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Osteopontin accelerates chondrocyte proliferation in osteoarthritis rats through the NF-κb signaling pathway

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Abstract. – OBJECTIVE: To explore the influence of osteopontin (OPN) on the chondrocyte proliferation in osteoarthritis (OA) rats.

MATERIALS AND METHODS: A total of 30 Sprague-Dawley rats were divided in the control group (n=10), model group (n=10), and OPN knockdown group (n=10). No treatment was performed in the control group, while OA rats were administrated with control adenovirus in the model group and OPN knockdown adenovirus in the OPN knockdown group. After sampling, the degree of OA was evaluated *via* hematoxylin-eosin (HE) staining, and the mRNA expression of OPN was detected. Moreover, the expression of the proliferation-associated protein cyclin D1 was detected using immunohistochemistry. The chondrocytes were isolated from the normal rats, cultured, and transfected with OPN overexpression vector or si-OPN. Methyl thiazolyl tetrazolium (MTT) assay was adopted to determine the proliferative capacity of chondrocytes, and Caspase3 activity was measured to evaluate the changes in the apoptotic capacity of chondrocytes. Meanwhile, Western blotting was performed to verify the influences of OPN on the pathways on chondrocyte proliferation.

RESULTS: After the OA model was established, the expression level of OPN significantly increased. According to HE staining results, OPN knockdown effectively inhibited the onset of OA. Compared with that in the control group, the expression level of cyclin D1 in the model group was raised. However, upregulated cyclin D1 in OA rats was repressed in OPN knockdown group. OPN overexpression promoted the proliferation of chondrocytes, but suppressed their apoptosis, while OPN knockdown had the opposite effects. Besides, OPN overexpression upregulated nuclear factor-κB (NF-κB), and NF-κB knockdown eliminated the regulatory effects of OPN on proliferation and apoptosis of chondrocytes.

CONCLUSIONS: OPN promotes the expression of NF-κB signals to accelerate chondrocyte proliferation, thereby inducing OA in rats.

Key Words:
Osteopontin, NF-κB, Chondrocyte, Osteoarthritis.

Introduction

Osteoarthritis (OA) is regarded as one of the most common chronic arthropathies and is characterized by degradation of articular cartilage, subchondral bone thickening, and synovitis[1-3]. OA mainly affects the middle-aged and elderly, with the morbidity rate up to 89% in the elderly older than 70 years. Chondrocytes, present in various cartilage tissues, are responsible for tissue growth and maintenance. Excessive proliferation of human chondrocytes may promote the pathological process of OA[4]. Osteopontin (OPN), a 44-75 kD multifunctional phosphoprotein, is related to the pathogenesis of OA. OPN regulates the expressions of a wide variety of OA-associated factors, including matrix metalloproteinase-13, ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs), TIMP-8, and interleukin-6/8/9[5-8]. However, researches on the regulation of the physiological function of chondrocytes by OPN now are lacked. Therefore, this paper aims to explore the role of OPN in the onset of OA *via* the *in vivo* experiments in OA rats administrated with OPN knockdown adenovirus. In addition, the influence of OPN on proliferation and apoptosis of chondrocytes were further analyzed.

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Materials and Methods

Experimental Reagents and Instruments

Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China), papain (Doing-Higher), TRIzol (Invitrogen, Carlsbad, CA, USA), fluorescence quantitative polymerase chain reaction (qPCR) instrument (Bio-Rad CFX96, Hercules, CA, USA), hematoxylin-eosin (HE) staining kit (Beyotime Biotechnology, Shanghai, China), cyclin D1 antibody (CST, Danvers, MA, USA), immunohistochemistry kit, optical microscope, and fetal bovine serum (FBS) (Sijiqing, Hangzhou, China), LipoFiter (HanBio, Shanghai, China), methyl thiazolyl tetrazolium (MTT) assay kit (Beyotime Biotechnology, Shanghai, China), Caspase3 activity assay kit (Nanjing, Kaigen, Nanjing, China), and OPN antibody and nuclear factor-κB (NF-κB) antibody (Abcam, Cambridge, MA, USA).

