miR-30 inhibits proliferation of trophoblasts in preeclampsia rats partially related to MAPK/ERK pathway

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Received November 13, 2019; Accepted April 1, 2020

DOI: 10.3892/etm.2020.8866

Abstract. Effect of micro ribonucleic acid (miR)-30 on the proliferation of trophoblasts in preeclampsia (PE) rats through the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway was studied. The miR-30 mimic was transfected into the trophoblast HTR8/SVNEO cell lines. The effects of expression level of miR-30 on the proliferation and hypoxia-induced apoptosis of HTR8/SVNEO cells were detected via methyl thiazolyl tetrazolium (MTT) assay and Annexin V/propidium iodide staining, respectively, using the flow cytometer. A total of 30 pregnant Sprague-Dawley rats were randomly divided into control group (CTL group, n=10), PE rat group (PE group, n=10) and PE + miR-30 Mimic group (PE+agomiR-30 group, n=10) using a random number table. The protein expression levels of phosphorylated ERK (p-ERK)1/2, ERK1/2, proliferating cell nuclear antigen (PCNA) and tubulin were determined using western blot analysis, and the mRNA expression level of ERK1/2 was detected via reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression level of PCNA in tissues was detected via immunohistochemistry. The results of MTT assay showed that the proliferation of HTR8/SVNEO cells significantly declined in hypoxic environment, while miR-30 promoted the proliferation of HTR8/SVNEO cells and alleviated the hypoxia-induced inhibition on cell proliferation. It was found that the trophoblast apoptosis rate was increased in hypoxia group compared with that in CTL group, while it was significantly decreased in miR-30 Mimic group compared with that in hypoxia group. PE group had obviously decreased p-ERK and PCNA expression levels as well as p-ERK/ERK ratio in placental tissues compared with CTL group, while PE+agomiR-30 group had an obviously increased expression level of PCNA as well as p-ERK/ERK ratio in placental tissues compared with PE group. MiR-30 activates the MAPK/ERK signaling pathway and increases the expression level of PCNA through raising the p-ERK level and p-ERK/ERK ratio, thereby inhibiting cell apoptosis and promoting cell proliferation.

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Key words: miR-30, MAPK/ERK signaling pathway, preeclampsia, cell proliferation

Introduction

Preeclampsia (PE) is a disease during pregnancy, and its major symptoms include hypertension, proteinuria, edema and systemic organ damage after 20 weeks of pregnancy (1). According to the epidemiological studies, the morbidity rate of PE is 9.4% in China and 7-12% worldwide, which is the main cause of maternal death, fetal growth restriction and high fetal mortality (2).

There are many causes of PE, with a complicated pathogenesis, and its specific mechanism of action remains unclear (3). At present, the theory of trophoblastic ischemia and hypoxia is widely recognized, which holds that during the placental formation of PE patients, excessive trophoblast apoptosis weakens the invasion ability of extravillous trophoblasts, leads to the disorders of vascular remolding of uterine spiral arterioles, and makes the maternal body unable to supply enough blood and oxygen to the placenta, ultimately resulting in the systemic inflammatory response and systemic vascular endothelial injury, and inducing various clinical symptoms of PE (4).

Micro ribonucleic acids (miRNAs) are endogenous non-coding single-stranded small-molecule RNAs with 19-22 nt in length. The major mechanism of action of miRNAs includes the complementary binding to the target gene mRNA 3'-untranslated region (3'-UTR) to directly degrade mRNA or inhibit its translation, thereby regulating the gene expression at the transcriptional level. Currently, studies have found that miRNAs play very important roles in such biological processes as cell proliferation, differentiation and apoptosis (2-4). As a widely-studied miRNA at present, miR-30 plays a vital role in the occurrence and development of cardiovascular diseases, metabolic diseases and tumors (5-7). There are studies showing that miR-30 can inhibit the transforming growth factor-β (TGF-β)-induced organ fibrosis, which is associated with epithelial cell apoptosis involving TGF-β (8,9).

Currently, studies have demonstrated that the occurrence and development of PE are closely related to the proliferation of trophoblasts. The present study, therefore, explored whether miR-30 can affect the proliferation ability of trophoblasts in PE through the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway.

