice were divided into 4 groups (control, control + 5-AZA-DC, PE and PE + 5-AZA-DC). Systolic blood pressure, 24-h urinary protein, APTT, D-D, PT, ALT, Scr, and BUN were determined. Placental blood flow velocity was detected by Doppler ultrasound, HE staining for kidney injury. Results: STAT3, PTEN and TSC2 were the dominantly differential expressed genes in preeclampsia. Aberrant STAT3 expression increased DNMT1 levels. STAT3 regulated PTEN promoter activity. STAT3 interacted with PTEN and TSC2. DNMT1 was increased while STAT3, PTEN and TSC2 were decreased by 5-AZA-DC. Cell proliferation was promoted and apoptosis was inhibited by 5-AZA-DC. PE-induced STAT3 down-regulation was restored by 5-AZA-DC. Systolic blood pressure, 24-h urinary protein, APTT, D-D, PT, ALT, Scr and BUN were increased, and velocity of placental blood flow was inhibited in PE compared with control mice, while 5-AZA-DC relieved these indicators. Conclusions: Preeclampsia symptoms was relieved by 5-AZA-DC, suggesting that 5-AZA-DC could be used as a potential drug for epigenetic treatment of preeclampsia.

Keywords: 5-aza-2'-deoxycytidine, preeclampsia, STAT3, PTEN, TSC2

Introduction

Preeclampsia is a multisystemic disorder of pregnancy, usually defined as hypertension and proteinuria diagnosed after 20 weeks of gestation [1, 2]. The incidence of preeclampsia is high, and the specific etiology and pathogenesis of this pregnancy complication are still not fully elucidated [3, 4]. Preeclampsia is thought to be due to inadequate placental trophoblast invasion followed by widespread maternal endothelial dysfunction [5, 6]. What's more, studies showed that excess anti-angiogenic factors soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng) are released from the placenta into the maternal bloodstream, causing extensive endothelial dysfunction and leading to systemic manifestations of preeclampsia

such as hypertension and proteinuria [5, 6]. Prophylactic aspirin or calcium supplements are recommended for women with a history of hypertension for preeclampsia prevention, while delivery is still the only definitive treatment [7, 8].

Signal transducer and activator of transcription 3 (STAT3) shows important roles in growth factor and cytokine signaling, which was implicated in nuclear translocation and activation of transcription of its target genes [9]. In preeclampsia, hypoxic condition activates STAT3 signaling pathway, hence increasing trophoblast cell viability and angiogenesis [10]. There is strong evidence that altered DNA methylation, especially within the regulatory region of the expressed gene, has a significant effect on

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transcription [11, 12]. Because STAT3 gene expression is thought to change in preeclampsia pregnancy, it is reasonable to assume that preeclampsia may also be associated with changes in DNA methylation in key regulatory processes [13, 14]. The mechanism of epigenetic abnormalities in preeclampsia, especially with regarding to DNA hypomethylation of the promoter region of the STAT3 gene in preeclampsia, is incompletely understood [15, 16].

5-aza-2'-deoxycytidine (5-AZA-DC) was a nucleoside antimetabolite firstly synthesized by Piskala and Sorm [17]. 5-AZA-DC can incorporate into DNA, showing cytotoxicity for cultured cells and animals [18, 19]. Recent findings showed that 5-AZA-DC modulates the methylation of tumor necrosis factor receptor associated factor 6 (TRAF6), and enhances the expression of proinflammatory cytokines [20]. Furthermore, 5-AZA-DC was utilized to target DNA and histone methylation for acute myeloid leukemia treatment [21]. In this study, we investigated the effects of DNA methylation inhibitor 5-AZA-DC on preeclampsia *in vivo* and *in vitro*. Besides, the roles of STAT3 gene in PTEN and TSC2 methylation were also explored.

Materials and methods

Cell lines and culture

HTR8/SVneo cells were provided by the Cell Resource Centre of the Shanghai Academy of Life Sciences (Shanghai, China). The human trophoblast cell lines JEG-3, JAR, and BeWo cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). HTR8/Svneo and JAR cells were grown in Roswell Park Memorial Institute-1640 medium (RPMI-1640; HyClone, South Logan, UT, USA). JEG-3 and BeWo cells were cultured in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12; Gibco, Rockville, MD, USA). The culture medium was supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). All cells were grown in an incubator containing 5% CO₂ at 37°C. To investigate the effects of 5-AZA-DC, the cells were treated with 10 µg/mL 5-AZA-DC for 12 h.

Cell transfection

The full sequence of STAT3 was constructed into pcDNA3.1 plasmid by the Gene Pharma

(Shanghai, China). Short hairpin RNAs (shRNAs) targeting STAT3 (shSTAT3) and PTEN (shPTEN) were generated and synthesized by the Gene Pharma. The cells were cultured in a serum-free medium (500 µL per well). The transfection was carried out using Opti-MEM reduced serum medium and Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's introduction. After 48 h, the cells were collected, and STAT3 and PTEN protein expression was examined by Western blot.

Western blot analysis

Placenta tissues and cells were lysed using RIP pyrolysis solution. Protein content in the lysates was determined with a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins. The separated proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was sealed with 5% non-fat milk powder in TBST for 1 h at room temperature. The proteins were probed with the antibody against DNMT1 (1:1000, sc-10222, Santa Cruz Biotechnology, Santa Cruz, CA, USA), STAT3 (1:1000, Cat#12640, CST, Danvers, MA, USA), PTEN (1:1000, Cat#9559, CST), TSC2 (1:1000, sc-44174, Santa Cruz Biotechnology) and β-actin (1:1000, Cat#3700, CST). The primary antibodies were detected with horseradish peroxidase (HRP)-labeled donkey anti-rabbit IgG (GE Healthcare, Mississauga, Canada) using an enhanced chemiluminescence detection kit (GE Healthcare, Piscataway, NJ, USA). The expression of β-actin was used as a normalization control. The Quantity One software (Bio-Rad, Hercules, CA, USA) was used to detect the band intensities for protein quantification.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from placenta tissues and cells was extracted using a RNAsimple Total RNA Kit (Tiangen Biotech, Beijing, China), and RNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription was carried out using a FastQuan RT Kit (Tiangen Biotech). qPCR was executed on a LightCycler 480 System II (Roche, Indianapolis, IN, USA) using SYBR Green real-time PCR mas-

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Table 1. RT-qPCR primers used in this study

Gene	Forward primer	Reverse primer
DNMT1	5'-AGATGTGGATCAGCAAGCA-3'	5'-GCGCAAGTTAGGTTTTGTCA-3'
STAT3	5'-ACCCAACAGCCGCGTAG-3'	5'-CAGACTGGTTGTTCCATTCAGAT-3'
PTEN	5'-CGACGGGAAGACAAGTTCAT-3'	5'-AGGTTTCCTCTGGTCCTGGT-3'
TSC2	5'-CACTGGTGAGGACGTCTG-3'	5'-CCGCAGCATCAGTGTGC-3'
β -actin	5'-AGATGTGGATCAGCAAGCA-3'	5'-GCGCAAGTTAGGTTTTGTCA-3'

ter mix (Applied Biosystems, Stockholm, Sweden) and the specific primers of DNMT1, STAT3, PTEN, TSC2 and β -actin. All primers used in this study are listed in **Table 1**. The procedure was set at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 59°C for 35 s and extension at 72°C for 1 min, and followed by the final extension at 72°C for 10 min. The relative expression of DNMT1, STAT3, PTEN, and TSC2 mRNA was calculated by $2^{\Delta\Delta CT}$ and normalized to β -actin mRNA.

