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*Original Article*

# Insulin-like growth factor-I maintains the phenotype of rat endplate chondrocytes *in vitro* via distinct signaling pathways

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**Abstract:** The goal of this study was to investigate whether the degeneration of rat endplate chondrocytes during *in vitro* monolayer culture is able to imitate that *in vivo* and whether insulin-like growth factor-I (IGF-I) is able to slow down this process. Rats were divided into three groups according to their age (young, adult, and older rats). From passage-2 (P2), the chondrocytes of young rats were passaged every third day, finally reaching P6, irrespective of whether IGF-I was used. Three cell staining methods were performed to examine the cell morphology and extracellular matrix. Chondrocyte protein and gene expression was examined by Western blot and immunofluorescence, and reverse-transcription quantitative PCR, respectively. The results indicated that cartilaginous end plates underwent age-associated degenerative changes. 40 ng/ml IGF-I was found to be the optimal concentration for enhancing the expression of COL-2A in endplate chondrocytes. With increasing passage, chondrocytes in monolayer cultures exhibited significant phenotypic and morphological changes. However, IGF-I maintained the chondrocyte phenotype and activated ERK and AKT signaling pathways during *in vitro* monolayer culture. Of note, the increased quantity of phosphorylated ERK and AKT was mainly located in the nucleus. Therefore, the degeneration of chondrocytes cultured *in vitro*, to a certain extent, may serve as a model of that caused in chondrocytes by age *in vivo*, and IGF-I delays the aging process, probably mediated by the ERK and AKT signaling pathways.

**Keywords:** IGF-I, chondrocytes, degeneration, ERK, PI3K/AKT

## Introduction

Intervertebral disc (IVD) degeneration (IDD) is regarded as the main cause of chronic neck and back pain [1, 2]. With increasing age, endplate thinning, and fracture lead to abnormal stress distribution and injury, which spread to the adjacent disc, increasing the risk of suffering from IDD [3, 4]. Vertebral endplate sclerosis and the reduction of endplate pore density and size may seriously limit the ancillary nutrient transport to IVD cells and thus increase the risk of IDD [5-7]. In view of the above, elucidating the mechanisms of cartilaginous end plate (CEP) aging may provide novel approaches for treating aging-associated IDD.

Increased expression of senescence markers, including senescence-associated β-galactosidase, p16INK4A, and decreased telomere length, were observed in aged as well as degenerated discs [8, 9]. Accordingly, it may be deduced that the quantity of aging cells was increased. Above all, the senescence of chondrocytes, as the only cell type in CEP, is a potential driver for CEP degeneration and CEP aging. These senescent chondrocytes displayed growth arrest and perturbed matrix homeostasis, as indicated by reduced matrix synthesis ability (anabolism) and increased matrix degradation ability (catabolism) [10]. In addition, the cell density decreased and the number of apoptotic cells raised rapidly in aging IVDs [11-13].

Chondrocytes also display obvious changes in their phenotype and morphology during monolayer culture *in vitro* [14, 15]. Various proteins are involved in the process of phenotypic changes of chondrocytes from polygonal to

fibroblastic. With increasing passage number, the expression of apoptotic genes and activation of senescence-associated pathways increases, while the proliferation and viability of the cells decreases [16]. However, to the best of our knowledge, no systematic and comprehensive study has been performed on endplate chondrocyte degeneration caused by natural passaging, and no *in vitro* model of the chondrocyte degeneration encountered *in vivo* has been developed.

The synthesis of collagen type IIα (COL-2A) determines the integrity of normal chondrocytes. Various *in vitro* and *in vivo* experiments on mice and humans have confirmed that (sex-determining region Y)-box 9 (Sox9) is a significant transcriptional regulator for chondrogenesis [17, 18]. COL-2A is not only one of the potent transcription factors for regulation, but also of great importance for the synthesis of aggrecan and core proteins, all of which are necessary for maintaining the normal chondrocyte phenotype. Senescent chondrocytes produce cartilage-degrading enzymes, such as matrix metalloproteinase 13 (MMP13) and aggrecanases, which accelerate the degradation of aggrecan and COL-2A [19, 20]. Activation of caspase-3 has a core position and is usually considered as a marker in the process of apoptosis [21]. The above indices, as important makers of maintaining the cell phenotype, were used as makers for chondrocyte degeneration in the present study.