Establishment of OA Model

Thirty Sprague Dawley (SD) rats (Shanghai Model Organisms Center, Inc., Shanghai, China) were randomly assigned into three groups, namely control group, model group, and OPN knockdown group. The rats in the control group received no treatment, and those in the model group were intra-articularly administrated with 0.2 mL 4% papain per rat once a day for 2 consecutive weeks. The rats in the OPN knockdown group were pre-injected with OPN knockdown adenovirus through the tail vein. This study was approved by the Animal Ethics Committee of Shandong University Animal Center.

Determination of Expression Level of OPN Via qPCR

After being ultrasonicated and homogenized, the tissues were added with 1 mL of TRIzol to extract total ribonucleic acid (RNA). 1 μg of the total RNA was reversely transcribed in the following steps: 37°C for 20 min, 98°C for 5 min, and standing at 4°C. The products were harvested and prepared into 10 μL of reaction system, with the reaction conditions as follows: pre-denaturation at 98°C for 10 min, denaturation at 95°C for 5 s, and annealing and extension at 60°C for 1 min for 35 cycles. The experimental results were calculated based on $2^{-\Delta\Delta Ct}$. The following primers were used: glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Forward: 5'-ACGGCAAGTTCAACGGCACAG-3', Reverse: 5'-GAAGACGCCAGTAGACTCCACGAC-3' and OPN: Forward: 5'-GTGGGAAGGACAGTTATGAA-3', Reverse: 5'-CTGACTTTGGAAAGTTCCTG-3'.

Immunohistochemistry

The tissues were embedded and sliced into 4 μm-thick sections, and then they were baked at 60°C, deparaffinized, and dehydrated in gradient alcohol, followed by antigen retrieval. After being rinsed, the tissue sections were added dropwise with endogenous peroxidase blocker for 10-min incubation, blocked using 5% bovine serum albumin (BSA), and incubated with cyclin D1 antibody (1:200) at 4°C overnight. On the next day, with the primary antibody discarded, the resulting sections were rinsed, reacted with peroxidase blocker for 10 min, and added dropwise with DAB for color development. Finally, the nuclei were stained with hematoxylin, and the sections were sealed and observed.

Hematoxylin-Eosin (HE) Staining

After being baked, the sections were deparaffinized, dehydrated in gradient alcohol, stained with hematoxylin for 10 min, hydrated, and washed using clean water. The sections were then stained with eosin for several seconds, decolored in gradient alcohol, and transparentized in xylene. Finally, the sections were sealed in neutral resin and observed.

Extraction and Transfection of Chondrocytes

A total of 10 normal newborn SD rats were obtained, and their cartilage tissue samples were sliced into pieces with the thickness less than 1 mm. Then, they were digested using 0.15% collagenase II (Invitrogen, Carlsbad, CA, USA) at 37°C for 5-6 h, while the mixture was stirred once every 20 min. After centrifugation, the chondrocytes were isolated and cultured in the DMEM-F12 containing 10% FBS and antibiotics for 5-7 d. Subsequently, the chondrocytes were grouped as follows: overexpression vector pcDNA-OPN group (OV-OPN group), OPN knockdown group [small interfering (si) OPN group], and NF-κB knockdown (siNF-κB group). The negative controls (siNCs) (Promega, Madison, WI, USA) were set. Finally, OA chondrocytes were transfected following the instructions of LipoFiter (Hanbio, Shanghai, China).

MTT Assay

Cell viability was determined *via* MTT assay. After the corresponding treatment, the cells were cultured with 10 μL of MTT (5 mg/mL) separately for 2-4 h until purple deposits were observed. With the medium removed, 5 μL of dimethyl sulfoxide was applied in each well, and the cells were incubated at room temperature in the dark for 2 h. Finally, the absorbance at 490 nm was recorded.

Western Blotting

An equal amount of protein was separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Burlington, MA, USA). After blockage in 5% skim milk in Tris-Buffered Saline with Tween 20 [20 mL of tris (pH=7.6), 150 mL of NaCl and 0.1% Tween 20] for 3 h, the membranes were incubated with anti-OPN antibody and anti-NF-κB antibody (Abcam, Cambridge, MA, USA) at room temperature for 1 h. Then, the membranes were incubated at 4°C overnight and with the HRP-conjugated secondary antibodies for another 1 h, with actin as an internal reference.

