Original Article

LncRNA *THRIL* is involved in the proliferation, migration, and invasion of rheumatoid fibroblast-like synoviocytes

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Background: Fibroblast-like synoviocytes (FLSs), which can migrate and directly invade the cartilage and the bone, are crucial players in joint damage in rheumatoid arthritis (RA). Nevertheless, the detailed mechanisms underlying the aberrant activation of RA FLSs remain unclear. Several studies have attempted to explore the relationship between long non-coding RNAs (lncRNAs) and RA pathology; however, the role of lncRNAs in RA is unknown. The present study aimed to determine the functions of tumor necrosis factor-α and heterogeneous nuclear ribonucleoprotein L-related immunoregulatory lincRNA (*THRIL*) in RA FLSs migration and invasion.

Methods: Small interfering RNA targeting *THRIL* or lentivirus overexpressing *THRIL* was used to knockdown or overexpress *THRIL*. Quantitative reverse transcription polymerase chain reaction (PCR) was employed for the detection of RNA expression. The proliferation rate of RA FLSs was measured using a 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay. Migration and invasion were detected using a transwell chamber. Downstream targets were identified using a human cell cycle real-time PCR array and a human cell motility real-time PCR array.

Results: A significant decrease in *THRIL* expression was found in RA FLSs compared with cells from healthy control (HC)patients. *THRIL* is mainly localized in the nucleus. Knockdown of *THRIL* increased the proliferation, migration, and invasion of RA FLSs. In contrast, *THRIL* overexpression had the opposite effect. *THRIL* knockdown increased interleukin-1β (IL-1β)-triggered expression of matrix metalloproteinase (*MMP*)-1, *MMP-3*, and *MMP-13*. *THRIL* overexpression led to a significant decrease in *MMP-13* expression in response to stimulation with IL-1β. Furthermore, we observed that the expression levels of cyclin-dependent kinase 1 (*CDK1*) and G2 and S phase-expressed-1 (*GTSE1*), both of which are associated with cellular mobility and proliferation, were downregulated with *THRIL* overexpression.

Conclusions: Reduced expression of lncRNA *THRIL* represses the proliferation, migration, and invasion of RA FLSs, suggesting that lncRNA *THRIL* might be a potential target for RA therapy.

Keywords: Rheumatoid arthritis (RA); long non-coding RNA (lncRNA); migration; invasion

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Introduction

As one of the most prevalent systemic rheumatoid diseases, rheumatoid arthritis (RA), which is characterized by synovitis and progressive joint damage, has been perceived as a major cause of extremity disability (1,2). To date, however, the etiology of RA has not yet been fully clarified. It has been well established that fibroblast-like synoviocytes (FLSs), located in the highly inflamed and invasive RA pannus, play vital roles in RA pathology (3-5). Previous studies have demonstrated that RA FLSs exhibit enhanced secretion of various proinflammatory cytokines, chemokines, and matrix metalloproteinases (MMPs) that participate in inflammatory infiltration and joint destruction. Interestingly, RA FLSs display an aggressive phenotype that surprisingly resembles that of tumor cells, including uncontrolled proliferation, resistance to apoptosis, invasiveness to cartilage, and migration to unaffected joints (3,5). Considerable research has been devoted to exploring the mechanisms underlying the "tumor-like transformation" of RA FLSs, but these mechanisms are still largely unknown. Therefore, identifying key regulators that control the pathogenetic behaviors of RA FLSs could shed light on novel FLS-targeted therapy for RA.

The past 2 decades have seen dramatic development in non-coding RNA research, especially in long non-coding RNAs (lncRNAs). Increasing evidence indicates that lncRNAs participate in regulating various cellular biological processes (6-8) and initiating and developing various diseases, including RA (9-11). Attempts have been made to investigate the function of lncRNAs in regulating the aberrant behaviors displayed by RA FLSs. Our previously published work identified a novel downregulated lncRNA, lowly expressed in rheumatoid fibroblast-like synoviocytes (*LERFS*), which suppresses the proliferation, migration, and invasion of RA FLSs by abrogating the expression of small GTPase proteins through interaction with hnRNP Q (12). In addition, lncRNA Fer-1-like protein 4 (*FER1L4*) has been found to modulate synovial inflammation by targeting nucleotide oligomerization domain-like receptors 5 (*NLRC5*) (13). However, several lncRNAs, including ZNFX1 antisense RNA 1 (*ZFAS1*) (14), gastric adenocarcinoma associated, positive CD44 regulator, long intergenic non-coding RNA (*GAPLINC*) (15), and P38 inhibited cutaneous squamous cell carcinoma associated lincRNA (*PICSAR*) (16), are involved in modulating the activated phenotype of RA by working as competing endogenous RNAs through sponging microRNAs. To date, accumulating evidence indicates that lncRNAs could be pivotal regulators and promising therapeutic targets in RA; however, little is currently known about the function of lncRNAs on synovial inflammation and articular damage.

Recently, lncRNA tumor necrosis factor-α (TNF-α) and heterogeneous nuclear ribonucleoprotein L (hnRNPL)-related immunoregulatory lincRNA (*THRIL*) has shown crucial regulatory effects on inflammation and immune response. For instance, *THRIL* was found to be required for the expression of a wide variety of cytokines, especially TNF-α, by interacting with hnRNPL to form a functional complex that binds to the promoter of target genes in human macrophages. In addition, the expression level of *THRIL* is associated with the severity of Kawasaki disease, an autoimmune-related vasculitis of children (17). T cells isolated from RA patients display increased expression of *THRIL* compared with T cells from healthy individuals (18).

Interestingly, a recent study reported the possible involvement of *THRIL* in regulating RA FLS growth and inflammatory response (19). However, it is still unclear whether *THRIL* is involved in modulating the transformed and invasive phenotype of RA FLSs. Therefore, in the present study, we primarily focused on the involvement of *THRIL* in the migration and invasion behaviors displayed by RA FLSs. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-1362).

