Effect of DJ-1 down-regulation on the functions of the first trimester extravillous trophoblasts

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Effect of DJ-1 down-regulation on the functions of the first trimester extravillous trophoblasts

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ABSTRACT

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DJ-1 (PARK7) has been reported to be causative gene of Parkinson's disease and also an oncogene. A loss in DJ-1 function can lead to cell death in neurodegenerative disease or a gain of it can cause unregulated cell survival in cancer, respectively. DJ-1 protein is known to be expressed mainly in trophoblastic cells in the placenta, with increased expression in the first trimester compared to later in term. However, its role in trophoblast regulation remains unknown. This study aimed to investigate the effect of DJ-1 regulation on a first trimester extravillous trophoblast cell line, HTR-8/SVneo. The effect of DJ-1 downregulation induced by small interfering RNA on cell apoptosis, migration, and the pathway to regulate the cell function were assessed. Data of this study showed that DJ-1 downregulation increased apoptosis and reduced migration by regulating MMP2 and MMP9 in HTR-8/SVneo cells under both ambient and oxidative stress. Changes in cell function were demonstrated to be at least partly dependent on the Akt/S6K1 pathway. In summary, DJ-1 might

play a protective role in maintaining trophoblastic cell functions through the Akt/S6K1-based pathway.

Key words: DJ-1, small interfering RNA, down-regulation, first trimester extravillous trophoblast, apoptosis, migration

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I. INTRODUCTION

During human pregnancy, adequate invasion of extravillous trophoblasts (EVTs) into uterine decidua, myometrium, and spiral arteries is essential for normal feto-placental development.[1] These extravillous trophoblasts are differentiated from villous cytotrophoblasts and are divided into two subtypes: (1) interstitial EVTs invading into maternal decidual stroma that are part of the myometrium and (2) endovascular EVTs migrating into spiral arteries that transform into the endothelial phenotype.[2] This remodeling of spinal arteries leads to increased blood flow at the maternal-fetal interface by lowering vascular resistance. Any abnormalities in this process may lead to abnormal placentation, a common pathophysiology of miscarriage, fetal growth restriction, and preeclampsia.[3,4]

The human Parkinson disease protein 7 gene (PARK7), encoding the oncogenic protein DJ-1, is located on the distal part of the short arm of chromosome 1 (1p36.12–1p36.33) and is ubiquitously expressed in over 20

human tissues, including placenta.[5] DJ-1 is currently thought to be a multifunctional protein, including roles as a redox-regulated chaperone[6], transcriptional coactivator[7], regulatory subunit of RNA-binding protein[8], and a cysteine protease.[9] Therefore, DJ-1 has been investigated in various research fields investigating biological processes, including antioxidative stress[10-12], transcriptional regulation[13], mitochondrial regulation[14], fertilization[15], and tumor TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis[16]. Kim et al.[17] also reported that DJ-1 can promote angiogenesis and osteogenesis in vitro and in vivo by activating fibroblast growth factor receptor-1 signaling. They found that recombinant DJ-1 induced immortalized human umbilical vein endothelium cell migration and angiogenesis in the formation of well-organized, capillary-like networks, in a dose-dependent manner.

DJ-1 has been reported to be causative gene of Parkinson's disease and also an oncogene. A loss in DJ-1 function can lead to cell death in neurodegenerative disease or a gain of it can cause unregulated cell survival in cancer, respectively.[5,9] In the first trimester, especially, trophoblasts have similar characteristics to cancer cells. They can rapidly proliferate, invade adjacent tissues, and be metastatic. Therefore, DJ-1 is expected to have regulatory roles in trophoblasts. However, there are few studies on the expression of DJ-1 in the placenta. Zhang et al. reported that first trimester villous placental DJ-1 immunostaining indices (26.6 ± 5.6; mean ± standard deviation) were significantly higher than those of

term placentas (2.5 ± 0.7).[18] Additionally, DJ-1 is reported to be mainly expressed in trophoblasts.[18,19] However, DJ-1's biological function in trophoblasts has not been investigated to date. The objective of our study was to investigate the effect of DJ-1 regulation on first trimester extravillous trophoblast function.

II. MATERIALS AND METHODS

1. HTR-8/SV neo cell culture and transfection

HTR-8/SVneo cells were obtained from ATCC (ATCC® CRL-3271™) and cultured in RPMI 1640 Medium (Invitrogen Life Technologies, Inc., Burlington, Canada) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin. For each experimental condition, cells were trypsinized and plated in triplicate wells at a density of 1×10^4 or 3×10^4 cells/well. The HTR-8/SVneo cell line used was obtained following immortalization of the first trimester short-lived extravillous trophoblast cell line HTR-8 after transfection with SV40 large T antigen, followed by selection for neomycin resistance.[20] Cells were seeded into 2-well chamber slides for the Hoechst 33342 staining assay, 6-well plates for western blot analysis, and 100-mm dishes for real-time quantitative polymerase chain reaction (qRT–PCR). Cells were maintained between passages 76–80 and incubated in a standard CO_2 incubator (5% CO_2, 37.8℃). For small interfering

RNA (siRNA) transfection, HTR-8/SVneo cells were seeded in 6-well culture plates with a density of 1×10^5 cells/well and transfected with 50 μM of DJ-1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Scrambled siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a control. Transfected cells were harvested after 48 h prior to assay analyses.

