2022년 2월
석사학위논문

# Chondrocyte oxiapoptophagy mediated by 7α,25-DHC-EBI2 axis in IL-1β-induced arthritis

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서 정 연

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관절염에서 7α,25-DHC-EBI2 경로로 매개되는
연골세포의 oxiapoptophagy

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이 논문을 이학 석사학위신청 논문으로 제출함.

2021년 10월

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서 정 연

# 서정연의 석사학위논문을 인준함

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# LIST OF ABBREVIATIONS

25-HC ····· 25-hydroxycholesterol
7α,25-DHC ····· 7α,25-dihydroxycholesterol
ANOVA ····· Analysis of variance
Bcl-2 ····· B-cell lymphoma 2
Bcl-xL ····· B-cell lymphoma extra-large
BSA ····· Bovine serum albumin
CH25H ····· Cholesterol-25-hydroxylase
Col II ····· Type II collagen
COX-2 ····· Cyclooxygenase-2
CYP ····· Cytochrome P450
CYP7B1 ····· Cholesterol by cytochrome P450 family 7 subfamily B member 1
DAPI ····· 4’,6-Diamidino-2-phenylindole dihydrochloride
DMEM/F-12 ····· Dulbecco’s modified eagls’s medium/nutrient mixture F-12
DMSO ····· Dimethyl sulfoxide
EBI2 ····· Epstein-Barr virus-induced G-protein-coupled receptor 2
ECM ····· Extracellular matrix
EDTA ····· Ethylenediaminetetraacetic acid
FBS ····· Fetal bovine serum
GPx ····· increase glutathione peroxidase
H&E ····· Hematoxylin & eosin
$H_2$DCFDA ····· 2′,7′-Dichlorodihydrofluorescein diacetate
IACUC ····· Institutional Animal Care and Use Committee
ICC ····· Immunocytochemistry
IHC ····· Immunohistochemistry
IL-1β ····· Interleukin-1β
iNOS ····· inducible nitric oxide synthase
LC3 ····· Microtubule-associated proteins 1A/1B light chain 3B
MMP ····· Matrix metalloproteinase

MTT ····· Dimethyl thiazolyl diphenyl tetrazolium salt
NO ····· Nitric oxide
OA ····· Osteoarthritis
PARP ····· Poly ADP-ribose polymerase
PBS ····· Phosphate buffered saline
PCR ····· Polymerase chain reaction
$PGE_2$ ····· Prostaglandin $E_2$
PVDF ····· Polyvinylidene fluoride
qPCR ····· Quantitative polymerase chain reaction
qRT-PCR ····· Quantitative real-time PCR
RA ····· Rheumatoid arthritis
RORα ····· Retinoic acid-related orphan receptor α
ROS ····· Reactive oxygen species
RT ····· Room temperature
S.D. ····· Standard deviation
SOD ····· Superoxide dismutase
SPSS ····· Statistical Package for the Social Sciences
SREBP-2 ····· Sterol regulatory element binding protein 2
TBS-T ····· Tris buffered saline with Tween 20
Δψm ····· Mitochondrial membrane potential

# 국 문 초 록

## 관절염에서 7α,25-DHC-EBI2 경로로 매개되는 연골세포의 oxiapoptophagy

서 정 연
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7α,25-dihydroxycholesterol(7α,25-DHC)은 염증 조건 하에서 cholesterol by cytochrome P450 family 7 subfamily B member 1(CYP7B1)이라는 효소에 의해 25-hydroxycholesterol(25-HC)로부터 합성되는 옥시스테롤이며, 이는 다양한 유형의 세포에서 세포사멸과 관련되어 있다. 본 연구에서는 interleukin-1β(IL-1β)와 7α,25-DHC가 유도하는 연골세포 oxiapoptophagy에서 7α,25-DHC 수용체 Epstein-Barr virus-induced G-protein-coupled receptor 2(EBI2)의 길항작용과 그 기전을 분석하였다.

IL-1β는 흰쥐 관절 연골에서 세포외 기질의 주요 구성요소인 aggrecan과 Type II collagen의 발현을 감소시켜 연골세포의 점진적인 퇴행을 증가시켰으며 세포에서 관절염의 주요 인자인 matrix metalloproteinase(MMP)-1, -3, -13과 염증 매개체인 inducible nitric oxide synthase, cycloxygenase-2, 산화질소 및 prostaglandin $E_2$의 발현을 증가시켰다. 또한, 흰쥐 관절 연골세포에서 IL-1β는 외인성 세포사멸 인자인 caspase-8과 내인성 세포사멸 인자인 caspase-9를 활성화시켜 caspase-3과 Poly ADP-ribose polymerase의 활성화를 유도하였으며, 이와 더불어 자가포식작용 바이오마커인 beclin-1과 microtubule-associated proteins 1A/1B light chain 3B의 발현 증가를 유도하여 연골세포의 생존력을 감소시켰다. 이러한 결과는 흰쥐 연골세포에서 IL-1β가 산화적 스트레스를 동반한 연속적인 caspase 활성화에 의한 세포사멸과 자가포식작용을 동시에 유도할 수 있음을 시사한다. 또한 IL-1β에 의한 연골세포 사멸 시 25-HC와 7α,25-DHC 등과 같은 옥시스테롤 합성이 동반됨을 제시하고 있

다. 이에 따라 본 연구에서는 염증 상태에서 콜레스테롤로부터 합성되는 25-HC와 그의 하위 옥시스테롤인 7α,25-DHC가 흰쥐 관절연골의 프로테오글리칸 소실의 가속화와 더불어 산화적 스트레스와 자가포식작용을 동반한 세포사멸인 oxiapoptophagy를 통해 관절연골 퇴행성을 유도한다는 것을 입증하였다. 반면 7α,25-DHC 수용체 EBI2 길항제인 NIBR189는 7α,25-DHC에 의해 유도되는 연골세포 산화작용, 세포사멸 및 자가포식작용을 억제하였다. NIBR189는 7α,25-DHC에 의해 증가된 pro-apoptotic factor 및 autophagy 바이오마커의 발현 억제를 통하여 연골세포사멸을 감소시켰으며, 생체 외 장기 배양 관절 연골에서 7α,25-DHC에 의해 매개되는 프로테오글리칸 소실을 억제하였다. 이러한 결과는 7α,25-DHC 수용체 억제 시 IL-1β에 의해 유도되는 관절연골퇴행성이 지연 또는 예방될 수 있음을 제시한다. 본 연구에서는 NIBR189는 흰쥐 관절연골에서 IL-1β가 유도하는 프로테오글리칸 소실 및 세포외 기질 성분 감소를 억제하였으며, 연골세포에서 IL-1β에 의한 연골 분해 효소 및 염증 매개체의 발현증가를 억제하였다. 또한 NIBR189는 연골세포에서 IL-1β로 유도된 세포독성과 세포사멸 및 자가포식작용을 억제하였다.

본 연구의 결과는 7α,25-DHC 수용체 EBI2의 길항작용이 IL-1β로 유도되는 연골세포 oxiapoptophagy를 억제시킬 수 있음을 시사한다. 따라서 본 연구의 결과로, EBI2 억제를 이용한 관절염 예방 및 치료전략의 방향을 제시할 수 있을 것으로 사료된다.

# Ⅰ. INTRODUCTION

Synovial joints located between bones permit the movement and mobility of the body through abduction, adduction, extension, and flexion of the body in mammals [1]. The joint is composed of two bones, articular cartilage, and synovial fluid, and is an organ that enables mobility for the human body [2].

Articular cartilage is a smooth white tissue that wraps around the ends of bones that join to form joints, and acts as a cushion that enables mobility by alleviating friction and distributing the load on the upper body [3]. Articular cartilage is mainly composed of water and chondrocytes, which are special cells with an extracellular matrix (ECM) composed of type II collagen (COL II) and aggrecan, which is a component of proteoglycan [4].

Chondrocytes maintain homeostasis, and an imbalance can lead to the degeneration of articular cartilage, inducing arthritis such as osteoarthritis (OA) and rheumatoid arthritis (RA) [5]. Although the pathophysiological risk of arthritis is multifactorial, including genetic predisposition, aging, obesity, traumatic joint injury, and joint misalignment, and is the closely related to chronic oxidative stress and inflammation in the synovial joint, its pathophysiological etiology is largely unknown [6]. Articular cartilage cell death is pathologically related to arthritis [7]. Therefore, although the survival of articular cartilage cells is a very important, and the relationship between apoptosis and inflammatory response has been discussed, the pathological mechanism of articular cartilage cell death has not been elucidated [8].

Arthritis is a chronic joint disease in which the articular cartilage of the synovial joint degenerates, leading to inflammation and pain making it difficult to keep the body moving and restricting activity [9]. Also, arthritis is a chronic joint disease in which the articular cartilage of the synovial joint degenerates, causing pain. Arthritis is caused by multifactorial factors such as aging, obesity, joint deformity, traumatic joint damage, and genetic predisposition, which is closely related to chronic oxidative stress and inflammation in the synovial joint within the synovial joint, but the exact mechanism has not been revealed [10].

Recently, a pathological association of chronic low-grade inflammation, caused by metabolic diseases and aging, with arthritis has been determined [11]. Cholesterol is an essential component of cells, and oxysterol, a cholesterol metabolite, is produced by auto-oxidation, an enzymatic reaction [12]. High levels of oxysterol are produced by metabolic diseases, such as Huntington's disease, Parkinson's disease, Alzheimer's disease, atherosclerosis, and carcinogenesis in various types of cancers, and chronic low-grade inflammation increases with aging [13-15].

In addition, oxysterol has recently been shown to play an important role in various biological processes, such as cholesterol homeostasis regulation, lipid metabolism regulation, autophagy, and apoptosis as an oxidative metabolite produced by oxidase or pro-oxygen in the cholesterol metabolism process [16]. A recent study reported that oxysterol associated with cholesterol homeostasis significantly increases in patients with arthritis [16], indicating, the relationship between oxysterol and apoptosis [17]. In addition, oxysterols overexpress reactive oxygen species (ROS) and increase glutathione peroxidase (GPx) and superoxide dismutase (SOD) [18]. Through this, the loss of mitochondrial membrane potential ($\Delta\psi m$), activation of caspase-3, degradation of poly (ADP-ribose) polymerase (PARP), reduction of B-cell lymphoma 2 (Bcl-2), and nuclei condensation and fragmentation occur, resulting in apoptosis caused by oxidative stress [19]. Autophagy also leads to the formation of large cytoplasmic vacuoles, considered autophagic vacuoles, and the activation of Microtubule-associated protein 1A/1B-light chain 3-I (LC3)-Ⅰ to LC3-Ⅱ, which is considered to be an autophagic criterion associated with autophagosome formation [20].

Recent studies have shown that 25-hydroxycholesterol (25-HC), an oxysterol synthesized by cholesterol from cholesterol 25-hydroxylase (CH25H), induces apoptosis in various types of cells, including motor neuron-like cell lines and head and neck squamous cell carcinoma FaDu cells [21,22]. Additionally, P2X7-dependent pyroptosis is induced by 25-HC in human keratinocytes NSC34 [23]. In addition 25-HC acts as an oxysterol to induce chondrocyte apoptosis through the CH25H-CYP7B1-retinoic acid-associated orphan receptor α (RORα) axis in OA [24].

Oxysterol 7α,25-dihydroxycholesterol (7α,25-DHC) is synthesized from cytochrome

P450 Family 7 Subfamily B Member 1 (CYP7B1) from 25-HC under inflammatory conditions, which is associated with apoptosis in various cell types [17]. The 7 α,25-DHC receptor epstein-Barr virus-induced G-protein-coupled receptor 2 (EBI2) is required for the generation of antibody responses and has been implicated in a variety of inflammatory diseases [25]. Regulatory elements of EBI2 expression are associated with cardiac inflammation, type 1 diabetes, and inflammatory bowel disease [26]. The expression of EBI2, CH25H, 7α,25-DHC, and CYP7B1 was found to be highly regulated under many inflammatory diseases [25]. Therefore, aim of this study was to investigate the antagonism of the 7α,25-DHC receptor EBI2 and its mechanism in 7α,25-DHC-induced chondrocyte oxiapoptophagy. In addition, this study analyzed a novel pathophysiological link between cholesterol metabolism and arthritis based on apoptosis, oxidative stress and oxiapoptophagy, another type of cell death accompanied by autophagy.

# II. MATERIALS AND METHODS

## II-1. Chemicals

Interleukin-1β (IL-1β) was purchased from ProSpec-Protein Specialists (Rehovot, Israel) and was dissolved in distilled water at stock concentration (10 μg/mL). 25-HC was purchased from Tocris Bioscience (Bristol, United Kingdom) and was dissolved in ethanol at stock concentration (8 mg/mL). 7α,25-DHC was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at stock concentration (5 mg/mL). NIBR189 was purchased from Sigma-Aldrich and was dissolved in DMSO at stock concentration (50 mM).

## II-2. Isolation and culture of primary rat chondrocytes

Primary rat chondrocytes were extracted from articular cartilage of rats (5-day-old; Sprague-Dawely, Damul Science Co., Daejeon, Republic of Korea) knee joins, in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Chosun University, Gwangju, Republic of Korea (CIACUC2020-A0011). Dissected cartilage tissues were washed in antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Welgene, Gyeongsan-si, Gyeongsangbuk-do, Republic of Korea). Subsequently, Trypsin-Ethylenediaminetetraacetic acid (EDTA; Welgene) was treated to leave only cartilage tissue. And then 0.02% collagenase P (Sigma-Aldrich) was treated to isolate chondrocyte. Isolated primary rat chondrocytes were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12; Welgene) supplemented with 10% fetal bovine serum (FBS; Welgene), antibiotics, and 50 μg/mL ascorbic acid (Sigma-Aldrich).

## II-3. *Ex vivo* organ culture of rat articular cartilage tissues

The articular cartilage tissue was isolated from the knee joint of 5-day-old Sprague-Dawley rats and then cultured in DMEM/F-12 supplemented with 10% FBS. Next, the articular cartilage tissue was treated defined treatment conditions for 14 days. At the end of the culture period, the sample was collected and washed in PBS. To perform histological analysis, the sample was fixed in 4% paraformaldehyde for 72 h.

## II-4. Safranin-O & fast green staining

To verify the loss of proteoglycan in articular cartilage, histological analysis using safranin-O & fast green staining was performed. Briefly, the articular cartilage tissue was decalcified in EDTA and embedded in paraffin. sequentially, the prepared paraffin block containing articular cartilage was serially sliced to 6 ~ 7 μm thickness using microtome (Leica Biosystems, Wetzlar, Hesse, Germany) and placed on slides. To assess proteoglycan loss in the articular cartilage ground substance, safranin-O & fast green (Thermo Fisher Scientific, Waltham, MA, USA) staining was performed. The slide was observed and imaged using Leica DM750 microscope (Leica Microsystems, Heerbrugg, Switzerland).

## II-5. Quantitative polymerase chain reaction (qPCR) and quantitative real-time PCR (qRT-PCR)

To investigate the alteration of mRNA induction, qPCR and qRT-PCR were performed. Chondrocytes were treated with defined treatment conditions. Thereafter, total RNA was isolated from the chondrocyte using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentrations of the isolated total RNA were measured using a Nanodrop 2000 (ThermoFisher Scientific). To synthesize cDNA, 1 μg RNA was reverse transcription using a

qPCRBIO cDNA Synthesis Kit (PCR Biosystems Ltd., London, UK) according to the manufacturer's instructions. For qRT-PCR, cDNA was amplified using an Eco Real-Time PCR system (Illumine Inc., San Diego, CA, USA). Relative gene expressions were determined using the $^{\Delta\Delta}$CT method, as detailed by the manufacturer (Illumine Inc). qPCR was performed using 2 x TOP simple DyeMIX-*n*Taq (Enzynomics, Seoul, Republic of Korea) and specific primers on a TaKaRa PCR Thermal Cycler Dice (TaKaRa Bio Inc., Kusatsu, Shiga, Japan). Thereafter, To determine the expression level of the target genes, the PCR product was electrophoresed on an agarose gel. β-actin was used as an internal control, the primer sequences and the used conditions were summarized in Table 1 and Table 2.