Methods

Preparation of human specimens

We used synovial tissue (ST) specimens from RA patients with active disease (n=10, 7 females and 3 males; age range: 46–61 years) and osteoarthritis patients (n=5, 3 females and 2 males; age range: 54–66 years) who underwent synovectomy, joint replacement of the knee joint, or Parker-Pearson needle synovial biopsy at the First Affiliated Hospital, Sun Yat-sen University, China. All RA patients were diagnosed based on the 2010 ACR/EULAR criteria for RA classification (20). STs of healthy controls (HCs) (n=9, 6 females and 3 males; age range: 32–66 years) were obtained from individuals who underwent orthopedic surgery because of trauma, meniscus injury, or cruciate ligament injury. Individuals with manifestations of acute or chronic arthritis were not included. The study was performed in compliance with the Declaration of Helsinki (as revised in 2013). The Medical Ethics Committee of the

Table 1 Primers used in this study for the detection of the indicated RNAs

Genes	Sense (5'-3')	Anti-sense (5'-3')
THRIL	AAACAGGTGCACGTTTCAGG	CCAGGTCTCAGTTTGGAGAAGA
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
Actin	GCAAAGACCTGTACGCCAA	GGAGGAGCAATGATCTTGATCTTC
MMP1	CTCTGGAGTAATGTCACACCTCT	TGTTGGTCCACCTTTCATCTTC
MMP3	CGGTTCCGCCTGTCTCAAG	CGCCAAAAGTGCCTGTCTT
MMP13	TCCTGATGTGGGTGAATACAATG	GCCATCGTGAAGTCTGGTAAAAT
IL6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
TNF	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
MKI67	AGAAGAAGTGGTGCTTCGGAA	AGTTTGCGTGGCCTGTACTAA
CDC20	GACCACTCCTAGCAAACCTGG	GGGCGTCTGGCTGTTTTCA
CDK1	AAACTACAGGTCAAGTGGTAGCC	TCCTGCATAAGCACATCCTGA
AUKRB	CAGTGGGACACCCGACATC	GTACACGTTTCCAAACTTGCC
GTSE1	CAGGGGACGTGAACATGGATG	ATGTCCAAAGGGTCCGAAGAA

AUKRB, aurora kinase B; *CDC20*, cell division cycle 20; *CDK1*, cyclin-dependent kinase 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GTSE1*, G2 and S phase-expressed-1; *IL-6*, interleukin 6; *MKI67*, markers of proliferation Ki-67; *MMP*, matrix metalloproteinase; *THRIL*, tumor necrosis factor-α and heterogeneous nuclear ribonucleoprotein L-related immunoregulatory lincRNA; *TNF*, tumor necrosis factor.

First Affiliated Hospital of Sun Yat-sen University approved all research protocols involved in this research {No. [2017]-049}. All participants provided written informed consent.

Cell isolation and culture

ST specimens were cut into 1-mm-diameter pieces and washed with phosphate-buffered saline (PBS). After digestion for 2 h at 37 ℃ with 1 mg/mL type I collagenase (Sigma-Aldrich, St. Louis, MO, USA), isolated RA FLSs were washed with PBS and then resuspended and cultured adherently in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) under 5% CO_2 at 37 ℃. RA FLSs from passages 4–6 were used in subsequent experiments.

Separation of nuclear and cytoplasm fractions

The nuclear and cytoplasmic fractions of FLSs were separated using a PARIS kit (Thermo Fisher Scientific, Waltham, MA, USA). In brief, RA FLSs at 90% confluence were trypsinized and lysed using cell fractionation buffer. After collecting the supernatant, the remaining nuclear pellet was washed and further lysed, and the nuclear fraction was collected.

Extraction of RNA and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the isolated RNA was quantified with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription of RNA was carried out with a PrimeScript RT kit (Takara, Tokyo, Japan). A SYBR Premix Ex Taq kit (Takara, Tokyo, Japan) was used for qRT-PCR, and the Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA) was employed. Details of the primers used in amplification are provided in *Table 1*. The PCR program was set as follows: 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 5 s and then 60 ℃ for 30 s. *GAPDH* was used as the internal reference gene, and the $2^{-\Delta\Delta Ct}$ method was employed to

Table 2 siRNA sequence targeting *THRIL*

Sequences	Target sequence for *THRIL* siRNAs
si*THRIL*-001	CCACCAATCCCTAAGCTGT
si*THRIL*-002	GTGTGTAGTTCCACGTCAA
si*THRIL*-003	GCCACTTTCTTGCTCAGTC
Negative control siRNA	Purchased from RiboBio (siN0000001-1-5)

siRNA, small interfering RNA; *THRIL*, tumor necrosis factor-α and heterogeneous nuclear ribonucleoprotein L-related immunoregulatory lincRNA.

calculate gene expression.

Transfection of small interfering RNA (siRNA)

siRNAs targeting *THRIL*, scramble control siRNA, and CY3-labeled siRNA were synthesized at RiboBio (Guangzhou, China). RA FLSs at 70% confluence were transfected with the indicated siRNA with Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA). The target sequences of the indicated genes are shown in *Table 2*.

Infection of overexpression lentivirus

Lentiviruses overexpressing *THRIL* and vector control lentiviruses were constructed at GeneChem (Shanghai, China). Cells at 60% confluence were infected with lentivirus particles (MOI =30) in the presence of 10 μg/mL polybrene.

EdU incorporation assay

The Cell-Light EdU Apollo567 In Vitro Kit (RiboBio, Guangzhou, China) was utilized to evaluate the proliferation rate. FLSs at a confluence of 80% were incubated with EdU solution (1:1,000) for 8 h and then subjected to EdU and Hoechst staining. EdU-positive cells were regarded as proliferating cells and counted under a fluorescence microscope.

Apoptosis assay

The cell apoptosis rate of FLSs was measured by annexin V and 7-AAD staining. Briefly, FLSs were washed with PBS and then suspended in 200 μL 1× binding buffer; 5 μL annexin V-APC (BD Bioscience, San Jose, CA, USA) was added to the cell suspension and incubated for 15 min at room temperature away from light. Then 5 μL 7-AAD (BD Biosciences, San Jose, CA, USA) was added, and the samples were analyzed by flow cytometry.

