Southern Medical University[1]; Department of Orthopaedic Surgery[2], Guangzhou Orthopedic Trauma Hospital; School of Basic Medical Science[3], Southern Medical University, Guangzhou; The Affiliated Putian Hospital of Southern Medical University (The Affiliated Hospital of Putian University) [4], Putian, China

MicroRNA-320a protects against osteoarthritis cartilage degeneration by regulating the expressions of BMI-1 and RUNX2 in chondrocytes

Hao Peng[1], Du Liang[2], Baolin Li[2], Chizhang Liang[2], Wenhua Huang[3,*], Haibin Lin[4,*]

Received October 25, 2016, accepted November 30, 2016

**Corresponding authors: Wenhua Huang, School of Basic Medical Science, Southern Medical University, No 1023-1063, Shatai South Road, Baiyun District, Guangzhou 510515, China*
huangwenhua732@126.com
Haibin Lin, The Affiliated Putian Hospital of Southern Medical University
(The Affiliated Hospital of Putian University), No 999, Dongzhen East Road, Licheng District, Putian 351100, China
linhaibin535@126.com

Pharmazie 72: 223–226 (2017) *doi: 10.1691/ph.2017.6890*

Osteoarthritis (OA) is one of the most common chronic degenerative diseases characterized by deterioration of articular cartilage. Many studies have demonstrated the role of microRNAs (miRNAs) in OA, but the role of miR-320a in OA remains elusive. The aim of this study was to identify the protective role of miR-320a in OA cartilage degeneration by regulating the expression of BMI-1 and RUNX-2 proteins in chondrocytes. Normal and OA chondrocytes obtained from patients were cultured *in vitro*. The chondrocytes (both normal and OA) were transfected with miR-320a inhibitor to investigate the effects of miR-320a on chondrocyte proliferation, and to identify the miR-320a target proteins. The results indicated that miR-320a expression was significantly higher ($P<0.05$) in OA chondrocytes than in normal chondrocytes. Inhibition of miR-320a effectively enhanced chondrocyte cell viability *in vitro* in a time-dependent manner. Inhibition of miR-320a showed a significant decrease ($P<0.05$) in the secretion of matrix metalloproteinase-13 (MMP-13). Furthermore, miR-320a could regulate the expression levels of BMI-1 and RUNX-2 proteins in OA chondrocytes ($P<0.05$). The data suggested that miR-320a protected against OA cartilage degeneration and regulated the expression levels of BMI-1 and RUNX2 proteins in chondrocytes. Our study might provide a new insight in the clinical treatment of OA.

1. Introduction

Osteoarthritis (OA) is a common chronic degenerative disease that seriously affects life quality of patients (Cui et al. 2016; Le et al. 2013). OA is characterized by deterioration of chondrocytes which is an integral part of the articular cartilage, subchondral bone and joint margins (Le et al. 2013). Studies have shown that proteolytic enzymes such as matrix metalloproteinases (MMP), secreted by chondrocytes are involved in the degradation of cartilage (Cui et al. 2016; Wu et al. 2014). Pathogenesis of OA is a complex process involving multiple genetic factors that contribute to the disease process and runs in families (Le et al. 2013; Wu et al. 2014). Gene expression alterations in chondrocytes are involved in the synthesis and degradation of cartilage, including microRNAs (miRNAs or miRs) (Aigner et al. 2006; Goldring and Goldring 2007; Lewis et al. 2005; Little et al. 2002; Malemud et al. 2003; Reynard and Loughlin 2012). Research into the regulatory mechanisms underlying the pathogenesis of OA is therefore warranted, and there was increased interest in the functional role of miRs.

