Upregulated ox40l Can Be Inhibited by miR-146a-5p in Condylar Chondrocytes Induced by IL-1β and TNF-α: A Possible Regulatory Mechanism in Osteoarthritis

Ding Yu¹ Wang Wei¹ Yang Hefeng¹ Li Weihao¹ Qu Qianqian¹ Li Song¹

¹Department of Dental Research, The Affiliated Stomatology Hospital of Kunming Medical University, Kunming, China; ²Department of Orthodontics, The Affiliated Stomatology Hospital of Kunming Medical University, Kunming, China

Keywords
Osteoarthritis · Chondrocytes · MicroRNA · OX40 ligand · Inflammation

Abstract
Introduction: Osteoarthritis (OA) is a common musculoskeletal disease characterized by pain, stiffness, limited activity, occasional effusion, and local inflammation. MiR-146 is one of the noncoding RNA closely related to OA, but the role of miR-146 in OA remains controversial. The tumour necrosis factor receptor OX40 is activated by its cognate ligand OX40L (TNFSF4) and functions as a T-cell costimulatory molecule. The T-cell functions, including cytokine production, expansion, and survival, are enhanced by the OX40 costimulatory signals. Methods: We established an inflammatory model of condylar chondrocytes induced by IL-1β and TNF-α and detected the expression of miRNA by miRNA sequencing. Then, cell transfection was used to study the role of miR146a-5p in OA. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and database analysis were used to screen out potential target genes of miR-146a-5p. A dual luciferase activity assay tested whether ox40l is the target gene of miR-146a-5p. Results: MiR-146a-5p and OX40L was upregulated after induced by IL-1β and TNF-α, miR-146a-5p reduced the production of inflammatory factors but had no effect on chondrophenotypic factors, and ox40l was targeted by miR-146a-5p. Conclusion: OX40L and miR-146a-5p of condylar chondrocytes in the inflammatory environment (induced by IL-1β and TNF-α) were significantly increased, miR-146a-5p is a protective factor in the inflammatory response, which can reduce the production of inflammatory factors, and miR-146a-5p may regulate T-cell-mediated immunity through targeting of ox40l in OA.

Introduction
Osteoarthritis (OA) is a common musculoskeletal disease characterized by pain, stiffness, limited activity, occasional effusion, and local inflammation, which adversely affect patients’ quality of life [1]. OA has a high incidence worldwide. Woolf and Pfelger [2] estimated that...
9.6% of men and 18% of women over 60 years old may suffer from OA. MicroRNA (miRNA) is an endogenous noncoding RNA with 20–24 nucleotides, which can interact with homologous mRNA and regulate gene transcription by enhancing degradation or inhibiting translation. In 2008, Iliopoulos et al. [3] proposed that miRNA may play key roles in OA development. Due to the popularization of miRNA sequencing technology, a large number of miRNA which are closely related to OA have been found [4].

miR-146 is one of the noncoding RNA closely related to OA. Yamasaki et al. [2] studied the expression of miR-146a in the joints of 15 patients with OA in 2009, and their results showed that the expression of miR-146a increased in the patients with mild disease (Mankin score level 1), suggesting that miR-146 may play a role in the development of OA [5]. Sun et al. [6] and Zhang et al. [7] proved that miR-146 could target cxc4 and camk2d to accelerate the progress of OA. Guan et al. [8] and Taganov et al. [9] suggested that miR-146 may play a role in the development of OA [5]. Sun et al. [6] and Zhang et al. [7] proved that miR-146a can reduce the inflammatory response by inhibiting the production of inflammatory factors such as Notch1, IL-6, and IL-1. Zhong et al. [10] found that miR-146a was targeted to TRAF6 in human chondrocytes of OA, thus blocking the NF-kb signaling pathway. It can be seen that, although the expression of miR-146 increased in OA lesions, the role of miR-146 in OA remains controversial.

