miR-137 decreases proliferation, migration and invasion in rheumatoid arthritis fibroblast-like synoviocytes

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Abstract. MicroRNA-137 (miR-137) is involved in cell proliferation, migration, invasion and apoptosis in a variety of cells. However, the role of miR-137 in rheumatoid arthritis (RA) remains unclear. The present study aimed to identify the biological roles of miR-137 in RA. The expression of miR-137 in RA fibroblast-like synoviocytes (RA-FLS) and in normal control FLS was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The effects of miR-137 on RA-FLS proliferation, migration and invasion were also determined using MTT, wound healing and Transwell invasion assays, respectively. The effects of miR-137 on inflammatory cytokine expression in RA-FLS were assessed by ELISA. Bioinformatics databases (TargetScan and miRanda), luciferase reporter assays, RT-qPCR and western blotting assays were conducted to identify potential target genes. miR-137 expression was decreased in RA-FLS compared with expression in normal control FLS. Overexpression of miR-137 resulted in a significant reduction in RA-FLS proliferation, migration and invasion, and decreased the expression of inflammatory cytokines of RA-FLS. In addition, bioinformatics analysis and luciferase reporter assays indicated that miR-137 may target the 3'-untranslated region of C-X-C motif chemokine ligand 12 (CXCL12), which was confirmed by RT-qPCR and western blot analyses. These results further demonstrated that miR-137 may serve an inhibitory role in RA by targeting CXCL12 expression, and miR-137 may be a potential target for the treatment of RA.

Introduction

Rheumatoid arthritis (RA) is a heterogenic and systemic autoimmune disease that is characterized by inflammation of the joint lining and subsequent joint destruction (1). Fibroblast-like synoviocytes (FLS) are the primary effectors of cartilage destruction in RA and have been reported to serve a crucial role in initiating and maintaining the inflammatory and destructive processes that occur in the rheumatoid joint (2,3). Therefore, a better understanding of the molecular mechanisms involved in the progression of RA, particularly in FLS, may facilitate the development of novel biomarkers and therapies for RA.

The roles of microRNAs (miRNAs) have been examined in a number of diseases (4). miRNAs are a class of small, non-coding RNAs that regulate gene expression by binding to the 3'-untranslated region (UTR), leading mRNA degradation or translation inhibition (5,6). Aberrant miRNA expression has been reported to regulate diverse biological processes, such as cell proliferation, differentiation and apoptosis, be have been associated with various diseases and cancers (7). In addition, an increasing number of reports have suggested that miRNAs serve crucial roles in the pathogenesis of RA, and may serve as diagnostic biomarkers and therapeutic agents (8-10).

The miRNA miR-137 is located on chromosome Ip22 and has been revealed to serve as a regulator of susceptibility genes in certain diseases, such as non-small cell lung cancer (10), gastric cancer (11), renal cancer (12), breast cancer (13), Alzheimer's disease (14) and schizophrenia (15). However, the biological functions and underlying molecular mechanisms of miR-137 in RA remain unclear. Therefore, the present study aimed to analyze miR-137 expression in RA-FLS, to examine the effects of miR-137 on proliferation, migration, invasion and the expression of inflammatory cytokines, and to investigate its role in RA-FLS.

Materials and methods

Animals and RA model preparation. A total of 10 male Wistar rats (weight, 160-180 g; age, 5-6 weeks) were obtained from the Animal Center of Jilin University (Changchun, China), and were maintained under specific pathogen-free conditions (SPF), fed standard chow and provided with tap water ad libitum at room temperature (20-25°C) and 12-h light dark cycle. Rats were randomly divided into 2 groups (n=5/group): The RA group and the normal control group. The RA mouse model was established as described previously (16). Briefly, rats were treated with Complete Freund's Adjuvant (0.1 ml/100 g body weight, administered via left paw injection; Sigma-Aldrich;
Merck KGaA, Darmstadt, Germany) for 21 days. Rats in the normal control group received injections of normal PBS (0.1 ml/100 g body weight) in the left paw. Animal experimental protocols were approved by The Animal Care and Use Committee of Jilin University (Changchun, China).

Cell culture and transfection. FLSs were obtained from the synovial tissues of RA and normal control rats as described previously (17). Briefly, synovial tissues were obtained and were cut into blocks of 1x1x1 mm in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) under sterile conditions. The tissues were aspirated using a sterile pipette and sprayed evenly onto the bottom of the 25 cm² flasks, then were cultured in DMEM supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂. The medium was replaced every two days. The tissue blocks were washed with PBS one week later and after the primary culture reached 70% confluence, the cells were passaged. Cells from passages 3-6 were used in the present study. FLSs were grown in cell culture flasks in 10% high-glucose DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (both from the Beyotime Institute of Biotechnology, Haimen, China) and maintained at 37°C in humidified air with 5% CO₂.