Caspase3 Activity Determination

At 48 h after transfection, Caspase3 activity was determined using the commercial kit (Nanjing Kaigen, Nanjing, China). The cells were first lysed in lysis buffer on ice for 20 min. After centrifugation, the supernatant was reacted with Caspase substrates in the reaction buffer at 37°C for 4 h. Finally, the absorbance of the cells was read at a wavelength of 405 nm, and the relative activity of Caspase3 was calculated based on the ratio of A_{405} in the OPN knockdown group to that in the control group.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD) and analyzed using Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as mean ± SD. The differences between the two groups were analyzed by the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

OPN Expression and Pathological Degree in OA Rats

According to the qPCR results of RNAs extracted from rat tissues, the expression level of OPN was upregulated in the model group and substantially decreased in the OPN knockdown group compared with that in the control group (Figure 1A). HE staining revealed that compared with the control group, the rats in the model group exhibited cartilage damage, while the modeling-induced damage was alleviated in OPN knockdown group (Figure 1B).

Expression of Cyclin D1 Detected Through Immunohistochemistry

According to the immunohistochemistry results (Figure 2), cyclin D1-positive cells were stained brown, indicating their strong proliferative capacity. In comparison with those in the control group, the chondrocytes in the model group had a stronger proliferative capacity, while those in the OPN knockdown group had a weaker proliferative capacity, showing statistically significant differences ($p<0.05$).

Identification Isolated Chondrocytes in Rats

At 24 h after adherence, the isolated chondrocytes were observed to be flagstone-shaped in morphology and linked closely (Figure 3).

Verification of Transfection Efficiency

24 h after transfection of chondrocytes with OPN plasmids, the transfection efficacy was determined. It was found that OPN was overexpressed successfully by transfection of pcDNA-OPN (Figure 4A). Moreover, at 48 h after transfection of si-OPN, the knockdown efficiency was verified (Figure 4B).

Influence of OPN on Chondrocyte Proliferation

According to MTT results (Figure 5), compared with that in the control group, the proliferative capacity of chondrocytes was enhanced by overexpressing OPN ($p<0.01$), but was weakened *via* knocking down OPN ($p<0.01$).

Impact of OPN on Chondrocyte Apoptosis

It was found that the overexpression of OPN weakened the activity of Caspase3 in chondro-

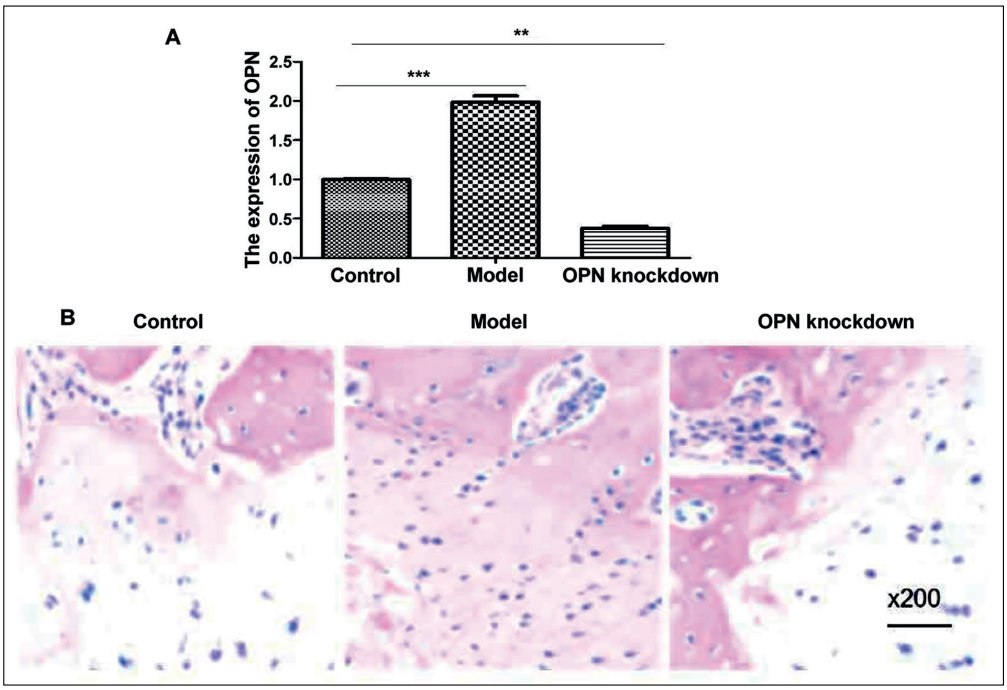

Figure 1. A, OPN expression detected *via* qPCR. **B,** Morphological changes in cartilage tissues identified through HE staining (×200). ***p*<0.05 *vs.* control group, ****p*<0.05 *vs.* control group.

