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Inhibition of Kruppel-like factor 7 attenuates cell proliferation and inflammation of fibroblast-like synoviocytes in rheumatoid arthritis through nuclear factor κB and mitogen-activated protein kinase signaling pathway

Jingjing CAO[1,2], Yanhui NI[3], Huaxing ZHANG[4], Xiaoran NING[2] and Xiaoyong QI[1,5]

[1]*Teaching and Research Section of Internal Medicine, Hebei Medical University, No. 361, Zhongshan East Road, Hebei, 050017, Shijiazhuang, P.R. China*
[2]*Department of Rheumatology and Immunology, Hebei General Hospital, No. 348, Heping West Road, Hebei, Shijiazhuang, 050051, P.R. China*
[3]*Department of Cardiology, Hebei General Hospital, No. 348, Heping West Road, Hebei, Shijiazhuang, 050051, P.R. China*
[4]*Division of Medical Service, Hebei General Hospital, No. 348, Heping West Road, Hebei, Shijiazhuang, 050051, P.R. China*
[5]*Department of Cardiology Center, Hebei General Hospital, No. 348, Heping West Road, Hebei, 050051, Shijiazhuang, P.R. China*

Abstract: Rheumatoid arthritis (RA) is an autoimmune disease, which can lead to joint inflammation and progressive joint destruction. Kruppel-like factor 7 (KLF7) is the member of KLF family and plays an important role in multiple biological progresses. However, its precise roles in RA have not been described. Present study aimed to investigate the role of KLF7 in RA-fibroblast-like synoviocytes (FLSs). Data showed that KLF7 expression was obviously upregulated in synovial tissues of rats with adjuvant-induced arthritis. Functional studies demonstrated that the loss of KLF7 may suppress cell proliferation and the expression of pro-inflammatory factors (IL-6, IL-1β, IL-17A) and matrix metalloproteinase (MMP-1, MMP-3, MMP-13) in FLSs through the inhibition of phosphorylation of nuclear factor κB (NF-κB) p65 and JNK. We further showed that miR-9a-5p specifically interacts with KLF7 to negatively regulate the expression of KLF7 in RA-FLSs. Taken together, our results demonstrated that KLF7 which targeted by miR-9a-5p might participate in the pathogenesis of RA by promoting cell proliferation, pro-inflammatory cytokine release and MMP expression through the activation of NF-κB and JNK pathways in RA-FLSs. Hence, KLF7 could be a novel target for RA therapy.

Key words: Kruppel-like factor 7 (KLF7), mitogen-activated protein kinase (MAPK), miR-9a-5p, nuclear factor κB (NF-κB), rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic, relapsing systemic autoimmune inflammatory disease accompanied by joint deformity and the loss of function. It is characterized by the destruction of articular cartilage and bone tissue caused by systemic inflammation of synovial membrane [1]. The pathologic hallmark of RA is chronic synovitis of single or multiple joints and vasculitis [2]. RA affects nearly about 1% of the world's population, with higher incidence rate among Europeans and Asians [3]. Fibroblast-like synoviocytes (FLSs) are vital effector cells in the pathogenesis of RA [4, 5]. The synovium is composed of synovial macrophages and

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Corresponding author: X. Qi. email: qxy201809@126.com

FLSs. In RA, activated-FLSs are the main cell population in synovial hyperplasia, and play an important role in mediating inflammatory responses, angiogenesis, and bone tissue invasion [6]. However, the specific mechanisms by which synovial fibroblasts are activated in the pathological setting of RA yet are not known.

Generally, various researches have suggested that genetic and environmental factors jointly promote the development of RA [7, 8]. In the pathogenesis of RA, FLSs obtain tumor-like phenotype and directly or indirectly mediate cartilage destruction through the production of pro-inflammatory cytokines, including IL-6, IL-1β and tumor necrosis factor α (TNF-α), which are main trigger factors of joint inflammation in RA [9]. Besides, these molecules are capable of increasing the synthesis of matrix metalloproteinase (MMPs). MMPs expressed by FLSs are proteolytic enzymes that degrade the extracellular matrix, and are implicated in several synovial joint pathologies [10]. Some evidences have shown that a variety of signaling pathways are involved in the regulation of the expression of inflammatory factors and chemokines during RA pathogenesis, such as nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) families [11]. NF-κB is an important nuclear transcription factor associated with joint inflammation, and is essential for the production of cytokines and proteases produced by FLSs [12]. Research showed that MAPK pathway is involved in the regulation of apoptosis, proliferation, cytokine and MMPs expression in RA [13].

Kruppel-like factors (KLFs) are a class of transcription factors present in animals, which consist of 17 members with zinc finger structures. It is widely involved in regulating multiple physiological functions, such as cell proliferation, differentiation and embryonic development [14, 15]. KLF7 is located in the nucleus and plays a role in transcriptional activation. Its high expression may play an important role in the development of obesity-induced inflammation in visceral adipose tissue [16]. Several studies have shown that KLF7 was involved in the transmission of palmitate-induced inflammatory Toll-like Receptor 4 (TLR4)/NF-κB/IL-6 pathway in adipocytes [17]. In epicardial adipose tissue from patients with coronary artery disease, KLF7 promoteed macrophage activation through activation of the NF-κB pathway [18]. The important role of TLRs/NF-κB & MAPK, cytokine and chemokine signaling pathways in regulating inflammatory and immune responses involved in RA pathogenesis have been documented [19–22]. However, the role of KLF7 in the development of RA has not been reported in previous studies.

MicroRNAs (miRs) are approximately 22 nucleotide-long non-coding RNAs that are involved in various physiological processes such as apoptosis, cell differentiation, and immune defense [23]. Thus far, numerous researches demonstrated that miRNAs participate in the pathogenesis of RA and could be potential therapeutic targets in the treatment of RA [24]. A recent study reported that miR-9 may attenuate RA by inhibiting the NF-κB/receptor activator of nuclear factor-kappa B ligand (RANKL) pathway in synovial fibroblasts [25]. The prediction from the database miRDB (http://mirdb.org/miRDB/) revealed that there were potential binding sites of miR-9a-5p in the sequence of *Klf7* 3'-UTR. Based on these, we investigated the role of *Klf7* in inflammatory response and proliferation of FLSs in RA, and whether its expression may be modulated by miR-9a-5p.

