

Lnc-IL7R promotes the growth of fibroblast-like synoviocytes through interaction with enhancer of zeste homolog 2 in rheumatoid arthritis

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Abstract. Rheumatoid arthritis (RA) is an inflammatory and autoimmune disease that affects ~1% of the world's population. Although the precise mechanism of RA has yet to be elucidated, accumulating evidence suggests that fibroblast-like synoviocytes (FLSs) serve critical roles in the initiation and progression of RA. However, the underlying molecular mechanisms of FLS proliferation have yet to be elucidated. Long noncoding-interleukin-7 receptor (lnc-IL7R) has been recently identified, which is activated by lipopolysaccharide (LPS) stimulation and diminishes the LPS-induced inflammatory response. In the present study, gain- and loss-of-function assays were performed in order to investigate the role of lnc-IL7R in FLS. It is demonstrated, to the best of the authors' knowledge for the first time, that lnc-IL7R promotes cell proliferation, cell cycle progression and inhibits apoptosis in FLS. Further investigation identified that lnc-IL7 interacts with enhancer of zeste homolog 2 (EZH2) and is required for polycomb repressive complex 2 (PRC2)-mediated suppression, including cyclin-dependent kinase inhibitor 1A and cyclin-dependent kinase inhibitor 2A. Lnc-IL7R may be a promising therapeutic target for the treatment of RA.

Introduction

Rheumatoid arthritis (RA) is an inflammatory and autoimmune disease that affects ~1% of the world's population. RA is characterized by chronic and invasive synovitis, damage to cartilage and bone, and cartilage destruction (1,2). Despite the advances in RA treatment, including aggressive immune suppression with biologics and modifying anti-rheumatic drugs, the general efficacy of treatment remains unsatisfactory (3). Accumulating evidence suggests that fibroblast-like synoviocytes (FLSs) serve

critical roles in the initiation and progression of RA (4). The abnormal proliferation of fibroblast-like synoviocytes (FLSs) leads to destructive invasion of the cartilage and bones through secreting digestive proteases and inflammatory cytokines (5,6). Furthermore, FLSs exhibit resistance to apoptosis induced by several apoptotic stimuli (7,8). Therefore, understanding the molecular mechanism of FLS proliferation and developing pro-apoptosis drugs for FLS is important.

The human transcriptome contains a large number of protein-coding mRNAs and noncoding transcripts (9). Among these, long noncoding RNA (lncRNA) is defined as a molecule of RNA >200 nucleotides in length (10). Accumulating studies have reported that numerous lncRNAs have important biological functions, including chromatin remodeling, transcriptional control and posttranscriptional modification (11,12). Dysregulation of lncRNA has been identified to be associated with human diseases, including inflammation and cancer (13-15). The abnormal expression of lncRNA may contribute to disease initiation and progression through regulating expression of the gene products (16,17). A recent study has reported that differentiation and activation of immune cells are associated with lncRNAs, which have an essential role in autoimmune diseases, including RA (18). However, the role of lncRNA in FLSs has yet to be elucidated.

Long noncoding-interleukin-7 receptor (lnc-IL7R) has been recently identified, and it overlaps with the 3' untranslated region (3'UTR) of the interleukin-7 receptor α -subunit gene (IL7R). Lnc-IL7R is activated by LPS stimulation, and reduces the LPS-induced inflammatory response (19). A previous study (20) demonstrated the differential expression of lncRNAs between RA patients and non-RA patients. From the different expression profiles, it was identified that lnc-IL7R is one of these lncRNAs. However, the role of lnc-IL7R in FLSs has yet to be elucidated. The present study demonstrates that lnc-IL7R promotes cell proliferation, cell cycle progression and inhibits apoptosis in FLSs. Further investigation identified that lnc-IL7 interacts with enhancer of zeste homolog 2 (EZH2), and is required for polycomb repressive complex 2 (PRC2)-mediated suppression.

Materials and methods

Isolation and culture of FLSs. Isolation and culture of FLSs was performed as previously described (21). In brief, synovial

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tissues were obtained during surgery from RA patients diagnosed according to American Rheumatism Association criteria. The present study was approved by the ethics committee of Cangzhou Central Hospital (Cangzhou, China) and written informed consent was provided by all patients. The synovial tissues were digested with 2.5 g/l trypsin for 2 h, and cells were centrifuged at $2,500 \times g$ for 10 min at 4°C and cultured in Dulbecco's minimum essential medium in 5% CO₂ at 37°C. Cells within the 3rd-8th passages were used for all experiments.

