BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-21-173

Title: Endoplasmin regulates differentiation of tonsil-derived mesenchymal stem cells into chondrocytes through ERK signaling.

Article Type: Article

Keywords: Tonsil-derived Mesenchymal Stem Cells (T-MSCs); Lizard Tail Extracts (LTEs); Differentiation; Chondrocytes; Endoplasmin

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Running title: The effects of endoplasmin on the chondrogenesis.

Keywords: Tonsil-derived Mesenchymal Stem Cells (T-MSCs), Lizard Tail Extracts (LTEs),

Differentiation, Chondrocytes, Endoplasmin

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ABSTRACT

It has been well known that some species of lizard have the exceptional ability, that is 'caudal autotomy', as the anti-predation mechanism. After the amputation was occurred, they regenerate their new tail in a few days. The new tail section is generally shorter than the original and is composed of cartilage rather than vertebrae bone, and the skin of the regenerated tail distinctly differs from its previous appearance. We performed a proteomics analysis in extracts-derived from regenerating lizard tail tissue after amputation and found the ENPL (Endoplasmin, GRP94) as the main factor among the proteins of which up-regulated expression during regeneration. In fact, ENPL has been known to affect cell survival or proliferation. Thus, we performed farther experiments to verify that ENPL could induce chondrogenesis of mesenchymal stem cells. Mesenchymal stem cells (MSCs) are capable of differentiating into some specific cells and have self-renewal ability. Although MSCs can obtained from various tissue, the MSCs derived from tonsil tissues (Tonsil-derived Mesenchymal Stem Cells, T-MSCs) have several advantages including superior proliferation rate and differentiation capabilities, compared with MSCs derived from other tissues. In this study, we found ENPL is involved in chondrogenic differentiation of T-MSCs through suppression of ERK phosphorylation.

INTRODUCTION

Stem cells are considered to be the most effective therapeutic material in regenerative medicine. Among the stem cells, mesenchymal stem cells (MSCs) are easy to acquire, and they display high proliferation rates as well as demonstrate self-renewal ability; thus, MSCs have been considered as a noticeable material in cell-based clinical therapy (1-3). Although MSCs can be obtained from various tissues, in this study, we used tonsil-derived mesenchymal stem cells (T-MSCs), which offer advantages such as superior proliferation rate and differentiation capabilities compared with MSCs derived from other tissues (3-6). 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) (7-10). Once chondrogenic differentiation proceed, various chondrocyte-specific polysaccharides and proteoglycans, including aggrecan (AGG) and collagen type Ⅱ (COL2), accumulate to form glycosaminoglycans (GAGs) (11-13).

Some lizard species demonstrate an exceptional ability known as caudal autotomy as their anti-predation mechanism. After the amputation was occurred, these species could regenerate their new tail within a few days (14,15). However, the new tail is generally shorter than the original, 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 a regenerating lizard tail tissue could induce the 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 these factors, we paid attention to endoplasmin (ENPL), which belongs to the chaperone heat shock protein 90 (HSP90) family and 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, and consequently we found that ENPL is indeed involved in chondrogenic differentiation of T-MSCs through the suppression of ERK phosphorylation. Our findings suggest a novel role for ENPL in chondrogenic differentiation. This study demonstrates for the first time that ENPL could be a solution for the limitation of MSC-based cell therapy.

RESULTS

LTEs induce the expression of chondrogenic specific cell markers in T-MSCs through regulation of ENPL

First, we performed WST-1 assay to confirm the cell viability of T-MSCs according to treatment of lizard tail extracts (LTEs). It was confirmed that the concentration of 0-20 μg/ml LTEs did not exert a significant effect on T-MSCs survival (Figure 1A) and we set the concentration of LTEs to be added to the differentiation induction medium with 0.5 μg/ml in this study. Through the alcian blue staining and real-time PCR, we found that the expression of chondrogenic specific cell markers was shown faster at the LTEs-treated cells than the LTEs-untreated cells (Figure 1B, C). Next, we conducted tandem mass spectrometry and then, we found the ENPL, whose expression was maximally increased between the day 6 (blastema-phase, which displays the characteristics of mesenchymal stem cells) and the day 12 (redifferentiation-phase) (Figure 1D). Also, the intracellular mRNA expression of ENPL in T-MSCs showed that the more inducing the chondrogenic differentiation, the more increasing of ENPL expression (Figure 1E).