The temporomandibular joint (TMJ), as the most important joint in the maxillofacial region, plays an important role in speech, swallowing, expression, and facial development. TMJ-OA is a degenerative disease characterized by progressive cartilage degeneration, subchondral bone remodeling, local inflammation, chronic pain, and dysfunction [11]. Current research about miRNA and OA is primarily focused on the knee, hip, and other joints; less research has focused on the role of miR-146 in TMJ-OA.

The tumor necrosis factor receptor OX40 (CD134) is activated by its cognate ligand OX40L (CD134L, CD252, and TNFSF4) and functions as a T-cell costimulatory molecule [12]. OX40L is expressed in many cells, including antigen-presenting cells (APC), T cells, vascular endothelial cells, mast cells, and natural killer cells [13]. According to the conventional understanding of OX40 costimulation, an interaction between OX40 and OX40L, occurs when activated T cells bind to APC. The T-cell functions, including cytokine production, expansion, and survival, are enhanced by the OX40 costimulatory signals [14]. The interaction between OX40 and OX40L is closely related to local inflammatory responses that appear critical for both effective T-cell-mediated responses and chronic immune pathologies [15]. In this study, we detected the expression of OX40L and miR-146a-5p in an vitro inflammatory model of rat condylar chondrocytes and proved that miR-146a-5p may reduce the inflammatory response by targeting ox40l.

**Table 1. Primers**

<table>
<thead>
<tr>
<th>Primer(Rat)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-146a-5p</td>
<td>5'-TGAGAAGCTGATTCCATGGTT-3'</td>
<td>-</td>
</tr>
<tr>
<td>Mmp9</td>
<td>5'-CTTACGAGCATGACGAAAGACAA-3'</td>
<td>5'-TGGTGACGGAGTGAAGGAGTG-3'</td>
</tr>
<tr>
<td>Mmp13</td>
<td>5'-GTGTCGAGAGGCTGAAGGCGAC-3'</td>
<td>5'-AGCAACAGTCTGACTAAGGAC-3'</td>
</tr>
<tr>
<td>Cox2</td>
<td>5'-GTCCGATGATACGCCGATGGCC-3'</td>
<td>5'-CCGTTGAGGGATGAGGTGTTG-3'</td>
</tr>
<tr>
<td>Adamts5</td>
<td>5'-GGACCAAGGACATTTGCTTAGATGTG-3'</td>
<td>5'-GTGTGAGCAAGAACGAGAAGCA-3'</td>
</tr>
<tr>
<td>Sox9</td>
<td>5'-CAGACCCGATGCCGATGG-3'</td>
<td>5'-CTCCTGAGGAAGGTTCT-3'</td>
</tr>
<tr>
<td>Col2a1</td>
<td>5'-AAAGGCAAGGAGAAGCAAGCA-3'</td>
<td>5'-GTGGAGGAGGTGGAGGAGGAG-3'</td>
</tr>
<tr>
<td>Tnfsf4</td>
<td>5'-TGCCGACGAGGTCTGTTG-3'</td>
<td>5'-TTGCGAGGTACGGAGGAC-3'</td>
</tr>
<tr>
<td>U6</td>
<td>5'-CTCGCTTCGCAACAGAACAGA-3'</td>
<td>5'-AAGCTTACAGATTCTCTC-3'</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5'-GATTGGCTGATTGCTAGTG-3'</td>
<td>5'-GTGGTCGAGGATGTCATTGCT-3'</td>
</tr>
</tbody>
</table>

**Fig. 1.** Cell culture and establishment of the inflammatory model. The chondrocytes were harvested using the cartilage tissue block method. a–d Conditions of the primary condylar chondrocyte cultures via the tissue method at 3, 5, 7, and 9 days, respectively. On day 3 the chondrocytes began to grow out of the tissue block (a), and on day 9 the cells were at > 90% confluence (d). e, f Toluidine blue staining; the results were positive. g, h Immunohistochemistry of type II collagen; the results were positive; WB (i) and RT-qPCR (j) results showed obvious changes about related factors in the experimental group compared with NC group: the expression of related inflammatory factors (MMP9, MMP13, COX2, and ADAMTS5) increased significantly, and chondrophenotypic factors (COL2a1 and SOX9) decreased significantly (*** p < 0.05; ** p < 0.1).
Materials and Methods

