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Metformin exhibits its therapeutic effect in the treatment of pre-eclampsia via modulating the Met/H19/miR-148a-5p/P28 and Met/H19/miR-216-3p/EBI3 signaling pathways

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ABSTRACT

Metformin (Met) has been found to modify the methylation of H19 and to alter its expression. In addition, IL-27, one of the downstream factors in the H19 signaling pathway, plays an important role in the pathogenesis of pre-eclampsia (PE). In this study, we investigated the molecular mechanism underlying the therapeutic effect of Met in the management of PE both in vivo and in vitro. The role of H19 signaling pathway in PE was validated using online bioinformatics tools, luciferase assays, real-time PCR and Western Blot. A tail-cuff method was used to examine the blood pressures in PE rats with or without Met treatment. Cells exhibited a dose-dependent increase of H19 methylation, which inhibited the expression of H19. Additionally, upon the Met treatment, levels of miR-148-5p and miR-216-3p were both elevated in a dose-dependent manner while levels of p28 mRNA and EBI3 mRNA were both inhibited by Met treatment. Also, H19 was found to regulate the expression of miR-148a-5p and miR-216-3p, while P28 and EBI3 were respectively identified as target genes of miR-148a-5p and miR-216-3p. Therefore, the Met/H19/miR-148a-5p/P28 and Met/H19/miR-216-3p/EBI3 signaling pathways were implicated in the pathogenesis of PE. Met was implicated in the pathogenesis of PE via modulating the H19 signaling pathway. The methylation of H19 reduced H19 expression, which in turn could up-regulate the expression of miR-148-5p/miR-216-3p. And the expressions of subunits of IL-27, P28 and EBI3, were thus suppressed. Therefore, Met-induced inhibition of H19 also led to the reduction of IL-27 expression, TNF-α and IL-6 in vivo.

1. Introduction

As a disorder frequently occurring in pregnancy, pre-eclampsia (PE) affects about 5% of pregnancies and can cause increased blood pressure as well as proteinuria [1]. Therefore, PE has become a major cause of neonatal, fetal, and maternal mortality, especially in developing countries [2].

As a recently discovered interleukin, IL-27 functions by forming a heterodimer that includes two protein sub-units encoded by the genes of Epstein-Barr virus-induced gene 3 (EBI3) and p28, respectively. In addition, IL-27 was shown to play a critical role in the stimulation of naive CD4 T cells in both mouse and human. Furthermore, the synergistic synthesis of interleukin 12 (IL-12) and interferons (IFN) is implicated in the proliferation of CD4 T cells [3]. Moreover, it has been reported in several studies that the susceptibility to PE can be affected by multiple cytokines, including transforming growth factor beta 1 (TGF-β1), TNF-α, IL-6, and interleukin 10 (IL-10) [4–7]. For example, Yin et al. showed that the expression of IL-27 as well as the expression of IL-27 receptor was apparently increased in trophoblastic cells collected from the placenta of PE patients [8]. In addition, Liu et al. investigated the correlation between the single nucleotide polymorphism (SNP) of IL27 and the risk of PE. The authors showed that the CC genotype of rs153109 SNP was associated with an increased risk of PE [9].

As the largest group of RNA transcripts, non-coding RNAs occupy up to 90% of the human genome. In particular, long non-coding RNAs (lncRNAs) were shown to contain microRNA responsible elements (MREs) that can potentially sponge their target miRNAs and impair the ability of miRNAs to mediate post-transcriptional regulation of their target mRNAs [10].

As an lncRNA that has been extensively studied in the past decade, H19 was previously shown to participate in the pathogenesis of many

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diseases. In addition, the SNPs discovered in the seed sequences of H19 were shown to alter H19 expression, thus affecting the susceptibility to PE [11].

Originally used in clinical applications as an anti-diabetic agent, metformin (Met) is now used in the treatment of a wide range of diseases, including polycystic ovarian disease, gestational diabetes mellitus, and prediabetes mellitus. Recently, several clinical trials have demonstrated the role of Met in the prevention and treatment of PE [12]. It was also demonstrated that the concomitant use of Met and insulin could result in lower weight gain and a decreased incidence of PE among pregnant women suffering from diabetes. Therefore, the application of Met as a supplementary drug in diabetic treatment of pregnant women may produce a more favorable prognosis, including a reduced incidence of PE. In addition, the abovementioned effect of Met is likely dependent on the age of the patients [13].

Met has been found to play a therapeutic role in the treatment of PE [13]. In addition, Met was shown to modify the methylation of H19 to alter its expression [14]. Furthermore, by searching the literature, we found that H19 functioned as a sponge of miR-148a and miR-216 [15,16]. In addition, two subunits of IL-27, P28 and EBI3, were found to be the target of miR-148a-5p and miR-216-3p, respectively. Interestingly, IL-27 plays an important role in the pathogenesis of PE [16]. Therefore, in this study, we investigated the molecular mechanism underlying the therapeutic effect of Met in the management of PE both in vivo and in vitro.

2. Materials and methods

2.1. Animal and treatment

To investigate the role of Met in the pathogenesis of PE, a rat model of PE was established and treated with Met. Briefly, healthy female and pregnant Sprague-Dawley (SD) rats were purchased from the animal center of our hospital and divided into the following groups: a Sham operated group, a PE group, and a PE + Met group. Through the entire experiment, all rats were housed in mesh cages and maintained at a temperature of 20–25 °C and relative humidity of 50–70%. In addition, the rats were subjected to under cycles containing 12 h of light (06:00–18:00) and 12 h of darkness (18:00–06:00) and had free access to water and a standard diet. The rats were adapted to the environment for a week before the start of model establishment. From 14 d to 20 d of gestation, L-NAME (Sigma-Aldrich, St. Louis, MO), a nitric oxide inhibitor, was added to the drinking water for the rats in the PE group at a concentration of 0.3 g/L to induce the onset of PE. In the PE + Met group, the rats were also given the L-NAME-containing water as those in the PE group from 14 d to 20 d. In addition, the rats in the PE + Met group were also given a daily intragastric administration of Met hydrochloride (Sigma-Aldrich, St. Louis, MO) at a dose of 300 mg/kg/day for 1 week (14d to 20 d). The systolic and diastolic blood pressure in each rat was recorded on 0 d, 12 d, and 19 d using a tail-cuff BP monitor (MRBP, IITC Life Sciences Inc., USA). And the data on 19 d was presented accordingly, In addition, on 20 d, peripheral blood samples were collected through the tail vein from all rats to measure the expression of target genes. This animal experiment was approved by the ethics committee at our institution. All animal experiments in this study were done in accordance with the principles of management and usage of local laboratory animals, and followed the guidelines published by the National Institutes of Health on the Management and Use of Laboratory Animals. In the process of the experiment, the disposal of rat carcasses was done in accordance with the basic principles of animal welfare.

2.2. RNA isolation and real-time PCR

Total RNA in collected samples was extracted using a Trizol kit (Invitrogen, Carlsbad, CA). Subsequently, cDNA was synthesized from extracted RNA using a BcaBest RNA PCR kit (TaKaRa, Tokyo, Japan). In the next step, the expression of H19, miR-148a-5p, miR-216-3p, P28 mRNA and EBI3 mRNA was measured using real-time qPCR and a SYBR Green Real-time PCR Master Mix (ABI, Foster City, CA) following the manufacturer's instructions. The relative expression of H19, miR-148a-5p, miR-216-3p, P28 mRNA and EBI3 mRNA was calculated using the $2^{-\triangle\triangle Ct}$ method, and the expression of GAPDH was used as the internal control. A set of primers, i.e., H19 Forward: 5′-GCTTCACTGGCAAGA CTACTCTTAG-3′, H19 Reverse: 5′-CACCTCGGGCTATTTGCTTTC TAA-3′, miR-148-5p Forward: 5′-GGGAAAGTTCTGAGACACTC-3′, miR-148a-5p Reverse: 5′-CAGTGCGTGTCGTGGAGT-3′, P28 mRNA Forward: 5′-CACTGTTGCTACAGAACCATCT-3′, P28 mRNA Reverse: 5′-TGGGAG ACTCCAGTAGACTCA-3′, EBI3 mRNA Forward: 5′-TCATTGCCACGTAC AGGCTC-3′, EBI3 mRNA Reverse: 5′-GGGTCGGGCTTGATGATGTG-3′, GAPDH Forward: 5′-TGTGGGCATCAATGGATTTGG-3′, GAPDH Reverse: 5′-ACACCATGTATTCCGGGTCAAT-3′.

2.3. Cell culture and transfection

Trophoblast and HUVEC cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/L penicillin and 100 mg/L streptomycin. The cell culture conditions included 37 °C, 5% CO_2, and saturated humidity. When the cells reached 90% confluence, they were trypsinized and adjusted to a concentration of 1×10^5 cells/mL. Subsequently, 0.2 mL of cell suspension was seeded into each well of a 24-well plate and cultured overnight. On the next day, the cells were divided into different groups and were treated by 5 nM and 10 nM of Met or transfected by pGL3-H19 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the instruction of the manufacturer. The cells were harvested at 48 h post treatment for subsequent assays.