2. Apoptosis assay

To investigate the effect of silencing of *PARK7* on programmed cell death, cells were stained with Hoechst 33342 dye (Sigma-Aldrich, St. Louis, MO) for a rapid fluorescence assay to detect condensed chromatin in apoptotic cells. This blue fluorescent dye stains chromatin with an excitation and emission maximum of 346 nm and 461 nm respectively, where the chromatin of apoptotic cells stains more brightly than the chromatin of normal cells. HTR-8/SVneo cells were seeded in 6-well plates with 3 mL of complete medium added to each well. Cells transfected with PARK7 (DJ-1) siRNA and control siRNA were incubated for 24 h and the media was removed before staining. Hoechst dye was added to each well and incubated for 20 min. Cells were evaluated using the Evos FL fluorescence microscope. The apoptotic cells were counted in four randomly selected fields and the percentage of cells undergoing apoptosis was calculated.

3. Cell migration assay

A total of 1×10^5 transfected cells were resuspended in 200 μL serum-free RPMI 1640 and added to a 24-well Millicell™ cell culture insert with 8-μm-sized pores (Millipore Sigma, Darmstadt, Germany) along with 600 μL complete culture medium in the lower chamber. After culturing at 37°C for 18–24 h, non-migrated cells on the upper surface were removed with a cotton swab, and the migrated cells on the lower surface of the filter were fixed were fixed in 80% ethanol for 10 min and stained with 0.2% crystal violet for 10 min. Migrating cells were counted under an inverted phase-contrast microscope (Olympus, Japan). The average number of five random fields was calculated for each insert.

4. Western blot analysis

To detect proteins, western blot was performed. The proteins were separated according to their molecular weights by two-dimensional gel electrophoresis (2-DE) analyses and quantified using the a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA). For immunoblotting, equal amounts of protein were subjected to electrophoresis on a 10–15% gradient polyacrylamide gel (Koma Biotech, Seoul, Korea) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) over 50 min at 15 V using a EZway blotting system (Koma Biotech, Seoul, Korea). Blots were blocked for 1 h at room temperature with 3% (w/v) non-fat dried milk in Tris-buffered saline

(TBS) solution (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.1% Tween-20 (TBS-T).

After three washes with TBS-T, the blots were incubated overnight at 48°C with primary antibody: mouse monoclonal antibody (3E8) to DJ-1 (1:500; Enzo Life Sciences, Inc. Farmingdale, NY, USA), mouse monoclonal antibody (4D3) to matrix metalloproteinase 2 (MMP2) (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal antibody (2C3) to MMP9 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal antibody to cellular tumor antigen p53 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit monoclonal antibody (50E3) to apoptosis regulator Bcl2 (1:200; Cell Signaling, Inc. Beverly, MA, USA), rabbit polyclonal antibody to apoptosis regulator Bax (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal antibody to caspase3 (CASP3) (1:500; Cell Signaling, Inc. Beverly, MA, USA), rabbit monoclonal antibody (138G6) to phosphatase and tensin homolog (PTEN) (1:500; Cell Signaling, Inc. Beverly, MA, USA), rabbit monoclonal antibody to protein kinase B (PKB)/Akt (C67E7) (1:1000; Cell Signaling, Inc. Beverly, MA, USA), rabbit monoclonal antibody (D9E) to phospho-Akt (p-Akt) (Ser473) (1:500; Cell Signaling, Inc. Beverly, MA, USA), rabbit monoclonal antibody (49D7) to ribosomal protein S6 kinase beta-1 (S6K1) (1:500; Cell Signaling, Inc. Beverly, MA, USA), or rabbit monoclonal antibody (108D2) to phospho-S6K1 (p-S6K1) (Thr389) (1:500; Cell Signaling, Inc. Beverly, MA, USA).

For visualization, membranes were washed with TBS-T solution and incubated with horseradish peroxidase-conjugated goat anti-mouse (1:5000; Thermo Scientific, Waltham, MA, USA) or anti-rabbit IgG (1:2000; Thermo Scientific, Waltham, MA, USA) at room temperature for 1 h. Immunoreactive proteins were detected by chemiluminescent reaction followed by exposure of membranes to hyperfilm ECL (Amersham Pharmacia Biotech, Inc., Little Chalfont, UK). The relative density of the protein bands was quantified using Gel Doc XR+ (Bio-Rad Laboratories, Hercules, CA, USA).

5. RNA isolation and real time quantitative polymerase chain reaction (qRT-PCR)

Total RNA from HTR-8/SVneo cells was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) and mRNA was reverse transcribed into cDNA by SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. The expression of mRNA was measured by SYBR green real-time quantitative polymerase chain reaction (qRT-PCR) with a ABI 7300 instrument (Applied Biosystems, Forster, CA). The PCR reaction was performed in 20 μl buffer containing 2 μl cDNA, 5 pM of each primer, and power SYBR green PCR master mix (Applied Biosystems). The thermal cycling conditions were preincubated for 2 min at 50℃, then denaturated for 10 min at 95℃, followed by 40 cycles of denaturation for 15 s at 95℃, and annealing and extension for 1 min at 60℃. The relative mRNA

abundance of each gene was normalized to the expression levels of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GADPH) and was defined by the ΔΔCt method.[21] The primers used are given in Table 1.

