Department of Orthopedics[1]; Department of Radiology[2], Urology Department[3], Hongqi Hospital Affiliated to Mudanjiang Medical University, Mudanjiang City, China

MiR-204 enhances the progression of osteoarthritis by suppressing the production of IL-1β

XianDong Song[1], Min Zhu[2*], Yuanbo Sun[3], Bo Liu[1], Zhaowei Yan[1], Yanwei Yin[2]

Received April 20, 2017, accepted May 26, 2017

[*]*Corresponding author: Dr. Min Zhu, Mudanjiang City, #5 Tongxiang Road, Aimin District, Hongqi Hospital Affiliated to Mudanjiang Medical University, Mudanjiang City, Heilongjiang Province, 157011, China*
zhumin420@sina.com

Pharmazie 72: 587–592 (2017) *doi: 10.1691/ph.2017.7588*

Recent studies suggest that cytokines and microRNAs play a key role in the destruction of cartilage matrix in osteoarthritis (OA) tissues. In the current study, we focused on miR-204, which has never been explored in OA. We found that the level of miR-204 was markedly reduced in the OA cartilage tissues compared with that of normal control. Real time PCR analysis demonstrated that the level of miR-204 was markedly decreased after IL-1β treatment for 3, 6, 12 h in the normal chondrocytes and OA chondrocytes, respectively. Furthermore, overexpression of miR-204 markedly suppressed the protein levels of IL-1β, COX-2 and IL6 in human OA chondrocytes and chondrogenic SW1353 cells. Dual luciferase reporter assay demonstrated that miR-204 significantly suppressed the relative luciferase activity of pmirGLO-IL-1β-3'UTR, indicating that IL-1β was a target gene of miR-204. More importantly, treatment with IL-1β significantly enhanced the protein levels of IL-1β, COX-2 and IL6. However, overexpression of miR-204 could partially abolish such effects. In conclusion, our data demonstrate that reduced miR-204 expression enhances the destruction of the cartilage tissues among OA patients mainly through targeting IL-1β.

1. Introduction

Osteoarthritis (OA) is a degenerative disease, which is characterized by articular cartilage damage and joint marginal osteophyte formation, accompanied by inflammation of synovium in different degree (Blom et al. 2007; Bowles et al. 2014). However, the pathogenesis is still not clear. Recent studies suggest that cytokines play a key role in the destruction of cartilage matrix (Blom et al. 2007; Calich et al. 2010).

Cytokines are non immunoglobulin proteins that are produced in living cells (Chambers et al. 1997). They mainly regulate the target cells through specific cell surface receptors, thereby transferring intracellular information (Chambers et al. 1997). Under normal circumstances, the catabolic and anabolic balance of articular cartilage matrix is maintained by cytokines decomposition and cytokine synthesis (Chen et al. 2015; Mabey et al. 2016). IL-1 is mainly derived from macrophages, fibroblasts, cartilage cells and osteoclasts (Lou et al. 2017). As the inflammation medium, IL-1β significantly destructs the tissues. In cultured normal chondrocytes and normal synovial tissue cells, only limited amounts of IL-1β are produced, but high levels of IL-1β could be detected in the supernatant of OA cells and tissues (Meulenbelt et al. 2010). Therefore, IL-1β could be closely related to the pathological process of synovium and cartilage.

Studies have found that miRs are closely related to the development of osteoarthritis (Buckland 2015; Chen et al. 2015). For instance, miR mediated gene expression inhibition plays an important role in the regulation of osteogenic differentiation. Moreover, the expression level of these endogenous miR has changed significantly in multiple clinical samples (Tornero-Esteban et al. 2015). It is reported that enhanced expression of miR-22 reduces the expression of aggrecan, increases IL-1βand matrix metalloproteinase 13 in cartilage cells. And miR are suggested to participate in the occurrence and development of osteoarthritis through three aspects, transcription suppression, chromatin modification and DNA methylation (Wang et al. 2013; Buckland 2015; Chen et al. 2015; Rasheed et al. 2017).

In the current study, we explored the expression of miR-204 in the tissues of OA patients. Our data showed that IL-1β subunit was a target gene of miR-204. Through targeting IL-1β, decreased miR-204 in the chondrocytes enhanced proinflammation response thereby triggering the inflammatory response.

2. Investigations and results

2.1. Expression of miR-204 and IL-1B in normal and OA cartilage

Firstly, we evaluated the expression of miR-204 and IL-1B in normal and OA cartilage tissues. As shown in Fig. 1A, the level of miR-204 was markedly reduced in the OA cartilage tissues compared with that of normal control. Furthermore, the level of IL-1B was found to be significantly higher than that of control (Fig. 1B).

