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MiR-27a alleviates osteoarthritis in rabbits *via* inhibiting inflammation

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Abstract. – OBJECTIVE: To investigate the regulatory effect of micro ribonucleic acid-27a (miR-27a) on the nuclear factor-kappa B (NF-κB) pathway and to explore its effect on rabbits with osteoarthritis (OA).

MATERIALS AND METHODS: Anterior cruciate ligament (ACL) cross-section method was adopted to establish OA rabbit models. Cartilage specimens were collected to detect expression levels of miR-27a in OA cartilage and normal cartilage tissues. Meanwhile, chondrocytes were isolated and cultured, and transfected with miR-27a mimics and miR-27a inhibitor. Blank control group was set up. Next, the changes in chondrocyte proliferation were detected using 5-ethynyl-2'-deoxyuridine (EdU) staining and cell counting kit-8 (CCK-8). Quantitative Real Time Polymerase Chain Reaction (PCR) was applied to detect the messenger RNA (mRNA) expression of inflammatory factors interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) in chondrocytes. Also, Western blot was adopted to detect the differential expression of NF-κB pathway-related proteins NF-κB and matrix metalloproteinase 13 (MMP-13).

RESULTS: Compared with that in normal cartilage tissues, miR-27a in OA cartilage tissues was decreased evidently ($p<0.05$). The expression level of miR-27a was higher in miR-27a mimics group than in control group, while it significantly declined in miR-27a inhibitor group ($p<0.05$). EdU staining and CCK-8 method results showed that miR-27a mimics could promote the proliferation of chondrocytes, while miR-27a inhibitor inhibited the proliferation of chondrocytes. Compared with those in control group, the expression levels of inflammatory factors TNF-α and IL-6 in chondrocytes in miR-27a inhibitor group were increased significantly ($p<0.05$). MiR-27a mimics could evidently reduce the expression of inflammatory factor IL-6 ($p<0.05$), but did not significantly reduce the expression of TNF-α. Besides, the results of Western blot suggested that the expression levels of MMP-13 and NF-κB proteins were decreased significantly in miR-27a mimics group ($p<0.05$) and increased significantly in miR-27a inhibitor group ($p<0.05$).

CONCLUSIONS: MiR-27a in OA cartilage tissues is evidently lower than in normal cartilage tissues. Transfection of miR-27a mimics can promote proliferation of chondrocytes, lower the expression of inflammatory factors, and reduce the expression of MMP-13 and NF-κB proteins. Therefore, the up-regulation of miR-27a can benefit the treatment of bone joints through the NF-κB pathway.

Key Words:
MiR-27a, NF-κB pathway, Osteoarthritis, Chondrocytes.

Introduction

Osteoarthritis (OA) is a common kind of chronic joint disease accompanied by pain and disability[1], affecting 15% of people worldwide[2]. OA can gradually lead to functional loss of patients, which is primarily manifested as articular cartilage injury, narrowing of joint clearance, synovitis, and dyskinesia[3]. The causes of OA may be related to heredity, cartilage metabolism, inflammation, immune, and other factors[4]. According to the pathogenesis of OA, the current research about treatment of OA mainly involves endogenous hormone, oxygen-free radicals, calcified cartilage zone structure, autoimmunity, and cytokines[5]; however, there are few works that can correctly find out the molecule mechanism of OA development. Previous studies[6] have shown that the occurrence of OA is correlated with the imbalance of the extracellular matrix and apoptosis of chondrocytes. Therefore, inhibiting substrate degradation and apoptosis of chondrocytes is considered as a therapeutic target of OA[7].

