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Human placenta-derived mesenchymal stem cells induce trophoblast invasion via dynamic effects on mitochondrial function

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Abstract

The trophoblast is a critical cell for placental development and embryo implantation in the placenta. We previously reported that placenta-derived mesenchymal stem cells (PD-MSCs) increase trophoblast invasion through several signaling pathways. However, the paracrine effects of PD-MSCs on mitochondrial function in trophoblasts are still unclear. Therefore, the objective of the study was to analyze the mitochondrial function of trophoblasts in response to cocultivation with PD-MSCs. The results showed that PD-MSCs regulate the balance between cell survival and death and protect damaged mitochondria in trophoblasts from oxidative stress. Moreover, PD-MSCs upregulate factors involved in mitochondrial autophagy in trophoblast cells. Finally, PD-MSCs improve trophoblast invasion. Taken together, the data indicate that PD-MSCs can regulate trophoblast invasion through dynamic effects on mitochondrial energy metabolism. These results support the fundamental role of mitochondrial energy mechanism in trophoblast invasion and suggest a new therapeutic strategy for infertility.

KEYWORDS
invasion, mitochondria, mitochondrial autophagy, placenta-derived mesenchymal stem cells, trophoblast

1 | INTRODUCTION

Normal placentation requires adequate invasion of trophoblast cells into the maternal endometrium of the uterus. During pregnancy, trophoblasts play a role in embryonic and placental development through effects on mononuclear cytotrophoblast cell fusion and differentiation into syncytiotrophoblasts, which form the maternal-fetal interface. Several studies have reported that numerous micro-environmental and cellular factors, including hypoxia inducible factor 1-alpha (HIF1-α), matrix metalloproteinase (MMP), and Rho family members, are involved in trophoblast invasion (Carter et al., 2015; Soares et al., 2017). First, transcription factors such as HIF1-α, which responds to hypoxia, are involved to placental development by stimulating trophoblast invasion. The overexpression of HIF1-α enhances trophoblast invasion via the autophagy pathway (Choi et al., 2012; Highet et al., 2015; Ietta et al., 2006). Second, MMPs are enzymes essential for invasion with the primary role of digesting the extracellular matrix. MMP-2 and MMP-9 are widely known as gelatinases that digest type IV collagen, the major collagen constituent of the basement membrane. MMP-2/9 deficiency leads to poor

placentation and gynecological diseases, such as preeclampsia, through vascular dysfunction (Espino et al., 2017; Li et al., 2018).

Mitochondria have multiple functions, including cellular metabolism, adenosine triphosphate (ATP) synthesis, reactive oxygen species (ROS) generation and calcium ion movement and signaling, that are important for cellular homeostasis. First, ROS generation is induced under hypoxic conditions and during cellular metabolism (e.g., cell survival signaling and ATP synthesis), leading to oxidative stress (Diebold & Chandel, 2016; Tarasov et al., 2012). High ROS levels lead to critical oxidative stress and result in preeclampsia, intrauterine growth restriction (IUGR), and cell death. However, antioxidants can manage moderate increases in ROS levels and selectively remove abnormal mitochondria (Khera et al., 2013; Youle & van der Bliek, 2012). Previous reports have suggested that heme oxygenase (HO) 1 and 2 and antioxidants promote the proliferation and invasion of various trophoblast cell lines, including human umbilical vein endothelial cells and human embryonic stem cells (Ha et al., 2015; Na et al., 2018). Second, cellular ATP production is regulated via calcium ion movement between mitochondria and the endoplasmic reticulum (ER). Ca^{2+} uptake into the mitochondrial matrix stimulates ATP production through the activation of dehydrogenases in the Krebs cycle (Decuypere et al., 2011). Ca^{2+} enters through inositol triphosphate receptor (IP3R) at ER-mitochondria junctions. IP3R, a membrane glycoprotein complex, is a well-known activator of Ca^{2+} channels and leads to proliferative arrest through effects on Ca^{2+} entry. Interestingly, IP3R is activated by PI3K and FGF signaling and upregulates cytosolic Ca^{2+}. Collectively, Ca^{2+} uptake into the mitochondria is necessary not only for determining cell fate (survival or death) but also for regulating mitochondrial function (Baczyk et al., 2011; Mound et al., 2013). Recent reports suggest a correlation between HIF1-α and mitochondrial calcium uniport (MCU). In MCU-deficient cells, mitochondrial ROS levels and HIF1-α gene expression are decreased. These data suggest that the signaling roles of mitochondrial ROS and HIF1-α are downstream of the mitochondrial calcium channel (Singh et al., 2017; Sonkar et al., 2016). In a similar study, Zhao et al. (2013) showed that mitochondrial dynamics, such as fission in breast cancer cells, regulates migration and invasion. Moreover, mitochondrial fragmentation by fission enhances cellular glycolysis and lactate production via upregulating DRP expression (Maycotte et al., 2017; Zhao et al., 2013).

Mitochondrial quality control systems regulate mitochondrial autophagy (mitophagy) and mitochondrial biogenesis and are thus involved in cellular homeostasis. Mitophagy is a major survival mechanism that balances mitochondrial fission and fusion. Persistent hypoxia induces selective mitophagy through the active destruction of damaged mitochondria (Chen & Dorn, 2013). Additionally, an imbalance between mitochondrial fission and fusion can induce trophoblast-related diseases. PTEN-induced putative kinase 1 (PINK1) protects cells from stress-induced mitochondrial damage and activates PARKIN, a cytosolic E3 ubiquitin ligase, to bind to depolarized mitochondria. PARKIN plays a role in recognizing proteins on the mitochondrial outer membrane and mediates the removal of damaged mitochondria via autophagy (Eiyama & Okamoto, 2015). The PINK1-PARKIN pathway is associated with the selective autophagy of damaged mitochondria. Chen & Dorn (2013) recently reported that PINK1 phosphorylates MFN2 and recruits PARKIN to dysfunctional mitochondria (Pickrell & Youle, 2015).

Human placenta-derived mesenchymal stem cells (PD-MSCs) have been in the spotlight recently for their high self-renewal capacity, immunomodulatory properties and therapeutic effects. Choi and et al. (2014) reported that mesenchymal stem cells (MSCs) and extracts isolated from normal term placenta promote trophoblast invasion and immunomodulation by altering HLA-G expression (Choi et al., 2014; Kim et al., 2018). Several reports, have suggested that MSCs improve trophoblast invasion through various mechanisms that influence cellular function, including integrin signaling (Chen, 2014; Huang et al., 2016). However, there is limited evidence for the detailed mechanism by which energy metabolism and mitochondrial function affect trophoblasts. Therefore, we investigated the effect of PD-MSCs on the proliferation, death and invasion of trophoblasts. We report the basic mechanism by which PD-MSCs regulate trophoblast invasion through effects on mitochondrial function.

2 | MATERIALS AND METHODS

2.1 | Cell culture and indirect coculture systems

Placentas were obtained from women without any medical, obstetrical, or surgical complications who delivered at term (38 ± 2 gestational weeks). It is approved by the Institutional Review Board of CHA General Hospital, Seoul, Korea (IRB 07-18). Briefly, PD-MSCs were isolated from as the inner side of the chorionic plate of the placentas obtained following Cesarean section, as described in previously reports (Lee et al., 2010). PD-MSCs were cultured in alpha-minimum essential medium (HyClone, GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 1% penicillin/streptomycin (Pen-Strep; Gibco-BRL), 25 μg/ml human fibroblast growth factor 4 (PeproTech Inc.) and 1 μg/ml heparin (Sigma-Aldrich) at 37°C in an incubator with a humidified atmosphere of 5% CO_2. The human extravillous trophoblast cell line HTR-8/SVneo was provided by Dr. Graham (Queen's University, Kingston, Ontario, Canada). HTR-8/SVneo cells were cultured in Roswell Park Memorial Institute 1640 medium (HyClone) supplemented with 5% FBS (Gibco-BRL) and 1% Pen-Strep (Gibco-BRL) under the same conditions as PD-MSCs.