2.4. Vector construction, mutagenesis and luciferase assay

A bioinformatics tool, TargetScan (www.targetscan.org), was used to predict the sites of interactions between H19/miR-148a-5p, H19/miR-216-3p, miR-148a-5p/P28, and miR-216-3p/EBI3. Subsequently, the full lengths of miR-148a-5p and miR-216-3p, as well as the 3′UTR of P28 and EBI3 containing the binding sites for miR-148a-5p and miR-216-3p, respectively, were amplified by PCR and cloned into pGL3 luciferase reporter vectors (Promega, Madison, WI) downstream of the firefly luciferase reporter gene. At the same time, site-directed mutagenesis was carried out in the H19 binding sites of miR-148a-5p and miR-216-3p, as well as in the 3′UTR of P28 and EBI3 corresponding to the binding sites for miR-148a-5p and miR-216-3p, respectively, to create mutant sequences of miR-148a-5p, miR-216-3p, P28 and EBI3. These mutant sequences were also amplified by PCR and respectively cloned into pGL3 luciferase reporter vectors. Subsequently, Trophoblast and HUVEC cells were co-transfected with H19, miR-148a-5p or miR-216-3p in conjunction with their target genes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the instruction of the manufacturer. At 48 h post transfection, the cells were collected and the luciferase activity in transfected cells was measured using a Dual luciferase reporter gene assay (Promega, Madison, WI). The relative luciferase activity was expressed as the ratio of the firefly luciferase activity to the activity of renilla luciferase (internal control). The experiment was repeated three times.

2.5. Western blot analysis

After being washed by PBS, cell samples were lysed and centrifuged at 1200 r/min for 30 min at 4 °C to remove the cell debris. The supernatant was collected to measure the total protein concentration according to the instructions of a bicinchoninic acid (BCA) kit. Subsequently, 50 μg proteins from each sample was mixed with a 2× sodium dodecyl sulfate loading buffer and boiled at 100 °C for 5 min, and then resolved using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In the next step, the protein was

transferred onto a polyvinylidene fluoride (PVDF) membrane and blocked with 5% skimmed milk for 1 h at room temperature. Subsequently, the membrane was incubated overnight at 4 °C with diluted (1: 100) anti-P28 (catalogue number: ab104068, Abcam, Cambridge, MA), anti-EBI3 (catalogue number: ab83896, Abcam, Cambridge, MA) and anti-IL27 primary antibodies (catalogue number: ab229139, Abcam, Cambridge, MA). Afterwards, the membrane was washed 3 times by Tris(-HCL)-buffered saline + Polysorbate 20 (Tween 20) (TBST) and further incubated for 1 h at room temperature with horseradish peroxidase (HRP)-labeled secondary antibodies. Finally, the membrane was developed in an enhanced chemiluminescence (ECL) reagent (Biomiga, San Diego, CA) and observed under an X-ray apparatus. The relative expression of P28, EBI3 and IL27 proteins was calculated using the ratio between the mean OD values of their protein band and that of the protein band of GAPDH internal reference.

2.6. ELISA

The levels of TNF-α, IL-6 and IL-27 in collected serum samples were measured using appropriate ELISA kits (Thermo Fisher Scientific, Waltham, MA) following the kit instructions. The optical density (OD) value in each well of the ELISA assays was measured at a wavelength of 450 nm using an instrument (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.7. Immunohistochemistry

Samples were fixed in 10% formaldehyde, dehydrated in gradient alcohol (95%, 80%, and 75%, 1 min each), embedded in paraffin, incubated for 1 h, dewaxed and dehydrated again by gradient alcohol. Subsequently, the sections were incubated with 3% H_2O_2 at 37 °C for 30 min, boiled in a 0.01 M citrate buffer at 95 °C for 20 min, and blocked with normal goat serum at 37 °C for 10 min. In the next step, the samples were incubated overnight at 4 °C with anti-IL-27 monoclonal primary antibodies (catalogue number: ab229139, 1:100, Abcam, Cambridge, MA), washed with PBS, and further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (catalogue number: ab181658, 1: 1000, Abcam, Cambridge, MA) at room temperature for 30 min. Afterwards, the sections were developed with diaminobenzidine (DAB), counterstained with hematoxylin, and mounted for observation. During the observation, the proportion of cells with positive expression of IL-27 was calculated.

2.8. Methylation analysis

A DNA extraction and purification kit (Promega, Madison, WI) was used to extract the DNA from the samples. Subsequently, 1 μl of DNA solution was diluted by 100 times and measured by ultraviolet spectroscopy to determine its DNA concentration. According to the instruction of an EZ DNA Methylation-Gold Kit (ZYMO, Los Angeles, CA), DNA was modified via a bisulfite reaction to produce BSP-DNA, which was then amplified by PCR. A set of primers, i.e., unmethylated forward: 5′-GTTTATGGGAGTTATATTATGTTTTTGTATTG-3′, unmethylated reverse: 5′-CAAAATTTACTATACTCATCACACAAATAA ACA-3′, methylated forward: 5′-GGGAGTTATATTACGTTTTCGTA TCG-3′, and methylated reverse: 5′-TACTATACTCATCACGCGAATAA ACG-3′, were used to amplify the BSP-DNA templates using PCR. The PCR reaction system contained 10 μl PCR Mix (2×), 0.4 μl forward primers and reverse primers, 1.0 μl BSP-DNA and 8.6 μ1 ddH_2O. The reaction procedures were as follows: pre-denaturation at 95 °C for 15 min, followed by 45 cycles of denaturation at 95 °C for 1 min and annealing at 57 °C for 1 min, and a final extension cycle at 72 °C for 10 min. and then reserving at 4 °C. After the PCR products were tested using 1.5% agarose gel electrophoresis, a gel extraction kit (Promega, Madison, WI) was used to purify and recover the DNA. Finally, the methylation analysis of H19 was conducted by real-time PCR using an

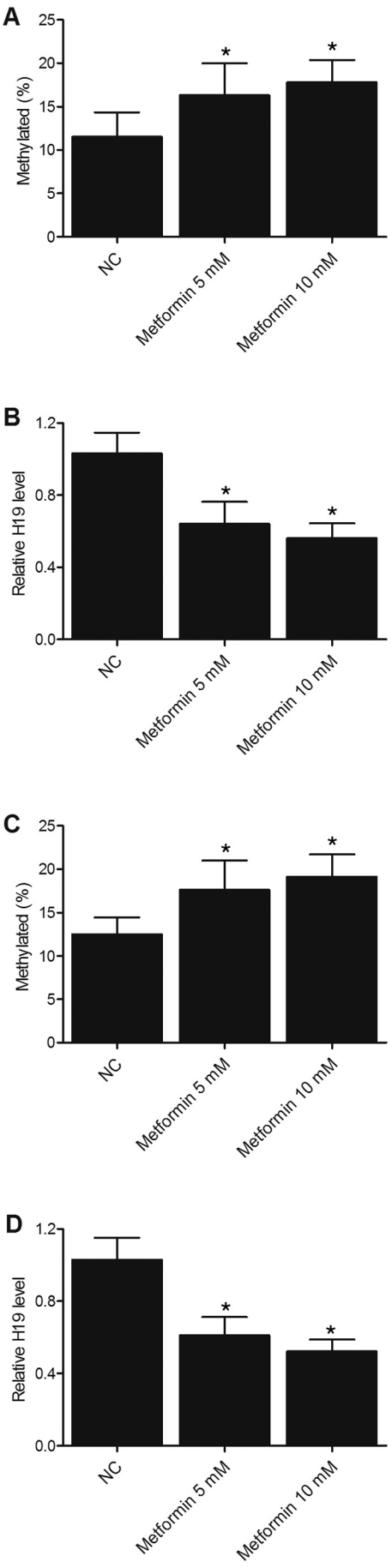

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Fig. 1. Metformin increased H19 methylation and inhibited H19 expression in a dose-dependent manner.
A. Methylation of H19 showed a dose-dependent increase in Trophoblast cells treated with 5 nM Met and 10 nM Met ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).
B. Relative H19 expression was inhibited dose-dependently in Trophoblast cells treated with 5 nM Met and 10 nM Met ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).
C. Methylation of H19 showed a dose-dependent increase in HUVEC cells treated with 5 nM Met and 10 nM Met (N = 3; *P value < 0.05, vs. NC; NC, negative controls).
D. Relative H19 expression was inhibited dose-dependently in HUVEC cells treated with 5 nM Met and 10 nM Met (N = 3; *P value < 0.05, vs. NC; NC, negative controls).

iQSYBRGreen kit (Bio-Rad, Hercules, CA) in conjunction with a Bio-Rad iCycler.

