Differentiation of adipose-derived stem cells into functional chondrocytes by Sox9-induced small molecule

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Directed by Professor Jong-Chul Park

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ABSTRACT

Differentiation of adipose-derived stem cells into functional chondrocytes by Sox9-induced small molecule

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Osteoarthritis (OA) is a common joint disease that results from the disintegration of joint cartilage and underlying bone. Upon damage to the cartilage, chondrocytes differentiate into hypertrophic chondrocytes and increase their expression of hypertrophic markers. The resulting hypertrophic chondrocytes release matrix-degrading enzymes, such as MMP13, ADAMTS4, and ADAMTS5, leading to cartilage disintegration and calcification. Because cartilage and chondrocytes lack the capability to self-regenerate, the effort has been made to utilize stem cells to treat OA. Various methods, including defined factors, microRNAs, and small molecules, have been used in attempts to differentiate stem cells into functional chondrocytes. However, currently available methods are not effective enough to induce stem cells to undergo

differentiation into chondrocyte-like cells without also inducing hypertrophic chondrocyte characteristics. Therefore, an optimized method to differentiate stem cells into chondrocytes that do not display undesired phenotypes is needed. Sox9 is expressed during cartilage development, which it plays an important role by increasing the expression of factors that can constitute cartilage cells. Previously studies have succeeded in differentiating stem cells into chondrocytes by inducing the overexpression of Sox9. Therefore, this study focused on differentiating adipose-derived stem cells (ASCs) into functional chondrocytes using a small molecule that regulated the expression of Sox9 and then explored its ability to treat OA. First, the expression of Sox9 was evaluated in chondrocytes and stem cells, and a drug capable of increasing the expression of Sox9 was then selected using a GFP-Sox9 promoter vector. Three candidate drugs were selected during the first screen. Among these, Drug 138 (Ellipticine, ELPC) was selected because it did not induce hypertrophic chondrocyte characteristics. The expression levels of mature chondrocyte markers, such as type II collagen and aggrecan, were increased in the chondrocytes differentiated from ASCs by ELPC, whereas the expression levels of hypertrophic chondrocyte markers, such as RUNX2 and type X collagen, and extracellular matrix-degrading enzymes, such as MMP13, ADAMTS4, and ADMATS5, were decreased. In addition, this result was showed that ELPC regulates the expression of Sox9 by increasing the expression of p53 and its translocation into the nucleus. Next, the recovery rate

of cartilage regeneration in ASCs-differentiation into chondrocytes by ELPC was confirmed in a collagenase-induced animal model of OA. The group injected with ASCs-differentiated into chondrocytes by ELPC recovered damaged cartilage faster than compared to the group injected with untreated ASCs. Taken together, these data confirm that Ellipticine induces ASCs to differentiate into mature chondrocytes that do not exhibit the characteristics of hypertrophic chondrocytes, thus overcoming a problem encountered in previous studies. Additionally, the mechanism by which chondrocytes are differentiated by ELPC and the recovery-inducing effect of ELPC-differentiated ASCs in OA was confirmed. These results indicate that ELPC is a novel chondrocyte differentiation-inducing drug that shows potential as a cell therapy for OA.

Keywords: osteoarthritis, adipose-derived stem cells, chondrocytes, small molecules

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I. INTRODUCTION

Osteoarthritis (OA), a major degenerative and common chronic disease caused mainly by aging, is a disease that affects most joints, including the knees, hips, back, neck, and the small joints of the fingers and toes.[1,2] Over 70% of people over the age of 50 years old suffer from OA because musculoskeletal changes rapidly occur beginning when individuals reach approximately 40 years old.[3-5] The joints of elderly individuals are affected by changes in the cartilage and connective tissue that cause the cartilage and cartilage components to become thinner and deformed. These changes, make the cartilage less resilient and more susceptible to damage. Therefore, the surfaces of cartilage tissues become rough as a result of damage and eventually cannot move smoothly. Additionally, because the connective tissue within ligaments and tendons becomes harder and more brittle, the joints also become stiffer. Moreover, this

change also limits the range of motion of joints and induces sarcopenia, in which muscle mass and muscle strength are gradually lost, and this increased bone turnover may contribute to the development of OA.[6-8] Thus, the change in the musculoskeletal system that occurs with age may also contribute to the development of OA by making joints more susceptible to other OA risk factors including abnormal biomechanics, joint injuries, genetics, and obesity.[9] OA is also more common in females than in males, and its frequency with age is higher in females.[9] Several factors that have previously been reported to contribute to the development of OA are related to aging. These include degenerative changes of the meniscus and degenerative ligaments, an increased bone turnover rate, and the increasing calcification of joint tissue. However, over the past few years, OA has also been observed in younger populations as a result of obesity, genetic factors, repeated injuries caused by high-intensity exercises, and acute joint injuries (e.g., a torn anterior cruciate ligament) in addition to other causes.[10-14]

Joints are composed of synovial fluid and articular cartilage, which cover the bone tip and act as a lubricant and shock absorber, respectively. Synovial fluid, a known also synovia, is a viscous, thick, non-Newtonian fluid found in the cavities of the synovial joint. It acts as a lubricant that assists in joint movements by reducing friction in the articular cartilage while the joint is moving.[15] Recent studies have shown that the expressions of factors associated with arthritis development are altered in the synovial fluid during OA.[16,17] For

example, a C-telopeptide fragment of Type II collagen, which is related to MMPs, was higher in OA patients than in a normal healthy reference group.[16] Calcium pyrophosphate dihydrate and apatite crystals and fibrils are also common in severe OA patients.[17] The articular cartilage is composed of chondrocytes and a dense extracellular matrix (ECM) and does not contain blood vessels, nerves, or lymphatics, unlike most other tissues.[18,19] The processes underlying OA include joint damage caused by aging, obesity, and high-intensity strength exercises, which occur during the first stage of OA and are followed by ECM damage in articular cartilage. Chondrocytes in cartilage are thus sequentially exposed to the environment, leading to the induction of the maturation of hypertrophic chondrocytes. Chondrocytes are eventually differentiated into hypertrophic chondrocytes as a result of a decrease in transforming growth factor-beta (TGF-β) which is known to repress chondrocyte hypertrophic differentiation.[20] Studies have reported that chondrocytes that differentiated into hypertrophic chondrocytes characteristically overexpress hypertrophic markers, such as runt-related transcription factor 2 (RUNX2), Type X collagen, Indian hedgehog, and Transglutaminase-2. Studies have also demonstrated that RUNX2 acts as a key regulator of endochondral ossification during development by regulating a unique set of cell cycle genes to control chondrocyte proliferation and differentiation.[21-23] Kielty et al. and K. Maerk et al. demonstrated that Type X collagen, a short, non-fibril-forming collagen, is only synthesized by

hypertrophic chondrocytes.[6,24] Type X collagen is expressed before endochondral bone formation begins, and it regulates and expedites matrix mineralization and compartmentalizes matrix components during endochondral ossification.[25] Hypertrophic chondrocytes are also known to synthesize various matrix-degrading enzymes, such as matrix metalloproteinases (MMPs), a disintegrin, and metalloproteinases containing thrombospondin motifs (ADAMTSs), which are enzymes that accelerate the decomposition of the ECM, leading to cartilage loss and calcification. The ADAMTS are numbered from 1 to 20 and belong to the metzincin protease superfamily, which includes MMPs and a disintegrin and metalloproteinases (ADAMs).[26,27] The ADAMTS family is composed of pro, catalytic, disintegrin-like, cysteine-rich, and spacer motifs and a variable number of TS type 1 motif. MMP13, a member of the matrix metalloproteinase family, and ADAMTS4, and ADAMTS5 play roles in chondrocyte enlargement and cartilage calcification and are commonly found in hypertrophic chondrocytes.[7,28] The articular cartilage is a key component of joints that have a limited capacity for intrinsic healing and repair. There is currently no way to reverse the onset of the OA process, and a variety of methods, including medications, physical therapy, surgical and other procedures, and alternative medicine have been implemented to relieve the pain and symptoms of affected patients. For example, topical pain relievers, anti-inflammatory painkillers, steroids, anti-rheumatic drugs, and chemotherapy have been used to reduce pain and treat OA.[5,29-32] In patients with more severe

joint damage, hyaluronic acid, which is similar to a component of joint fluid, has been directly injected into joints as a therapy aimed at improving lubrication.[2,32,33] Although hyaluronic acid has been shown to reduce pain in the knee, it has little effect on OA and a short reapplication time, meaning that this treatment must be periodically reapplied. Joint replacement surgery (arthroplasty) is a last therapeutic step for severe joint damage that involves removing the joint surface and replacing it with plastic or metal parts.[3] However, since artificial joints will begin to show wear or loosen over time, they must eventually be replaced with a new artificial joint.[10] It is often difficult to choose which artificial joint to use because the cost of the surgery can be high, the lifespan of the artificial joint can be shortened by poor management, and there is therefore a risk of reoperation and intraoperative infection.

The most recent therapeutic approaches for OA involve the use of stem cells.[11] Stem cells include embryonic stem cells, adult stem cells (tissue-specific stem cells and mesenchymal stem cells), and induced pluripotent stem cells. All stem cells have the ability to self-renew and can differentiate into every cell type. Embryonic stem cells, which are obtained from the blastocyst inner cell mass, can differentiate into all of the specialized cells in our tissues and organs. However, the use of embryonic stem cells is complicated by ethical issues, which it is therefore difficult to obtain embryos that can be used for this type of research.[34] To overcome this problem, Yamanaka produced induced pluripotent stem cells by injecting specific factors such as Sox2, c-Myc, Klf4, and Oct-4

into somatic cells, which are not pluripotent.[35] However, the major concern associated with induced pluripotent stem cells is that they can form tumors.[36] Adult stem cells are undifferentiated cells that are found in some mature tissues and organs, such as the bone marrow, brain, peripheral blood, skin, and adipose tissue. They share properties with embryonic stem cells. Among adult stem cells, bone marrow-derived stem cells and adipose-derived stem cells are the most commonly used.[37,38] Bone marrow-derived stem cells are more effectively differentiated into bone, cartilage, fat, or muscle than are adipose-derived stem cells.[39] Adipose-derived stem cells (ASCs) have recently been widely used because they are easier to obtain than bone marrow. ASCs are separated from fat, and large numbers of ASCs can be acquired.[38] Previous studies have demonstrated the possibility of using stem cells for cartilage formation.[40,41] However, one of the major problems of the protocols currently available to generate chondrocytes from stem cells is that the differentiated chondrocytes present the characteristics of hypertrophic chondrocytes, including the overexpression of ECM-degrading enzymes and the induction of calcification. Therefore, to optimize stem cell-based therapeutics for OA, it is necessary to identify alternative methods to generate chondrocytes from stem cells without inducing any of the undesired characteristics of hypertrophic chondrocytes.