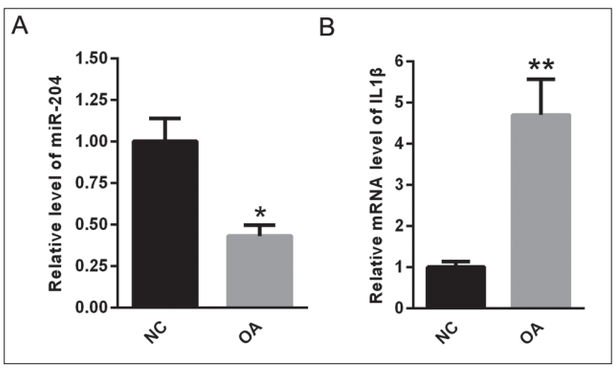

Fig. 1: Decreased miR-204 level and enhanced IL-1β in the OA cartilage tissues compared with that of normal control. (A) Real time PCR analysis of miR-204 level. (B) The level of IL-1B analyzed by ELISA. *P<0.05, **P<0.01, vs. control.

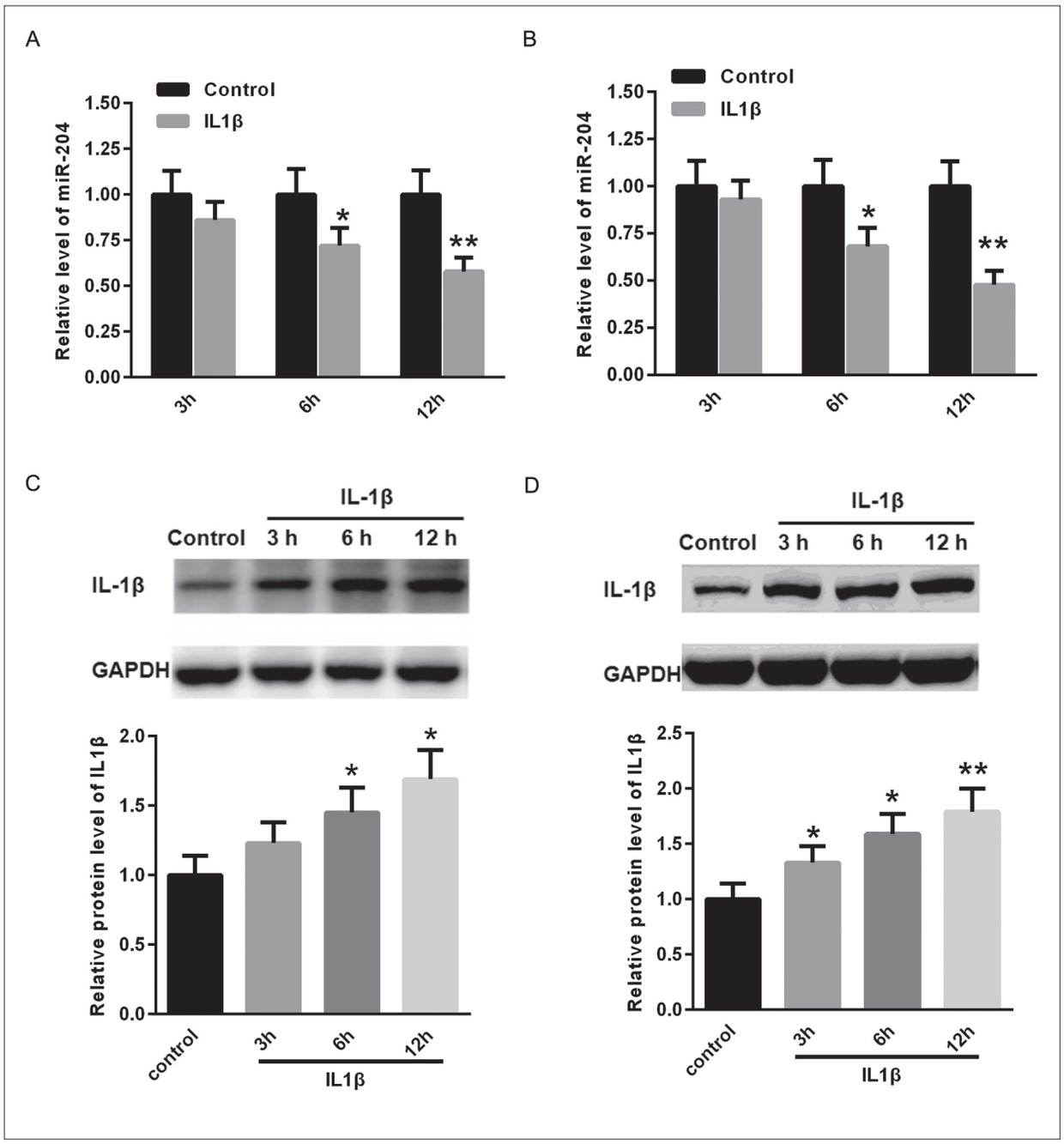

Fig. 2: Reverse correlation between IL-1β-regulated NF-κB and miR-204 expression in normal and OA chondrocytes. Real time PCR analysis of miR-204 in the normal chondrocytes (A) and OA chondrocytes (B). The protein expression of IL-1β was increased IL-1β treatment for 3, 6, 12 h in the normal chondrocytes (C) and OA chondrocytes (D). *P<0.05, **P<0.01, vs. control.

