miR-let-7c-5p and miR-149-5p inhibit proinflammatory cytokine production in osteoarthritis and rheumatoid arthritis synovial fibroblasts

Yat-Yin Law1,2, Wei-Fang Lee3, Chin-Jung Hsu4,5, Yen-You Lin6, Chun-Hao Tsai5,7, Chien-Chung Huang6,8, Min-Huan Wu9,10, Chih-Hsin Tang6,11,12, Ju-Fang Liu13

1Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan
2Department of Orthopedics, Chung Shan Medical University Hospital, Taichung, Taiwan
3School of Dental Technology, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan
4School of Chinese Medicine, China Medical University, Taichung, Taiwan
5Department of Orthopedic Surgery, China Medical University Hospital, Taichung, Taiwan
6School of Medicine, China Medical University, Taichung, Taiwan
7Department of Sports Medicine, College of Health Care, China Medical University, Taichung, Taiwan
8Division of Immunology and Rheumatology, Department of Internal Medicine, China Medical University Hospital, Taichung, Taiwan
9Bachelor of Science in Senior Wellness and Sports Science, Tunghai University, Taichung, Taiwan
10Tunghai University Sports Recreation and Health Management Degree Program, Tunghai University, Taichung, Taiwan
11Chinese Medicine Research Center, China Medical University, Taichung, Taiwan
12Department of Medical Laboratory Science and Biotechnology, College of Medical and Health Science, Asia University, Taichung, Taiwan
13School of Oral Hygiene, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan

Correspondence to: Chih-Hsin Tang, Ju-Fang Liu; email: chtang@mail.cmu.edu.tw, jufangliu@tmu.edu.tw
Keywords: miR-let-7c-5p, miR-149-5p, osteoarthritis, rheumatoid arthritis, inflammation
Received: March 2, 2021 Accepted: June 4, 2021 Published: July 1, 2021

ABSTRACT

Osteoarthritis (OA) and rheumatoid arthritis (RA) are two of the most common types of arthritis. Both are characterized by the infiltration of a number of proinflammatory cytokines into the joint microenvironment. miRNAs play critical roles in the disease processes of arthritic disorders. However, little is known about the effects of miRNAs on critical inflammatory cytokine production with OA and RA progression. Here, we found higher levels of proinflammatory cytokines including interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) in human OA and RA synovial fibroblasts (SFs) compared with normal SFs. Searches of open-source microRNA (miRNA) software determined that miR-let-7c-5p and miR-149-5p interfere with IL-1β, IL-6 and TNF-α transcription; levels of all three proinflammatory cytokines were lower in human OA and RA patients compared with normal controls. Anti-inflammatory agents dexamethasone, celecoxib and indomethacin reduced proinflammatory cytokine production by promoting the expression of miR-let-7c-5p and miR-149-5p. Similarly, ibuprofen and methotrexate also enhanced miR-let-7c-5p and miR-149-5p expression in human SFs. The evidence suggests that increasing miR-let-7c-5p and miR-149-5p expression is a novel strategy for OA and RA.
INTRODUCTION

Osteoarthritis (OA) and rheumatoid arthritis (RA) feature synovial inflammation and damage to articular cartilage, as well as pathological changes in subchondral bone [1]. Anti-inflammatories (NSAIDs and corticosteroids) are typically used to reduce ongoing inflammation and relieve the pain induced by arthritis [2, 3]. Patients with arthritis live with low-grade, chronic joint inflammation that perpetuates the release of inflammatory mediators, with ever-worsening damage to cartilage, bone and synovium [4, 5].

The activities of proinflammatory cytokines interleukin 1 beta (IL-1β), IL-6 and tumor necrosis factor alpha (TNF-α) contribute to the pathogenesis of arthritis by promoting proteolytic enzyme activity that damages the cartilage extracellular matrix [6, 7]. IL-1β, IL-6 and TNF-α levels in human arthritic serum and synovial fluid are higher than those of healthy controls and have been targeted by therapies including the IL-1β inhibitor canakinumab, the IL-6 inhibitor tocilizumab, and the TNF-α inhibitor infliximab [8]. Reducing proinflammatory cytokine activity has shown merit as a therapeutic strategy to reduce arthritis progression [6].

