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Effect of STAT3 mediated epigenetic regulation in pre-eclampsia: an analysis of trial data

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Abstract. – OBJECTIVE: To study the role of the PI3K-AKT/mTor signalling pathway, mediated by the STAT3 gene, in the epigenetic pathogenesis of pre-eclampsia.

MATERIALS AND METHODS: HTR8/Svneo, JEG-3, JAR, and Bewo trophoblast cell lines were divided into a PE group and a control group. STAT3 gene overexpression and interference silencing, immunofluorescence, Real Time-PCR and Western blot, GST-pull down, CHIP, Dual-Luciferase reporter gene test, Rescue experiment, LC-MS/MS analysis. Methods and materials also comprised CCK-8, transwell invasion, MSP and BSP, flow cytometry and wound-healing assay.

RESULTS: Compared with the control group, the expression of DNMT1 mRNA and protein was increased, while the expression of STAT3, PTEN, TSC2 mRNA and protein was decreased. There was improvement after 5-Aza-2'-deoxycytidine (5-AZA-DC) intervention.GST-pull down confirms that STAT3 interacts with PTEN and TSC2. CHIP verifies that STAT3 binds directly to PTEN, and double luciferase reporter experiments verify that STAT3 regulates PTEN promoter activity. STAT3 expression of PTEN is, meanwhile, saved by knock down of PTEN in HTR8/SVneo cells. LC-MS/MS analysis showed upregulation of STAT3, PTEN, TSC2 and TSC2. MSP and BSP analysis screened out the significant difference genes of STAT3, PTEN and TSC2. Following 5-AZA-DC intervention, apoptosis downregulation, invasion upregulation and cell-proliferation upregulation were evinced.

CONCLUSIONS: Trophoblast STAT3 was an important gene mediating the pathogenesis of pre-eclampsia induced by the PI3K-AKT/mTor signal axis. In the context of epigenetic therapy for pre-eclampsia, STAT3 may be deployed as a potential target.

Key Words:
STAT3, PI3K-AKT/mTor signal axis, Pre-eclampsia, Epigenetic pathogenesis.

Introduction

Pre-eclampsia is a serious hypertensive disorder of pregnancy that increases the morbidity and mortality of pregnant women and parturients. Some ambiguity persists regarding the specific pathogenesis and etiology[1,2]. The morbidity of pre-eclampsia in puerpera worldwide was 35%, and in developing countries, maternal mortality caused by pre-eclampsia accounts for 25% of the total maternal mortality rate[3,4].

Pre-eclampsia causes severe adverse pregnancy outcomes for both mothers and children. It is thus imperative that the disorder is diagnosed and addressed as soon as possible[5]. Placental dysfunction is considered to be one of the causes of the disease of pre-eclampsia[6]. Both albuminuria and high maternal blood pressure are typical of the condition[7]. Placental DNA methylation can occur in pregnancy complications[8,9], including pre-eclampsia[10], gestational diabetes mellitus[11] and intrauterine growth restriction[12]. Although the relationship between DNA methylation and gene expression at the transcriptional level is complex, there is strong evidence that changed DNA methylation, especially in the regulatory region of expressing genes, has a significant effect on transcription[13]. In our previous research, we found a plausible connection between pre-eclampsia and DNA-methylation change within the key regulatory region[14,15]. Thus far, our understanding of epigenetic changes in pre-eclampsia is relatively limited, and research should be carried out to elucidate the progress of DNA methylation and pre-eclampsia.

In the context of pre-eclampsia, there is a continuing lack of clarity regarding DNA hypomethylation, both in terms of its clinical significance and its mechanism[16,17]. In this study, we discovered that the expression and functional regulation of STAT3 and PTEN genes in placen-

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tal trophoblasts of pre-eclampsia may be related to the methylated status of cytosine-guanine dinucleotide (CpG) islands in the promoter region of pre-eclampsia. The clinical manifestations of pre-eclampsia are varied, since it is itself a heterogeneous condition[18,19]. Indeed, temporary alterations in gene expression may stem from many causes[20]. In contrast, DNA methylation is more stable. Our study has, moreover, addressed the function of in-depth epigenetic regulation molecule analysis. The latter cannot only elucidate the mechanism of action in pre-eclampsia, but also provides a potential therapeutic object for targeted pre-eclampsia therapy.

Materials and Methods

Antibodies and Reagents

Anti-DNMT1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-10222), anti-STAT3 (1:1000, Cell Signalling Technology, Danvers, MA, USA, Cat#9145), STAT3 (1:1000, Cell Signalling Technology, Danvers, MA, USA,Cat#12640), Anti-PTEN (1:1000, Cell Signalling Technology, Danvers, MA, USA, Cat#9559), Anti-TSC2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-44174), Anti-PI3K (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-61340), pAKT (1:1000, Cell Signalling Technology, Danvers, MA, USA, Cat#9271), AKT (1:1000, Cell Signalling Technology, Danvers, MA, USA, Cat#9272), mTor (1:1000, Cell Signalling Technology, Danvers, MA, USA, Cat#2983), LY294002 and A-443654 (Cell Signalling, Danvers, MA, USA, Cat#9901,Cat#13055), rapamycin, 5–AZA-DC and L-NAME (Sigma, Cat#553210, Cat#189825, Cat#N5751), anti-Actin (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-47778), anti-GAPDH (1:5000,Cell Signalling Technology, Danvers, MA, USA, Cat#2118), GAPDH (1:2000, Abcam, Cambridge, MA, USA, Cat#ab8245) Alexa Fluor 488 goat anti-mouse IgG (1:5000, Abcam, Cambridge, MA, USA, Cat#ab150117). Foetal bovine serum (FBS; GIBCO, Rockville, MD, USA). The Luciferase assay system (Promega, Madison, WI, USA). Annexin V/7AAD apoptosis detection kit Annexin V (BD, Franklin Lakes, NJ, USA), Biosciences. Anti-GST (Sangon, Shanghai, China, D199985).