MiRNAs are small (17-25 nucleotide long) non-coding RNAs, which can regulate expression levels of multiple target genes and approximately one-third of the mammalian genes were regulated by these miRNAs (Lewis et al. 2005; Yang et al. 2005). A study by Kobayashi et al. (2008) found that chondrocyte proliferation and differentiation were regulated by dicer-dependent pathways. Jones et al. (2009), identified 17 human miRNAs which showed greater differential expressions (about 4-fold) between normal and OA cartilage. A study found different degrees of expressions of seven human miRNAs in normal and OA cartilage (Díaz-Prado et al. 2012). The results of these studies revealed the possibility of involvement of miRNA in OA pathogenesis. Some miRNAs promote while some suppress the chondrocyte events in OA pathogenesis. Hsa-miR-148a overexpression promoted cartilage production while inhibition of it led to cartilage degradation by OA chondrocytes (Kobayashi et al. 2008; Vonk et al. 2014). miR-33a regulated cholesterol synthesis and cholesterol efflux-related genes in OA chondrocytes (Jones et al. 2009; Kostopoulou et al. 2015). MiR-149 downregulation in OA chondrocytes showed the increased expression of pro-inflammatory cytokines (TNFα, IL1β, and IL6) (Santini et al. 2014). Subsequently, microRNA-125b regulated the expressions of aggrecanase-1 (ADAMTS-4) in human OA chondrocytes (Matsukawa et al. 2014). Therefore, miRNAs could exert positive or negative effects on OA chondrocyte metabolism. The identification of the effect of miRNAs on chondrocyte metabolism is beneficial in understanding OA etiology and for provide potential approach for OA therapy.

Many clinical studies have found that miR-320a was highly expressed in patients with osteoarthritis (Deugarte et al. 2015; Meng et al. 2016). It is also involved in the development of cartilage and homeostasis by directly targeting MMP-13 (Meng et al. 2016). Direct target genes that which pivotal role in the pathogenesis of OA include Runt-related transcription factor 2 (RUNX2) and BMI-1. RUNX2 is a key transcription factor involved in the osteogenic differentiation which was regulated by miR-320a (Hamam et al. 2014; Meng et al. 2016; Yu et al. 2011). According to previous studies, overexpression of BMI-1 was found to resolve miR-320a induced biological functions (Qi et al. 2014). However, the role of miR-320a in the pathogenesis of OA cartilage degeneration is still not clear.

Therefore, we conducted this study to explore the role of miR-320a in the pathogenesis of OA. In this study, the expression of miR-320a and its target genes were investigated in human normal and OA chondrocytes. The effects of miR-320a on expression of RUNX2 and BMI-1 in chondrocytes of OA cartilage were also explored.

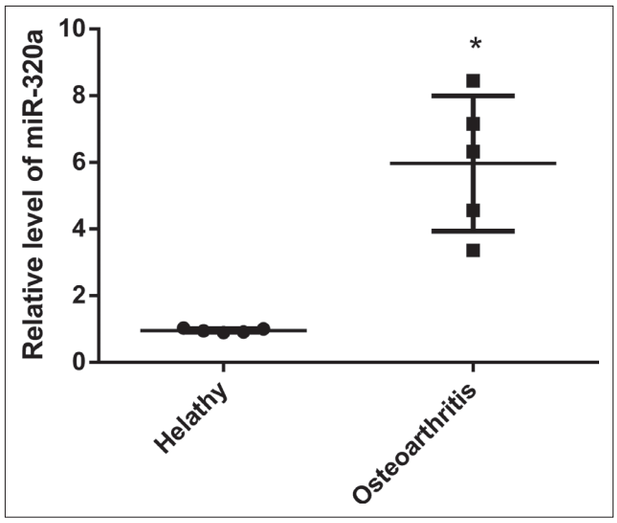

Fig. 1: Expression of miR-320. miR-320a expression levels in the chondrocytes were detected by RT-PCR. The results showed that the expression of miR-320a in the OA chondrocytes was significantly higher than that of the healthy controls.

