MiRNA-6089 inhibits rheumatoid arthritis fibroblast-like synoviocytes proliferation and induces apoptosis by targeting CCR4

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ABSTRACT
Several studies have suggested that fibroblast-like synoviocytes (FLSs) and miRNAs are implicated in the pathogenesis of rheumatoid arthritis (RA). This study aimed to evaluate the function of miR-6089 in the regulation of RA-FLSs. The levels of miR-6089 were detected to be significantly lower in the synovial tissues and FLSs of RA than in the healthy synovial tissues and FLSs. The miR-6089 up-regulation in RA-FLSs significantly inhibited the proliferation and promoted cell apoptosis accompanied with an increase protein expression of cleaved-Caspase-3, -8 and -9. Furthermore, CCR4 was determined to target miR-6089 directly, and its expression was significantly increased in the synovial tissues of RA than in the healthy synovial tissues. The overexpression of CCR4 reversed the effect of miR-6089 on proliferation and apoptosis in RA-FLSs effectively. In conclusion, our study suggests that the miR-6089 may be a potential target for prevention and treatment of RA.

Introduction
Rheumatoid arthritis (RA) is a chronic, systemic inflammatory joint disease that is primarily characterised by the inflammation of synovial joint and the destruction of cartilage and bone (Yue et al. 2019). The incidence of RA in developing countries reaches 5–50 per 100,000, while the RA affects between 0.5 and 1% of adults in developed countries (Zhang et al. 2017). The prevalence of RA is uncommon in age under 15 and from there the occurrence rises with age (Wroblewski et al. 2007). Fibroblast-like synoviocytes (FLS) are the specialised cell type forming the inner layer of synovial membrane. It is well known that the abnormal activation of FLS plays a crucial role in the initial stages of synovial inflammation and joint destruction of RA, as well as in the progressive development stage (Li et al. 2017; Wang et al. 2020). Therefore, it is essential to establish the effective therapeutic strategies to treat RA-FLS.

MicroRNAs (miRNAs) are endogenous small RNAs of about 20–24 nucleotides in length that can bind to the 3’ untranslated region (UTR) of a target mRNA through complete or incomplete complementary base-pairing, followed by either transcripts degradation or translation inhibition (Li et al. 2015). Although miRNAs comprise only 2% of the human genome, they regulate the expression of nearly one-third of the genes, especially those involved in cell differentiation, cell proliferation, apoptosis and immune response (Li et al. 2020). Recently, the abnormal expression of miRNAs have been found to be involved with the development of RA, which means miRNAs are the diagnostic biomarker of RA (Zhao et al. 2019). miR-6089, a newly discovered miRNA, have not been reported in regard of its role and molecular mechanisms in RA.

Therefore, the purpose of this study is to detect the expression of miR-6089 in synovial tissues of healthy FLSs and RA-FLSs, and evaluate the effects of miR-6089 on proliferation, apoptosis and inflammatory response in RA-FLS. In addition, we also explored the role of miR-6089 in RA-FLSs, and the underlying molecular mechanisms were determined in order to provide a basis for future clinical applications for RA.

Materials and methods
Samples of tissues
The synovial tissues from 25 patients with RA were obtained during knee replacement surgery, representing as RA group. RA was diagnosed as per classification criteria from the American College of Rheumatology. Healthy synovial tissues from 16 patients with a traumatic knee condition were collected as the control group. The study was approved by the Ethics Committee of The Third Clinical College of Wenzhou Medical University, Wenzhou People’s Hospital (approval no. 2017-95), and informed written consents were obtained from all subjects.
**Cell culture and transfection**

The normal human FLSs and RA-FLSs were obtained from Cell Applications (San Diego, CA, U.S.A.). The cells were cultured in DMEM medium containing 10% foetal bovine serum (FBS) with 5% CO₂ in 37°C. RA-FLSs cells were transfected with miR-6089 mimic, negative control mimic (miR-NC), lentiviral vectors CCR4 (siRNA1, siRNA2) and empty lentiviral vectors (Scramble) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions.