Glutathione S-transferase (GST) pull-down assay

PTEN protein-coding sequence was cloned into pGEX-KG vector (Novagen, Darmstadt, Germany) to generate GST-tagged PTEN protein. The constructs were transduced into BL21 competent cells, and protein expression was induced with isopropyl thiogalactoside (IPTG) at 37°C for 4 h. The bacterial pellets were collected in STE buffer, followed by incubation with glutathione beads (Amersham Bioscience, Amersham, UK) at 4°C overnight. The pre-bound GST-beads were incubated with lysates from the cells transfected with pcDNA3.1-STAT3 at 4°C overnight. Then the beads were extensively washed in TBSN buffer, followed by Western blot analysis.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with an EZ-ChIP kit (Millipore). Formaldehyde cross-linked chromatin was sonicated to generate different fragments. To immuno-precipitate the chromatin fragments, antibody against STAT3 was incubated with the chromatin fragments. Anti-rabbit IgG was used as a negative control. The precipitated chromatin DNA was analyzed by RT-qPCR.

Luciferase reporter assay

The putative binding sites of PTEN promoter (wild type) were cloned into the pGL3-Basic/Luciferase vector purchased from Promega

(Madison, WI, USA). The plasmids were transfected into shSTAT3 and STAT3^{OE} cells. The cells were seeded into 96-well plates and incubated for 48 h. Luciferase fluorescence was determined with a dual-luciferase reporting system kit (Promega) according to the manufacturer's instruction.

LC-MS/MS proteomics and data analysis

The cells were excised and digested with trypsin (Thermo Fisher Scientific) for 16 h at 37°C. Protein extracts were subjected to SDS-PAGE, and the separated proteins were stained with Coomassie brilliant blue. Appropriately 100 μ g of peptide segments was subjected to iTRAQ reagents (Applied Biosystems). The peptides were dissolved in solvent buffer A (0.1% formic acid and 2% acetonitrile). The labeled samples were separated on an ACQUITY UPLC BEH C18 RP column (1.7 μ m particle size, 2.1 \times 100 mm; Waters, USA) under a gradient of 3-45% buffer B (0.1% formate and 80% acetonitrile) at a flow rate of 250 nL/min for 120 min. An Agilent 6500 Q-TOF mass spectrometry (Agilent, Santa Clara, CA, USA) was used to detect proteins after chromatographic separation. The parameters of instrument were set as follows, ion spray voltage floating: 5500 V in positive mode and -4500 V in negative mode, respectively; curtain gas: 35 psi; ionization temperature: 500°C; gas1: 50 psi; gas2: 60 psi; collision energy: 45 \pm 20 eV; declustering potential: 50 V. The samples were scanned from m/z 300 to 1800 under a positive ionization mode with an acquisition rate of 4 spectra per second. Spectrum Mill MS Proteomics Workbench (Agilent) was used to perform peptide quantification and protein identification. MS/MS spectrum was aligned with the Uniprot-Swiss-Prot database. Protein was relatively quantified using iTRAQ.

Methylated DNA immunoprecipitation sequencing (MeDIP-Seq)

Genomic DNA was sonicated into DNA fragments with 100-500 dp by sonication. The son-

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icator (Diagenode, Denville, New Jersey, USA) was set to high, and genomic DNA was sonicated 3-4 pulses of 10-15 seconds each, followed by 30-40 seconds rest on ice between each pulse. DNA fragments were then denatured at 95°C for 10 min, and then immediately cooled on ice. Thereafter, the denatured sonicated DNA was incubated with monoclonal mouse anti-5-methyl cytidine (Diagenode) on a rotator overnight at 4°C. Next, 500 µL of DNA-antibody mixture was mixed with 50 µL of beads, followed by incubation for 2 h. The bead-DNA mixture was resuspended in 250 µL digestion buffer, and then supplemented with 250 µL of phenol-chloroform isoamylalcohol solution. The supernatant was collected, and incubated with 2 µL of glycoblue (20 mg/mL), 20 µL of 5 mol/L NaCl, and 500 µL ethanol at -40°C overnight for precipitation. The precipitated pellet was collected for sequencing on Illumina HiSeq 2500 with a PE50 application.

Cell proliferation

Cell proliferation was measured with a cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's description. Assays were conducted at 24 h, 36 h, 48 h, 60 h, and 72 h. The optical density (OD) was measured at 450 nm with a microplate reader (Molecular Devices, Palo Alto, CA, USA).

Apoptosis assay

The cells were detached by trypsinization and then centrifugated at 4°C and 1000 rpm for 5 min. Cellular pellets were washed in PBS. Apoptotic cells were examined with Annexin V-FITC kit (Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's description. Briefly, appropriately 1×10^6 cells were washed in Annexin V binding buffer and then transferred into 5 µL of Annexin V-FITC, followed by an incubation for 15 min in the dark. The fluorescence was detected on a FACScan using the Cell Quest software (BD Biosciences, Franklin Lake, NJ, USA).

Establishment of high specificity preeclampsia mouse model and grouping

Healthy, 8-week old C57BL/6J mice (SPF grade, 18-33 g) were purchased from the Experimental Animal Center of Southern Medical University (Guangzhou, China). Animals were maintained

at 20-25°C and 40-70% humidity, with standard rodent chow available ad libitum under a semi-natural light dark cycle (12:12 h). *PIGF*^{-/-} and *VEGF*^{-/-} mice were constructed by CRISPR/Cas9 technology [22]. The experiment was approved by the Ethics Committee of the affiliated Huadu Hospital, Southern Medical University (Approval number: 2019130) and complied with the International Regulations for the Administration of Laboratory Animals.

C57BL/6J females impregnated by C57BL/6J males constituted the control group (Con group, n = 30). C57BL/6J females impregnated by *PIGF*^{-/-} and *VEGF*^{-/-} C57BL/6J males were the preeclampsia group (PE group, n = 30). *PIGF* and *VEGF* show proangiogenic effects on the maternal endothelium, and *PIGF*^{-/-} and *VEGF*^{-/-} mice show the phenotypes of the preeclampsia mice [23]. We analyzed the pre-experiment and related literature. Innovative animal models such as preeclampsia are constructed. Con mice and PE mice were treated with 5-AZA-DC (2.5 mg/kg i.p.) from 1-18 days post-conception. On day 10, all pregnant rats were anesthetized with chloral hydrate and euthanized by cervical dislocation.

Blood pressure, 24 h-urine protein, APTT, D-D, PT, ALT, Scr and BUN detection

A CODA™ monitoring system (Kent Scientific Corporation, Torrington, CT, USA) was used for non-invasive monitoring of tail artery blood pressure in mice, and the blood pressure was recorded on day 0-18. At 08:00 am, the pregnant mice were placed in a metabolic cage to collect urine on day 0-17. All urine was measured with an automatic biochemical analyzer (Bayer Company, Tarrytown, NY, USA). The orbital blood, activated partial thrombin time (APTT), D-dimer (D-D), and prothrombin time (PT) of pregnant mice were analyzed with an automatic thrombus/hemostatic analyzer (Dade Behring, Schwalbach, Germany). Concentrations of alanine aminotransferase (ALT), serum creatinine (Scr) and urea nitrogen (BUN) were determined with an automatic biochemical analyzer (Roche).

Doppler ultrasound examination of placental blood flow velocity

Pregnant mice were anesthetized with 3.5-5% isoflurane on day 18 and placed on a heated surgical blanket. Doppler ultrasound (Esaote

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MyLab™ One/Touch, Esaote Biomedica, Genova, Italy) was used to detect blood flow velocity. The blood flow velocity of umbilical artery and uterine spiral artery was measured for three consecutive cardiac cycles.

Assessment of placental and fetal mice development

After ultrasound examination on day 18, the mice were anesthetized in a chamber containing 2.5% ether in oxygen. The mice were subjected to euthanasia by rapid cervical dislocation. The mice were disinfected with 75% ethanol on the abdominal surface. Each placenta was rapidly collected and washed in pre-cold sterile phosphate buffer saline, then frozen in liquid nitrogen, and stored at -80°C.

