Comparative analysis of human umbilical cord blood derived mesenchymal stem cells between preeclampsia and normal pregnant women

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Comparative analysis of human umbilical cord blood derived mesenchymal stem cells between preeclampsia and normal pregnant women

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Han-Sung Hwang

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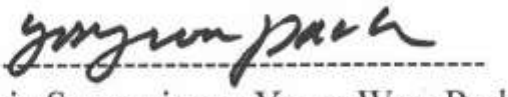
This certifies that the Doctoral Dissertation of Han-Sung Hwang is approved.



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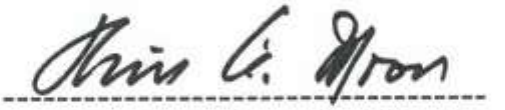

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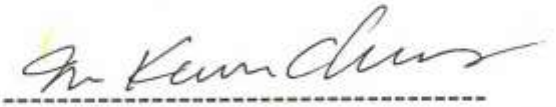

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First of all, I have to admit that this dissertation represents much more than the culmination of many years of research. This thesis includes the great knowledge and inspiration of many people that I have met since I started the division of maternal fetal medicine, the department of Obstetrics and Gynecology.

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Han Sung Hwang

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Comparative analysis of human umbilical cord blood derived mesenchymal stem cells between preeclampsia and normal pregnant women

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Preeclampsia is a syndrome characterized by deterioration of either the maternal condition or the fetal condition. The adverse intrauterine environment made by preeclampsia results into intrauterine growth restriction, and increased risk of a variety of diseases in future life. Given the adverse environment of fetal circulation made in the preeclamptic condition, and the role of mesenchymal stem cell (MSC) as a multipotent progenitor cell, it is reasonable to hypothesize that MSCs derived from human umbilical cord blood (hUCB-MSCs) obtained from preeclampsia may be perturbed compared with normal pregnancy. The aim of this study was to analyze the biological characteristics, and compare the functional abilities and gene expression patterns of hUCB-MSCs originating from pregnant women with and without preeclampsia. hUCB-MSCs were isolated, and cultured from 28 pregnant women with severe preeclampsia and 30 normal pregnant women.

Characterization analysis of hUCB-MSCs including flow cytometry, semi quantitative real time quantitative polymerase chain reaction (RT-qPCR), immunofluorescence staining, and in vitro differentiation studies, comparative analysis including proliferation assay, senescence-associated β-galactosidase (SA-β-gal) assay, telomerase activity assay, and reactive oxygen species (ROS) activity assay, and comparison of gene expression including gene expression pattern using microarray, hierarchical cluster analysis of differentially expressed genes, gene ontology classification, and pathway network analysis between two groups were performed. hUCB-MSCs obtained from women with preeclampsia were less proliferative, more senescent, and had lower telomerase activity and higher ROS activity than cells from women with normal pregnancy. Many senescence-related DEGs were identified by analysis of gene expression profiles, and significantly associated with the gene ontology term cell aging. In conclusion, hUCB-MSCs obtained from women with preeclampsia are functionally defective compared with cells from women with normal pregnancy.

Key words : Human umbilical cord blood, Mesenchymal stem cell, Preeclampsia, Senescence, Microarray, Network analysis

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I. INTRODUCTION

The discovery of mesenchymal stem cells (MSCs) by Fridenstein et al. in 1976 suggested a potentially useful model for gene therapy, regenerative medicine, and better and more advanced treatment strategies for various diseases, even those that seem to be incurable[1]. An increasing number of reports indicate that MSCs have extensive proliferative potential and the ability to differentiate into various cell types, including osteoblastic, adipogenic, chondrogenic, myogenic, and neurogenic cells[2-5]. Because of these properties, numerous laboratories are studying the clinical safety and efficacy of MSCs for the treatment of a number of pathological conditions, such as heart failure[6], spinal cord injury[7], and bone and cartilage diseases[8]. Whereas bone marrow was the first main source of MSCs, recent studies have suggested that MSCs can be obtained from many other tissues of the human body, such as fat[9], umbilical

cord blood, chorionic villi of the placenta[10], amniotic fluid[11], peripheral blood[12], lung[13], skeletal muscle[14], synovial membrane[15], hepatic tissue[16], and even exfoliated deciduous teeth[17]. In particular, recent studies showed that MSCs derived from human umbilical cord blood (hUCB-MSCs) could be isolated more efficiently and are more primitive than MSCs derived from adult tissues[18]. For hematopoietic stem cells of umbilical cord blood, the various senescent stages and their regulatory pathways are well known[19-21]. In contrast, the mechanisms of senescence and functional impairment of MSCs remain unknown, although several recent studies have shown that MSCs isolated from older donors are more senescent than those isolated from younger donors[22,23], and that MSCs have a replicative senescence pathway involving intracellular superoxide accumulation[24,25].

Preeclampsia is a complication found in 3% of pregnancies and a major cause of maternal and perinatal morbidity and mortality. Preeclampsia is a syndrome characterized by deterioration of either the maternal condition (hypertension and proteinuria with or without multiorgan abnormalities) or the fetal condition (intrauterine growth restriction, decreased amniotic fluid)[26,27]. Intrauterine growth restriction is a major fetal complication of preeclampsia. Although reduced placental blood flow[28,29], and increased sensitivity of the human placental vasculature to vasoconstrictors have been suggested as possible causes[30], the pathophysiology of intrauterine growth restriction in preeclampsia is still unclear. Moreover, children born at term to mothers with preeclampsia

have an increased risk of a variety of diseases, such as endocrine, nutritional, and metabolic diseases, as well as diseases of the blood and blood-forming organs[31]. These findings in the preeclamptic condition may originate through adaptations of the fetus to an adverse intrauterine environment. Previous studies have given explanations for this adverse condition comparison of umbilical cord blood with and without preeclampsia. As compared with the normal pregnancy group, increased anti-angiogenic factors, reduced expression of pro-angiogenic signal, elevated oxidative stress, and increased inflammatory response have been founded in fetal serum during preeclampsia[32-34].

Given the adverse environment of fetal circulation made in the preeclamptic condition, and the role of MSC as a multipotent progenitor cell, it is reasonable to hypothesize that hUCB-MSCs obtained from preeclampsia may be perturbed compared with normal pregnancy. The aim of this study was to analyze the biological characteristics, and compare the functional abilities and gene expression patterns of hUCB-MSCs originating from pregnant women with and without preeclampsia.

II. MATERIALS AND METHODS

1. Study participants and sample collection

We studied the cord blood of pregnant women who visited Konkuk University Hospital, 30 of whom had no pregnancy complications (normal group), and 28 of whom had severe preeclampsia (preeclampsia group). Only women delivered by cesarean section without labor were enrolled in this study. Umbilical cord blood was obtained from each pregnant woman at the time of cesarean section. The indications of cesarean section for pregnant women without preeclampsia were previous cesarean section, previous myomectomy, breech presentation, or transverse lie. All subjects were enrolled in this study after signing an informed consent document approved by the institutional review board (IRB No: KUH1040005). Severe preeclampsia was defined as the presence of hypertension (systolic blood pressure ≥ 160 mmHg and/or diastolic pressure ≥ 110 mmHg) and proteinuria (≥ 3+ on dipstick test or 5g per 24 hours) beyond the 20^{th} week of pregnancy[35]. At least two consecutive measurements were required for diagnosis. Subjects were excluded from this study if they had known fetal or maternal complications, such as multiple gestation, fetal structural or genetic problems, maternal chronic hypertension, cardiovascular disease, renal disease, hepatic disease, diabetes mellitus, infectious disease, connective tissue disease, and autoimmune disease.

