Ligustrazine-induced microRNA-16-5p inhibition alleviates preeclampsia through IGF-2

Yuan Yuan^{1,*}, Ling Zhao^{2,*}, Xiaoying Wang¹, Feng Lian³ and Yan Cai¹

¹Department of Obstetrics, The Fourth Affiliated Hospital of Harbin Medical University, Harbin, China, ²The Second Department of Respiratory, Harbin Medical University Cancer Hospital, Harbin, China and ³The Third Department of Orthopaedics, The Fourth Affiliated Hospital of Harbin Medical University, Harbin, China

Correspondence should be addressed to Y Cai; Email: caiyang650318@163.com

*(Y Yuan and L Zhao contributed equally to this work)

Abstract

Preeclampsia (PE), a serious complication of pregnancy, is associated with abnormal trophoblast cell differentiation and autophagy. Herein, we investigated the molecular mechanism underlying the function of ligustrazine (2,3,5,6-tetramethylpyrazine, TMP), a constituent of the traditional Chinese plant medicine *Ligusticum wallichii*, in PE. Lipopolysaccharide (LPS) was applied to induce a PE rat model, followed by tail vein injection of TMP or lentiviral vector overexpressing microRNA-16-5p (miR-16-5p). Human trophoblast cell line JEG3 was cultured in vitro to construct a PE cell model, followed by the treatment with different concentrations of TMP, miR-16-5p mimic/inhibitor, or shRNA (shRNA) against insulin growth factor-2 (IGF-2) (sh-IGF-2). Formation of autophagosomes and autophagy-related proteins were then examined. Cell counting kit-8 (CCK-8) and Transwell assays were applied to measure trophoblast cell viability and migration. The binding affinity between miR-16-5p and IGF-2 was verified by dual luciferase report assay. After TMP treatment, autophagosome formation was reduced in trophoblast cells of placental tissue of PE rats, along with downregulation of autophagy-related proteins Light Chain 3 (LC3)-II/LC3-I, Beclin1 (BECN1), and SQSTM1. Moreover, TMP repressed JEG3 cell autophagy, promoted viability and migration concentration-responsively. MiR-16-5p was upregulated in PE, and TMP inhibited miR-16-5p expression. Besides, miR-16-5p downregulated IGF-2 expression to promote cell autophagy and inhibit the viability and migration of JEG3 cells. Further, in vivo experiments validated that TMP impeded PE progression in rats by regulating the miR-16-5p/IGF-2 axis. In summary, TMP inhibits trophoblast cell autophagy and promotes its viability and migration in PE rat model through regulating the miR-16-5p/IGF-2 axis.

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Introduction

Preeclampsia (PE) is a frequent complication of pregnancy, which arises in the placenta and results in a wide array of maternal and fetal disorders (Redman & Sargent 2005). According to the World Health Organization, 10% of pregnant women are diagnosed as having PE, and PE accounts for one-seventh of maternal deaths in China, which is equivalent to an incidence rate of 5% (Say et al. 2014, Liang et al. 2019, Yang et al. 2020). PE is a pregnancy complication mainly occurring after 20 weeks of gestation, which is characterized by maternal hypertension and proteinuria (Phillips et al. 2020, Rozas-Villanueva et al. 2020). PE usually first manifests with hypertension followed by proteinuria in the third trimester, and the pathogenesis and impact on both mother and fetus make it a tremendously challenging condition (Rana et al. 2019). Previous research links PE to insufficient extravillous cytotrophoblast invasion and aberrantly excessive apoptosis in the placental

trophoblast cells (Leach et al. 2002). Thus, the abnormal interplay of trophoblast and decidua-membrane is a major cause underlying PE (Vento-Tormo et al. 2018). Notably, traditional Chinese medicine (TCM) such as the herb *Uncaria rhynchophylla* has been widely applied for PE in China (Wang et al. 2018).

Ligustrazine (2,3,5,6-tetramethylpyrazine, TMP) is extracted from a traditional Chinese medicine *Ligusticum wallichii*, which has been proven to alleviate microcirculatory dysfunction and lung injury (Zhang & Dang 2006). A prior study has suggested that TMP injections led to remarkable therapeutic effects on pregnancy-induced hypertension through repressing fibrinolysis and enhancing microcirculation (Wang & Zhao 2003). Moreover, TMP administration attenuates placental endoplasmic reticulum stress in PE rats (Gu et al. 2019). However, the molecular mechanisms underlying the pharmacological actions of TMP remain uncharacterized. Recent microRNA (miRNA) profiling studies have implicated the potential use of miRNAs

as diagnostic and prognostic indicators in pregnancy complications, including PE (Choi *et al.* 2013). MiRNAs represent a series of noncoding 21- to 25-nucleotide RNAs which can inversely modulate gene expression posttranscriptionally (Wang *et al.* 2012a). Interestingly, microRNA-16 (miR-16) is expressed in the placental tissue and can modulate placental cell survival and development pathways (Maccani *et al.* 2011). A prior study by Wang *et al.* has pointed out the involvement of miR-16 in the pathogenesis of PE (Wang *et al.* 2012b), which sheds some light on the potential role of miR-16 in the clinical setting of PE. In this study, an initial bioinformatics analysis predicted the targeting relationship between miR-16-5p and insulin-like growth factor 2 (IGF-2). Previous work has shown IGF-2 to have a pivotal role in fetal development, and its abnormal expression is linked with maternal blood pressure dysfunction during pregnancy (Bozadjieva 2011). Previous work has shown an association between IGF-2 expression and PE, yet the underlying mechanism needs further study (Jung *et al.* 2019). We thus predicted that TMP would affect PE progression in rats by regulating the miR-16-5p/IGF-2 axis. Therefore, we aimed in this study to test this hypothesized mechanism of the miR-16-5p/IGF-2 axis underlying the pharmacological actions of TMP in a PE rat model.

Materials and methods

Ethics statement

The study was conducted under the approval of the ethics committee of the Fourth Affiliated Hospital of Harbin Medical University. All animal experiments were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Experimental animals

Healthy clean grade female Sprague Dawley (SD) rats (200–250 g) and male SD rats (250–300 g) of 8–10 weeks old were purchased from Experimental Animal Center of The Secondary Military Medical University (SCXK (Hu)-2013-0016). All rats were routinely housed in an SPF environment for 1 week's acclimation. For breeding, female rats and male rats were housed in the same constant temperature cages at a gender ratio of 2:1. Cohabitation time was from 16:00 to 08:00 h the next day. After separating the rats, the vaginal plugs were examined, and vaginal smears were taken to observe sperm and epithelial cells. If sperm were present on the smear, that day was considered the first day of rat pregnancy.

