

Effect of Immortalization-Upregulated Protein-2 (IMUP-2) on Cell Death of Trophoblast

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ABSTRACT : Trophoblasts, in the placenta, play a role for placental development as well as implantation in the early pregnancy. The characteristics and functions of trophoblast are identified by their localization and potency for proliferation, differentiation, and invasion. Thus, inadequate trophoblast cell death induces trophoblast dysfunction resulting in abnormal placental development and several gynecological diseases. Recently, it was reported that increased immortalization-upregulated protein-2 (IMUP-2) by hypoxia influences trophoblast apoptosis. However, IMUP-2 function on autophagy, which is type II programmed cell death remains unclear. In this study, we analyzed IMUP-2 expression in trophoblast cells (HTR8-SVneo) and compared IMUP-2 effects on cell death including apoptosis and autophagy in trophoblast regardless of IMUP-2 expression. Increased IMUP-2 in trophoblast by IMUP-2 gene transfection induces cell death, especially, apoptosis increases more than autophagy ($p < 0.05$). However, the decreased IMUP-2 in trophoblasts after siRNA treatment decreased apoptosis with the decreased activities of caspase 3 and 7. The expressions of LC3 and MDC as an autophagosome makers and phosphorylated mTOR, which is a negative regulator for autophagy, increased. In addition, the S phase of cell cycle increased in trophoblasts when IMUP-2 expression decreased. Taken together, the alteration of IMUP-2 can control the balance between apoptosis and autophagy of trophoblasts resulting in functional involvement in placental development and in gynecological diseases by regulating the function of trophoblasts.

Key words : Trophoblast, Immortalization-upregulated protein-2, Apoptosis, Autophagy

INTRODUCTION

The trophoblast constitutes an undifferentiated single layer of cytotrophoblast forming the outer layer of a blastocyst and the multinucleated double layer of syncytiotrophoblast that is fused from cytotrophoblast at the period of embryo implantation (Huppertz, 2008). Multinucleated syncytiotrophoblasts come into contact with villous basement membrane and differentiate into villous syncytiotrophoblasts that proliferate with a fibroblast growth factor-4. Those differentiated cells form syncytial knots, induce

cell death, and are released through placental blood vessels (Scifres & Nelson, 2009). Also, cytotrophoblast can differentiate into another type of trophoblasts called extravillous trophoblasts. These are formed by an invasion of trophoblastic cell columns into the uterine stroma. Extravillous trophoblast stem cells in the cell columns penetrate into the myometrium and can differentiate into the endovascular extra villous trophoblast and interstitial extra villous trophoblast forming a normal placenta. This process provides an adequate blood supply to the placenta with spiral arteries and facilitates the exchange of nutrients, wastes,

Manuscript received 6 May 2013, received in revised form 20 May 2013, accepted 22 May, 2013

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and gases between the maternal and fetal systems. This proliferation, differentiation, and adequate cell death play a significant role to maintain placental development. Inadequate trophoblast cell deaths can induce trophoblast dysfunction by lowering trophoblast numbers resulting in abnormal placentation and several gynecological diseases (Huppertz et al., 2006; Na et al., 2010).

Immortalization-upregulated proteins (IMUPs) are novel genes identified in SV40-immortalized human fibroblast cell lines, encoding two novel nuclear proteins, IMUP-1 (321 bp, 10.9 kDa) and IMUP-2 (258 bp, 8.5 kDa). IMUP-1 and IMUP-2 arise during transcriptional processing through an RNA alternative splicing procedure (Kim et al., 2000; Ozer, 2000; Kim et al., 2008). In addition, IMUPs influence cellular distortion, proliferation, cell death, and tumorigenesis including lungs, colon, and ovarian cancer (Kim et al., 1998; Kim et al., 2003). Although IMUPs are known for their cellular proliferation and cell death, however, trophoblastic effectiveness is not well known. A recent study showed placentas with an obstetrical disease, such as pre-eclampsia, have an excess of IMUPs compared with normal placentas and the excess IMUPs can cause cell death (Jeon et al., 2010).

Programmed cell death is classified as necrosis, apoptosis (Type I programmed cell-death), and autophagy (Type II programmed cell-death) (Bildirici et al., 2012). Apoptosis is the process of programmed cell death that may occur in a homeostatic context of the cell organisms or during growing morphology. The genetically engaged survival process plays an important role in physiological development, homeostasis, and immunity facilitating appropriate growth and differentiation for the normal placental development (Lockshin & Zakeri, 2004). Conversely, autophagy is the catabolic mechanism that maintains the turnover rate of organelles. It involves the cell degradation of unnecessary cellular components through lysosomal machinery or ubiquitin-like conjugation reactions, which fuses the isolated cells with lysosomes. Recent studies show that the autophagosome formation occur through a nucleation complex of Class III PI3K and Beclin from Bcl₂ as well

as from the reaction of LC3-II and Atg5-12 complexes that are independently generated protein conjugation cascades (Singh, 2011). Another study showed that expression of LC3 II was in fact more elevated in cesarean section placentas than in vaginal deliveries. Also, the expression of LC3 II was increased in pre-eclamptic placentas than in normal placentas (Oh et al., 2008). However, how IMUP-2 expression changes affect cell death and functions are not clear. Therefore, the objective of study is to analyze the effect of IMUP-2 on apoptosis and autophagy in trophoblast cell.