Cell Culture and Identification

Condylar chondrocytes were obtained from 1- to 3-day-old healthy Sprague-Dawley rats (purchased from the experimental animal center of Kunming Medical University). The complete culture medium included Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT, USA), 10% fetal bovine serum (HyClone), and 1% penicillin-streptomycin (HyClone), maintained in 5% CO₂ at 37 °C. The culture medium was changed every 3 days. Stably cultured cells with more than 75% confluence were split 1:2 using 0.25% trypsin (Gibco, Thermo Scientific, Rockford, IL, USA). Condylar chondrocytes were identified using a Toluidine Blue Staining Kit (Solarbio, Beijng, China) and a Type II Collagen Immunohistochemistry Kit (Solarbio).

Establishing the Chondrocyte Inflammatory Model

The first generation of condylar chondrocytes was inoculated in a 25 T culture bottle at 5 × 10⁶ cells/mL. When the confluence reached 75%, cells were starved for 12 h. Next, IL-1β, TNF-α, and

Fig. 2. MiRNA sequencing results. The experimental and control groups were set with 3 repetitions. The heat map (a) showed that the experimental group had 7 upregulated and 6 downregulated miRNA (p < 0.01). The most obviously upregulated miRNA (miR-146a-5p) was verified by RT-qPCR (b); KEGG analysis (c) showed that the signals related to T-cell receptors were obviously enriched. The results of gene ontology analysis (d) showed there was obvious enrichment in the membrane and cell surface (**p < 0.05; nc, negative control).
phosphate-buffered saline were added into two 25 T culture bottles as follows: (1) IL-1β (Sino Biological, Beijing, China) and TNF-α (Sino Biological) at a final concentration of 5 ng/mL, respectively, and (2) the same amount of phosphate-buffered saline (HyClone). After 24 h, RT-qPCR and WB were used to detect the expressions of related inflammatory factors (MMP9, MMP13, COX2, and ADAMTS5) and chondrophenotypic factors (SOX9 and COL2a1).

**RT-qPCR**

Total RNA and cDNA were prepared as per the instructions of the RNAiso Plus Kit (Takara, Kusatsu, Japan) and the PrimeScript RT Master Mix Kit (Takara). RT-qPCR detection was performed using TB Green® Premix Ex Taq™II (Takara). For detection of miRNA, an miRcute Extraction and Separation Kit (Tiangen, Beijing, China) and an miRcute Increase First-Strand Synthesis Kit (Tiangen) were used to extract the miRNA and prepare the cDNA. RT-qPCR was performed with an miRcute enhanced miRNA Fluorescence Quantitative Detection Kit (Tiangen). Reverse transcription was performed on an s1000tm Thermal Cycler (BioRad, Hercules, CA, USA) platform, and RT-qPCR was performed on an mx3005p (Agilent, Santa Clara, CA, USA) platform. During the RT-qPCR, each sample was provided with 3 attachment holes, with 3 repetitions per group. The experimental results were analyzed using 2-ΔΔCT. GAPDH and U6 were selected as internal parameters. The primers were designed and synthesized by Shanghai Bioengineering Technology Service Co., Ltd. Table 1 shows the main primer sequence.

