Enhanced Anti-cancer Effects of Conditioned Medium From Hypoxic Cultured Human Adult Dermal Fibroblasts

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Abstract

This study investigates the anti-cancer effects of cervical cancer (HeLa) cells in a conditioned medium (CM) obtained from normoxic and hypoxic cultured human adult dermal fibroblasts (HDFs). The HeLa cells showed decreased cell viability, arrested cell cycles, and increased apoptosis in CM from hypoxic cultured HDFs (H-CM) compared with normoxic cultured HDFs (N-CM). In up-regulated (> 2-fold) proteins of H-CM compared with N-CM, the top enriched term of biological process of gene ontology (GO) was GO:0006955~immune response. In intracellular down-regulated (> 2-fold) proteins of HeLa cells treated with H-CM compared with N-CM, the top enriched term of biological process of GO was GO:0016579~protein deubiquitination, and the terms of the KEGG pathway were determined to be hsa05166:HTLV-I infection, hsa03410:base-excision repair, and hsa05340:primary immunodeficiency. Among down-regulated hub proteins with ≥ 5 edges (ESR1 MCL1, TBP, CD19, LCK, PCNA, CHEK1, and POLA1) of HeLa cells treated with H-CM, the top enriched term of biological process of GO and the KEGG pathway were GO:0006272~leading strand elongation and hsa05166:HTLV-I infection. H-CM displayed not only enhanced anti-cancer effects on HeLa cells compared with N-CM, but also induced intracellular signaling patterns with 9 hub proteins.

Introduction

Hypoxic (low oxygen) conditions reportedly induce cancer proliferation or metastasis[1,2] or cause severe injury in a variety of diseases[3–6]. Beneficial effects of hypoxia have also been reported, in the form of enhanced wound healing[7], angiogenesis[8], anti-aging[9], and anti-cancers effects[10], depending on the kind of cells.

In the case of fibroblasts, hypoxia plays an important role in construction and repair of organs and tissues by secretory factors and the extracellular matrix (ECM) reorganization[11], and in cancer initiation, progression, metastasis through direct interaction signaling[12]. When fibroblasts were exposed to hypoxic conditions, contradictory results have been reported. Hypoxic fibroblasts exhibited increased cell viability and proliferation[13] and stimulated invasive activity of cancer cells[14], but proliferation of severely hypoxic fibroblast was regulated[15], and fibroblast-mediated cancer stiffness and metastasis were impaired[16].

In a previous study, epithelial cells[17], mesenchymal stem cells[18], embryonic stem cells[19] and immune cells[20] all suppressed cancer cells. However, the anti-cancer effects of hypoxic fibroblasts have not yet been studied. In this study, we investigated whether a conditioned medium (CM) from hypoxic human adult dermal fibroblast (HDFs) (H-CM), compared with normoxic CM (N-CM) enhanced anti-cancer effects on cervical cancer (HeLa) cells. We also profiled secretory proteins in H-CM and determined the intracellular signaling pattern in HeLa cells induced by H-CM, compared with N-CM, using protein antibody array analysis.

Results

Enhanced reduction in cell viability of HeLa cells with H-CM. The ratio of proliferation cell viability between normoxic and hypoxic HDFs at passage 6 did not change (Fig. 1A, B). Viability of HeLa cells was significantly reduced by H-CM treatment compared with C-CM or N-CM at 48 to 72 h (Fig. 1C). In contrast, viability of HUVECs was significantly increased by H-CM compared with C-CM or N-CM at 72 h (Fig. 1D). In the case of HUC-MSCs, viability was increased by both N-CM and H-CM compared with C-CM at 48 h, but increased only by N-CM compared with C-CM at 72 h, with no statistically significant different evident between N-CM and H-CM (Fig. 1E).

Increased apoptosis of HeLa cells with H-CM. When HeLa cells were treated for 48 h, the proportion of live cells indicating annexin-V(−)/PI(−) significantly decreased. When treated with H-CM compared with C-CM and N-CM (Fig. 2A-B), early apoptotic cells indicating annexin-V(+)/PI(−) increased by N-CM or H-CM compared with C-CM (Fig. 2A-C). Between N-CM and H-CM treatment conditions, early apoptotic cells were decreased by H-CM (Fig. 2A-C). Late apoptotic cells indicating annexin-V(+)/PI(+) were strongly increased when treated with H-CM compared with N-CM and C-CM (Fig. 2A-D). The representative apoptotic marker, caspase-3/7 activity in HeLa cells, increased when treated with H-CM compared with C-CM and N-CM at 12 to 48 h (Fig. 2E). MMPs of HeLa cells decreased when treated with N-CM or H-CM compared with CM at 24 h, and decreased strongly with only H-CM compared with C-CM and N-CM at 48 h (Fig. 2F).

