Interleukin-Iβ promotes cartilage degeneration by regulating forkhead box protein O4 and type II collagen

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Abstract. Osteoarthritis (OA) is one of the most prevalent pain-inducing and disabling diseases globally. Aging is a primary contributing factor to the progression of OA. Forkhead box protein O4 (FOXO4) is known to be involved in the cell cycle and apoptosis regulation. The aim of the present study was to investigate the association between FOXO4 expression and chondrocyte degeneration in rats. Chondrocytes were assigned to the control (4-week-old rats), natural degeneration (16-week-old rats) or induced degeneration (IL-1β-treated chondrocytes from 4-week-old rats) groups. Immunocytochemical analysis with β-galactosidase staining revealed a greater number of stained cells present in the natural and induced degeneration groups than in the control group. PCR analysis indicated lower mRNA expression levels of collagen type II α1 chain (Col2α1) and higher levels of FOXO4, and western blotting revealed reduced col2α1 protein expression levels and significantly elevated FOXO4 levels in the natural and induced degeneration groups, compared with those in the control group. The results of the present study revealed that FOXO4 expression was altered in the natural and induced degeneration groups, and further research and exploration are needed to clarify the underlying mechanism.

Introduction

Articular cartilage that is damaged or undergoing degeneration is unable to repair itself to a healthy state, which may lead to osteoarthritic alterations (1). Osteoarthritis (OA) is one of the most common pain-inducing and disabling diseases worldwide (2). Patients living with OA experience significantly compromised quality of life and increased health-care costs (3). Age is a primary contributing factor to OA progression; however, various other factors are also involved in the development of age-related OA, including articular cartilage damage, articular space stenosis, osteophyte formation, subchondral bone alteration and soft tissue degeneration (4). The prevalence of OA in China is increasing with the aging of the population (1). Despite its high prevalence, the pathogenesis of OA is still relatively unknown, and there are currently no therapeutic cures for the disease (5,6). Due to the lack of effective treatments for the reversal of articular cartilage degradation, current treatments aim to improve pain symptoms and delay disease progression. Although prosthetic joint replacement is an effective treatment for end-stage OA, the functioning of the prosthetic joint is inferior to that of its healthy counterpart. Accordingly, the development or identification of conservative treatments that effectively prevent OA progression is critical (2).

Gene therapy for OA has become a research focus, as studies continue to identify genes that play important roles in the development and progression of OA (3,7). Zhou et al (8) found that conditional deletion of Indian hedgehog (Ihh) protein in mice chondrocytes attenuates OA progression, suggesting the possibility that blocking Ihh signaling can be used as a therapeutic approach to prevent or delay cartilage degeneration. Therefore, gene-targeted therapy may be an effective approach to repairing cartilage damaged by OA.

The forkhead box (FOX)O proteins are a subfamily of the FOX family of transcription factors. They are transcriptional activators that are also considered to be longevity factors (9). This protein family serves important roles in the regulation of tissue homeostasis through autophagy and oxidative stress (9,10), and is essential for maintenance of the hematopoietic stem cell pool (11). FOX protein O4 (FOXO4) has been shown to be involved in the cell cycle, apoptosis regulation and tumorigenesis through transcriptional regulation (12-14). Baar et al (15) previously reported increased FOXO4 mRNA expression and a decreased rate of apoptosis after the induced aging of IMR-90 fibroblast cells with ionizing radiation. Conversely, inhibiting FOXO4 expression decreased the
expression levels of FOXO4 mRNA and increased the rate of apoptosis.

In summary, FOXO4 is closely associated with cellular aging and apoptosis; however, potential associations between FOXO4 and cartilage degeneration or OA are yet to be elucidated, and past studies have reported controversial findings. Akasaka et al (16) revealed that young articular cartilage expressed FOXO1 and 3 proteins, but not FOXO4 (16). Additionally, Ludikhuize et al (17) found that FOXO4 was expressed and phosphorylated in the knee joint tissues of patients with rheumatoid arthritis and OA. Matsuzaki et al (18) also reported that FOXO1, 3 and 4 may be key to cartilage development and maturation, and may prevent OA-associated cartilage damage (18).

In the present study, an effective chondrocyte degeneration model was established in Sprague-Dawley (SD) rats. The model was used to experimentally determine the expression levels of FOXO4 and collagen type II α1 chain (Col2α1) in normal and degenerated rat cartilage cells, and to investigate potential associations between FOXO4 expression levels and chondrocyte degeneration.