Pregnant rats were randomly classified into the blank group ($n=10$, from the 7th day to the 18th day of pregnancy, physiological saline was injected through the tail vein at a dose of 1.0 $\mu\text{g/kg/day}$) or subjected to lipopolysaccharides (LPS) treatment ($n=80$, from the 7th day to the 18th day of pregnancy, the LPS solution (L-2880, Sigma) was injected

through the tail vein at a dose of 20 $\mu\text{g/kg/day}$) (Li *et al.* 2018). From the 3rd day of pregnancy, a BP-2010 A automatic noninvasive blood pressure monitor (Softron Co., Beijing, China) was used to monitor the arterial blood pressure of rats (before LPS injection) every 3 days. In addition, an automatic biochemical analyzer (Roche) was used to measure the 24-h urine protein of rats (before LPS injection) every 3 days. The presence of elevated arterial pressure exceeding 16 kPa (normal blood pressure value = 14.5 ± 0.5 kPa), and a concurrent large increase in urinary protein at 24 h indicated the successful establishment of the PE model in rats.

Animal treatment

The LPS-induced PE model rats were assigned into:

- (1) the LPS group (untreated); LPS+DMSO group: during PE modeling, DMSO solution (Sigma-Aldrich) was injected intraperitoneally from the 7th to 18th day of pregnancy at a dose of 60 mg/kg/day ;
- (2) LPS+TMP group: during PE modeling, TMP injection (20 mg/mL , Wuxi No. 7 Pharmaceutical Factory, China) was injected intraperitoneally from the 7th to 18th day of pregnancy at a dose of 60 mg/kg/day (Gu *et al.* 2019);
- (3) LPS+agomir-NC group: during PE modeling, lentiviral vectors containing negative control sequence of miR-16-5p agomir were injected into a rat tail vein from the 13th day to 18th day at a dose of 0.5 mg/kg/day ;
- (4) LPS+agomir-16-5p group: during PE modeling, lentiviral vectors overexpressing miR-16-5p were injected into a rat tail vein from the 13rd day to 18th day at a dose of 0.5 mg/kg/day ;
- (5) agomir-NC+DMSO group: during PE modeling, the rats were injected intraperitoneally with DMSO solution at the dose of 60 mg/kg/day from the 7th to the 18th day of pregnancy, and the negative control of miR-16-5p agomir was injected into a tail vein at the dose of 0.5 mg/kg/day ;
- (6) agomir-16-5p+DMSO group: during PE modeling, rats were injected intraperitoneally with DMSO solution from the 7th to the 18th day of pregnancy at a dose of 60 mg/kg/day . From the 13th day to 18th day, lentiviral vectors overexpressing miR-16-5p was injected into a tail vein at a dose of 0.5 mg/kg/day ;
- (7) agomir-16-5p+TMP group: during PE modeling, TMP solution was injected intraperitoneally at a dose of 60 mg/kg/day on the 7th to 18th days of pregnancy, and the lentiviral vectors overexpressing miR-16-5p was injected at a dose of 0.5 mg/kg/day through a tail vein from the 13th day to 18th day. The agomir-16-5p and agomir-NC were purchased from GenePharma (Shanghai, China). From the 3rd day of pregnancy, the arterial blood pressure and 24-h urine protein of the rats were monitored every 3 days. On the 19th day, all rats were killed by overdose with intraperitoneal injection of sodium pentobarbital. The rats were given a Cesarean section, and the placenta tissue was removed and rinsed with normal saline. One half of the sample was fixed by immersion in 4% formaldehyde solution, embedded in paraffin and sectioned for HE staining (Zhou *et al.* 2017). The other half was stored at -80°C for molecular analysis.

Human trophoblast cell culture

Human trophoblast cell line JEG3 was purchased from the Medical Research Center of Beijing Chaoyang Hospital. JEG-3 cells were cultured in DMEM (4.5 g/L Glucose) (Gibco) containing 10% FBS (HyClone, Logan, UT, USA) and cultured at 37°C with 5% CO₂. The culture medium was renewed every 2 days and passaged every 5 days (Li *et al.* 2019).

TMP treatment of JEG3 cells

JEG3 cells in logarithmic growth phase were digested with 0.25% trypsin to make a single cell suspension. The cells were plated at 5×10^4 cells/well in a 24-well plate and cultured overnight. After that, TMP solutions with a final concentration of 0 (the same volume of PBS solution), 2.5, 5 and 10 nM were added to the medium. Then the cells were first cultured in a closed container at 37°C, 5% CO₂, 94.5% N₂, and 0.5% O₂ for 8 hours, and then transferred to a constant temperature incubator at 37°C, 5% CO₂, and 20% O₂ for 16 h.

Cell transfection and grouping

The mimic-NC, miR-16-5p mimic (Catalog No. miR10000069-1-5), inhibitor-NC and miR-16-5p inhibitor (Catalog No. miR20000069-1-5) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China), and transduced using Lipofectamine 2000 (Invitrogen) at a concentration of 100 nm/1000 cells. ENLs reagent (enhanced infection solution; Catalog No. REVG0002, GeneChem Co. Ltd., Shanghai, China) was applied according to the manufacturers' protocols to transfect recombinant lentiviruses containing IGF-2-1 shRNA, IGF-2-2 shRNA, and sh-NC empty vector (all lentiviral vectors were purchased from GeneChem Co. Ltd., Shanghai, China). The transfected JEG3 cells were all cultured at 37°C and 5% CO₂ for 48 h for subsequent experiments.