During skeletal development in embryogenesis, chondrogenesis refers to the process by which cartilage forms from condensed mesenchymal cells.[41] In the early stage of chondrogenesis, sex-determining region Y-type high mobility

group box (Sox) genes such as Sox9, Sox5, and Sox6 are known to play crucial roles by acting as transcription factors during the cartilaginous skeleton formation.[8,30,32] During embryonic development, the expression of Sox9 is increased and activated during the prechondrocytic mesenchymal condensation of mesenchymal cells, which is an early process during cartilage formation.[42,43] Sox9 functions as a master transcriptional activator that regulates chondrocyte-specific matrix proteins such as Type II collagen and Aggrecan and can activate these enhancers in non-chondrocytic cells. The expression of Sox5 and Sox6 is also controlled by Sox9.[44,45] Cartilage does not development in teratomas derived from Sox9-knockout embryonic stem cells.[42] Furthermore, the expression of Sox9 was down-regulated in terminal differentiating growth plate chondrocytes and OA cartilage.[46] Previous studies have shown that differentiation into chondrocytes can be induced by controlling the expression of sox genes in embryonic stem cells.[32,47] In particular, among these factors, Sox9 has been shown to be overexpressed during chondrogenesis in mouse embryos.[29,31] Another study demonstrated that Sox9 is important during chondrogenesis by confirming that miRNAs that regulate the expression of Sox9 inhibit the differentiation of stem cells into chondrocytes.[9]

In order to regulate the expression of critical gene and protein of stem cell differentiation, previous studies have used a system such as viral vector, microRNA, siRNA, and shRNA.[48,49] However, the conventional system has a limitation about cytotoxicity, delivery, and maintenance.[50,51] Therefore, it is

possible to control the expression of genes and proteins by using small molecules which are stable and transferable in the cells.

Consequently, a specific vector system (GFP-Sox9) that contained the Sox9 promoter region to screen for small molecule drugs was used to induce differentiation from ASCs into chondrocytes in this study. In addition, the ultimate goal of this study was to develop a new small molecule-based method to induce the optimal differentiation of ASCs into mature chondrocytes. Additionally, the mechanisms underlying the activities of these small drugs during chondrocyte differentiation was investigated.

II. MATERIALS AND METHODS

1. Materials

A. Reagents

Ellipticine (ELPC, 5,11-Dimethyl-6H-pyrido[4,3-b]carbazole) was purchased by Enzo Life Science Inc. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were made by the same corporation (Life Technologies, Grand Island, NY, USA). For the polymerase chain reaction (PCR), oligonucleotide synthesized from Bioneer (Bioneer, Daejeon, South Korea), RNA was extracted using chloroform and 2-Propanol (Sigma-Aldrich, St. Louis, MO, USA), reverse transcription for cDNA synthesis was conducted using the reverse transcription system (Promega Corporation, Madison, WI, USA), and PCR experimented with Ex Taq, dNTP mixture (2.5 mM each), and a 10 x Ex Taq buffer (Takara Bio Inc., Otsu, Japan). A kind of antibodies of western blot, immunofluorescence are type II collagen, aggrecan, and type X collagen, p53, p-p53, and β-actin (Santa Cruz Biotechnology, USA). Secondary antibody usage was divided into 2 types: mouse or rabbit (Enzo Life Sciences, Inc., Farmingdale, NY, USA) western blot analysis (Horse-radish peroxidase (HRP)-conjugated) and mouse, rabbit, or goat (Jackson Immunoresearch Laboratories, West Grove, PA, USA; Vector

Laboratories, Burlingame, CA, USA; Abcam, USA) immunostaining (fluorescein isothiocyanate, FITC; phycoerythrin, PE; or allophycocyanin, APC-conjugated).Western blotting detection systems were obtained from GE healthcare Life Sciences (Uppsala, Sweden). 4',6-diamidino-2-phenylindole was obtained from Life Technologies (USA).

B. Animals

Adult 8-wkke-old Sprague-Dawley male rats were used (Koatech, Pyeongtaek, Korea) from Harlan USA. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee, Yonsei University College of Medicine, and the Association for Assessment and Accreditation of Laboratory Animal Care were performed in accordance with the Guidelines and Regulations for Animal Care.

2. Methods

A. Adipose-derived stem cells culture

Human ASCs were purchased from Invitrogen and cultured in a growth media (DMEM supplemented with 10% FBS, 100 unit/ml of penicillin, and 100 μg/ml of streptomycin) at 37°C, 5% CO_2. Experiments used between passage 3 and 7 for differentiation.

B. Sox9 promoter gene assay

The human Sox9 proximal promoter region information (-1034bp to

+67bp) relative to the transcriptional start site was obtained in a previous study.[52] The PCR product of promoter region was cloned to become pAcGFP1-1 vector. The conjugated Sox9 promoter GFP vector was transfected in the ASC using TransIT-X2 Dynamic Delivery system. After transfection, GFP intensity was examined under the fluorescence microscopy.

C. Pellet culture system

ASCs have been cultured by an improved pellet culture system by Johnstone et al..[28] $2.5x10^5$ ASCs were centrifuged 600g for 5min in a 15ml polypropylene tube. The resulting pellets were treated witn an ELPC final concentration of 1 μM in the 10% FBS DMEM media for 16days. The medium and the ELPC were replaced with a fresh medium and ELPC dosage once every 3 days.

D. Cell viability assay

ASCs were plated in 96-well cell culture plate triplicate at $1x10^4$ cells/well. After treating with ELPC for 24hr or 48hr in ASCs, EZ-cytox reagent (DoGEN, Seoul, Korea) was added to each well and incubated at 37°C for 2hr to react reagent reactions. The sample absorbance was measured using a microplate reader (Thermo Fisher Scientific, MA, USA) at 450nm.

E. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using a TRIzol. Chloroform was added to seperate each sample into layers of RNA, DNA, proteins to distinguish and

then each sample were spun in a centrifuge at 12,000 rpm, 4 °C for 15 min. Next, the RNA from each sample was collected in a new tube and 2-propanol was added to gain the pellet after which repeated the Centrifugation at 12,000 rpm, at 4 °C for 10 min. The pellet washed in 75% (v/v) ethanol and mixed with diethylpyrocarbonate (DEPC) dissolved in water. After centrifugation at about 12000 rpm, at 4 °C for 5 min, pellet dried at room temperature. Finally, the pellet was dissolved in 30 μL nuclease-free water (NFW). The quality and quantity of RNA were estimated by calculation of OD260/OD280 ratios using a spectrophotometer. Complementary DNA (cDNA) was synthesized using the RTsystem kit. The RNA was added to the oligo dT primer, dNTP mixture, Rtase, RNase inhibitor, and buffer. The composed cDNA was mixed with each primer, dNTP mixture, Taq polymerase, and reaction buffer in the PCR tube. PCR conditions consisted of denaturation at 94 °C for 3 min, followed by 30 cycles each featuring denaturation at 94 °C for 30 sec, annealing at 48-60 °C for 30 sec, and elongation at 72 °C for 30 sec and then the reaction was maintained at 72 °C for 10 min. PCR products were separated by electrophoresis on 1.2% (w/v) agarose gels. Gel-Doc was used to visualize the bands.

Table 1. RT-PCR primers list used in this study

Primer	Sequence
Sox9	F: GAGGAAGTCGGTGAAGAACG
	R: ATCGAAGGTCTCGATGTTGG
Aggrecan	F: TGAGGAGGGCTGGAACAAGT
	R: GGAGGTGGTAATTGCAGGGA
Type II collagen	F: TGGAGAAACCATCAATGGTGG
	R: TGGAGAAACCATCAATGGTGG
Type X collagen	F: ATGACCCAAGGACTGGAATCTTTA
	R: CTGAGAAAGAGGAGTGGACATAC
RUNX2	F: AAGGGTCCACTCTGGCTTTG
	R: CTAGGCGCATTTCAGGTGCT
ADAMTS4	F: TTTCCCTGGCAAGGACTATG
	R: GGAGGAGAACTGGACACCAC
ADAMTS5	F: TGACCATGAGGAGCACTACG
	R: TGGGAGAGGCCAAGTAAATG
MMP13	F: GTGGTGTGGGAAGTATCATCA
	R: GCATCTGGAGTAACCGTATTG
p53	F: GAAACTACTTCCTGAAAACAACGT
	R: GCCTCACAACCTCCGTACT
GAPDH	F: CATGGGTGTGAACCATGAGA
	R: GGTCATGAGTCCTTCCACGA

F. Nuclear extraction

Nuclear and cytoplasmic of ASCs was extracted using NE-PER nuclear and cytoplasmic extraction reagents kit. (Thermo Scientific, IL, USA) ASCs were harvested with trypsin-EDTA and centrifuged at 500g for 5min to collect cell pellet. And then, the cell pellet was washed with PBS and 1-10x10^6 cells were transferred to a 1.5ml microcentrifuge tube and pellet by centrifugation at 500g for 3min. After the PBS was removed, cytoplasmic extraction reagent I (CER I) was added to the cell pellet. The cell pellet was vortexed to fully suspend on the highest setting for 15 sec and then incubated on ice for 10min. And then cytoplasmic extraction reagent II (CER II) was added in the sample, vortexed for 5sec, placed on ice for 1min, vortexed for 5 sec, and centrifuged for 5min at maximum speed in a microcentrifuge (~16,000g). The supernatant was immediately transferred to a new tube and store it on ice until use. The supernatant contained cytoplasmic proteins, and the pellet contained the nuclear fraction. After adding the ice-cold nuclear extraction reagent (NER) to the pellet, the vortex for 15 sec and on ice for 10 min was repeated for a total 4 times. And then, the sample was centrifuged at maximum speed in a microcentrifuge (~16,000g) for 10min then was transferred the supernatant fraction to a new tube. The supernatant included the nuclear extract.

G. Western blot analysis

The cells were washed with PBS and lysed in lysis buffer with proteinase

and phosphatase inhibitor. Protein concentrations were determined using the BCA Protein Assay Kit. After, same concentration proteins were separated on a sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After the membrane was blocked in the 5% skim milk mixed 0.1 % Tween 20 in TBS buffer for 1 hr at room temperature, the membrane was rinsed twice with TBS buffer and incubated with primary antibody for overnight at 4 °C. Next, the membrane was washed three times with 0.1 % tween 20 TBS buffer for 10 minutes and incubated for 1 hr at room temperature with HRP-conjugated secondary antibodies. After extensive washing, the bands were detected with an enhanced chemiluminescence reagent. The band intensities were quantified using NIH Image J version 1.34e software.

H. ADAMTS4 and ADAMTS5 ELISA verification

The protein activities of ADAMTS4 and ADAMTS5 which are released as active protease form in cell culture supernatant were measured using ADAMTS4 and ADAMTS5 ELISA kit (R&D system). The ADAMTS4 and ADAMTS5 capture antibodies were coated a 96 well microplate during an overnight at room temperature. After washing 3 times, blocking with reagent diluent was performed for 1 hr, added standard and sample, and react at room temperature for 2 hr. Repeat wash the plate 3 times, incubate with detection antibody for 2 hr, wash again, add streptavidin-HRP was added and reacted for 20 min in the dark. After the

substrate solution was added for 20min, stop the reaction using stop solution, and the value was read at 450nm using a microplate reader.

I. Osteoarthritis induction model and ASCs injection

The osteoarthritis was produced using 250g male Sprague-Dawley (SD) rats. Rats were anesthetized by intraperitoneal administration of 20 mg/kg Zoletil and 5 mg/kg Rumpun. The depth of anesthesia was performed the twice intra-articular injection for 1 weeks, where 30 μl of either sterile saline (control group) and a 250 U or 500 U of type II collagenase dissolved in saline and filtered a 0.22 μm membrane was injected. ASCs induce differentiation into chondrocytes by treating ELPC for 16 days prior to injection. One week after the injection of Type II collagenase, normal ASCs and differentiated ASCs are injected with $1x10^6$ cells in PBS.

J. Alcian blue stain analysis

After 16days, the cell was rinsed once with PBS and fixed cells with 4 % formaldehyde solution for 1 hr at room temperature. And then alcian blue staining solution with 3 % acetic acid solution (pH 2.5) was added to each sample for 30 min. After staining, samples were washed in running tap water for 2 min and finally mounted with mounting solution.

K. Safranin O staining

The knee joint paraffin section was stained using safranin O staining kit. First, the section was deparaffinized, rehydrated, and rinsed with tap water and then was stained 0.1 % fast green solution for 5 min. The sample was

rinsed with 1 % acetic acid for a 10 sec then stained using 0.1 % safranin o staining solution for 30 min. Finally, the section was dehydrated and mounted with mounting solution.

