

Effect of autophagy induced by dexamethasone on senescence in chondrocytes

ENXING XUE^{1,2}, YU ZHANG², BING SONG¹, JUN XIAO¹ and ZHANJUN SHI¹

¹Department of Orthopedic Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515;

²Department of Orthopedic Surgery, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000, P.R. China

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Abstract. The aim of the current study was to explore the effects of dexamethasone (DXM) on autophagy and senescence in chondrocytes. Collagen II and aggrecan were examined in normal chondrocytes isolated from Sprague-Dawley rats. Following stimulation with DXM, LysoTracker Red staining, monodansylcadaverine (MDC) staining, green fluorescent protein-red fluorescent protein-light chain 3 (LC3) and western blotting were used to detect autophagy levels in the chondrocytes. Mechanistic target of rapamycin (mTOR) pathway-associated molecules were investigated by western blotting. Cell senescence was analyzed by senescence-associated (SA)- β -galactosidase (β -gal) staining. A dose-dependent increase in the number of autophagic vacuoles was observed in the DXM-treated chondrocytes, as demonstrated by LysoTracker Red and MDC staining. A dose-dependent increase in autophagosome formation was observed in the DXM-treated chondrocytes. Expression of LC3-II and beclin-1 was increased by DXM, in particular in the cells treated with DXM for 4 days. However, P62 expression was reduced as a result of treatment. SA- β -gal staining indicated that DXM increased cell senescence. Notably, DXM-induced cell senescence was exacerbated by the autophagic inhibitor 3-MA. Autophagy induced by DXM protected chondrocytes from senescence, and it is suggested that the mTOR pathway may be involved in the activation of DXM-induced autophagy.

Introduction

Osteoarthritis (OA) is a common disease, and its incidence rate in people over 25 years old is 14%, with ~400,000 patients diagnosed with OA per year in the Catalonia region of

Spain (1). Clinically, local intra-articular injection of DXM has an anti-inflammatory function, relieving the symptoms of knee OA, such as pain and swelling. However, long-term and repeated injections of DXM can disrupt the metabolic balance in chondrocytes, leading to the catabolism in the cartilage with increase of matrix metalloproteinases (MMPs), and can accelerate cell apoptosis and death. This may result in a reduction in chondrocyte number and further deterioration of articular cartilage in OA. Therefore, a novel method to maintain the survival of chondrocytes under DXM stimulation is required.

Autophagy, a self-protective mechanism, has been suggested to maintain cellular homeostasis by removing protein aggregates and damaged organelles by the fusion of autophagosomes and lysosomes. Previous studies have demonstrated that autophagy is a protective process preventing against chondrocyte apoptosis and cartilage degeneration (2,3). In a mouse model, local injection of rapamycin, an inducer of autophagy, has been demonstrated to impede the progress of arthritis (2,4). It is widely accepted that autophagy in mammalian cells can be adaptively activated by different stresses (5-7), therefore, it was hypothesized that DXM may be able to activate autophagy in chondrocytes.

Previous studies have demonstrated that the ability of joint tissues to restore the articular surface is reduced with age; patients with intra-articular fractures of the knee >50 years have a greater risk of developing OA than younger patients (8,9). Age also increases the risk of post-traumatic OA in patients with anterior cruciate ligament tears (9). With the aging of cartilage, senescent chondrocytes may accumulate in the cartilage, leading to the loss of ability to maintain and restore articular cartilage effectively (10). A previous study additionally observed a clear correlation between increasing age and incidence of OA (11). Aging is the largest risk factor for OA, and cell senescence serves an important role in the occurrence and development of OA. However, the exact mechanism involved in the effect of DXM on chondrocyte senescence remains unclear.

A previous study demonstrated that autophagy is a protective mechanism in human chondrocytes with mitochondrial dysfunction (3). These observations suggest that autophagy serves an important role to protect chondrocytes from oxidative stress, and additionally supported the theory that pharmacological interventions targeting autophagy may

Correspondence to: Professor Zhanjun Shi or Professor Jun Xiao, Department of Orthopedic Surgery, Nanfang Hospital, Southern Medical University, 1838 North Guangzhou Road, Guangzhou, Guangdong 510515, P.R. China
E-mail: nfgk@sohu.com
E-mail: orthopaedxj@163.com

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prevent cartilage degradation (3). The effect of autophagy on chondrocyte senescence remains to be fully elucidated. Therefore, the current study aimed to culture rat chondrocytes treated with DXM, in order to determine the effect of DXM on chondrocyte senescence and autophagy and the association between these two events.

Materials and methods

All procedures involving Sprague-Dawley rats were performed under the approval and guidance of the Animal Care and Use Committee at Southern Medical University (Guangzhou, China).