Cell Lines and Cell Culture

The Cell Resource Centre of the Shanghai Academy of Life Sciences was the commercial supplier of the HTR8/SVneo cells required for the research. The human trophoblast cell lines JEG-3, JAR, BeWo were purchased from an American type-culture collection (ATCC; Manassas, VA, USA). The culture medium of the HTR8/SVneo and JAR cells was Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA), and the culture medium of the JEG-3 and BeWo cells was Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12; Gibco, Rockville, MD, USA). 10% FBS and double antibody (100 U/mL penicillin and 100 μg/mL streptomycin) were added to the cell-culture medium. An incubator was used for all cell culture, at 5% CO_2 and at a temperature of 37°C.

Construction of Pre-Eclampsia Cell Model by CRISPR/cas9 and Hypoxia

We used CRISPR/Cas9 to select suitable targets for the placental growth factor (PIGF) and VEGF genes, and to transfer the tool plasmid (Santa Cruz Biotechnology, Santa Cruz, CA, USA) into cell lines. Gene knockout was accomplished via the expressed enzyme digestion effect. The cell lines were cultured in a mixed gas of 1% O_2 (1% O_2, 5% CO_2 and 94% N_2). After changing the fluid, the intake pipe was connected to the low-oxygen-mixture gas of 1% O_2, and the low-oxygen mixture of 1% O_2 was affused for 15 min.

PI3K/AKT/mTor Inhibitor Treatment

The cells were incubated with the PI3K inhibitor LY294002 (30 μmol/L), the AKT inhibitor A-443654 (600 nmol/L), and the mTor inhibitor RAPA (90 ng/mL) for 24 h and for Western blot verification results.

Overexpression and Silencing of STAT3

Pre-eclampsia cell model cell lines were cultured with a serum-free medium of 500 μL per well. The target lentivirus and the no-loaded lentivirus 20 μL were added to each well for 24-hour incubation. Western blot duly confirmed the STAT3-protein expression.

Cell Lines Divided into Groups and 5-AZA-DC Treatment

The normal cultured HTR8/SVneo, JEG-3 and JAR, BeWo cell lines were a blank control group (Con group). The pre-eclampsia cell model cell lines were divided into the shSTAT3 and STAT3^{OE} groups. We added a 5 μm 5-Aza-2'-deoxycytidine (5-AZA-DC) fresh medium, and the cells

were collected 72 hours later. The Con group was treated with a culture solution of 0.01% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA).

Immunofluorescence

4% paraformaldehyde was fixed for 30 min, and was incubated at 1% bovine serum albumin (BSA) for 10 min at room temperature. After being added to the slide, the rabbit anti-mouse STAT3 (1:100) was incubated throughout the night at 4°C. We added the 4', 6-diamidino-2-phenylindole (DAPI) for 20 min and dyed the nucleus. A laser confocal microscope (Olympus, Tokyo, Japan) was employed to undertake the fluorescence quantitative analysis.

Real Time-PCR Analysis

The cells were placed in a 1.5 mL Eppendorf (EP; Hamburg, Germany) tube and then the TRIzol solution was added (Invitrogen, Carlsbad, CA, USA) for1.0 mL. After the homogenate, chloroform to the quantity of 0.2 mL was added. At 4°C, we carried out centrifuging at 12000 rpm for 15 min, and added 0.5 mL isopropanol. We carried out oscillatory washing of the RNA precipitation once, with 7500 rpm centrifugation for 5 min, and we made the necessary calculations using the $2^{-\Delta\Delta CT}$ formula (Table I).

Western Blot Analysis

The cells were lysed with radioimmunoprecipitation assay (RIPA) lysate after transfection to extract the total protein, and the protein concentration was detected with the bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Waltham, MA, USA). After adding five xloading buffers, we mixed and boiled for 5 min. The protein concentration was adjusted for sodium dodecyl sulphate (SDS)-gel electrophoresis, and the protein was transferred to the polyvinylidene difluoride (PVDF) membrane by wet transfer (Millipore, Billerica, MA, USA). 5% BSA was closed for 1 h, then added to the configured antibody. US protein-tech companies supplied all the first antibodies: STAT3 (15400-1-ap), DNMT1 (10373-2-ap), PTEN (10375-2-ap), TSC2 (15400-1-ap), Vimentin (10366-1-ap), N-cadherin (20874-1-ap), E-cadherin (20874-1-ap), β-actin (10494-1-ap). Incubation took place overnight at 4°C. There were three rounds of PBST washing, each lasting 15 min. We then add diluted sheep anti-rabbit antibody (ab205718, Abcam, Cambridge, MA, USA) to incubate for 90 min. A PBST wash was conducted at room temperature, three times, for 15 min on each occasion. Enhanced chemiluminescence (ECL) involved a medium amount of A and B solution from the fluorescence detection kit (Thermo Fisher Scientific, Waltham, MA, USA). This was mixed in the dark chamber, dropped in "drop form" onto the film and put into the gel imager for exposure imaging. Image j grey level analysis was undertaken.

GST Pull-Down Assay

For the GST pull-down assay, His-tag-STAT3, GST- PTEN, GST- TSC2 and GST proteins were added. 20 μL glutathione agarose beads were added to the output group. Glutathione agarose beads containing GST empty protein were used as control. Western blot provided the test analysis.

Luciferase Reporter Assay

Upstream of the pGL3-Basic/Luciferase vector Luciferase, we inserted the gene that comprised the PTEN promoter sequence. Briefly, the fluorescence intensity of Luciferase was detected by the Dual-Luciferase reporting system kit (Promega, Madison, WI, USA).