ENPL promotes the chondrogenic differentiation in T-MSCs

To confirm the effect of ENPL on chondrogenic differentiation, we used ENPL inhibitor, Luminespib (MedChemExpress, New Jersey, USA), which inhibits the activation of ENPL (24). Furthermore, we confirmed the expression of activating transcription factor 6 (ATF6), which is activated by ER stress and is considered to induce the expression of ENPL (25-29). We conducted to investigate the chondrogenesis specific cell markers and then, the results showed that target markers were expressed higher in cells treated with LTEs only than those in LTEs-

nontreated cells (Figure 2A, B). However, when cells were treated with Luminespib, the expression of target markers was significantly suppressed in both of mRNA and protein levels. Next, we overexpressed the ENPL gene in T-MSCs by transfecting with ENPL overexpression vector; OV_0 means that the cells were cultured for 24 hours in a normal medium after transfection with the ENPL overexpression vector. OV_4, OV_8, and OV_12 indicate that cells were cultured for 4, 8, and 12 days in differentiation induction medium respectively (Figure 2C). The results showed that the vector was successfully transfected and also the mRNA and protein expression levels of chondrogenesis-specific markers were significantly increased according to the differentiation induction.

ENPL inhibition decreases the chondrogenic differentiation through ERK signaling.

We conducted experiments to identify the effects when ENPL was unactivated. '+I' means that the cells were cultured in Luminespib(inhibitor)-treated medium. When Luminespib was treated on cells, the all target markers were significantly suppressed compared to inhibitor-untreated groups (Figure 3A, B). In addition, the results of staining showed that GAGs accumulated relatively few in the Luminespib-treated groups (Figure 3C). Finally, we investigated that the mechanism of ERK signal pathway, which is closely associated with ER stress, during chondrogenesis (30,31). The results confirmed that phosphorylated ERK 1/2 showed at fairly low levels in the Luminespib-untreated groups; on the contrary, in the Luminespib-treated groups, ERK 1/2 phosphorylation was considerably high (Figure 3D).

ENPL may promote chondrogenic differentiation of T-MSCs by inhibition of ERK 1/2 phosphorylation through autocrine or paracrine manner.

After the endogenous ENPL was verified to induce chondrogenesis, exogenous ENPL (MyBioSource, Inc.) was investigated as to whether it could also induce differentiation. First of all, we confirmed whether the cytotoxicity of T-MSCs was induced according to treatment of exogenous ENPL through MTT assay; the results confirmed that ENPL, except at the 500 ng/ml concentration, did not induce the cytotoxicity of T-MSCs (Figure 4A). Next, to verify the effect of exogenous ENPL, the cells were cultured in the differentiation induction medium, excluding the TGF-β. In alcian blue staining, all ENPL-treated cells stained more intensely than those not treated with ENPL (0 μg/ml) (Figure 4B). In addition, we confirmed that the mRNA and protein expression of chondrogenesis-specific markers was increased in the ENPL-treated groups (Figure 4C, D). Moreover, the phosphorylation of ERK1/2 was also suppressed in a concentration-dependent manner (Figure 4D). These results demonstrated that exogenous ENPL could also induce chondrogenic differentiation of the cells.

DISCUSSION

In this study, we paid attention to the ER stress as the cause of the increase in ENPL. Commonly, ER stress has been known to be the main cause of various diseases as it induces abnormal responses of cells, but recently, it has been confirmed that appropriate and beneficial ER stress provides a significant signal in cells. In addition, ER stress has been 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, which is localized in the membrane of the endoplasmic reticulum, acts as a transcription factor in the nucleus that helps the transcription of ER chaperone genes, including ENPL (30-33). However, the initial mechanism as to how ENPL bind to cells or how ENPL stimulus initiates ER-stress has not yet been elucidated. Also, certain cell signal pathway involved in chondrogenic differentiation remains controversial about its roles, especially ERK signaling pathway (34-36). The ERK acts in an activated or inactivated state depending on the 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 the 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 (Gibco^{TM}) supplemented with 10% fetal bovine serum (Capricorn Scientific) and 1X Antibiotic-Antimycotic (Gibco^{TM}). The cells were incubated at 37℃ and 5% CO_2 conditions. The cells were detached by 0.25% Trypsin-EDTA (Capricorn Scientific) and then centrifuged at 2000 rpm for 5 min.

