European Review for Medical and Pharmacological Sciences 2019; 23: 5051-5058

MiR-9 facilitates cartilage regeneration of osteoarthritis in rabbits through regulating Notch signaling pathway

H.-T. YU[1], C.-Z. GU[2], J.-Q. CHEN[3]

[1]Department of Orthopedics, Baoding No.1 Central Hospital, Baoding, China
[2]Department of Orthopedics, Liaocheng Central Hospital, Liaocheng, China
[3]Department of Orthopedics, The Third Hospital of Hebei Medical University, Shijiazhuang, China

Abstract. – OBJECTIVE: To explore the effect of micro ribonucleic acid (miR-9) on cartilage regeneration of osteoarthritis in rabbits through the regulation of the Notch signaling pathway.

MATERIALS AND METHODS: A total of 30 specific pathogen-free Sprague-Dawley rabbits were randomly divided into control group (healthy rabbits, n=10), model group (osteoarthritis model, n=10) and miR-9 group (osteoarthritis model + miR-9 interference, n=10). The degeneration degree of rabbit knee articular cartilage in three groups was assessed through the Mankin's score. The morphology of cartilage tissues was observed under an optical microscope. Expressions of the Notch1, B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax) proteins, and collagen II (CII) in chondrocytes were detected via the immunohistochemical assay.

RESULTS: In rabbits of control group, the articular cartilage had a smooth surface and complete structure, and the cells were arranged orderly with a clear tidal line. A large number of articular chondrocytes died in model group, while it was improved in miR-9 group. The Mankin's score was 3.52±0.79 points in control group, 6.73±0.95 points in model group, and 5.37±0.61 points in miR-9 group, showing significant differences among the three groups ($p<0.05$). Results of immunohistochemistry showed that the protein expressions of Notch1 and Bax were higher in model group, but lower in control group and miR-9 group ($p<0.05$). The protein expression of Bcl-2 was lower in model group but was upregulated in control group and miR-9 group ($p<0.05$). The results of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) revealed that the expressions of Notch1 and Bax in control group were lower than those in model group and miR-9 group ($p<0.05$), while the expression of Bcl-2 in model group was lower than that in control group and miR-9 group ($p<0.05$). According to the results of immunohistochemistry, the CII optical density (OD) value was 0.18±0.013, 0.25±0.05 and 0.22±0.009 in control group, model group, and miR-9 group, respectively. It could be seen that the CII OD value was the highest in model group, indicating that the CII expression in articular cartilage in osteoarthritis was negatively correlated with the severity of osteoarthritis.

CONCLUSIONS: MiR-9, through the down-regulation of the expressions of Notch and Bax, can activate the Bcl-2 to promote the differentiation and regeneration of chondrocytes. It can facilitate the cartilage regeneration of osteoarthritis in rabbits through the mediation of the CII expression.

Key Words:
MiR-9, Osteoarthritis, Notch signal, Bax, Bcl-2.

Introduction

The morbidity rate of knee osteoarthritis (KOA) in females is 1.3 times than in males, and it constantly rises with aging. According to the data of WHO, more than 60% of global osteoarthritis patients are the elderly over 50 years[1,2]. About 77% of osteoarthritis patients suffer from limited mobility, and disability occurs in 25% of them[3,4]. KOA results from degenerative changes of the knee, senescence, blocked proliferation and differentiation of knee articular chondrocytes, and the sharply declined number of chondrocytes. The factors leading to KOA are related to age, metabolic function, immune function, obesity, genetics, and environment. Articular cartilage is mainly composed of cartilage tissues. The metabolism of articular cartilage mainly depends on the proliferation and differentiation of articular chondrocytes. It is found that articular chondrocytes account for 2-3% of total articular cartilage tis-

Corresponding Author: Jingqing Chen, MD; e-mail: rde303a@163.com

sues, which can secrete many important substances required for articular cartilage such as collagen, phosphorus, and alkaline phosphatase[5-7].