Table I. Primers used for real-time PCR.

Primer set		Sequence	PCR product (bp)
DNMT1	Sense	5-AGATGTGGATCAGCAAGCA-3	119
	Antisense	5-GCGCAAGTTAGGTTTTGTCA-3	
STAT3	Sense	5-ACCCAACAGCCGCCGTAG-3	143
	Antisense	5-CAGACTGGTTGTTTCCATTCAGAT-3	
PTEN	Sense	5-CGACGGGAAGACAAGTTCAT-3	125
	Antisense	5-AGGTTTCCTCTGGTCCTGGT-3	
TSC2	Sense	5-CACTGGTGAGGACGTCTG-3	131
	Antisense	5-CCGCAGCATCAGTGTGTC-3	
β-actin	Sense	5-AGATGTGGATCAGCAAGCA-3	87
	Antisense	5-GCGCAAGTTAGGTTTTGTCA-3	

Rescue Assay

The cells were inoculated on a 24-well plate, and divided into groups: pcDNA3+pcDNA3 (0.6 μg+0.4 μg); pcDNA3+pSTAT3 (0.6 μg+0.4 μg); pSTAT3+p sh PTEN (0.6 μg+0.4 μg). A solution: take 1 μg plasmid, add serum-free medium Opti-MEM to 50 μL volume. B solution: take 1.5 μL liposomes lipofectamine2000 reagent, add serum-free medium Opti-MEM to 50 μL volume. The two solutions, A and B, were gently mixed, before Western blot was deployed for the test analysis.

LC-MS/MS Proteomics

The cells evinced SDS-PAGE electrophoresis and Coomassie brilliant blue was stained. The 100 μg peptide segment was subjected to iTRAQ isotope labelling (AB SCIEX). Mass spectrometry analysis was undertaken after chromatographic separation (Q-Exactive). The time required for the mass spectrometry analysis was 60 min, while the scanning range was 300-1800 m/z.

Methylation-Specific PCR (MSP) and Bisulphite Sequencing (BSP)

Each 20 μL DNA sample was mixed with a 130 μL conversion reagent for MSP reaction on the PCR instrument. The MSP product was identified by 2% agarose gel electrophoresis. The DNA was treated with sodium bisulphite. The determination of the CpG Island of the PTEN gene was undertaken via online Software. A 1.0% agarose gel electrophoresis was deployed to identify the specificity and size of the PCR products.

Cell Proliferation, Apoptosis, Invasion and Migration Assay

The cells were seeded on 96 well plates and cultured for 24 h. This was measured via cell-counting kit-8 (CCK-8) assays (Dojindo Molecular Technologies, Kumamoto, Japan). The testing times were 24 h, 36 h, 48 h, 60 h and 72 h. Measurements were conducted at OD 450 nm with the BioTek Gen5 system (BioTek, Biotek Winooski, VT, USA). The cells were centrifuged at 4°C and 1000 rpm for 5min. We added 10 μL Annexin V-FITC fluorescent probe and 5 μL propidium iodide (PI). The Annexin V and PI signals were measured after incubation. Data were analyzed using FlowJo software (Tree Star Inc., San Carlos, CA, USA). The mitomycin c was added to the HTR8/ SVneo cells, which were incubated at 37°C for 2 h. 200 μg/mL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was pre-treated in the transwell chamber, and added to a new well containing 0.1% crystal purple. The cells were added to a DMEM-F12 medium which contained 2% FBS. The culture was left at 37°C for a 48-h period. We duly made observations at 0 h and 24 h, and took photographs at these points.

Statistical Analysis

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

Results

Signal Transduction Pathway Inhibitor and Overexpression and Silencing of STAT3 Gene in Cell Line

In the HTR8/SVneo cells of the constructed PE cell model, the cells were incubated separately with the pi3k inhibitor LY294002 (30 μmol/L), the AKT inhibitor A-443654 (600 nmol/l) and the mTor inhibitor RAPA (90 ng/mL) for 24 hours. Results demonstrated that Ly294002 produced no significant change to STAT3. PTEN and TSC2 were significantly down regulated. A-443654 made no significant change to STAT3 and PTEN. The TSC2 was significantly down regulated. Meanwhile, the RAPA produced no significant change in TSC2 or PTEN, and the STAT3 was significantly down regulated (Figures 1A and 1B).

We consider the demethylation of 5-aza-dc to have increased the STAT3 content in the PE cell model (Figure 1C). It was overexpressing STAT3 in the HTR8/SVneo, JEG-3 and JAR,BeWo cell lines. Western blot detected that STAT3 protein bands were significantly higher than those of the empty vector transfected cells. It was found that the overexpression of the STAT3 cell line was successfully constructed (Figure 1D). The HTR8/SVneo, JEG3, JAR and BeWo cells were transfected with shRNA and routine culture for 48 h. The establishment of a silent STAT3 cell line was demonstrated via Western blot detection (Figure 1E). The relationship obtaining between the PI3K/ AKT/mTor signal pathway and STAT3 was illustrated via these data.

