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Mitofusin 2 Expression in Placental Tissues of Preeclamptic Patients and Its Correlations with Trophoblast Invasion and Placental Hypoxia

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KEYWORDS Invasion. Mitofusin 2. Placental Hypoxia. Preeclampsia. Trophoblast

ABSTRACT The researchers aimed to detect mitofusin 2 (Mfn2) expression in the placental tissues of preeclampsia (PE) patients and correlations with trophoblast invasion and placental hypoxia. Mfn2 mRNA expressions in placental tissues from PE and healthy pregnant women were detected by qRT-PCR. Trophoblast cells JEG-3 were transfected with small-interfering RNA-Mfn2 (si-Mfn2). Proliferation was tested by CCK-8 and colony formation assays. Invasion and migration were determined with Transwell assay. Mfn2, HIF-1α, HPH-1 and leptin protein expressions were detected by Western blotting. Mfn2 protein had strong positive and weak negative expressions in normal and PE women, respectively. Mfn2 mRNA expression in PE women was lower than that in normal women ($P<0.05$). Compared with control and si-NC groups, si-Mfn2 group had lower proliferation, migration and invasion abilities, as well as HIF-1α, HPH-1 and leptin expressions ($P<0.05$). Mfn2 expression in the placental tissues of PE women obviously decreases, which inhibits trophoblast invasion and triggers placental hypoxia.

INTRODUCTION

Preeclampsia (PE) is a specific disease during pregnancy, which is typified by hypertension, proteinuria and other life-threatening complications such as dysfunction of the kidney, liver and other multiple organs, as a major cause for the death of pregnant women and unborn foetuses (Malha and August 2015). At present, the pathogenesis of PE remains unclear, and most studies have focused on trophoblast infiltration abnormalities and vascular endothelial cell injury or dysfunction. Oxidative stress reaction occurs in the placenta in the second and third trimesters of pregnancy due to ischemia and hypoxia, which is clinically manifested as various symptoms of PE and eclampsia (Zárate et al. 2014). Additionally, the onset of PE has been closely associated with endothelial cell injury (Zhao et al. 2019). Mitofusin 2 (Mfn2), as a crucial dynein of mitochondria, is mainly distributed on the outer membrane and able to facilitate mitochondrial fusion, which is crucial to the regulation of mitochondrial function and morphology. Besides, Mfn2 also participates in key biological processes such as cell energy metabolism, signal transduction, proliferation and apoptosis (Naon et al. 2017). After Mfn2 expression decreases, the apoptosis of trophoblasts is facilitated, thus probably being one of the mechanisms by which Mfn2 affects the invasion ability of trophoblasts (Yu et al. 2016; Cai et al. 2018). However, whether there is a correlation between Mfn2 and the onset of PE has never been reported hitherto.

Objective

Therefore, the researchers herein studied the Mfn2 expression changes in the placental tissues of PE women as well as the effects on trophoblast invasion and placental hypoxia, aiming to explore the correlation between Mfn2 and PE occurrence.

MATERIAL AND METHODS

Baseline Clinical Data

A total of 40 pregnant women, who were diagnosed as PE (PE group) in the hospital from

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January 2018 to December 2019 were selected as subjects. Their clinical data are shown as follows. That is, they met the diagnostic criteria for PE stipulated by Obstetrics and Gynecology (8th Edition). They were primiparas with singleton pregnancy. They were aged between 25 and 34 years old, and the gestational age at delivery was 36-38 weeks. Besides, 40 healthy pregnant women who underwent prenatal examination and gave birth in the same period were selected as a control group. Their clinical data are shown below. That is, obstetric complications, such as gestational diabetes mellitus, placenta previa, placental abruption and premature rupture of membranes, were excluded through prenatal examination. They were primiparas with singleton pregnancy. They were aged between 23 and 35 years old, and the gestational age at delivery was 37-40 weeks. The age of the pregnant women was 31.8 ± 4.5 years old in the PE group, and 30.3 ± 4.5 in the control group, and the gestational age was 37.5 ± 4.6 weeks in the PE group, and 38.1 ± 4.8 in the control group, without significant differences (P>0.05).