MTT assays. A total of 1×10^3 cells per well were seeded in 96-well plates and the viability of the cells was ascertained from three replicates in three independent experiments using an MTT assay [Vazyme Biotech (Nanjing) Co., Ltd., Nanjing, Jiangsu, China] after 3 days.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1 µg complementary DNAs (cDNAs) were synthesized using a Prime Script RT Reagent kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. Quantification of gene expression was determined using FastStart Universal SYBR Green Master mix in the StepOne Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression of RNAs was calculated using the comparative C_q method (or the $2^{-\Delta\Delta C_q}$ method) (22). Gene-specific primers were as follows: lnc-IL7R: 5'-CCAGCCTTTGCCTCTTCC TTCAAT-3' (forward), 5'-CCGTACCAAGTCTCTTAGCCC CTC-3' (reverse); p16: 5'-GCCCCAACGCACCGAATAG TTA-3' (forward), 5'-ACGGGTCGGGTGAGAGT-3' (reverse); p21: 5'-CCTCATCCCGTGTCTCCTTT-3' (forward), 5'-GTA CCACCCAGCGGACAAGT-3' (reverse).

Transfection. The cDNA encoding lnc-IL7R was subcloned into pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were transfected with the plasmids pcDNA3.1 or pcDNA-lnc-IL7R using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were transfected and grown for 48 h prior to the subsequent assays. The small interfering RNA (siRNA) against lnc-IL7R was purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The target sequence was as follows: AAG AGATATATTTCATCGAGA. Cells were transfected with 10 nM siRNA for 48 h prior to subsequent assays.

Cell-cycle analysis. Cell-cycle analysis was performed as previously described (23). In brief, cells were permeabilized with 75% ethanol, and DNA was stained with 50 µg/ml propidium iodide. Cellular DNA content was measured using flow cytometric analysis using a FACScan flow cytometer, and the data were analyzed with Kaluza software (version 1.20; Beckman Coulter, Inc., Brea, CA, USA).

Apoptosis analysis. Cells were trypsinized, resuspended and stained with fluorescein isothiocyanate-conjugated annexin V and 7-aminoactinomycin D (7-AAD; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's

protocol. Cells were then measured by flow cytometric analysis using the FACScan flow cytometer with the data analyzed with Kaluza software.

Western blot analysis. Cells were lysed in RIPA lysis buffer with cocktail protease inhibitor (Selleck Chemicals, Houston, TX, USA). Samples were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). The PVDF membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated with primary antibodies (1:1,000) raised against CDK4 (cat. no. 12790), CDK6 (cat. no. 13331), caspase 3 (cat. no. 9661), poly (ADP-ribose) polymerase (PARP; cat. no. 5625) or GAPDH (cat. no. 2118) overnight at 4°C, followed by incubation with anti-rabbit secondary antibodies (1:10,000; cat. no. 7074) coupled to horseradish peroxidase (all antibodies purchased from Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Signals were visualized with an ECL chemiluminescence system (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific, Inc.).

RNA immunoprecipitation (RIP) and chromatin immunoprecipitation (ChIP) assays. RIP and ChIP assays were performed using an EZ-Magna RIP RNA-binding protein immunoprecipitation kit and an EZ-Magna ChIP kit, according to the manufacturer's protocol (Merck Millipore). RIP and ChIP products were analyzed by RT-qPCR. ChIP-enriched promoter of target genes was quantified by RT-qPCR using the following primers: p16: Forward: 5'-CGCTAAGTGCTCGGA GTTAATA-3', reverse: 5'-CGACCCTGTCCCTCAAATC-3'; p21: Forward: 5'-GTGTCCAGCGCACCAAC-3', reverse: 5'-TGAGCCTGGCCGAGTTC-3'.

RNA pull-down assays. RNA pull-down assays were performed as described previously (24). Briefly, RNAs were biotin-labeled with the Biotin RNA Labeling mix (Roche Diagnostics, Basel, Switzerland) and transcribed *in vitro* using T7 RNA polymerase (Roche Diagnostics, Switzerland). Biotinylated RNAs were mixed and incubated with cell lysates for 2 h at 4°C. Streptavidin-agarose beads (Thermo Fisher Scientific, Inc., Waltham, MA, USA) were added to each binding reaction, and incubated for 1 h at room temperature. The beads were then boiled in sodium dodecyl sulfate (SDS) buffer. The eluted proteins were detected by western blot analysis.