Table 1. Primer sequences specific to the target genes

Gene	Direction	Sequence
DJ-1	Sense	5'-GTCATTTGTCCTGATGCCAG-3'
	Anti-sense	5'-TCAGATAAATTCTGTGCGCCC-3'
MMP2	Sense	5'-ACCGCGACAAGAAGTATGGC-3'
	Anti-sense	5'-CCACTTGCGGTCATCATCGT-3'
MMP9	Sense	5'-CGATGACGAGTTGTGGTCCC-3'
	Anti-sense	5'-TCGTAGTTGGCCGTGGTACT-3'
Ki67	Sense	5'-GAAAGAGTGGCAACCTGCCTTC-3'
	Anti-sense	5'-GCACCAAGTTTTACTACATCTGCC-3'
GAPDH	Sense	5'-ACCACAGTCCATGCCATCAC-3'
	Anti-sense	5'-TCCACCACCCTGTTGCTGTA-3'

6. Hydrogen peroxide (H_2O_2) treatment

HTR-8/SVneo cells were exposed to H_2O_2 in complete medium to study the effects of DJ-1 downregulation on cell functions under oxidative stress. Initially, HTR-8/SVneo cells were treated with 0–1,000 μM H_2O_2 concentrations for 48 h and the viability of cells was assessed using the 3-(4, 5-Dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. According to the MTT assay results, 50 μM of H_2O_2 was used in the subsequent experiments.

7. MTT assay for evaluating cell viability

The MTT assay using the CellTiter 96® Non-Radioactive cell proliferation assay kit (Promega, WI, USA) was used to evaluate the proliferation capacity of the HTR-8/SVneo cells according to the manufacturer's instructions. A total of 1×10^4 cells in 100 μL medium per well were seeded into 96-well plates and incubated for 48 h. 15 μL of MTT was then added to each well, and the plates were incubated for another 4 h. The medium was then removed, and 100 μL of stop solution was added. The absorbance was measured at 570 nm (650 nm as a reference). Cell viability was determined based on the optical density ratio of the siDJ-1 treated culture relative to the siControl treated culture.

8. Statistical analysis

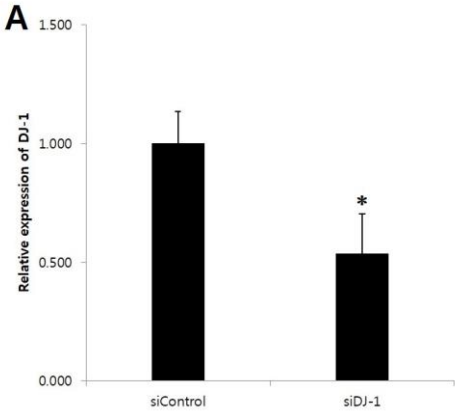
The differences in mean values among the groups were evaluated and expressed as the mean ± standard deviation. Statistical calculations were

performed using a Student's t-test in Microsoft Excel 2016 (Redmond, WA, USA). The Kolmogorov-Smirnov test showed that the distribution of each data was normal. A P value less than 0.05 was considered statistically significant.

III. RESULTS

1. DJ-1 siRNA transfection efficiency

HTR-8/SVneo cells were transfected with DJ-1 siRNA and the scrambled control (Fig. 1). qRT-PCR was performed to assess tranfection efficacy. About 54% of DJ-1 mRNA was inhibited in comparison with the siControl (Fig 1A, P=0.00017) and DJ-1 siRNA resulted in a significant decrease in DJ-1 protein levels to 67% of that observed in the siControl. (Fig. 1B; P = 0.000016).



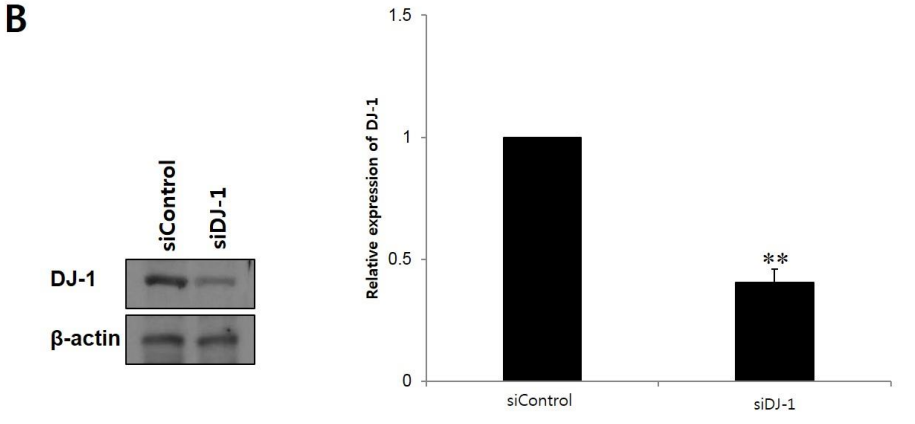


Figure 1. Transfection efficiency of siRNA of DJ-1 in HTR-8/SVneo cells. A. Relative expression of DJ-1 mRNA after transfection of siRNA of DJ-1 determined by RT-PCR. B Relative expression of DJ-1 protein after transfection of siRNA of DJ-1 determined by Western blot. Data are shown as represent % of control. *: $P < 0.01$, **: $P < 0.0001$, n = 3 in triplicate for RT-PCR and n = 3 in duplicate for Western blotting.

2. Effect of DJ-1 inhibition on apoptosis

To assess whether DJ-1 inhibition by siRNA could induce apoptosis, Hoechst 33342 staining was used. After identifying increased apoptosis in cells treated with siRNA in comparison with controls, confirmatory western blots for apoptosis-related proteins such as CASP3, Bax, and Bcl2 were assessed.

A. Hoechst 33342 staining for detecting apoptotic cells

Compared with the control, siDJ-1 treatment increased cell apoptosis. A significant difference was found between treatment with siDJ-1 and siControl, indicating that DJ-1 may have protective effects against apoptosis. (siControl 1.06±0.14%, siDJ-1 13.8±1.45%, $P = 0.0000003$).

