



Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

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Original article

MicroRNA-186 ameliorates Knee osteoarthritis via regulation of P2X7-mediated Cathepsin-K/Runx2/ADAMTS5 signalling axis in articular chondrocytes

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ARTICLE INFO

Article history:

Received 24 May 2021

Revised 26 June 2021

Accepted 27 June 2021

Available online 3 July 2021

Keywords:

Chondrocytes

Knee osteoarthritis

Cathepsin-K

Cartilage degeneration

ABSTRACT

Knee osteoarthritis (KOA) is a chronic joint disorder involving the articular cartilage and tissues around the synovial joint. The key objective of this study was to determine the effect of miR-186-5p administration on the expression of pathogenic signalling in the chondrocytes using a surgical destabilization of the medial meniscus (DMM) model of KOA, and to testify the mechanism of P2X7-mediated regulation of RUNX2/ADAMTS5 axis by miR-186 in the KOA rats. After eight weeks of intra-articular injection of the miR-186-5p and negative control lentivirus samples, the knee cartilage tissues were subjected to histopathological analysis Safranin-O/Fast green staining. Further, the articular chondrocytes were separated and analysed for various proteins including P2X7, cathepsin-K, RUNX2 and ADAMTS5 using Western blotting method. We observed that the protein expressions of P2X7, cathepsin-K/RUNX2/ADAMTS5, and also MMP-13 were upmodulated in the KOA rats, while intra-articular miR-186-5p lentivirus administration prevented these aberrations.

Hence, the study concludes that miR-186 orchestrates P2X7 expression and the P2X7-mediated cathepsin-K/RUNX2/ADAMTS5 axis and regulates the pathogenesis of KOA. In light of this evidence, we propose that molecular therapeutic interventions targeting miR-186 activation might attenuate osteoarthritic cartilage degeneration.

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1. Introduction

Knee osteoarthritis (KOA) is a chronic progressive joint deterioration disease involving the articular cartilage and tissues around the synovial joint (Livings et al., 2020). KOA is associated with an increased death risk when compared to the normal population (Heidari, 2011). Further, apart from the geriatric population, even the younger obese women are more likely to get KOA, when compared to their healthy population counterpart. To add fuel to the

fire, global restrictions and the fear about COVID-19 contagion pose challenges to the physical and mental well-being of the osteoarthritic patients, who are mainly reliant on appropriate mobility. Chondrocyte senescence, aberrant proteolytic extracellular matrix erosion, periarticular muscle deterioration, subchondral bone sclerosis, osteophyte formation, ligament rupture and pain are the common manifestations of KOA. Despite the deep knowledge about KOA, clinical researchers are still striving to fathom out the pivotal reason behind the pathogenesis of KOA (Ragni et al., 2020).

Although an array of chemically-induced and spontaneous animal models of osteoarthritis are used widely, surgical models offer the merits of rapid disease onset, reduced inconsistency, and reduced inclination on genetic predisposition (Glasson et al., 2007). Various studies based on quantitative magnetic resonance imaging and 3D- finite-element analysis (Berthiaume et al., 2005; Li et al., 2019) revealed that medial meniscus damage is robustly associated with the exacerbation of KOA. Recently, a meta-analysis report by Faucett et al. (2019) reported that surgical med-

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Peer review under responsibility of King Saud University.



ial meniscus repair could be a desirable intervention, when compared to meniscectomy or non-operative therapies for KOA. Hence, understanding of the KOA pathophysiology using a rodent model of surgical medial meniscus destabilization (MMD) offers deeper insights (Glasson et al., 2007).