Materials and methods

Cell culture. The human choriocarcinoma HTR8/SVNEO cell lines used in this study were purchased from American Type Culture Collection (ATCC), and cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Invitrogen; Thermo Fisher Scientific, Inc.; batch no: 991015) containing 10% fetal bovine serum (FBS) (Invitrogen; Thermo Fisher Scientific, Inc.; batch no: 423257) in an incubator with 5% CO_2 at 37˚C.

Cell experimental scheme. In the present study, all cells were divided into hypoxia group, miR-30 Mimic group (hypoxia + transfection with miR-30 mimic) and CTL group (normal HTR8/SVNEO cells). In hypoxia group, HTR8/SVNEO cells were transfected with 50 nM of miRNA negative control while in miR-30 Mimic group, HTR8/SVNEO cells were transfected with 50 nM of miR-30 mimic (Shanghai Genechem Co., Ltd.) using the Lipofectamine 2000 kit (Invitrogen; Thermo Fisher Scientific, Inc.; batch no: 11573019) in strict accordance with the instructions. Cells were seeded in 24-well plates for 24 h before transfection. miRNA negative control or miR-30 mimics were diluted and then mixed with Lipofectamine® 2000. The mixture was added to each well after maintained at room temperature for 20 min. The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C for 6 h, then the medium was replaced. At 48 h after transfection, the cells were cultured in a tri-gas incubator (1% O_2 and 99% N_2) for 24, 48 and 72 h, followed by subsequent experiments. The sequences are as follows: miRNA negative control F: 5'-TCTGAGGCTA ACCACGGTCTGTA-3' and R: 5'-CTGATTAAGTGTCAT ACTCATAC; miR-30 mimics F: 5'-TGTAAACATCCTACAC TCTCAGC-3' and R: 5'-CTCGCTTCGGCAGCACACCG ACT-3'.

Detection of cell proliferation via methyl thiazolyl tetrazolium (MTT) assay. First, the cells treated in each group were cultured *in vitro*, paved onto the plate, digested and counted. Then the cells were diluted to $5x10^4$/ml, and 100 μl of diluent was added to a well of a 96-well plate, with the number of cells controlled at 5,000 cells/well. At different time points during growth, 5 mg/ml MTT stock solution (Sigma-Aldrich; Merck KGaA) was prepared, diluted at 1:10 and added into cells for reaction in the incubator for 4 h. After MTT solution was removed, the wells were dried, 150 μl of DMSO was added into each well, and the plate was placed in the incubator for 15 min. Finally, the optical density (OD) value of cells was measured at 490 nM using a microplate reader to evaluate the number of viable cells. The higher OD value corresponds to more cells.

Detection of apoptosis via flow cytometry. The cells were suspended, directly centrifuged at 500 x g at 4˚C for 5 min and collected. The adherent cells were digested with trypsin containing EDTA (endothelial cell medium) for an appropriate time, and the reaction was terminated with complete medium. Then the cells were rinsed with phosphate buffered saline (PBS), counted and centrifuged at 500 x g at 4˚C for 5 min. Cells ($1-5x10^5$) were collected, resuspended with 500 μl of binding buffer, and mixed evenly with 5 μl of Annexin V-Light 650 and 10 μl of propidium iodide (PI), followed by reaction at room temperature in the dark for 5-15 min. Flow cytometry was performed within 1 h, and the Annexin V-Light 650 fluorescence signal and PI fluorescence signal were detected through the FL4 channel, and the FL2 or FL3 channel, respectively. Finally, the Annexin V-Light 650 single positive tube and PI single positive tube were detected simultaneously to determine the fluorescence compensation value and the position of cross quadrant gate.

Animal feeding, treatment and grouping. A total of 30 healthy wild-type pregnant Sprague-Dawley (SD) rats aged 5-6 weeks and weighing 200-250 gr purchased from Shanghai BRL Biotechnology Co., Ltd. were fed in the specific pathogen-free animal room at 22˚C, relative humidity of 60% and 12/12 h light/dark cycle, and they had free access to food and water. The day when sperms were found in the vagina of rats and the vaginal plug fell was determined as the first day of pregnancy. The SD rats were randomly divided into control group (CTL group, n=10), PE rat group (PE group, n=10) and PE+miR-30 Mimic group (PE+agomiR-30 group, n=10). AgomiR-30 control group was not included in this study. In PE+agomiR-30 group, agomiR-30 was injected via the caudal vein (10 nmol/day) once every 2 days for a total of 7 days. This study was approved by the Animal Ethics Committee of Qinghai Provincial People's Hospital (Qinghai, China).