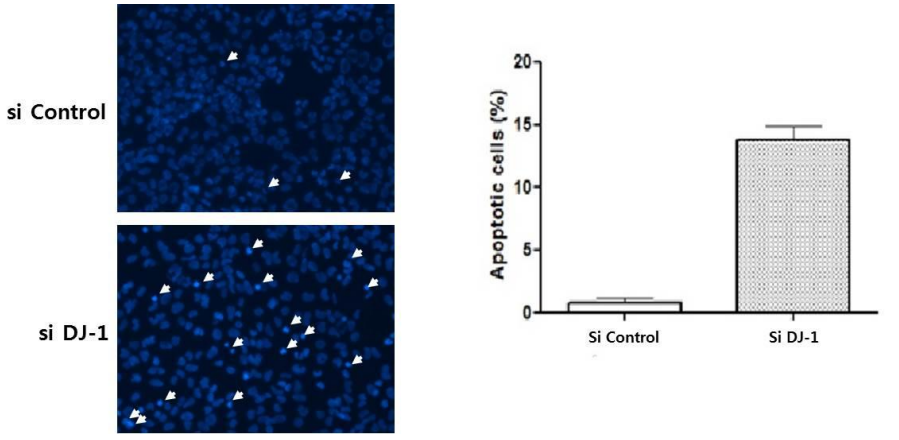


Figure 2. Hoechst 33342 staining for assessing cell apoptosis at a magnification of × 200. Typical apoptotic cells were visualized with condensed chromatin and nuclear fragments (arrows). The percentage of apoptotic cells is shown indicating much higher percentage of apoptotic cells among DJ-1 siRNA‐transfected cells compared to the control cells, Data are shown as represent mean ± SEM. $P < 0.001$.

B. Western blot analysis of apoptosis-related proteins

The effect of treatment with siDJ-1 on Bcl2, Bax, and CASP3 protein expression in cultured HTR-8/SVneo cells was assessed by western blot analysis (Fig. 3). Compared with siControl-treated cells, treatment with siDJ-1 significantly ($P = 0.000075$) decreased 26 kDa anti-apoptosis Bcl2 expression. However, Bax a member of the Bcl2 family that promotes apoptosis, was significantly increased in siDJ-1 treated cells in comparison with siControl-treated cells ($P = 0.0028$). The Bax/Bcl2 ratio was also as much as

20-fold higher in siDJ-1 treated cells than siControl treated cells (P = 0.000019). Treatment with siDJ-1 significantly (P = 0.00022) decreased CASP3, known to play a central role in the execution-phase of cell apoptosis.

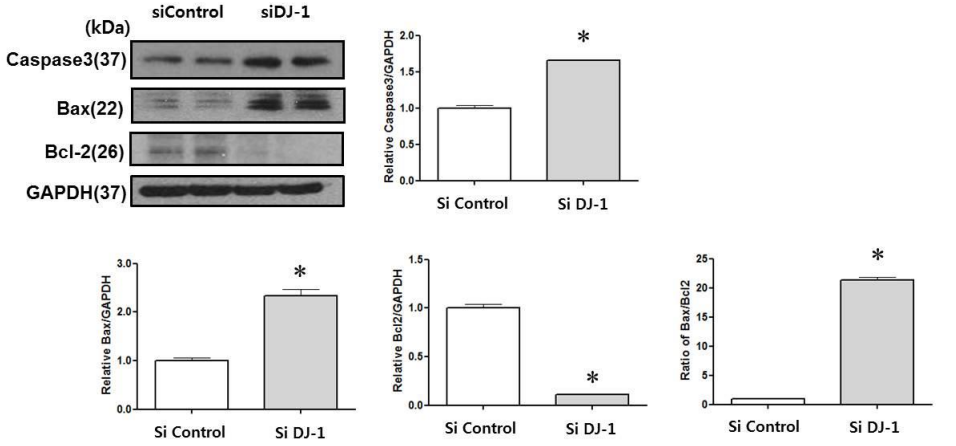


Figure 3. HTR-8/SVneo cell lysates were analyzed for the expression of apoptosis-related proteins such as caspase3, Bax and Bcl2 by Western blot analysis. Caspase 3 and Bax protein expression were increased and Bcl 2 was decreased in siDJ-1 treated cells, compared to control. Marked increase of Bax/Bcl 2 ratio was shown in siDJ-1 treated cells. Data are shown as % of control. *: $P < 0.05$, n = 3 in duplicate

3. Ki 67 expression for evaluating cell proliferation

Proliferation marker protein Ki-67 is used as a molecular marker for assessing the cell proliferation index.[22] Compared with controls, Ki-67 mRNA expression was significantly (P = 0.031) decreased to 33% in siDJ-1 treated cells. This indicates that DJ-1 inhibition in HTR-8/SVneo cells can cause a

reduction in the proliferative potential.

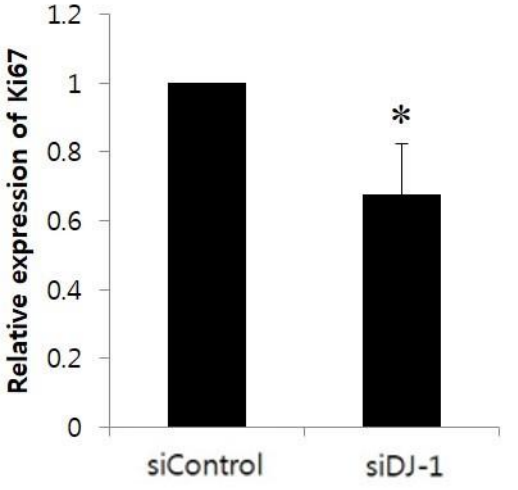


Figure 4. The expression level of Ki 67 mRNA in HTR-8/SVneo cells Ki 67 expression was decreased in siDJ-1 treated cells, compared to control. Data are shown as % of control. *: $P < 0.05$, n = 3.

