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MiR-20a regulates fibroblast-like synoviocyte proliferation and apoptosis in rheumatoid arthritis

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Abstract. – OBJECTIVE: STAT3 expression is elevated in the synovial tissue of patients with rheumatoid arthritis (RA). MiR-20a plays a role in mediating synovial inflammation in RA. Bioinformatics analysis has identified a binding site between miR-20 and the 3'-UTR of STAT3 mRNA. This study aimed to investigate the role of miR-20a in the regulation of STAT3 expression and synovial cell proliferation as well as apopt[illegible]

PATIENTS AND METHODS: Synovial [illegible] were collected from RA patients and os[illegible]r-thritis (OA) patients to measure miR-20a, S[illegible] p-STAT3, and Ki-67 expressions. Fibroblast[illegible] synoviocytes (FLS) were treated with IL-17 ([illegible] ng/ml) and then Ki-67 expres[illegible]d cell cy[illegible]cle were evaluated by flow [illegible]. The tar-geting relationship betwee[illegible]R-20a [illegible] STAT3 was assessed by dual l[illegible]ase re[illegible] gene assay. FLS cells were d[illegible] into [illegible] miR-NC, miR-20a mi[illegible] s[illegible]TAT3, and miR-20a mimic + si[illegible]T3 gro[illegible].

RESULTS: In R[illegible]atients, sig[illegible]ntly low-er MiR-20a exp[illegible] and substa[illegible]y high-er STAT3, p-S[illegible]3, [illegible]i-67 expres[illegible]on were found in the [illegible]novial [illegible]s compared with those in C[illegible]atients. IL-[illegible]atment marked-ly prom[illegible] FLS cell prolife[illegible], inhibited cell apopt[illegible] reduc[illegible]d miR-20a expression, as well as u[illegible]gulate[illegible]els of STAT3, p-STAT3, and Bc[illegible]iR-20[illegible]ayed a[illegible]egulatory function on the e[illegible] of STA[illegible]MiR-20a mimic and/or si-STAT[illegible]fectio[illegible]parently downregulated [illegible], p-S[illegible] Bcl-2 expression, attenu-ate[illegible]17A-in[illegible] cell proliferation promotive and[illegible]anced ce[illegible]apoptosis in FLS cells.

C[illegible]IONS: The expression of miR-20a w[illegible]n synovial tissue of RA patients [illegible] the increased level of STAT3. Downregu-[illegible]n of miR-20a promoted the expression of [illegible] p-STAT3, and Bcl-2, facilitated FLS cell pro[illegible]ation, reduced apoptosis and, thereby, played a critical role in RA.

Key W[illegible] STAT3, Bcl-2, RA, [illegible]S.

[illegible]roduction

R[illegible]thritis (RA) is a type of arthro-pathy [illegible]terized by chronic inflammatory [illegible]novial tissue hyperplasia[1]. Fibroblast-like sy-[illegible]s (FLS) are the main cell type in the [illegible] tissue. Their excessive proliferation and activation play an important role in RA patho-genesis[2]. Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway play a critical role in multiple biological processes, including cell proliferation, apopto-sis, inflammation, and homeostasis[3]. STAT3 is a member of STAT family in JAK-STAT signaling pathway. It can promote the expression of seve-ral multiple target genes, such as Bcl-2, Cyclin D1, and VEGF, to facilitate cell proliferation and restrain cell apoptosis[4]. It has been shown that STAT3 is abnormally elevated in the synovial tis-sue in RA patients compared with that in normal control[5,6]. A previous clinical study has revealed that attenuation of STAT3 activity can alleviate the inflammation in RA patients[7]. In addition, the role of Bcl-2 reduction in mediating synovial cell excessive proliferation and promoting RA pro-gression, has also been confirmed by several stu-dies[8,9]. MicroRNAs are a type of small noncoding RNAs that play a key role in the epigenetic gene regulation. They can regulate more than 30% of human gene expression, thus serving as a critical modulator in controlling cell growth, apoptosis, and immunologic function maintenance. It has been reported that miR-20a was downregulated in

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multiple autoimmune diseases, including multiple sclerosis[10] and systemic lupus erythematosus[11]. In addition, miR-20a was demonstrated to play a regulatory role in the inflammation of synovial cells in RA[12-14]. Bioinformatics analysis has identified a binding site between miR-20 and the 3'-UTR of STAT3 mRNA. This study investigated the role of miR-20a in regulating STAT3 expression, as well as synovial cell proliferation and apoptosis.