As one of the important growth factors for cartilage, insulin-like growth factor-I (IGF-I) has specific functions in endocrine, paracrine, and autocrine signaling mechanisms. Primarily, IGF-I increases cell proliferation, strengthens the synthesis of matrix-associated proteins, and restrains cell apoptosis through adjusting downstream signals via the phosphoinositide 3-kinase (PI3K) pathway and extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) cascade [22-24]. In rat endplate chondrocytes, the effect of IGF-I on COL-2A expression is mostly mediated via the PI3K pathway, while the effect of IGF-I on MMP-13 expression proceeds via the ERK pathway [23]. At the same time, as for the *in vitro* culture of chondrocytes, IGF-I activates and promotes molecular interactions between phosphorylated (p)-ERK1/2 and Sox9, thus delaying cell apoptosis and finally stabilizing the chondrogenic potential [24]. The present study further investigated the degeneration of endplate chondrocytes during *in vitro* monolayer culture and explored the effect of IGF-I on these processes.

## Materials and methods

### *Animals*

Fifteen male Sprague Dawley rats with different body weights and ages were obtained from the Experimental Animal Center of Qinglongshan (Nanjing, China), and were housed in a pathogen-free rat colony. The rats were divided into three groups (n=5 in each): i) Young rats (age, 3 months; body weight, 160±20 g); ii) adult rats (age, 12 months; body weight, 300 ±20 g); iii) older rats (age, 24 months; body weight, 650±20 g). The experimental protocol of the present study was approved by the ethics committee of Yijishan Hospital (Wannan Medical College; Wuhu, China).

### *Chondrocyte isolation and culture*

The primary chondrocytes were isolated from the young rats. Endplate cartilage of the L1-L5 was carefully removed from the vertebrae and minced into small pieces (<0.03 $mm^3$). The procedure was carried out as described in a previous study by our group [25]. Cells at the second passage (P2) were used for the subsequent experiments.

### *IGF-I treatment*

The P2 chondrocytes in *in vitro* monolayer culture were washed three times with serum-free medium. To confirm the optimal IGF-I concentration for enhancing the expression of COL-2A by endplate chondrocytes from rats, the P2 chondrocytes were treated with various concentrations of IGF-I (Peprotech, Inc., Rocky Hill, NJ, USA) for 24 h. Following stimulation with the optimal concentration of IGF-I, cells in *in vitro* monolayer culture were passaged every third day to finally reach P6. The chondrocytes from each passage were plated at a density of $2\times10^5$ cells/$cm^2$ on six-well plates in 2 ml Dulbecco's modified Eagle's medium/F-12 and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and grown

**Table 1.** Primer sequence

| Genes | Forward Primer (5'-3') | Reverse Primer (5'-3') | Size |
|---|---|---|---|
| COL-2A | CCTGAAACTCTGCCACCCAG | GTTCTTCCGAGGCACAGTCG | 151 |
| Sox9 | CGTCAACGGCTCCAGCA | TGCGCCCACACCATGA | 69 |
| MMP13 | AAAGAACATGGTGACTTCTACC | ACTGGATTCCTTGAACGTC | 283 |
| GAPDH | CTCAACTACATGGTCTACATGTTCCA | CTTCCCATTCTCAGCCTTGACT | 81 |

at 37°C in a humidified atmosphere with 5% $CO_2$, and growth medium was changed once in three days. The same steps were used for chondrocytes treated without IGF-I.

*Histological staining and cell staining*

Lumbar spines from young, adult, and older rats were fixed in 10% paraformaldehyde, decalcified, and embedded in paraffin. Serial sections (7 mm thick) were stained with hematoxylin and eosin (H&E). Furthermore, endplate chondrocytes from P2 and P6 at a density of $2\times10^3$ cells per well were seeded on six-well plates, and then stained with H&E and toluidine blue.

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

Total RNA was isolated from chondrocytes using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, 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) ac cording 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) were shown in **Table 1**. The data was analyzed using the $2^{-\Delta\Delta Ct}$ method and expressed as the fold change compared with the control [26].