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Micro ribonucleic acids (miRNAs) are non-coding endogenous RNAs, which can be taken as key regulators of the gene expression[8]. Now it has been proved that miRNAs play important roles in many human diseases, including OA[9,10]. MiR-146a is one of the miRNAs related to OA cartilage[11]. Previous researches[12,13] suggested that it is closely related to relevant pathophysiology of OA. Moreover, the expression level of miR-16A in OA cartilage is low, while its expression is caused by the stimulation of interleukin-1β (IL-1β)[14]. Song et al[15] found that the expression of miR-9 in OA chondrocytes is significantly decreased compared with that in control group, while the apoptosis of chondrocytes is regulated by the miR-9-targeted protein. Besides, the research[16] indicated that miR-9 can bind to corresponding genes, such as nuclear factor-kappa B1 (NF-κB1) and the activation of the NF-κB1 signal pathway will trigger the release of proinflammatory cytokines, which play a critical role in altering the degree of cartilage injury. Moreover, some evidence[17-20] has indicated that IL plays a key role in the onset of OA and affects the expression and regulation of the related genes in OA. In previous reports[21], it has been proven that miR-27b regulates the expression of related inflammatory factors of human OA chondrocytes; however, how miR-27a regulates NF-κB and whether such regulation will affect the development of OA have not been clearly studied yet.

The present work aims to systematically illuminate the effect of miR-27a on rabbits with OA through the NF-κB pathway, which may help develop new diagnosis and treatment strategies for knee OA.

Materials and Methods

Establishment of OA Rabbit Models

Establishment of OA rabbit models: the rabbits were injected intraperitoneally with chloral hydrate (3.5 mL/kg). A 1.5 cm-long vertical incision was made on the medial collateral ligament and anterior-posterior cruciate ligament of the knee joint, respectively. Also, the medial meniscus was removed. After the articular cavity was washed with normal saline, it was sutured to establish the OA models. The front traction test proved that the ligament was completely ruptured. Then, the rabbits were fed separately and injected with penicillin every day. This investigation was approved by the Animal Ethics Committee of Medical College of the Northwest Minzu University Animal Center.

Culture and Transfection of Chondrocytes

Cartilage samples of bone joints were taken out under aseptic condition. One part was used to extract the total RNA of cartilage tissue for detection of miR-27a expression, while the other part was used for extraction of chondrocytes, which were incubated with the culture medium containing 10% fetal bovine serum (FBS) and 1% double-antibody in an incubator. The culture solution was replaced every 48 h. The second generation and the third generation of chondrocytes were used in the experiments. Before transfection, chondrocytes were divided into miR-27a mimics group, miR-27a inhibitor, and control group. Also, it was transfected according to the instructions of the transfection kit. The following experiments were performed after the verification of transfection efficiency.

5-Ethynyl-2'-Deoxyuridine (EdU) Staining

Chondrocytes were stained using the Click-iT EdU kit (Invitrogen, Carlsbad, CA, USA) at 24 h after transfection according to the instructions of kits. Finally, they were photographed to verify the number of EdU positive cells.

Detection of Proliferation Ability of Chondrocytes Via Cell Counting Kit-8 (CCK-8) Method

Cells in the logarithmic phase were cultured for 0, 12, 24, and 48 h. Each well was added with 110 μL developing a solution. After incubation in the incubator at 37°C for 2 h, the absorbance of each group was determined using an ultraviolet spectrophotometer (450 nm).

Expression of Inflammation-Related Genes in Chondrocytes Via Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cartilage tissues of the knee joints in healthy rabbits and OA models and the total RNA of transfected cells in different groups were extracted using TRIzol reagents (Invitrogen, Carlsbad, CA, USA). Once the RNA purity and concentration were qualified, they were reversely transcribed into complementary deoxyribose nucleic acid (cDNA) with the use of isopropanol. The primer sequences of target genes and internal

reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed according to the GenBank (Table I). The expression levels of target genes were determined *via* qRT-PCR.