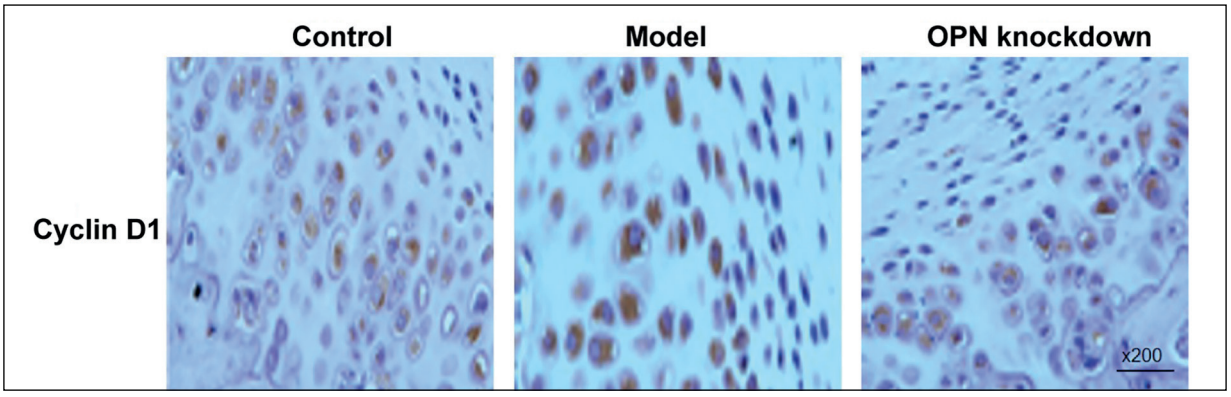

Figure 2. Expression of cyclin D1 detected *via* immunohistochemistry (×200).

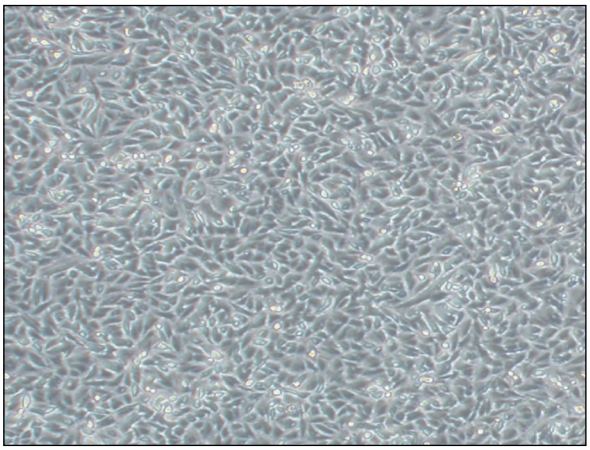

Figure 3. Morphological identification image of rat chondrocytes (×200).

cytes (p<0.01), while the knockdown of OPN enhanced the activity of Caspase3 to promote their apoptosis (Figure 6).

Influence of OPN on NF-κB Signals

Western blotting results showed that OPN overexpression increased the protein expression level of NF-κB, whereas OPN knockdown decreased its protein expression level (Figure 7).

Impact of NF-κB on OPN Overexpression-Promoted Chondrocyte Proliferation

According to MTT results (Figure 8), NF-κB knockdown repressed the cell proliferation accelerated by OPN overexpression.