Patients and Methods

Patients

We enrolled a total of 49 RA patients in Baise People's Hospital between December 2015 and August 2016, including 19 males and 30 females with a mean age of 60.8 ± 12.9 years (range: 49-73 years). No patients received vitamin D, glucocorticoid, or immunosuppressor treatment before surgery. Twenty-one patients with osteoarthritis (OA) in Baise People's Hospital during the same period were selected as controls. The synovial tissues and articular cavity synovia were collected.

Main Reagents and Materials

Dulbecco's Modified Eagle Medium (D M) and fetal bovine serum (FBS) were purch from Hyclone (South Logan, UT, USA). IL- cytokine and antibody were obta from R& systems (Minneapolis, MN, U primers were synthetized by Gene (Shan i, China). QuantiTect SYBR C RT-PC kit was purchased from Qiagen ncia STAT3 and p-STAT3 ibo purchased from Abcam (Cam ge, MA, Bcl-2 and β-actin were pu d by Santa C iotechnology (Santa USA). Goat ti-rabbit and mouse Ig econda tibody were purchased from vector (Sha China). STAT3 siRNA negative contro e synthesized by Sa Cruz B technology (Santa Cruz, CA, US iR-20 mic and negative control were obta fro E Dh acon (Lafayette, CO, USA). p ciferas porter vector was pur- d fro Middlesex, UK). Endonuclea SacI an were purchased from New Eng Biolabs Shanghai, China). Dual-Luciferase Assay System was provided by Promega (Madison, WI, USA). Annexin V-FITC/PI cell apoptosis detection kit was purchased from Multi Sciences (Beijing, China). IL-17A ELISA kit was purchased from RayBiotechnology (Norcross, GA, USA).

Immunofluorescence

The frozen section of synovial tissue w re- pared to measure STAT3 expression was fixed with formaldehyde and eabilize by Triton X. After the section w locked with BSA, it was incubated overni th STAT3 monoclonal antibody (1:300 dilutio n, the section was incubated with xa Flou be led secondary antibody a dilution o Next, the section was ed wi API and e- served under an inv micr pe.

Flow Cytome Dete of K Expression

The syn ue was digest type II collagenase try The tissue was then incubated with PECy5 l Ki-67 at 4°C and was dete flow cytom

Cell Separation and Cultivation

The synovial ue was digested by 0.1% type llagenase a 05% trypsin. Next, the cells w ltured i MEM. The cells in the 4th generation were used for the experiment.

ELISA

IL-17A level in articular cavity synovia was d cted by ELISA. The plate was incubated sequentially with primary antibody and biotin labeled secondary antibody. After washing and development, the absorbance of each well was detected by a spectrometer at 450 nm.

IL-17A Treatment

FLS cells were seeded into a 6-well plate and treated with IL-17A (10 ng/ml) to simulate inflammation condition. The cells were then collected after 48 h treatment.

Luciferase Reporter Assay

The full-length fragment or mutant fragment of STAT3 3'-UTR was amplified by PCR. The product was treated with SacI and XbaI, and further cloned to pIS2 luciferase vector. After sequencing, the plasmid with correct sequence was named as pIS2-STAT3-wt and pIS2-STAT3-3'-mut, respectively. pIS2-STAT3-wt or pIS2-STAT3-3'-mut was co-transfected to HEK293T cells using Lipofectamine 2000 together with miR-20a mimic or miR-NC. Luciferase activity was measured after 48 h by the kit.

FLS Cell Transfection and Grouping

FLS cells in the 4th generation were seeded into 10-cm dish and transfected using Lipofectamine

2000 transfection reagent. FLS cells were divided into five groups, including miR-NC, miR-20a mimic, si-NC, si-STAT3, and miR-20a mimic + si-STAT3 groups. After 48 h of cultivation, the cells were treated with 10 ng/ml IL-17A for 48 h.

qRT-PCR

QuantiTect SYBR Green RT-PCR Kit was used for qRT-PCR detection. The primers used were as follows. $STAT3P_F$: 5′-ATCACGCCTTCTACAGACTGC-3′, $STAT3P_R$: 5′-CATCCTGGAGATTCTCTACCACT-3′; $Bcl\text{-}2P_F$: 5′-GGTGGGGTCATGTGTGTGG-3′, $Bcl\text{-}2P_R$: 5′-CGGTTCAGGTACTCAGTCATCC-3′; $\beta\text{-actin}P_F$: 5′-GAACCCTAAGGCCAAC-3′, and $\beta\text{-actin}P_R$: 5′-TGTCACGCACGATTTCC-3′.