MATERIALS AND METHODS

1. HTR-8/SVneo trophoblast cell line culture

HTR-8/SVneo trophoblastic cells at the early stage of pregnancy were provided by Dr. Charles H. Graham (Queen's University, Canada). They were exposed to an atmosphere consisting of 5% CO₂ at 37°C maintained in RPMI-1640 medium (Gibco BRL) supplemented with 100 U/ml penicillin and streptomycin (Gibco BRL, NY, USA), 5% fetal bovine serum (FBS, Gibco BRL), and 100 U/ml penicillin/streptomycin (Gibco).

2. Transfection of pcDNA3/IMUP-2 and siRNA/IMUP-2 into the trophoblast cell line

To extract plasmid DNA containing IMUP-2 genes, *E. coli* containing IUMP-2 plasmid was incubated for 16 to 18 hours using rotator shaking and a HiYield Plasmid mini kit (RBC Bioscience Corp, Taipei, Taiwan) was used to extract pcDNA3/IMUP-2 from *E. coli*. HTR-8/SVneo trophoblast cells (1×10^6) were seeded onto 100 mm culture dishes (Thermo Scientific, MA, USA). When the cells reached 70% confluence, they were mixed with 500 ng pcDNA3/IMUP-2, 1 nM siRNA/IMUP-2, and 2 μ l Lipofectamin 2000 (Invitrogen, CA, USA), exposed at room temperature for 20 minutes. They were switched to Opti-MEM (Gibco BRL), a reduced antibiotics and serum media. Then they were transfected with a 500 ng of pcDNA3/IMUP-2 and 1 nM siRNA/IMUP-2 to be cultured

for 24 hours.

3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) analysis

We conducted a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) analysis to analyze cell death, depending on the expression of IMUP-2, with pcDNA3/IMUP-2 and siRNA/IMUP-2 transfected cells. 1×10^4 HTR-8/SVneo trophoblast cells were seeded onto 96-well plates (BD Biosciences, California, USA), transfected with 500 ng of pcDNA3/IMUP-2 and 1 nM of siRNA/IMUP-2 using 2 μ l of Lipofectamin mix, then cultured for 24 hours to perform the MTT analysis. 20 μ l of MTT liquid with 5 mg/ml density was added and maintained at 37°C for 2 hours and 30 minutes after MTT liquid was lysed. Then, 200 μ l of dimethyl sulfoxide (DMSO) was added. To test reaction, they were placed on a gyratory shaker for 5 minutes. Next, optical densities were measured with a wavelength of 562 nm.

4. Total RNA isolation and RT-PCR analysis

To detect the change of IMUP-2 expression in trophoblast cells with pcDNA3/IMUP-2 and siRNA/IMUP-2 transfection, a RT-PCR analysis was performed. The total amount of RNA was extracted using RNeasy RNA isolation kit (QIAGEN, California, USA) and Trizol reagent (Invitrogen). The extracted RNA densities were quantified by a Nano Drop spectrophotometer (Thermo Scientific). 1 μ g of the extracted total RNA, 0.5 μ g of oligo dT, 10mM dNTP mix (Invitrogen), and DEPC-D.W (Invitrogen) were blended, and the transcription reaction was performed at 65°C for 5 minutes. Afterwards, the transcription reaction was performed at 50°C for an hour and at 72°C for 15 minutes using a 5 \times first-strand buffer, 0.1 m DTT, RNase-out, and 200 U/ μ l of superscript III RT (Invitrogen) to synthesize cDNA. The synthesized cDNA was amplified by PCR, using 20 pmol/ μ l of IMUP-2 primers and a H-Taq polymerase kit (Solgent Co., Ltd, Daejeon, South Korea). PCR products were visualized by electrophoresis on a 1.2% (w/v) agarose gel, and the amplified DNA was

indicated by a quantity video image analyzer (Bio-Rad, CA, USA). Then the housekeeping genes of the 28s rRNA bands were quantified using the Quantity One program to conduct a comparative quantitative analysis on the degree of expression of each DNA group with the control group of IMUP-2. The sequences for IMUP-2 primer used in the study are 5'- ATG GAG TTC GAC CTG GG-3' (Forward), 5'-ACT TCA CAT CCG TG TCCG-3' (Reverse), and the sequences for 28s rRNA primer are 5'-TTG AAA ATC CGG GGG AGA G-3' (Forward), 5'-ACA TTG TTC CAA CAT GCC AG-3' (Reverse).

5. Western blot analysis

To analyze how IMUP-2 expression affects the cellular proliferation, and cell death, we performed a western blot analysis on the expression of the correlated factors of cell cycle, apoptosis, and autophagy. 24 hours after transfecting pcDNA3/IMUP-2 and siRNA/IMUP-2 into the trophoblast cells, protein was lysed in 200 μ l RIPA buffer (50 mmol/l Tris-HCl, pH 7.6, 150 mmol/l NaCl, 1% NP40, 0.25% deoxycholic acid, 0.1% SDS, 1 mmol/l EGTA, 1 mmol/l EDTA, 10 mmol/l NaF, 1 mmol/l Na_3VO_4) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Protein extracts were quantified by BCA assay kit (Pierce, Texas, USA). The total of 40 μ g protein extracts were heated at 95°C for 5 minutes, loaded onto 8 to 15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) for 2 hours, and electrotransferred onto PVDF membranes (Bio-Rad) at 70V. The membranes were blocked by TBS-T, made from TBS (Abel Bio, Seoul, South Korea) containing 5% albumin serum bovine (BSA; Amresco, Ohio, USA) and 0.1% Tween 20 (Sigma-Aldrich, Missouri, USA), for 80 minutes and then reacted with various antibodies overnight at 4°C (Table 1). The next day, after washing them with TBS-T, we tested their reaction at room temperature for an hour using horseradish peroxidase (HRP)-conjugated rabbit (1:25,000) (Bio-Rad) or anti-mouse IgG (1:25,000) (Bio-Rad) secondary antibodies. The protein was detected by visualizing peroxidase