Hematoxylin and eosin staining

Renal tissues were dissected, fixed in 4% paraformaldehyde at 4°C overnight and washed in phosphate buffer saline. The washed tissues were successively dehydrated in 75%, 85%, 95%, 100% and 100% ethanol. Then the tissues were soaked in xylene before being embedded in soft wax (60°C) and paraffin (80°C). The sections (5- μ m thick) were de-waxed with xylene, followed by rehydration with 100%, 100%, 95%, 85% and 75% ethanol. The sections were incubated in hematoxylin solution at 60°C for 3 minutes, then in eosin solution for 20 s, and rinsed with distilled water. The sections were air-dried and examined for pathological changes under an Olympus IX81 microscope (Olympus, Tokyo, Japan). The images were captured with Olympus CellSens Standard Software (Olympus) and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Fluorescence in situ hybridization

Placental tissue sections were soaked in acetone solution for 20 min and rinsed in SSC (pH 7.0 \pm 0.2) solution. FISH assay was performed using a Ribo™ Fluorescent In Situ Hybridization Kit and Ribo™ STAT3 FISH Probe Mix (Ribo, Guangzhou, China) according to the manufacturer's instructions. DAPI was used to stain cell nucleus. The sections were examined under a BZ8000 fluorescence microscope (Keyence, Osaka, Japan).

Gene score enrichment analysis (GSEA)

GSEA was carried out with the java program (<http://www.broadinstitute.org/gsea>). The function of the STAT3-regulated genes was then analyzed using the MSigDB. GSEA results were sorted by enrichment fraction (enrichment score, ES) for all genes. The significance threshold was set as false discovery rate (FDR) < 0.25.

Statistical analysis

Statistical analysis was performed with the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). The data are presented as the mean \pm SEM (Standard error of mean). Student's *t*-test was deployed to evaluate the differences between the two groups. A comparison between multiple groups was carried out using One-way ANOVA followed by a Post-Hoc Test (Least Significant Difference). *P* < 0.05 was considered statistically significant.

Results

Effects of 5-AZA-DC intervention on normal pregnant and PE mice

Compared to Con mice, systolic blood pressure, urine protein, APTT, PT, D-D, ALT, BUN, and Scr were significantly higher in PE mice from day 1 to day 18 without 5-AZA-DC intervention (*P* < 0.05) (**Figure 1A-H**). After 5-AZA-DC intervention, the PE group showed decreased systolic blood pressure from day 10 to day 18 compared with PE without 5-AZA-DC intervention (*P* < 0.05) (**Figure 1A-H**). High-resolution ultrasound was used to detect changes in blood flow velocity and dynamics in the umbilical artery and uterine spiral artery of pregnant mice on day 18. PE mice showed a significant decrease in flow rate in the umbilical artery and uterine spiral artery compared with Con mice (*P* < 0.05) (**Figure 1I**). After 5-AZA-DC intervention, the blood flow velocity of the umbilical artery and uterine spiral artery of the PE group was evidently elevated compared to PE mice without 5-AZA-DC intervention (*P* < 0.05) (**Figure 1I**). Compared to Con mice, the placenta weight, diameter, crown-rump length and fetal weight were all significantly reduced in PE group, while 5-AZA-DC intervention relieved these changes induced by PE (*P* <

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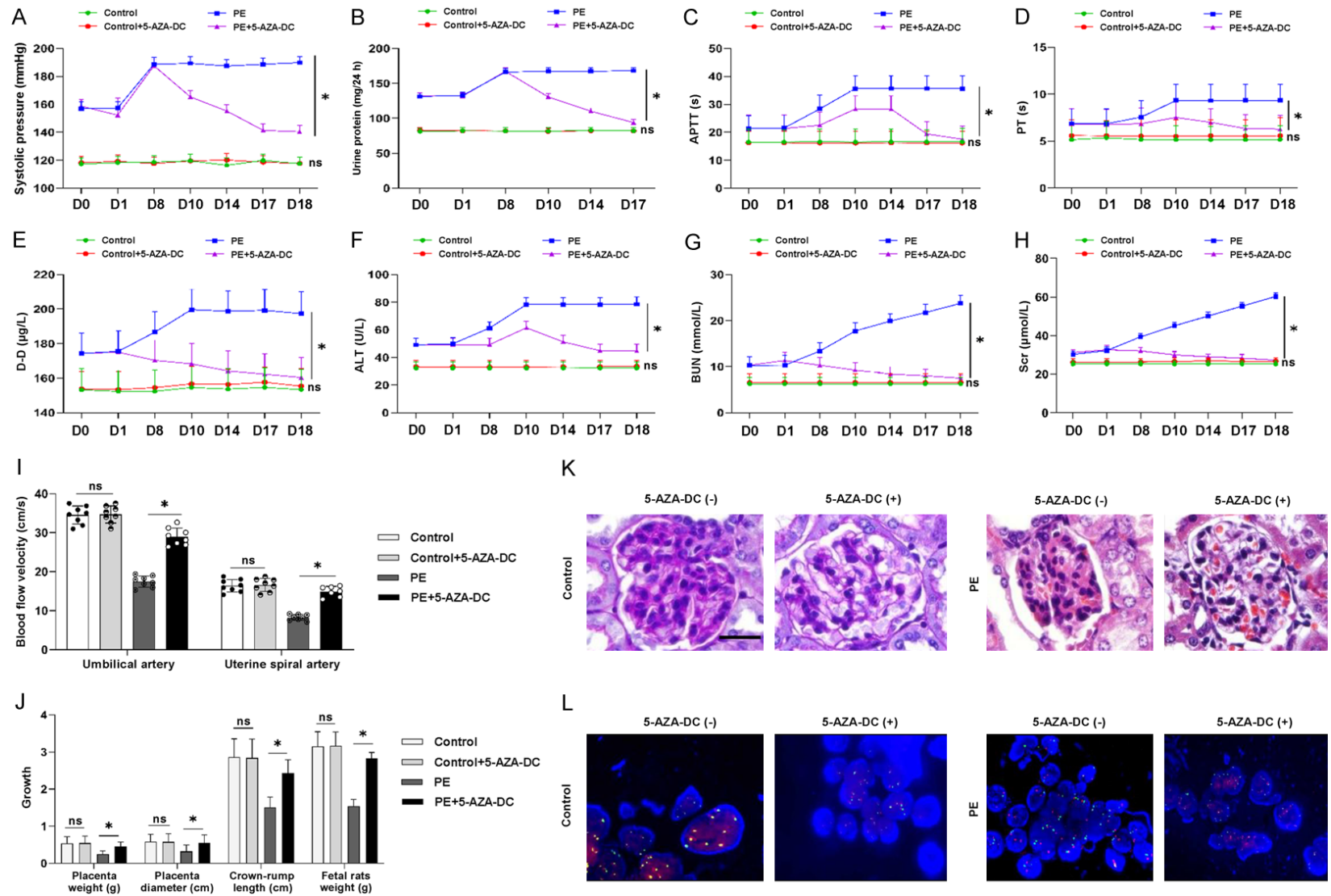


Figure 1. Effects of 5-AZA-2'-DC intervention on normal pregnant and preeclampsia mice at different stages of pregnancies. (A) Systolic blood pressure, (B) 24 h-urinary protein, (C) APTT, (D) PT, (E) D-D, (F) ALT, (G) BUN, and (H) Scr were examined in normal pregnant mice or mice with preeclampsia 0-18 days after 5-AZA-2'-DC administration. Effects of 5-AZA-DC intervention on (I) blood flow velocity of umbilical artery and uterine spiral artery, and (J) the growth of placenta and fetus were examined 18 days after 5-AZA-2'-DC administration. (K) Hematoxylin-eosin staining of kidney tissues ($\times 200$) and (L) fluorescence in situ hybridization of STAT3

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in placental tissue ($\times 100$) were examined 18 days after 5-AZA-DC treatment. Data represent mean \pm SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test ($*P < 0.05$). n = 5-8. 5-AZA-2'-deoxycytidine, 5-AZA-DC; activated partial thrombin time, APTT; prothrombin time, PT; D-dimer, D-D; alanine aminotransferase, ALT; blood urea nitrogen, BUN; serum creatinine, Scr; no significant difference, ns.