In this study, indirect coculture was performed with a six-well cell culture trans well system (8 μm pore insert; Falcon, BD Biosciences). HTR-8/SVneo cells (1×10^5) were seeded in the lower chamber with 2 ml medium, PD-MSCs (2.5×10^5) were seeded in the upper chamber with 0.7 ml serum-free medium, and the system was incubated for 12, 24, and 48 h.

2.2 | Trypan blue staining

To analyze the survival rate of HTR-8/SVneo cells according to PD-MSC cocultivation, HTR-8/SVneo cells (1×10^5) were seeded in the lower chamber with 2 ml medium, and PD-MSCs (2.5×10^5) were

seeded in the upper chamber with 0.7 ml serum-free medium. The cells (including adherent and floating cells) were harvested at 12, 24, and 48 h and stained with trypan blue (Gibco-BRL); the positively stained cells were counted by using a hemocytometer.

2.3 | Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from harvested HTR-8/SVneo cells using TRIzol reagent (Ambion) according to the manufacturer's protocol. The isolated RNA concentration was quantified by a Nanodrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with 1 μg total RNA, 20 pmol oligo dT, 10 mM dNTP mix, RNase OUT, and SuperScript III Reverse Transcriptase (Invitrogen). The messenger RNA (mRNA) levels of specific genes were analyzed by qRT-PCR using SYBR Green Master Mix (Rox; Roche Diagnostics). The mRNA amplification conditions were as follows: denaturation at 95°C for 5 min, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The human glyceraldehyde 3-phosphate dehydrogenase gene was used as an internal control for normalization. All reactions were performed in duplicate or triplicate. The relative mRNA expression levels were analyzed by the comparative CT method. The human-specific primers used are presented in Table 1.

2.4 | The western blot analysis

Protein lysates were isolated from HTR-8/SVneo cells cocultured or not with PD-MSCs by using protein lysis buffer containing a protease inhibitor cocktail (mini-tablet; Roche) and phosphatase inhibitor cocktail II (A.G. Scientific). The protein lysates were then loaded onto 6%–15% sodium dodecyl sulphate (SDS) polyacrylamide gels for the detection of specific gene products. Separated proteins were transferred onto polyvinylidene difluoride membranes, which were then incubated for 1 h at room temperature with 5% bovine serum albumin (Amresco). After this blocking step, the membranes were incubated overnight at 4°C with antibodies against p-ERK (1:1000; Cell Signaling), BAX (1:500; Santa Cruz), Bcl-2 (1:500; Santa Cruz), HO-1 (1:1000; Novus), HO-2 (1:1000; Novus), superoxide dismutase (SOD; 1:1000; Novus), heat shock protein 60 (HSP60; 1:1000; Cell Signaling), prohibitin 1 (PHB1; 1:1000; Cell Signaling), voltage-dependent anion channel (VDAC; 1:1000; Cell Signaling), PINK1 (1:1000; Abcam), and PARKIN (1:1000; Abcam). The membranes were then incubated with secondary antibody or anti-rabbit immunoglobulin G for 1 h at room temperature in an orbital shaker. After a washing step, the bands were detected using enhanced chemiluminescence reagents (Bio-Rad Laboratories Inc.). All experiments were performed in duplicate or triplicate.

2.5 | Fluorescence-activated cell sorting (FACS) analysis

Trophoblast cells cocultured or not with PD-MSCs were treated with 0.05% trypsin and TrypLE Express solution (Gibco-BRL) containing 1 mM ethylenediaminetetraacetic acid. The extracted cells were immersed in 100% ethanol (Merck Millipore) at room temperature for 10 min and then in 0.5 μg/μl RNase A (Sigma-Aldrich) solution for 15 min. Next, the cells were stained with propidium iodide solution (1:200; Sigma-Aldrich). The cell cycle distribution of the stained cells was examined using a FACScan flow cytometer (BD Biosciences).

2.6 | Trans well assay

The invasiveness of trophoblasts according to PD-MSC cocultivation was analyzed by using inserts (8-μm pores; Falcon) in 24-well plates.

TABLE 1 mRNA primers used in this study

Gene	Sequence	NM number
HIF1-a	F: 5′-GTTTACTAAAGGACAAGTCA-3′ R: 5′-TTCTGTTTGTTGAAGGGAG-3′	001243084.1
HO-1	F: 5′-TGGTGATGGCCTCCCTGTACCACATCT-3′ R: 5′-AGAGCTGGATGTTGAGCAGGAACGCAGTCT-3′	001199033.1
HO-2	F: 5′-ATGTCAGCGGAAGTGGAA-3′ R: 5′-GGGAGTTTCAGTGCTCGC-3′	001202681.2
SOD	F: 5′-ATGGCGACGAAGGCCGTGTGCGTGCTGAAG-3′ R: 5′-TGCCTCTCTTCATCCTTTGGC-3′	000454.4
IP3R	F: 5′-CGGAGCAGGGTATTGGAACA-3′ R: 5′-GTCCACTGAGGGCTGAAACT-3′	002224.3
MCU	F: 5′-CGTTTCCAGTTGAGAGATGGC-3′ R: 5′-GATCCTCTGGTGTACCGTCC-3′	001270679.1
hGAPDH	F: 5′-CTCCTCTTCGGCAGCACA-3′ R: 5′-ACCGCTTCACCTAATTTGCGT-3′	001256799.2

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCU, mitochondrial calcium uniport; mRNA, messenger RNA; SOD, superoxide dismutase.

HTR-8/SVneo cells (1×10^4) were seeded in the upper chamber with serum-free medium, and PD-MSCs (5×10^4 cells) were seeded in the lower chamber with culture medium containing FBS. After incubation for 12, 24, and 48 h, all the cells in the upper wells were removed with a cotton swab. The invading cells that had attached to the bottom side of the filter were fixed with methanol for 20 min and stained with Mayer's hematoxylin (Dako) at room temperature. The cell invasion ability was determined by counting the number of stained cells in nine randomly selected fields on the membranes at ×100 magnification. The number of invaded trophoblast cells cocultivated with PD-MSCs was normalized to that of control cells, and the data are presented as the mean invasion.

2.7 | Zymography

HTR-8/SVneo cell culture supernatants were harvested to determine MMP-2/9 activity by zymography. The conditioned medium was separated in a 12% SDS-polyacrylamide gel electrophoresis gel supplemented with 0.5% gelatin (Sigma-Aldrich). The separated proteins were incubated for 30 min in renaturation buffer (Bio-Rad Laboratories), rinsed and incubated in development buffer (Bio-Rad Laboratories) for 24 h at 37°C in an orbital shaker. The gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) solution for 3 h at room temperature and then distained with a buffer containing 10% acetic acid and 30% methanol (Merck) until the zymogen bands could be visualized. MMP-2 and MMP-9 activity was analyzed based on the intensity of the unstained bands. All experiments were performed in duplicate.

2.8 | Mito SOX and Mito Tracker immunofluorescence

HTR-8/SVneo cells were seeded on cover slips and then cocultured with PD-MSCs using inserts for 12, 24, and 48 h. Next, the cells were washed with 1X cold PBS and incubated with MitoSOX and MitoTracker (Invitrogen) at 37°C. The cells were then washed with 1X cold PBS and incubated with 1 μg/ml diamidino-phenylindole hydrochloride (Sigma-Aldrich) at room temperature for 1 min. The cells were mounted with mounting solution (Dako), and images were obtained by using an EVOS microscope (Thermo Fisher Scientific).