Table 1. Antibodies used in the study

Source	Antibody	Dilution factor	Company
Rabbit	IMUP2	1:500	AbFRONTIER
	Bcl ₂	1:1,000	Santa Cruz Biotechnology (cat: SC-492)
	Bak	1:1,000	EPITOMICS (cat:1542-1)
	PI3K kinase class III	1:1,000	cell signaling (cat: 3811)
	mTor	1:1,000	cell signaling (cat: 2983)
	phospho mTor	1:1,000	cell signaling (cat: 2971)
	LC3	1:1,000	Novus (cat: NB100-2220)
	Beclin 1	1:1,000	Santa Cruz (cat: Sc-11427)
	cyclinA	1:1,000	Abcam (cat: ab7956)
	ATG5-12	1:1,000	Santa Cruz (cat: SC271688)
	Cyclin E	1:1,000	Biolegend (cat: 630702)
	p53	1:1,000	Santa Cruz (cat: SC-126)
	p21	1:1,000	Abcam (cat: ab7960)
	Mouse	Caspase-9	1:1,000
GAPDH		1:1,000	Abfrontier(cat: LF-PA0212)

activity using an ECL advance western blotting detection kit (Amersham Bioscience, Buckinghamshire, UK).

6. Apoptosis analysis

To explore the effect of IMUP-2 expression on trophoblastic apoptosis, caspase activities in pcDNA3/ IMU-2 and siRNA-IMUP-2 transfected trophoblastic cells were analyzed using a Capsase- Glo 3/7 assay kit (Promega Corp, Wisconsin, USA). The cell lysates were quantified using a protease inhibitor-free lysis buffer (Culture Lysis Reagent; Promega), 50 µg of protein, and reacted solution, placed in wells, were exposed to the ordinary temperature for 2 hours and 40 minutes. Fluorescence of the reacted samples was measured with a wavelength of 470 nm using a SpectraMax L microplate reader (Molecular Devices, California, USA). Also, to indicate the fragmented DNA from apoptosis, after washing incubated trophoblastic cells on glass cover slip (Nunc, FL, USA) with PBS, the slides were fixed in methanol (Merck, New York, USA) at room temperature for 10 minutes. They were stained with 1 µg/ml PI (Sigma) at room temperature for 5 minutes to detect nuclear segmentation, washed, and mounted in glycerol-based mounting solution (DAKO, Glostrup, Denmark).

Then, they were observed with an EVOS fl microscope (AMG, Washington, USA).

7. Fluorescence-activated cell sorter (FACS) analysis

The pcDNA3/IMUP-2 and siRNA-IMUP-2 transfected trophoblastic cells were treated with 0.05% of trypsin and TrypLE Express solution (Gibco BRL) containing 1mM of EDTA. The extracted cells were immersed in 100% ethanol (Merck) at room temperature for 10 minutes and in 0.5 µg/µl RNase A (Sigma) solution for 15 minutes. Then they were stained with Propidium Iodide (PI) solution (1:200, Sigma). The cycle of stained cells was examined using a FACScan flow cytometer (BD Biosciences).

8. IMUP-2 expression and autophagosome analysis using immunofluorescence

The pcDNA3/IMUP-2 and siRNA-IMUP-2 transfected trophoblastic cells were incubated on 24-well plates with glass cover slips (Nunc) for 24 hours, washed with PBS (Sigma), and then mounted in 100% methanol (Merck) for 15 minutes. The cells were blocked by protein block serum-free buffer (Dako) at room temperature for 30 minutes. Rabbit anti-IMUP2 (provided by Jinkyong Kim,

CHA University, Korea, 1: 100×) for IMUP-2 expression analysis and rabbit anti-human LC3 (Norvus Biologicals, CO, USA, 1:200×) for the detection of autophagosome formation were separately incubated overnight at 4°C. The next day, after washing them with PBS, they were subsequently incubated in a humidified chamber with a secondary antibody conjugated to Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (Invitrogen, 1:400) at the ordinary temperature for 30 minutes. Then, they were counterstained in DAPI-containing mounting solution (Dako) and analyzed using an EVOS fl microscope (AMG). To detect autophagosome using MDC staining, the 24 hours incubated trophoblastic cells were fixed in 4% paraformaldehyde solution (Usb Corporation, Ohio, USA) after being reacted with 0.05 mM MDC (Sigma) at 37°C for 30 minutes. They were mounted in fluorescence mounting medium (Dako) and were observed with an EVOS fl microscope (AMG).

9. Statistical analysis

All experiments were repeated thrice, data representing the mean±standard error bar (SE). The data was analyzed for statistical significance of p -value<0.05 using student's t -test and ANOVA method from SAS software (SAS Institute, North Carolina, USA).