Strongly induced cell-cycle arrest HeLa cells with H-CM. After 24 h of CM treatment, cell-cycle arrest of HeLa cells was induced by H-CM compared with C-CM and N-CM. Not only did the G0/G1 phase increase (Fig. 3A-B), but the S phase decreased (Fig. 3A-C) when treated with H-CM compared with C-CM and N-CM. The G2/M phase did not significantly change after CM treatments (Fig. 3A-D). After 48 h of CM treatment, the G0/G1 phase increased strongly (Fig. 3E-F), and the S and G2/M phases decreased strongly (Fig. 3E-H) after treatment with H-CM compared with C-CM and N-CM.

Profiling of up- and down-regulated proteins in H-CM compared with N-CM. To investigate secretory proteins in H-CM compared with N-CM, a protein antibody array was performed. Two-fold up-regulated (red) or down-regulated (blue) proteins in H-CM compared with N-CM were identified among 10,000 proteins of the antibody array. Twenty proteins of up-regulated and down-regulated proteins (> 2-fold) were analyzed in H-CM compared with N-CM (Fig. 4A, Supplemental Table 1). To categorize up- and down- regulated proteins in H-CM, a GO analysis using DAVID was performed ($p < 0.01$), and the data were described as the $-\log_{10}$ p value.

In up-regulated proteins, the highest enriched term of biological process of GO was GO:0006955 ~ immune response (8.062). In the next, GO:0006954 ~ inflammatory response (6.948), GO:0007165 ~ signal transduction (5.766), GO:0070374 ~ positive regulation of ERK1 and ERK2 cascade (4.408), GO:0070098 ~ chemokine-mediated signaling pathway (4.175), GO:0050930 ~ induction of positive chemotaxis (3.899), GO:0048754 ~ branching morphogenesis of an epithelial tube (3.520), GO:0043547 ~ positive regulation of GTPase activity (3.478), GO:0006935 ~ chemotaxis (3.478), GO:0050918 ~ positive chemotaxis (3.152), GO:0001666 ~ response to hypoxia (3.043), GO:0002548 ~ monocyte

chemotaxis (2.993), GO:0001541 ~ ovarian follicle development (2.993), GO:0042127 ~ regulation of cell proliferation (2.951), GO:0071346 ~ cellular response to interferon-gamma (2.729), GO:0009612 ~ response to mechanical stimulus (2.700), GO:0060326 ~ cell chemotaxis (2.617), GO:0030593 ~ neutrophil chemotaxis (2.603), GO:0071347 ~ cellular response to interleukin-1 (2.541), GO:0050729 ~ positive regulation of inflammatory response (2.517), GO:0071356 ~ cellular response to tumor necrosis factor (2.170), GO:0045766 ~ positive regulation of angiogenesis (2.133), GO:0033209 ~ tumor necrosis factor-mediated signaling pathway (2.111), GO:0048842 ~ positive regulation of axon extension involved in axon guidance (2.103), and GO:0019221 ~ cytokine-mediated signaling pathway (2.024) were categorized (Fig. 4B, Supplemental Table 2).

In the case of down-regulated proteins, terms of biological process of GO included GO:0002576 ~ platelet degranulation (6.370), GO:0022617 ~ extracellular matrix disassembly (4.844), GO:0044267 ~ cellular protein metabolic process (4.271), GO:0018149 ~ peptide cross-linking (3.328), GO:0033209 ~ tumor necrosis factor-mediated signaling pathway (2.588), GO:0007596 ~ blood coagulation (2.211), GO:0051918 ~ negative regulation of fibrinolysis (2.185), and GO:0030198 ~ extracellular matrix organization (2.158) (Fig. 4C, Supplemental Table 3).

