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EGR1 promotes the cartilage degeneration and hypertrophy by activating the Krüppel-like factor 5 and β-catenin signaling

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ABSTRACT

Osteoarthritis is one of the most common orthopedic diseases in elderly people who have lost their mobility. In this study , we observed abnormally high EGR1 expression in the articular cartilage of patients with osteoarthritis. We also found significantly high EGR1 expression in the articular cartilage of mice with destabilized medial meniscus (DMM)-induced osteoarthritis and 20-month-old mice. In vitro experiments indicated that IL-1β could significantly enhance EGR1 expression in primary mouse chondrocytes. EGR1 over-expression in chondrocytes using adenovirus could inhibit COl2A1 expression and enhance MMP9 and MMP13 expression. And silencing EGR1, using RNAi, had the opposite effects. Moreover, EGR1 over-expression accelerated chondrocyte hypertrophy in vitro, and EGR1 knockdown reversed this effect. We then explored the underlying mechanism. EGR1 over-expression increased Kruppel-Like Factor 5 (KLF5) protein level without influencing its synthesis. Enhanced EGR1 expression induced its integration with KLF5, leading to suppressed ubiquitination of KLF5. Moreover, EGR1 prompted β-catenin nuclear transportation to control chondrocyte hypertrophy. Ectopic expression of EGR1 in articular cartilage aggravated the degradation of the cartilage matrix in vivo. The EGR1 inhibitor, ML264, protected chondrocytes from IL-1β-mediated cartilage matrix degradation in vitro and DMM-induced osteoarthritis in vivo. Above all, we demonstrate the effect and mechanisms of EGR1 on osteoarthritis and provide evidence that the ML264 might be a potential drug for treating osteoarthritis in the future.

1. Introduction

Osteoarthritis (OA) is a common chronic and age-related degenerative joint disease. Previous reports indicate that about 10% of the population suffer from this disease worldwide [1,2]. Many factors play a role in the development of OA, including age, obesity, injury and genetics, yet the pathogenesis of OA remains unclear [3]. Due to the lack of early diagnosis and intervention in OA, this disease seriously affects patient's quality of life and social economy. Therefore, it is of practical significance to clarify the pathogenesis of OA and to identify effective treatments, to reduce the economic burden on families and society [2–4].

Typical pathological features of OA include degeneration and loss of cartilage, accompanied by the formation of osteophytes and changes in subchondral bone [5]. The formation of subchondral bone may be an initial event in the development of OA, and its role in the pathological development of OA has been confirmed. Interaction between the subchondral bone and the articular cartilage leads to the continuous development of OA [6]. Articular cartilage consists of chondrocytes and extracellular matrix. The extracellular matrix of the cartilage is composed of a fibrous collagen network, rich in type II collagen and proteoglycans, and accounts for approximately 95% of the articular cartilage in the human body [7]. In the development of OA, the content and activity of proteolytic enzymes increases, which accelerates the degradation of the cartilage matrix [8]. The balance of cartilage matrix synthesis and degradation was destroyed in OA, directly leading to the continuous development of OA and clinical symptoms.

Chondrocytes, the only cells in the adult cartilage matrix, play a major role in maintaining the stability and function of the cartilage matrix structure. Under normal conditions, articular chondrocytes do

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not undergo terminal differentiation, and weak cartilage matrix metabolism maintains the internal stability of cartilage [9]. However, under pathological conditions, such as OA, articular chondrocytes begin to express MMP13 and COLX (markers of chondrocyte hypertrophy). Subsequently, expression of other chondrocyte hypertrophy-related genes is enhanced (osteocalcin, IHH, and CD36), accompanied by degradation of the cartilage matrix and chondrocyte apoptotic calcification [10]. Therefore, it is important to determine the underlying mechanism of articular cartilage matrix degradation and abnormal hypertrophy of articular chondrocytes. Signaling pathways including Wnt-βcatenin, TGF-β, MAPK, NF-κb, and specific genes such as ITGBL1 and RUNX2, and tyrosine kinase encoding Fyn , have been implicated in the progress of OA [11–14]. At present, OA pathogenesis research tends to be focused on articular cartilage matrix degradation, or on the mechanism of abnormal articular chondrocytes hypertrophy.

EGR1(Early Growth Response 1) belongs to the EGR family of C2H2-type zinc-finger proteins. As a transcriptional regulator, EGR1 controls many target genes involved in cell proliferation, survival and differentiation. EGR1 plays a central role in regulating the response to growth factors, DNA damage and ischemia [15,16]. Moreover, EGR1 binds P53 to regulate cell growth, proliferation, and differentiation. Additionally, the Δ133p53/EGR1/KLF5 axis controls the SRSF1 (serine/arginine-rich splicing factor 1)-mediated vascular smooth muscle cell proliferation and growth [17]. EGR1 also plays a dominant role in the differentiation of stem cells into tendons. It binds to the promoter region of Col1a1 and Col2a1 in vivo and regulates tendon differentiation and repair through interacting with TGF-β [18]. In the large sample screening of the myeloma mutation spectrum, patients with EGR1 mutations had an increased survival rate [19]. EGR1 can also mediate inflammatory responses by regulating the expression and function of inflammatory mediators including IL-1β, TNF-α, mPGES-1 and PGE2 [20]. Krüppel-like factor 5 (KLF5) is a member of the Kruppel-like factor subfamily of zinc finger proteins [21]. KLF5 is a transcriptional activator that binds the promoter of target genes or acts downstream of multiple signaling pathways [22,23]. KLF5 causes cartilage degradation via MMP9 [24]. Although EGR1 induces activation of KLF5 [25], the specific mechanism by which EGR1 regulates KLF5 expression is unclear and the role of EGR1 in OA has not been clearly elucidated.

In this study, we demonstrate the effect of EGR1 on OA, clarify the underlying mechanism, and identify a novel target of OA.

2. Materials and methods

2.1. Reagents

DMEM (Dulbecco's Modified Eagle Medium), penicillin/streptomycin, and FBS (fetal bovine serum) were purchased from Gibco-BRL (Gaithersburg, MD, USA). ML264 and XAV939 were purchased from MCE(USA).They were dissolved in DMSO and stored in −20 °C. ML264 and XAV939 were then diluted by DMEM before cell culture to make sure the DMSO was < 0.1% to the total medium.IL-1β was purchased from R&D system. Antibodies specific for EGR1、Actin、Ub、KLF5、β-catenin and LaminA were purchased from cell signaling technology (Danvers,MA,USA). Antibodies specific for MMP9、MMP13、COLX、Collagen II、ADAMTS4 and ADAMTS5 were purchased from Abcam(Cambridge, MA, USA). Calcium cobalt staining and Alizarin Red S were purchased from beyotime(Shanghai China).RNA interference was purchased from GenePharma(Shanghai China) All other chemicals used were of analytical grade and indicated in the article.

2.2. Plasmids and adenoviruses

Full-length EGR1 cDNA amplified by PCR was subcloned into BamHI and *Mlu*I sites of the pADM-FH-GFP expression vector. EGR1 adenoviruses were generated using an Adenovirus Generation Kit (Takara), following the manufacturer's instructions. An empty adenovirus vector was used as a control. The obtained EGR1 adenovirus was used for over-expression experiments.

2.3. Human articular cartilage samples and experimental OA in mice

Articular cartilages were collected from 3 patients suffering from hip osteoarthritis at the time of total hip arthroplasty. Cartilage fragments were dissected from each individual and subjected to histological analysis. Normal articular cartilage samples (N = 3) were prepared from people who had femoral neck fracture without hip osteoarthritis. Use of human materials has been approved by the institutional review board of the Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang university. And the informed consents from patients were obtained before the study.

C57BL/6 J mice were used for the experimental OA studies. The animal experiments in this study were carried out according to the principles and procedures of the National Institutes of Health (NIH) Guide and the guidelines for animal treatments of Sir Run Run Shaw Hospital. The experimental protocols are approved by the Ethics Committee of Sir Run Run Shaw Hospital. As indicated, male mice of various ages (4 and 20 months old) were used for age-related degeneration OA model. And male mice of 8 weeks old mice were killed at 8 weeks after DMM surgery or at 8 weeks after the first intra-articular (IA) injection of adenovirus unless noted. ML264 (20 mg/kg body weight) or DMSO was injected into the intraarticular spaces of the knee joints every 2 days for 6 weeks, beginning 2 weeks after DMM surgery. The mice were subjected to histological and biochemical analyses. OARSI scoring were carried out according to the previous study [26].

2.4. Infection and intraarticular (IA) injection of adenovirus

Mouse articular chondrocytes were cultured for 2 days, infected with 800 multiplicity of infection (MOI) of adEGR1(pAdM-FH-GFP-EGR1), and cultured in DMEM with 10% fetal bovine serum (FBS) for further analysis. For IA injection of adenovirus, Ad-EGR1 and Ad-GFP (1 × 109 PFUs in a total volume of 10 μL) were injected into the knee joints of mice once per week for 3 weeks. ML264 (20 mg/kg body weight) or XAV939 (5 mg/kg body weight) was injected into the intraarticular spaces of the knee joints every 2 days for 4 weeks, beginning 1 week after the first injection of adenovirus. Cartilage destruction was examined by safranin-O staining and analyzed using the OARSI scoring system. Genes expression were examined using Realtime-PCR.