4. Migration assay

Cell migration ability was analyzed by the transwell chamber assay. 0.2% crystal violet-stained migrated cells were counted after culturing for 18–24 h. MMP2 and MMP9 protein expression was measured using western blot.

A. Increased migration in siDJ-1-treated HTR-8/SVneo cells

To compare the migration of siDJ-1- and siControl-treated HTR-8/SVneo cells a transwell migration assay was performed. The migration of

HTR-8/SVneo cells treated with siDJ-1 was significantly reduced in comparison with siControl-treated cells (Fig. 5). The data demonstrate that HTR-8/SVneo cells treated with siDJ-1 have a much lower migration potential than siControl-treated cells(Mean of migrated cells/field; siControl 300 *vs* siDJ-1 150) .

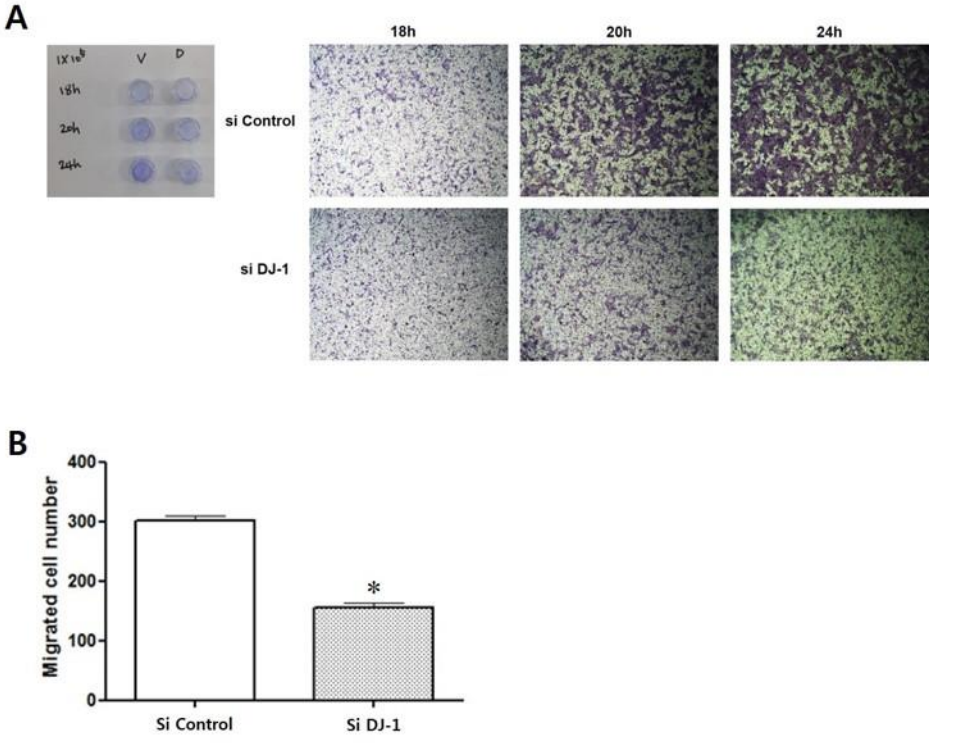


Figure 5. Effects of siDJ-1 on HTR-8/SVneo cell migration. A. Representative images of crystal violet stained HTR-8/SVneo migratory cells transfected with siDJ-1 and siControl at a magnification of ×200. siDJ-1 inhibits the migration of HTR-8/SVneo cells 18, 20, and 24 hours after transfection in time-dependent manner. B. Quantification of the migratory cells. Data are shown as represent

mean ± SEM, *: P<0.05

B. Western blot analysis of MMPs

MMP2 and MMP9 are known to play an important role in the trophoblast migration process.[23] The protein expression of the inactive, propeptide form of MMP2 (Pro-MMP2; 72 kDa), MMP2 (63 kDa), and MMP9 (92 kDa) in cultured HTR-8/SVneo cells was assessed by western blot analysis (Fig. 6). Both pro-MMP2 (P = 0.034) and MMP2 (P = 0.009) were significantly decreased in cells with siDJ-1 treatment in comparison with siControl-treated cells. Expression of MMP9 protein was also lower in siDJ-1-treated cells than control (P = 0.000019).

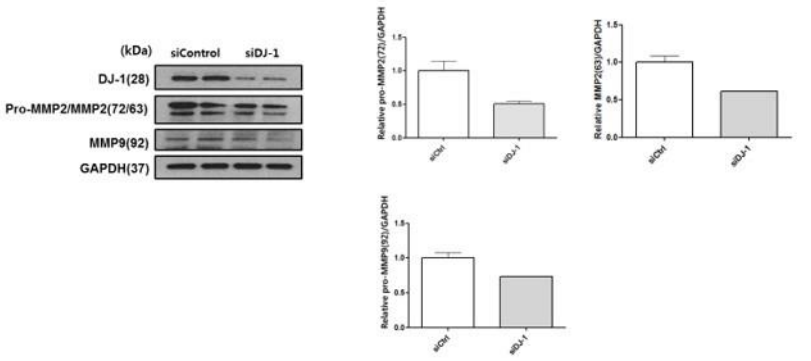


Figure 6. The protein expression analysis of MMP2 and MMP9 including pro-MMP by Western blot. Pro-MMP2, MMP2, pro-MMP9, and MMP9 protein expression were decreased in siDJ-1 treated cells, compared to control. Data were normalized using GAPDH. Data are shown as % of control. *: P < 0.05, n = 3 in duplicate.