Gu et al[8] have demonstrated that micro ribonucleic acid (miR-9) can promote chondrocyte regeneration and self-renewal in osteoarthritis through the inhibition of the PRTG protein. Some studies have also found that miR-9 is involved in bone metabolism and plays an important role in the development of bone tissues. Notch signaling pathway, as a widely studied signaling pathway, participates in cellular metabolism, embryonic development, and maturation. The process of bone remodeling includes bone formation and bone resorption[9,10]. Bone formation derived from mesenchymal stem cells interacts with bone resorption of osteoclasts. This interaction is significant in bone development and remodeling[11]. The inactivation of the Notch signaling pathway can promote bone development and regulate the differentiation and commitment of stromal stem cells, which has a direct relation with osteocyte reconstruction and regulation of osteogenic tissues[12]. In the present study, the rabbit model of osteoarthritis was established to study the effect of miR-9 on the bone of the right knee. The potential correlation between the Notch signaling pathway and miR-9 was further analyzed to provide a theoretical basis for the biological researches on bone health.

Materials and Methods

Laboratory Animals and Grouping

A total of 30 specific pathogen-free Sprague-Dawley rabbits aged 8 weeks old were purchased from the Hebei Medical University Animal Center. This investigation was approved by the Animal Ethics Committee of Hebei Medical University Animal Center.

Instruments and Reagents

The instruments and reagents used in this experiment were as follows. The goat anti-rabbit antibody was purchased from Abcam (Cambridge, MA, USA), reverse transcription kit from TaKaRa (Otsu, Shiga, Japan), rabbit Notch1 polyclonal antibody, rabbit B-cell lymphoma-2 (Bcl-2) polyclonal antibody and Bcl-2 associated X protein (Bax) polyclonal antibody from Sigma-Aldrich (St. Louis, MO, USA), trypsin and medium from Hyclone (South Logan, UT, USA), hematoxylin-eosin (HE) staining kit from Maxim (Fuzhou, China), ultrasonic imager from PHILIPS (Amsterdam, Netherlands), polyvinylidene difluoride (PVDF) membrane from Millipore (Billerica, MA, USA), Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum (FBS) from Sangon (Shanghai, China), and rabbit immunoglobulin G antibody from DAKO (Glostrup, Denmark).

Establishment of Animal Model and Grouping

A total of 30 rabbits were randomly divided into control group (healthy rabbits, n=10), model group (osteoarthritis model, n=10) and miR-9 group (osteoarthritis model + miR-9 interference, n=10). After fasting for 12 h, the rabbits were anesthetized *via* abdominal injection of anesthetics. The knee joint of the rabbit was forced to bend at right angles. The *villous* around the knee joint was shaved off, and the medial joint was cut to expose the subcutaneous tissues. Surrounding soft tissues were separated to expose the knee joint. Then, the medial and lateral ligaments were cut off, and the wound was sutured. Post-operative anti-inflammation was performed. The operation should be performed carefully and the injury to the articular cartilage surface should be avoided as far as possible. After the operation, the rabbits were fed in separate cages, and they had free access to the activity. The morphology of the knee articular cartilage in each group was observed. The successful preparation of osteoarthritis model was verified through pathological examinations.

Drug Intervention and Observation

At 4 weeks after the operation, different reagents were injected into the knee joint cavity of rabbits on Tuesday and Friday every week. The miR-9 solution was injected in rabbits of miR-9 group, while an equal volume of phosphate-buffered saline (PBS) was injected in control group and model group for 8 consecutive weeks.

Specimen Collection

After the reagents were injected for 8 weeks, the rabbits in each group were executed, and the cartilage tissues of the right knee were taken. The articular cartilage tissues on the weight-bearing surface of the distal femoral medial and lateral condyle were taken using the sharp surgical blade and preserved.

Mankin's Score

The degree of cartilage injury was quantitatively analyzed using the Mankin's score. Pathologi-

cal indexes observed included cartilage structure score, cell count score, cartilage color score, and tidal line score (Table I).

HE Staining

The cartilage tissues of rabbits were fixed in 4% paraformaldehyde overnight, dehydrated, embedded in paraffin and placed for 24 h. The collagen deposition in cartilage tissues was observed using the Masson method, and the cartilage structure of rabbits was detected *via* HE staining. After antigen retrieval, the tissue sections were sealed with serum for 30 min. Sections were incubated with the primary antibody at 4°C overnight and the secondary antibody for 40 min using the two-step method on the next day. Color development was performed using diaminobenzidine (DAB), and the sections were observed under the light microscope to quantify the protein expressions of Notch1, Bax, and Bcl-2 in each group.