Materials and methods

**Animals.** A total of 30 male SD rats (4- and 16-week-old) were purchased from Hunan Silaike Jingda Laboratory Animal Co., Ltd. The weight of the SD rats at 4 and 16 weeks were 70-100 g and 380-480 g, respectively. They were housed at 20-22˚C and 40-70% humidity with normal circadian rhythm light/dark cycle. They were fed sterilized feed (10g/100 g weight) and water (10-15 ml/100g weight) ad libitum. There were 10 rats in each group. All animal experiments were approved by the Animal Research and Care Committee of Nanchang University (approval no. NCXK-2019-21; Hangzhou, China). All efforts were made to minimize suffering and the number of animals used in the study. All rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg).

**Isolation and culture of chondrocytes from the femoral heads of SD rats.** All rats were euthanized by an overdose of pentobarbital sodium (120 mg/kg, intraperitoneal administration). The absence of respiration, heartbeat and the corneal/palpebral reflex indicated animal death. Following routine dissection, the femoral head was exposed using surgical forceps and ophthalmic scissors. The cartilage tissue covering the femoral head was minced into pieces of ~1.0 mm², and then digested with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C for 30 min. The precipitate was collected after centrifugation at 300 x g for 10 min at room temperature, and further digested with 0.2% type II collagenase (Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C for 3 h. The resulting precipitate was then agitated for 2 min and transferred to a clean centrifuge tube. After centrifugation at 300 x g for 10 min at room temperature, the precipitate containing the chondrocytes was collected and the entire process was repeated once more. The dissociated chondrocytes were repeatedly pipetted, washed with phosphate-buffered saline (PBS; HyClone; Cytiva), and centrifuged three times at 300 x g for 5 min at room temperature. The cells were then seeded into 50-cm² culture flasks filled with Dulbecco’s modified Eagle’s medium (DMEM; HyClone; Cytiva) supplemented with 15% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The flasks were incubated at 37˚C (5% CO₂) and the medium was changed every 3-4 days until the cells had reached confluency. The cells were divided into groups for use in the following experiments, and proliferation was periodically observed and images were captured.

**Morphological identification of SD rat chondrocytes.** On reaching ~80% confluence, chondrocytes at passage 1 and passage 2 underwent morphological identification. Chondrocytes were stained using Toluidine blue (Shanghai Macklin Biochemical Co., Ltd.) and Alcian blue (Beijing Solarbio Science & Technology Co., Ltd.) for cellular identification, and were examined under a light microscope (TS100-F; Nikon Corporation). Cells were fixed with 4% paraformaldehyde in phosphate buffer. Cells were fixed at room temperature for 10-20 min, washed with running water for 3 times for 2 min and then stained. Cells were respectively stained with 1% toluidine blue and 0.1% Alcian blue for 30 min at 37˚C, the excess dye washed away with double distilled water, dehydrated with absolute ethanol and sealed with neutral gum.

**Col2α immunocytochemical staining.** The second generation of chondrocytes was used for Col2α immunocytochemical staining following adhesion to the flask wall after culture for 24 h. The second-generation chondrocytes were cultured in a 6-well plate for 24 h, and then fixed with 4% paraformaldehyde for 30 min at room temperature. Then, 30% H₂O₂ and methanol (1:50) were added for 30 min at room temperature, after which the chondrocytes were blocked with 5% bovine serum albumin (BSA; Gibco; Thermo Fisher Scientific, Inc.) in PBS for 1 h at room temperature. Monoclonal rat anti-Col2α antibody (1:200; cat. no. 28459; ProteinTech Group, Inc.) was added and the cells were incubated for 2 h at 37˚C. After washing with PBS, the appropriate biotin-conjugated secondary antibodies (1:200; cat. no. SA00004-8; ProteinTech Group, Inc.) were applied for 30 min at 37˚C. The chondrocytes were washed with PBS once more, and then incubated with avidin-biotin peroxidase conjugate (SABC kit; Wuhan Boster Biological Technology, Ltd.) for 30 min at 37˚C. Diaminobenzidine tetrahydrochloride (0.3%; DAB kit; Wuhan Boster Biological Technology, Ltd.) was used as the substrate for the peroxidase reaction. Staining was visualized and images were captured with an immunofluorescence microscope (magnification, x100; TS100-F; Nikon Corporation).