Untransfected JEG3 cells or those transfected for 48 h were plated at 5×10^4 cells/well in 24-well plates and cultured overnight. JEG3 cells are grouped as follows: (1) control group (untransfected cells as a control); (2) mimic-NC group (cells transfected with miR-16-5p negative control plasmid); (3) miR-16-5p mimic group (cells transfected with miR-16-5p mimic plasmid); (4) inhibitor-NC group (cells transfected with miR-16-5p negative control plasmid); (5) miR-16-5p inhibitor group (cells transfected with miR-16-5p inhibitor plasmid); (6) sh-NC group (cell transfected with sh-NC empty vector); (7) sh-IGF-2 group (cell transfected with IGF-2 shRNA recombinant lentivirus); (8) sh-IGF-2-1 group (cells transfected with recombinant lentivirus expressing IGF-2-1 shRNA); (9) sh-IGF-2-2 group (Cells transfected with recombinant lentivirus expressing IGF-2-2 shRNA); (10) inhibitor-NC+sh-NC group (cells transfected with inhibitor-NC plasmid+sh-NC empty vector); (11) inhibitor-NC+sh-IGF-2 group (cells transfected with inhibitor-NC plasmid+sh-IGF-2 recombinant lentivirus); (12) miR-16-5p inhibitor+sh-IGF-2 group (cells transfected with miR-16-5p inhibitor plasmid+sh-IGF-2 recombinant lentivirus); (13) miR-16-5p mimic+DMSO group (cells were transfected with miR-16-5p mimic plasmid and 10 nM vehicle

control DMSO was added); (14) miR-16-5p mimic+TMP group (cell were transfected with miR-16-5p mimic plasmid and added with 10 nM TMP); (15) sh-IGF-2+DMSO group (cells were transfected with sh-IGF-2 recombinant lentivirus and added with 10 nM DMSO); (16) sh-IGF-2+TMP group (cells transfected with sh-IGF-2 recombinant lentivirus and treated with 10 nM TMP).

EdU assay

The cells to be tested were seeded in a 24-well plate, and three replicates were made for each group. Cells were incubated with EdU (concentration=10 µmol/L) for 2 h. The medium was aspirated, and the cells were fixed at room temperature in a PBS solution containing 4% paraformaldehyde. DAPI was employed to stain the nucleus. After mounting, randomly selected 6–10 fields were observed under a fluorescence microscope, and the number of EdU positive cells in each field was recorded.

Transmission electron microscope (TEM) observation

The placental tissue was cut into pieces, and these tissue pieces or JEG3 cells were fixed with a fixing solution (2% paraformaldehyde, 2.5% glutaraldehyde) at 4°C for 1 h, and then in 1% osmic acid for 1.5 hours. Tissues were dehydrated with gradient alcohol, dipped into epoxy resin overnight, embedded, and polymerized for 24 h at 35, 45, and then 60°C. After ultra-thin sectioning and lead-uranium staining, slices were observed using a transmission electron microscope (JEM-1011; JEOL, Tokyo, Japan) under an acceleration voltage of 80 kV. Photographs were taken using a side-mounted Camera-Megaview III (Soft Imaging System, Münster, Germany).

Western blots

Tissues or cells were added with pre-cold lysate (R0010, solarbio) containing PMSF and phosphatase inhibitors, ground thoroughly, and lysed on ice. After that, the lysed samples were centrifuged at 6037 *g* and 4°C for 10 min to harvest the supernatant. The total protein concentration was measured using a BCA kit (Pierce). Then, 20 µg of protein was dissolved in 2 × SDS loading buffer and electrophoresed on 10% SDS-PAGE. The protein was transferred to a PVDF membrane by a wet transfer method, and the membrane was blocked with 5% skim milk powder at room temperature for 1 h. Membranes were immunoblotted at 4°C overnight with diluted primary antibodies rabbit anti-IGF-2 (ab63984, 1:500, Abcam), LC3-II (ab48394, 1 µg/mL, Abcam), LC3-I (ab128025, 2 µg/mL, Abcam), Beclin1 (ab62557, 1 µg/mL, Abcam), SQSTM1 (ab91526, 1 µg/mL, Abcam), Ki67 (ab92472, 1:5000, Abcam) and GAPDH (ab8245, 1: 10,000). After washing three times with TBST, the membrane was incubated with HRP-labeled secondary antibody IgG (1:1000) for 1 h. Immunoreactive bands were visualized using ECL detection kit (BB-3501, Amersham Biosciences). Data were analyzed with the Molecular Imager (Gel DocTM XR, 170-8170) and the associated software Quantity One-4.6.5 (Bio-Rad Laboratories).

Table 1 Primer sequences for RT-qPCR.

Gene	Forward (5'–3')	Reverse (5'–3')
miR-16-5p	TGGGGTAGCAGCACGTAA	CTCAACTGGTGTCTGGAGTC
U6	CTCGCTTCGGCAGCACAT	AACGCTTCACGAATTGCGT
IGF-2	GTGACCAGCAAGGCACAAATC	CACCAAGTAGGCACCACTAAG
GAPDH	TCATGAAGTGTGACGTTGACATCCGT	CCTAGAAGCATTTGCGGTGCACGATG

RNA isolation and quantitation

Total RNA was purified from cells using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. IGF-2 was reverse transcribed using PrimeScript RT reagent Kit (Promega). After this, gene expression was quantified using SYBR Green Master Mix (Life Technologies). For the detection of miR-16-5p, cDNA was obtained by RT using the Mir-XTM miRNA First Strand Synthesis Kit (Takara). Quantification of miR-16-5p level was checked by Mir-XTM miRNA qRT-PCR TB GreenTM Kit (Takara). The miR-16-5p (normalized to U6) or IGF-2 gene (normalized to GAPDH and β -actin) levels were determined using $2^{-\Delta\Delta Ct}$ method (Table 1) with the formula as $\Delta\Delta Ct = (CT_{\text{target}} - CT_{\text{internal control}})_{\text{Subject}} - (CT_{\text{target}} - CT_{\text{internal control}})_{\text{Control}}$. $CT_{\text{internal control}}$ is the geometric mean of β -actin and GAPDH CTs. Outliers were excluded if the normalized (delta Ct) values were N2 standard deviations from the group mean. Relative expression levels are reported as fold change and ΔCt values are used for further statistical analysis (Applied Biosystems User Bulletin No. 2).