L. Immunofluorescence

The knee joint was fixed overnight with 10 % (v/v) formaldehyde. The knee joint embedded in paraffin and transversely sectioned into serial thick sections. After deparaffinization, rehydration, and rinsing with tap water, sodium citrate antigen retrieval was performed using 10 mM sodium citrate (pH 6.0) in a microwave for 10 min. Sections were incubated in 1 % H2O2 for quenching endogenous peroxidase. The tissue sections were treated with 0.1 % sodium borohydride to remove autofluorescence and were blocked in 2.5 % normal horse serum. After blocking, the sections were incubated with primary antibody overnight. The sections were analyzed with DAPI, Aggrecan, collagen type 2, and 10 by confocal laser scanning microscope LSM700.

M. Statistical analysis

Results are expressed as mean ± SD from at least three independent experiments. Statistical analyses were performed using Student's t-test. Comparisons between more than two groups were performed by one-way ANOVA using Bonferroni's correction. Relationships were considered statistically significant when the p-value was less than 0.05.

III. RESULTS

1. The crucial role of Sox9 in the differentiation of stem cells into chondrocytes

A. Differential expression of Sox9 between Adipose-derived stem cells and chondrocytes

Sox9 is known to be a master regulator of cartilage development, especially during chondrogenesis. First, the ability of Sox9 to induce cell differentiation and the basal expression levels of Sox9 in ASCs and chondrocytes was confirmed. The mRNA and protein levels of Sox9 were confirmed in healthy, untreated ASCs and chondrocyte. Both the mRNA and protein expression levels of Sox9 were higher in chondrocytes than in ASCs, as shown in Figure 1A and 1B.

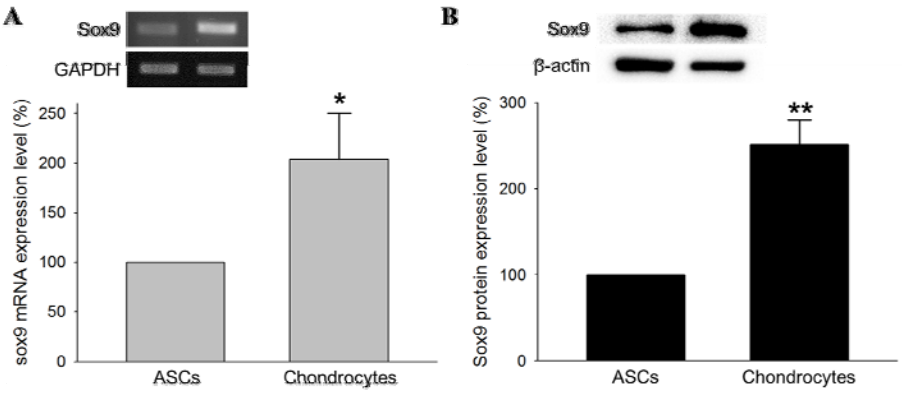


Figure 1. Differential expression of Sox9 in ASCs and chondrocytes. (A) mRNA expression of Sox9 in ASCs and chondrocytes was determined by RT-PCR. mRNA expression level of Sox9 was normalized by GAPDH expression. (B) The protein expression level of Sox9 in ASCs and chondrocytes were detected by western blot analysis. Sox9 expression was normalized by β-actin. *$p<0.05$ **$p<0.001$ compared to ASCs.

B. Critical regulation of Sox9 in chondrocyte differentiation

To confirm that Sox9 plays an important role during the differentiation of ASCs into chondrocytes, it was explored whether the differentiation of ASCs into chondrocytes was affected when Sox9 was inhibited in ASCs. A siRNA for Sox9 was used to inhibit Sox9 expression, and chondrocyte differentiation medium was used to induce the differentiation of ASCs into chondrocytes. ASCs were transfected with 50 nM of Sox9 siRNA for 24 hr and cultured in chondrocyte differentiation induction medium (DM). The cells were transfected with the Sox9 siRNA every 3 days for 16 days while cultured in DM. The expression levels of Sox9, Aggrecan, and Type II collagen were higher in the DM-treated ASC group but inhibited in the Sox9 siRNA-transfected ASC group. (Figure 2)

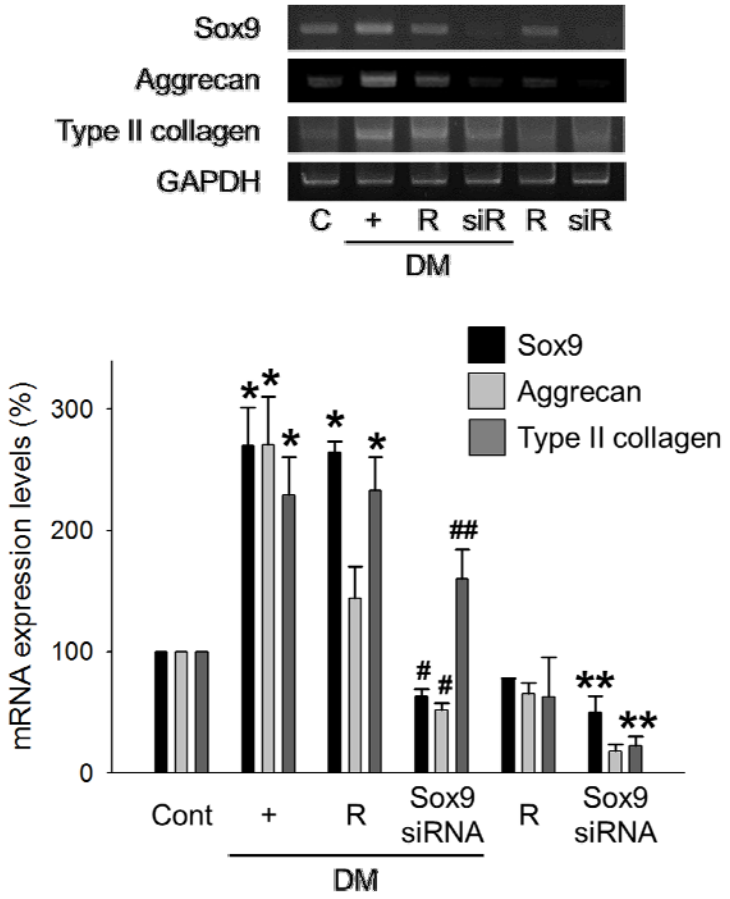


Figure 2. Suppression of chondrocyte differentiation by Sox9 downregulation. The mRNA expression of Sox9, Aggrecan, and Type II collagen was determined by RT-PCR. mRNA expression level of Sox9 was normalized by GAPDH expression. C and Cont: Control, R: Reagent-transfected ASCs, siR: Sox9 siRNA-transfected ASCs, DM: chondrocyte differentiation induction medium. *p<0.001, **p<0.05 compared to Control. #p<0.001, ##p<0.05 compared to Reagent-transfected and DM-treated ASCs.

2. Screening of small molecules that increase the expression of Sox9 in Adipose-derived stem cells

A. Production of vectors used to confirm the expression of Sox9

To obtain a drug that increases the expression of Sox9, a specific vector system containing the Sox9 promoter region and GFP was constructed (Figure 3A). The promoter region for Sox9 used in this study was based on the promoter region described in DC Colter et al..[53] The cloned promoter region of Sox9, which contained the entire -1034 bp to + 67 bp sequence of the Sox9 promoter, was inserted into a GFP-tagged vector and then transfected into ASCs. After the ASCs were transfected with the Sox9 promoter-containing, GFP-tagged vector (Sox9 promoter-GFP vector), they were treated with 1 μM of a library of seven different types of small molecules that have previously been shown to modulate protein kinase activities and natural compounds. These drugs were GFP-fluorescent, and the results were therefore analyzed based on the overexpression of GPF. When the results were analyzed at 24 hr after cells were transfected with the Sox9 promoter-GFP vector, it was found that GFP expression was increased in cells transfected with three of the drugs (Figure 3B). The same results were also observed after 72 hr (data not shown). Finally, these three drugs were selected as candidates based on the intensity of GFP fluorescence they induce.

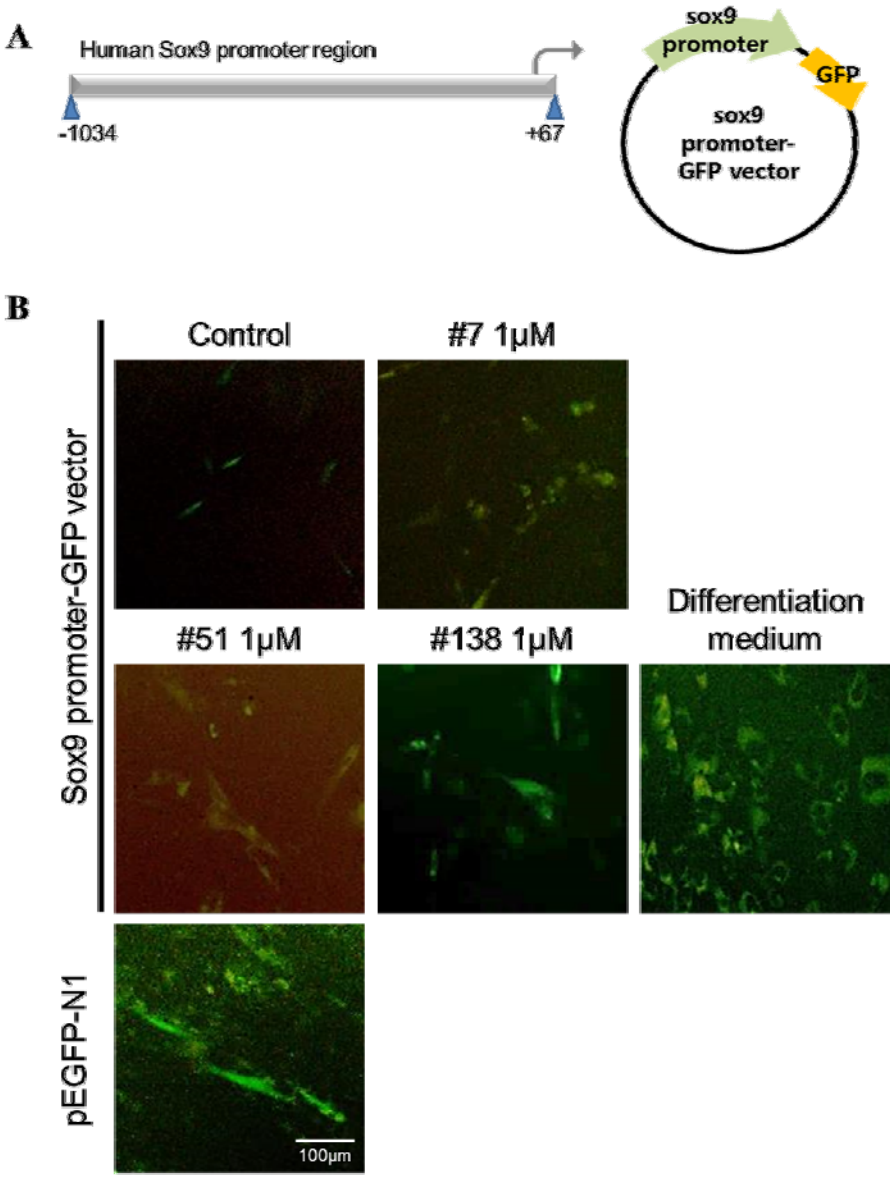


Figure 3. GFP expression in the Sox9 promoter GFP-tagged vector transfected-ASCs. (A) Vector generation strategy containing Sox9 promoter region and GFP tag (B) The Sox9 promoter-GFP vector-transfected ASCs were treated with small molecules library. GFP Expression in the ASCs transfected

with Sox9 promoter-GFP vector was observed by fluorescence microscopy. A pEGFP-N1 vector was used as a positive control to evaluate the transfection efficiency. And chondrocyte differentiation induction medium (Differentiation medium) was also used as a positive control to evaluate the Sox9 expression. #7: Drug 7, #51: Drug 51, #138: Drug 138-treated ASCs, Scale bar: 100 μm.