Profiling of induced intracellular proteins in HeLa cells by H-CM compared with N-CM. To investigate intracellular signaling patterns of HeLa cells with respect to the enhanced anti-cancer effects of H-CM compared with N-CM, a protein antibody array was performed. Compared with N-CM, H-CM induced up-regulation of 20 proteins and down-regulation of 56 proteins (> 2-fold) among 1,358 proteins of the array of HeLa cells (Fig. 5A, Supplemental Table 4). These proteins were categorized by GO analysis and the KEGG pathway using DAVID, and the data were described as the $-\log_{10}$ p value. In up-regulated proteins, the biological process of GO and the KEGG pathway were not determined. In the GO analysis ($p < 0.01$) of down-regulated proteins, the top enriched term of biological process was GO:0016579 ~ protein deubiquitination (2.448), and the next enriched terms were GO:0042981 ~ regulation of apoptotic process (2.373), GO:0006366 ~ transcription from RNA polymerase II promoter (2.311), and GO:0006272 ~ leading strand elongation (2.033) (Fig. 5B, Supplemental Table 5). In the case of the KEGG pathway ($p < 0.01$) of down-regulated proteins, hsa05166:HTLV-I infection (2.422), hsa03410:base-excision repair (2.078), and hsa05340:primary immunodeficiency were determined (2.053). (Fig. 5C, Supplemental Table 5).

PPI network and hub protein selection. To identify PPI and select hub proteins in HeLa cells treated with H-CM compared with N-CM, the STRING database and Cytoscape software were used. A total of 47 nodes (proteins) and 74 edges (protein interaction lines) were determined in PPI of up- and down-regulated intracellular proteins in HeLa cells treated with H-CM compared with cells treated with N-CM (Fig. 5D, Table 1). When the interacting proteins had more than 5 edges in PPI, they were defined as hub proteins. Based on this definition, TNF (3.002 + fold), ESR1 (2.142- fold), MCL1 (2.035- fold), TBP (2.355- fold), CD19 (2.257- fold), LCK (2.030- fold), PCNA (2.172- fold), CHEK1 (2.205- fold), and POLA1 (2.022- fold) were determined to be hub proteins (Fig. 5E, Table 1). GO and the KEGG pathway were applied to these hub proteins to determine the signal pathway patterns, and the data are described as the $-\log_{10}$ p value.

With only one up-regulated protein, TNF, GO and the KEGG pathway were not determined. In the other 8 down-regulated hub proteins, biological process of GO analysis included GO:0006272 ~ leading strand elongation (2.903), GO:0006260 ~ DNA replication (2.763), GO:0006271 ~ DNA strand elongation involved in DNA replication (2.205), GO:0016032 ~ viral process (2.204), and GO:0000083 ~ regulation of transcription involved in G1/S transition of mitotic cell cycle (2.020) (Fig. 5F, Table 2), and for the KEGG pathway analysis, only hsa05166:HTLV-I infection (2.807) was determined (Fig. 5G, Table 2).

Table 1
Nodes and edges in PPI of up- and down-regulated intracellular proteins in HeLa cells treated with H-CM compared with N-CM (> 2-fold)

Node	Number of edges	Fold change
TNF	18	3.002 (+)
ESR1	12	2.142 (−)
MCL1	7	2.035 (−)
TBP	7	2.355 (−)
CD19	6	2.257 (−)
LCK	6	2.030 (−)
PCNA	6	2.172 (−)
CHEK1	5	2.205 (−)
POLA1	5	2.022 (−)
ACTG2	4	2.338 (−)
APAF1	4	2.327 (−)
HPRT1	4	2.468 (−)
PTH	4	2.113 (−)
REN	4	2.297 (+)
USP13	4	2.200 (−)
BCL10	3	2.018 (−)
CD8A	3	2.181 (−)
GATA1	3	2.055 (−)
PLK2	3	2.206 (+)
ACTR3	2	2.082 (−)
ADRA2A	2	2.096 (−)
ANXA6	2	2.433 (−)
ALDH3B1	2	2.358 (−)
COL3A1	2	2.315 (+)
ADGRE1	2	2.042 (−)
HMGB1	2	2.070 (−)

Node	Number of edges	Fold change
S100A6	2	2.027 (−)
TFAP2A	2	2.465 (−)
POU2F2	2	2.073 (−)
USP19	2	2.089 (−)
USP30	2	2.140 (−)
ADH7	1	2.264 (−)
DNM1	1	2.071 (-)
ELK1	1	2.165 (-)
GAD1	1	2.075 (+)
GPRIN2	1	3.252 (+)
GPR18	1	2.408 (−)
GRTP1	1	3.786 (+)
IAPP	1	2.501 (−)
IP6K2	1	2.393 (−)
NFE2L2	1	2.217 (−)
PTGS1	1	2.427 (+)
POLL	1	2.090 (−)
TNNI3	1	2.246 (−)
TNXB	1	2.044 (−)
TUBGCP3	1	2.331 (+)
USP24	1	2.122 (−)