Detection of Expression of NF-κB Pathway-Related Proteins in Chondrocytes in Each Group Via Western Blot

After the culture medium was discarded, chondrocytes were blown and washed with phosphate-buffered saline (PBS) for 3 times, as well as added with the lysis buffer prepared proportionally to lyse cells completely and release the cell protein. The supernatant was collected after centrifugation. Then, the total protein concentration in each group was detected using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). After that, the samples and gel were prepared for protein electrophoresis. The protein was transferred onto a membrane, sealed, incubated with the primary antibody overnight, and incubated again with the secondary antibody for 1 h. The protein band was scanned and quantified using the Odyssey scanner, while the level of protein to be detected was corrected using GAPDH. The Western blot bands were quantified by the Image Lab software. The expression levels of the corresponding proteins in each group were calculated.

Statistical Analysis

All original data obtained in the experiments were statistically analyzed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) and expressed as mean ± standard deviation. The *t*-test was used for the intergroup comparison of data and $p<0.05$ suggested a statistically significant difference.

Table I. PCR primers.

mRNA	Sequence
miR-27a	F: 5'-UUCACAGUGGCUAAGUUCCGC-3'
	R: 5'-AGGGCUUAGCUGCUUGUGAGCA-3'
NF-κB	F: 5'-CTGAACCAGGGCATACCTGT-3'
	R: 5'-GAGAAGTCCATGTCCGCAAT-3'
MMP-13	F: 5'-CCCCAACCCTAAACATCCAA-3'
	R: 5'-AACAGCTCCGCATCAACCT-3'
IL-6	F: 5'-CAATGAGGAGACTTGCCTGG-3'
	R: 5'-GCACAGCTCTGGCTTGTTCC-3'
TNF-α	F: 5'-CGCTACGACCGCCAG ATTG-3'
	R: 5'-ACACCGTTCACCAGCAAGTC-3'
GAPDH	F: 5'-TGACTTCAACAGCGACACCCA-3'
	R: 5'-CACCCTGTTGCTGTAGCCAAA-3'

Results

Expression of MiR-27a in Cartilage Tissues of OA Models and Normal Rabbits

QRT-PCR was adopted to determine the expression of miR-27a in cartilage tissues of OA rabbit models. Also, it was found that the expression level of miR-27a in OA models was evidently lower than in normal rabbits ($p<0.05$) (Figure 1).

Transfection Efficiency of Chondrocytes in Different Groups

QRT-PCR was used to determine the relative expression of miR-27a in chondrocytes after transfection. Results revealed that compared with that in control group, the expression level of miR-27a was increased evidently in miR-27a mimics group ($p<0.05$) and significantly decreased in miR-27a inhibitor group ($p<0.05$) (Figure 2).

EdU Staining Results of Cells in Different Groups

The proliferation ability of cells in different groups was evaluated using the EdU staining. Compared with control group, miR-27a mimics could promote the proliferation of chondrocytes ($p<0.05$), while miR-27a inhibitor could inhibit the proliferation of chondrocytes ($p<0.05$) (Figure 3).

Proliferation Ability of Chondrocytes Detected Via CCK-8 Method

The proliferation ability of chondrocytes was detected using the CCK-8 method. It was found that compared with that in control group, the

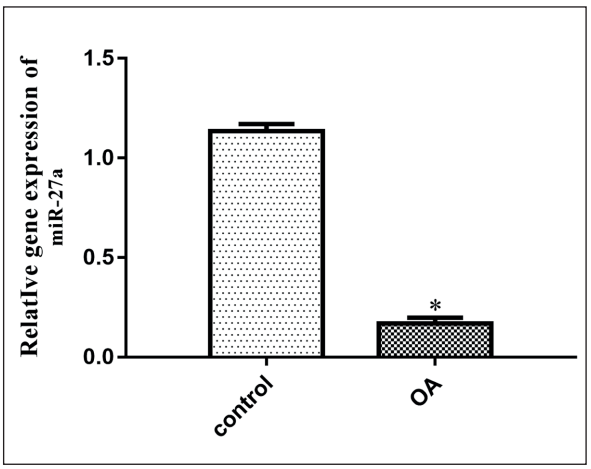

Figure 1. Expression of miR-27a in cartilage tissues of OA models and normal rabbits. In OA models, the expression level of miR-27a declines evidently ($p<0.05$).