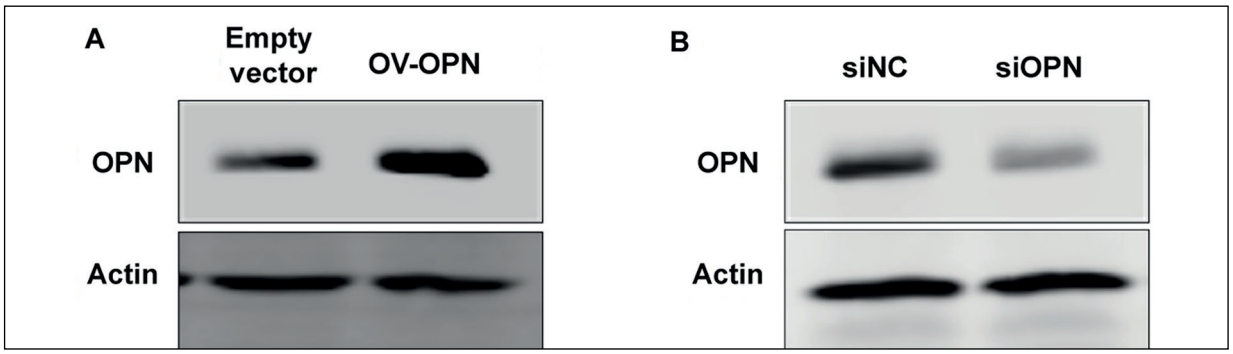

Figure 4. A, Verification of efficiency of OPN overexpression in chondrocytes. **B,** Verification of efficiency of OPN knockdown in chondrocytes.

Influence of NF-κB on OPN Overexpression-Inhibited Chondrocyte Apoptosis

Based on the Caspase3 activity assay results (Figure 9), NF-κB knockdown reversed the OPN overexpression-induced decline in chondrocyte apoptosis.

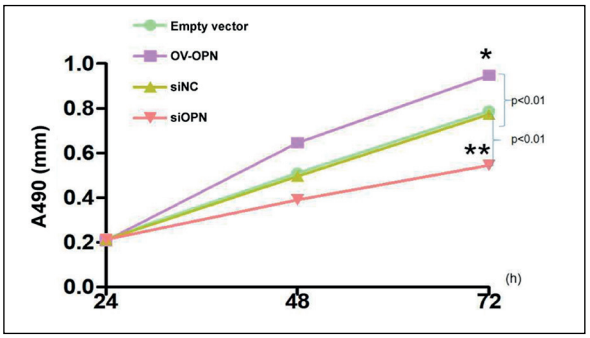

Figure 5. Influences of OPN overexpression and knockdown on chondrocyte proliferation detected *via* MTT assay. *p<0.01 *vs.* empty vector group, **p<0.01 *vs.* siNC group.

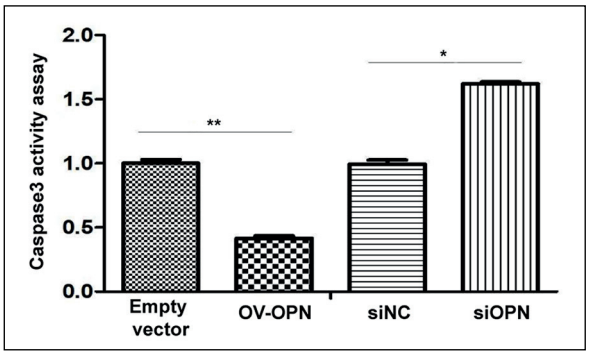

Figure 6. Impacts of OPN overexpression and knockdown on chondrocyte apoptosis examined through Caspase3 activity assay. *p<0.01 *vs.* empty vector group, **p<0.01 *vs.* siNC group.

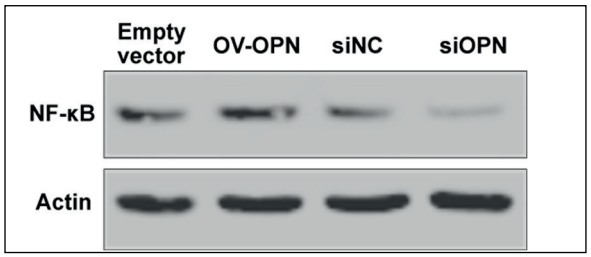

Figure 7. Influences of OPN overexpression and knockdown on NF-κB signals detected *via* Western blotting.

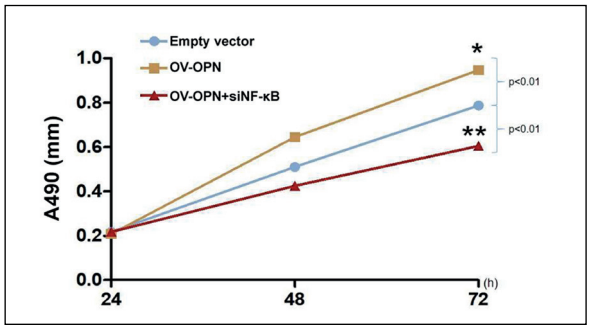

Figure 8. Impact of NF-κB knockdown on chondrocyte proliferation after OPN overexpression detected *via* MTT assay. *p<0.01 *vs.* empty vector group, **p<0.01 *vs.* empty vector group.