**Experimental grouping.** The experiments were conducted using three groups of chondrocytes. Chondrocytes in the control group were obtained from 4-week-old rats, while those in the natural degeneration group were obtained from 16-week-old rats. Chondrocytes in the induced degeneration group were obtained from 4-week-old rats and treated with IL-1β (PeproTech, Inc.) at 37˚C for one week. For this treatment, 10 ng/ml IL-1β solution was added to DMEM/F-12 medium containing 15% FBS, which was used to culture the chondrocytes of 4-week-old rats from passage 1 until subsequent experimentation.

**β-galactosidase staining.** β-galactosidase staining was conducted using the β-galactosidase staining kit (Beijing
Solarbio Science & Technology Co., Ltd.) per the manufacturer's protocol. Staining at room temperature for 15 min was performed and images were captured using a fluorescence microscope (TS100-F; Nikon Corporation).

**RNA extraction and reverse transcription (RT)-semi-quantitative PCR.** Chondrocytes from each group were washed with cold PBS and preserved at -80˚C. Total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.), and 500 ng RNA was reverse transcribed into cDNA using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo Life Science). The RT kit was used according to the manufacturer's protocol. The generated cDNA was then used for mRNA screening in RT-semi-quantitative PCR assays, which were performed in 20 µl with 1 µl prepared cDNA, 0.5 µl each primer and 10 µl SYBR Green PCR Master Mix (Toyobo Life Science) using the C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc.) under the following conditions: 95˚C for 1 min, 25 cycles at 95˚C for 15 sec, 58˚C for 30 sec and 72˚C for 30 sec. The relative expression levels of each mRNA were normalized to those of GAPDH. The primer sequences for Col2α, FOXO4 (both PeproTech, Inc.) and GAPDH are listed in Table I.

| Primer       | Primer sequence                  | Product length, bp |
|--------------|----------------------------------|--------------------|
| GAPDH forward| 5'-GATGCTGGTGCCGAGTAC-3'         | 104                |
| GAPDH reverse| 5'-GCTAGATGAGTACCTTTTGG-3'       |                    |
| Col2α forward| 5'-CGAGGTGACAAAGGAGAAGC-3'       | 453                |
| Col2α reverse| 5'-CTGGTTGGTCAGCAGACTTG-3'       |                    |
| FOXO4 forward| 5'-CCAGAGAATAAGAAGTCAAGCGACAG-3' | 147                |
| FOXO4 reverse| 5'-CTCCACCTCGGACGTTCCG-3'        |                    |

FOXO4, forkhead box protein 4; Col2α, collagen type II α1 chain.

### Results

**Isolation and culture of SD rat chondrocytes.** Femoral head cartilage from the 4-week-old rats was thick, tough, brittle, elastic and easy to completely remove (Fig. 1A and B). The femoral head cartilage from the 16-week-old rats was thin and densely combined with the subchondral bone (Fig. 1C), making it difficult to completely remove from the femoral bone.

After incubation for 48 h, a large number of adhering primary chondrocytes were obtained from the 4-week-old rats. Adherent cells were uniformly distributed (Fig. 2A) with a 'paving stone'-shaped appearance (Fig. 2B). After 7 days, the cells were overconfluent for the first passage (Fig. 2C). After 48 h of incubation, a small number of primary chondrocytes from the 16-week-old rats had adhered to the flask wall in a scattered arrangement, before forming cell clusters and gradually expanding outwards (Fig. 2D and E). At ~2 weeks, the multi-cluster cell groups merged to form a fully confluent monolayer, which indicated that the cells were proliferating correctly (data not shown). The second passage was performed after 3 and 4-5 days on cells from the 4- and 16-week-old rats, respectively.

**Identification and morphological changes in SD rat chondrocytes.** There were no notable morphological differences...
between the two groups of chondrocytes, which appeared to be either triangular or polygonal in shape with an abundant cytoplasm. The chondrocytes of 4-week-old rats were shown in Fig. 3A. Toluidine blue staining revealed blue-colored cytoplasm with a darker blue nucleus (Fig. 3B). The cytoplasm appeared pale blue following Alcian blue staining (Fig. 3C). Immunocytochemical staining revealed cytoplasm with a brownish-yellow appearance, while the nucleus appeared dark brown, indicating Col2α staining (Fig. 3D). The three staining methods yielded positive results for chondrocyte identification.

Second-generation cells in the control group appeared spindle-shaped with a rich cytoplasm and good cell refractive index (Fig. 4A). By contrast, second-generation cells from the natural and induced degeneration groups appeared fusiform
or irregular in shape with a reduced cell refractive index (Fig. 4B and C).