2.2. Reverse correlation between IL-1β-regulated NF-κB and miR-204 expression in normal and OA chondrocytes

To investigate whether miR-204 was involved in IL-1β-induced NF-kB activation, normal chondrocytes and OA chondrocytes were treated with 10 nM of IL-1β. Real time PCR analysis demonstrated that the level of miR-204 was markedly decreased after IL-1β treatment for 3, 6, 12 h in the normal chondrocytes and OA chondrocytes, respectively (Fig. 2A and 2B). Moreover, we determined the protein level of IL-1β after IL-1β treatment. As shown in Fig. 2C and 2D, the protein expression of IL-1β was increased IL-1β treatment for 3, 6, 12 h in a time-dependent manner in the normal chondrocytes and OA chondrocytes. These data indicated a reverse correlation between IL-1β-regulated NF-κB and miR-204 expression in normal and OA chondrocytes.

2.3. Negative regulation of pro-inflammatory factors production by miR-204 in human chondrocytes

Next, we explored the effects of miR-204 on NF-κB signaling activation in both human OA chondrocytes in the presence of 10 nM IL-1β. Western blot analysis demonstrated that overexpression of miR-204 markedly suppressed the protein levels of IL-1B, COX-2 and IL6 in human OA chondrocytes (Fig. 3A). In contrast, the protein levels of IL-1β, COX-2 and IL6 were significantly increased in human OA chondrocytes transfected with miR-204 inhibitors (Fig. 3B). We also collected the supernatant from the culture. As shown in Fig. 3C, overexpression of miR-204 significantly decreased the production of pro-inflammatory factors, including IL-1B, IL-6 and IL-8 in human OA chondrocytes. In contrast, inhibition of miR-204 markedly enhanced the levels of