0.05) (**Figure 1J**). HE staining of renal tissues showed that the kidneys of Con mice exhibited complete glomerular structures and clear boundaries. However, PE mice had a disordered glomerular structure, increased inflammatory cell infiltration, an unclear boundary with surrounding tissue, swelling of endothelial cells, and mild cloud degeneration of renal tubules. A significant improvement was observed in the kidneys of PE mice after 5-AZA-DC intervention (**Figure 1K**). FISH results suggested that 5-AZA-DC intervention in Con mice did not affect the amplification of STAT3 genes, but STAT3 amplification was promoted in PE mice after 5-AZA-DC intervention (**Figure 1L**).

5-AZA-DC changed the expression and methylation of STAT3, PTEN and TSC2 in placental tissues

Preeclampsia caused the up-regulation of DNMT1 expression while decreased the expression of STAT3, PTEN and TSC2 at protein and mRNA levels compared with control mice ($P < 0.05$) (**Figure 2A, 2B**). It was noticed that 5-AZA-DC showed no effects on DNMT1, STAT3, PTEN, and TSC2 expression compared with control mice ($P > 0.005$). However, 5-AZA-DC significantly decreased the expression of DNMT1, and increased the expression of STAT3, PTEN and TSC2 in mice with preeclampsia compared with the non-treated mice ($P < 0.05$) (**Figure 2A, 2B**). Through Gene Set Enrichment Analysis (GSEA), we obtained 43 significant enrichment terms with false discovery rates (FDR) < 0.25 ; particularly, the obtained results showed that STAT3 may be associated with PI3K/AKT/mTOR signaling pathway in preeclampsia, and 5-AZA-DC may modulate the expression of DNMT1, STAT3, PTEN and TSC2 in dependence on PI3K/AKT/mTOR pathway (**Figure 2C**). Results from MeDIP-Seq showed that the CpG islands of STAT3, PTEN and TSC2 genes were located on the promoter region, implying that the methylation of CpG islands may be included in the down-regulation of STAT3, PTEN and TSC2 induced by preeclampsia, and the restoration of STAT3, PTEN and TSC2 expression elicited by 5-AZA-DC (**Figure 2D**). Continually, we examined the methylation

of STAT3, PTEN and TSC2. As shown in **Figure 2E**, STAT3, PTEN and TSC2 methylation was significantly upregulated in mice with preeclampsia after 5-AZA-DC intervention relative to untreated mice.

Abnormal expression of STAT3 changed protein expression

Western blot results showed that STAT3 was significantly up-regulated in HTR8/Svneo, JEG-3, JAR and BeWo cells transfected with the recombinant plasmid pcDNA3.1-STAT3 (**Figure 3A**) and down-regulated in the cells transfected with shSTAT3 (**Figure 3B**), which confirmed the successful construction of STAT3-overexpressed or STAT3-silenced cells. HTR8/Svneo, JEG-3, JAR and BeWo cells were routinely cultured for 48 h after transfection. HPLC-MS was used to detect protein expression in the transfected cells. As shown in **Figure 3**, a total of 42 proteins were differentially expressed in shSTAT3 or STAT3^{OE} cells compared with Con cells (**Figure 3C**). Thereinto, DNMT1 was elevated by STAT3 overexpression or down-regulation, and PTEN and TSC2 were positively modulated by STAT3 (**Figure 3C**).

Effects of 5-AZA-DC on apoptosis, proliferation and protein expression in STAT3 aberrantly expressing cells

The proliferative ability of shSTAT3 and STAT3^{OE} groups was significantly inhibited ($P < 0.05$) (**Figure 4A-D**), and meanwhile STAT3 down-regulation and overexpression contributed to apoptosis of HTR8/Svneo, JEG-3, JAR, and BeWo cells ($P < 0.05$) (**Figure 4E-H**). After 5-AZA-DC intervention, the proliferative activity of the cells transfected with shSTAT3 and STAT3^{OE} was significantly enhanced, and apoptosis was retarded ($P < 0.05$) (**Figure 4E-H**). Thereafter, we evaluated whether 5-AZA-DC affects protein expression in STAT3 aberrantly expressing cells. As presented in **Figure 5A**, 5-AZA-DC intervention relieved the abnormal expression of 42 proteins initiated by STAT3 overexpression or down-regulation. Next, Western blot was used to detect the changes in DNMT1, STAT3, PTEN, and TSC2 expression in HTR8/Svneo cells. The results showed that DNMT1 mRNA and protein in shSTAT3 and

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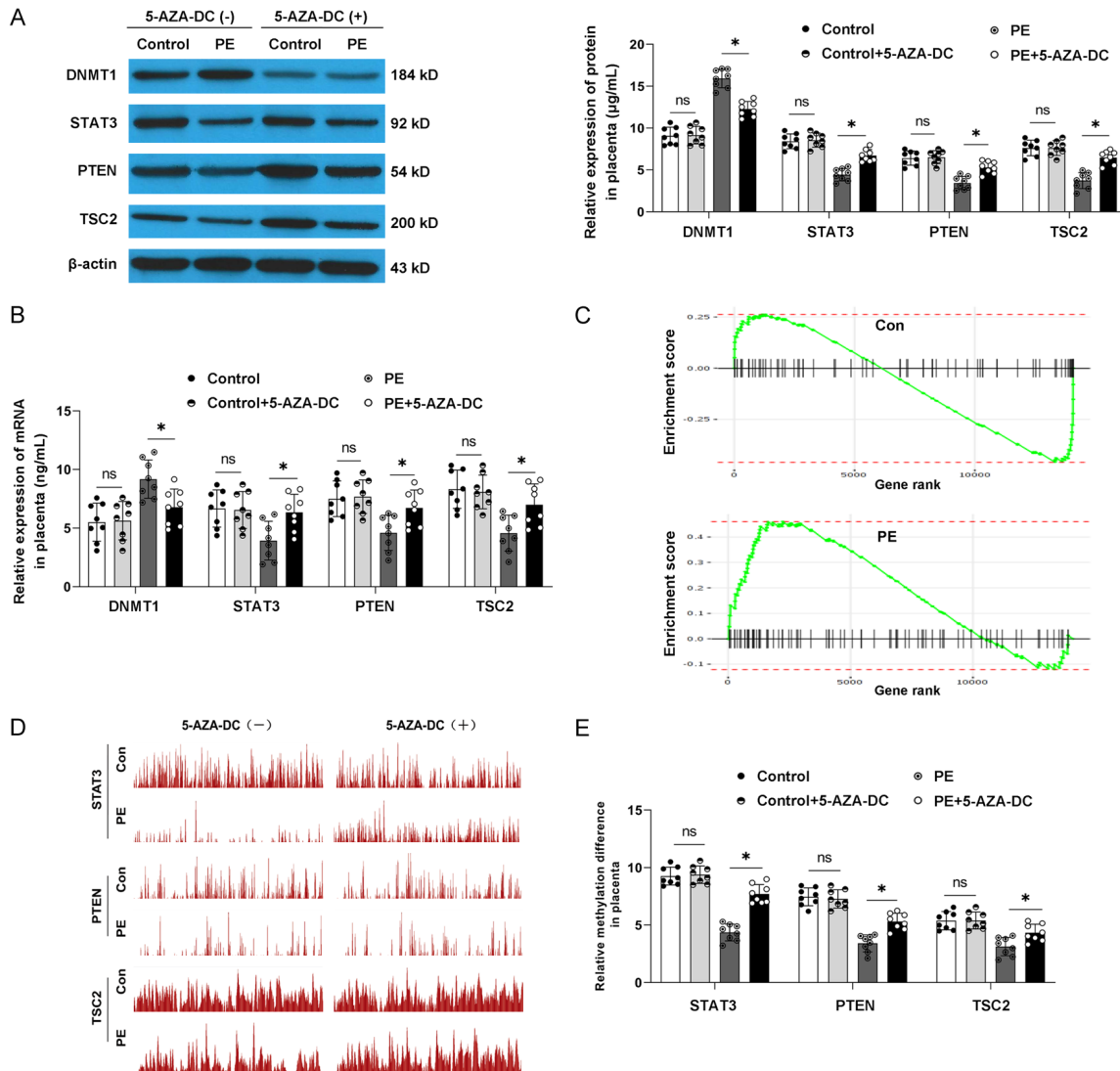


Figure 2. Effects of 5-AZA-DC intervention on the expression and methylation of DNMT1, STAT3, PTEN, and TSC2 in placental tissues of preeclamptic mice. **A.** Western blots of DNMT1, STAT3, PTEN and TSC2 in placental tissues from normal and preeclamptic mice. **B.** qRT-PCR assay of DNMT1, STAT3, PTEN and TSC2 mRNA in placental tissue from normal and preeclamptic mice. **C.** GSEA showed 43 significant enrichment terms with FDR < 0.25. **D, E.** MedIP-Seq of STAT3, PTEN and TSC2 promoter methylation in placental tissue from normal and preeclamptic mice. Placental tissue from normal and preeclamptic mice were collected 18 days after 5-AZA-DC treatment. Data represent mean \pm SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's *t*-test ($*P < 0.05$). $n = 5-8$. 5-AZA-2'-deoxycytidine, 5-AZA-DC; no significant difference, ns; DNA methyltransferase 1, DNMT1; signal transducer and activator of transcription 3, STAT3; phosphatase and tensin homolog, PTEN; TSC complex subunit 2, TSC2; gene set enrichment analysis, GSEA; Methylated DNA immunoprecipitation, MedIP.

