Int J Clin Exp Pathol 2015;8(12):15599-15610
www.ijcep.com /ISSN:1936-2625/IJCEP0017801

Original Article

IGFBP-3 may trigger osteoarthritis by inducing apoptosis of chondrocytes through Nur77 translocation

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Received October 13, 2015; Accepted November 25, 2015; Epub December 1, 2015; Published December 15, 2015

Abstract: Osteoarthritis is not an uncommon disease worldwide and it is characterized by chondrocytes apoptosis in articular cartilages. Previous researches had discovered that insulin-like growth factor binding protein-3 (IGFBP-3) was abundant inside the osteoarthritic cartilages and the more IGFBP-3, the worse of osteoarthritis. However, there is still little knowledge of the association between the onset of osteoarthritis and the yield of IGFBP-3 in cartilages. In consideration of the apoptotic effect of IGFBP-3 on other types of cells, we had hypothesized that IGFBP-3 may induce the chondrocytes apoptosis, which was highly considered as the origin of the osteoarthritis. Exposing the cultured chondrocytes to exogenous recombinant IGFBP-3, we were able to observed the apoptotic chondrocytes under microscope and figured out an increased proportion ($P<0.05$) of them by both CCK-8 assay and flow cytometry. Under laser confocal microscope, we also found that the apoptosis of chondrocytes induced by IGFBP-3 were committed to the nucleus-mitochondria translocation of Nur77, which is nuclear protein, and this phenomena was similar as the one described in malignant cells only. In conclusion, our work suggested that IGFBP-3 may trigger osteoarthritis by inducing the chondrocytes apoptotic through nucleus-mitochondria translocation of Nur77.

Keywords: Insulin-like growth factor binding protein-3, IGFBP-3, chondrocytes, Nur77, nucleus-mitochondria translocation

Introduction

Osteoarthritis (OA) is a common joint disease, which is characterized by cartilage loss, osteophyte formation, subchondral sclerosis and periarticular weakness. It not only damages knees, hips and interphalangeal joints mainly, but also causes pain and stiff in those joints. Chondrocyte apoptosis and extracellular matrix degradation, as known as cartilage degeneration, are both believed as the onset of OA. As the only cell inside the joint cartilage, chondrocyte maintain the balance of anabolism and catabolism of cartilage. Based on the up-to-date researches, there is a correlation between the chondrocyte apoptosis and the onset of OA [1]: (1) a much more ratio of apoptotic chondrocytes could be observed in the OA cartilage rather than the normal one; (2) a positive correlation could be calculated between the remaining alive chondrocytes and severity of OA. Now, a consensus is achieved that multiple factors together damage joint cartilage then causally induce both chondrocyte apoptosis and extracellular matrix degeneration [2]. Insulin-like growth factor binding protein-3 (IGFBP-3), especially its IGF independent effect, has been provoked as a novel way to study the onset of OA [3].

IGFBP-3 is one of the insulin-like growth factor binding proteins family. Insulin like growth factor 1 (IGF-1), the other half of the IGF/IGFBPs system, is the most important protein in this system. The free IGF-1, which is activated by conjugating to IGF-1 receptor (IGF-1R), is not only able to stimulate cell proliferation by activating MAPK and PI3K pathway, but also able to prevent cell apoptosis through blocking the Caspase pathway with PI3K activation [4]. However, most of IGF-1 is deactivated because of the conjugation of IGFBP-3. As the most protein of IGFBPs family and the major carrier of the IGF, IGFBP-3 can be secreted by various

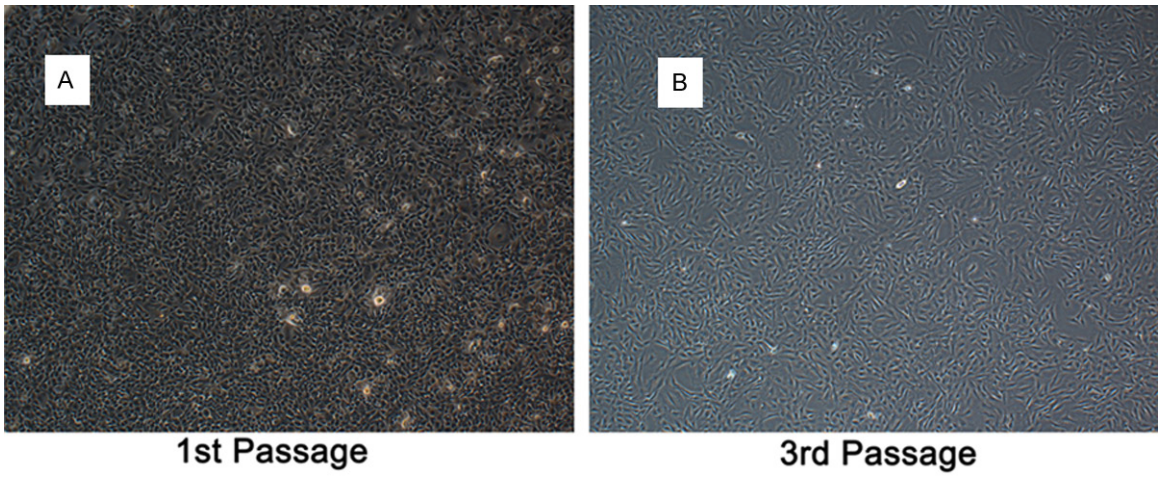

Figure 1. The morphological observation of the cultured chondrocytes under bright field microscope. The 1st generation chondrocytes (A) were irregular polygon shape and were stone road like when they got confluent in the flask. When they underwent 2 times or more passage (B), the polygon chondrocytes transformed into fibroblast-like shape.

types of cell, prolonging the half-time of IGF. IGFBP-3 possesses a higher conjugation to IGF-1 than that of IGF-1R. It means that IGFBP-3 can competitively conjugate free IGF-1 with IGF-1R and block IGF-1's cell proliferation effect by deactivating MAPK and PI3K pathway, both of which can be initiated by combination of IGF-1 and IGF-1R (16, 17). This is known as the IGF-dependent effect of IGFBP-3.