Statistical analysis. All statistical analyses were performed by using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA). Student's *t*-test was performed for the results from all assays. In all cases, $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Overexpression of lnc-IL7R promotes FLS proliferation, induces cell cycle progression and decreases cell apoptosis. To determine the role of lnc-IL7R in FLSs, gain-of-function studies were performed. The cell proliferation of FLSs with empty vector or lnc-IL7R overexpression was detected by performing an MTT assay. It was identified that lnc-IL7R

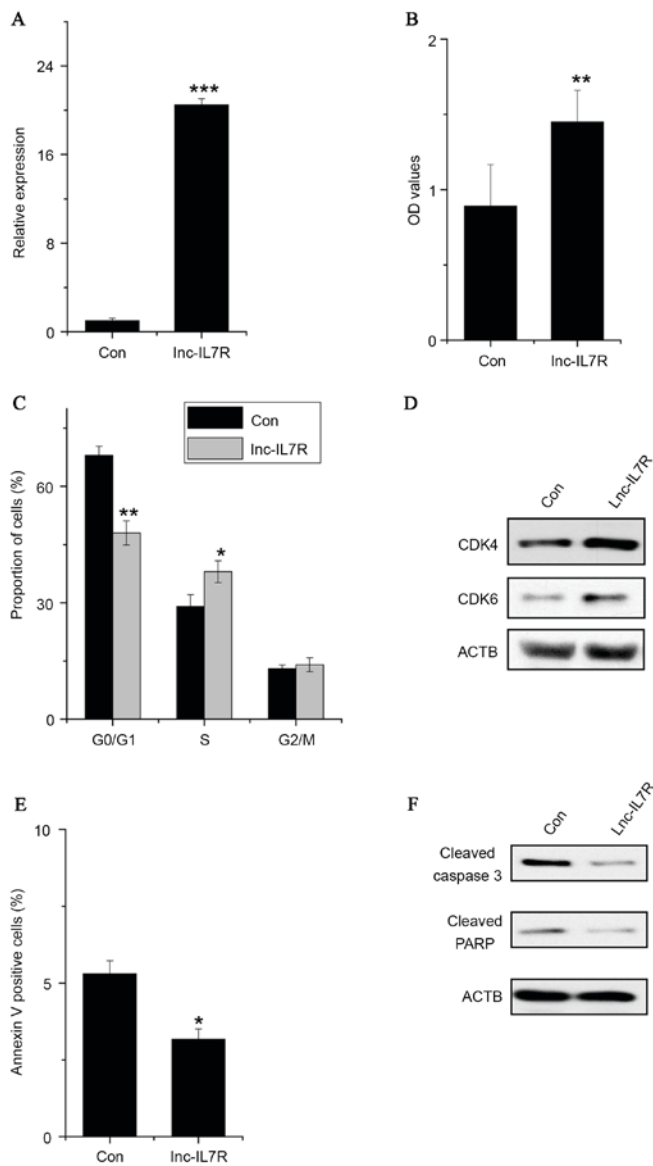


Figure 1. Overexpression of lnc-IL7R promotes FLS proliferation, induces cell cycle progression and decreases cell apoptosis. (A) Relative expression of lnc-IL7R in FLSs expressing control and lnc-IL7R. (B) Cell growth determined by MTT proliferation assays. (C) Data derived from the FACS analysis, illustrating increases or decreases of the cells in S or G1 phases, respectively, in FLSs with lnc-IL7R overexpression. (D) CDK4 and CDK6 expression levels detected by western blot analysis following lnc-IL7R overexpression. (E) Cells with lnc-IL7R overexpression stained with a combination of annexin V and 7-AAD and analyzed using FACS; cells positive for annexin V staining were counted as apoptotic cells. (F) Caspase 3 and PARP expression levels were detected by western blot analysis following lnc-IL7R overexpression. Data are shown as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 (Student's *t*-test). lnc-IL7R, long noncoding-interleukin-7 receptor; FLSs, fibroblast-like synoviocytes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FACS, fluorescence-activated cell sorting; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; 7-AAD, 7-amino-actinomycin D; PARP, poly(ADP) ribose polymerase; Con, control.