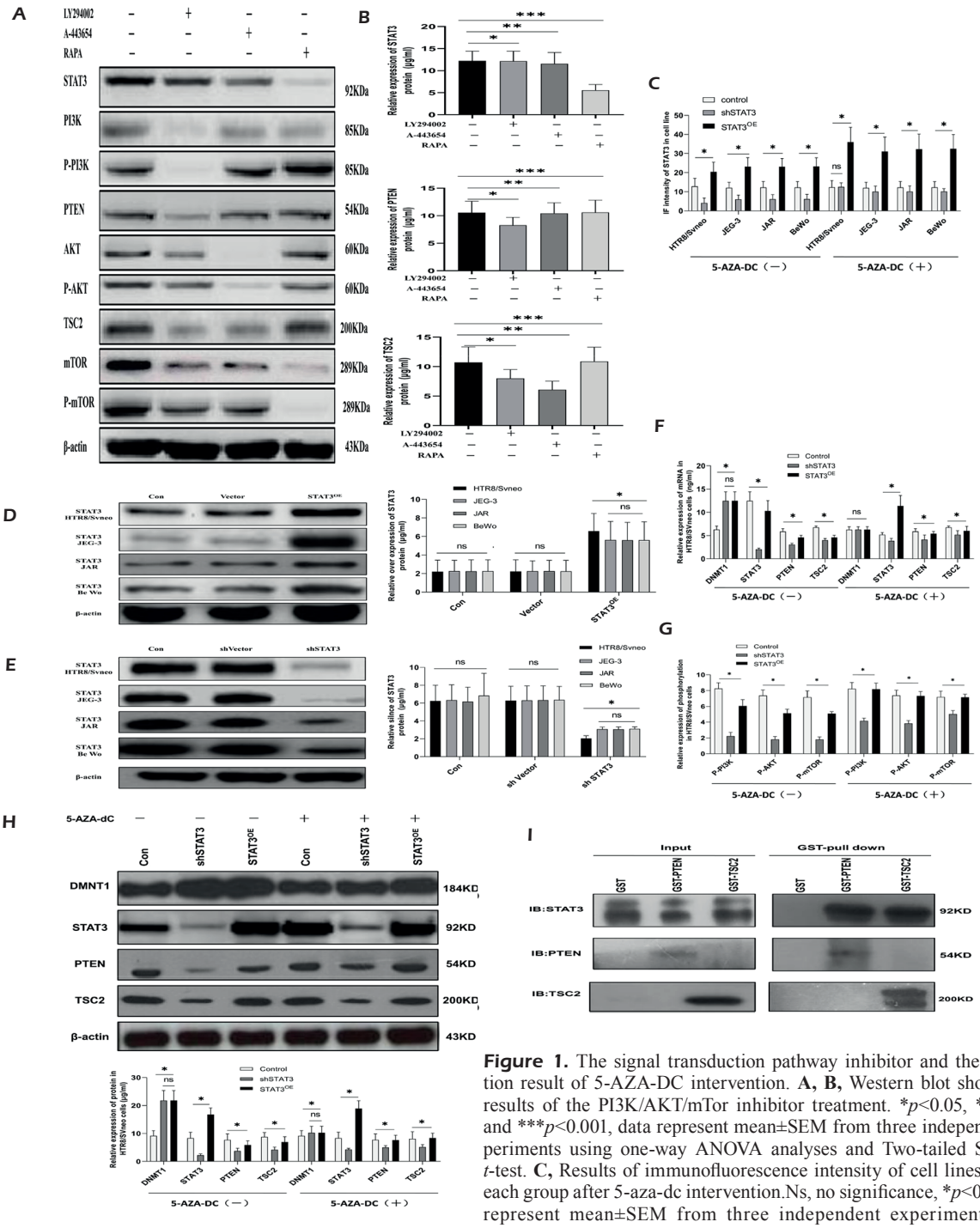

Figure 1. The signal transduction pathway inhibitor and the interaction result of 5-AZA-DC intervention. **A, B,** Western blot showed the results of the PI3K/AKT/mTor inhibitor treatment. *p<0.05, **p<0.01 and ***p<0.001, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **C,** Results of immunofluorescence intensity of cell lines stat3 in each group after 5-aza-dc intervention.Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **D,** Detection result of overexpression protein of STAT3 gene in cell lines by Western blot. Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **E,** Detection result of silencing protein of STAT3 gene in cell lines by Western blot. Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **F,** Detection result of DNMT1, STAT3, PTEN, TSC2 mRNA in HTR8/SVneo cells by Real time PCR after 5-aza-dc intervention. Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **G,** Following 5-aza-dc intervention, the phosphorylation greyscale of P- PI3K, P-AKT and P- mTor in HTR8/SVneo cells were identified via Western blot. *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **H,** Detection of STAT3, PTEN, TSC2 protein difference in HTR8/SVneo cells by Western blot after 5-aza-dc intervention. Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **I,** The deployment of GST-pull down assay to identify the interaction of STAT3, PTEN and TSC2 protein *in vitro.*

The Difference Between and Interaction of DNM1, STAT3, PTEN and TSC2 in Each Group of Cell Lines After 5-aza-dc Intervention

5-aza-dc intervention did not affect the STAT3 fluorescence intensity of each cell line in the Con group. After such intervention, the fluorescence intensity of STAT3 in the shSTAT3 and $STAT3^{OE}$ groups increased significantly. Between these two, the increase in the $STAT3^{OE}$ group was more significant. Real time PCR was used to detect the difference of DNMT1, STAT3, PTEN and TSC2 mRNA in the HTR8/SVneo cells. We confirmed that 5-aza-dc intervention deepened the degree of demethylation in each group. There was a decrease in the expression level of DNMT1 mRNA. Meanwhile, the expression levels of STAT3, PTEN and TSC2 mRNA increased (Figure 1F).