The progression of arthritis disease is regulated by several microRNAs (miRNAs), including miR-92a, miR-129-3p, miR-141-3p and miR-199a-5p [9–12], while the proinflammatory mediators IL-1β, IL-6, TNF-α and matrix metalloproteinases (MMPs) account for histological changes that occur with arthritis [13–15]. However, how miRNAs might regulate the progression of OA and RA remains unclear. We describe finding higher levels of several microRNAs (miRNAs), including miR-92a, miR-129-3p, miR-141-3p and miR-199a-5p [9–12], while the proinflammatory mediators IL-1β, IL-6, TNF-α and matrix metalloproteinases (MMPs) account for histological changes that occur with arthritis [13–15]. However, how miRNAs might regulate the progression of OA and RA remains unclear. We describe finding higher levels of several microRNAs (miRNAs), including miR-92a, miR-129-3p, miR-141-3p and miR-199a-5p [9–12], while the proinflammatory mediators IL-1β, IL-6, TNF-α and matrix metalloproteinases (MMPs) account for histological changes that occur with arthritis [13–15]. However, how miRNAs might regulate the progression of OA and RA remains unclear. We describe finding higher levels of several microRNAs (miRNAs), including miR-92a, miR-129-3p, miR-141-3p and miR-199a-5p [9–12], while the proinflammatory mediators IL-1β, IL-6, TNF-α and matrix metalloproteinases (MMPs) account for histological changes that occur with arthritis [13–15]. However, how miRNAs might regulate the progression of OA and RA remains unclear. We describe finding higher levels of several microRNAs (miRNAs), including miR-92a, miR-129-3p, miR-141-3p and miR-199a-5p [9–12], while the proinflammatory mediators IL-1β, IL-6, TNF-α and matrix metalloproteinases (MMPs) account for histological changes that occur with arthritis [13–15].
transcriptional regulation of target protein expression [21, 22]. However, little is known about the effect of miRNAs on critical inflammatory cytokine production and OA and RA progression. Our study results describe how OASFs and RASFs contain higher levels of proinflammatory cytokines compared with NSF levels. We also found that miR-let-7c-5p and miR-149-5p negatively regulate levels of IL-1β, IL-6 and TNF-α expression. The therapeutic effects of anti-inflammatory agents used in this study were associated with their inhibition of miR-let-7c-5p and miR-149-5p expression, indicating that modulation of these miRNAs may be a novel strategy for reducing OA and RA inflammation.

MiRNAs post-transcriptionally regulate gene expression [23]. During OA and RA progression, aberrant miRNA expression mediates inflammatory pathway signaling [24, 25]. Our examination of open-source database records identified that miR-let-7c-5p and miR-149-5p potentially interfere with the transcription of IL-1β, IL-6 and TNF-α. The levels of both miRNAs were lower in OA and RA patients than in normal controls, indicating negative correlations between miR-let-7c-5p and miR-149-5p and proinflammatory cytokine expression. There was no evidence of sequence similarity between these miRNAs. Other evidence has shown that miR-let-7c-5p inhibits the proliferation and migration of cervical carcinoma cells [26] and negatively regulates NLRC5.
Figure 3. MiR-let-7c-5p and miR-149-5p regulate IL-1β, IL-6 and TNF-α expression in OASFs and RASFs. After transfecting OASFs (A, B) and RASFs (C, D) with the miR-let-7c-5p and miR-149-5p mimics or their respective inhibitors, IL-1β, IL-6 and TNF-α expression was examined by qPCR.

Figure 4. Anti-inflammatory agents upregulate miR-let-7c-5p and miR-149-5p expression. OASFs (A–C) and RASFs (D–F) were treated with dexamethasone, celecoxib, or indomethacin (1–50 μM), then subjected to qPCR quantification of miR-let-7c-5p and miR-149-5p expression.
protein expression in ethanol-induced hepatic injury [27]. Interestingly, miR-149-5p is capable of inhibiting the growth of gastric cancer, cholangiocarcinoma and glioblastoma [28–30]. MiR-149-5p can also inhibit M1 macrophage-associated inflammation in experimental abdominal aortic aneurysm formation [31]. In this study, we enhanced miR-let-7c-5p and miR-149-5p levels in OASFs and RASFs by transfecting them with their respective miRNA mimics, which also reduced IL-1β, IL-6 and TNF-α production. In contrast, transfection of OASFs and RASFs with miR-let-7c-5p and miR-149-5p inhibitors downregulated the levels of both miRNAs. Thus, our evidence has identified novel anti-inflammatory functions in association with miR-let-7c-5p and miR-149-5p. Numerous signaling pathways, including the MAPK, PI3K/Akt and PKC pathways,

Figure 5. Anti-inflammatory agents reduce proinflammatory cytokine production in OASFs and RASFs. After treating OASFs (A–F) and RASFs (G–L) with dexamethasone, celecoxib, or indomethacin (1–50 μM), Western blot and qPCR assays quantified the levels of IL-1β, IL-6 and TNF-α expression.
control miRNA synthesis during the progression of arthritis disease [9–12]. We did not include the upstream molecules of miR-let-7c-5p and miR-149-5p in this investigation. Further research is needed to determine whether their expression is regulated by the same signaling cascades.