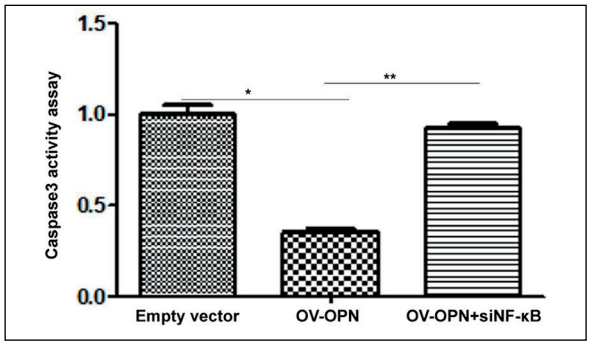

Figure 9. Influence of NF-κB knockdown on chondrocyte apoptosis after OPN overexpression according to Caspase3 activity assay. *p<0.01 *vs.* empty vector group, **p<0.01 *vs.* OV-OPN group.

Discussion

OA results in pathological lesions in particular cartilages. In addition to the changes in cartilage with aging, cartilage degeneration may occur in inappropriate mechanical stress and low-grade systemic inflammation associated with trauma, obesity, and genetic susceptibility, which is the leading risk factor for the development and progression of OA[9]. Chondrocytes provide frames for the bones in development and regulate the growth of long bones *via* the growth plate. The chondrocytes expressed in articular cartilage maintain tissue stability through the synthesis and degradation of extracellular matrix (ECM). Cell growth, differentiation, death, and ECM remodeling are modulated by the cellular signaling pathway networks to respond to various extracellular stimuli[10,11]. The disorders in these processes may greatly contribute to cartilage-related diseases, including OA.

OPN, known as an early T-cell activation gene-1, is secreted by many types of cells, such as macrophages, lymphocytes, epithelial cells, vascular smooth muscle cells, chondrocytes, and synovial cells[12-13]. Notably, the messenger RNA (mRNA) expression and protein abundances of OPN are correlated with the onset of OA. A study[14] initially found that the mRNA expression level of OPN in human OA cartilage is higher than that in normal cartilage. Besides, OA patients have higher OPN content in the plasma, synovia, and articular cartilage. The above findings suggested that OPN expression is related to progressive joint injury and the severity and progression of OA.

In the present study, the OA model was established in SD rats. OPN was upregulated in OA rats than controls. Rats administrated with OPN knockdown adenovirus showed much-alleviated cartilage lesions. Meanwhile, the proliferative capacity of chondrocytes was enhanced after OA modeling, and OPN knockdown inhibited the modeling-induced enhancement of the proliferative capacity of chondrocytes. Moreover, OPN overexpression promoted the proliferation, but inhibited the apoptosis of primary chondrocytes, whereas OPN knockdown had the opposite consequences.

OPN can activate NF-κB to promote the secretion of ECM in chondrocytes[15]. NF-κB is able to activate the genes that maintain cell proliferation, which is composed of two subunits p65 and p50. Silencing NF-κB expression is believed to hinder the proliferation of HeLa cells, while its upregulation is likely to directly cause the oncogene HRAS to induce cell proliferation. However, the expression of NF-κB is upregulated in OA tissues in knee joints due to the action of inhibitors[16,17]. MiR-9 expression is associated with the increased proliferation of OA cells in the knee. MiR-9 has been corroborated to negatively regulate the expression of NF-κB. Besides, NF-κB exerts anti-apoptosis and anti-proliferation effects[18,19]. Therefore, it is speculated in this study that OPN may promote the proliferation and further accelerate the development of OA by facilitating NF-κB signal expression. The results of the present study revealed that the regulatory effects of OPN on proliferation and apoptosis of OA-related chondrocytes were dependent on the activation of NF-κB.