2.5. Histology analysis and immunohistochemistry

Human OA cartilage was frozen, and sectioned at a thickness of about 5 μm. The samples were fixed in paraformaldehyde. Cartilage destruction was examined by safranin O-fast green staining. Briefly, knee joints were fixed in 4% paraformaldehyde, decalcified in 0.5 M EDTA, and embedded in paraffin. The specimens were sectioned at a thickness of about 5 μm. They were then deparaffinized in xylene, hydrated with graded ethanol, and stained with safranin O-fast green staining for cartilage destruction. Cartilage destruction was scored by five observers under blinded conditions using the OARSI scoring system (grade 0–6). As for the analysis of EGR1, immunohistochemistry was carried out using a histo-stain SABC kit (CWBIO, Shanghai) according to the manufacturer's instructions. Rabbit anti-ERG1 was used for this study at a dilution of 1:100. Three pathologists (CJ, ZHJ, ZBY), who were blind to the experiment, were responsible for scoring of EGR1 for each of the five sections in each specimen according to the standard: negative (0 point); light yellow (1 point); light brown (2 points); dark brown (3 points). The total scores were added from the three pathologists.

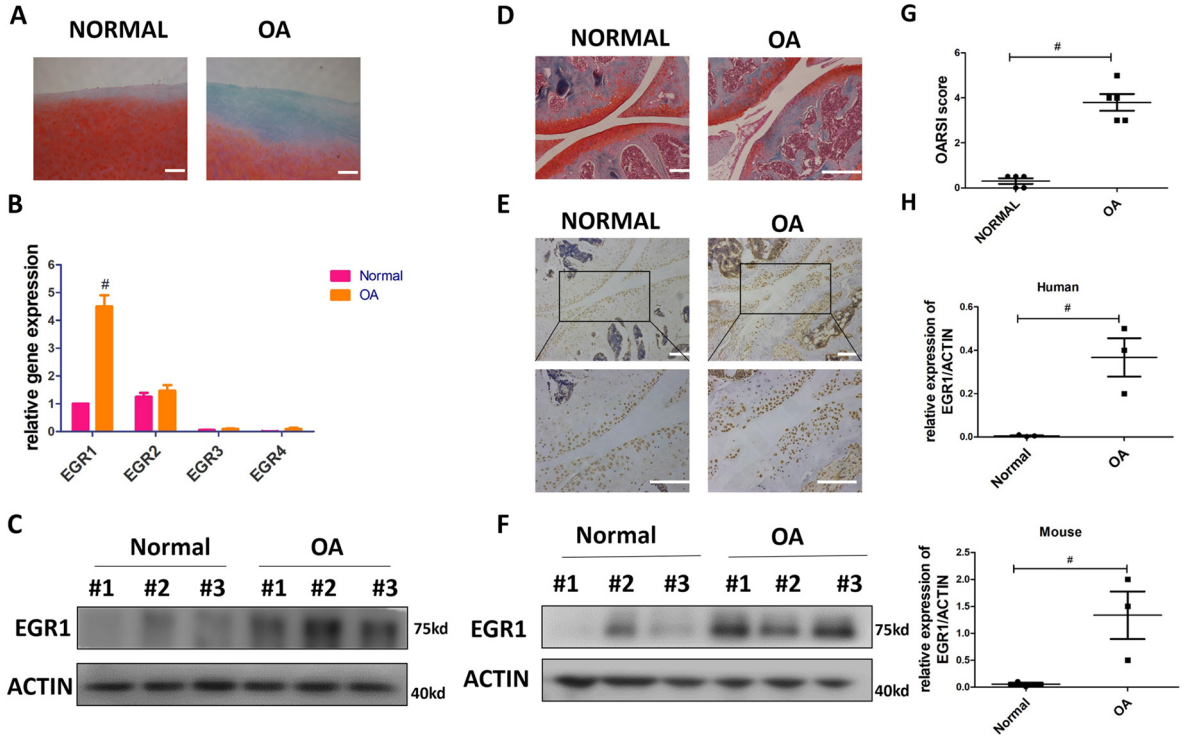


Fig. 1. EGR1 expression in human and mice cartilage. (A)Human normal and OA articular cartilage was obtained (N = 3). Cartilage destruction analyzed using safranin O-fast green staining. Original scale bars: 100 μm.(B) mRNA was isolated from human normal and OA articular cartilage (N = 3). Expression of EGR1, EGR2, EGR3, and EGR4 was detected using real-time PCR. (C) Protein was isolated from human normal and OA articular cartilage (N = 3). EGR1 protein level was analyzed using western blotting. (D) The DMM-induced OA model was established. Eight weeks after surgery, the knee joints from the Sham group (Normal) and DMM group (OA) were obtained (N = 5). Cartilage destruction was analyzed using safranin O-fast green staining. Original scale bars: 200 μm. (E) EGR1 expression was detected in articular cartilage using immunohistochemistry. Original scale bars: 200 μm. (F) EGR1 expression in the Sham (Normal) and DMM (OA) groups was assessed via western blotting. The protein was isolated from the articular cartilage in both groups (N = 3). (G) Cartilage destruction in DMM-induced osteoarthritis was confirmed via OARSI score analysis. (H) EGR1 expression in human and mouse OA samples was quantitatively analyzed. All data in bar graphs are expressed as mean ± SEM. P* < 0.05, P# < 0.01.

2.6. Primary chondrocytes

Primary chondrocytes from human and mice were obtained as described previously [13,20]. In brief, human articular chondrocytes were harvested by overnight incubation of 1 mm^2 cartilage slices with collagenase P (2 mg/mL) in DMEM supplemented with 10% FBS and 50 μg/mL gentamicin. After resuspension and filtration through a filter (0.7 μm), cells were cultured in a 24-well plate at a seeding density of approximately 10^5 cells/mL. Mouse chondrocytes were obtained from primary articular cartilage isolated from the femoral condyles and tibial plateaus of mice on postnatal day 5–6 or from mice with experimental OA as described previously [20].

2.7. Protein extraction from human cartilage and mice joints

Human cartilage or mice joints were first dissected into small pieces (approximately 0.5 mm × 0.5 mm) with a knife, and milled in 200 μL radioimmune precipitation assay (RIPA) lysis buffer with protease inhibitor cocktail. The mixture was then centrifuged at 10000 *g* and the supernatant was collected for further analysis.

2.8. Cell transfection

Cells were transfected with siRNA using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. In brief, the day before transfection, primary chondrocytes were seeded in 6-well plates at a density of approximately 2 × 104 cells/well and transfected with 20 nM siEGR1 and control siRNA. After 6 h, the medium was replaced with normal DMEM containing 10% FBS and cultured for the indicated days for analysis.

2.9. Immunofluorescence staining

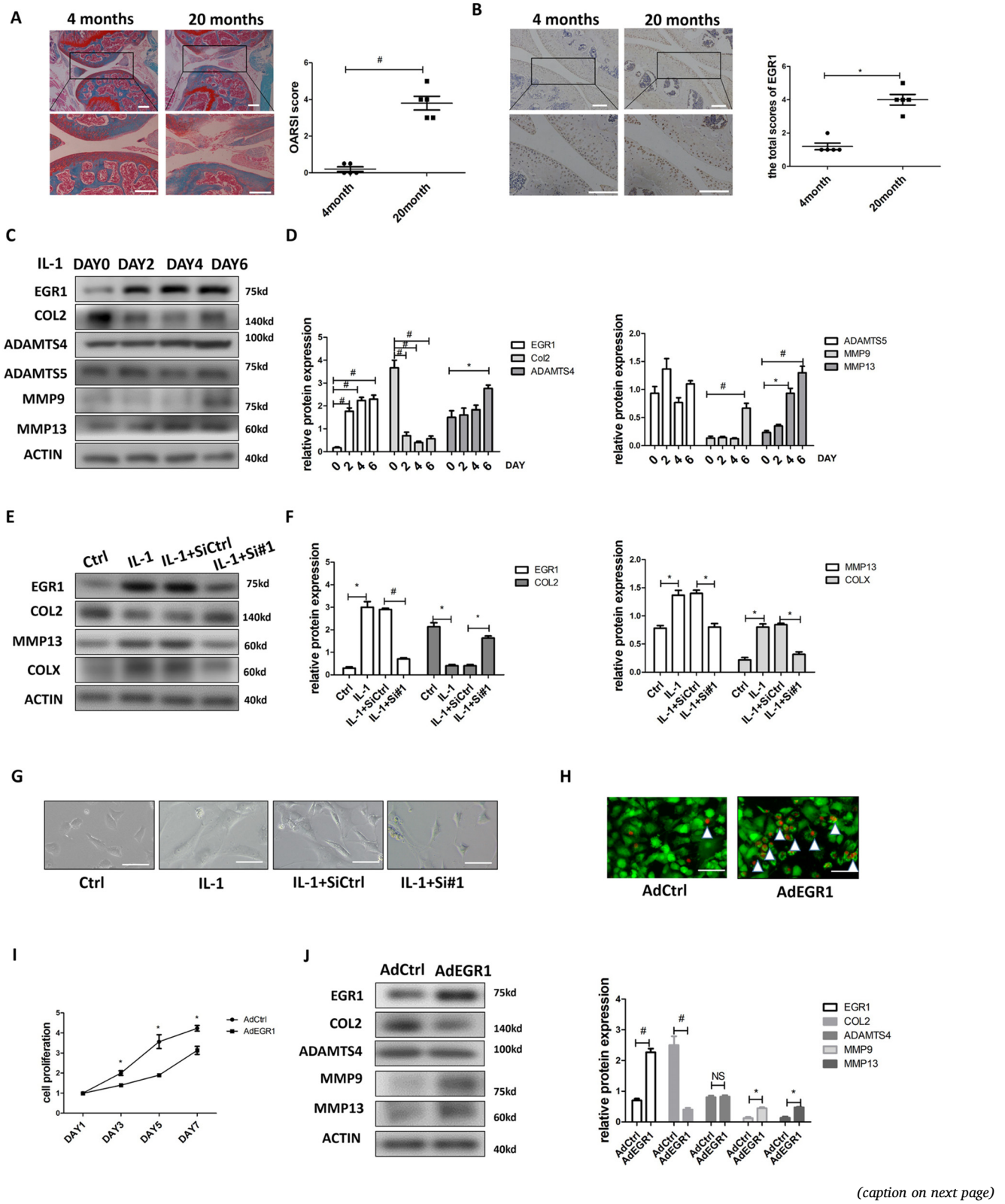
Cultured cells were firstly washed with PBS three times and fixed in 4% paraformaldehyde for 30 mins. Cells were blocked with 5% (*w/v*) BSA in PBST, and immunostained with anti-β-catenin (Rabbit) antibody overnight at 4 °C followed by a gota-anti-rab bit Alexa Fluor-568-conjugated secondary antibody(Invitrogen). Cells were then washed with PBS for three times, stained with DAPI (Beyotime Institute of Biotechnology, Shanghai,China) and observed under a fluorescence microscope.