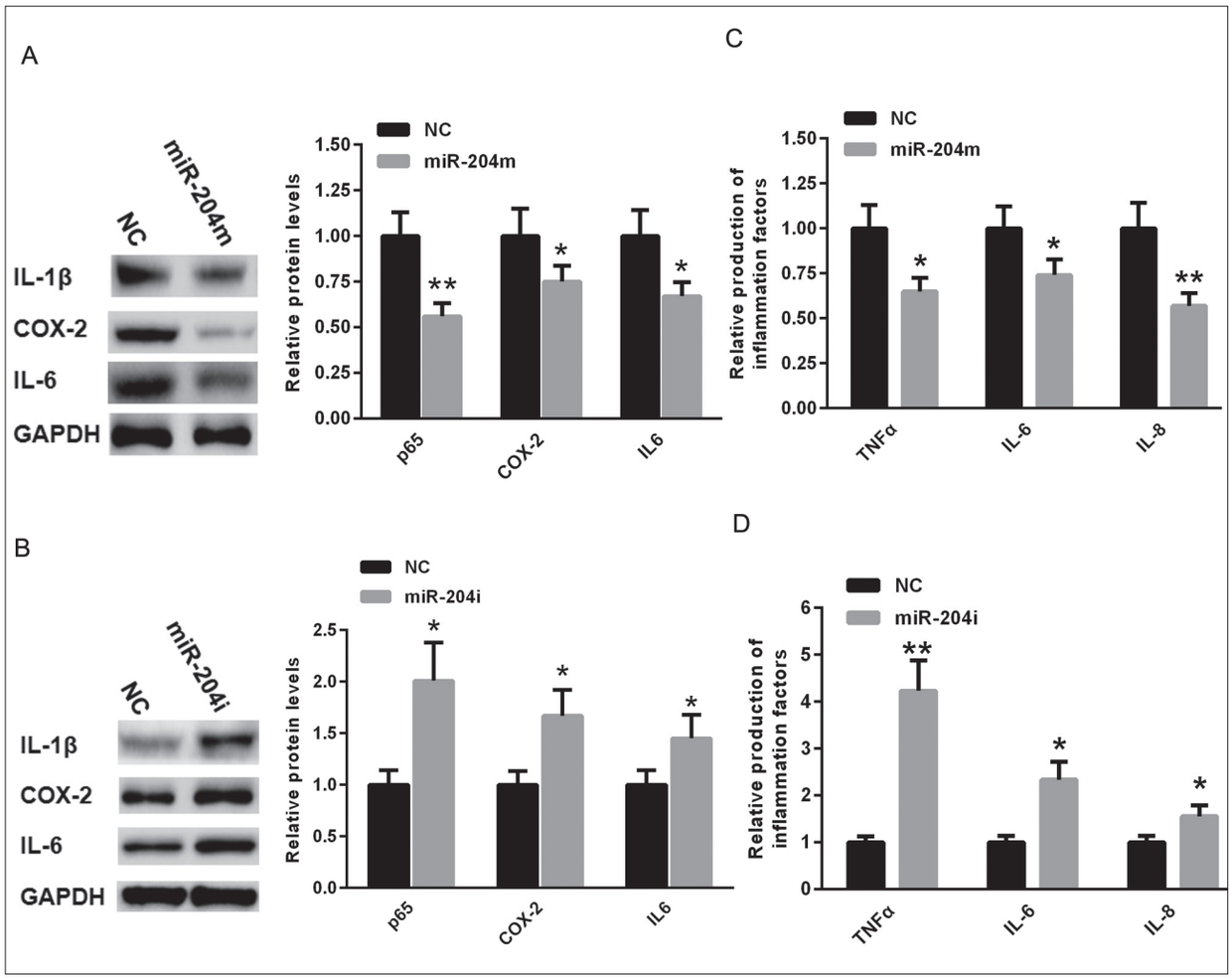

Fig. 3: Negative regulation of pro-inflammation factors production by miR-204 in human chondrocytes. (A) Overexpression of miR-204 markedly suppressed the protein levels of IL-1β, COX-2 and IL6 in human OA chondrocytes. (B) The protein levels of IL-1B, COX-2 and IL6 were significantly increased in human OA chondrocytes transfected with miR-204 inhibitors. (C) Overexpression of miR-204 significantly decreased the production of pro-inflammatory factorin human OA chondrocytes. (D) Inhibition of miR-204 markedly enhanced the levels of IL-1β, IL-6 and IL-8 in human OA chondrocytes. *P<0.05, **P<0.01, vs. control.

IL-1B, IL-6 and IL-8 in human OA chondrocytes (Fig. 3D). These data indicated the anti-inflammation effects of miR-204 in human OA chondrocytes.

2.4. IL-1β was a target gene of miR-204

Then, we analyzed the possible target gene of miR-204. According to TargetScan, a conserved binding site of miR-204 in the 3' untranslated region (3'UTR) of IL-1β was identified (Fig. 4A).

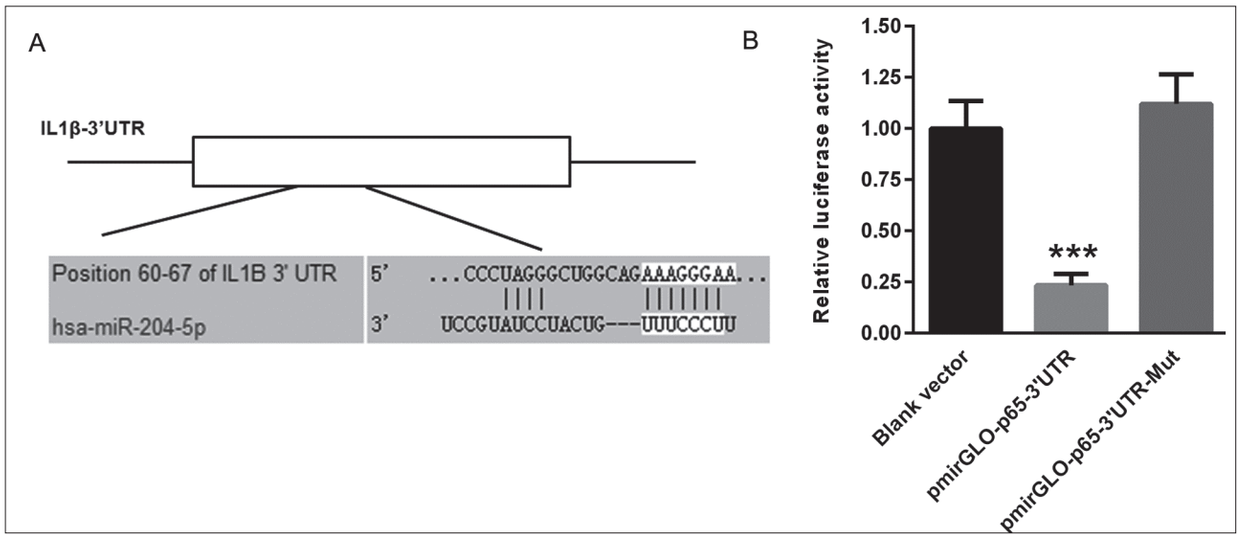

Fig. 4: IL-1β was a target gene of miR-204. (A) One conserved binding site was identified in the 3'UTR of IL-1β. (B) Dual luciferase reporter assay demonstrated that miR-204 significantly suppressed the relative luciferase activity of pmirGLO- IL-1β-3'UTR. *P<0.05, **P<0.01, vs. control.