CCK-8 assay

JEG3 cell viability as measured using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, USA). After 48 h transfection, cells (5×10^3 cells per well) were seeded in a 96-well plate, and incubated in a 37°C, 5% CO₂ incubator. Each group was repeated three times. The 96-well plates were removed at 24, 48, and 72 h. After adding 10 μ L/well CCK-8 solution under sterile conditions, the incubation was continued in a 37°C, 5% CO₂ incubator for about 1.5 h. The OD value was measured with an automatic microplate reader (Molecular Devices, USA) at a wavelength of 450 nm.

Transwell migration assay

Cells in logarithmic growth phase were digested with 0.25% trypsin, resuspended in serum-free DMEM and adjusted to a cell density of 5×10^4 cells/mL. Cell suspension (100 μ L) was added to the upper chamber of a 24-well Transwell chamber (BioCoat; BD Biosciences, San Jose, CA, USA). Then, 500 μ L of 10% FBS-containing DMEM was added in advance to the lower chamber, and incubated in a 37°C, 5% CO₂ incubator for 24 h. The chamber was fixed with methanol for 10 min and stained for 10 min using crystal violet. A cotton swab was employed to carefully wipe the cells from the upper surface of chamber. The chamber polycarbonate membrane was sealed in a glass slide with a neutral resin, and cells were counted in six random fields under a microscope.

Luciferase activity assay

The luciferase reporter gene assay was adopted to verify the targeting relationship between IGF-2 and miR-16-5p. IGF-2 3'UTR gene fragment was artificially synthesized, and the complementary sequence mutation site of the seed sequence was designed on the IGF-2 wild type, and introduced into the pMIR-reporter (Promega) via the endonuclease sites *SpeI* and *HindIII* to construct the target gene IGF-2 dual-luciferase reporter gene vector and mutants with mutations in the binding site to miR-16-5p: PGLO-IGF-2 Wt and PGLO-IGF-2 Mut. Wild type (WT) and mutant (MUT) luciferase reporter plasmids were co-transfected with miR-16-5p mimic plasmid/mimic-NC plasmid and Renilla vector (pRL-TK; Promega), respectively, into HEK-293T cells. Twenty-four hours after transfection, the cells were lysed according to the Dual-Luciferase Reporter Assay Kit (Promega), incubated at 25°C for 20 min, and the supernatant was collected. The Dual-Luciferase Reporter Assay Kit was employed to measure firefly luciferase activity and Renilla luciferase activity. The relative luciferase activity was calculated as the ratio of fire fly luciferase to Renilla luciferase.

Statistical analysis

All data were processed using SPSS 21.0 statistical software (IBM Corp). Measurement data were expressed as mean $\pm$ s.d. Conforming to normal distribution and homogeneous variance, data between two groups were compared using unpaired *t* test. The comparison between multiple groups was analyzed by one-way ANOVA with Tukey's *post hoc* test. Data comparison between groups at different time points was performed by two-way ANOVA. Pearson correlation analysis was carried out for the correlation between miR-16-5p and IGF-2. $P < 0.05$ indicates that the difference is statistically significant.

Results

TMP has a good therapeutic effect on PE rat models

We employed tail vein injection of LPS to develop a PE model in rats, and monitored the arterial pressure and 24 h urine protein content of the rats from the 3rd to the 18th day of their pregnancy. There was no significant change in arterial pressure and 24 h urine protein in rats of the blank group from the 3rd to the 18th day ($P > 0.05$). Relative to the blank group, the arterial pressure of LPS-induced rats increased with the passage of time (>30 mmHg) from the 6th day, and the 24 h urine protein content also rose ($P < 0.05$) (Fig. 1A and B). These results indicated that the PE rat model was successfully established.

Figure 1 Therapeutic effect of TMP on LPS-induced PE rat model. (A) Changes in arterial pressure of rats with/without LPS induction at various time points monitored by a blood pressure monitor; (B) changes of 24 h urine protein content of rats with/without LPS induction at various time points monitored by an automatic biochemical instrument; (C) the impact of TMP treatment on arterial pressure of LPS-induced rats monitored by a blood pressure monitor; (D) the impact of TMP treatment on 24-hour urine protein content in LPS-induced rats monitored by an automatic biochemical instrument; (E) HE staining to observe the pathological characteristics of placental tissue ($\times 400$); (F and G) Western blot assay to measure autophagy-related proteins LC3-II/LC3-I, Beclin1, and SQSTM1 and proliferation-related proteins Ki67 expression in placental tissues. $*P < 0.05$ vs blank group or LPS + DMSO group, $n = 10$. Measurement data are expressed as mean $\pm$ s.d. Comparisons between groups were performed using one-way ANOVA with Tukey's post hoc test. Data comparison between groups at different time points was performed by two-way ANOVA.

To unveil the effect of TMP on the development of PE in rats, we injected TMP into PE rats and used DMSO as a vehicle control. DMSO vehicle had no significant effect on the arterial pressure and 24 h urine protein content of LPS-induced rats at any time point ($P > 0.05$). However, TMP treatment reduced the arterial pressure and 24 h urine protein content of LPS-induced rats at various time points ($P < 0.05$) (Fig. 1C and D). TEM observation showed that DMSO did not change the number of autophagosomes in the trophoblast cells of LPS-induced rats. However, TMP treatment reduced the number of autophagosomes in trophoblast cells of placental tissue in LPS-induced rats ($P < 0.05$) (Supplementary Fig. 1A, see section on [supplementary materials](#) given at the end of this article). The HE staining results displayed obvious placental tissue cysts, basal trophoblast giant cell proliferation, elevated vacuole cells, thickened basal bands, and obvious inflammatory cell infiltration in the LPS-induced rats with or without DMSO. After TMP treatment, the incidence of cysticity, basement thickening, and inflammatory cell infiltration were lower in placental tissues of LPS-induced rats (Fig. 1E). Western blot assay showed that DMSO could not affect the expression of autophagy-related proteins LC3-II/LC3-I, Beclin1, and SQSTM1, and the expression of proliferation-related protein Ki67 ($P > 0.05$). However, TMP treatment reduced the protein expression of

LC3-II/LC3-I, Beclin1, and SQSTM1, and elevated the protein expression of Ki67 ($P < 0.05$) (Fig. 1F and G). The above results suggest that TMP has an alleviatory effect on PE rat models.