B. Expression of mature and hypertrophic chondrocyte related markers in Adipose-derived stem cells treated with the three selected drugs

To explore the ability of the three candidate drugs to induce differentiation, the mRNA expression levels of mature chondrocyte and hypertrophic chondrocyte markers were measured using PCR. ASCs were treated with a 1 μM solution of each of the three selected candidate drugs every 3 days for 16 days. The expressions levels of mature chondrocyte markers such as Sox9, Aggrecan, and Type II collagen were higher in ASCs treated with each of the three drugs, whereas the expressions levels of the hypertrophic chondrocytes markers RUNX2 and Type X collagen were increased only in cells treated with two of the drugs, Drug 7 and 51, and not in ASCs treated with Drug 138, as shown in Figure 4. As for the differentiation of chondrocytes, the increased expression levels of markers of mature chondrocytes are important. However, it is also important to identify a drug that did not induce the characteristics of hypertrophic chondrocytes. In this experiment, only Drug 138 increased the expression of markers of mature chondrocyte while not inducing markers characteristic of hypertrophic chondrocytes.

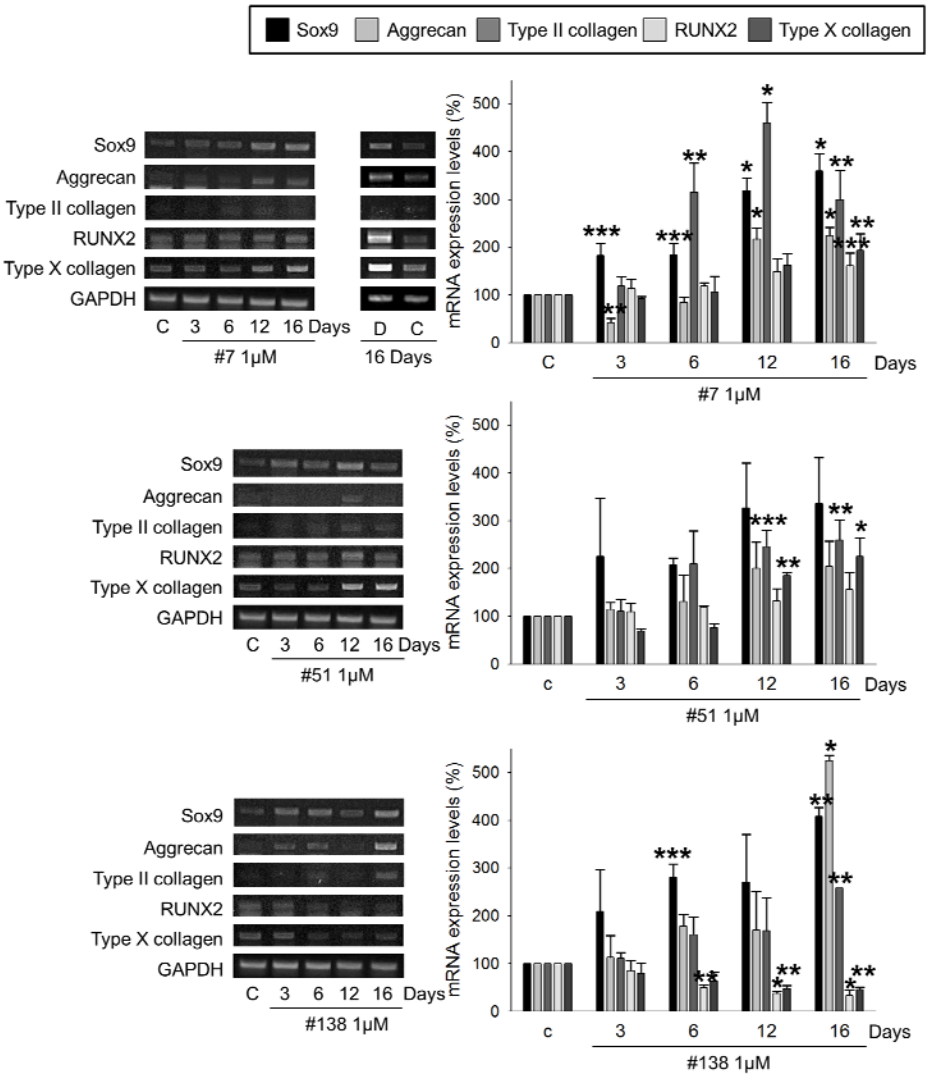


Figure 4. Expression of the mature and hypertrophic chondrocyte marker in Drug 7, Drug 51, and Drug 138 treated ASCs. The mRNA expression of mature chondrocyte marker; Sox9, Aggrecan, and Type II collagen, and hypertrophic chondrocyte marker; RUNX2, Type X collagen, and GAPDH is detected by RT-PCR. mRNA expression levels were normalized by GAPDH.

#7, #51, #138: Drug 7, Drug 51, Drug 138-treated ASCs, C: control, D: DMSO.

*p<0.001 **p<0.01***p<0.05 compared to Control.

3. Drug 138 (ELPC) induces chondrocyte differentiation in Adipose-derived stem cells.

A. Chondrocyte differentiation of Adipose-derived stem cells by ELPC in 2D cultures

Drug 138, a drug is also known as Ellipticine (ELPC), is a natural tetracyclic compound that is known to inhibit topoisomerase II via intercalative binding to DNA (Figure 5A). In cancer, ELPC has been used as a drug to induce cell death because it inhibits topoisomerase II.[54] Therefore, it was first confirmed the cell cytotoxicity using ASCs prior to continuing experiment. Because a concentration of 5 μM or higher is generally used to induce cell death, a wide range, from low to high concentration was selected. Cell viability was evaluated for up to 48 hr, and 80% or more of ASCs were induced to undergo cell death was induced in 10μM of ELPC-treated ASCs. However, it was confirmed that cell death was observed to be less than 10% in 1μM of ELPC-treated ASCs. (Figure 5B)

To confirm the chondrocyte differentiation ability of ELPC to induce the differentiation of ASCs into chondrocytes, an Alcian blue staining was performed after ASCs were incubated with ELPC for 16 days. Alcian blue was a blue-stained drug associated with the sulfated glycosaminoglycan of the cartilage matrix, confirming that ELPC-treated ASCs showed much more blue positive and condensed area than that of untreated ASCs in Figure 6.

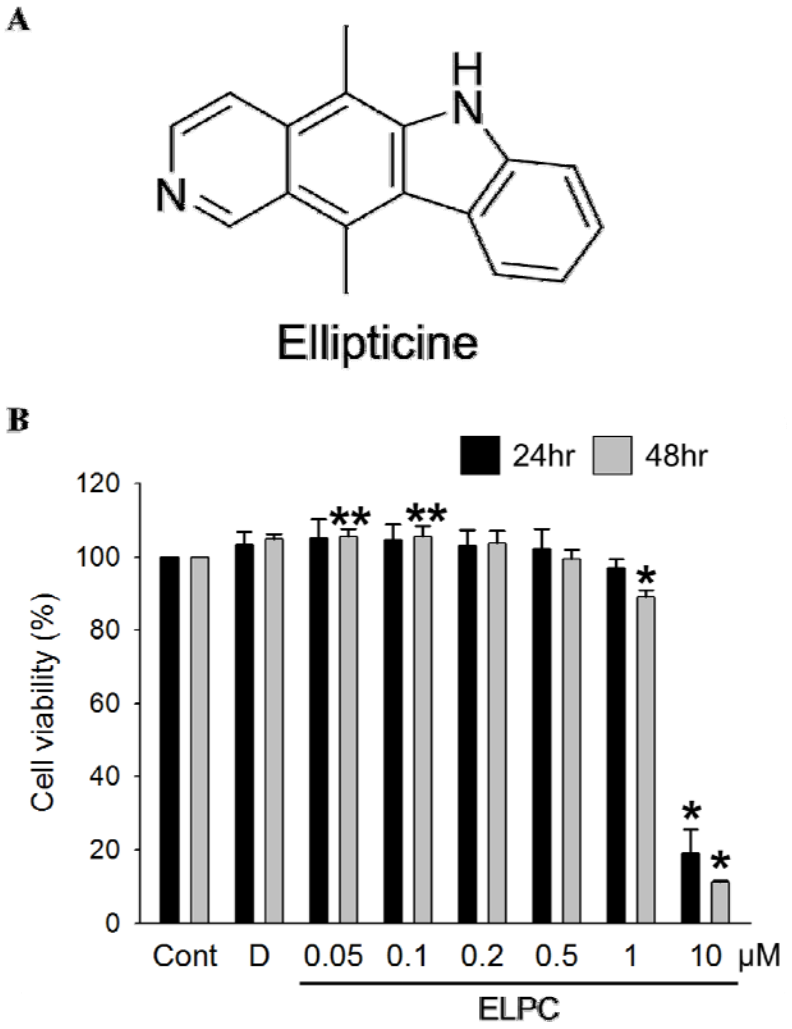


Figure 5. Structure of Ellipticine and concentration-dependent cell viability.

(A) The figures about the structure of Ellipticine (B) Cell viability was measured by cell viability assay. Cont: control, D: DMSO, *p<0.001, **p<0.05 compared to control.

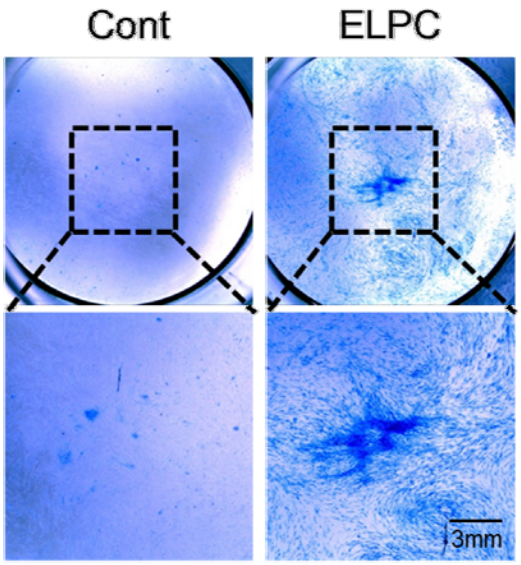
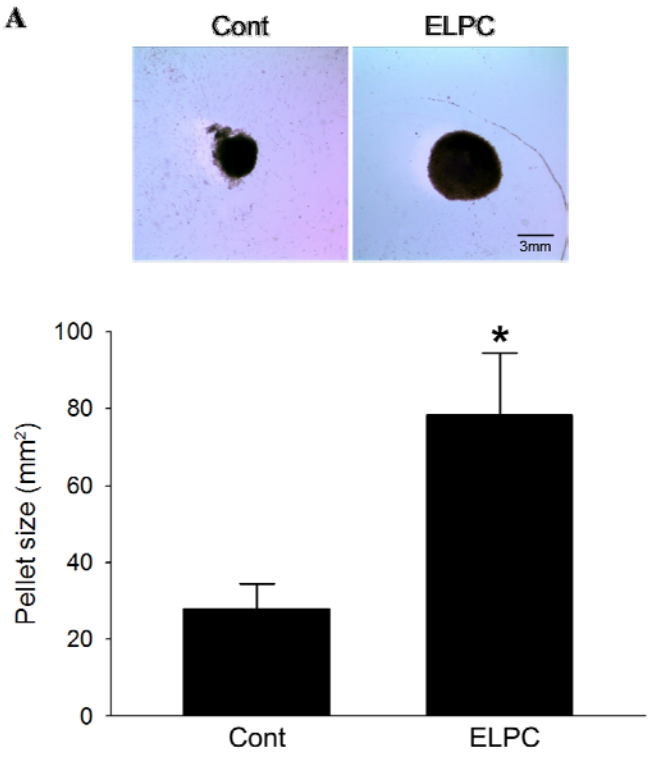


Figure 6. Induction of chondrocyte differentiation in ELPC-treated ASCs.