In our study, we chose to explore the influence of microRNA-186 (miR-186) in modulating the progression of KOA using a mouse model of MMD. MicroRNAs—a family of miniature non-coding RNA molecules with a short nucleotide sequence—are known to interfere with the functions of downstream target genes in protein biosynthesis. MiR-186, originally discovered from Saos-2 (a human osteoblast sarcoma cell line), is known to exhibit anti-apoptotic and anti-inflammatory effects in cardiomyocytes and intestinal-immune cells respectively (Xu et al., 2017; Nadorp and Soreq, 2015). In addition, miR-186 was shown to repress interleukin-2 (IL-2)-mediated inhibition of Janus kinase/signal transducers and activators of transcription (JAK-STAT) axis and abrogate neuronal apoptosis in Alzheimer's disease (Wu et al., 2018). A clinical study by Chilton et al. (2014) demonstrated that miR-186 plays a pivotal role in enhancing the immune functions in healthy volunteers via regulation of telomere homeostasis. A recent study revealed that miR-186 enhances fracture healing via modulation of bone morphogenetic protein (BMP)-SMAD6 signalling axis (Wang et al., 2019). Besides, upmodulation of miR-186 abrogated chondrocyte apoptosis and promoted chondrocyte proliferation in osteoarthritic mice via inhibition of the PI3K-AKT pathway (Lin et al., 2019). However, the researchers used papain as the osteoarthritis (OA) inducing agent, which has a lesser clinical relevance than the destabilization of the medial meniscus (DMM) model (Thyssen et al., 2015).

A seminal study by Glasson et al. (2005) demonstrated that genetic deletion of a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5; aggrecanase-2) averts cartilage degradation in osteoarthritis. Besides, Larkin et al. (2015) proposed that GSK2394002, a humanized monoclonal antibody selective for ADAMTS5, effectively impedes cartilage degradation and alleviates allodynia in OA patients. In addition to these beneficial effects, GLPG1972, a selective ADAMTS5 inhibitor, showed anti-catabolic subchondral bone protection effects (Clement-Lacroix et al., 2017). However, the molecular link between miR-186 and ADAMTS5 has not yet been elucidated in any model of osteoarthritis. This study was designed to investigate the plausible molecular mechanism that connects miR-186 and ADAMTS5 and thus, may throw light on the potential therapeutic target in the management of KOA.

2. Materials and methods

2.1. Animals and treatment

KOA was triggered by surgical destabilization of the medial meniscus (DMM) in 8–10 weeks old male rats. Sham-operated rats, operated without any medial meniscus destabilization, served as the normal control group. After eight weeks of intra-articular injection of the miR-186-5p containing and negative control lentivirus samples, the cartilage tissues was separated and analysed. The care and use of animals used in the study were approved by our Institutional Animal Care and Use Committee. Besides, international animal care and handling guidelines were followed as per the protocol of National Institute of Health.

2.2. Preparation of lentiviral vectors

The transfection reagent Lentifectin (ABM) was used to transfect miR-186-5p and negative control lentiviruses into human

293FT cells and were cultured overnight in the incubation medium. Followed by that, concentrated lentiviral vectors were prepared for the injections.

2.3. Dual-luciferase reporter gene assay

The miR-186 binding sites of the sequences in the 3'-untranslated region (UTR) of the P2X7 gene were predicted using microRNA.org platform and the luciferase assay was performed. After 48 h of incubation, cells were assayed with the Dual-Luciferase Assay (Promega).

2.4. Western blot analysis

The sodium dodecyl sulphate – polyacrylamide gel electrophoresis was used for the separation of proteins from the knee articular chondrocytes, which are then impregnated onto a polyvinylidenedifluoride (PVDF) membrane (Millipore, Billerica, MA). Blocking solution was applied to block the membrane after washing, followed by overnight incubation with specific primary antibodies: P2X7, Cathepsin-K, RUNX2, MMP-13, and ADAMTS5 (Abcam, USA). The antibody-blotted membranes were rinsed and subsequently incubated with secondary HRP (horseradish peroxidase)-conjugated antibodies (Santa Cruz Biotechnology, Inc., CA). At the end, quantitative analysis of the chemiluminescent bands was done by using Tanon imaging system.

2.5. Histological analysis

Histological investigation of all the groups including sham, KOA, miR-186-5p-treated and miR control treated groups was done by using Safranin-O/Fast green staining.