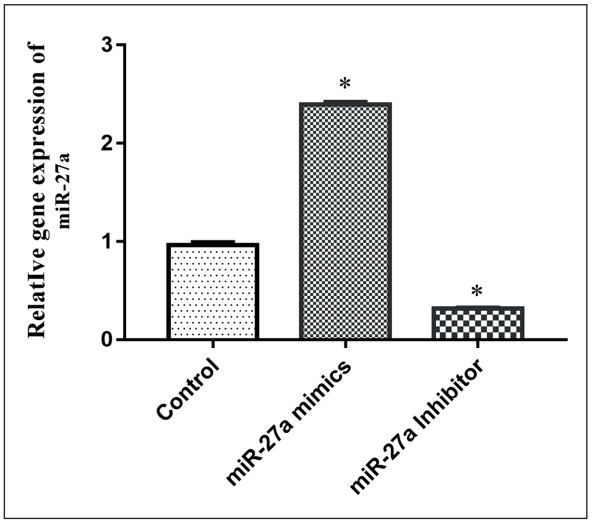

Figure 2. Expression of miR-27a in chondrocytes of different groups after transfection. *p<0.05 *vs.* control group.

cell proliferation was higher in miR-27a mimics group (p<0.05) and lower in miR-27a inhibitor group (p<0.05) (Figure 4).

Expression of Inflammation-Related Genes in Chondrocytes

As shown in Figure 5, the expression levels of IL-6 and TNF-α in chondrocytes of miR-27a inhibitor group were significantly increased (p<0.05). MiR-27a mimics could evidently reduce the expression level of inflammation gene IL-6 (p<0.05); however, it did not significantly reduce the expression of TNF-α.

Expression of NF-κB Pathway-Related Proteins in Chondrocytes

Compared with those in control group, the expression levels of MMP-13 and NF-κB in chondrocytes were markedly decreased in miR-27a mimics group (p<0.05) and significantly increased in miR-27a inhibitor group (p<0.05), indicating that the transfection of miR-27a mimics could reduce the expression of MMP-13 and NF-Kb (Figure 6).

Discussion

OA is a kind of joint disease affecting articular cartilage and its adjacent tissue. The occurrence of OA will further result in cartilage injury and ultimately lead to disability[22]. However, few researchers can explain its inner mechanism comprehensively. MRNAs are considered as potential biomarkers and are involved in the pathogenesis of OA by controlling target genes and regulating the gene expression. It has been found in some reports about OA patients that such mRNAs as miR-9, miR-27, miR-140, and miR-146 are in the