ELPC-treated ASCs were stained using Alcian blue staining analysis after 16 days. Cont: control, ELPC: Ellipticine-treated ASCs, Scale bar: 3 mm.

B. Chondrocyte differentiation of Adipose-derived stem cells by ELPC in 3D pellet cultures

A 3D pellet culture system was used to confirm that stable cartilage would form in an appropriate differentiation environment. When cartilage formation proceeds in a stable and smooth manner, the pellet will keep a rounded shape while it grows. ASCs were centrifuged in a 15 ml conical tube, and the resulting pellet was treated with ELPC every 3 days. The size of the pellet was observed for 16 days. A rounded pellet was maintained better in the ASCs treated with ELPC than in the untreated ASCs. The diameter and total size of the pellet were also increased by treatment with ELPC (Figure 7A). The expression levels of Aggrecan, Type II collagen, and Type X collagen were confirmed in the untreated and ELPC-treated ASC pellets using immunofluorescence. In the ASC pellets treated with ELPC, the expression levels of Aggrecan and Type II collagen were increased. However, the expression of Type X collagen was not altered, similar to the results of the *in vitro* experiments, as shown in Figure 7B.



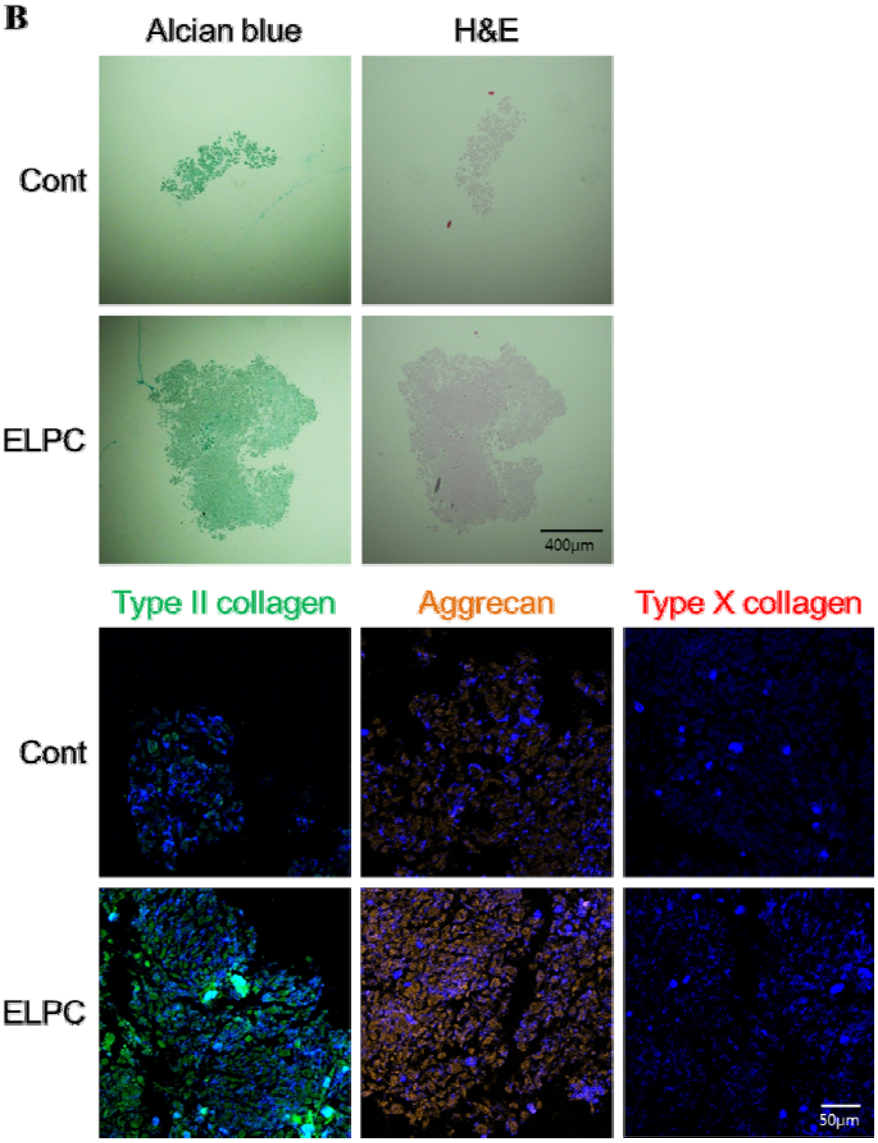


Figure 7. 3D pellet formation and expression of the chondrocyte marker in the pellet. (A) 3D pellet culture was performed using ASCs or ELPC-treated ASCs during 16 days. (B) The ASCs and ELPC-treated ASCs pellet were stained using Alcian blue and H&E. And the expression of Type II collagen,

Aggrecan, and Type X collagen was detected by immunofluorescence. Nuclei were stained with DAPI (Blue). Cont: control, ELPC: ELPC-treated ASCs, Scale bar: upper 400 μm and lower 50 μm. *$p<0.01$ compared to control.

C. The regulation of various extracellular matrix-degrading enzymes by ELPC

Hypertrophic chondrocytes are known to release ECM-degrading enzymes, such as MMPs and ADAMTSs. MMPs and ADAMTSs consist of various subtypes, and of these, MMP13, ADAMTS4, and ADAMTS5 are enzymes that are characteristic of hypertrophic chondrocytes.[55,56] Therefore, it was confirmed whether the mRNA expression levels of these three ECM-degrading enzymes were regulated by ELPC. The mRNA expression levels of MMP13, ADAMTS4, and ADAMTS5 were decreased in a time-dependent manner by treatment with ELPC (Figure 8). ADAMTS, which are released as active proteases, play a role in turning over aggrecan and procollagen during physiological and pathological processes including angiogenesis and arthritis.[57,58] To evaluate the activity of the ADAMTS4 and ADAMTS5 molecules that were secreted in the medium, the cells were treated with ELPC every 3 days. After 16 days, the medium of the differentiated ASCs was collected, and ELISA was performed. The activity levels of ADAMTS4 and ADAMTS5 was decreased in the medium of ASCs treated with ELPC and increased in the ASCs treated with only DM (Figure 9).

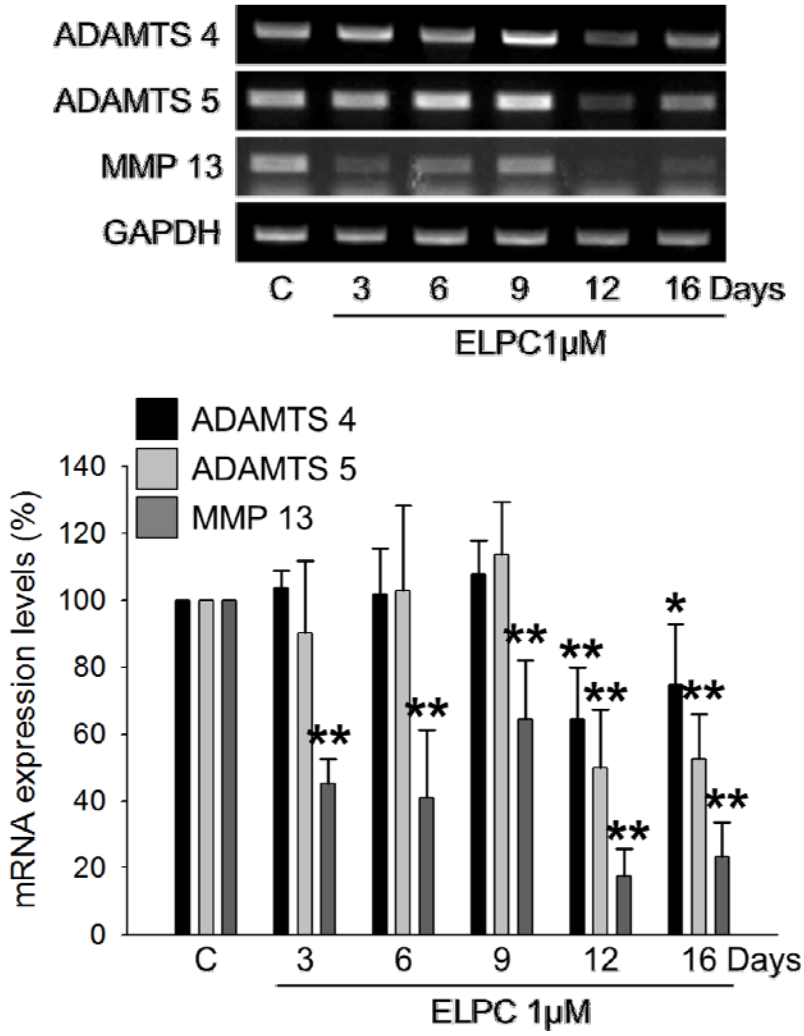


Figure 8. The expression of extracellular matrix degradation enzymes The expression of ADAMTS4, ADAMTS5, and MMP13 were measured by RT-PCR. mRNA expression was normalized by GAPDH. C: control, *p<0.05 **p<0.001 compared to control.

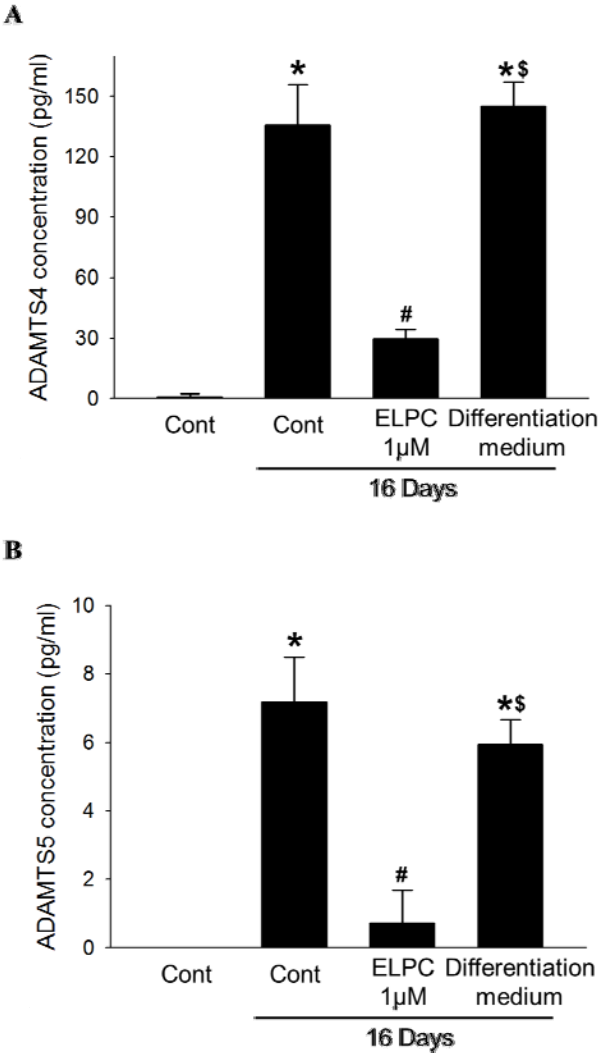


Figure 9. The activity of ADAMTS4 and ADAMTS5. The ADAMTS4 and ADAMTS5 protein activity of the cell culture supernatant were measured by ELISA. Cont: control. *p<0.001 compared to control, #p<0.001 compared to 16 days control, $p<0.001 compared to ELPC-treated ASCs.