Western Blot

Total protein was extracted and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the protein was transferred to polyvinylidene fluoride (PVDF) membrane and blocked with skim milk at room temperature for 60 min. Next, the membrane was sequentially incubated with primary antibody (STAT3, p-STAT3, Bcl-2, and β-actin at a dilution of 1:300, 1:200, 1:300, 1:3 1:600, respectively) and secondary antib at a dilution of 1:5000. At last, the membrane developed by ECL and scanned.

Flow Cytometry Detectio Apoptosis and Prolifera

The cells were collecte d incub with 5 µl of Annexin V and 5 PI. T ls were tested on AC No flow cytometry to evaluate apoptosis cells were collected and in d with Ki-6 body at 4°C. Cell prol as detected ACEA NovoCyte™ cyto

Statisti Analysis

All a anal s were performed by SPSS 18.0 tware M SPSS, Chicago, IL, USA). The r t data presented as mean ± standar ion an mpared by *t*-test. $p<0.05$ consid tat ally significant.

Results

R20a Downregulated, while STAT3 ted in the Synovial Tissue of RA

A result showed that the level of IL-17A was significantly increased in the articular cavity synovia of RA compared with that in OA (Figure 1A). qRT-PCR analysis demonstrat that miR-20a expression was declined, w mRNA expression was enhanced i A compared with that in OA (Figure 1B) munofluorescence analysis revealed that ST otein level was markedly higher in the synovia of RA than that in OA (Figure Western gested that STAT3 and AT3 protein sions in RA were app y hig han thos OA (Figure 1D). F ytom exhibi d that Ki-67 level was gnifi gher in than that in OA, ind g the sive eration of synovial ti in RA pati e 1E). Pearson corre alysis showe the level of IL-17A i sy was positively correlated with STAT3 mRN ession, and negatively corre ith miR-20 ession (Table I).

7A Downregulated miR-20a and vated STA Expressions in FLS Cells

study the r f IL-17A, flow cytometry de- te was empl and revealed that IL-17A signifi Ki-67 expression, facilitated cell proliferation, and reduced cell apoptosis (Figure 2A and B). Moreover, qRT-PCR exhibited that IL-17A lated miR-20a expression and elevated AT xpression in FLS cells (Figure 2C). Western blot confirmed the finding and demonstrated that STAT3, p-STAT3, and Bcl-2 protein levels after IL-17A treatment were markedly higher compared with before treatment (Figure 2D). Bioinformatics analysis revealed the binding site between miR-20 and the 3'-UTR of STAT3 mRNA (Figure 2E), which was later confirmed by the reporter assay revealing that miR-20a mimic transfection significantly declined the relative luciferase activity in HEK293T cells after transfected with pIS2-STAT3-wt (Figure 2F), indicating the targeted regulatory effect of miR-20a on STAT3.

Upregulation of miR-20a Inhibited STAT3 Expression, Attenuated Cell Proliferation, and Increased Cell Apoptosis

To elucidate the function of miR-20a on the expression of STAT3, we used miR-20a mimic

Table I. Correlation analysis of IL-17A with miR-20a and STAT3.