2.6. Statistical analysis

SPSS software was applied for the analysis of experimental results and was indicated as mean \pm standard deviation (SD) (V13.0; SPSS, Inc., USA). One-way analysis of variance (ANOVA) was applied using Tukey's post-hoc test and the results were compared among experimental and control groups. Significant level was kept at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. MiR-186-5p treatment attenuated cartilage degeneration and chondrocyte apoptosis in KOA rats

In this study, DMM surgery was performed to mimic human KOA in a rat model. We observed that intra-articular injection of lentiviral miR-186-5p particles significantly mitigated the cartilage degeneration, manifested as increased chondrocyte cellularity, improved OARSF medial femoral and tibial plateau scores against DMM-provoked KOA ($p < 0.05$) (Fig. 1). Further assessment of chondrocyte apoptosis depicted increased chondrocyte apoptosis in the KOA rats, when compared to the sham-operated rats. However, miR-186-5p treatment significantly alleviated the chondrocyte apoptosis against KOA pathogenesis ($p < 0.05$) (Fig. 2). However, no significant difference was observed between miR-control and KOA group rats ($p > 0.05$).

3.2. MiR-186-5p administration inhibited P2X7 expression in KOA rats

P2X7 play a pivotal role in eliciting inflammatory joint damage and pain in KOA. In our study, we noticed that P2X7 expression was upmodulated in the chondrocytes of DMM-provoked KOA rats,

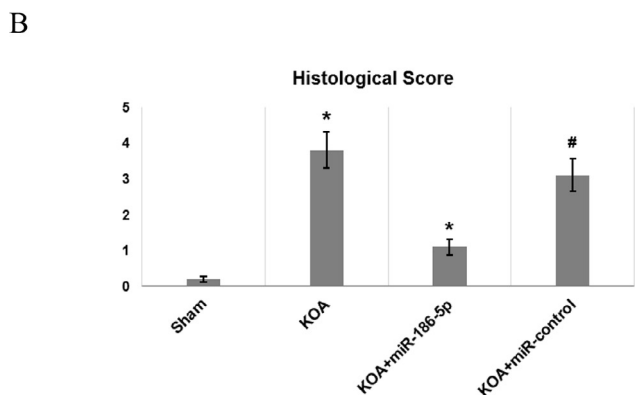
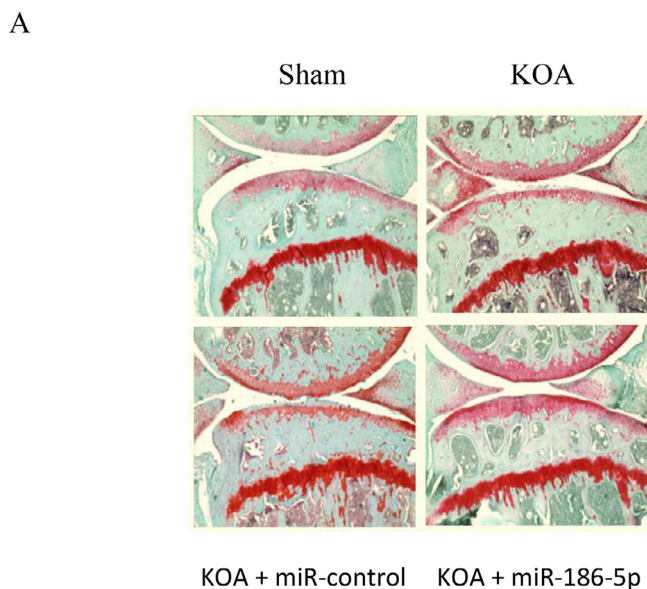


Fig. 1. (A) Representative histopathological images of safranin-O/fast green stained sections of sham, KOA, miR-186-5p-treated and miR control treated groups. (B) Histological scoring of sections across all the groups. Shown data were indicated as mean ± SEM. *Sham vs. KOA, *KOA vs. miR-186-5p-treated, #miR-control vs. KOA; $p < 0.05$.

when compared to the sham-operated rats. In contrary, P2X7 expression was significantly downmodulated in the miR-186-5p injected group ($p < 0.05$) (Fig. 3). Nevertheless, no significant difference was observed between miR-control and KOA group rats.