Animal modeling method. It is currently recognized that placental ischemia and hypoxia are important causes of PE, so the chronic placental hypoxia model was established in this study using L-nitro-arginine methyl ester (L-NAME), a NOS inhibitor (10). L-NAME was subcutaneously injected (100 mg/kg/d) in PE group and PE+agomiR-30 group for 7 days from 12th day after pregnancy, while the same volume of normal saline was subcutaneously injected in CTL group. The rats were sacrificed using 5% isoflurane followed by cervical dislocation after 7 days of treatment.

Detection of protein expression using western blot analysis. The placental tissues of SD rats were cut into pieces, homogenized and added with lysis buffer, followed by centrifugation in 4,000 x g and 4˚C for 30 min. The total protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce Protein Biology; Thermo Fisher Scientific, Inc.). After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gel), the protein (10 μl) was transferred onto a polyvinylidene fluoride (PVDF) membranes (Merck Millipore; IPVH00010), and incubated with phosphorylated ERK (p-ERK)1/2 (1:1,000; cat no 4370), ERK1/2 (1:1,000; cat no 4695), PCNA (1:1,000; cat no 13110) and tubulin (1:1,000; cat no 2148) primary antibodies (Cell Signaling Technology, Inc.) at 4˚C overnight. After washing, the protein was incubated

Table I. Primer sequences.

Genes	Forward (5'-3')	Reverse (5'-3')
ERK	AGAGTTGAAGGATGATGACT	CACTCATGCAGCACCTGCAG
β-actin	GCAGAAGGAGATTACTGCCCT	GCTGATCCACATCTGCTGGAA

ERK, extracellular signal-regulated kinase.

again with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000; cat no 7075; Cell Signaling Technology, Inc.) for 1 h. Finally, the electrochemiluminescence (ECL) mixture was added to obtain images using the fluorescence development technique. Quantity One (version 4.0; Bio-Rad Laboratories, Inc.) was used for densitometric analysis. Protein expression levels were calculated as the relative band density to tubulin.

Detection of mRNA expression levels of ERK1/2 via reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The mRNA was extracted from tissues in each group using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., cat no. 10296028), and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Takara PrimeScript™ RT Master Mix kit (Takara Biotechnology Co., Ltd.; cat no RR036A). Reverse transcription reaction was performed at 50˚C for 60 min, then the reverse transcriptase was inactivated at 85˚C for 5 min. A total of 2 μl of 5xPrimeScript RT Master Mix was added into 500 ng of RNA, and the total reaction system was 10 μl. Then PCR amplification was performed using SYBR® Green Master Mix (Takara Bio, Inc.; cat no MB3353). cDNA (2 μl) of was added with 10 μl of SYBR Premix Ex Taq II (Tli RNaseH Plus) (2x), 0.8 μl of forward primers, 0.8 μl of reverse primers, and 0.4 μl of ROX Reference Dye II (50x), and deionized water was added to total volume of 20 μl. PCR amplification conditions: pre-denaturation at 94˚C for 5 min, followed by 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 1 min and 30 sec for 40 cycles. The mRNA expression level was calculated using the cycle threshold, with β-actin as an internal reference. The $2^{-\Delta\Delta Cq}$ method was used to determine the relative expression levels (11). The primer sequences are shown in Table I.

Immunohistochemistry (IHC). First, the placenta tissue was obtained from the rat and paraffin sections were routinely prepared, deparaffinized, incubated with 3% H_2O_2 - 60% methanol at room temperature for 30 min, and washed with PBS 3 times, followed by membrane permeabilization with 0.1% Triton X 100+PBS for 20 min, and incubation with 5% normal goat serum at room temperature for 20 min, rabbit anti-mouse PCNA monoclonal antibody (1:200; cat no 13110) in a refrigerator at 4˚C overnight, and biotinylated goat anti-rabbit IgG secondary antibody at 37˚C for 1 h. After washing with PBS 3 times, the sections were incubated with HRP-labeled streptavidin antibody at 37˚C for 30 min, followed by diaminobenzidine (DAB) staining (Beijing Solarbio Science & Technology Co., Ltd.) in the dark at room temperature, hematoxylin counterstaining for 30 min, dehydration with gradient ethanol, transparentization with xylene and sealing with neutral balsam. Finally, the sections were observed under an inverted fluorescence microscope.