Materials and Methods

Animals

Twelve Sprague Dawley (SD) male rats, weighting 160–180 g (provided by Liaoning Changsheng Biotechnology Co., Ltd., Benxi, China), were used in this study. Animals were housed under climate-controlled condition (12 h light/dark cycle, 22 ± 1°C and 45–55% relative moisture). Rats were fed with rodent chow and drank tap water ad libitum. The experimental procedures were approved by the ethic committee of Hebei General Hospital (Approval No: 2021-71) and carried out according to the National Institutes of Health Guide for the care and use of laboratory animals. Rats were anesthetized by intraperitoneal injection of 50 mg/kg sodium barbituratea and euthanized by intraperitoneal injection of 200 mg/kg sodium barbiturate.

The experimental process of adjuvant-induced arthritis (AIA) rat model was referred to Yu *et al*. with minor modifications [26]. After 7 days of adaptive feeding, the rats were randomly divided into two groups (n=6 per group): control group and AIA group. Complete Freund's adjuvant (CFA) was used to induce AIA in rats. Rats were intradermal injected with 0.1 ml CFA (BioFroxx, Einhausen, Germany) into the right hind paw in AIA group, while equal volume of saline in the control group. The blood pressure (systolic pressure) of rats in each group was measured once a week from adaptive feeding to execution, and the fasting blood glucose was measured by glucose test strips once a week. After treatment for 28 days, the hind paws of the rats were photographed and scored for arthritis [27], the volume of left hind paw of each rat was measured, then the rats were executed and the blood and ankle synovial tissues were kept.

Hematoxylin-Eosin staining

The fixed synovial tissue of ankle joint was embedded in paraffin, followed by deparaffinized and rehydrated. Subsequently, the de-waxed sections were immersed in hematoxylin solution (Solarbio, Beijing, China) for 5 min, soaked in double distilled water for 5 min. Then dissimilation caused by 1% hydrochloric acid ethanol for 3 s, and rinsed with running water for 20 min. After soaked in double distilled water for 2 min, the sections were stained by eosin solution (Sangon, Shanghai, China) for 3 min. At last, the slices were dehydrated, transparent and sealed. The pathomorphological changes of synovial tissues were observed under microscope.

Cell culture and transfection

Cell culture and treatment: Rat Synovial Fibroblasts Cells, purchased from iCell Bioscience Inc. (Shanghai, China), were cultured in primary fibroblast culture medium (iCell) containing 10% fetal bovine and 1% penicillin-streptomycin. Cells were cultured at 37°C, 5% CO_2 thermostatic incubator (Heal Force, Shanghai, China). TNF-α was used to stimulate FLS cells at a concentration of 20 ng/ml for 24 h [28].

Transfection: The FLS cells were seeded into 6-well plates and divided into control, TNF-α, NC small interfering RNA (siRNA), KLF7 siRNA-1 and KLF7 siRNA-2 group. After adherence, cells were transfected with siRNA (NC siRNA, KLF7 siRNA-1 or KLF7-siRNA2) using Lipofectimine3000 (Invitrogen, Carlsbad, CA, USA). After 24 h validated transfection, cells were treated with 20 ng/ml TNF-α for 24 h for further analyzed.

MTT assay

FLSs in each group after transfection were collected and seeded into 96-well plates at a density of 3×10^3 cells per well under TNF-α (20 ng/ml) stimulation for 24, 48 and 72 h. A total of 20 μl 3-(4, 5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide (MTT) (KeyGEN, Nanjing, China) solution was added to each well at the corresponding time point, and cells were further cultured in incubator for 4 h. Subsequently, the supernatant was gently removed and replaced with 150 μl DMSO. After incubation for 10 min in dark, the absorbance at 570 nm was measured using microplate reader (BioTek, Winooski, VT, USA).

ELISA

The cell culture supernatants were collected and then cleared through centrifugation to assess the secretion of IL-1β, IL-6 and IL-17A. Mouse IL-1β, IL-6 and IL-17A kits (LIANKE Biotech, Hangzhou, China) were used respectively. Assays were performed in accordance with the manufacturer's instructions.

Real-Time quantitative polymerase chain reaction (RT-qPCR)

The total RNA from animal tissues and cells were lysed with TRIpure reagent (Bioteke, Beijing, China) according to manufacturer's protocol. The RNA purity and concentration were quantified by NanoDrop 2000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). Extracted RNA was reversed-transcribed under BeyoRT II M-MLV reverse transcriptase (Beyotime, Shanghai, China) to synthesize cDNA. RT-qPCR was performed using SYBR Green PCR Master Mix kit (Solarbio) in 20 μl reactions containing 10 μl Master Mix, 0.3 μl SYBR Green, 0.5 μl primers of each forward and reverse, 1 μl cDNA template and 7.7 μl ddH_2O on Exicycler 96 PCR System (Bioneer, Daejon, Korea). Primer sequences, designed by Genscript (Nanjing, China). *Gapdh* was used as internal control. Relative gene expression was calculated by the comparative cycle threshold method ($2^{-\Delta\Delta CT}$) method. PCR primer sequences were as follows: KLF7: F: GGA AAT GCC GTG ACC AGA, R: TGG CTT CCT CCT TCC TTG. GAPDH: F: CGG CAA GTT CAA CGG CAC AG, R: CGC CAG TAG ACT CCA CGA CAT.

Western blotting analysis

Animal tissues and cells were harvested and protein was lysed by lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF) (Solarbio) in radioim munoprecipitation assay (RIPA) (Solarbio) to extract. The protein concentration was tested by BCA protein assay kit (Solarbio). Approximately 10–20 μg protein was heated for 5 min at 100°C, separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated protein was electro-blotted to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocked in 5% skimmed milk (Sangon) or BSA (Biosharp, Hefei, China) for 1 h at room temperature, the membranes were cut out and each band were incubated with corresponding primary antibodies at 4°C overnight. Membranes were washed by TBST and then followed by incubation with secondary antibodies conjugated with horseradish peroxidase (HRP) at 37°C for an hour. The chemiluminescence signals of membranes were detected with enhanced chemiluminescence (ECL) kit (Solarbio) by Gel imaging system. Antibodies used in the experiment were as follows: anti-KLF7 (sc-398576) (Santa Cruz, CA, USA ; 1:300 dilution), anti-MMP-1 (DF6325), anti-MMP-3 (AF0217), anti-MMP-13 (AF5355), anti-p65 (AF5006),

anti-p-p65 (AF2006), anti-ERK1/2 (AF0155), anti-p-ERK1/2 (AF1015), anti-JNK (AF6318), anti-p-JNK (AF3318), anti-p38 (BF8015), anti-p-p38 (AF4001) (Affinity, Changzhou, China; 1:1,000 dilution), anti-GAPDH (60004-1-Ig) (Proteintech, Rosemont, IL, USA ; 1:10,000 dilution). GAPDH was used as loading control. Second antibodies: goat anti rabbit IgG (SE134), goat anti mouse IgG (SE131) (Solarbio; 1:3,000 dilution).