2.9 | Immunofluorescence

To analyze PINK1 and PARKIN expression in trophoblast cells according to PD-MSC cocultivation, HTR-8/SVneo cells (1×10^4) were cultured with serum-free medium on cover slips in 24-well culture plates, and PD-MSCs (5×10^4) were cultured with cell culture medium containing FBS (Gibco-BRL) in 24-well inserts for 12, 24, and 48 h. After the trophoblasts on the cover slips were washed with 1X cold PBS, they were incubated with blocking solution (Dako) at room temperature for 1 h and then with primary antibody (1:200) at 4°C overnight. The next day, the cells were washed with 1X cold PBS and incubated with secondary antibody at room temperature for 1 h. The cells were mounted with mounting solution, and images were obtained by using confocal microscopy (Carl Zeiss).

2.10 | DCFDA/H2DCFDA assay

To analyze ROS levels in HTR-8/SVneo cells according to PD-MSC cocultivation, HTR-8/SVneo cells and PD-MSCs were cocultured using an indirect coculture system. After removing the inserts, 50 μM H2DCFDA (DCF-DA; Thermo Fisher Scientific) was added to the wells containing HTR-8/SVneo cells, which were incubated at 37°C for 30 min. After the cells were washed twice with HBSS, the fluorescence intensity was determined at 535/485 nm using an Infinite 200 Microplate Reader (Tecan m200; Tecan Trading).

2.11 | XF assay

To analyze the glycolytic flux and mitochondrial stress levels in HTR-8/SVneo cells according to PD-MSC cocultivation, the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were determined using an XF-24 cell culture assay (Seahorse Bioscience) according to the manufacturer's protocol. Briefly, HTR-8/SVneo cells (4×10^3) were seeded in the appropriate culture plates, and PD-MSCs (1.6×10^4) were seeded in 24-well inserts. After 12, 24, and 48 h, the cells were exposed to compounds that affect glycolysis (glucose: 10 mM; oligomycin: 1.0 μM; 2-deozyglucose, 2-DG: 50 mM) or mitochondrial stress as measured by oxygen consumption (oligomycin: 1.0 μM; carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone, FCCP: 0.5 μM; rotenone/AA: 0.5 μM) in culture medium at pH 7.4. The ECAR and OCR were analyzed using an XF analyzer (Seahorse Bioscience). All experiments were performed in triplicate.

2.12 | Statistical analysis

Student's *t* tests were performed for groupwise comparisons, and a p value less than .05 was considered to indicate statistical significance. All experiments were performed in duplicate or triplicate.

3 | RESULTS

3.1 | PD-MSC cocultivation induced the rapid turnover of trophoblast cells

To determine the effect of PD-MSC cocultivation on trophoblast proliferation and death, we stained trophoblasts using trypan blue solution and counted the positively stained cells. After 12 h of culture, there was no difference in the number of stained trophoblast cells between the coculture and individual culture groups. However, the numbers of

stained trophoblast cells at 24 and 48 h were lower in the group co-cultured with PD-MSCs than in the group cultured alone (Figure 1a). In addition, the cell cycle distribution of trophoblasts was analyzed by using FACS analysis. As shown in Figure 1b, the population of cells in the sub-G1 phase, which related to DNA fragmentation and apoptosis, not different according to PD-MSC cocultivation. However, the population of cells in G1, S and G2/M phase, which related to cell growth and DNA replicate, were slightly increased in the group cocultured with PD-MSCs than in the group cultured alone (Figure 1b). Furthermore, we analyzed the expression of genes related to cell survival and death by western blotting. The levels of p-ERK, which is involved in cell survival and proliferation, were increased in trophoblasts cocultured with PD-MSCs compared with trophoblasts cultured alone at 24 and 48 h. The gene expression of BAX and Bcl2, which regulate apoptotic cell death, was clearly different according to PD-MSC cocultivation. Interestingly, BAX gene expression in trophoblasts cocultured with PD-MSCs was significantly increased at 12 h but decreased at 24 and 48 h. Bcl2 gene expression was increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone (*$p < .05$, Figure 1c-f). These results show that PD-MSC cocultivation reduces trophoblast cell death and increases cell proliferation.

3.2 | PD-MSC cocultivation increased HIF1-α expression and ROS levels in trophoblast cells

The HIF1-α gene is a key factor in dynamic cellular metabolism and invasion that is regulated by cellular ROS levels. HIF1-α gene expression was determined by qRT-PCR and enzyme-linked immunosorbent assay (ELISA) in trophoblast cells cocultured with PD-MSCs. HIF1-α mRNA expression was significantly increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone, as shown by qRT-PCR and ELISA (*$p < .05$, Figure S1a,b). These findings suggest that PD-MSC cocultivation induces the expression of HIF1-α, a transcription factor, in trophoblasts.

To determine the effect of PD-MSC cocultivation on ROS generation in trophoblast cells, ROS levels were analyzed by DCF-DA ELISA and MitoSOX/Tracker staining. ROS levels were markedly increased in trophoblasts cocultured with PD-MSCs compared with trophoblasts cultured alone at 24 and 48 h (*$p < .05$, Figure 2a). Similarly, superoxide accumulation was notably increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone at 24 h, as shown in Figure 2b. The HO-1/2 and SOD genes encode catalytic enzymes with cellular protective functions that regulate oxidative stress through effects on ROS. HO-1/2 mRNA expression was increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone (Figure S2a,b). The western blot analysis showed that HO-1 protein expression was increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone at 24 and 48 h (Figure 2c,d). In addition, HO-2 protein expression was significantly increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone at 12, 24, and 48 h (*$p < .05$, Figure 2c,e). Similar to the mRNA expression results, SOD1 protein expression was decreased in trophoblasts cocultured with PD-MSCs (*$p < .05$, Figure S2c and Figure 2c,ff). These findings suggest that PD-MSC cocultivation induces HIF1-α expression and regulates ROS levels in trophoblast cells.

