Endoplasm regulates differentiation of tonsil-derived mesenchymal stem cells into chondrocytes through ERK signaling

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It is well-known that some species of lizard have an exceptional ability known as caudal autotomy (voluntary self-amputation of the tail) as an anti-predation mechanism. After amputation occurs, they can regenerate their new tails in a few days. The new tail section is generally shorter than the original one and is composed of cartilage rather than vertebrae bone. In addition, the skin of the regenerated tail distinctly differs from its original appearance. We performed a proteomics analysis for extracts derived from regenerating lizard tail tissues after amputation and found that endoplasmic (ENPL) was the main factor among proteins up-regulated in expression during regeneration. Thus, we performed further experiments to determine whether ENPL could induce chondrogenesis of tonsil-derived mesenchymal stem cells (T-MSCs). In this study, we found that chondrogenic differentiation was associated with an increase of ENPL expression by ER stress. We also found that ENPL was involved in chondrogenic differentiation of T-MSCs by suppressing extracellular signal-regulated kinase (ERK) phosphorylation. [BMB Reports 2022; 55(5): 226-231]

INTRODUCTION

Stem cells are considered as the most effective therapeutic materials in regenerative medicine. Among stem cells, mesenchymal stem cells (MSCs) are easy to acquire and display high proliferation rates with a self-renewal ability (1-3). MSCs are most often used in research on joint-related diseases as they can differentiate into cells of mesodermal lineages (e.g., bone, fat, and cartilage) (4-7). Although MSCs can be obtained from various tissues, we used tonsil-derived mesenchymal stem cells (T-MSCs) in this study. T-MSCs have better proliferation rate and differentiation capabilities than MSCs derived from other tissues (8-10). Once chondrogenic differentiation proceeds, various chondrocyte-specific polysaccharides and proteoglycans, including aggrecan (AGG) and collagen type II (COL2), accumulate to form glycosaminoglycans (GAGs) (11-13).

Some lizard species have an exceptional ability known as caudal autotomy as their anti-predation mechanism. After amputation occurs, these species could regenerate their new tails within a few days (14, 15). However, the new tail is generally shorter than the original one, and it consists of cartilage rather than vertebrae bone. Therefore, we assumed that lizards possess a remarkable capability for chondrogenic differentiation (16, 17).

In this study, we investigated whether extracts derived from regenerating lizard tail tissues could induce chondrogenic differentiation of MSCs. In a previous study involving proteomics analysis, we have identified some factors in extracts derived from a regenerating lizard tail following an amputation (18). Among various factors, endoplasmic (ENPL) attracted our attention. ENPL, which belongs to the chaperone heat shock protein (HSP90) family, is known to affect the folding of specific proteins as well as the survival and proliferation of cells (19-23). Thus, we investigated whether human ENPL could induce chondrogenesis. Consequently we found that ENPL was indeed involved in chondrogenic differentiation of T-MSCs by suppressing ERK phosphorylation. Our findings suggest a novel role of ENPL in chondrogenic differentiation. For the first time, this study demonstrates that ENPL could be a solution to over the
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Fig. 1. LTEs induce the expression of chondrogenic specific cell markers in T-MSCs by regulating ENPL. (A) Potential cytotoxicity to T-MSCs after treatment with LTEs. It was confirmed that LTEs at concentrations of 0-20 μg/ml did not exert a significant effect on T-MSCs survival (Fig. 1A). The concentration of LTEs to be added to the differentiation induction medium was set to be 0.5 μg/ml in this study. Through alcian blue staining and real-time PCR, we found that chondrogenic specific cell markers expressed earlier in LTEs-treated cells than in LTEs-untreated cells (Fig. 1B, C). Next, we conducted tandem mass spectrometry and we found that ENPL expression was maximally increased between day 6 (blastema-phase, which displays the characteristics of mesenchymal stem cells) and day 12 (redifferentiation-phase) (Fig. 1D). Regarding intracellular mRNA expression of ENPL in T-MSCs, more induction of chondrogenic differentiation was associated with a more increase of ENPL expression (Fig. 1E).