Dual luciferase reporter assay

HEK-293T cells (Zhong Qiao Xin Zhou Biotechnology Co., Ltd., Shanghai, China) were grown in 12-well plate to 70% confluence and serum starved for 1 h. Then they were co-transfected with miR-9a-5p mimic or control mimic and wild type 3'UTR of *Klf7* or 3'UTR mutation of *Klf7* using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection, luciferase activities were measured by Dual Luciferase kit (KeyGen, Nanjing, China).

For following experiment, the FLS cells were transfected with miR-9a-5p inhibitor, inhibitor control, miR-9a-5p mimic or mimic control to evaluate the role of miR-9a-5p in regulating KLF7 expression. Then FLS cells were transfected with negative control inhibitor, miR-9a-5p inhibitor, miR-9a-5p inhibitor and NC siRNA, or miR-9a-5p inhibitor and KLF7 siRNA followed by TNF-α stimulation for 24 h to verify whether the biological function of KLF7 was mediated by miR-9a-5p. The miR-9a-5p mimics, miR-9a-5p inhibitors and their negative control were purchased from General Biological System (Anhui) Co., Ltd. (Anhui, China).

Statistical analysis

GraphPad Prism 8.0 software (GraphPad Software, USA) was used for statistical analyses and data were all presented as mean ± SD. Student's *t*-test, one-way ANOVA or two-way ANOVA were used for the comparisons. Differences with $P<0.05$ were considered to be statistically significant.

Results

KLF7 is highly expressed in AIA rats

After subcutaneous injection of CFA into the right hind paw of rats for 28 consecutive days, it was observed that compared with the control group, the hind paw of rats in the AIA model group showed conspicuous foot swelling (Fig. 1A) accompanied by higher arthritis scores (Fig. 1B). In addition, CFA induced paw oedema (changes in volume) (Fig. 1C). The pathological changes of synovial tissues in each group were observed by H&E staining, and tissue inflammation was scored. According to the results, we found that rats in the AIA model group displayed a large number of inflammatory cell infiltration, synovial tissue hyperplasia, joint narrowing, and increased inflammation score compared with normal group (Fig. 1D). These indicated that the rat model of AIA was successfully constructed. The KLF7 level in the synovial tissue of the hind paw was detected by RT-qPCR and Western blotting, and it was found that the mRNA and protein expression of KLF7 was significantly increased in synovial tissue of rats in AIA model group, compared with control group (Fig. 1E).

KLF7 expression in TNF-α-stimulated FLSs

To simulate the inflammatory state of FLS in RA synovium, FLS cells were stimulated by 20 ng/ml TNF-α. *In vitro*, the KLF7 expression in untreated FLS cells was in low expression (Figs. 2A and B). The expression of KLF7 in untreated FLS cells were arbitrarily set as 1. The relative expression of KLF7 at both mRNA and protein levels was clearly increased in TNF-α-stimulated cells. Further, KLF7 was silenced using siRNA in TNF-α-stimulated FLSs. RT-qPCR and Western blotting were used to access transfection efficiency of siRNA-mediated knockdown of KLF7 (Figs. 2A and B).

Effect of KLF7 on the proliferation of FLSs stimulated by TNF-α

To investigate the role of KLF7 in RA-FLSs, si-KLF7 was transfected into cells to construct KLF7 knockdown FLSs. Firstly, MTT assay was used to evaluate the proliferation of FLSs. Results showed that the proliferation of FLS cells were obvious increased in a time-dependent manner with TNF-α (20 ng/ml) treatment for 24, 48, 72 h compared with control group (Fig. 3). Treatment with KLF7-siRNA significantly decreased the viability of RA-FLSs at each point (Fig. 3), suggesting that siRNA-mediated downregulation of KLF7 could effectively reduce the proliferation of RA-FLSs. Based on the results obtained at 24, 48 and 72 h of TNF-α treatment, we chose 24 h as the observation for subsequent studies.

KLF7 deficiency reduces inflammatory factor release and MMP expression in TNF-α-stimulated FLSs

RA synovial fibroblasts not only display an increased growth rate, but also spontaneously secrete inflammatory cytokines and chemokines [29]. Thus, our study investigated whether KLF7 plays a role in the process that regulates the production of inflammatory cytokines of FLSs by ELISA. As shown in Fig. 4A, TNF-α stimulation resulted in increased release of cytokines in RA-