RESULTS

1. IMUP-2 expression in HTR-8/SVneo trophoblast cell line

To detect the change of IMUP-2 expression with pcDNA3/IMUP-2 and siRNA/IMUP-2 transfection in HTR-8/SVneo trophoblasts, reverse transcription polymerase chain reaction (RT-PCR) analysis was performed. We observed that quantitative mRNA expression of IMUP-2 significantly increased in the pcDNA3/IMUP-2 transfected cells but

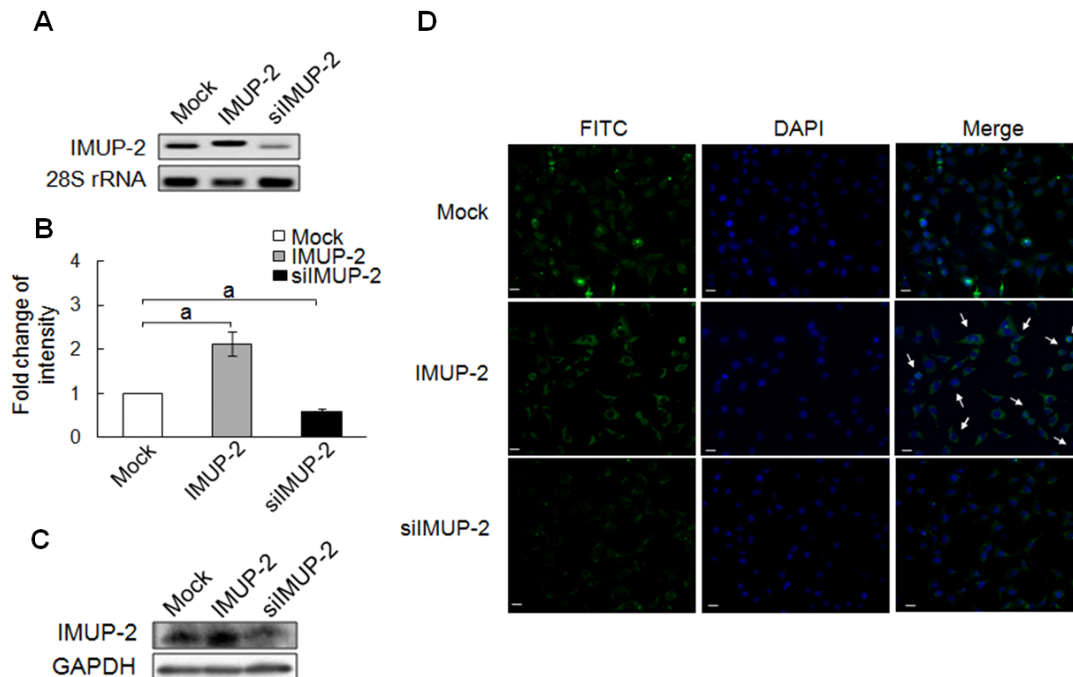


Fig. 1. Expression of IMUP-2 in HTR-8/SVneo trophoblast. (A) Expression of IMUP-2 in HTR-8/SVneo trophoblast using RT-PCR. (B) Densitometric analysis of IMUP-2 mRNA expression relative to 28s rRNA mRNA expression. Data are expressed as means±standard error (SE). Statistical analysis was carried out by Student's t -tests ($*P<0.05$). (C) Expression of IMUP-2 in HTR-8/SVneo trophoblast using Western blot analysis. GAPDH was used as a internal control. (D) Immunofluorescence for IMUP-2 in HTR-8/SVneo trophoblast cells after transfection of pcDNA3/IMUP-2 and siRNA/IMUP-2 ($\times 200$). Arrow means positive signals for IMUP-2 in HTR-8/SVneo trophoblast. (Mock: lipofectamin transfection; IMUP-2: pcDNA3/ IMUP-2 transfection; siIMUP-2: siRNA/IMUP-2 transfection).

decreased in siRNA/IMUP-2 transfected trophoblastic cells ($p < 0.05$, Fig. 1A, B). Similarly with the RT-PCR result, the protein expression of IMUP-2 increased in the pcDNA3/IMUP-2 transfected trophoblastic cells when compared with mock but decreased in siRNA/IMUP-2 transfected trophoblastic cells (Fig. 1C). Also, when observed with immunofluorescence for IMUP-2 expression in HTR-8/SVneo trophoblastic cells, the cellular localization of IMUP-2 was translocated from cytoplasm into nuclear in pcDNA3/IMUP-2 transfected trophoblasts, comparing to the Mock group, but no nuclear translocation was observed in siRNA/IMUP-2 transfected trophoblasts (Fig. 1D).

2. Cell cycle analysis depend on IMUP-2 expression

In cell cycle analysis using FACS analysis, generally, the sub G1 phase of trophoblasts were increased by IMUP-2 expression through up and down-regulation of IMUP-2 ($p < 0.05$, Fig. 2A). However, the sub G1 phase of pcDNA3/IMUP-2 transfected trophoblasts shows a tendency to increase more than siRNA/IMUP-2 transfected trophoblasts ($p < 0.05$). In case of the S phase, there are no statistical significances in pcDNA3/IMUP-2 expression, although they increased from 13.44% to 16.54% compared

to the mock control. However, we observed statistical significance in the S phase of siRNA/IMUP-2 expression showing the increase from 13.44% to 19.37% ($p < 0.05$, Fig. 2A). Also, for the G2/M phase, there is no difference although pcDNA3/IMUP-2 expression decreased from 27.65% to 25.85% comparing to the mock control. However, siRNA/IMUP-2 expression, compared to the mock control, showed significant decrease from 27.65% to 21.00% in G2/M phase than pcDNA3/IMUP-2 ($p < 0.05$, Fig. 2A).

