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Mitochondrial fusion protein 2 regulates endoplasmic reticulum stress in preeclampsia

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Preeclampsia (PE) refers to a group of dysfunction syndromes associated with elevated blood pressure and proteinuria in women with previously normal blood pressure after 20 weeks of pregnancy, and it may be accompanied by symptoms including headache, vertigo, nausea and vomiting, and epigastric discomfort (Steegers et al., 2010). It is affected by a variety of risk factors and follows a distinctive disease progression, although its pathogenesis is still unknown. Upon termination of a PE pregnancy, the clinical symptoms of PE improve rapidly, suggesting that PE is a disease originating in the placenta. Recent studies have shown that excessive apoptosis of trophoblast cells and reduced trophoblast infiltration capacity can lead to superficial implantation of the placenta, a lack of spiral arteriole remodeling, and insufficient placental perfusion leading to placental ischemia and hypoxia, all of which are important factors that cause the onset of PE (Eddy et al., 2019). Therefore, there appears to be a close relationship between excessive trophoblast cell apoptosis and development of PE. Recently, increasing evidence has shown that in patients with PE, endoplasmic reticulum (ER) stress can cause trophoblast cell apoptosis, and this finding has become an important feature of the placental pathology of PE (Lorenzon-Ojea et al., 2020).

Mitochondrial fusion protein 2 (Mfn2) is a mitochondrial membrane protein, which is involved in the promotion of mitochondrial fusion, the regulation of mitochondrial morphology and function, cell energy metabolism, signal transduction, proliferation, apoptosis, and many other cellular processes (Chan, 2006). The latest research shows that Mfn2 also plays a key role in a variety of ER functions, such as calcium balance and protein synthesis (Chandhok et al., 2018). More than this, studies have found that Mfn2 expression is abnormal in the tissues of patients with PE and that Mfn2 plays an important role in embryo development during the first trimester (Yu et al., 2016). However, while Mfn2 protein expression levels are known to be altered in the placentas of pregnant women with PE, the role of Mfn2 in trophoblasts and the question of whether it can regulate ER stress to inhibit PE remain unclear.

To explore the role of Mfn2 in PE, we analyzed the expression levels of Mfn2 in samples from 20 women with normal pregnancies and 20 women with severe PE using western blot. The clinical characteristics of all patients are summarized in Table 1. The results indicate that the expression of Mfn2 was lower in PE tissues than in normal tissues (Fig. 1), meaning that decreased Mfn2 levels may be associated with the development of PE. Furthermore, we transfected JEG-3 cells with either a small interfering RNA (siRNA) control or siRNA-Mfn2. The expression levels of Mfn2 were significantly down-regulated in cells transfected with siRNA-Mfn2 compared with the siRNA control group cells after 48 h (Fig. 2a). The cholecystokinin-8 (CCK8) and flow cytometry assays showed that decreasing Mfn2 levels inhibited the proliferation (Fig. 2b) and promoted the apoptosis (Fig. 2e) of JEG-3 cells, while wound healing assays revealed that decreased expression of Mfn2 greatly inhibited the migration of JEG-3 cells (Fig. 2c). In addition, we further tested the effect of Mfn2 on the invasion capacity of trophoblast cells. As shown in Fig. 2d, decreased

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Table 1 Clinical characteristics of patients with or without preeclampsia

Group	Number	Age (year)	Gestational age at delivery (week)	Blood pressure		Birth weight (g)	Fetal gender, male/female
				Systolic (mmHg)	Diastolic (mmHg)		
Preeclampsia	20	30.65±5.16	33.35±3.28	157.45±19.84	99.70±7.36	2445.00±530.62	8/12
Normal	20	29.20±4.27	39.10±0.85	118.95±12.40	71.85±10.64	3626.00±387.49	11/9
P		0.339	<0.001	<0.001	<0.001	<0.001	0.355

Data (except number, fetal gender, and *P*) were expressed as mean±standard deviation. 1 mmHg=0.133 kPa.