**Western Blot**

Total protein was extracted using an RIPA buffer (Beyotime, Shanghai, China), and the protein concentration was detected using Q3000 (Thermo Fisher Scientific). The Western blot (WB) was established as per the instructions of the PAGE Gel Kit (Solarbio) and PVDF membrane. The primary antibody was incubated overnight at 4 °C; the secondary antibody was incubated at room temperature for 1 h, and the protein band density was determined using Quantity One software (Bio Rad). WB was performed using the primary antibodies MMP9 (ab38898), MMP13 (ab39012), COX2 (ab15191), ADAMTS5 (ab41037), GAPDH (ab181602), SOX9 (ab185230), COL2a1 (ab34712), and TNFSF4 (ab156285) and secondary antibody (ab205718).

**miRNA Sequencing**

The experiment was performed according the instructions provided by Illumina (San Diego, CA, USA), including library preparation and sequencing. The small RNA sequencing library was prepared using TruSeq Small RNA Sample Prep Kits (Illumina). Next, the constructed library was sequenced using Illumina HiSeq2500, and the length of the sequenced library was 1 × 50 bp. Acgt101 MIR (LC Sciences, Houston, TX, USA) software was used for the data analysis. The original data were subjected to quality control to obtain clean reads, from which the 3’ connector was removed, and the length was screened. Sequences with a base length of 18–26 nt were retained. The remaining sequences were compared with other RNA databases, including the mRNA, rfam (for rRNA, tRNA, snRNA, and snoRNA) and RepBase databases, and filtered to obtain effective data, with 3 repetitions per group.

**Cell Transfection**

The first-generation condylar chondrocytes were inoculated in 6-well plates at 5 × 10^4 cells/well; after being grown to 75% confluency, cells were transfected with rno-miR-146a-5p mimic (RIBOBIO, Guangzhou, China) according to the manufacturer’s instructions. The results of cell transfection were validated using RT-qPCR and WB.

**Dual Luciferase Activity Assay**

Detection of the inhibitory effect of miRNA (rno-mir-146a-5p) on target gene ox40l (tnfsf4) by a dual luciferase reporter gene system. The constructed pMiR-luciferase report vector (H306, Ohio, Shanghui, China) with the ox40l 3’UTR carrying the rno-miR-146a-5p binding sites (AGTTCTCT) were amplified by PCR referred to as ox40l-wt (H15570, Ohio). To mutate the binding site of rno-miR-146a-5p in the ox40l 3’UTR, the sequence of the binding site was replaced by TACGAAAG and referred to as ox40l-mut (H15571, Ohio). The 293T cell line came from the cell bank of Chinese Academy of Sciences, the cell culture conditions were DMEM (HyClone) with 10% FBS (HyClone) maintained in 5% CO2 at 37 °C. 293T cells were transfected with the reporter constructs and rno-miR-146a-5p using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

**Statistical Analysis**

GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical analysis. All experimental data are expressed as means ± SD. A t test was used for between-group comparisons.

**Results**

**Cell Culture and Establishment of the Inflammatory Model**

Chondrocytes were harvested through the cartilage tissue block culture method and identified via toluidine blue and type II collagen immunohistochemical staining. The results showed that the cultured cells were irregular, polygonal, and rich in cytoplasm with obvious nuclei; the toluidine blue and type II collagen immunohistochemical staining results were positive (Fig. 1a–h). A model of chondrocyte inflammation was established by combined induction of IL-1β and TNF-α. RT-qPCR and WB results showed that inflammatory factors (MMP9, MMP13, COX2, and ADAMTS5) increased, and chondrophenotypic factors (SOX9 and COL2a1) were reduced, significantly, compared to values in the NC group (Fig. 1i, j).