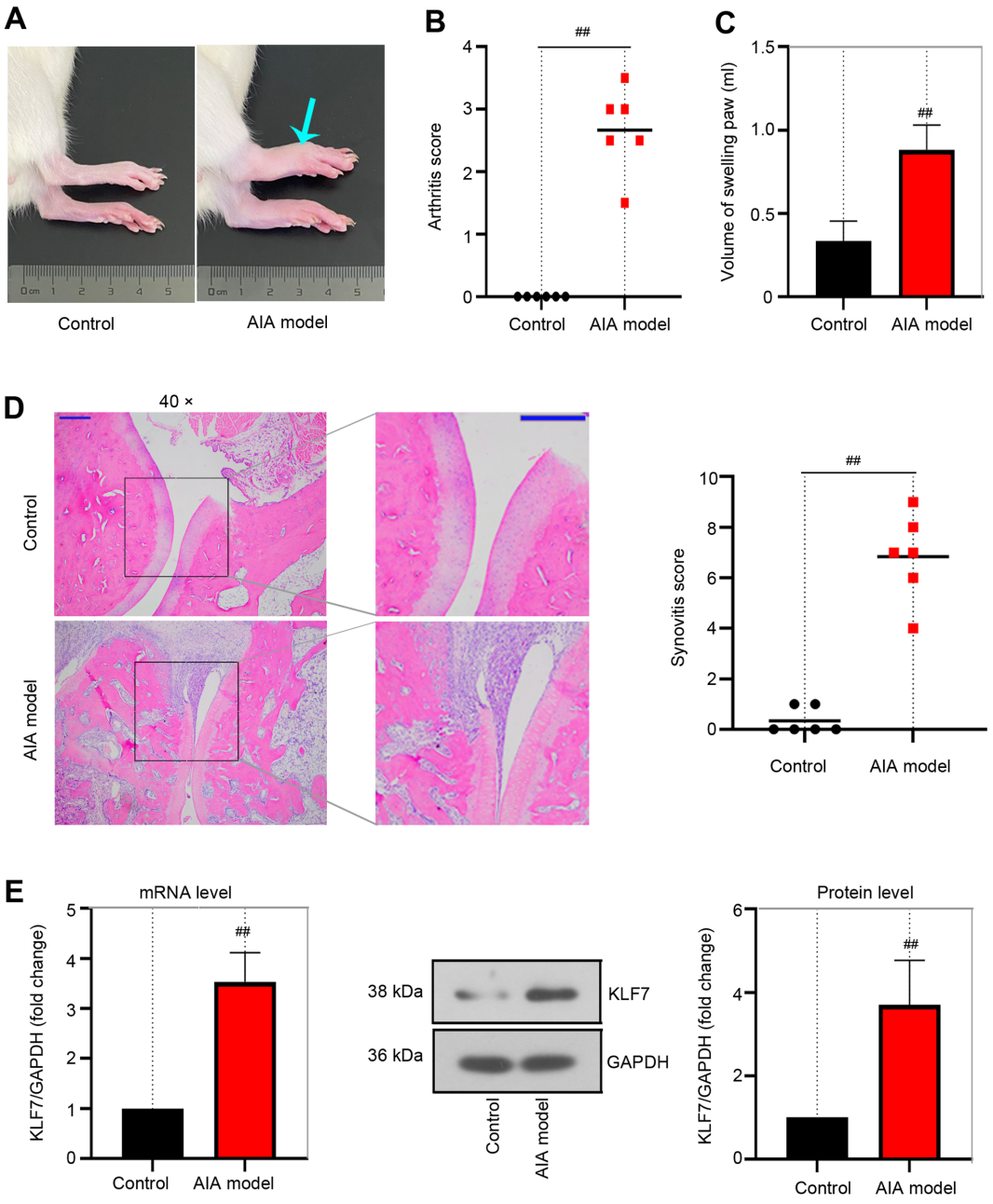

Fig. 1. KLF7 is highly expressed in AIA rats. A rat model of AIA was established by a single intradermal injection of 0.1 ml CFA into the right hind paw. After 28 days, the rats were euthanized, and blood and ankle joints were harvested. (A) The photographs of hind paw were shown. (B) The average score of arthritis in each group. (C) The volume of hind paw swelling was calculated. (D) Representative images (500 μm) of H&E-stained histological sections of the synovial tissues in rats were displayed, and the inflammation score were performed. (E) The expression of KLF7 at mRNA and protein levels was determined by RT-qPCR and Western blotting, respectively. Relative gene expression was normalized with GAPDH. AIA, adjuvant-induced arthritis; CFA, complete Freund's adjuvant; H&E, haematoxylin and eosin. Values are means ± SD, with 6 rats/group. $^{\#\#}P<0.01$ vs. Control.

FLSs, including IL-1β, IL-6, and IL-17A compared to the control group. Nevertheless, KLF7 siRNAs significantly depressed the secretion of IL-1β, IL-6, and IL-17A in FLSs with TNF-α stimulation. The MMPs are vital regulators of invasive phenotype of FLSs, which are correlated with the severity of disease [30]. We further investigated whether KLF7 could regulate the activity of MMPs of FLS. The expression of MMP-1, MMP-3 and MMP-13 were remarkable raised in TNF-α-stimulated cells compared with control group. Consistent with the results of inflammatory factors, the expression levels of MMPs were lower in si-KLF7-transfected FLS cells than those in the NC-KLF7-transfected group (Figs. 4B and C).

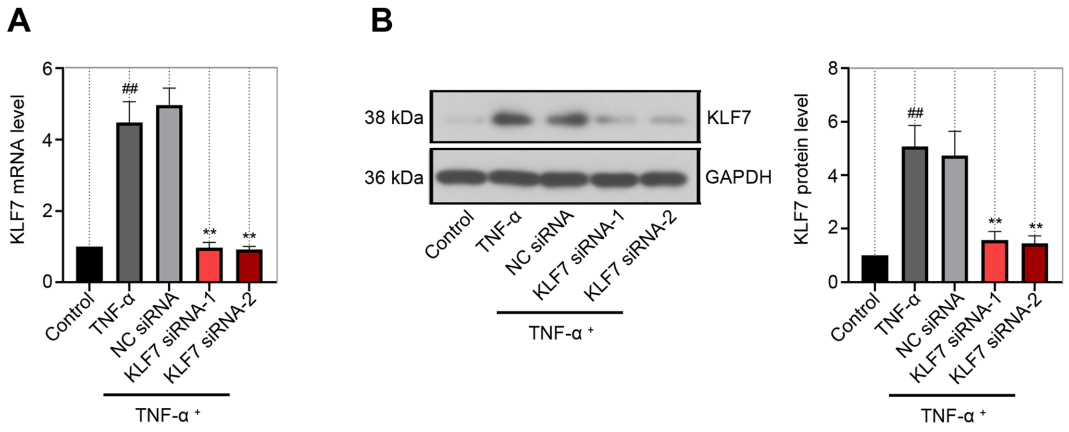

Fig. 2. KLF7 expression in TNF-α- *stimulated* FLSs. FLSs was transfected with NC siRNA or KLF7 siRNA for 24 h, and then stimulated with TNF-α (20 ng/ml) for another 24 h. (A) The mRNA level of *Klf7* was measured using RT-qPCR assay. (B) The protein level of KLF7 was determined by Western blotting analysis. FLSs, fibroblast-like synoviocyte. Values are mean ± SD, with three data points/group. $^{\#\#}P<0.01$ vs. Control; $^{**}P<0.01$ vs. TNF-α+NC siRNA.