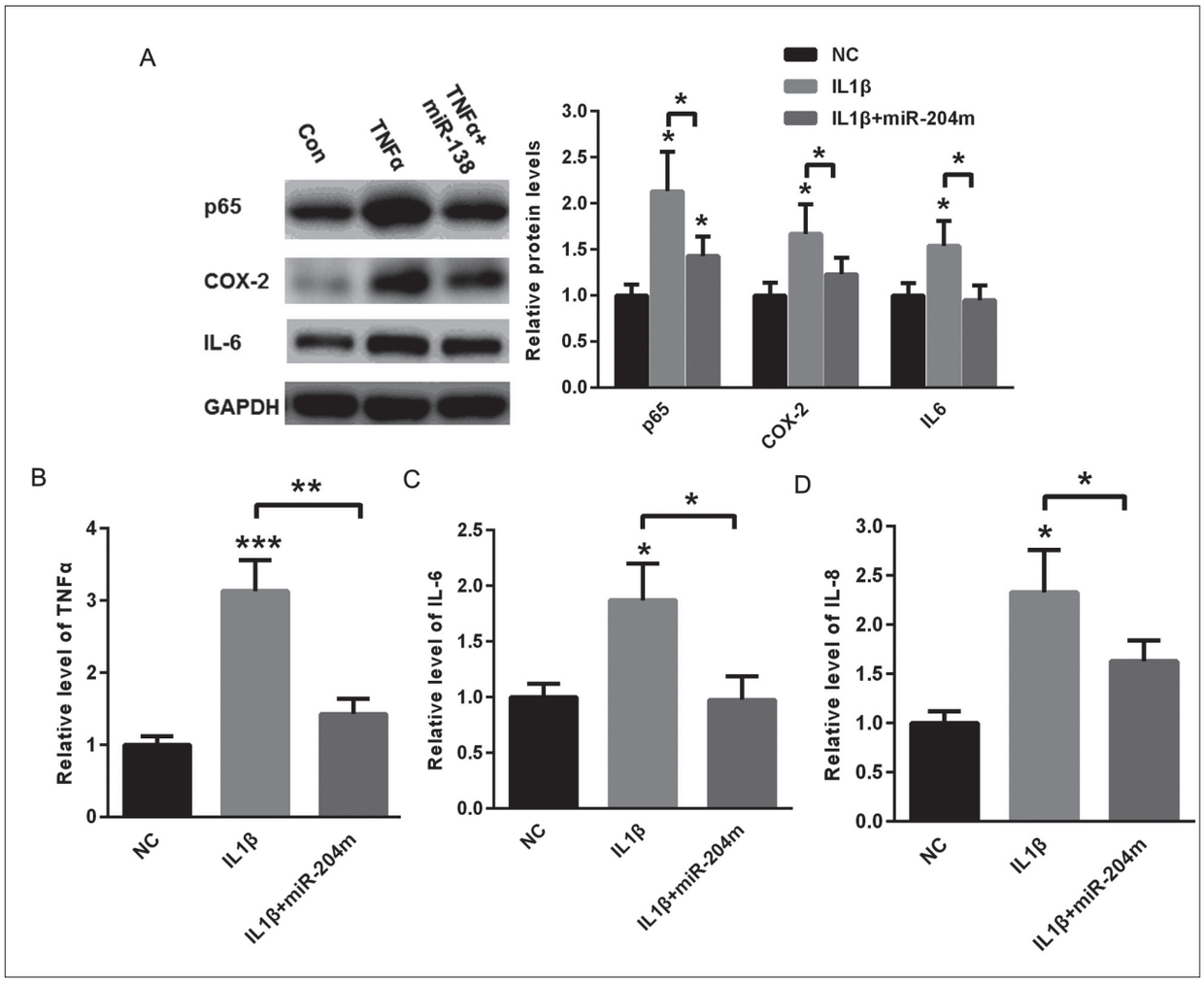

Fig. 5: Overexpression of miR-204 could partially abolish IL-1B-induced inflammation responses. (A) Overexpression of miR-204 could partially abolish IL-1B-induced up-regulation of IL-1β, COX-2 and IL6 protein levels. miR-204 overexpression could reverse the production of IL-1β(B), IL-6 (C) and IL-8 (D) induced by IL-1β. *P<0.05, **P<0.01, vs. control.

Then, the 3'UTR of IL-1β containing the binding site was cloned into the luciferase reporter vector, pmirGLO plasmid, pmirGLO-IL-1β-3'UTR. Dual luciferase reporter assay demonstrated that miR-204 significantly suppressed the relative luciferase activity of pmirGLO- IL-1β-3'UTR. However, when the possible binding sites were muted, no changes of luciferase activity were identified (Fig. 4B).

2.5. Overexpression of miR-204 could partially abolish IL-1β-induced inflammation responses

Next, the effect of miR-204 on IL-1β-induced NF-κB activation was analyzed. As shown in Fig. 5A, treatment with IL-1β significantly enhanced the protein levels of IL-1β, COX-2 and IL6. However, overexpression of miR-204 could partially abolish such effects. Furthermore, we evaluated the inflammation factors. In line with the protein expression changes, IL-1β significantly increased the levels of inflammatory factors, while miR-204 overexpression could reverse the production of IL-1β, IL-6 and IL-8 induced by IL-1β (Fig. 5B, 5C and 5D).