Immunohistochemistry

The cartilage tissue sections were deparaffinized, immersed in 3% of methanol-HZOZ for 20 min, washed with PBS once every 10 min for 3 times and sealed with goat serum at room temperature for 0.5 h. Subsequently, the sections were incubated with 100 μg of primary antibody at 35°C overnight, rewarmed at 37°C for 35 min and washed with PBS for 3 times (5 min/time). Sections were then incubated with 100 μg of the secondary antibody and washed again with PBS at room temperature once every 5 min for 3 times. Color development was conducted using DAB, and the staining was observed under the microscope after 2 min. The sections were washed with PBS once every 2 min for 3 times and counterstained with HE for 15 min. Finally, the Notch1, Bcl-2, Bax, and collagen II (CII)-positive cells were observed, and the absorbance of CII expression was calculated.

Detection of Notch1, Bcl-2, and Bax Expression via RT-PCR

The cartilage tissues of rabbits were digested with trypsin, washed with PBS and added with 0.9% of sodium chloride solution to obtain the chondrocytes. 20 mg of chondrocytes were placed in the Eppendorf (EP) tube and lysed in 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to extract RNA. Then, RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the A3500 reverse transcription kit. Expressions of target genes were detected with nucleic acid gel dye using the CFX-96 qRT-PCR instrument. The primers used were designed by the NCBI/Primer (Table II). Reaction conditions were as follows: 98°C for 6 min, and a total of 55 cycles at 8°C for 28 s, 75°C for 30 s, and 80°C for 4 min.

Statistical Analysis

Data were analyzed by the *t*-test in Statistical Product and Service Solutions (SPSS) 15.0 software (IBM, Armonk, NY, USA). Univariate analysis was adopted for the intragroup comparison. All data were expressed as median ±SD. Comparison between groups was done using the One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

Articular Cartilage in Each Group

The calcified layer, radiating layer, migration layer, and the superficial layer of the articular cartilage had a clear boundary and the tidal line was complete. It was observed that the articular cartilage had a smooth surface and complete structure without defects, and the chondrocytes were

Table I. Mankin scoring criteria.

Cartilage structure score		Cell count score		Cartilage color score		Tidal line score	
Criteria	Score	Criteria	Score	Criteria	Score	Criteria	Score
Normal	0	Normal	0	Normal	0	Normal	0
Arranged disorderly but with clear layer	1	Mild hyperplasia	1	Slight fading	1	Multilayer	1
Arranged irregularly and with disordered layer	2	Moderate hyperplasia	2	Moderate fading	2	Blurred	2
Serious disorder	3	Severe hyperplasia	3	Severe fading and no staining	3	Blood vessels pass through	3

Table II. Primer sequences.

Protein	Gene	Primer sequence
Notch1	Forward	5'-GACTCCAAGATGAAGAAGATGTG-3'
	Reverse	5'-GAGCATTCGCAGGTVCAAGCC-3'
Bax	Forward	5'-TCCACCAAGAAGCTGAGCGAG-3'
	Reverse	5'-GTCCAGCCCATGATGGTTCT-3'
Bcl-2	Forward	5'-TTCTTTGAGTTCGGTGGGGTC-3'
	Reverse	5'-TGCATATTTGTTTGGGGCAGG-3'
GAPDH	Forward	5'-CAACGGGAAAGCCATCACCA-3'
	Reverse	5'-ACGCCAGTAGACTCCACGACAT-3'

orderly arranged with a definite boundary, and a clear tidal line in control group. In model group, there was focal hyperplasia on the surface of articular cartilage. Pronounced joint defects, irregularly arranged chondrocyte, and a blurred boundary were observed in model group. In addition, a large number of chondrocytes died, and the tide line disappeared. In miR-9 group, the surface of articular cartilage was rough, and the joint defects were improved compared to those in model group (Figure 1).

Mankin's Score

The Mankin's score was 3.52±0.79 points in control group, 6.73±0.95 points in model group, and 5.37±0.61 points in miR-9 group, showing significant differences among groups ($p<0.05$) (Figure 2).