2.10. Chondrocyte calcification in vitro

Cells were trypsinized and seeded onto a 24-wells plate. After transfection with siEGR1 and AdEGR1, cells calcification was induced for another 2 weeks with calcification medium, containing 1% ITS + (BD Biosciences, USA), 1% antibiotic-antimycotic solution,50 μg/mL ascorbate-2-phosphate (Sigma), 40 μg/mL L-proline (Sigma), 100 nM dexamethasone (Sigma) and 1 nM triiodothyronine (T3) (Sigma). Von kossa staining and alizarin red S staining was carried out according to the protocol to detect the calcification.

2.11. Co-immunoprecipitation (IP) assay

In brief, cell extracts were pre-cleared with 25 μL of protein A/G-agarose (50% v/v). The supernatants were then immunoprecipitated using 2 μg of anti-KLF5 or/and anti-EGR1 antibodies overnight at 4 °C,



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followed by incubation with protein A/G-agarose at 4 °C for approximately 4 h. Protein A/G-agarose-antigen-antibody complexes were collected by centrifugation at 13,600 *g* for 1 min at 4 °C. The pellets were then washed five times with 1 mL of IPH buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 0.1 mM PMSF) at 4 °C. Bound proteins were resolved by SDS-PAGE, followed by western blotting with anti-EGR1, anti-ubiquitin, and anti-KLF5.

2.12. Quantitative real-time PCR

The cultured cells in each experiment were washed three times with cold PBS and lysed with TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The extracted RNA was then reverse-transcribed and used for quantitative real-time PCR performed in an ABI Prism 7500 system (Applied Biosystems, Foster City, CA,

Fig. 2. EGR1 expression of in age-related articular cartilage in mice and IL-1β-induced primary mice chondrocytes. (A) The joints from 4 and 20-month-old mice were obtained (N = 5). Cartilage destruction was analyzed using safranin O-fast green staining. Original scale bars: 300 μm. OARSI score analysis was performed to evaluate the degeneration of articular cartilage. (B) EGR1 expression in the articular cartilage of mice of different ages was analyzed using immunohistochemistry and quantified. Original scale bars: 200 μm. (C) Primary mouse chondrocytes were isolated from C57 mice and cultured in DMEM with IL-1β (10 ng/mL) stimulation. EGR1, COL2A1, ADAMTS4, ADAMTS5, MMP9, and MMP13 expression was analyzed by western blotting at the indicated time points. (D) Quantitative analysis of western blot results. (E) Primary chondrocytes were transfected with SiEGR1(Si#1)and SiCtrl for 3 days and stimulated with IL-1β for another 6 days. EGR1, COL2, MMP13, and COLX expression was analyzed using western blotting. (F) Quantitative analysis of western blot results. (G) Cellular morphology was observed under the microscope. Original scale bars: 20 μm. (H) Primary chondrocytes were transfected with AdEGR1 and AdCtrl for 7 days. The effect of EGR1 on cell death was analyzed using live-dead viability/cytotoxicity Kit (Thermo, L3224) according to the manufacturer's instructions. Live cells were stained with Calcein-AM (green) and dead cells with EthD-1 (red). Original scale bars: 20 μm. (I) CCK8-assay was performed to test the effect of EGR1 on cell proliferation. (J) Primary chondrocytes were transfected with AdEGR1 and AdCtrl for 7 days. EGR1, COL2, ADAMTS4, MMP9, and MMP13 expression was analyzed by western blotting. Quantitative analysis confirmed the results. All data in bar graphs are expressed as mean ± SEM. P* < 0.05, P# < 0.01.

USA) using the cycling conditions: 95 °C for 10 min, followed by 35 cycles at 95 °C for 15 s and 60 °C for 1 min. The whole reaction was consisted of 2ul cDNA,10ul UltraSYBR Mixture (CWBIO, Beijing, China), 6ul ddH20, and 2ul primers (10uM). The values were normalized to the ACTIN. The primers sequences were used according to the previous report unless noted [27].

Mouse (MMP9): F: GCAGAGGCATACTTGTACCG; R: TGATGTTAT GATGGTCCCACTTG.

Mouse (COL2A1): F: GGGTCACAGAGGTTACCCAG; R: ACCAGGGG AACCACTCTCAC.

Human (EGR1): F: GGTCAGTGGCCTAGTGAGC; R: GTGCCGCTGA GTAAATGGGA.

Human (EGR2): F: TCAACATTGACATGACTGGAGAG; R: AGTGAA GGTCTGGTTTCTAGGT.

Human (EGR3): F: GACATCGGTCTGACCAACGAG; R: GGCGAACT TTCCCAAGTAGGT.

Human (EGR4): F: TCCTCGTCAAGTCCACTGAAG; R: CAGGAGTC GGCTAAGTCCC.

2.13. Western blotting

Proteins were extracted using RIPA lysis buffer (Sigma-Aldrich) according to the manufacturer's protocol. Briefly, cultured cells with different treatments were washed with cold PBS for three times. RIPA lysis buffer together with PMSF was used to split the cells for 20mins on ice. Cell lysates were collected and centrifuged at 12000 *g* for 20mins. The supernatants were collected and dissolved in 5× loading buffer and separated on 10% SDS-PAGE gels, and then transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). After washed with Tris-buffered saline-Tween 30 (TBST) three times for 10 min each, the membranes were then blocked with 5% nonfat dry milk at room temperature for 1–2 h and incubated with primary antibody overnight at 4 °C with gentle shaking. The next day, the membranes were washed with PBS for three times and then incubated with secondary HRP-conjugated IgG (Abcam, Cambridge, MA, USA) for 1 h at room temperature. After washed with TBST for three times (10 min each), protein bands were detected by electrochemical luminescence reagent (Millipore, Billerica, MA, USA) and observed using the LAS-4000 Science Imaging System (Fujifilm, Tokyo, Japan). The grey levels of bands were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.14. Statistical analysis

All quantitative data are presented as mean ± SEM. Statistical significance was analyzed with the unpaired, two-tailed Student's *t*-test or ANOVA for multiple comparisons. The value of $p < 0.05$ was considered to be statistically significant. Significance level was presented as either *p < 0.05 or #$p < 0.01$.

3. Results

3.1. EGR1 expression in human and mice cartilage

Cartilage from OA and normal human tissue was analyzed using safranin O-fast green staining. We observed serious cartilage destruction and degeneration in OA samples. OA samples had less safranin O positive staining and more fast green positive staining than did the normal samples (Fig. 1A). EGR family gene expression was detected using real-time PCR. EGR1 expression was enhanced the most in human OA cartilage (Fig. 1B). Western blot analysis showed that EGR1 protein expression was significantly enhanced in OA cartilage (Fig. 1C). We then established a DMM-induced OA in mice (Fig. 1D). Immunohistochemistry and western blot analyses showed that EGR1 expression was upregulated in OA mice (Fig. 1E and F). The OARSI score was analyzed in DMM-induced OA mice. The DMM-induced group obtained the high scores (Fig. 1G). And quantitatively analysis confirmed the results of western blot (Fig. 1H).

3.2. EGR1 expression in age-related articular cartilage in mice and IL-1β-induced primary mice chondrocytes

We used immunohistochemistry to examine EGR1 expression in the normal degeneration of articular cartilage in 20-month old mice. Safranin-O staining revealed age-related cartilage destruction in older mice when compared with the 4-month old mice. And 20-month old mice obtained the higher OARSI scores (Fig. 2A). Immunohistochemistry revealed that EGR1 expression was enhanced in the 20-month-old mice, which was confirmed by quantitatively analysis (Fig. 2B). Low-grade inflammation existed and accelerated OA progress. Primary chondrocytes from mice were cultured and stimulated with IL-1 β for the indicated time. EGR1, COL2A1(COL2), ADAMTS4, ADAMTS5, MMP9, and MMP13 expression was assessed using western blotting. This analysis showed that COL2 expression was suppressed, while the levels of EGR1, ADAMTS4, MMP9, and MMP13 increased after stimulation with IL-1 β. The protein level of ADAMTS5 was not changed (Fig. 2C and D). To test the effect of EGR1 on IL-1β-induced chondrocyte hypertrophy, we silenced EGR1 expression using small interfering RNA (SiRNA). Primary chondrocytes were transfected with SiEGR1(Si#1)or SiCtrl for 3 days and then stimulated with IL-1β for another 6 days. Expression of EGR1, COL2, COX, and MMP13 was analyzed using western blotting. SiRNA downregulated EGR1 expression, which enhanced COL2 expression and suppressed MMP13 and COLX expression (Fig. 2E and F). Chondrocyte size was observed under the microscope. After stimulation with IL-1β for 6 days, the chondrocytes became big and stretched. Silencing EGR1 attenuated the effect of IL-1β since smaller chondrocytes were observed (Fig. 2G). To confirm the vital effect of EGR1 on cell degeneration, primary chondrocytes were transfected with adneovirus-EGR1 (AdEGR1) and adenovirus-vector (AdCtrl) for 7 days without the stimulation of IL-1β. Over-expression of EGR1 (AdEGR1) promoted cell death, which was confirmed by live-dead assay (Fig. 2H). Moreover, CCK-8 assay results indicated that EGR1 over-expression significantly suppressed cell