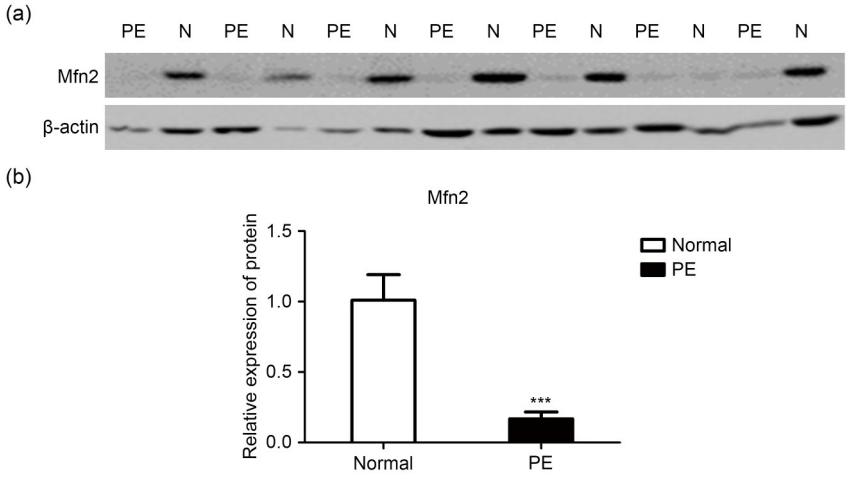

Fig. 1 Mitochondrial fusion protein 2 (Mfn2) expression is down-regulated in preeclampsia (PE) tissues. (a) Western blot analysis of the expression of Mfn2 in PE tissues (group PE) and normal pregnancy (group N) (randomly selected seven groups); (b) Relative expression of Mfn2 in PE and normal groups. Values are expressed as mean±standard deviation (n=20). *** P<0.001, vs. normal group.

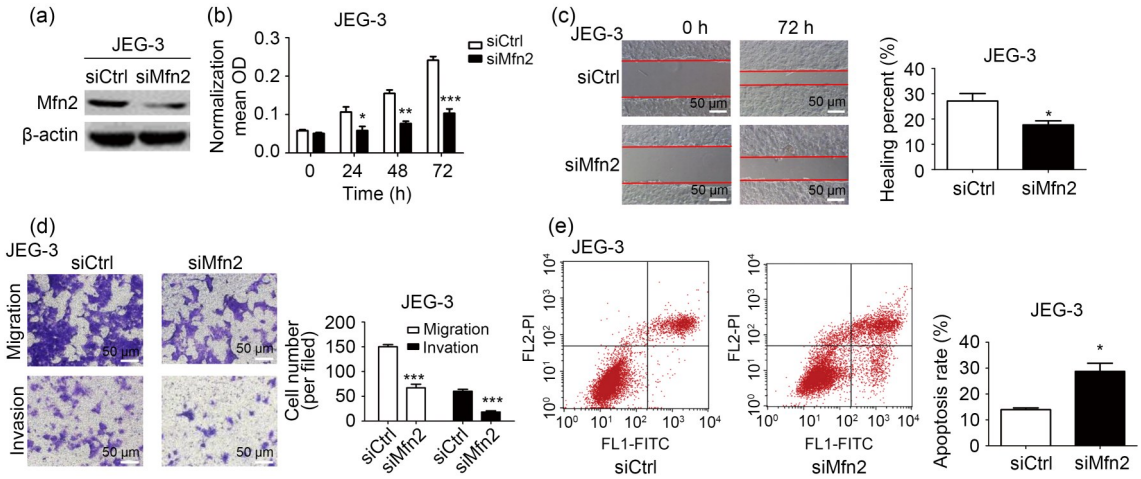

Fig. 2 Decrease of mitochondrial fusion protein 2 (Mfn2) suppress the proliferation, migration, and invasion of JEG-3 cells. (a) The levels of Mfn2 were in JEG-3 cells after transfection with small interfering RNA (siRNA) control (siCtrl) or siRNA-Mfn2 (siMfn2) by western blot; (b–e) Cholecystokinin-8 (CCK8) assay (b), wound healing (100× magnification) (c), Transwell assay (100× magnification) (d), and flow cytometry assay (e) were used to observe proliferation, migration, and invasive and apoptosis capacity of trophoblast cells after transfection with siCtrl or siMfn2, respectively. Data are expressed as mean±standard error of the mean (n=3). * P<0.05, ** P<0.01, *** P<0.001, vs. siCtrl.

expression of Mfn2 markedly repressed the invasive capacity of JEG-3 cells. Meanwhile, we obtained similar results in JAR cells (Fig. S1). These findings indicate that decreased Mfn2 expression may effectively

suppress the proliferation, migration, and invasion of trophoblast cells in vitro.