Comparisons of Protein Expressions of Notch1, Bax, And Bcl-2 in Rabbits

The results of immunohistochemistry showed that the protein expressions of Notch1 and Bax were higher in model group but lower in control group and miR-9 group ($p<0.05$). The protein expression of Bcl-2 was lower in model group, but was significantly higher in control group and

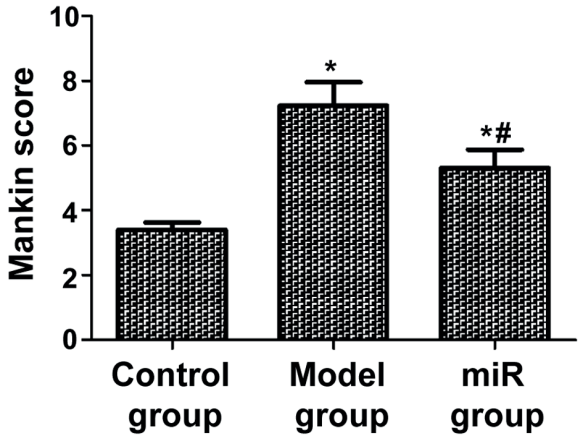

Figure 2. Mankin's score of rabbits in each group. *$p<0.05$ *vs.* control group. #$p<0.05$ *vs.* model group.

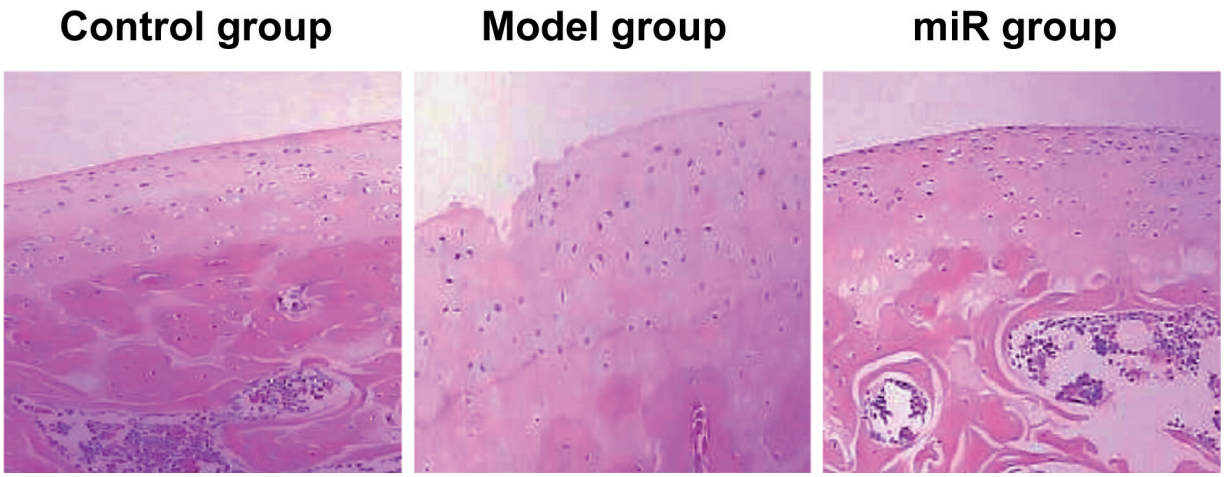

Figure 1. Articular cartilage morphology of rabbits in each group (Magnification 40x).

miR-9 group (p<0.05). It can be seen that the expressions of Notch1 and Bax were inversely proportional to Bcl-2, i.e., the increased expressions of Notch1 and Bax correspond to the decreased expression of Bcl-2 (Figure 3).

Relative Expressions of Notch1, Bax, And Bcl-2 in Each Group of Rabbits

The results of RT-PCR revealed that the expressions of Notch1 and Bax in control group were lower than in model group, and miR-9 group (p<0.05), while the expression of Bcl-2 in model group was lower than in control group and miR-9 group (p<0.05) (Figures 4 and 5).

CII Expression in Rabbits Detected Via Immunohistochemistry

According to the results of immunohistochemistry, the CII OD value was 0.18±0.013, 0.25±0.05, and 0.22±0.009 in control group, model group and miR-9 group, respectively. It could be seen that the CII OD value was the highest in model group, indicating that the CII expression in articular cartilage in osteoarthritis was negatively correlated with the severity of osteoarthritis.