TMP inhibits autophagy and enhances viability and migration in JEG3 cell

We added TMP at different concentrations (0, 2.5, 5, and 10 nM) to the medium to probe the effect of TMP on the progress of PE in JEG3 cells. TEM results demonstrated that relative to 0 nM TMP, TMP at 2.5, 5, and 10 nM reduced the number of autophagosomes formed in JEG3 cells, and higher concentrations led to the formation of fewer autophagosomes, thus showing certain concentration dependence (Supplementary Fig. 1B). CCK-8 assay (Fig. 2A) and Transwell assay (Fig. 2B and C) showed that JEG3 cell viability and migration ability were elevated with increasing TMP concentration, showing certain concentration dependence ($P < 0.05$). Western blot assay showed that versus 0 nM TMP, TMP at 2.5, 5, and 10 nM diminished the protein expression of LC3-II/LC3-I, Beclin1, and SQSTM1 in JEG3 cells, and elevated the protein expression of Ki67, indicating a certain concentration dependence ($P < 0.05$) (Fig. 2D). The above results show that TMP can repress autophagy of JEG3 cells and promote viability and migration concentration-responsively.

Figure 2 TMP inhibits autophagy and enhances viability and migration in JEG3 cells. (A) CCK-8 assay to detect the effect of TMP at different concentrations on JEG3 cell proliferation; (B and C) Transwell assay to detect the effect of TMP at different concentrations on JEG3 cell migration ($\times 200$); (D) Western blot assay to detect the effect of TMP at different concentrations on the protein expression of LC3-II/LC3-I, Beclin1, SQSTM1 and Ki67 in JEG3 cells. $*P < 0.05$ vs TMP at 0 nM. Measurement data are expressed as mean $\pm$ s.d. Comparisons between groups were performed using one-way ANOVA with Tukey's post hoc test. Data comparison between groups at different time points was performed by two-way ANOVA. Cellular experiment was repeated three times.

MiR-16-5p enhances autophagy and inhibits viability and migration of JEG3 cells

LPS-induced rats with/without DMSO treatment showed upregulated miR-16-5p in rat placental tissue ($P < 0.05$), but TMP treatment reduced miR-16-5p expression in rat placental tissue ($P < 0.05$) (Fig. 3A). In TMP-treated JEG3 cells, versus TMP at 0 nM, TMP at 2.5, 5 and 10 nM reduced miR-16-5p expression ($P < 0.05$) (Fig. 3B). These results indicate that miR-16-5p was highly expressed in PE rats, and TMP can reduce the expression of miR-16-5p in PE rats. To investigate whether miR-16-5p participated in the development of PE, we overexpressed or inhibited miR-16-5p expression in JEG3 cells, respectively. qRT-PCR results showed that relative to mimic-NC, miR-16-5p expression was elevated in the presence of miR-16-5p mimic ($P < 0.05$), while relative to inhibitor-NC, the expression of miR-16-5p was downregulated in the presence of miR-16-5p inhibitor ($P < 0.05$) (Fig. 3C), indicating that miR-16-5p expression was successfully up or downregulated in JEG3 cells.

TEM observations showed that miR-16-5p mimic elevated the number of autophagosome formation, while miR-16-5p inhibitor reduced the number of autophagosome formation (Supplementary Fig. 1C). CCK-8 assay (Fig. 3D) and Transwell assay (Fig. 3E and F)

showed that miR-16-5p mimic inhibited cell viability and migration capacity, while miR-16-5p inhibitor enhanced cell viability and migration capacity ($P < 0.05$). Western blot assay revealed that miR-16-5p mimic elevated the protein expression of LC3-II/LC3-I, Beclin1 and SQSTM1, and reduced the protein expression of Ki67, but that miR-16-5p inhibitor reversed these changes ($P < 0.05$) (Fig. 3G). The above results indicate that miR-16-5p strengthened autophagy of JEG3 cells and repressed their viability and migration.

IGF-2 is a target gene of miR-16-5p

The miRNA target gene prediction website miRDB (<http://www.mirdb.org/>) showed that miR-16-5p could target the IGF-2 gene (Fig. 4A). The luciferase reporter gene assay results verified the binding relationship, showing that versus mimic-NC, the luciferase activity of miR-16-5p mimic and IGF-2 3'UTR-wt fusion was reduced ($P < 0.05$), but the luciferase activity of its fusion with IGF-2 3'UTR-mut was hardly affected ($P > 0.05$) (Fig. 4B). In addition, miR-16-5p mimic diminished the expression of IGF-2 ($P < 0.05$), and miR-16-5p inhibitor elevated the expression of IGF-2 ($P < 0.05$) (Fig. 4C, D and E). qRT-PCR and Western blot identified that the expression of IGF-2 diminished in placental tissue of LPS-induced rats ($P < 0.05$), and that TMP enhanced IGF-2

Figure 3 MiR-16-5p promotes autophagy and inhibits viability and migration of JEG3 cells. (A) qRT-PCR to detect miR-16-5p expression in PE rat placental tissue; (B) qRT-PCR to detect miR-16-5p expression in TMP-treated JEG3 cells; (C) qRT-PCR to detect miR-16-5p expression in JEG3 cells in the presence of miR-16-5p mimic or inhibitor; (D) CCK-8 assay to detect the effects of miR-16-5p on JEG3 cell viability; (E and F) Transwell assay to detect the effects of miR-16-5p on JEG3 cell migration capacity ($\times 200$); (G) Western blot assay to detect the effects of miR-16-5p on protein expression of LC3-II/LC3-I, Beclin1, SQSTM1 and Ki67 in JEG3 cells; * $P < 0.05$ vs. blank group, TMP at 0 nM or mimic-NC group; # $P < 0.05$ vs LPS+DMSO group, TMP at 2.5 nM or inhibitor-NC group; & $P < 0.05$ vs TMP at 5 nM. Measurement data are expressed as mean $\pm$ s.d. Comparisons between groups were performed using one-way ANOVA with Tukey's post hoc test. Data comparison between groups at different time points was performed by two-way ANOVA, $n = 10$ in panel A. Cellular experiments were repeated three times.