Migration and invasion assay

Transwell inserts (Corning, NY, USA) with 8 μm pores were used to evaluate migration. Briefly, after counting and resuspending in serum-free medium, RA FLSs (2.5×10^4/well) were seeded into transwell inserts, and cell culture medium supplemented with 10% FBS was added to the wells below the chambers for directed chemotaxis. Six hours later, after removing the cells remaining above the transwell membrane using cotton swabs, RA FLSs that migrated across the transwell membrane were visualized by crystal violet staining and counted manually under an optical microscope (magnification 100×). To detect the invasion rate, transwell chambers precoated with Matrigel (BD Biosciences, San Jose, CA, USA) were utilized, 5×10^4 cells were seeded, and a medium with 15% FBS was used as a chemoattractant. After 30 h, the number of invading cells was counted as described for the migration assay.

qRT-PCR array

To identify target genes regulated by *THRIL*, RT² Profiler PCR Array Human Cell Cycle and RT² Profiler PCR Array Human Cell Motility (Kang Chen Biotech, Shanghai, China) were used. In brief, RNA of RA FLSs was extracted with TRIzol and cleaned using the RNeasy MinElute Cleanup Kit (Qiagen, Duesseldorf, Germany) to remove contaminating DNA. RNA was then reverse transcribed to produce cDNA using the RT2 First Strand Kit (Invitrogen, Carlsbad, CA, USA) and amplified by PCR using RT2 SYBR Green qPCR Master Mix (Invitrogen, Carlsbad, CA, USA). Five housekeeping genes, including glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin beta (*ACTB*), ribosomal protein lateral stalk subunit P0 (*RPLP0*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), and beta-2-microglobulin (*B2M*), were incorporated in the assay, and the average Ct values were calculated.

Statistical analysis

GraphPad Prism 8 for Windows (GraphPad, La Jolla, CA, USA) was used to carry out statistical analyses and draw

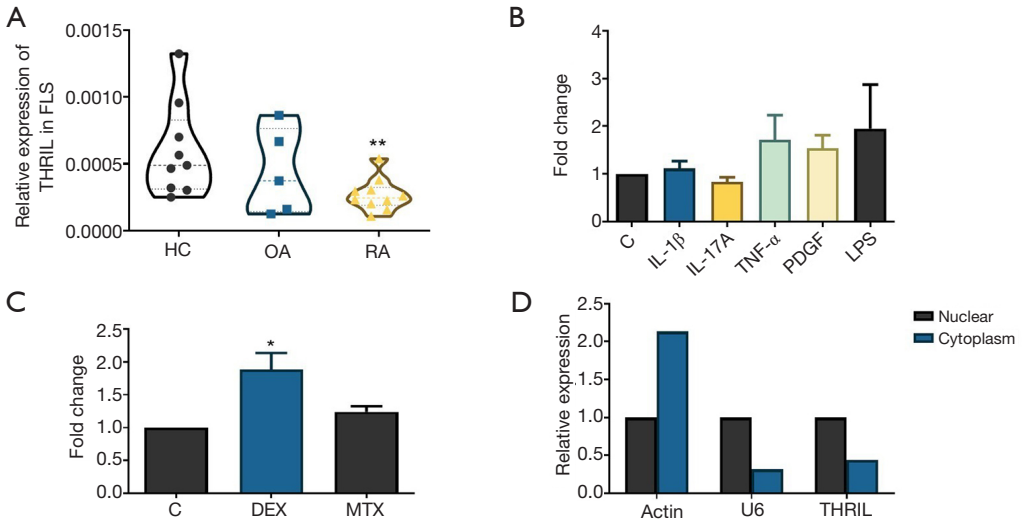

Figure 1 Expression of tumor necrosis factor-α and heterogeneous nuclear ribonucleoprotein L-related immunoregulatory lincRNA (*THRIL*) in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs) and healthy control (HC) FLSs. (A) Differential expression of *THRIL* in FLSs from HCs (n=9), osteoarthritis (OA) patients (n=5) and RA patients (n=10). ● HC individuals, ■ OA patients, ▲ RA patients. **P<0.01 *vs.* HC. (B) Expression of *THRIL* under treatment with various cytokines. RA FLSs were stimulated with interleukin (IL)-1β (10 ng/mL), IL-17A (10 ng/mL), TNF-α (10 ng/mL), platelet derived growth factor(PDGF) (10 ng/mL), or Lipopolysaccharide (LPS) (100 ng/mL) for 24 h. (C) Effect of dexamethasone (DEX) or methotrexate (MTX) on *THRIL* expression in RA FLSs. RA FLSs were treated with DEX (1 μg/mL) or MTX (10 μg/mL) for 24 h. Ct values were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Data shown are the mean ± standard error of mean from at least 3 independent experiments. *P<0.05 *vs.* control (C). (D) *THRIL* expression in cytoplasmic and nuclear fraction of FLSs. Actin served as a cytoplasmic marker and U6 served as a nuclear marker.

statistical charts. The values shown were pooled from at least 3 independent experiments and presented as the mean value ± standard error of the mean. To minimize variability between independent experiments using FLSs from different RA patients, some data were normalized and presented as the fold change over the indicated control group. A 2-tailed Student's *t*-test was employed for comparisons between 2 groups. For comparisons between 3 or more different groups, 1-way analysis of variance with Bonferroni's post-hoc test was employed. P<0.05 was considered to be statistically significant.

Results

Decreased expression of lncRNA THRIL in FLSs from RA patients

First, qRT-PCR was employed for the quantification of *THRIL* expression in FLSs. As shown in *Figure 1A*, RA FLSs displayed significantly decreased expression of *THRIL* compared with HC FLSs. It is now well established that pro-inflammatory cytokines are key components that form the inflammatory microenvironment in the rheumatoid synovium. We evaluated the influences of cytokine stimulation on the expression of *THRIL* in RA FLSs. However, unexpectedly, *THRIL* expression in RA FLSs was not influenced by stimulation with interleukin (IL)-1β, IL-17A, TNF-α, PDGF, or LPS for 24 h (*Figure 1B*). In addition, treatment with dexamethasone, but not methotrexate, increased *THRIL* expression in RA FLSs (*Figure 1C*).

Next, to explore the expression pattern and intracellular localization of *THRIL*, we separated the nuclear and cytoplasmic fractions of RA FLSs. We found that *THRIL* was primarily expressed in the nucleus (*Figure 1D*), suggesting that *THRIL* probably exerts its regulatory function at the transcription level in RA FLSs, as previously reported for macrophages (17).

THRIL is a negative regulator of RA FLS proliferation

siRNAs targeting *THRIL* were transfected into RA FLSs, and the silencing efficiency was confirmed (*Figure 2A,2B*).