*Western blot analysis*

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 (Pierce; Thermo Fisher Scientific, Inc.). For Western blot analysis, 20 μg protein samples were fractionated by 10% SDS-PAGE and then electro-transferred onto nitrocellulose membranes (Whatman, Piscataway, NJ, USA). The primary antibodies were rabbit anti-active caspase-3 antibody (1: 5,000 dilution; Abcam, Cambridge, UK), rabbit anti-Sox9 antibody (1:10,000 dilution; Abcam), rabbit anti-MMP13 antibody (1:5,000 dilution; Abcam), rabbit anti-collagen II antibody (1: 5000 dilution; Abcam), rabbit anti-total ERK 1/2 antibody (1:10,000 dilution; Abcam), rabbit anti-p-ERK1/2 antibody (1:10,000 dilution; Abcam), rabbit anti-total AKT (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-p-AKT (1:2,000 dilution; Cell Signaling Technology, Inc.). Protein levels were normalized to GAPDH using an anti-GAPDH antibody (1:10,000 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 1:5,000, room temperature for 2 h. The bound complex was detected using an enhanced chemiluminescence detection system (Millipore, Billerica, MA, USA).

*Immunofluorescence*

Chondrocytes at P2 or P6 were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized in 0.25% (v/v) Triton-X 100 in 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-p-AKT antibody (1:200; Cell Signaling Technology, Inc.) and anti-p-ERK antibody (1:200 dilution; Abcam). After washing, the cells were incubated with a goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (1:500 dilution; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. For F-actin staining,

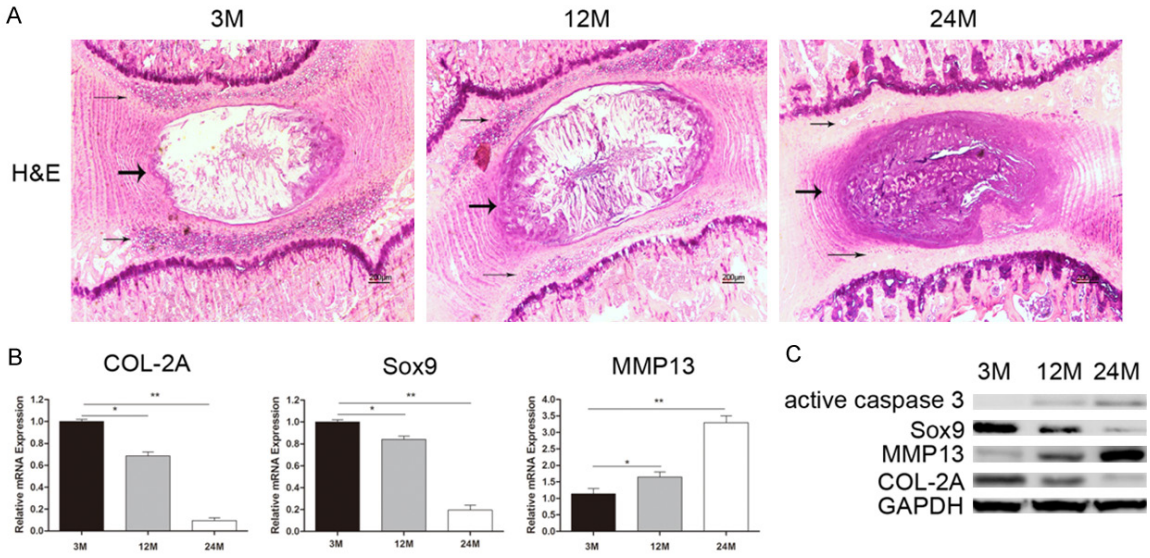


**Figure 1.** Cartilaginous end plates undergo age-associated degenerative changes. A. H&E histological staining of disc sections from young, adult, and older rats (original magnification, ×40; scale bar, 200 μm). Images representative of three samples are displayed. Cartilaginous end plates (thin arrows) and nucleus pulposus (thick arrows) are indicated. B. RT-qPCR revealed alterations in the expression levels of COL-2A, Sox9, and MMP13 in cartilaginous end plates of the three groups. C. Expression of active caspase-3, Sox9, MMP13, and COL-2A were determined by Western blot analysis in endplates of the three groups. GAPDH was used as an internal control for the RT-qPCR and Western blots. Each experiment was performed in triplicate. $^{*}P<0.05$, $^{**}P<0.01$. H&E, hematoxylin and eosin; M, age of rat in months; COL-2A, collagen type IIα; MMP, matrix metalloproteinase; Sox, (sex-determining region Y)-box 9; RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

the cells were washed and then incubated with Texas Red®-X phalloidin (1:500 dilution; Invitrogen; Thermo Fisher Scientific, Inc.) 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 performed by 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