Figure 4 IGF-2 is a target gene of miR-16-5p. (A) microRNA.org (<http://www.microrna.org/>) to predict and analyze the binding site of miR-16-5p and IGF-2; (B) dual luciferase assay to verify the binding of miR-16-5p and IGF-2; (C) qRT-PCR to detect IGF-2 mRNA expression in JEG3 cells after transfection; (D and E) Western blot assay to detect IGF-2 protein expression in JEG3 cells after transfection; (F) qRT-PCR to detect IGF-2 mRNA expression in placental tissue; (G and H) Western blot assay to detect IGF-2 protein expression in placental tissue; (I) Pearson correlation analysis to evaluate correlation of miR-16-5p level and IGF-2 mRNA in placental tissue; * $P < 0.05$ vs mimic-NC group or blank group; # $P < 0.05$ vs inhibitor-NC group or LPS+DMSO group. Measurement data are expressed as mean $\pm$ s.d. The differences between groups were analyzed using unpaired t test. Comparisons between groups were performed using one-way ANOVA with Tukey's post hoc test. Data comparison between groups at different time points was performed by two-way ANOVA with Bonferroni post hoc test, $n = 10$ in panel F, G, H. Cellular experiments were repeated three times.

expression in LPS-induced rats ($P < 0.05$) (Fig. 4F, G and H). Pearson correlation analysis showed that the level of miR-16-5p in JEG3 cells was negatively correlated with the expression of IGF-2 mRNA ($r = -0.743$, $P < 0.05$) (Fig. 4I). The above results indicate that IGF-2 is a target gene of miR-16-5p.

MiR-16-5p enhances autophagy and represses viability and migration of JEG3 cells by targeting IGF-2

After knocking down IGF-2 expression in JEG3 cells, qRT-PCR results showed that, relative to sh-NC, IGF-2

expression was diminished by sh-IGF-2, sh-IGF-2-1, and sh-IGF-2-2 treatments, with the most pronounced effect caused by sh-IGF-2 (Fig. 5A). We then downregulated the expression of miR-16-5p and IGF-2 in JEG3 cells. Relative to inhibitor-NC+sh-NC, inhibitor-NC+sh-IGF-2 only downregulated the expression of IGF-2, while miR-16-5p inhibitor+sh-NC repressed miR-16-5p and upregulated IGF-2; meanwhile, miR-16-5p inhibitor+sh-IGF-2 downregulated both miR-16-5p and IGF-2 expression ($P < 0.05$) (Fig. 5B). Thus, the intended downregulation of miR-16-5p and IGF-2 expression in JEG3 cells was successful.

Figure 5 MiR-16-5p enhances autophagy and represses viability and migration of JEG3 cells by targeting IGF-2. (A) qRT-PCR to detect the efficiency of shRNA targeting IGF-2 in JEG3 cells; (B) qRT-PCR to detect the expression of miR-16-5p and IGF-2 in the presence of miR-16-5p inhibitor and sh-IGF-2 in JEG3 cells; (C) CCK-8 assay to detect the viability of JEG3 cells in the presence of miR-16-5p inhibitor and sh-IGF-2; (D and E) Transwell assay to detect the migration capacity of JEG3 cells in the presence of miR-16-5p inhibitor and sh-IGF-2 ($\times 200$); (F) Western blot assay to detect protein expression of LC3-II/LC3-I, Beclin1, SQSTM1 and Ki67 in JEG3 cells in the presence of miR-16-5p inhibitor and sh-IGF-2; * $P < 0.05$ vs sh-NC group or inhibitor-NC+sh-NC group; # $P < 0.05$ vs inhibitor-NC+sh-IGF-2 group. Measurement data are expressed as mean $\pm$ s.d. Comparisons between groups were performed using one-way ANOVA with Tukey's post hoc test. Data comparison between groups at different time points was performed by two-way ANOVA. Cellular experiments were repeated three times.

TEM observation (Supplementary Fig. 1D) showed that relative to inhibitor-NC+sh-NC, treatment with miR-16-5p inhibitor+sh-NC diminished the number of autophagosomes formed in cells, while inhibitor-NC+sh-IGF-2 elevated autophagosome formation. In comparison with inhibitor-NC+sh-IGF-2, treatment with miR-16-5p inhibitor+sh-IGF-2 suppressed the autophagosome formation in cells ($P < 0.05$). The CCK-8 assay (Fig. 5C) and Transwell assay (Fig. 5D and E) showed that miR-16-5p inhibitor+sh-NC enhanced cell viability and migration compared to inhibitor-NC+sh-NC, while inhibitor-NC+sh-IGF-2 reduced cell viability and migration ability ($P < 0.05$). In comparison with inhibitor-NC+sh-IGF-2, treatment with miR-16-5p inhibitor+sh-IGF-2 augmented cell viability and migration ability ($P < 0.05$). Western blot assay results showed that miR-16-5p inhibitor+sh-NC reduced the expression of LC3-II/LC3-I, Beclin1 and SQSTM1, and elevated the expression of Ki67, as compared to inhibitor-NC+sh-NC, and that inhibitor-NC+sh-IGF-2 treatment elevated the protein expression of LC3-II/LC3-I, Beclin1, and SQSTM1, and diminished the protein expression of Ki67 ($P < 0.05$). In comparison with inhibitor-NC+sh-IGF-2, treatment with miR-16-5p inhibitor+sh-IGF-2 reduced protein expression of LC3-II/LC3-I, Beclin1, and SQSTM1, and

elevated protein expression of Ki67 ($P < 0.05$) (Fig. 5F). The above results suggest that miR-16-5p augments autophagy of JEG3 cells and curbs their viability and migration by targeting IGF-2.

TMP inhibits autophagy and enhances viability and migration of JEG3 cells by regulating the miR-16-5p/IGF-2 axis

Next, we attempted to investigate whether TMP affected the PE process by regulating the miR-16-5p/IGF-2 axis. We added TMP to JEG3 cells transfected with miR-16-5p mimic or sh-IGF-2, respectively. TEM observations showed that TMP reduced the autophagosome formation in miR-16-5p mimic-treated and sh-IGF-2-treated JEG3 cells (Supplementary Fig. 1E). CCK-8 assay (Fig. 6A) and Transwell assay (Fig. 6B and C) showed that TMP enhanced the viability and migration capacity of miR-16-5p mimic-treated and sh-IGF-2-treated JEG3 cells. Western blot assay results showed that TMP diminished the expression of LC3-II/LC3-I, Beclin1 and SQSTM1, and elevated the expression of Ki67 protein in miR-16-5p mimic-treated and sh-IGF-2-treated JEG3 cells ($P < 0.05$) (Fig. 6D). The above results indicate that TMP inhibits autophagy and augments viability and migration of JEG3 cells by regulating the miR-16-5p/IGF-2 axis.