IGFBP-3 had come into the horizon of OA researchers. Tavera et al had reported that [5], in primary OA joint cartilages, despite there was more IGF-1R than those in the normal cartilages, chondrocytes from the OA joints did not respond to the stimulus of IGF-1 correlatively. Meantime, those researchers also found an elevated express of IGFBPs in OA cartilage, including IGFBP-2, IGFBP-3, IGFBP-4, especially the middle one elevated significantly. Factors which could reduce the proteolysis of IGFBP-3 were observed by Whellams in the OA synovial fluid [6] and he thought maybe there were a relationship between those factors he and OA. Beside the latent relationship of IGFBP-3 and OA based on the tissue, it is possible that they may be linked genetically: a study suggested that a low IGFBP-3 level in serum had a positive correlation with a low prevalence of OA [7]. When combining the studies above, some researchers had supposed that [9] overexpress IGFBP-3 in cartilage could induce chondrocyte apoptosis through its IGF-dependent effect, and then the chondrocyte proliferation and its proteoglycan secretion could also be interfered, and causally extracellular matrix may be degraded. Those factors may be the causes of the OA onset.

When the studies of OA and IGFBP-3 went further, more researchers found that IGFBP-3 could induce cell apoptosis by exerting its IGF-independent effect [10, 11], which is RXRα-Nur77 dependent [12, 13]. RXRα is a member of RXRs family (the other two are RXRβ and γ), which is nuclei receptor and exerts their biological effect by homodimerizing or heterodimerizing other nuclei receptors [26]. Nur77 which is also known as TR3 or nerve growth factor-induced clone B (NGFI-B) is an orphan receptor and combines to RXRα [26]. IGFBP-3 possesses nucleus location sequence (NLS) and RXRα binding domain, transporting into cell nucleus and combining RXRα-Nur77 heterodimer. Then Nur77 is induced into cytoplasm from nuclear and co-locates to mitochondria [13], initiating intrinsic Caspase pathway: (1) Nur77 enhances the permeability of mitochondrial membrane; (2) cytochrome c leaks into cytoplasm from mitochondrial matrix [13] and compound into Apaf-1 by heterodimerizing with dATP; (3) Apaf-1 cleaves pro-Caspase-9 into Caspase-9 and the activated Caspase-9 then cleaves pro-Caspase-3, which is the precursor of Caspase-3 the activated Caspase-3 induces apoptotic effect.