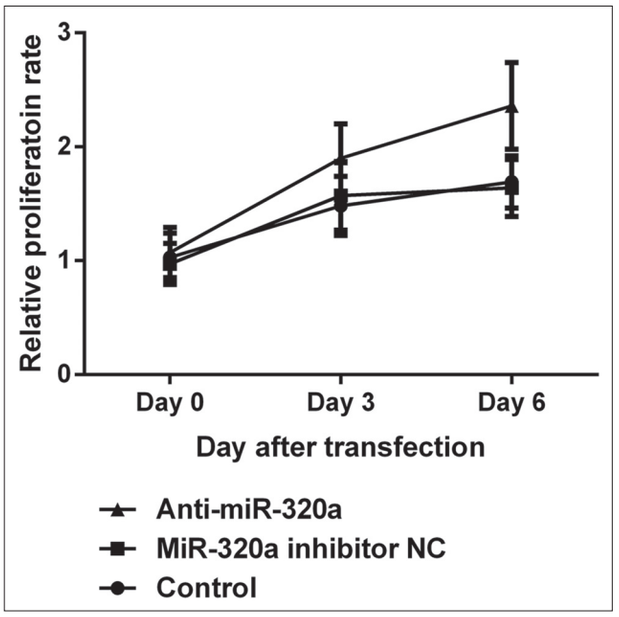

Fig. 2: Cell viability of chondrocytes. The chondrocytes were transfected with miR-320a inhibitor, and then the cell viability was analyzed. The results showed that miR-320a inhibitor can effectively enhance cell viability of chondrocytes *in vitro*.

2. Investigations and results

2.1. Expression of miR-320a in human normal and OA chondrocytes

Cartilage samples were selected from two groups, one including patients with normal cartilage and another one which included patients with OA. Cells were cultured, followed by the detection of the gene level using real-time PCR (RT-PCR) technique. As shown in Fig. 1, the gene level of miR-320a in the OA chondrocytes group was significantly higher compared to the normal healthy controls (*P<0.05). The data thus indicated that the levels of miR-320a in OA chondrocytes were higher than those in normal chondrocytes.

2.2. Cell viability of chondrocytes

The loss-of-function approach was employed to explore the role of miR-320a in cultured chondrocytes. The chondrocytes were transfected with anti-miR-320a and their negative control (NC) and then the cell viability was analyzed on days 0, 3, and 6. Both miR-320a inhibitor and miR-320a inhibitor-NC showed a time-depended proliferation rate of OA chondrocytes. These results suggested that miR-320a inhibitor effectively enhanced cell viability of chondrocytes *in vitro* (Fig. 2).

2.3. MMP-13 levels

The levels of MMP-13 in cultured chondrocytes were detected by ELISA assay. The cells were transfected with a specific inhibitor of miR-320a (anti-miR-320a) or NC. Next, analysis of MMP-9 protein expression in cells and its secretion in the culture medium were done. Treatment with anti-miR-320a led to a significant decrease in the secretion (*P<0.05) of cellular MMP-13 protein by the chondrocytes. These results revealed that over expression of miR-320a was accompanied by an increased secretion of MMP-13, while inhibition of miR-320a reduced the level of MMP-13 (Fig. 3). These results suggested that miR-320a modulates the secretion of MMP-13.

2.4. BMI-1 mRNA levels

Further studies were conducted to explore the possible target genes of miR-320a for regulating chondrocytes. We found that there was a negative correlation between BMI-1 and miR-320a (Fig. 4), indicating that miR-320a significantly (*P<0.05) inhibited the development of OA which might decrease the expression of BMI-1 in chondrocytes.

