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Original Article

IGF-1 increases production of extracellular matrix in human endplate chondrocytes via distinct signaling pathways

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Abstract: To uncover the role and mechanism of IGF-1 in regulation of extracellular matrix in human lumbar endplate chondrocytes. First chondrocytes were treated with IGF-1, then the expression of extracellular matrix was examined by Western blotting and RT-qPCR. Multiple strategies were used to examine the signaling pathways after IGF-1 treatment. Finally, the effects of inhibitors of signaling on extracellular matrix in cultured endplate chondrocytes were analyzed by RT-qPCR and Western blotting. IGF-1 (40 ng/ml) treatment for 24 h could significantly increase the mRNA and protein of COL-2A and Sox9, but decreased that of MMP-13 in chondrocytes. The phosphorylation level of AKT and ERK1/2 primarily happened in the nucleus, and both peaked at 15 min with IGF-1 treatment, subsequently decreased to control levels after 60 min and 30 min respectively. Inhibition of PI3K with LY294002 significantly blocked the effects of IGF-1 on expression of the COL-2A mRNA, while inhibition of ERK using U0126 significantly blocked the effects of IGF-1 on repression of MMP-13 expression and upregulation of Sox9. IGF-1 stimulates COL-2A expression mainly through the PI3K pathway, but upregulation of Sox9 expression and downregulation of MMP-13 expression is regulated primarily by the ERK pathway in human endplate chondrocytes.

Keywords: IGF-1, human, endplate chondrocyte, PI3K/AKT, ERK

Introduction

Low back pain (LBP) is a common chronic complaint and is associated with dramatic effects on quality of life, as well as increasing economic strain on our society. LBP is thought to be caused by degeneration of intervertebral discs (IVDs) of the lumbar spine, a primary cause of disc herniation, spinal instability and stenosis, and facet joint osteoarthritis [1, 2]. Endplate cartilage is an important structural component of IVDs and is composed of a thin layer of hyaline cartilage. Vertebral endplate sclerosis and the accompanying reduction in endplate pore density and size can seriously limit ancillary nutrient transport to intervertebral disc cells, thus increasing the risk of IVD degeneration [3-5]. What's more, the phenotypic stability of endplate chondrocytes primarily arises from balanced expression of COL-2A, Sox9, MMP13 [6, 7].

As an important growth factor for cartilage, Insulin-like growth factor-1 (IGF-1) increases cell proliferation, enhances synthesis of matrix-associated proteins, and prevents apoptosis of chondrocytes, which may be an effective treatment for LBP [8-12].

Zhang M et al found that the PI3K pathway mainly transduces IGF-1 effects on col2 expression while the ERK pathway mediates IGF-1 effects on MMP-13 expression in rats' endplate chondrocytes [10]. Similarly, we concluded from earlier research that the degeneration of rats' endplate chondrocytes cultured *in vitro*, to a certain extent, may serve as a model of that caused in chondrocytes by age *in vivo*, and IGF-1 delays the aging process, probably mediated by the ERK and AKT signaling pathways [11]. Shakibaei et al isolated human chondrocytes from articular cartilage of femoral heads, and then cultured the primary chondrocytes up to 10 passages. They discovered that treatment of chondrocyte cultures with IGF-I stabilizes chondrogenic potential, stimulates Sox9 and promotes molecular interactions between ERK and Sox9, which may be regulated by the

Table 1. Sequences of primers used in RT-qPCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Product length (bp)
COL-2A	GGAGCAGCAAGAGCAAGGAGAAG	TGGACAGCAGGCGTAGGAAGG	138
Sox9	CACACGCTGACCACGCTGAG	GCTGCTGCTGCTCGCTGTAG	100
MMP13	TCCTGGCTGCCTTCCTCTTCTTG	AGTCATGGAGCTTGCTGCATTCTC	182
GAPDH	CAACGTGTCAGTGGTGGACCTG	GTGTCGCTGTTGAAGTCAGAGGAG	156

integrin/MAPK signaling pathways [12]. Nevertheless, the precise mechanisms by which IGF-1 regulates expression of COL-2A, Sox9, and MMP-13 in human endplate chondrocytes have not been elucidated.