## II-6. Immunoblotting

Immunoblotting was performed to investigate the alteration of protein expression. Chondrocytes were cultured in a 12-well plate and treated with defined treatment conditions. Thereafter, the total protein was extracted from the chondrocytes using a PRO-PREP (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Replic of Korea) included Phosphatase Inhibitor Cocktail III (AG scientific, San Diego, CA, USA), according to the manufacturer's instructions. Protein concentrations were determined using a bicinchoninic acid protein assay (ThermoFisher Scientific). Equal amount of each protein sample was electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel and subsequently transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Burlington, MA, USA) at 4℃. Thereafter, the PVDF membrane was blocked using 5% (v/v) bovine serum albumin (BSA; Bioshop, Burlington, Ontario, Canada), prepared in tris buffered saline with Tween 20 (TBS-T; Santa Cruz Biotechnology Inc., Dallas, TX, USA). Sequentially, membranes were incubated with the primary antibodies at 4℃ and incubated with the secondary antibodies in TBS-T containing 5% (v/v) BSA. The immunoreactive band was visualized using the ECL System (Amersham Biosciences, Piscataway, NJ, USA), exposed on radiographic film or MicroChemi 4.2 (Dong-Il Shimadzu Corp., Seoul, Republic of Korea). The antibodies and the conditions used are summarized in Table 3.

## II-7. Gelatin zymography

To assess the activation of matrix matalloprotoeinases (MMPs) in primary rat chondrocytes, gelatin zymography was performed. Briefly, primary rat chondrocytes were treated with defined treatment conditions. Thereafter, an equal volume of conditioned medium was electrophoresed on a 8% polyacrylamide gel containing co-polymerized 0.2% (1 mg/mL) porcine skin gelatin. After electrophoresis, the gel was incubated in zymogram renaturating buffer (50 mM Tris-HCI [pH 7.6], 10 mM $CaCl_2$, 50 mM NaCl, and 0.05% Brij-35) at 37℃ for 72 h. After renaturation of MMPs, the gel was stained by 0.1% Coomassie Brilliant Blue R250. Gelatinolytic band was revealed as clear bands on a background uniformly stained light blue and then imaged using a digital camera (Canon, Ota, Tokyo, Japan).

## II-8. Measurement of prostaglandin $E_2$ ($PGE_2$)

To measure $PGE_2$, primary chondrocytes were seeded at $1 \times 10^6$ cells/mL in a 12 well culture plate. Chondrocytes were treated with defined treatment conditions. The production of $PGE_2$ was measured using a $PGE_2$ Parameter Assay Kit (R&D SYSTEMS, Minneapolis, MN, USA), Diluent RD5-56 calibrator added to the remaining standard and conditioned wells. And 50 μL of primary antibody solution was reacted to each well. The plate was covered tightly with sealant and incubated for 1 h. Then, 50 μL of $PGE_2$ Conjugate was added to each well and incubated for 2 h. Subsequently, the well was rinsed and added substrate solution to each well, then incubate for 30 min in a dark condition. Finally, 100 μL of stop solution was reacted to each well. Absorbance was measured at 450 nm using a spectrophotometer (Epoch microplate Spectrophotometer; BioTek, Winooski, VT, USA).

## II-9. Measurement of nitric oxide (NO)

Primary rat chondrocytes were treated with defined treatment conditions. Thereafter,

the conditioned medium was reacted with each equal amount of sulfanilamide (Duksan Company, Ansan, Gyeonggi-do, Republic of Korea) containing 5% (v/v) phosphoric acid (Duksan company) and *N*-1-naphthylethylenediamine dihydrochloride (Sigma-Aldrich). Absorbance was measured at 540 nm wavelength using a spectrophotometer.

## II-10. Cell viability assay

Cell viability was measured by 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Alfa Aesar, Haverhill, MA, USA). Chondrocytes ($1 \times 10^6$ cells/mL) were cultured in 48-well culture plates, and then treated with defined treatment conditions. Following addition MTT solution, the chondrocytes were further incubated for 4 h. After incubation, the MTT crystal that formed was suspended completely in DMSO and the absorbance was read at 570 nm using a spectrometer.

## II-11. Hematoxylin & Eosin (H&E) staining

H&E staining was performed to observe the morphological alteration of rat primary chondrocytes. Chondrocytes ($1 \times 10^6$ cells/mL) were cultured in 8-well chamber slide (Nunc Lab-Tek II Chamber Slide system; Sigma-Aldrich) and treated with defined treatment conditions. Subsequently, cells were fixed in 4% paraformaldehyde for 30 min at 4℃ and washed with phosphate buffered saline (PBS; Sigma-Aldrich). After staining with Haematoxylin (YD-Diagnostics, Yongin-si, Gyeonggi-do, Republic of Korea) and Eosin (Junsei Chemical Co., Tokyo, Japan), cells were observed and imaged using Leica DM750 microscope.

## II-12. Cell survival assay

To measure the survival of chondrocyte treated with IL-1β, cell survival assay was performed using a Live/Dead Viability/Cytotoxicity kit (Invitrogen), which consists of

green calcein AM for labeling live cells and ethidium homodimer-1 for labeling dead cells. Chondrocytes were cultured in 8-well chamber slides (Nunc Lab-Tek II Chamber Slide system; Sigma-Aldrich), and then treated with defined treatment conditions. Thereafter, the stained cell was imaged using a fluorescence microscope.

## II-13. Nuclear staining

To detection the condensed nucleus of the cell, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) staining was performed. Chondrocytes were cultured in 8-well chamber slide and treated with defined treatment conditions. Thereafter, the cells were rinsed three times with PBS and stained with DAPI. Nuclear condensations were observed and imaged using a fluorescence microscope.

## II-14. Immunocytochemistry (ICC)

ICC was performed to observe the alteration of target protein expression in chondrocyte. Chondrocytes ($1 \times 10^6$ cells/mL) were cultured in 8-well chamber slide, then cells were treated with defined treatment condition for 24 h. Thereafter, the cells were post-fixed in 4% paraformaldehyde for 10 min and reacted in endogenous peroxidase block 10 min. Slides were incuated using a Vectastain® ABC Kit (Vector Laboratories, Burlingame, CA, USA) at 4°C with primary antibody (caspase-3). Beclin-1 antibody was treated overnight, and then slides were incubated with peroxidase-conjugated goat anti-mouse antibody for 1 h at room temperature (RT). Slides were subsequently counterstained using hematoxylin. Subsequently, slides were transferred to the mounting reagent and examined by microscopy.

## II-15. Immunohistochemistry (IHC)

IHC was performed to observe the alteration of target protein expression in *ex vivo* organ-cultured articular cartilage, using Vectastain® ABC Kit. *Ex vivo*

organ-cultured articular cartilage was post-fixed in 4% paraformaldehyde for 7 days and dehydrated in a series of ethanol solutions (70, 80, 90, and 100%; 2 h per step). sequentially, samples were submerged in xylene twice for 2 h and in paraffin twice for 2 h. Embedded tissue block was prepared and cutted using a microtome. The 7-μm-thick sections were placed on glass slides. The section was deparaffinized using two changes of xylene for 3 min, rehydrated with two washes each of 100, 90, 80, and 70% ethanol for 3 min. Rehydrated section was rinsed with tap water for 3 min. The sections were incubated with caspase-3, beclin-1 antibody at 4°C overnight and then incubated with peroxidase-conjugated goat anti-mouse antibody for 1 h at RT. The section was subsequently counterstained using hematoxylin, transferred to the mounting reagent, and examined by microscopy.

## II-16. Caspase-3/-7 activity assay

Chondrocytes were cultured in 8-well chamber slide, and treated with defined treatment conditions. Thereafter, the activity of caspase-3/-7 was examined using the cell permeable fluorogenic substrate PhiPhiLux-$G_1D_2$ (OncoImmunin Inc., Gaithersburg, MD, USA). After cell staining, caspase-3/-7 activity was imaged using fluorescence microscopy.

## II-17. Measurement of 25-HC

To measure 25-HC, chondrocytes were seeded at $1 \times 10^6$ cells/mL in a 12 well culture plate. Chondrocytes were treated with defined treatment conditions. The production of 25-HC was measured using a Rat 25-Hydroxycholesterol ELISA Kit (My BioSource, San Diego, CA, USA). 50 μL of Sample Diluent was added to the remaining standard and conditioned wells. And 50 μL of standard solution was added to each well, 50 μL of cell pellet in PBS was added to each well. 100 μL of horseradish peroxide (HRP)-conjugate reagent was added to each well, covered the plate tightly with sealant in incubated for 1 h at 37°C. And then samples were

rinsed 1X washing solution to each well, four repetitions. Subsequently, 50 μL chromogen solution was added to each well, incubated for 15 min in a dark place at 37°C. Finally, 50 μL of stop solution was reacted to each well. Absorbance was measured at 450 nm using a spectrophotometer.

## II-18. ROS staining

2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA; Sigma-Aldrich) staining was performed to investigate the production of ROS. Chondrocytes were cultured in 8-well chamber slide and were treated with defined treatment conditions. Thereafter, the cells were rinsed three times with PBS and stained with $H_2$DCFDA. ROS was observed and imaged using a fluorescence microscope.

## II-19. Statistical analysis

All experimental were performed at least 3 times, and all experimental data was expressed as mean ± standard deviation (S.D.). The statistical significance was analyzed by using Student's *t*-test for two groups and one way analysis of variance (ANOVA) for multi-group comparisons. All statistical analyses was performed using Statistical Package for the Social Sciences (SPSS) software package version 22.0 (IBM, Armonk, NY, USA). A $p$-value $< 0.05$ was considered statistically significant.

**Table 1. qPCR primer sequences used in this study**

| Gene | Primer sequences | NCBI gene no. |
|---|---|---|
| *aggrecan* | Forward : 5'-CAGAGGAACACACCGAAAGT-3'<br>Reverse : 5'-GCACACTGGCTCCATCTATT-3' | NM_022190.1 |
| *ch25h* | Forward : 5'-CCCTTCTTCCCAGTCATCTTT-3'<br>Reverse : 5'-TCCCAGACGCTCATGTATTG-3' | NM_001025415.1 |
| *col II* | Forward : 5'-GGTCCTGGCATCGACAT-3'<br>Reverse : 5'-GTGCGAGCGGGATTCTT-3' | NM_012929.1 |
| *cox-2* | Forward : 5'-CCCTTCCTCCTGTGGCTGAT-3'<br>Reverse : 5'-CCCAGGTCCTCGCTTCTGAT-3' | NM_017232.3 |
| *cyp7b1* | Forward : 5'-CGGGCATGAAGAGTTTGAAATAG-3'<br>Reverse : 5'-AGACTTCTGGGTCATTGTGTATC-3' | NM_019138.1 |
| *inos* | Forward : 5'-GCATCGGCAGGATTCAGTGG-3'<br>Reverse : 5'-TAGCCAGCGTACCGGATGAG-3' | NM_012611.3 |
| *mmp-1* | Forward : CAACGCAGATTTAGCCTCCGA<br>Reverse : GAGATGCCCAGGACCACAGT | NM_001134530.1 |
| *mmp-3* | Forward : 5'-TCCTACCCATTGCATGGCAGTGAA-3'<br>Reverse : 5'-GCATGAGCCAAGACCATTCCAGG-3' | NM_133523.2 |
| *mmp-13* | Forward : 5'-GGCAAAAGCCATTTCATGCTCCCA-3'<br>Reverse : 5'-AGACAGCATCTACTTTGTCGCCA-3' | NM_133530.1 |
| *β-actin* | Forward : 5'-CGATAAAGGAAGGCTGGAAGAG-3'<br>Reverse : 5'-GTGCCCATCTATGAGGGTTATG-3' | NM_019212.2 |

**Table 2. qRT-PCR primer sequences used in this study**

| Gene | Primer sequences | NCBI gene no. |
|---|---|---|
| *aggrecan* | Forward : 5'-CCCCAAATCCCTCATACTCAG-3'<br>Reverse : 5'-CTGTTTCTCCTGACCCTTCTG-3' | NM_032893390.1 |
| *ch25h* | Forward : 5'-CACTCACCATCCTCGTCTTTC-3'<br>Reverse : 5'-GGAAAGTCGTAACCTGAGTGG-3' | NM_001025415.1 |
| *col II* | Forward : 5'-AGCACATCTGGTTTGGAGAG-3'<br>Reverse : 5'-CAGTGGTAGGTGATGTTCTGG-3' | NM_012929.1 |
| *cox-2* | Forward : 5'-CAACCCATGTCAAAACCGTG-3'<br>Reverse : 5'-TTGTCAGAAACTCAGGCGTAG-3' | NM_017232.3 |
| *cyp7b1* | Forward : 5'-AATTGTTCAGGAGAGGCAGG-3'<br>Reverse : 5'-CATAGCTGGAATGGTGTTTGC-3' | NM_019138.1 |
| *inos* | Forward : 5'-CGGTGTTCTTTGCTTCTGTG-3'<br>Reverse : 5'-TGAAGGCGTAGCTGAACAAG-3' | NM_012611.3 |
| *mmp-1* | Forward : 5'-CAACGCAGATTTAGCCTCCGA-3'<br>Reverse : 5'-GAGATGCCCAGGACCACAGT-3' | NM_001134530.1 |
| *mmp-3* | Forward : 5'-GACCCTGAGACCT1TACCAATG-3'<br>Reverse : 5'-AAAGAACAAGACTTCTCCCCG-3' | NM_133523.3 |
| *mmp-13* | Forward : 5'-GATGAAGACCCCAACCCTAAG-3'<br>Reverse : 5'-GGAGACTAGTAATGGCATCAAGG-3' | NM_133530.1 |
| *β-actin* | Forward : 5'-ATGGGTGTGAATGAGAAGGAC-3'<br>Reverse : 5'-GTCATTAGCCCTTCCACGATC-3' | NM_023964.1 |

**Table 3. Immunoblot antibodies used in this study**

| Antibodies | Molecular weight (kDa) | Dilution Concentration | Secondary Antibody | |
|---|---|---|---|---|
| β-actin | 43 | 1 : 5000 | Mouse | Santa Cruz Biotechnology Inc. |
| Beclin-1 | 60 | 1 : 2000 | Mouse | Santa Cruz Biotechnology Inc. |
| CH25H | 36 | 1 : 2500 | Mouse | Santa Cruz Biotechnology Inc. |
| Cleaved-caspase-3 | 17 | 1 : 2000 | Rabbit | Cell Signaling Technology |
| Cleaved-caspase-8 | 41 | 1 : 2500 | Rabbit | Cell Signaling Technology |
| Cleaved-caspase-9 | 37, 39 | 1 : 2500 | Rabbit | Cell Signaling Technology |
| COX-2 | 74 | 1 : 1500 | Rabbit | Cell Signaling Technology |
| CYP7B1 | 48 | 1 : 2500 | Rabbit | Bioss |
| iNOS | 130 | 1 : 1000 | Rabbit | Cell Signaling Technology |
| LC3 | 16, 18 | 1 : 2000 | Rabbit | Cell Signaling Technology |
| MMP-1 | 46 | 1 : 2500 | Rabbit | MBL |
| MMP-3 | 57 | 1 : 2000 | Mouse | Santa Cruz Biotechnology Inc. |
| MMP-13 | 48 | 1 : 2000 | Mouse | Santa Cruz Biotechnology Inc. |
| Cleaved-PARP | 89 | 1 : 2000 | Rabbit | Cell Signaling Technology |

# III. RESULTS

## III-1. IL-1β induces the progressive degeneration of articular cartilage through the acceleration of proteoglycan loss and the activation of cartilage-degrading enzymes

To determine whether IL-1β accelerates proteoglycan loss, the explant of articular cartilage was dissected from the knee joint of 5-day-old rats were cultured *ex vivo* with 10 and 50 ng/mL IL-1β for 14 days. Thereafter, the explant was fixed with 4% paraformaldehyde and safranin-O & fast green staining was performed to stain the proteoglycan. As shown in Fig. 1A, proteoglycans were decreased in articular cartilage treated with 10 and 50 ng/mL IL-1β compared with the untreated controls. Furthermore, IL-1β significantly suppressed the induction of mRNAs associated with the major components of the ECM such as aggrecan and Col II, in primary rat chondrocytes (Fig. 1B).