STAT3^{OE} cells were significantly higher than that of Con group ($P > 0.05$). However, DNMT1 mRNA and protein in shSTAT3 group and STAT3^{OE} group were down-regulated significantly after 5-AZA-DC intervention ($P > 0.05$), and there was no significant difference from the Con group ($P > 0.05$) (Figure 5B, 5C). STAT3, PTEN, and TSC2 at mRNA and protein levels were significantly upregulated after 5-AZA-DC

intervention in shSTAT3 and STAT3^{OE} cells (Figure 5B, 5C). To confirm that 5-AZA-DC mediate PTEN expression by regulating STAT3, we transduced pcSTAT3 into shSTAT3-transfected cells, and PTEN was silenced to prove the effect of 5-AZA-DC. The obtained results from Western blot and qRT-PCR proved that PTEN knockdown can save the effects of STAT3 on PTEN expression (Figure 5D, 5E).

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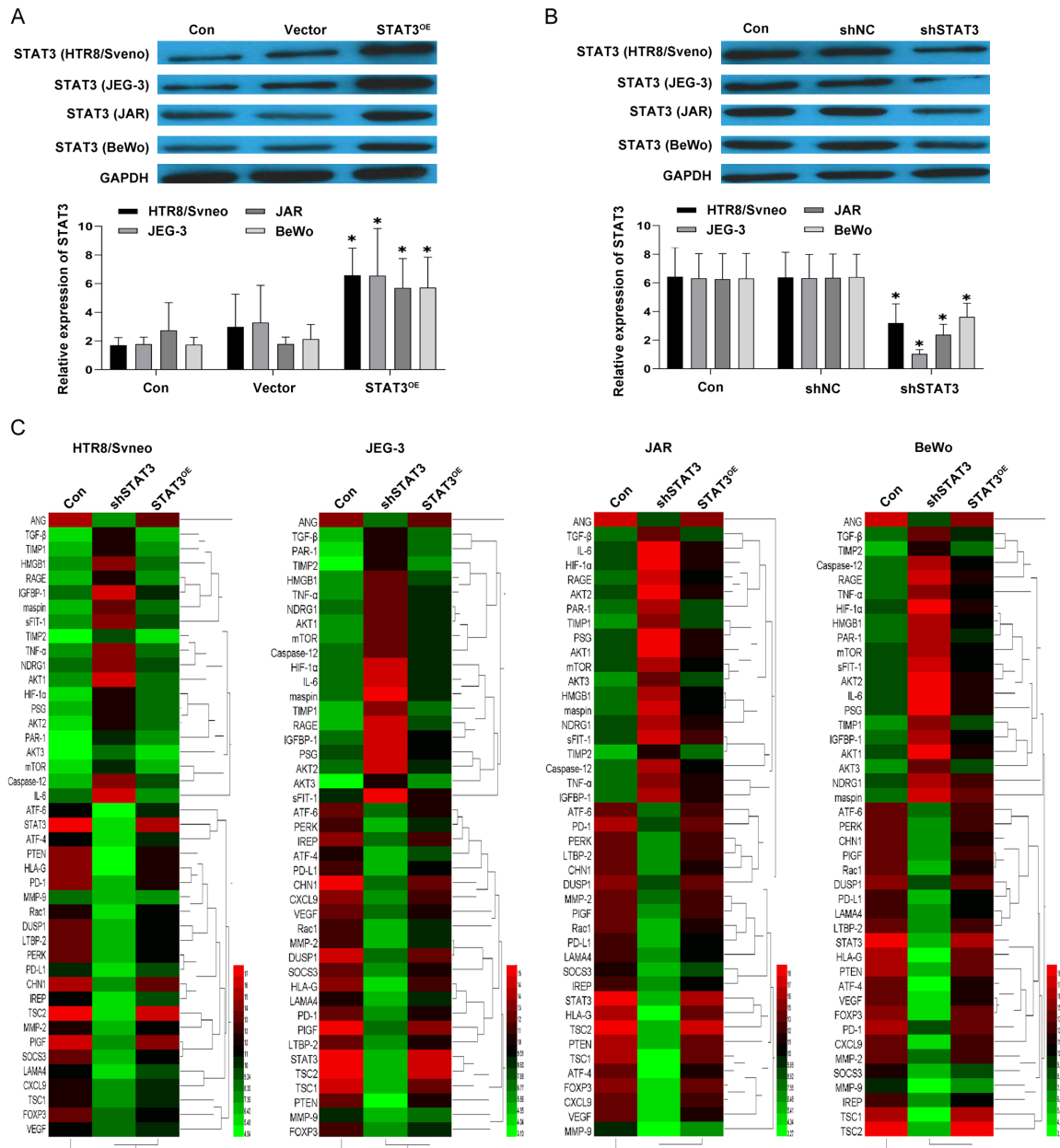


Figure 3. STAT3 knockdown or overexpression changed protein expression in HTR8/Svneo, JEG-3, JAR and BeWo cells. Western blot assay of STAT3 expression in HTR8/Svneo, JEG-3, JAR and BeWo cells transfected with (A) the recombinant plasmid pcDNA3.1-STAT3 (STAT3^{OE}) and (B) short-hairpin RNA targeting STAT3 (shSTAT3). (C) Protein expression in HTR8/Svneo, JEG-3, JAR and BeWo cells transfected with pcDNA3.1-STAT3 or shSTAT3, assayed by HPLC-MS. The cells were collected 48 h after transfection, followed by administration with 5-AZA-DC. Data represent mean \pm SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's *t*-test (**P* < 0.05). n = 5-8. signal transducer and activator of transcription 3, STAT3; no significant difference, ns. 5-AZA-2'-deoxycytidine, 5-AZA-DC.

STAT3 bound to PTEN and TSC2 and retarded the transcription process in HTR8/Svneo cells

GST pull-down assay proved that STAT3 interacted with PTEN and TSC2 protein *in vitro* (Figure 6A). What's more, STAT3 can signifi-

cantly enrich PTEN gene in HTR8/Svneo, JEG-3, JAR and BeWo cells compared with normal rabbit IgG (Figure 6B). This result suggested that transcription factors STAT3 directly bound to PTEN gene. To verify whether STAT3 affects PTEN expression at the transcriptional level, we