For transfections, FLSs were seeded (1x10⁴ cells/well) in 6-well plates and cultured for 24 h. RA-FLS were transfected with miR-137 mimic (5'-GAUGCCGAUAAGAUAUCG UUAU-U3') or the corresponding negative control (miR-NC; 5'-UCGCUGUUGGCAGGUGCGGAA-A3'; both from Shanghai GenePharma Co., Ltd., Shanghai, China) at final concentration 100 nM using the Lipofectamine 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Then transfected cells were cultured at 37°C in humidified air with 5% CO₂ for 48-72 h to test miR-137 role in RA-FLS. Transfection efficiency was determined by RT-qPCR at 24 h post-transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA including miRNAs was isolated from 2x10⁴ cultured cells and 100 mg frozen fresh tissues using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The purity and concentration of total RNA were determined with a dual-beam ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany). To quantify miR-137, cDNA was synthesized using the TaqMan miRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.), then was quantified using TaqMan Human MicroRNA Assay kit (Thermo Fisher Scientific, Inc.) on an ABI7900 Real-Time PCR System. The CXCL12 and GAPDH primers used in the present study were described previously (19). The primer of CXCL12 was as follows: Forward 5'-CTCTGCATCGTGAC GTTAAAG-3' and reverse, 5'-AATCTGAGGCGACAGTT TGG-3'; the primer for GAPDH was as follows: Forward 5'-TGTAATCCACTGGCCTTC-3' and reverse, 5'-GCT TACGCCCCATCACAA-3'. The PCR amplification conditions were as follows: 95°C for 40 sec and 40 cycles of 95°C for 5 sec, 60°C for 40 sec, finally extend for 72°C 5 min. U6 small nuclear RNA was used to normalize miR-137 expression levels, and GAPDH was used to normalize CXCL12 mRNA levels. The relative expression levels were evaluated using the 2⁻ΔΔCq method (20).

Cell proliferation. RA-FLS were seeded (2x10⁴ cells/well) in 96-well plates and cultured in DMEM medium containing 10% FBS at 37°C in humidified air with 5% CO₂ for 24 h prior to transfection with miR-137 mimic or miR-NC for an additional 48 h at 37°C. Following the addition of MTT solution (20 µl, 5 g/l; Sigma-Aldrich; Merck KGaA) to each well, plates were cultured for 4 h at 37°C. The medium was removed, 150 µl dimethylsulfoxide (Sigma-Aldrich; Merck KGaA) was added and the plates were oscillated for 10 min. The absorbance of each well was measured at 490 nm using a Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Cell migration and invasion. The migratory ability of miR-137 mimic- or miR-NC-transfected RA-FLS was examined by wound-healing assay. Briefly, RA-FLS (2x10⁴ cells/well) were seeded into 24-well tissue culture plates for 24 h at 37°C in humidified air with 5% CO₂ and grown to ~80% confluence, a linear wound was made in the cellular monolayer with a 200 µl pipette tip. Following wounding, the debris was removed, DMEM was added and the cells were incubated for 24 h. Wound closure was observed and imaged at 0 and 24 h using an IX71 inverted light microscope (Olympus Corporation, Tokyo, Japan). The migration index was calculated as follows: Experimental group scratch distance (0-24 h)/control group (miR-NC) scratch distance (0 h-24 h).

For the Transwell invasion assay, RA-FLS (2x10⁴) were suspended in serum-Free DMEM medium and seeded in the upper Transwell chamber that was pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). DMEM containing 20% FBS was added to the lower chamber as the chemoattractant and the cells were incubated for 48 h. Non-invading RA-FLS were removed from the upper membrane surface with cotton swabs, and the RA-FLS that migrated to the lower membrane were fixed with 100% methanol at room temperature for 30 min and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature for 5 min. Cells were imaged with an IX71 inverted light microscope (Olympus Corporation) and counted in five randomly selected fields.

Measurement of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-8 and IL-1β protein expression. Following transfection, protein expression levels of TNF-α, IL-6, IL-8
and IL-1β were measured in each FLSs using ELISA. Briefly, transfected RA-FLS were seeded into a 24-well plate at a density of 2x10^5 cells/well and grown in DMEM medium with 10% FBS for 48 h at 37°C. The supernatants were collected by centrifugation at 1,000 x g for 5 min at 4°C. TNF-α, IL-6, IL-8 and IL-1β production in supernatants were determined using human Cytokine 25-Plex Panel (Invitrogen; Thermo Fisher Scientific, Inc.) at the density of 2x10^5 cells/well and grown in DMEM medium with 10% FBS for 48 h at 37°C. The supernatants were collected by centrifugation at 1,000 x g for 5 min at 4°C. TNF-α, IL-6, IL-8 and IL-1β production in supernatants were determined using human Cytokine 25-Plex Panel (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The concentrations of each protein were determined using ELISA multi-well spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA).