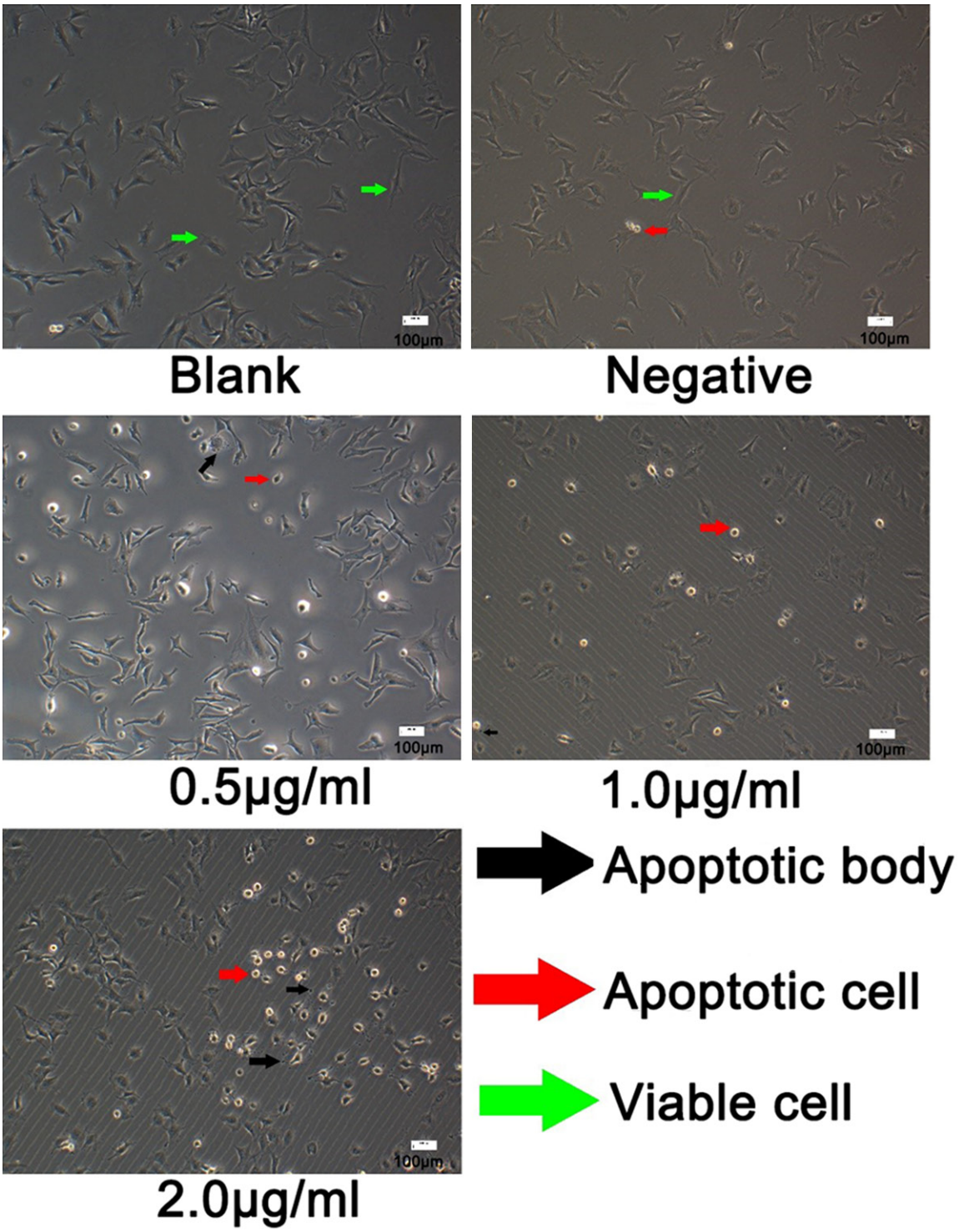

Figure 2. The morphological analysis of the chondrocytes in different groups. The viable chondrocytes of blank and negative groups were just the same as the ones cultured in complete culture medium (**Figure 1**), which were polygon shape or fibroblast-like and indicated by green arrows. They could be induced to apoptosis when exposed to IGFBP-3 (0.5 μg/ml~2.0 μg/ml), because the shrinking morphologic change of cells (red arrow) and the apoptotic bodies (black arrow) were both captured by us. The proportion of apoptotic chondrocytes correlated with the concentration of IGFBP-3. (the scale bar was 100 μm).

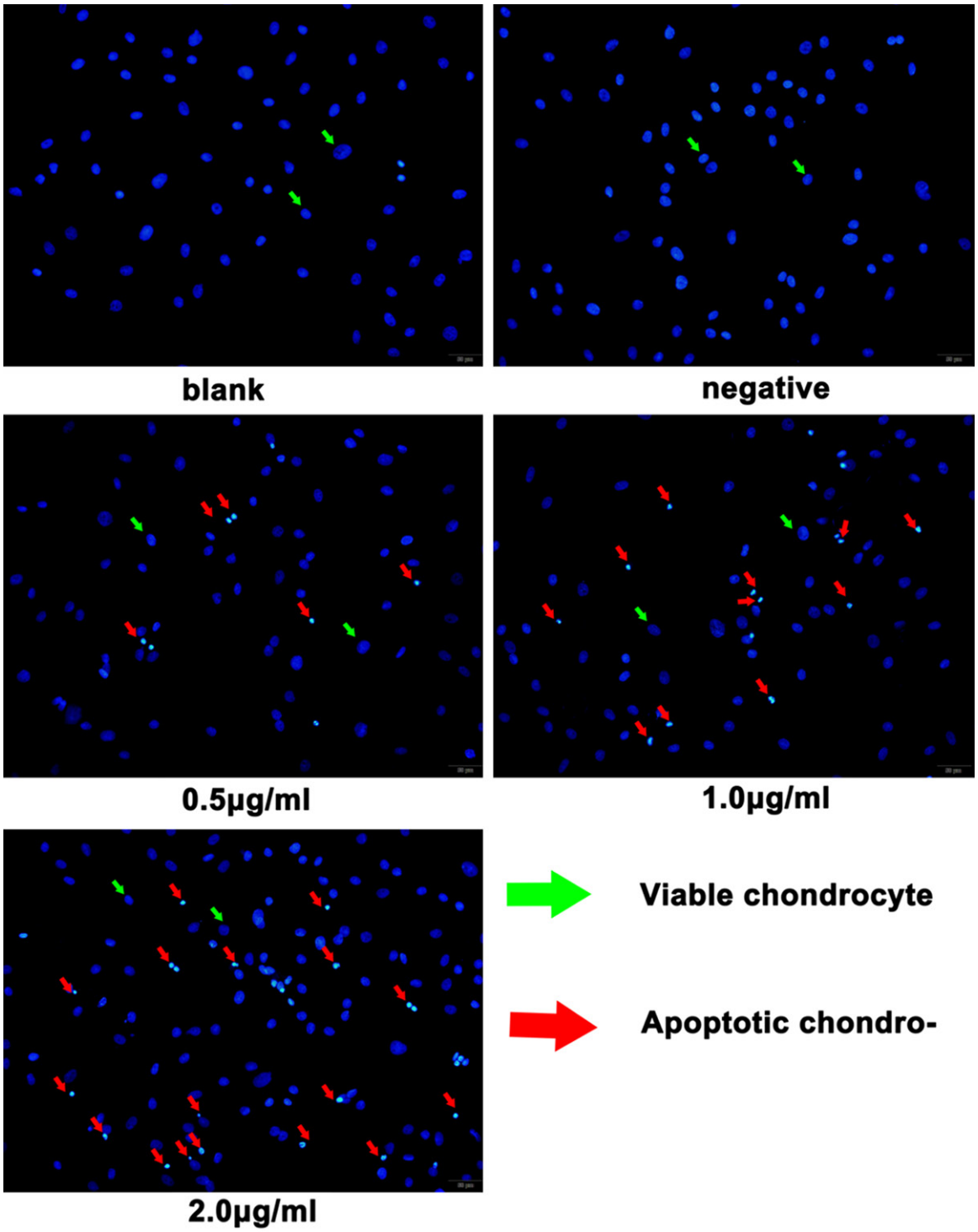

Figure 3. Hoechst 33342 stain of the nuclei of chondrocytes. The green arrows indicated the nuclei of the viable chondrocytes, which were round or stain in homogeneous blue color under fluorescence microscope, while the red arrows showed the apoptotic chondrocytes whose nuclei were shrinking and stained in high intensity fluorescence. The apoptotic cells in blank and negative groups were little. Nevertheless, when exposed to IGFBP-3 (0.5 μg/ml~2.0 μg/ml), the viable cells could be induced into apoptotic and this percentage was positive associated with the concentration of IGFBP-3. (The scale bar was set as 50 μm).