Positive expression as dark brown particles in cells. The mean OD value of IHC-positive particles was determined using ImageJ professional image analysis system, and the PCNA protein expression was semi-quantitatively analyzed.

Statistical analysis. GraphPad Prism 6.0 (La Jolla) was used for the statistical analysis of data. Three batches of the cell culture were carried out and the data were expressed as mean ± SEM (standard error of the mean). Comparisons between multiple groups was performed using one-way analysis of variance test followed by Least Significant Difference post hoc test. $P<0.05$ indicates statistically significant difference.

Results

Effect of miR-30 on proliferation of trophoblast HTR8/SVNEO cells. First, MTT assay was performed to detect the cell proliferation in each group. It was found that the number of proliferating cells in hypoxia group was significantly smaller than that in CTL group at 24, 48 and 72 h, while the cell proliferation ability was stronger in miR-30 Mimic group than that in hypoxia group at 48 and 72 h (Fig. 1). The above findings suggest that the proliferation of HTR8/SVNEO cells significantly declines in hypoxic environment, while miR-30 can promote the proliferation of HTR8/SVNEO cells and alleviate the hypoxia-induced inhibition on cell proliferation.

Effect of miR-30 on hypoxia-induced apoptosis of HTR8/SVNEO cells. The effect of miR-30 on hypoxia-induced apoptosis of HTR8/SVNEO cells was detected via Annexin V-FITC and PI fluorescence labeling using a flow cytometer. The results showed that the trophoblast apoptosis rate was increased in hypoxia group compared with that in CTL group, while it was significantly decreased in miR-30 Mimic group compared with that in hypoxia group (Fig. 2), indicating that miR-30 can inhibit hypoxia-induced trophoblast apoptosis.

Effect of miR-30 on expression level of PCNA in placental tissues. To explore the effect of miR-30 expression on the proliferation of placental tissues, western blot analysis was performed to detect the protein expression of PCNA in placental tissues in the three groups. The results revealed that the PCNA level evidently declined in PE group compared with that in CTL group ($P<0.01$), while it was evidently increased

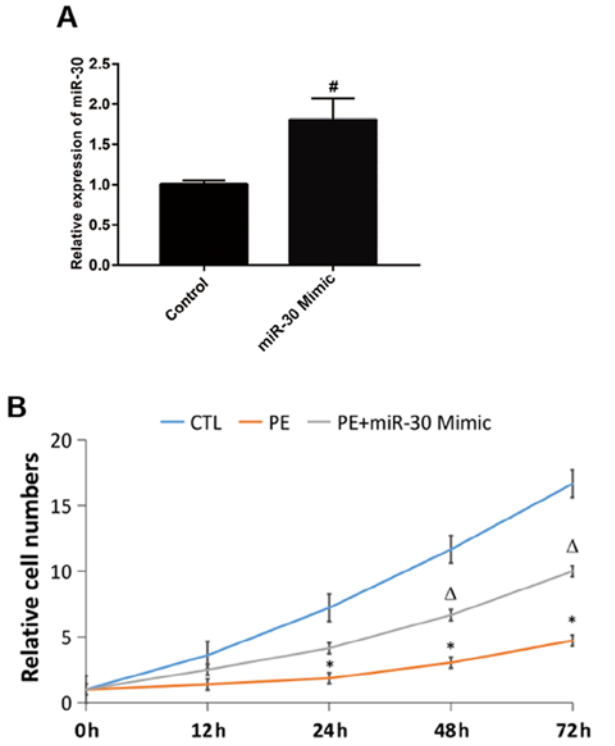

Figure 1. Effect of miR-30 on trophoblast proliferation in PE rats. (A) miR-30 expression was markedly elevated in miR-30 mimic-transfected cells. (B) The number of cells in each group was detected via MTT assay at 0, 12, 24, 48 and 72 h to compare the cell proliferation ability (n=6). The values are expressed as mean ± SEM. #P<0.05, Control group vs. miR-30 Mimic group; *P<0.01, hypoxia group vs. CTL group; ΔP<0.05, miR-30 Mimic group vs. hypoxia group. SEM, standard error of the mean.