2. Isolation and cultivation of hUCB-MSCs

Umbilical cord blood samples (about 50 mL each) with anticoagulant were collected from umbilical cord vein attached to placenta by gravity flow after delivery. Mononuclear cells (MNCs) were isolated from cord blood samples by density gradient centrifugation over Biocoll (Biochrom, Berlin, Germany) for 30 minutes at 400×*g*, washed three times in phosphate-buffered saline (PBS) (Biochrom). Among the MSCs, CD133/c-kit-positive cells were selected to differentiate into MSCs. CD133/C-kit-positive cells were enriched using the MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, MNC were washed and resuspended in PBS buffer. Cells were incubated with anti-CD133/C-kit microbeads in the presence of human IgG as blocking reagent at 4℃ for 30 min. Labeled cells were loaded onto a column installed in a magnetic field. The column was rinsed with PBS buffer and negative cells passed through. Trapped cells were eluted after the removal of column from the magnet. Isolated CD133/C-kit positive cells were seeded 1×10^6 cells on 6-well plates, coated with human fibronectin (Sigma-Aldrich Chemie, Munich, Germany) in endothelial basal medium-2 (EBM-2) (Clonetics, Cell Systems, St Katharinen, Germany). The medium was supplemented with endothelial growth medium-2 (EGM-2; Clonetics, Cell Systems) containing fetal bovine serum, human VEGF-A, human fibroblast growth factor-B, human epidermal growth factor, insulin-like growth factor 1 (IGF1), and ascorbic acid in appropriate amounts. After 3 days, non-adherent

cells were removed and fresh culture medium was added. Cultures were maintained with EGM-2 supplement. Phenotypical analysis of the cells was performed on days 7, 13 and 15.

3. Characterization of hUCB-MSCs

A. Flow cytometric analysis

The primary cultured cells were treated with 0.25% trypsin, washed once with PBS, and collected. The following fluorescently labeled antibodies were used for flow cytometric characterization of hUCB-MSCs: anti-CD29 (Molecular Probes, Eugene, OR), anti-CD73 (BD Pharmingen, San Diego, CA), and anti-CD90 (Abcam, Cambridge, MA). Detached cells were washed twice with Dulbecco's PBS, centrifuged, washed in ice-cold Dulbecco's PBS supplemented with 1% bovine serum albumin (FCM buffer), and fixed in 2% paraformaldehyde in FCM buffer. Cells were then incubated with antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (Pharmingen, BD Biosciences Europe, Heidelberg, Germany) for 15 minutes on ice in a dark room at concentrations recommended by the manufacturer. We used anti-IgG-FITC (33814X; Pharmingen, BD Biosciences Europe) and anti-IgG-PE (33815X; Pharmingen, BD Biosciences Europe) as isotypic controls. After being washed, cells were analyzed on COULTER EPICS XL-MCL flow cytometer (Beckman Coulter, Krefeld, Germany) by using EXPO-32 software. Instrument settings for scatter conditions and background fluorescence were adjusted for untreated cells.

B. RNA isolation and semi quantitative real time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cells with a TRIzol reagent kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, first-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA with SuperScript II reverse transcriptase, oligo(dT) primer, and 10 mM dNTP mixture (Invitrogen, Carlsbad, CA). The cDNA mixture (1 μL) was used for PCR. Amplification of the GAPDH gene was carried out in parallel for normalization. The PCR was performed in a DNA thermal cycler (model PTC-200; MJ Research, Scientific Support, Inc, Waltham, MA) under the following conditions: denaturation at 94℃ for 5 minutes for the first cycle and for 30 seconds thereafter, annealing at 60℃ for 30 seconds, and extension at 72℃ for 30 seconds for 40 cycles. All results were normalized to GAPDH mRNA. The primers used are given in table 1.

Table 1. Primer sequences specific to the target genes

Gene	Direction	Sequence
CD133	Sense	5’-CCCGCAGGAGTGAATCTTTT-3’
	Anti-sense	5’-AGGAAGGACTCGTTGCTGGT-3’
c-kit	Sense	5’-TCTCTTTAGGAAGCAGCCCC-3’
	Anti-sense	5’-ACATTTCAGCAGGTGCGTGT-3’
CD29	Sense	5’-GTAGCTGGTGTGGTTGCTGG-3’
	Anti-sense	5’-TGTCCCATTTGGCATTCATT-3’
CD44	Sense	5’-GGTGCATTTGGTGAACAAGG-3’
	Anti-sense	5’-CACCCCAATCTTCATGTCCA-3’
CD73	Sense	5’-TGGATGGCTCCTCTCAATCA-3’
	Anti-sense	5’-GCACATGGATACGTGGTTCC-3’
CD90	Sense	5’-TCTCCTCCCAGAACGTCACA-3’
	Anti-sense	5’-GAGAGGGAGAGCAGGAGCAG-3’
CD105	Sense	5’-GAGGCGGTGGTCAATATCCT-3’
	Anti-sense	5’-GTAGAGGCCCAGCTGGAAAG-3’
GAPDH	Sense	5’-ATGGGGAAGGTGAAGGTCG-3’
	Anti-sense	5’-GGGGTCATTGATGGCAACAATA-3’

C. Immunofluorescence staining

Cells were put on glass coverslips at the bottom of wells in 24-well culture plates, fixed with 4% formaldehyde solution, and permeabilized with 0.3% Triton X-100. They were incubated with 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity and then washed twice in PBS for 5 minutes. Cells were incubated overnight at 4 ℃ with the following primary antibodies: mouse anti-human monoclonal antibody against α-SMC (Sigma-Aldrich, St. Louis, MO), 1:200; mouse anti-human monoclonal antibody against CD90 (Abcam, Cambridge, MA), 1:100; and mouse anti-human monoclonal antibody against CD73 (BD Pharmingen, San Diego, CA), 1:500. Cells were washed three times with PBS containing Triton X-100 and mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). After being washed twice for 15 minutes in PBS, slides were incubated (30 minutes at 37℃) with secondary antibody (anti-goat IgG antibody conjugated to Alexa 488 or 555; Molecular Probes), and then with streptavidin-conjugated horseradish peroxidase. Slides were developed with 3,3′-diaminobenzidine tetrahydro chloride.

4. In vitro differentiation studies of hUCB-MSCs

A. Osteogenic differentiation

To induce osteogenic differentiation, we plated cells in a 24-well plate at 5×10^3 cells/cm^2. At 70% confluency, cells were treated with osteogenic induction medium (low glucose Dulbecco's modified Eagle's medium, 10% FBS, 10 mm β-glycerophosphate, 10 nm dexamethasone, 50 μm ascorbate, and antibiotics). The medium was changed every 3 - 4 days, and cell morphology was assessed visually every day for up to 3 weeks. At the end of differentiation, cells were stained for alkaline phosphatase and with Von Kossa stain[36].

B. Adipogenic differentiation

To induce adipogenic differentiation, we treated cells with adipogenic medium (1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 μg/mL recombinant human insulin, 0.2 mM indomethacin, and 10% fetal calf serum) for 3 weeks. Maintenance medium including only recombinant human insulin and 10% fetal calf serum was replaced twice weekly, and adipogenesis was assessed at weekly intervals. Control cells were kept in adipogenic maintenance medium. Cells were fixed with 10% formalin, washed, and stained with 0.18% Oil Red O solution for 5 minutes. Adipogenic differentiation was confirmed by intracellular accumulation of lipid-rich vacuoles that stained with Oil Red O.

C. Chondrogenic differentiation

To induce chondrogenic differentiation, we cultured 3×10^5 cells/well in chondrogenic medium (high glucose Dulbecco's modified Eagle's medium, 1×insulin-transferrin-selenium pre-mix, 0.1 mM ascorbic acid 2-phosphate, 10 mM sodium pyruvate, 10 ng/mL transforming growth factor-β1, and 100 nM dexamethasone) for three weeks. Medium changes were carried out twice weekly and chondrogenesis was assessed at 2 ~ 3 day intervals. Cells were fixed in 4% formaldehyde, dehydrated in an ethanol series, and embedded in paraffin blocks. Blocks were cut and sections were stained for sulphated proteoglycans with Safranin-O (0.1% aqueous solution) (Sigma-Aldrich) to evaluate chondrogenic differentiation.