After 5-aza-dc intervention, the phosphorylation of P-PI3K, P-AKT and P-mTor was up-regulated (Figure 1G). The difference of DNMT1, STAT3, PTEN and TSC2 protein in the HTR8/SVneo cells was detected by Western blot. DNMT1 protein was significantly down regulated, following 5-aza-dc intervention, in both the $STAT3^{OE}$ and sh STAT3 groups. STAT3, PTEN and TSC2 protein were significantly upregulated (Figure 1H). GST-pull down provided confirmation of the interaction of the STAT3 with PTEN, and TSC2 *in vitro* expression proteins. Western blot results showed that the STAT3 interaction with PTEN, TSC2, STAT3 could be pulled down by the GST fusion protein PTEN, TSC2. The STAT3, PTEN and TSC2 were all expressed. There was, however, no interaction between STAT3 and GST, and the GST empty protein lacked the capacity to pull down STAT3 (Figure 1I). These results suggested an effect of 5-aza-dc on the production of cells.

The Relationship Between Transcription Factor STAT3 and Target Gene PTEN, and the Effect of 5-aza-dc Intervention

The total chromatin input was a positive control, while the chromatin precipitated with IgG was a negative control. The CHIP result indicated that the transcription factor STAT3 could be directly bound to the target gene PTEN (Figure 2A) and the quantitative results (Figure 2B). We used the Dual-Luciferase reporter system to measure the expression of plasmid lucifer. The HTR8/SVneo, JEG-3, JAR, and BeWo cell lines were transfected into a successful PTEN promoter plasmid pGL3-PTEN-luc. The fluorescence intensity of the negative control group was significantly lower than that of the transfected pGL3-PTEN-luc plasmid group (Figure 2C). Tto verify that the transcription factor STAT3 was capable of regulating the activity of the PTEN promoter, we had co-rotated p CD3/Flag/STAT3 and p GL3-PTEN-luc in the HTR8/SVneo, JEG-3, JAR, BeWo cells in the Con group, sh STAT3 group and $STAT3^{OE}$group. The promoter activity of PTEN in the $STAT3^{OE}$ group was higher than that in the Con group. Conversely, in the shSTAT3 group, the PTEN-promoter activity was lower than in the Con group (Figure 2D).

To prove that PTEN was a functional target gene of STAT3, we had designed a rescue experiment involving HTR8/SVneo cells. Overexpression of STAT3 could promote the expression of endogenous PTEN. On this basis, after exogenous knockout of PTEN,the expression level of endogenous PTEN was rescued. The results showed that the knockout of PTEN could rescue the promoting effect of STAT3 on the expression of PTEN (Figure 2E). The quantitative proteome analysis of HTR8/SVneo cells by lc-ms/ms utilized the itraq technique. Following in-depth analysis, we found 44 significantly differentially expressed proteins. Among these, 25 were down regulated while 19 were up regulated. Cluster Analysis results reflected differential proteins after 5-aza-dc intervention (Figure 2F). The differential gene methylated region was indicated via Circos (Figure 2G). We selected a HTR8/SVneo cell line with relatively low expression of PTEN as the object of study. The methylated status of the cell-line PTEN promoter after 5-aza-dc intervention was detected by MSP. The PTEN gene was hypermethylated in HTR8/SVneo cells. BSP sequencing confirmed the results of MSP methylation analysis. In the absence of 5-aza-dc intervention, the methylation of the shSTAT3 and $STAT3^{OE}$ groups was significant. Moreover, following 5-aza-dc intervention, both the $STAT3^{OE}$ and shSTAT3 groups were non-methylated (Figure 2H). Therefore, our results implied a relationship between transcription Factor STAT3 and Target Gene PTEN.

Cell Proliferation, Apoptosis, Invasion and Migration

CCK-8 detected each group of cells. Whether or not 5-aza-dc intervention had little effect on the Con group, the proliferation abilities of shSTAT3 and $STAT3^{OE}$ were decreased in the context of 5-aza-dc non-intervention. The prolif-