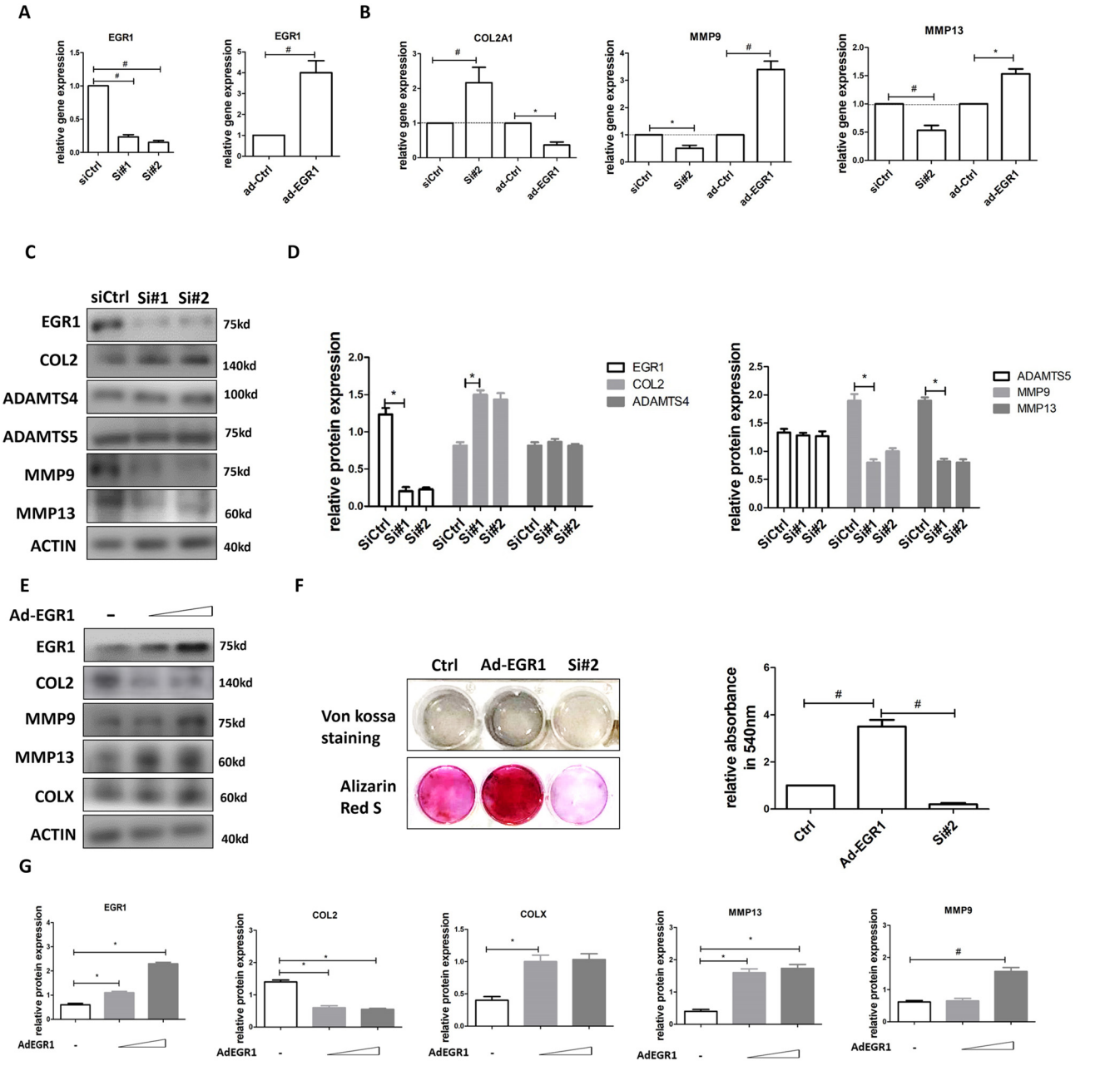

Fig. 3. EGR1 is involved in the regulation of cartilage degeneration and hypertrophy related gene expression. (A) Primary chondrocytes were transfected with SiCtrl, SiEGR1 (Si#1), and SiEGR1(Si#2). Silencing efficiency was analyzed using real-time PCR. Primary mice chondrocytes were transfected with AdCtrl and AdEGR1. The over-expression efficiency was analyzed using real-time PCR. (B) Primary mice chondrocytes were cultured in calcification medium and transfected with Si#2 or AdEGR1 for 48 h. mRNA was isolated for real-time PCR to measure COL2A1, MMP9, and MMP13 expression. (C) Primary mice chondrocytes were transfected with SiCtrl and SiEGR1 for 72 h and cultured in calcification medium for another 4 days. EGR1, COL2, ADAMTS4, ADAMTS5, MMP9, and MMP13 expression was detected by western blot. (D) Quantitative analysis confirmed the results of western blotting. (E) Primary mice chondrocytes were transfected with AdCtrl and AdEGR1(400 MOI and 800 MOI) for 72 h and cultured in calcification medium for another 4 days. Protein was isolated for western blot to detect EGR1, COL2, COLX, MMP9, and MMP13 expression. (F) Primary mice chondrocytes were cultured in calcification medium and transfected with SiEGR1(Si#2) and AdEGR1 (400 MOI) for 14 days. Chondrocyte calcification was analyzed using Von kossa staining and Alizarin Red S staining. Mineralization was quantitatively assessed from Alizarin Red S staining by measuring the absorbance of the extracted stain at 562 nm using a microplate reader. (G) Quantitative analysis confirmed the AdEGR1-mediated effect of protein expression. All data in bar graphs are expressed as mean ± SEM. P* < 0.05, P# < 0.01.

proliferation (Fig. 2I). Additionally, EGR1 over-expression inhibited COL2 expression and enhanced the MMP9 and MMP13 expression as was confirmed by western blot and quantitative analysis (Fig. 2J).

3.3. EGR1 is involved in the regulation of cartilage degeneration and hypertrophy related gene expression

We used RNAi (si#1 and si#2) and AdEGR1 to explore the function of EGR1 in OA. Transfection efficiency was analyzed using real-time PCR (Fig. 3A). Si#2 was further used to knockdown the expression of EGR1. Primary mice chondrocytes were cultured in the calcification medium and transfected with Si#2 or AdEGR1 for 48 h. Real-time PCR indicated COL2 expression was inhibited following EGR1 over-expression and increased following EGR1 RNAi. MMP9 and MMP13 expression was upregulated following EGR1 over-expression and down-regulated following EGR1 RNAi (Fig. 3B). Western blot analysis revealed the