2.9. Statistical analysis

The data were analyzed by SPSS statistical software version 21.0. All measurement data were expressed as mean ± standard deviations. The *t*-test was used for comparing two groups, while one-way analysis of variance (ANOVA) was used for comparing multiple groups. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. Met promoted H19 methylation while inhibiting H19 expression

To explore the effect of Met on the methylation and expression of H19, we measured the methylation status (%) and expression of H19 in Trophoblast and HUVEC cells treated by different doses of Met. As demonstrated in Fig. 1, both Trophoblast (Fig. 1A) and HUVEC (Fig. 1C) cells showed an increased level of H19 methylation in a dose-dependent manner when the cells were treated with 5 nM and 10 nM of Met, respectively. At the same time, the Met treatment also inhibited the expression of H19 in both Trophoblast (Fig. 1B) and HUVEC (Fig. 1D) cells. Therefore, it could be concluded that Met could promote the methylation of H19 as well as inhibit the expression of H19.

3.2. Met was involved in the pathogenesis of PE by regulating the activation of the H19 signaling pathway

Subsequently, to investigate the role of Met in H19-induced pathogenesis of PE, we compared the expression of miR-148-5p, miR-216-3p, P28 mRNA and EBI3 mRNA in Trophoblast and HUVEC cells treated with different doses of Met. The results showed that the levels of miR-148-5p (Fig. 2A) and miR-216-3p (Fig. 2B) were both elevated in the Trophoblast cells treated with Met, whereas the levels of both P28 mRNA (Fig. 2C) and EBI3 mRNA (Fig. 2D) were decreased. In addition, the expression of above genes changed in a dose-dependent manner according to the concentration of Met. Moreover, because P28 and EBI3 are subunits of IL-27, the inhibited expression of P28 and EBI3 also decreased the expression of IL-27 in Trophoblast cells (Fig. 2E). In addition, similar results were also obtained from HUVEC cells (Fig. 2F–J), further confirming that effect of Met on the expression of factors involved in the H19 signaling pathway. Subsequently, Trophoblast and HUVEC cells were transfected with plasmids carrying H19 before the expression of PE-related factors was measured. As revealed by the results, the transfection of H19 significantly down-regulated the expression of miR-148a-5p (Fig. 3A) and miR-216-3p (Fig. 3B), while up-regulating the expression of P28 mRNA (Fig. 3C) and EBI3 mRNA (Fig. 3D) in Trophoblast cells. Furthermore, the expression of IL-27 was also increased (Fig. 3E) in Trophoblast cells transfected with H19. When the experiments were repeated in HUVEC cells (Fig. 3F–J), similar results were obtained, indicating that Met was involved in the pathogenesis of PE via regulating the H19 signaling pathway.

3.3. H19 functioned as a sponge of miR-148a-5p and miR-216-3p

Using online bioinformatics tools, we found putative binding sites of H19 in miR-148a-5p (Fig. 4A) and miR-216-3p (Fig. 4D), respectively. The luciferase activity of Trophoblast cells co-transfected with miR-148a-5p mimics and wild-type H19 was inhibited compared with that in Trophoblast cells co-transfected with control mimics or mutant H19 (Fig. 4B). In addition, the Trophoblast cells co-transfected with miR-216-3p mimics and wild-type H19 showed reduced luciferase activity compared with the Trophoblast cells co-transfected with control mimics or mutant H19 (Fig. 4E). Moreover, same results were obtained in HUVEC cells (Fig. 4C, F), validating the role of H19 as a sponge of miR-148a-5p and miR-216-3p.

3.4. Met/H19/miR-148a-5p/P28 and Met/H19/miR-216-3p/EBI3 signaling pathways were established

In addition, P28 mRNA and EBI3 mRNA were identified as potential target genes of miR-148a-5p and miR-216-3p, respectively, with the miRNA binding sites located in the 3′UTR of wild-type P28/EBI3 mRNA (Fig. 5A, D). Subsequent luciferase assays showed reduced luciferase activity in Trophoblast (Fig. 5B) and HUVEC cells (Fig. 5C) co-transfected with miR-148a-5p mimics and wild-type P28, as well as in Trophoblast (Fig. 5E) and HUVEC cells (Fig. 5F) co-transfected with miR-216-3p mimics and wild-type EBI3. Therefore, it could be concluded that P28 was a target of miR-148-5p while EBI3 was a target of miR216-3p. In this way, the Met/H19/miR-148a-5p/P28 and Met/H19/miR-216-3p/EBI3 signaling pathways were established.

3.5. Met reduced the blood pressure and inhibited the expression of TNF-α/IL-6 in PE rats

To validate the presence of above signaling pathways in vivo, a PE rat model was established and treated with Met. The blood pressure of PE rats from different treatment groups was measured and compared. The results showed reduced systolic blood pressure, SBP (Fig. 6A) and diastolic blood pressure, DBP (Fig. 6B) in the PE rats treated with Met compared with those in untreated PE rats. In addition, the plasma level of TNF-α (Fig. 6C) was obviously higher in untreated PE rats, while the Met treatment helped to decrease the TNF-α level close to that in the sham-operated group. Moreover, IL-6 level (Fig. 6D) was also increased in PE rats compared with that in sham-operated rats, while Met treatment showed suppressive effect on the expression of IL-6.

3.6. Met exhibited a therapeutic effect in PE treatment via modulating the H19 signaling pathway

Finally, the expression of various factors involved in the Met/H19 signaling pathways, as well as the expression of their downstream protein IL-27, was compared among Met treated PE rats, untreated PE rats and sham-operated rats. As shown in Fig. 7, the expression of H19 (Fig. 7A), P28 (Fig. 7D) and EBI3 (Fig. 7E) was inhibited in Met treated PE rats compared with that in untreated PE rats. In addition, the results of Western Blot analyses (Fig. 7F) also showed decreased intensity of P28/EBI3 protein bands in Met treated PE rats. Furthermore, the levels of miR-148a-5p (Fig. 7B) and miR-216-3p (Fig. 7C) were evidently increased upon Met treatment. Since P28 and EBI3 proteins are subunits of IL-27, the Met treatment also down-regulated IL-27 expression by inhibiting the expression of P28/EBI3 proteins, and the effect of Met on IL-27 expression was also validated by the reduced intensity of IL-27 band in both Western Blot (Fig. 7F) and IHC (Fig. 8) assays. Therefore, the involvement of Met in the pathogenesis of PE was confirmed. By reducing the expression of H19 via promoting its methylation, Met

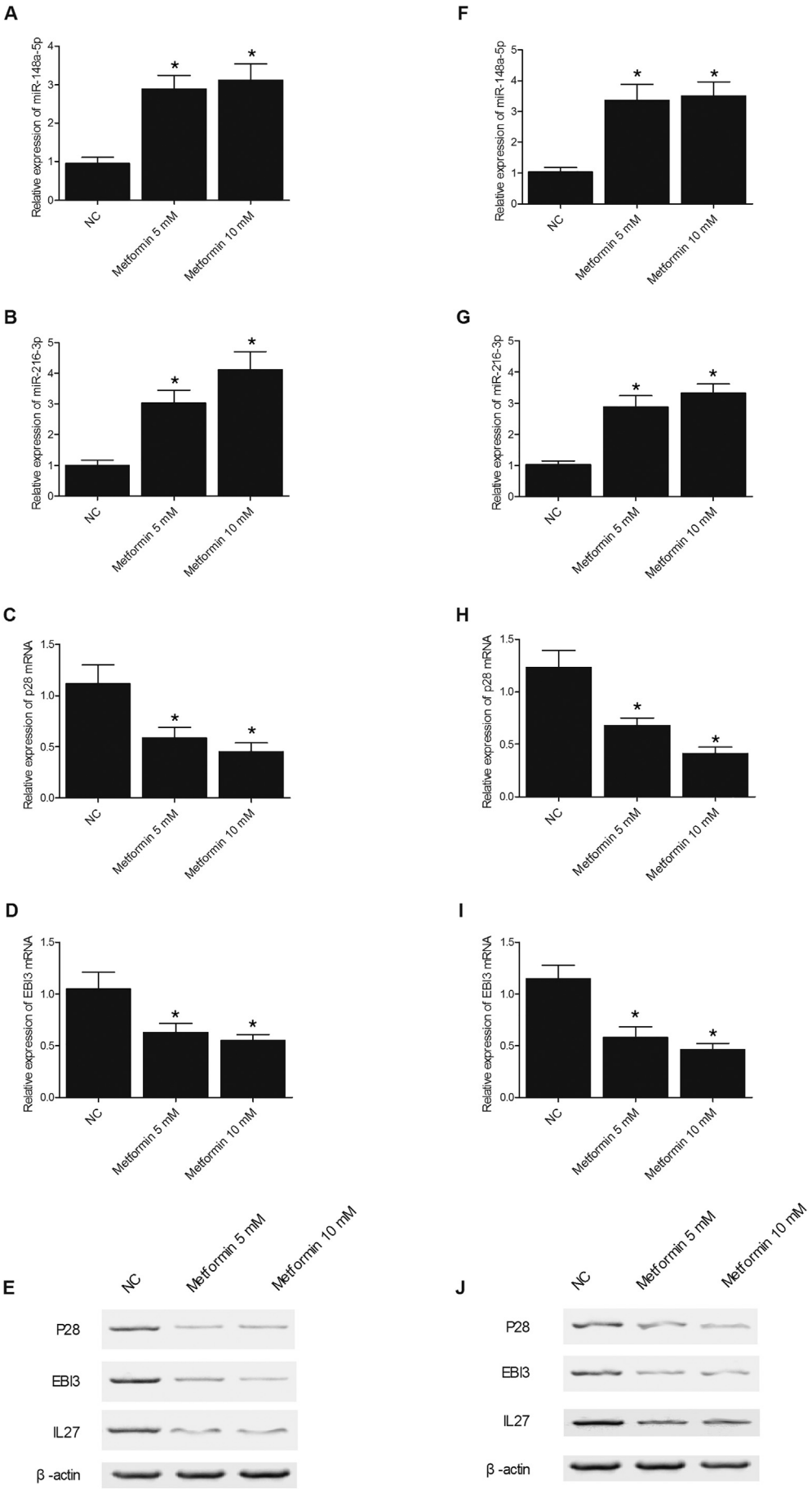

Fig. 2. Met treatment elevated expressions of miR-148-5p and miR-216-3p while inhibiting expressions of P28 and EBI3 mRNA/protein in a dose-dependent manner.