Table 1. Absorbance of viable cells exposed to exogenous IGFBP-3 (mean ± SD, n=3)

Groups	Values of absorbance
Blank	1.43±0.03Δ
Negative	1.44±0.05Δ
0.5 μg/ml	1.31±0.03**,#
1.0 μg/ml	1.06±0.07**,#
2.0 μg/ml	0.82±0.10**,#

**between IGFBP-3 subgroups, P<0.05; #between IGFBP-3 subgroups and blank group or negative, P<0.05; Δbetween blank and negative groups (P>0.05).

The onset of OA is tight link with the elevated permeability of mitochondrial membrane, which had been suggested by previous researches [14]. After reviewing the results announced by the researchers above, which were overexpress IGFBP-3 level inside the OA cartilage or synovial fluid as well as the announcement that IGFBP-3 could induce cell apoptosis by elevating the permeability of mitochondrial membrane with IGF-1 independent effect, we have speculated that the chondrocyte apoptosis observed in the OA cartilage may be exerted by IGFBP-3. Up to date, no similar articles were published.

Materials and methods

Animals

Sprague-Dawley rats certificated and provided by animal experiment center of Wuhan University, class SPF.

Harvest and primary culture of chondrocytes

Rats were executed by cervical dislocation, and were disinfected by 75% alcohol. The cartilage of knee and elbow in both sides were obtained in sterile conditions. We carefully scraped the soft tissue on the cartilage and fragmented the cartilage into 1 mm^3 size. These fragments were rinsed by sterile PBS solution in tubes and centrifuged for 5 min at 1000 rpm. Trypsin with EDTA was mixed with residue after removing the supernatants, and then a digestion was performed by shaker for 80 rpm at 60 min, 37°C. When this program was done, we rinsed this mixture with sterile PBS solution and afterwards centrifuged again for removing the supernatant. Next, we added 0.2% type II collagenase solution and shake under 80 rpm at 4 hrs, 37°C. When the shake done, we centrifuged again and discarded the supernatants then mixed DMEM/F-12 medium with the residue as suspension. This suspension was filtered 3 times with sterile stainless net and was centrifuged once more for discarding the DMEM/F-12 medium. At last, we suspended the residue by complete culture medium (with 10% FBS and 1% Penicillin-Streptomycin). The chondrocyte intensity was adjusted as 1×10^6 per milliliter then was seeded into 25 cm^2 flask at 5% CO_2 wet incubator. We changed the complete medium every 24 h at the first three days. The primary cultured chondrocyte was marked as P0.

Passage

When the chondrocyte reached an 80~90% aggregation observed under microscope, the complete medium was pipetted out and the internals of culture flask were rinsed by sterile PBS solution. After PBS rinse, 1 ml trypsin with EDTA was pipetted into flask, and the cultured chondrocytes was observed carefully under microscope. When the adherent polygonal chondrocytes became round, 2 ml complete medium was added into flask for neutralizing trypsin. This chondrocyte suspension was pipetted and transferred into new tubes then was centrifuged for 1000 rpm at 5 min. We discarded the supernatants and suspended the chondrocytes with new complete medium and counted those cells as $1\sim2\times10^5$/ml then transferred into new flasks. The first passage was marked as P1. P1 to P4 was used throughout our works.

IGFBP-3 compound and controls

As direction from manual, IGFBP-3 lipid was dissolved into sterile ddH_2O, compounding an IGFBP-3 solution at 1 mg/ml. This solution was mixed with sterile 0.1% BSA solution for a 20 μg/ml IGFBP-3 stock solution. DMEM/F-12 serum-free medium plus chondrocytes as blank group, DMEM/F-12 plus 0.1% BSA solution as negative group, and IGFBP-3 stock solution mixed with DMEM/F-12 serum-free medium compounding three concentrations, 0.5 μg/ml, 1.0 μg/ml, and 2 μg/ml, as positive group respectively, we had set five groups.