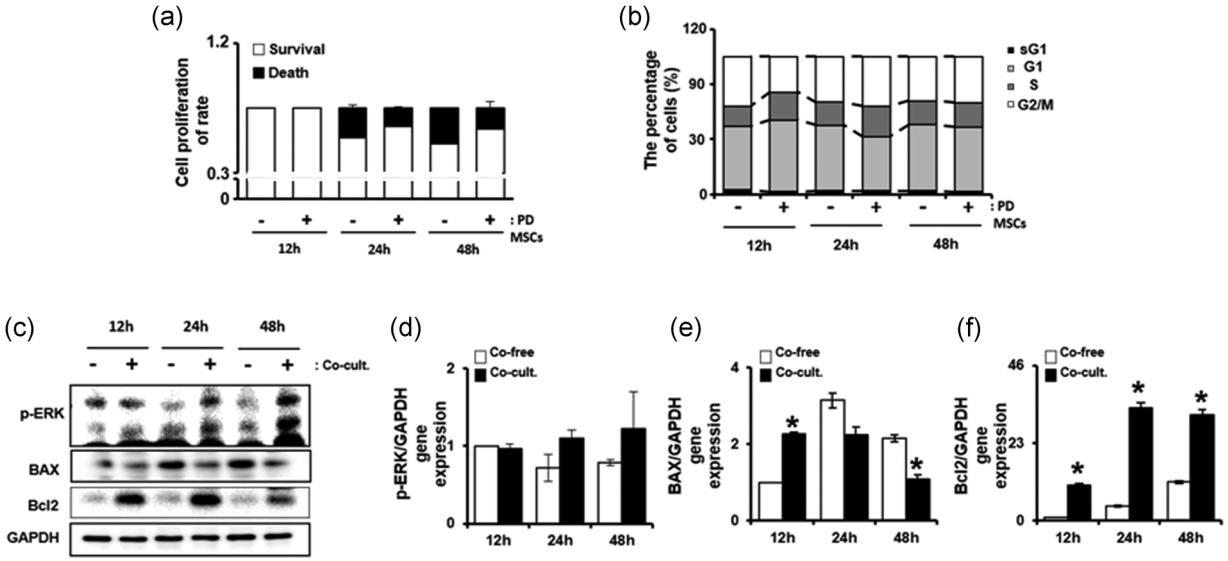

FIGURE 1 PD-MSC cocultivation did not affect the trophoblast cell survival rate through rapid cell cycle progression. HTR-8/SVneo cells were cocultured with or without PD-MSCs for 12, 24, and 48 h and subjected to trypan blue staining, FACS analysis and western blot analysis. (a) Fold change (dead/live rate) in trypan blue-stained cells according to PD-MSC cocultivation. (b) Quantification of trophoblast cells in Sub-G1, G1, S, and G2/M phase according to PD-MSC cocultivation. (c) Levels of p-ERK, Bax and Bcl2, which are markers of cell survival and death, in trophoblast cells according to PD-MSC cocultivation. (d–f) Fold change in the expression of specific genes normalized to the internal control (i.e., GAPDH). Values represent the mean ± *SD* of three independent experiments; *$p < .05$, *t* test. White bar: control; black bar: PD-MSC cocultivation. *: Without versus with PD-MSC cocultivation at each time point. Control: trophoblasts cultured alone.
FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PD-MSC, placenta-derived mesenchymal stem cell

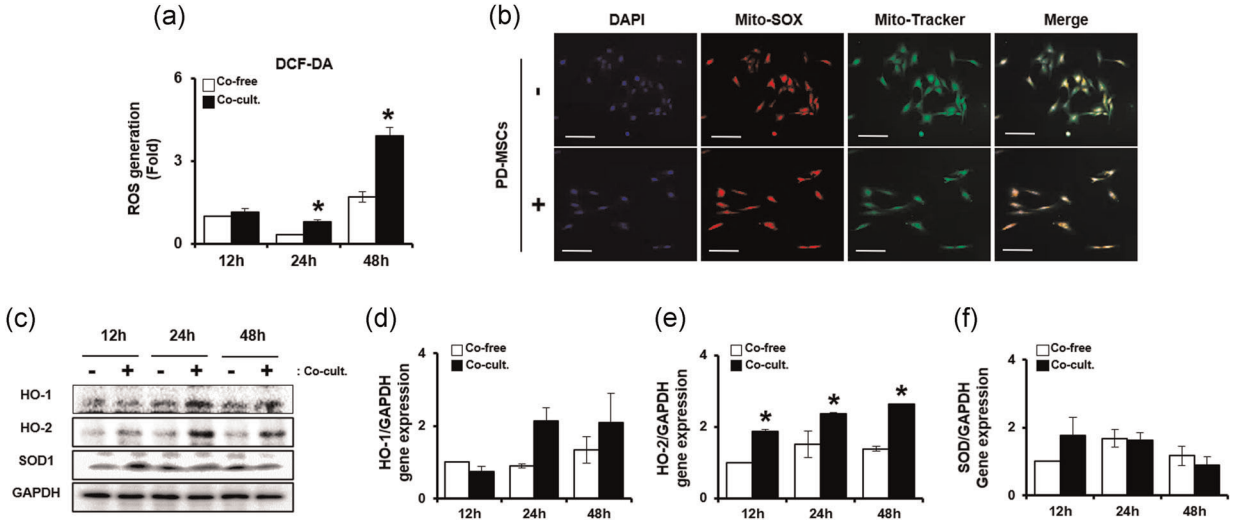

FIGURE 2 PD-MSC cocultivation induced the expression of genes related to oxidative stress in trophoblasts. HTR-8/SVneo cells were cocultured with or without PD-MSCs for 12, 24, and 48 h and subjected to DCF-DA assays, MitoSOX/Tracker staining and western blot analysis. (a) Fold change in ROS levels in trophoblast cells according to PD-MSC cocultivation. (b) The MitoSOX/Tracker ratio in trophoblast cells according to PD-MSC cocultivation was determined as described in the Section 2. Representative confocal images are shown (magnification: ×200). (c) Expression of HO-1, HO-2, and SOD1, which are markers of oxidative stress, in trophoblast cells according to PD-MSC cocultivation. (d–f) Fold change in the expression of specific genes normalized to the internal control (i.e., GAPDH). Values represent the mean ± *SD* of three independent experiments; *$p < .05$, *t* test. White bar: control; black bar: PD-MSC cocultivation. *: Without versus with PD-MSC cocultivation at each time point. Control: trophoblasts cultured alone. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PD-MSC, placenta-derived mesenchymal stem cell; SOD, superoxide dismutase

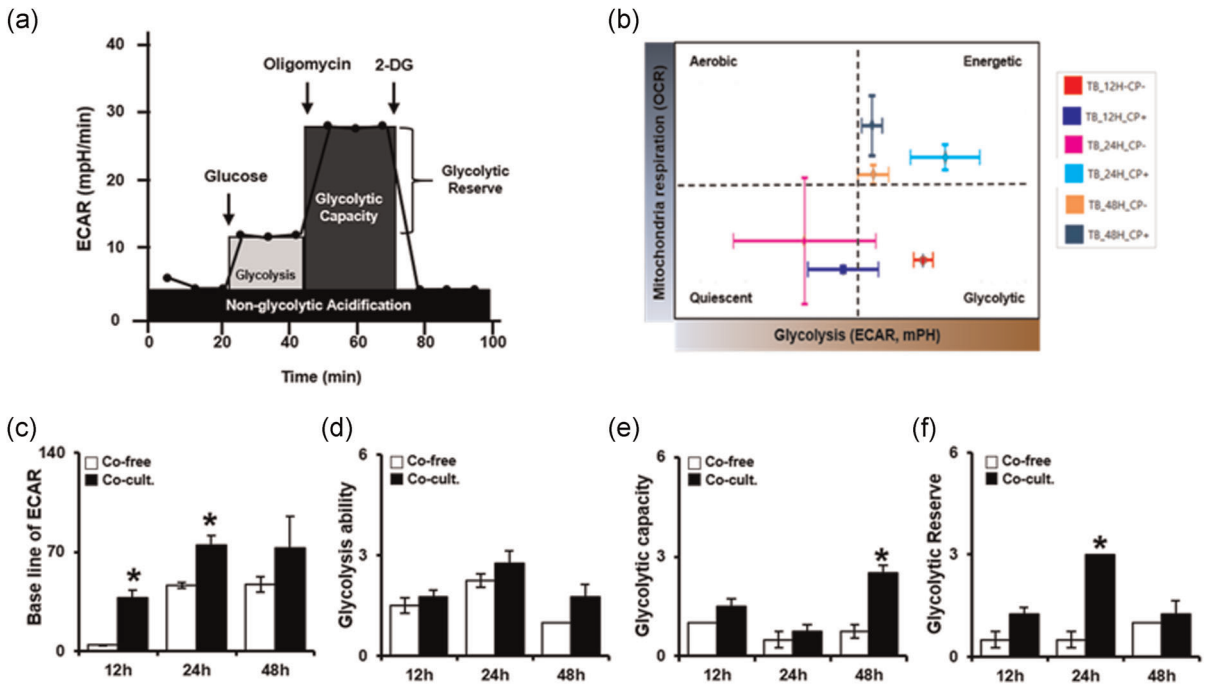

FIGURE 3 3 PD-MSC cocultivation increased glycolysis in trophoblasts. HTR-8/SVneo cells were cocultured with or without PD-MSCs for 12, 24, and 48 h, and XF analysis was performed to measure glycolysis. (a) Schematic diagram of the glycolysis experiment using XF analysis. ECAR levels were measured by XF assays under basal conditions and after the sequential addition of glucose (10 mM), oligomycin (1 μM) and 2-DG (50 mM), as indicated. (b) The phenotype of trophoblast cells according to PD-MSC cocultivation. (c) Baseline ECAR, (d) glycolysis ability, (e) glycolytic capacity and (f) glycolytic reserve in trophoblasts according to PD-MSC cocultivation. Values represent the mean ± *SD* of three independent experiments; *$p < .05$, *t* test. White bar: control; black bar: PD-MSC cocultivation. *: Without versus with PD-MSC cocultivation at each time point. Control: trophoblasts cultured alone. ECAR, extracellular acidification rate; PD-MSC, placenta-derived mesenchymal stem cell