4. ELPC regulates the expression of Sox9 by regulating p53 during chondrogenesis

A. Selection of transcription factors attached to the Sox9 promoter region by ELPC

To investigate whether ELPC increases the expression of Sox9 by increasing the expression of the transcription factors that attach to the promoter region of Sox9, transcription factors that are expected to adhere to the promoter of Sox9 were identified. Several transcription factors were selected that were predicted to bind to the Sox9 promoter region by the PROMO 3.0 program (Figure 10).

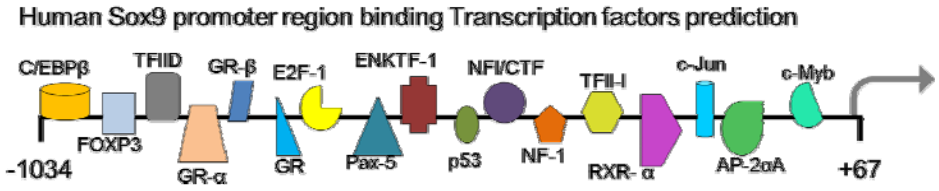


Figure 10. Prediction of transcription factors binding to Sox9 promoter.

The figure about list of transcription factors predicted to bind a Sox9 promoter region.

B. The induction of p53 in the ELPC-treated ASCs

First, it was determined whether a relationship exists between ELPC and the transcription factors predicted to attach to the Sox9 promoter region. Among the predicted transcription factors, only p53 was found to be associated with ELPC. ELPC was previously reported to regulate p53 translocation in cancer cells, leading to apoptosis.[13] Another study demonstrated that ELPC recovered the transcriptional function of a mutant p53.[59] Therefore, it was sought to determine whether p53 is regulated by ELPC in ASCs. After ASCs were treated with ELPC, the mRNA and protein expression levels of p53 were measured over time. Both the mRNA and protein expression levels of p53 increased for up to 12 hr (Figure 11).

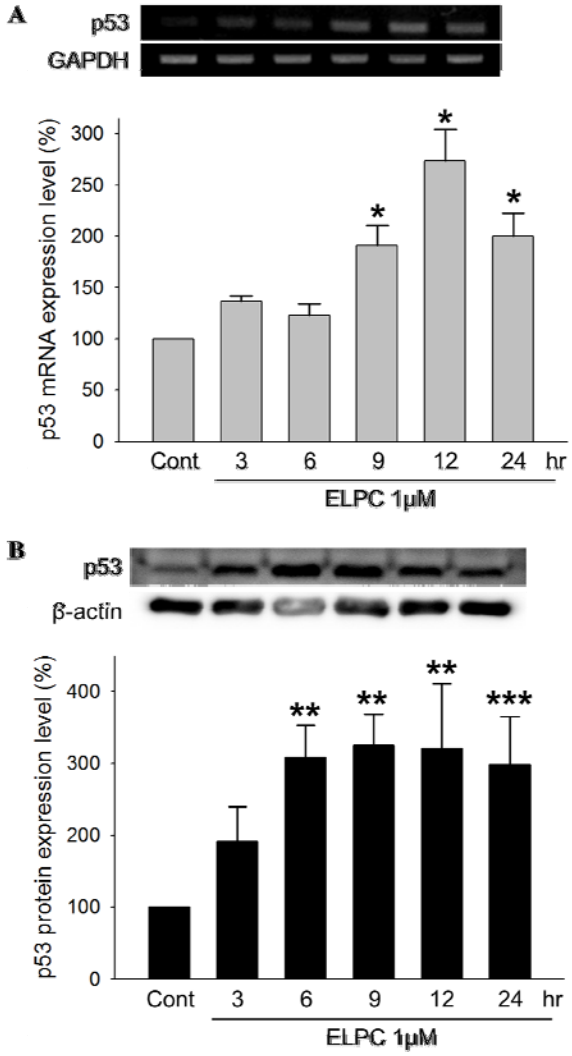


Figure 11. The expression of p53 in ELPC-treated ASCs. (A) and (B) The p53 mRNA and protein expressions were measured by RT-PCR and western blot analysis, respectively. p53 mRNA and protein expressions were normalized by GAPDH and β-actin. Cont: control, *p<0.001 **p<0.01***p<0.05 compared to Control.

C. The nucleus translocation of p53 in ELPC-treated ASCs

The expression of p53 was evaluated in isolated nuclear and cytosolic samples to confirm its expression and that it is transferred to the nuclear compartment, where it acts as a transcription factor. When the translocation of p53 was tested at the same time, I confirmed that its expression level was shifted toward the nucleus, similar to its previously observed increasing pattern. The expression of p53 was initially increased in both the nucleus and the cytosol, but it was decreased in both the nucleus and the cytosol at 12 hr (Figure 12).

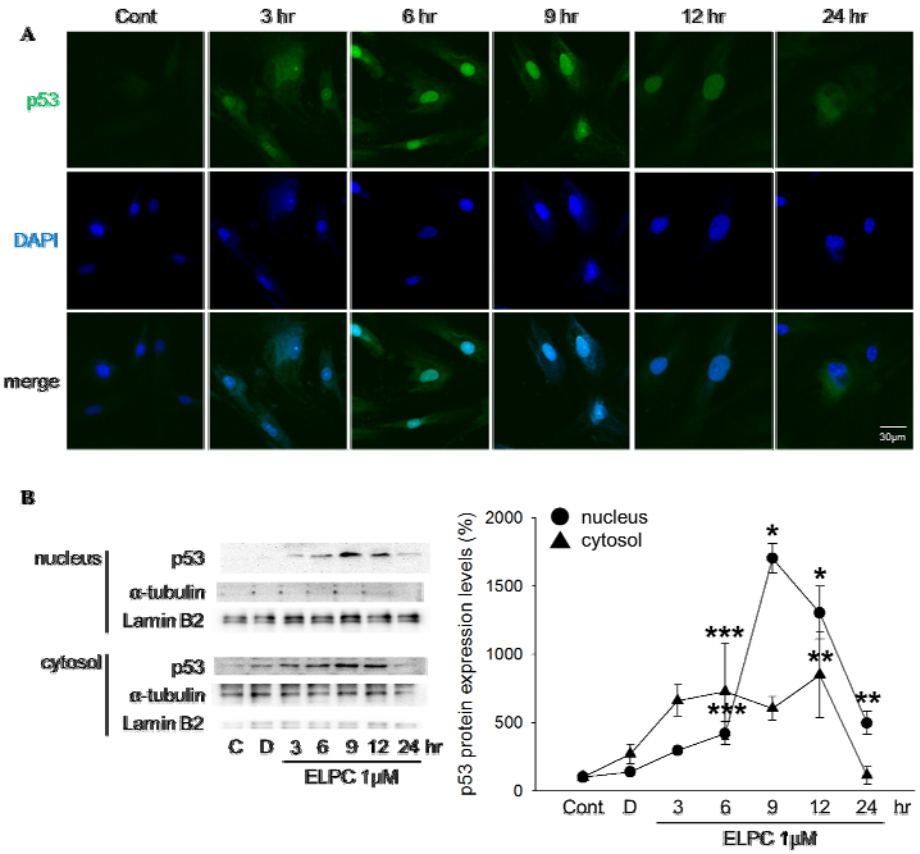


Figure 12. The translocation of p53 in ELPC-treated ASCs. (A) The translocation of p53 was confirmed by immunofluorescence staining. p53 was detected by FITC green fluorescence. The nucleus was stained with DAPI (Blue). Scale bar: 30 μm (B) ELPC-treated ASCs for 24hr were separated into nucleus and cytosol fractions. p53 was quantified and normalized with Lamin B2 and α-tubulin for the nucleus and cytosol, respectively. C and Cont: control, D: DMSO-treated ASCs, *p<0.001 **p<0.01 *p<0.05 compared to Control.

D. ELPC increase the expression of Sox9 by regulating of p53

To confirm the association between ELPC and both p53 and Sox9, an experiment was conducted in which a siRNA system was used to inhibit the expression of p53. First, a p53 siRNA was applied at various concentrations to determine which concentration would most effectively suppress p53 expression. The expression of p53 decreased as the concentration of the p53 siRNA increased (Figure 13A and 13B). Alternatively, the expression of Sox9 was inhibited by the inhibition of p53, and the expression of Sox9 was suppressed in ASCs transfected with the p53 siRNA (Figure 13C). The expression of Type II collagen was also decreased in ASCs transfected with p53 siRNA (Figure 13D). The results confirmed that the p53-induced inhibition of Sox9 reduced the production of Type II collagen and consequently inhibited differentiation into chondrocytes. These results indicate that ELPC induces Sox9 expression by regulating the expression of p53.

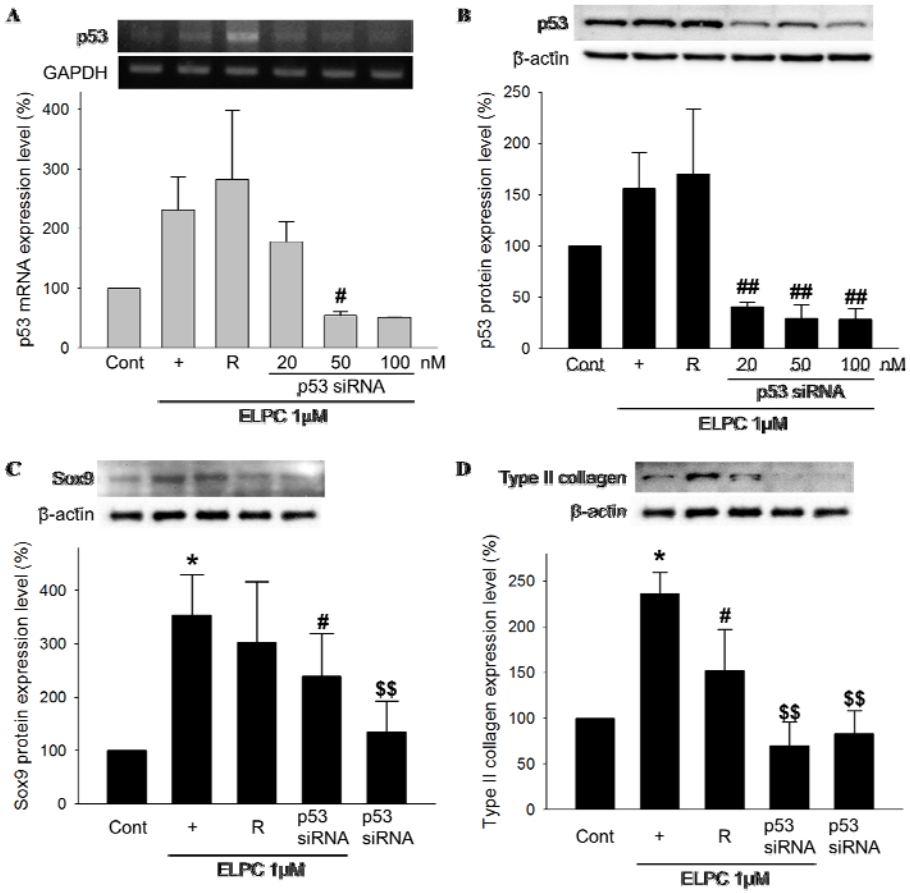


Figure 13. The regulatory role of p53 in Sox9 expression. ASCs were transfected with p53 siRNA for 24 hr and then treated with ELPC during 12 hr. (A) mRNA and (B) protein expression levels of p53 were measured by RT-PCR and western blot analysis, respectively. (C) The 50 nM of p53 siRNA-transfected ASCs for 24 hr were treated with ELPC during additional 24 hr. The protein expression level of Sox9 was measured using western blot analysis. (D) The 50 nM of p53 siRNA-transfected ASCs for 24 hr were then treated with ELPC every 3 days for 16 days. mRNA and protein expression

levels were normalized by GAPDH and β-actin, respectively. Cont: control, R: reagent, *p<0.001 compared to Control, #p<0.05 ##p<0.001 compared to ELPC-treated ASCs, $$p<0.001 compared to Reagent-transfected and ELPC-treated ASCs.