Figure 6 TMP inhibits autophagy and enhances viability and migration of JEG3 cells by regulating the miR-16-5p/IGF-2 axis. (A) CCK-8 assay to detect the viability of JEG3 cells in the presence of miR-16-5p, sh-IGF-2 and TMP; (B and C) Transwell assay to detect the migration ability of JEG3 cells in the presence of miR-16-5p, sh-IGF-2 and TMP ($\times 200$); (D) Western blot assay to detect the protein expression of LC3-II/LC3-I, Beclin1, SQSTM1 and Ki67 in JEG3 cells in the presence of miR-16-5p, sh-IGF-2 and TMP; * $P < 0.05$ vs miR-16-5p mimic + DMSO group; # $P < 0.05$ vs sh-IGF-2 + DMSO group. Measurement data are expressed as mean $\pm$ s.d. Comparisons between groups were performed using one-way ANOVA with Tukey's post hoc test. Data comparison between groups at different time points was performed by two-way ANOVA. Cellular experiments were repeated three times.

TMP hinders the progression of PE in rats by regulating the miR-16-5p/IGF-2 axis

We further injected lentiviral vectors containing agomir-16-5p or sh-IGF-2 into PE model rats to verify whether TMP affects PE progression in rats by regulating the miR-16-5p/IGF-2 axis. On the 19th day of pregnancy, rats were subjected to cesarean section, and placental tissues were removed. The expression of miR-16-5p in placental tissues of rats was detected by RT-qPCR. RT-qPCR results showed that agomir-16-5p elevated miR-16-5p expression and reduced IGF-2 mRNA expression in rat placental tissue ($P < 0.05$). Meanwhile, sh-IGF-2 did not change the expression of miR-16-5p but reduced the expression of IGF-2 in rat placental tissue ($P < 0.05$). TMP treatment reduced miR-16-5p expression and elevated IGF-2 mRNA expression in agomir-16-5p-treated and sh-IGF-2-treated rats ($P < 0.05$) (Fig. 7A). Western blot assay showed that agomir-16-5p or sh-IGF-2 reduced IGF-2 protein expression in rat placental tissue ($P < 0.05$). TMP treatment can increase the expression of IGF-2 protein in agomir-16-5p-treated and sh-IGF-2-treated rats ($P < 0.05$) (Fig. 7B and C).

HE staining results showed that compared with agomir-NC or sh-NC, rats injected with lentiviral vector containing agomir-16-5p or sh-IGF-2 had swollen and thickened uterine decidua with obvious inflammatory cell infiltration, giant cell proliferation in the trophoblast of the placenta basal zone, increase in vacuole cells, and thickening of the baseband. Compared with agomir-

16-5p+DMSO or sh-IGF-2+DMSO treatments, rats treated with agomir-16-5p+TMP or sh-IGF-2+TMP had alleviation of the cysticity of the placental tissue, the degree of basal zone thickening, and the degree of inflammatory cell infiltration (Fig. 7D).

TEM observations showed that agomir-16-5p or sh-IGF-2 augmented autophagosome formation in rat placental tissue. TMP treatment suppressed autophagosome formation in rat placental tissue in agomir-16-5p-treated and sh-IGF-2-treated rats (Supplementary Fig. 1F).

Western blot assay showed that agomir-16-5p or sh-IGF-2 elevated the protein expression of LC3-II/LC3-I, Beclin1, and SQSTM1, and diminished the protein expression of Ki67 in rat placental tissue ($P < 0.05$). TMP treatment reduced the protein expression of LC3-II/LC3-I, Beclin1, and SQSTM1, and elevated the protein expression of Ki67 in rat placental tissue ($P < 0.05$) (Fig. 7E). These results indicate that TMP hinders the progression of PE in rats by regulating the miR-16-5p/IGF-2 axis.

Discussion

PE is a frequently occurring disorder in pregnancy with some genetic inheritance, which is associated with maternal hypertension and proteinuria (Kaartokallio *et al.* 2016). Trophoblast cells are key regulators during the process of placental development,

Figure 7 TMP hinders the progression of PE in rats by regulating the miR-16-5p/IGF-2 axis. (A) qRT-PCR to detect miR-16-5p and IGF-2 expression in placental tissue in the presence of agomir-16-5p, sh-IGF-2 and TMP; (B and C) Western blot assay to detect IGF-2 protein expression in placental tissue in the presence of agomir-16-5p, sh-IGF-2 and TMP; (D) HE staining to detect pathological changes in placental tissue in the presence of agomir-16-5p, sh-IGF-2 and TMP (400x); (E) Western blot assay to detect LC3-II/LC3-I, Beclin1, SQSTM1 and Ki67 protein expression in placental tissue in the presence of agomir-16-5p, sh-IGF-2 and TMP; * $P < 0.05$ vs agomir-NC group; # $P < 0.05$ vs sh-NC group; & $P < 0.05$ vs agomir-16-5p+DMSO group; \$ $P < 0.05$ vs sh-IGF-2+DMSO group. Measurement data are expressed as mean $\pm$ s.d. Comparisons between groups were performed using one-way ANOVA with Tukey's post hoc test, $n = 10$.

and abnormal trophoblast cell activities are associated with pregnancy complications including PE (Straszewski-Chavez *et al.* 2005). In China, physicians apply many TCMs, such as *Uncaria rhynchophylla*, in the treatment of PE and hypertension (Wang *et al.* 2018). Increased expression of certain miRNAs plays a critical role in modulating trophoblast behaviors, which may have potential as therapeutic biomarkers in PE (Niu *et al.* 2018). In this study, we clarified that TMP inhibits trophoblast cell autophagy and promotes trophoblast cell viability and migration in PE through regulating the miR-16-5p/IGF-2 axis (Fig. 8).