Next, to verify IL-1β-induced catabolic effect such as the expression and activation of cartilage-degrading enzymes, primary rat chondrocytes were cultured with 10 and 50 ng/mL IL-1β for 24 h. Thereafter, qPCR, qRT-PCR, immunoblotting, and gelatin zymography were performed to assess the alteration of cartilage-degrading enzymes. As shown in Fig. 2A - 2C, the expression of MMP-1, -3, and -13 mRNAs was significantly increased in the chondrocytes treated with IL-1β. As shown in Fig. 2D, the activity of cartilage-degrading enzymes was increased in chondrocytes treated with 10 and 50 ng/mL IL-1β. These data indicate that IL-1β induces the progressive degeneration of articular cartilage through an increase in proteoglycan loss, which is mediated by both the activation of cartilage-degrading enzymes and the suppression of ECM components in primary rat chondrocytes.

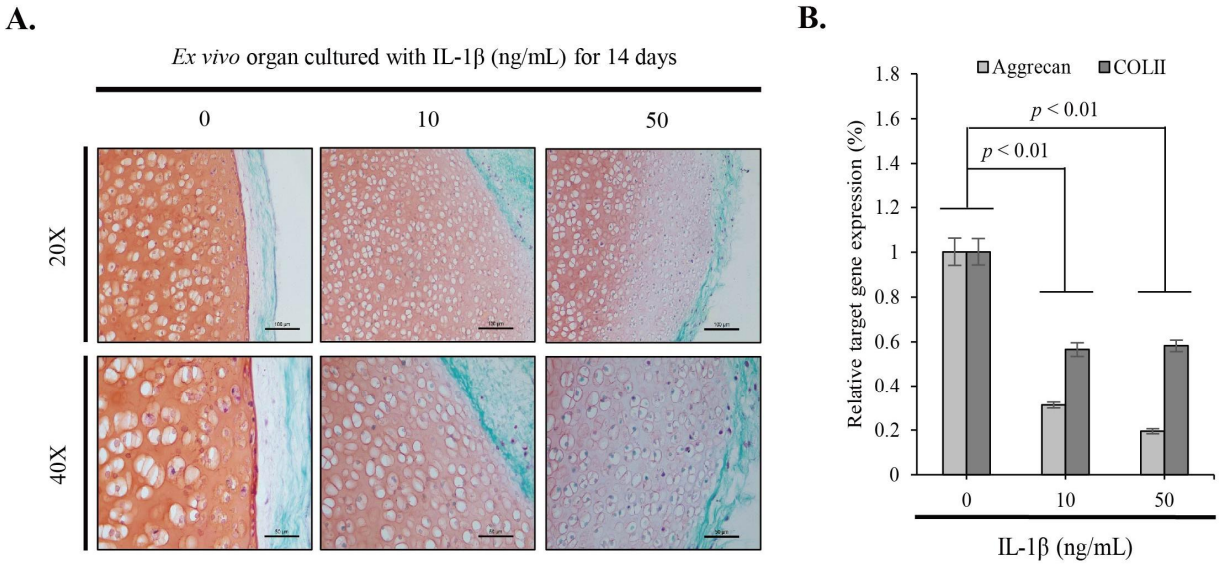



**Fig. 1. IL-1β induces the progressive degeneration of articular cartilage via the acceleration of proteoglycan depletion and the suppression of ECM synthesis.** A, IL-1β accelerates the proteoglycan loss from articular cartilage dissected from rat knee joint. Explants of articular cartilage tissues were dissected from rat knee joint treated with 10 and 50 ng/mL IL-1β for 14 days. Thereafter, explants fixed with 4% paraformaldehyde were exposed to safranin-O & fast green staining to stain the proteoglycan. Images were captured using a microscope. B, the synthesis of ECM components, such as aggrecan and col II, was significantly decreased in the primary rat chondrocytes treated with IL-1β. Primary rat chondrocytes were isolated from the articular cartilage of rat knee joint treated with 10 and 50 ng/mL IL-1β for 24 h. Thereafter, total RNA was isolated to perform the qRT-PCR after the synthesis of cDNA.

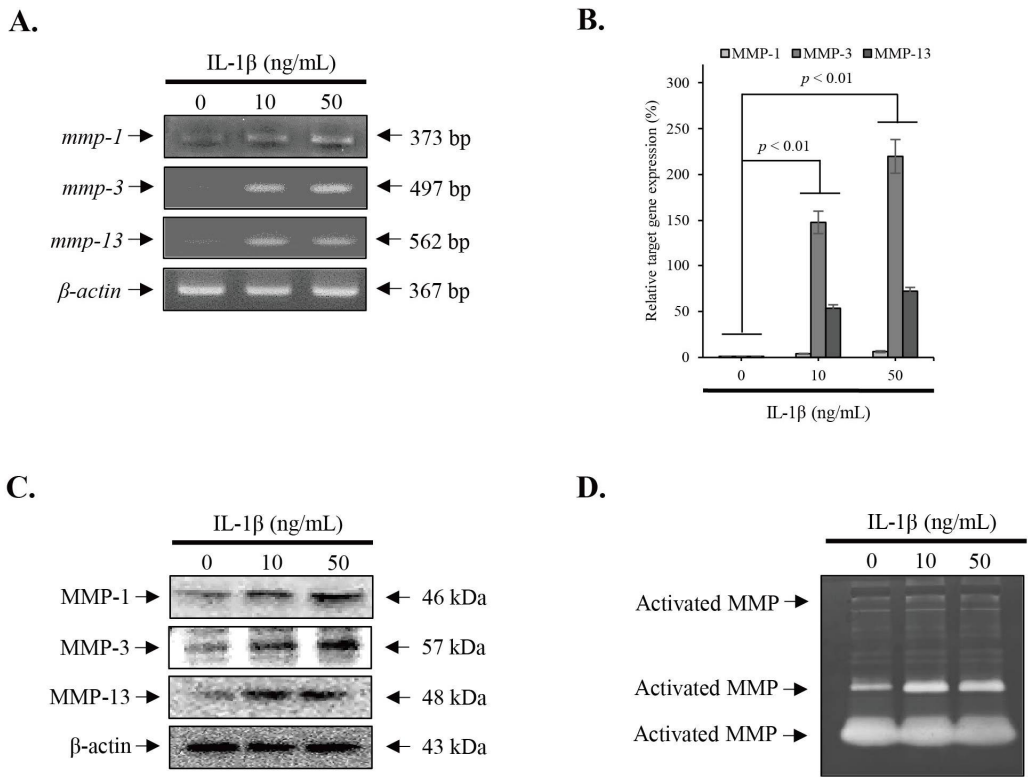



**Fig. 2. IL-1β induces progressive degeneration of articular cartilage through the activation and expression of cartilage-degrading enzymes.** Primary rat chondrocytes were treated with 10 and 50 ng/mL IL-1β for 24 h. Thereafter, total RNA, total protein, and conditioned media were collected for qPCR (A), qRT-PCR (B), and immunoblotting (C) using specific antibodies associated with cartilage-degrading enzymes, such as MMP-1, -3, and -13, and gelatin zymography (D). A & B, the mRNAs encoding *mmp-1*, *-3*, and *-13* were significantly increased by IL-1β in primary rat chondrocytes. C, the expression of MMP-1, -3, and -13 proteins was significantly upregulated in primary rat chondrocytes treated with IL-1β. D, the activation of MMPs associated with cartilage-degrading enzymes was increased in the conditioned media collected from primary rat chondrocytes treated with IL-1β.

## Ⅲ-2. Inflammatory mediators associated with arthritis risk factors increase in the chondrocytes treated with IL-1β

To determine whether IL-1β induces inflammatory mediators, an arthritis risk factors, primary rat chondrocytes were cultured with 10 and 50 ng/ml IL-1β for 24 h. Thereafter, the mRNA and protein level of inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), were assessed. As shown in Fig. 3A - 3C, the mRNA and protein level of iNOS and COX-2 significantly increased after IL-1β treatment in chondrocytes. The relative production of NO increased by 224.44 ± 9.45% and 333.38 ± 5.34% in the chondrocytes treated with 10 and 50 ng/mL IL-1β, respectively, compared with that in control cells (100.01 ± 1.94%) (Fig. 3D). The production of $PGE_2$ significantly increased by 4,242.21 ± 905.97 pg/mL and 7,194.30 ± 1,210.73 pg/mL in the chondrocytes treated with 10 and 50 ng/mL IL-1β, respectively, compared with that in the control cells (1,471.09 ± 572.78 pg/mL). These data indicate that IL-1β upregulates the production of inflammatory mediators, such as iNOS, COX-2, NO, and $PGE_2$, which act as arthritis risk factors in primary rat chondrocytes.

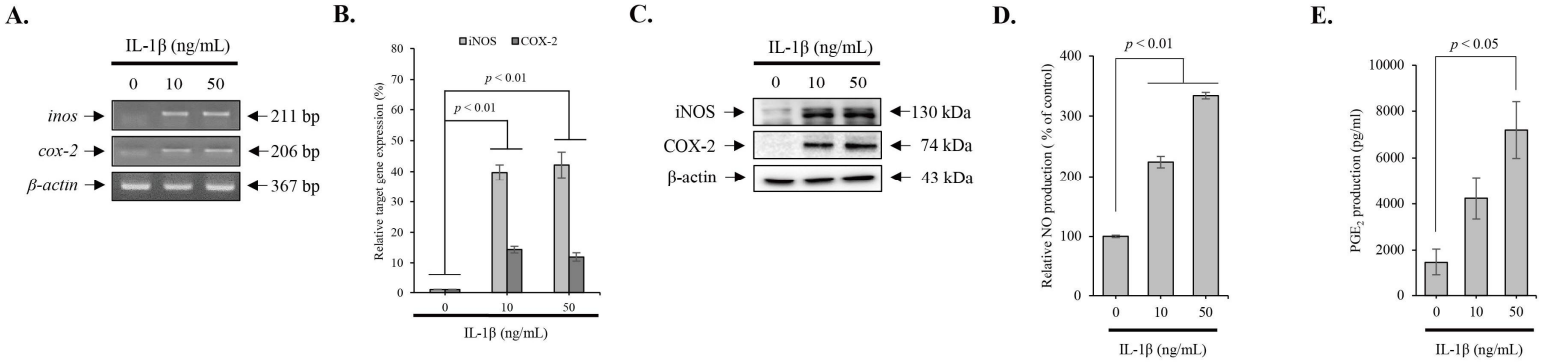



**Fig. 3. IL-1β upregulates the inflammatory mediators and acts as a pathophysiological factor in arthritis.** Primary rat chondrocytes were treated with 10 and 50 ng/mL IL-1β for 24 h. Thereafter, total RNA and total protein were collected for qPCR (A), qRT-PCR (B), and immunoblotting (C) using specific antibodies associated with inflammatory mediators, NO assay (D), and $PGE_2$ ELISA (E). A & B, the mRNAs encoding *inos* and *cox-2* were significantly increased by IL-1β in primary rat chondrocytes. C, expression of iNOS and COX-2 proteins was significantly upregulated in primary rat chondrocytes treated with IL-1β. D, NO production was significantly increased by IL-1β in primary rat chondrocytes. E, IL-1β upregulated the production of $PGE_2$ in primary rat chondrocytes.

## Ⅲ-3. IL-1β induces apoptotic cell death via the cascade activation of caspases in chondrocytes

Primary rat chondrocytes were treated with 10 and 50 ng/mL IL-1β for 24 h. Thereafter, MTT assay, cell live/dead staining, and DAPI staining were performed to verify cell viability, cell survival, and the alteration of chromatin in primary rat chondrocytes, respectively. As shown in Fig. 4A, relative cell viability was measured to be 87.92 ± 2.22% and 83.05 ± 1.61% in the chondrocytes treated with 10 and 50 ng/mL IL-1β, respectively, compared with that of the untreated control (100.01 ± 1.68%). Moreover, cell live/dead staining showed that the number of living chondrocytes decreased because of IL-1β in a dose-dependent manner (Fig. 4B upper panel). Furthermore, the results of H&E staining showed that IL-1β not only decreased the number of chondrocytes in a dose-dependent manner but also increased the altered condensed morphology in chondrocytes (Fig. 4B middle panel). In addition, the result of DAPI staining showed that the number of chondrocytes with condensed chromatin, a typical feature of apoptosis, was increased by IL-1β in a dose-dependent manner (Fig. 4B lower panel). These results indicate that IL-1β -induced chondrocyte death is involved in apoptosis.

Hence, to verify whether IL-1β-induced chondrocyte death is mediated by apoptosis, immunoblotting using caspase-8, -9, -3, and PARP specific antibodies was performed. The expression of pro-apoptotic factors, including cleaved caspase-8, -9, -3, and PARP, significantly increased in primary rat chondrocytes treated with IL-1β (Fig. 4C). Furthermore, the results of caspase-3/-7 activity assay and ICC using caspase-3 specific antibodies showed that IL-1β not only upregulated the activity of caspase-3 but also increased the expression of caspase-3 in primary rat chondrocytes (Fig. 4D). Taken together, these data consistently indicate that IL-1β induces chondrocyte apoptosis through the cascade activation of caspases associated with the death receptor-mediated extrinsic and mitochondria-dependent intrinsic apoptosis pathways in primary rat chondrocytes.