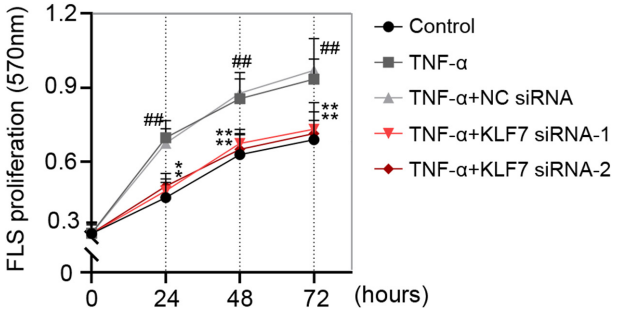

Fig. 3. Effect of KLF7 on the proliferation of FLSs *stimulated* by TNF-α. (A) The proliferation of FLSs was examined by MTT assay on 0, 24, 48 and 72 h. MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide. The results are expressed as mean ± SD (n=3). $^{\#\#}P<0.01$ vs. Control; $^{*}P<0.05$, $^{**}P<0.01$ vs. TNF-α+NC siRNA.

Effect of KLF7 on the activation of NF-κB and MAPK signaling pathways

The activation of NF-κB and MAPK signaling pathways are vital in FLSs to promote cytokines release, thereby accelerating RA progression [31]. To access the effect of KLF7 on NF-κB and MAPK signaling pathways, the expression levels of p65, p-p65, ERK1/2, p-ERK1/2, JNK, p-JNK, p38 and p-p38 were detected by Western blotting. As shown in Fig. 5A, the p-p65 expression levels were markedly elevated after TNF-α stimulation, while were suppressed in KLF7-knockdown cells. For MAPK pathway-related protein expression, the expression levels of p-ERK1/2, p-JNK and p-p38 were increased in TNF-α group, and the total protein expression of ERK1/2, JNK and p-38 showed no effect compared with control group (Fig. 5B). Whereas, KLF7 knockdown robustly inhibited p-JNK, and had no significant effect on p-ERK1/2, p-p38, ERK1/2, JNK and p-38 expression (Fig. 5B), indicating that KLF7 boosted the activation of NF-κB and JNK pathway leading to inflammatory responses in RA-FLSs.

Effect of KLF7 on the proliferation and inflammatory response of TNF-α-stimulated FLSs may be regulated by miR-9a-5p

As miRNAs exert their function by targeting the 3'UTR of specific genes, potential targets of miR-9a-5p were predicted from database (Fig. 6A). Dual-luciferase target experiment tested the correlation between miR-9a-5p and KLF7. Luciferase assays confirmed the binding of miR-9a-5p to *Klf7* 3'UTR (Fig. 6B). More to the point, there was a remarkable decrease in KLF7 expression in miR-9a-5p mimic transfected cells compared with negative transfected cells, while an opposite trend was observed of this molecule with miR-9a-5p inhibitors (Fig. 6C), indicating a negative correlation between miR-9a-5p and KLF7. For research whether miR-9a-5p mediated physiological activities in FLSs by KLF7, si-KLF7 was used to knockdown the expression of KLF7 in miR-9a-5p inhibitor-treated FLSs. The results showed that the inhibition of miR-9a-5p promoted FLS cell proliferation by targeting KLF7 (Fig. 6D). Inhibition of miR-9a-5p significantly increased the expression levels of p-p65 and p-JNK, while obviously reversed by KLF7 knockdown (Figs. 7E and H). Moreover, KLF7-knockdown inhibited the secretion of IL-6, IL-17A and MMP-13 in miR-9a-5p inhibitor-treated FLSs (Figs. 6D and E). Taken together, these results indicated that miR-9a-5p was negative correlated with KLF7, and was involved in cell proliferation and inflammatory response through interacting with KLF7.