Rat knee chondrocyte culture. A total of 34 3-month-old Sprague-Dawley male rats, ranging in weight between 300 and 340 g, were obtained from the Laboratory Animal Center of Southern Medical University. These rats were anesthetized by 10% chloral hydrate and sacrificed by cervical dislocation, and the articular cartilage was separated from the femoral condyles and tibial plateaus under a microscope. Cartilage slices were incubated with trypsin (0.5 mg/ml) (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 30 mins at 37°C. Subsequent to removal of trypsin, the cartilage slices were incubated with 0.1% collagenase, type II (Sigma-Aldrich; Merck Millipore) in Dulbecco's modified Eagle's medium (DMEM; Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 5% fetal calf serum (FCS; Life Technologies; Thermo Fisher Scientific, Inc.) for 4 h at 37°C with shaking. The isolated chondrocytes were recovered and plated in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. The chondrocytes were incubated at 37°C in a humidified gas mixture containing 5% CO₂ balanced with air. In the current study, all cells used were second-generation chondrocytes. DXM (Sigma-Aldrich; Merck Millipore) was added to the chondrocytes at various concentrations (0, 0.1, 1, 25 and 50 µg/ml).

3-MA and DXM were added to chondrocytes at various concentrations (0 µg/ml, 10 mmol/l 3-MA, 10 mmol/l 3-MA + 25 µg/ml DXM and 25 µg/ml DXM).

Alcian blue staining. Chondrocytes were fixed with 4% paraformaldehyde for 30 min at 37°C. Chondrocytes were stained with 1% Alcian Blue 8GX (Sigma-Aldrich; Merck Millipore) dissolved in glacial acetic acid for 30 min at room temperature. The sections were counterstained with 0.1% nuclear red dissolved in 5% aluminium sulfate for 20 sec followed by routine dehydration. The stained sections and cells were washed three times with phosphate-buffered saline (PBS) and observed by light microscopy.

LysoTracker Red staining. Chondrocytes (3x10⁵ cells/well) were fixed with various concentrations of DXM for 4 days at 37°C in 24-well plates and rinsed with DMEM three times. LysoTracker Red culture medium (66 mM; 1 ml) was added to each well and then the cells were cultured for 30 min at 37°C. Subsequent to washing with PBS three times, the chondrocytes were observed using an inverted fluorescence microscope.

Monodansylcadaverine(MDC)staining. Monodansylcadaverine is a specific *in vivo* marker for autophagic vacuoles. Chondrocytes

(3x10⁶ cells/well) were fixed at different concentrations of DXM for 4 days at 37°C in 6-well plates and rinsed with PBS three times. Subsequently, chondrocytes were incubated at 37°C in 0.05 mM MDC (Sigma-Aldrich; Merck Millipore) for 30 min. Following incubation, chondrocytes were washed three times with PBS at 37°C and fixed for 30 min in 4% paraformaldehyde. Following fixation, chondrocytes were washed four times with PBS and observed under a fluorescence microscope. To observe the rate of autophagy, stained chondrocytes were tested using flow cytometry.

Western blotting. Chondrocytes incubated with various concentrations of DXM (0, 0.1, 1, 25 and 50 µg/ml) were washed with PBS. Total proteins were isolated, and were mixed with a standard protein solution from the Bicinchoninic Acid (BCA) assay kit to produce the BCA working fluid. Each well was filled with the working fluid (200 µl) for 15 min at room temperature. The protein concentration was determined according to the measured optical density values. According to the protein concentration of each group (sample quantity, 30 µg; total volume, 20 µl), the total amount of protein in each group was calculated. Subsequent to heating with loading buffer (Beyotime Institute of Biotechnology, Haimen, China) and PBS at 100°C for 5 min, protein samples were prepared. Equal quantities (30 µg) of protein from each sample were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following blocking with 5% nonfat milk, the membranes were incubated with the following rabbit anti-rat primary antibodies overnight at 4°C: Polyclonal anti-beclin-1 (1:1,000; Abcam, Cambridge, MA, USA; cat. no. ab55878), mouse monoclonal anti-p62 (1:1,000; Abcam; cat. no. ab56416), rabbit polyclonal anti-LC3 (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse polyclonal anti-β-actin (1:1,000; Beyotime Institute of Biotechnology; cat. no. AA128). Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit; 1:2,000; cat. no. A0208; Beyotime Institute of Biotechnology) for 2 h at 37°C. The bands were detected using ECL plus reagent (Invitrogen; Thermo Fisher Scientific, Inc.) on an enhanced chemiluminescence detection system (PerkinElmer, Inc., Waltham, MA, USA). In addition, the intensity of the bands was quantified using Alpha Ease FC software, version 4.0 (Alpha Innotech Corporation; ProteinSimple, Santa Clara, CA, USA).