5. Comparative analysis of hUCB-MSCs between groups

A. Proliferation assay

To compare the doubling time of hUCB-MSCs between groups, cells were seeded in six T-25 flasks. On each of six consecutive days, the cells from one flask were obtained and enumerated. Mean counts were calculated. The mean population doubling time (PD) was calculated with the following formula: PD = $t \times \lg 2 / (\lg N_t - \lg N_0)$, where N_0 is the inoculum cell number, N_t is the number of harvested cells, and t is the duration of culture (in hours)[37].

B. Senescence-associated β-galactosidase assay

The senescence-associated β-galactosidase (SA-β-gal) assay was performed to distinguish senescent cells[38]. SA-β-gal activity of hUCB-MSCs at passage 3 was measured and compared between two groups. Briefly, hUCB-MSCs were washed in PBS, fixed for 3 minutes (at room temperature) in 2% paraformaldehyde, washed and incubated for 24 hours at 37℃ with fresh SA-β-gal staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40). hUCB-MSCs were counterstained with DAPI (0.2 μg/mL in 10 mM Tris-HCl, pH 7.0, 10 mM EDTA, and 100 mM NaCl) for 10 minutes. Distinctly stained cells were observed by phase contrast microscopy. The mean

staining intensity of SA-β-gal-positive cells was calculated from four randomly selected microscopic fields (×200 magnification) by densitometry.

C. Telomerase activity assay

To analyze the telomerase activity of hUCB-MSCs quantitatively, we conducted a telomeric repeat amplification protocol assay using a TeloTAGGG Telomerase PCR ELISA kit (Roche Molecular Biochemicals, Brussels, Belgium) according to the manufacturer's protocol. The telomerase activity of hUCB-MSCs at passage 3 was measured and compared between two groups. Briefly, 2×10^5 hUCB-MSCs were pelleted at 3000*g* for 10 minutes at 4℃, washed twice with cold PBS, incubated for 20 minutes at 4°C with 200 μL of precooled lysis buffer (solution 1 of the kit), and centrifuged at 16,000*g* for 20 minutes. Telomeric repeats were added to a biotinlabeled primer during the first reaction, and then the elongation products were amplified by PCR. Finally, the immobilized PCR product was detected with an anti-digoxigenin-peroxidase antibody and visualized as a colored reaction product with the substrate 3,3′,5,5′-tetramethyl benzidine. The absorbance was measured in triplicate at 450 nm, by reading against a blank (reference absorbance at 690 nm). Samples were regarded as telomerase-positive if the difference in absorbance (A_{450} - A_{690}) was greater than 0.2.

D. Reactive oxygen species (ROS) activity assay

Endogenous superoxide production was evaluated using the oxidative fluorescent dye dihydroethidium (DHE). ROS activity of hUCB-MSCs at passage 3 was measured and compared between two groups. Cells were plated on 12-well plates, washed with Krebs-HEPES buffer (pH 7.4), and stained with DHE for 15 minutes at 37℃ in an incubator. After fixation with paraformaldehyde, slides were coverslipped with mounting medium and photos were taken.

E. Densitometric analysis

SA-β-gal-positive cells and DHE-stained cells were visualized by densitometric scanning using a luminescent image analyzer (LAS-1000, Fuji Photo Film Co. Ltd, Tokyo, Japan) and digital analysis software (Image Reader LAS-1000 Lite, Fuji Photo Film Co. Ltd).

6. Microarray expression analysis of hUCB-MSCs

Five micrograms of total RNA from hUCB-MSCs was hybridized to the Human-GE 4 × 44K v2 Microarray (human whole genes; Agilent Technologies, Santa Clara, CA). Two types of hUCB-MSCs were analyzed and compared: N3 (cells at passage 3, normal pregnancy), and P3 (cells at passage 3, preeclampsia). The standard protocol used for sample preparation and microarray processing is available from Agilent Technologies. Expression data were analyzed using Agilent's GeneSpring GX software (Genomic tree Inc. Daejeon, Korea).

7. Pathway network analysis of differentially expressed genes (DEGs)

The functional interactions between differentially expressed genes (DEGs) were analyzed by GeneMANIA webserver[39]. The GO term was used to create the interaction network between the DEGs and additional genes by using human as a source species. DEGs were mapped to the GeneMANIA to investigate how these genes interact with each other and additional genes that are related to a set of query genes by using a very large set of functional interaction data. By integrating these relationships, a network between DEGs and additional related genes was constructed for intersection of DEG sets N3 vs. P3. To confirm the gene network created with DEGs, the GO term enrichment analysis was performed among the

genes in the network.

8. Statistical analysis

Reported data are mean ± standard deviation (SD). Patients' characteristics, cell population doubling time, densitometric values for SA-β-gal-positive cells, telomerase activity, and ROS activity were compared between groups by Mann-Whitney U test. Other variables, including cell number, were compared by Student *t*-test using SPSS, version 12.0 (SPSS, Chicago, IL). A p value less than 0.05 was considered statistically significant.

III. RESULTS

1. Clinical characteristics of participants in the normal and preeclampsia groups

The clinical characteristics of the patients who provided cord blood for the study are presented in Table 2. There was no significant difference in maternal age, or gestational age at delivery between the normal and the preeclampsia groups. Birth weight in the preeclampsia group was significantly lower than that in the normal group. Systolic and diastolic blood pressure were significantly higher in the preeclampsia group than in the normal group.

Table 2. Clinical characteristics of study participants

Variable	Normal group (n = 30)	Preeclampsia group (n = 28)
Maternal age (years)	31 ± 2.1	32 ± 2.5
Gestational age at delivery (weeks)	36 ± 1.6	35 ± 1.7
Birth weight (kg)	3.14 ± 0.31	2.81 ± 0.48**
Systolic blood pressure (mmHg)	115 ± 5	173 ± 12**
Diastolic blood pressure (mmHg)	69 ± 6	106 ± 9**
Proteinuria	none	28/28*

Data are mean ± SD. * ≥ 2+ on a urine dipstick test.

**Mann-Whitney *U* test, $p < 0.05$ (statistically significant)

2. Isolation, and cultivation of hUCB-MSCs

Primary cultured cells were obtained after 7 days of culture of MNCs obtained from umbilical cord blood. Among these cells, CD133/c-kit-positive cells were selected for differentiation into MSCs. CD133/c-kit-positive cells were differentiated into MSCs, as indicated by their characteristic shape (Fig. 1).

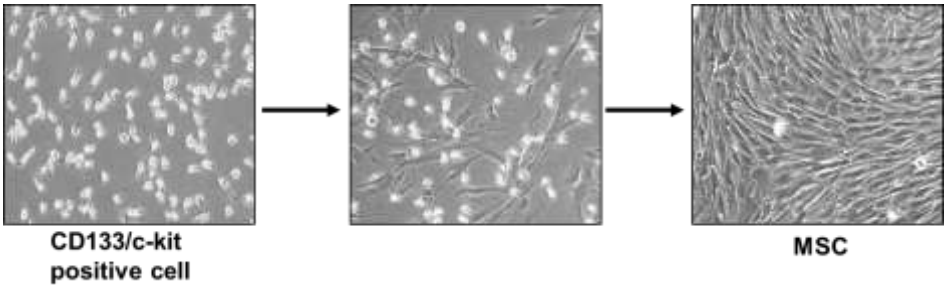

Figure 1. Differentiation of CD133/c-kit-positive cells from human umbilical cord blood into mesenchymal stem cells (MSCs).

3. Characterization of hUCB-MSCs

hUCB-MSCs obtained from 20 normal pregnancies were used for MSC characterization analysis including flow cytometry, semi quantitative RT-qPCR, immunofluorescence staining, and in vitro differentiation studies.

A. Flow cytometric analysis

Flow cytometric analysis of hUCB-MSCs was performed. Representative results for the hUCB-MSCs are shown in Figure 2. The cells were positive for the MSC markers CD29, CD73, and CD90.