The aim of this study was to comprehensively and systematically confirm the roles of these signaling pathways in modulating differential effects of IGF-1 on COL-2A, Sox9, and MMP13 expression in human endplate chondrocytes.

Materials and methods

Patient samples

Endplate cartilage samples were obtained from 15 patients with acute traumatic lumbar fractures (mean age: 35 years; eight females and seven males) who were treated with open vertebral surgery at our department. No cases had tumor, tuberculosis, diabetes, infectious disease, or bone metabolic disease. All cases were routinely performed with magnetic resonance imaging (MRI) before surgery, and those of whose discs in the experimental group had no evident degeneration from the results of MRI according to Pfirrmann's classification were included. This study was approved by the Ethics Committee of Shaoxing Hospital of China Medical University. Informed consent was obtained from all participants.

Chondrocyte isolation and culture

Primary chondrocytes were isolated from the lumbar spine endplate cartilage just as described in our previous research [7]. The first passage of cells was with a density of 1.5×10^5 cells/well on six-well culture plates cultured in 2 ml Dulbecco's Modified Eagle's medium/F-12 medium (DEME, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 10% fetal bovine serum (FBS, Gibco) were used for the following tests. The cells were cultured for 72 h to 80-90% confluence, flushed three times with serum-free medium, then cultured in serum-free medium for 12 h and treated with IGF-1 (Austral Biologicals, San Ramon, CA). LY294002 (PI3K/AKT signaling inhibitor; Sigma-Aldrich, St. Louis, MO, USA) and U0126 (ERK signaling inhibitor; Sigma-Aldrich) were used in some tests to block specific pathways. All chondrocytes were cultured at 37°C and 5% CO_2.

Cell viability

Primary cells were cultured to 80-90% confluence, trypsinized and subcultured at 5×10^3 cells per well in 96-well plate containing 100 μl DMEM/F-12 medium with 10% FBS, then treated with excessive amount of either LY294002 (20 μM) or U0126 (1 μM) for 24 h. To the plate, 20 μl of MTT (5 mg/ml, Sigma-Aldrich) was directly added, then incubated at 37°C for 4 h. After removing the fluid in each well, 150 μl Dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to the plate, covered with tinfoil and agitated on orbital shaker for 15 min. The absorbance of each well was measured by enzyme immunoassay under OD 490 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from chondrocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and was reverse transcribed using the SuperScript III First-Strand synthesis system (Life Technologies, Carlsbad, CA, USA) with oligo (dT) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using SYBR Premix Ex Taq™ (Takara, Dalian, China) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as an internal control utilizing a Roche LightCycler 480 real-time PCR System (Roche, Basel, Switzerland). The reactions were incubated in 96-well plates at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec, followed by a dissociation curve. The primer sequences (Sangon Biotech Co. Ltd., Shanghai, China) are shown in **Table 1**. The data were analyzed using the $2^{-\Delta\Delta Ct}$

method and expressed as the fold change compared with the control [13].

Western blotting

Cells were lysed in RIPA extraction solution (15 mM Tris, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM dithiothreitol, 0.5% Triton X-100, and protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO, USA]). The protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (KeyGen Biotech, Nanjing, China). For western blot analysis, 20 μg protein samples were fractionated by 10% SDS-PAGE and then electrotransferred onto nitrocellulose membranes (Whatman, Piscataway, NJ, USA). The primary antibodies were composed of rabbit anti-Sox9 antibody (1:10000; Abcam, Cambridge, UK), rabbit anti-MMP13 antibody (1:5000; Abcam), rabbit anti-total ERK1/2 antibody (1:10000; Abcam), rabbit anti-phospho ERK1/2 antibody (1:10000, Abcam), rabbit anti-total AKT (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-phospho AKT (1:2000; Cell Signaling Technology). Protein levels were normalized to GAPDH using an anti-GAPDH antibody (1:10000 dilution; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 4°C overnight. Followed by horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Biosharp, Hefei, China) at a dilution of 1:5000 at room temperature for 2 h. The bound complex was detected using Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). The images were analyzed using Odyssey Application Software, version 1.2 (Li-Cor) to obtain the integrated intensities.