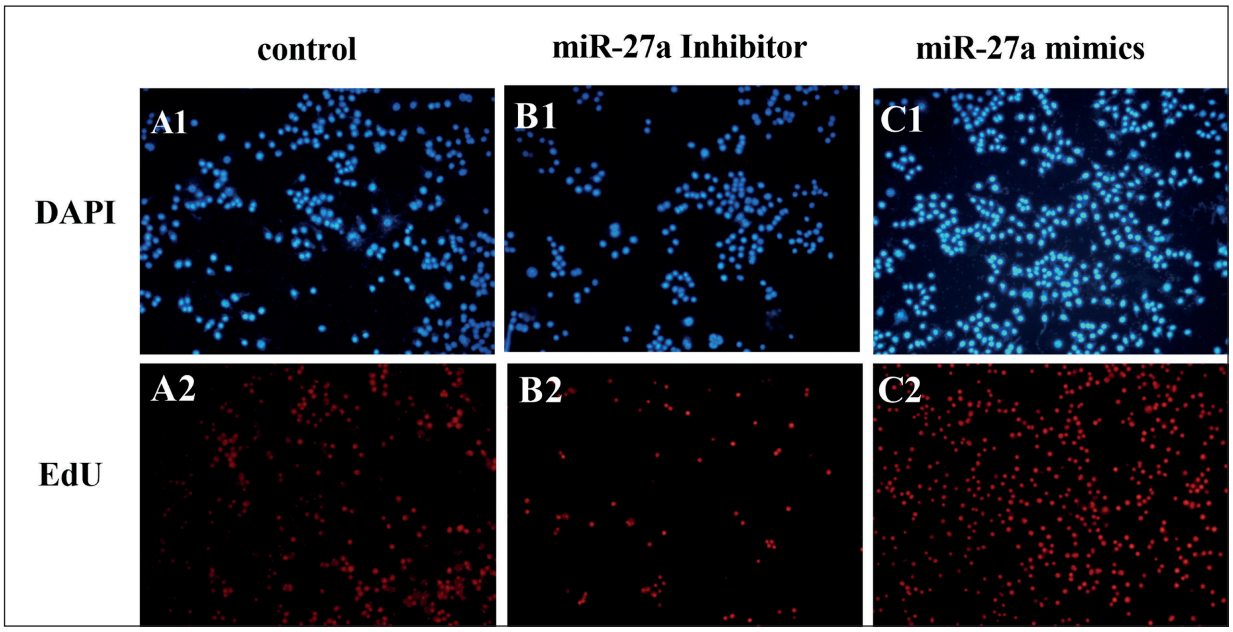

Figure 3. EdU staining results of cells in different groups (magnification: 100×). ***A,*** Control group, ***B,*** MiR-27a inhibitor group, ***C,*** MiR-27a mimics group. MiR-27a mimics can promote proliferation of chondrocytes (p<0.05). *p<0.05 *vs.* control group.

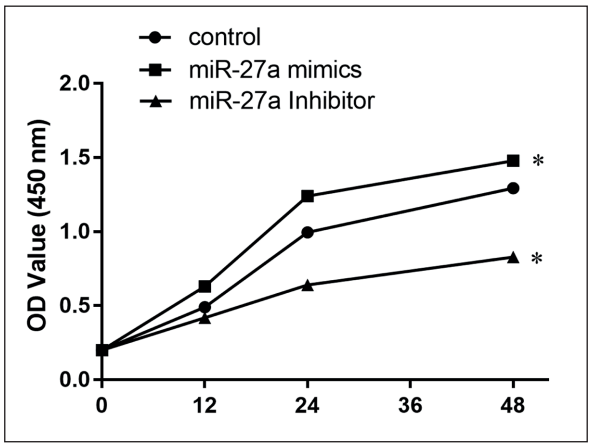

Figure 4. Detection of proliferation ability of chondrocytes *via* CCK-8 method. *p<0.05 *vs.* control group.

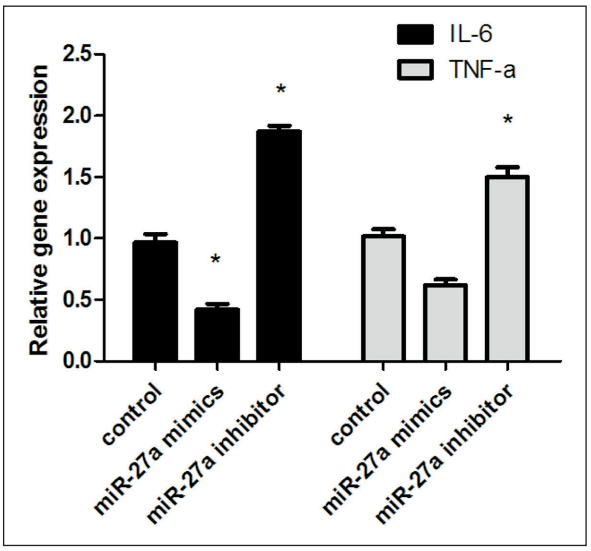

Figure 5. Expression of inflammation-related gene mRNA in chondrocytes. MiR-27a mimics can evidently reduce the expression of inflammation gene IL-6 (p<0.05). *p<0.05 *vs.* control group.

abnormal expression. Similarly, some genes (like NF-κB, IL-6, and MAP-13) are reported to be overexpressed in OA patients, especially in the early stage of OA[23,24]. The objective of this study is to explore the effects of miR-27a on OA rabbits through the NF-κB pathway on the basis of establishing internal relations of miR-27a, IL-6, MAP-13, and NF-κB in the cartilage of bone joints.