Induction of chondrogenic differentiation

T-MSCs were cultured in 60 mm cell culture dishes at 1 X 10^5 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-β) human (Sigma-Aldrich) and 50 nM Insulin (Roche). The cells were incubated at 37℃ and 5% CO_2, 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 Zools (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-iodo-phenyl)-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 X 10^4 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 dispensed into the wells, respectively. The cells were incubated at 37℃ and 5% CO_2 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. 200μl 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/Acetonitrile (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% Trypsin-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 composed 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 deposition 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 hours. Subsequently, to distain 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^{TM} 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'-TGAGCCATGATTCGCCTCGG-3', R 5'-CACAGACACAGATCCGGCA-3', SOX9: F 5'-CTGAACGAGAGCGAGAAGCG-3', R 5'-CCCGTTCTTCACCGACTTCC-3', AGG: F 5'-GAAGGAGGTAGTGCTGCTGG-3', R 5'-GGGTAGTTGGGCAGTGAGAC-3', MMP13: F 5'-CGCCAGACAAATGTGACCCT-3', R 5'-TACGGTTGGGAAGTTCTGGC-3', ENPL: F 5'-TCTGGAAATGAGGAACTAACAGTC-3', R 5'-ACTCGCTTGTCCCAGATTTG-3', ATF6: F 5'-TTCAGTCTCGTCTCCTCGGT-3', R 5'-ATCTTCCTTCAGTGGCTCCG-3', Actin: F 5'-CCCTGGAGAAGAGCTACGAG-3', R 5'-AGGTAGTTTCGTGGATGCCA-3'.

Western blotting

T-MSCs lysates were prepared in RIPA buffer (iNtRON 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 an NC membrane (Cytiva, formerly GE Healthcare) at 25 V and 400 mA for 45 min using a semi-dry transfer (Bio-Rad). Subsequently, the membrane was blocked with 5% or 7.5% skimmed milk and probed with primary antibodies to AGG (diluted 1:300, Proteintech Group, Inc.), 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℃. The primary antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted 1:5000, Enzo Life Sciences, Inc.) at 37℃ 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 or untreatment groups. The P-value < .05 was considered significant.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF-2020R1F1A1070475) and the Korea Research Institute of Bioscience and Biotechnology (KRIBB-20200288010100).

CONFLICTS OF INTEREST

The authors disclose no potential conflict of interest.

FIGURE LEGENDS

Figure 1. LTEs induce the expression of chondrogenic specific cell markers in T-MSCs through regulation of ENPL. (A) The potential cytotoxicity according to the concentration of LTEs in T-MSCs was measured through WST-1 assay. (B) GAGs were relatively more accumulated in 0.5μg/ml LTEs-treated cells, especially since after day 8. 100X magnification. Scale bar=100 ㎛. (C) Results of qRT-PCR showed the relative mRNA levels of SOX9, AGG, COL2 and MMP13. (D) Up- or Down-regulated proteins confirmed by tandem mass spectrometry of regenerating lizard tail on the 6 and 12 days respectively. (E) Results of qRT-PCR showed the relative mRNA expression of ENPL in T-MSCs during chondrogenic differentiation. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, $^{\#\#\#}P < 0.001$.

Figure 2. ENPL promotes the chondrogenic differentiation in T-MSCs. Differentiation of T-MSCs into chondrocyte was induced for 8 days. DI, Differentiation Induction. (A) The mRNA levels of ATF6 and ENPL were detected using qRT-PCR. (B) The mRNA and protein levels of chondrogenesis- specific markers were detected using qRT-PCR and Western blotting, respectively. (C) OV_0, 4, 8 and 12 means the cells that cultured in differentiation medium for certain days after transfection, respectively. Increased expression of ENPL mRNA in T-MSCs transfected by ENPL overexpression vector. Also, the relative mRNA and protein levels of chondrogenesis specific markers were detected using qRT-PCR and Western blotting, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Figure 3. ENPL inhibition decreases the chondrogenic differentiation through ERK signaling. 'OV+I' means the cells that cultured in inhibitor-treated medium after transfection. (A) The mRNA levels of ATF6 and ENPL were detected by qRT-PCR. (B) The relative mRNA and protein levels of chondrogenesis specific markers were detected using qRT-PCR and Western blotting, respectively. (C) Alcian blue staining was conducted to confirm the GAGs deposition following the transfection of ENPL overexpression vector. 40X magnification. Scale bar=200 μm. (D) The protein levels of phosphorylated ERK were detected by Western blotting. *P <0.05, **P <0.01 and ***P <0.001.