E. Regulation of p53 phosphorylation site by ELPC

For p53 to become active, it must be phosphorylated. The p53 protein has many phosphorylation sites, and different results are induced by activity at each phosphorylation site.[60] To determine which p53 phosphorylation site is activated by ELPC, each of the p53 phosphorylation sites was checked. A reaction was confirmed at 2 out of the 8 phosphorylation sites. One was ser15, and the other is ser392. The phosphorylation of ser15 was decreased, whereas that of ser392 was increased (Figure 14).

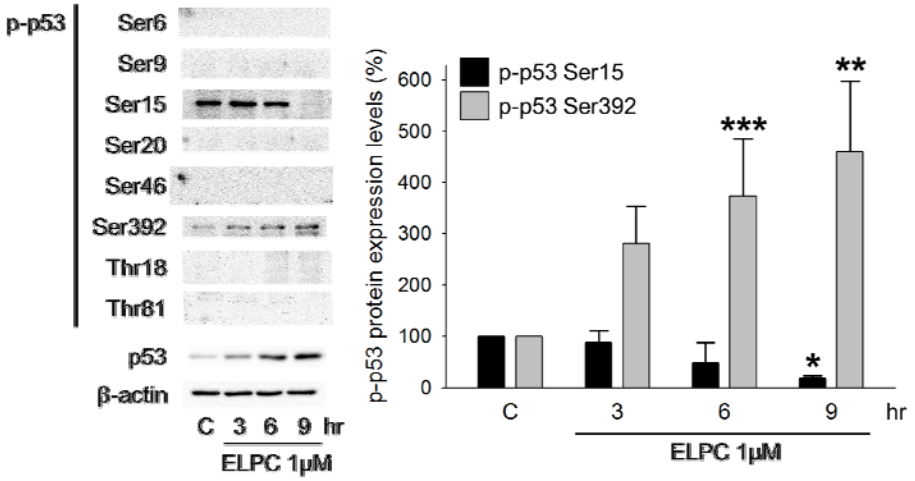


Figure 14. Different phosphorylation of p53 in ELPC-treated ASCs. The expression and phosphorylation status of p53 was detected by western blot analysis in the ELPC-treated ASCs. Phosphorylated p53 protein expression levels were normalized by β-actin. C: control, *p<0.001**p<0.01***p<0.05 compared to control.

5. Protective effects of ELPC in an osteoarthritic animal model

Next, it was sought to confirm that ELPC exerts a cartilage-protecting effect on ASCs, which can be induced to differentiate into chondrocytes, in an induced-OA animal model. ASCs or ASCs differentiated by ELPC into chondrocytes were injected into an induced-OA animal model after one week of treatment with Type II collagenase. After six weeks, safranin O staining was performed to evaluate the level of cartilage damage and to identify any protective effects. In the OA-induced animal model, cartilage damage was more severe than that observed in control animals. In addition, less cartilage damage was observed in both the untreated ASC- and ELPC-treated ASC-injected groups (Figure 15). Markers of normal and damaged cartilage were also evaluated in the same model. The expression levels of both type II collagen and Aggrecan were higher while the expression level of Type X collagen was lower in the ELPC-treated ASC-injected group than in the untreated ASC-injected group (Figure 16).

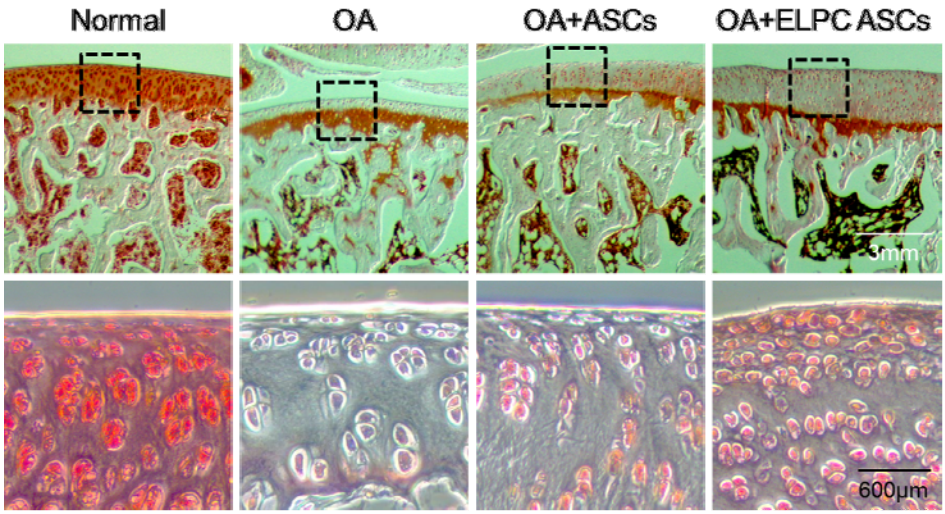


Figure 15. Recovery of damaged cartilage by ELPC-treated ASCs in OA-induced animal model. Safranin O staining in paraffin sections of OA induced animal model. Representative images show cartilage damage after 6 weeks of OA induction. Safranin O binds to glycosaminoglycan in cartilage and stains its red color. The lower figure shows an enlargement of cartilage part in the upper figure. Scale bar: upper 3mm and lower 600μm.

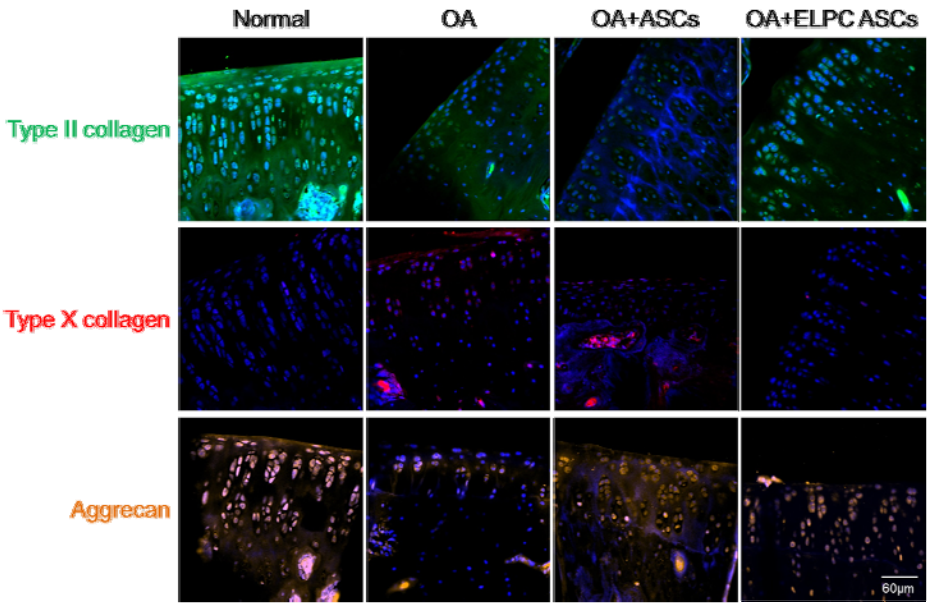


Figure 16. Detection of chondrocyte and hypertrophic chondrocyte marker in OA-induced animal model. Type II collagen (FITC, Green), Aggrecan (Texas red, Red), and Type X collagen (Rhodamine, Orange) expressions were measured by Immunofluorescence staining and visualized with a confocal microscope. Each section was co-stained with DAPI (Blue) for nucleus staining. The images in the same region of tissue were merged into the overlapped image. Scale bar: 60 μm.

IV. DISCUSSION

The results of this study suggest that ELPC plays crucial roles in regulating ASC differentiation into chondrocytes. It was founded that ELPC upregulates the expression of Sox9 by increasing the expression and activation of p53. In addition, ASCs differentiated by ELPC did not exhibit the characteristics of hypertrophic chondrocytes.

Articular cartilage is a type of permanent hyaline cartilage (i.e., the types being hyaline, elastic, and fibrous cartilage). Unlike temporary cartilage, which is present during embryonic development and in growth plates and eventually differentiates into bone, permanent cartilage is maintained as an unossified cartilage and does not undergo terminal differentiation.[61] Both temporary and permanent cartilage has a similar cellular origin. Chondrocytes are found only in the articular cartilage, are located separate from the cartilage matrix and retain the tensile strength and flexibility to the articular surface by secreting ECM proteins, such as collagen, proteoglycan, and glycosaminoglycan, which constitute the ECM.[40,53] The chondrocytes located in the growth plate produce a matrix capable of undergoing mineralization. These functional differences account for the differences in the phenotypes of mature and hypertrophic chondrocytes.[62]

The damage to articular surface collagens is initiated by injury or excessive mechanical load and enzymatic cleavage. This phenomenon leads the cells to change phenotypes from mature into hypertrophic chondrocytes. Recent studies

have also shown that changes in ECM molecules or their activation by inflammatory cytokines may play roles indirectly activating the phenotypic change from chondrocytes into hypertrophic chondrocytes.[28,34] The change into hypertrophic chondrocytes observed in OA results in the production and release of enzymes, such as MMP13 and aggrecanases (ADAMTS) that digest ECM components.[63] The increases in alkaline phosphatase and pyrophosphate levels observed in the hypertrophic chondrocyte layer have been associated with tissue calcification.[37,64-66] Finally, type X collagen is a standard marker of hypertrophic chondrocytes that have been detected in OA cartilage and is not expressed in normal healthy articular cartilage.[6,37]

In an effort to treat OA, cartilage tissue engineering techniques have explored methods to separate chondrocytes or chondrocyte precursors obtained from tissue biopsies, to increase the number of cells they form in cell cultures, to place the cells on a 3D scaffold that can be incubated for a period of time to form a structure that can be placed inside the articular cartilage of patients. However, chondrocytes tend to dedifferentiate into fibroblastic chondrocytes during subculture. The fibroblastic chondrocytes that were converted from chondrocytes express type I collagen, type III collagen, and low molecular weight proteoglycans (i.e., biglycan and decorin) instead of type II collagen and high molecular weight proteoglycans (Aggrecan).[67,68] To minimize their conversion to fibroblastic chondrocytes, chondrocytes must be cultured in a single layer, which requires many cells.[69] A number of problems occur when

chondrocytes are isolated *in vitro*, as described above, and this is an issue that must be resolved before clinical applications can be developed.