overexpression increased the proliferation of FLSs compared with control cells (Fig. 1A and B). To understand the mechanism by which lnc-IL7R promotes cell proliferation, the apoptosis and cell cycle distribution of lnc-IL7R-overexpressed FLSs was detected by fluorescence-activated cell sorting (FACS). It was identified that lnc-IL7R overexpression

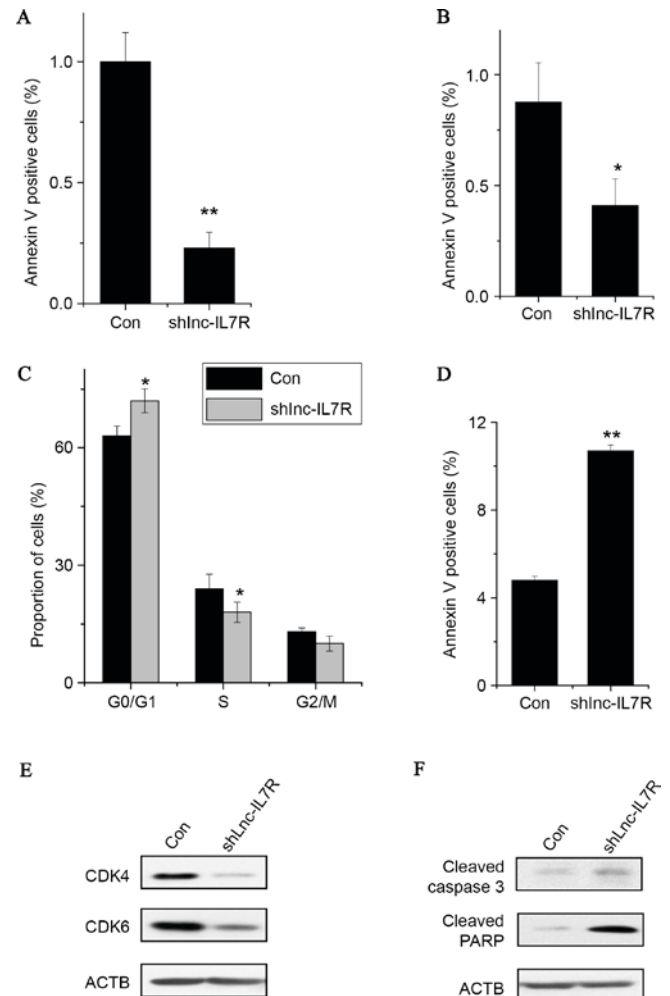


Figure 2. Knockdown of lnc-IL7R inhibits FLS proliferation, induces cell cycle arrest and promotes cell apoptosis. (A) Relative expression of lnc-IL7R in FLSs expressing Con and lnc-IL7R shRNA. (B) Cell growth determined by MTT proliferation assay. (C) FACS analysis showed a marked decrease or increase of cells in the S or G1 phase, respectively, in FLSs with lnc-IL7R shRNA. (D) Cells with lnc-IL7R shRNA stained with a combination of annexin V and 7-AAD and analyzed by FACS. Cells positive for annexin V staining were counted as apoptotic cells. (E) CDK4 and CDK6 expression levels detected by western blot analysis following lnc-IL7R silencing. (F) Caspase 3 and PARP expression levels detected by western blot analysis following lnc-IL7R silencing. Data are shown as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01 (Student's *t*-test). lnc-IL7R, long noncoding-interleukin-7 receptor; FLS, fibroblast-like synoviocyte; shRNA, small hairpin RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Con, control; FACS, fluorescence-activated cell sorting; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; 7-AAD, 7-amino-actinomycin D; PARP, poly(ADP) ribose polymerase.

significantly promoted G1/S transition in FLSs (Fig. 1C). Consistent with the FACS data, the expression of G1/S-phase checkpoint markers, including CDK4 and CDK6, were upregulated in lnc-IL7R-overexpressed FLSs (Fig. 1D). However, lnc-IL7R-overexpressed FLSs showed a significantly higher percentage of annexin V-positive cells than did control cells (Fig. 1E). Consistent with the FACS results, apoptosis markers, including cleaved caspase 3 and PARP, markedly decreased in FLSs with lnc-IL7R overexpression (Fig. 1F). Taken together, the results demonstrated that lnc-IL7R promotes FLS proliferation, via driving cell cycle progression and decreasing cell apoptosis.