β-galactosidase (β-gal) staining. Chondrocytes were treated with 3-MA and DXM at various concentrations (0 µg/ml, 10 mmol/l 3-MA, 10 mmol/l 3-MA + 25 µg/ml DXM and 25 µg/ml DXM) for 24, 48 and 72 h; then, chondrocytes were fixed using a Senescence β-Galactosidase Staining kit for 15 min at room temperature. Chondrocytes were stained with β-gal dye for 12 h and then washed three times. The stained cells were observed using a phase-contrast microscope to determine the percentage of positive cells out of the total chondrocytes under magnification of x200.

Red fluorescent protein (RFP)-green fluorescent protein (GFP)-light chain 3 (LC3) assay. Prior to treatment with glucocorticoids, chondrocytes were transfected with monomeric

(m)RFP-GFP-LC3 when the confluence was 50-70% using RFP-GFP-LC3 adenoviral vectors (HanBio Technology Co., Ltd., Shanghai, China). The multiplicity of infection was 100. The chondrocytes were incubated with the adenovirus in DMEM with no serum for 2 h at 37°C. The transfected chondrocytes were incubated with 10% DMEM supplemented with fetal bovine serum overnight prior to glucocorticoid treatment to eliminate the effect of starvation on an autophagic level. Following treatment with different doses of glucocorticoids for 4 days, autophagosomes and autolysosomes in chondrocytes were observed under a confocal microscope (SP8; Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. Statistical analyses were performed using SPSS statistical software, version 16 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to analyze the differences between groups. Tukey's significance test was used to detect differences between two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Chondrocyte identification. Chondrocytes were stained with toluidine blue. The extracellular matrices of the chondrocytes were stained blue and the nuclei were stained dark blue, which is consistent with typical chondrocyte characteristics (Fig. 1A).

DXM promote chondrocyte autophagy. Chondrocytes were incubated with different concentrations of DXM for 4 days and stained with LysoTracker and MDC to observe autophagic vacuoles. Compared with the control group, only a small number of chondrocytes displayed LysoTracker-positive staining (Fig. 1B). It was observed that the intensity of LysoTracker-positive cells increased markedly with increasing concentrations of DXM (Fig. 1B). The majority of chondrocytes were incubated with 50 $\mu\text{g/ml}$ DXM for 4 days and were positively stained with LysoTracker. Therefore, DXM is able to promote autophagous vesicles in the chondrocytes.

The results of LysoTracker staining were similar to that of MDC staining. In the control group, only certain cells were stained positive for MDC (Fig. 2A) and the intensity of MDC staining in the cells was markedly increased with increasing concentrations of DXM. The MDC-stained chondrocytes were observed by flow cytometry, and it was demonstrated that the cells that were incubated with the different concentrations of DXM had higher incidences of autophagy compared with the control group after 4 days ($P < 0.05$). In the 50 $\mu\text{g/ml}$ group, autophagy occurred with an average incidence of 56.74%.

RFP-GFP-LC3 assay. Autophagy is a recycling process including the maturation of autophagosomes, fusion of autophagosomes and lysosomes, autolysosome formation and degradation. The total process is termed autophagic flux. *In vitro*, autophagic flux can be determined by the transfection of an adenovirus harboring mRFP-GFP-LC3. Subsequent to transfection, the autophagosomes in cells are presented as yellow dots (the combination of red and green fluorescence),

and the autolysosomes are presented as red dots (the extinction of GFP in the acid environment of lysosomes). Following culture for 4 days, the yellow autophagosomes were increased in the cytoplasm of chondrocytes with increasing DXM dose (Fig. 2G), indicating the stimulatory role of DXM on autophagic flux in chondrocytes.

DXM upregulates autophagy-associated proteins. To determine the role of DXM in regulating chondrocyte autophagy, western blotting was conducted in order to observe the expression levels of LC3, beclin-1 and P62. When chondrocytes were incubated with different concentrations of DXM for 2 days, no significant differences in the expression levels of LC3-II/ β -actin in the different groups were observed. However, after 4 days, the expression levels of LC3-II/ β -actin were significantly increased with increasing concentrations of DXM, and after 6 days, the expression levels of LC3-II/ β -actin were reduced at the highest concentration of DXM (Fig. 3). Changes in the expression levels of P62 and beclin-1 were also similar, however, notably, the expression levels of P62 reduced with increasing concentrations of DXM after 2 days.