Anti-inflammatory agents (e.g., corticosteroids, COX-2 selective inhibitors and NSAIDs), are well recognized for their modulation of inflammatory responses in different inflammatory diseases, including arthritis [16]. Our study results confirm that dexamethasone, celecoxib and indomethacin effectively lower the synthesis of arthritic inflammatory cytokines, including IL-1β, IL-6 and TNF-α, in both OASFs and RASFs, by upregulating miR-let-7c-5p and miR-149-5p expression. Our genetic and pharmacologic investigations suggest that enhancing the levels of these miRNAs may be a novel avenue for treating OA and RA.

MATERIALS AND METHODS

Materials

Antibodies for IL-1β, IL-6 and TNF-α were obtained from GeneTex International Corporation. Lipofectamine® 2000 and Trizol® were acquired from Life Technologies. The miRNA mimic, inhibitor and negative control and Dharmafect1 were purchased from Dharmacon. β-Actin antibody and all other chemicals not already mentioned were acquired from Sigma-Aldrich.

Cell preparation

Human NSFs (primary fibroblast-like synoviocytes; 408-05A) were obtained from Cell Applications, Inc. (San Diego, CA, USA). Human RASFs were purchased from the Riken Cell Bank (Ibaraki, Japan). Human OASFs were isolated from synovial tissues of 10 OA patients by collagenase treatment, using previously detailed procedures [32]. All cells were maintained in DMEM (containing 10% FBS and antibiotics) in a 5% CO2 incubator (at 37°C).

Human synovial tissues

Study approval was granted by the Institutional Review Board of China Medical University Hospital (Taichung, Taiwan) and all patients provided written informed consent before participating in the study. Synovial tissue samples were obtained from patients undergoing total knee arthroplasty for OA or RA and also from those undergoing arthroscopic procedures for trauma/joint derangement (healthy controls).

Western blot

SDS-PAGE was used to resolve the extracted proteins, which were transferred to PVDF membranes, as described in our previous publications [33–35]. Membranes were blocked for 1 h with PBST containing 4% non-fat milk, then treated with antibodies targeting IL-1β, IL-6 and TNF-α for 1 h, before being incubated

Figure 6. Schematic diagram summarizes the effects of miR-let-7c-5p and miR-149-5p in OA ad RA. Treatment of OASFs and RASFs with anti-inflammatory agents upregulates levels of miR-let-7c-5p and miR-149-5p expression, leading to decreases in the expression of proinflammatory cytokines (IL-1β, IL-6 and TNF-α). Thus, enhancing miR-let-7c-5p and miR-149-5p expression may be a novel therapeutic avenue for halting the progression of OA and RA disease.
for 1 h with HRP-conjugated secondary antibodies. We visualized the blot membranes using a Fujifilm LAS-3000 imaging system.

**Quantitative real-time PCR**

All RNA was collected from SFs using TRIzol™ Reagent. We generated cDNA using an Invitrogen reverse transcription kit. qPCR analysis was conducted with the Taqman® One-Step RT-PCR Master Mix. Mir-X™ miRNA First-Strand Synthesis and the SYBR® RT-PCR kit were used for reverse transcription of miRNA. Analysis was carried out according to a previous protocol [36–38].

**Transfection**

Synthetic miRNA mimics and inhibitors (10 nM) were transfected into OASFs and RASFs following the Dharmafect1 transfection protocol. After 24 h of transfection, inflammatory cytokine expression was examined by qPCR.

**Statistics**

All values are presented as the mean ± standard deviation of 5 independent experiments. Differences between two experimental groups were assessed for significance using the Student’s t-test and considered to be significant if the p value was < 0.05.

**AUTHOR CONTRIBUTIONS**

Y.-Y. Law, J.-F.L. and C.-H.T. conceived and designed the experiments, which were performed by J.-F.L., W.-F.L. and Y.-Y.L. Reagents, materials and analysis were provided by C.J.H., C.-Y.H., C.-C.H., C.-H.T. and M.-H.W. The paper was written by J.-F.L. and C.-H.T. All authors have read and agreed to the published version of the manuscript.

**ACKNOWLEDGMENTS**

We thank Iona J. MacDonald from China Medical University, Taichung, Taiwan, for her English language revision of this manuscript.

**CONFLICTS OF INTEREST**

The authors declare that they have no conflicts of interest.