Apparatus

JEG-3 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (China). Lipofectamine 2000 transfection reagent, phosphate-buffered saline (PBS), minimum Eagle's medium (MEM), pancreatin, 0.1 percent crystal violet, immunohistochemical kit for Mfn2 and TRIzol kit were bought from Invitrogen (USA). ELISA kits for Mfn2 and vascular endothelial growth factor (VEGF) were provided by Beijing Solarbio Life Sciences Co., Ltd. (China). Primary antibodies against matrix metalloproteinase-2 (MMP-2), MMP-9, hypoxia-inducible factor 1-alpha (HIF-1α) and GAPDH were offered by Abcam (USA). Transwell chambers were purchased from Millipore (USA). Small-interfering RNA-Mfn2 (si-Mfn2) and its negative control si-NC vector were designed and synthesised, and normal goat serum working solution (SP-9001) for blocking and horseradish peroxidase (HRP)-labelled secondary antibody were bought from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (China). The multifunctional microplate reader was offered by Thermo Fisher Scientific (USA). ABI 7500 fluorescence quantitative real-time polymerase chain reaction (qRT-PCR) system was bought from ABI (USA). Inverted microscope was bought from Leica (Germany).

Collection and Storage of Placental Samples

Thirty minutes after delivery, a villus tissue mass (about 1 cm × 1 cm × 1 cm) was cut from the central part of the placenta's maternal surface away from calcified and necrotic areas. Then the tissue mass was rinsed repeatedly with normal saline until the residual blood was completely washed off, and water was sucked out on a filter paper. Subsequently, the tissue mass was frozen briefly in liquid nitrogen in a freezing tube for 10 minutes. After being taken out, it was stored in an -80°C refrigerator. All tissue samples were collected after the subjects signed the informed consent, and the procedures were reviewed and approved by the Ethics Committee of the hospital.

Detection of Mfn2 Protein Expression in Placental Tissue by Immunohistochemistry

The placental tissues fixed with four percent formaldehyde solution were taken out for the detection of the protein expression level of Mfn2 in each group of placental tissue by immunohistochemistry. First, the tissues were put into citrate buffer for antigen retrieval after dehydration with dimethylbenzene and ethanol, and blocked with three percent hydrogen peroxide for 10 minutes at room temperature. Then the tissues were incubated with the Mfn2 primary antibody overnight at 4°C, washed with PBS the next day, incubated with biotin-labelled secondary antibody for 30 minutes at room temperature and washed again. Subsequently, an appropriate amount of diaminobenzidine was added for 4 minutes of reaction, which was terminated by deionised water, followed by counterstaining with hematoxylin for 2 minutes. After hematoxylin was washed off, the tissue sections were placed in ethanol for gradient dehydration, dried in xylene, and sealed with neutral balsam. Finally, the sections were observed under a microscope using the HRP-labelled streptavidin-biotin method. Image-Pro Plus software was em-

ployed to measure the optical density in the positively stained area.

Detection of Mfn2 mRNA Expression in Placental Tissue by qRT-PCR

RNA was extracted from 100 mg of each placental tissue sample according to the instructions of TRIzol RNA extraction kit, and its integrity was identified by agarose gel electrophoresis. Afterwards, the expression level of Mfn2 was determined by qRT-PCR under the following reaction conditions, that is, pre-denaturation at 95°C for 30 seconds, denaturation at 95°C for 60 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 50 seconds, 40 cycles in total. The procedure was repeated three times for each group, using β-actin as the internal reference. Upstream primer for Mfn2: 5'-CTCGAGATGTCCCTGCTCTTCT-3' and downstream primer: 5'-CTCCTCGGCCATTGCAGTC-3'. Upstream primer for U6: 5'-CTCGCTTCGGCAGCACA-3' and downstream primer: 5'AACGCTTCACGAATTTGCGT-3'. The relative expression was represented as $2^{-\Delta\Delta Ct}$. Each sample was tested in triplicate independently.