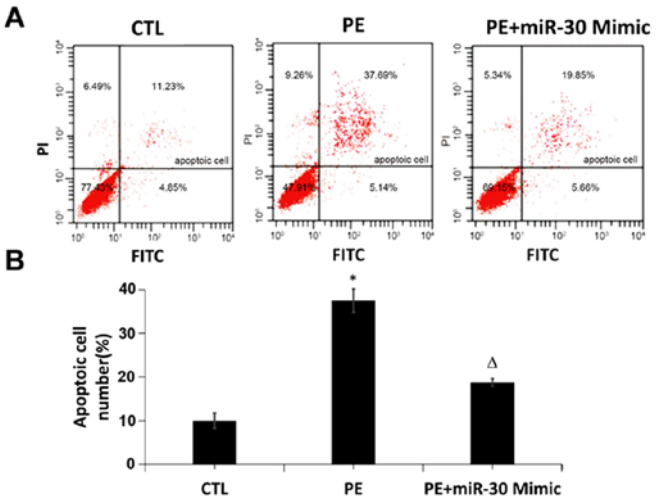

Figure 2. Effect of miR-30 on hypoxia-induced trophoblast apoptosis. (A) Apoptosis detected via Annexin V-FITC and PI double labeling and flow cytometry, the double positive cells in the right upper quadrant are the apoptotic cells. (B) Quantification of results (n=3). The values are expressed as mean ± SEM. *P<0.01, hypoxia group vs. CTL group; ΔP<0.05, miR-30 Mimic group vs. hypoxia group. SEM, standard error of the mean.

in PE+agomiR-30 group compared with that in PE group (P<0.01) (Fig. 3), suggesting that the cell proliferation level in placental tissues was significantly decreased in PE group, and significantly increased in PE+agomiR-30 group.

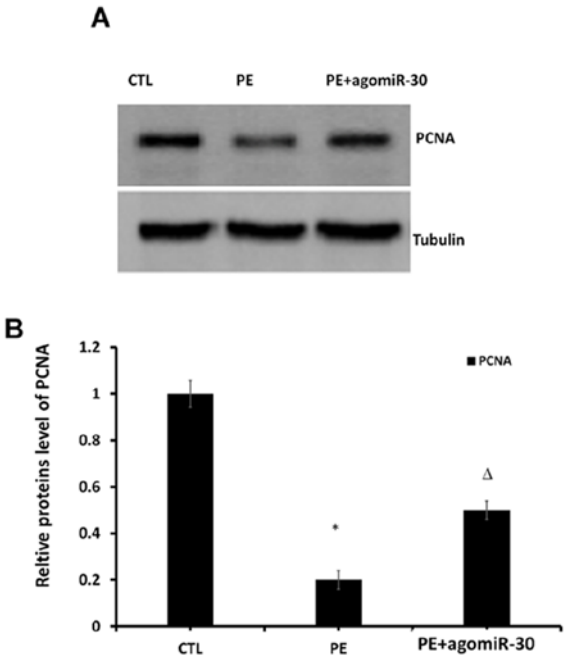

Figure 3. Effect of miR-30 on expression level of PCNA in placental tissues. (A) PCNA protein expression level in each group detected using western blot analysis. (B) Quantification of results (n=3). The values are expressed as mean ± SEM. *P<0.01, PE group vs. CTL group; ΔP<0.05, PE + agomiR-30 group vs. PE group. SEM, standard error of the mean; PCNA, proliferating cell nuclear antigen.

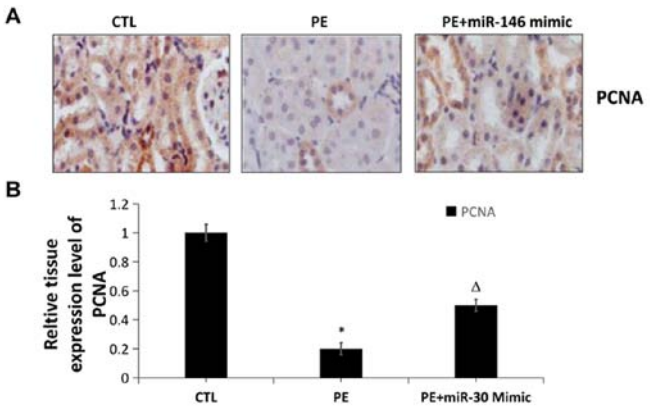

Figure 4. Effect of miR-30 on PCNA level determined using IHC. (A) PCNA protein expression level in each group detected using IHC. (B) Quantification of results in each group detected using IHC (n=3). The values are expressed as mean ± SEM. *P<0.01, PE group vs. CTL group; ΔP<0.05, PE + agomiR-30 group vs. PE group. PCNA, proliferating cell nuclear antigen; IHC, immunohistochemistry.