3. Discussion

Osteoarthritis is a degenerative disease, which refers to all lesions involving joint cartilage, subchondral bone, synovium, joint capsule, ligaments and muscles around the joint (Miller et al. 2014). The pathophysiological process is very complex and the etiology is not very clear. For the normal articular cartilage, matrix degradation and repair is a continuous process of balance, which depends on the normal function of cartilage cells (Mabey et al. 2016; Lou et al. 2017). Cartilage cell function may be affected by the decomposition of multiple cytokines. The morphology and function of normal cartilage depends on the ratio of cartilage matrix synthesis and decomposition of cartilage cells. Many studies have confirmed the role of cytokines in the pathophysiology of osteoarthritis. Most research on OA and decomposition of cytokines mainly focus on IL-1β, IL-6 and TNFα (Schlaak et al. 1996; Meulenbelt et al. 2010; Moser 2010; Lv et al. 2012).

MicroRNA is a kind of small non coding RNAs from mammalian cells, which plays a very important role in the regulation of gene expression, especially the regulation mechanism of transcription (Wang et al. 2013, Xu et al. 2016). In recent years, the role of miRNA in disease development has become a research hotspot. Osteoarthritis is a common senile disease, and its etiology is not yet entirely clear (Xu et al. 2015; Wu et al. 2017). The latest research shows that inflammatory mediators are closely associated with miRNA, suggesting that miRNA plays an important role in the occurrence and development of osteoarthritis (Yu et al. 2011; Xu et al. 2016). Thus, by changing the relevant miRNA expression, the therapeutic purpose for osteoarthritis may be achieved. In the current study, we mainly focused on miR-204, which has never been explored in OA patients. Our data showed that the level of

miR-204 was significantly decreased in cartilage tissues of OA patients compared with that of normal control. Further study demonstrated that suppressed miR-204 levels contributed to the inflammatory responses in chondrocytes, indicating an anti-inflammation role of miR-204 in the progression of OA.

By binding to the cell surface specific target IL-1R, IL-1β could cause by OA. IL-1β can promote MMP production and secretion, thereby causing degradation of cartilage matrix, inhibiting the synthesis of and proteoglycan and collagen II, promoting the formation of the type I collagen, so as to promote cartilage cell degeneration (Hess 1990; Kardel et al. 2003; Blom et al. 2007). At the same time, the production of glycoprotein, collagen and macromolecular substances secreted by cartilage is released into the synovial fluid. IL-1β can also promote the production of prostaglandin E2 (PGE2), aggravating joint inflammation, increasing osteoclast activity, and promoting bone absorption (Chambers et al. 1997; Hampel et al. 2013). IL-1β can stimulate the production of MMP and PGE in synovial cells, which further leads to cartilage matrix destruction and enhanced inflammation of synovial membrane (Furuzawa-Carballeda et al. 2008). Here, we first demonstrated that IL-1β was silenced by miR-204. Through suppressing the expression of IL-1β, reduced miR-204 expression contributed to the progression of inflammation responses in chondrocytes. Furthermore, we found that overexpression of miR-204 significantly suppressed the production of IL-1β, IL-6 and IL-8. In contrast, inhibition of miR-204 markedly enhanced the levels of IL-1β, IL-6 and IL-8.

Undoubtedly, cytokines are the mediators of OA articular cartilage damage. However, the theory of cytokines induced cartilage destruction cannot fully explain the mechanism of local cartilage destruction in OA. Because the pathogenesis of 0A is not very clear, the clinical treatment is still symptomatic and the effect is not satisfactory. Our data demonstrate miR-204 expression was decreased in the cartilage tissues of OA patients. Reduced miR-204 levels induced the inflammatory responses in the chondrocytes by targeting IL-1β, which may offer opportunities for the treatment of OA.

4. Experimental

4.1. Patients

Human tissue was obtained from the Hongqi Hospital Affiliated to Mudanjiang Medical University. OA cartilage samples were collected from 22 patients (mean±SD age 64.12±5.75 years) who underwent total joint arthroplasty at Hongqi Hospital Affiliated to Mudanjiang Medical University. OA was diagnosed according to the Chinese College of Rheumatology criteria. Normal cartilage samples were obtained from trauma patients with no known history of OA or RA (n = 3). This investigation was approved by the Human Ethics Committee of Hongqi Hospital Affiliated to Mudanjiang Medical University. Each donor signed an informed consent.

4.2. Preparation of chondrocytes

Firstly, the specimens (cartilage and subchondral bone) were washed with sterile phosphate buffered saline (PBS). Then, the macroscopic cartilage degeneration was examined by India ink staining (Armstrong and Mow 1982). To prepare chondrocytes, the cartilage with a smooth articular surface were selected and then achieved by enzymatic digestion as previously described (Singh et al. 2002). Primary OA chondrocytes at 80% confluence were applied for the study.