DXM inhibits the mechanistic target of rapamycin (mTOR) signaling pathway. The mechanism of activating autophagy is predominantly associated with mTOR-dependent and mTOR-independent pathways, with p70S6K and 4EBP1 involved as downstream effectors in mTOR-dependent pathways. In the 1 and 25 $\mu\text{g/ml}$ treatment groups, the expression levels of P-p70S6K and 4EBP1 were reduced when compared with the control group, and the difference was identified to be statistically significant (Fig. 3G), indicating that DXM inhibits autophagy through an mTOR-dependent pathway.

The role of DXM and autophagy in chondrocyte senescence. When the cells become senescent, β -gal-positive staining occurs. The proportion of β -gal-positive chondrocytes significantly increased with time upon treatment with 25 $\mu\text{g/ml}$ glucocorticoid, and was greatest following treatment for 72 h (Fig. 4A-E). However, the proportion of β -gal-positive chondrocytes increased significantly when 3-MA, which can inhibit autophagy, was added in comparison with the DXM group alone after 72 h. Furthermore, 3-MA alone was not able to increase the proportion of β -gal-positive chondrocytes (Fig. 4F).

Discussion

In the current study, it was investigated whether DXM is able to inhibit chondrocyte autophagy through an mTOR-dependent pathway and induce senescence. Senescence activated by DXM was observed to be inhibited by autophagy, and it was identified that chondrocyte senescence increased with the inhibition of autophagy.

DXM is widely used to relieve a variety of symptoms caused by OA, however long-term and repeated treatment often results in complications (12,13). DXM can lead to chondrocyte apoptosis, growth inhibition, and a reduction in biological activity (13,14). Apoptosis may reduce the number of chondrocytes and reduce the extracellular