Cell Culture

JEG-3 cells were cultured in MEM containing a mixture of ten percent foetal bovine serum and one percent penicillin-streptomycin in an incubator with five percent CO_2 at 37°C, and subcultured every 3 days. Trypsin digestion was performed when the cells were in the logarithmic growth phase. Then si-Mfn2 and si-NC were transfected into the cells using transfection reagents. The cells were collected following 48 hours of culture, and the Mfn2 level in each group was measured by Western blotting.

Detection of Cell Proliferation by CCK-8 and Colony Formation Assays

The si-NC group, si-Mfn2 group and untransfected JEG-3 cells were seeded into 12-well plates at 5×10^3/well. Five repeated wells were set for each group, and the original medium was replaced once a week. Two weeks later, Coomassie brilliant blue was used for cell staining, and the cell colonies were counted under the microscope.

The cells were grouped as above, with 5 repeated wells for each group. After the density was adjusted to 5×10^4/mL, the cells were inoculated into 96-well plates (100 μL per well). Following culture for 24 hours, each well was added to 10 μL of CCK-8 solution and incubated in the incubator at 37°C for 2 hours in the dark. Finally, the optical density at 450 nm was measured using the microplate reader.

Detection of Cell Migration by Scratch Assay

The density of the above cells was adjusted to 5×10^4/mL with MEM containing ten percent foetal bovine serum, and then the cells were seeded into 12-well plates. Afterwards, each well was added with 1 mL of cell suspension, followed by 24 hours of incubation in the incubator at 37°C. After the cells basically reached confluence, the cell layer was scratched with a 200-μL pipette tip, and the cell debris was washed away with PBS 3 times. The cell migration was observed under the inverted microscope after incubation for 24 hours. The cell migration rate (%) = (distance before migration – distance after migration)/distance before migration × 100 percent.

Detection of Cell Invasion by Transwell Assay

Twelve hours before the experiment, the cell culture medium was replaced with serum-free medium for starvation. Then the cells were plated at a density of 2×10^5/mL. Afterwards, 200 mL of cell suspension was sucked out and inoculated into the upper Transwell chamber pre-coated with Matrigel, while 500 mL of MEM was added into the lower Transwell chamber. Following 24 hours of culture in the incubator, the chambers were taken out and washed twice with PBS, followed by fixing with methanol for 30 minutes. Subsequently, the cells that did not migrate from the upper Transwell chamber to the lower one were gently wiped off with cotton swabs and placed upside down for drying. The migrating cells were stained with 0.1 percent crystal violet, observed under the inverted microscope, and counted in 5 randomly selected visual fields.

Detection of Hypoxia-Related Protein Expressions by Western Blot

The above-mentioned cells were collected and lysed with 300 mL of RIPA lysis buffer for 10

minutes, followed by centrifugation at 15,000 rpm for 3 minutes to obtain the supernatant. Next, BCA protein kit was utilised to determine the protein concentration in the supernatant. After ten percent gel SDS-PAGE, the proteins were transferred onto a polyvinylidene fluoride membrane that was blocked with five percent skim milk at room temperature for 2 hours. The membrane was thereafter incubated with primary antibodies against HIF-1α (diluted at 1: 500), HIF proline hydroxylation-1 (HPH-1) (diluted at 1: 1500), leptin (diluted at 1: 1000) and GAPDH (diluted at 1: 500) overnight at 4°C. After rewarming the next day, the PVDF membrane was washed and incubated with diluted HRP-labelled goat anti-mouse IgG antibody at 37°C for 2 hours. Images were developed using ECL reagent. The membrane was photographed, and the gray values of target bands were analysed by ImageJ software, with GAPDH as the internal reference.