β-galactosidase staining. β-galactosidase staining of cells in the control group did not elicit a notably positive response (Fig. 5A). By contrast, β-galactosidase staining of cells in the natural and induced degeneration groups resulted in a positive reaction, with dark blue staining in a large number of cells (Fig. 5B and C). There were no notable differences in β-galactosidase staining intensity between the natural and induced degeneration groups.

Col2α and FOXO4 transcription and protein expression. Each group contained 5S, 18S and 28S bands, and the intensity of the 28S rRNA band was approximately twice that of the 18S rRNA band, indicating sufficient total RNA extraction from chondrocytes in each group (Fig. 6).

Compared with the control group, Col2α mRNA expression was significantly reduced in chondrocytes from the natural and induced degenerative groups (0.07±0.02 vs. 0.53±0.06, and 0.15±0.03 vs. 0.53±0.06; Fig. 7A and B). Additionally, FOXO4 mRNA expression was significantly elevated in both groups compared with the control group (0.58±0.03 vs. 0.08±0.02, and 0.83±0.03 vs. 0.08±0.02; Fig. 7A and C).

Furthermore, the protein expression levels of Col2α in chondrocytes from the natural and induced degeneration groups were significantly lower than those in the control group (0.14±0.01 vs. 0.51±0.02, and 0.11±0.03 vs. 0.51±0.02; Fig. 8A and B). FOXO4 protein expression was significantly

Figure 3. Morphological representation of chondrocytes at passage one. (A) Unstained, (B) toluidine blue staining, (C) Alcian blue staining and (D) type II collagen immunocytochemical staining. Magnification, x100.

Figure 4. Microscopic evaluation of second-generation chondrocytes. (A) Control, (B) natural degeneration and (C) induced degeneration groups. Magnification, x100.
elevated when compared with the control group (0.16±0.02 vs. 0.08±0.01, and 0.21±0.02 vs. 0.08±0.01; Fig. 8A and C).

Discussion

Aging is a natural process involved in the pathogenesis of numerous diseases, and is considered a significant risk factor for the development of OA (7). OA affects >50% of the world’s population >65 years of age (2), but the association between OA and aging remains to be elucidated. An enhanced understanding of the mechanisms relevant to OA development is critical for the investigation of novel treatment strategies. A well-established hypothesis is that chondrocyte degeneration causes premature aging due to excessive mechanical load or oxidative stress, leading to stress-induced aging, and ultimately, the onset of OA (19). Therefore, insights into the underlying mechanisms of chondrocyte degeneration are critical to researching OA development. The present study introduced the establishment of an effective chondrocyte degeneration model, and investigated the association between the expression of related proteins and cartilage degeneration.

The selection of effective modeling methods is an important part of disease research (20). In vivo, such animal methods include artificially-induced and spontaneous models. Relatively speaking, spontaneous models more adequately simulate the process of natural articular cartilage degeneration, which was the ideal model for the present study. Spontaneous OA can occur in guinea pigs at ~3 months of age, and is characterized by an uneven or absent cartilage surface and collagen dissolution in chondrocytes (21). In the present study, cartilage taken from 16-week-old SD rats displayed observable thinning of the surface, decreased elasticity, hardening and deformation in the shape of cultured cells after passage. These observations suggested that key characteristics of cartilage degeneration, such as collagen reduction and calcification, can occur in rats at 16 weeks of age. The in vitro cell model for cartilage degeneration is commonly established through the use of inducing agents, such as ascorbate, rat serum and IL-1β (22).

In the present study, the experiments successfully replicated the model described by Ding et al (23), which established a cellular model of arthritis in rabbits using media with a final concentration of 10 ng/ml IL-1β.

Some aging cells express senescence-associated β-galactosidase (SA-βgal), which is easily detected at pH 6.0, but undetectable in young stationary-phase, immortal and tumor cells (24). SA-βgal activity is closely associated with aging cells and is widely used in research as a biomarker for cellular aging (25). Type II collagen is a major component of cartilage that is specifically expressed by chondrocytes; together with proteoglycans, type II collagen responsible for maintaining the strength and hardness of chondrocytes (26). Sufficient production of type II collagen is essential to maintaining the biomechanical properties of cartilage, although its expression gradually decreases as chondrocyte degeneration progresses (27). In the present study, a large number of cells in the natural and induced degeneration groups stained positive for β-galactosidase, while no such staining was observed in the control cells. As confirmed by RT-semi-quantitative PCR and western blotting, the mRNA and protein expression levels of Col2α were decreased in the natural and induced degeneration groups compared with in the control group.
Figure 7. mRNA expression levels in chondrocytes of the control, natural degeneration and induced degeneration groups. (A) PCR electropherogram. (B) Col2α and (C) FOXO4 mRNA expression levels. GAPDH was used as the internal parameter. Data are expressed as the mean ± SD; n=3. ***P<0.001. FOXO4, forkhead box protein O4; Col2α, collagen type II α1 chain.