ER stress is a characteristic pathological feature of PE (Chaiworapongsa et al., 2014). To determine whether ER stress affects Mfn2 expression, JEG-3 cells were treated with different concentrations of tunicamycin (Tm) in culture for 24 h, after which the protein expression levels of Mfn2 and ER stress markers such as protein kinase R-like ER kinase (PERK), CCAAT/enhancer-binding protein homologous protein (CHOP), and 78-kDa glucose-regulated protein (GRP78) were detected by western blot, while quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to determine the expression level of Mfn2 messenger RNA (mRNA). The results of these analyses showed that when compared with untreated controls, the expression of Mfn2 in JEG-3 cells was significantly reduced under conditions of ER stress (Fig. 3). Also, to further determine whether Mfn2 supports trophoblast cell viability and inhibits accumulation of cellular oxidative stress during ER stress, we established a stable Mfn2-overexpression JEG-3 cell line. Puromycin was used to select stably transfected cells. The overexpression of Mfn2 was confirmed by western blot analysis (Fig. 4a). The stable cell lines were then incubated with 2.5 μmol/L Tm for 24 h. Following treatment, cell viability and oxidative stress levels in trophoblast cells were measured using a CCK8 assay (Fig. 4b), flow cytometry (Fig. 4d), and a superoxide dismutase (SOD) detection assay (Fig. 4c). The results of these analyses indicated that Mfn2 overexpression can increase cell viability and inhibit accumulation of cellular oxidative stress in trophoblast cells in the context of ER stress.

At present, there are many hypotheses regarding the pathogenesis of PE revolving around immunological factors, genetic influences, oxidative stress, and vascular damage, but these hypotheses cannot fully explain the pathological progression of PE. The symptoms of PE significantly worsen as pregnancy progresses, but upon delivery of the placenta, they improve rapidly, suggesting that PE is related to the presence of the placenta. Placental factors are also considered to be an important cause of PE onset (Chaiworapongsa et al., 2014). Women suffering from placental trophoblastic disease can have symptoms similar to those of PE, which further suggests that placental trophoblast function is related to the onset of PE. In early pregnancy, the remodeling of spiral arterioles depends on the apoptosis of vascular endothelial cells and smooth muscle cells mediated by extravillous trophoblasts (EVTs). The role EVTs play in promoting the invasion of the spiral arteries, and the destruction of the vascular wall is similar to that of malignant tumor cells, but this invasive behavior is restricted to the first trimester and the inner 1/3 of the myometrium (Genbacev and Miller, 2000). When EVTs undergo excessive apoptosis, their invasive ability is weakened, leading to insufficient remodeling of spiral arterioles and shallow implantation of the placenta and also causing local trophoblast cells to be ischemic and hypoxic in the placenta; this drives the development of the first stage of PE. Placental ischemia and hypoxia caused by insufficient spiral arteriole remodeling can induce syncytiotrophoblast cell apoptosis through various apoptotic pathways. This releases syncytiotrophoblast microparticles (STBMs) into the maternal bloodstream, causing inflammation throughout the body. Eventually, the inflammation damages

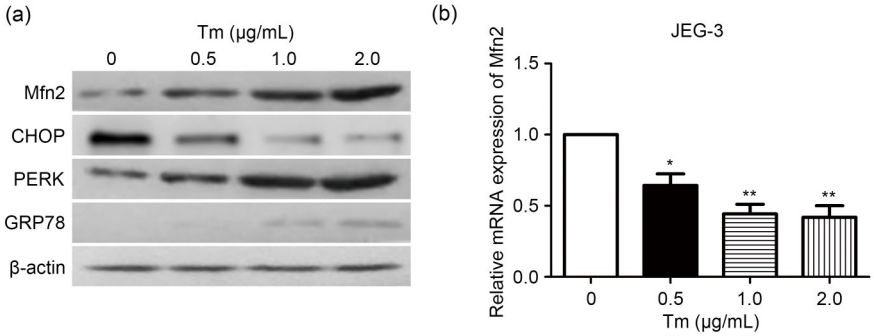

Fig. 3 Effect of endoplasmic reticulum stress on mitochondrial fusion protein 2 (Mfn2) expression in trophoblast cells. The expression of Mfn2 protein was detected western blot (a) and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (b) after JEG-3 cells were cultured in different concentrations of tunicamycin (Tm) for 24 h. Data are expressed as mean±standard error of the mean (n=3). * P<0.05, ** P<0.01, vs. control (0 μg/mL Tm). CHOP: CCAAT/enhancer-binding protein homologous protein; PERK: protein kinase R-like endoplasmic reticulum kinase; GRP78: 78-kDa glucose-regulated protein; mRNA: messenger RNA.