Next, we analyzed the expression of cell cycle-related genes to determine the IMUP-2 effect. The expressions of cell cycle-related gene such as Cyclin A and Cyclin E in siRNA/IMUP-2 treated trophoblasts were increased comparing to the mock group. However, the expression of p53, which is a checkpoint of the cell cycle, did not show significant difference even though it slightly increased in both cells of pcDNA3/IMUP-2 and siRNA/IMUP-2 when compared to the mock. Also, the expression of p21 generally decreased in both pcDNA3/IMUP-2 and siRNA/IMUP-2 comparing to the mock, but siRNA/IMUP-2 transfected cells decreased more than the pcDNA3/IMUP-2 transfected cells (Fig. 3C). The results suggest that the increased expression of IMUP-2 can induce apoptosis. But, the decreased expression of IMUP-2 is associated

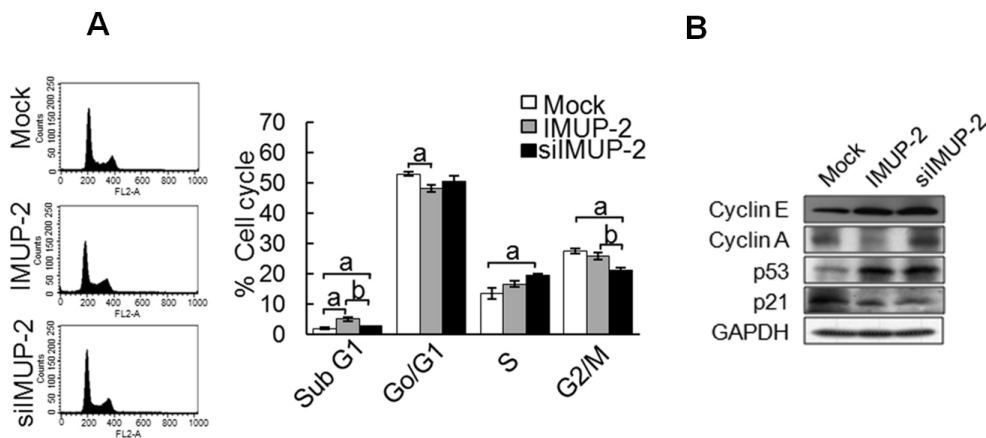


Fig. 2. Cell cycle analysis in HTR-8/SVneo trophoblast depend on IMUP-2 expression. (A) FACS analysis on cell cycle in HTR-8/SVneo trophoblast after transfection of pcDNA3/IMUP-2 and siRNA/IMUP-2. (B) Western blot analysis of cell cycle-related genes in HTR-8/SVneo trophoblast after transfection of pcDNA3/IMUP-2 and siRNA/IMUP-2. GAPDH was used as a loading control (Mock: lipofectamin transfection; IMUP-2: pcDNA3/IMUP-2 transfection; siIMUP-2: siRNA/IMUP-2 transfection).

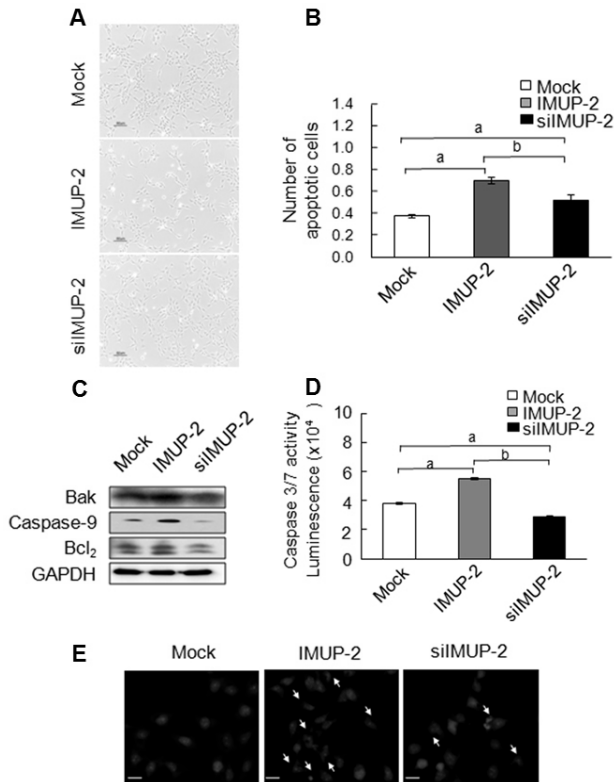


Fig. 3. Effect of IMUP-2 on apoptosis in HTR-8/SVneo trophoblast depend on IMUP-2 expression. (A) Morphologies of HTR-8/SVneo trophoblast depend on IMUP-2 expression. ($\times 400$). scale bar, 80 μm . (B) The number of apoptotic cells in HTR-8/SVneo trophoblast depend on IMUP-2 expression. Statistical analysis was carried out by Student's *t*-tests ($*P < 0.05$). (C) Western blot analysis of apoptosis-related genes in HTR-8/SVneo trophoblast depend on IMUP-2 expression. GAPDH was used to internal control. (D) Caspase3/7 activity in HTR-8/SVneo trophoblast cells depend on IMUP-2 expression. (E) Apoptosis analysis in HTR-8/SVneo trophoblast using PI staining after transfection of pcDNA3/IMUP-2 and siRNA/IMUP-2 ($\times 400$). Arrow means nuclear abnormalities in HTR-8/SVneo trophoblast after transfection of pcDNA3/IMUP-2 and siRNA/IMUP-2 (Mock: lipofectamin transfection; IMUP-2: pcDNA3/IMUP-2 transfection; siIMUP-2: siRNA/IMUP-2 transfection).

with cellular proliferation although the expression of IMUP-2 seems to be involved in cell death.