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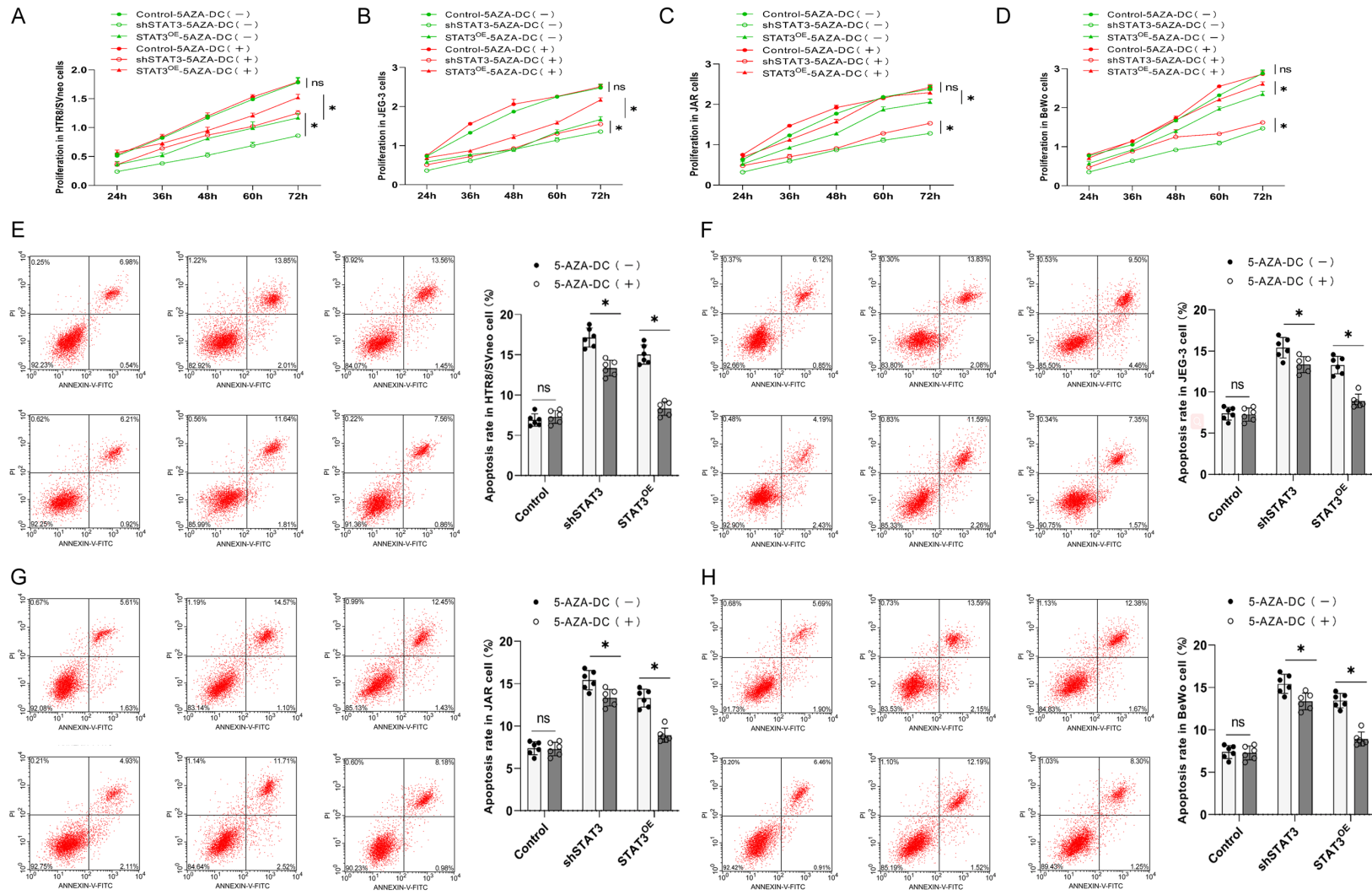


Figure 4. Effects of 5-AZA-DC intervention on proliferation and apoptosis of HTR8/Svneo, JEG-3, JAR, and BeWo cell lines. Proliferative activities of (A) HTR8/Svneo, (B) JEG-3, (C) JAR, and (D) BeWo cells assayed using CCK-8. Apoptosis rate of (E) HTR8/Svneo, (F) JEG-3, (G) JAR, and (H) BeWo cells were examined by a flow cytometry. The cells were collected 48 h after transfection, followed by administration with 5-AZA-DC. Data represent mean \pm SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's *t*-test ($*P < 0.05$). *n* = 5-8. signal transducer and activator of transcription 3, STAT3; 5-AZA-2'-deoxycytidine, 5-AZA-DC; no significant difference, ns.

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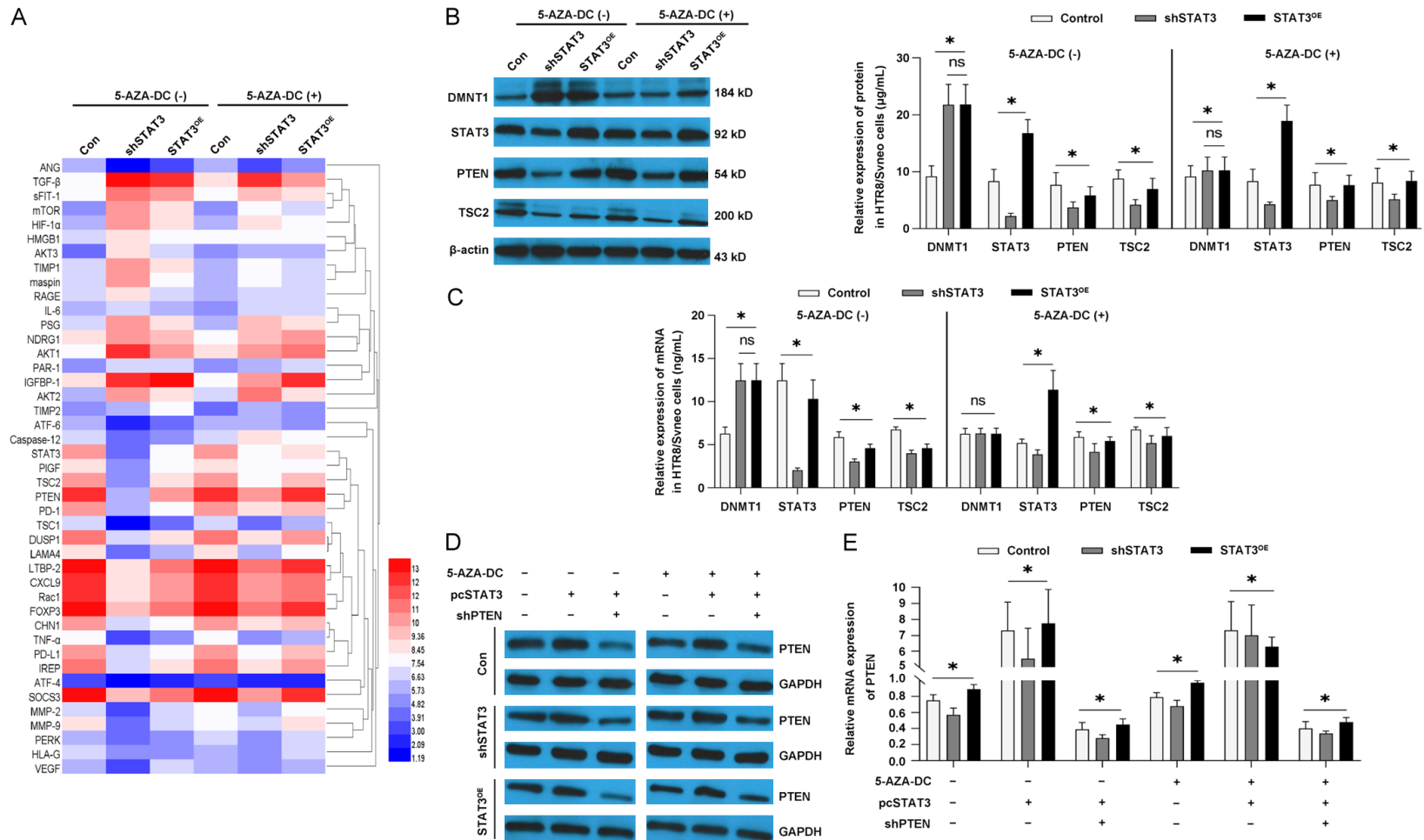
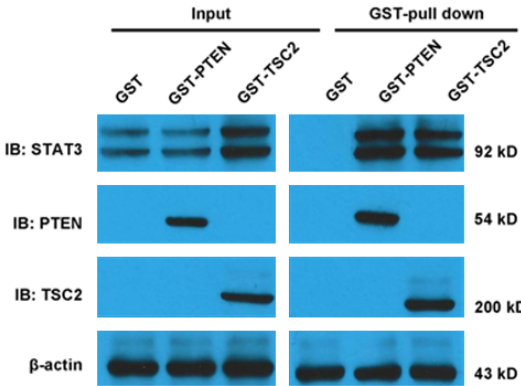