Discussion

Hyperplasia at the joint margin, functional degeneration, and injury of articular cartilage are

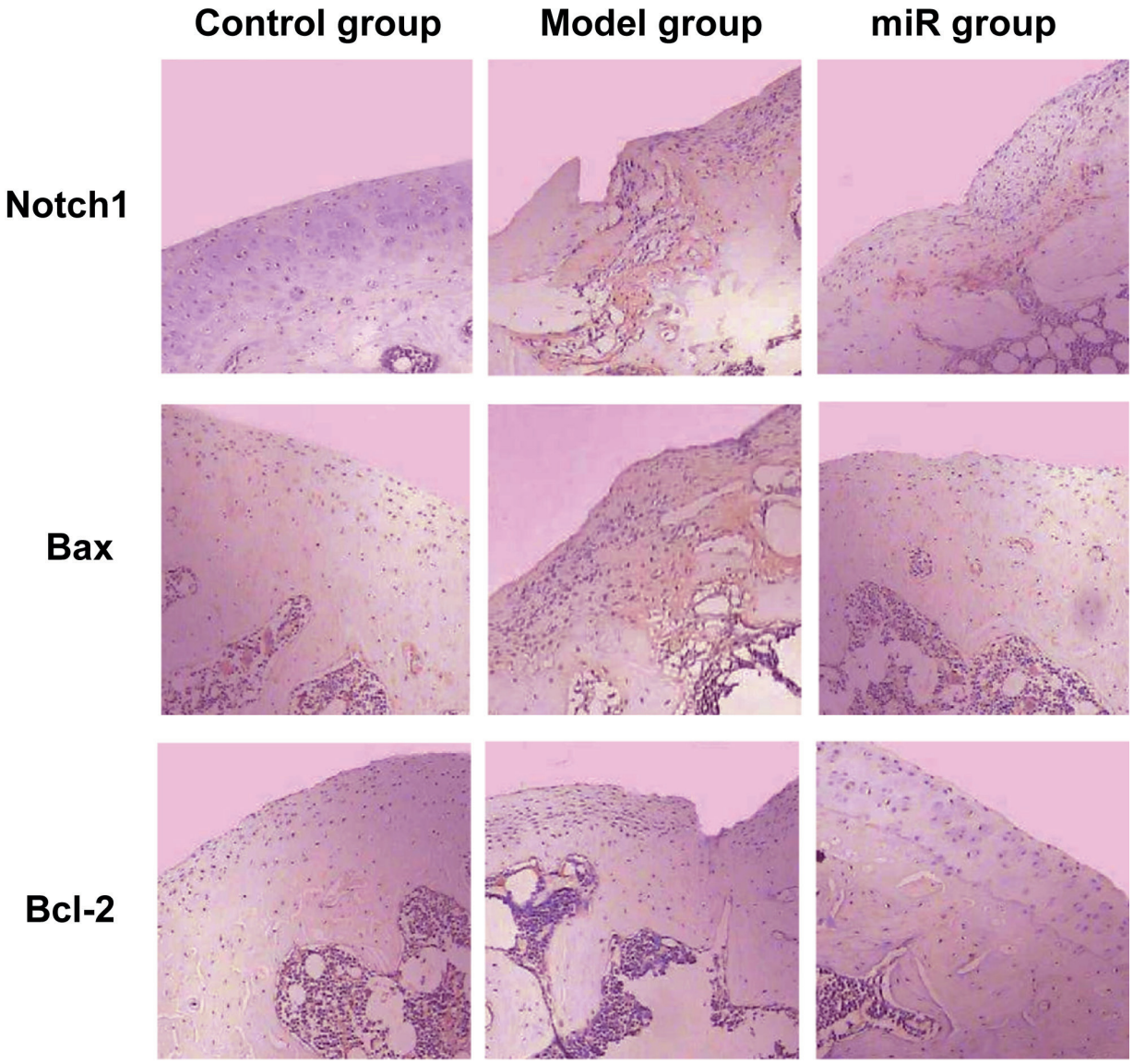

Figure 3. Protein expressions of Notch1, Bax, and Bcl-2 detected *via* immunohistochemistry (Magnification 20x).