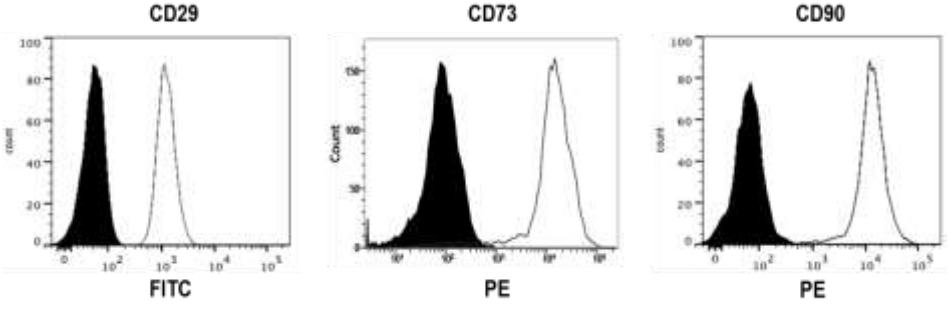

Figure 2. MSCs derived from human umbilical cord blood were positive for the MSC markers CD29, CD73, and CD90. FITC, fluorescein isothiocyanate; PE, phycoerythrin

B. Semi quantitative RT-qPCR for MSC markers

RT-qPCR confirmed that hUCB-MSCs expressed the MSC markers CD29, CD44, CD73, CD90, and CD105, but did not express CD133 and c-kit (Fig. 3). MNCs obtained from human umbilical cord blood had highly expression of CD133 and c-kit.

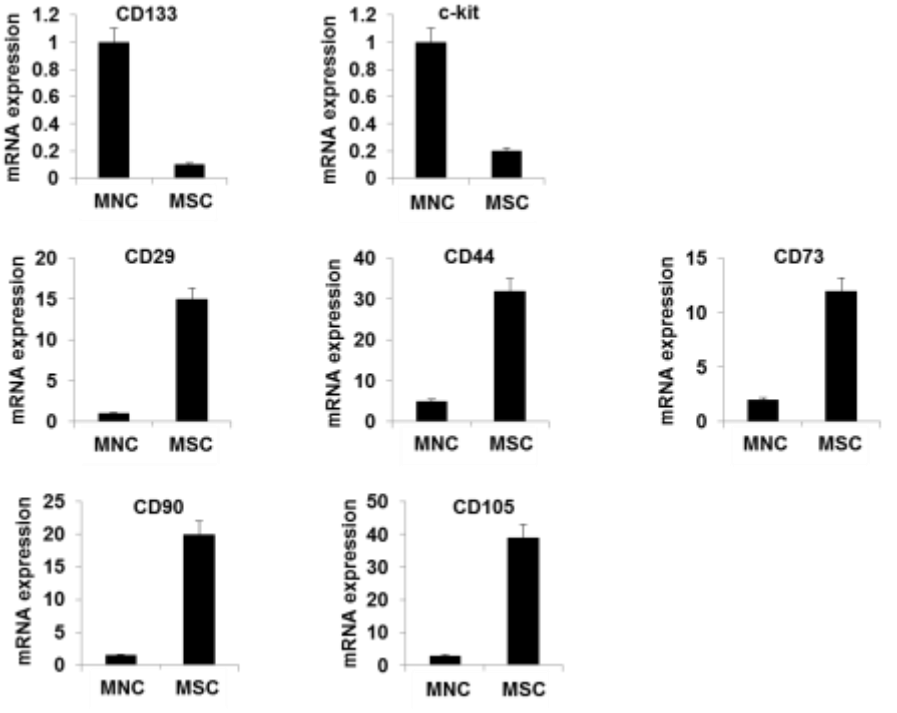

Figure 3. Characterization of CD marker profile of MCSs derived from human umbilical cord blood (hUCB-MSCs) by RT-qPCR. hUCB-MSCs were positive for the expression of CD29, CD44, CD73, CD90, and CD105, but negative for CD133, and c-kit.

C. Immunofluorescence staining for hUCB-MSC markers

hUCB-MSCs had similar levels of expression of the MSC markers α-SMA, CD90, and CD73 (Fig. 4).

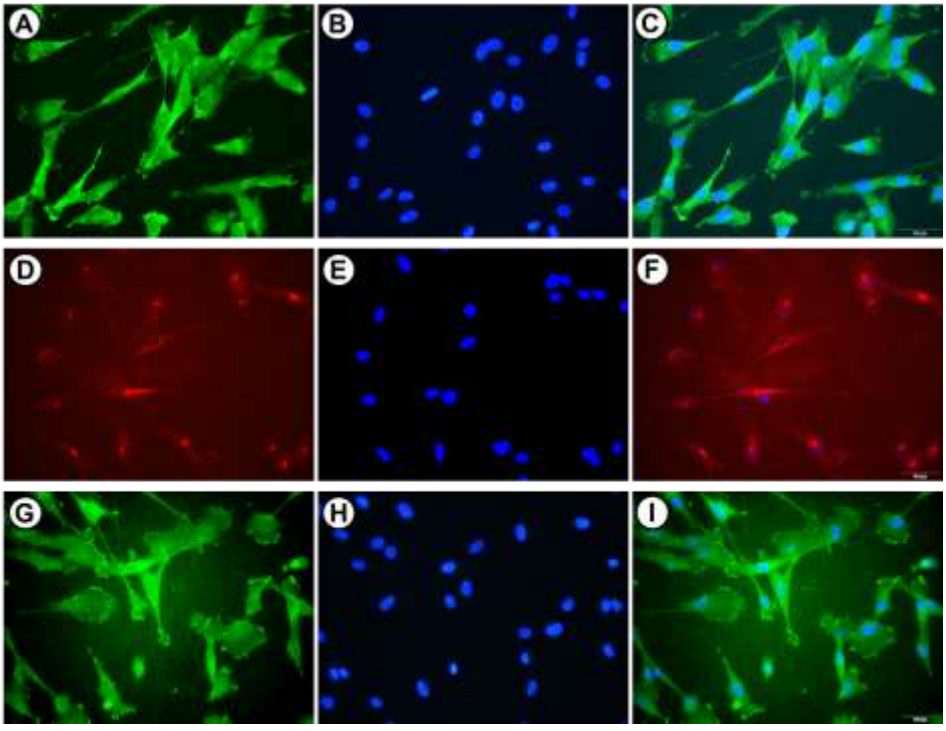

Figure 4. Cytoskeletal protein expression in hUCB-MSCs. hUCB-MSCs were stained for α-SMA (A,C), CD90 (D,F), and CD73 (G,I). Protein expression is indicated by green and red fluorescence, and nuclei are indicated by DAPI staining (blue; B, E, H).

4. In vitro differentiation studies of hUCB-MSCs

Osteogenic differentiation of hUCB-MSCs was confirmed by the detection of an osteogenic phenotype consisting of increased expression of alkaline phosphatase and by the deposition of a silver-stained mineralized matrix. Adipogenic differentiation of the cells was demonstrated by the accumulation of neutral lipid vacuoles stained by Oil Red O. Chondrogenic differentiation was confirmed by the formation of a sphere in the micromass culture and the secretion of cartilage specific proteoglycans stainable with Safranin O (Fig. 5).