A. Expression of miR-148-5p was elevated in a dose-dependent manner in Trophoblast cells treated with Met ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).

B. Expression of miR-216-3p was elevated in a dose-dependent manner in Trophoblast cells treated with Met ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).

C. Expression of P28 mRNA was inhibited in a dose-dependent manner in Trophoblast cells treated with Met ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).

D. Expression of EBI3 mRNA was inhibited in a dose-dependent manner in Trophoblast cells treated with Met ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).

E. The intensity of P28, EBI3 and IL-27 protein bands was reduced in Trophoblast cells treated with Met.

F. Expression of miR-148-5p was elevated in a dose-dependent manner in HUVEC cells treated with Met (N = 3; *P value < 0.05, vs. NC; NC, negative controls).

G. Expression of miR-216-3p was elevated in a dose-dependent manner in HUVEC cells treated with Met (N = 3; *P value < 0.05, vs. NC; NC, negative controls).

H. Expression of P28 mRNA was inhibited in a dose-dependent manner in HUVEC cells treated with Met ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).

I. Expression of EBI3 mRNA was inhibited in a dose-dependent manner in HUVEC cells treated with Met ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).

J. The intensity of P28, EBI3 and IL-27 protein bands was reduced in HUVEC cells treated with Met.

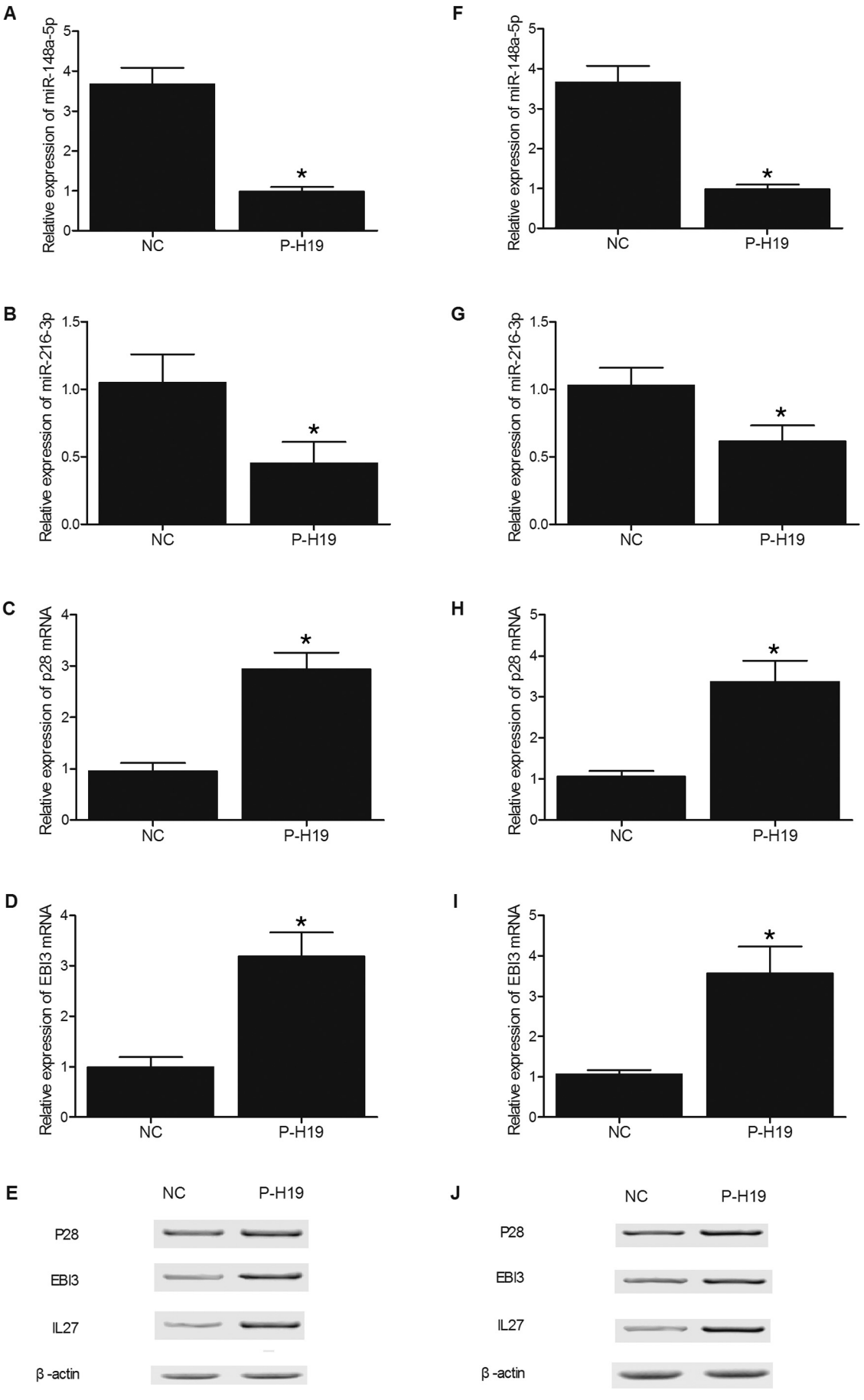

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Fig. 3. Transfection of H19 reduced expressions of miR-148-5p and miR-216-3p, while the expressions of P28 and EBI3 mRNA/protein were increased.
A. Expression of miR-148-5p was reduced in Trophoblast cells transfected with H19 ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).
B. Expression of miR-216-3p was reduced in Trophoblast cells transfected with H19 ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).
C. Expression of P28 mRNA was increased in Trophoblast cells transfected with H19 (N = 3; *P value < 0.05, vs. NC; NC, negative controls).
D. Expression of EBI3 mRNA was increased in Trophoblast cells transfected with H19 ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).
E. The intensity of P28, EBI3 and IL-27 protein bands was increased in Trophoblast cells transfected with H19.
F. Expression of miR-148-5p was reduced in HUVEC cells transfected with H19 ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).
G. Expression of miR-216-3p was reduced in HUVEC cells transfected with H19 ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).
H. Expression of P28 mRNA was increased in HUVEC cells transfected with H19 ($N = 3$; *P value < 0.05, vs. NC; NC, negative controls).
I. Expression of EBI3 mRNA was increased in HUVEC cells transfected with H19 (N = 3; *P value < 0.05, vs. NC; NC, negative controls).
J. The intensity of P28, EBI3 and IL-27 protein bands was increased in HUVEC cells transfected with H19.