Conclusions

In summary, we first verified that OPN can regulate the NF-κB signaling pathway to affect the proliferation of chondrocytes, thereby alleviating OA, which lays a theoretical foundation for the treatment of OA.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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References

1) Dulay GS, Cooper C, Dennison EM. Knee pain, knee injury, knee osteoarthritis & work. Best Pract Res Clin Rheumatol 2015; 29: 454-461.
2) Mahmoud SAAH, Atoum MF, Al-Hourani HM, Bateineh S, Abderrahman S, Alzoughool F. Vitamin d deficiency and rs731236(taq1) vitamin d receptor gene polymorphism as possible risk factors for rheumatoid arthritis and osteoarthritis. Acta Medica Mediterr 2018; 34: 209-213.
3) Zhang J, Liu Z, Wang H. The effect of basic fibroblast growth factor (BFGF) on repairing intestinal mucosa in acute colitis rat. Acta Medica Mediterr 2019; 35: 1609-1614.
4) Yu SP, Hunter DJ. Managing osteoarthritis. Aust Prescr 2015; 38: 115-119.

5) Giachelli CM, Steitz S. Osteopontin: a versatile regulator of inflammation and biomineralization. Matrix Biol 2000; 19: 615-622.

6) Kahles F, Findeisen HM, Bruemmer D. Osteopontin: a novel regulator at the cross roads of inflammation, obesity and diabetes. Mol Metab 2014; 3: 384-393.

7) Gao SG, Zeng C, Song Y, Tian J, Cheng C, Yang T, Li H, Zhang FJ, Lei GH. Effect of osteopontin on the mRNA expression of ADAMTS4 and ADAMTS5 in chondrocytes from patients with knee osteoarthritis. Exp Ther Med 2015; 9: 1979-1983.

8) Ichikawa H, Imano M, Takeyama Y, Shiozaki H, Ohyanagi H. Involvement of osteopontin as a core protein in cholesterol gallstone formation. J Hepatobiliary Pancreat Surg 2009; 16: 197-203.

9) Poole AR. An introduction to the pathophysiology of osteoarthritis. Front Biosci 1999; 4: D662-D670.

10) Gao SG, Cheng L, Zeng C, Wei LC, Zhang FJ, Tian J, Tu M, Luo W, Lei GH. Usefulness of specific OA biomarkers, thrombin-cleaved osteopontin, in the posterior cruciate ligament OA rabbit model. Osteoarthr Cartilage 2013; 21: 144-150.

11) Laslett LL, Quinn SJ, Winzenberg TM, Sanderson K, Cicuttini F, Jones G. A prospective study of the impact of musculoskeletal pain and radiographic osteoarthritis on health related quality of life in community dwelling older people. BMC Musculoskelet Disord 2012; 13: 168.

12) Standal T, Borset M, Sundan A. Role of osteopontin in adhesion, migration, cell survival and bone remodeling. Exp Oncol 2004; 26: 179-184.

13) Xu M, Zhang L, Zhao L, Gao S, Han R, Su D, Lei G. Phosphorylation of osteopontin in osteoarthritis degenerative cartilage and its effect on matrix metalloprotease 13. Rheumatol Int 2013; 33: 1313-1319.

14) Blagojevic M, Jinks C, Jeffery A, Jordan KP. Risk factors for onset of osteoarthritis of the knee in older adults: a systematic review and meta-analysis. Osteoarthritis Cartilage 2010; 18: 24-33.

15) Brasier AR. The NF-kappaB regulatory network. Cardiovasc Toxicol 2006; 6: 111-130.

16) Benito MJ, Murphy E, Murphy EP, van den Berg WB, FitzGerald O, Bresnihan B. Increased synovial tissue NF-kappa B1 expression at sites adjacent to the cartilage-pannus junction in rheumatoid arthritis. Arthritis Rheum 2004; 50: 1781-1787.

17) Kaltschmidt B, Kaltschmidt C, Hehner SP, Droge W, Schmitz ML. Repression of NF-kappaB impairs HeLa cell proliferation by functional interference with cell cycle checkpoint regulators. Oncogene 1999; 18: 3213-3225.

18) Song J, Kim D, Chun CH, Jin EJ. MicroRNA-9 regulates survival of chondroblasts and cartilage integrity by targeting protogenin. Cell Commun Signal 2013; 11: 66.

19) Wan HY, Guo LM, Liu T, Liu M, Li X, Tang H. Regulation of the transcription factor NF-kappaB1 by microRNA-9 in human gastric adenocarcinoma. Mol Cancer 2010; 9: 16.