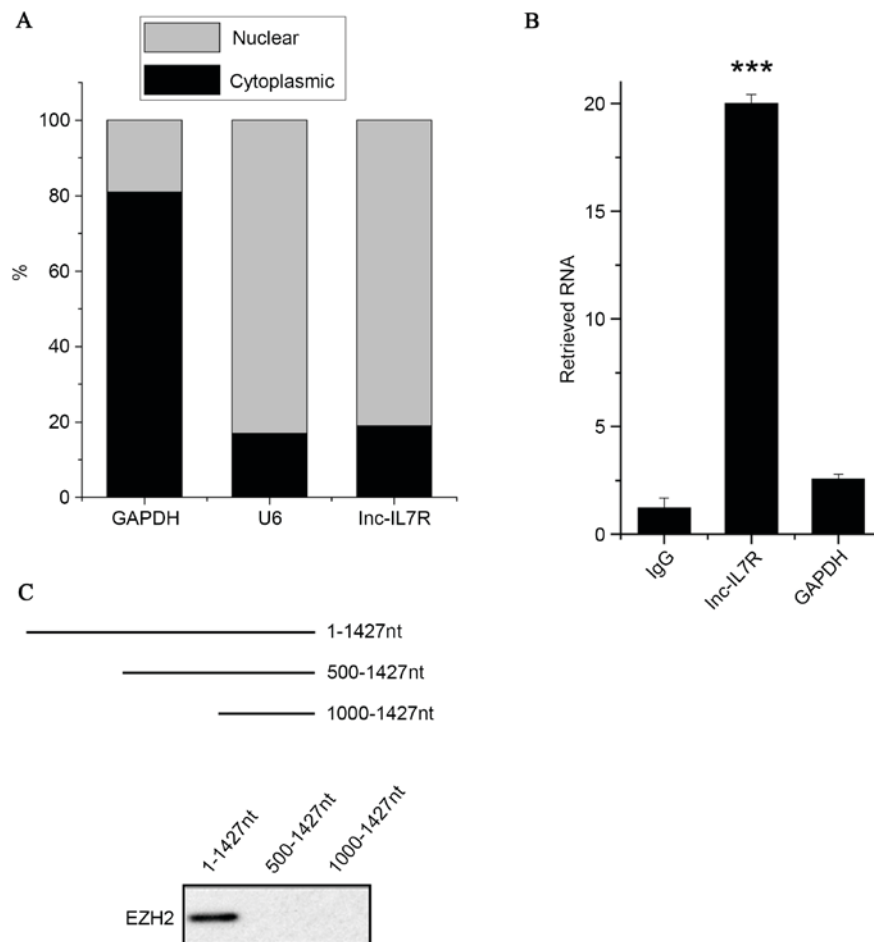


Figure 3. Lnc-IL7R interacts with EZH2. (A) Representative analysis of Lnc-IL7R distribution by cellular fractionation of FLSs. GAPDH mRNA and U6 RNA served as controls for cytoplasmic and nuclear RNAs, respectively. (B) Results from RIP and subsequent RT-qPCR assays. Relative quantification of Lnc-IL7R and GAPDH in RNA-protein complexes immunoprecipitated with IgG or EZH2 antibodies from nuclear extracts. (C) Deletion mapping of EZH2-binding domain in Lnc-IL7R. Western blot of EZH2 in protein samples pulled down by different Lnc-IL7R fragments. Data are shown as the mean \pm standard deviation ($n=3$). *** $P<0.001$ (Student's t -test). Lnc-IL7R, long noncoding-interleukin-7 receptor; EZH2, enhancer of zeste homolog 2; FLSs, fibroblast-like synoviocytes; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RIP, RNA immunoprecipitation; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; IgG, immunoglobulin G.

Knockdown of Lnc-IL7R inhibits FLS proliferation, induces cell cycle arrest and promotes cell apoptosis. Lnc-IL7R expression in FLSs was silenced (Fig. 2A) and an MTT assay was performed to detect the cell proliferation of FLSs with Lnc-IL7R small hairpin RNA (shRNA). It was identified that knockdown of Lnc-IL7R decreased the proliferative capacity of FLSs compared with control cells (Fig. 2B). Similarly, a FACS assay demonstrated that Lnc-IL7R knockdown significantly inhibited G1/S transition and promoted cell apoptosis (Fig. 2C and D). Consistent with the FACS data, the expression of G1/S phase checkpoint markers were markedly downregulated, while the apoptosis markers were upregulated in Lnc-IL7R knockdown FLSs (Fig. 2E and F). Collectively, these data indicate that Lnc-IL7R serves an important role in FLS proliferation, cell cycle and apoptosis.