3.3. MiR-186-5p administration inhibited cathepsin-K mediated activation of RUNX2/ADAMTS5 and RUNX2/MMP-13 signalling axes in KOA rats

Cathepsin-K has a well-established role in the pathogenesis of inflammatory joint diseases like KOA, which is harmony with our observation in the KOA rats. Cathepsin-K being a downstream executor of RUNX2/ADAMTS5 and RUNX2/MMP-13 signalling activation, blocking the activation of P2X7-mediated of RUNX2/ADAMTS5 and RUNX2/MMP-13 signalling by miR-186-5p administration, further significantly down modulated the protein levels of RUNX2, ADAMTS5 and MMP-13 against surgical DMM-provoked KOA in the rats ($p < 0.05$) (Figs. 4–6). On the other side, miR-control injected rats displayed no significant difference between the KOA modelled rats.

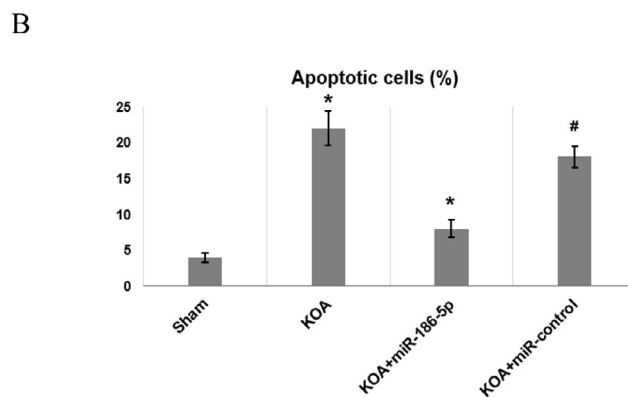
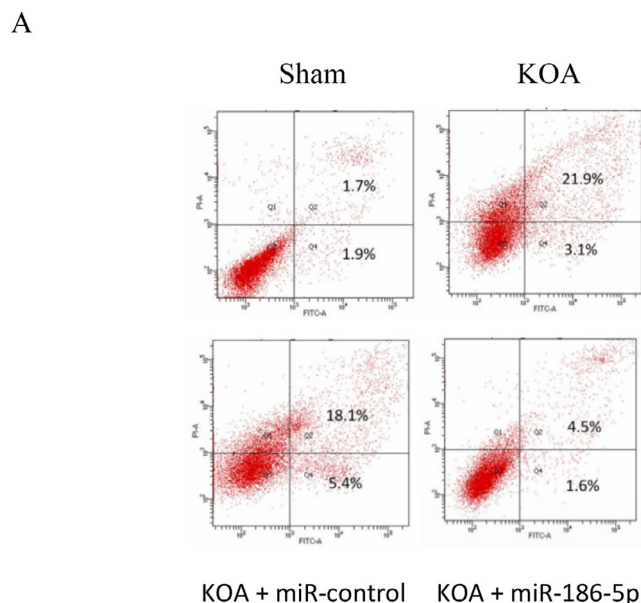


Fig. 2. (A) Flow cytometry images showing percentage of apoptotic cells in sham, KOA, miR-186-5p treated and miR control treated groups. (B) Quantification of apoptotic cells in sham, KOA, miR-186-5p-treated and miR control treated groups. Shown data were indicated as mean ± SEM. *Sham vs. KOA, *KOA vs. miR-186-5p-treated, #miR-control vs. KOA; $p < 0.05$.

4. Discussion

A gamut of scientific reports accentuated that chondrocyte-associated detrimental changes are pivotal in the pathogenesis of KOA. The pathological manifestations of chondrocyte malfunction include oxidative stress, inflammation, mitochondrial dysfunction, hypertrophy, autophagy, senescence, chondroptosis, altered chondrocyte volume and disrupted chondrocyte-pericellular matrix interaction (Qin et al., 2019; Hall, 2019; Guilak et al., 2018). Of note, diverse interconnected and independent molecular signalling pathways are associated with these aberrations. In this study, we chose to analyze the plausible molecular connections between miR-186, a microRNA with anti-osteoarthritic potential (Lin et al., 2019) and ADAMTS5, one of the most central regulators of OA pathology (Glasson et al., 2005; Larkin et al., 2015; Clement-Lacroix et al., 2017).