Immunofluorescence

The chondrocytes were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized in 0.25% (v/v) Triton X-100/PBS for 15 min, and then blocked with 5% (v/v) goat serum for 1 h. Chondrocytes were incubated with primary antibodies overnight at 4°C in a humidified chamber. Primary antibodies included a rabbit anti-phospho AKT antibody (1:200, Cell Signaling Technology) and anti-phospho ERK antibody (1:200, Abcam). After washing, the cells were incubated with a goat anti-rabbit fluorescein isothiocyanate (FITC) secondary antibody (1:500 dilution; Invitrogen) for 1 h at room temperature. Finally, the cells were incubated with 1.5 mg/ml DAPI (Shanghai Mai Bio Co, Shanghai, China) for 8 min and then visualized under a confocal microscope (TCSSP5; Leica, Wetzlar, Germany).

Statistical analysis

Values are expressed as the mean ± standard deviation. Statistical analysis was performing by a two-tailed independent Student's t-test using GraphPad Prism v5.0 (GraphPad software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

IGF-1 stimulates production of extracellular matrix by chondrocytes

Treatment of chondrocytes with IGF-1 induced a dose-dependent increase in COL-2A mRNA expression. The maximum level of induction (almost 4-fold) was observed at a dose of 40 ng/ml IGF-1 ($P < 0.01$) (**Figure 1A**). Time-dependent analysis of COL-2A expression revealed that expression peaked at 24 h after IGF-1 treatment (**Figure 1B**).

In order to further analyze the effects of IGF-1 on the production and degradation of extracellular matrix by chondrocytes, expression of the Sox9 and MMP-13 mRNAs and proteins were analyzed after 24 h of IGF-1 treatment. Results showed that IGF-1 treatment increased the expression of Sox9 at both the mRNA and protein level. In contrast, MMP-13 mRNA and protein levels were decreased by IGF-1 treatment (**Figure 1C**, **1D**). Together, these results indicate that IGF-1 stimulates production of extracellular matrix by chondrocytes.

IGF-1 induces activation of ERK1/2 and AKT in chondrocytes

In order to investigate the mechanisms underlying the effects of IGF-1 on endplate chondrocytes, we analyzed activation of the AKT and ERK signaling pathways. Western blot analysis was performed using antibodies directed against phospho-ERK1/2, total ERK1/2, phospho-AKT, and total AKT. Treatment with 40 ng/ml IGF-1 increased expression of phospho-ERK1/2, which peaked at 15 min, followed by a return to control levels by 30 min (**Figure 2A**). Similarly, phospho-AKT expression also peaked