**Luciferase reporter assay.** Prediction of miR-137 targets was performed using two publicly available algorithms: TargetScan (www.targetscan.org) and miRanda (www.microrna.org). Human CXCL12 3'UTR oligonucleotides containing the wild-type (Wt) or mutant (Mut) binding site of miR-137 were synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China) and inserted into the pGL3-control vector (Ambion; Thermo Fisher Scientific, Inc.) at the NheI (Sigma-Aldrich; Merck KGaA) and Xhol (Sigma-Aldrich; Merck KGaA) sites. For luciferase reporter assay, RA-FLS were seeded into 24-well plates for 24 h, and transiently co-transfected with 100 ng of either Wt-CXCL12 or Mut-CXCL12 plasmid, and 100 nM of either miR-137 mimic or mi-NC using Lipofectamine 2,000. The cells were incubated for 48 h, harvested and lysed. Luciferase activities were determined using the Dual-Luciferase Reporter 1000 Assay System (Promega Corporation, Madison, WI, USA). Renilla-luciferase was used for normalization.

**Western blotting.** Cultured FLS (2x10^5 cells) were harvested and lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). The supernatants were collected by centrifugation at 1,000 x g for 5 min at 4°C, and protein concentrations were determined using the Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). A total of 30 μg protein were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, and incubated overnight at 4°C with the following primary antibodies: Mouse monoclonal anti-human CXCL12 (1:1,000; cat no. sc-74271) and mouse monoclonal anti-human GAPDH (1:5,000; cat no. sc-293335); both were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Following primary antibody incubation, the membranes were washed three times in TBS/0.1% Tween-20 (TBST; Sigma-Aldrich; Merck KGaA) and incubated for 1 h with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10,000; cat no. 516102; Santa Cruz Biotechnology, Inc.) in TBS/Tween-20 at room temperature. Protein bands were detected using the Enhanced Chemiluminescence-Plus kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. GAPDH was used as an internal control.

**Statistical analysis.** Data are presented as the mean ± standard deviation of at least three separate experiments, and were analyzed using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA). Statistical differences were determined by using two-tailed Student's t-test for two comparisons or one-way analysis of variance followed by a post hoc Tukey's test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

**Results**

miR-137 expression levels are upregulated in RA-FLS. The levels of miR-137 expression in RA-FLS and normal FLS were examined using RT-qPCR (Fig. 1). The miR-137 expression level was significantly higher in RA-FLS compared with the level of miR-137 expression in normal FLS. This result indicated that miR-137 maybe involved in RA pathogenesis.

miR-137 overexpression inhibits RA-FLS proliferation, migration and invasion. To investigate the biological functions of miR-137, RA-FLS were transfected with miR-137 mimic or mi-NC. RA-FLS transfected with miR-137 mimics exhibited a significant increase in miR-137 expression compared with RA-FLS transfected with miR-NC (Fig. 2A). Cell proliferation, migration and invasion were determined in by MTT, wound healing and Transwell invasion assays, respectively. miR-137 overexpression significantly inhibited RA-FLS proliferation, migration and invasion compared with RA-FLS transfected with miR-NC (Fig. 2B-D). These results suggested that miR-137 may serve an inhibitory role in RA-FLS.

miR-137 overexpression inhibits TNF-α, IL-6, IL-8 and IL-1β protein expression in RA-FLS. To investigate the effects of miR-137 on inflammatory cytokines, TNF-α, IL-6, IL-8 and IL-1β protein expression levels were measured in RA-FLS by ELISA (Fig. 3). The results revealed that RA-FLS transfected with miR-137 mimic exhibited significantly decreased expression levels of TNF-α, IL-6, IL-8 and IL-1β compared with cytokine expression levels in RA-FLS transfected with miR-NC.

CXCL12 mRNA is a direct target of miR-137 in RA-FLS. Potential miR-137 targets were predicted with two bioinformatics databases (TargetScan and miRanda), CXCL12
was selected for further analysis as it has been previously reported to serve crucial roles in RA development (21). To verify whether CXCL12 is a direct target of miR-137 in RA-FLS and luciferase activities were determined. miR-137 overexpression in RA-FLS co-transfected with miR-137 mimics and Wt-CXCL12 resulted in a significant decrease in luciferase activity compared with the luciferase activity in RA-FLS co-transfected with miR-NC and Wt-CXCL12 (Fig. 4B). No significant differences were identified between RA-FLS co-transfected with Mut-CXCL12 and either miR-137 mimic or miR-NC (Fig. 4B). These results suggested that CXCL12 maybe a direct target of miR-137.