ENPL promotes chondrogenic differentiation in T-MSCs
To determine the effect of ENPL on chondrogenic differentiation, we used luminespib (MedChemExpress, New Jersey, USA), an ENPL inhibitor by inhibiting the activation of ENPL (24). Furthermore, we investigated the expression of activating transcription factor 6 (ATF6), which is activated by ER stress and related to the expression of ENPL (25-29). We conducted experiments to investigate chondrogenesis specific cell markers. Results showed that target markers were expressed higher in cells treated with LTEs than in control cells not treated with LTEs (Fig. 2A, B). However, when cells were treated with luminespib, the expression of target markers was significantly suppressed at both mRNA and protein levels. Next, we overexpressed the ENPL gene in T-MSCs by transfecting cells with an ENPL overexpression vector. OV_0 meant that cells were cultured for 24 hours in a normal medium after transfection with the ENPL overexpression vector. OV_4, OV_8, and OV_12
indicated that cells were cultured for 4, 8, and 12 days in differentiation induction medium, respectively (Fig. 2C). Results showed that the vector was successfully transfected and that mRNA and protein expression levels of chondrogenesis-specific markers were significantly increased after differentiation induction.

**ENPL inhibition decreases chondrogenic differentiation through ERK signaling**

We conducted experiments to determine effects when ENPL was inactivated. ‘+I’ meant that cells were cultured in luminespib (inhibitor)-treated medium. When luminespib was used to treat cells, all target markers were significantly suppressed compared to those in inhibitor-untreated groups (Fig. 3A, B). In addition, results of alcian blue staining showed that GAGs accumulated relatively few in luminespib-treated groups (Fig. 3C). Finally, we investigated the mechanism of ERK signal pathway closely associated with ER stress during chondrogenesis (30, 31). Results revealed that phosphorylated ERK 1/2 showed fairly low levels in luminespib-untreated groups. On the contrary, ERK 1/2 phosphorylation was considerably high in luminespib-treated groups (Fig. 3D).

**ENPL may promote chondrogenic differentiation of T-MSCs by inhibiting ERK 1/2 phosphorylation in an autocrine or a paracrine manner**

After endogenous ENPL was verified to be able to induce chondrogenesis, exogenous ENPL (MyBioSource, Inc.) was used to investigate whether it could also induce differentiation. First of all, we determined whether exogenous ENPL was cytotoxic to T-MSCs through MTT assay. Results confirmed that ENPL did not induce cytotoxicity of T-MSCs except when it was used at a very concentration at 500 ng/ml (Fig. 4A). Next, to determine the effect of exogenous ENPL, cells were cultured in a differentiation induction medium excluding TGF-β. Alcian blue staining revealed that all ENPL-treated cells were stained more intensely than those not treated with ENPL (0 μg/ml) (Fig. 4B). In addition, we found that mRNA and protein expression levels of chondrogenesis-specific markers were increased in ENPL-treated groups (Fig. 4C, D). Moreover, the phosphorylation of ERK1/2...
was suppressed by exogenous ENPL in a concentration-dependent manner (Fig. 4D). These results demonstrate that exogenous ENPL could also induce chondrogenic differentiation of cells by suppressing the ERK1/2 phosphorylation.

DISCUSSION

In this study, we paid attention to ER stress as the cause of an increase in ENPL. Commonly, ER stress is known to be the main cause of various diseases as it can induce abnormal responses of cells. However, it has been recently confirmed that appropriate and beneficial ER stress provides a significant signal in cells (25, 26). In addition, ER stress is known to be involved in the differentiation process of various cells such as chondrocytes (25, 26). When ER stress is induced by a specific stimulus, ATF6 localized in the membrane of the endoplasmic reticulum can act as a transcription factor in the nucleus that helps transcription of ER chaperone genes including ENPL (30-33). However, the initial mechanism as to how ENPL can bind to cells and how ENPL stimulus can initiate ER-stress has not been elucidated yet. In addition, certain cell signal pathways involved in chondrogenic differentiation remain controversial, especially the ERK signaling pathway (34-36). ERK can act in an activated or an inactivated state depending on MSCs derived from specific tissues. In this study, we confirmed that ERK phosphorylation was inhibited in cells that highly expressed chondrogenesis specific markers. It is meaningful for the authentication of the relationship between human ENPL and chondrogenic differentiation of MSCs in vitro.