Table 2
GO and KEGG pathways of down-regulated hub proteins (≥ 5 edges) in HeLa cells with H-CM compared with N-CM ($p < 0.01$)

DAVID	Category	Term	Protein	$-\log_{10}$ p value
GO analysis	Biological process	GO:0006272 ~ leading strand elongation	POLA1, PCNA	2.903
		GO:0006260 ~ DNA replication	POLA1, PCNA, CHEK1	2.763
		GO:0006271 ~ DNA strand elongation involved in DNA replication	POLA1, PCNA	2.205
		GO:0016032 ~ viral process	POLA1, TBP, LCK	2.204
		GO:0000083 ~ regulation of transcription involved in G1/S transition of mitotic cell cycle	POLA1, PCNA	2.020
KEGG pathway analysis		hsa05166:HTLV-I infection	PCNA, TBP, LCK, CHEK1	2.807

Discussion

We demonstrated that H-CM treatment resulted in enhanced anti-cancer effects in HeLa cells, profiled secretory proteins in H-CM compared with N-CM, and determined intracellular signaling patterns and hub proteins related to the enhanced anti-cancer effects.

In a previous study, hypoxic culture conditions (1% or 5% O_2) increased cell viability and proliferation of human pulmonary fibroblasts[13]. Another research team reported that moderate hypoxic conditions (2% O_2) increased DNA synthesis and proliferation of human lung fibroblasts, whereas severe hypoxic conditions (0.1% O_2) decreased human lung fibroblasts[15]. In our experiment, proliferation of HDFs did not increase in hypoxic culture conditions (1% O_2). These contradictory results may be caused by different kinds of cells or oxygen concentrations of the hypoxic culture condition.

Among up-regulated secretory proteins in H-CM compared with N-CM, researchers have found that IL37[21], LECT2[22], and TNFSF15[23] suppress tumor growth, but the other up-regulated proteins were not directly associated with anti-cancer effects. These 3 proteins may be candidates for development of effective anti-cancer drug cocktails.

In intracellular signaling patterns of down-regulated proteins in HeLa cells treated with H-CM, the terms of biological process of GO analysis included GO:0016579 ~ protein deubiquitination, GO:0042981 ~ regulation of apoptotic process, GO:0006366 ~ transcription from RNA polymerase II promoter, and

GO:0006272 ~ leading strand elongation. In the case of terms of the KEGG pathway, the highest enriched term was hsa05166:HTLV-I infection, and hsa03410:base-excision repair and hsa05340:primary immunodeficiency were determined. HTLV-I infection plays an essential role in cellular transformation and tumorigenesis from CD4 + T-lymphocytes to adult T-cell leukemia/lymphoma[24], base-excision repair induced proliferation of prostate cancer[25], and primary immunodeficiency was related to malignancies in patients[26]. These terms may be considered multiple targets for effective anti-cancer therapies. Based on this knowledge, the relationship between down-regulated proteins and the terms of GO and the KEGG pathway in HeLa cells treated with H-CM indicates that, compared with N-CM, H-CM strongly influences intracellular signaling related to enhanced anti-cancer effects corresponding to our *in vitro* results, including reduced cell viability, increased apoptosis, and inducement of cell-cycle arrest in HeLa cells.

In a PPI network of up- and down-regulated proteins in HeLa cells treated with H-CM, 1 (TNF) up-regulated and 8 (ESR1, MCL1, TBP, CD19, LCK, PCNA, CHEK1, and POLA1) down-regulated hub proteins were determined by selecting proteins with more than 5 edges. TNF was the only up-regulated hub protein to play an opposite role as a tumor stimulator or suppressor[27] depending on differences in organs, cells, and carcinogens. As down-regulated hub proteins, ESR1[28], MCL1[29], LCK[30], TBP[31], and PCNA[32] play roles in the proliferation or survival of cancer cells, and CHEK1[33] is associated with the cell-cycle checkpoint in cancer cells. In addition, CD19[34] and POLA1[35] have been reported to be targets for anti-cancer therapy. The known functions of selected hub proteins are consistent with our data on enhanced anti-cancer effects. Furthermore, the 8 down-regulated hub proteins were categorized as terms including GO:0006272 ~ leading strand elongation, GO:0006260 ~ DNA replication, GO:0006271 ~ DNA strand elongation involved in DNA replication, and GO:0000083 ~ regulation of transcription involved in G1/S transition of the mitotic cell cycle in biological process of GO analysis. Only one term in the KEGG pathway from down-regulated hub proteins was categorized as hsa05166:HTLV-I infection, which was mentioned with respect to tumorigenesis[24]. These terms of GO and the KEGG pathway in down-regulated hub proteins are related to our *in vitro* results showing enhanced anti-cancer effects of H-CM as well.