**miRNA Sequencing**

Seven upregulated and six downregulated miRNA were selected after library preparation, computer sequencing, and data analysis (Fig. 2a). The most obviously upregulated miRNA (miR-146a-5p) was verified by RT-qP-
Fig. 3. Cell transfection and dual luciferase activity assay. a, b Expression of inflammatory factors and chondrophenotypic factors after transfection; results showed that miR-146a-5p reduced the production of inflammatory factors but had no effect on chondrophenotypic factors. c The expression of 4 genes (tnfsf4, thrb, traf6, and cxcr4) in 11 predicted target genes related to OA decreased significantly. d Dual luciferase activity assay (H306: pMIR-REPORT luciferase, H15570: pMIR-REPORT luciferase-ox40l 3′UTR-WT, and H15571: pMIR-REPORT luciferase-ox40l 3′UTR-MUT) results showed that miR-146a-5p could regulate the expression of luciferase with 3′UTR in ox40l; after mutation of the binding site, the regulatory relationship was significantly weakened. It was confirmed that rno-miR-146a-5p targeted ox40l. Expression of OX40L (e, f) after inflammatory stimulation and transfection; the results showed that the expression of OX40L increased significantly in an inflammatory environment and decreased significantly after being transfected by rno-miR-146a-5p.
CR (6 miRNA were selected for validation, some of which are not listed; Fig. 2b). KEGG analysis showed that the signals related to T-cell receptors were obviously enriched (Fig. 2c) under the effect of differential expression miRNA. The results of gene ontology analysis showed that targets of differential expression miRNA had obvious enrichment in the membrane and cell surface (Fig. 2d).

**Cell Transfection and Dual Luciferase Activity Assay**

The first-generation condylar chondrocytes were transfected with rno-miR-146a-5p mimic. Then, mmp9, mmp13, cox2, adams5, col2a1, and sox9 were detected by qPCR and WB, and the results showed that miR-146a-5p reduced the production of inflammatory factors but had no effect on chondrophenotypic factors (Fig. 3a, b). The 11 predicted targets were from iRanda, TargetScan, and PicTar (RT-qPCR; Fig. 3c). A dual luciferase activity assay, qPCR, and WB proved that ox40l (tnfsf4) was inhibited by miR-146a-5p (Fig. 3d–f).

**Discussion**

**Condylar Chondrocytes and T-Cell-Mediated Immunity**

OX40L is a member of the tumor necrosis factor superfamily, mainly expressed in APC, activated T cells, and others including lymphoid tissue inducer cells, some endothelia, and mast cells [12]; its receptor OX40 is expressed in activated CD4+ T cells and CD8+ T cells [16]. Interactions between OX40 and OX40L could promote T-cell survival [17], increase effector cytokine production [18], and enhance T-cell mobility [12]. Our miRNA sequencing results and KEGG analysis suggested that T-cell-mediated immunity may play a role in OA, which was consistent with other studies; Nakamura et al. [19] found T-cell infiltration in the perivascular area in the OA early stage, and some of them were CD4+ T cells. Dzhambazov et al. [20] proved that type II collagen is a target for autoreactive T cells in both rheumatoid arthritis and the murine model collagen-induced arthritis. Zhu et al. [21] conclude that TLR3 (Toll-like receptor 3) upregulation of FLS (fibroblast-like synoviocytes) activated by T cells results in articular inflammation [21]. We observed that the expression of OX40L of condylar chondrocytes increased significantly after stimulation of TNF-α and IL-1β (Fig. 3e, f). It suggests that condylar chondrocytes may have the ability of antigen presentation after inflammatory stimulation; in other words, chondrocytes may activate T cells through the interaction between OX40L and OX40. Some studies have also confirmed that chondrocytes have the ability of antigen presentation after the stimulation of some factors [20, 22, 23]. According to the experimental results and previous research, we speculate that condylar chondrocytes in the inflammatory environment can act as a kind of nonprofessional APC, which affects T-cell-mediated immunity through the interaction of OX40L-OX40. To verify this hypothesis, more antigen presentation experiments are needed.