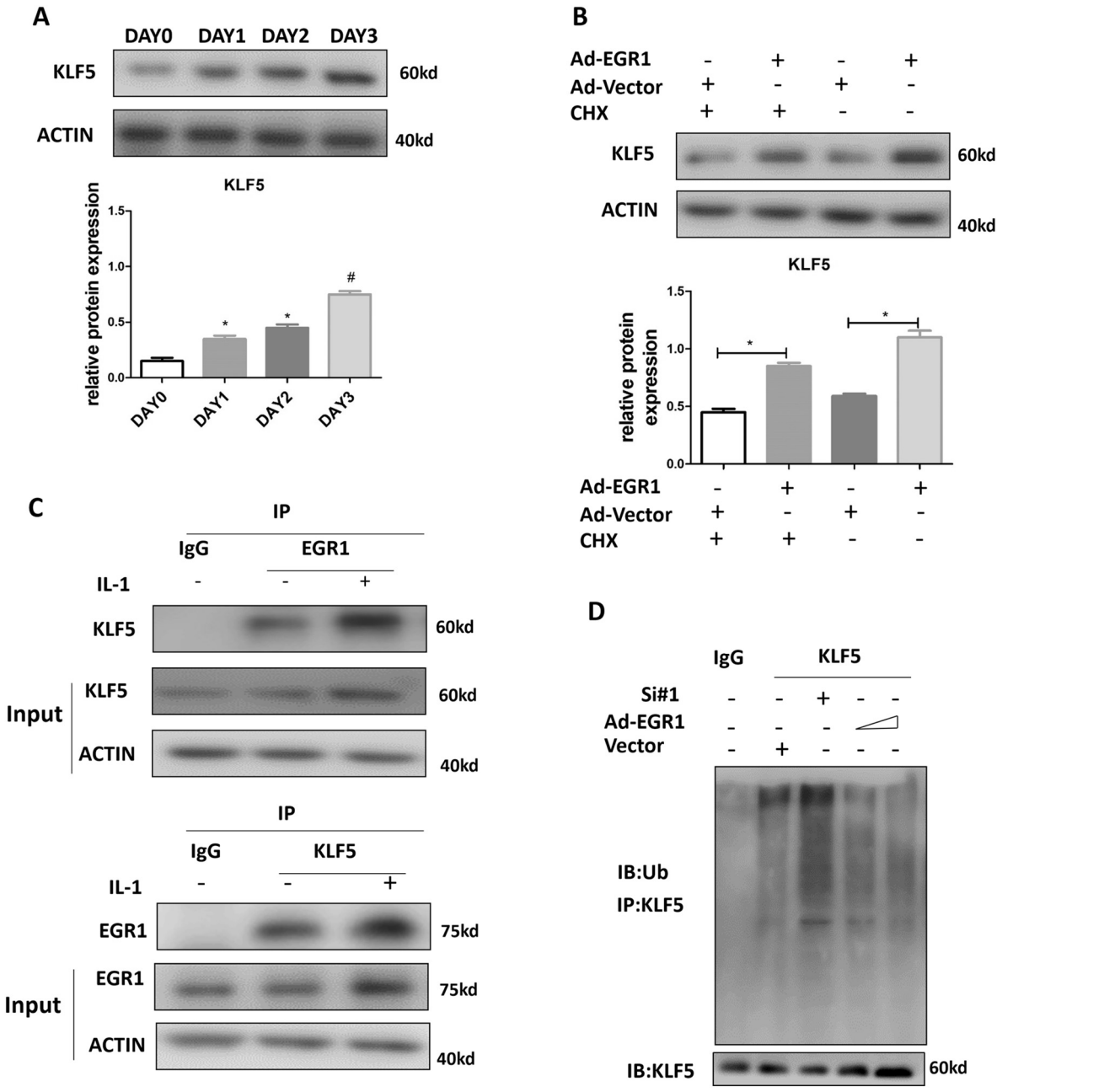

Fig. 4. EGR1 enhances KLF5 expression via suppressing its binding with ubiquitin. (A) Primary mice chondrocytes were cultured in DMEM with the IL-1β (10 ng/mL) stimulation for the indicated time. KLF5 expression was assessed using western blotting and quantitative analyzed. (B) Primary mice chondrocytes were pre-treated with cycloheximide (CHX) for 6 h. Chondrocytes were transfected with AdEGR1 for 48 h. KLF5 expression was analyzed using western blotting and quantitative analyzed. (C) Primary mice chondrocytes were stimulated with IL-1 β for 12 h. The connection between EGR1 and KLF5 was assessed by immunoprecipitation assay. (D) Primary mouse chondrocytes were cultured in DMEM and transfected with SiEGR1(Si#1)and AdEGR1 (400 MOI and 800 MOI). To explore the effect of EGR1 on ubiquitination-mediated degradation of KLF5, immunoprecipitation assays were performed to detect the direct connection between KLF5 and ubiquitin. All data in bar graphs are expressed mean ± SEM. P* < 0.05, P# < 0.01.

similar results that silencing the EGR1 expression increased COL2 protein expression and decreased MMP9 and MMP13 protein expression. Silencing EGR1 expression did not affect the expression of ADAMTS4 and ADAMTS5 (Fig. 3C). Quantitative analysis confirmed our results (Fig. 3D). Over-expression of EGR1 suppressed COL2 expression and increased MMP9, MMP13, and COLX expression (Fig. 3E). To further examine the effect of EGR1 on chondrocyte hypertrophy, primary mice chondrocytes were cultured in calcification medium for 14 days. Von kossa staining and Alizarin red S staining indicated that EGR1 over-expression of accelerated cell calcification while inhibition of EGR1 expression suppressed this process (Fig. 3F). Quantitative analysis confirmed the results of western blot (Fig. 3G).

3.4. EGR1 enhanced KLF5 expression via suppressing its connecting with ubiquitin

KLF5 expression was also upregulated in IL-1β treated primary chondrocytes in a time-dependent manner. Quantitative analysis confirmed this result (Fig. 4A). EGR1 is a potent transcriptional activator of KLF5. EGR1 over-expression significantly increased the levels of KLF5 protein. Cycloheximide (CHX) was used to inhibit protein synthesis. Results indicated that EGR1 over-expression still enhanced the level of

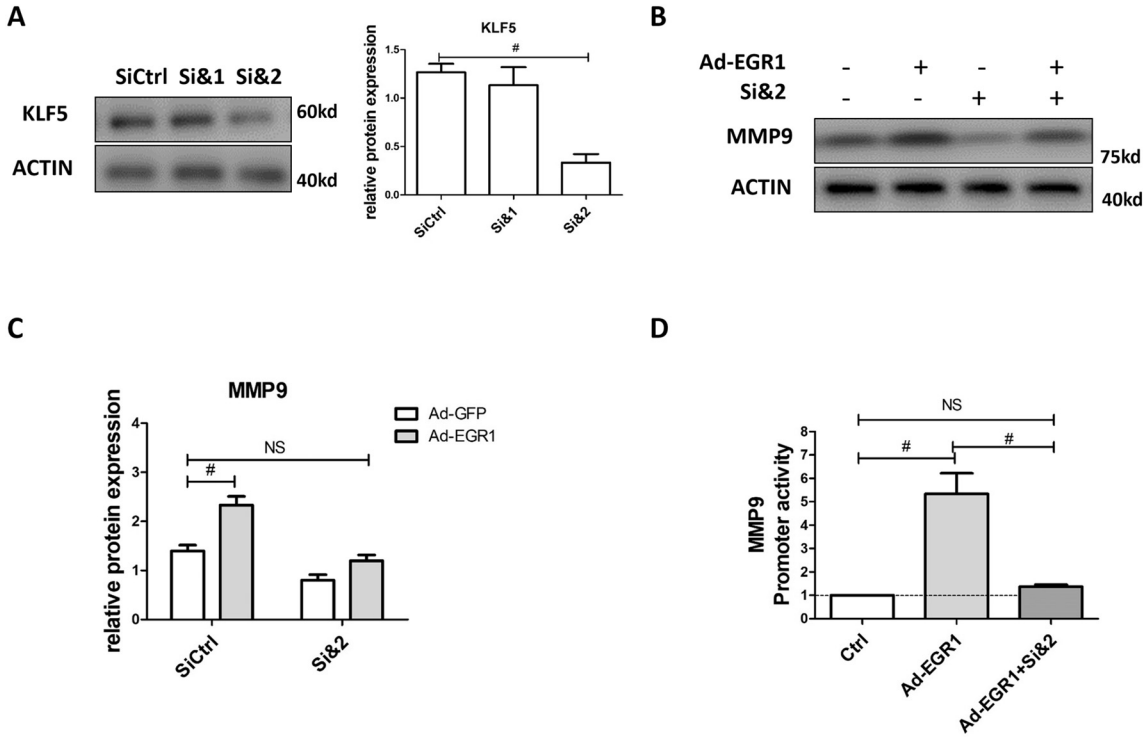

Fig. 5. KLF5 is involved in the EGR1-mediated increase of MMP9 expression. (A) KLF5 gene expression was silenced using SiKLF5 (Si&1 and Si&2). The silencing efficiency was assessed by western blotting. (B) Primary mouse chondrocytes were cultured in DMEM and transfected with SiKLF5 (Si&2) and AdEGR1 for 48 h. Cells were cultured for another 48 h. And protein was isolated and MMP9 expression was analyzed using western blotting. (C) Quantitative analysis confirmed the effect of KLF5 on EGR1-mediated expression of MMP9. (D) The MMP9-luciferase reporter plasmid was transfected into primary mouse chondrocytes. After 24 h, chondrocytes were transfected with AdEGR1 with or without SiKLF5(Si&2) for another 24 h. Luciferase expression was analyzed using the Firefly Luciferase Reporter Gene Assay Kit (Beyotime) following the manufacturer's instructions. All data in bar graphs are expressed mean ± SEM. P* < 0.05, P# < 0.01. NS: no significance.

KLF5 protein, even in the presence of CHX. Quantitative analysis confirmed our results (Fig. 4B). These results indicate that EGR1 induces KLF5 expression via inhibiting its degeneration. Co-IP assay demonstrated that after stimulation with IL-1 β the connection between EGR1 and KLF5 was stronger (Fig. 4C). Since KLF5 degradation is ubiquitin-dependent, we performed an IP assay to test the effect of EGR1 on the connection between KLF5 and ubiquitin. We observed that RNAi knock down of EGR1 enhanced binding between KLF5 and ubiquitin, while EGR1 over-expression attenuated this process (Fig. 4D).

3.5. KLF5 is involved in the EGR1-mediated increase of MMP9 expression

We tested the effect of KLF5 on EGR1-mediated gene expression during chondrocyte degeneration. We firstly silenced the expression of KLF5 using RNAi in primary mice chondrocytes. Si&2 significantly suppressed the expression of KLF5 and was used for further analysis (Fig. 5A). We found that over-expression of EGR1 induced MMP9 expression in primary mice chondrocytes. Silencing KLF5 expression attenuated the EGR1-mediated increase in MMP9 expression (Fig. 5B and C). We then transfected the MMP9-luciferase reporter plasmid into primary mice chondrocytes to explore the effect of KLF5 on MMP9 transcription. Over-expression of EGR1 induced MMP9 transcription via KLF5 as knockdown of KLF5 expression attenuated EGR1-mediated promoter activity (Fig. 5D). Taken together our results show that KLF5 is involved in EGR1-mediated MMP9 expression, which contributes to cartilage degeneration in OA.