MATERIALS AND METHODS

Primary cell culture

Primary T-MSCs were obtained from Professor Jae-Ho Kim of Pusan National University Graduate School of Medicine. T-MSCs (passage 10) were usually cultured in α-MEM, no nucleosides medium (GibcoTM) supplemented with 10% fetal bovine serum (Capricom Scientific) and 1X Antibiotic-Antimycotic (GibcoTM). The cells were incubated at 37°C and 5% CO₂. These results demonstrate that exogenous ENPL could also induce chondrogenic differentiation of cells by suppressing the ERK1/2 phosphorylation.

Induction of chondrogenic differentiation

T-MSCs were cultured in 60 mm cell culture dishes at 1 × 10⁵ cells/dish in differentiation induction medium, which consisted of α-MEM containing 10% FBS, 1X Antibiotic-Antimycotic, 0.1 μM dexamethasone (Sigma-Aldrich), 50 μM L-Ascorbic acid (Sigma-Aldrich), 1% Sodium Pyruvate (Invitrogen), 10 ng/ml Transforming Growth Factor-β1 (TGF-β1) human (Sigma-Aldrich) and 50 mM Insulin (Roche). The cells were cultured at 37°C and 5% CO₂, and the differentiation induction medium was changed every 2 days. Especially, TGF-β1 was added up to 4 days of differentiation induction, after that, it was excepted from the medium components.

Preparation of lizard tail extracts (LTEs)

Lizards (Hemitheconyx caudicinctus) were purchased from Zoos (Seoul, Korea). All experiments using lizard tail were approved by the Institutional Animal Care and Use Committee of Dong-A University. Also, we followed the guidelines of the Animal Care Committee of Dong-A University. After cutting the lizard’s tail to about 3 cm in length, we carefully peeled off only the skin of the cut tail. The peeled tail tissue was minced, placed in a homogenizer tube containing 1 ml 1X phosphate buffered saline (PBS, pH 7.4), and then homogenized using a homogenizer. The tissue was centrifuged at 4°C and 13000 rpm for 11 min, and then the supernatant was transferred into a new tube. To decompose the DNA and RNA in the supernatant, it is pulverized for 5 times at 2 watt, 2-second intervals using a sonicator and then centrifuged at 4°C and 13000 rpm for 11 min; the supernatant was transferred into a new tube by using a syringe filter (0.2 μm).

Cell cytotoxicity assay

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) assay were performed to verify the cells survival. T-MSCs were plated into 96 well plate at 1 × 10⁵ cells/dish in α-MEM containing 10% FBS, 1X Antibiotic-Antimycotic and then incubated for 24 hours. After that, the medium was removed, and a fresh medium containing LTEs or ENPL was added into the plate. After one more 24-hour incubation, the medium was gently removed and WST-1 reagent and MTT solution were prepared beforehand, and 110 μl of these solutions were dispersed into the wells, respectively. The cells were incubated at 37°C and 5% CO₂ for 2 hours. Then, especially, the formazan, which formed in ENPL-treated experiment using an MTT reagent, was solubilized by adding 100 μl dimethyl sulfoxide (DMSO) into each well. The absorbance of formazan, which formed in both assays, was measured at 440 nm in quintuplicate.