Lnc-IL7R interacts with EZH2. The molecular mechanisms by which Lnc-IL7R promotes cell proliferation were subsequently investigated. First, cellular fractionation assays were performed to analyze the subcellular localization of Lnc-IL7R. It was identified that Lnc-IL7R was localized in the nuclei of FLSs (Fig. 3A). This suggested that Lnc-IL7R may be involved in transcriptional regulation.

A previous study (25) reported that 20% of human lncRNAs are associated with PRC2. It was hypothesized that Lnc-IL7R may interact with EZH2, a core component of PRC2, and regulate gene expression. To confirm this, an RIP assay was performed with an EZH2 antibody from nuclear extracts of FLSs. A marked enrichment of Lnc-IL7R by the EZH2 antibody was identified, but not any enrichment of GAPDH compared with the IgG control antibody (Fig. 3B). Consistent with the RIP assay, the association between EZH2 and Lnc-IL7R was validated through a RNA pull-down assay (Fig. 3C). The deletion-mapping assay showed that 500 nucleotides at the 5' end of Lnc-IL7R are required for its interaction with EZH2. Collectively, the results demonstrated that there is an interaction between Lnc-IL7R and EZH2.

Lnc-IL7R promotes FLS proliferation, induces cell cycle progression and decreases cell apoptosis via association with EZH2. To investigate the practical relevance of the interaction of Lnc-IL7R and EZH2, EZH2 was silenced in Lnc-IL7R-overexpressed FLSs (Fig. 4A). EZH2 knockdown reversed the effects of promotion of cell proliferation and cell cycle progression, and the anti-apoptosis effects, induced