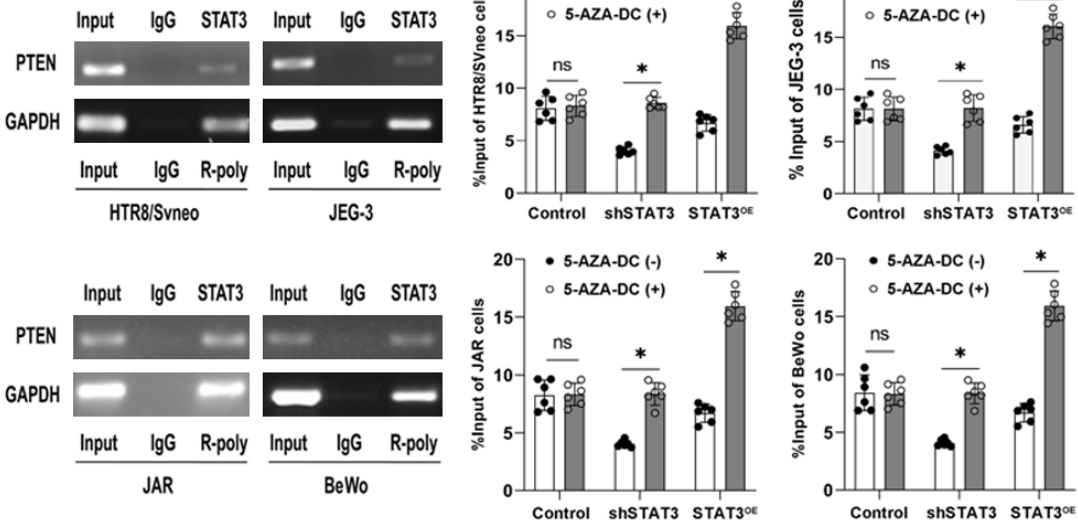
Figure 5. 5-AZA modulated gene expression in dependence on STAT3 in HTR8/Svneo cells. (A) Protein expression in HTR8/Svneo cells transfected with pcDNA3.1-STAT3 or shSTAT3, assayed by HPLC-MS. (B) Western blot assay and (C) qRT-PCR assay for DNMT1, STAT3, PTEN, and TSC2 in HTR8/Svneo cells transfected with pcDNA3.1-STAT3 or shSTAT3, assayed by HPLC-MS. shSTAT3- or pcDNA3.1-STAT3-transfected cells were sequentially transfected with pcDNA3.1-STAT3 or shPTEN, followed by 5-AZA-DC administration, and the cells were collected for (D) Western blot and (E) qRT-PCR assay. Data represent mean \pm SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's *t*-test ($*P < 0.05$). $n = 5-8$. 5-AZA-2'-deoxycytidine, 5-AZA-DC; no significant difference, ns; DNA methyltransferase 1, DNMT1; signal transducer and activator of transcription 3, STAT3; phosphatase and tensin homolog, PTEN; TSC complex subunit 2, TSC2.

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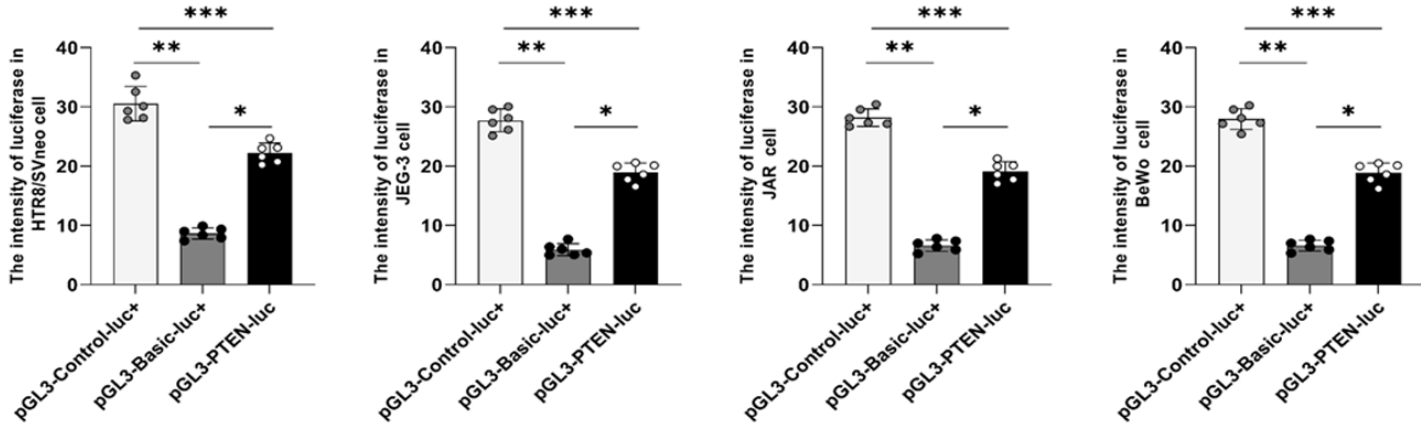
A



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STAT3-mediated effect on preeclampsia

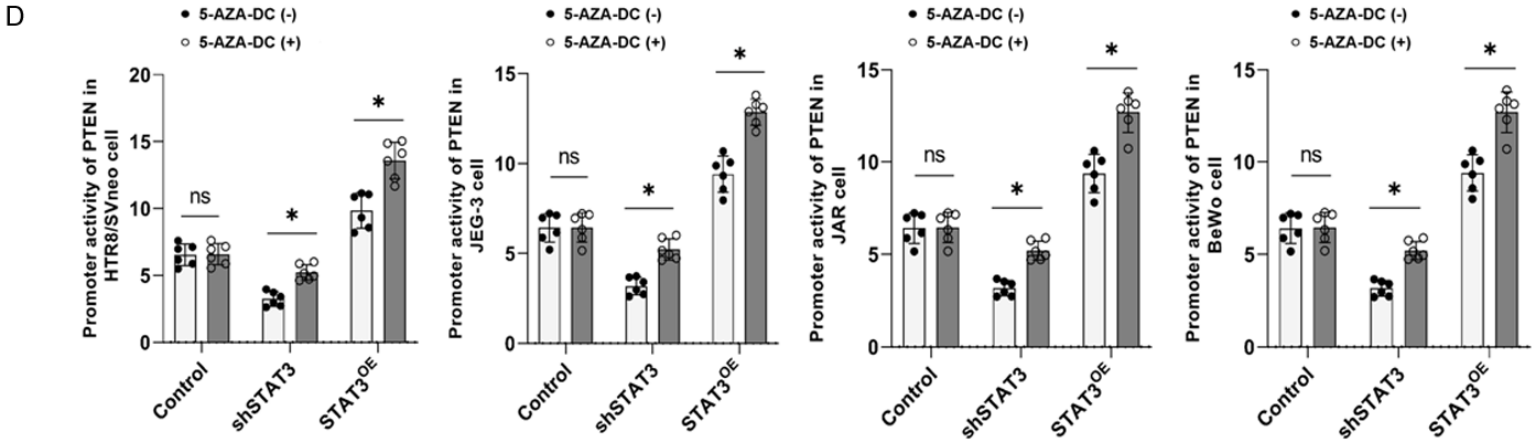


Figure 6. STAT3 bound to PTEN and TSC2 and retarded the transcription process in HTR8/Svneo cells. A. GST-pull down verified that STAT3 interacted with PTEN and TSC2 protein in in vitro. B. ChIP assay was used to verify STAT3 binds to PTEN in HTR8/Svneo, JEG-3, JAR, and BeWo cells. C. Fluorescence intensity of luciferase plasmids carrying PTEN sequence was assayed in HTR8/Svneo, JEG-3, JAR, and BeWo cells transfected with pcDNA3.1-STAT3. D. PTEN promoter activity was assayed in the cells transfected with shSTAT3 or pcDNA3.1-STAT3 and administrated with 5-AZA-DC. Data represent mean \pm SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's *t*-test ($*P < 0.05$). *n* = 5-8. 5-AZA-2'-deoxycytidine, 5-AZA-DC; no significant difference, ns; phosphatase and tensin homolog, PTEN; TSC complex subunit 2, TSC2.