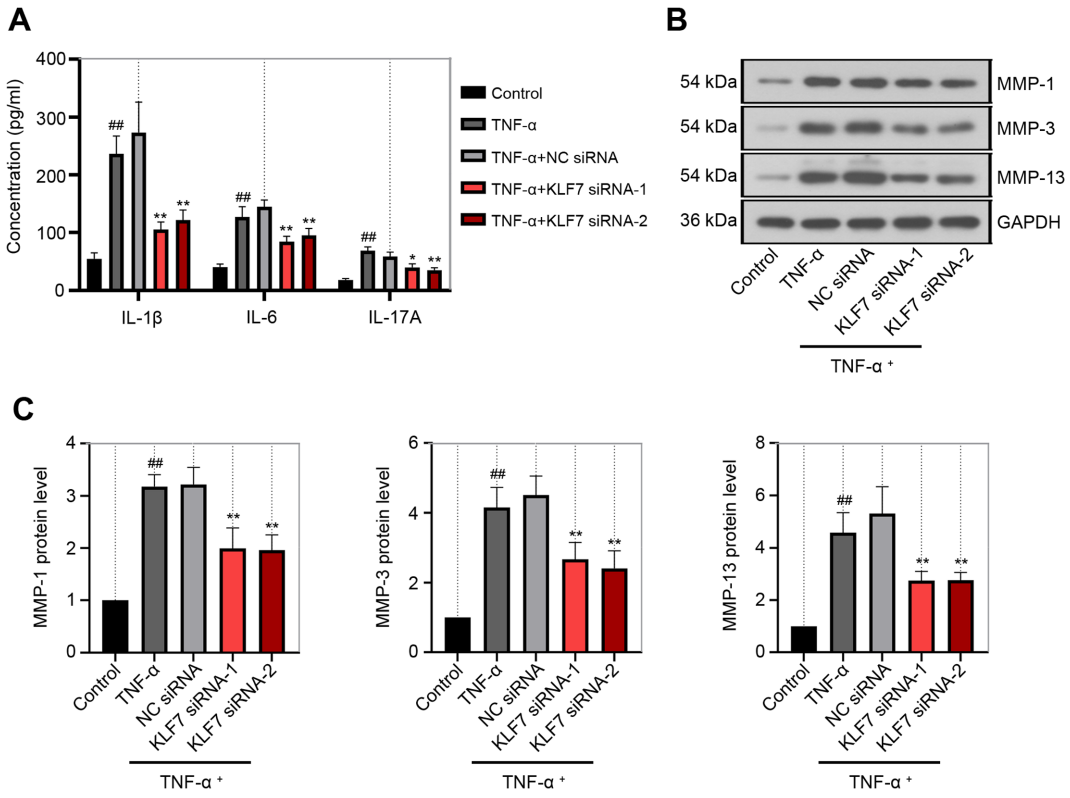

Fig. 4. KLF7 deficiency reduces inflammatory factor release and MMP expression in TNF-α-*stimulated* FLSs. (A) ELISA was used to measure the levels of IL-1β, IL-6 and IL-17A in the cell supernatant. (B–C) Relative expression levels of MMP-1, MMP-3 and MMP-13 in FLSs. MMP, matrixmetalloproteinase; ELISA, enzyme-linked immunosorbent assay. The data are mean ± SD of three independent experiments. $^{\#\#}P<0.01$ vs. Control; $^{*}P<0.05$, $^{**}P<0.01$ vs. TNF-α+NC siRNA.

Discussion

In this study, the roles of KLF7 on the pathogenesis of RA were investigated. KLF protein plays an important role in regulating a variety of biological processes and human diseases, such as cardiovascular disease and cancer [32]. Some of the studies showed that KLF7 is a crucial component of the disease process, such as sepsis and obesity-induced inflammation [16, 33], while the role of KLF7 in the pathogenesis of RA is generally uniformed. Here we used AIA models to examine the role of KLF7 in disease pathogenesis. Our results firstly showed a stronger expression of KLF7 in the synovium of CFA rats, suggesting a relationship between KLF7 and RA.

RA is a systemic chronic inflammatory disease characterized by cytokine production, synovial lining hyperplasia and joint destruction. It was reported that FLS proliferation is one of the major reasons for hyperplasia of synovial membrane. In the process of pathogenesis, FLSs could proliferate and secrete a variety of pro-inflammatory cytokines that mediate cartilage destruction and trigger joint inflammation. In RA, IL-1β is thought to be a major part in synovial inflammation and is also a pivotal cytokine that induces the expression of other inflammatory cytokines, such as IL-6, IL-17A [34, 35]. Previous studies demonstrated that increased levels of IL-1β in synovial tissue associated with histological features of arthritis [36, 37]. TNF-α is one of the most mature effectors that regulate synovial inflammation involved in RA [38]. Previous study has demonstrated that stimulation of TNF-α resulted in the activation of FLSs and the increase of inflammatory cytokines release [39]. In our study, 20 ng/ml TNF-α were used and similar results were obtained. KLF7 siRNA depressed both cell viability and the release of IL-8, IL-17A and IL-1β. Among them, the IL-1β trend is more obvious. A previous study researched the effect of KLF4 on the pathogenesis of inflammatory arthritis *in vivo*, which showed obvious increase in cell proliferation and secretion of IL-1β, IL-6 and MMPs [40]. Kawamura *et al*. [41] found that the over-expression of KLF7 could up-regulate IL-6 expression in human preadipocytes.

The invasive characteristic of FLSs is one of the main causes of bone and cartilage destruction in RA, and MMPs are the primary effectors for cell invasion in RA [42]. Meanwhile, previous studies reported that inflammatory cytokines could enhance the expression of

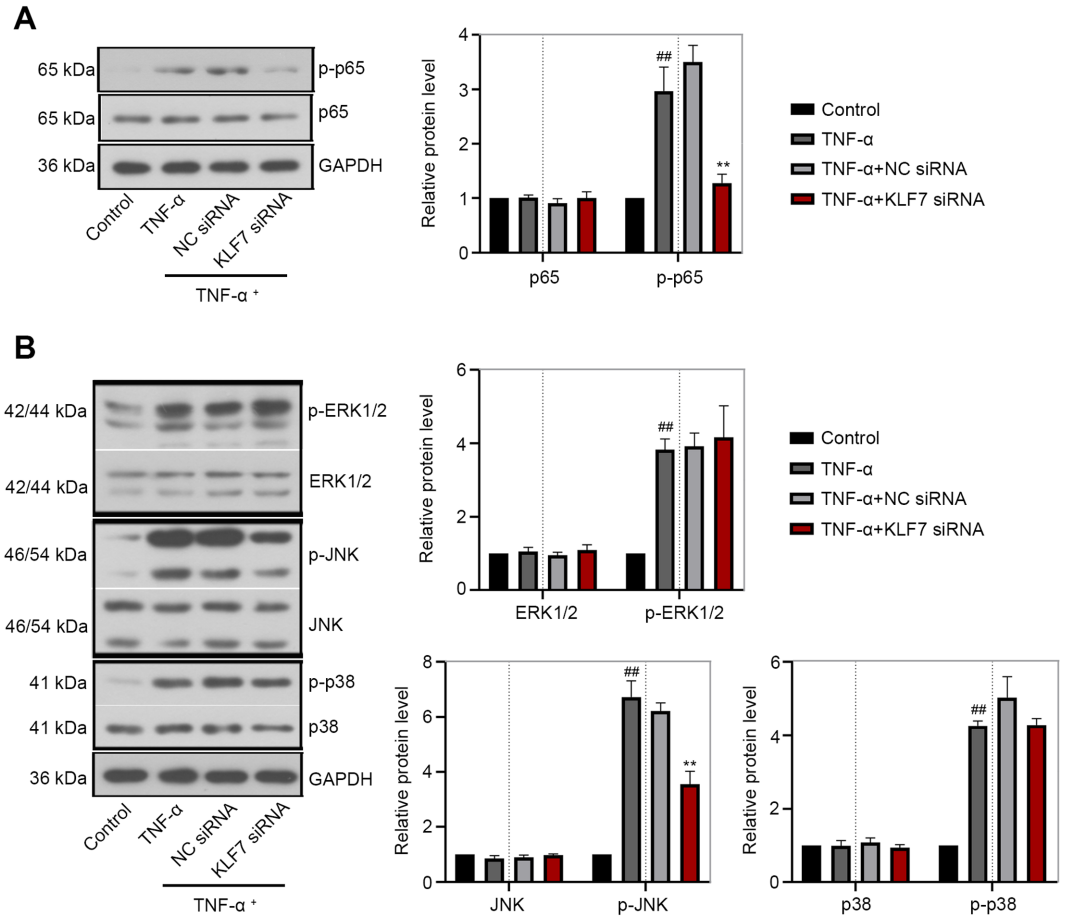

Fig. 5. Effect of KLF7 on the activation of NF-κB and MAPK signaling pathways. (A) Western blotting was performed to detect the expression of p65 and p-p65 in TNF-α-stimulated FLSs. (B) Expression levels of ERK1/2, p-ERK1/2, JNK, p-JNK, p38 and p-p38. NF-κB, nuclear factor-kappa B; MAPK, mitogen-activated protein kinase. The data represent the mean ± SD (n=3). $^{\#\#}P<0.01$ vs. Control; $^{**}P<0.01$ vs. TNF-α+NC siRNA.