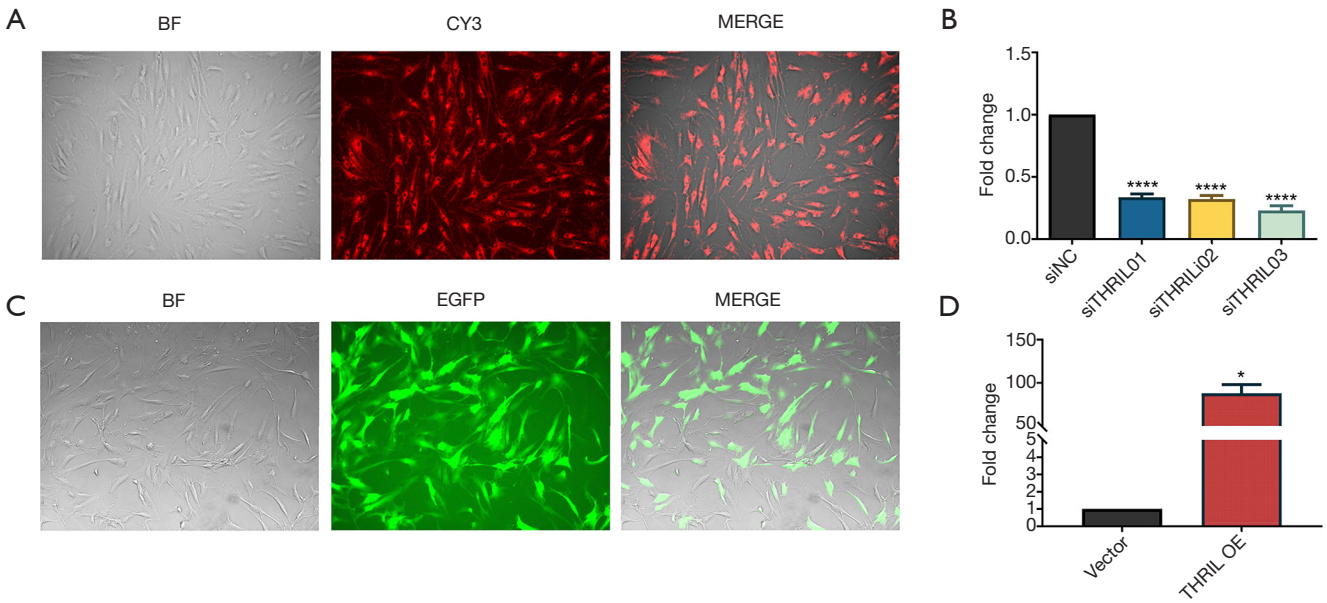

Figure 2 Efficiency of tumor necrosis factor-α and heterogeneous nuclear ribonucleoprotein L-related immunoregulatory lincRNA (*THRIL*) knockdown or overexpression in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs). (A) CY3-labeled small interfering RNA (siRNA) was transfected to RA FLSs at a final concentration of 50 nM and visualized under a fluorescence microscope after 48. Red fluorescence represents CY3-labeled siRNA. Original magnification 100×. (B) RA FLSs were transfected with scramble siRNA (siNC) or *THRIL* siRNA (si*THRIL*), and efficiency of *THRIL* silencing was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). (C) RA FLSs were infected with Green Fluorescent Portein (GFP)-carrying lentivirus at a Multiplicity of Infection (MOI) of 30 and observed after 72 h. Green fluorescence represents GFP-positive cells. Original magnification 100×. (D) Expression level of *THRIL* in RA FLSs after infection with empty vector lentivirus (Vector) or *THRIL* overexpression lentivirus (*THRIL* OE) was measured by qRT-PCR. Data shown are the mean ± standard error of mean from at least 3 independent experiments. *P<0.05, ****P<0.0001 *vs.* control small interfering RNA (siNC) or Vector.

Transfection of all 3 siRNAs successfully decreased the expression level of *THRIL*, and si*THRIL*03, which showed the greatest silencing efficiency, was chosen for the subsequent experiments. A lentivirus-based overexpression strategy was employed to increase the expression of *THRIL* (*Figure 2C*,*2D*).

Because the significant proliferation and reduced apoptosis of RA FLSs contribute to synovial hypertrophy in the articular cavity, we investigated the role of *THRIL* in controlling the proliferation rate of RA FLSs. We found that silencing *THRIL* with siRNA significantly increased the percentage of proliferating cells, as measured by EdU staining (*Figure 3A*). In contrast, as shown in *Figure 3B*, *THRIL* overexpression by lentivirus markedly decreased the proliferation rate compared with that of the empty vector control in RA FLSs. However, we observed that the proliferation of HC FLSs with *THRIL* knockdown or overexpression was not different from that of the control siRNA or empty vector control group (*Figure 3C*,*D*). We further demonstrated that *THRIL* knockdown or overexpression did not influence the apoptosis of RA FLSs (*Figure 3E*,*3F*) and HC FLSs (*Figure 3G*,*3H*).

THRIL represses the migration and invasion of RA FLSs

Another key pathogenic feature of RA FLSs is tumor-like aggressive behavior characterized by enhanced migration and invasion ability, which is the main cause of bone and cartilage destruction in joints. To establish whether the aberrant expression of *THRIL* modulates the migration of RA FLSs, we performed a transwell assay to measure chemotaxis migration. We found that siRNA-mediated knockdown of *THRIL* promoted the migration of RA FLSs (*Figure 4A*). We then evaluated the influence of *THRIL* on RA FLSs invasion through a transwell chamber precoated with Matrigel. The findings indicated that knockdown of