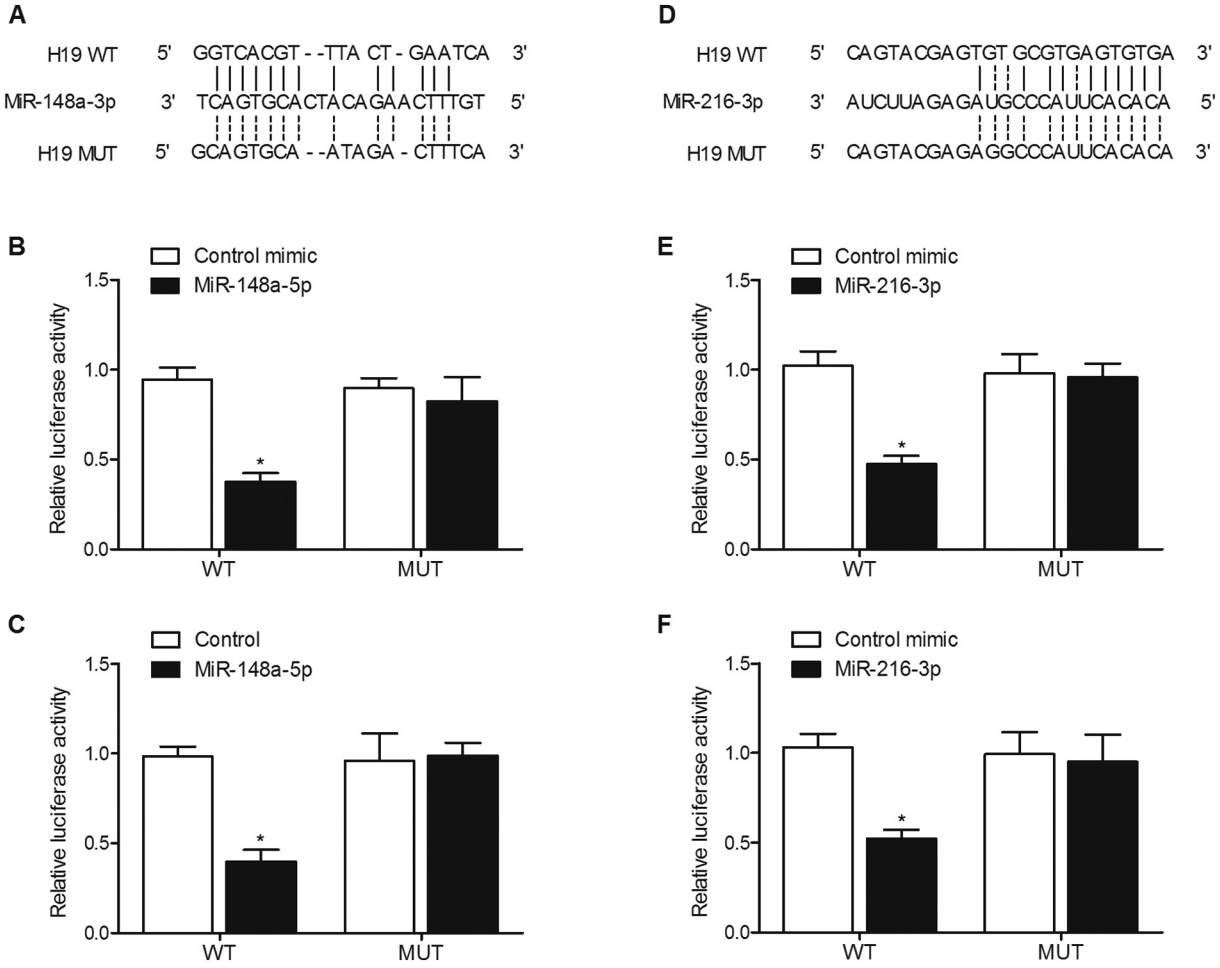

Fig. 4. H19 functioned as a sponge of miR-148a-5p and miR-216-3p, which reduced the relative luciferase activity of cells transfected with miR-148-5p or miR-216-3p.
A. MiR-148a-5p was found to bind to H19 via computational analysis.
B. Reduced luciferase activity was observed in Trophoblast cells co-transfected with miR-148a-5p mimics and wild-type H19 ($N = 3$; *P value < 0.05, vs. control mimics; WT, wild-type; MUT, mutant).
C. Reduced luciferase activity was observed in HUVEC cells co-transfected with miR-148a-5p mimics and wild-type H19 ($N = 3$; *P value < 0.05, vs. control mimics; WT, wild-type; MUT, mutant).
D. MiR-148a-5p was found to bind to H19 via computational analysis.
E. Reduced luciferase activity was observed in Trophoblast cells co-transfected with miR-148a-5p mimics and wild-type H19 ($N = 3$; *P value < 0.05, vs. control mimics; WT, wild-type; MUT, mutant).
F. Reduced luciferase activity was observed in HUVEC cells co-transfected with miR-216-3p mimics and wild-type H19 (N = 3; *P value < 0.05, vs. control mimics; WT, wild-type; MUT, mutant).

inhibits the activation of the H19 signaling pathway, suppresses the expression of P28 and EBI3, and up-regulates the expression of miR-148-5p and miR-216-3p, thus decreasing the levels of IL-27, TNF-α and IL-6, and exhibiting an therapeutic effect during PE treatment.

4. Discussion

As a disorder observed in up to 5% of all pregnancies, PE is featured by proteinuria and hypertension [17,18]. In addition, PE has become a main contributor of perinatal and maternal mortality and morbidity [11]. Interestingly, PE often initiates with local dysfunctions in the

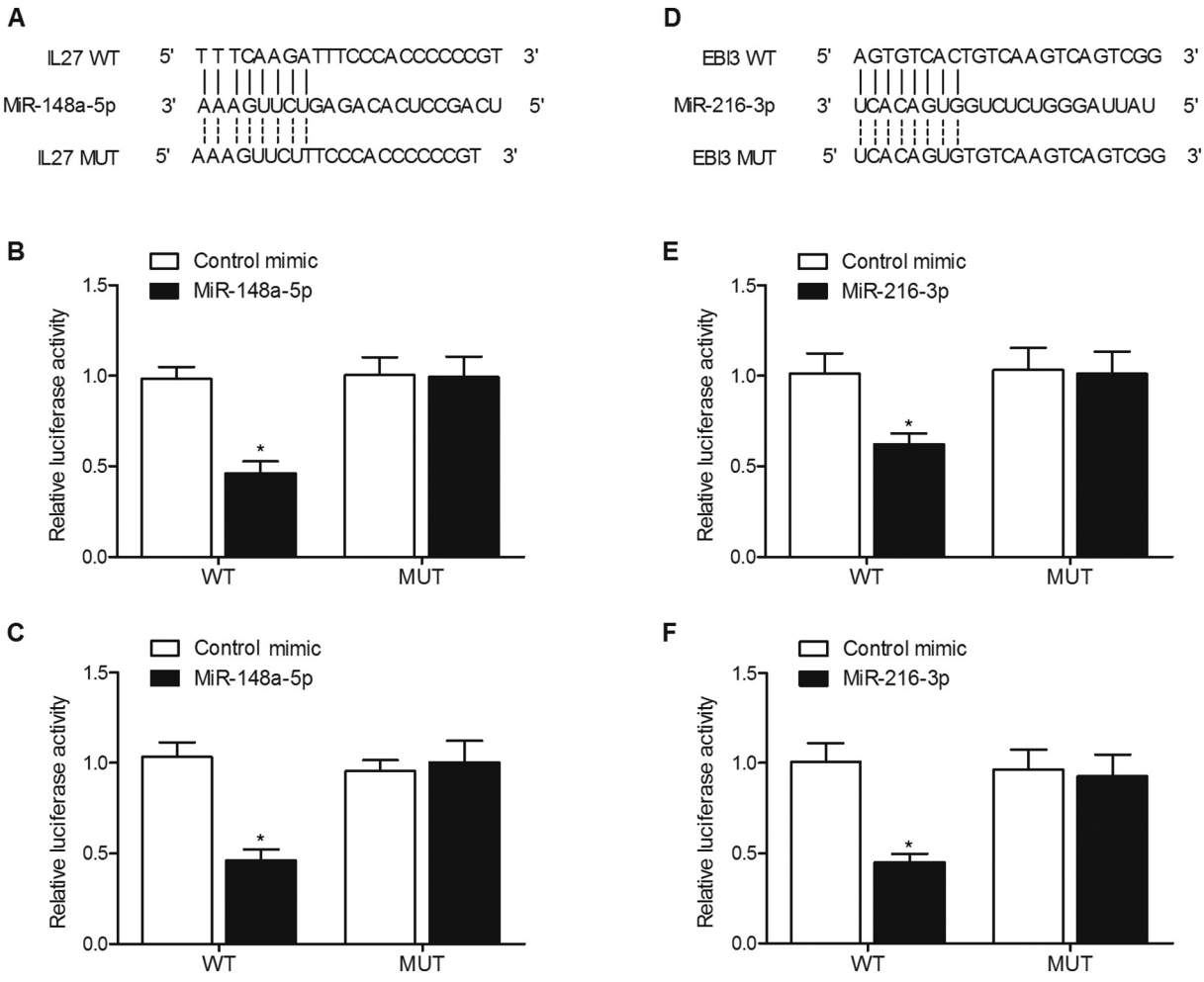

Fig. 5. P28 mRNA reduced relative luciferase activity of cells transfected with miR-148-5p while EBI3 mRNA reduced relative luciferase activity of cells transfected with miR-216-3p.

A. P28 mRNA was identified as potential target gene of miR-148a-5p via computational analysis.

B. Reduced luciferase activity was observed in Trophoblast cells co-transfected with miR-148a-5p mimics and wild-type P28 (N = 3; *P value < 0.05, vs. control mimics; WT, wild-type; MUT, mutant).

C. Reduced luciferase activity was observed in HUVEC cells co-transfected with miR-148a-5p mimics and wild-type P28 (N = 3; *P value < 0.05, vs. control mimics; WT, wild-type; MUT, mutant).

D. EBI3 mRNA were identified as potential target gene of miR-216-3p via computational analysis.

E. Reduced luciferase activity was observed in Trophoblast cells co-transfected with miR-216-3p mimics and wild-type EBI3 (N = 3; *P value < 0.05, vs. control mimics; WT, wild-type; MUT, mutant).

F. Reduced luciferase activity was observed in HUVEC cells co-transfected with miR-216-3p mimics and wild-type EBI3 (N = 3; *P value < 0.05, vs. control mimics; WT, wild-type; MUT, mutant).

placenta and subsequently develops into maternal proteinuria and hypertension. Due to the lack of placental development caused by PE, the inflamed and stressed placenta cannot meet the nutritional demands of gradually growing fetus. Nevertheless, the molecular mechanisms underlying the development of PE remains poorly understood [19,20].