MMPs, and activate the transcription factors [43], thus further aggravating inflammatory arthritis and joint damage [44]. The expression levels of MMP-1, MMP-3 and MMP-13 were detected, which are the major types of MMPs contributing to the process of joint destruction in RA. Results found that knockdown of KLF7 could decrease the expression of these MMPs. Similar to our result, Yusuke *et al*. revealed that KLF5 could activate MMPs to cause cartilage degration [45]. The above data indicated that KLF7 may promote the invasiveness of FLSs by promoting MMPs production. Therefore, our results supported the role of KLF7 in promoting cell proliferation, pro-inflammatory cytokine and chemokine release in RA-FLSs.

Next, we investigated the underlying molecular mechanism of the regulative effects of KLF7 on RA-FLSs. NF-κB is a transcription factor that express proteins functioning in inflammation, and previous studies have reported that constitutively activated NF-κB pathway was observed in the synovial tissue of RA patients and animal models [46–48]. In addition to NF-κB, MAPK is also considering as a target that contribute to RA. MAPK is a widely conserved serine/threonine protein kinase family containing p-JNK/JNK, p-ERK/ERK, and p-p38/p38. Some researchers have suggested that NF-κB & MAPK are involved in regulating the expression of genes controlling cell proliferation and apoptosis and genes in response to inflammation and immune responses [49–51]. Thus, we further studied to verify whether KLF7 exerts pro-inflammatory effects through the modulation of NF-κB and MAPK signaling pathway. We analyzed the expression of related proteins, and observed that KLF7 siRNA blocked the phosphorylated activation of NF-κB and JNK significantly stimulated by TNF-α, but not the ERK and p38 in RA, indicating that the NF-κB and JNK pathway may mediate the action of KLF7 in FLSs. JNK is thought to be an important contributor to the pathological changes of RA because it can phosphorylate c-jun and then initiate the expression of MMPs [52]. Zhao *et al*. [53] have reported that JNK is the key