The levels of CXCL12 mRNA and protein expression in FLS were analyzed by RT-qPCR and western blotting, respectively. CXCL12 mRNA expression was significantly increased in RA-FLS compared with normal FLS (Fig. 4C); similarly, CXCL12 protein expression was notably higher in RA-FLA compared with normal FLS (Fig. 4D). To determine whether miR-137 regulated CXCL12 expression in FLS, RA-FLS were transfected with miR-137 mimics or miR-NC, and CXCL12 mRNA and protein expression levels were determined in by RT-qPCR and western blotting, respectively. As expected, overexpression of miR-137 in RA-FLS resulted in a significant decrease in CXCL12 mRNA expression (Fig. 4E), and a notable decrease in CXCL12 protein expression (Fig. 4F). These results indicated that CXCL12 was a direct target of miR-137 in RA-FLS.

Discussion

An increasing number of studies have indicated that the aberrant expression of miRNAs was involved in RA progression through regulating cell proliferation, migration and invasion (8,9). For example, a recent study reported that increased miR-21 expression in FLS in RA model rats promoted cell proliferation by facilitating the nuclear translocation of NF-κB (17). Another recent study demonstrated that decreased miR-221 expression led to a significant reduction in the expression of pro-inflammatory cytokines, and inhibited FLS migration and invasion through the inhibition of vascular endothelial growth factor, matrix metalloproteinase (MMP)-3 and MMP-9 expression (22). miR-20a has been revealed to regulate the formation of the NACHT, LRR and PYD domains-containing protein 3 inflammasome and the release of cytokines in RA-FLS by targeting thioredoxin-interacting protein expression (23). miR-137 has previously been reported to be involved in the development of certain human diseases and cancers (10-15). However, whether miR-137 serves a role in RA development remains unclear. The present study demonstrated that the expression levels of miR-137 were significantly lower in FLS from RA model rats compared with normal FLS, and that overexpression of miR-137 (via miR-137 mimics transfection) significantly inhibited proliferation, migration and invasion, and reduced the expression of inflammatory cytokines. These results indicated that miR-137 may serve an inhibitory role in RA pathogenesis.

A number of in vitro and in vivo studies have reported that certain inflammatory cytokines, including TNF-α, IL-1β, IL-18 and IL-6, were closely related to RA development (24-26).
Figure 3. miR-137 overexpression inhibits TNF-α, IL-6, IL-8 and IL-1β protein expression in RA-FLS. ELISA was performed to measure the protein expression levels of the inflammatory cytokines (A) TNF-α, (B) IL-6, (C) IL-8 and (D) IL-1β in RA-FLS transfected with miR-137 mimic or miR-NC by ELISA. *P<0.05 and **P<0.01 vs. miR-NC. FLS, fibroblast-like synoviocytes; IL, interleukin; miR, microRNA; NC, negative control; RA, rheumatoid arthritis; TNF, tumor necrosis factor.

Figure 4. CXCL12 is a direct target of miR-137 in RA-FLS. (A) Predicted binding site for miR-137 in the 3'UTR of Wt-CXCL12; mutations in the binding sites in the Mut-CXCL12 sequence are also indicated. (B) Relative luciferase activity of in RA-FLS co-transfected with either Wt-CXCL12 or Mut-CXCL12 3'UTR reporter plasmid and either miR-137 mimic or miR-NC. **P<0.01 vs. miR-NC. The expression levels of CXCL12 (C) mRNA and (D) protein were detected in FLS isolated the RA model rat group and the normal control group by RT-PCR and western blotting, respectively. GAPDH was used as an internal control. **P<0.01 vs. normal FLS. (E and F) RA-FLS were transfected with either miR-137 mimic or miR-NC, and CXCL12 (E) mRNA and (F) protein expression levels were determined. GAPDH was used as an internal control. **P<0.01 vs. miR-NC. CXCL12, C-X-C motif chemokine ligand 12; FLS, fibroblast-like synoviocytes; miR, microRNA; Mut, mutant; NC, negative control; RA, rheumatoid arthritis; UTR, untranslated region; Wt, wild-type.
These cytokines stimulate synovial fibroblast hyperplasia, leading to the secretion of chemokines and cytokines and subsequent joint damage (24, 27-29). Therefore, the present study aimed to determine whether miR-137 effected inflammation in RA-FLS, and the results revealed that the overexpression of miR-137 substantially decreased the expression of TNF-α, IL-6, IL-8 and IL-1β in RA-FLS, suggesting that miR-137 may have potential as an anticytokine therapy for RA.

CXCL12 is a potent chemotactant that has been identified for T-cell-intrinsic role in 1-3317