Statistical Analysis

All data were statistically analysed by GraphPad Prism 5 software and expressed as mean ± standard deviation. Intergroup comparisons were performed by a one-way analysis of variance. $P<0.05$ was considered statistically significant.

RESULTS

Mfn2 was Lowly Expressed in Placental Tissues of PE Pregnant Women

Mfn2 exhibited a strong positive protein expression in the placental tissues of normal pregnant women, but a weak negative protein expression in those of PE pregnant women (Fig. 1). The Mfn2 mRNA expression in placenta tissue of normal pregnant women was (0.82 ± 0.11), and that of PE women was (0.24 ± 0.12). Compared with that in the placental tissues of normal pregnant women, the relative mRNA expression of Mfn2 in the placental tissues of PE pregnant women was significantly reduced ($P<0.05$) (Fig. 2).

Mfn2 Levels in Cells After Transfection

The Mfn2 protein expression levels of control, si-NC and si-Mfn2 groups were (1.21 ± 0.11), (1.13 ± 0.12) and (0.25 ± 0.03), respectively. The protein level of Mfn2 in cells declined significantly in si-Mfn2 group compared with that in control group ($P<0.05$), while it had no significant difference between si-NC group and control group ($P>0.05$) (Fig. 3).

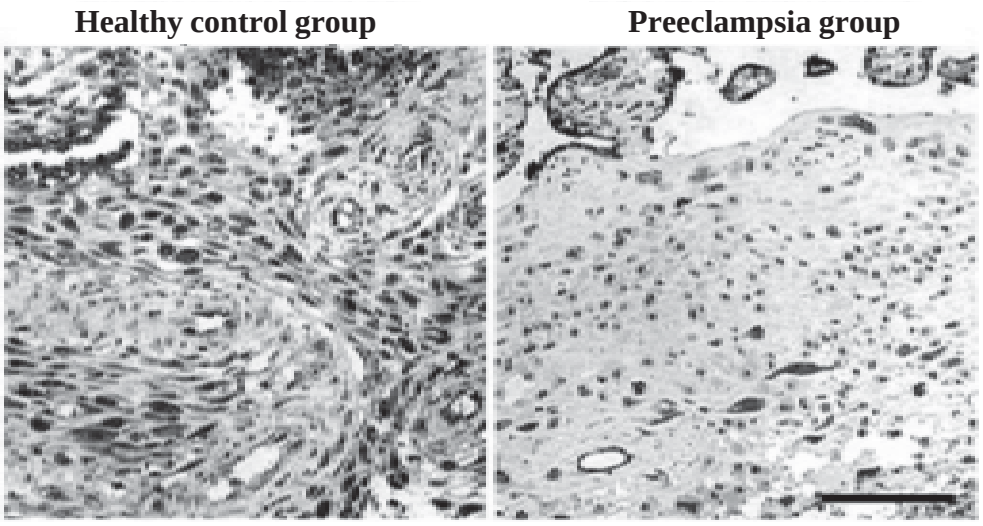

Fig. 1. Mfn2 expressions in placental tissues of normal and PE pregnant women observed by immunohistochemistry. Left: Placental tissue of normal pregnant woman in which Mfn2 had strong positive expression; right: placental tissue of PE woman in which Mfn2 had weak negative expression

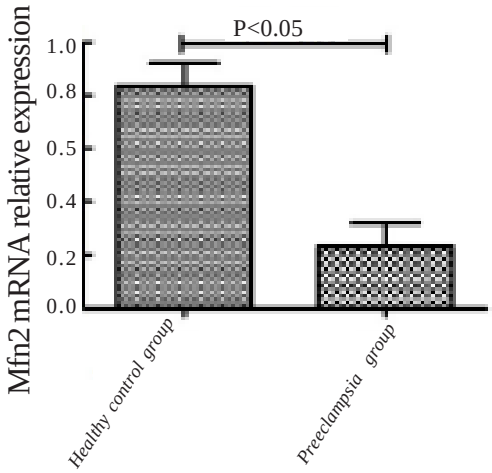

Fig. 2. Mfn2 expressions in placental tissues of normal and PE pregnant women detected by RT-PC

Mfn2 Promoted JEG-3 Cell Proliferation

Si-Mfn2 group exhibited a significantly weaker cell proliferation ability (P<0.05) and a significantly smaller number of colonies (P<0.05) than control group, but there were no significant differences in those indicators between control group and si-NC group (P>0.05) (Fig. 4).