3.3 | PD-MSC cocultivation enhanced glycolysis in trophoblast cells

To measure glycolysis in trophoblast cells according to PD-MSC cocultivation, the glycolysis XF analyzer was used to analyze live trophoblast cells. Glycolysis was measured based on the ECAR, which indicates a change in pH of the culture media of trophoblast cells. After PD-MSCs and trophoblast cells were cocultivated, the trophoblast cells were treated with glucose, oligomycin or 2-DG to analyze each step-in glycolysis individually. XF analysis was performed as shown in Figure 3a. The energetic phenotype was determined in trophoblasts cocultured with PD-MSCs compared to that in trophoblasts cultured alone at 24 and 48 h (Figure 3b). Comparative quantitative analysis of the results showed that the basal ECAR was significantly increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone at 12, 24, and 48 h (*$p < .05$, Figure 3c). Among trophoblasts treated with glucose, those cocultivated with PD-MSCs showed an increase in glycolysis ability compared to those cultured alone (Figure 3d). Among the trophoblast cells treated with oligomycin, those cocultivated with PD-MSCs showed an increase in glycolysis capacity at 48 h compared to those cultured alone (*$p < .05$, Figure 3e). After 2-DG treatment, the trophoblast cells cocultivated with PD-MSCs had an increased glycolytic reserve at 24 h (*$p < .05$, Figure 3f). These findings suggest that PD-MSC cocultivation activates glycolysis in trophoblast cells.

3.4 | PD-MSC cocultivation induced mitochondrial oxygen consumption in trophoblast cells

To investigate the effect of PD-MSC cocultivation on the mitochondrial OCR of trophoblast cells, the mitochondrial OCR was analyzed in live trophoblast cells. Mitochondrial stress levels can be deduced by the OCR, which is detected based on changes in the pH of trophoblast cell culture media. Trophoblast cells cocultivated with PD-MSCs were treated with oligomycin, FCCP or rotenone/antimycin A to analyze each step of mitochondrial oxygen consumption individually. XF analysis was performed as shown in Figure 4a. The results revealed the energetic and glycolytic phenotypes of trophoblasts cocultured with PD-MSCs compared to those of trophoblasts cultured alone (Figure 4b). The comparative quantitative analysis revealed that the basal OCR at 24 and 48 h was significantly increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone (*$p < .05$, Figure 4c). After oligomycin treatment, trophoblast cells cocultivated with PD-MSCs showed significantly increased ATP production at 12 h (Figure 4d). After FCCP treatment, the maximal respiration of trophoblast cells was markedly increased in the PD-MSC cocultivation group compared to the individual culture group at 12 h (*$p < .05$, Figure 4e). In contrast, the spare capacity of trophoblast cells was not significantly different under the two culture conditions (Figure 4f). These findings suggest

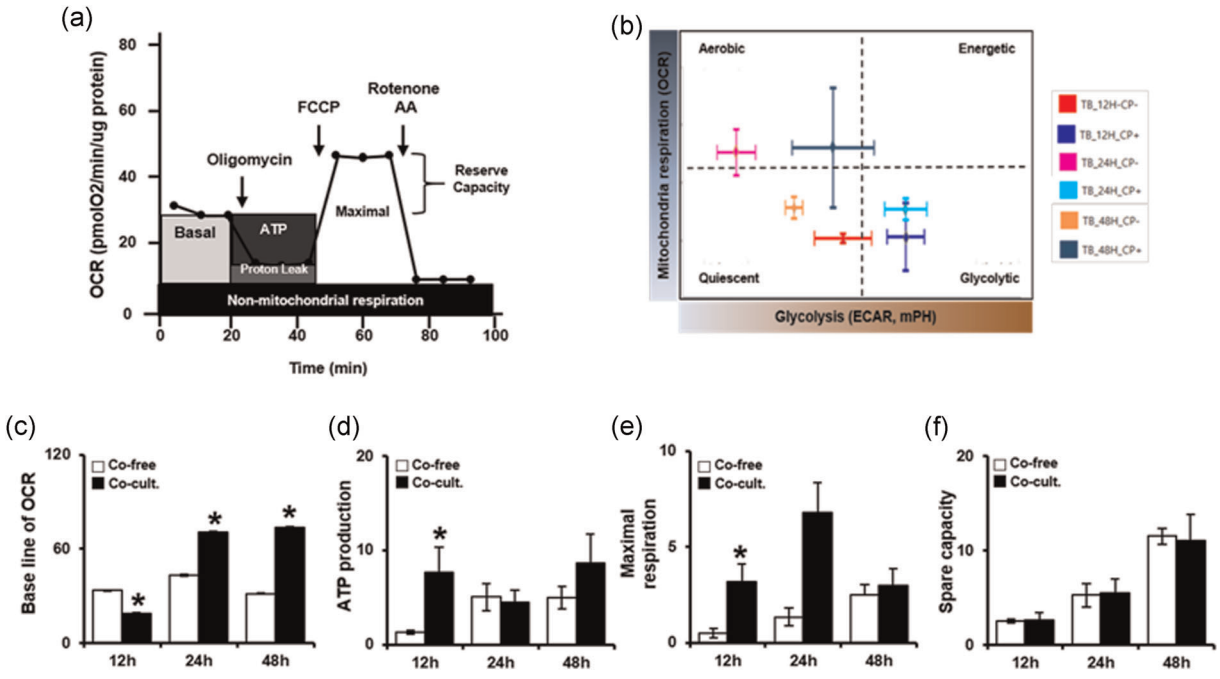

FIGURE 4 4 PD-MSC cocultivation regulated mitochondrial respiration in trophoblasts. HTR-8/SVneo cells were cocultured with or without PD-MSCs for 12, 24, and 48 h, and XF analysis was performed to measure mitochondrial respiration. (a) Schematic diagram of the mitochondrial stress experiment (i.e., mitochondrial respiration) using XF analysis. The OCR was measured by XF assay under basal conditions and after the sequential addition of oligomycin (1 μM), FCCP (0.5 μM) and rotenone A/A (0.5 μM), as indicated. (b) The phenotype of trophoblast cells according to PD-MSC cocultivation. (c) Baseline OCR, (d) ATP production, (e) maximal respiration and (f) spare capacity were analyzed in trophoblasts according to PD-MSC cocultivation. Values represent the mean ± *SD* of three independent experiments; *$p < .05$, *t* test. White bar: control; black bar: PD-MSC cocultivation. *: Without versus with PD-MSC cocultivation at each time point. Control: trophoblasts cultured alone. ATP, adenosine triphosphate; OCR, oxygen consumption rate; PD-MSC, placenta-derived mesenchymal stem cell

that PD-MSC cocultivation induces mitochondrial oxygen consumption and ATP production in trophoblast cells.

3.5 | PD-MSC cocultivation affected mitochondrial function in trophoblast cells

To investigate the effect of PD-MSC cocultivation on mitochondrial function in trophoblasts, we performed JC-1, NAO and ATP production assays. The JC-1 assay results indicated that the mitochondrial membrane potential was significantly decreased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone (*$p < .05$, Figure S3a). The NAO assay revealed an increased mitochondrial mass in trophoblasts cocultured with PD-MSCs at 12 and 24 h but a decreased mass in trophoblasts cocultured with PD-MSCs at 48 h compared to trophoblasts cultured alone (*$p < .05$, Figure S3b). These findings suggest that PD-MSC cocultivation influences mitochondrial function in trophoblasts, including mitochondrial membrane potential and mass.