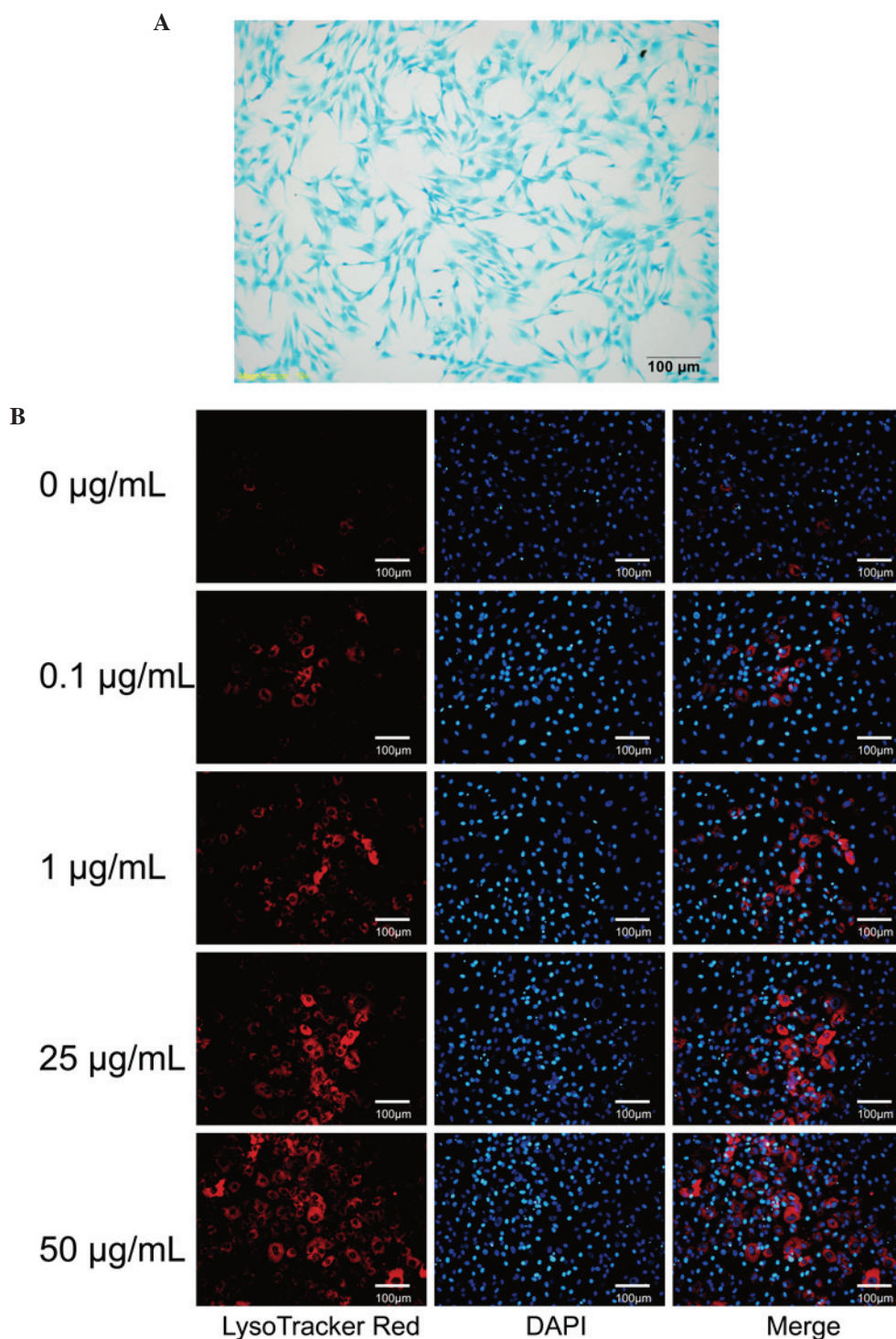


Figure 1. (A) Chondrocytes were stained with alcian blue staining. (B) The LysoTracker Red staining of chondrocytes treated with dexamethasone at different concentrations for 4 days. A dose-dependent increase in the intensity of LysoTracker Red staining in chondrocytes stimulated with dexamethasone was observed. Scale bar, 100 μm .

matrix, including proteoglycan and collagen type II, thus it is suggested that chondrocyte apoptosis is an important factor in the pathogenesis of OA (15). While autophagy is a programmed cell death program similar to apoptosis, the effects of glucocorticoid treatment on chondrocyte autophagy remain to be investigated.

Autophagy is not only similar to apoptosis but also has a protective effect on chondrocytes in OA (2,3,16). Autophagy

has gained research focus due to its potential in regulating the aging process. A previous study has reported that the activity of autophagy was reduced in senescent tissues (17). When cells encounter certain stress situations or bacterial invasion, double membranous vesicles called autophagosomes are formed. These autophagosomes either fuse with endosomes or lysosomes, leading to the formation of autolysosomes. The process begins with the formation of a double membranous