3.6. EGR1 regulates chondrocyte hypertrophy via activating β-catenin signaling

Primary chondrocytes were transfected with AdEGR1 for 24 h. Immunofluorescence indicated that EGR1 over-expression induced the nuclear transport of β-catenin (Fig. 6A). We then isolated the nuclear protein. Western blot analysis confirmed that over-expression of EGR1 increased nuclear expression of β-catenin. Quantitative analysis confirmed the western blot result (Fig. 6B). To further determine the effect of β-catenin on EGR1-mediated chondrocyte hypertrophy, we silenced β-catenin expression using SiRNA. Western blotting confirmed the silencing efficiency (Fig. 6C). Primary chondrocytes were transfected with Siβ-catenin (Siβ1) or SiCtrl and AdEGR1 or AdCtrl. The expression of chondrocyte hypertrophy-related genes was evaluated. Silencing β-catenin attenuated the promotion effect of EGR1 on COLX, MMP13, and RUNX2 expression (Fig. 6D and E). We also isolated the mRNA to explore the expression of hypertrophy related genes MMP13, COLX, and Runx2. The β-catenin signaling inhibitor XAV939 was used to test the effect of β-catenin signaling on EGR1-mediated chondrocyte hypertrophy. Real-time PCR results showed that EGR1 over-expression enhanced the expression of MMP13, COLX, and Runx2, while the β-catenin signaling inhibitor, XAV939, attenuated this process (Fig. 6F).

3.7. IA injection of AdEGR1 induced cartilage destruction in mice

To explore the effect of EGR1 on OA in vivo, we ectopically expressed EGR1 in knee joint tissues of 8-week-old male mice via IA injection of AdEGR1. Joint tissue specimens were observed under a fluorescence microscope to confirm that EGR1 was effectively over-expressed in cartilage. Safranin-O staining indicated loss of glycosaminoglycans in articular cartilage after IA injection of AdEGR1 for 4 weeks. After 8 weeks, joint tissues injected with AdEGR1 showed more severe erosion of cartilage (Fig. 7A). OARSI scores confirmed the that ectopically expressed EGR1 in knee joint tissues induced cartilage destruction (Fig. 7B). Real-time PCR indicated that IA injection of AdEGR1 induced the expression of hypertrophy and degeneration-related genes (MMP9, MMP13, and COLX) (Fig. 7C). We then explored

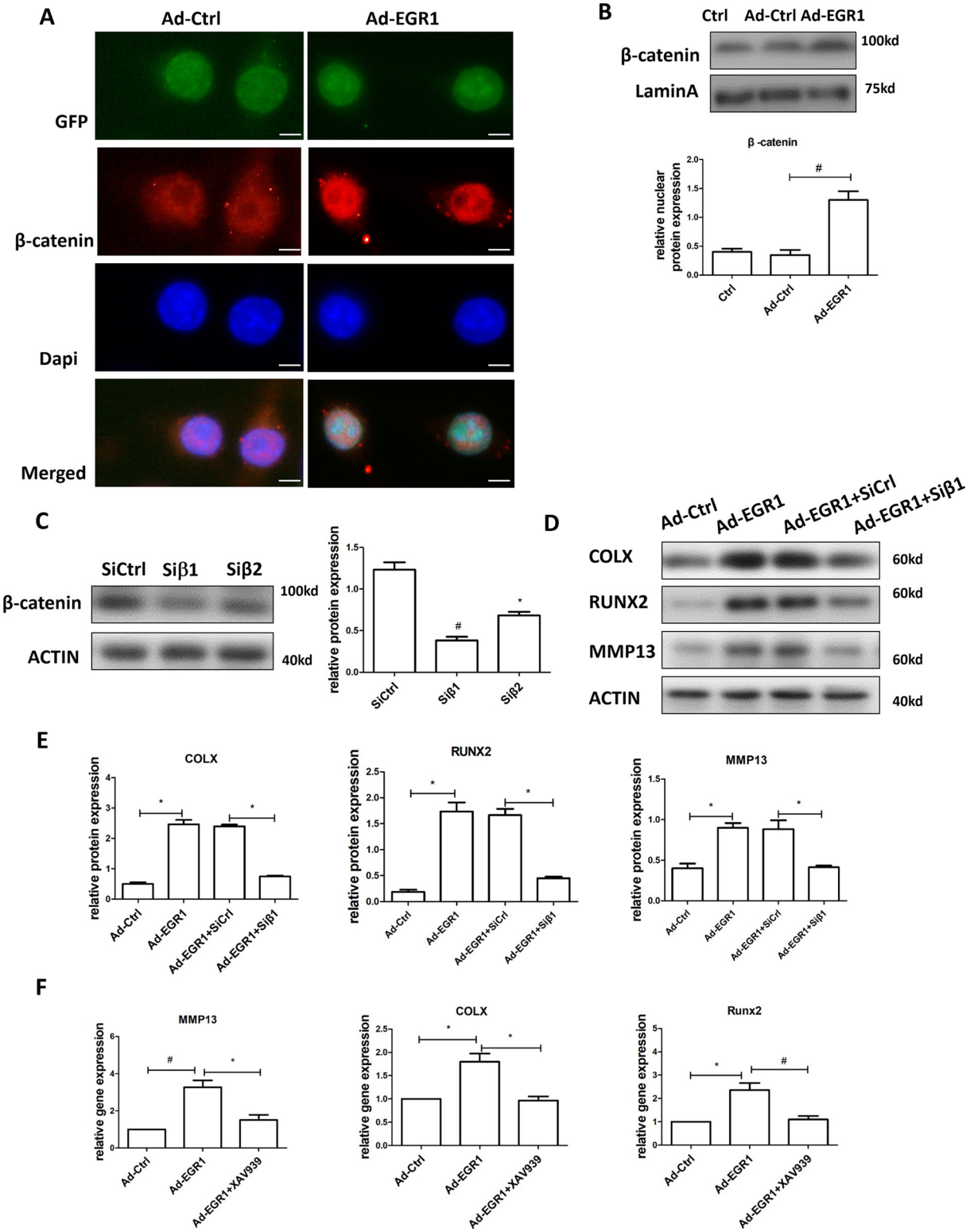

Fig. 6. EGR1 regulates chondrocyte hypertrophy by activating β-catenin signaling. (A) Primary mouse chondrocytes were transfected with AdVector and AdEGR1 for 12 h. Immunofluorescence was performed according to the material and methods to observe the intracellular location of β-catenin. Original scale bars: 15 μm. (B) Primary mice chondrocytes were transfected with AdVector and AdEGR1 for 12 h. Nuclear protein was isolated using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). β-catenin expression was analyzed by western blotting and was normalized to that of LaminA. (C) Primary chondrocytes were transfected with Siβ-catenin(Siβ1 and Siβ2) for 3 days. The silencing efficiency was analyzed by western blotting. (D) Primary chondrocytes were transfected with Siβ-catenin (Siβ1) or SiCtrl for 2 days and then transfected with AdEGR1 or AdCtrl for another 4 days. COLX, RUNX2, and MMP13 expression was detected using western blotting. (E) Quantitative analysis confirmed the results of western blotting. (F) Primary mouse chondrocytes were pre-treated with β-catenin inhibitor XAV939. Cells were then transfected with AdEGR1 for 24 h. Gene expression of MMP13, COLX, and Runx2 was assessed using real-time PCR. All data in bar graphs are expressed mean ± SEM. P* < 0.05, P# < 0.01.