5. MTT assay for assessing the effect of H_2O_2 on cell viability in HTR-8/SVneo cells

A. MTT assay for assessing the effect of H_2O_2 on cell viability in HTR-8/SVneo cells

H_2O_2 was used as a model for oxidative stress in HTR-8/SVneo in order to evaluate cell viability using an MTT assay. 1×10^4 cells were seeded in each well of a 96-well plate. After incubation for 24 h, cells were treated with different concentrations of H_2O_2 (0–1,000 μM) for 48 h, and cell viability was measured. As shown in Figure 1, the viability of H_2O_2-treated cells significantly decreased in a dose-dependent manner. At a concentration of 50 μM H_2O_2, cell viability was reduced by 26% (24 h) and 37% (48 h). This was considered to be the optimum concentration of H_2O_2 as the effect of siRNA on cell viability and was used for subsequent experiments.

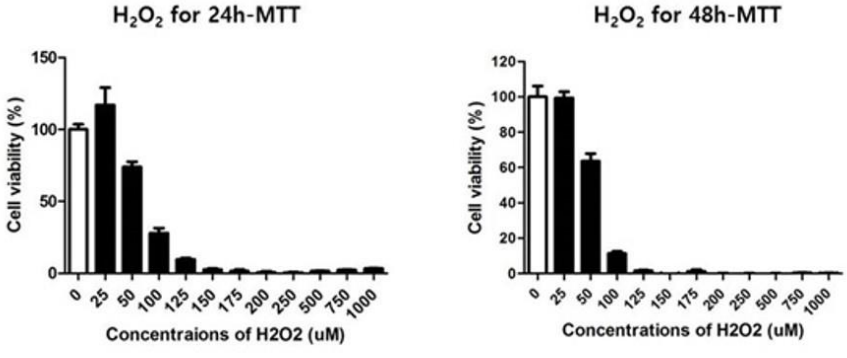


Figure 7. Effect of H_2O_2 on cell viability in HTR-8/SVneo cells determined using an MTT assay.

B. Effect of DJ-1 down-regulation on HTR-8/SVneo in H_2O_2 induced oxidative stress condition

As mentioned above, DJ-1 is known to be an antioxidant and DJ-1 deficiency increased susceptibility to apoptosis in the first trimester extravillous trophoblast cell line, HTR-8/SVneo. The MTT assay was performed to investigate the effect of DJ-1 downregulation on cell viability under oxidative stress induced by 50 μM H_2O_2. Representative results are shown in Figure 8. Cell viability was calculated based on the optical density ratio of siDJ-1-treated culture relative to the siControl, revealing that the viability of the HTR-8/SVneo cells was lower in siDJ-1 treated cells ($P < 0.05$) (Fig. 8)

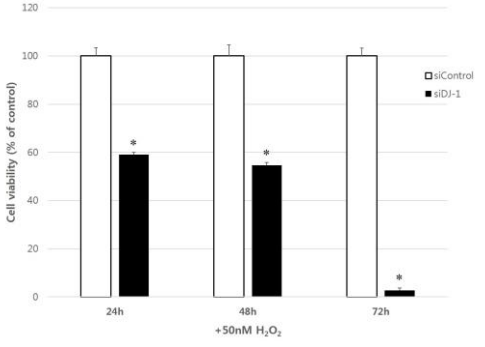


Figure 8. HTR-8/SVneo cell viability measured by MTT assay. DJ-1 siRNA transfection inhibited the viability of HTR-8/SVneo cells. 1×10^4 cells were seeded and 50 μM H_2O_2 was treated after 24 hours incubation. Data are shown

as % of control, *: $P < 0.05$, n=5

6. Effect of siDJ-1 on MMP2 and MMP9 mRNA expression in H_2O_2 induced oxidative stress

The mRNA expression levels of MMP2 and MMP9 were measured by qRT-PCR. DJ-1 downregulation using siRNA significantly decreased MMP2 and MMP9 expression in HTR-8/SVneo cells (Fig. 9). The data revealed that in comparison with controls, MMP2 and MMP9 expression was lowered by 83% and 73%.

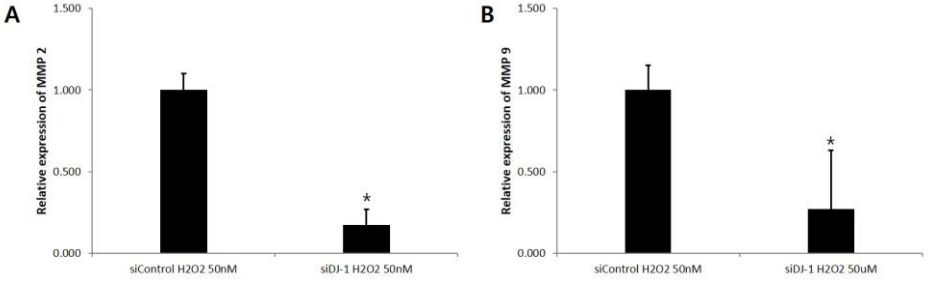


Figure 9. Inhibitory effect of siDJ-1 on MMP 2 and MMP 9 mRNA expression in 50 μM H_2O_2 induced oxidative stress condition. Expression of MMP2 (A) and MMP9 (B) mRNA were determined by RT-PCR and normalized using GAPDH. Data are shown as represent mean ± standard error of mean (SEM). *: $P < 0.05$, n = 3 in triplicate.