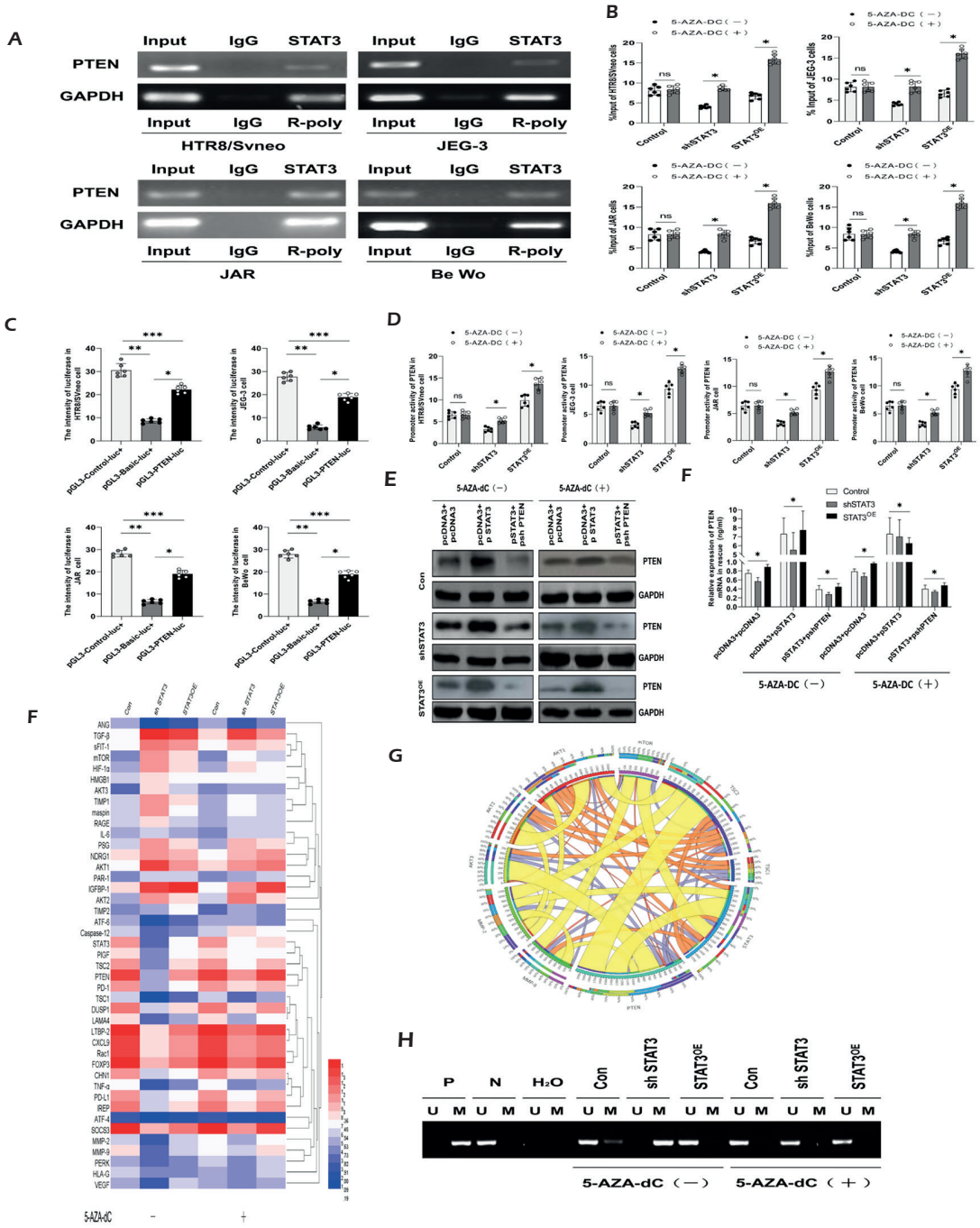

Figure 2. The impact of 5-aza-dc intervention and the relationship between PTEN and STAT3. **A,** CHIP assay validated the binding of the transcription factor STAT3 to target gene PTEN. **B,** CHIP assay resulted in quantitative verification that the transcription factor STAT3 binds to the target gene PTEN in the cells. Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **C,** Expression of plasmid luciferase fluorescence intensity by Dual-Luciferase reporting system in cells. *p<0.05, **p<0.01 and ***p<0.001, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **D,** Following 5-aza-dc intervention, the regulation by STAT3 of PTEN-promoter activity in cell lines. Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **E,** Detection of PTEN protein level in HTR8/SVneo cells by Western blot, and the mRNA results by real time PCR quantitative, in the rescue experiment following 5-aza-dc intervention. *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **F,** Clustered heatmap analysis of Proteome differential expression in HTR8/SVneo cells after 5-aza-dc intervention. **G,** The circos plot presents the proteomic differential protein expression of the top 10 genes upregulated or downregulated. **H,** Detection of PTEN Promoter methylation by Methylation-specific PCR in HTR8/SVneo cells after 5-aza-dc intervention. U was a non-methylated primer and M was a methylation primer. p was the positive control group and N was the negative control group.

eration of the shSTAT3 and STAT3^{OE} groups was significantly improved after 5-aza-dc intervention (Figure 3A). The results of flow cytometry showed that the apoptosis rate of the Con group had no effect after 5-aza-dc intervention, and the apoptosis rate of the shSTAT3 and STAT3^{OE} groups decreased (Figure 3B). Transwell was deployed to detect the invasive capacity of the HTR8/SVneo cells. The results showed that after 5-aza-dc intervention, the invasive ability of the shSTAT3 and STAT3^{OE} groups was significantly improved, although that of the shSTAT3 group was lower than the STAT3^{OE} group (Figure 3C). The effect, or otherwise, of 5-aza-dc intervention on the Con group is reflected in the quantitative results (Figure 3D). The detection of the migration ability of the HTR8/SVneo cells was undertaken via Wound healing assay. The results showed that after 5-aza-dc intervention, the migration ability of the shSTAT3 and STAT3^{OE} groups was significantly improved. The STAT3^{OE} group had stronger migration ability (Figure 3E), as reflected in the Quantitative results (Figure 3F). These sets of data suggest that the 5-aza-dc interferes with the HTR8/SVneo cells, while the difference in terms of the STAT3 gene produces different results for proliferation, apoptosis, invasion and migration.

Discussion

Main Findings

A disease occurring during pregnancy, pre-eclampsia can cause widespread organ damage in expectant mothers. Within the postpartum period, the condition plays a significant role in heightening levels of both infant and maternal mortality. The pathogenesis of the disease is still unclear. It has been suggested, nonetheless, that the condition stems from an excessive inflammatory reaction to pregnancy on the part of the mother[21].

Placental dysfunction may be due to early epigenetic abnormalities in gametes[22]. It has been suggested that abnormal epigenetic regulation increases susceptibility to pre-eclampsia, both before and during pregnancy. Our results detected that the expression of DNMT1 in pre-eclampsia placentae was significantly higher than in normal mature placenta, and the expression of STAT3 was significantly lower than that in normal mature placenta. After treatment with methylated inhibitor, the expression of DNMT1 is significantly down regulated, while STAT3 expression is significantly upregulated. It has been posited that DNA methylation serves to regulate STAT3 expression.

Strengths and Limitations

Signal transduction and transcriptional activator 3 (STAT3) is a protein of about 92 kda. There are a number of functions involved in the processes of cell proliferation, apoptosis, migration and differentiation[23]. The activation of STAT3 requires the assistance of tyrosine phosphate kinase, while tyrosine phosphatase also has dephosphorylation. The dephosphorylation of STAT3 that has already undergone phosphorylation, results in loss of activity. Inactivated STAT3 molecules can be re-transported back to the cytoplasm, Once the phosphorylation of the tyrosine phosphatase is encountered, they will be activated again[24,25]. The phosphatase and tension protein homologous gene (PTEN), which is deleted on chromosome 10, is a tumour inhibitor gene with double phosphatase activity of lipid phosphatase and protein phosphatase[26]. Accumulation of PIP3 within cells occurs as a result of the deletion of PTEN. The PTEN-PI3K/AKT signal transduction pathway is unbalanced and AKT is continuously activated. Further activation of the rapamycin target protein (mTor) occurs in mammalians[27,28]. In the context of a protein with a molecular weight of about 200k Da of the TSC2 gene, the phosphorylation of AKT inhibited the activation of mTor.