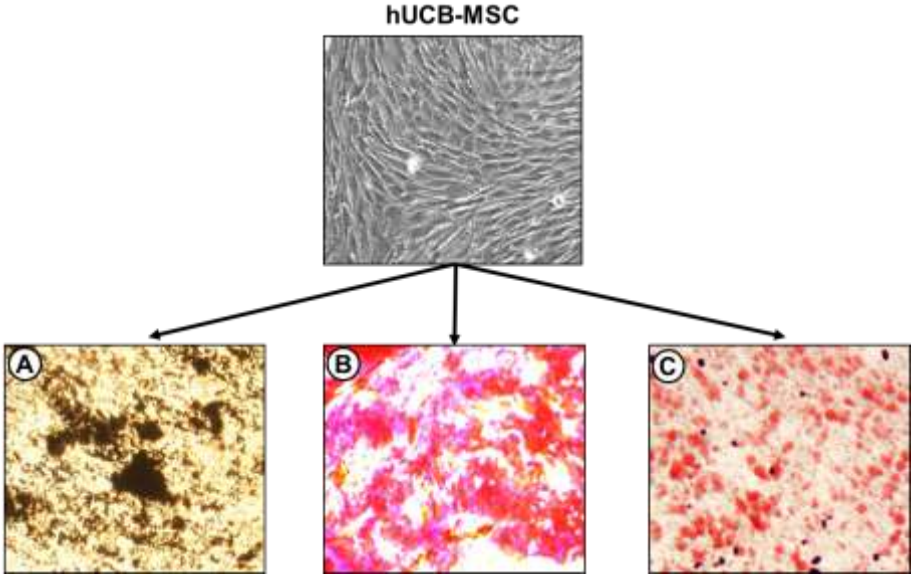

Figure 5. Multilineage differentiation capacity of hUCB-MSCs. The MSCs obtained from umbilical cord blood were investigated for their in vitro multilineage differentiation capacity (A, osteogenesis; B, chondrogenesis; C, adipogenesis).

5. Comparative analysis of hUCB-MSCs obtained from pregnant women with or without preeclampsia

hUCB-MSCs obtained from 10 normal pregnancies and 10 preeclampsia were used for comparative analysis including proliferation assay, SA-β-gal assay, telomerase activity assay, and ROS activity assay between two groups.

A. Decreased proliferative potential of hUCB-MSCs from preeclampsia

To compare the proliferative ability of hUCB-MSCs from women with normal and preeclampsia, a proliferation assay was performed. It was apparent that the proliferation of hUCB-MSCs from women with preeclampsia was significantly reduced in comparison with normal pregnancy (Fig. 6). The data demonstrate that hUCB-MSCs from women with preeclampsia have a much lower expansion potential than those from women with normal pregnancy.

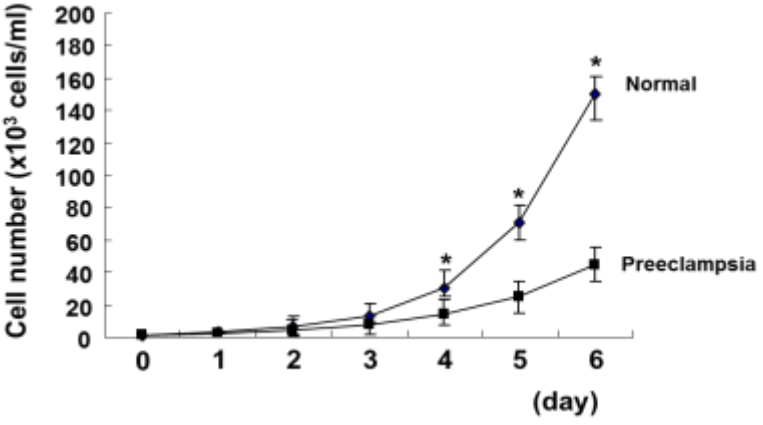

Figure 6. Proliferation assay of hUCB-MSCs from the two groups. After 6 days, the number of cell was significantly lower in cultures of hUCB-MSCs derived from women with preeclampsia than in cultures of hUCB-MSCs derived from women with normal pregnancy. Data are mean ± SD. *Student's *t*-test, $p < 0.01$.

B. Increased senescence of hUCB-MSCs from women with preeclampsia

A SA-β-gal assay of hUCB-MSCs obtained from women with normal pregnancy or preeclampsia was performed to assess the characteristics of cellular aging in vitro. The number of SA-β-gal-positive cells was significantly higher in hUCB-MSCs from women with preeclampsia (64.5%; range, 58.8-70.2%) than in those from women with normal pregnancy (39.8%; range, 35.0-44.6%; $p < 0.001$) (Fig. 7A). The mean staining intensity was significantly higher in the preeclampsia group than in the normal group (129.5 ± 12.3 % vs. 100.0 ± 11.1 %; $p < 0.001$) (Fig. 7B).

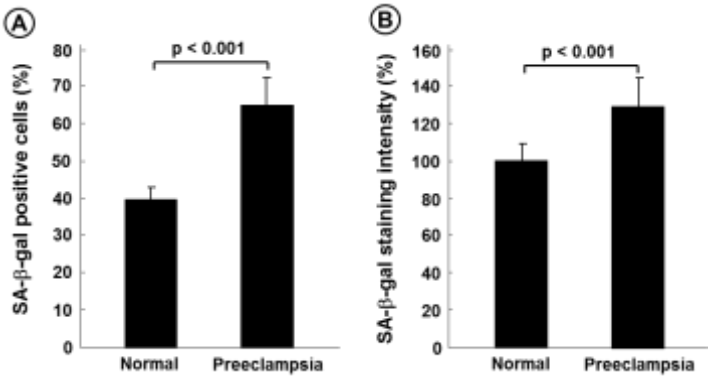

Figure 7. Increased senescence of hUCB-MSCs from women with preeclampsia. (A) The number of SA-β-gal-positive cells was counted from at least 200 cells. The percentage of cells that were clearly SA-β-gal-positive was significantly higher in the preeclampsia group (n=10) than in the normal group (n=10). (B) The staining intensity of SA-β-gal-positive cells was determined by densitometry. The relative staining intensity was significantly higher in the preeclampsia group. Values are mean ± SD. $p < 0.001$ by Mann-Whitney *U* test.

C. Decreased telomerase activity in hUCB-MSCs from women with preeclampsia

Preeclampsia-related alterations of telomerase activity in hUCB-MSCs were evaluated. Telomerase activity was lower by 40% in hUCB-MSCs from the preeclampsia group compared with those from the normal group (Fig. 8).

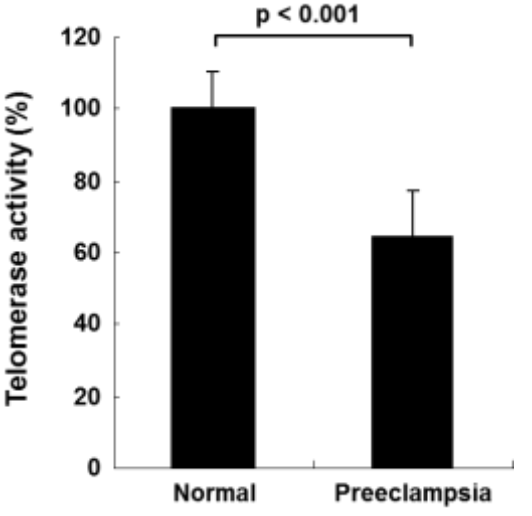

Figure 8. Quantitative analysis of preeclampsia-related alterations of telomerase activity. Mean telomerase activity of hUCB-MSCs was significantly lower in the preeclampsia group (n=10) than in the normal group (n=10). Values are mean ± SD. $p < 0.001$ by Mann-Whitney *U* test.

D. Increased ROS activity in hUCB-MSCs from women with preeclampsia

ROS can bring about cellular senescence, apoptosis, or carcinogenesis. ROS-induced cellular damage also contributes to stem cell aging[40]. As shown in Figure 9, ROS were significantly increased in hUCB-MSCs from women with preeclampsia.