Mfn2 Promoted JEG-3 Cell Migration

The cell migration rates of control, si-NC and si-Mfn2 groups were 47.82 ± 1.14 percent, 45.42 ± 2.35 percent and 15.21 ± 2.23 percent, respectively. The cell migration ability was attenuated significantly in si-Mfn2 group compared with that in control group and si-NC group (P<0.05), whereas it was not significantly different in the control group from that in si-NC group (P>0.05) (Fig. 5).

Mfn2 Promoted JEG-3 Cell Invasion

The numbers of invasive cells in control, si-NC and si-Mfn2 groups were (267 ± 21), (260 ± 18) and (87 ± 7), respectively. The number of invasive cells in si-Mfn2 group was decreased significantly compared with that in control group and si-NC group (P<0.05), while the invasion ability of cells displayed no significant difference between si-NC group and control group (P>0.05) (Fig. 6).

Mfn2 Inhibited Expressions of Hypoxia-Related Proteins HIF-1α, HPH-1 and Leptin

The protein expressions of HIF-1α, HPH-1 and leptin in cells were significantly higher in si-Mfn2 group than those in control group (P<0.05), but the differences in such protein expressions were not significant between si-NC group and control group (P>0.05) (Fig.7).

DISCUSSION

PE is not only an idiopathic disease in women during pregnancy but also a severe compli-

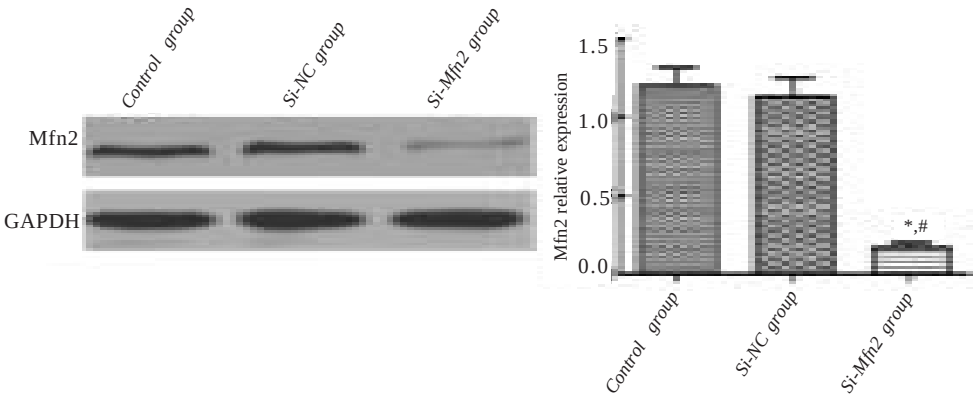

Fig. 3. Mfn2 levels in cells after transfection. Compared with control group, *P<0.05; compared with si-NC group, #P<0.05