Primary chondrocytes and human chondrogenic SW1533 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) supplemented with streptomycin (100 μg/mL) and penicillin (100 U/mL) (Life Technologies, Rockville, MD, USA).

4.3. Chondrocyte treatment and preparation of microRNA

Before IL-1β (20 nM) treatment, OA chondrocytes were serum starved overnight and total RNA was prepared using TRIzol reagent (Invitrogen). To purify microRNAs, the mirVana Kit (Applied Biosystems) was used according to the manufacturer's instructions. For some studies, microRNAs were isolated from normal and OA cartilage samples. In brief, cartilage was ground to a fine powder in liquid nitrogen and then the microRNA was purified as described above.

4.4. Transfection of miRNAs

Human chondrocytes were transfected with the mature type of hsa-miR-204 or the antisense inhibitor, anti-miR-204, at a concentration of 50 nM, using the calcium phosphate precipitation method. SW1353 cells were seeded in 12- or 24-well plates. The following day, cells were transfected with miR-204 or anti-miR-204 at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen, CA, USA), according to the manufacturer's instructions. For the following experiments, cells were used 48 h after transfection. Nonspecific control miR (miR-Control, Dharmacon) was used as a control for off-target effects.

4.5. Reverse transcription-PCR (RT-PCR) analysis

TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) were used to analyze the expression of miR-204. U6 snRNA was used to normalize as a loading control (Cheng et al. 2013). Real-time PCR for mRNAs was performed with the SYBR Green PCR mixture (Invitrogen) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

4.6. Western blotting

Total proteins were isolated from tissues using a total protein extraction kit (Keygen, Nanjing, China). A total of 20 μg protein was separated using SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes and then blocked with 5% fat-free milk at room temperature for 2 h. The immune-blot was incubated with primary antibody detecting IL-1β(1 : 1000 dilution; CST) and GAPDH (1 : 1000 dilution; CST) was used as a control. The signals were detected using a Super ECL Plus Kit (Keygen) and determined by quantitative analysis using UVP software (UVP, LLC, Upland, CA, United States).

4.7. Luciferase reporter assay

HEK293T cells were seeded in 24-well plate at the density of 5×104 cells/well. After 24 h, wild-type or mutated IL-1β-3'-UTR luciferase reporter vector and miR-217 mimic or inhibitor were transfected into cells with Vigofect transfection reagent according to the manufacturer's instruction. After transfection for 48h, the Dual-luciferase reporter assay system (Promega) was applied to determine the changes of relative luciferase units (RLU). Renilla activity was used as the internal control.

4.8. Statistical analysis

Comparisons were performed using the Origin 6.1 software package (1 paired 2-tailed t-test with one-way analysis of variance and Tukey's post hoc analysis). P values less than 0.05 were considered significant, and P values less than 0.001 were considered highly significant.

Conflicts of interest: None declared.

References

Armstrong CG, Mow VC (1982) Variations in the intrinsic mechanical properties of human articular cartilage with age, degeneration, and water content. J Bone Joint Surg Am 64: 88-94.

Blom AB, van der Kraan PM, van den Berg WB (2007) Cytokine targeting in osteoarthritis. Curr Drug Targets 8: 283-292.

Bowles RD, Mata BA, Bell RD, Mwangi TK, Huebner JL, Kraus VB, Setton LA (2014) In vivo luminescence imaging of NF-kappaB activity and serum cytokine levels predict pain sensitivities in a rodent model of osteoarthritis. Arthritis Rheumatol 66: 637-646.

Buckland J (2015) Osteoarthritis: MicroRNA-mediated meniscal injury repair in rats. Nat Rev Rheumatol 11: 64.

Calich AL, Domiciano DS, Fuller R (2010) Osteoarthritis: can anti-cytokine therapy play a role in treatment? Clin Rheumatol 29: 451-455.

Chambers MG, Bayliss MT, Mason RM (1997) Chondrocyte cytokine and growth factor expression in murine osteoarthritis. Osteoarthritis Cartilage 5: 301-308.

Chen D, Shen J, Hui T (2015) Epigenetic and microRNA regulation during osteoarthritis development. F1000Res 4.

Chen J, Sun G, Chen F, Fang Y, Gates MH, Yang S (2015) T-cell immunoglobulin domain and mucin domain 3 polymorphism affects cytokine expression in different cells and is associated with increased susceptibility to knee osteoarthritis. Gene 566: 32-36.

Furuzawa-Carballeda J, Macip-Rodriguez PM, Cabral AR (2008) Osteoarthritis and rheumatoid arthritis pannus have similar qualitative metabolic characteristics and pro-inflammatory cytokine response. Clin Exp Rheumatol 26: 554-560.

Hampel U, Sesselmann S, Iserovich P, Sel S, Paulsen F, Sack R (2013) Chemokine and cytokine levels in osteoarthritis and rheumatoid arthritis synovial fluid. J Immunol Methods 396: 134-139.