**Cell proliferation**

Cell proliferation was detected by Cell Counting Kit-8 (CCK-8; Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China). To be specific, transfected cells were seeded in a 96-well plate (2 × 10³ cells/well) and incubated overnight at 37°C. A 10 μL CCK-8 reagent was then added to each well and the detection of absorbance at 450 nm was done after culturing for 24, 48, and 72 h in accordance with the manufacturer’s protocol.

**Cell apoptosis**

Cells were collected and washed twice with cold 1× PBS, followed by resuspend in 100 μL binding buffer. The cells were incubated with Annexin V-FITC and propidium iodide for 15 min at room temperature in the dark. A 200 μL of binding buffer was added, and flow cytometry was used to analyse the cells.

**Luciferase reporter assay**

According to TargetScan (http://www.targetscan.org) and MiRanda (http://www.microrna.org/microrna/home.do) databases, CCR4 was assumed as a putative target of miR-6089, which was measured using the dual luciferase reporter assay. 2 × 10⁴ cells/well were cultured overnight, then 100 ng wild-type (WT) CCR4-3'UTR (WT-CCR4-3'-UTR), and mutant CCR4-3'UTR (MUT-CCR4-3'UTR) luciferase reporter plasmid was co-transfected and cultured with 50 ng miR-6089 mimic or miR-NC using Lipofectamine 3000 for 48 h. The relative luciferase activity was determined according to the dual luciferase assay kit instructions. This experiment was repeated for five times.

**qRT–PCR analysis**

Total RNA was extracted from cultured FLSs and tissue samples utilising a Trizol reagent (Invitrogen, Carlsbad, CA), and a spectrophotometer was used to determine the purity and concentration of RNA. The RNA was then transcribed reversely into cDNA templates for real time PCR. The RT-PCR was performed on a 7500 real-time RT-PCR system (ABI, Foster City, CA) using SYBR Premix kit (TaKaRa, Tokyo, Japan) to measure the relative expression of, miR-6089, and CCR4 according to the 2^(-ΔΔCt) method. The primer sequences were as follows: miR-6089-F-5' CCG TGC GCC AGT GG-3', miR-6089-R-5' AGG CGG TGG GC-3'; CCR4-F-5' CCC ACG GAT ATA GCA GAC ACC-3', CCR4-R-5' GTG CAA GGC TTG GGG ATA CT-3'; U6-F-5' CGT TCG GCA GCA CAT CTA AAA T-3'; U6-R-5' CCC CTG TTC ACG AAT TTG CGT GT-3'; GAPDH-F-5' AGA AGG CTG GGG CTC ATT TG-3', GAPDH-R-5' AGG GCC CAT CCA CAG TCT TC-3'. U6 or GAPDH were used as the internal reference for miR-625 or CCR4. The relative expression levels of miR-6089 and CCR4 were analysed using the 2^(-ΔΔCt) method.

**ELISA assays**

The concentration of MMP-1, TNF-α and IL-6 in the cell culture supernatant were detected using ELISA kits (Affymetrix, Santa Clara, CA) following the manufacturer's recommendations.

**Western blot analysis**

To prepare protein extract, harvested cells were washed three times with cold PBS, and lysed with RIPA buffer supplemented with protease inhibitors. The extracted protein was then quantified using the BCA method, and the cell lysates were diluted in 5× loading buffer at the ratio of 1:4. After denaturing at 95°C for 10 min, 30 μg protein per sample was subjected to SDS-PAGE. The protein bands were transferred to PVDF membranes and the latter were blocked with 5% skim milk at room temperature for 2 h. The blots were incubated overnight with primary antibodies against CCR4 (1:1000), cleaved-Caspase-3(1:1000), cleaved-Caspase-8(1:1000), cleaved-Caspase-9 (1:1000), and GAPDH (1:5000) at 4°C on a shaker. After washing with TBST buffer for three times, the blots were probed with HRP-conjugated secondary antibody (1:5000) for 1 h at room temperature, followed by another washing with TBST for three times. ECL chromogenic substrate was used to visualise the bands, and the grey value of each band was determined using Image J software. The content of each protein was calculated relative to GAPDH.