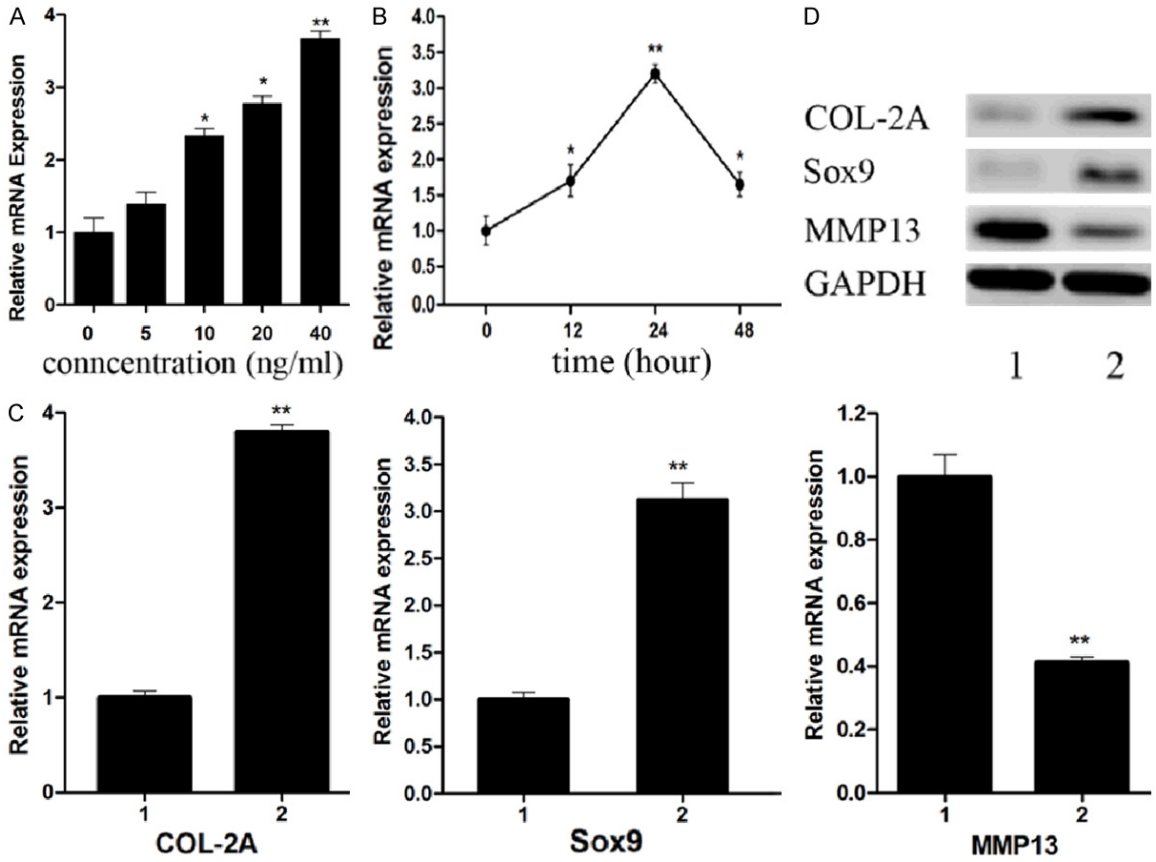

Figure 1. IGF-1 stimulates the production of extracellular matrix of chondrocytes. Chondrocytes in monolayer cultures were switched to serum-free media for 12 h, then treated with IGF-1. A. COL-2A mRNA expression was assessed by RT-qPCR. IGF-1 stimulated COL-2A mRNA expression in a dose-dependent manner. B. IGF-1 stimulated COL-2A mRNA expression in a time-dependent manner. C. Endplate chondrocytes were treated with IGF-1 (40 ng/ml) for 24 h. COL-2A mRNA, Sox9 mRNA, MMP13 mRNA expression was assessed by RT-qPCR respectively. D. Protein samples were collected and western blot was performed with an antibody against COL-2A, Sox9 and MMP13. GAPDH was used as an internal control for the RT-qPCR and Western blot. Each experiment was performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control. 1: control, 2: cultured in IGF-1. IGF, insulin-like growth factor; COL-2A, collagen type IIα; MMP, matrix metalloproteinase; Sox, (sex-determining region Y)-box 9; RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

at 15 min of IGF-1 treatment, although levels did not return to baseline until 60 min (**Figure 2B**). Immunofluorescence analysis of phospho-ERK1/2 and phospho-AKT localization in chondrocytes stimulated with IGF-1 for 15 min revealed that the activated proteins primarily localized to the nucleus of IGF-1-treated cells (**Figure 2C**, **2D**).

Distinct roles of the AKT and ERK pathways in IGF-1-treated chondrocytes

To analyze the distinct functions of IGF-1 induction of the AKT and ERK pathways in chondrocytes, we observed the effects of inhibition of each pathway. We began by determining the dose of each inhibitor required to block IGF-1-induced pathway activation. Treatment with U0126, an inhibitor of MEK, a kinase upstream of ERK, almost completely blocked IGF-1-induced ERK1/2 phosphorylation at a concentration of 1 μM (**Figure 3A**). Inhibition of PI3K, a kinase upstream of AKT, using LY29-4002 effectively blocked IGF-1-induced AKT phosphorylation at a concentration of 20 μM (**Figure 3B**). No significant changes in chondrocyte viability were observed at these doses (**Figure 3C**).