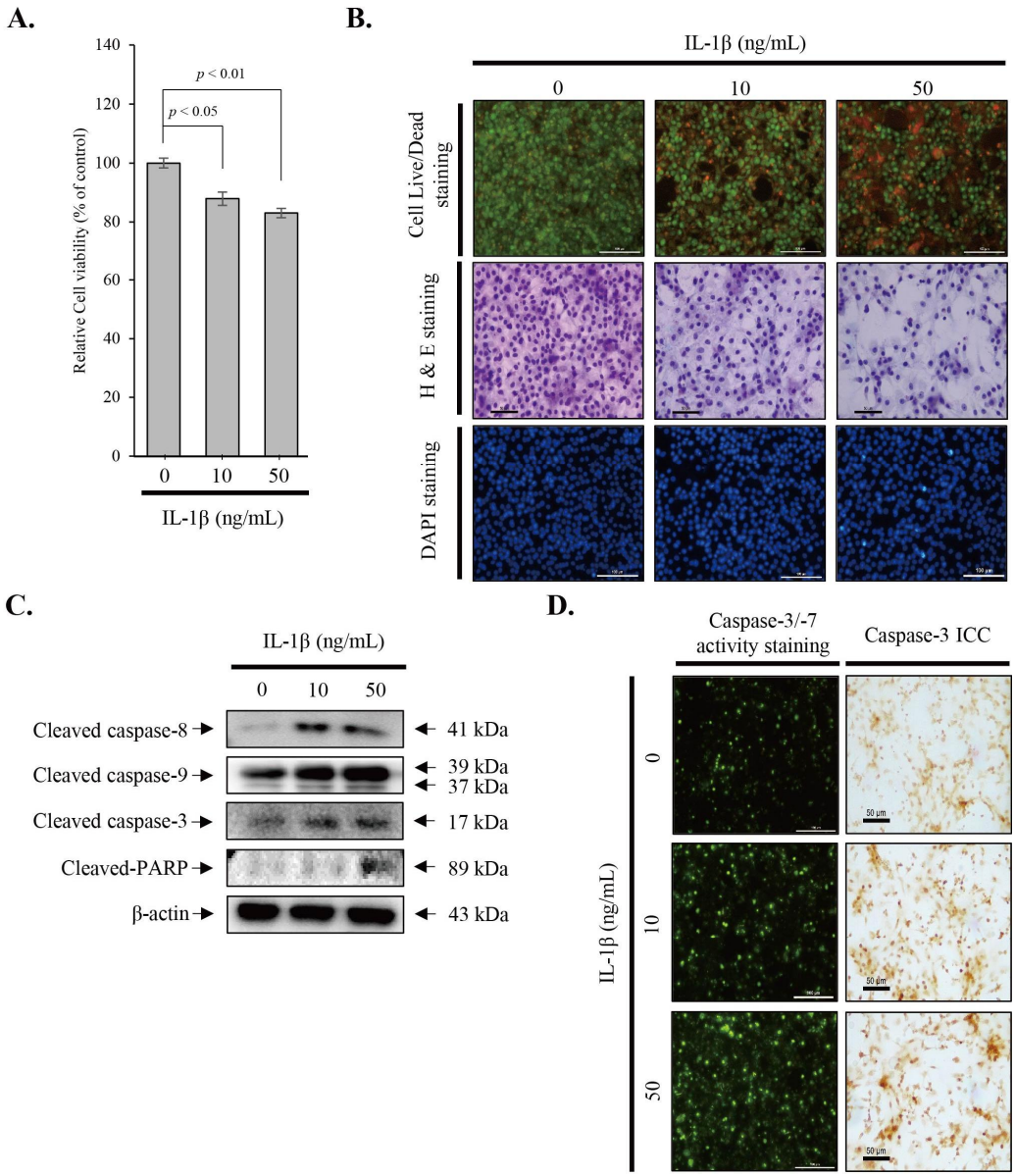



**Fig. 4. IL-1β induces the apoptosis of chondrocytes through the cascade activation of caspases.** Primary rat chondrocytes were treated with 10 and 50 ng/mL IL-1β for 24 h. Thereafter, MTT assay (A), cell live/dead staining (B, upper panel) using ethidium homodimer-1 to stain the dead cells with red fluorescence and green calcein AM to stain live cells with green fluorescence, and DAPI staining (B, lower panel) were performed to investigate the viability, survival, and chromatin condensation of primary

chondrocytes. Total proteins extracted from primary rat chondrocytes treated with IL-1 β were analyzed to verify the expression of caspases including caspase-8, -9, and -3 by immunoblotting (C). In addition, to verify the activation and expression of caspase-3, caspase-3/-7 activity staining (D, left panel) using PhilphiLux$G_1$/$G_2$ substrate, and ICC (D, right panel) with caspase-3 specific antibody was performed in primary rat chondrocytes. A, The viability of primary rat chondrocytes was decreased by IL-1β in a dose-dependent manner. B, The number of dead cells with condensed chromatin was increased in primary rat chondrocytes treated with IL-β. C, the expression of cleaved caspases-8, -9, and -3 was increased by IL-1β in primary rat chondrocytes. D, the activity and expression of caspase-3 was increased in the primary rat chondrocytes treated with IL-1β.

## Ⅲ-4. IL-1β-induced chondrocyte death is involved with autophagy

Autophagy is a cellular process for protein degradation to remove a damaged organelles by lysosomes [27]. Therefore, an immunoblotting assay using autophagy-specific antibodies, such as beclin-1 and LC3, was performed to verify whether IL-1β-induced chondrocyte death is related in autophagy. As shown in Fig. 5A, the expression of beclin-1 and LC3 was significantly increased in chondrocytes treated with IL-1β. Furthermore, ICC and IHC results showed that the immunoreactivity of beclin-1 was increased by IL-1β in primary rat chondrocytes and *ex vivo* organ-cultured articular cartilage (Fig. 5B). These results indicate that IL-1β -induced cell death is accompanied by autophagy in chondrocytes.

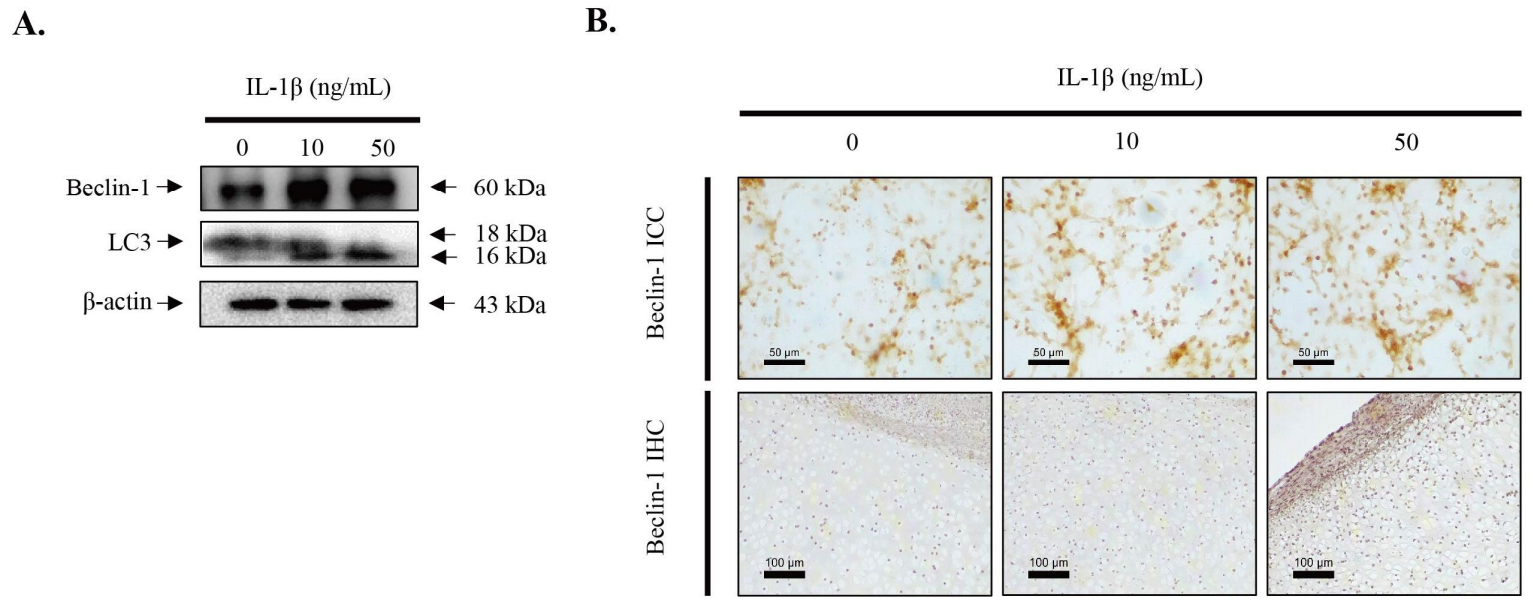



**Fig. 5. IL-1β-induced chondrocytes death is involved with autophagy.** Primary rat chondrocytes were treated with 10 and 50 ng/mL IL-1β for 24 h. Thereafter, total proteins were extracted to perform the immunoblotting (A) using autophagy biomarkers such as beclin-1 and LC3. Primary rat chondrocytes cultured on 8-well chamber slides were treated with IL-1β for 24 h. Thereafter, to verify the expression of beclin-1 in the primary rat chondrocytes treated with IL-1β, ICC (B, upper panel) was performed. In addition, to verify the expression of beclin-1 in the articular cartilage treated with IL-1β, explants of articular cartilage, dissected from rat knee joint, were treated with 10 and 50 ng/mL IL-1β for 14 days. Then, IHC (B, lower panel) was performed. A, the expressions of autophagy biomarkers, such as beclin-1 and LC3, were increased by IL-1β in primary rat chondrocytes. B, the expression of beclin-1 was upregulated by IL-1β in both primary rat chondrocytes and articular cartilage.

## Ⅲ-5. IL-1β induces the synthesis of 25-HC and its downstream oxysterol 7α,25-DHC in chondrocytes

To determine whether oxysterol is synthesized under inflammatory condition, primary rat chondrocytes were cultured with 10 and 50 ng/mL IL-1β for 24 h. As shown in Fig. 6A and 6B, the qPCR and qRT-PCR results consistently showed that the level of *ch25h* mRNA, which encodes a CH25H enzyme that converts cholesterol to 25-HC, was significantly increased in the chondrocytes treated with IL-1β. Sequentially, the level of *cyp7b1* mRNA, which encodes a CYP7B1 enzyme that converts 25-HC to 7α,25-DHC, was increased by IL-1β in primary rat chondrocytes (Fig. 6A and 6B). Furthermore, immunoblotting assay showed that the expression of CH25H and CYP7B1 was upregulated in the chondrocytes treated with IL-1β (Fig. 6C). As shown in Fig. 6D, the ICC results showed that immunoreactivities of CH25H and CYP7B1 were increased by IL-1β in primary rat chondrocytes. Moreover, the relative production of 25-HC was increased by 1,084.50 ± 120.21 pg/mL and 1,204.50 ± 106.10 pg/mL in the chondrocytes treated with 10 and 50 ng/mL IL-1β, respectively, compared with that of control cells (632.50 ± 3.54 pg/mL) (Fig. 6E). These data indicate that IL-1β induces the production of oxysterols such as 25-HC and 7α,25-DHC, through an increase in oxysterol synthases such as CH25H and CYP7B1, respectively, in primary rat chondrocytes.

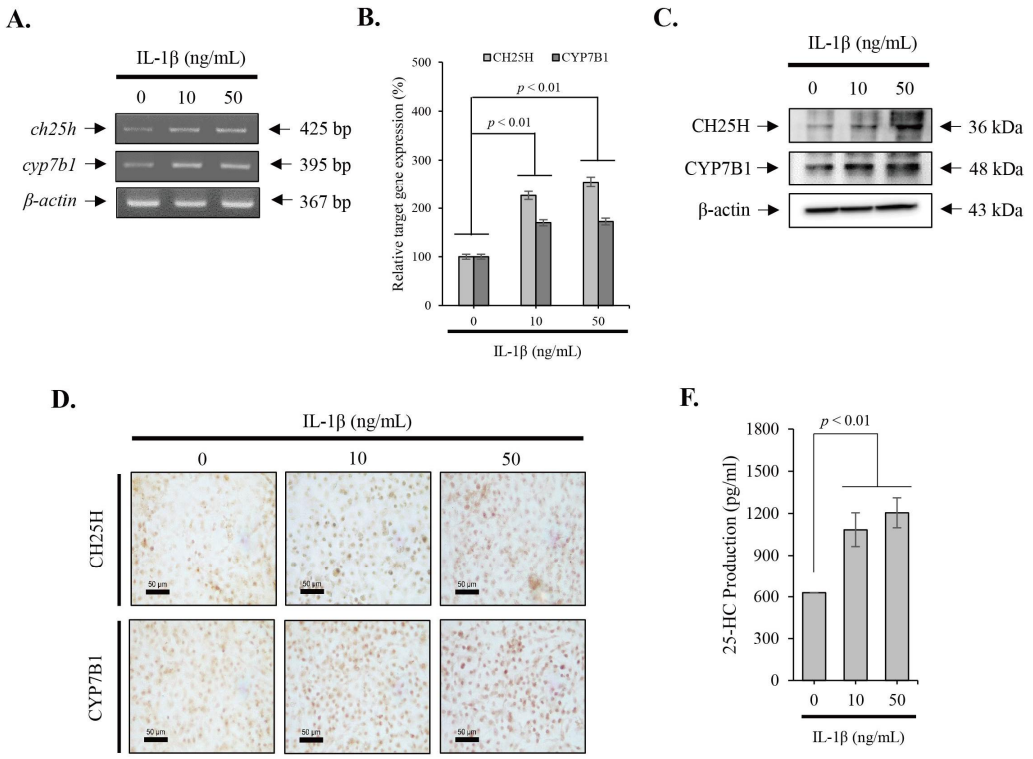



**Fig. 6. IL-1β upregulates the production of 25-HC and its downstream oxysterol 7 α,25-DHC through the expression of the CH25H-CYP7B1 axis in primary rat chondrocytes.** Primary rat chondrocytes were treated with 10 and 50 ng/mL IL-1β for 24 h. Thereafter, total RNA and total protein were isolated for qPCR (A), qRT-PCR (B), and immunoblotting (C) using specific antibodies oxysterol synthases, such as CH25H and CYP7B1. Primary rat chondrocytes cultured on the 8-well chamber slides were treated with 10 and 50 ng/mL IL-1β for 24 h. Thereafter, ICC (D) was performed to verify the expression of CH25H and CYP7B1 in primary rat chondrocytes treated with IL-1β. In addition, 25-HC ELISA (E) was performed to verify the production of 25-HC in primary rat chondrocytes treated with IL-1β. A & B, mRNAs expression of *ch25h* and *cyp7b1* was induced by IL-1β in primary rat chondrocytes. C & D, CH25H and CYP7b1 protein expression was upregulated in primary rat chondrocytes treated with IL-1β. E, the production of 25-HC was increased by IL-1β in primary rat chondrocytes.

## Ⅲ-6. 25-HC induces the progressive degeneration of articular cartilage via chondrocyte oxiapoptophagy

It has been reported that some oxysterols induce cell death via oxiapoptophagy [28]. Hence, to verify whether oxysterol 25-HC-induced chondrocyte death is involved in oxiapoptophagy, primary rat chondrocytes were treated with 10 and 20 μg/mL 25-HC for 24 h. As shown in Fig. 7A, the relative cell viability was measured to be 95.00 ± 2.50% and 81.78 ± 2.42% in chondrocytes treated with 10 and 20 μg/mL 25-HC, compared with that of the untreated control (100.04 ± 3.08%). Moreover, the results of cell live/dead staining showed that the number of living chondrocytes was decreased by 25-HC in a dose-dependent manner (Fig. 7B). Sequentially, 25-HC not only upregulated the expression of cleaved caspase-3 and cleaved PARP (Fig. 7C) but also increased of caspase-3 activity in primary rat chondrocytes (Fig. 7D). In addition, ROS production was gradually upregulated by 25-HC in primary chondrocytes (Fig. 7E). The expression of autophagy biomarkers such as beclin-1 and LC3 increased in a dose-dependent manner by 25-HC in primary rat chondrocytes (Fig. 7F). Finally, the result of safranin-O & fast green staining showed that proteoglycan loss was significantly increased by 25-HC in the *ex vivo* organ-cultured explant of articular cartilage dissected from rat knee joints (Fig. 7G). These data suggest that chondrocyte death by IL-1β-induced oxysterol 25-HC is involved in progressive articular degeneration through the acceleration of proteoglycan loss, which is mediated by oxiapoptophagy accompanied by oxidative stress, apoptosis and autophagy in primary rat chondrocytes.