In our previous study, CM from hypoxic hUC-MSCs showed enhanced anti-cancer effects on HeLa cells, with the exception of HDFs[10]. In our current study, H-CM showed enhanced anti-cancer effects only on HeLa cells, with the exception of HUC-MSCs and HUVECs. These results suggest that hypoxic conditions may be a useful tool to screen candidates for anti-cancer drugs and develop effective anti-cancer therapies. Further study will be required to determine the different effects and mechanisms involved in treatment of HeLa cells and HUC-MSCs or HUVECs with H-CM.

Our study makes it clear that CM from hypoxic HDFs not only enhances anti-cancer effects but also induces anti-cancer–related intracellular signaling patterns and hub proteins related with these effects in HeLa cells. It also suggests that hypoxic culture conditions for HDFs offer a useful alternative approach to developing effective anti-cancer therapies.

Materials And Methods

Cell culture. Human adult dermal fibroblasts (PromoCell GmbH, Heidelberg, Germany), HeLa cells (ATCC, Manassas, VA, USA) and human umbilical-cord–derived mesenchymal stem cells (HUC-MSCs) (PromoCell GmbH, Heidelberg, Germany) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 0.1% antibiotics (Gibco, Grand Island, NY, USA) at 37 ºC in a 5% CO_2 incubator (APM-30D; ASTEC, Fukuoka, Japan)[10]. Human umbilical vein endothelial cells (HUVECs) (Lonza, Warkerville, MD, USA) were cultured in endothelial cell growth medium-2 (PromoCell GmbH, Heidelberg, Germany). An oxygen level of 21% as a normoxic condition and 1% O_2 as a hypoxic condition were applied to the culture condition of HDFs from passage 4 to passage 6[10]. When cell confluency of all cells reached 90%, the cells were passaged using 0.25% Trypsin-EDTA (Gibco, Grand Island, NY, USA). For the proliferation assay of both culture conditions of HDFs, 2×10^5 HDFs at passage 6 were cultured in a 100-mm culture plate for 5 days, and cell numbers were measured using Trypan blue 0.5% solution (Biowest, Riverside, MO, USA) staining[10].

Preparation of CM from normoxic and hypoxic HDFs. Normoxic and hypoxic HDFs (2×10^5 cells) at passage 6 were cultured in 100-mm culture plates with complete medium. When cell confluency reached 90% at day 5, the cultured medium was removed and 1× phosphate-buffered saline (PBS) was added to wash the cells. Next, 6 mL of DMEM without FBS or antibiotics was added to the cells. After incubation for 24 h, N-CMs and H-CMs were harvested and centrifuged at 300 *g* for 5 minutes. The supernatant was transferred to new 15 mL tubes and stored at − 80 ºC. For a control, CM (C-CM), DMEM without FBS or antibiotics was used[10].

Cell viability assay. Normoxic and hypoxic HDFs (1×10^3 cells) at passage 6 were seeded in 96-well white plates. After 5 days of culture, 100 µL of CellTiter-Glo assay 2.0 reagents (Promega, Madison, WI, USA) was applied to the cells. After 10 min of incubation, the luminescence ratio indicating cell viability was measured using a GLOMAX Multi Detection System (Promega Biosystems Sunnyvale, CA, USA)[10]. For analysis of HeLa cell viability and HUC-MSCs and HUVECs treated with CM from HDFs, 1×10^4 cells were seeded in 96-well white plates. The cultured medium was removed the following day and CMs were applied to the cells. After 48 or 72 h, the same procedure using CellTiter-Glo assay 2.0 reagents (Promega, Madison, WI, USA) was followed[10].

Apoptosis assay. HeLa cells (1.5×10^5) were seeded in 6-well culture plates[10]. The next day, the culture medium was removed and cells were treated with 2 mL of C-CM, N-CM and H-CM. After 48 h of incubation, the cells were harvested with 0.25% Trypsin-EDTA (Gibco, Grand Island, NY, USA) and stained with a fluorescein isothiocyanate annexin-V apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA). Stained cells were analyzed with a caliber flow cytometer (Becton-Dickinson, San Jose, CA, USA) and Flowjo software (Treestar, San Carlos, CA, USA)[10].