We also analyzed the effect of PD-MSC cocultivation on HSP60, PHB1, and VDAC gene expression in trophoblasts (Figure 5a). The expression of HSP60, which protects against cell damage, is induced under mitochondrial stress conditions; trophoblasts cocultured with PD-MSCs had markedly lower HSP60 expression than trophoblasts cultured alone at 12, 24, and 48 h (*$p < .05$, Figure 5b). Among the proteins involved in mitochondrial stabilization and ion channel signaling, PHB1 and VDAC were analyzed by western blotting. The expression of PHB1, which is related to mitochondrial stabilization, was extremely high in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone at 12, 24, and 48 h (*$p < .05$, Figure 5c). The expression of VDAC, an ion channel in the mitochondrial outer membrane related to ATP transport and synthesis, was markedly increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone (*$p < .05$, Figure 5d). IP3R and MCU are involved in Ca^{2+} signaling between the ER and mitochondria to regulate ATP synthesis (Shanmughapriya et al., 2015). Therefore, we analyzed the expression of genes related to Ca^{2+} signaling and ATP synthesis that may play a role in trophoblast

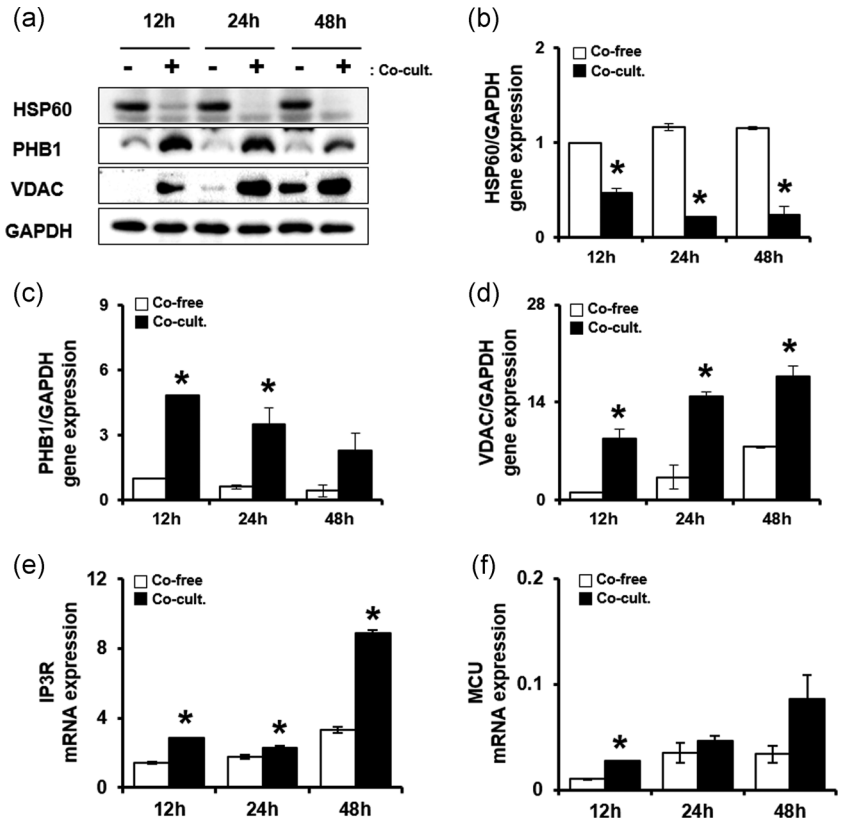

FIGURE 5 PD-MSC cocultivation changed gene expression and mitochondrial function with effect on stress levels and ion channels. HTR-8/SVneo cells were cocultured with or without PD-MSCs for 12, 24, and 48 h and subjected to western blotting and qRT-PCR analyses. (a) Expression of HSP60, PHB1, and VDAC, which are markers of mitochondrial stress, in trophoblast cells according to PD-MSC cocultivation. (b–d) Fold change in the expression of specific genes normalized to the internal control (i.e., GAPDH). (e, f) IP3R and MCU mRNA expression in trophoblast cells according to PD-MSC cocultivation. Values represent the mean ± *SD* of three independent experiments; *$p < .05$, *t* test. White bar: control; black bar: PD-MSC cocultivation. *: Without versus with PD-MSC cocultivation at each time point. Control: trophoblasts cultured alone. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCU, mitochondrial calcium uniport; mRNA, messenger RNA; PD-MSC, placenta-derived mesenchymal stem cell; qRT-PCR, quantitative real-time polymerase chain reaction

invasion. IP3R mRNA expression was significantly increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone (*$p < .05$, Figure 5e), and MCU mRNA expression was significantly increased in trophoblasts cocultured with PD-MSCs at 12 h (*$p < .05$, Figure 5f). These findings suggest that PD-MSC cocultivation activates ATP metabolism through effects on calcium channels and enhances mitochondrial stabilization, resulting in reduced mitochondrial damage in trophoblast cells.

3.6 | PD-MSC cocultivation induces mitophagy factors in trophoblast cells

To evaluate mitochondrial autophagy, which provides quality control in trophoblast cells, we analyzed the effect of PD-MSC cocultivation on the expression of genes related to mitophagy in trophoblast cells by western blotting (Figure 6a). PINK1 protein expression was significantly increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone at 12 and 24 h (*$p < .05$, Figure 6a,b). In addition, PARKIN expression was markedly increased in trophoblasts cocultured with PD-MSCs compared to trophoblasts cultured alone at 12, 24, and 48 h (*$p < .05$, Figure 6a,c). The results for PINK1 and PARKIN expression in trophoblasts are shown in Figure 6d. PD-MSC cocultivation markedly increased PINK1 and PARKIN expression in trophoblasts (Figure 6d). These findings suggest that PD-MSC cocultivation regulates mitochondrial damage in trophoblasts by upregulating PINK1 and PARKIN gene expression.

3.7 | PD-MSC cocultivation improved trophoblast cell invasion

To ascertain the effect of PD-MSC cocultivation on trophoblast invasion, we performed an invasion assay using a trans well insert system. The invasion assays showed that trophoblast cell invasion was markedly increased in the PD-MSC cocultivation group compared to the individual culture group, as shown in Figure 7a. We counted the number of invaded cells through hematoxylin staining. The number of invaded cells significantly increased after PD-MSC cocultivation at 12, 24, and 48 h (*$p < .05$, Figure 7b). In accordance with previous reports, we also analyzed the activity of MMP-2/9, which are involved in trophoblast invasion. MMP-2/9 activity in the supernatant was analyzed by zymography. MMP-2 activity was not different between the PD-MSC cocultivation and individual culture groups at 12 h. Interestingly, MMP-2 activity was