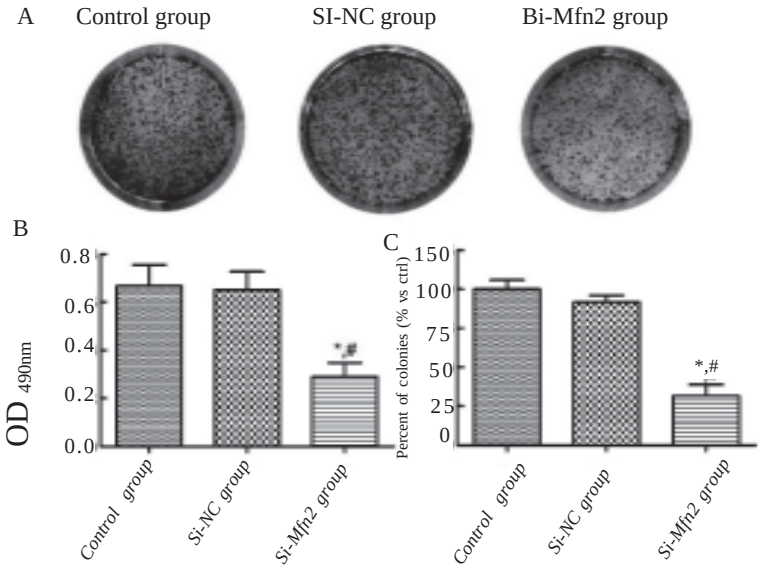

Fig. 4. Effects of Mfn2 on proliferation capacity of trophoblasts. A: Cell colonies; B: cell proliferation capacities; C: percentages of colonies. Compared with control group, *P<0.05; compared with si-NC group, #P<0.05

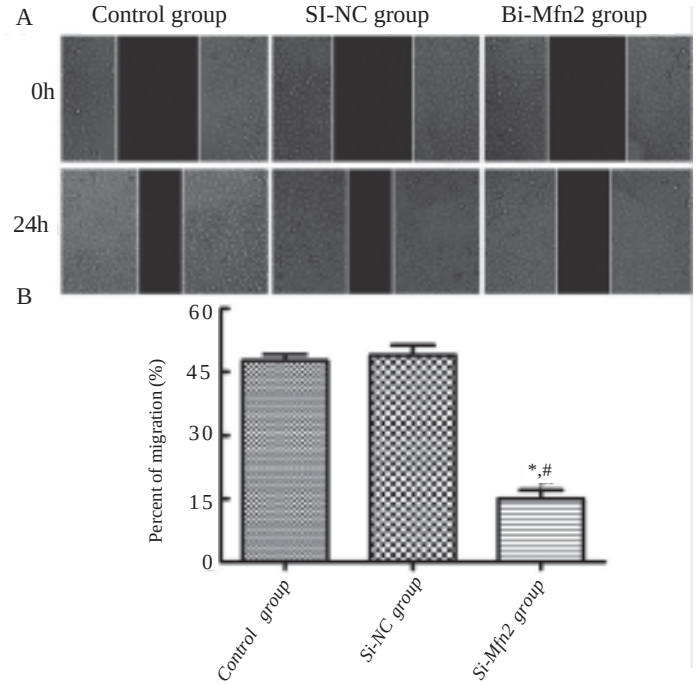

Fig. 5. Effects of Mfn2 on migration capacity of trophoblasts. A: Cell migration; B: percentage of migrating cells. Compared with control group, *P<0.05; compared with si-NC group, #P<0.05