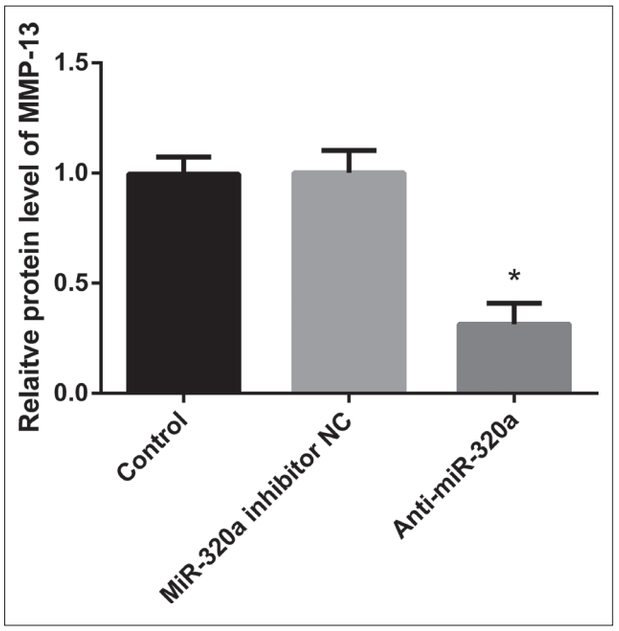

Fig. 3: MMP-13 level. The levels of MMP-13 in cultured chondrocytes were detected by ELISA. We found that the high expression of miR-320a was accompanied by increased secretion of MMP-13, while inhibition of miR-320a could reduce the level of MMP-13. *p<0.05 compared to the control group.

2.5. RUNX2 protein levels

Previous studies have shown that RUNX2 was a key transcription factor involved in OA and might be a potential target protein of miR-320a. Therefore, we sought to assess the expression levels of RUNX2 in OA cartilage degeneration. The cells treated with anti-miR-320a showed a significant increase in the protein levels of RUNX2 (*P<0.05). The results revealed that the expression levels of RUNX2 protein were significantly increased after miR-320a inhibition (Fig. 5).

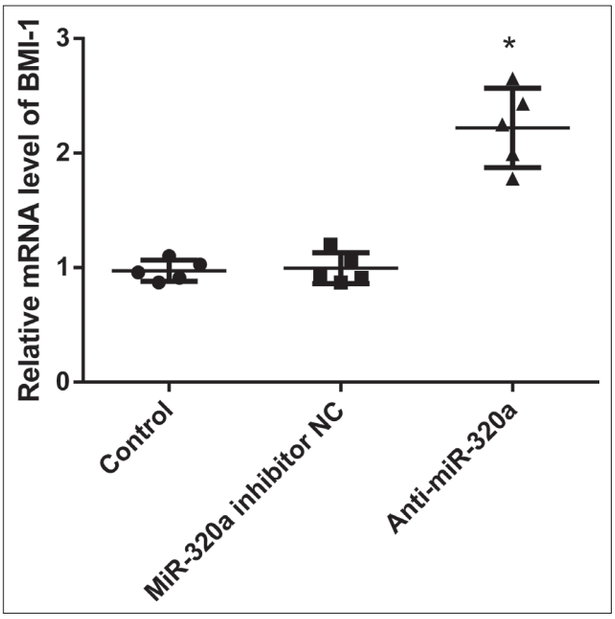

Fig. 4: BMI-1 mRNA levels. Further studies were done to explore the possible target genes of miR-320a. We found there was a negative correlation between BMI-1 and miR-320a, indicating that miR-320a could inhibit the development of osteoarthritis might be by decreasing the expression of BMI-1 in the chondrocytes. *p<0.05 compared to the control group.