**FUNDING**

This work was supported by grants from the Ministry of Science and Technology of Taiwan (108-2320-B-039-065 and MOST 107-2320-B-039-019-MY3), China Medical University Hospital (DMR-110-106 and DMR-110-022) and Shin Kong Wu Ho-Su Memorial Hospital (SKH-8302-106-0402).

**REFERENCES**


9. Kuo SJ, Liu SC, Huang YL, Tsai CH, Fong YC, Hsu HC, Tang CH. TGF-β1 enhances FOXO3 expression in human synovial fibroblasts by inhibiting miR-92a
https://doi.org/10.18632/aging.102038
PMID:31232696

enhances aggrecan expression in chondrocytes via the
https://doi.org/10.1002/jcp.26451 PMID:29319178

11. Wu MH, Tsai CH, Huang YL, Fong YC, Tang CH. Visfatin
Promotes IL-6 and TNF-α Production in Human
Synovial Fibroblasts by Repressing miR-199a-5p
through ERK, p38 and JNK Signaling Pathways. Int J Mol
https://doi.org/10.3390/ijms19010190 PMID:29316707

12. Tsai CH, Liu SC, Wang YH, Su CM, Huang CC, Hsu CJ,
Tang CH. Osteopontin inhibition of miR-129-3p
enhances IL-17 expression and monocyte migration in
rheumatoid arthritis. Biochim Biophys Acta Gen Subj.
2017; 1861:15–22.
https://doi.org/10.1016/j.bbagen.2016.11.015
PMID:27851983

2018; 7:92.
https://doi.org/10.3390/cells7080092 PMID:30071609

K, Chen Q. Evidence that miR-146a attenuates aging-
and trauma-induced osteoarthritis by inhibiting
Notch1, IL-6, and IL-1 mediated catabolism. Aging Cell.
2018; 17:e12752.
https://doi.org/10.1111/ace1.12752 PMID:29575548

15. Huang J, Zhao L, Fan Y, Liao L, Ma PX, Xiao G, Chen D.
The microRNAs miR-204 and miR-211 maintain joint
https://doi.org/10.1038/s41467-019-10753-5 PMID:31253842

16. Tang CH. Research of Pathogenesis and Novel
https://doi.org/10.3390/ijms20071646 PMID:30987068

17. Mclnnes IB, Schett G. The pathogenesis of rheumatoid
https://doi.org/10.1056/NEJMra1004965 PMID:22150039

18. Smolen JS, Aletaha D, Mclnnes IB. Rheumatoid
https://doi.org/10.1016/S0140-6736(16)30173-8 PMID:27156434

19. Catrina AI, Svensson CI, Malmström V, Schett G,
Klareskog L. Mechanisms leading from systemic
https://doi.org/10.1038/nrrheum.2016.200 PMID:27974851

20. Lefevre S, Meier FM, Neumann E, Muller-Ladner U.
https://doi.org/10.2174/138161282066614082512203
PMID:25163744

21. Tüfekçi KJ, Oner MG, Meuwissen RL, Genç S. The role
https://doi.org/10.1007/978-1-62703-748-8_3 PMID:24272430

22. Wilson RC, Doudna JA. Molecular mechanisms of RNA
https://doi.org/10.1146/annurev-biophys-083012-
130404 PMID:23654304

23. Nugent M. MicroRNAs: exploring new horizons in
https://doi.org/10.1016/j.joca.2015.10.018 PMID:26576510

https://doi.org/10.1016/j.jaut.2020.102438 PMID:32184036

RNAs in Rheumatoid Arthritis: From Bench to Bedside.
https://doi.org/10.3389/fimmu.2019.03129 PMID:32047497

Sun G, Hu J, Sun H, Xie S, Li Y. Delivery of MicroRNA-let-
7c-5p by Biodegradable Silica Nanoparticles Suppresses
https://doi.org/10.1166/jbn.2020.2989 PMID:33461652

27. Wang Q, Li M, Shen Z, Bu F, Yu H, Pan X, Yang Y, Meng
X, Huang C, Li J. The Long Non-coding RNA MEG3/miR-
let-7c-5p Axis Regulates Ethanol-Induced Hepatic
https://doi.org/10.3389/fphar.2018.00302 PMID:29692724

Y, Li M, Wang L, Dong C, Yin F. Circular RNA
Circ_006282 Promotes Cell Proliferation and


Supplementary Figure 1. Ibuprofen and methotrexate upregulate miR-let-7c-5p and miR-149-5p expression. Synovial fibroblasts were treated with ibuprofen and methotrexate, then subjected to qPCR quantification of miR-let-7c-5p and miR-149-5p expression.