Figure 8. Protein expression levels in chondrocytes of the control, natural degeneration and induced degeneration groups. (A) Western blot analysis, and (B) Col2α and (C) FOXO4 protein expression levels. Data are expressed as the mean ± SD; n=3. *P<0.05, **P<0.01 and ***P<0.001. FOXO4, forkhead box protein O4; Col2α, collagen type II α1 chain.
Due to its role as a transcriptional activator, FOXO protein is considered to be a pro-longevity factor (28). FOXO4 is a member of the FOXO protein family that promotes longevity via its subcellular localization and transcriptional activity, which are primarily regulated by phosphorylation and acetylation (29). Baar et al (15) demonstrated that FOXO4 mRNA expression was increased in aging-induced IMR90 fibroblasts, whereas apoptotic rate was decreased. Conversely, FOXO4 mRNA expression was decreased and the apoptotic rate was increased following FOXO4 lentiviral inhibition. Furthermore, apoptosis was induced by INK-ATTAC gene-mediated or cell-permeable FOXO4-DRI peptides, which can eliminate aging cells. Overall, this may reduce aging-related damage and dysfunction, while enhancing cellular performance and longevity (30).

The aim of the present study was to investigate the association between FOXO4 expression and chondrocyte degeneration in rats. The study was predicated by that of Akasaki et al (16), which revealed that FOXO4 was not expressed in the articular cartilage of young subjects, and Matsuzaki et al (18), which indicated that FOXO4 may serve a protective role against OA, and was critical for cartilage development and maturation. It is controversial whether FOXO4 expression has a protective effect in OA. The aim of the present study was to reveal the relationship between FOXO4 expression and OA. Accordingly, it was hypothesized that FOXO4 may be associated with the chondrocyte degeneration process. RT-semi-quantitative PCR and western blotting revealed elevated FOXO4 mRNA and protein expression levels in the natural and induced degeneration groups compared with in the control group, although elucidation of the specific mechanisms requires further investigation.

The pathogenesis of OA is still being studied, and the relationship between the matrix metalloproteinase (MMP) family and OA has received increasing attention. The OA progression was slowed in MMP13 Col2ER mice 8, 12 and 16 weeks post-surgery. Cartilage grading by blinded observers confirmed decreased articular cartilage degeneration in MMP13 Col2ER mice at 8, 12 and 16 weeks compared with negative mice. MMP13 is the main enzyme that targets cartilage degradation (31). The expression levels of MMP13 in OA have been reported to be 10 times higher than those in normal individuals (32). Furthermore, the increased expression of MMP13 has been shown to be positively correlated with the development of OA, which may be caused by inflammation that stimulates synovial hypersecretion of MMP13, which degrades collagen and breaks the balances of bone matrix synthesis and decomposition, thus in turn leading to OA occurrence (31). Studies (33,34) have shown that FOXO4 may activate the transcription of the MMP9 gene in response to tumor necrosis factor-α signals; the transactivation domains of FOXO4 are required for FOXO4 to activate MMP9 transcription. However, to the best of our knowledge, at present there are no reports on the effects of FOXO4 on the expression of MMP13, and further research is needed.

In conclusion, the natural and induced degeneration groups described in the present study represent effective models of chondrocyte degeneration for use in experiments associated with cartilage degeneration or OA. The transcription factor FOXO4 is expressible in, and associated with, the degeneration of rat chondrocytes, the expression level trend of chondrocytes was consistent with the degree of cartilage degeneration. As a study limitation, the effect of FOXO4 on chondrocyte degeneration needs to be verified by more experiments. We acknowledge that the causal relationship between FOXO4 and the pathogenesis of OA is not yet clear. Follow-up work will continue to develop the effects of FOXO4 on the pathogenesis of OA by inducing knockout or overexpression of FOXO4 in chondrocytes.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JW, HZ and XF planned the experiments; JW, HZ, RD and LX performed the experiments; JW, LX and MH analyzed the data; JW, HZ and XF wrote the manuscript. JW, HZ and XF confirm the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Research and Care Committee of Nanchang University (approval no. NCXK-2019-21; Hangzhou, China). All efforts were made to minimize suffering and the number of animals used in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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