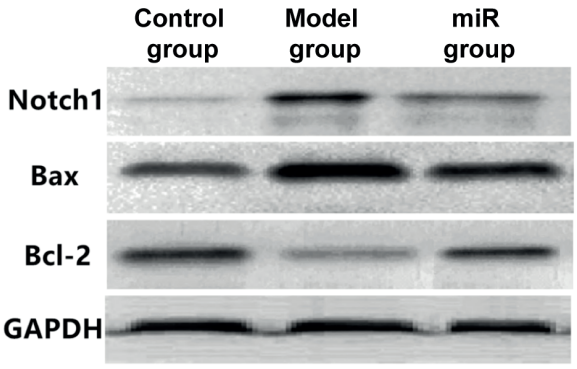

Figure 4. Expressions of Notch1, Bax, and Bcl-2 in rabbits detected *via* RT-PCR.

generally considered to be the pathogenic factors of osteoarthritis[13]. The morbidity rate of osteoarthritis is positively correlated with ageing[14]. The pathogenesis of osteoarthritis is a complex process involving multiple signaling pathways, genes, cytokines, cartilage health, slow metabolism, stress disorder, and effect of enzymes on cartilage matrix[15,16]. In this paper, the articular cartilage had a smooth surface and complete structure, and the chondrocytes were arranged orderly with a clear tidal line in control group. In model group, pathological morphology of articular cartilages was much more pronounced. In miR-9 group, the surface of articular cartilage was rough, and the joint defects were improved compared to those in model group. Xiang et al[17] have revealed that chondrocytes are the only cell type in mature cartilage tissues, which contribute to maintain the stability of the environment in cartilage. In the present study, the Mankin's score was much higher in model group compared to that of control group and miR-9 group. Moreover, studies have found that miR-9, through the down-regulation of the expression of PRTG, may inhibit the chondrocyte apoptosis in osteoarthritis, relieve the symptoms of osteoarthritis, and exert a protective effect in osteoarthritis[18]. Our study obtained consistent results that miR-9 could promote the maturation and development of chondrocytes.

The findings of immunohistochemistry showed that the protein expressions of Notch1 and Bax were higher in model group than in control group and miR group ($p<0.05$). The protein expression of Bcl-2 achieved the opposite trend as those of Notch1 and Bax. Subsequent qRT-PCR further validated these results. It can be seen that expression changes of Bax and Notch1 were similar, while Bcl-2 was the opposite. The Notch signaling pathway plays an important role in bone development and self-renewal[19], and it is also involved in the formation and growth of cartilage. Inactivation of the Notch signal can effectively promote the formation of bone and cartilage, facilitate the early development of bone tissues and repair of bone defects, and control the occurrence of some bone diseases[20,21]. Wang et al[22] showed that silence of the Notch signal can downregulate Bax and upregulate Bcl-2, thereby inhibiting chondrocyte apoptosis and exerting a protective effect on osteoarthritis. Among the apoptosis-related genes, Bcl-2 and Bax have the most prominent features, and they are negatively correlated with each other. Besides, Wang et al[23] argued that the silence of the Notch signal will contribute to the therapeutic effect on osteoarthritis, which is consistent with the results in this study.

According to the results of immunohistochemistry, CII OD value was the highest in model group, indicating that the CII expression in articular cartilage in osteoarthritis was negatively correlated with the severity of osteoarthritis. During the development of osteoarthritis, the increased degradation of CII has a direct relation with the imbalanced secretion. The destroyed extracellular matrix fiber network damages the chondrocytes[24,25]. According to the study of Bagur-Calafat et al[26], inhibiting the the Notch signaling pathway can significantly inhibit the CII expression in articular cartilage in osteoarthritis. Osteocytes

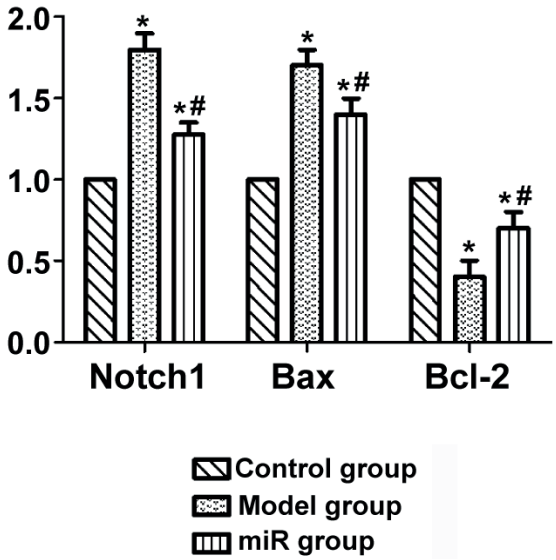

Figure 5. Differences in the relative expressions of Notch1, Bax, and Bcl-2 in rabbits. *$p<0.05$ *vs.* control group. #$p<0.05$ *vs.* model group.