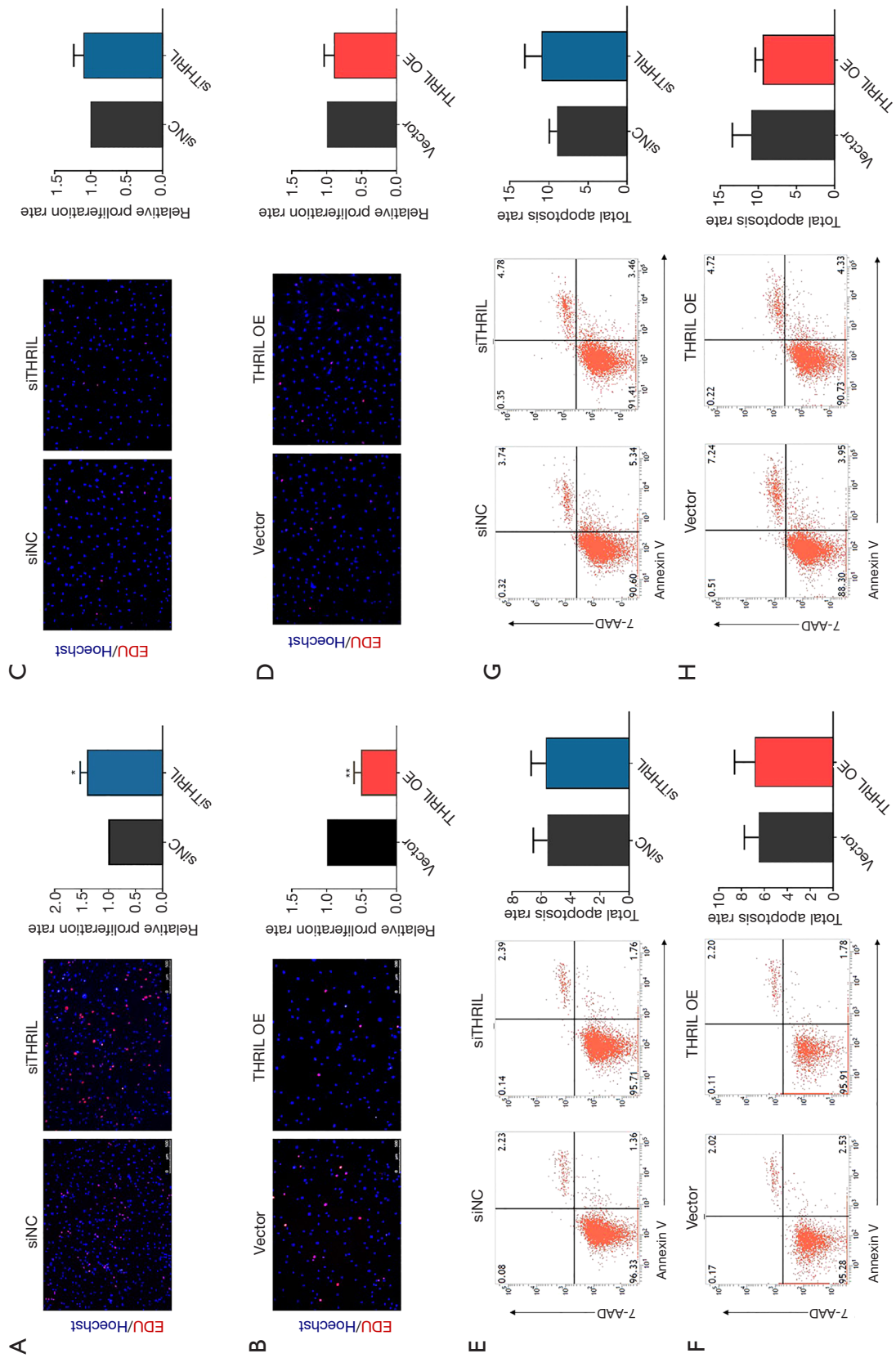

Figure 3 Tumor necrosis factor-α and heterogeneous nuclear ribonucleoprotein L-related immunoregulatory lincRNA (*THRIL*) represses rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs) proliferation. (A,B) Effect of *THRIL* knockdown or overexpression on proliferation of RA FLSs. 5-ethynyl-2′-deoxyuridine(EdU) incorporation assay was performed for the measurement of the proliferation rate of RA FLSs. Red fluorescence represents proliferating cells labeled with EdU and the blue fluorescence represents nuclei stained with Hoechst 33342 (original magnification 100×). (C,D) Effect of *THRIL* knockdown or overexpression on proliferation of healthy control (HC) FLSs. (E,F) Effect of *THRIL* knockdown or overexpression on apoptosis of RA FLSs. Apoptosis was measured using flow cytometry after staining with annexin V-APC and 7-AAD. (G,H) Effect of *THRIL* knockdown or overexpression on apoptosis of HC FLSs. Graphs represent mean ± SEM from at least 3 independent experiments. *P<0.05, **P<0.01 *vs.* control small interfering RNA (siNC) or empty vector lentivirus (Vector).

THRIL significantly increased the invasion of RA FLSs (*Figure 4B*). We also observed that RA FLSs overexpressing *THRIL* exhibited significantly decreased migration and invasion rates than the control group (*Figure 4C,4D*). However, we found that the migration and invasion of HC FLSs with *THRIL* knockdown or overexpression were not different from that of the control siRNA or empty vector control group (*Figure 4E-4H*).

Modulation of the expression of MMPs and proinflammatory cytokines by THRIL in RA FLSs

RA FLSs have been found to secrete various MMPs in response to inflammatory stimulation, which directly degrade the extracellular matrix and facilitate invasion of RA FLSs into the articular cartilage and bones. Suppression of MMP expression might be an efficient strategy to block joint destruction. Interestingly, we found that silencing *THRIL* remarkably increased the mRNA expression of *MMP-1*, *MMP-3*, and *MMP-13* in IL-1β-treated RA FLSs (*Figure 5A*). However, *THRIL* overexpression resulted in a significant decrease in the IL-1β-triggered expression of *MMP-13* (*Figure 5B*). *THRIL* overexpression also slightly reduced the expression of *MMP-1* and *MMP-3* but had no statistical significance (*Figure 5B*). We also observed that silencing of *THRIL* increased the IL-1β-triggered expression of MMP-1 mRNA in HC FLSs; however, we found that *THRIL* knockdown or overexpression did not affect the expression of *MMP-3* and *MMP-13* in IL-1β-treated HC FLSs (*Figure 5C,5D*). In addition, we found that *THRIL* knockdown increased the expression of IL-6 and TNF-α, 2 important pro-inflammatory cytokines in RA (*Figure 5E*).

Downstream molecular targets of THRIL in RA FLSs

To further explore how *THRIL* regulates RA FLSs function, 2 RT-PCR arrays designed to measure 84 genes related to the human cell cycle and 84 genes associated with human cell motility were used to identify target genes that are regulated by *THRIL* overexpression. As shown in *Figure 6A,6B*, a large panel of genes involved in cell proliferation and motility was found to be regulated by *THRIL*. The top 5 differentially expressed genes regulated by *THRIL* overexpression were further confirmed by qRT-PCR in RA FLSs infected with *THRIL* overexpression lentivirus. We found that *THRIL* overexpression decreased the expression of *MKI67*, *CDC20*, *CDK1*, *AURKB*, and *GTSE1* (*Figure 6C*).

Discussion

In the current study, we reported that the lower-than-normal expression of lncRNA *THRIL* negatively regulated the proliferation rate, migration, and invasion of RA FLSs. In addition, *THRIL* modulated the expression of MMP-1, MMP-3, and MMP-13. Furthermore, we showed that *THRIL* regulated various genes associated with the cell cycle and cell motility. Our findings suggest that downregulated *THRIL* may contribute to excessive hyperplasia and invasive behaviors of rheumatoid pannus, ultimately leading to joint destruction.