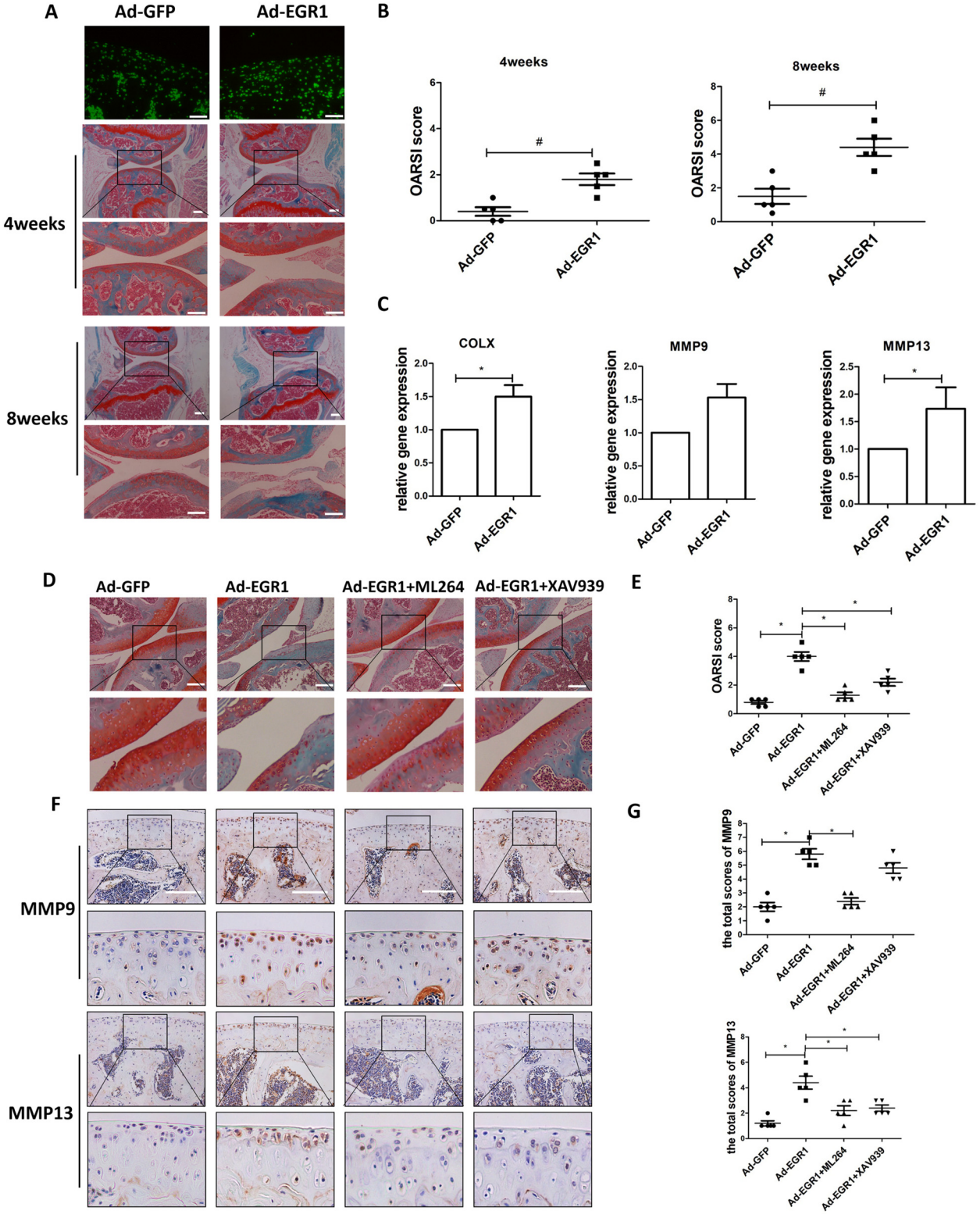

Fig. 7. IA injection of AdEGR1-induced the cartilage destruction in mice. (A) EGR1 was ectopically expressed in knee joint tissues of 8-week-old male mice (N = 5). Two weeks after the first injection, the joint tissue specimen was observed under a fluorescence microscope to detect the ectopic EGR1 expression. Four and eight weeks later, the joint tissue specimen was collected for Safranin O-fast green staining. Original scale bars: 300 μm. (B) The OARSI score analysis was carried out to detect the cartilage destruction of knee joints after the IA injection of AdEGR1 for 4 and 8 weeks. (C) EGR1 was ectopically expressed in knee joint tissues for 4 weeks (N = 5). mRNA was isolated from the whole humerus and real-time PCR was used to detect the expression of MMP9, MMP13, and COLX. (D)ML264 (20 mg/kg body weight) or XAV939 (5 mg/kg body weight) was injected into the intraarticular spaces of the knee joints 1 week after the first AdEGR1 or Ad-GFP injection. Cartilage destruction was examined by safranin-O staining and analyzed using the OARSI scoring system (E). Original scale bars: 300 μm.(F) MMP9 and MMP13 expression was assessed using immunohistochemistry and was quantitative analyzed (G). Original scale bars: 300 μm. All data in bar graphs are expressed mean ± SEM. P* < 0.05, P# < 0.01. NS: no significance.

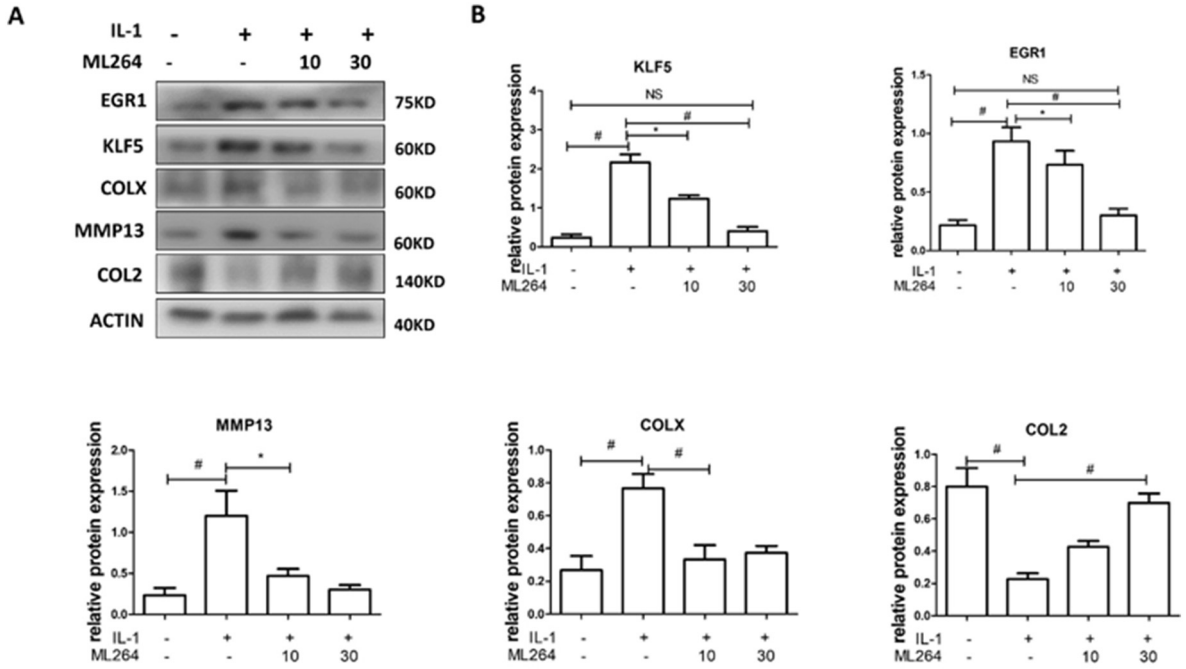

Fig. 8. ML264, an EGR1 inhibitor, attenuates IL-1β-induced gene expression in vitro. (A) Primary chondrocytes were pre-treated with EGR1 inhibitor ML264 (10 μM or 30 μM) for 6 h before IL-1β (10 ng/uL) stimulation and cultured for another 48 h. EGR1, KLF5, COLX, COL2 and MMP13 expression was analyzed using western blotting. (B) Quantitative analysis confirmed the results of western blotting. All data in bar graphs are expressed mean ± SEM. P* < 0.05, P# < 0.01.

the effect of ML264 and XAV939 in this model. One week after the first AdEGR1 injection, ML264 or XAV939 was added by intra-articular injection for another 4 weeks. Safranin-O staining showed that ML264 and XAV939 significantly suppressed EGR1-mediated loss of glycosaminoglycans in articular cartilage (Fig. 7D). The ML264 and XAV939-treated groups obtained higher OSARI scores than did the AdEGR1 group (Fig. 7E). Immunohistochemistry analysis demonstrated that EGR1 promoted the expression of MMP9 and MMP13, while ML264 significantly inhibited their expression and XAV939 greatly suppressed the MMP13 expression (Fig. 7F). Quantitative analysis confirmed the results of immunohistochemistry (Fig. 7G). Moreover, we detected KLF5 and β-catenin expression in DMM-induced OA cartilage and human cartilage using immunohistochemical staining. We found that KLF5 and β-catenin expression was higher in human OA cartilage than in normal cartilage (FigS1A). Quantitative analysis confirmed this results (FigS1B). Similar results were observed in mice cartilage as DMM induced OA cartilage had higher KLF5 and β-catenin expression than SHAM group (FigS1C, D). These results further demonstrated that KLF5 and β-catenin was involved in the EGR1 mediated cartilage degeneration and hypertrophy.

3.8. EGR1 inhibitor, ML264, attenuates IL-1β-induced gene expression in vitro

Primary chondrocytes were pre-treated with ML264 for 6 h before IL-1β stimulation. Western blot analysis showed that ML264 could attenuate the IL-1β-induced expression of EGR1, KLF5, COLX, MMP9, and MMP13 in a dose-dependent manner. Additionally, ML264 reversed the inhibitory effect of IL-1β on the expression of COL2 (Fig. 8A and B).

3.9. ML264 attenuated the DMM induced OA in vivo

We established DMM-induced OA to explore the effect of ML264 on treating OA in vivo. Safranin-O staining indicated that ML264 effectively attenuated the cartilage destruction in vivo (Fig. 9A). OARSI scores were relatively lower in the ML264-treated group than in the DMSO-treated group (Fig. 9B). The chondrocyte and matrix-related genes from each group were analyzed using real-time PCR. COL2A1 gene expression was upregulated in the ML264-treated group and MMP9, MMP13, and COLX expression was correspondingly downregulated (Fig. 9C). Immunohistochemistry analysis of MMP9 and MMP13 expression revealed that ML264 significantly suppressed DMM-induced expression of MMP9 and MMP13 (Fig. 9D). Quantitative analysis confirmed our results (Fig. 9E). Taken together, these results indicate that ML264 can protect articular cartilage from DMM-induced OA.

4. Discussion

In this study , we demonstrate that EGR1 is upregulated in human and mouse OA articular cartilage. EGR1 accelerated cartilage hypertrophy and degeneration via regulating KLF5 expression and β-catenin signaling. Moreover, the EGR1 inhibitor, ML264, significantly attenuated IL-1β-induced matrix degradation in vitro and DMM-induced OA in vivo. Thus, EGR1 might be a promising target for treating OA. Interestingly, studies in mice indicate that ML264 displays 47% oral bioavailability, a very promising therapeutic advantage for compound development [28].