In the STAT3/PI3K/PTEN/AKT/TSC2/mTor signal transduction pathway, PI3K can activate its downstream signal molecules[29,30], resulting in cell proliferation and anti-apoptosis[31-33]. In the context of a range of varying transcription factors, activated AKT may lead to phosphorylation[34,35]. It can, via the regulation of these transcription factors, regulate apoptosis, as well as the survival and proliferation of cells[36-38].

MeDIP-seq is based on the principle of antibody enrichment[39-41]. In our placental tissue study, there was a deviation caused by capture and enrichment dependence in low-methylation-level and low-CpG-density gene fragments. For this reason, we also used the unoriented whole genome shotgun sulphite sequencing (WGBS) for higher throughput and high-resolution differentially methylated region (DMR) screening[42]. We speculated that, within pre-eclampsia placental tissue, DNA methylation may not be restricted to a gene-specific level, but may occur overall.

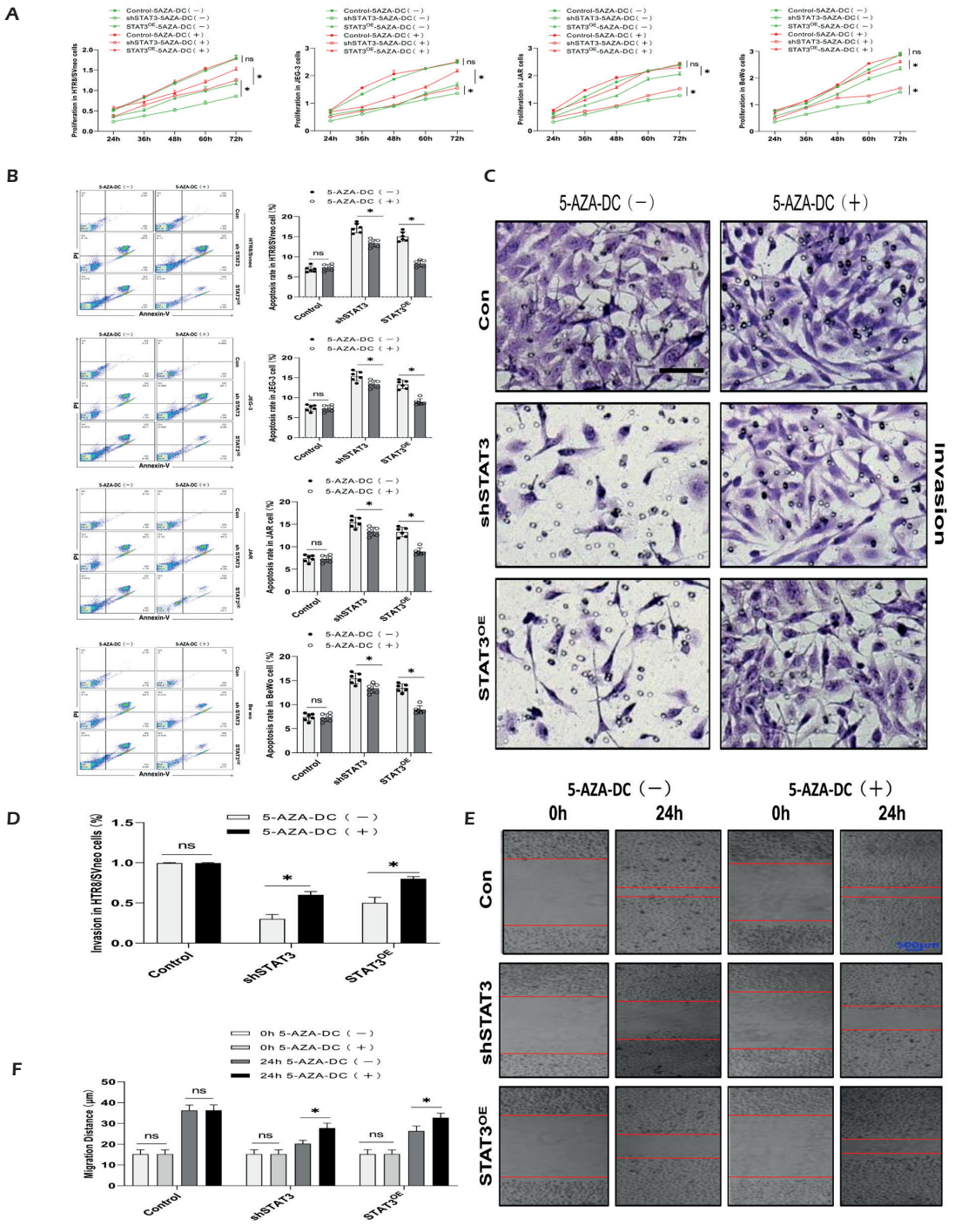

Figure 3. Cell proliferation, apoptosis, invasion and migration. **A,** The identification, following 5-aza-dc intervention, of cell-line proliferation via CCK-8 assay. Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **B,** Double-stained with Annexin V-FITC and PI, and assessed by flow cytometry analysis of cell lines to evaluate apoptosis. Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **C,** Following 5-aza-dc intervention, the identification of the invasion of HTR8/SVneo cells via transwell. Scale bars, 200 μm (magnification: 200×). ***D,*** Quantitative results of invasion detected by transwell in HTR8/SVneo cells after 5-AZA-DC intervention. Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test. **E,** Following 5-aza-dc intervention, the use of wound-healing assay to identify HTR8/SVneo-cell migration (magnification: 20×). **F,** Quantitative results of migration detected by wound-healing assay in HTR8/SVneo cells after 5-AZA-DC intervention. Ns, no significance, *p<0.05, data represent mean±SEM from three independent experiments using one-way ANOVA analyses and Two-tailed Student's t-test.