**Statistical analyses**

SPSS 19.0 software was used for statistical analysis. Descriptive statistics were carried out to determine the group means and standard deviations in the numerical data. Independent sample t-test was applied to compare the data between two groups, and one-way ANOVA along with Dunnett’s or Bonferroni’s test was used for multiple groups. The level of significance was p < .05.

**Results**

**Down-regulated miR-6089 in RA synovial tissues and FLSs**

qRT-PCR was conducted to measure the expression levels of miR-6089, which was significantly lower in synovial tissues of RA patients than the healthy control group (p < .01; Figure 1A). Consistent with the patient samples, the RA-FLSs also showed lower expression level of miR-6089 compared with...
the normal FLSs \( (p < .01; \text{Figure } 1\text{B}) \). Hence, those data indicated that miR-6089 might play an important role in the pathogenesis of RA.

**Up-regulation of miR-6089 suppresses the proliferation and promotes the apoptosis in RA-FLSs**

The transfection of RA-FLSs with miR-6089 mimic was conducted to determine the effects of miR-6089 on RA-FLSs proliferation and apoptosis, which showed a 5.7-fold increase in miR-6089 expression \( (p < .01; \text{Figure } 2\text{A}) \). The results from CCK-8 assay suggested that miR-6089 overexpression significantly suppressed the proliferation of RA-FLSs (Figure 2B). In addition, flow cytometry was performed to detect the extent of apoptosis after miR-6089 overexpression in RA-FLSs, indicating that miR-6089 overexpression significantly increased apoptosis ratio compared to the miR-NC group \( (p < .01; \text{Figure } 2\text{C}) \). We also found the increased protein expression levels of cleaved-Caspase-3, -8, and -9 in overexpression miR-6089 of RA-FLSs (Figure 2D). Overall, our findings indicated that miR-6089 could inhibit the proliferation and trigger apoptosis of RA-FLSs.

**Up-regulation of miR-6089 inhibits the release of inflammatory cytokines in RA-FLSs**

Next, we determined the role of miR-6089 in the response to the inflammatory cytokines of RA-FLSs. As shown in Figure 3, miR-6089 overexpression in RA-FLSs significantly reduced the production of MMP-1, TNF-\( \alpha \), and IL-6, suggesting that miR-6089 might protect RA through inhibiting the inflammatory secretion.

**miR-6089 acts directly on CCR4 in RA-FLSs**

The TargetScan database was used to predict the 3′-UTR of the CCR4 gene was complementary to miR-6089, indicating the CCR4 is a putative target of miR-6089 (Figure 4A). As shown in Figure 4B, the possible relationship was confirmed by dual luciferase gene reporter assay which showed the luciferase activity significantly reduced when co-transfected with the miR-6089 mimic and WT CCR4, whereas co-transfection with miR-6089 mimic and MUT CCR4 had no significant effect on the luciferase activity. Moreover, in comparison with the miR-NC group, there was a lower level of CCR4 in miR-6089 overexpression group on both expression levels of mRNA and protein (Figure 4CD). Interestingly, CCR4 expression was significantly increased in RA synovial tissues (Figure 4E). These results suggested that CCR4 is a target gene of miR-6089 and is negatively regulated by miR-6089.

**Knocking down of CCR4 inhibits the proliferation and promotes the apoptosis in RA-FLSs**

Firstly, the CCR4 expression in RA-FLSs was silenced by transfection of siRNA of CCR4. Two independent CCR4 (siRNA1 and siRNA2) were used in case of off-target effect. Both siRNAs successfully knocked down CCR4 expression, with a scrambled sequence as control (Figure 5A). The proliferation was detected with the CCK8 assay, which demonstrated the silencing of CCR4 could significantly inhibit the cell proliferation (Figure 5B). Flow cytometry was carried out 48 h after the transfection of siRNAs. Both siRNAs of CCR4 increased the cell apoptosis (Figure 5C), indicating the important role of CCR4 in RA-FLSs.