Caspase 3/7 activity assay. HeLa cells (1×10^4) cells were seeded in 96-well white plates[10]. The next day, the culture medium was removed and 100 µL of C-CM, N-CM and H-CM was applied to the cells. After 12, 24, and 48 h of incubation, 100 µL of Caspase-Glo 3/7 Assay reagent (Promega, Madison, WI, USA) was

added to the cells, which were then incubated for 1 h. The luminescence ratio indicating caspase 3/7 activity was analyzed using a GLOMAX Multi Detection System (Promega Biosystems Sunnyvale, CA, USA)[10].

Mitochondrial membrane potential assay. HeLa cells (1×10^4) were seeded in 96-well clear plates[10]. The culture medium was removed the next day and 100 µL of C-CM, N-CM, and H-CM was applied to the cells. After 12, 24, and 48 h of incubation, the cells were stained with an Orange Mitochondrial Membrane Potential Assay Kit (Abcam, Cambridge, UK). The fluorescence ratio (Ex/Em = 540/590 nm) indicating matrix metalloproteinases (MMPs) was measured using a Mithras^2 LB 943 Multimode Reader (Berthold Biotechnologies, Bad Wildad, Germany)[10].

Cell-cycle assay. HeLa cells (1.5×10^5) were seeded in 6-well culture plates[10]. After overnight incubation, the culture medium was removed and cells were treated with 2 mL of C-CM, N-CM, and H-CM. After 24 and 48 h of incubation, cells were harvested with 0.25% Trypsin-EDTA and fixed with 70% alcohol at 4°C for 1 h. Fixed cells were stained with 20 µg/mL propidium iodide (PI; Abcam) and 1% RNase A (QIAGEN, Valencia, CA, USA) for 30 min at 37°C. Stained cells were suspended in PBS and analyzed using a FACSVerse flow cytometer (BD Biosciences) and Flowjo software (Treestar, San Carlos, CA, USA)[10].

Analysis of secretory protein by protein antibody array. Secretory proteins in N-CM and H-CM were analyzed using a RayBio Label-based (L-Series) Human L1000 Antibody Array (Raybiotech, Inc., Norcross, GA, USA) by E-biogen (Kyung Hee Business Center, Kyung Hee University, Seoul, Korea), and data were analyzed in Genowiz 4.0 (Ocimum Biosolutions, India)[10]. Up- and down-regulated proteins in H-CM compared with N-CM (> 2-fold) were described using UniProt DB, and GO and KEGG pathway of proteins were determined using Database for Annotation, Visualization and Integrated Discovery (DAVID) ($p < 0.01$)[10].

Analysis of intracellular signaling pathways by protein antibody array. HeLa cells (2×10^5) cells were cultured in 100-mm culture plates with complete medium. When cell confluency reached 90%, the culture medium was removed and 1× PBS was added to wash the cells. After removing the PBS, 10 mL of N-CM and H-CM was applied to the cells for 24 h. The cells were then harvested and intracellular proteins analyzed with a Signaling Explorer antibody array (Full Moon BioSystems, Sunnyvale, CA, USA) by E-biogen (Kyung Hee Business Center, Kyung Hee University, Seoul, Korea)[10]. The data were analyzed using Genowiz 4.0), and up- and down-regulated proteins were described using UniProt DB. GO and the KEGG pathways of up- and down-regulated proteins were analyzed using DAVID ($p < 0.01$)[10]. Protein-protein interaction (PPI) was analyzed using the STRING database (string-db.org) and Cytoscape software (www.cytoscape.org). The number of nodes (protein) and edge (protein interaction line) were analyzed, and nodes with more than 5 edges in PPI were defined as hub proteins.

Statistical analysis. All experimental data were analyzed by the t test. A p value < 0.05 was considered statistically significant. All analyses were carried out using GraphPad Prism version 6.01 (San Diego, CA, USA).

Declarations

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Kyu-Hyun Han: Study design, performing experiments, interpretation of data, and manuscript writing and review. Ae-Kyeong Kim: performing experiments, interpretation of data. Dong-ik Kim: Study design, interpretation of data, manuscript writing and review, supervision. All authors read and approved the manuscript.

Conflicts of interest

The authors declare no potential conflict of interest.

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