3. Apoptosis analysis according to the IMUP-2 expression

From the MTT analysis on cellular proliferation de-

pending on the IMUP-2 expression in trophoblasts, we observed that the cell numbers of pcDNA3/IMUP-2 transfection were more significantly decreased than those of siRNA/IMUP-2 (Fig. 3A, Fig. 3B, $p < 0.05$). Also, we analyzed other apoptosis-related factors to explain the reason for cell number decrease. The representative pro-apoptotic markers such as Bak and caspase-9 were increased in pcDNA3/IMUP-2 transfected trophoblasts, but the expression of Bcl₂ as an anti-apoptosis marker did not show a significant difference. However, the expressions of Bak, caspase 9 and Bcl₂ were decreased in siRNA/IMUP-2 transfected trophoblasts (Fig. 3C). Caspase 3/7 activities showed the highest activities in the pcDNA3/IMUP-2 transfected trophoblasts ($p < 0.05$), but the least activities in the siRNA/IMUP-2 transfected trophoblasts (Fig. 3D). Additionally, to analyze the morphological characteristics on trophoblastic apoptosis, propidium iodide (PI) staining was used. The results show that excessive expression of IMUP-2 induces nuclear condensation, abnormal phenotypes of the nucleus, and a number of nuclear fragmentations, but relatively decreased expression of IMUP-2 reduces morphological changes in the nucleus (Fig. 3E). These results mean that excessive expression of IMUP-2 induces apoptosis of trophoblast through increased caspase 3/7 activities and abnormal morphological changes comparing to those of down-regulated IMUP-2.

4. Autophagy analysis according to the IMUP-2 expression

Autophagy, another different form of programmed cell death, was analyzed to determine its function on the trophoblasts depending on the IMUP-2 expression. The expression of mTOR phosphorylated protein as an anti-autophagy marker was increased in pcDNA/IMUP-2 transfected trophoblasts comparing to the mock, otherwise, they was significantly decreased in siRNA/IMUP-2 transfected trophoblasts. Furthermore, we observed that the expressions of PI3KIII, ATG 5-12c, ATG 5-12m, LC3 I, and LC3 II were increased in siRNA/IMUP-2 transfected trophoblasts (Fig. 4A). Therefore, to investigate whether

DISCUSSION

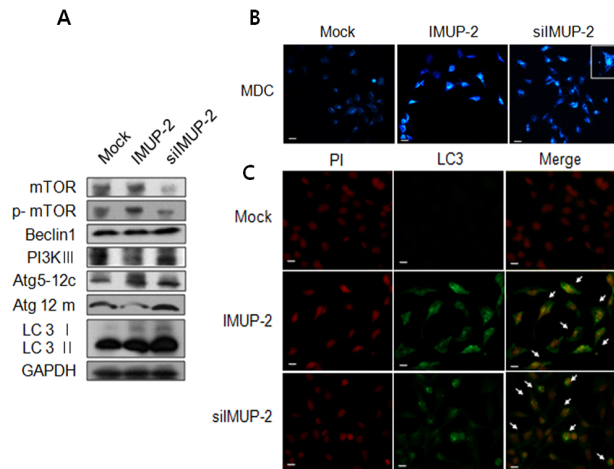


Fig. 4. Effect of IMUP-2 on autophagy in HTR-8/SVneo trophoblast depend on IMUP-2 expression. (A) Western blots analysis of autophagy-related genes in HTR-8/SVneo trophoblast after transfection of pcDNA3/IMUP-2 and siRNA/IMUP-2. GAPDH was used as a loading control. Representative with (B) Monodansylcadaverine (MDC) staining to detect autophagosomes in HTR-8/SVneo trophoblast after transfection of pcDNA3/IMUP-2 and siRNA/IMUP-2. (C) Cellular LC3, which is the final molecule induced during autophagy, was detected by immunofluorescence in HTR-8/SVneo trophoblast after transfection of pcDNA3/IMUP-2 and siRNA/IMUP-2. PI was used for nuclear staining. scale bar, 80 μ m. (Mock: lipofectamin transfection; IMUP-2: pcDNA3/IMUP-2 transfection; siIMUP-2: siRNA/IMUP-2 transfection).

autophagy is activated by alternative expression of IMUP_2 in trophoblast, we performed an immunofluorescence assay evaluating LC3, which is an early marker for autophagosomes. Additionally, we employed MDC, which is a fluorescent compound that becomes incorporated into the autophagosomal vacuoles. As shown in Fig. 4B, immunofluorescence also revealed the presence of many cup-shaped and ring-like structures in both types, which represent autophagosomes. We confirmed that the formation of autophagosomes with the MDC incorporation assay in siRNA/IMUP-2 transfected trophoblasts more increased than pcDNA/IMUP-2 transfected trophoblasts (Fig. 4B). Also, the expression of LC3 II was more increased in siRNA/IMUP-2 transfected trophoblasts than cDNA/IMUP-2 transfected trophoblasts.

The placental development at the early stage of pregnancy is carried out through balance between the cellular proliferation, differentiation, and adequate cell death of trophoblast (Smith et al., 1997b). During hypoxia condition (\sim 20 mmHg) at the time, trophoblast differentiates into cell layers resulting induce cellular proliferation and oxygen supply as well as apoptosis of the trophoblast (Ray et al., 2010). During placental development, many factors induce cell death were found (Ray et al., 2009). A recent study showed that anti-apoptotic factors in Bcl₂ groups slow cell cycle, but pro-apoptotic factors in Bcl₂ groups facilitate the cell cycle (Ray et al., 2010). Especially, Bak was reported to involve the cellular proliferation by controlling the cell cycle, but it turned out to be more related to cell death than to the cell cycle (De Falco et al., 2001). The single layer of mononuclear villous cytotrophoblast fuses between cells to differentiated into syncytiotrophoblast, which does not maintain cell proliferation (Hughes et al., 2004). Therefore, excessive apoptosis in syncytiotrophoblast causes negative effects such as decrease in adequate number of trophoblasts during pregnancy, degradation in functioning leading to damage on fetal growth with placental dysfunction and cause gynecological diseases such as preeclampsia or intra-uterine growth restriction (IUGR) (Smith et al., 1997a; Tomas et al., 2011). Therefore, a delicate balance between proliferation and apoptosis of trophoblasts plays a significant role in maintaining a healthy placenta and fetus.