*CEPs undergo age-associated degenerative changes*

Compared with those of young rats, the IVDs of adult rats did not display any obvious changes, but the nucleus pulposus (NP) of older rats did, as fissures appeared. In addition, with advancing age, the cell density in the CEP decreased but it increased in the trabecular bone (**Figure 1A**). With increasing age of the rats, the mRNA and protein levels of Sox9 and COL-2A decreased, while MMP13 increased in the CEP (**Figure 1B** and **1C**). In addition, active caspase-3 protein was detected in the CEPs of the adult rats and was increased in that of the older rats (**Figure 1C**).

*Chondrocytes undergo degeneration with increasing passage number*

Within six passages, the morphology of the chondrocytes cultured *in vitro* changed from a polygonal or round to a flattened, amoeboid-like shape, which could be deduced from the results of H&E staining and Texas Red®-X phalloidin staining. In addition, the brightness of the extracellular matrix became weakened in toluidine blue-stained samples. (**Figure 2A**). When P2 chondrocytes were treated with IGF-I for 24 h, the mRNA expression of COL-2A in the rat's endplate chondrocytes was significantly increased if IGF-I concentrations of ≤10 ng/ml were used. Treatment with IGF-I at 40 ng/ml resulted in maximum COL-2A levels,

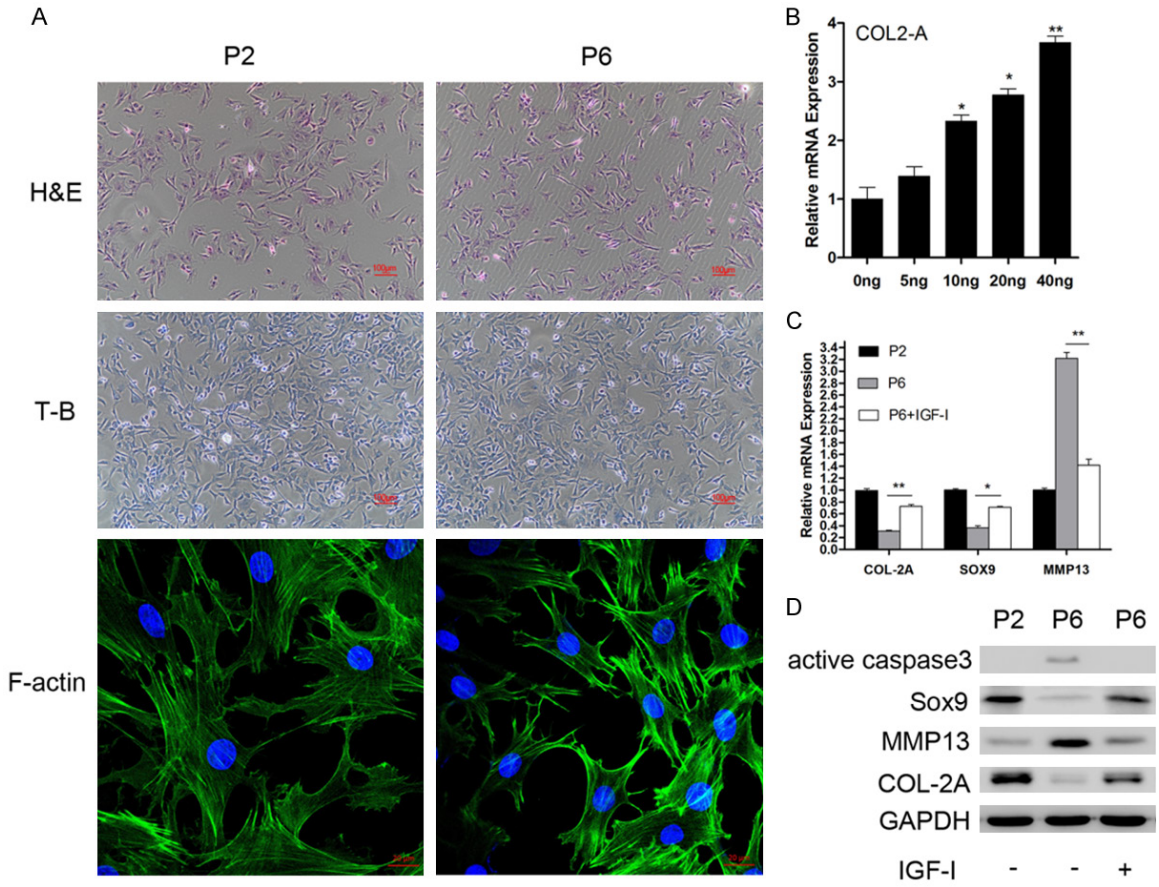


**Figure 2.** IGF-I maintains the phenotype of chondrocytes during *in vitro* monolayer culture. A. Cellular morphology and staining. Images of H&E and T-B representative of three samples are displayed (original magnification, ×100; scale bar, 100 μm). F-actin staining revealed changes in the P6 chondrocyte cytoskeleton. Representatives of three samples are shown; green indicates F-actin and blue indicates DAPI staining (scale bar, 20 μm). B. P2 chondrocytes were serum-starved for 12 hours and then treated with IGF-I at various concentrations for 24 h. qRT-PCR revealed alterations in the expression levels of COL-2A in an IGF-I