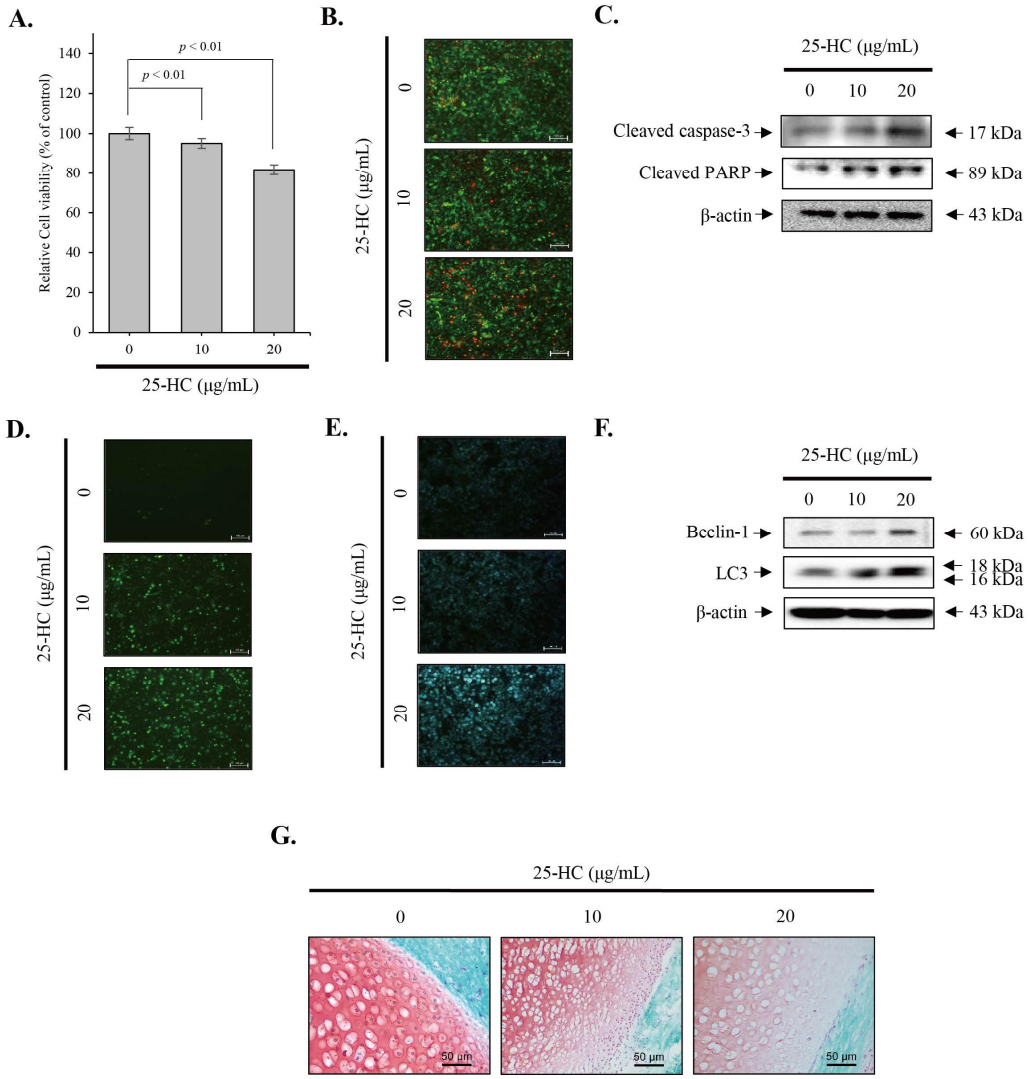



**Fig. 7. 25-HC induces the progressive degeneration of articular cartilage through chondrocyte death involving oxiapoptophagy, which is accompanied by apoptotic cell death, oxidative stress, and autophagy.** Primary rat chondrocytes were treated with 10 and 20 μg/mL 25-HC for 24 h. Thereafter, MTT assay (A), cell live/dead staining (B), immunoblotting (C), caspase-3/-7 activity staining (D) and $H_2$DCFDA staining (E) were performed to investigate cell viability, cell survival, activated caspase, and ROS production in primary chondrocytes treated with 25-HC. Total proteins were extracted from the primary chondrocytes treated with 25-HC to perform

immunoblotting (C & F). Explants of articular cartilage dissected from rat knee joints were cultured *ex vivo* in the presence of 10 and 20 μg/mL 25-HC for 14 days. Thereafter, safranin-O & fast green staining (G) was performed to verify the 25-HC-induced proteoglycan loss in the explant of articular cartilage. A, 25-HC treatment decreased the viability of primary rat chondrocytes. B, cell survival was decreased in primary rat chondrocytes treated with 25-HC. C, 25-HC induced apoptosis in primary rat chondrocytes. D, the activity of caspases-3 was increased by 25-HC in primary rat chondrocytes. E, ROS production increased in primary rat chondrocytes treated with 25-HC. F, the expression of autophagy biomarkers was increased in primary rat chondrocytes treated with 25-HC. G, proteoglycan depletion was accelerated by 25-HC in the explants of articular cartilage dissected from rat knee joints.

## Ⅲ-7. 7α,25-DHC accelerates the progressive degeneration of articular cartilage via chondrocyte oxiapoptophagy

7α,25-DHC is a downstream oxysterol of 25-HC and is catalyzed by CYP7B1. To verify whether oxysterol 7α,25-DHC-induced chondrocyte death is involved in oxiapoptophagy, chondrocytes were treated with 25 and 50 μg/mL 7α,25-DHC for 48 h. As shown in Fig. 8A, relative cell viability was 79.00 ± 1.96% and 77.52 ± 2.18% in the chondrocytes treated with 25 and 50 μg/mL 7α,25-DHC, respectively, compared with that of the untreated control (100.04 ± 3.29%). Moreover, the result of cell live/dead staining showed that the number of living chondrocytes was decreased by 7α,25-DHC in a dose-dependent manner (Fig. 8B). Sequentially, 7 α,25-DHC not only upregulated the expression of cleaved caspase-3 and cleaved PARP (Fig. 8C) but also increased of caspase-3 activity in primary rat chondrocytes (Fig. 8D). In addition, ROS production was gradually upregulated by 7α,25-DHC in primary chondrocytes (Fig. 8E). Expressions of autophagy biomarkers were increased by 7α,25-DHC in primary rat chondrocytes (Fig. 8F). Finally, the result of safranin-O & fast green staining showed that proteoglycan loss was significantly increased by 7 α,25-DHC in the *ex vivo* organ-cultured explant of articular cartilage dissected from rat knee joints (Fig. 8G). These results indicate that chondrocyte death by IL-1β -induced oxysterol 7α,25-DHC is similar to chondrocyte death induced by 25-HC.

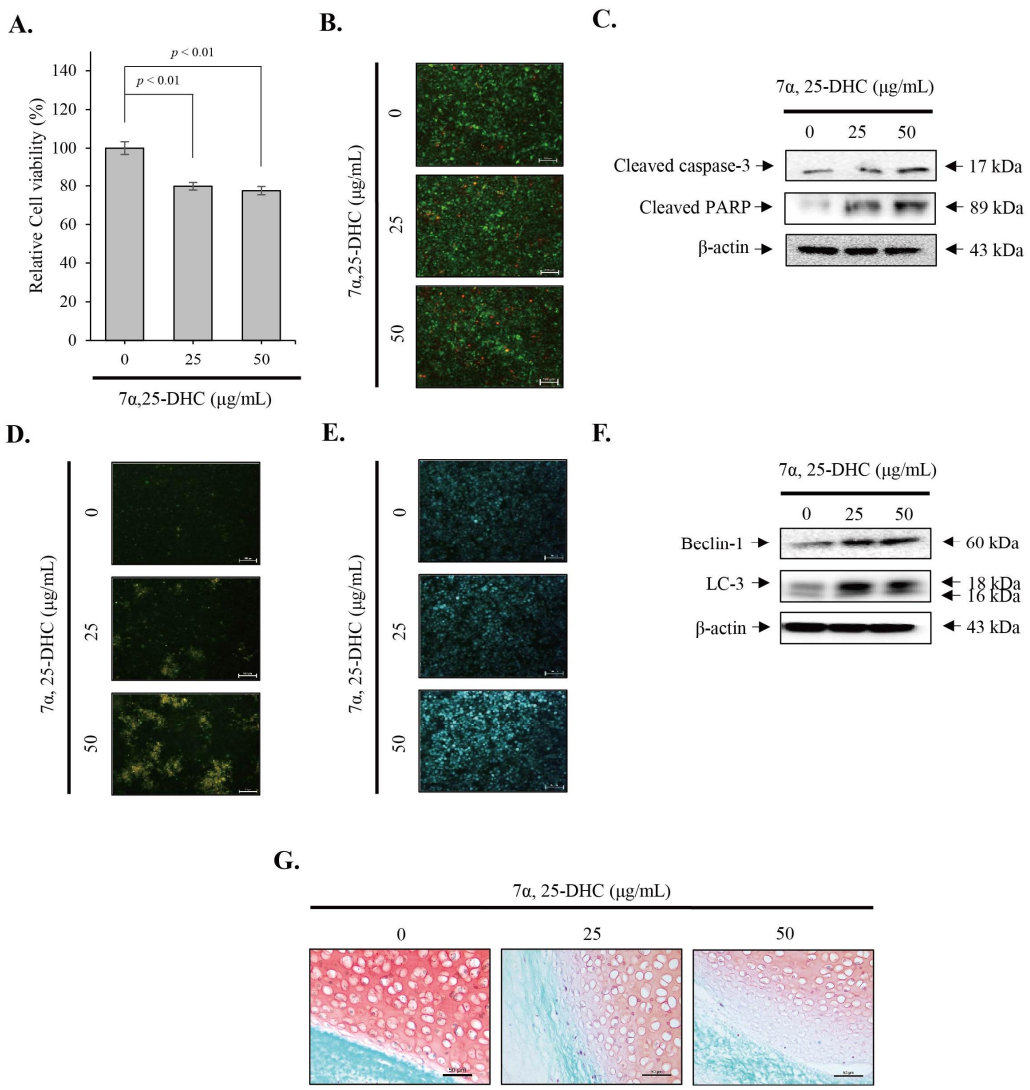



**Fig. 8. 7α,25-DHC induces the progressive degeneration of articular cartilage through chondrocyte oxiapoptophagy.** Primary rat chondrocytes were treated with 10 and 20 μg/mL 7α,25-DHC for 48 h. Thereafter, MTT assay (A), cell live/dead staining (B), immunoblotting (C), caspase-3/-7 activity staining (D), and $H_2$DCFDA staining (E) were performed to investigate cell viability, cell survival, activated caspase, and ROS production in the primary chondrocytes treated with 7α,25-DHC. Total proteins were extracted from the primary chondrocytes treated with 7α,25-DHC to perform immunoblotting (C & F). Explants of articular cartilage dissected from rat knee joints were cultured *ex vivo* in the presence of 10 and 20 μg/mL 7α,25-DHC for 14 days.

Thereafter, safranin-O & fast green staining (G) was performed to verify the 7 α,25-DHC-induced proteoglycan loss in articular cartilage. A, 7α,25-DHC treatment decreased the viability of primary rat chondrocytes. B, cell survival was decreased in primary rat chondrocytes treated with 7α,25-DHC. C, 7α,25-DHC induced apoptosis in primary rat chondrocytes. D, the activity of caspases-3 was increased by 7 α,25-DHC in primary rat chondrocytes. E, ROS production was increased in primary rat chondrocytes treated with 7α,25-DHC. F, the expression of autophagy biomarkers was increased in primary rat chondrocytes treated with 7α,25-DHC. G, proteoglycan depletion was accelerated by 7α,25-DHC in the explants of articular cartilage dissected from rat knee joints.

## Ⅲ-8. NIBR189 counteracts 7α,25-DHC-induced catabolic effects

To investigate whether the inhibition of EBI2, a receptor of 7α,25-DHC, counteract the 7α,25-DHC-induced oxiapoptophagy, primary rat chondrocytes were treated with 7 α,25-DHC in the presence or absence of EBI2 antagonist NIBR189. As shown in Fig. 9A, results of MTT assay showed that relative viability of primary rat chondrocytes was measured by 100.00 ± 2.64% and 91.16 ± 3.21% in the absence or presence of NIBR189, respectively. The relative viability was decreased by 71.30 ± 1.47% in primary chondrocytes treated with 7α,25-DHC (Fig. 9A). However, relative viability was measured by 75.28 ± 1.67% in the chondrocytes co-treated with 7α,25-DHC and NIBR189 (Fig. 9A). Hence, these data demonstrate that NIBR189 counteracts 7α,25-DHC-induced cytotoxicity in primary chondrocytes. Moreover, cell live/dead staining showed that 7α,25-DHC decreased the number of live cells stained with green fluorescence, but it was counteracted by NIBR189 in primary rat chondrocytes. Sequentially, the expression of pro-apoptotic factors such as cleaved caspase-3 and cleaved PARP was upregulated in the primary rat chondrocytes treated with 7α,25-DHC, but it was counteracted by NIBR189 (Fig. 9C). In addition, the upregulation of caspase-3/-7 activity by 7α,25-DHC was also reduced by NIBR189 in primary rat chondrocytes (Fig 9D). NIBR189 not only suppress 7α,25-DHC-induced ROS production (Fig. 9E) but also counteracted the expression of 7α,25-DHC-induced autophagy biomarkers in primary rat chondrocytes (Fig. 9F). Finally, the result of safranin-O & fast green staining showed that NIBR189 suppressed proteoglycan loss by 7α,25-DHC in *ex vivo* organ-cultured articular cartilage (Fig. 9G).

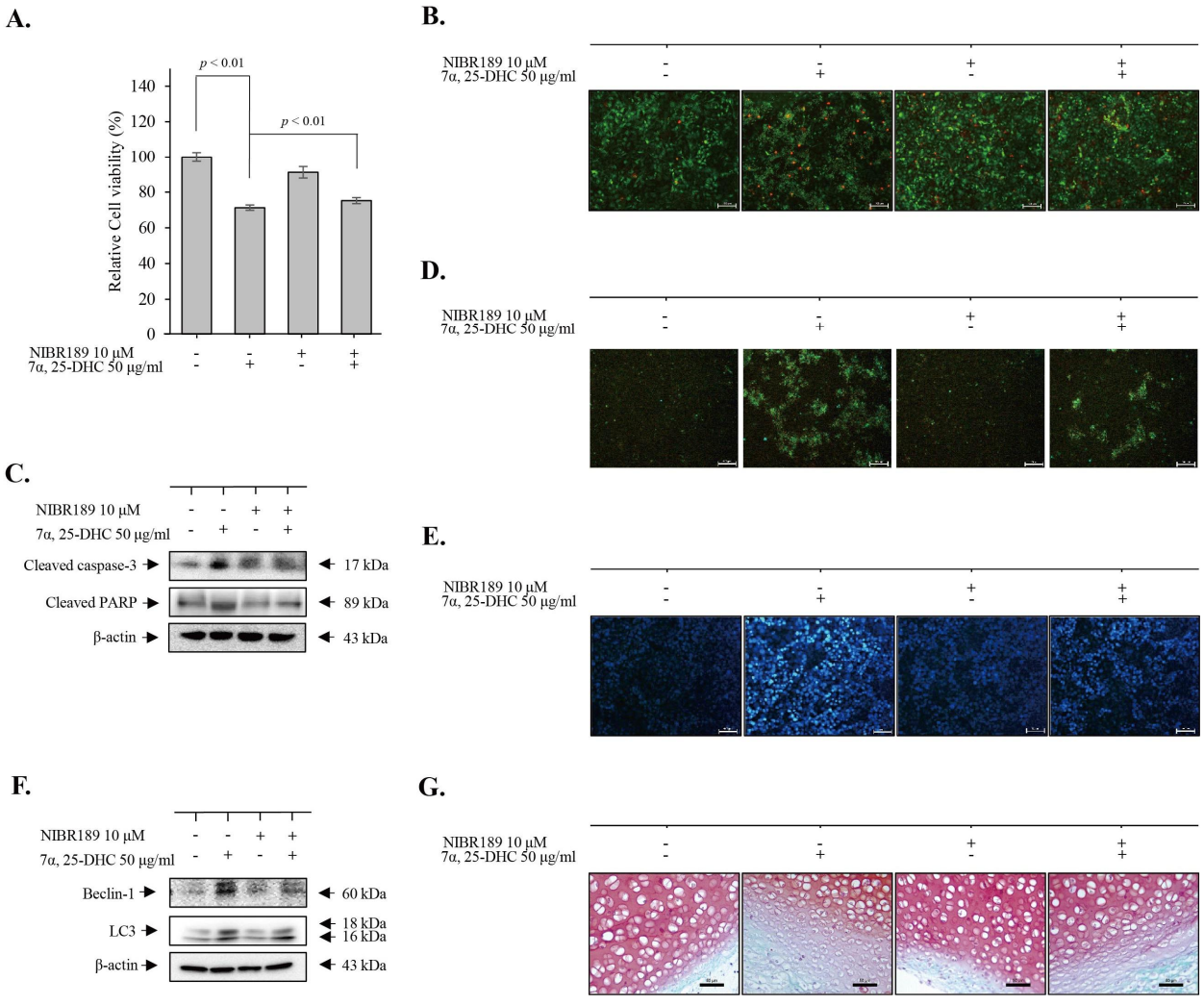


**Fig. 9. NIBR189 counteracts 7α,25-DHC-induced catabolic effects.** Primary rat chondrocytes were treated with 50 μg/mL 7α,25-DHC in the presence or absence of NIBR189 for 48 h. Thereafter, MTT assay (A) and cell live/dead staining were performed to investigate the viability and survival of chondrocytes, respectively. Total protein was collected to perform the immunoblot (C & F), caspase-3/-7 activity staining (D) and $H_2$DCFDA staining (E) were performed to investigate the activated caspase and ROS production in the primary chondrocytes treated with 50 μg/mL 7α,25-DHC in the presence or absence of NIBR189. Explants of articular cartilage dissected from rat knee joint were incubated with 50 μg/mL 7α,25-DHC in presence or absence of NIBR189, a receptor antagonist of 7α,25-DHC, for 14 days. Thereafter, safranin-O & fast green staining (G) was performed to verify the proteoglycan loss in articular cartilage. A, NIBR189 rescued the viability of primary rat chondrocytes in the presence of 7α,25-DHC. B & C, the expressions of apoptosis biomarkers, such as cleaved caspase-3 and PARP, were decreased by NIBR189 in the rat primary chondrocytes treated with 7α,25-DHC. D, NIBR189 suppressed the activation and expression of caspase-3 in the primary rat chondrocytes treated with 7α,25-DHC. E, the increase of ROS associated with oxidative stress was rescued by NIBR189 in the rat primary chondrocytes treated with 7α,25-DHC. F, NIBR189 significantly suppressed the expression of autophagy biomarkers such as LC3 and beclin-1 in the primary rat chondrocytes treated with 7 α,25-DHC. G, 7α,25-DHC-induced proteoglycan depletion was significantly suppressed by NIBR189 in the *ex vivo* organ-cultured articular cartilage.