	IL-17A	
	r	p
miR-20a	-0.583	0.037
STAT3	0.615	0.031

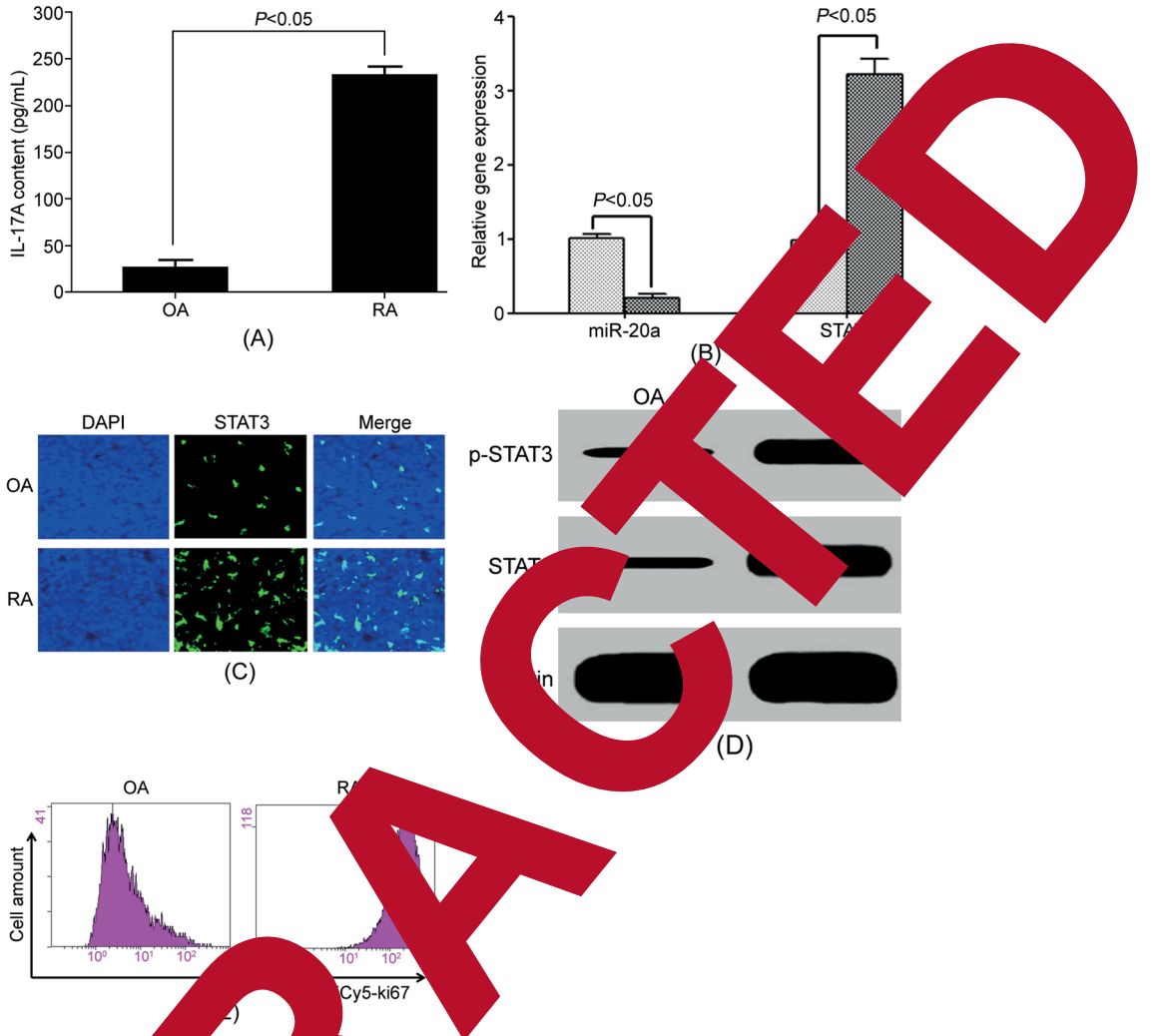

Figure 1. The synovial tissue displa 20a, but enhanced STAT3 expression. ***(A)*** ELISA detection of IL-17A content in the articula vity qRT-PCR detection of miR-20a and STAT3 mRNA expressions in the synovial tissue; ***(C)*** immunofluor ce dete STAT3 expression in the synovial tissue; ***(D)*** Western blot detection of protein expression in the syno issue; ***(E)*** flo metry detection of Ki-67 expression in the synovial tissue.

and/or si-S T3 transfec nd found apparently re ed expressions o T3, p-STAT3, and B after t tment (Figure 3A). MiR-20 attenu IL-17 effect on the promotion of cell prol le II), enhanced cell apoptosis in F (Figu B).

Discussion

K-STA signaling pathway can be mediated various cytokines, including IL-6, TNF-α, IL-17, IFN-γ and plays a key role in regulating a v of pathophysiological processes, such as cell proliferation, apoptosis, inflammation, and homeostasis[15,16]. STAT3 is one of the most important members of STAT family and participates in promoting cell proliferation and inhibiting cell

Table II. Mean fluorescence intensity of Ki-67 expression in each group.