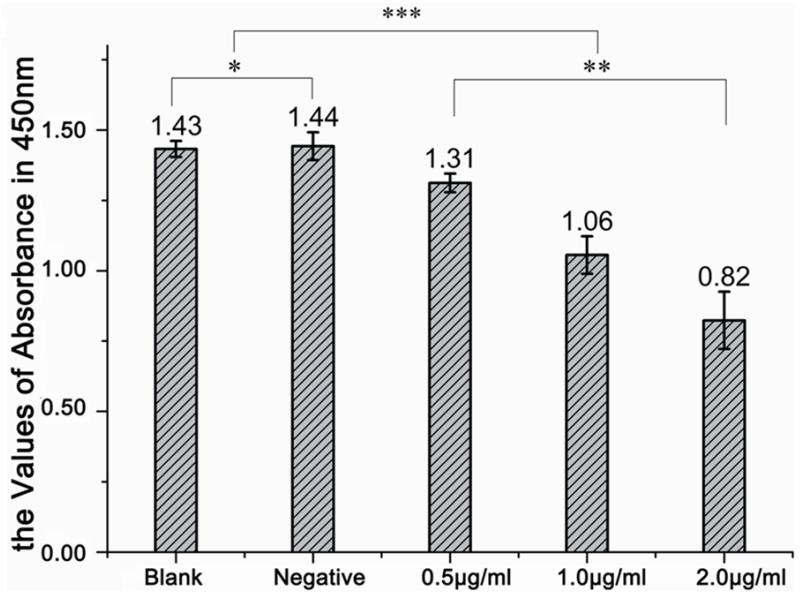

Figure 4. Absorbance values of CCK-8 assays. IGFBP-3 caused the decrease of absorbance value of CCK-8 of chondrocytes: blank vs. negative, P>0.05*; 0.5 μg/ml vs. 1.0 μg/ml vs. 2.0 μg/ml, respectively, P<0.05**; blank or negative vs. 0.5 μg/ml or 1.0 μg/ml or 2.0 μg/ml, P<0.05***.

IGFBP-3 assay

Chondrocytes (1×10^5/well) were seeded in 6 well plate and adhered for 24 hrs. We discarded the complete culture medium and washed the adherent chondrocytes with sterile PBS to eliminate the IGF-1 from the complete culture medium. The blank, negative and positive group was set as previously description. The morphological changes of chondrocytes were examined under bright field of microscope.

Hoechst33342 stain assay

Chondrocytes (1×10^5 cells per well) was seeded into the 6 well plate which contained a sterile coverslip inside each well and adhered for 24 hrs. After this period, we rinsed each well with sterile PBS solution, and set blank, negative and positive groups, incubating another 24 hrs period. Discarding the medium of all groups, fixing the chondrocytes with 4% paraformaldehyde at room temperature for 15 min. Rinsing the fixed cells with PBS solution for 3 times, each time with 5 min. Then 10 μg/ml Hoechst33342 dye was added to stain chondrocytes for 15 min at room temperature. The excess was finally discarded and the coverslips were washed with PBS and were mounted. The morphological changes of chondrocyte nucleus were observed under fluorescence microscope.

CCK-8 cell viability assay

8000 Chondrocytes per well were seeded in the 96 well plate. After those cells adhered 24 hrs later, blank group, negative group and positive groups was set. Each of these groups contained three individual wells, and another 3 wells were set as control group which contained no chondrocyte. After another 24 hrs, the complete culture medium of each well was removed, and 10 μl of CCK-8 was added into each well. The chondrocytes was then incubated at 37°C in dark for 1 h. Finally, we measured the absorbance of each well at 450 nm.

Annexin V-FITC/propidium iodide dual staining assay

Chondrocytes (1×10^5/well) were seed into 6 well plate and adhered for 24 hrs. Rinsed by sterile PBS solution 3 times, we set blank, negative and positive groups and incubated for 24 hrs. The chondrocytes were washed with cold binding buffer of Annexin-V/PI cell apoptosis kit and then cells were collected and suspended by the same binding buffer at a concentration of 1×10^5 cells per mL and incubated with Annexin V-FITC and PI for 15 min in dark on ice. Then these samples were analyzed by BD flow cytometry. 1×10^4 chondrocytes of each sample were analyzed.

Immunofluorescence staining assay

Chondrocytes (2×10^4 cells per well) were seeded on 12 well plate which contained a sterile coverslip in each well. When chondrocytes adhered for 24 hrs, the three groups were set as previous ones. After 24 hrs later, we pipetted out the medium and rinsed the chondrocytes with sterile PBS solution for 3 times. MitoTracker® Deep Red FM stock solution was