**CCR4 overexpression reverses the impact of miR-6089 on the proliferation and apoptosis of RA-FLSs**

The biological relevance of the miR-6089/CCR4 axis in RA-FLSs was further analysed by co-transfecting with miR-6089 mimic and the CCR4 overexpression plasmid in RA-FLSs. As shown in Figure 6A, the miR-6089 mimic significantly decreased the levels of CCR4 \( (p < .01) \). In addition, overexpression of CCR4 also abrogated the decreased proliferation and increased apoptosis induced by miR-6089 in RA-FLSs (Figure 6BC). Thus, miR-6089 might play a suppressive role in RA-FLSs by inhibiting CCR4.
Discussion

Accumulating evidences suggest that miRNAs regulate 1/3rd of the human genome via translational inhibition or degradation of the cognate genes, and form complex regulatory networks that have been implicated in malignant progression (Chen et al. 2019; Hunter et al. 2019). Distinct expression of miRNAs has been detected in RA-associated inflammatory autoimmune responses, which were considered as prognostic biomarkers and therapeutic targets (Liu H., et al., 2020; Simon and Kleyer 2020). According to the study that conducted by Yu et al., the miR-92a could target AKT2 to inhibit fibroblast-like synoviocyte proliferation and migration in RA (Yu et al. 2018). Furthermore, another paper have found a decreased miR-125 level in synovial tissues, and miR-125 may regulate PI3K/Akt/mTOR pathway by directly inhibiting PARP2 expression, thereby weakening the development of
Figure 3. Up-regulation of miR-6089 suppresses inflammatory cytokines release of RA-FLSs. FLSs transfected with either miR-6089 or miR-NC were cultured for 24 h. RA-FLS transfected with miR-6089 mimic significantly reduced the production of MMP-1, TNF-α, and IL-6 compared with RA-FLSs transfected with miR-NC. *p < .05; **p < .01 vs. miR-NC group.

Figure 4. CCR4 is a direct target of miR-6089 in RA-FLSs. (A) Putative miR-6089-binding sites in the 3'-UTR of CCR4. (B) Relative luciferase activity was detected in RA-FLSs co-transfected with WT or MUT 3'-UTR CCR4 reporter plasmids and miR-6089 mimic or miR-NC. (C, D) CCR4 expression on mRNA and protein levels was examined in RA-FLS. RA-FLSs transfected with miR-6089 mimic or miR-NC mimic. GAPDH was used as an internal control. (E) Relative CCR4 mRNA expression was detected in synovial tissues from RA patients and health donor. GAPDH was used as an internal control. *p < .05; **p < .01 vs. miR-NC group or Healthy.
RA (Liu et al. 2019). Li et al. also reported that miR-19 expression was significantly decreased in FLS from RA patients compared with control groups. MiR-19 inhibits the release of cytokine in FLS by targeting TLR2 in RA (Li et al. 2016). Dysregulation of miRNAs is established to be involved in many diseases. miR-6089 serves as a tumor-suppressive miRNA in ovarian cancer, which directly targets MYH9 to inactivate the Wnt/β-catenin and its downstream EMT (Liu L., et al., 2020). In our study, we proved that miR-6089 was significantly decreased in synovial tissues and FLS of RA patients compared with healthy donors. The in vitro studies further showed that the recovery of miR-6089 significantly inhibited the proliferation accompany with an increased apoptosis in RA-FLSs. These results indicated that miR-6089 might play an important role in progression of RA. Moreover, the overexpression of miR-6089 has notably increased the apoptosis with the apoptosis-related protein expression of caspase-3, -8, and -9 in RA-FLSs. We also found a lower level of inflammatory cytokines in RA-FLSs transfected with miR-6089 mimic compared with the negative control. Thus, miR-6089 possesses the inhibitive effect in the development of RA-FLSs.