Group	Ki-67 protein mean fluorescence intensity (n=3)
miR-NC	218.7±19.5
miR-20a mimic	31.5±2.4
si-NC	235.6±21.4
si-STAT3	19.8±1.1
miR-20a mimic+ si-STAT3	9.2±0.5

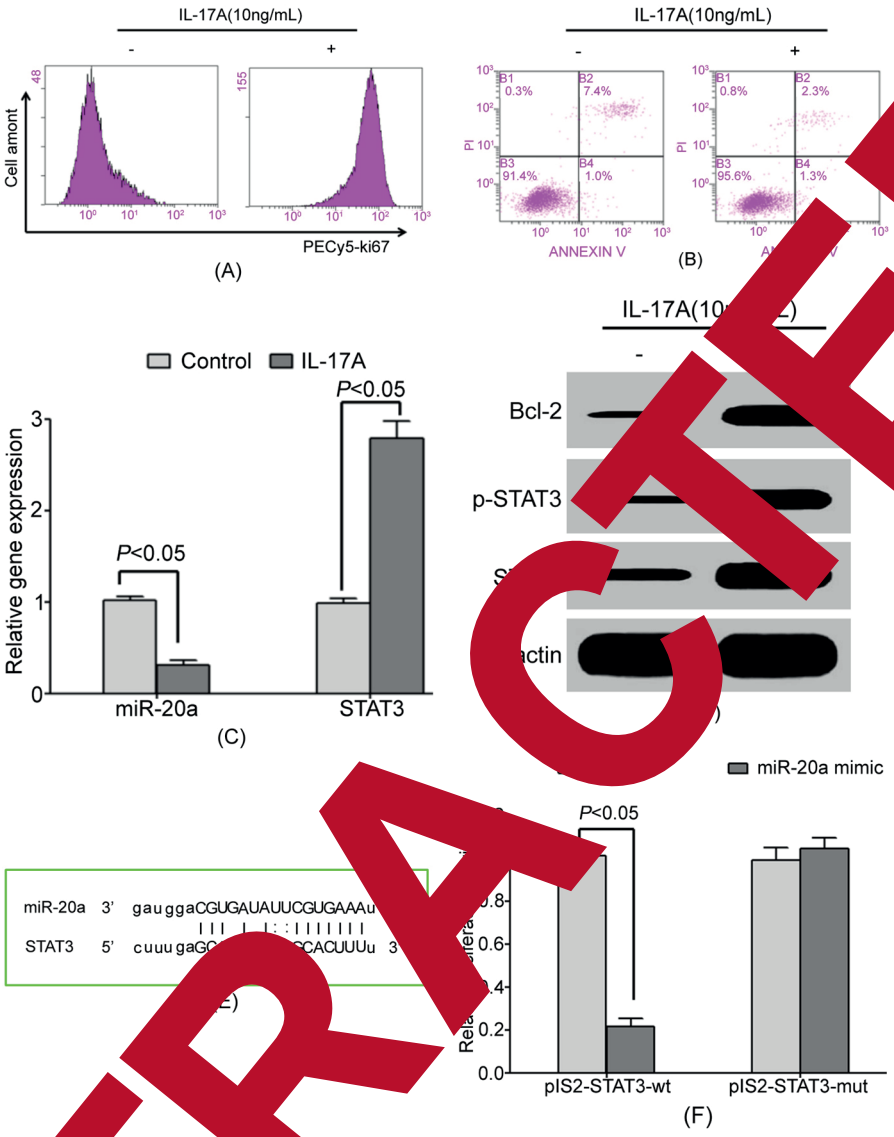

Figure 2. IL-17A d[illegible]ulated miR-20[illegible]regulated STAT3 expression in FLS cells. ***(A)*** flow cytometry detection of Ki-67 expression[illegible] ***(B)*** flow cyto[illegible]y detection of FLS cell apoptosis; ***(C)*** qRT-PCR detection of miR-20a and STAT3 mRNA[illegible]ssio[illegible]S cells; ***(D)*** Western blot detection of protein expression in FLS cells; ***(E)*** the binding site between miR[illegible]20a and the 3'-[illegible]STAT3 mRNA; ***(F)*** Luciferase reporter gene assay.