As a biguanide compound, Met has been commonly used in the intervention of type 2 diabetes due to its low side effects and high efficacy [21]. Recently, It has been shown that fms-like tyrosine kinase 1 (sFlt-1), an anti-angiogenic factor present in the circulating blood, could increase the incidence of PE in pregnancies. In addition, it was recently found that Met could reduce the synthesis of sFlt-1 in placental explants and placental tissues [22]. Therefore, Met may become a candidate for the preventive intervention of PE [13]. A previous study also showed that Met induced the degradation of H19 by activating the MAPK pathway [21]. Furthermore, the exposure of endometrial cancer cells in a Met solution changed the pattern of DNA methylation in these cells, presumably mediated by the activity of the H19/SAHH axis. Interestingly, Met could also induce the inhibition of H19 and subsequent methylation of multiple genes in patients with endometrial cancer [21]. These results confirmed that Met is associated with a potential that not only can treat diabetes, but also can treat a wide range of tumors by regulating the MAPK signaling [21]. In this study, the treatment of cells by Met increased the level of H19 methylation and suppressed H19 expression in a dose-dependent manner. Therefore, Met was confirmed to inhibit H19 expression via promoting H19 methylation.

Previously, microRNA miR-148a-3p has been identified as a virtual target of H19, and miR-148a-3p expression was shown to be inhibited by H19. In addition, the suppression of miR-148a-3p expression has been linked to increased invasiveness, migration and proliferation of LSCC. Interestingly, the suppression of LSCC growth could be achieved by silencing the activity of H19. In this study, using computational analyses and luciferase reporter assays, we found that miR-148a-5p and miR-216-3p could bind to H19. Furthermore, Met treatment elevated the levels of miR-148-5p and miR-216-3p while inhibiting the expression of p28 mRNA and EBI3 mRNA in a dose-dependent manner. Moreover, the intensity of IL-27, P28 and EBI3 protein bands was all

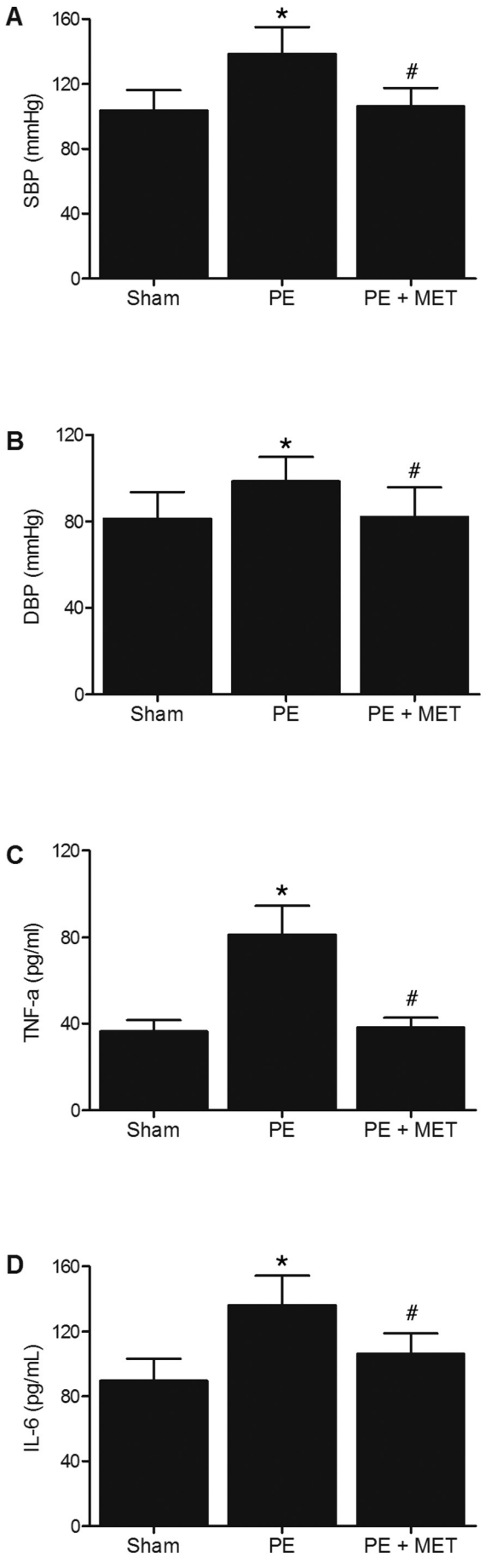

Fig. 6. Met reduced the increased SBP and DBP of PE rats, and the up-regulated expressions of TNF-α/IL-6 in PE rats were also inhibited upon Met treatment.
A. SBP was reduced in PE rats treated with Met compared with that in untreated PE rats (N = 3; *P value < 0.05, vs. sham group; #P value < 0.05, vs. PE group).
B. DBP was reduced in PE rats treated with Met compared with that in untreated PE rats (N = 3; *P value < 0.05, vs. sham group; #P value < 0.05, vs. PE group).
C. TNF-α expression was obviously elevated in PE rats, and the high expression level of TNF-α was evidently reduced by Met treatment (N = 3; *P value < 0.05, vs. sham group; #P value < 0.05, vs. PE group).
D. IL-6 expression was obviously elevated in PE rats, and the high expression level of IL-6 was evidently reduced by Met treatment (N = 3; *P value < 0.05, vs. sham group; #P value < 0.05, vs. PE group).

decreased by the treatment of Met. In addition, the transfection of H19 significantly down-regulated the expression of miR-148a-5p and miR-216-3p, while up-regulating the expression of P28/EBI3 mRNA and protein, confirming the role of Met in the pathogenesis of PE via regulating the H19 signaling pathway.

It is widely recognized that IL-27 functions by forming a heterodimer consisting of EBI3 and p28. On the other hand, the receptor of IL-27 contains a unique binding motif for IL-27 as well as gp130, which can also be found in the receptor of IL-6R [23,24]. It was previously shown that EBi3 is mainly synthesized by B cells infected by Epstein-Barr viruses. However, latest studies suggested that EBi3 can also be produced by keratinocytes, antigen-presenting cells, and T cells [25–27]. Since EBi3 can interact with a wide range of cytokines including IL-27 and IL-35, it can elicit various immune responses mediated by T cells. For example, EBi3 was shown to inhibit the development of Th17 and Th2 cells [28,29]. In addition, IL-35 was shown to induce the secretion of IL-10 by CD8 T cells and Th1 cells, as well as inducing the synthesis of Tim3 by Th1 cells [30–32]. In this study, we established an animal model of PE and treated PE rats with Met. The results showed that the Met treatment helped to decrease the levels of TNF-α and IL-6 in PE rats, although the effect of Met on IL-6 was not significant. Meanwhile, the expression of H19, as well as its downstream targets P28 and EBI3, was inhibited in Met treated PE rats. Furthermore, the levels of miR-148a-5p and miR-216-3p were evidently increased upon Met treatment compared with those in the untreated group. Therefore, Met exhibited a therapeutic effect during PE treatment by reducing the expression of P28 and EBI3 proteins, which in turn reduced the expression of IL-27. Therefore, the SNPs in IL-27 may affect the risk of PE. Previous studies have suggested that the rs17855750 and rs153109 SNPs in IL-27 acted as biomarkers for the genetic risk of PE. In addition, since the glycoprotein encoded by EBI3 shares a similar structure with the p40 subunit in IL-12, it can interact effectively with IL-35 and IL-27 by binding to IL-12P35 and IL-27P28 [33]. Interestingly, EBI3 was also demonstrated to regulate the cytokine productions in T cells and to affect the initiation of infection and inflammation [34]. It was also demonstrated that the level of EBI3 mRNA was increased in macrophages and T cells collected from PE patients, indicating the critical role of EBI3 in PE development.

5. Conclusion

In conclusion, our study revealed that Met could regulate the pathogenesis of PE. In this research, we have demonstrated that the Met/H19/miR-148a-5p/P28 and Met/H19/miR-216-3p/EBI3 pathways played important roles in the regulation of PE.