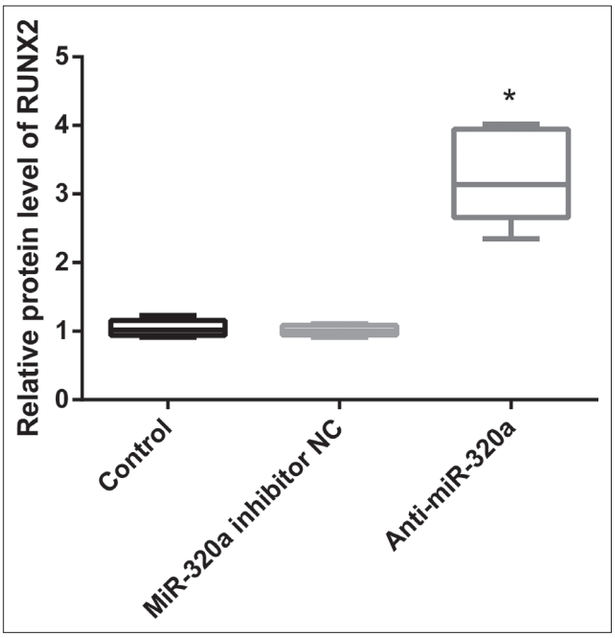

Fig. 5: RUNX2 protein levels. We analyzed the expression levels of RUNX2 and the results showed that the expression levels of RUNX2 protein were significantly increased after miR-320a inhibition. *p<0.05 compared to the control group.

3. Discussion

Previous studies have reported that miRNAs expressed differentially in normal and OA chondrocytes (Díaz-Prado et al. 2012; Jones et al. 2009). According to research by Meng et al. (2016), miR-320 was found to target MMP13, and downregulation of this miRNA was predicted to contribute to OA pathogenesis. Consistent with these results, our data indicated a higher expression of miR-320a in OA chondrocytes compared with normal chondrocytes. For the first time, our study found that high expression of miR-320a in primary cultured human osteoarthritis chondrocytes, which was in line with a previous study (Meng et al. 2016). We then found that miR-320a inhibitor could effectively enhance of chondrocytes and decrease MMP-13 levels. The results indicated that miR-320a protected against OA cartilage degeneration. Further study suggested that miR-320a could regulate the expression of BMI-1 and RUNX2 in the chondrocytes. Therefore, it is confirmed that miR-320a is up-regulation in OA chondrocytes. Our study might provide a new insight into clinical treatment of osteoarthritis.

Some studies have demonstrated the involvement of miRNAs in regulating OA pathological progression. For example, miR-149 reduction in OA chondrocytes is associated with increased expression of pro-inflammatory cytokines such as TNFα, IL1β and IL6 (Santini et al. 2014). Especially, the association between miRNA and the extracellular matrix of chondrocytes has been investigated that miR-127-5p regulated MMP13 in human chondrocytes and might contribute to the development of OA (Park et al. 2013). Here, our data also indicated that miR-320a could regulate the expression of main biological markers in matrix metabolism, such as MMP13 in OA chondrocytes. Meanwhile, miR-320a was involved in regulating chondrocyte survival, which is similar to the effect of other miRNAs. Similarly, an increased apoptosis rate in chondrocytes was observed by miR-146 regulation (Li 2012). The miR-223 overexpression stimulates apoptosis in human articular chondrocytes and deteriorates the cartilage in db/db mice (Kim et al. 2014). Hence, we suggested that miR-320a might be involved in regulating cell survival and matrix synthesis of OA chondrocytes.

However, our data indicated that decreased miR-320a exerted inhibitory effects on matrix synthesis in OA chondrocytes, which seems to be a contrary effect on cell events. Based on our data of the high expression of miR-320a in OA chondrocytes, we hypothesize that the increase of miR-320a might contribute to the development of OA pathogenesis. Whereas, the miR-320a decrease in the OA chondrocytes contributes to spontaneous resistance of chondrocytes in the pathological microenvironment of OA, or a more complicated regulatory mechanism on miR-320a. Moreover, the inhibitory effect of the overexpression of miR-320a on cell survival in cancer cells has already been demonstrated, that the overexpression of miR-320a activates the apoptotic pathway in mitochondria by targeting genes associated with mitochondrial homeostasis, anti-apoptosis, antioxidant ability and autophagy in prostate and endometrial cancers, and that miR-320a is considered as an effective miRNA which induces apoptosis and inhibit the level of p-Akt in cancer (Deugarte et al. 2015). These findings support our data that miR-320a suppressed the survival of OA chondrocytes. The role of miR-320a in the whole OA progression needs to be further studied.