In the present paper, the results of RT-PCR showed that the expression of NF-κB in chondrocytes was affected by miR-27a. Also, the decline in miR-27a in OA chondrocytes may result in the increased expression of NF-κB in chondrocytes. Therefore, it was concluded that the expression of NF-κB is regulated by miR-27a, and miR-27a affects rabbits with OA through the NF-κB pathway. Besides, miR-27a mimics could also promote the proliferation of chondrocytes and inhibit the expression of related inflammatory factors. It could be assumed that the interaction between miR-27a and NF-κB inhibits cell apoptosis during the development process of cartilage, while the apoptosis of chondrocytes transfected with miR-27a mimic delinces[25]. To explain how miR-27a regulates OA through the signaling pathway, the expression of IL-6 and TNF-α in chondrocytes in miR-27a mimics and miR-27a inhibitor group was compared in the present study. Previous research about miR-9 showed that IL-6 and MMP-

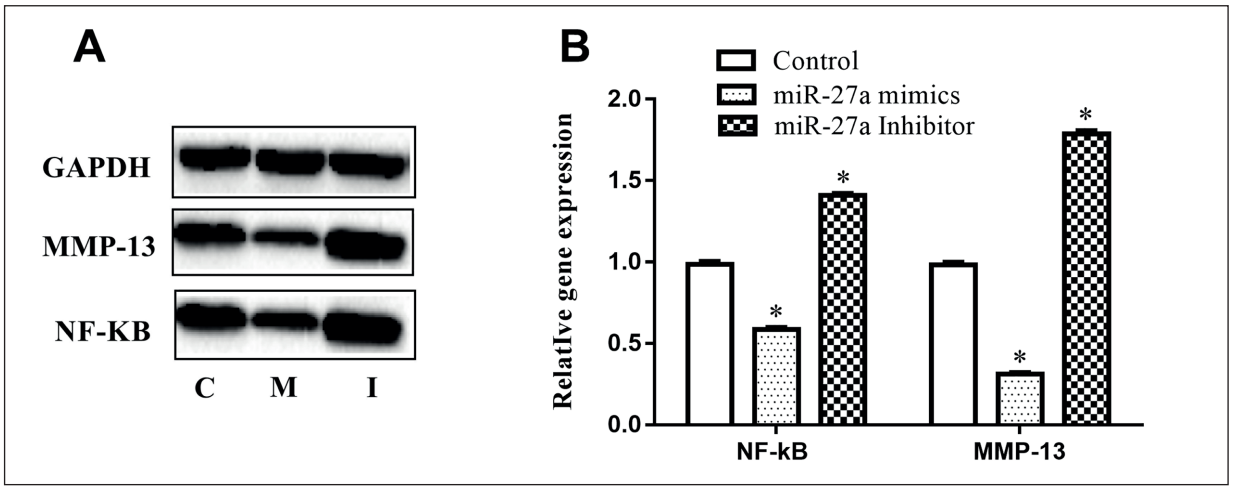

Figure 6. Expression of NF-κB pathway-related proteins in chondrocytes. C: control group, M: miR-27a mimics group, I: miR-27a Inhibitor group. *p<0.05 *vs.* control group.

13 were both overexpressed in cartilage tissues of bone joints. Besides, IL-6 and MMP-13 both declined significantly after chondrocytes were transfected with NF-κB1 siRNA. The results of this study revealed that the decreased expression of IL-6 and MMP-13 was due to the NF-κB expression under targeted regulation of miR-9. Besides, it was determined that miR-9 regulates the NF-κB expression negatively. Therefore, the down-regulation of miR-9 will promote NF-κB expression and inhibit cell proliferation[26], which is consistent with the trend observed in miR-27a mimics group.