Interpretation

We suggested that the STAT3 gene may also be involved in the regulation of trophoblast migration, invasion and other biological functions during human placental development. To undertake multigroup research, therefore, we collected placental tissue from pregnant women both with and without pre-eclampsia. Our results reveal the expression of the STAT3 gene during placental development. It is suggested that STAT3 is involved in the pathogenesis of pre-eclampsia. The abnormal expression may be related to the biological dysfunction of trophoblast. We showed that the down regulation of TSC2 expression induced the activation of mTor. The STAT3 is then activated to up-regulate the expression of the PTEN, while the activated STAT3 binds to a specific site on the PTEN gene promoter, and the expression of the PTEN is regulated at the transcription level. In addition, our study detects that in the PI3K/PTEN/AKT/mTor signalling pathway, the activity of AKT was regulated by the upstream PTEN gene and the downstream TSC2 gene. The increase in the methylation level of the CpG island in the gene promoter region will affect the transcription of the gene, and then inhibit the expression and function of the gene. Gene expression and reactivation may be induced by demethylation[43].

We previously showed that STAT3 may be an important negative regulator in the early maternal and fetal interface, to regulate the migration and invasion, proliferation and apoptosis of trophoblast. Hence, this regulation may in turn be effected by regulating, either directly or indirectly, the activity of TSC2 and PTEN. The significance of upregulating and downregulating the expression of the STAT3 gene was confirmed in the present study. These results suggest that hypoxia can significantly inhibit the proliferation and migration of trophoblast and promote its apoptosis, which is consistent with the functional changes of placental trophoblast in pre-eclampsia. We further found that the methylated status of the STAT3-gene promoter region may be altered via demethylation drugs. This improves the proliferation and migration ability of trophoblast, and significantly inhibits its apoptosis.

In the experimental study of animals *in vivo*, we deployed methyltransferase inhibitor 5-aza-dc intervention. The blood pressure and 24 h urinary protein of pre-eclampsia in pregnant mice were significantly alleviated, which suggests that methylated transferase inhibitors may play a role in improving the clinical symptoms of pre-eclampsia. In addition, we also observed that there were significant differences in the number and body weight of fetal mice among pregnant mice. These results suggest that methyltransferase inhibitors may have effects on the development and maturation of fetal mice.

Our data provide strong support for the proposition that the degree of DNMT1 methylation in pre-eclampsia is upregulated. The level of upregulation is higher than in pregnant women without the condition. STAT3 was down-regulated, to an extent that was significantly lower than for the normal pregnant women. Although PI3K was upregulated, it was also lower than for the normal pregnant women. PTEN decreased significantly, and was lower than for the normal pregnant women. AKT was upregulated, but lower than the normal pregnant women. TSC2 decreased significantly, at a level lower than the normal pregnant women. Although mTor was upregulated, it was lower than that of normal pregnant women. Within trophoblast, apoptosis increased while there was a decrease in the capacity for invasion, migration and proliferation. Our study pinpointed the fact that the STAT3 did not change, but PTEN and TSC2 were significantly upregulated after the application of the PI3K specific pathway inhibitor. Moreover, while no change was evinced by PTEN or STAT3, the application of the AKT specific pathway inhibitor led to significant upregulation for TSC2. After the application of the mTor specific pathway inhibitor, STAT3, PTEN and TSC2 were significantly upregulated. Our study also pinpointed the fact that, after 5-aza-dc intervention, the degree of DNMT1 methylation was downregulated, to a level lower than that of normal pregnant women. Conversely, while STAT3 was upregulated, the level was similar to that of pregnant women without pre-eclampsia. Although PI3K was down-regulated, it was also close to the level of normal pregnant women, which was higher than that before intervention. PTEN was significantly upregulated, close to the level of normal pregnant women.

Although AKT was downregulated, it was close to the level of normal pregnant women, and thus higher than that before intervention. As regards TSC2, this saw significant upregulation, for a level marginally higher than before intervention, but still lower than that of normal pregnant women. Although mTor was downregu-

lated, it was close to the level of normal pregnant women, which was higher than that before intervention. The capacity for proliferation, invasion and migration increased and apoptosis decreased in trophoblast.

Nonetheless, our sample was relatively small. A restricted sample size, of course, impacts credibility and generalizability – hence, we plan to deploy larger samples in future. Further research and statistical analysis regarding other complications and related factors are required, in order to determine the related factors for pre-eclampsia. Such investigations will afford a more secure theoretical foundation for the diagnosis and treatment of the condition.

Conclusions

In summary, our findings demonstrated the significance of placental trophoblast methylation at the onset of pre-eclampsia. Within trophoblast cells, STAT3-gene down regulation was shown to be significant. STAT3 changes are regulated by the PI3K/ AKT/ mTor signal transduction pathway, mediated in trophoblast. In terms of ameliorating the symptoms of pre-eclampsia, we further identified the potentially significant role of 5-aza-dc. The STAT3 gene can be used as a potential target for the epigenetic therapy of pre-eclampsia. This study also suggests that epigenetic regulation as mediated by the STAT3 gene may play a key role in the molecular mechanism of pre-eclampsia.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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