Also, many target genes of miRNAs were detected in the OA pathogenesis. For example, miR-146a with the help of VEGF and TGF-β signaling pathway by targeting Smad4 inhibition in cartilage contributes to the pathogenesis of OA (Li 2012). MiR-21 controls by targeting GDF-5 in chondrocytes contributes to the development of OA (Zhang et al. 2014). MiR-210 in OA inhibits inflammatory signaling pathway, NF-κB, by targeting DR6 (Zhang et al. 2015). Our results based on Western blotting and quantitative RUNX2 analysis showed an inverse relationship between miR-320a and BMI-1 and RUNX-2 expression in OA chondrocytes. Therefore, we suggested that BMI-1 and RUNX-2 acted as putative target genes that inhibited by miR-320a. Similar BMI-1 results were observed in nasopharyngeal cancer cells (Qi et al. 2014).

Hence, there is increasing evidences which suggested that miRNA-320a convey a novel and efficient means in gene expression regulation. Understanding and gaining knowledge on its expression and dynamic regulation helps in enhancing chondrogenic differentiation. This might affect the direct treatments in OA which is aimed at preventing tissue destruction and driving repair. Unraveling the role of miRNA-320a in joint physiology and pathology will shed light upon the diagnosis, prevention, and treatment of OA.

4. Experimental

4.1. Human chondrocyte isolation and culture

Human articular cartilages were minced and digested with 0.2% collagenase II in Dulbecco's modified Eagle's medium (DMEM). Chondrocytes were maintained in DMEM containing 10% fetal bovine serum (FBS, Gibco, USA) for 24 h at 37 °C. The cells were filtered through a 0.075 mm cell strainer and washed before culturing or miRNA/mRNA isolation with sterile phosphate buffered saline (PBS). First passage chondrocytes were obtained after two weeks. All the experiments were done within three days of first passage. During the culture period, cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air, and the medium was changed at an interval of two days.

4.2. Cell transfection or modulation of miRNA expression

Chondrocytes were transfected with 100 nM of anti-miR-320a (Cat#MH11621, Ambion) or negative control anti-miR miRNA inhibitors #1 (Cat#4464076) using TransIT-TKO transfection reagent (Cat#MIR2154, Mirus Bio) for 48 h according to the manufacturer's instructions. Cellular MMP-9 expression was assessed by Western blotting. The concentrations of MMP-9 and IL-6 in culture supernatants were measured by ELISA (cat#DY911 and DY206, respectively, R&D systems). Transfection efficiency was evaluated using Dy547-labeled miRIDIAN microRNA mimic transfection control (cat#CP-004500-01-05, Thermo Scientific). Flow cytometry analysis showed that 85% of chondrocytes were transfected.

4.3. Cell viability assay

Cells after transfection for 48 h were plated in 96-well plates (1×10^3 cells/well) and cultured for different times (0, 3, 6 days). 10 µl Cell Counting Kit-8 (CCK8) solution (Beyotime, Shanghai, China) was then added per well and the cultures were incubated at 37 °C for a further 3 h as described in previous study. The end product was quantified spectrophotometrically at a wavelength of 450 nm. The OD values correspond to the number of viable cells.

4.4. MMP-13 enzyme-linked immunosorbent assay

The human chondrocyte culture supernatants were harvested following 29-kDa FN-f stimulation for 24 h, and the MMP-13 protein level was determined by enzyme-linked immunosorbent assay (ELISA) using a pro-MMP-13 immunoassay kit according to the manufacturer's instructions (R&D Systems). Plates were read at 450 nm using a Thermo Scientific Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland).