Several studies have proven that NF-κB can activate the genes that maintain cell proliferation. For example, inhibiting expression of NF-κB will hinder the proliferation of HeLa cells, while up-regulating NF-κB may indirectly result in cell proliferation induced by H-ras cancer gene[27,28]. Indeed, the expression of miR-27a in chondrocytes of OA is relatively lower, which is related to the apoptosis of chondrocytes[15]. NF-κB can control various genes of different functions such as anti-apoptosis and anti-proliferation[29]. Also, it can promote the expression of anti-apoptosis genes in chondrocytes more easily than anti-proliferation genes, while it turned out just the opposite in other cells. Although the relation between miR-27a and NF-κB, and the formation of OA has been proved, the sample size in this work was small, and only chondrocytes of the same donor were used in the cell experiments. Therefore, chondrocytes from different donors should be used and the NF-κB pathway should be further explored combined with the unique analysis of the pathway to make the roles of miR-27a and NF-κB in the development of OA more reliable. Therefore, the molecular mechanism of miR-27a and NF-κB pathway in the formation and development of OA should be further explored.

Conclusions

We showed that the expression level of miR-27a in OA tissues of bone joints is decreased significantly compared with that in normal tissues. Furthermore, targeting NF-κB with miR-27a can enhance proliferation and inhibit apoptosis of chondrocytes. Therefore, miR-27a and NF-κB may become therapeutic targets for patients with OA. This work provides important experimental support for the treatment of OA and a theoretical basis for further research.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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References

1) Soder S, Roach HI, Oehler S, Bau B, Haag J, Aigner T. MMP-9/gelatinase B is a gene product of human adult articular chondrocytes and increased in osteoarthritic cartilage. Clin Exp Rheumatol 2006; 24: 302-304.
2) Akama H, Saito S. Glucosamine and chondroitin for osteoarthritis. Arthritis Rheum 2001; 45: 107.
3) Greene MA, Loeser RF. Aging-related inflammation in osteoarthritis. Osteoarthritis Cartilage 2015; 23: 1966-1971.
4) Yu SP, Hunter DJ. Managing osteoarthritis. Aust Prescr 2015; 38: 115-119.
5) Roos EM, Arden NK. Strategies for the prevention of knee osteoarthritis. Nat Rev Rheumatol 2016; 12: 92-101.
6) Thomas CM, Fuller CJ, Whittles CE, Sharif M. Chondrocyte death by apoptosis is associated with cartilage matrix degradation. Osteoarthritis Cartilage 2007; 15: 27-34.
7) Felson DT, Kim YJ. The futility of current approaches to chondroprotection. Arthritis Rheum 2007; 56: 1378-1383.
8) Zhang D, Wang J, Wang Z, Zhang T, Shi P, Wang X, Zhao F, Liu X, Lin X, Pang X. MiR-136 modulates TGF-beta1-induced proliferation arrest by targeting PPP2R2A in keratinocytes. Biomed Res Int 2015; 2015: 453518.
9) Le LT, Swingler TE, Clark IM. Review: the role of microRNAs in osteoarthritis and chondrogenesis. Arthritis Rheum 2013; 65: 1963-1974.
10) Mirzamohammadi F, Papaioannou G, Kobayashi T. MicroRNAs in cartilage development, homeostasis, and disease. Curr Osteoporos Rep 2014; 12: 410-419.
11) Wang JH, Shih KS, Wu YW, Wang AW, Yang CR. Histone deacetylase inhibitors increase microRNA-146a expression and enhance negative regulation of interleukin-1beta signaling in osteoarthritis fibroblast-like synoviocytes. Osteoarthritis Cartilage 2013; 21: 1987-1996.
12) Li X, Gibson G, Kim JS, Kroin J, Xu S, van Wijnen AJ, Im HJ. MicroRNA-146a is linked to pain-related pathophysiology of osteoarthritis. Gene 2011; 480: 34-41.
13) Li X, Kroin JS, Kc R, Gibson G, Chen D, Corbett GT, Pahan K, Fayyaz S, Kim JS, van Wijnen AJ, Suh J, Kim SG, Im HJ. Altered spinal microRNA-146a and the

microRNA-183 cluster contribute to osteoarthritic pain in knee joints. J Bone Miner Res 2013; 28: 2512-2522.