Figure 4. ENPL may promote chondrogenic differentiation of T-MSCs by inhibition of ERK 1/2 phosphorylation through autocrine or paracrine manner. (A) The potential cytotoxicity of ENPL at T-MSCs was measured through MTT assay. (B) Alcian blue staining was conducted to confirm the GAGs deposition by exogenous ENPL. 100X magnification. Scale bar=100 μm. (C) The mRNA levels of chondrogenesis specific markers were detected using qRT-PCR. (D) The protein levels of chondrogenesis specific markers and phosphorylated ERK1/2 were detected by Western blotting. DI, Differentiation Induction. *P <0.05, **P <0.01, ***P <0.001 and P >0.05 is indicated by "ns" for not significant.

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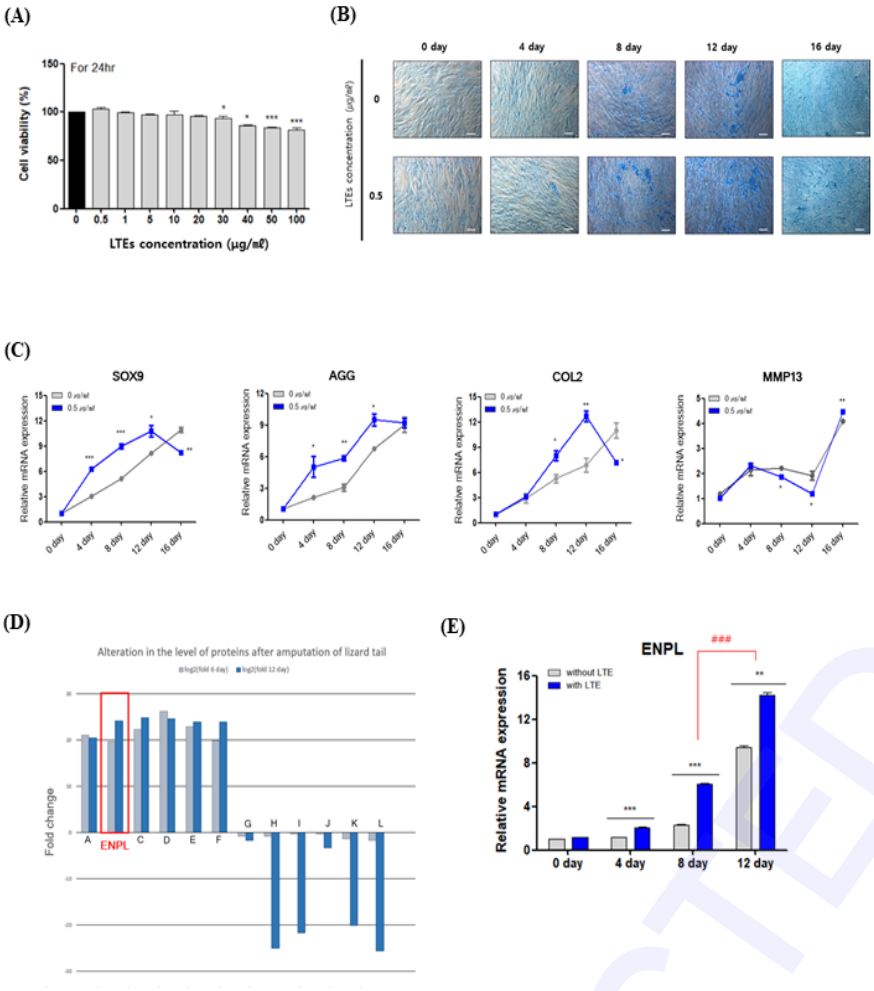

Fig. 1.