**MiR-146a-5p and OA**

MiR-146 is one of the most studied microRNA; the latest research shows that it plays a complex role in im-
mune regulation [24–27], inflammatory response [28–31], and tumor progression [32–34] through affecting the PI3K/AKT/MAPK [34], NF-kappaB [35], Notch [36], CXCR/CXCL [37], and TLR [38] signal pathways. The miRNA sequencing results showed that miR-146a-5p was upregulated after induction by IL-1β and TNF-α (Fig. 2), and this result is consistent with many other studies; Nakasa et al. [39] analyzed clinical specimens of OA [5] and rheumatoid arthritis [39], and the results showed that the content of miR-146a increased significantly. Shao et al. [19] demonstrated that miR-146a-5p was significantly upregulated in osteoarthritic tissues. According to cell transfection, miR-146a-5p reduced the expression of inflammatory factors (Fig. 3a, b), suggesting that miR-146a-5p is a protective factor, attenuating the severity of inflammation in OA. However, there is still some controversy surrounding the role of miR-146 in OA; Sun et al. [6] and Zhang et al. [7] proved that miR-146 could target cxcr4 and camk2d to accelerate the progress of OA. Shao et al. [40] showed that miR-146a-5p induces chondrocyte apoptosis by targeting the TRAF6-mediated NF-kB signaling pathway in OA [40], Guan et al. [8] and Taganov et al. [9] prove that miR-146a reduced the inflammatory response by inhibiting the production of inflammatory factors such as Notch1, IL-6, and IL-1. Zhong et al. [10] found that miR-146a was targeted to TRAF6 in human OA chondrocytes, thus blocking the NF-kB signaling pathway. These results showed that the role of miR-146a-5p in OA is extensive and complex. To further understand the mechanism of miR-146a-5p in OA, we confirmed that ox40l (tnfsf4) is another target of miR-146a-5p by dual luciferase activity assay. OX40L can activate a T-cell-mediated immune response by interacting with its receptor OX40, and thus miR-146a-5p may reduce the inflammatory response by inhibiting the expression of OX40L in chondrocytes (Fig. 4). These findings explain other possible roles of miR-146a-5p and chondrocytes in OA, which may improve the pathogenesis of OA.

**Conclusion**

The expression of OX40L and miR-146a-5p of condylar chondrocytes in the inflammatory environment (induced by IL-1β and TNF-α) was significantly increased, miR-146a-5p is a protective factor in TMJ-OA, which can reduce the production of inflammatory factors, and miR-146a-5p may regulate T-cell-mediated immunity through targeting of OX40L.

**Acknowledgement**

This work was finished at the Department of Dental Research, The Affiliated Stomatology Hospital of Kunming Medical University. Thanks to Yang Rongqiang, Huang Guobin, Dai Zichao, and Chen Rui for their great help with this study.

**Statement of Ethics**

Experimental research on animals have been approved by the Animal Experimental Ethical Committee of Kunming Medical University (No. kmmu2020183).

**Conflict of Interest Statement**

The authors have no conflict of interests to declare.

**Funding Sources**

This work was supported by grants from the National Natural Science Foundation of China (No. 81660178 and 81360163) and Scientific Research Project of Yunnan Provincial Education Bureau (No. 2020J0211).

**Author Contributions**

Conceptualization and methodology: Ding Yu and Li Song. Software and investigation and visualization: Wang Wei. Validation: Ding Yu and Wang Wei. Formal analysis, writing, review, and editing, and project administration: Ding Yu. Resources: Ding Yu and Yang Hefeng. Data curation: Ding Yu and Li Weihao: writing and preparation of the original draft: Ding Yu and Qu QianQian. Supervision and funding acquisition: Li Song. All of the authors read and approved the published version of this paper

**References**

Inhibited by miR-146a-5p in OA

Cui D, Li H, Xu X, Ye L, Zhou X, Zheng L, Taganov KD, Boldin MP, Chang KJ, Balti...

Webb GJ, Hirschfield GM, Lane PJ. OX40, Gough MJ, Weinberg AD. OX40 (CD134) ...

Sun T, Li X, Song H, Gao F, Zhou G, Li X, et al. MiR-146a aggravates LPS-induced inflam...

Zhou Y. Mesenchymal stem cells for cartilage regeneration of TMJ osteoarthritis. Stem ...