dose-dependent manner. C. From P2, chondrocytes were cultured and incubated with or without IGF-I (40 ng/ml). Every third day, the confluent cells in monolayer culture were passaged, finally reaching P6. RT-qPCR analysis of P2 and P6 chondrocytes as well as P6 chondrocytes that had been cultured in the presence of IGF-I revealed differences in the expression levels of COL-2A, Sox9, and MMP13 between the groups. D. Expression of active caspase-3, COL-2A, Sox9, and MMP13 protein was determined by Western blot analysis in each group. GAPDH was used as an internal control for the qRT-PCR and Western blot analyses. Each experiment was performed in triplicate. $^{*}P<0.05$, $^{**}P<0.01$ vs P2. T-B, toluidine blue; H&E, hematoxylin and eosin; COL-2A, collagen type IIα; MMP, matrix metalloproteinase; Sox, (sex-determining region Y)-box 9; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; P2, passage 2. IGF, insulin-like growth factor.

which were almost four times those in IGF-I-untreated cells (**Figure 2B**). In subsequent experiments, 40 ng/ml was used as the optimal concentration of IGF-I to stimulate gene expression by chondrocytes. The chondrocytes at P2 had a high expression of COL-2A and Sox9, which decreased with increasing passage number, while MMP13 expression was initially low and was elevated with increasing passage number. Furthermore, P6 chondrocytes contained active caspase 3, which was not detectable in P2 chondrocytes (**Figure 2C** and **2D**).

*IGF-I maintains the phenotype of chondrocytes during in vitro monolayer culture*

Compared with that in the control group, treatment of P6 chondrocytes with IGF-I increased the protein and mRNA expression of COL-2A and Sox9 but decreased the protein and mRNA expression of MMP13. However, IGF-I treatment of P6 chondrocytes did not fully restore the expression levels of these genes to those in P2 cells (**Figure 2C** and **2D**). Western blot analysis was used to examine the protein levels of activated caspase-3 in P2 and P6 chon-