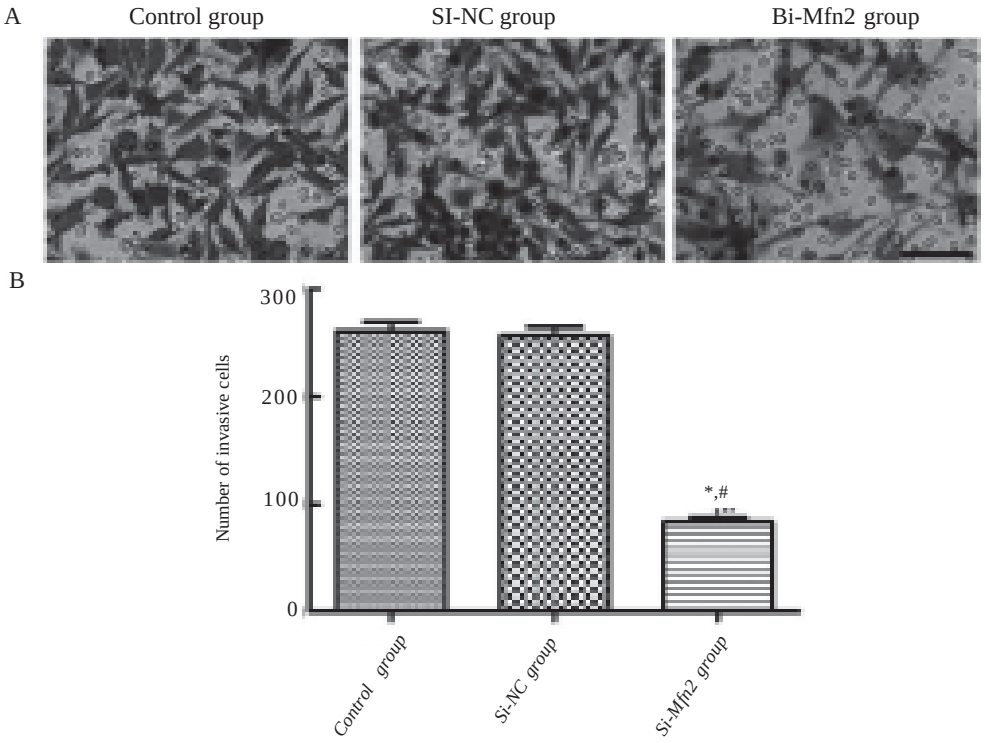

Fig. 6. Effects of Mfn2 on invasion capacity of trophoblasts. A: Cell invasion; B: number of invasive cells. Compared with control group, *P<0.05; compared with si-NC group, #P<0.05

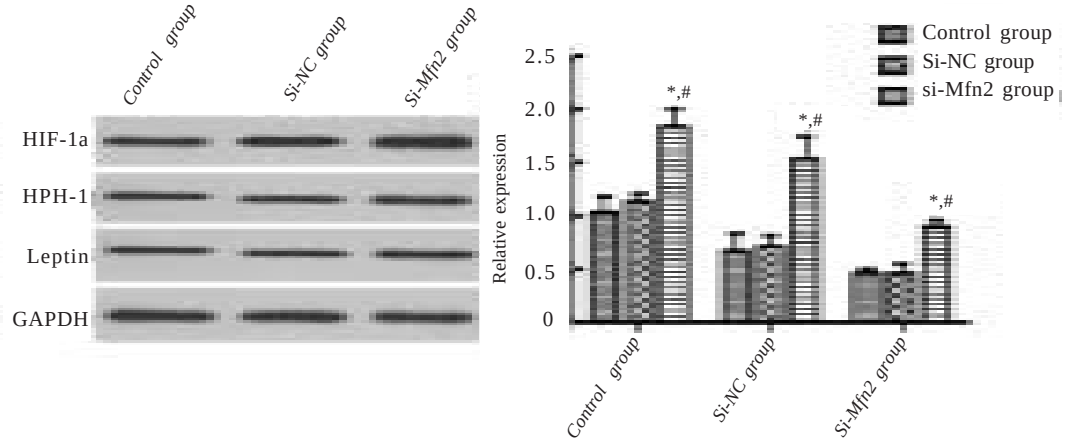

Fig. 7. Expression levels of hypoxia-related proteins measured by Western blot. Compared with control group,

cation in obstetrics, whose major clinical manifestations are proteinuria, edema and hypertension (Zhuang et al. 2019). Currently, the incidence rate of PE is rising year by year in China (Xiao et al. 2014). However, the pathogenesis of PE remains unclear at present, and the clinical treatments of PE are limited to symptomatic therapy and prevention of related complications. Moreover, the termination of pregnancy is the most efficacious treatment method for the disease in clinical practice, as otherwise various emergencies threatening the lives of puerperae and foetuses may occur (Smyth et al. 2017).