Chondrocytes were then cultured in serum-free medium for 12 h, followed by stimulation with 40 ng/ml IGF-1 alone or in combination with either 1 μM U0126 or 20 μM LY294002. Co-treatment with LY294002, but not U0126,

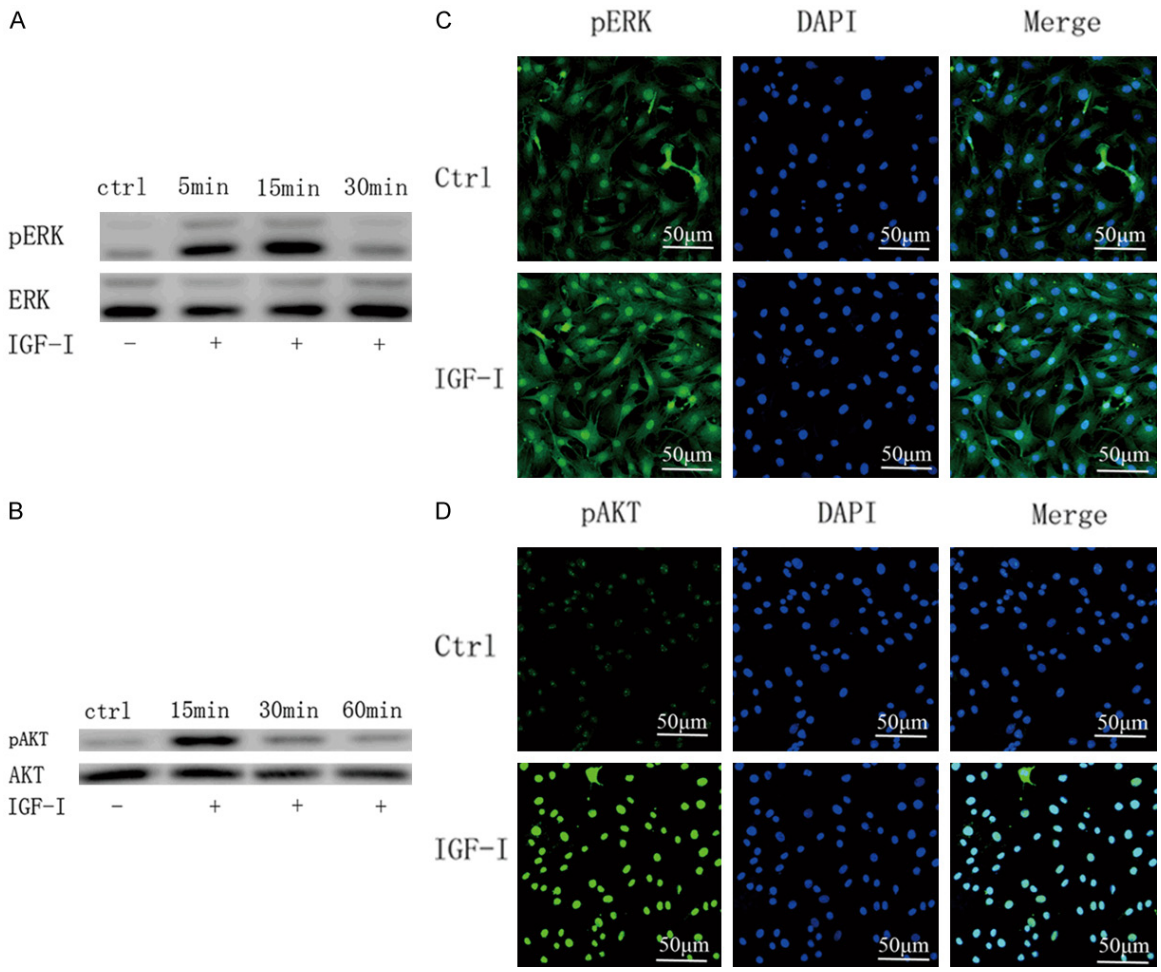

Figure 2. IGF-1 induces activation of ERK and AKT in chondrocytes. Chondrocytes in monolayer cultures were switched to serum-free media for 12 h, then treated with IGF-1 (40 ng/ml) for different time points. A. Cell lysates were prepared and western blot was done with an antibody against phospho ERK1/2 and total ERK1/2. B. Cell lysates were prepared and Western blot was done with an antibody against phospho AKT and total AKT. C. Immunofluorescence staining was used to reveal the expression and distribution of the phospho ERK1/2 after chondrocytes were stimulated with IGF-1 for 15 min. D. Immunofluorescence staining was used to reveal the expression and distribution of the phospho AKT after chondrocytes were stimulated with IGF-1 for 15 min. GAPDH was used as an internal control for western blot. Each experiment was performed in triplicate. pERK, phosphorylated extracellular signal-regulated kinase; ERK, total ERK1/2.