TMP, a purified and chemically identified component of a TCM, is an effective medicine to block vasoconstriction and to rescue damage from oxygen free radicals (Feng *et al.* 2004). After TMP treatment, the autophagosomes formed in trophoblast cells of PE rat placental tissues was appreciably diminished, and the expression of autophagy-related proteins LC3-II/LC3-I, BECN1, and SQSTM1 was reduced, while the expression of proliferation-related protein Ki67 was elevated. These results indicate that TMP can impede the progression of PE in rats. To further explore the effect of TMP on autophagy of JEG3 cells, we performed Western blot analysis to detect the protein expression of autophagy-related genes LC3-II/LC3-I, Beclin 1 and SQSTM1. LC3 is also known as microtubule-associated protein 1 light chain 3 (MAP1LC3), which is a homolog of Atg8 in mammals, including two mutually transformable forms, LC3-I and LC3-II involved in the formation of autophagosome membranes. Atg4 cleaves early synthesized pro-LC3 exposing C-terminal glycine to form a cytosolic soluble form of LC3-I. After induction of autophagy, LC3-I is coupled with the substrate PE on the surface of autophagosome membranes under the joint action of E1-like enzyme Atg7, E2-like enzyme Atg3 and E3-like enzyme Atg5-Atg12-Atg16L complex to form a membrane-bound form of LC3-II. This LC3-II is an important marker molecule of autophagosome and accumulates with

increasing abundance of autophagosome membranes. Beclin1 (BECN1), a homologous gene of yeast Atg6 in mammals, is phosphorylated by ULK1 and acts as an integral scaffold for the PI3K complex, promoting the localization of autophagic proteins to autophagic vacuoles. The autophagic system is substrate selective; the most widely used substrate for autophagic degradation is p62/SQSTM1, which can be coupled to microtubule-associated protein light chain 3, and participates as a regulator in the formation of autophagosomes, being degraded in the middle and late stages of autophagy (Glick *et al.* 2010, Pyo *et al.* 2012, Cicchini *et al.* 2015, Kuma *et al.* 2017). TMP was observed to ameliorate pregnancy-induced hypertension (Liu 1994). The application of TMP in hypertension syndrome of pregnancy has been highlighted to dilate blood vessel, to restore kidney function and to rescue microcirculatory function and rheology (Qian *et al.* 1991). Previous evidence has suggested that TMP can alleviate pulmonary pathological changes as well as systemic inflammatory reactions (Zhang & Dang 2006). Moreover, our *in vitro* experiments also confirmed that TMP treatment can hinder the autophagy and augment viability and migration of human trophoblast cell line JEG3 concentration-responsively.

Recent studies have revealed the associations of miRNAs with the molecular mechanism in relation to TCM application (Dai *et al.* 2016, Liu *et al.* 2017). A prior study of human umbilical vein endothelial cells under hypoxic conditions has suggested that TMP augmented cell viability and repressed cell apoptosis through elevating miR-135b (Wei & Wang 2019). Our observations have identified that miR-16-5p was upregulated in placental tissue of the PE rat model, and that TMP can repress the miR-16-5p expression in JEG3 cells. A similar study has uncovered that TMP can reduce cardiomyocyte apoptosis and restore their viability in ischemic heart disease through elevating miR-499a and diminishing its downstream target Sirt1 (Zhang *et al.* 2019a). The current study unveiled that

Figure 8 TMP inhibits trophoblast cell autophagy and promotes trophoblast cell viability and migration in PE through regulating the miR-16-5p/IGF-2 axis. In PE trophoblast cells, TMP may promote IGF-2 expression by inhibiting miR-16-5p. On the one hand, upregulated IGF-2 can inhibit the expression of LC3II/LC3I, Beclin-1, and SQSTM1, thereby inhibiting cell autophagy. On the other hand, upregulated IGF-2 can promote the expression of the proliferation-related protein Ki67 protein, thereby promoting cell proliferation and migration.

overexpression of miR-16-5p accelerated autophagy in JEG3 cells and restricted their viability and migration. Other evidence shows that miR-16 is associated with the anti-cancer effect of the TCM artesunate against bladder cancer through modulating proliferative behaviors of cancer cells (Zuo *et al.* 2014). Treatment with another TCM, Pien Tze Huang, upregulated miR-16 expression in hepatocellular carcinoma cells, which leads to repression of cancer cell growth (Qi *et al.* 2017). In severe PE, miR-16 was found to halt proliferation and migration of trophoblast cells through directly binding to cyclin E1 (Wang *et al.* 2012b).

In addition, our mechanistic investigations illuminated that miR-16-5p can target the IGF-2 gene and downregulate IGF-2 expression, thereby potentiating autophagy and abrogating viability and migration in JEG3 cells. The evidence presented by Chen *et al.* documented that miR-16 could bind to IGF1R and inversely modulated its expression, thereby exerting tumor suppressive activities in osteosarcoma (Chen *et al.* 2013). The importance of IGF-2 has been previously highlighted in the paracrine interplay at the maternal-fetal interface during pregnancy, which could affect trophoblast cell invasion in PE (Irwin *et al.* 1999). TMP administration was observed to function through triggering the activation of IGF1R survival signaling, which thereby halted the apoptosis of cardiomyocyte apoptosis under hypoxic conditions (Lin *et al.* 2015). Also, further exploration in the present study verified that TMP inhibited JEG3 cell autophagy and augmented cell viability and migration by regulating the miR-16-5p/IGF-2 axis in vitro and in vivo.

Based on the present findings, we suggest that therapeutic strategies with TMP for PE should be directed toward the ablation of miR-16-5p, which may potentially be clinically viable targets in the treatment of PE. TMP inhibits trophoblast cell autophagy and promotes trophoblast cell viability and migration in PE through upregulating miR-16-5p and downregulating IGF-2. A better understanding of molecular mechanisms associated with the alleviatory effects of TMP in PE via miR-16-5p could enable the development of targeted therapies accompanied by TCM for pregnancy complications. Besides, existing literature has reported that PE patients are accompanied by liver dysfunction, such as increased alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activity (Zhang *et al.* 2019b), but we did not detect the effect of TMP/miR-16-5p on liver function in rats in our experiment. We will explore the impairment of TMP/miR-16-5p on liver function in future clinical experiments.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0309>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Availability of data and materials

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

Author contribution statement

Yuan Yuan conceived and designed research. Ling Zhao performed experiments and interpreted results of experiments. Xiaoying Wang analyzed data and prepared figures. Feng Lian drafted paper. Yan Cai edited and revised manuscript. All authors read and approved the final manuscript.

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