apo[illegible]s thro[illegible]regulating Bcl-2, Cyclin D1, and [illegible]F4[illegible]has be[illegible]hown that inflammatory cy[illegible]-medi[illegible]FLS excessive proliferation and [illegible]osi[illegible]ockage was an important pa[illegible]ysiolog[illegible]ure of RA[17,18]. JAK-STAT3 sign[illegible]g pathwa[illegible] may be involved in RA pathe[illegible]dering its role in regulating cell [illegible]leration[illegible]d apoptosis. More importantly, it [illegible]been confirmed that STAT3 was abnormal[illegible]egulated in the synovial tissue of RA patie[illegible]On the contrary, attenuation of STAT3 activity led to alleviation of inflammation in RA, which further elucidates the role of JAK-STAT3 in RA[7]. MiR-20a was downregulated in multiple autoimmune diseases, including multiple sclerosis and systemic lupus erythematosus[10,11]. It has been shown that miR-20a played a regulatory role in mediating synovial cell inflammation in RA[12,13,15]. Bioinformatics analysis has revealed a binding site between miR-20 and the 3'-UTR of STAT3 mRNA. This study investigated the role of miR-20a in regulating STAT3 expression, as well as synovial cell proliferation and apoptosis. Flow cytometry exhibited that Ki-67 level was signi-

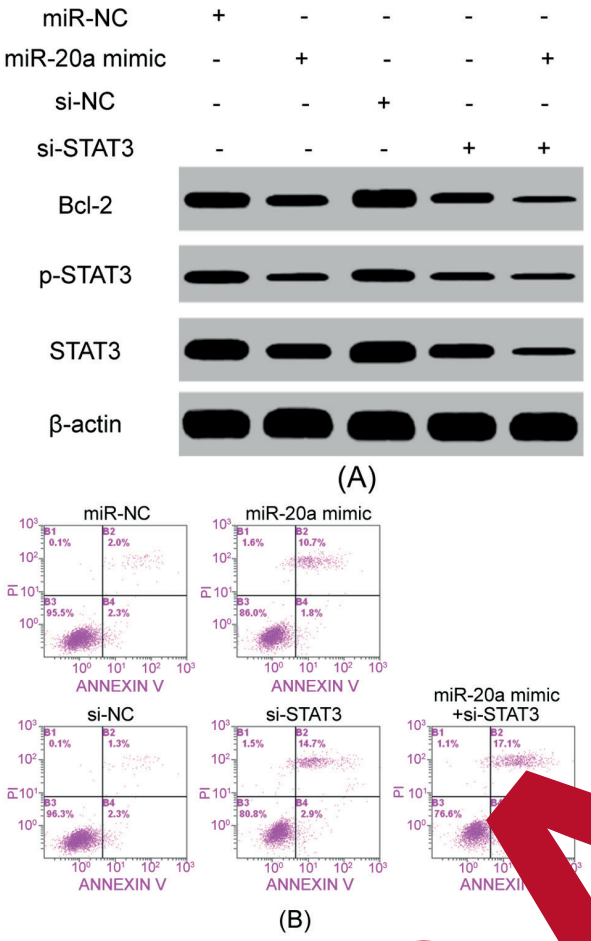

Figure 3. Upregulation of miR-20a in[illegible]T3 expression, attenuated cell proliferation, a[illegible]l apoptosis. ***(A)*** Western blot detection of[illegible]in expr[illegible]n in FLS cells; ***(B)*** flow cytometry detect[illegible] FLS cel[illegible]