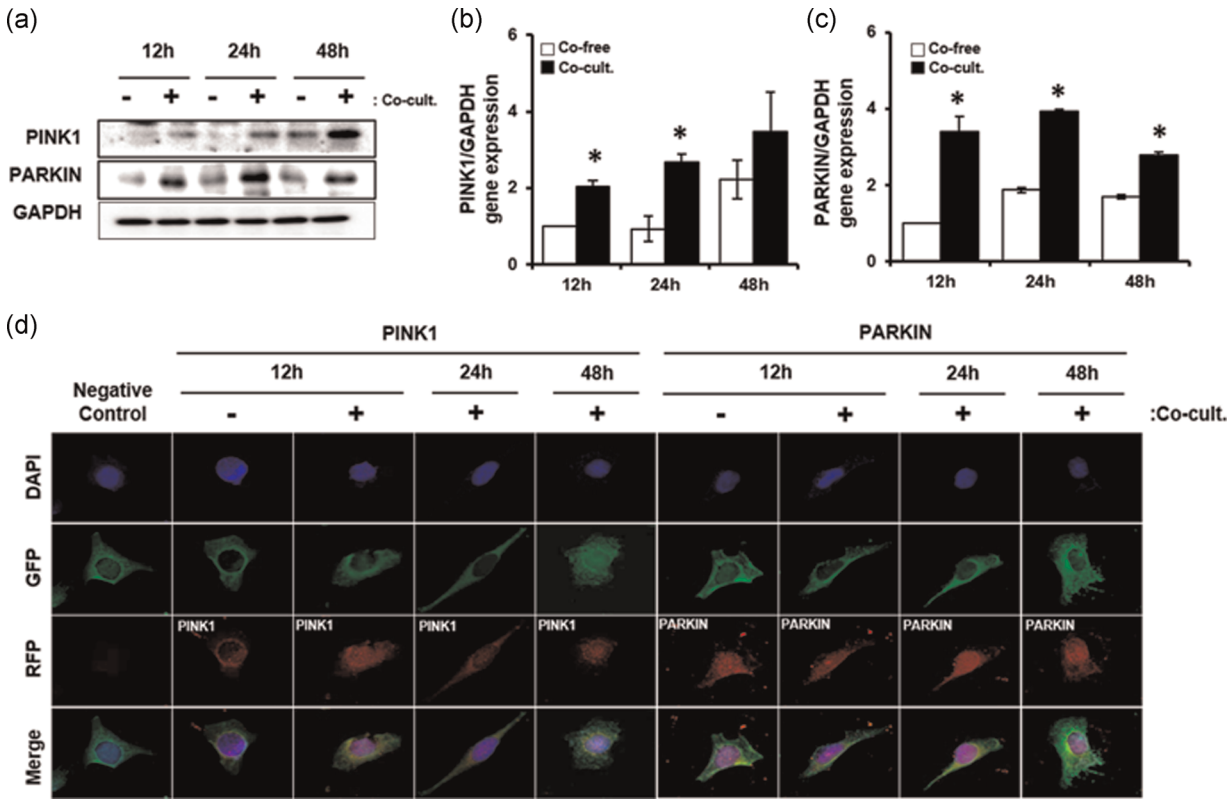

FIGURE 6 PD-MSC cocultivation increased markers of mitophagy. HTR-8/SVneo cells were cocultured with or without PD-MSCs for 12, 24, and 48 h, and western blotting and immunofluorescence were performed. (a) Expression of PINK1 and PARKIN, which are markers of mitophagy, in trophoblast cells according to PD-MSC cocultivation. (b, c) Fold change in the expression of specific genes normalized to the internal control (i.e., GAPDH). (d) PINK1 and PARKIN expression in trophoblast cells according to PD-MSC cocultivation. Values represent the mean ± *SD* of three independent experiments; *$p < .05$, *t* test. White bar: control; black bar: PD-MSC cocultivation. *: Without versus with PD-MSC cocultivation at each time point. Control: trophoblasts cultured alone. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PD-MSC, placenta-derived mesenchymal stem cell; PINK1, PTEN-induced putative kinase 1

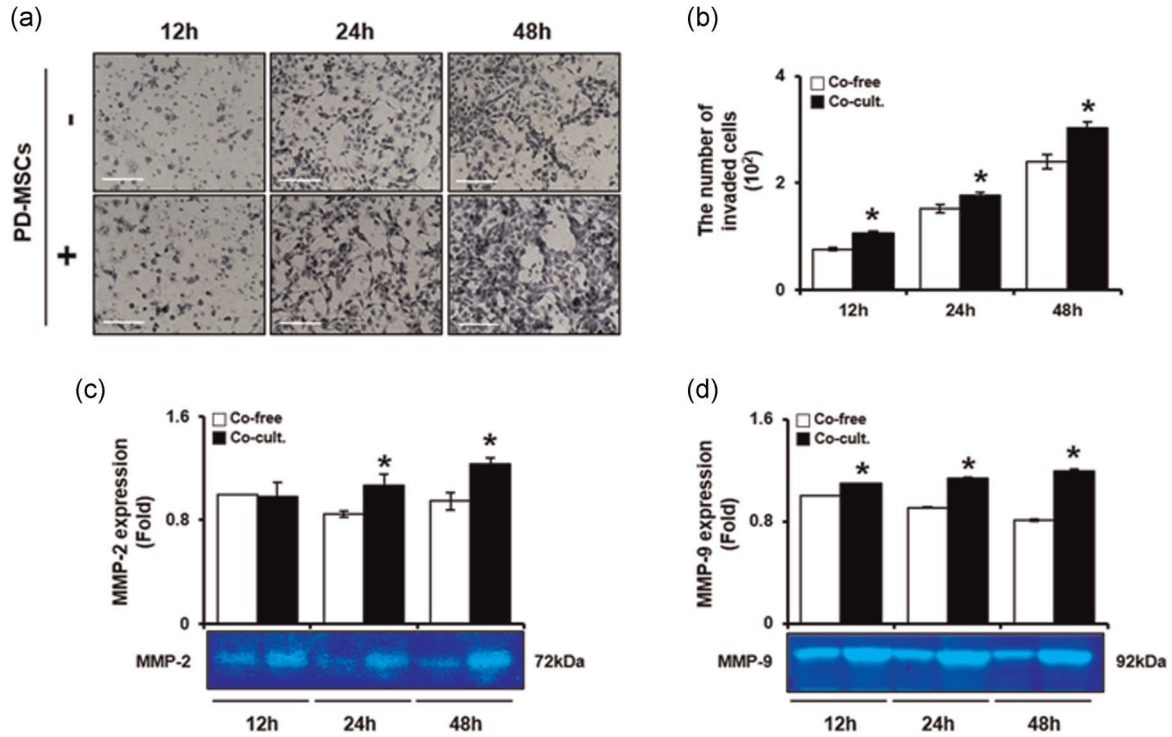

FIGURE 7 PD-MSC cocultivation increased trophoblast invasion. HTR-8/SVneo cells were cocultured with or without PD-MSCs for 12, 24, and 48 h and then subjected to invasion assays and zymography. (a) Trophoblast cell invasion was determined according to PD-MSC cocultivation (magnification, ×100). (b) Quantification of invaded cells by ImageJ software. (c,d) MMP-2 and MMP-9 expression in the supernatant of trophoblast cells according to PD-MSC cocultivation. Values represent the mean ± *SD* of three independent experiments; $^*p < .05$, *t* test. White bar: control; black bar: PD-MSC cocultivation. *: Without versus with PD-MSC cocultivation at each time point. Control: trophoblasts cultured alone. MMP, matrix metalloproteinase; PD-MSC, placenta-derived mesenchymal stem cell

increased in the supernatant from trophoblast cells cocultivated with PD-MSCs for 24 and 48 h compared to that from trophoblasts cultured alone ($^*p < .05$, Figure 7c). Moreover, MMP-9 activity was significantly increased in the PD-MSC cocultivation group at 12, 24, and 48 h compared to the individual culture group ($^*p < .05$, Figure 7d). The MMP-9 concentration in trophoblast cells was analyzed by ELISA and found to be increased in trophoblasts cocultured with PD-MSCs at 12, 24, and 48 h ($^*p < .05$; Figure S4a). In addition, we performed the effect of PD-MSCs cocultivation on invasion ability and MMPs activities of other placental cells. As shown in Figure S5, the invasion ability and MMPs activities of three types placental cells (e.g., primary trophoblast cells (PTB), JEG3 and BeWo), as well as trophoblast (i.e., HTR-8/SVneo cells) were significantly increased in the PD-MSCs cocultivation group compared to the individual culture group ($^*p < .05$, Figure S5a–i). These findings suggest that PD-MSC cocultivation induces trophoblast invasion by activating MMP-2/9.