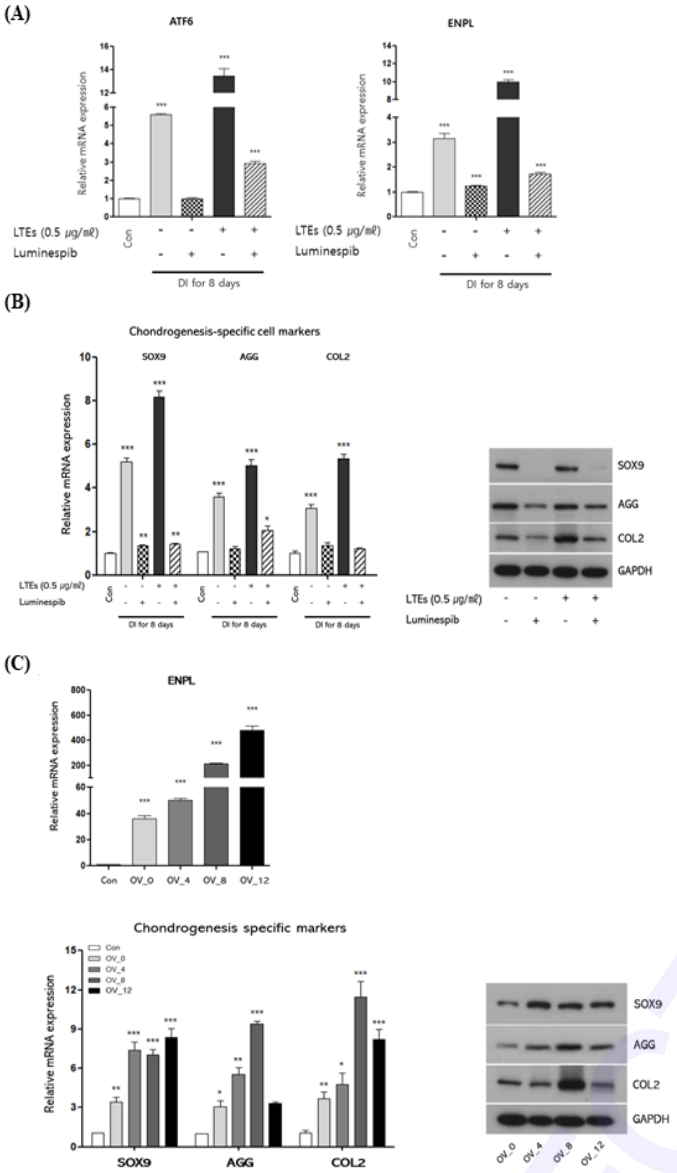

Fig. 2.