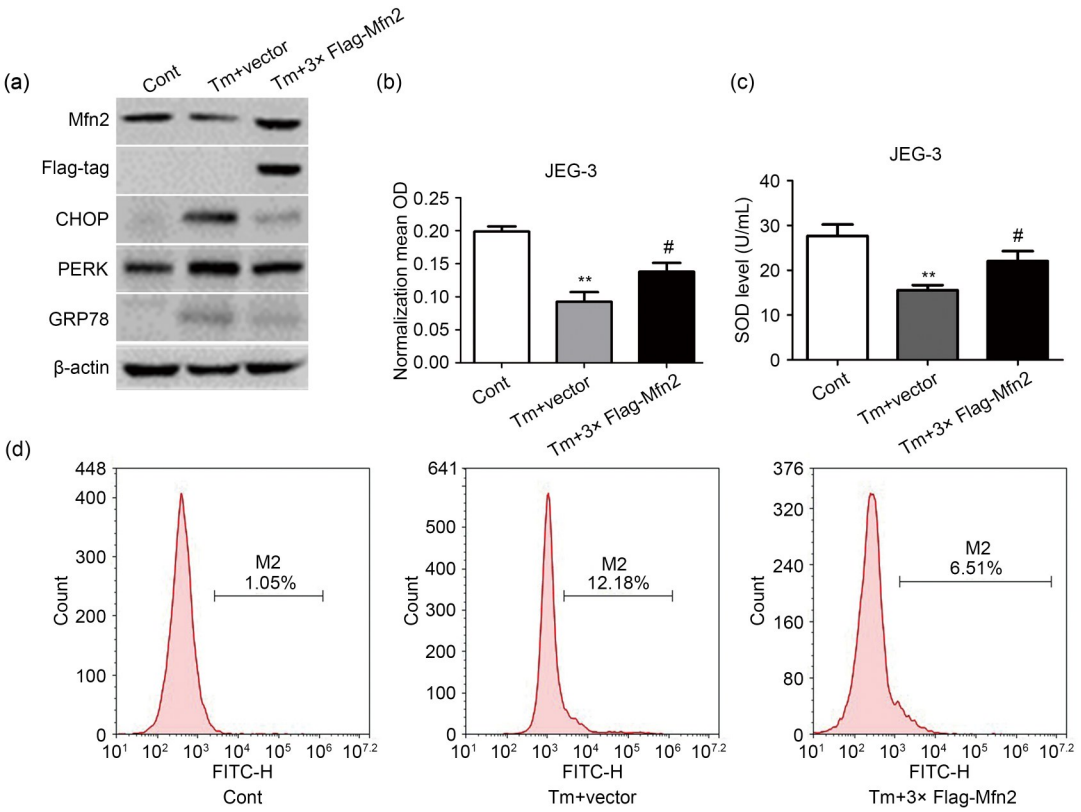

Fig. 4 Regulation of trophoblast viability and cellular oxidative stress by mitochondrial fusion protein 2 (Mfn2) under endoplasmic reticulum stress. (a) Levels of Mfn2 were assessed in the Mfn2-overexpressed stable cell line and control stable cell line; (b) Cholecystokinin-8 (CCK-8) assay was used to detected cell viability; (c, d) Superoxide dismutase (SOD) detection assay (c) and flow cytometry (d) were used to detected cellular oxidative stress of trophoblast cells under endoplasmic reticulum stress. Data are expressed as mean±standard error of the mean (n=3). ** P<0.01, vs. control; $^{\#}$ P<0.05, vs. Tm+vector. Cont: control; Tm: tunicamycin; CHOP: CCAAT/enhancer-binding protein homologous protein; PERK: protein kinase R-like endoplasmic reticulum kinase; GRP78: 78-kDa glucose-regulated protein.

the vascular endothelial cells, causing various clinical symptoms of more progressed PE (Brosens et al., 2019).

The reduced invasive ability of placental trophoblast cells, along with placental ischemia and hypoxia, is regarded as key events in PE. Excessive apoptosis severely diminishes the invasive ability of trophoblast cells. Trophoblast apoptosis is widespread in placental tissues at different stages of normal pregnancy, making trophoblast apoptosis essential for a normal healthy pregnancy, and this apoptosis is strictly regulated by a series of mechanisms to ensure the normal progression of the pregnancy. However, pathological apoptosis can lead to the occurrence of various diseases. Previous studies examining the mechanisms of apoptosis and related signaling pathways have focused on mitochondrial damage (intrinsic pathway) and extracellular death receptors (extrinsic pathway). In recent years, some studies have reported that the ER may be another important organelle that induces apoptosis and this type of apoptosis may not depend on mitochondria or death receptor pathways. There are many chaperone proteins and folding enzymes present in the ER. When the ER is stimulated by various pathological factors, unfolded or misfolded proteins in the ER lumen will aggregate and damage the normal physiological functions of the cell, leading to ER stress. As a new mechanism of apoptosis, the ER stress apoptotic pathway has been found to be closely related to degenerative neurological diseases, cancers, and osteoporosis. However, there are few studies on the relationship between ER stress-related apoptosis and PE. Burton et al. (2010) have reported that there is oxidative stress in the placental tissue of patients with PE, and that induction of sustained ER stress will promote excessive apoptosis of trophoblast cells, thereby resulting in maternal-fetal injury.