In recent years, a growing number of studies have demonstrated that mitochondrial dysfunction is closely correlated with the onset of such diseases as cardiovascular disease (Chistiakov et al. 2018) as well as the invasion and migration of tumour cells (Hsu et al. 2016; Han et al. 2018). The division and fusion of mitochondria themselves not only play crucial roles in maintaining normal mitochondrial morphology but also participate in sustaining the mitochondrial function, thereby keeping the life activities of cells such as energy metabolism, migration, invasion, infiltration and apoptosis (Nagy et al. 2015). In addition, aberrant mitochondrial dynamics and endothelial injury are detected in a variety of pregnancy-related diseases, suggesting that the dysfunction has close associations with the development and progression of the pregnancy-related diseases (Vaka et al. 2018). Mfn2, a mitochondrial dynamics-related gene discovered by Professor Guanghui Chen, is structurally composed of a GTPase domain in the N-terminus and a transmembrane protein domain in the C-terminus. There is a hydrophobic region with heptad repeats on either side of the transmembrane domain, which is a structural basis for the formation of most coiled coils (Ausman 2017). The GTPase domain in the N-terminus and the heptad repeats in the C-terminus of Mfn2 are both located in the cytoplasm and have pivotal effects on mitochondrial fusion (Dorn 2020). Besides, the N-terminus of Mfn2 protein has binding sites to Ras, which may be involved in mediating other biological functions in addition to mitochondrial fusion (Du et al. 2020). Located on the outer membrane of mitochondria, Mfn2 is capable of promoting the mitochondrial fusion. Meanwhile, it is implicated in the regulation of mitochondrial morphology and function as well as the proliferation and energy metabolism of different types of cells. Moreover, Mfn2 has been widely investigated as an important marker and molecular therapeutic target of cardiovascular diseases (Yu et al. 2018). Decreased protein expression level of Mfn2 in mice has an adverse effect on placentation, prominently manifested as apparent reduction of trophoblast cell count, and further affects mouse embryonic development, ultimately inducing second-trimester abortion (Zhao et al. 2015). In this study, therefore, the protein level of Mfn2 in the placental tissues of PE pregnant women and normal pregnant women was first determined, and it was shown that the Mfn2 expression was lowered remarkably in the placental tissues of PE pregnant women. Later, with the JEG-3 trophoblast cell line as the model, the correlations of the Mfn2 expression with trophoblast cell invasion and placental hypoxia were explored. The results manifested that the cells transfected with si-Mfn2 exhibited a notably lower expression level of Mfn2, proving the successful cell transfection. Furthermore, the proliferation, migration and invasion abilities of cells were weakened evidently in si-Mfn2 group compared with those in control group and si-NC group. HIF-1α, HPH-1 and leptin have been widely applied in hypoxia research as hypoxia-related indicators (Iriyama et al. 2015; Kalinderis et al. 2015), so the indicators related to placental hypoxia were measured in this study. It was found that si-Mfn2 group had distinctly elevated expression levels of HIF-1α, HPH-1 and leptin in cells, illustrating that the placental hypoxia is aggravated. All these results demonstrate that the decrease in the Mfn2 expression attenuates the invasion ability of trophoblast cells and increase the degree of placental hypoxia in the case of PE.

CONCLUSION

In conclusion, the reduced Mfn2 expression is strongly related to the incidence of PE, which can weaken the invasion ability of trophoblast cells and increase the degree of placental hypoxia, seriously impairing the health of pregnant women and foetuses.

RECOMMENDATIONS

Abnormal trophoblast invasion is a crucial factor for the pathogenesis of PE, and Mfn2 affects the invasion ability of trophoblasts. Hence, the detection and regulation of Mfn2 expression in early pregnancy provide new ideas for the diagnosis and treatment of PE. Meanwhile, many other pregnancy-related diseases (for example, recurrent miscarriage, gestational diabetes and foetal growth restriction) are related to abnormal trophoblast cell function. For these patients, detecting Mfn2 after excluding organic diseases may help clarify the pathogenesis.

LIMITATIONS

However, the specific mechanism of Mfn2 in PE onset still needs to be deeply explored.

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