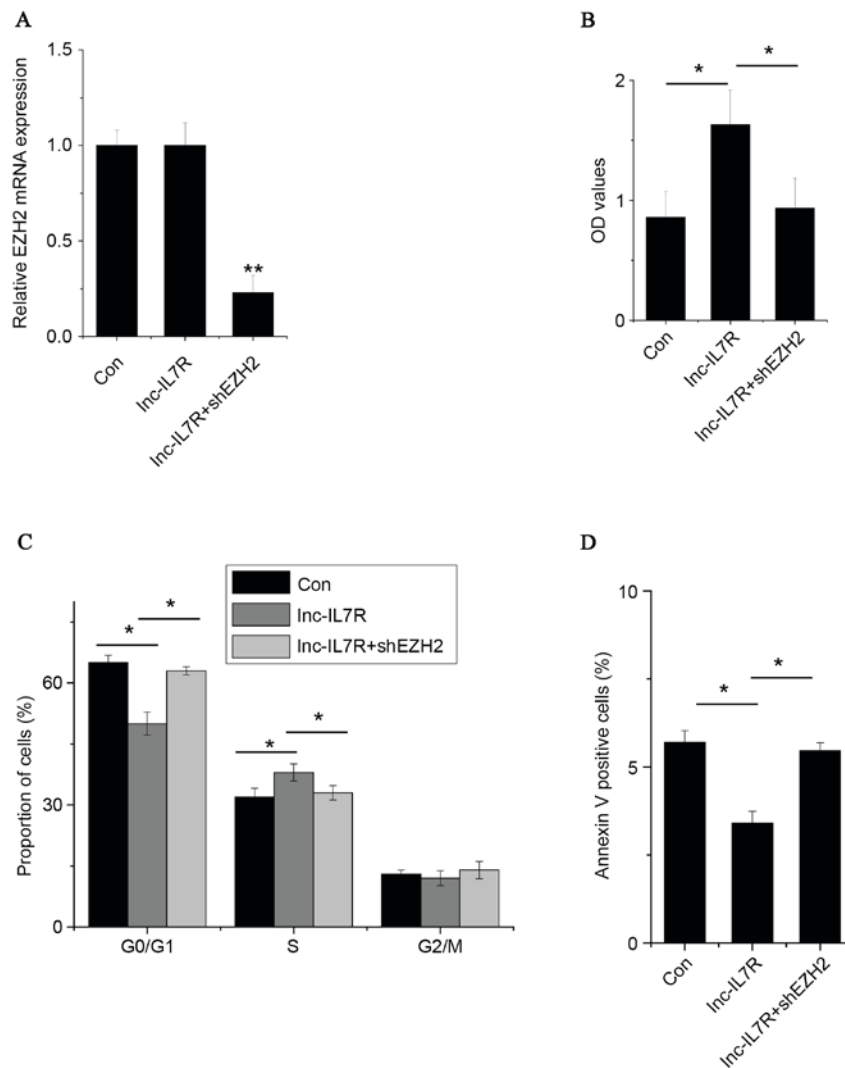


Figure 4. Lnc-IL7R promotes FLS proliferation, induces cell cycle progression and decreases cell apoptosis via association with EZH2. (A) Relative expression of EZH2. (B) Cell growth of indicated groups determined by MTT proliferation assays. (C) Cell cycle distribution detected by FACS analysis. (D) Cell apoptosis detected by FACS analysis. Data are shown as the mean \pm standard deviation (n=3). *P<0.05 (Student's *t*-test). Lnc-IL7R, long noncoding-interleukin-7 receptor; FLSs, fibroblast-like synoviocytes; EZH2, enhancer of zeste homolog 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FACS, fluorescence-activated cell sorting; Con, control.

by Lnc-IL7R (Fig. 4B-D). These data suggest that Lnc-IL7R exerts its function, at least in part, through interaction with EZH2.

Lnc-IL7R regulate target gene expression via epigenetic mechanisms. To further corroborate the practical relevance of the association between Lnc-IL7R and EZH2, PRC2 target genes, which regulate the cell cycle, were detected, including cyclin dependent kinase inhibitor 2A (p16) and cyclin dependent kinase inhibitor 1A (p21) (Fig. 5A). p16 and p21 expression levels were decreased by Lnc-IL7R overexpression, and reversed by knockdown of EZH2. By contrast, both p16 and p21 expression levels were increased by Lnc-IL7R knockdown (Fig. 5B). A ChIP assay in Lnc-IL7R-overexpressed FLSs was performed to test whether Lnc-IL7R is involved in transcriptional regulation by recruiting EZH2 to target genes. The results demonstrated that Lnc-IL7R increased the binding of EZH2 and H3K27me3 levels across the p16 and p21 promoter regions (Fig. 5C and D).

Discussion

Development of RA is closely associated with characteristic changes of the synovium, including FLS hyperplasia, abnormal angiogenesis and infiltration of immune cells (26,27). Previous studies (28,29) have reported that the excessive proliferation of FLSs is associated with aberrant activation of the inflammatory signal pathway. Noncoding RNA (ncRNA) is also involved in regulating FLS proliferation. For example, microRNA (miR)-152 overexpression significantly decreases FLS proliferation through the upregulation of SFRP4, a negative regulator of the Wnt signaling pathway (30). However, the function of LncRNA in FLSs has yet to be elucidated. The present study demonstrated that Lnc-IL7R promotes cell proliferation and cell cycle progression, and inhibits apoptosis in FLSs. Although the present study has demonstrated that Lnc-IL7R diminishes the LPS-induced inflammatory response in human monocytes, this phenomenon was not observed in FLSs (data not shown).