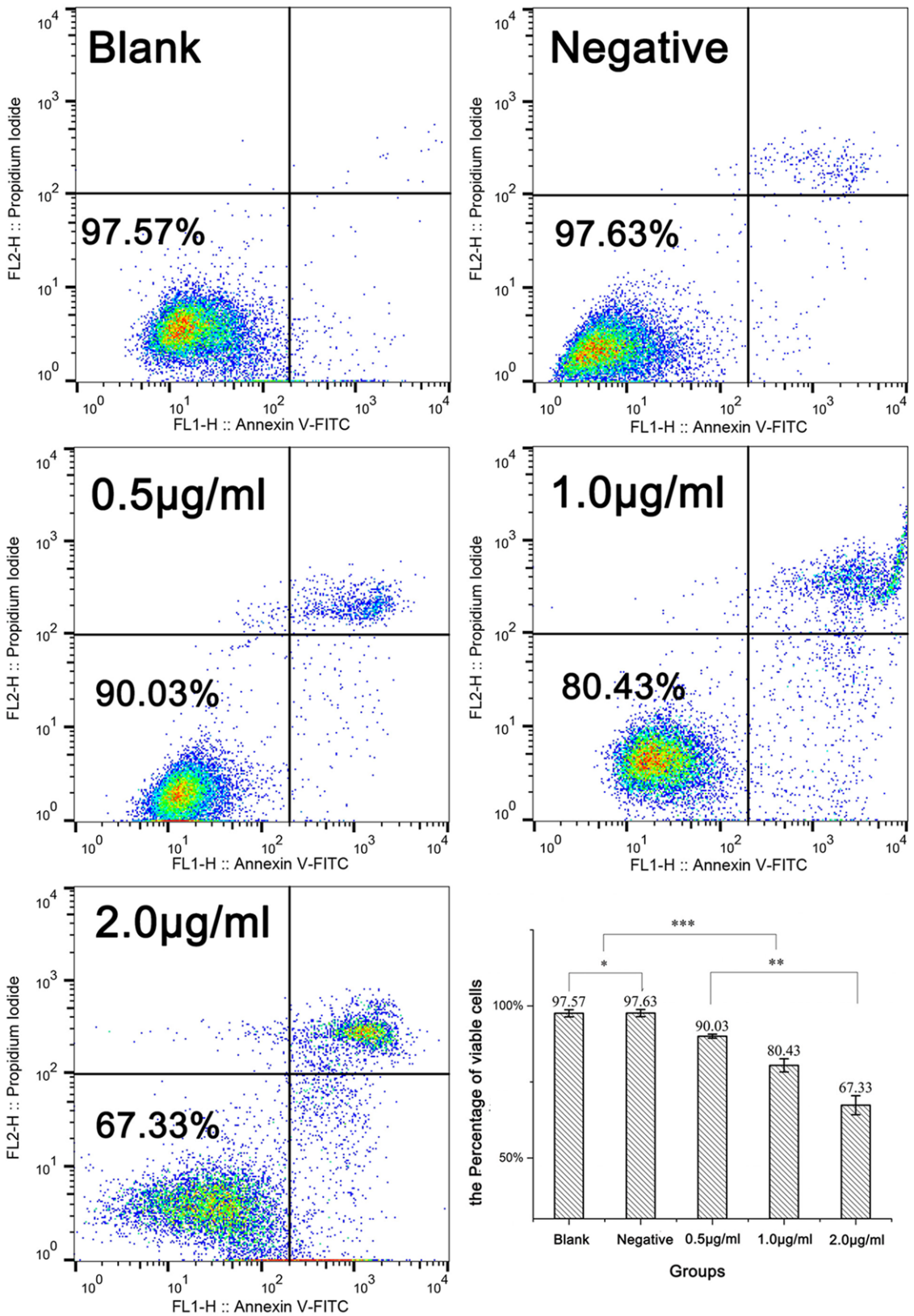

Figure 5. Percentage of viable cells was examined by flow cytometry. IGFBP-3 caused the reduction of this percentage: blank vs. negative, P>0.05*; 0.5 μg/ml vs. 1.0 μg/ml vs. 2.0 μg/ml, respectively, P<0.05**; blank or negative vs. 0.5 μg/ml or 1.0 μg/ml or 2.0 μg/ml, P<0.05***.