4.5. RT-PCR (Real time-polymerase chain reaction)

Total RNAs were extracted using Trizol (Invitrogen) according to the manufacturer's instructions and reverse-transcribed by M-MLV First Strand cDNA Synthesis Kit (Invitrogen). The expression of miRNAs was measured by quantitative real-time PCR according to the manufacturer's protocol (Ribobio Co.) using Power SYBR Green PCR Master Mix (Invitrogen), and U6 small nuclear RNA was used as an internal normalized reference. Each reaction was performed in triplicate, and the results were analysed using $2^{-\Delta\Delta Ct}$ method.

4.6. RUNX2 quantification

For quantification of RUNX2 protein, human chondrocyte were transfected with pre-miR-Neg or pre-miR-320a (30 nM), and 72 h later cells were collected and washed with PBS. Cells were lysed in 100 µl PBS containing protease inhibitors using five freeze-thaw cycles. Cell lysate was subsequently spun down at maximum speed for 10 min, and supernatant was collected and stored at −80 °C. Subsequently, RUNX2 was quantified using the RUNX2 ELISA kit according to the manufacturer's recommendation (Uscn Life Science Inc., Wuhan, PRC).

4.7. Statistical analysis

All collected data were first tested for normal distribution using one-sample K-S test. Enumeration of data was done by chi-square test or rank-sum test. Data measurement was performed by student t-test (for two groups) or analysis of variance (ANOVA, for more than three groups). Furthermore, between the group comparisons were then done by post-hoc Tukey test. $P<0.05$ was considered as statistically significant.

Acknowledgements: This research received no funding support.

Conflicts of interest: All authors declare no conflict of interest.

References

Aigner T, Fundel K, Saas J, Gebhard PM, Haag J, Weiss T, Zien A, Obermayr F, Zimmer R, Bartnik E (2006) Large-scale gene expression profiling reveals major pathogenetic pathways of cartilage degeneration in osteoarthritis. Arthritis Rheumatol 54: 3533-3544.

Cui X, Wang S, Cai H, Lin Y, Zheng X, Zhang B, Xia C (2016) Overexpression of microRNA-634 suppresses survival and matrix synthesis of human osteoarthritis chondrocytes by targeting PIK3R1. Scientific Reports 6: 23117.

Díaz-Prado S, Cicione C, Muiños-López E, Hermida-Gómez T, Oreiro N, Fernández-López C, Blanco FJ (2012) Characterization of microRNA expression profiles in normal and osteoarthritic human chondrocytes. Osteoarthritis Cartilage 13: 144.

Deugarte L, Yoskovitz G, Balcells S, Güerrifernández R, Martinezdiaz S, Mellibovsky L, Urreizti R, Nogués X, Grinberg D, Garcíagiralt N (2015) MiRNA profiling of whole trabecular bone: identification of osteoporosis-related changes in MiRNAs in human hip bones. BMC Med Genom 8: 1-11.

Goldring MB, Goldring SR (2007) Osteoarthritis. J Cell Physiol 231: 626-634.

Hamam D, Ali D, Vishnubalaji R, Hamam R, Alnbaheen M, Chen L, Kassem M, Aldahmash A, Alajez NM (2014) microRNA-320/RUNX2 axis regulates adipocytic differentiation of human mesenchymal (skeletal) stem cells. Cell Death Dis 5: e1499-e1499.

Jones SW, Watkins G, Good NL, Roberts S, Murphy CL, Brockbank SMV, Needham MRC, Read SJ, Newham P (2009) The identification of differentially expressed microRNA in osteoarthritic tissue that modulate the production of TNF-⊠ and MMP13. Osteoarthritis Cartilage 17: 464-472.

Kim D, Song J, Ahn C, Kang Y, Chun CH, Jin EJ (2014) Peroxisomal dysfunction is associated with up-regulation of apoptotic cell death via miR-223 induction in knee osteoarthritis patients with type 2 diabetes mellitus. Bone 64: 124-131.

Kobayashi T, Lu J, Cobb BS, Rodda SJ, McMahon AP, Schipani E, Merkenschlager M, Kronenberg HM (2008) Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. Proc Natl Acad Sci USA 105: 1949-1954.