## Ⅲ-9. NIBR189 counteracts IL-1β-induced arthritis through the inhibition of chondrocyte oxiapoptophagy

In accordance with previous results of this study, IL-1β-induced oxysterols such as 25-HC and 7α,25-DHC were involved in the progressive degeneration of articular cartilage through the induction of chondrocyte oxiapoptophagy. Hence, to verify whether the inhibition of EBI2 might attenuate IL-1β-induced arthritis, explants of articular cartilage dissected from rat knee joints were *ex vivo* cultured with 50 ng/mL IL-1β in the presence or absence of 10 μM NIBR189 for 14 days. Thereafter, safranin-O & fast green staining was performed to verify the alteration of proteoglycans in articular cartilage. As shown in Fig. 10A, proteoglycan loss was significantly increased by IL-1β, but counteracted by NIBR189. Furthermore, mRNAs associated with ECM components, such as aggrecan and col II, decreased by 50 ng/mL IL-1β, compared with the untreated control (Fig. 10B). However, it was significantly counteracted by 10 μM NIBR189 in primary rat chondrocytes (Fig. 10B). Therefore, these data demonstrate that the inhibition of EBI2 attenuates the progressive degeneration of articular cartilage through the inhibition of IL-1β-induced proteoglycan loss in articular cartilage and the loss of ECM components by IL-1β in primary rat chondrocytes.

To verify whether NIBR189 suppresses IL-1β-induced catabolic effects owing to the progressive degeneration of articular cartilage mediated by the expression and activation of cartilage-degrading enzymes, primary rat chondrocytes were treated with IL-1β in the presence or absence of NIBR189. Thereafter, qPCR, qRT-PCR, immunoblotting, and gelatin zymography were performed to assess alterations in cartilage-degrading enzymes. As shown in Fig. 11A - 11C, the expression of MMP-1, -3, and -13 significantly increased in the chondrocytes treated with IL-1β, compared with that in the untreated control, whereas it was significantly counteracted by NIBR189. As shown in Fig. 11D, the upregulated activity of cartilage-degrading enzymes in the primary rat chondrocytes treated with IL-1β was also decreased by NIBR189. These data indicate that the inhibition of EBI2 has an anti-catabolic effect

through the suppression of cartilage-degrading enzymes in primary rat chondrocytes treated with IL-1β.

Next, to determine whether NIBR189 suppresses the IL-1β-induced inflammatory mediators, an arthritis risk factor, primary rat chondrocytes were cultured with 50 ng/mL of IL-1β in the presence or absence of 10 μM NIBR189 for 24 h. Thereafter, mRNA and protein level of inflammatory mediators, such as iNOS and COX-2, were assessed by qPCR, immunoblotting, NO measurements, and $PGE_2$ measurements. As shown in Fig. 12A - 12C, mRNA and protein level of iNOS and COX-2 were significantly increased by 50 ng/mL IL-1β, but were counteracted by NIBR189 in chondrocytes. Sequentially, the increase of NO and $PGE_2$ production by IL-1β was also counteracted by NIBR189 in primary rat chondrocytes (Fig. 12D and 12E). Hence, these data suggest that the inhibition of EBI2 has anti-catabolic effects mediated by the suppression of inflammatory mediators in the chondrocytes treated with IL-1β.

To verify whether NIBR189 suppresses IL-1β-induced chondrocyte apoptosis, primary rat chondrocytes were treated with IL-1β and NIBR189. As shown in Fig. 13A, the MTT assay showed that the relative viabilities of primary rat chondrocytes were measured to be 100.00 ± 0.37% or 94.82 ± 1.42% in the absence or presence of NIBR189, respectively. The relative viability decreased by 84.90 ± 0.40% in primary chondrocytes treated with IL-1β (Fig. 13A). However, relative viability was measured to be 87.72 ± 1.25% in chondrocytes co-treated with IL-1β and NIBR189 (Fig. 13A). Hence, these data demonstrate that NIBR189 counteracts IL-1β-induced cytotoxicity in primary chondrocytes. Cell live/dead staining showed that IL-1β decreased the number of live cells that presented green fluorescence, but it was counteracted by NIBR189 in primary rat chondrocytes (Fig. 13B). Sequentially, the expression of pro-apoptotic factors such as cleaved caspase-8, -9, and −3, and cleaved PARP were upregulated in primary rat chondrocytes treated with IL-1β (Fig. 13C). In contrast, the expression of pro-apoptotic factors was significantly downregulated in the primary rat chondrocytes co-treated with IL-1β and NIBR189 (Fig. 13C). In addition, the upregulation of caspase-3/-7 activity by IL-1β was also decreased by NIBR189 in primary rat chondrocytes (Fig. 13D upper panel). Furthermore, ICC and

IHC results showed that the immunoreactivity of caspase-3 was increased by IL-1β, but it was counteracted by NIBR189 in chondrocytes and *ex vivo* organ-cultured articular cartilage, as shown in Fig. 13D (middle and lower panel). Therefore, these data indicate that the inhibition of EBI2 has an anti-catabolic effect against IL-1β -induced chondrocyte apoptosis.

To determine whether NIBR189 suppresses IL-1β-induced chondrocyte autophagy, primary rat chondrocytes were treated with IL-1β in the presence or absence of NIBR189. NIBR189 counteracted the expression of IL-1β-induced autophagy biomarkers such as beclin-1 and LC3 in the primary rat chondrocytes with IL-1β (Fig. 14A). Finally, the results of ICC and IHC showed that the upregulation of beclin-1 by IL-1β was reduced by NIBR189 in *ex vivo* organ-cultured articular cartilage in the presence of IL-1β (Fig. 14B). Therefore, these data indicate that the inhibition of EBI2 suppresses IL-1β-induced autophagy in primary rat chondrocytes.

Overall, these results consistently suggest that the inhibition of EBI2 may attenuate IL-1β-induced arthritis through the suppression of oxiapoptophagy in primary rat chondrocytes.

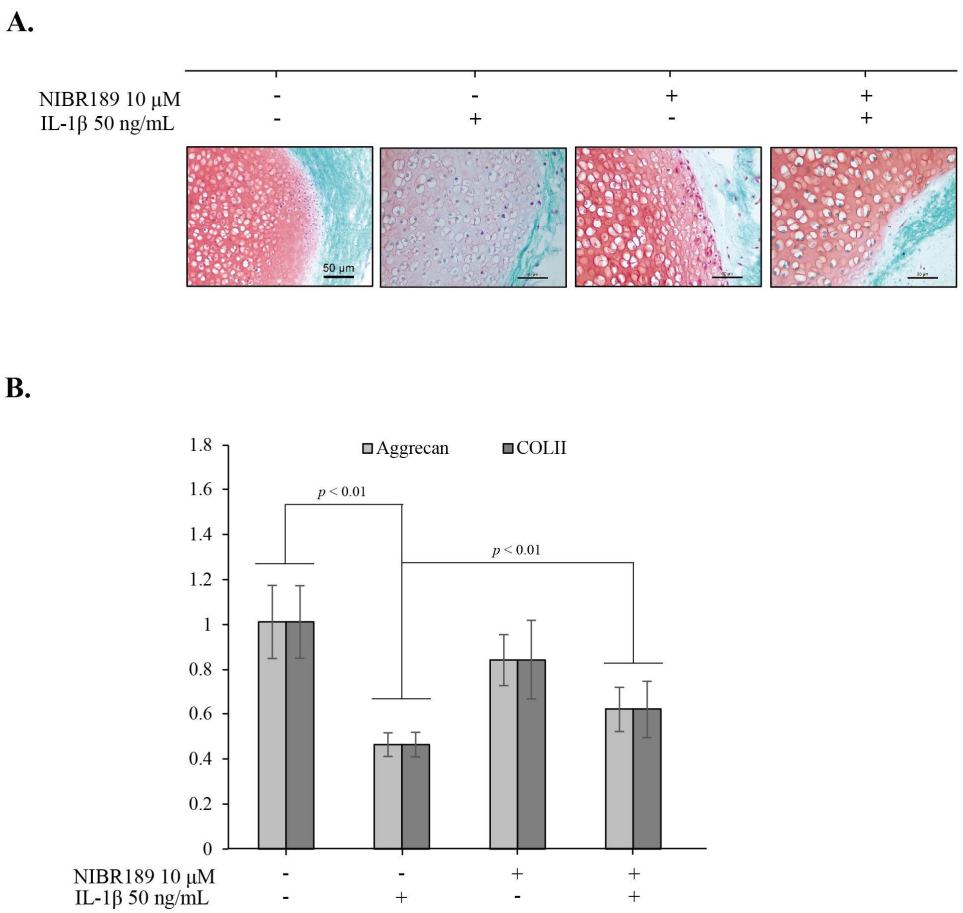



**Fig. 10. NIBR189 counteracts IL-1β-induced catabolic effects.** Explants of articular cartilage dissected from rat knee joint were incubated with 50 ng/mL IL-1β in presence or absence of NIBR189, a receptor antagonist of 7α,25-DHC, for 14 days. Thereafter, safranin-O & fast green staining (A) was performed to verify the proteoglycan loss in articular cartilage. In addition, to verify the alteration of mRNAs associated with the ECM components, rat primary chondrocytes were treated with 50 ng/mL IL-1β in presence or absence of NIBR189 for 24 h. Thereafter, mRNAs were isolated to perform the qPCR (B). A, IL-1β-induced proteoglycan depletion was significantly suppressed by NIBR189 in *ex vivo* organ-cultured articular cartilage. B, the reduction of mRNAs associated with ECM components was rescued by NIBR189 in the rat primary chondrocytes treated with IL-1β.

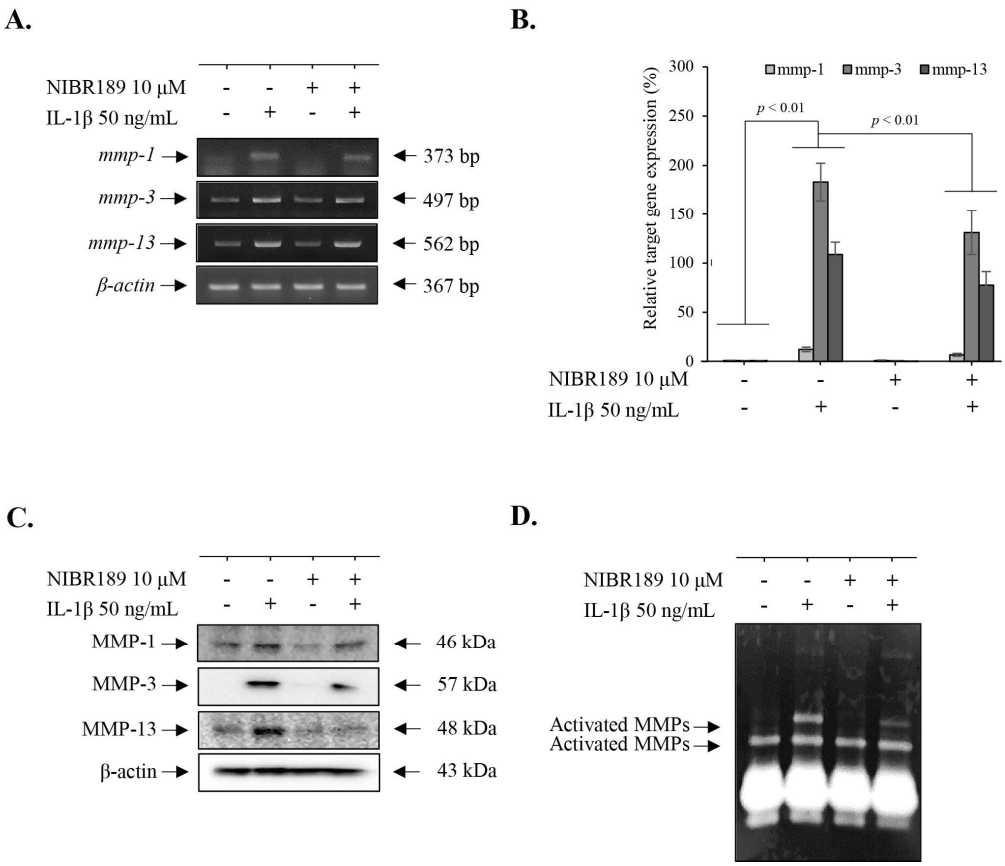



**Fig. 11. NIBR189 suppresses the expression and activation of cartilage degrading enzymes in the primary rat chondrocytes treated with IL-1β.** Primary rat chondrocytes were treated with 50 ng/mL IL-1β in the presence or absence of NIBR189 for 24 h. Thereafter, total RNA, total protein, and conditioned media were collected to perform the qPCR (A), qRT-PCR (B), immunoblotting (C) using specific antibodies associated with cartilage degrading enzymes, such as MMP-1, -3, and -13, and gelatin zymography (D). A & B, the mRNAs encoded the *mmp-1*, *-3*, and *-13* were significantly rescued by NIBR189 in primary rat chondrocytes treated with IL-1β. C, the expressions of MMP-1, -3, and -13 proteins were significantly decreased by NIBR189 in primary rat chondrocytes treated with IL-1β. D, the activation of MMPs were decreased by NIBR189 in the conditioned media collected from primary rat chondrocytes treated with IL-1β.