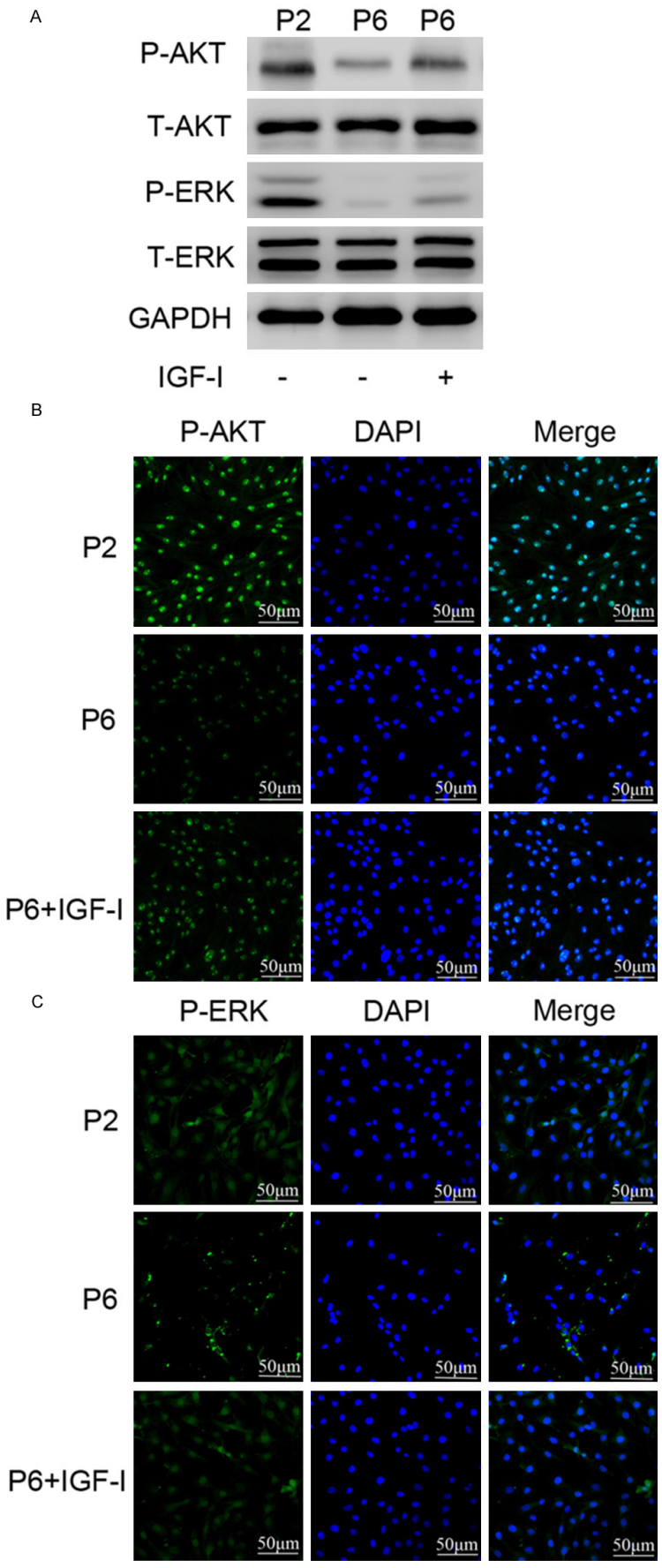


**Figure 3.** IGF-I induces activation of ERK and AKT in chondrocytes. From P2, chondrocytes were cultured and incubated with or without IGF-I (40 ng/ml). Every third day the confluent cells in the monolayer culture were passaged, finally reaching P6. (A) Levels of T-ERK and P-ERK, T-AKT, and P-AKT in P2 and P6 chondrocytes as well as P6 chondrocytes that had been cultured in the presence of IGF-I were determined by Western blot analysis. GAPDH was used as an internal control. Levels of (B) P-ERK and (C) P-AKT were also detected by immunofluorescence staining in each group (scale bar, 50 μm). Each experiment was performed in triplicate. P2, passage 2; IGF, insulin-like growth factor; P-AKT, phosphorylated AKT; T-ERK, total extracellular signal-regulated kinase.

drocytes with or without IGF-I treatment. The IGF-I-treated chondrocytes at P6 had lower levels of activated caspase-3 than the untreated ones, just as the same as that of P2 chondrocytes (**Figure 2D**).

*IGF-I activates the ERK and AKT signaling pathways in chondrocytes*

In order to investigate the signaling pathways involved in the effect of IGF-I on chondrocytes, the protein levels of ERK and AKT were detected in the P2, P6, and P6+IGF-I chondrocytes. Overall, there appeared to be no noticeable changes in the protein expression levels of total ERK or AKT (**Figure 3A**). However, compared with that in P2 chondrocytes, the P6 chondrocytes displayed a decrease in p-ERK1/2 and p-AKT. Furthermore, treatment with IGF-I markedly increased the phosphorylation levels of ERK and AKT proteins, and these changes were also obvious in the cell nuclei on immunohistochemistry, which indicate nuclear translocation of p-ERK and p-AKT stimulated by IGF-I. How-

ever, IGF-I treatment did not restore the p-ERK1/2 and p-AKT levels in P6 chondrocytes to those in P2 chondrocytes (**Figure 3A-C**).