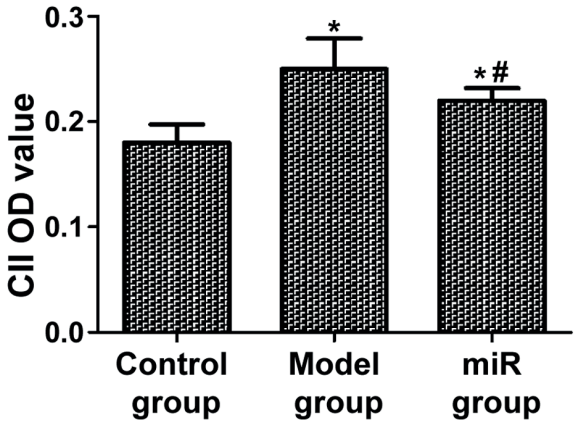

Figure 6. Comparison of CII OD value of rabbits in each group. *p<0.05 *vs.* control group. #p<0.05 *vs.* model group.

synthesize and secrete collagen and proteoglycan outside the cells, forming the fiber network structure to protect the chondrocytes[27].

Conclusions

We demonstrated that miR-9, through the down-regulation of the expressions of Notch and Bax, can activate Bcl-2 to promote differentiation and regeneration of chondrocytes. It can facilitate cartilage regeneration of osteoarthritis rabbits by regulating the CII expression.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

Shandong province medicine health science and technology development project (2017WS277).

References

1) Liu YP, Li J, Xin SB, X J. Study the relevance between inflammatory factors and estradiol and their association with knee osteoarthritis in postmenopausal women. Eur Rev Med Pharmacol Sci 2018; 22: 472-478.
2) Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, Evans DJ, Haughton L, Bayram Z, Boyer S, Thomson B, Wolfe MS, Archer CW. The surface of articular cartilage contains a progenitor cell population. J Cell Sci 2004; 117: 889-897.
3) Blanco FJ, Guitian R, Vázquez-Martul E, de Toro FJ, Galdo F. Osteoarthritis chondrocytes die by apoptosis. A possible pathway for osteoarthritis pathology. Arthritis Rheum 1998; 41: 284-289.
4) Ferreira AC, Suriano G, Mendes N, Gomes B, Wen X, Carneiro F, Seruca R, Machado JC. E-cadherin impairment increases cell survival through Notch-dependent upregulation of Bcl-2. Hum Mol Genet 2012; 21: 334-343.
5) Yanke AB, Chubinskaya S. The state of cartilage regeneration: current and future technologies. Curr Rev Musculoskelet Med 2015; 8: 1-8.
6) Chubinskaya S, Otten L, Soeder S, Borgia JA, Aigner T, Rueger DC, Loeser RF. Regulation of chondrocyte gene expression by osteogenic protein-1. Arthritis Res Ther 2011; 13: R55.
7) Chu CR, Beynnon BD, Buckwalter JA, Garrett WE Jr, Katz JN, Rodeo SA, Spindler KP, Stanton RA. Closing the gap between bench and bedside research for early arthritis therapies (EARTH): report from the AOSSM/NIH U-13 Post-Joint Injury Osteoarthritis Conference II. Am J Sports Med 2011; 39: 1569-1578.
8) Gu S, Zhang R, Gu J, Li X, Lv L, Jiang J, Xu Z, Wang S, Shi C, Wang DP, Wu C. HES5 promotes cellular proliferation of non-small cell lung cancer through STAT3 signaling. Oncol Rep 2017; 37: 474-482.
9) Kuem N, Song SJ, Yu R, Yun JW, Park T. Oleuropein attenuates visceral adiposity in high-fat diet-induced obese mice through the modulation of WNT10b- and galanin-mediated signalings. Mol Nutr Food Res 2014; 58: 2166-2176.
10) Peng GL, Tian Y, Lu C, Guo H, Zhao XW, Guo YW, Wang LQ, Du QL, Liu CP. Effects of Notch-1 down-regulation on malignant behaviors of breast cancer stem cells. J Huazhong Univ Sci Technolog Med Sci 2014; 34: 195-200.
11) Smirnova L, Gräfe A, Seiler A, Schumacher S, Nitsch R, Wulczyn FG. Regulation of miRNA expression during neural cell specification. Eur J Neurosci 2005; 21: 1469-1477.
12) Huang Y, Mucke L. Alzheimer mechanisms and therapeutic strategies. Cell 2012; 148: 1204-1222.
13) Tinkle CL, Haas-Kogan D. Hepatocellular carcinoma: natural history, current management, and emerging tools. Biologics 2012; 6: 207-219.
14) Rapisarda V, Loreto C, Malaguarnera M, Ardiri A, Proiti M, Rigano G, Frazzetto E, Ruggeri MI, Malaguarnera G, Bertino N, Malaguarnera M, Catania VE, Di Carlo I, Toro A, Bertino E, Mangano D, Bertino G. Hepatocellular carcinoma and the risk of occupational exposure. World J Hepatol 2016; 8: 573-590.
15) Li H, Wang D, Yuan Y, Min J. New insights on the MMP-13 regulatory network in the pathogenesis of early osteoarthritis. Arthritis Res Ther 2017; 19: 248.
16) Knapik JJ, Pope R, Hoedebecke SS, Schram B, Orr R. Effects of oral chondroitin sulfate on osteoarthri-