A preclinical study by Hoshi et al. (2017) displayed that combined silencing of ADAMTS5 and MMP13 using their corresponding small interfering RNAs (siRNAs), has the same cartilage protection

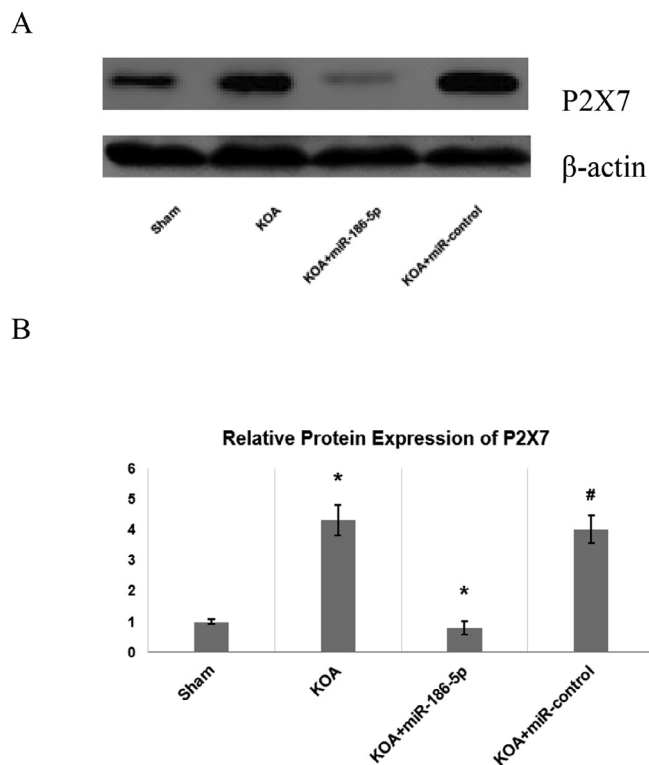


Fig. 3. (A) Representative western blot image of P2X7 expression. (B) Quantification of P2X7 protein expression level. Shown data were indicated as mean ± SEM. *Sham vs. KOA, *KOA vs. miR-186-5p-treated, #miR-control vs. KOA; p < 0.05.

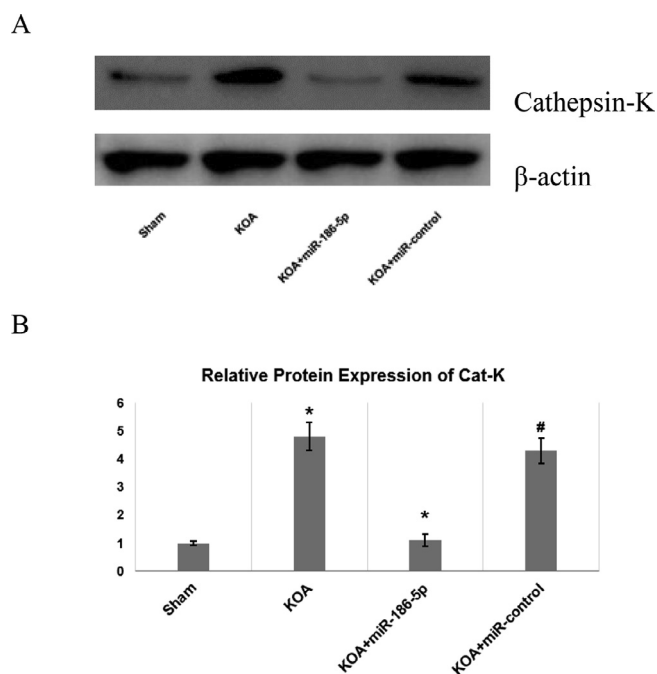


Fig. 4. (A) Representative western blot image of Cathepsin-K expression. (B) Quantification of Cathepsin-K protein expression level. Shown data were indicated as mean ± SEM. *Sham vs. KOA, *KOA vs. miR-186-5p-treated, #miR-control vs. KOA; p < 0.05.

effect as that of MMP13 siRNA alone. This implies that repression of MMP13 might be an effective strategy that repression of aggregase functions in the early phase of OA. In this line, pharmacological inhibition of P2X purinoceptor7 (P2X7) receptor (P2X7R)