Effect of miR-30 on PCNA level. To obtain further evidence, the changes in the protein expression of PCNA in placental tissues in the three groups were determined using IHC. The PCNA level evidently declined in PE group compared with that in CTL group (P<0.01), while it was evidently increased in PE+agomiR-30 group compared with that in PE group (P<0.01), consistent with the results of western blot analysis (Fig. 4). The cell proliferation level in placental tissues significantly declined in PE group, and significantly increased in PE+agomiR-30 group.

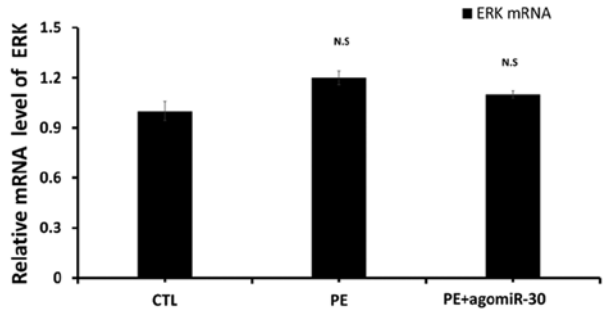

Figure 5. Effect of miR-30 on mRNA level of ERK. The mRNA level of ERK was detected using RT-qPCR (n=3). The values are expressed as mean ± SEM. [N.S]There is no significant difference among the three groups. ERK, extracellular signal-regulated kinase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean.

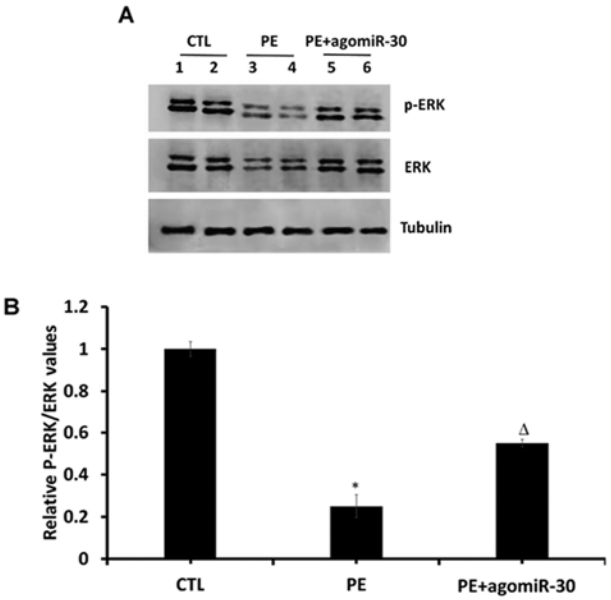

Figure 6. Effect of miR-30 on MAPK/ERK signaling pathway. (A) Protein levels of P-ERK, ERK and tubulin in each group detected using western blot analysis. (B) Quantification of p-ERK/ERK ratio (n=3). The values are expressed as mean ± SEM. [*]P<0.01, PE group vs. CTL group; [Δ]P<0.05: PE + agomiR-30 group vs. PE group. MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; P-ERK, phosphorylated ERK; SEM, standard error of the mean.

Effect of miR-30 expression on mRNA level of ERK1/2. To explore the specific mechanism of miR-30 in promoting the proliferation of placental tissues, RT-qPCR was performed, and the results showed that there was no significant difference in the mRNA level of ERK among CTL, PE and PE+agomiR-30 group (Fig. 5).

Effect of miR-30 on MAPK/ERK signaling pathway in placenta tissues. Protein levels of P-ERK1/2, ERK1/2 and tubulin in placenta tissues were detected using western blot analysis. The results showed that PE group had extremely decreased p-ERK and p-ERK/ERK ratio in placental tissues compared with CTL group (P<0.01), while PE+agomiR-30 group had obviously increased p-ERK as well as p-ERK/ERK ratio in placental tissues compared with PE group (P<0.05) (Fig. 6), demonstrating that the MAPK/ERK signaling pathway is suppressed in PE rats, and it can be activated by miR-30.