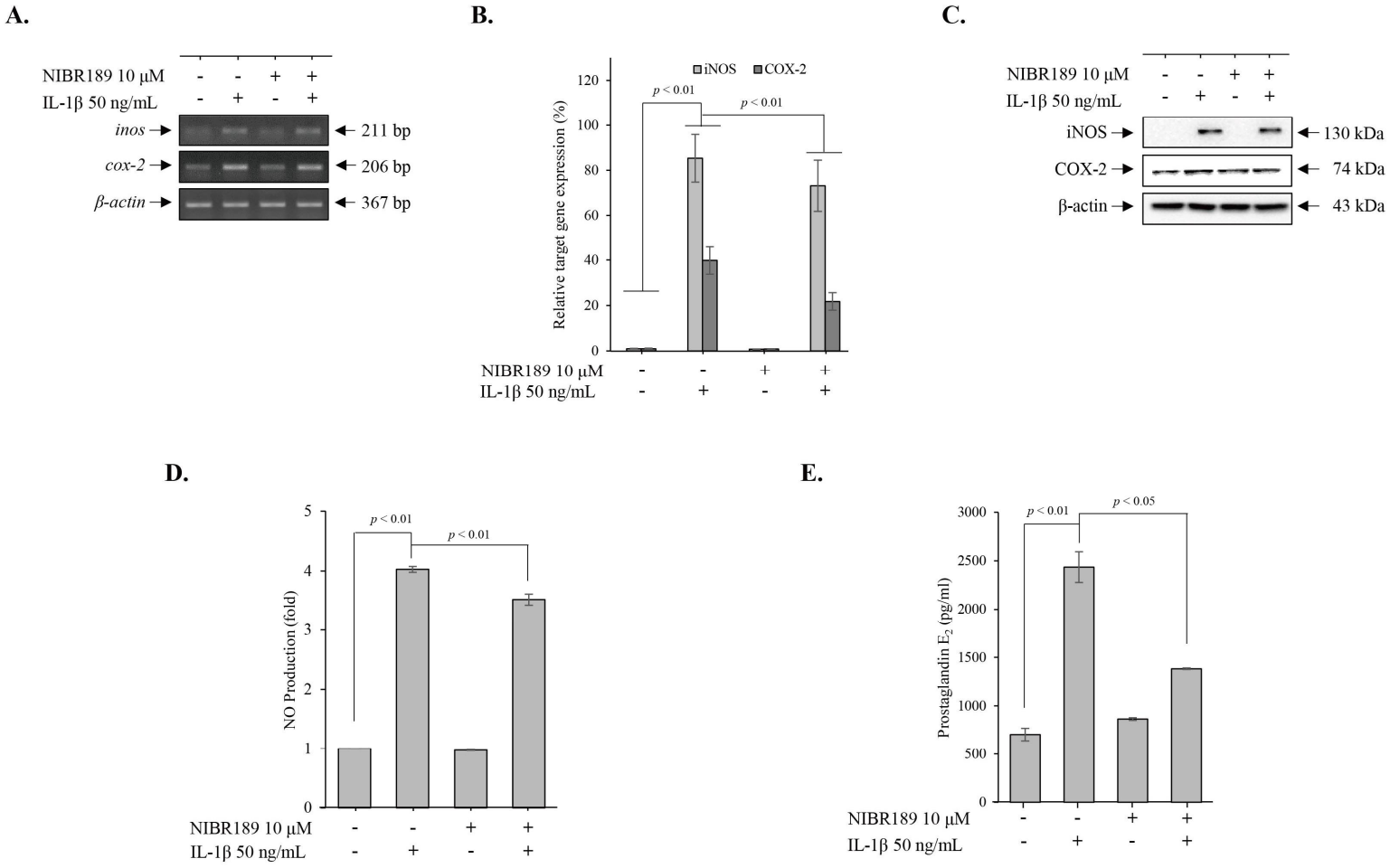


**Fig. 12. NIBR189 suppresses the expression and production of inflammatory mediators in the primary rat chondrocytes treated with IL-1β.** Primary rat chondrocytes were treated with 50 ng/mL IL-1β in the presence or absence of NIBR189 for 24 h. Thereafter, total RNA and total protein were collected to perform the qPCR (A), qRT-PCR (B), immunoblotting (C) using specific antibodies associated with inflammatory mediators, NO assay (D), and $PGE_2$ ELISA (E). A & B, the mRNAs encoded the *inos* and *cox-2* were significantly decreased by NIBR189 in the primary rat chondrocytes treated with IL-1β. C, the expression of iNOS and COX-2 was significantly downregulated by NIBR189 in primary rat chondrocytes treated with IL-1β. D, the production of NO was significantly decreased by NIBR189 in primary rat chondrocytes treated with IL-1β. E, the production of $PGE_2$ was significantly decreased by NIBR189 in the primary rat chondrocytes treated with IL-1β.

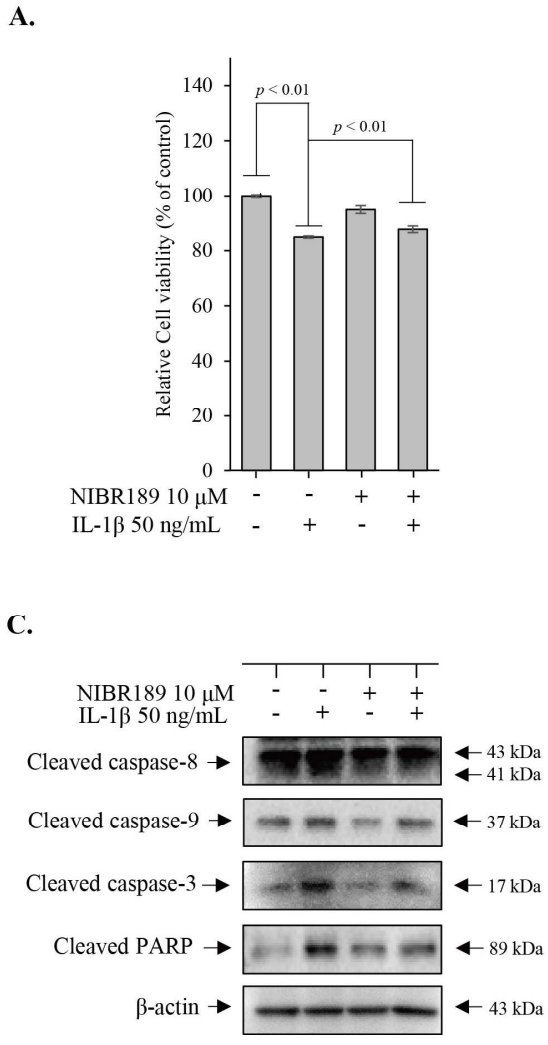


**Fig. 13. NIBR189 suppresses IL-1β-induced chondrocytes apoptosis.** Primary rat chondrocytes were treated with 50 ng/mL IL-1β in the presence or absence of NIBR189 for 24 h. Thereafter, MTT assay (A) and cell live/dead staining (B) were performed to investigate viability and survival of chondrocytes, respectively. Total proteins were extracted to perform the immunoblotting (C) using apoptotic specific biomarkers such as caspase-8, -9, -3, and PARP. In addition, caspase-3/-7 activity (D, upper panel) and ICC (D, middle panel) were performed to investigate activation and expression of caspase-3 in the primary rat chondrocytes treated with IL-1β and NIBR189. The explants of articular cartilage dissected from rat knee joint were treated with IL-1β in the presence or absence of NIBR189 for 14 days. Thereafter, IHC (D, lower panel) was performed to investigate the expression of caspase-3. A & B, NIBR189 rescued the viability of primary rat chondrocyte in the presence of IL-1β. C, expression of apoptosis biomarkers was decreased by NIBR189 in rat primary chondrocytes treated with IL-1β. D, NIBR189 suppressed the activation and expression of caspase-3 in both primary rat chondrocytes and *ex vivo* organ-cultured articular cartilage treated with IL-1β.

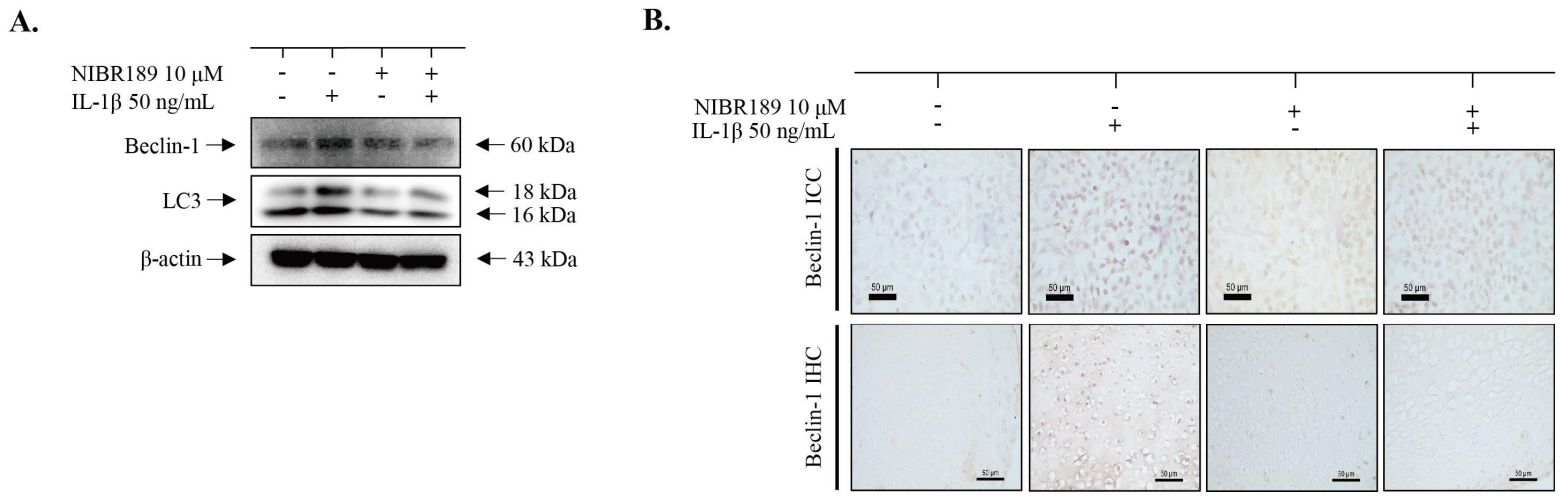



**Fig. 14. NIBR189 suppresses IL-1β-induced autophagy in primary rat chondrocytes.** Primary rat chondrocytes were treated with 50 ng/mL IL-1β in the presence or absence of NIBR189 for 24 h. Thereafter, total proteins were collected to perform immunoblotting (A) using specific autophagy biomarkers. To verify the NIBR189-mediated autophagy suppression in both primary rat chondrocytes and articular cartilage, ICC (B, upper panel) and IHC (B, lower panel) were performed using autophagy specific biomarker beclin-1. A, NIBR189 significantly suppressed the IL-1β-induced LC3 and beclin-1 expressions in primary rat chondrocytes. B, NIBR189 suppressed the expression of beclin-1 in both primary rat chondrocytes and *ex vivo* organ-cultured explants of articular cartilage dissected from rat knee joint.

# Ⅳ. DISCUSSION

Recent studies have reported several representative catabolic risk factors of arthritis, including ROS (such as NO and superoxide anions), pro-inflammatory cytokines (such as IL-1β, Interleukin-6, Interleukin-8 and tumor necrosis factor α), and pain-associated molecules (such as substance-P and $PGE_2$) [18, 29]. These catabolic risk factors can initiate the progressive degeneration of articular cartilage through the accelerating breakdown of the ECM by either the upregulation of cartilage-degrading enzymes and the suppression of tissue inhibitor of metalloproteinases and ECM, components such as Col II and aggrecan, which are regulated by chondrocytes [30].

The pathophysiological etiology of arthritis has been reported to be closely related to aging, traumatic joint injury, severe mechanical joint movement, obesity, individual genetic characteristics, and sex [31]. However, the pathophysiological etiology of arthritis remains largely unknown because it is caused by multiple factors such as one or a combination of more pathophysiological etiologies [32].

Aging, a representative pathophysiological etiology of arthritis, induces chronic low-grade inflammation, referred to as inflammaging [11]. Furthermore, traumatic joint injury and severe mechanical joint movement are the pathophysiological etiology of arthritis as they increase the inflammatory conditions due to chronic overload on the articular cartilage of synovial joints [6]. In addition, genetic disorders associated with the immune system also act as a pathophysiological etiology of arthritis through the upregulation of inflammation at the synovial joint [33]. The menopause also acts as a risk factor for arthritis as it increases inflammation due to hormonal change-mediated stress in elderly women [34]. Hence, inflammation caused by these pathophysiological factors at the synovial joint is a crucial risk factor for arthritis.

In the present study, the representative pro-inflammatory cytokine IL-1β induced arthritis, mediated by the progressive degeneration of articular cartilage via the acceleration of proteoglycan loss was studied in an *ex vivo* organ-cultured explant of articular cartilage dissected from a rat knee joint (Fig. 1A). Sequentially, IL-1β -induced arthritis was mediated by the suppression of ECM components, such as

aggrecan and Col II (Fig. 1B), the expression and activation of cartilage-degrading enzymes such as MMP-1, -3, and -13 (Fig. 2), and an increase of inflammatory mediators, including iNOS, COX-2, NO, and $PGE_2$ (Fig. 3) in primary rat chondrocytes.

Apoptosis is a precisely regulated process associated with cell death during development, homeostasis, and aging [8]. Furthermore, dysregulation of apoptosis is closely related to the pathogenesis of degenerative diseases [8]. In particular, the apoptosis of chondrocytes, which are specialized cells for the maintenance of the ECM, leads to the progressive degeneration of articular cartilage and is observed in the articular cartilage during the late-stage of arthritis [8, 35]. Del Carlo et al. reported that inflammatory mediators, such as NO and superoxide anions, are the key factors that induce the apoptosis of chondrocytes [36]. In addition, the IL-1 family, including Interleukin-1α and IL-1β, the representative pro-inflammatory cytokine mediating the progressive degeneration of articular cartilage, is reported to decrease the viability of chondrocytes [37]. Hence, these results suggest that the apoptosis of chondrocytes is related to persistently high levels of inflammation, which could augment the progressive degeneration of articular cartilage [8]. As shown in Fig. 4, present study demonstrated that IL-1β decreased the viability of chondrocytes through apoptosis mediated by the cascade activation of pro-apoptotic caspases, such as caspase-8, -9, and -3. Furthermore, the expression of both cleaved caspase-8 and -9 was gradually increased by IL-1β in primary rat chondrocytes. These results indicate that IL-1β-induced chondrocyte apoptosis is mediated by the mitochondrial-dependent intrinsic apoptosis pathway.

In addition, present study demonstrated that the expression of autophagy-related biomarkers, such as beclin-1 and LC3, was significantly increased in both chondrocytes and articular cartilage under IL-1β-induced inflammatory conditions (Fig. 5). Autophagy, a cellular homeostatic mechanism, regulates cell death and inflammation through interaction with various types of stimuli, including metabolic stress and oxygen depletion [37-39]. Furthermore, autophagy has been associated with a complex cross-talk with apoptosis through the sharing with beclin-1, which is identified as a Bcl-2 interacting protein possessing a Bcl-2 homology 3 domain to bind Bcl-2 and

B-cell lymphoma-extra large (Bcl-xL) [40, 41]. Hence, the upregulation of the anti-apoptotic factor Bcl-2 can suppress the autophagic function of beclin-1 [42]. Moreover, the inhibition of death receptor-mediated extrinsic apoptosis, mediated by the caspase-8 and -3 axis, suppresses autophagy through a direct interaction with autophagy-relevant factors, such as LC3 [43]. Therefore, many studies have shown that autophagy accompanied by apoptosis is closely associated with the pathogenesis of arthritis [35, 44]. Taken together, these data suggest that IL-1β-induced arthritis is involved in apoptosis and autophagy in chondrocytes.

Recent studies have shown that metabolic syndromes, including obesity, hyperlipidemia, hypercholesterolemia, and hypertension, act as the pathophysiological risk etiologies of arthritis, including OA [45], RA [46-48], psoriatic arthritis [49], gout [50], and juvenile idiopathic arthritis [51]. Metabolic syndromes are closely associated with systemic inflammation [52]. Hence, recent studies associated with the pathophysiological etiology of arthritis have been conducted to investigate the aspects of metabolic diseases [53, 54]. In accordance with this attempt, cholesterol metabolism associated with metabolic syndrome, is attracting attention as a crucial risk factor for arthritis [55]. Aksu et al. reported that the levels of oxysterols, the metabolites of cholesterol, are significantly increased in patients with silicosis-related complications, including RA, systemic sclerosis, and vasculitis, which indicates a correlation between cholesterol oxidation, lipid peroxidation, and silicosis [56]. More recently, Choi et al. reported that cholesterol metabolism, mediated by the CH25H-CYP7B1-RORα axis, is closely associated with the pathogenesis of OA [24]. These reports consistently indicate that the pathogenesis of arthritis is involved in the biological linkage between inflammation and cholesterol metabolism [24, 56]. Hence, in this study, the expression and synthesis of oxysterols were investigated in both articular cartilage and chondrocytes under representative pro-inflammatory cytokine IL-1β-induced arthritic conditions.