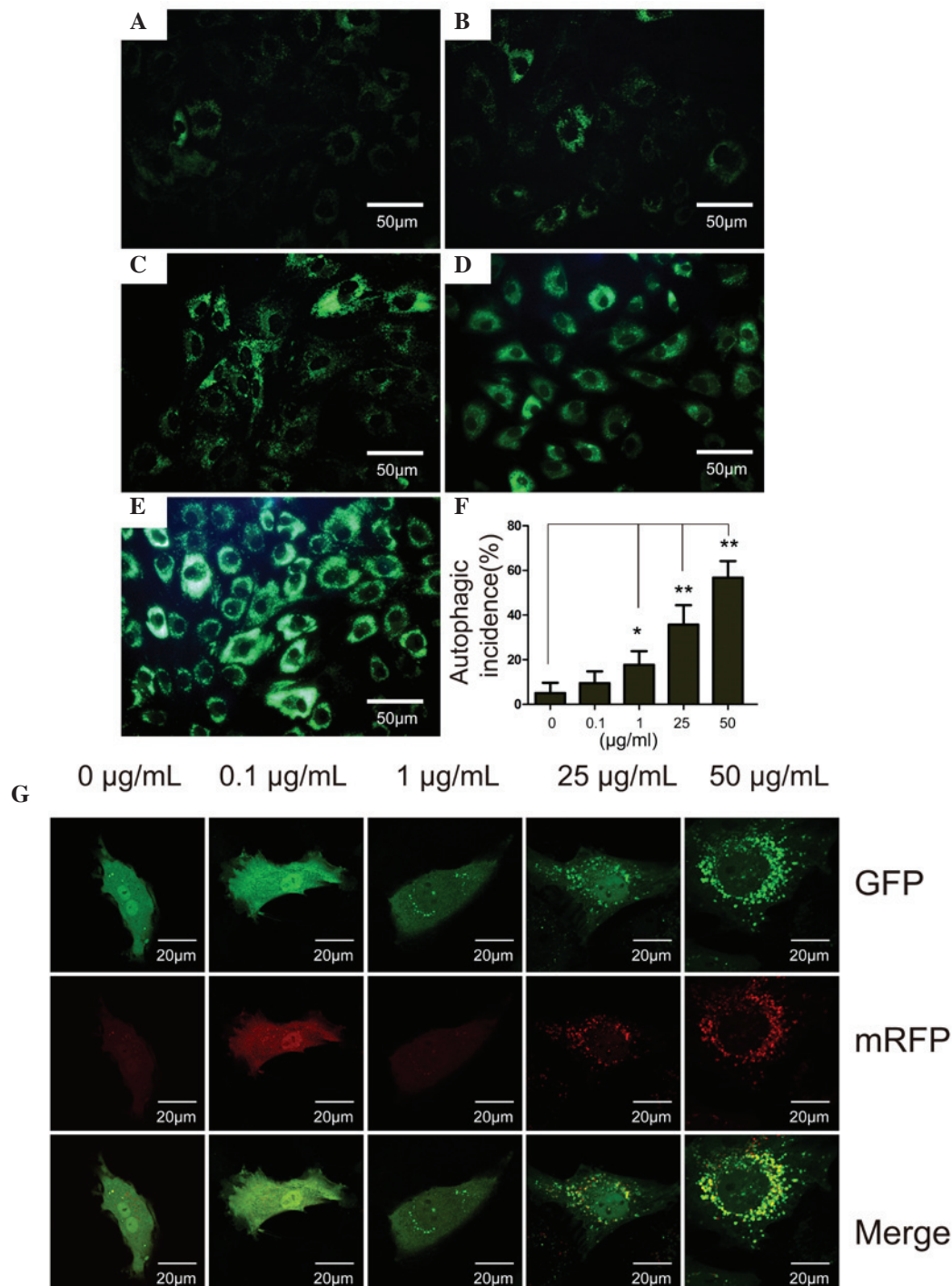


Figure 2. The MDC staining, flow cytometry analysis and RFP-GFP-LC3 assay of chondrocytes treated with DXM. MDC staining of chondrocytes treated with different concentrations of DXM: (A) 0 µg/ml, (B) 0.1 µg/ml, (C) 1 µg/ml, (D) 25 µg/ml and (E) 50 µg/ml. Scale bar, 50 µm. (F) Autophagic incidence analyzed by flow cytometry. Mean ± standard deviation. n=3. *P<0.05 vs. control; **P<0.01 vs. control. (G) Prior to DXM treatment, chondrocytes were transfected with RFP-GFP-LC3 adenoviral vectors. A dose-dependent increase in the number of autophagosomes formed was observed in the DXM-treated chondrocytes. MDC, monodansylcadaverine; GFP, green fluorescent protein; RFP, red fluorescent protein; DXM, dexamethasone; LC3, light chain 3.

phagophore that elongates into an autophagosome, during which cellular material becomes enclosed (18). In this way, autophagy can transport larger organelles or pathogens. *In vitro*, autophagy can inhibit chondrocyte apoptosis and prevent degradation of the extracellular matrix induced by interleukin 1β (19). *In vivo*, it remains unclear whether intraperitoneal injection of rapamycin or local intra-articular injection of rapamycin can promote chondrocyte autophagy

and reduce the progress of degeneration of articular cartilage (2,4). In the current study, it was identified that DXM significantly activates chondrocyte autophagy, thus it was hypothesized that autophagy may be compensatory to the chondrocyte damage induced by DXM.

mTOR is one of the components of mTOR complex 1 (mTORC1) and serves as a key switch in regulating autophagy, thus the autophagic signal pathway can be divided into