Discussion

miRNAs are a class of endogenous non-coding single-stranded small-molecule RNAs 19-22 nt in length, which regulate and inhibit the target gene expression mainly at the transcriptional level (12). The maturation of miRNAs involves two processes. First, the pri-RNA molecules in the nucleus are processed by Drosha protein and DGCR8 protein into pre-miRNA with 70 nt in length. Then under the action of nuclear export protein Exportin5/Ran GTP, pre-miRNA is transported from the nucleus to the cytoplasm, and further processed by Dicer into mature miRNA about 21 nt in length. miRNA molecules form the RNA-induced silencing complex through the target gene mRNA, thus inhibiting or degrading the target gene expression (13).

As a widely-studied miRNA at present, miR-30 plays an important role in the occurrence and development of cardiovascular diseases, metabolic diseases and tumors. MiR-30 includes 5 family members: miR-30a, miR-30b, miR-30c, miR-30d and miR-30e (14). Studies have shown that miR-30 can inhibit the TGF-β-induced organ fibrosis, which is associated with epithelial cell apoptosis involving TGF-β. Overexpression of TGF-β can increase Caspase-3 expression to facilitate apoptosis, while miR-30 is able to inhibit epithelial cell apoptosis through suppressing TGF-β expression (15).

MAPK is a kind of serine/threonine protein kinase in cells (16). Multiple parallel MAPK signaling pathways are found in lower prokaryotic cells and higher mammalian cells, and they mediate different cellular biological responses, in which MAPK/ERK signaling pathway is an important one participating in and playing a vital role in cell proliferation, differentiation and apoptosis (17-19). PE is an idiopathic placenta-derived disease during pregnancy, as well as a polygenic disease whose pathogenesis has not been fully clarified yet (20,21). Currently, studies on signaling pathways in PE mostly focus on the adhesion protein, PI3K and TGF-Smad pathways. PI3K is related to the differentiation and invasion of trophoblasts, and MAPK is an intracellular serine/threonine protein kinase. MAPK signals are activated by different molecules, thereby inducing such biological reactions as cell proliferation, differentiation, transformation and apoptosis (22). Moreover, the MAPK signaling pathway is suppressed in PE patients, and it can be activated by miR-30.

In the present study, the effect of miR-30 on the trophoblast proliferation in PE through the MAPK/ERK signaling pathway was explored. The effect of expression level of miR-30 on the proliferation of HTR8/SVNEO cells under hypoxia was detected via MTT assay. Results showed that the trophoblast proliferation significantly declined in hypoxic environment, while miR-30 promoted cell proliferation and alleviate hypoxia-induced inhibition on cell proliferation. It was found through flow cytometry that the level of hypoxia-induced trophoblast apoptosis obviously declined in miR-30 Mimic group compared with that in hypoxia group. The results of the *in vivo* western blot analysis and IHC manifested that the protein expression of PCNA in placental tissues was evidently decreased in PE group, while it was evidently raised by miR-30. In addition, the cell proliferation ability in placental tissues was remarkably

weakened in PE group, whereas it was remarkably enhanced in PE+agomiR-30 group. According to the results of RT-qPCR and western blot analysis, miR-30 phosphorylated ERK1/2 to activate the MAPK/ERK signaling pathway, which may play an important role in the enhanced proliferation of placental tissues. However, there are some limitations in this study. AgomiR-30 control was not used in any of the groups to exclude non-specific effects, which could impact on the conclusion. MAPK signals contain EKR, JNK and p38, whether miR-30 affects JNK and p38 phosphorylation is still unknown.

In conclusion, miR-30 activates the MAPK/ERK signaling pathway and increases the expression level of PCNA through raising the p-ERK level and p-ERK/ERK ratio, thereby inhibiting cell apoptosis and promoting cell proliferation.

Acknowledgements

Not applicable.

Funding

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YW, LJ and HGu designed the study and performed the experiments. YW and HGo established the animal models. LJ and YuL collected the data. AX and YaL analyzed the data. YW, LJ and HGu prepared the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Qinghai Provincial People's Hospital (Qinghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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