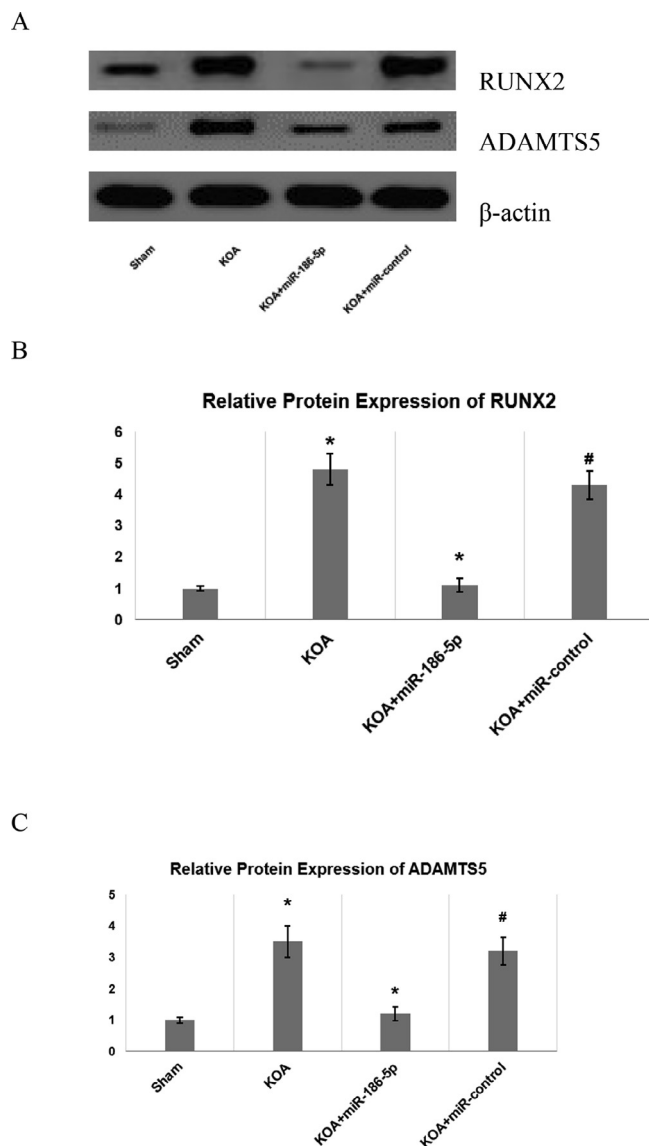


Fig. 5. (A) Representative western blot images of RUNX2 and ADAMTS5 expressions. (B) Quantification of RUNX2 and ADAMTS5 protein expression levels. Shown data were indicated as mean ± SEM. *Sham vs. KOA, *KOA vs. miR-186-5p-treated, #miR-control vs. KOA; p < 0.05.

is known to avert cartilage degradation via suppression of MMP13 and NF-κB pathways (Hu et al., 2016). In our study, we found that the expression of P2X7 and MMP13 were upmodulated in the KOA rats. In contrary, miR-186 activation, using a miR-186-5p mimic, prevented the upmodulation of P2X7 and MMP13 protein levels in the articular chondrocytes of KOA rats. Interestingly, KOA rats depicted severe cartilage injury, while miR-186-5p administration stalled the cartilage degeneration. These observations are in concordance with the earlier reports, wherein miR-186 expression was shown to exhibit anti-apoptotic effect through down-modulation of P2X7 expression (Zhou et al., 2008; Sha et al., 2015).