7. PTEN/Akt/mTOR signaling pathway assessed by Western blotting

DJ-1 has been reported to be involved in tumorigenesis by negatively

regulating the phosphatase and tensin homolog (PTEN), which antagonize phosphatidylinositol 3-kinase-Akt-mammalian target of rapamycin (mTOR) pathway.[24] And Akt-mTOR pathway is involved in intracellular signaling for cell survival and this has been described as one of the important pathways in human trophoblast.[25] So we hypothesized that the Akt-mTOR signaling pathway may be inhibited by inhibition of DJ-1. Ribosomal protein S6 kinase beta-1 (S6K1), also known as p70S6 kinase, is the direct substrate of mTOR. As shown in Figure 10, expression of PTEN was increased and expression of phosphorylated Akt (p-Akt), and p-S6K1 was lowered by DJ-1 siRNA ($P < 0.05$) especially in H_2O_2-treated conditions when compared with siControls.

Additionally, H_2O_2 treatment induced reduced PTEN expression and promoted p-Akt/p-S6K1 expression, likely representing a compensatory mechanism to protect against oxidative stress and promote cell survival.

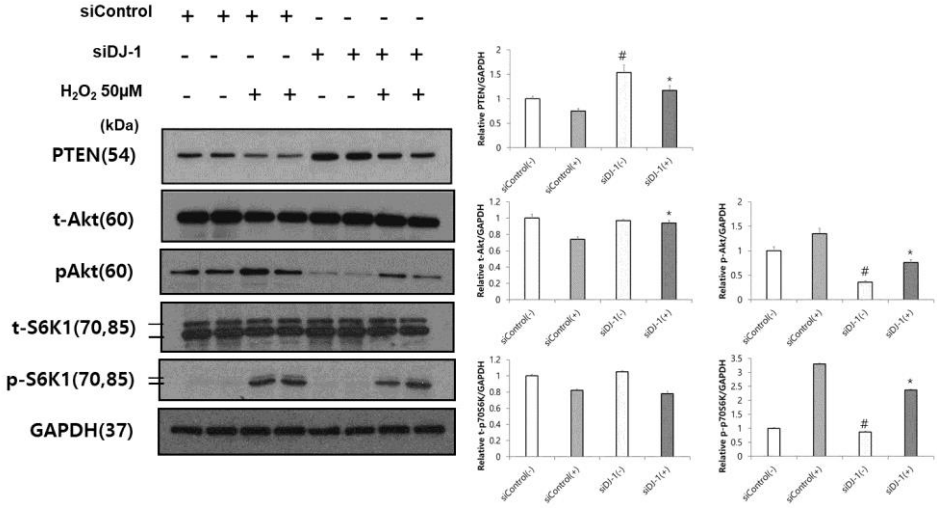


Figure 10. DJ-1 downregulation promotes PTEN expression and suppresses the

Akt-S6K1/p70S6K (S6K1) pathway, especially under 50 μM H_2O_2-induced oxidative stress. Data are presented as the percentage of control and normalized to α-tubulin. (-): not treated with H_2O_2, (+): treated with H_2O_2, Data represent the mean SEM. *P < 0.05 when compared with siControl (+), and #P < 0.05 when compared with siControl (-), n=3 in duplicate

IV. DISCUSSION

In previous reports, DJ-1 acts as an oncogene that is overexpressed in many cancers.[26-28] DJ-1 promotes proliferation of cancer cells through anti-apoptotic and proliferative activity,[29,30] and the migration and invasion involved in the metastasis of cancer cells via MMPs.[30,31] DJ-1 is also expressed in the placenta, especially in cytotrophoblasts and syncytiotrophoblasts and its expression is higher in the first trimester than in the third trimester.[18,19] It is therefore reasonable to speculate that DJ-1 may have some biological functions associated with cancer cells, such as cell apoptosis, proliferation, and migration.

The data from the current study reveals the effects of DJ-1 on the first trimester extravillous trophoblast cell line HTR-8/SVneo through downregulation of its expression. Apoptosis of HTR-8/SVneo was increased after DJ-1 siRNA treatment, as confirmed by Hoechst staining and western blot that showed decreased levels of Bcl2 and increased levels of CASP3 and Bax protein. The level of cell proliferation marker, Ki-67, was decreased slightly at the transcriptional level. Furthermore, migration of HTR-8/SVneo cells was

significantly impaired after inhibition of DJ-1 via regulation of MMP2 and MMP9, proteases reported to be required for trophoblast migration.[32] The current study revealed that downregulation of DJ-1 inhibited the expression of MMP2 and MMP9 at the protein level. The results are in accordance with previous studies on cancer.[30,31] Zhu et al. found that the expression of DJ-1 was significantly upregulated in gastric cancer specimens with peritoneal metastasis compared to those without peritoneal metastasis. Additionally, knockdown of DJ-1 expression significantly inhibited invasion and migration in vitro, as well as the in vivo peritoneal metastatic abilities of gastric cancer cells, and also diminished the expression of MMP2 and MMP9 via the Akt pathway.[31]