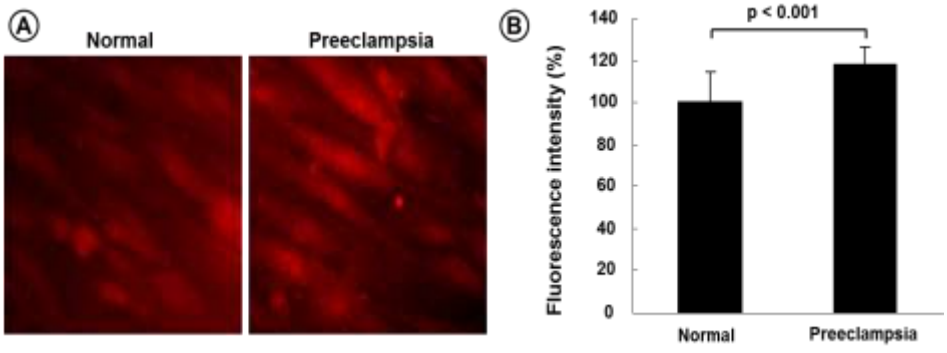

Figure 9. Increased reactive oxygen species production in hUCB-MSCs from women with preeclampsia. (A) Representative photomicrographs showing hUCB-MSCs stained for ROS, from women with normal pregnancy and preeclampsia. (B) Quantification of fluorescence intensity of hUCB-MSCs. The staining intensity was determined by densitometry (n=10 in each group). Relative staining intensity was significantly higher in the preeclampsia group. Values are mean ± SD. $p < 0.001$ by Mann-Whitney *U* test.

6. Comparisons of gene expression in hUCB-MSCs from the normal and preeclampsia groups using microarray analysis

hUCB-MSCs obtained from 2 normal pregnancies and 2 preeclampsia were used for comparison of gene expression including gene expression pattern using microarray, hierarchical cluster analysis of differentially expressed genes, gene ontology classification, and pathway network analysis between two groups.

A. Gene expression pattern of hUCB-MSCs from the two groups

After data processing, expression profiles were analyzed by scatter plot and MA (log ratio and mean) plot (Fig. 10). hUCB-MSCs at passage 3 from the normal group (N3) were compared with cells at passage 3 from the preeclampsia group (P3). In the plots, red spots represent genes with higher signal intensity in hUCB-MSCs from the preeclampsia group than in the hUCB-MSCs from the normal group. Green spots represent decreased signal intensity. The scatter plot and MA plot show the differentially expressed genes (DEGs) between N3 and P3.

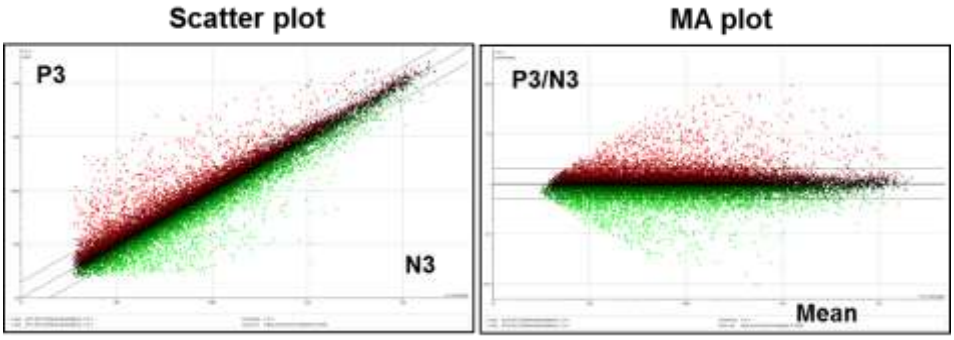

Figure 10. Microarray analysis of hUCB-MSCs from the normal and preeclampsia groups. The scatter plot and MA plot show the DEGs between N3 and P3 cells. In the scatter plot, the median line indicates no difference in signal intensity between the two groups. The upper gray line indicates a two-fold higher signal intensity, and the lower gray line indicates a two-fold lower signal intensity for hUCB-MSCs from the preeclampsia group as compared to the normal group. In the MA plot, the median line represents N signal/P signal = 1. The upper gray line represents N signal/P signal = 2, and the lower gray line represents N signal/P signal = 0.5. N3, hUCB-MSCs at passage 3, normal pregnancy; P3, hUCB-MSCs at passage 3, preeclampsia.

B. Screening of differentially expressed genes (DEGs)

Expression of genes was compared between hUCB-MSCs from the normal and preeclampsia groups, and genes were clustered by expression pattern (Fig. 11). In the comparison between N3 and P3 cells, twofold and fourfold differences in expression (either up- or down-regulation) were detected for 2684 up-regulated and 259 down-regulated genes, respectively.

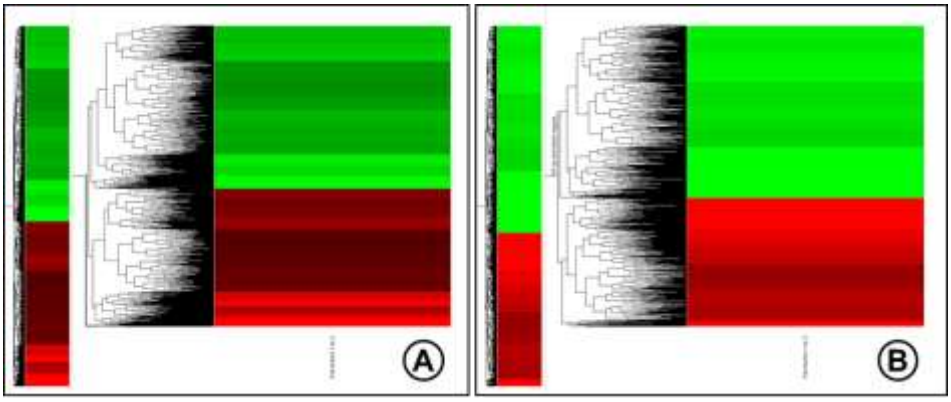

Figure 11. Differentially expressed genes (DEGs) of hUCB-MSCs from the normal and preeclampsia groups. Hierarchical cluster analysis of DEGs between N3 and P3 cells. Red represents up-regulated gene clusters, and green represents down-regulated gene clusters. The panels show genes up- or down-regulated twofold (A) or fourfold (B) in P3 (versus N3) cells. N3, hUCB-MSCs at passage 3, normal pregnancy; P3, early hUCB-MSCs at passage 3, preeclampsia.

C. Comparison of DEGs between N3 and P3 cells

Genes differentially expressed between N3 and P3 cells were compared to identify specific DEGs in each group and DEGs common to both groups (Fig. 12). Twofold up- and down-regulated genes were used in these comparisons. We identified 1227 up-regulated and 1457 down-regulated genes that were common between N3 vs. P3.

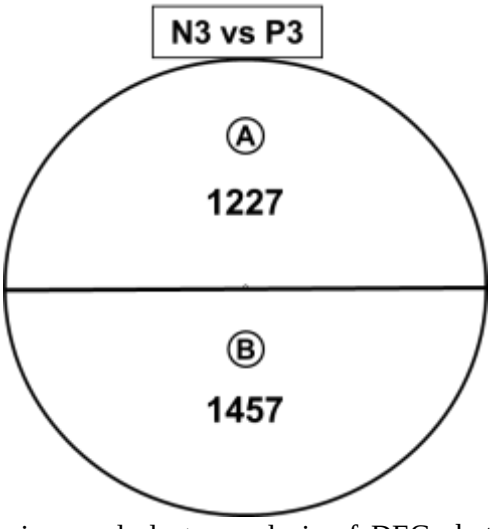

Figure 12. Comparison and cluster analysis of DEGs between N3 and P3. Twofold up-regulated genes (A) and twofold down-regulated genes (B) were identified between N3 vs. P3. N3, hUCB-MSCs at passage 3, normal pregnancy; P3, hUCB-MSCs at passage 3, preeclampsia.

D. Analysis of senescence related gene expression in N3 vs. P3

After the hierarchical clustering of senescence-related DEGs, we constructed a dendrogram to display the clusters (Fig. 13). Forty senescence-related DEGs were identified.