Interestingly, Lopez-Castejon et al. (2010) proposed that activation of P2X7 receptor triggers the release of various cathepsins including cathepsin K, leading to tissue damage in joint diseases. Further, Ma et al. (2018) showed that miR 186 mimics protect against osteoporotic bone erosion via repression of cathepsin K. Another study revealed that cathepsin K knock-out stalled the progression of osteoarthritis via down-modulation of ADAMTS5

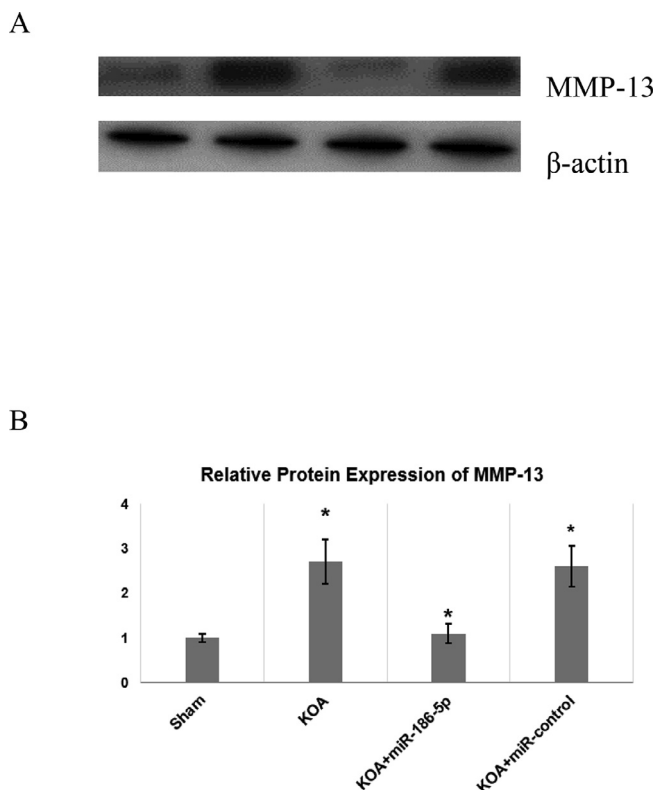


Fig. 6. (A) Representative western blot image of MMP-13 expression. (B) Quantification of MMP-13 protein expression level. Shown data were indicated as mean \pm SEM. *Sham vs. KOA, *KOA vs. miR-186-5p-treated, #miR-control vs. KOA; $p < 0.05$.

expression in chondrocytes (Kozawa et al., 2012). However, some studies accentuate that Runt-related transcription factor 2 (RUNX2), a regulator of chondrocyte development and hypertrophy, is an upstream regulator of ADAMTS5 (Mokuda et al., 2019). Besides, repression of RUNX2 is known to downregulate the protein expression of ADAMTS5 in articular chondrocytes and thus, offers protection from osteoarthritic articular cartilage damage (Thirunavukkarasu et al., 2007). In our study, miR-186-5p administration down-modulated the protein expression levels of cathepsin K, RUNX2 and ADAMTS5 against the abnormally increased protein expression levels of these parameters in the articular chondrocytes of KOA rats. A study by Yi et al. (2017) supported our finding that blockage of cathepsin K inhibits the downstream RUNX2/ADAMTS5 axis in KOA. Besides, transcriptional activation of RUNX2 positively modulates expression of MMP-13 and ADAMTS5 in chondrocyte-like cells (Tetsunaga et al., 2011), indicating that downregulation of RUNX2 by miR-186-5p administration might have suppressed the expression of 13 and ADAMTS5 in the chondrocytes of KOA rats. However, inhibitory effect of miR-186-5p on the protein expression of cathepsin K might have directly or through RUNX2 downmodulated the protein expression of MMP-13 and ADAMTS5 in the chondrocytes of KOA rats (Tetsunaga et al., 2011).

5. Conclusions

On the basis of our study outcomes, we propose that miR-186 activation represses P2X7-mediated activation of cathepsin K and its direct/indirect downstream targets: RUNX2, ADAMTS5 and MMP-13 leading to chondrocyte protection against osteoarthritic cartilage degeneration. Hence, molecular therapeutic interventions targeting miR-186 activation might abrogate osteoarthritic cartilage damage. However, further studies are warranted to ascertain

the long-term clinical benefits of miR-186 modulation in osteoarthritic patients.

6. Data availability

All the data can be acquired by reasonable request from the corresponding author.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81860399), the Science and Technology Plan Project of Jiangxi Provincial Health Commission(202130504), Jiangxi Provincial Key Research and Development Program (20202BBG72001) and the Provincial Youth Science Fund of Jiangxi (20142BAB215072).

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