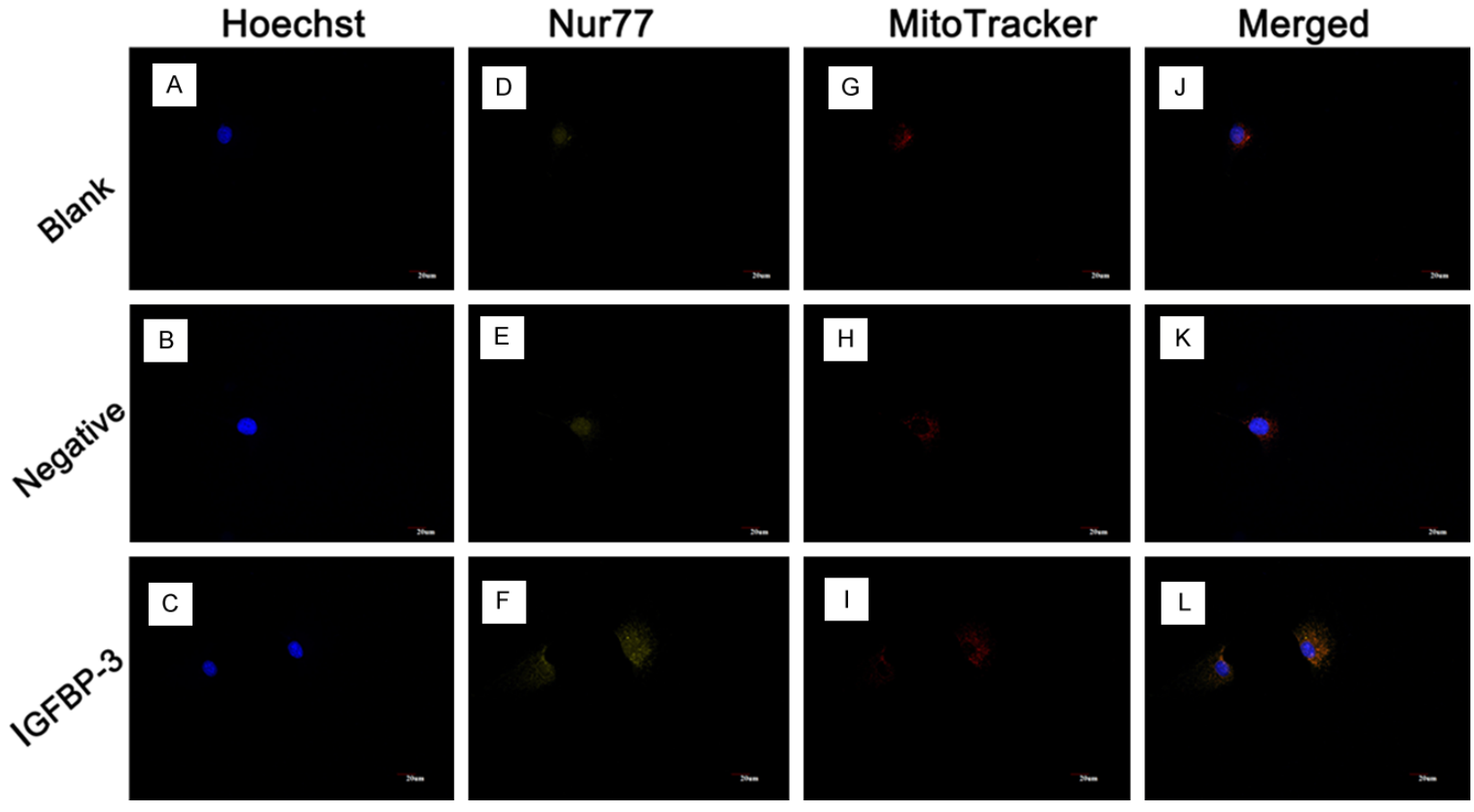

Figure 6. Nucleus-mitochondria translocation of Nur77 induced by IGFBP-3. (A-C) Represented the nuclei of chondrocytes stained by Hoechst 33342. Without IGFBP-3, the nuclear protein, Nur77 was located in the nuclei (D, E), and was not translocated to mitochondria (G-H). IGFBP-3 could caused Nur77 translocated to mitochondria in cytoplasm (C, F, I, L). (The scale bar was set as 20 μm).

compounded with complete culture medium at the concentration of 0.5 μM and was incubated with chondrocytes in dark at room temperature for 45 minutes. Then we discarded them and fixed cells with 4% paraformaldehyde for 15 min. After fixation, the 15 min permeation with 0.5% Tritox-X100 and the 30 min blockage with 2% BSA was performed step by step. Next, Nur77 conjugated primary antibody (rabbit anti-rat antibody, 1:50 dilution) was added to chondrocytes and was incubated in dark at 37°C for 2 h. Then we removed the antibody and washed with PBST for 3 times (5 min each). At last, cells were counter stained with 10 μg/ml Hoechst33342 solutions for 15 min and were mounted by mounting medium. These cells were observed and captured under laser confocal microscope.

Statistical analysis

All the assays were performed at least three times independently. The data were presented as a mean ± SD and were analyzed with ANOVA test with SPSS(ver. 17.0). A *P*-value less than 0.05 was considered statistically significant.

Results

Chondrocytes culture

The shape of primary and the 1st passage chondrocytes were both irregular polygon and adhered in flasks as stone road like (the left of **Figure 1**). After several passages (2~4 generations), their appearances extended and transform into fibroblast-like shape (the right of **Figure 1**).

Morphological analysis

The morphological changes of chondrocytes exposed with IGFBP-3 were shown as **Figure 2**. The chondrocytes in blank group and negative group remained fibroblast-like and the proportion of apoptotic cells were rare. The apoptotic chondrocytes, which were indicated by shrinkage of cytoplasm and cell volume as well as apoptotic bodies, were observed in the all subgroups of positive one (0.5 μg/ml~2.0 μg/ml) and the percentage of them were dose-dependent with the concentration.

Hoechst33342 staining analysis

As shown on **Figure 3**, the viable chondrocytes nucleus (green arrow) in blank group and negative group were round were stained equally, while the apoptotic ones (red arrow) in positive group were shrinking and stained with high intensive blue fluorescence, indicating apoptotic cells. Additionally, the proportion of chondrocytes was correlated with the concentration of exogenous IGFBP-3.

CCK-8 cell viability analysis

The chondrocytes viability was shown as **Table 1** and **Figure 4**. No statistical differences ($P>0.05$) between blank group (1.43±0.03) or negative group (1.44±0.05), while the subgroups of positive (1.31±0.03, 1.06±0.07, 0.82±0.10) got statistical differences with each other ($P<0.05$) and with the blank one or the negative one respectively ($P<0.05$). The absorbance values were negatively correlated with the concentration of exogenous IGFBP-3.

Flow cytometry analysis

The apoptotic chondrocytes measured by flow cytometry were shown as **Figure 5**. The viable chondrocytes proportion in blank group and negative group were similar, which was 97.57±1.17% and 97.63±1.18% respectively, and no significant difference ($P>0.05$) was observed. The significant statistical differences ($P<0.05$) were not only observed among subgroups of positive one, which was 90.03±0.65%, 80.03±2.18% and 67.33±3.13% respectively, but also between each subgroups and blank or negative one. The proportion of viable cells was dose-dependent decreased with the concentration of exogenous IGFBP-3.

Immunofluorescence analysis

As observed under laser confocal microscope (**Figure 6**), in the blank group and the negative group, Nur77 signal (yellow) co-located with Hoechst33342 signal (blue) and separated with mitochondrial signal (red), indicating that Nur77 expressed in chondrocyte nucleus only when unexposed to IGFBP-3. However, in positive group, Nur77 signal in nucleus decreased while the counter one increased in cytoplasm, and it co-located with the mitochondrial signal. These suggested the Nur77 translocated from nucleus to mitochondria, when exposed to IGFBP-3.

Discussion

Despite these study groups reviewed by this article all announced the result that IGFBP-3 exerted cell apoptosis by IGF-1 independent effect, those announcements were all based on malignant cell [10-13]. Yet, some other articles reported IGFBP-3 may inhibit apoptosis in normal cell rather than stimulated that [15, 16]. Differ from the malignant cell, which is a rapidly mitotic cell, chondrocyte is a quiescent cell type [17], and it possesses a lower mitochondrial mass than other types of normal cell and malignant cell [18]. So, it is necessary to restudy whether IGFBP-3 could exert chondrocyte apoptosis as well as the mechanism.