## Discussion

During the process of spine movement, IVDs bear and distribute the mechanical load. The CEP is an integral part of IVDs and supplies nutrition to avascular IVDs. The phenotype of endplate chondrocytes is mainly characterized by the expression of genes encoding cartilage-specific extracellular matrix proteins or their regulators, such as COL-2A and Sox9, which have a major role in maintaining cartilage anabolism. Certain studies have indicated that the degeneration of endplate cartilage and loss of the chondrogenic phenotype increases the internal disruption of discs, thus promoting degeneration [4, 27]. The present study discovered that CEP underwent age-associated degenerative changes. Furthermore, in accordance with the findings of a previous study, the age-associated structural aberrations of CEP appeared earlier than those of IVDs [11].

During disc aging, cells undergo functional and phenotypic changes, which were mostly found in the nucleus pulposus (NP) cell subpopulations [28]. In the aging process, disc cells undergo numerous phenotypic changes, including the transformation from an anabolic to a catabolic phenotype; for instance, collagen and proteoglycan matrix anabolism decreases, while the levels of matrix proteoglycan degradation products and certain MMPs increase [29, 30]. Similarly, following isolation from native cartilage and culture *in vitro* as a monolayer, they also lose their phenotype and various senescence and de-differentiation-mediating genes are expressed [31]. The present study found that the morphology of chondrocytes changed from polygonal or round to a flattened, amoeboid-like shape, and at the same time, during the aging process of chondrocytes in monolayer culture, the expression of COL-2A and Sox9 decreased, while MMP13 and active caspase 3 increased. It was therefore hypothesized that chondrocyte degeneration during *in vitro* monolayer culture resemble those encountered *in vivo*.

The IGF-I pathway regulates various cellular processes, which include cell growth, proliferation and survival, protein synthesis, and transcription mechanisms. Even though it is known that IGF-I induces the anabolic activity of discs, the signaling pathways associated with disc aging have remained to be fully elucidated [23, 24]. It has been reported that IGF-I extends the chondrogenic potential of de-differentiated chondrocytes and delays the appearance of active casapase-3 during *in vitro* monolayer culture [32]. However, to the best of our knowledge, the present study was the first to demonstrate that during *in vitro* monolayer culture, IGF-I delays endplate chondrocyte senescence via stimulating Sox9 and COL-2A, while decreasing MMP13, and reduces the apoptosis of chondrocytes through decreasing active caspase-3. Furthermore, the effect of IGF-I on chondrocytes decreased from P2 to P6, which might be due to resistance of older cells to respond to IGF influence [33].

As a member of the MAPK cascade, ERK1/2 is involved in regulating chondrocyte apoptosis, proliferation, and de-differentiation, while augmentation of p-ERK1/2 indicates the activation of the signaling pathway [34, 35]. Studies have reported that during monolayer culture of chondrocytes, the loss of chondrogenic capacity is associated with a reduction in the synthesis of cartilage markers and the decline of key signaling proteins in the Ras/MAPK pathway (SH-containing protein and ERK1/2), which eventually causes apoptosis [36, 37]. Considered as a vital effector kinase downstream of the PI3K pathway, AKT is capable of regulating a number of cellular processes, including cell growth, death, differentiation, and migration [38], with changes of p-AKT activating associated signaling pathways. A previous study by our group reported that activation of the PI3K/AKT signaling pathway was suppressed in chondrocytes after six passages in monolayer culture [39]. The experimental results led to the speculation that the stress responses caused by *in vitro* culture conditions repress the activation of AKT and ERK signaling pathways, and that these changes mainly occur in the nucleus, leading to degeneration of cells.

It is widely accepted that PI3K/AKT and ERK are two major pathways via which IGF-I exerts its effects on chondrocytes [22-24]. The PI3K pathway mainly mediates the effects of IGF-I on COL-2A expression, while the reduction of

MMP-13 expression as well as induction of Sox9 expression and caspase-3 activation by IGF-I are mediated via the ERK pathway. IGF-I-induced signaling was reported to specifically activate ubiquitination of p-AKT mediated by neural precursor cell expressed developmentally down-regulated protein 4-1, without changing total AKT ubiquitination, and the ubiquitinated p-AKT then translocates to perinuclear regions, where it is either released into the cytoplasm, imported into the nucleus or subjected to proteasomal degradation [40, 41]. Another study demonstrated that upon its activation and nuclear translocation, ERK interacts with Sox9 stimulated by IGF-I [24]. Based on the results of the present study, IGF-I stimulated ERK and AKT signaling pathways, and p-AKT and p-ERK translocated to the nucleus to directly interact with targets to delay cell degeneration.

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## Disclosure of conflict of interest

None.

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