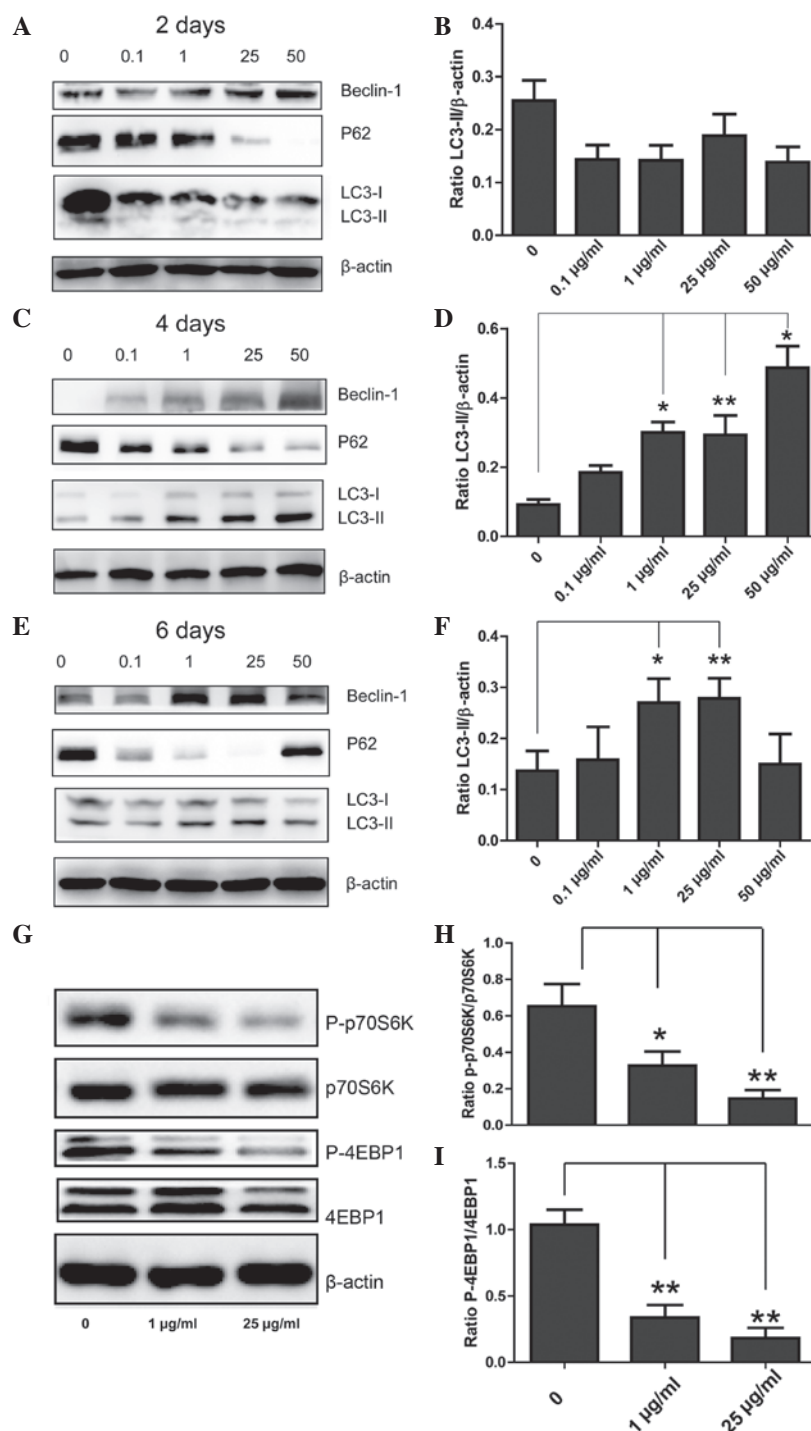


Figure 3. The expression of autophagy-associated protein and mechanistic target of rapamycin pathway-associated proteins in chondrocytes treated with dexamethasone. (A, C and E) The expression of beclin-1, P62 and LC3 in chondrocyte analyzed by western blotting. (B, D and F) The corresponding optical densities of LC3-II/β-actin were analyzed. (G) The expression of P-p70S6 K and P-4EBP1 in chondrocytes analyzed by western blotting. (H and I) The corresponding optical densities of P-p70S6K/p70S6K and P-4EBP1/4EBP1 were analyzed. Data are presented as the mean ± standard deviation; n=3; *P<0.05 vs. control; **P<0.01 vs. control. LC3, light chain 3; P-, phosphorylated.

mTOR-dependent and mTOR-independent pathways (20). Commonly, the stimulation of autophagy begins with the inhibition of mTOR, and the most important of two downstream targets of mTORC1 are p70S6K and 4EBP1. It was identified that DXM can reduce p70S6K and 4EBP1 phosphorylation and activity, and thus, DXM activates autophagy in chondrocytes through an mTOR-dependent pathway. Similarly, glucosamine, which is the current treatment for

OA, also activates autophagy through an mTOR-dependent pathway (21). These results demonstrate that the predominant signal pathway of autophagy activation in chondrocytes is mTOR-dependent.

DXM can reduce cell growth and inhibit cell activity. Poulsen *et al* (22) identified that DXM can reduce tendon cell growth and induce senescence. However, whether DXM can induce chondrocyte senescence remains to be determined.