Additionally, previous studies have used pluripotent stem cells to regenerate cartilage because they can be differentiated into various components of musculoskeletal systems, including bone cells, chondrocytes, and adipocytes. There is a method to insert isolated treated or untreated stem cells to induce differentiation into chondrocytes. In addition, recent cell-based techniques that utilize various three-dimensional carrier systems, such as alginate, fibrin, hyaluronate, collagen gels, or biodegradable macromolecular polymers that mimic the physiological milieu, have demonstrated promising results.[46] Directly injecting stem cells has resulted in problems including low tissue compatibility, which causes calcification and the formation of fibrous tissues in addition to a low rate of differentiation into chondrocytes. Consequently, recent studies have explored factors that can potentially induce the formation of chondrocyte-like cells. Certain cocktails containing several growth factors have been shown to induce chondrocytes to form from stem cells.[25,43,70,71] Chondrocytes differentiated from stem cells express many biomolecules typical of hyaline cartilage, such as type II collagen, proteoglycans, and aggrecan. However, incorrect proportions of some combinations of these growth factors failed to produce normal articular cartilage. The amounts of collagen and proteoglycan expressed by differentiated chondrocyte-like cells are only about half the amounts that are found in normal cartilage, and this reduction negatively affects

its tensile strength and load-carrying capacity.[42,72,73] In addition, studies have been conducted to identify small molecules that selectively induce stem cells to differentiate into chondrocytes to support cartilage reconstruction.[74,75] However, the chondrocyte-like cells that were differentiated from stem cells in these experiments exhibited hypertrophic chondrocyte characteristics.

The small molecule used in this study was screened from the library of kinases activity modulators and natural products derived compounds. The kinase activity modulators used in the experiments are inhibitors of six major subfamilies; TK (tyrosine kinase families), TKL (tyrosine kinase-like families), CMGC (CDK, MAPK, GSK3, CLK families), CAMK (Ca/calmodulin dependent protein kinase), AGC (PKA, PKG, and PKC families), and CKI (Casein kinase family). And these protein kinases are known to modulate wide range of biological activities, including cell metabolism, cell cycle regulation, apoptosis, and importantly differentiation.[76-78] For example, the small molecule which inhibited glycogen synthase kinase 3, has been shown to induce neurogenesis.[79,80] Mitogen-activated protein kinase also regulate differentiation of stem cell into the osteogenic and adipogenic lineage.[81]

In this study, three candidate small molecules were selected because they increased the expression of Sox9 (Figure 3 and 4). I found that ELPC caused ASCs to differentiate into chondrocytes. These differentiated chondrocytes exhibited the characteristics of chondrocytes in normal cartilage. The expression levels of Sox9, type II collagen, and aggrecan and the rate of

aggregation were higher in the differentiated chondrocytes. Furthermore, the ELPC-induced chondrocytes did not exhibit the characteristics of hypertrophic chondrocytes (Figure 4). The mRNA expression levels of runx2 and type X collagen in addition to mmp13 and ADAMTS4 and ADAMTS5, which are matrix-degrading enzymes, were lower in the ELPC-treated ASCs. These results indicate that ELPC induced the differentiation of ASCs into chondrocytes and that the differentiated chondrocytes displayed the characteristics of mature chondrocytes in normal cartilage and not the characteristics of hypertrophic chondrocytes.

Drug 7 and 51 are an adenosine kinase inhibitor and GSK-3β inhibitor, respectively. Adenosine kinases have been studied for their ability to induce apoptosis when they are excessively produced by chondrocytes. A previous study confirmed that this decrease in cell death was inhibited when adenosine kinase was suppressed by inhibitors.[82] Additionally, inhibiting adenosine kinase reduced the amount of cartilage damage that was induced by inflammatory cytokines such as IL-1β and LPS.[83] However, while previous studies have failed to found any association with Sox9, it has been shown that apoptosis can be reduced by inhibiting adenosine kinase in chondrocytes. GSK-3β induced the phosphorylation of Sox9, which resulted in binding between FBW7 and Sox9. This led to an increase in the ubiquitination of Sox9 and consequently decreased Sox9 expression, leading to cell death.[84] In this study, the authors confirmed that the inhibition of Sox9 expression was suppressed when GSK-3β was

inhibited by an inhibitor. By confirming the relationship between GSK-3β and Sox9, it was confirmed that the expression of Sox9 could be increased by suppressing GSK-3β in these cells. These data confirm that it is possible to increase the expression of Sox9 using a GSK-3β inhibitor and that these markers may represent additional candidates as long as they do not also induce hypertrophic characteristics.

Drug 138 is an ellipticine and an alkaloid that was first extracted from the species *Ochrosia elliptica* and *Rauvolfia sandwicensis*. Ellipticine has been reported to inhibit the enzyme topoisomerase II via intercalative binding to DNA[54,85], to inhibit p53 ubiquitination and to increase the nuclear localization of endogenous p53.[59] Hence, most previous studies of ellipticine have explored its effects on a variety of cancers.

p53 is an important tumor suppressor that affects the cellular response to DNA damage and apoptosis when cells are exposed to genotoxic stressors. Recent studies have also confirmed that p53 regulates the differentiation of embryonic stem cells and induces pluripotent stem cells in response to genomic damage. p53 uses a different mechanism to regulate activated and repressed genes.[70] In differentiated embryonic stem cells and induced pluripotent stem cells, p53 induces the expression of differentiation-linked genes by suppressing the expression of pluripotent-linked genes.[71] p53 also acts as a transcription factor to affect the expression of various proteins. The p53 protein has several phosphorylation sites, including ser6, ser9, ser15, ser392, and thr18, and each

phosphorylation site is affected by several different protein kinases.[86]

In ASCs treated with ELPC, it was found that the p53 phosphorylation sites that were affected were ser15 and ser392. In these cells, the phosphorylation of ser15 was decreased while the phosphorylation of ser392 was increased by ELPC in a time-dependent manner (Figure 14).

In the cartilage, ser15 of p53 is activated by p38 kinase and plays a role in shear strain-and NO-induced apoptosis in chondrocytes.[87,88] In shear strain-induced apoptosis, the expression levels of p53 and p53AIP-1 were higher in OA chondrocytes than in normal chondrocytes. In addition, in NO-induced apoptosis, the ser15 region of p53 was phosphorylated by p38, resulting in the induction of chondrocyte apoptosis. However, I also found that the phosphorylation of ser15 of p53 was decreased in ELPC-treated ASCs, and this did not seem to affect activated p38-induced apoptosis. Although the results of this study demonstrate that among several known p53 phosphorylation sites, only ser392 phosphorylation was increased, this result has not yet been explored in chondrocytes and other cell types. Therefore, further study is needed to explore the effects induced by the phosphorylation of ser392 in chondrocytes.

in vivo studies demonstrated that cartilage damage was able to restored in rats injected with ASCs differentiated by ELPC (Figure 15). In addition, the factors of mature chondrocytes were increased and the factor of hypertrophic chondrocytes was not increases, and it was confirmed that OA was inhibited by suppression of the decrease of hypertrophic chondrocytes (Figure 16). Although

it has not been confirmed whether the differentiated ASCs are directly adhered to the cartilage to regenerate or to be improved by the secreted molecules from the transplanted ASCs, further investigation is needed to confirm this. In addition, since there is a stem cell in synovium, it is also necessary to confirm the effect of treating ELPC alone to recover OA in *in vivo* study.

V. CONCLUSION

In the present study, the results demonstrate that ELPC induces ASCs to differentiate into chondrocytes by increasing Sox9, which was itself mediated via the over-expression of p53 and its translocation into the nucleus. In addition, it has been confirmed that ELPC exerted a protective effect in an OA-induced animal model by inducing ASCs to differentiate into the appropriate type of chondrocytes. Taken together, these results show that ELPC is a potentially new therapeutic agent for OA. The therapeutic efficacy of this drug should encourage further studies of the effects of small molecular compounds on chondrocyte differentiation from ASCs. The results of this study may, therefore, be useful in future therapeutic strategies aimed at treating OA.

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ABSTRACT (IN KOREAN)

Sox9 발현유도 저분자 화합물에 의한 지방유래 줄기세포의 기능성 연골세포로의 분화

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이 지 윤

퇴행성 관절염은 관절 연골 및 관절 밑의 뼈의 붕괴로 인해 발생되는 일반적인 관절 질환이다. 관절 연골에 손상이 발생할 때, 연골세포는 비대화 연골세포로 형질변화가 유도되어 RUNX2 및 X 형 콜라겐의 발현을 증가시키게 된다. 비대화 연골세포는 세포 외 기질을 분해하는 효소인 MMP13 및 ADAMTS4 와 ADMATS5를 분비하여 관절의 분해 및 석회화를 유도한다. 성인의 연골과 연골세포는 자체적으로 재생 능력이 없기 때문에 퇴행성 관절염을 치료하기 위하여 다양한 방법들이 시행되어 왔다. 최근 연구들에서 줄기세포를 사용한 퇴행성 관절염 치료법을 연구해오고 있다. 지금까지 줄기세포를 기능성 연골세포로 분화 유도하기 위해 특정 인자들의 발현조절, microRNA, 저분자 화합물을 이용하여 다양한 방법을 시도해왔다. 그러나, 현재까지 줄기세포에서 기능성 연골세포로의 분화 유도 연구에 있어 가장 큰 문제점은 분화된 연골세포에서 비대화 연골세포의 특성이 나타나는 점이다. 따라서, 줄기세포를 이용한 비대화 연골세포의 특성을 갖지 않는 기능성 연골세포로 분화 유도하기 위한 최적의 방법을 개발하는 것이 중요하다. 관절 연골의 발달 과정 초기에 Sox9이 발현되어 연골과

연골세포를 구성하는 표지인자들의 발현을 증가시키는 중요한 역할을 한다. 이전의 연구에서 Sox9의 발현을 제거한 마우스 모델을 사용하여 연골형성이 발생하지 않음을 증명하였다. 또한 Sox9의 과발현을 통해 줄기세포에서 연골세포로의 분화를 유도하였다. 따라서 본 연구에서는 퇴행성 관절염의 치료를 위해 Sox9의 발현을 조절 할 수 있는 저분자 화합물을 이용한 줄기세포로부터 기능성 연골세포로의 분화 유도에 초점을 맞추었다. 먼저, 연골세포와 줄기세포에서의 기본적으로 발현되는 Sox9의 양적 비교를 통해 Sox9의 발현 증가와 연골세포로의 분화 유도의 연관성을 확인하였다. Sox9 프로모터가 붙은 GFP 벡터를 이용하여 Sox9의 발현을 증가시키는 약물들을 1차 선별하였고, 선별된 약물을 처리하였을 때 연골세포로의 분화유도 여부와 비대화 연골세포의 특성을 나타내지 않는 Ellipticine이라고 알려진 138번이 최종 선별되었다. Ellipticine 처리된 줄기세포는 성숙된 연골세포에서 발현되는 II 형 콜라겐과 Aggrecan의 발현이 증가되어있었으며, 비대화 연골세포에서 발현되는 RUNX2와 X형 콜라겐, 세포 외 기질은 분해하는 효소인 MMP13 및 ADAMTS4와 ADAMTS5의 발현이 감소되어 있는 것을 확인할 수 있었다. 또한 Ellipticine은 p53의 발현을 증가시키고 핵으로의 전좌를 증가시킴으로써 p53의 활성을 조절하여 Sox9의 발현을 증가시킨 다는 것을 확인하였다. Collagenase에 의해 유도된 퇴행성 관절염 동물 모델에서, 줄기세포로부터 분화 유도된 연골세포를 처리한 그룹이 처리되지 않은 줄기세포를 주입한 그룹보다 효과적으로 손상된 연골을 회복시키는 것을 확인하였다. 따라서 본 연구 결과는 새로운 연골세포로의 분화 가능한 새로운 저분자 화합물의 확인을 통해 퇴행성 관절염 치료에 사용 가능한 줄기세포로부터 분화된 기능성 연골세포의 치료제로써의 가능성을 제시하였다.

핵심되는 말: 퇴행성 관절염, 지방유래 줄기세포, 연골세포, 저분자 화합물