Next, we investigated the effect of DJ-1 downregulation on cell functions under oxidative stress considering that DJ-1 is a known antioxidant. Ambient oxygen concentrations of 21% can result in hyperoxic exposure in first trimester trophoblasts, having substantial effects on the behavior of the early placental explants associated with placenta-related diseases of pregnancy.[33,34] H_2O_2 was used as an additive and definite oxidative stress model. As mentioned above, cell viability and MMPs levels were lowered after DJ-1 transfection in HTR-8/SVneo cells under H_2O_2-induced oxidative stress. In our previous publication, DJ-1 was over-expressed in the placentas of severe preeclampsia patients, which was thought to be a compensatory mechanism in response to hypoxia or oxidative stress.[19] Based on current findings, we can confirm that DJ-1 has a protective role in trophoblast cells. However, the sustained increase

in DJ-1 from the first trimester to the third trimester can be interpreted in different way. DJ-1 is an oncogene and upstream regulator of hypoxia-inducible factor 1-alpha (HIF1A) by regulation of Akt and mTOR, increasing the cell resistance to hypoxic stress.[35] Kanasaki et al. demonstrated that deficiency in catechol O-methyltransferase (COMT), an enzyme that converts 17-hydroxyestradiol into 2-methoxyestradiol (2-ME), results in reduced 2-ME levels and a preeclampsia-like syndrome that was associated with placental HIF1A upregulation in pregnant mice.[36] In a recent review article, increased HIF-1 was suggested to promote the production of soluble Fms-like tyrosine kinase 1 (sFLT1), soluble endoglin, and endothelin-1 that cause endothelial dysfunction and the clinical manifestation of preeclampsia.[37]

Therefore, DJ-1 may play a pivotal role in the pathogenesis of preeclampsia. In other words, DJ-1 may be elevated in the preeclamptic placenta to protect trophoblasts, as demonstrated in our investigations, and may also upregulate HIF1A to cause preeclampsia. Additional research is required to prove this hypothesis. Moreover, oxidative stress may be involved in the pathogenesis of miscarriage and fetal growth restriction as well as preeclampsia.[38] The role of DJ-1 in these pregnancy complications has not yet been elucidated and is deserving of further research.

The molecular pathways involved in trophoblastic cell function were examined. The Akt/mTOR signaling pathway is a common pathway involved in trophoblast migration, invasion, and apoptosis.[31,36] Previous research

demonstrated that DJ-1 can activate these pathways by negatively regulating the activity of PTEN in cancer cells[24,36] and T cells.[39] The current study revealed that DJ-1 downregulation enhanced PTEN expression, thus reducing Akt/mTOR signaling-based S6K1/p70S6K phosphorylation, where DJ-1 functions in the first trimester trophoblasts may operate via these pathways.

V. CONCLUSION

To the best of our knowledge this is the first study to investigate the role of DJ-1 in trophoblasts. DJ-1 downregulation using siRNA promoted apoptosis and reduced migration by regulating MMP2 and MMP9 in a first trimester extravillous trophoblast cell line (HTR-8/SVneo) under both ambient and oxidative stress conditions. These changes in cell function may be at least partly dependent on the Akt/mTOR pathway via S6K1. Though DJ-1 is thought to play a protective role in maintaining trophoblast functions in preeclamptic placentas, considering previous reports, it can also lead to other placenta-related complications such as miscarriage and fetal growth restriction.

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ABSTRACT (IN KOREAN)

DJ-1의 발현 억제가 임신 제 1삼분기 융모외성 영양막세포의 기능에 미치는 영향

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권 한 성

DJ-1 (PARK7)은 파킨슨병의 원인 유전자 중 하나이며, 종양유전자 (oncogene)로도 알려져 있다. 지금까지 항산화 효과 등 여러가지 생물학적 기능들이 보고되었다. 특히 퇴행성 신경계 질환에서 DJ-1의 기능 소실은 세포사 (cell death)를 유발할 수 있으며, 암질환의 경우 이 유전자의 기능 항진이 암세포의 조절되지 않는 생존을 유발하기도 한다고 알려져 있다. DJ-1은 여러 장기에 걸쳐 발현되며, 태반의 영양막세포에서도 발현된다. 또한, 임신 제1삼분기의 영양막세포에서 임신 후기에 비해 그 발현이 증가하는 것으로 알려져 있다. 영양막세포의 이동, 침습 및 전이 등 그 생물학적 기능이 암세포와

유사함에도 불구하고 영양막세포에서 DJ-1의 생물학적 기능에 대해서는 알려진 바가 없다. 따라서, 이 연구는 임신 제1삼분기 융모외 (extravillous) 영양막세포주인 HTR-8/SV neo에서 DJ-1의 발현 조절이 세포 기능에 미치는 영향과 그 기전에 대해 알아보고자 하였다. siRNA (small interfering RNA)로 유도된 DJ-1 의 발현 억제가 세포자멸사 (apoptosis), 세포 이동 및 이러한 기능들을 조절하는 기전에 미치는 영향을 평가하였다. 이 연구에서 DJ-1의 발현 억제가 대기 환경과 산화스트레스 조건에서 세포자멸사를 증가시키고, 기질금속단백분해효소를 억제하여 세포이동이 감소하였으며 이러한 세포 기능의 변화가 Akt-S6K1 경로를 통해 일어남을 확인하였다. 결론적으로 DJ-1은 산화스트레스 환경에서 영양막세포의 기능을 보존하는 데 중요한 역할을 하는 것으로 생각되며, 향후 DJ-1의 발현 이상이 산과적 합병증에 미치는 영향에 대해서는 추가적인 연구가 필요할 것이다.

핵심되는 말 : DJ-1, 발현억제, 세포기능, 세포자멸사, 세포이동