significantly decreased IGF-1-mediated induction of COL-2A expression ($P < 0.01$; **Figure 3D** and **3E**). In contrast, LY294002 co-treatment did not affect IGF-1-mediated induction of Sox9 expression, while U0126 treatment blocked Sox9 induction ($P < 0.01$; **Figure 3D**, **3E**). U0126 treatment also significantly blocked IGF-1-induced downregulation of MMP-13 mRNA expression ($P < 0.01$), while LY294002 had no effect (**Figure 3D**, **3E**). Together, these data suggest that IGF-1 regulates COL-2A expression through activation of the PI3K/AKT signaling pathway, while IGF-1-mediated regulation of Sox9 and MMP-13 expression occurs through the ERK pathway.

Discussion

IGF-1 has long been known to be important for growth and differentiation of cartilage tissue. The present study investigated the underlying mechanisms, revealing that IGF-1 upregulates the mRNA and protein levels of COL-2A that can determine the integrity of normal chondrocytes in human endplate chondrocytes isolated from the human lumbar spine.

A number of *in vivo* and *in vitro* experiments in mice and humans have confirmed that Sox9 is a major transcriptional regulator of chondrogenesis [14, 15]. Not only is Sox9 a potent tran-

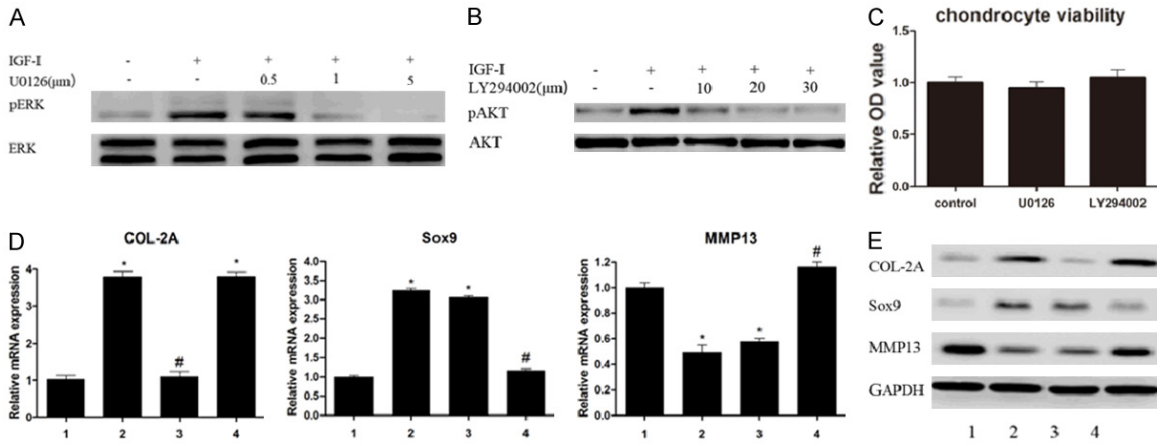

Figure 3. The mechanism of the effect of IGF-1 on chondrocytes. Chondrocytes in monolayer cultures were switched to serum-free media for 12 h, then treated with IGF-1. A. U0126 inhibited ERK activation in a dose-dependent manner after cells were treated with IGF-1 for 15 min. Cell lysates were prepared and western blot was done with an antibody against phospho ERK1/2 and total ERK1/2. B. LY294002 inhibited AKT activation in a dose-dependent manner after cells were treated with IGF-1 for 15 min, Cell lysates were prepared and western blot was done with an antibody against phospho AKT and total AKT. C. The cell viability assay using the MTT reagent expressed as relative OD value. D. Subconfluent chondrocytes were cultured in serum-free medium for 12 h and stimulated by IGF-1 (40 ng/ml) alone or in combination either with U0126 (1 μl) or LY294002 (20 μl). After 24 h incubation, COL-2A, Sox9 and MMP13 expression were assessed by RT-qPCR. E. COL-2A, Sox9 and MMP13 expression were assessed by Western blot. GAPDH was used as an internal control for the RT-qPCR and Western blot. Each experiment was performed in triplicate. $^{*}P < 0.05$ vs control, $^{\#}P < 0.05$ vs IGF-1 treatment. 1: control, 2: IGF-1 alone, 3: IGF-1 plus LY294002, 4: IGF-1 plus U0126. IGF, insulin-like growth factor; COL-2A, collagen type IIα; MMP, matrix metalloproteinase; Sox, (sex-determining region Y)-box 9; RT-qPCR, reverse-transcription quantitative polymerase chain reaction. pERK, phosphorylated extracellular signal-regulated kinase; ERK, total ERK1/2.