Kostopoulou F, Malizos KN, Papathanasiou I, Tsezou A (2015) MicroRNA-33a regulates cholesterol synthesis and cholesterol efflux-related genes in osteoarthritic chondrocytes. Arthritis Res Ther 17: 1-13.

Le LTT, Swingler TE, Clark IM (2013) Review: the role of microRNAs in osteoarthritis and chondrogenesis. Arthritis Rheumatol 65: 1963-1974.

Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20.

Li J (2012) miR-146a, an IL-1β responsive miRNA, induces vascular endothelial growth factor and chondrocyte apoptosis by targeting Smad4. Arthritis Res Ther 14: 1-13.

Little CB, Hughes CE, Curtis CL, Janusz MJ, Bohne R, Wang-Weigand S, Taiwo YO, Mitchell PG, Otterness IG, Flannery CR (2002) Matrix metalloproteinases are involved in C-terminal and interglobular domain processing of cartilage aggrecan in late stage cartilage degradation. Matrix Biol 21: 271-288.

Malemud CJ, Islam N, Haqqi TM (2003) Pathophysiological mechanisms in osteoarthritis lead to novel therapeutic strategies. Cells Tissues Organs 174: 34-48.

Matsukawa T, Sakai T, Yonezawa T, Hiraiwa H, Hamada T, Nakashima M, Ono Y, Ishizuka S, Nakahara H, Lotz MK (2014) MicroRNA-125b regulates the expression of aggrecanase-1 (ADAMTS-4) in human osteoarthritic chondrocytes. Orthoped Surg 65: 685-688.

Meng F, Zhang Z, Chen W, Huang G, He A, Hou C, Long Y, Yang Z, Zhang Z, Liao W (2016) MicroRNA-320 regulates matrix metalloproteinase-13 expression in chondrogenesis and interleukin-1β-induced chondrocyte responses. Osteoarthritis Cartilage 24: 932-941.

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

Qi X, Li J, Zhou C, Lv C, Tian M (2014) MicroRNA-320a inhibits cell proliferation, migration and invasion by targeting BMI-1 in nasopharyngeal carcinoma. FEBS Lett 588: 3732-3738.

Reynard LN, Loughlin J (2012) Genetics and epigenetics of osteoarthritis. Maturitas 71: 200-204.

Santini P, Politi L, Vedova PD, Scandurra R, Scotto dAA (2014) The inflammatory circuitry of miR-149 as a pathological mechanism in osteoarthritis. Rheumatol Int 34: 711-716.

Vonk LA, Kragten AHM, Dhert WJA, Saris DBF, Creemers LB (2014) Overexpression of hsa-miR-148a promotes cartilage production and inhibits cartilage degradation by osteoarthritic chondrocytes. Osteoarthritis Cartilage 22: 145-153.

Wu C, Tian B, Qu X, Liu F, Tang T, Qin A, Zhu Z, Dai K (2014) MicroRNAs play a role in chondrogenesis and osteoarthritis (review). Int J Mol Med 34: 13-23.

Yang M, Ying L, Padgett RW (2005) MicroRNAs: Small regulators with a big impact. Cytokine Growth Factor Rev 16: 387-393.

Yu F, Cui Y, Zhou X, Zhang X, Han J (2011) Osteogenic differentiation of human ligament fibroblasts induced by conditioned medium of osteoclast-like cells. Bioscience Trends 5: 46-51.

Zhang D, Cao X, Li J, Zhao G (2015) MiR-210 inhibits NF-κB signaling pathway by targeting DR6 in osteoarthritis. Scientific Reports 5: 12775.

Zhang Y, Jia J, Yang S, Liu X, Ye S, Tian H (2014) MicroRNA-21 controls the development of osteoarthritis by targeting GDF-5 in chondrocytes. Exper Mol Med 46: e79-e79.