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Funding

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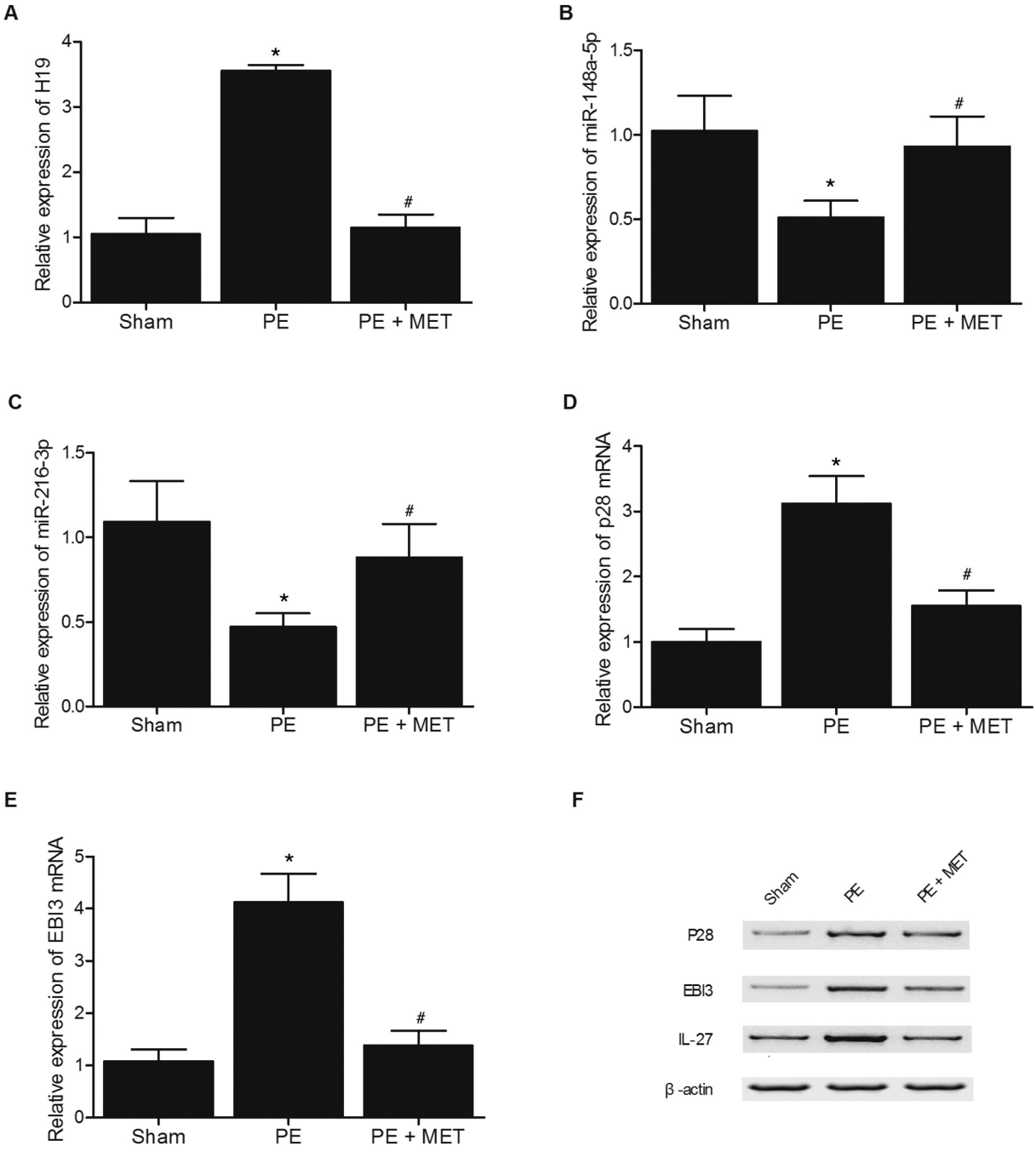

Fig. 7. Met were observed to exhibit therapeutic effects upon PE rats via modulating the signaling pathways of Met/H19/miR-148a-5p/P28 and Met/H19/miR-216-3p/EBI3.

A. H19 expression was evidently up-regulated in PE rats, and Met treatment could reduce H19 expression (N = 3; *P value < 0.05, vs. sham group; #P value < 0.05, vs. PE group).

B. MiR-148a-5p expression was evidently down-regulated in PE rats, and Met treatment could increase miR-148a-5p expression (N = 3; *P value < 0.05, vs. sham group; #P value < 0.05, vs. PE group).

C. MiR-216-3p expression was evidently down-regulated in PE rats, and Met treatment could increase miR-216-3p expression (N = 3; *P value < 0.05, vs. sham group; #P value < 0.05, vs. PE group).

D. P28 mRNA expression was evidently up-regulated in PE rats, and Met treatment could reduce P28 mRNA expression (N = 3; *P value < 0.05, vs. sham group; #P value < 0.05, vs. PE group).

E. EBI3 mRNA expression was evidently up-regulated in PE rats, and Met treatment could reduce EBI3 mRNA expression (N = 3; *P value < 0.05, vs. sham group; #P value < 0.05, vs. PE group).

F. P28, EBI3 and IL-27 expression was significantly repressed in PE rats treated with Met.

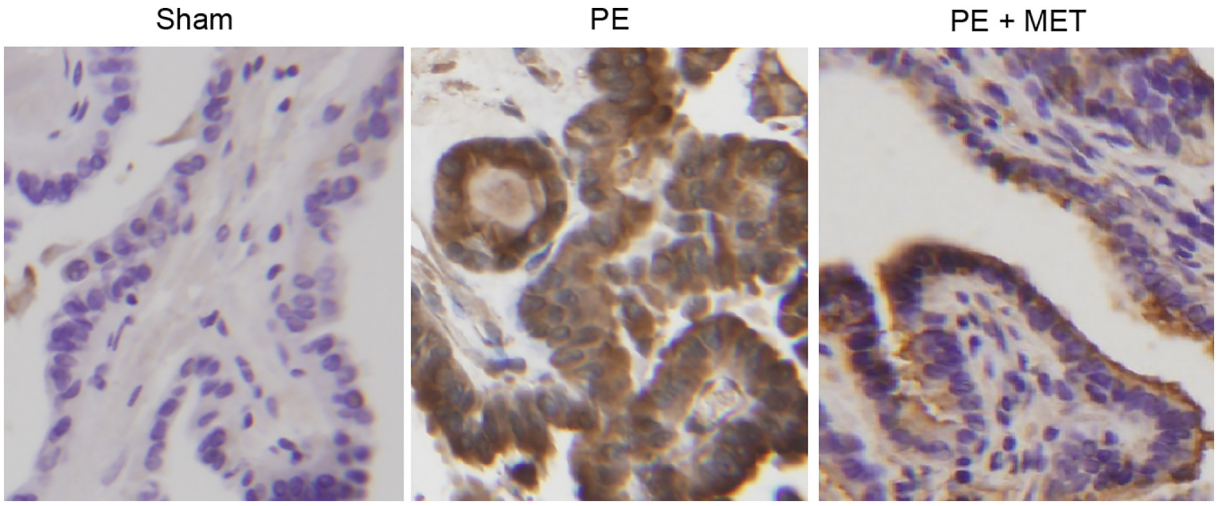

Fig. 8. Met treatment could suppress the expression of IL-27 in PE rats, as shown by the IHC assay.

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Ethical approval

This animal experiment was approved by the ethics committee at the First Hospital of Jilin University. All animal experiments in this study were done in accordance with the principles of management and usage of local laboratory animals, and followed the guidelines published by the National Institutes of Health on the Management and Use of Laboratory Animals.

Declaration of Competing Interest

None.