Although the aetiology and pathological mechanism of RA is still unknown, more and more reports confirm that miRNAs are closely related to the pathological process of RA (Arias De La Rosa et al. 2020; Yang et al. 2020). Different miRNAs can target and regulate different target genes, which affect the pathological process of RA (Iwamoto and Kawakami 2019). Thus, to further understand the underlying mechanisms by which miR-6089 inhibits the growth of RA-FLSs, the bioinformatics analysis was used to identify its direct target gene. The CCR4 was predicted as a target of miR-6089, and the dual luciferase gene reporter assay further confirmed that CCR4 was negatively regulated by miR-6089. Meanwhile, miR-6089 overexpression dramatically decreased CCR4 expression in RA-FLSs. All of these results suggested the targeted binding correlation between miR-6089 and CCR4 in RA-FLSs.

Recently, several studies showed that CCR4 is highly expressed on circulating Tregs (Watanabe et al. 2019). Tregs is recruited at tumour sites in many cancers, such as breast cancer and colorectal cancer (Sasidharan Nair et al. 2020). The anti-CCR4 antibody, alone or in combination with other immune modulators may potentially be used in the treatment of a human solid cancer with high levels of CCR4 expression in tumour-infiltrating leukocytes and abnormal plasma CCR4 ligand levels (Berlato et al. 2017). Importantly, CCR4 has been widely implicated in the pathogenesis of inflammatory diseases such as asthma and atopic dermatitis due to its expression on Th2 cells (Anderson et al. 2020). In our study, we found an increased mRNA level of CCR4 in synovial tissues, and the overexpression CCR4 reversed the effects of miR-6089 on proliferation and apoptosis in RA-FLSs. Our results showed that miR-6089 performed its inhibitory role in the development of RA-FLSs, at least in part, by targeting CCR4.

In conclusion, miR-6089 is downregulated in RA synovial tissues and FLSs. The overexpression of miR-6089 suppresses

Figure 5. Silencing of CCR4 inhibits cell proliferation of RA-FLSs. RA-FLSs were transfected with control (scramble) or CCR4-targeting siRNAs (CCR4 siRNA1 and 2) for 48 h. (A) Western blot analysis of CCR4 protein levels in RA-FLSs. (B) CCK8 assay was performed to detect cell proliferation after culturing for 24, 48, or 72 h. (C) Cell apoptosis was determined by flow cytometry. *p < .05; **p < .01 vs. miR-NC group.
proliferation, promotes apoptosis associated with the apoptosis protein expression (caspase-3, -8, and -9), as well as decreases the secretion of inflammatory cytokines in RA-FLSs by targeting CCR4. Our findings provide new insights into the roles of miR-6089 and CCR4 in RA, and identify potential prognostic biomarkers and therapeutic targets.

Author contributions
S.L. wrote the manuscript and operated the experiments; Z.Z., S.W., Y.L., and M.Y. operated molecular experiments; P.C. and M.W. analysed the data; S.L. and M.W. designed the experiments and edited the manuscript. S.L. and C.L. revised the manuscript.

Disclosure statement
No potential conflict of interest has been reported by the author(s).

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Date availability statement
All the required data are present in the article.

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Figure 6. The impact of miR-6089 on the proliferation and apoptosis of RA-FLSs was reversed by CCR4 overexpression. (A) CCR4 protein expression was determined in RA-FLSs transfected with miR-6089 mimic or miR-NC and with/without CCR4 overexpression vector (lack of 3′-UTR). GAPDH was used as an internal control. (B, C) The effect of miR-6089 on cell proliferation and apoptosis in RA-FLSs was reversed under the condition of overexpression of CCR4. *p < .05; **p < .01; ***p < .001.
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