It has been shown that some lncRNAs participate in RA pathogenesis (10,21); however, their contribution to RA remains largely elusive. In the current investigation, we established decreased expression levels of lncRNA *THRIL* in RA FLSs compared to HC cells. *THRIL* knockdown or overexpression increased or decreased the migration, invasion, and proliferation rates of RA FLSs. Collectively, our data suggest that *THRIL* negatively regulates the tumor-like aggressiveness of RA FLSs. Our previous work demonstrated that the downregulated lncRNA LERFS negatively regulated migration and invasion by interacting with hnRNP Q in RA FLSs (12). Increasing evidence indicates an important role of FLSs in promoting rheumatoid synovial aggression (3,22); therefore, our findings provide a potential novel target for controlling synovitis and joint damage in RA. Consistent with our results, *THRIL* was reported to be downregulated in gastric cancer tissues and non-small cell lung cancer tissues obtained from males compared with adjacent non-cancerous tissues (23,24). A recent report showed that *THRIL* modulated the migration and invasion of H9C2 cells (25). Another study indicated that *THRIL* was involved in the epithelial-to-mesenchymal transition phenotype of osteosarcoma cells (26).

MMPs, primarily secreted by FLSs in rheumatoid ST, are involved in the degradation of the extracellular matrix, leading to cartilage damage and bone erosion in RA joints. Pro-inflammatory cytokines, especially IL-1β, are important inducers of MMP production. Nonetheless, the detailed mechanisms responsible for the expression of MMPs in RA FLSs are not well understood. Here, we found that *THRIL* knockdown or overexpression increased or decreased the expression of *MMP-13* with IL-1β stimulation of RA FLSs. Our findings indicated that *THRIL* negatively regulates

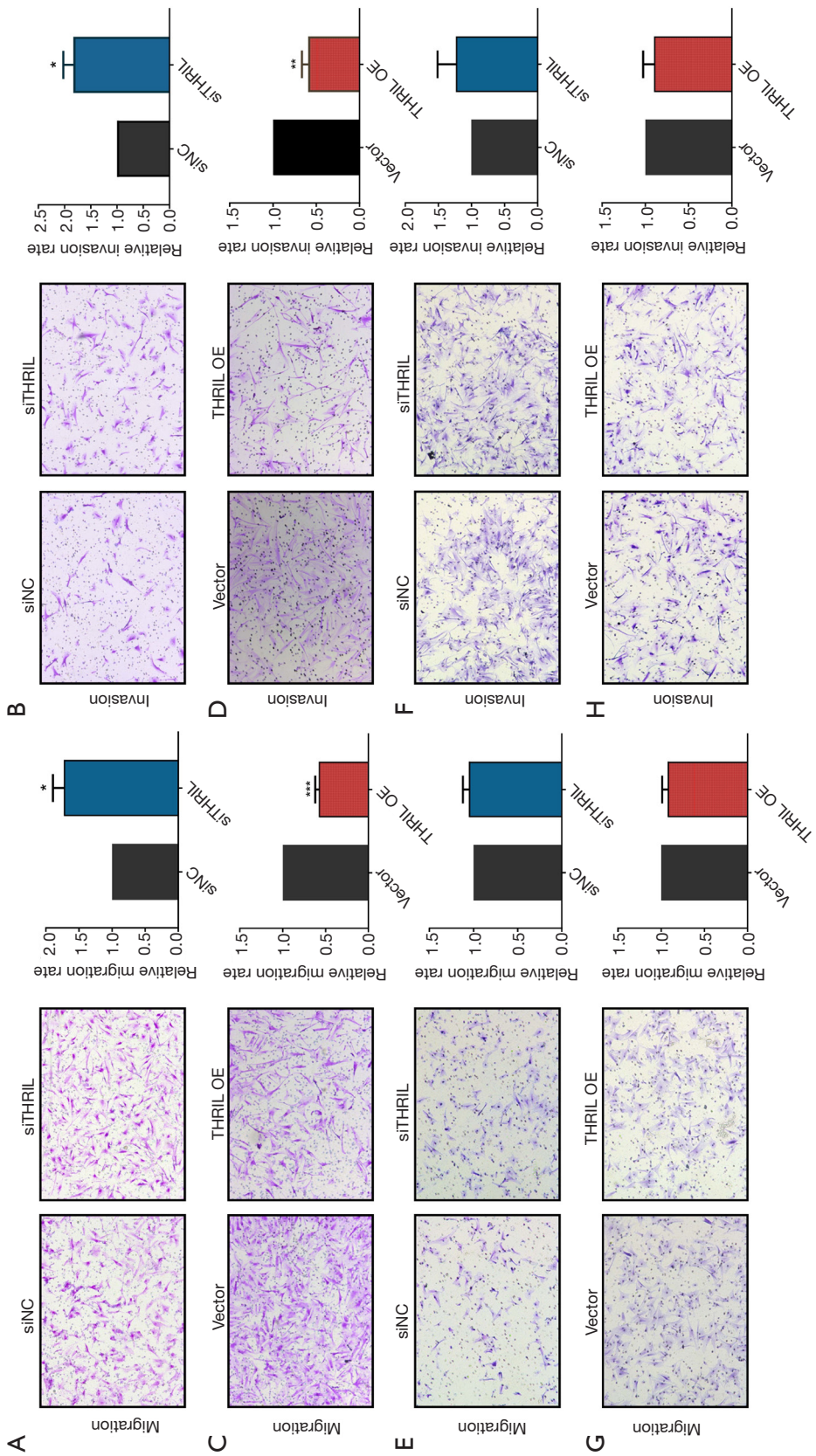

Figure 4 Tumor necrosis factor-α and heterogeneous nuclear ribonucleoprotein L-related immunoregulatory lincRNA (*THRIL*) negatively regulates the migration and invasion of rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs). Cell migration was performed using transwell assay, and 10% fetal bovine serum (FBS) was used as a chemoattractant. For invasion assay, transwell chambers precoated with Matrigel matrix were adopted, and FBS (15%) was used as a chemoattractant. (A,B) Effect of *THRIL* knockdown on the migration (A) and invasion (B) of RA FLSs. (C,D) Effect of *THRIL* overexpression on the migration (C) and invasion (D) of RA FLSs. (E,F) Effect of *THRIL* knockdown on the migration (E) and invasion (F) of HC FLSs. (G,H) Effect of *THRIL* overexpression on the migration (G) and invasion (H) of HC FLSs. Migrated and invaded cells were stained with 0.1% crystal violet and appear purple (original magnification 100×). Data are presented as mean ± standard error of mean from at least 3 independent experiments. *P<0.05, **P<0.01, and ***P<0.001 *vs.* control small interfering RNA (siNC) or empty vector lentivirus (Vector).