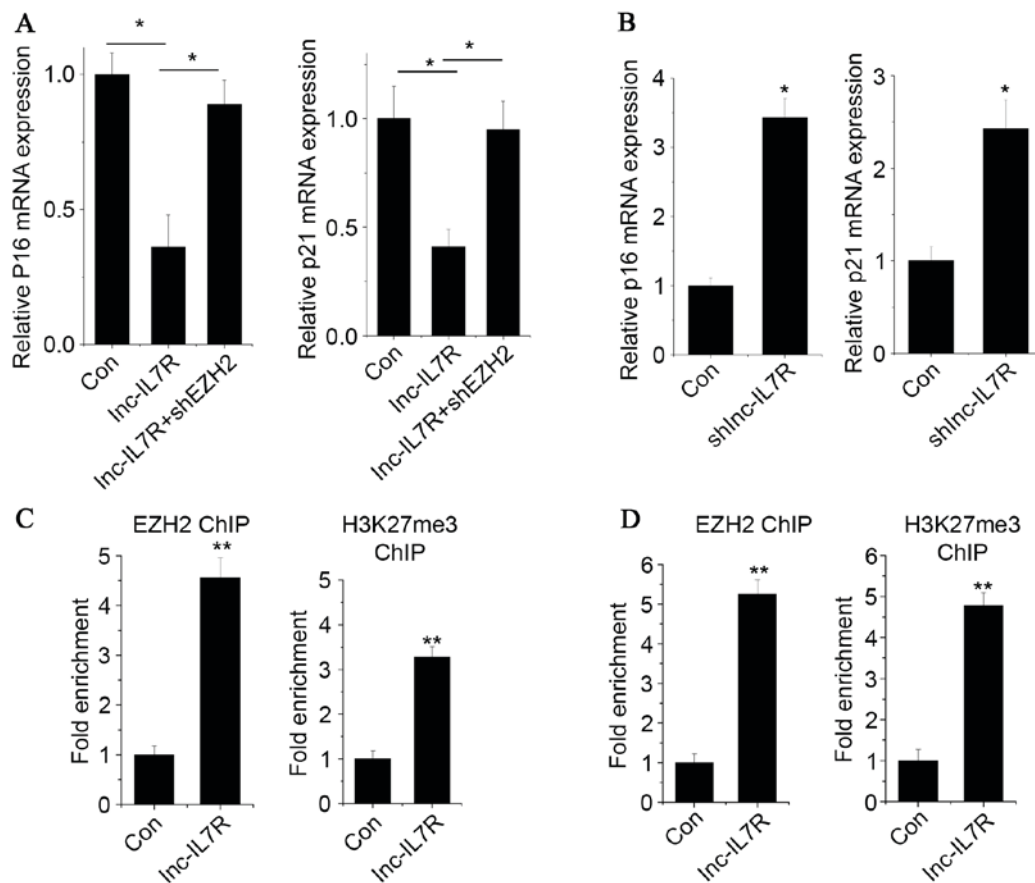


Figure 5. Lnc-IL7R regulate target gene expression via epigenetic mechanisms. The (A) p16 and (B) p21 expression levels of the groups as detected by RT-qPCR. (C) The occupancy of EZH2 and H3K27me3 in the promoter regions of p16 measured by EZH2 and H3K27me3 ChIP assay followed by RT-qPCR. (D) The occupancy of EZH2 and H3K27me3 in the promoter regions of p21 was measured by EZH2 and H3K27me3 ChIP assay followed by RT-qPCR. Data are shown as mean \pm standard deviation; $n=3$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (Student's *t*-test). Lnc-IL7R, long noncoding-interleukin-7 receptor; p16, cyclin-dependent kinase inhibitor 2A; p21, cyclin-dependent kinase inhibitor 1A; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; EZH2, enhancer of zeste homolog 2; H3K27me3, histone 3 lysine 27 trimethylation; ChIP, chromatin immunoprecipitation; Con, control.

Lnc-IL7R may therefore exert its function in a tissue-specific manner.

Aberrant epigenetic modifications have been thoroughly elucidated in human diseases. Epigenetic modifications, including DNA methylation and histone modification, regulate gene expression (31,32). Previous studies (33,34) have shown an extensive aberrant methylation of gene promoters in FLSs, including C-X-C motif chemokine ligand 12, interleukin 10 and interleukin 6. Many of these genes are involved in the immune response and in inflammation (35,36). However, histone modifications involved in RA have yet to be elucidated (37,38). A previous study (39) demonstrated that EZH2 is overexpressed in FLSs, and inhibits the expression of secreted frizzled-related protein 1. However, none of the PRC2 core components are responsible for the sequence-specific DNA-binding activity. Previous studies (40,41) have reported that PRC2 may be recruited to target genes by lncRNA. In the present study, it was demonstrated that lnc-IL7R associates with EZH2. Lnc-IL7R depletion leads to upregulation of genes which are suppressed by EZH2, including p16 and p21. Additionally, lnc-IL7R overexpression increases the binding of EZH2 and H3K27me3 levels across p16 and p21 promoters. Overall, the present study proposes a model in which lncRNA regulates proliferation via association with EZH2. Whether lnc-IL7R is

associated with other proteins requires further investigation. Understanding the precise molecular mechanisms by which lnc-IL7R functions in FLSs will aid the exploration of new therapies for RA.

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