ficantly higher in[illegible]han that in c[illegible]licating the role of exc[illegible]iferation in th[illegible]synovial tissue in RA[illegible]thogen[illegible]MiR-20a expression was declin[illegible]while STAT[illegible]-STAT3 expressions w[illegible]nhanced in RA, [illegible]pared with that in OA[illegible]uggest[illegible]that p-STAT3 elevation might resu[illegible]om ST[illegible]upregulation and JAK-STAT3 signi[illegible]cement[illegible]maki et al[6] reported that ST[illegible]pressi[illegible]as significantly increased T ce[illegible]l m[illegible]cytes from peripheral blood [illegible]synovi[illegible]A. Lee et al[19] found that the expr[illegible]ion of STA[illegible]3 and p-STAT3 was enhanced in[illegible] RA compared with that of OA. [illegible]results[illegible]re similar to those in Isomaki et and Lee et al[19], which confirmed that STAT3 [illegible]lation was associated with the pathogenesis[illegible]A. Li et al[12] showed that miR-20a level in FLS from rat RA model was markedly lower than the control. Our study found that miR-20a expression in synovial tissue of RA was lower th[illegible]hat in OA, indicating that miR-20a dow[illegible] might be involved in the pathogenes[illegible]RA, wh[illegible]ch was consistent with the study[illegible]ducted by Li et al[12]. ELISA showed that IL-17[illegible]nt was significantly increased in the articula[illegible]synovia of RA compared with[illegible]in OA. [illegible]al[2] revealed that IL-17 conte[illegible]as abnormally[illegible]ted in the articular cav[illegible]ynovia[illegible]RA. Pavi[illegible]c et al[21] exhibited tha[illegible]7 leve[illegible]s incr[illegible]sed in the peripheral bl[illegible]d [illegible]ang et[illegible]found that IL-17 expre[illegible]n abu[illegible]was[illegible]ficantly increased in[illegible]nonocytes[illegible]. Correlation analy[illegible]d that IL-17[illegible]ntent in the synovia[illegible]po[illegible]correlated with STAT3 mRNA expression a[illegible]atively correlated with miR-2[illegible]pression in[illegible]ial tissue. It suggest[illegible]L-17A might pla[illegible]a role in downregulating miR-20a, upregulating STAT3, and promo[illegible] RA. Flow c[illegible]metry detection revealed that [illegible]7A significa[illegible]enhanced Ki-67 expression, i[illegible]ing that[illegible]7A facilitated cell proliferation[illegible]demonstrated that IL-17 facilitated the p[illegible]iferation of synovial cells from RA *in vitro*. Hashizume et al[22] reported that FLS cell [illegible]ion was markedly enhanced after treated [illegible]. We observed that IL-17A apparently accelerated FLS cell proliferation, which was consistent with the results obtained by Lee et al[19] and Hashizume et al[22]. Moreover, IL-17A significantly downregulated miR-20a expression, while elevated STAT3, p-STAT3, and Bcl-2 levels in FLS cells. Philippe et al[13] used LPS to treat FLS cells *in vitro*, and found that miR-20a expression was reduced in FLS cells, revealing that miR-20a might be involved in the pathogenesis of RA. In this study, miR-20a expression was markedly repressed in FLS cells after treated with IL-17A, which was consistent with Philippe et al[13] findings. Lee et al[8] demonstrated that IL-17 significantly facilitated STAT3 activation in FLS cells. Lee et al[19] reported that IL-17 can upregulate Toll-like receptor 3 expression via promoting STAT3 activation. Also, the present study found the enhancement of IL-17 on STAT3 expression, of which miR-20a downregulation may be a reason of STAT3 elevation. Lee et al[8] revealed that IL-17 can induce Bcl-2 expression in synovial cells. Dual luciferase reporter gene assay confirmed that miR-20a can bind to the 3'-UTR of STAT3 mRNA and regulate STAT3 expression. Both Kim et al[23] and Lee et al[8] suggested the role of STAT3 activation in upregulating Bcl-2 expression, accelerating FLS

cell proliferation, and antagonizing apoptosis. In this study, IL-17A-mediated Bcl-2 elevation may be achieved by enhancing STAT3 and p-STAT3, which was similar with Kim et al[23] and Lee et al[8] results. Besides, it has been demonstrated that miR-20a induced the apoptosis of HepG2 cells and subsequently reduced cell proliferation via the activation of caspase-8 and caspase-3[23]. Therefore, we further explored the potential role of miR-20a on the proliferation and apoptosis of FLS cells. MiR-20a mimic and/or si-STAT3 transfection apparently downregulated STAT3, p-STAT3, and Bcl-2 expressions, attenuated the proliferation promotion effect of IL-17A, and enhanced cell apoptosis in FLS cells. Lee et al[8] found that STAT3 inhibitor STA21 induced synovial cell apoptosis and attenuated the effect of IL-17 on the promotion of synovial cells growth. Kim et al[24] showed that melittin accelerated FLS cell apoptosis via inhibiting STAT3 activation and downregulating Bcl-2 expression. Also, our study demonstrated that reduction of STAT3 might suppress FLS cell proliferation and increase apoptosis, which was consistent with Lee et al[8] and Kim et al[24] findings.

Conclusions

Decreased level of miR-20a ex[illegible]sion and increased STAT3 expression we[illegible] synovial tissue of RA patients. Dow[illegible]gulati[illegible]of miR-20a promoted STAT3, p-S[illegible]3, and [illegible]levels, facilitated FLS cell proli[illegible]re[illegible]sis and, thereby, play[illegible]cri[illegible]n RA.

Conflict of in[illegible]est

The authors[illegible]re no conflicts[illegible]est.

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