Identification of proteins in extracts using tandem mass spectrometry

Tandem mass spectrometry was performed to identify each substance present in the LTEs. After separating the factors in LTEs obtained on the 6 and 12 days of regenerating through SDS-PAGE, we collected each selected protein spots and transferred them into a 1.5 ml tube. 200 μl of Sterile distilled water was added and stirred to wash the proteins three times for 10 min each. Then, 200 μl of 100 mM Ammonium bicarbonate was added and stirred to wash the proteins three times for 10 min each. Then, 200 μl of 100 mM Ammonium bicarbonate (1:1 volume ratio) was added and then incubated for 30 min. Subsequently, 500 μl of Acetonitrile was added and stirred until the gel became faint and then dried for 15 min using a vacuum dryer. 0.25% Trystpin-EDTA 50 μl was added into a 1.5 ml tube containing dried gel pieces, and then the tube was allowed to stand on ice for 30 min. Subsequently, 20 μl of 50 mM Ammonium bicarbonate was added into the tube and then incubated at 37°C for 16 hours. Then, 20 μl of solution com-
posed of 50% Acetonitrile, 47.5% water and 2.5% Trifluoroacetic acid, was added into the tube, incubated for 10 min, and then centrifuged at 10,000 rpm for 10 min. The supernatant was transferred into a new tube, and the sample was analyzed using mass spectrometry.

**Alcian blue staining**
Glycosaminoglycans (GAGs), which are formed from the degradation of cartilage-related proteoglycans, were detected through alcian blue staining. After the medium was removed, 1 ml 10% formalin was added into the plate and incubated for 20 min to fix the cells. After fixation, the cells were washed three times with 1X PBS and 1% alcian blue solution (Sigma-Aldrich) was added into each well, and then the plate was gently shaken for 1 hour. Subsequently, to discard the unnecessary stain, 0.1 M HCl was added. After the HCl was removed, the cells were washed twice with 1X PBS.

**Cell transfection**
For the overexpression of the ENPL gene, an ENPL overexpression vector that was prepared from the coding sequences of ENPL gene and pcDNA6/V5-His B vector was transfected into the T-MSCs. When the T-MSCs density exceeded 70%, transfection was performed according to the protocol of Lipofectamine™ 2000 Transfection Reagent (Invitrogen).

**Quantitative real-time polymerase chain reaction (qRT-PCR)**
Total RNA was isolated and the relative mRNA expression levels of markers were determined using qRT-PCR. Primers was used as follows. COL2: F 5'-TGAGCCATGTTGGCTCCGG-3', R 5'-CACAGACAGACAGTCCCGCA-3', SOX9: F 5'-GGTAGGATGCTGCTGGG-3', R 5'-GGTAGGATGCTGCTGGG-3', TACGAG-3', R 5'-AGGTAGTTTCGTGGATGCCA-3'.

**Western blotting**
T-MSCs lysates were prepared in RIPA buffer (iNiRON Biotechnology, Gyeonggi, Republic of Korea) supplemented with protease inhibitors and phosphatase inhibitors (Thermo Scientific™). Protein concentrations were measured using a Pierce™ BCA Protein Assay Kit (Thermo Scientific™), and proteins were separated by 10% SDS-PAGE. And then, proteins were transferred onto a PVDF membrane (Cytiva, formerly GE Healthcare) at 25 V and 400 mA for 2 hours using a semi-dry transfer (Bio-Rad). Subsequently, the membrane was blocked with 5% or 7.5% skimmed milk, and then washed twice with 1X PBS and incubated with primary antibodies to COL2 (diluted 1:500, Proteintech Group, Inc.), SOX9 (diluted 1:100, Cell Signaling Technology Co.), ERK/p-ERK (diluted 1:1000, Cell Signaling Technology Co.) and GAPDH (diluted 1:1000, Enzo Life Sciences, Inc.) overnight at 4°C. The primary antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted 1:5000, Enzo Life Sciences, Inc.) at 37°C for 2 hours, and blots were visualized by D-Plus™ ECL Pico System (DonginLS, Seoul, Republic of Korea).

**Statistical analysis**
All experiments were replicated at least three times. The results were presented as mean ± SEM, and statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software). The significance of difference was evaluated by t-test between control and experimental groups. The P-value < 0.05 was considered significant.

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**CONFLICTS OF INTEREST**
The authors have no conflicting interests.

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