Mfn2 is located on the outer mitochondrial membrane and plays a key role in fusion for this membrane. Chan (2006) has found that Mfn2 not only

participates in the regulation of mitochondrial morphology, but also plays key roles in cell metabolism, proliferation, and apoptosis. Recent studies have also shown that Mfn2 can regulate the morphology of the ER and participate in the regulation of ER stress. Mfn2 expression is increased in the late stages of ER stress, while other mitochondrial fusion-related proteins such as Mfn1, optic atrophy 1 (OPA1), dynamin-related protein 1 (Drp1), and mitochondrial fission 1 (Fis1) are not significantly changed (Ngoh et al., 2012). In Mfn2-knockout mouse embryonic fibroblasts and adult mouse cardiomyocytes, the expression of intracellular chaperone proteins was increased (Song et al., 2015), indicating that Mfn2 can regulate ER stress. Related studies found evidence that mitochondrial dysfunction can also induce ER stress. In order to investigate whether Mfn2 is involved in directly regulating ER stress or indirectly regulating it by modulating mitochondrial morphology and function, Debattisti et al. (2014) used *Drosophila* with either an OPA1 knockout (OPA1 is an important protein for mitochondrial inner membrane fusion) or an Mfn2 knockout, and found that only the Mfn2 knockout induced ER stress in the flies, indicating that Mfn2 may directly participate in the regulation of ER stress. Muñoz et al. (2013) further demonstrated that Mfn2 can bind to PERK and inhibit its function. When Mfn2 levels are decreased, PERK is continuously activated, resulting in ER stress. Similarly, Mfn2 knockout has also been shown to induce ER stress in hypothalamic anorexigenic pro-opiomelanocortin (POMC) neurons (Schneeberger et al., 2013).

Although previous studies have shown that the expression of Mfn2 in the tissues of patients with PE is abnormal, these studies focused on the mRNA expression level (Yu et al., 2016). The protein levels of Mfn2 in the placenta of patients with PE and the potential effects of its expression on the biological phenotype of trophoblast cells have not been studied. In the present study, we analyzed the expression of Mfn2 in PE tissues and found that Mfn2 protein expression was significantly decreased in PE tissues compared to healthy placental tissues. We also examined the effects of Mfn2 knockdown on proliferation, invasion, and metastasis of trophoblast cells. The expression of Mfn2 in trophoblasts was significantly decreased following the induction of ER stress via Tm treatment, and the overexpression of Mfn2 rescued trophoblast cell viability and could inhibit accumulation of cellular oxidative stress during ER stress; together, these findings suggest that aberrant placental Mfn2 expression may be involved in the pathogenesis of PE. However, the present study has some limitations. First, we focused only on the influence of Mfn2 on trophoblast cells in vitro and thus were unable to draw conclusions on the effects of Mfn2 in an animal or in vivo model of PE. Second, the study did not thoroughly analyze the molecular mechanism of Mfn2 which regulates ER stress in PE. In addition, the present study was carried out with a relatively small sample size, which only included women with severe PE. It is unknown whether Mfn2 is also decreased in other kinds of PE, such as mild and critical cases. Therefore, further research is needed to analyze the molecular mechanism of Mfn2 in PE, and we recommend a larger sample size to investigate the relation between Mfn2 and both mild and severe manifestations of PE.

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Author contributions

Jihua JIN conceived the study and reviewed the manuscript. Dandan SUN searched the literature, collected the data, and drafted the manuscript. Hanbing WU, Ling AI, Yanting WU, and Hui ZHU performed the statistical analysis. All authors have read and approved the final paper, and they have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Dandan SUN, Hui ZHU, Ling AI, Hanbing WU, Yanting WU, and Jihua JIN declare that they have no conflict of interest.

The present study was approved by the Ethics Committee of Jiaxing Municipal Maternal and Child Health Care Hospital (Jiaxing, China). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

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Supplementary information

Materials and methods; Fig. S1