The expression of IMUP-2 correlates with cellular proliferation and oncogenesis in various cancer cell lines. Its overexpression in NIH/3T3 cells acts as an important factor to activate the cellular proliferation, increasing levels of S and G2/M phase cells and inducing p53 pathway-related mutations, which involves DNA formation, cell cycle control, and MAK kinase cascade (Ryoo et al., 2006). However, increased IMUP-2 by hypoxia induced apoptosis in trophoblasts of placenta. In previous reports, we demonstrated that IMUP-2 mRNA was expressed in

syncytiotrophoblasts, syncytial knots, and decidual cells of the placental villi, however, IMUP-2 mRNA expression was strong in syncytiotrophoblasts and syncytial knots within pre-eclamptic placentas, which induced apoptosis of the trophoblast (Jeon et al., 2010). Syncytial fusion by caspase 8, 9, and 10 activities in cytotrophoblasts and the formation of syncytial knots in syncytiotrophoblasts has been reported to induce apoptosis of trophoblasts (Huppertz et al., 1998; Huppertz et al., 1999). So, we analyzed cellular proliferation in trophoblasts and programmed cell death including apoptosis and autophagy to detect the fluctuation in IMUP-2 expression under the normal oxygen tension because there has been a lot of controversy on the roles of IMUP-2 in the balance between proliferation and cell death.

From the FACS analysis, we confirmed that the G₂/M phase was decreased in both pcDNA3/IMUP-2 transfected trophoblastic cells and siRNA/IMUP-2 treated cells ($p < 0.05$), but, the S phase was only increased in siRNA/IMUP-2 treated cells ($p < 0.05$). In addition, cyclin E of cell cycle forms a complex with cdk2, involved with the process from G₁ phase to S phase. This complex is inhibited by p21^{CIP}, p27^{KIP}, p57^{KIP2} in CIP/KIP group among CDK regulators. Also, p53-independent p21 function was reported to be involved in cellular proliferation and normal development in organelles (Tamura et al., 2007). In this study, the similar results were observed showing the increased expression in p53 and p21 in pcDNA3/IMUP-2 cells and the increase of p53 and decrease of p21 can facilitate the cellular proliferation in siRNA/IMUP-2 cells.

Excessive expression of IMUP-2 in trophoblasts increased the activities of caspase 3, 7, and 9, which indicates apoptosis in trophoblasts occurs through two pathways of initiator stages and execution stages. Anti-apoptotic Bcl-2 protein in a placenta at the early stage of pregnancy appears in cytotrophoblasts inhibits autophagy, thereby functioning as a cell proliferators (LeBrun et al., 1993). Also, our data showed that cell death occurred in both pcDNA3/IMUP-2 cells and siRNA/IMUP-2 cells, but the difference lies in that apoptosis by the activities of caspase

3, 7, and 9 were proactive with increased IMUP-2, but on the contrary, decreased IMUP-2 degraded the activities of Bak and Bcl₂ meaning cell death by the activities of caspase 3, 7 and 9 can be controllable.

Autophagy is a mechanism that involves cell growth, cellular proliferation, and tumorigenesis through phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) /mTOR pathway (Nicholson & Anderson, 2002). Mammalian target of rapamycin (mTOR), in particular, inhibits autophagosome formation by initiating kinase activity and dysfunctioning sub-cellular targets working essentially in the early stage of trophoblasts (Roos et al., 2009). In this study, it was predicted that excessive expression of IMUP-2 would induce mTOR increase and Class III PI3K decrease leading to inhibit autophagosome formation, but MDC expression and LC3 also turned out to induce autophagosome formation. However, decreased IMUP-2 expression decreases mTOR significantly and increases the expressions of Beclin, Class III PI3K, LC3 II, and Atg5-12, and facilitates autophagosome formation in MDC and LC3 II staining analysis.

In conclusion, the alteration of IMUP-2 in trophoblasts can control the balance between apoptosis and autophagy of trophoblast by acting as a apoptosis factor when the increased IMUP-2 expression in trophoblasts and also as a autophagy factor to facilitate cellular proliferation when decreased IMUP-2 expression reducing p21 but activating cycline A and E. Therefore, these results will help further studies on functional involvement of IMUP-2 in placental development and gynecological diseases.

ACKNOWLEDGMENTS

We would like to thank Dr. Charles H. Graham (Queen's University, Canada) who provided HTR-8/SVneo trophoblastic cells. This research was supported by the Academic Research Funding Project (2012R1A1A3009776) at the Department of Education and Science Technology in the National Research Foundation of Korea.