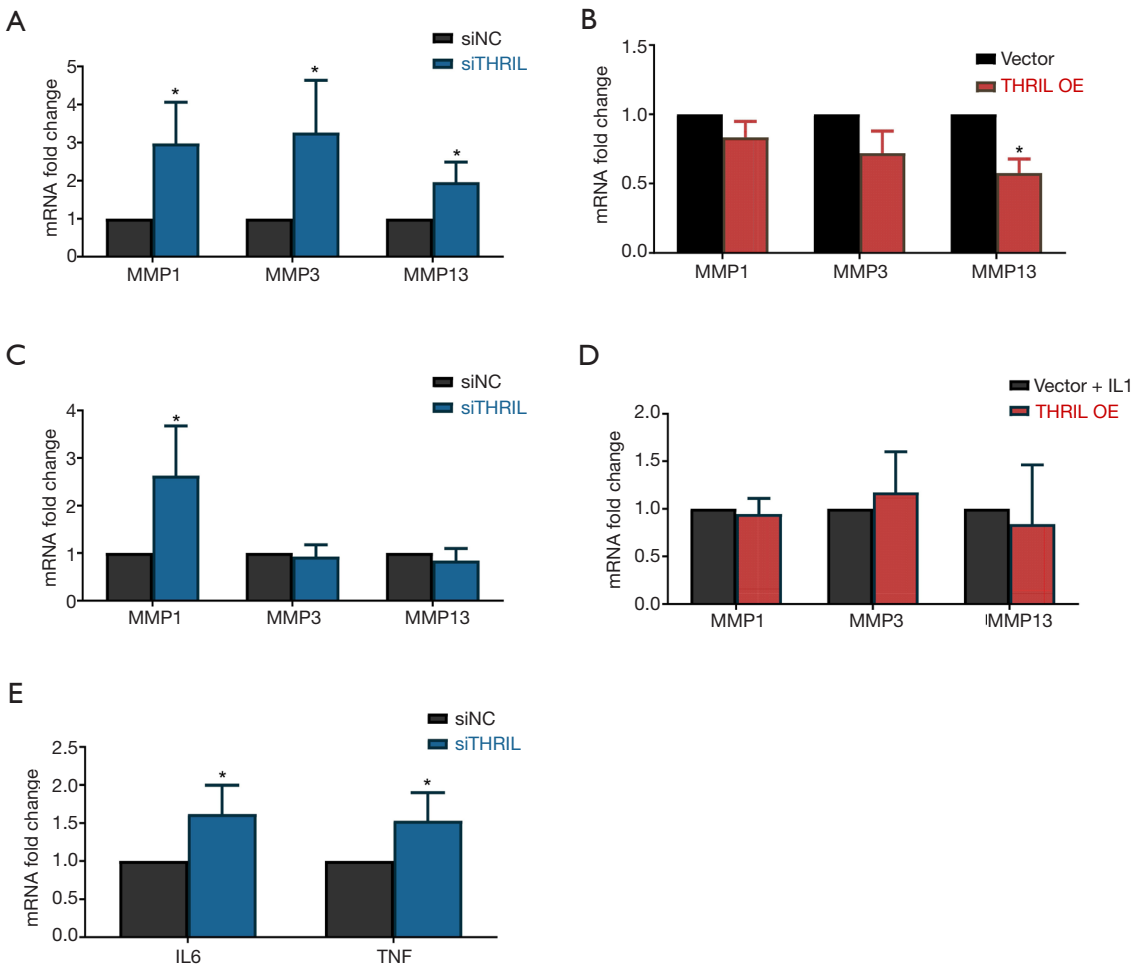

Figure 5 Modulation of interleukin (IL)-1β-induced expression of matrix metalloproteinases (MMPs) and pro-inflammatory cytokines in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) by tumor necrosis factor-α (TNF-α) and heterogeneous nuclear ribonucleoprotein L-related immunoregulatory lincRNA (*THRIL*). Cells were transfected with *THRIL* small interfering RNA (si*THRIL*) or control siRNA (siNC) for 48 h or infected with *THRIL* overexpression lentivirus (*THRIL* OE) or empty vector lentivirus (Vector) and then stimulated with IL-1β (10 ng/mL) for 24 h. mRNA expression of MMPs and cytokines was quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR). (A,B) Effect of *THRIL* knockdown (A) or overexpression (B) on the expression of MMP-1, MMP-3, and MMP-13 in RA FLSs. (C,D) Effect of *THRIL* knockdown (C) or overexpression (D) on the expression of MMP-1, MMP-3, and MMP-13 in HC FLSs. (E) Effect of *THRIL* knockdown on the expression of IL-6 and TNF-α in RA FLSs. Data are presented as mean ± standard error of mean from at least 3 independent experiments. *P<0.05 *vs.* siNC or Vector.

MMPs expression, which further supports the notion that *THRIL* is a powerful regulator in rheumatoid synovial aggression and joint damage.

In addition, we showed the suppressive effect on the proliferation of RA FLSs by *THRIL*. Similarly, a recent study indicated that silencing *THRIL* reversed TNF-α-induced reduction of cell viability and enhancement of apoptosis (19). The unrestrained proliferation of resident FLSs is one of the leading contributors to the formation of rheumatoid pannus and joint destruction in RA; therefore, our findings suggest that the decreased level of *THRIL* in RA FLSs might be associated with abnormal synovial hyperplasia in RA. In line with our findings, it has been shown that *THRIL* is involved in the proliferation of endothelial progenitor cells and osteosarcoma cells (26,27).