scriptional regulator of COL-2A expression, but it is also highly important for synthesis of aggrecan and core proteins, molecules necessary to maintain the normal chondrocyte phenotype. In contrast, senescent chondrocytes produce cartilage-degrading enzymes, such as matrix metalloproteinase-13 (MMP-13) and aggrecanases, which promote degradation of COL-2A and aggrecan, respectively [16]. Our findings demonstrate that IGF-1 treatment upregulates Sox9 expression and downregulates MMP-13 expression in human endplate chondrocytes. Therefore, IGF-1-induced upregulation of COL-2A expression may be due to a direct signaling effect, as well as indirect effects of increasing expression of Sox9 and decreasing expression of MMP-13. What's more, these results also suggest that IGF-1 plays a key role in maintaining the human endplate cartilage phenotype and inhibiting abnormal maturation and ossification by stimulating the synthesis of ECM proteins.

In order to further analyze the mechanisms underlying the effects of IGF-1 on endplate chondrocytes, we analyzed activation and function of the PI3K/AKT and MAPK/ERK pathways in IGF-1-treated cells. Both of these pathways have previously been implicated in the effects of IGF-1 in chondrocytes [9-12]. Western blot analysis and immunofluorescence staining with phospho-ERK and -AKT antibodies confirmed that IGF-1 activates both signaling pathways. In control cells not treated with IGF-1 phospho-ERK and phospho-AKT staining was predominately cytoplasmic, but stimulation with IGF-1 for 15 min induced nuclear translocation of both proteins.

Despite the observation that both pathways were activated by IGF-1 treatment, our results demonstrate that activation of the AKT pathway, not that of the ERK pathway, increases expression of COL-2A. Moreover, we also show that treatment with the MAPK pathway inhibitor U0126 completely reverses the effects of IGF-1 on MMP-13 expression. Interleukin-1 (IL-1) has previously been reported to increase the production of MMP-13 via the ERK pathway in chondrocytes [17]. Therefore, IL-1 and IGF-1 appear to achieve differing effects through activation of the same signaling pathway, likely through stimulation of different downstream targets. Not only did U0126 reverse the effects

of IGF-1 on MMP-13 expression, but it also reversed IGF-1-mediated upregulation of Sox9. Therefore, we hypothesize that functional activation and nuclear translocation of ERK may have a direct effect on Sox9 and MMP-13 expression, while functional activation and nuclear translocation of AKT may have a direct effect on COL-2A expression.

Interpretation of our results is limited by the fact that other known IGF-1-induced effects, such as promotion of chondrocyte survival, were not impacted by inhibitors of the PI3K/AKT or ERK signaling pathways (**Figure 3C**). However, IGF-1 has been reported to stimulate cell survival through activation of PI3K/AKT signaling, although other pathways also seem to contribute to survival [11, 18]. Furthermore, numerous studies have confirmed that IGF-1 can prevent chondrocyte apoptosis via the ERK signaling pathway [12]. Thus, we speculate that other compensatory mechanisms may also be present in chondrocytes.

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Disclosure of conflict of interest

None.

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