tis-related pain and joint structural changes: systematic review and meta-analysis. J Spec Oper Med 2019; 19: 113-124.

17) Xiang X, Huang J, Mo W, Jiang L, Sun W, Li P. Long non-coding RNA cartilage injury-related promotes malignancy in bladder cancer. Oncol Lett 2018; 15: 3049-3055.

18) Soyocak A, Kurt H, Ozgen M, Turgut Cosan D, Colak E, Gunes HV. MiRNA-146a, miRNA-155 and JNK expression levels in peripheral blood mononuclear cells according to grade of knee osteoarthritis. Gene 2017; 627: 207-211.

19) Akbarzadeh M, Majidinia M, Fekri Aval S, Mahbub S, Zarghami N. Molecular targeting of Notch signaling pathway by DAPT in human ovarian cancer: Possible anti metastatic effects. Asian Pac J Cancer Prev 2018; 19: 3473-3477.

20) Zhou H, Luo Y, Chen JH, Hu J, Luo YZ, Wang W, Zeng Y, Xiao L. Knockdown of TRB3 induces apoptosis in human lung adenocarcinoma cells through regulation of Notch 1 expression. Mol Med Rep 2013; 8: 47-52.

21) Lan L, Jiang Y, Zhang W, Li T, Ying B, Zhu S. Expression of Notch signaling pathway during osteoarthritis in the temporomandibular joint. J Craniomaxillofac Surg 2017; 45: 1338-1348.

22) Wang Z, Yin B, Wang B, Ma Z, Liu W, Lv G. MicroRNA-210 promotes proliferation and invasion of peripheral nerve sheath tumor cells targeting EFNA3. Oncol Res 2013; 21: 145-154.

23) Ma WH, Liu YJ, Wang W, Zhang YZ. Neuropeptide Y, substance P, and human bone morphogenetic protein 2 stimulate human osteoblast osteogenic activity by enhancing gap junction intercellular communication. Braz J Med Biol Res 2015; 48: 299-307.

24) Isaacson BM, Potter BK, Bloebaum RD, Epperson RT, Kawaguchi BS, Swanson TM, Pasquina PF. Link between clinical predictors of heterotopic ossification and histological analysis in combat-injured service members. J Bone Joint Surg Am 2016; 98: 647-657.

25) Cao J, Wang L, Du ZJ, Liu P, Zhang YB, Sui JF, Liu YP, Lei DL. Recruitment of exogenous mesenchymal stem cells in mandibular distraction osteogenesis by the stromal cell-derived factor-1/chemokine receptor-4 pathway in rats. Br J Oral Maxillofac Surg 2013; 51: 937-941.

26) Bagur-Calafat C, Farrerons-Minguella J, Girabent-Farres M, Serra-Grima JR. The impact of high level basketball competition, calcium intake, menses, and hormone levels in adolescent bone density: a three-year follow-up. J Sports Med Phys Fitness 2015; 55: 58-67.

27) Lv YF, Dai H, Yan GN, Meng G, Zhang X, Guo QN. Downregulation of tumor suppressing STF cDNA 3 promotes epithelial-mesenchymal transition and tumor metastasis of osteosarcoma by the Wnt/GSK-β/β -catenin/Snail signaling pathway. Cancer Lett 2016; 373: 164-173.