To further investigate the downstream molecular mechanisms and pathways through which *THRIL* modulates the pathogenic behaviors of RA FLSs, we employed real-

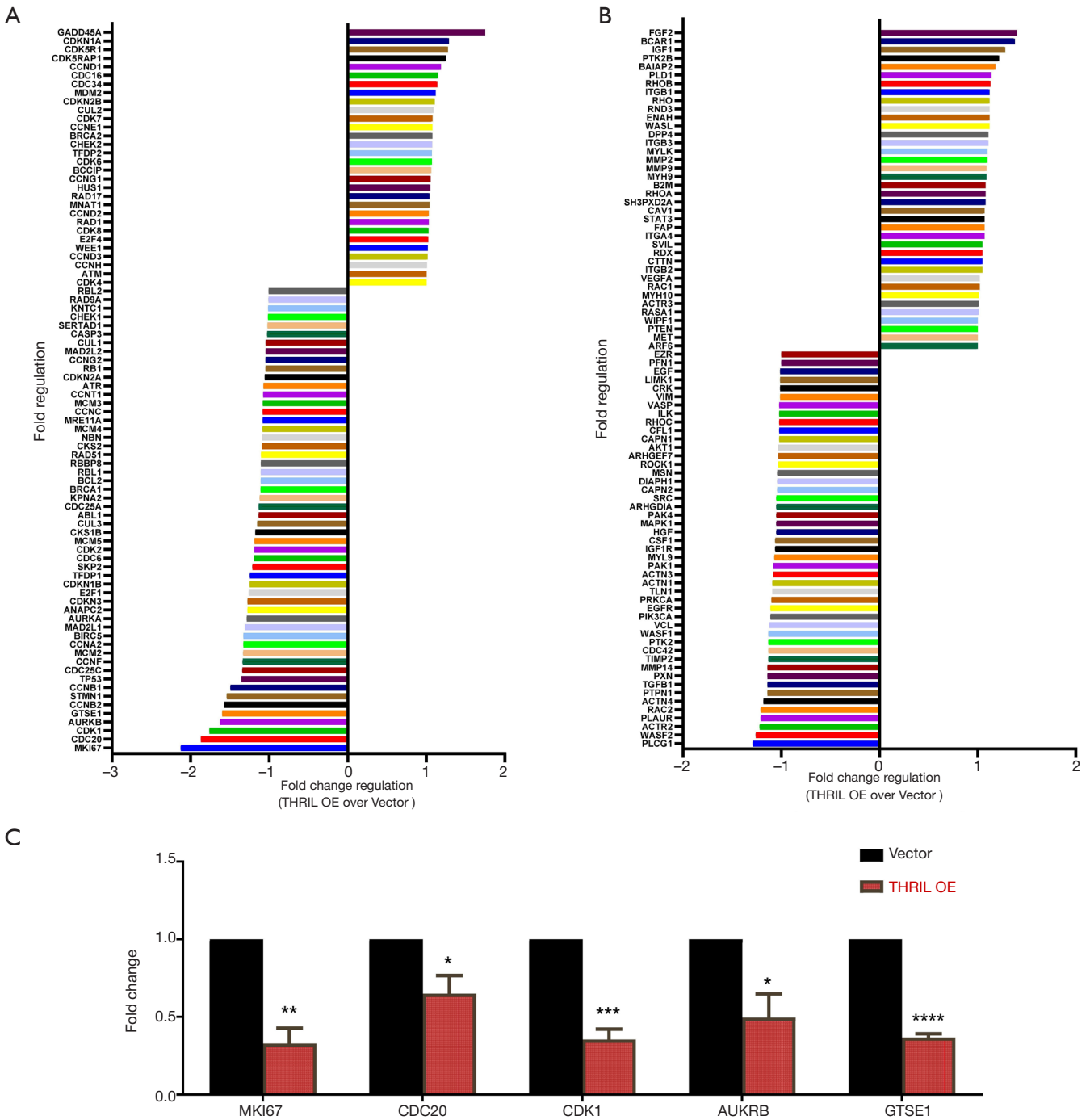

Figure 6 Downstream target genes regulated by tumor necrosis factor-α and heterogeneous nuclear ribonucleoprotein L-related immunoregulatory lincRNA (*THRIL*). Rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLSs) infected with control lentivirus (Vector) or *THRIL* overexpression lentivirus (*THRIL* OE) were subjected to human cell cycle real-time polymerase chain reaction (PCR) array (A) or human cell motility real-time PCR array (B). (C) Top 5 differentially expressed genes regulated by *THRIL* overexpression were further validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The results are shown as mean ± standard error of mean. *P<0.05, **P<0.01,***P<0.001, and ****P<0.0001 *vs.* Vector.

time PCR arrays to identify genes modulated by *THRIL*. A series of typical cell cycle-related genes were shown to be regulated by *THRIL* overexpression, which was further confirmed by qRT-PCR performed in RA FLSs infected with *THRIL* overexpression lentivirus. Among those differentially expressed genes, markers of proliferation Ki-67 (*MKI67*), cell division cycle 20 (*CDC20*), cyclin-dependent kinase 1 (*CDK1*), aurora kinase B (*AUKRB*), and G2 and S phase-expressed-1 (*GTSE1*) were confirmed to be downregulated by overexpression of *THRIL*. *MKI67* is a nuclear protein required to disperse mitotic chromosomes during mitosis and proliferation (28). *CDC20* and *AUKRB* are important regulators participating in mitotic checkpoints and cell cycle regulation (29,30). *CDK1*, also known as *CDC2*, a member of the cyclin-dependent kinase family, is essential for controlling the cell cycle and mitosis. Constitutive and deregulated *CDK1* activation has been found to contribute to the aberrant proliferation of cancer cells by driving cell cycle progression (31,32).

Interestingly, in addition to driving cell cycle progression, CDKs also carry out important roles in the modulation of the actin cytoskeleton and cell migration (33). Moreover, small-molecule inhibitors of CDKs are effective in treating experimental models of RA (34,35), suggesting a potential treatment strategy for RA by targeting CDK-related pathways. *GTSE1* has been previously reported to contribute to cancer progression and metastasis by promoting cell proliferation and invasion (36,37).

As previously reported, *THRIL* is a multifunctional lncRNA that has important roles in various cellular processes by transcriptional regulation of gene expression (17) and post-transcriptional regulation, such as targeting microRNAs (25,38). Our data indicate that the modulation of *THRIL* in the invasion and proliferation of RA FLSs might be associated with its role in regulating the gene expression of *CDK1* and *GTSE1*. Nevertheless, we do not rule out the possibility that some other mechanisms might be engaged in the regulation of RA FLSs by *THRIL*. Further studies are required to address the precise molecular mechanisms by which *THRIL* regulates the aggressive and proliferative behaviors of RA FLSs.

Conclusions

Our findings provide new evidence that the downregulated expression of *THRIL* negatively regulates the aggressive behavior of RA FLSs, suggesting that *THRIL* could be a novel key regulator controlling synovial hyperplasia and joint damage in RA.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). All study procedures were approved by the Medical Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University {No. [2017]-049}. Informed consent was provided by all patients.

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