References

[1] C.V. Ananth, K.M. Keyes, R.J. Wapner, Pre-eclampsia rates in the United States, 1980–2010: age-period-cohort analysis, BMJ 347 (2013) f6564.
[2] S. Saleem, E.M. McClure, S.S. Goudar, A. Patel, F. Esamai, A. Garces, E. Chomba, F. Althabe, J. Moore, B. Kodkany, O. Pasha, J. Belizan, A. Mayansyan, R.J. Derman, P.L. Hibberd, E.A. Liechty, N.F. Krebs, K.M. Hambidge, P. Buekens, W.A. Carlo, L.L. Wright, M. Koso-Thomas, A.H. Jobe, R.L. Goldenberg, A prospective study of maternal, fetal and neonatal deaths in low- and middle-income countries, Bull. World Health Organ. 92 (2014) 605–612.
[3] S. Pflanz, J.C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hibbert, T. Churakova, M. Travis, E. Vaisberg, W.M. Blumenschein, J.D. Mattson, J.L. Wagner, W. To, S. Zurawski, T.K. McClanahan, D.M. Gorman, J.F. Bazan, R. de Waal Malefyt, D. Rennick, R.A. Kastelein, IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells, Immunity 16 (2002) 779–790.
[4] T.H. de Lima, N. Sass, R. Mattar, A.F. Moron, M.R. Torloni, C.S. Franchim, S. Daher, Cytokine gene polymorphisms in preeclampsia and eclampsia, Hypertens. Res. 32 (2009) 565–569.
[5] S. Daher, N. Sass, L.G. Oliveira, R. Mattar, Cytokine genotyping in preeclampsia, Am. J. Reprod. Immunol. 55 (2006) 130–135.
[6] F. Stonek, E. Hafner, M. Metzenbauer, S. Katharina, I. Stumpflen, C. Schneeberger, H. Zeisler, P. Husslein, K. Philipp, Absence of an association of tumor necrosis factor (TNF)-alpha G308A, interleukin-6 (IL-6) G174C and interleukin-10 (IL-10) G1082A polymorphism in women with preeclampsia, J. Reprod. Immunol. 77 (2008) 85–90.
[7] X. Wang, F. Jiang, Y. Liang, L. Xu, H. Li, Y. Liu, S. Liu, Y. Ye, Interleukin-1beta-31C/T and -511T/C polymorphisms were associated with preeclampsia in Chinese Han population, PLoS One 9 (2014) e106919.
[8] N. Yin, H. Zhang, X. Luo, Y. Ding, X. Xiao, X. Liu, N. Shan, X. Zhang, Q. Deng, B. Zhuang, H. Qi, IL-27 activates human trophoblasts to express IP-10 and IL-6: implications in the immunopathophysiology of preeclampsia, Mediat. Inflamm. 2014 (2014) 926875.
[9] B. Liu, Y. Li, Y. Yao, H. Li, H. Liang, M. Xin, L. Wang, L. Zhao, J. Lin, S. Liu, Polymorphisms of the IL27 gene in a Chinese Han population complicated with preeclampsia, Sci. Rep. 6 (2016) 23029.
[10] K. Arun, G. Arunkumar, D. Bennet, S.M. Chandramohan, A.K. Murugan, A.K. Munirajan, Comprehensive analysis of aberrantly expressed lncRNAs and construction of ceRNA network in gastric cancer, Oncotarget 9 (2018) 18386–18399.
[11] M. Harati-Sadegh, L. Kohan, B. Teimoori, S. Salimi, The long non-coding RNA H19 rs217727 polymorphism is associated with PE susceptibility, J. Cell. Biochem. 119 (2018) 5473–5480.
[12] R. Romero, O. Erez, M. Huttemann, E. Maymon, B. Panaitescu, A. Conde-Agudelo, P. Pacora, B.H. Yoon, L.I. Grossman, Metformin, the aspirin of the 21st century: its role in gestational diabetes mellitus, prevention of preeclampsia and cancer, and the promotion of longevity, Am. J. Obstet. Gynecol. 217 (2017) 282–302.
[13] H.T. Ang, K.K. Lim, Y.H. Kwan, P.S. Tan, K.Z. Yap, Z. Banu, C.S. Tan, W. Fong, J. Thumboo, T. Ostbye, L.L. Low, A systematic review and meta-analyses of the association between anti-hypertensive classes and the risk of falls among older adults, Drugs Aging (2018), https://doi.org/10.1007/s40266-018-0561-3.
[14] L. Yan, J. Zhou, Y. Gao, S. Ghazal, L. Lu, S. Bellone, Y. Yang, N. Liu, X. Zhao, A.D. Santin, H. Taylor, Y. Huang, Regulation of tumor cell migration and invasion by the H19/let-7 axis is antagonized by metformin-induced DNA methylation, Oncogene 34 (2015) 3076–3084.
[15] Y. Li, J. Zhang, D. Zhang, X. Hong, Y. Tao, S. Wang, Y. Xu, H. Piao, W. Yin, M. Yu, Y. Zhang, Q. Fu, D. Li, X. Chang, M. Du, Tim-3 signaling in peripheral NK cells promotes maternal-fetal immune tolerance and alleviates pregnancy loss, Sci. Signal. 10 (2017).
[16] E. Dokmeci, L. Xu, E. Robinson, K. Golubets, K. Bottomly, C.A. Herrick, EBI3 deficiency leads to diminished T helper type 1 and increased T helper type 2 mediated airway inflammation, Immunology 132 (2011) 559–566.
[17] P.H. Andraweera, K.L. Gatford, G.A. Dekker, S. Leemaqz, R.W. Jayasekara, V.H.W. Dissanayake, L. McCowan, C.T. Roberts, The INSR rs2059806 single nucleotide polymorphism, a genetic risk factor for vascular and metabolic disease, associates with pre-eclampsia, Reprod. BioMed. Online 34 (2017) 392–398.
[18] A. Mohammadpour-Gharehbagh, S. Salimi, F. Keshavarzi, F. Saeidian, M. Mousavi, B. Teimoori, M. Esmaeilipour, M. Mokhtari, Genetic variants in 3'-UTRs of MTHFR in the pregnancies complicated with preeclampsia and bioinformatics analysis, J. Cell. Biochem. 119 (2018) 773–781.
[19] R. McGinnis, V. Steinthorsdottir, N.O. Williams, G. Thorleifsson, S. Shooter, S. Hjartardottir, S. Bumpstead, L. Stefansdottir, L. Hildyard, J.K. Sigurdsson, J.P. Kemp, G.B. Silva, L.C.V. Thomsen, T. Jaaskelainen, E. Kajantie, S. Chappell, N. Kalsheker, A. Moffett, S. Hiby, W.K. Lee, S. Padmanabhan, N.A.B. Simpson, V.A. Dolby, E. Staines-Urias, S.M. Engel, A. Haugan, L. Trogstad, G. Svyatova, N. Zakhidova, D. Najmutdinova, A.F. Dominiczak, H.K. Gjessing, J.P. Casas, F. Dudbridge, J.J. Walker, F.B. Pipkin, U. Thorsteinsdottir, R.T. Geirsson, D.A. Lawlor, A.C. Iversen, P. Magnus, H. Laivuori, K. Stefansson, L. Morgan, Variants in the fetal genome near FLT1 are associated with risk of preeclampsia, Nat. Genet. 49 (2017) 1255–1260.
[20] S. Verlohren, A. Galindo, D. Schlembach, H. Zeisler, I. Herraiz, M.G. Moertl, J. Pape, J.W. Dudenhausen, B. Denk, H. Stepan, An automated method for the determination of the sFlt-1/PlGF ratio in the assessment of preeclampsia, Am. J. Obstet. Gynecol. 202 (161) (2010) 425–430.
[21] T. Zhong, Y. Men, L. Lu, T. Geng, J. Zhou, A. Mitsuhashi, M. Shozu, N.J. Maihle, G.G. Carmichael, H.S. Taylor, Y. Huang, Metformin alters DNA methylation genome-wide via the H19/SAHH axis, Oncogene 36 (2017) 2345–2354.
[22] F.C. Brownfoot, R. Hastie, N.J. Hannan, P. Cannon, L. Tuohey, L.J. Parry, S. Senadheera, S.E. Illanes, T.J. Kaitu'u-Lino, S. Tong, Metformin as a prevention and treatment for preeclampsia: effects on soluble fms-like tyrosine kinase 1 and soluble endoglin secretion and endothelial dysfunction, Am. J. Obstet. Gynecol. 214 (2016) (356 e351-356 e315).
[23] R.G. Titus, M. Marchand, T. Boon, J.A. Louis, A limiting dilution assay for quantifying Leishmania major in tissues of infected mice, Parasite Immunol. 7 (1985) 545–555.
[24] P.A. Buffet, A. Sulahian, Y.J. Garin, N. Nassar, F. Derouin, Culture microtitration: a

sensitive method for quantifying Leishmania infantum in tissues of infected mice, Antimicrob. Agents Chemother. 39 (1995) 2167–2168.
[25] F. Arias, L. Rodriquez, S.C. Rayne, F.T. Kraus, Maternal placental vasculopathy and infection: two distinct subgroups among patients with preterm labor and preterm ruptured membranes, Am. J. Obstet. Gynecol. 168 (1993) 585–591.
[26] B. Huppertz, Placental origins of preeclampsia: challenging the current hypothesis, Hypertension 51 (2008) 970–975.
[27] C.W. Redman, I.L. Sargent, Pre-eclampsia, the placenta and the maternal systemic inflammatory response—a review, Placenta 24 (Suppl A) (2003) S21–S27.
[28] B. Cox, Bioinformatic approach to the genetics of preeclampsia, Obstet. Gynecol. 124 (2014) 633.
[29] L.C. Sanchez-Aranguren, C.E. Prada, C.E. Riano-Medina, M. Lopez, Endothelial dysfunction and preeclampsia: role of oxidative stress, Front. Physiol. 5 (2014) 372.
[30] C.P. Chen, Placental villous mesenchymal cells trigger trophoblast invasion, Cell Adhes. Migr. 8 (2014) 94–97.
[31] T. Cotechini, C.H. Graham, Aberrant maternal inflammation as a cause of pregnancy complications: a potential therapeutic target? Placenta 36 (2015) 960–966.
[32] A. Szarka, J. Rigo Jr., L. Lazar, G. Beko, A. Molvarec, Circulating cytokines, chemokines and adhesion molecules in normal pregnancy and preeclampsia determined by multiplex suspension array, BMC Immunol. 11 (2010) 59.
[33] E. Tirotta, P. Duncker, J. Oak, S. Klaus, M.R. Tsukamoto, L. Gov, T.E. Lane, Epstein-Barr virus-induced gene 3 negatively regulates neuroinflammation and T cell activation following coronavirus-induced encephalomyelitis, J. Neuroimmunol. 254 (2013) 110–116.
[34] R. Zheng, H. Liu, P. Song, Y. Feng, L. Qin, X. Huang, J. Chen, H. Yang, Z. Liu, Z. Cui, Z. Hu, B. Ge, Epstein-Barr virus-induced gene 3 (EBI3) polymorphisms and expression are associated with susceptibility to pulmonary tuberculosis, Tuberculosis (Edinb) 95 (2015) 497–504.