REFERENCES

- Bildirici I, Longtine MS, Chen B, Nelson DM (2012) Survival by self-destruction: a role for autophagy in the placenta? *Placenta* 33:591-598.
- De Falco M, De Luca L, Acanfora F, Cavallotti I, Cottone G, Laforgia V, De Luca B, Baldi A, De Luca A (2001) Alteration of the Bcl-2:Bax ratio in the placenta as pregnancy proceeds. *Histochem J* 33:421-425.
- Hughes M, Dobric N, Scott IC, Su L, Starovic M, St-Pierre B, Egan SE, Kingdom JC, Cross JC (2004) The Hand1, Stra13 and Gcm1 transcription factors override FGF signaling to promote terminal differentiation of trophoblast stem cells. *Dev Biol* 271:26-37.
- Huppertz B (2008) The anatomy of the normal placenta. *J Clin Pathol* 61:1296-1302.
- Huppertz B, Frank HG, Kingdom JC, Reister F, Kaufmann P (1998) Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. *Histochem Cell Biol* 110:495-508.
- Huppertz B, Frank HG, Reister F, Kingdom J, Korr H, Kaufmann P (1999) Apoptosis cascade progresses during turnover of human trophoblast: analysis of villous cytotrophoblast and syncytial fragments *in vitro*. *Lab Invest* 79:1687-1702.
- Huppertz B, Kadyrov M, Kingdom JC (2006) Apoptosis and its role in the trophoblast. *Am J Obstet Gynecol* 195:29-39.
- Jeon SY, Lee HJ, Park JM, Jung HM, Yoo JK, Lee HJ, Lee JS, Cha DH, Kim JK, Kim GJ (2010) Increased immortalization-upregulated protein 2 (IMUP-2) by hypoxia induces apoptosis of the trophoblast and pre-eclampsia. *J Cell Biochem* 110:522-530.
- Kim JK, An HJ, Kim NK, Ahn JY, Kim KS, Kang YJ, Ko JJ, Oh D, Lee C, Kim SJ, Cha KY (2003) IMUP-1 and IMUP-2 genes are up-regulated in human ovarian epithelial tumors. *Anticancer Res* 23:4709-4713.
- Kim JK, Ryll R, Ishizuka Y, Kato S (2000) Identification of cDNAs encoding two novel nuclear proteins, IMUP-1 and IMUP-2, upregulated in SV40-immortalized human fibroblasts. *Gene* 257:327-334.
- Kim SH, Banga S, Jha KK, Ozer HL (1998) SV40-mediated transformation and immortalization of human cells. *Dev Biol Stand* 94:297-302.
- Kim SJ, An HJ, Kim HJ, Jungs HM, Lee S, Ko JJ, Kim IH, Sakuragi N, Kim JK (2008) Imup-1 and imup-2 overexpression in endometrial carcinoma in Korean and Japanese populations. *Anticancer Res* 28:865-871.
- LeBrun DP, Warnke RA, Cleary ML (1993) Expression of bcl-2 in fetal tissues suggests a role in morphogenesis. *Am J Pathol* 142:743-753.
- Lockshin RA, Zakeri Z (2004) Apoptosis, autophagy, and more. *Int J Biochem Cell Biol* 36(12):2405-2419.
- Na KH, Lee HJ, Jung J, Kim GJ (2010) Expression of norepinephrine transporter in pre-eclamptic placenta. *Dev Reprod* 14:65-74.
- Nicholson KM, Anderson NG (2002) The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* 14:381-395.
- Oh SY, Choi SJ, Kim KH, Cho EY, Kim JH, Roh CR (2008) Autophagy-related proteins, LC3 and Beclin-1, in placentas from pregnancies complicated by pre-eclampsia. *Reprod Sci* 15:912-920.
- Ozer HL (2000) SV40-mediated immortalization. *Prog Mol Subcell Biol* 24:121-153.
- Ray J, Jurisicova A, Caniggia I (2009) IFPA Trophoblast Research Award Lecture: the dynamic role of Bcl-2 family members in trophoblast cell fate. *Placenta* 30 Suppl A:S96-100.
- Ray JE, Garcia J, Jurisicova A, Caniggia I (2010) Mtd/Bok takes a swing: proapoptotic Mtd/Bok regulates trophoblast cell proliferation during human placental development and in preeclampsia. *Cell Death Differ* 17:846-859.
- Roos S, Lagerlof O, Wennergren M, Powell TL, Jansson T (2009) Regulation of amino acid transporters by glucose and growth factors in cultured primary human trophoblast cells is mediated by mTOR signaling. *Am J Physiol Cell Physiol* 297:C723-731.
- Ryoo ZY, Jung BK, Lee SR, Kim MO, Kim SH, Kim HJ, Ahn JY, Lee TH, Cho YH, Park JH, Kim JK

- (2006) Neoplastic transformation and tumorigenesis associated with overexpression of IMUP-1 and IMUP-2 genes in cultured NIH/3T3 mouse fibroblasts. *Biochem Biophys Res Commun* 349:995-1002.
- Scifres CM, Nelson DM (2009) Intrauterine growth restriction, human placental development and trophoblast cell death. *J Physiol* 587:3453-3458.
- Singh R (2011) Hypothalamic lipophagy and energetic balance. *Aging (Albany NY)* 3:934-942.
- Smith SC, Baker PN, Symonds EM (1997a) Increased placental apoptosis in intrauterine growth restriction. *Am J Obstet Gynecol* 177:1395-1401.
- Smith SC, Baker PN, Symonds EM (1997b) Placental apoptosis in normal human pregnancy. *Am J Obstet Gynecol* 177:57-65.
- Tamura M, Sawabata N, Kobayashi S, Umezu H, Seki N, Yoshii N, Karube Y, Araki O, Ishihama H, Nagai S, Miyoshi S (2007) Prognostic significance of p21 protein expression in patients with pulmonary squamous cell carcinoma following induction chemotherapy. *Ann Thorac Cardiovasc Surg* 13:9-14.
- Tomas SZ, Prusac IK, Roje D, Tadin I (2011) Trophoblast apoptosis in placentas from pregnancies complicated by preeclampsia. *Gynecol Obstet Invest* 71:250-255.