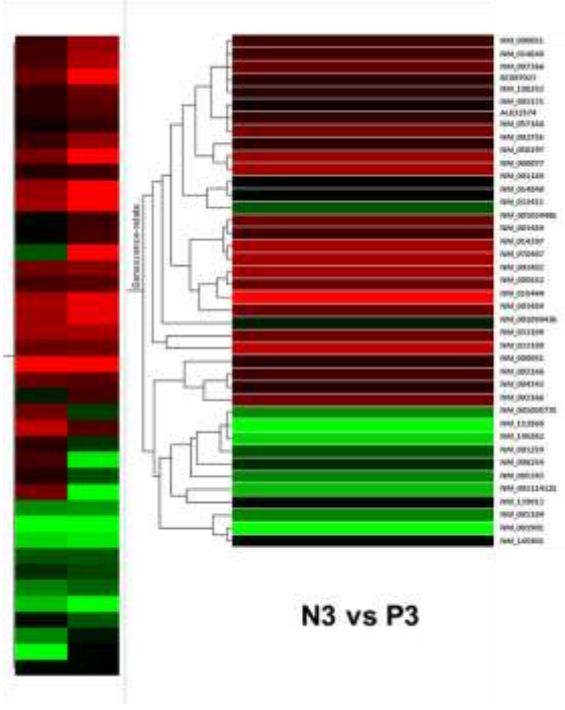

Figure 13. Dendrogram showing differential senescence-related gene expression in hUCB-MSCs from women with normal pregnancy or preeclampsia. DEGs related to senescence were compared between N3 and P3 cells. N3, early hUCB-MSCs at passage 3, normal pregnancy; P3, early hUCB-MSCs at passage 3, preeclampsia.

7. Functional categorization and pathway network analysis of senescence-related differentially expressed genes (DEGs)

A. Gene ontology classification of the senescence-related differentially expressed genes

The GO term was used to create the interaction network between the senescence-related DEGs and additional genes by using human as a source species. The relationship between the genes in the network includes co-expression, physical interactions, pathways, co-localization and protein domain similarity. The list of senescence-related DEGs was enriched for certain GO terms. Among the GO terms that have a significant relationship with senescence, determined by low FDR, were genes associated with cell cycle, which showed a very strong relationship with the selected genes (Table 3).

Table 3. Gene ontology classification of the senescence-related differentially expressed genes in hUCB-MSCs from the normal and preeclampsia groups

GO name	List numbers	Total numbers	FDR
cell cycle phase	122	414	1.78E-37
cell cycle	170	776	2.96E-34
M phase	103	329	4.64E-34
cell cycle process	140	565	5.40E-34
mitotic cell cycle	107	370	4.92E-32
M phase of mitotic cell cycle	80	224	7.77E-31
nuclear division	79	220	1.23E-30
mitosis	79	220	1.23E-30
organelle fission	79	229	2.74E-29
cell division	79	295	2.02E-21
DNA replication	56	190	2.30E-17
chromosome segregation	35	81	1.11E-16
regulation of cell cycle	73	331	8.05E-15
DNA metabolic process	95	506	2.13E-14
DNA packaging	37	117	1.25E-12
protein-DNA complex assembly	30	91	7.44E-11
cell cycle checkpoint	30	91	7.44E-11
regulation of mitotic cell cycle	39	152	2.96E-10
response to DNA damage stimulus	68	373	6.05E-10
spindle organization	20	45	6.12E-10
chromatin assembly	28	87	6.55E-10
chromosome organization	81	485	8.97E-10

List numbers, numbers of DEGs belonging to specific GO terms; Total numbers, total numbers of genes belonging to specific GO terms; FDR, false discovery rate

B. Pathway network analysis of senescence-related DEGs in hUCB-MSCs

Among 40 senescence related DEGs, we identified eight genes with filtering conditions of differential expression with more than twofold in N3 vs. P3, and then performed a GO term enrichment analysis with these genes. Especially, we investigated any relationship of those genes. The eight senescence-related DEGs had two networks. GeneMANIA network analysis for those genes suggested enrichment of 7 genes related to 'cell aging' GO term, including NM_078467, NM_058197, NM_001114121, NM_145862, NM_003483, NM_014397, and NM_003483 (genebank with large red circle in Fig. 14). Most of relations between genes were co-expressed. In the network, GO term 'cell aging' is significantly enriched with FDR-corrected p-value $2.89e^{-8}$. Among 57 genes related with 'cell aging', seven genes were covered.

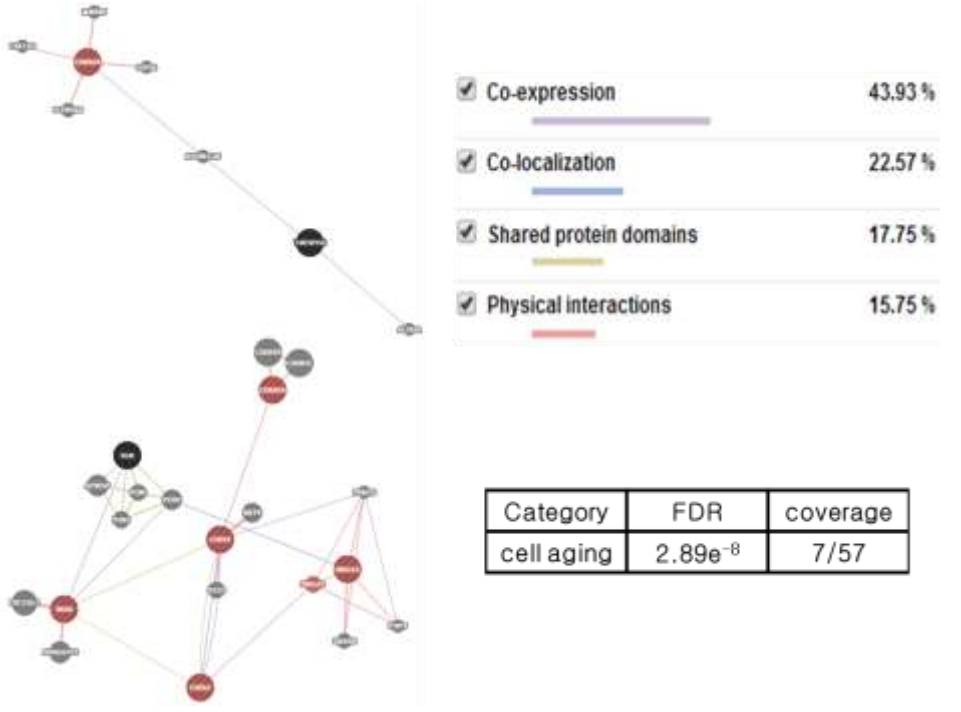

Figure 14. Network analysis of senescence-related DEGs. Genes represented by large circle are senescence-related DEGs obtained from microarray analysis. Red ones are genes related with senescence as well as cell aging. FDR, false discovery rate

IV. DISCUSSION

Preeclampsia is a disease characterized by pregnancy-induced hypertension and proteinuria that affects 2-8% of all pregnancies[26-28]. In women with this condition, the intrauterine environment is modified by changes in signaling patterns and substrate transport to the fetus[41,42]. This modification can lead to fetal growth restriction, and increased susceptibility to diseases later in life, such as cardiovascular, endocrine, nutritional, metabolic, and blood-related disease. The change of intrauterine environment made in the preeclampsia may be explained by the researches for the umbilical cord blood from two groups. The fetal circulation during preeclampsia may be associated with an increase in circulating anti-angiogenic factors such as sFlt-1 (soluble fms-like tyrosine kinase 1) and soluble endoglin, or reduced expression and activity of proangiogenic signals such as vascular endothelial growth factor or adenosine[43,44]. Some studies founded higher concentration of protein oxidation product (ex, protein carbonyl), and an increase of oxidative stress and lipid peroxidation in the cord blood of preeclamptic pregnancy compared to normotensive[45]. The umbilical serum level of inflammatory markers (interleukin-6, interleukin-8, and tumor necrosis factor-alpha) in pregnancies complicated by preeclampsia was significantly increased compared with normal[46]. Because of these pathologic conditions in the umbilical cord blood of women with preeclampsia, it is reasonable to hypothesize that circulating hUCB-MSCs in preeclamptic pregnancy cannot but be functionally impaired.