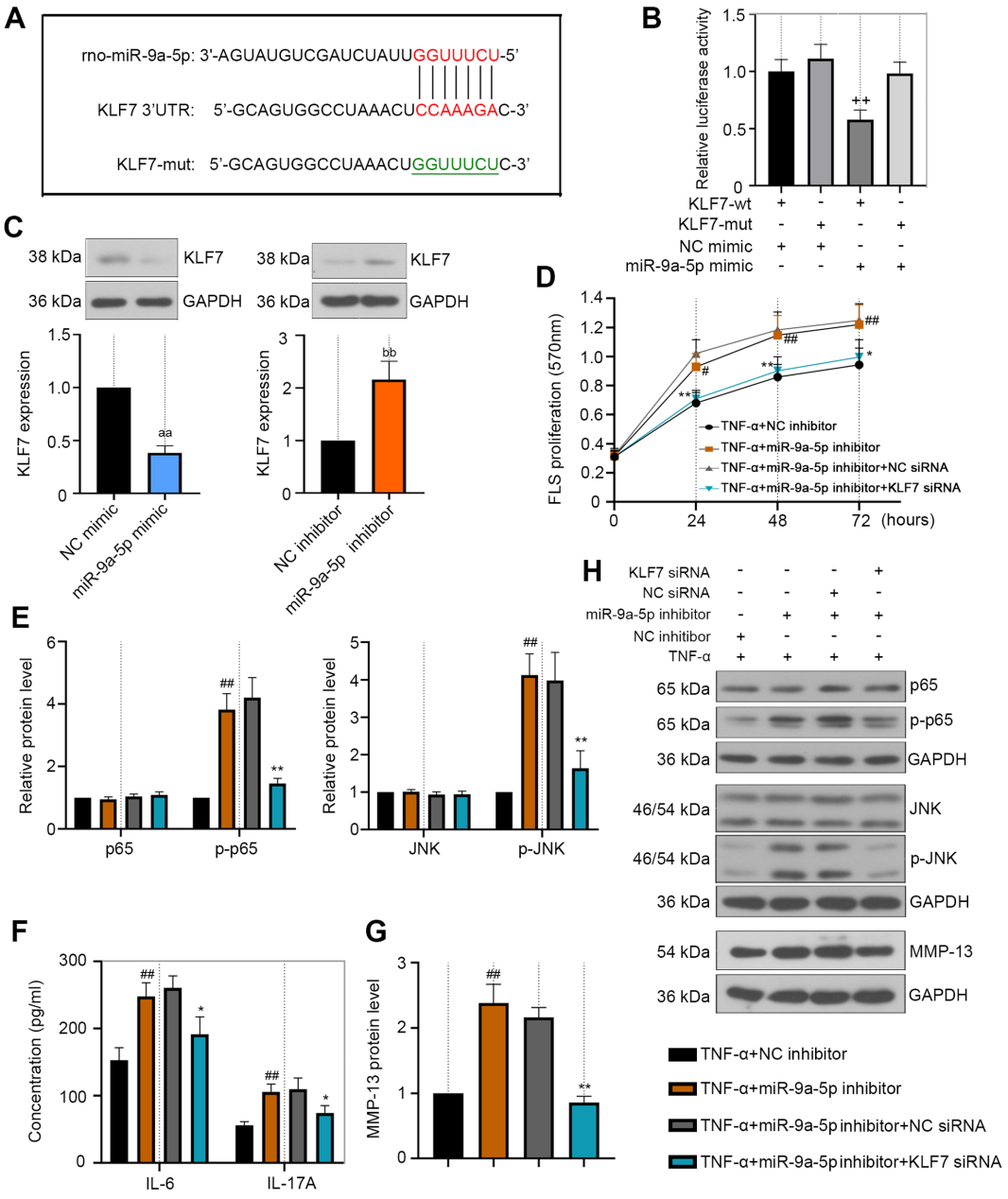

Fig. 6. Effect of KLF7 on the proliferation and inflammatory response of TNF-α-stimulated FLSs may be regulated by miR-9a-5p. (A) The putative miR-9a-5p target sites on the 3'UTR of *Klf7* were shown. (B) 293T cells were co-transfected with NC mimic or miR-9a-5p mimic and *Klf7* 3'UTR (wt or mut). Dual luciferase reporter assay showing the effect of miR-9a-5p on 3'UTR of KLF7. Wt, wild type; mut, mutant type. (C) FLSs was transfected with NC mimic, miR-9a-5p mimic, NC inhibitor, or miR-9a-5p inhibitor for 48 h. The transfection efficiency was confirmed by Western blotting assay. (D) FLSs was transfected with NC inhibitor or miR-9a-5p inhibitor and NC siRNA or KLF7 siRNA for 24 h, followed by TNF-α stimulation for another 24 h. MTT was carried out for FLSs proliferation. (E, H) Expression levels of p65, p-p65, JNK, p-JNK and MMP-13. (F) Levels of inflammatory factors IL-6 and IL-17A was evaluated by ELISA. (G) MMP-13 expression was analyzed by Western blotting. Data were shown as mean ± SD. ++P<0.01 vs. miR-9a-5p mimic+KLF7-mut. aa P<0.01 vs. NC mimic; bb P<0.01 vs. NC inhibitor. #P<0.05, ##P<0.01 vs. TNF-α+NC inhibitor; *P<0.05, **P<0.01 vs. TNF-α+miR-9a-5p inhibitor+NC siRNC.

MAPK pathway of collagenase gene expression in synovial cells and arthritis, and concluded that JNK is an important therapeutic target of RA. Other study found that inhibition of NF-κB activation resulted in reduced inflammation, but no change in bone destruction [54]. These data showed that KLF7 might modulate JNK and NF-κB pathway. They complement each other to regulate destruction or inflammatory in RA. Moreover, some researchers reported that NF-κB inhibition decreased pro-inflammatory cytokine levels such as IL-1β, TNF-α,

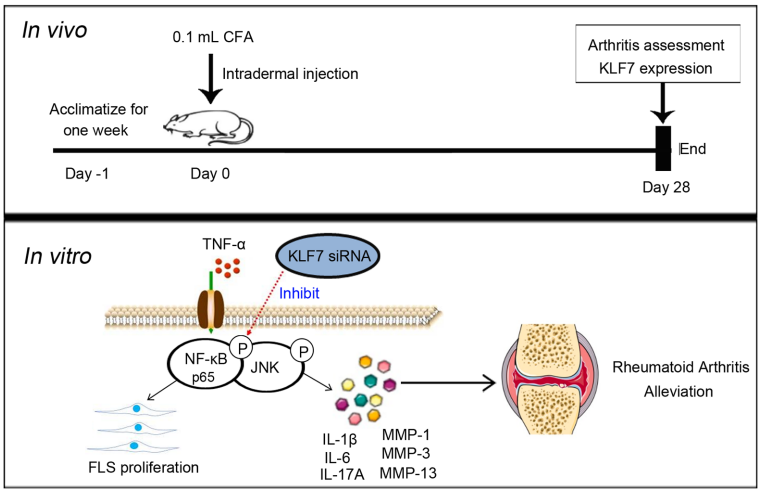

Fig. 7. Possible mechanism diagram of KLF7 in rheumatoid arthritis. KLF7 silencing alleviated the pathogenesis of RA by inhibiting cell proliferation, pro-inflammatory cytokine release and MMP expression through the inhibition of NF-κB and JNK pathways in RA-FLSs.

and IL-6 and MMPs to attenuate RA [55, 56]. Kanai *et al*. also showed that JNK inhibition in FLSs could decrease the decrease the secretion of IL-6 and MMP-3 [57], suggesting that KLF7 silence might reduce the release of inflammatory factors and chemokine release by inhibiting JNK and NF-κB pathways in FLSs, thereby attenuating RA.

A growing number of studies have focused on the expression of miRNA in RA. Therefore, understanding the disease-related mechanisms of these small noncoding RNAs may provide a new method for the diagnosis and treatment of RA. A recent study showed that miR-9a-5p plays a vital role in the regulation of HSC proliferation and migration, during liver fibrosis [58]. Li *et al*. revealed that miR-9 inhibitor increased the proliferation of FLSs and promoted inflammatory state [25]. In addition, it has been reported that miR-9a targets NF-κB gene in bone-marrow-derived mesenchymal stem cells, hence inhibiting the NF-κB pathway [59]. In our study, we aimed to test and verify a hypothesis that there is a target relationship between KLF7 and miR-9a-5p, which can be explained by bioinformatics-based prediction. Dual-luciferase reporter gene assay confirmed that KLF7 was the target gene of miR-9a-5p. Moreover, miR-9a-5p in low expression could inhibit the effects of KLF7 knockdown on the cell proliferation and the production of IL-6, IL-17A, and MMP-13, which mainly through the NF-κB and JNK pathway. This is the first report of miR-9a-5p to prove that miR-9a-5p could activate JNK and NF-κB signal pathway in RA-FLSs. These results suggested for the first time that the role of KLF7 may be modulated by miR-7a-5p. Although the contribution of KLF7 and miR-9a-5p to the whole immune system is unclear, inhibiting KLF7 or inducing miR-9a-5p may be a potential treatment for RA.

Taken together, our research proved for the first the time that the reduction of KLF7 could alleviate TNF-α-stimulated synovial inflammatory response, which might be related to the inactivation of NF-κB and JNK signal pathway. In addition, miR-9a-5p could negatively regulate KLF7 expression, which can be a potential therapeutic target for RA. The mechanism is shown in Fig. 7. However, whether KLF7 and miR-9a-5p can be used as diagnostic markers and prognostic indicators of RA, and how KLF7 and miR-9a-5p can be used in clinical treatment need to be further studied.

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