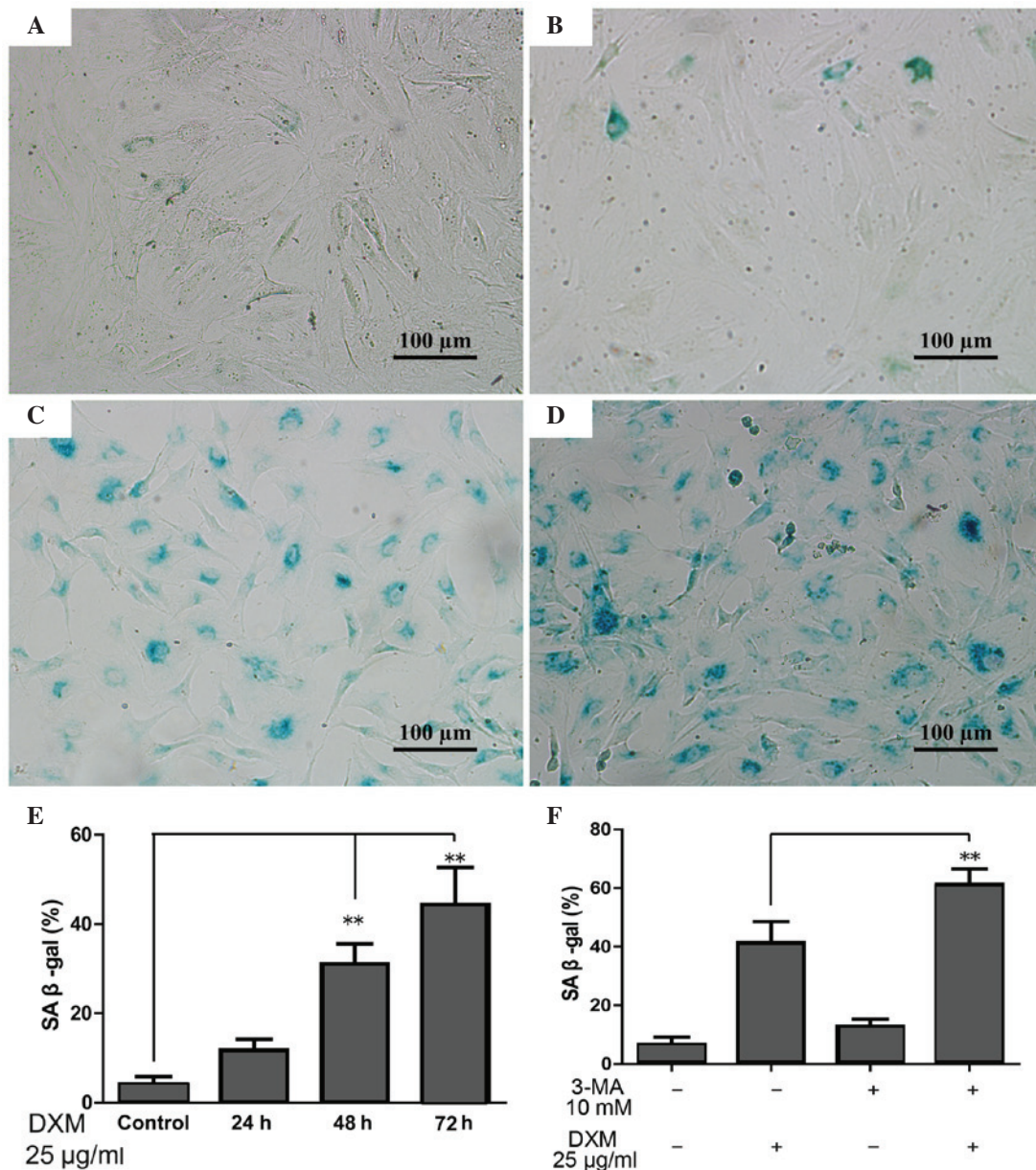


Figure 4. β -gal staining of the chondrocytes treated with DXM and/or 3-MA. (A-D) β -gal staining of the chondrocytes treated with 25 μ g/ml for 25, 48 and 72 h. (E) The quantitative analysis of the incidence of the cells with positive β -gal staining. $^{**}P<0.01$ vs. control. (F) The chondrocytes treated with DXM and/or 3-MA. The incidence of positive β -gal staining was analyzed. $^{**}P<0.01$ vs. cells treated with DXM alone. Data are presented as the mean \pm standard deviation; n=3. β -galactosidase, β -gal; DXM, dexamethasone; 3-MA, 3-methyladenine; SA, senescence-associated.

When cell-cycle progression is arrested, the cell can be in a static state or become senescent. When the cell senesces, cell proliferation stops, however its volume loss continues to increase, resulting in aging cells being larger than normal cells (23). In the current study, it was identified that DXM can promote chondrocyte senescence and that this effect is more marked as time progresses. Chondrocyte senescence has been considered to be a correlative factor for OA, thus it is suggested that the long-term use of glucocorticoid-induced senescence may be an explanation for chondrocytes undergoing degeneration and necrosis.

The association between autophagy and senescence remains controversial. Kamalakannan *et al* (24) identified that autophagy can prevent mononuclear cell senescence caused by heat shock protein, however it induces bronchial

epithelial cells to undergo senescence caused by oxidative stress (25). For chondrocytes, senescence was activated when 3-MA was used to inhibit autophagy, which indicates that glucocorticoid-induced autophagy may be a compensatory protective effect against senescence.

In conclusion, the current study identified that DXM can inhibit chondrocyte autophagy through an mTOR-dependent pathway and induce senescence. Autophagy may therefore serve as a protective process against senescence.

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