Only after an autocrine or a paracrine release from cell and retake, can IGFBP-3 act on cell [19]: IGFBP-3 firstly synthesized inside cell and afterwards autocrine or paracrine release into extracellular matrix, and next it integrates with transferrin-transferrin-receptor, which is located onto cell membrane, to form a trimer, then IGFBP-3 is internalized into cell again by meditation of transferrin-transferrin-receptor [20]. Alternately, it can be retaken into cell through endocytosis meditated by caveolae [20]. When transmitted into the cell, IGFBP-3 integrates with importin through nuclear localization sequence (NLS), and importin will meditate it into cell nucleus [19]. Maybe the mechanism which IGFBP-3 is absorbed by cell varies dependent on cell types, but Daniela et al had reported IGFBP-3 could combine onto the chondrocyte membrane [21]. After referring the above works, we believed the steps of IGFBP-3 transportation into cell from extracellular matrix may be simulated by adding exogenous IGFBP-3.

Exogenous IGFBP-3 protein was purchased from Perprotech Inc. (catalog number: 100-08), which is classified as recombinant human cytokines and formed by 264 amino acids. We had ran a BLAST program on the website UniProt (http://www.uniprot.org/), comparing this exogenous IGFBP-3 and the counterpart of rat. We found our exogenous IGFBP-3 is homogeneous high enough with the one in rat that the differences just located in signal sequence because this recombinant human IGFBP-3 removed signal sequence (1MHPARPALWAAALTALTLLRGPPVARA27). We believed it is reasonable to replace the original IGFBP-3 protein generated in rat chondrocyte by exogenous recombinant human IGFBP-3.

The IGF-1 independent effect of IGFBP-3 is plausibly explained by two existent theories: (1) IGFBP-3 integrates the death receptors (TGF-βRV, LRP-1 and Stat1) directly, activating Caspase-8 then the Caspase-3 pathway, also known as the exogenous apoptosis pathway and inducing cell apoptosis [6]; (2) IGFBP-3 internalized by cell, it induces a nucleus-to-mitochondria translocation of Nur77 and this translocated protein exerts an escalated permeability of mitochondria membrane, leading a leak of cytochrome c from mitochondrial matrix to cytoplasm and causally inducing cell apoptosis by activating Caspase-9/Caspase-3 pathway, which is called endogenous apoptosis pathway [22]. The second one preferred by most researchers [12, 13, 23], however, they transfected the recombinant IGFBP-3 plasmids and overexpressed this protein into various types of cell, instead of simulating the program which IGFBP-3 autocrine or paracrine released to extracellular matrix and was retook by cell. Therefore, those works did not analyze whether IGFBP-3 could activate exogenous apoptosis pathway technically. Therefore, after referring this article [21], our work concluded that exogenous IGFBP-3 may induce a nucleus-to-mitochondria translocation of Nur77 and exerted chondrocyte apoptosis probably through activated endogenous Caspase pathway which was initiated by increased permeability of mitochondria membrane.

IGF-1 is worldwide in FBS which is one of the components of complete medium. It can bond to IGFBP-3 rather than IGF-1R because the former one has a higher affinity so that the cell proliferation meditated by MAPK and PI3K/AKT pathways both of which are activated by conjugation of IGF-1 and IGF-1R is inhibited [4]. This is called the IGF-1 dependent effect of IGFBP-3. It will interfere our results because both IGF-1 dependent and independent effect induce cell apoptosis. To neutralize the interference, an IGFBP-3 working solution in concentration gradient was compounded by IGFBP-3 stock solution and serum-free DMEM/F-12 then we applied the working solution to cultured chondrocyte. Although mass chondrocyte apoptosis was not detected respectively in the blank group (DMEM/F-12 only) and negative group

(0.1% BSA & DMEM/F12) because of the lack of FBS which contained lots of IGF-1, this results may be explained by the phenomenon that IGF-1 could be autocrine or paracrine secretion from cultured chondrocyte [24]. Concerning this phenomenon, chondrocyte apoptosis induced by IGF-1 dependent effect of IGFBP-3 was not yet neutralized and this proportion was not able to exactly detect or analyze, despite the result did suggest chondrocyte apoptosis was induced by IGF-1 independent effect which initiated Nur77 translocated from nucleus to mitochondria and increased the permeability of mitochondrial membrane and finally activated the endogenous Caspase pathways.

This flaw could be removed in further studies by biosynthesizing the IGFBP-3 protein which lack the ability to integrate with free IGF-1. Zhang et al had offered the needed method [25]: Leu106, Leu109, Leu110, Gly245 and Gln251 on rat IGFBP-3 respectively mutated into Gln106, Gln109, Gln110, Ser245 and Ala251, a new recombinant IGFBP-3 which was not able to integrate IGF-1 could be made.

Our works showed that exogenous IGFBP-3 could be imported into chondrocyte and exerted IGF-1 independent effect, meditating a translocation of Nur77 from nucleus to mitochondria and causally inducing chondrocyte apoptosis. This result accorded with previous study [14], which had suggested that correlations between the onset of OA and an increased chondrocyte mitochondria membrane permeability. Based on our works and the previous ones, which had reported an overexpression of IGFBP-3 in OA joint cartilage, IGFBP-3 may be one of factor that triggers OA.

Acknowledgements

This work was funded by National Natural Science Foundation of China (NSFC), and the project number was 81171760, which could be checked on this website (http://isisn.nsfc.gov.cn/egrantindex/funcindex/prjsearch-list).

Disclosure of conflict of interest

None.

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