4 | DISCUSSION

In modern world, the early abortion, infertility and inadequate placentation are increasing problem due to unknown factor including environment. For overcome these problem, various placental cells including PTB, BeWo, JEG-3, JAR, and HTR-8/SVneo were used for infertility research. BeWo and JEG3 are choriocarcinoma origin with extra-villous trophoblast (EVT) phenotype, and used in invasion and syncytialization. HTR-8/SVneo cells is first trimester villous explants origin with EVT and villous cytotrophoblast phenotype, and used in adhesion, migration, invasion and proliferation (Hannan et al., 2010). To confirmed that effect of PD-MSC cocultivation on invasion ability of various placental cells as well as HTR-8/SVneo, we used PTB with positive expression of KRT7, CG, CGR, HLAG, and CD9, including BeWo and JEG-3 cells. Our data showed that PD-MSC cocultivation induced higher invasion ability of various placental cells via activated MMP-2/-9.

MSCs secrete many biologically active factors, including cytokines, chemokines, growth factors, exosomes and microRNAs, that have considerable therapeutic potential in several diseases (Kusuma et al., 2017; Spees et al., 2016). Yajing and colleagues recently reported that UC-MSCs regulate human chorionic gonadotropin, placental growth factor, and soluble Endoglin levels and promote the proliferation, migration, and invasion of trophoblasts through cell–cell interactions (Huang et al., 2016). Chen et al. (2013) reported that trophoblast invasion is dependent on paracrine signaling from PD-MSCs, and hepatocyte growth factor derived from PD-MSCs triggers trophoblast invasion by upregulating cAMP and downregulating Rap1 expression. Recently reported studies were demonstrated MSCs improve the migration and proliferation via antiinflammatory effects on excessive inflammation induced

trophoblast cells by lipopolysaccharide treatment (Yang et al., 2019). In our previously repots, PD-MSCs were reported the secreted various immunomodulatory cytokines, such as secreted protein acidic and cysteine rich (SPARC), granulocyte colony-stimulating factor (GCSF), monocyte chemoattractant protein 1/3 (MCP-1/3), interferon gamma-induced protein-10 (IP-10), MCP-1/-3 and so on. (Shin et al., 2010). Several studies demonstrated that SPARC, GCSF, and IP-10 were induced higher migration ability in trophoblast cells including various cell line. The SPARC regulates radiation induced HSP expression by inhibiting mitochondrial function, including mitochondrial mass and membrane potential in neuroblastoma cells, and by modulating the extracellular matrix (Furmento et al., 2016; Jiang et al., 2013; Tanpure et al., 2017; Zipin-Roitman et al., 2007). The MCP-1/-3 is related to macrophage migration and infiltration through effects on mitochondrial lipid homeostasis. It is reported that MCP induces cancer cell invasion and adhesion through the ERK pathway and MMP activity (Furukawa et al., 2013; Ito et al., 2015; Tan et al., 2013). In our data, PD-MSC cocultivation induced higher invasion ability of trophoblast cells including Through reported studies, we expect that higher invasion ability of trophoblast cells is controlled by SPARC, GCSF, IP-10, and so on, which are secreted by PD-MSCs. However, paracrine effect of MSCs on invasion ability and mitochondrial function of trophoblast cells have yet to be unclear.

Dynamic processes, including mitochondrial function and ATP synthesis, are required to increase trophoblast invasion and establish a successful pregnancy. Mitochondria, the ER and dynamic organelles are vulnerable to oxidative stress. Oxidative stress-induced mitochondrial damage results in dysfunctional mitochondria, the activation of mitochondria-dependent apoptosis and cell death. Moreover, the suppression of trophoblast invasion by mitochondrial dysfunction leads to disorders such as preeclampsia and IUGR. Several studies reported that placenta samples from women with preeclampsia showed mitochondrial dysfunction, including higher ROS levels and smaller mitochondria, in trophoblast cells. Therefore, mitochondrial function has an important role in trophoblast invasion to establish a successful pregnancy (Zsengeller et al., 2016).

Several studies have demonstrated the involvement of various regulatory factors, including HIF1-α and MMPs, and mitochondrial function in trophoblast invasion. Especially, HIF1-α is multiple functional transcription gene related to angiogenesis, cell survival, invasion and energy metabolism. Under hypoxia conditions. HIF1-α expression generates ROS and stimulates cell migration, invasion, proliferation and dynamic metabolism through the ERK pathway (Liu et al., 2014). Moreover, Choi et al. (2012) showed that upregulating HIF1-α expression induces trophoblast invasion by activating MMP-2/9. In our study, PD-MSC cocultivation promoted trophoblast invasion by activating MMP-2/9 and inducing HIF1-α expression. Collectively, these findings demonstrate that PD-MSCs regulate several factors related to migration to enhance the invasion ability of trophoblasts. Recently reports suggested that HIFs is transcription factor, but it is presented in outside the nucleus under hypoxic conditions (Briston et al., 2011). Also, HIF1-a is a regulator on mitochondrial metabolism including mitochondrial autophagy as well as ROS generation (Chandel et al., 2000; Kim et al., 2006; Semenza, 2011; Zhang et al., 2008). In our data, PD-MSC cocultivation induced mitochondrial function on trophoblast including cellular ROS levels and mitochondrial membrane potential/mass. The correlation between mitochondrial metabolism and HIF1-α expression through glycolysis and the tricarboxylic acid cycle has been reported in several publications. Recent reports stated that the HIF1-α gene is involved in glucose/ion metabolism through the upregulation of Glut1 gene expression in various cell types. Maloyan et al and colleagues demonstrated that HIF1-α upregulation in trophoblasts by DFO reduced mitochondrial respiration (i.e., basal respiration, ATP-coupled respiration, maximum respiration and reserve capacity; Maloyan et al., 2012; Muralimanoharan et al., 2012; Semba et al., 2016). Moreover, previous reports suggest that in contrast to syncytiotrophoblasts, cytotrophoblasts depend on both aerobic and glycolytic energy production (Bax & Bloxam, 1997). Our data showed that PD-MSC cocultivation improved mitochondrial function, including glycolysis and respiration, in trophoblast cells. Collectively, these findings suggest that PD-MSCs stimulate mitochondrial metabolism, including glycolysis and respiration, during trophoblast cell invasion.

Mitochondrial autophagy (mitophagy) is a selective event that targets damaged mitochondria in cells. The high energy requirement of invading cells induces mitochondrial damage in trophoblast cells. PINK1 interacts with PARKIN to activate mitophagy and thus protect mitochondria. Mitochondrial autophagy is initiated after the accumulation of PINK1 and phosphorylated PARKIN on the mitochondrial membrane. Therefore, PINK1 and PARKIN expression promote the upregulation of autophagy biomarkers (e.g., p62 and LC3-I/II) during mitophagy. During mitochondrial autophagy, damaged mitochondria are selectively removed to protect cells. Heeman B et al. recently reported that PINK1 modulates the mitochondrial network, energy maintenance and calcium homeostasis (Heeman et al., 2011). Our data showed that PD-MSC cocultivation reduced mitochondrial damage by downregulating HSP70 expression and enhanced mitochondrial protection by upregulating PHB1 in trophoblast cells. Moreover, PD-MSC cocultivation increased the expression of mitophagy regulators, such as PINK1 and PARKIN, in trophoblast cells. Taken together, these findings suggest that PD-MSCs attenuate mitochondrial damage in trophoblast cells.

In conclusion, PD-MSCs induce mitochondrial activity (e.g., glycolysis and mitochondrial respiration rate), ion channel signaling and/mitophagy factors and stabilize the mitochondrial membrane in trophoblasts. Additionally, PD-MSCs enhance trophoblast invasion. The findings of this study suggest that PD-MSCs have a positive paracrine effect on trophoblast function through effects on mitochondrial function. Although our data is normal physiological system, it is can help understanding for therapeutic effect of PD-MSCs on early implantation as well as liver disease and ovary dysfunction.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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