We firstly explored the EGR family in OA and normal human cartilage. We found that EGR1 expression was abundant in normal human cartilage. Real-time PCR results showed that its expression was enhanced in OA cartilage. To confirm EGR1 expression in OA, the DMM-induced OA and age-related articular cartilage degeneration models were established. Consistent with the results observed in human OA cartilage, EGR1 expression was significantly increased in these models. Consistent with our results, recent study pointed out that EGR1 expression was higher in OA rats than in sham rats and increased angiogenesis in cartilage [29]. They described the effect of EGR1 on Netrin-1 receptor DCC expression without exploring the exact role of EGR1 on angiogenesis in vitro and during OA progress. The effect of EGR1 on angiogenesis needs to be further examined using ERG1 overexpression studies in vivo or using human samples. Other study identified EGR1 as one of the upstream regulators for synovial expression signature genes in osteoarthritis [30] and higher level EGR1 was

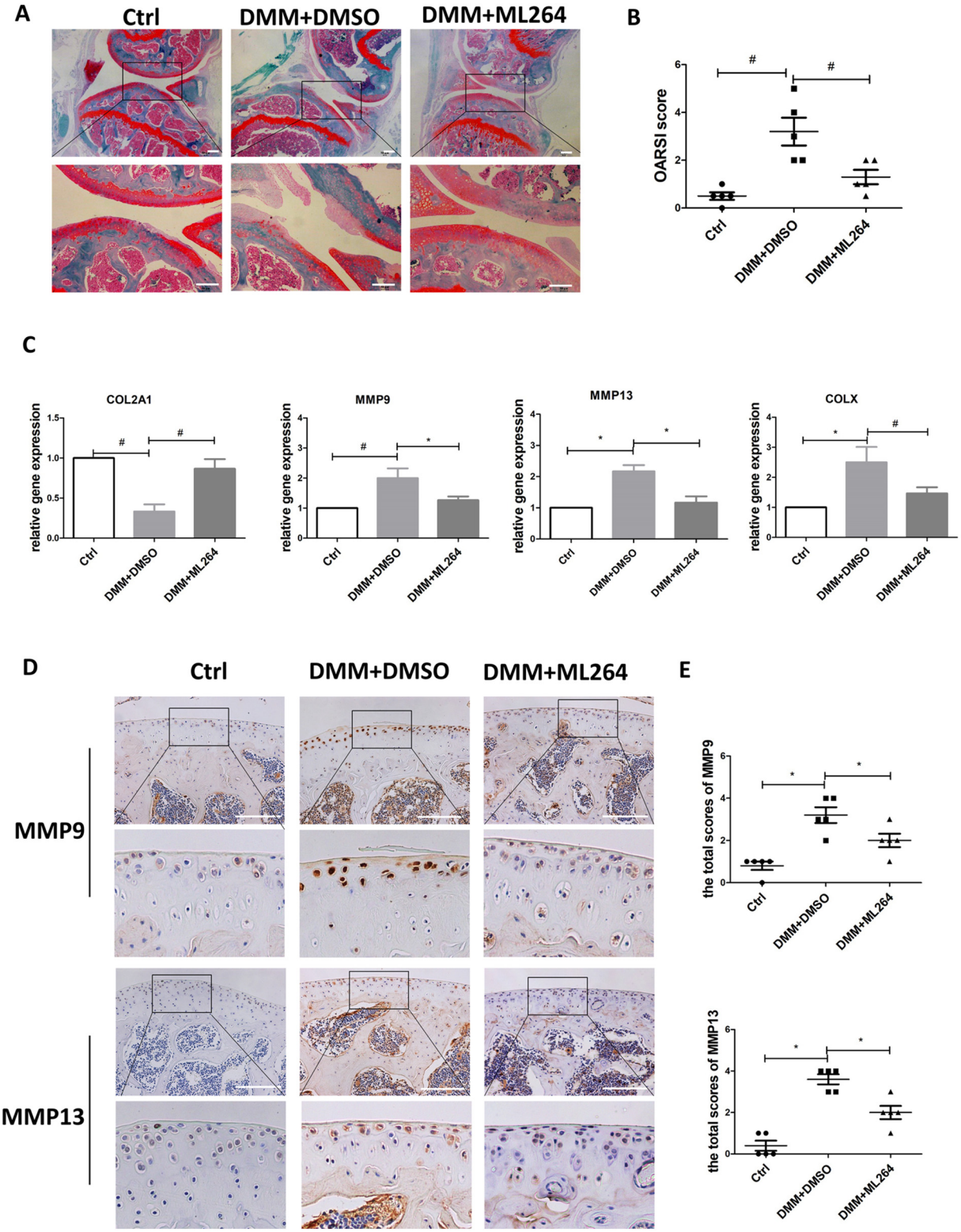

Fig. 9. ML264 attenuated DMM-induced osteoarthritis in vivo. (A) A DMM-induced osteoarthritis model was established. Mice were divided into three groups (N = 5): Ctrl (Sham), DMM + DMSO, and DMM + ML264. The mice joints were collected for Safranin O-fast green staining 8 weeks later. Original scale bars: 300 μm. (B) OARSI score analysis was carried out to detect the cartilage destruction of knee joints. (C) mRNA was isolated from the whole humerus and real-time PCR was used to measure expression of MMP9, MMP13, COL2A1, and COLX. (D) MMP9 and MMP13 expression was detected in each group using immunohistochemistry and was quantitatively analyzed (E). Original scale bars: 300 μm. All data in bar graphs are expressed mean ± SEM. P* < 0.05, P# < 0.01.

observed in OA cartilage [20]. However, the function of EGR1 in OA and its underlying mechanism have not been clarified in these studies. Here, we have described the exact role of EGR1 in accelerating chondrocyte degeneration and hypertrophy in vitro and in vivo. DMM-induced OA and human OA samples were used to confirm our results. Moreover, the EGR1 inhibitor ML264 attenuated the progress of DMM-

induced OA in vivo, which confirmed the effect of EGR1 on OA and identified a potential drug and drug target for the treatment of OA.

Cartilage degeneration and hypertrophy are two main process in the pathogenesis of OA [31]. Our results show that EGR1 is involved in both of these processes. Western blot analysis indicated that EGR1 inhibited COL2 expression and induced the expression of the cartilage matrix-degrading enzyme (MMP9 and MMP13). Hypertrophy-related gene expression (COLX) was enhanced by EGR1. Von kossa staining and Alizarin red staining confirmed the function of EGR1 in chondrocyte calcification in vitro.

Previous studies have demonstrated a close relationship between KLF5 and EGR1 [15,17], and EGR1 induces the transcriptional activity of KLF5 via binding to its promoter [28]. Moreover, KLF5 directly regulates the expression of MMP9, which is involved in cartilage degeneration [32]. In our study, we observed that IL-1β induced KLF5 expression. EGR1 over-expression also upregulated KLF5 expression. This process was not suppressed by CHX, indicating that the increased KLF5 level was partially resulted from decreased KLF5 protein degeneration. Co-IP results indicated that IL-1β facilitated the connection between EGR1 and KLF5. KLF5 degradation is ubiquitin-dependent. Therefore, we postulate that increased EGR1 facilitated the connection between KLF5 and EGR1, and reduced the connection between KLF5 and ubiquitin. Immunoprecipitation demonstrated that over expression of EGR1 reduced the connection between KLF5 and ubiquitin while EGR1 knockdown, using RNAi, had the opposite effect. This is the first report demonstrating that EGR1 inhibits the ubiquitination-mediated degradation of KLF5. Luciferase reporter assays confirmed that EGR1 regulates the transcriptional activity of MMP9 via KLF5. Thus, we have shown that EGR1 regulates cartilage degeneration partially through regulating the transcriptional activity of MMP9 via its connection with KLF5. We further investigated the effect of KLF5 on chondrocyte hypertrophy. Chondrocytes were transfected with plasmid carrying flag tagged KLF5 and cultured in calcification medium for 7 days. We found that induced KLF5 had no effect on the hypertrophy related genes expression (MMP13, RUNX2 and COLX). Von kossa staining and Alizarin Red S staining also confirmed that KLF5 could not promote chondrocyte calcification directly. Thus, other signaling might be involved in EGR1 mediated chondrocyte hypertrophy.

Many studies have demonstrated that Wnt-β-catenin is involved in the pathogenesis of OA via regulating cartilage hypertrophy [33,34]. In our study, we observed that EGR1 over-expression induces β-catenin nuclear transportation. Isolation of nuclear protein and subsequent western blotting analysis confirmed that EGR1 over-expression enhanced β-catenin nuclear expression. The knock down of β-catenin or Wnt-β-catenin inhibitor, XAV939, attenuated the enhanced expression of MMP13, COLX, and RUNX2. These results indicate that EGR1 regulates cartilage hypertrophy partially through activating Wnt-β-catenin signaling.

Ectopically expressed EGR1 in knee joint tissues aggravated the degeneration of articular cartilage in a time-dependent manner. Articular cartilage mRNA expression analysis revealed that ectopically expressed EGR1 enhanced COLX, MMP9, and MMP13 expression. We studied the effect of ML264, an ERG1 inhibitor [28], on IL-1 β-induced gene expression in primary chondrocytes in vitro. We found that ML264 inhibited the IL-1 β-induced expression of EGR1 and KLF5. Moreover, ML264 attenuated the enhanced expression of MMP13 and COLX. COL2 expression was rescued by ML264. Using the DMM-induced OA model, we observed that ML264 could significantly inhibit the destruction of articular cartilage. This was confirmed by Safranin O-fast green staining and OARSI score analysis. Immunohistochemistry analysis of MMP9 and MMP13 expression revealed that ML264 significantly suppressed ectopically expressed EGR1- and DMM-induced expression of MMP9 and MMP13 in vivo.

Collectively, our results show that EGR1 is involved in the development of cartilage degeneration and hypertrophy in OA. EGR1 enhances KLF5 binding to accelerate cartilage degeneration and activates β-catenin signaling to regulate cartilage hypertrophy. The EGR1/KLF5 inhibitor, ML264, might be a promising drug for the treatment of OA.

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Transparency document

The Transparency document associated this article can be found, in online version.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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Author contributions

P. Shi, X. Sun and Z. Chen designed this study. Study conduct: X.Sun、Z. Xie and B.Hu. In vitro experiments: X. Sun、S. Li and Z. Xie. In vivo experiments: Z. Chen and Z. Xie. Data collection and analysis: Z.Xie and X.Pan. Data interpretation: All authors. Drafting manuscript: X.Sun. Revising manuscript content: Y. Ma and H. Huang. Approving final version of manuscript: All authors. J. Wang performed the funding management. P. Shi take responsibility for the integrity of the data.

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