As shown in Fig. 6, IL-1β not only increased the expression of oxysterol synthases, such as CH25H and CYP7B1, but also upregulated the synthesis of 25-HC in chondrocytes. Hence, these data indicate that IL-1β-induced arthritis is accompanied by the synthesis of oxysterols such as 25-HC and 7α,25-DHC. Furthermore, these results

indicate that IL-1β-induced chondrocyte death involves oxiapoptophagy, which is a cell death accompanied by oxidative stress, apoptosis, and autophagy.

Previous studies have reported that oxysterol induces apoptosis in various types of cells, such as human monocytic cell lines (e.g., U-937 and HL-60) [57], human lymphoblastic leukemia CEM cells [58], human vascular smooth muscle cells [59], and presumptive endothelial cell line ECV-304 cells [60]. Additionally, it has been reported that some oxysterols induce oxiapoptophagy, a type of cell death involved in oxidative stress, apoptosis, and autophagy [61]. In particular, Kostopoulou et al. reported that sterol regulatory element binding transcription factor 2 (SREBP-2), a transcription factor associated with the pathogenesis of OA, was upregulated in OA chondrocytes treated with 25-HC [16]. Furthermore, Seo et al. reported that 25-HC is a metabolic pathophysiological factor of OA through an increase in chondrocyte apoptosis [62]. Taken together, the previous studies and the results of this study suggest that IL-1β-induced arthritis could be involved in oxysterol-induced chondrocyte oxiapoptophagy during the progressive degeneration of articular cartilage.

Oxysterols, cholesterol derivatives with 27 carbon atoms, are natural materials that are regulated by cholesterol metabolism in the human body and are involved in many physiological functions, such as several signaling pathways, membrane fluidity, and activity for some membrane proteins at very low concentrations [63-65]. However, some oxysterols are associated with the pathogenesis of serveral human diseases, such as atherosclerosis, Alzheimer's disease, Parkinson's disease, and even cancer [66]. Generally, oxysterols are synthesized from cholesterol via enzymatic and non-enzymatic pathways [67]. The enzymatic pathways are mainly mediated by the synthesis of side-chain oxysterols by the cytochrome P450 (CYP) family and CH25H [65]. In contrast, non-enzymatic pathways associated with the generation of ring-oxysterols are mediated by ROS [67]. Hence, various types of oxysterols are synthesized from cholesterol via enzymatic and non-enzymatic pathways [67].

The oxysterol 25-HC is generated from cholesterol by CH25H and ROS under inflammatory conditions [65]. Recent studies have reported that 25-HC not only amplifies the production of IL-1β in microglial cells [68] but also contributes to cerebral inflammation of X-linked adrenoleukodystrophy via the activation of the

NLRP3 inflammasome [69]. In addition, previous studies have reported that 25-HC induces apoptosis in various types of cells such as human aortic smooth muscle cells [70], human leukemic cell line CEM [71], oligodendrocytes [72], rat Leydig cells [73], and rat phaeochromocytoma cell line PC12 [74]. Furthermore, Olivier et al. reported that 25-HC induces both P2X7-dependent pyroptosis, a type of cell death mediated by inflammation, and caspase-dependent apoptosis in human keratinocytes [21]. In particular, Kostopoulou et al. reported that SREBP-2, a transcription factor associated with the pathogenesis of OA, is upregulated in OA chondrocytes treated with 25-HC [16]. Seo et al. reported that 25-HC acts as a metabolic pathophysiological factor of OA by increasing chondrocyte apoptosis [62]. Therefore, previous studies have indicated that 25-HC induces chondrocyte death mediated by inflammation-mediated oxidative stress, apoptosis, and autophagy in chondrocytes.

The present study demonstrated that 25-HC induced caspase-dependent apoptosis (Fig. 7A-7D), upregulation of ROS production (Fig. 7E), and expression of autophagy biomarkers (Fig. 7F). Finally, 25-HC induced progressive degeneration of articular cartilage via the acceleration of proteoglycan loss (Fig. 7G). Hence, these data suggest that 25-HC induces the degeneration of articular cartilage through oxiapoptophagy accompanied by oxidative stress, apoptosis, and autophagy in chondrocytes.

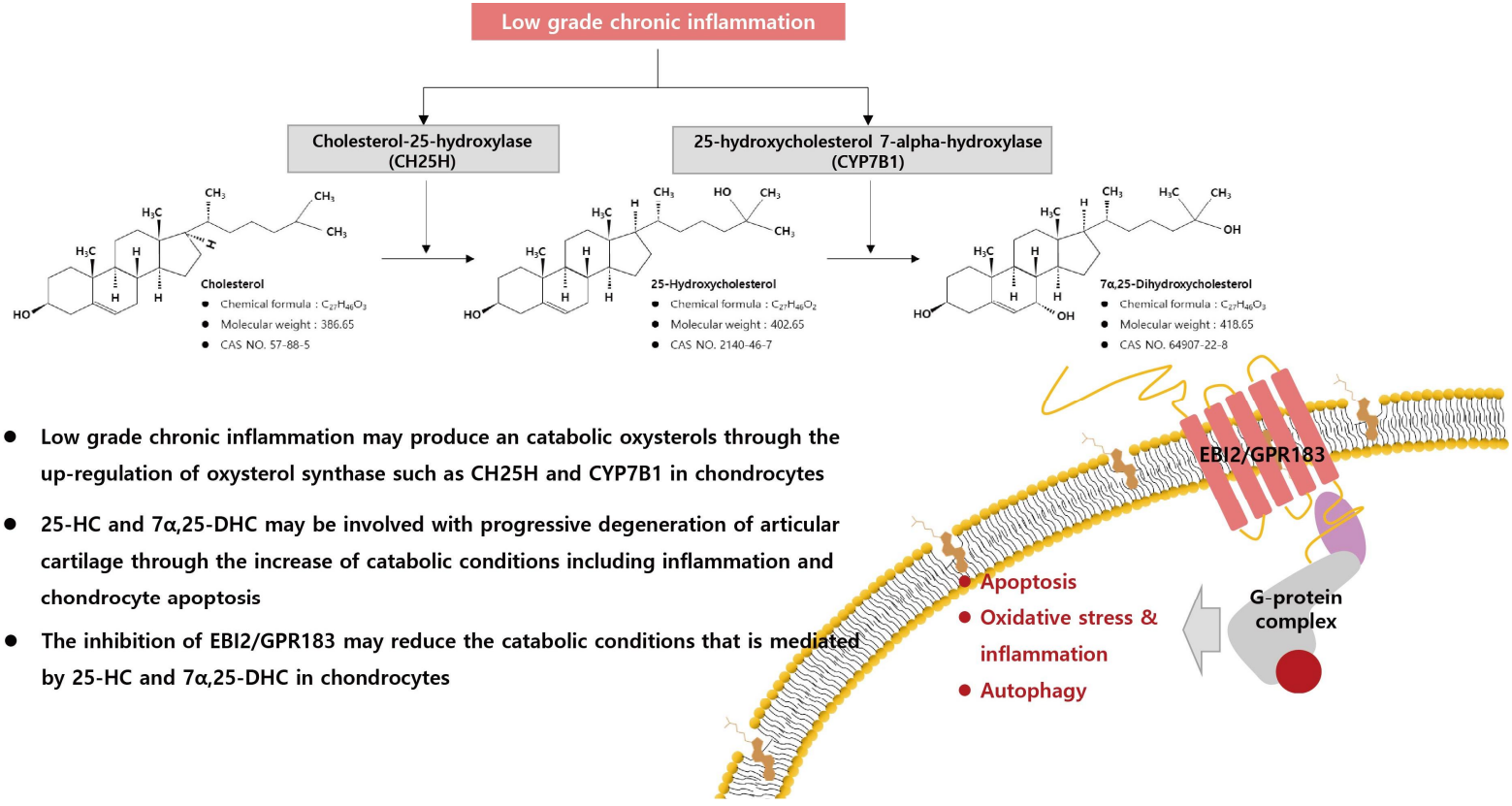
As shown in Fig. 6, the expression of CYP7B1 was significantly increased in primary rat chondrocytes treated with IL-1β. CYP7B1, a member of the CYP family, synthesizes 7α,25-DHC from 25-HC [75]. Furthermore, 7α,25-DHC is known as the physiological ligand of EBI2 [25] and was recently discovered as immune regulator [76]. More recently, Choi et al. reported that the CH25H-CYP7B1 axis regulates the pathogenesis of OA [24]. Therefore, this study was undertaken to investigate whether 7α,25-DHC, a downstream oxysterol of 25-HC, could induce progressive degeneration of articular cartilage through chondrocyte oxiapoptophagy.

Similar to 25-HC, 7α,25-DHC not only decreased the viability of chondrocytes (Fig. 8A and 8B) but also induced the caspase-dependent apoptosis through the cascade activation of pro-apoptotic caspases in chondrocytes (Fig. 8C and 8D). Furthermore, the production of ROS (Fig. 8E) and the expression of autophagy

biomarkers (Fig. 8F) were significantly increased in chondrocytes treated with 7α,25-DHC. Finally, 7α,25-DHC induced progressive degeneration of articular cartilage via the acceleration of proteoglycan depletion. Furthermore, as shown in Fig. 9, NIBR189, an antagonist of EBI2, not only counteracted the 7α,25-DHC-induced cytotoxicity and apoptosis but also suppressed both the production of ROS and the expression of autophagy biomarkers in chondrocytes. Moreover, NIBR189 ameliorated the 7α,25-DHC-induced proteoglycan depletion in *ex vivo* organ-cultured explants of articular cartilage (Fig. 9). These data consistently demonstrate that the inhibition of EBI2 attenuates the progressive degeneration of articular cartilage through the suppression of 7α,25-DHC-induced chondrocyte oxiapoptophagy, indicating that 7α,25-DHC is a catabolic risk factor of arthritis through the oxiapoptophagy of chondrocytes. Moreover, these results suggest that the inhibition of EBI2 may have an anti-catabolic effect to prevent the progressive degeneration of articular cartilage by counteracting 7α,25-DHC-induced chondrocyte oxiapoptophagy in the synovial joint under inflammatory conditions.

In addition, the inhibition of EBI2 counteracted significantly against IL-1β-induced severe catabolic effects, such as proteoglycan depletion in *ex vivo* organ-cultured explants of articular cartilage (Fig. 10A), the suppression of ECM components (Fig. 10B), the expression and activation of cartilage-degrading enzymes (Fig. 11), and the upregulation of inflammatory mediators (Fig. 12). Furthermore, the inhibition of EBI2 not only ameliorated the IL-1β-induced cytotoxicity and caspase-dependent apoptosis in chondrocytes (Fig. 13) but also counteracted IL-1β-induced autophagy (Fig. 14). Furthermore, these data suggest that IL-1β-induced arthritis may be involved in chondrocyte oxiapoptophagy by upregulating 25-HC and 7α,25-DHC under inflammatory conditions. Therefore, these results consistently demonstrate that the inhibition of EBI2 has an anti-catabolic effect in preventing inflammation-induced arthritis.

In conclusion, the present study demonstrates that the pathogenesis of arthritis is associated with the oxiapoptophagy of chondrocytes, which is mediated by the catabolic oxysterols 25-HC and 7α,25-DHC under inflammatory conditions.




- Low grade chronic inflammation may produce an catabolic oxysterols through the up-regulation of oxysterol synthase such as CH25H and CYP7B1 in chondrocytes
- 25-HC and 7α,25-DHC may be involved with progressive degeneration of articular cartilage through the increase of catabolic conditions including inflammation and chondrocyte apoptosis
- The inhibition of EBI2/GPR183 may reduce the catabolic conditions that is mediated by 25-HC and 7α,25-DHC in chondrocytes

**Fig. 15. Schematic diagram of chondrocyte oxiapoptophagy regulated by 25-HC-7α,25-DHC-EBI2 axis in arthritis.**

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# ABSTRACT

## Chondrocyte oxiapoptophagy mediated by 7α,25-DHC-EBI2 axis in IL-1β-induced arthritis

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Oxysterol 7α,25-dihydroxycholesterol (7α,25-DHC) is synthesized by cytochrome P450 family 7 subfamily B member 1 (CYP7B1) from 25-hydroxycholesterol (25-HC) under inflammatory conditions, associated with the apoptosis of various cell types. In this study, the antagonism of the 7α,25-DHC receptor Epstein-Barr virus-induced G-protein-coupled receptor 2 (EBI2) and its mechanism were analyzed in interleukin-1β (IL-1β)- or 7 α,25-DHC-induced chondrocyte oxiapoptophagy.

This study showed that IL-1β induced the progressive degeneration of articular cartilage though the acceleration of proteoglycan loss in the *ex vivo* organ cultured explant of articular cartilage dissected from rat knee joint. Sequentially, IL-1β-induced arthritis was mediated by the suppression of extracellular matrix (ECM) component such as aggrecan and type II collagen, the expression and activation of cartilage degrading enzymes such as matrix metalloproteinase -1, -3, and -13, and even the increase of inflammatory mediators including inducible nitric oxide synthase, cyclooxygenase-2, nitric oxide, and prostaglandin $E_2$ in primary rat chondrocytes. Furthermore, IL-1β decreased the viability of chondrocytes through apoptosis mediated by the cascade activation of pro-apoptotic caspases, such as caspase-8, -9, and -3. In addition, the expression of autophagy-related biomarkers, such as beclin-1 and Microtubule-associated protein 1A/1B-light chain 3 (LC3), was significantly increased in both chondrocytes and articular cartilage under IL-1β-induced inflammatory condition. Additionally, IL-1β did not only increase the expression of oxysterol

syntheses such as CH25H and CYP7B1, but also up-regulate the synthesis of 25-HC in chondrocytes. Hence, these data indicate that IL-1β-induced arthritis is not only mediated by apoptosis of chodnrocytes, but also is accompanied with the synthesis of oxysterols such as 25-HC and 7α,25-DHC under inflammatory condition.

Next, the present study demonstrated that both 25-HC and 7α,25-DHC induced the caspase dependent apoptosis, the upregulation of ROS production, and the expression of autophagy biomarkers such as beclin-1 and LC3. In addition, both oxysterols induced the progressive degeneration of articular cartilage through the acceleration of proteoglycan loss. Hence, these data indicate that both oxysterols induce the degeneration of articular cartilage through the oxiapoptophagy accompanied with oxidative stress, apoptosis, and autophagy in chondrocytes. Furthermore, the antagonism of EBI2, a receptor activated by 7α,25-DHC, did not only counteract the 7α,25-DHC-induced cytotoxicity and apoptosis, but also suppress both the production of ROS and the expression of autophagy biomarkers in chondrocytes. Moreover, NIBR189, an EBI2 antagonist, ameliorated the 7 α,25-DHC-induced proteoglycan depletion in the *ex vivo* organ cultured explants of articular cartilage. These data suggest that antagonism of EBI2 might have an anti-catabolic effect to prevent the progressive degeneration of articular cartilage through the counteraction against to 25-HC-7α,25-DHC-induced chondrocyte oxiapoptophagy in the synovial joint under inflammatory conditions.

Hence, it was hypothesized that the inhibition of EBI2 might counteract the IL-1β -induced arthritis through the suppression of chondrocyte oxiapoptophagy. NIBR189 counteracted significantly against to IL-1β-induced severe catabolic effects such as the proteoglycan depletion in *ex vivo* organ cultured explants of articular cartilage, the suppression of ECM components, the expression and activation of cartilage degrading enzymes, and the upregulation of inflammatory mediators. Furthermore, NIBR189 did not only ameliorate the IL-1β-induced cytotoxicity and caspase dependent apoptosis in chondrocytes, but also counteract against to IL-1β-induced autophagy.

Taken together, these results suggest that antagonism of the 7α,25-DHC receptor EBI2 can inhibit 7α,25-DHC-induced chondrocyte oxiapoptophagy. Finally, as a result of this study, it is thought that the application of EBI2 inhibition can provide directions for arthritis prevention and arthritis treatment strategies.