Recent studies showed that in women with preeclampsia, cord blood endothelial progenitor cells (EPCs) or circulating endothelial colony-forming cells were decreased, and functionally perturbed, and this might contribute to an increased risk of future cardiovascular events[20,47]. However, fetal growth restriction and diseases occurring after birth cannot be explained simply by alteration of endothelial stem cells derived from umbilical cord blood. There are many kinds of multipotent stem cells in umbilical cord blood including EPCs and MSCs. Because MSCs can self-renew, have a high proliferative capacity, and can differentiate into various cell types such as chondrocytes, osteocytes, adipocytes, myocytes, and neurons, hUCB-MSCs are highly likely to be functionally impaired in women with preeclampsia. In the present study, it was shown that hUCB-MSCs obtained from women with preeclampsia were less proliferative and more senescent than cells from women with normal pregnancy, and many senescence-related DEGs were identified by analysis of gene expression profiles.

Despite being a promising tool in regenerative medicine, MSCs remain controversial. This is because the clinical usefulness of MSCs, resulting from their multipotency and wide accessibility, is countered by their finite proliferative ability. Many factors affect the proliferation and senescence of MSCs in vitro, such as replicative senescence, donor age, and culture condition[48-50]. Therefore, many studies of MSCs have generated conflicting data showing a tremendous variance in growth potential. The results of this study

showed that hUCB-MSCs from women with preeclampsia were poorly proliferative, more senescent, and had decreased telomerase activity and increased ROS activity. These preeclampsia-associated changes in hUCB-MSCs were not related to donor age, replicative senescence, and culture condition. Therefore, there may be an unknown pathway associated with MSC senescence. Hierarchical clustering identified 40 senescence-related DEGs. These genes will be potential research targets in the future studies of MSCs.

At present, MSCs are extensively characterized in a culture-expanded state, and relatively little is known of their biological properties in vivo. Generally, ROS can bring about cellular senescence, apoptosis, or carcinogenesis. ROS-induced cellular damage also contributes to stem cell aging[40]. A recent study showed that human MSCs had high resistance to oxidative-stress induced death, which correlated with a low level of intracellular reactive species due to effective ROS scavenging, constitutive expression of enzymes required to manage oxidative stress and high levels of total intracellular glutathione[51]. Also, many studies suggest that telomeres and telomerase have important roles in senescence in vitro and in vivo[52]. Telomerase, a ribonucleoprotein complex containing a template RNA subunit, extends telomere length by adding telomeric repeats to the chromosome ends[54]. The high production of ROS results in a state of oxidative stress, which subsequently leads to senescence with the shortening of telomeres[53]. So, telomerase has telomere-independent anti-apoptotic, cytoprotective and pro-proliferative effects of telomerase or

protection of mitochondrial DNA against oxidative stress in addition to telomere elongation[55]. In this study, all cells were cultured under the same normoxic conditions. Nevertheless, hUCB-MSCs from women with preeclampsia were consistently more senescent and had higher ROS activity and lower telomerase activity than in women from the normal group. These findings can give explanations for the senescence of hUCB-MSCs from preeclampsia.

Comparison and cluster analysis of genes differentially expressed between N3 and P3 cells showed that 1227 up-regulated and 1457 down-regulated DEGs were common to both sets and were related the reduced function of hUCB-MSCs from women with preeclampsia. Through intersection analysis of microarray data, we eliminated the false-up or down-regulated DEGs, which could have caused misinterpretation of the microarray data. Through GO term categorization and pathway network analysis, we confirmed that the selected genes are highly related to proliferation, and cell cycle, all of which are the important causes or effects of cellular senescence. The senescence-related DEGs in two networks may be mainly associated with increased senescence of preeclamptic hUCB-MSCs. Those genes showed a network with coexpression pattern, and some of them are definitely involved in cell aging process. The senescence-related genes identified in this study can be further analyzed in many different ways.

Further studies are needed. A potential new pathway for MSC senescence

should be studied through the verification and analysis of senescence-associated genes. If hUCB-MSC markers related to intrauterine growth restriction and diseases occurring later in life are found, these pathological consequences of preeclamptic pregnancy may be resolved. Future studies of MSCs should focus on the effective promotion of long-term cell expansion, identification of pathways relevant to replicative exhaustion, and maximum growth capability without loss of the ability to differentiate.

V. CONCLUSION

In conclusion, the pathologic condition in the umbilical cord blood of women with preeclampsia causes fetal growth restriction, and increased susceptibility to diseases later in life. hUCB-MSCs obtained from women with preeclampsia are poorly proliferative, more senescent, have increased ROS activity and decreased telomerase activity compared with cells from women with normal pregnancy, and are related with many senescence-related DEGs identified by analysis of gene expression profiles. Because these preeclampsia-associated changes in hUCB-MSCs are not related to donor age, replicative senescence, and culture condition, another pathway associated with MSC senescence should be studied in the near future. 40 senescence-related DEGs identified in this study will be potential research targets in the future studies of MSCs.

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ABSTRACT (IN KOREAN)

정상산모와 임신중독증 산모의 제대혈액에서 유도된 중간엽줄기세포의 비교연구

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임신중독증은 산모 및 태아의 건강에 영향을 주는 임신관련 질환이다. 임신중독증을 앓고 있는 산모의 자궁 내 환경은 좋지 않아서, 태아의 자궁내성장지연이나 출생 후 성인이 되면서 다양한 질환을 앓게 될 가능성이 높다. 중간엽줄기세포는 다양한 세포로 분화할 수 있는 능력이 있으며, 태아의 성장 및 다양한 장기의 성장에 관여한다. 임신중독증산모의 제대혈액 내에 만들어지는 부적절한 환경은 혈액 내 다양한 줄기세포에

영향을 줄 수 있고, 특히 중간엽줄기세포의 특성을 고려해 볼 때, 정상에 비해서 임신중독증산모의 제대혈액 내 중간엽줄기세포의 기능에 이상이 발생했을 가능성이 높다. 따라서 본 연구의 목적은 제대혈액에서 유도된 중간엽줄기세포의 특성을 분석하고, 이를 바탕으로 정상산모와 임신중독증산모의 제대혈액에서 유도된 중간엽줄기세포의 기능적 특성과 유전자 발현의 차이를 비교하는 것이다. 30명의 정상산모와 28명의 임신중독증 산모가 본 연구에 포함되었으며, 각각에서 제대혈액에서 중간엽줄기세포를 분리, 배양하였다. 제대혈액에서 분리 배양된 중간엽줄기세포의 특성은 유세포분석법, 반정량 중합효소연쇄반응법, 면역형광염색법, 생체 외 분화유도의 방법을 사용하여 분석하였다. 두 군간의 중간엽줄기세포의 특성은 세포증식비교, 노화 관련 β-galactosidase 염색법, 텔로머라아제 활성도 비교, 활성산소 방법을 이용하여 비교하였고, 마이크로 어레이, 위계적 군집분석, 유전자 온톨로지분석, 유전자 pathway network 분석의 방법을 사용하여 유전자 발현의 차이를 비교하였다. 였다. 임신중독증 산모에서 배양된 중간엽줄기세포는 정상에

비해서 증식속도가 늦으며, 더 노화되어 있고, 텔로머라아제 활성도는 낮고, 활성산소는 높아져 있었다. 많은 수의 노화관련 유전자들이 유전자 분석을 통하여 확인 되었으며, 특히, 이들 유전자들은 유전자 온톨로지 분석에서 세포의 노화와 밀접한 관련이 있는 것으로 확인되었다. 결론적으로 임신중독증 산모의 제대혈액에서 유도된 중간엽줄기세포는 정상에 비해서 기능적으로 결함이 많다. 이러한 변화는 임신중독증 산모에게서 나타나는 태아의 성장 장애나 그 아이가 출생 후 다양한 질환에 이환 될 가능성이 높아지는 것이 자궁 내 환경이 좋지 않아서 생기는 현상이라는 것을 보여주는 충분한 근거가 될 수 있을 것이다.

핵심되는 말 : 제대혈액, 중간엽줄기세포, 임신중독증, 노화, 마이크로 어레이, 네트워크 분석