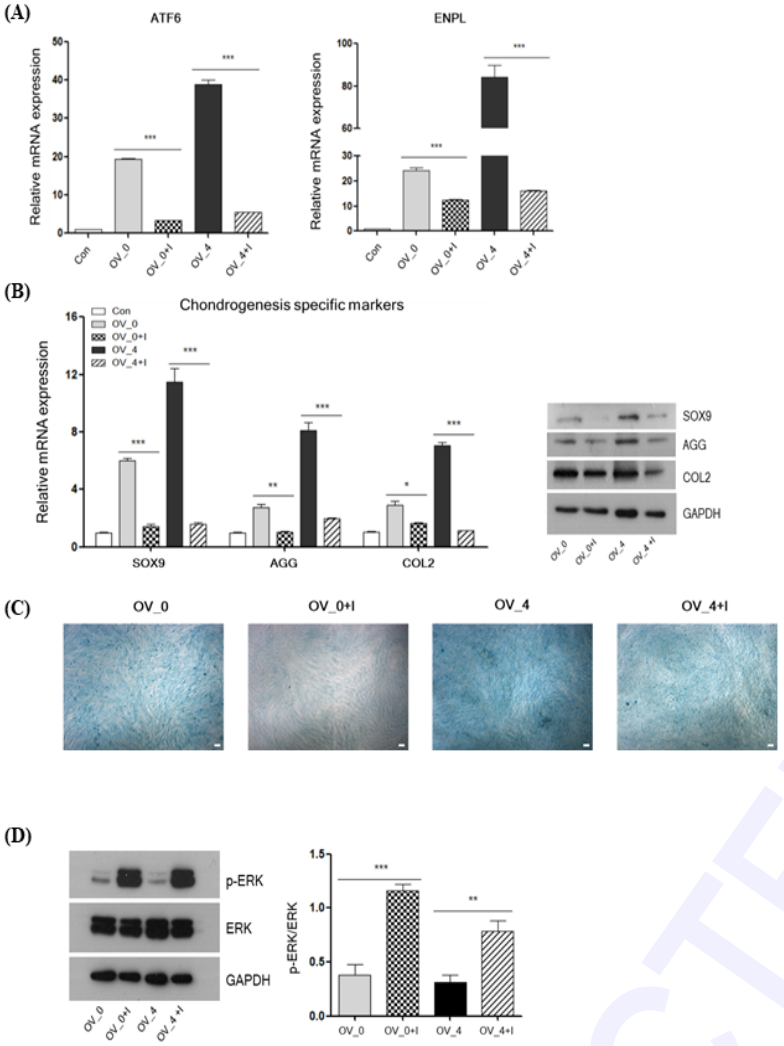

Fig. 3.

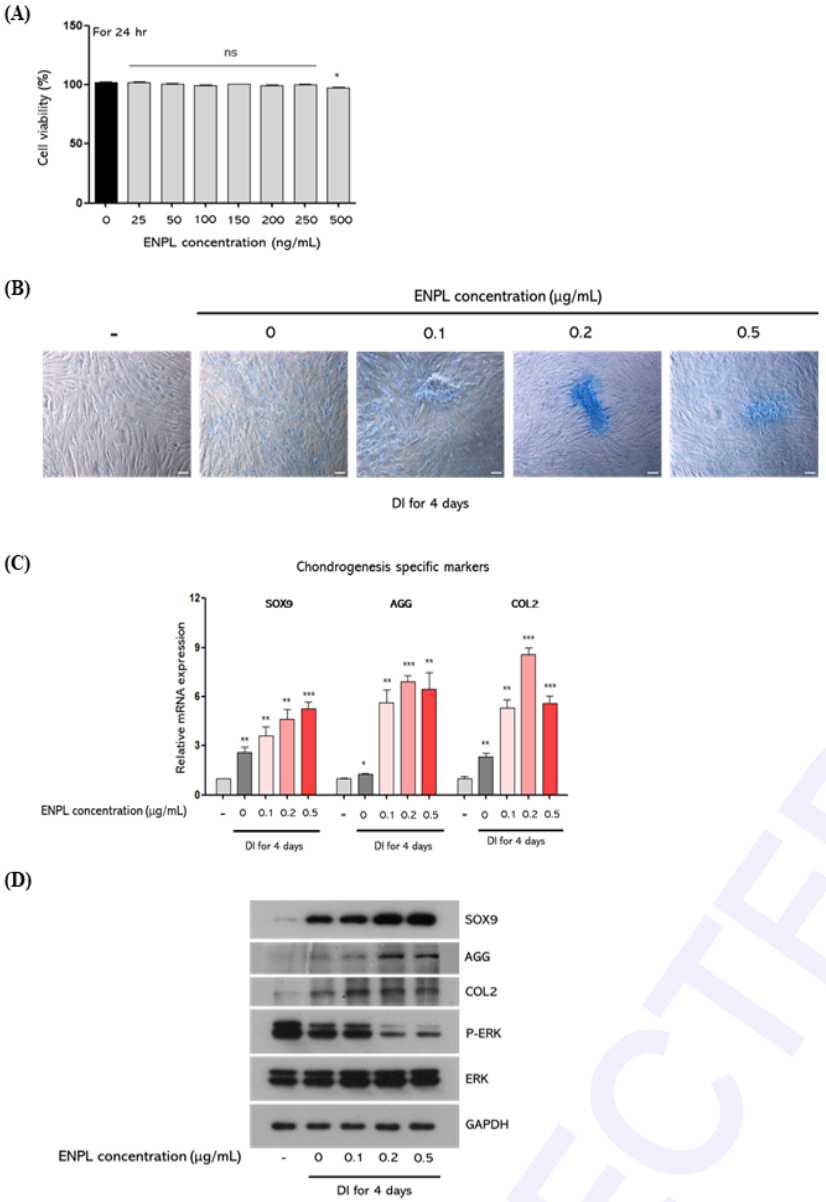

Fig. 4.