14) Yamasaki K, Nakasa T, Miyaki S, Ishikawa M, Deie M, Adachi N, Yasunaga Y, Asahara H, Ochi M. Expression of MicroRNA-146a in osteoarthritis cartilage. Arthritis Rheum 2009; 60: 1035-1041.

15) 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.

16) Toegel S, Wu SQ, Otero M, Goldring MB, Leelapornpisid P, Chiari C, Kolb A, Unger FM, Windhager R, Viernstein H. Caesalpinia sappan extract inhibits IL1beta-mediated overexpression of matrix metalloproteinases in human chondrocytes. Genes Nutr 2012; 7: 307-318.

17) Daheshia M, Yao JQ. The interleukin 1beta pathway in the pathogenesis of osteoarthritis. J Rheumatol 2008; 35: 2306-2312.

18) Park SJ, Cheon EJ, Lee MH, Kim HA. MicroRNA-127-5p regulates matrix metalloproteinase 13 expression and interleukin-1beta-induced catabolic effects in human chondrocytes. Arthritis Rheum 2013; 65: 3141-3152.

19) Park SJ, Cheon EJ, Kim HA. MicroRNA-558 regulates the expression of cyclooxygenase-2 and IL-1beta-induced catabolic effects in human articular chondrocytes. Osteoarthritis Cartilage 2013; 21: 981-989.

20) Makki MS, Haqqi TM. MiR-139 modulates MCPIP1/IL-6 expression and induces apoptosis in human OA chondrocytes. Exp Mol Med 2015; 47: e189.

21) Akhtar N, Rasheed Z, Ramamurthy S, Anbazhagan AN, Voss FR, Haqqi TM. MicroRNA-27b regulates the expression of matrix metalloproteinase 13 in human osteoarthritis chondrocytes. Arthritis Rheum 2010; 62: 1361-1371.

22) Bennell KL, Wrigley TV, Hunt MA, Lim BW, Hinman RS. Update on the role of muscle in the genesis and management of knee osteoarthritis. Rheum Dis Clin North Am 2013; 39: 145-176.

23) Nugent M. MicroRNAs: exploring new horizons in osteoarthritis. Osteoarthritis Cartilage 2016; 24: 573-580.

24) 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.

25) Tardif G, Hum D, Pelletier JP, Duval N, Martel-Pelletier J. Regulation of the IGFBP-5 and MMP-13 genes by the microRNAs miR-140 and miR-27a in human osteoarthritic chondrocytes. BMC Musculoskelet Disord 2009; 10: 148.

26) Liu N, Sun Q, Chen J, Li J, Zeng Y, Zhai S, Li P, Wang B, Wang X. MicroRNA-9 suppresses uveal melanoma cell migration and invasion through the NF-kappaB1 pathway. Oncol Rep 2012; 28: 961-968.

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

28) Jo H, Zhang R, Zhang H, McKinsey TA, Shao J, Beauchamp RD, Ballard DW, Liang P. NF-kappa B is required for H-ras oncogene induced abnormal cell proliferation and tumorigenesis. Oncogene 2000; 19: 841-849.

29) Gu R, Liu N, Luo S, Huang W, Zha Z, Yang J. MicroRNA-9 regulates the development of knee osteoarthritis through the NF-kappaB1 pathway in chondrocytes. Medicine (Baltimore) 2016; 95: e4315.