predicted the promoter sequence of PTEN using a bioinformatics tool (Prediction Software: promoter 2.0 prediction server and promoter scan). We successfully constructed the PTEN promoter sequence into the pGL3 plasmid (pGL3-PTEN-luc), and a double luciferase reporting system was used to determine the expression of plasmid luciferase. The PTEN promoter plasmid pGL3-PTEN-luc, positive control plasmid pGL3-Control-luc⁺, and negative control plasmid pGL3-Basic-luc⁺, were successfully transfected into HTR8/Svneo, JEG-3, JAR and BeWo cells ($P < 0.001$) (Figure 6C). The promoter activity of PTEN in shSTAT3 group was lower than that in Con group, while the promoter activity of PTEN in STAT3^{OE} group was higher than that in Con group ($P < 0.05$) (Figure 6D). Most noteworthy, 5-AZA-DC intervention elevated the promoter activity of PTEN both in shSTAT3 group and STAT3^{OE} group ($P < 0.05$) (Figure 6D).

Discussion

Preeclampsia is a unique disorder of pregnancy that causes damage to maternal organs [24, 25]. Although preeclampsia is one of the main causes of high perinatal mortality, the pathogenesis of the disease is still unclear [26, 27]. Recently, a growing number of researchers have concluded that preeclampsia is fundamentally a maternal hyperinflammatory response to pregnancy [28, 29].

Our study shows that the occurrence of functional defects in the placenta may be due to epigenetic abnormalities in early gamete stages, and we also believe that epigenetic regulatory abnormalities increase susceptibility before or during the pregnancy [30, 31]. Our results confirmed that DNMT1 expression in preeclampsia placental tissue was significantly higher than that in normal-term placental tissue. However, STAT3 expression was significantly lower than that in normal-term placental tissues. DNMT1 expression was significantly down-regulated by treatment with a methylation inhibitor while STAT3 expression was significantly up-regulated, indicating that the expression of STAT3 was regulated by DNA methylation [32, 33].

Signal transduction and transcriptional activation factor 3 (STAT3) is an approximately 92 kD protein that plays roles in cell proliferation,

apoptosis, migration, and differentiation [34-36]. Our previous studies have shown that tyrosine phosphokinase activates STAT3 [37-39]. PTEN deletion from chromosome 10 is a tumor suppressor gene with lipid phosphatase and protein phosphatase dual phosphatase activity [40, 41]. The deletion of PTEN leads to intracellular PIP3 accumulation, PTEN-PI3K/AKT imbalance of signal transduction pathways, continuous activation of AKT, and further activation of mammalian rapamycin target protein (mTOR) [42]. TSC2 protein is considered to be one of the causes of preeclampsia [43], which is further consolidated in our study.

The results of *in vitro* trophoblastic cell line experiments confirmed that STAT3 genes are involved in the regulation of biological functions such as trophoblast proliferation and apoptosis, suggesting that STAT3 may be involved in the pathogenesis of preeclampsia. Further, the abnormal expression of STAT3 may be related to the biological dysfunction of trophoblasts. Our study also demonstrated that downregulation of TSC2 expression was induced by the decrease of STAT3, as well as the expression of PTEN. We continually proved that STAT3 binds to the specific sites of PTEN promoter and then regulates PTEN expression at the transcriptional level [44]. The increase of CpG island methylation in the target gene PTEN and the TSC2 promoter region will affect transcription of upstream transcription factor STAT3, then inhibit the expression and function of STAT3 gene, while demethylation induces the expression and reactivation of STAT3 gene [45].

In *in vivo* animal experiments, blood pressure, 24-hour urine protein, and other specific indexes of preeclampsia pregnant mice were significantly improved after 5-AZA-DC intervention, suggesting that methyltransferase inhibitors may play a role in improving the clinical symptoms of preeclampsia. In addition, we also observed significant differences in the number and weight of fetal mice, indicating that methyltransferase inhibitors may also affect the development and maturation of fetal mice. Intra-peritoneal injection of the methyltransferase inhibitor 5-AZA-DC can also increase the expression of STAT3 gene mRNA and proteins in placental tissue. Our study suggests that, at the early maternal-fetal interface, STAT3 may be involved as an important negative regulator of trophoblast proliferation and apoptosis,

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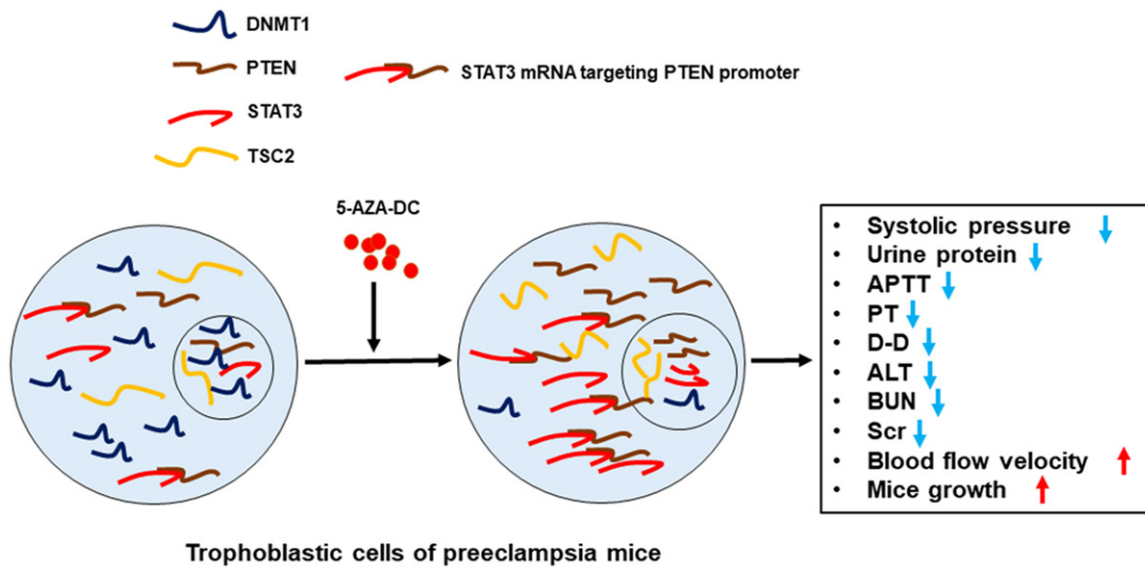


Figure 7. Trophoblastic cells of preeclampsia mice. The systolic blood pressure, urine protein and APTT, PT, DD, ALT, BUN, Scr of the preeclampsia mice model were decreased after 5-AZA-DC intervention. The blood flow velocity and the growth degree of the fetal mice were improved obviously.

which may be achieved by directly or indirectly regulating the activity of PTEN and TSC2. By using normal cell lines and a control group as reference, the significance of up-regulation and down-regulation of STAT3 gene expression was clarified. We also found that demethylation drugs can increase the expression of STAT3 mRNA in trophoblasts, improve proliferation, and significantly inhibit apoptosis by changing the methylation state of STAT3.

Conclusions

Here, we confirmed the important role of the methyltransferase inhibitor 5-AZA-DC in mitigating preeclampsia symptoms. We concluded that STAT3-mediated epigenetic regulation may play a key role in the molecular mechanism of preeclampsia (**Figure 7**). However, it is still not completely understood whether 5-AZA-DC-induced demethylation of STAT3 affects the association between STAT3 and PTEN or TSC2. Further research is needed to elucidate how STAT3 methylation affects preeclampsia symptoms.

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ence and Technology Project of Guang-dong Province (No. 20191256).

Disclosure of conflict of interest

None.

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