Hess EV (1990) Cytokine inhibitors and osteoarthritis. J Rheumatol 17: 1123-1124.

Kardel R, Ulfgren AK, Reinholt FP, Holmlund A (2003) Inflammatory cell and cytokine patterns in patients with painful clicking and osteoarthritis in the temporomandibular joint. Int J Oral Maxillofac Surg 32: 390-396.

Lou Y, Wang C, Zheng W, Tang Q, Chen Y, Zhang X, Guo X, Wang J (2017) Salvianolic acid B inhibits IL-1beta-induced inflammatory cytokine production in human osteoarthritis chondrocytes and has a protective effect in a mouse osteoarthritis model. Int Immunopharmacol 46: 31-37.

Lv C, Xu X, Wang J, Zhang Z, Zhang S, Guo C, Geng C, Sun Y (2012) Combined effect of cytokine gene polymorphisms on end-stage knee osteoarthritis from Chinese Han population. Rheumatol Int 32: 3625-3629.

Mabey T, Honsawek S, Tanavalee A, Yuktanandana P, Wilairatana V, Poovorawan Y (2016) Plasma and synovial fluid inflammatory cytokine profiles in primary knee osteoarthritis. Biomarkers 21: 639-644.

Meulenbelt I, Bos SD, Kloppenburg M, Lakenberg N, Houwing-Duistermaat JJ, Watt I, de Craen AJ, van Duijn CM, Slagboom PE (2010) Interleukin-1 gene cluster variants with innate cytokine production profiles and osteoarthritis in subjects from the Genetics, Osteoarthritis and Progression Study. Arthritis Rheum 62: 1119-1126.

Miller RE, Miller RJ, Malfait AM (2014) Osteoarthritis joint pain: the cytokine connection. Cytokine 70: 185-193.

Moser C (2010) Response to: cytokine profile of autologous conditioned serum for treatment of osteoarthritis, in vitro effects on cartilage metabolism and intra-articular levels after injection. Arthritis Res Ther 12: 410; author reply 411.

Rasheed Z, Rasheed N, Al-Shaya O (2017) Epigallocatechin-3-O-gallate modulates global microRNA expression in interleukin-1beta-stimulated human osteoarthritis chondrocytes: potential role of EGCG on negative co-regulation of microRNA-140-3p and ADAMTS5. Eur J Nutr doi: 10.1007/s00394-016-1375-x

Schlaak JF, Pfers I, Meyer Zum Buschenfelde KH, Marker-Hermann E (1996) Different cytokine profiles in the synovial fluid of patients with osteoarthritis, rheumatoid arthritis and seronegative spondylarthropathies. Clin Exp Rheumatol 14: 155-162.

Singh R, Ahmed S, Islam N, Goldberg VM, Haqqi TM (2002) Epigallocatechin-3-gallate inhibits interleukin-1beta-induced expression of nitric oxide synthase and production of nitric oxide in human chondrocytes: suppression of nuclear factor kappaB activation by degradation of the inhibitor of nuclear factor kappaB. Arthritis Rheum 46: 2079-2086.

Tornero-Esteban P, Rodriguez-Rodriguez L, Abasolo L, Tome M, Lopez-Romero P, Herranz E, Gonzalez MA, Marco F, Moro E, Fernandez-Gutierrez B, Lamas JR (2015) Signature of microRNA expression during osteogenic differentiation of bone marrow MSCs reveals a putative role of miR-335-5p in osteoarthritis. BMC Musculoskelet Disord 16: 182.

Wang JH, Shih KS, Wu YW, Wang AW, Yang CR (2013) Histone deacetylase inhibitors increase microRNA-146a expression and enhance negative regulation of interleukin-1beta signaling in osteoarthritis fibroblast-like synoviocytes. Osteoarthritis Cartilage 21: 1987-1996.

Wang M, Liu C, Zhang Y, Hao Y, Zhang X, Zhang YM (2013) Protein interaction and microRNA network analysis in osteoarthritis meniscal cells. Genet Mol Res 12: 738-746.

Wu W, He A, Wen Y, Xiao X, Hao J, Zhang F, Guo X (2017) Comparison of microRNA expression profiles of Kashin-Beck disease, osteoarthritis and rheumatoid arthritis. Sci Rep 7: 540.

Xu B, Li YY, Ma J, Pei FX (2016) Roles of microRNA and signaling pathway in osteoarthritis pathogenesis. J Zhejiang Univ Sci B 17: 200-208.

Xu JF, Zhang SJ, Zhao C, Qiu BS, Gu HF, Hong JF, Cao L, Chen Y, Xia B, Bi Q, Wang YP (2015) Altered microRNA expression profile in synovial fluid from patients with knee osteoarthritis with treatment of hyaluronic acid. Mol Diagn Ther 19: 299-308.

Yu C, Chen WP, Wang XH (2011) MicroRNA in osteoarthritis. J Int Med Res 39: 1-9.