FOXC1-mediated TRIM22 regulates the excessive proliferation and inflammation of fibroblast-like synoviocytes in rheumatoid arthritis via NF-κB signaling pathway

YAZHI WEI[1], XINMIN HUANG[2], YANMEI MA[3] and LIPING DAI[2]

Departments of [1]Clinical Laboratory and [2]Rheumatology; [3]The Science and Education Division, Shenzhen Futian Hospital for Rheumatic Diseases, Shenzhen, Guangdong 518040, P.R. China

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Abstract. Rheumatoid arthritis (RA) is a common systemic autoimmune disorder of unknown etiology, which threatens public health. The regulatory role of tripartite motif-containing 22 (TRIM22) has been reported in multiple types of cancers and disease, but not in RA. The aim of the present study was therefore to elucidate the potential roles and underlying mechanisms of TRIM22 in fibroblast-like synoviocytes (FLSs) in RA. The Gene Expression Omnibus database was used to examine TRIM22 mRNA expression levels in synovial tissue samples of patients with RA and healthy controls. TRIM22 and forkhead box C1 (FOXC1) mRNA and protein expression levels in normal FLSs and RA-FLSs were assessed using reverse transcription-quantitative PCR (RT-qPCR) and western blotting, respectively. The Cell Counting Kit-8 assay was used to assess cell proliferation. Cell apoptosis was analyzed using flow cytometry. The migratory and invasive abilities of RA-FLSs were assessed using Transwell assays. Western blotting was used to analyze the protein expression levels of apoptosis-related factors, MMP2, MMP9 and NF-κB signaling pathway-related proteins. Inflammatory factors levels were assessed via ELISA and RT-qPCR. Furthermore, the JASPAR database, chromatin immunoprecipitation and the dual-luciferase reporter assays were used to determine the interaction between FOXC1 and the TRIM22 promoter. The results of the present study demonstrated that TRIM22 expression levels were significantly elevated in the synovial tissue samples of patients with RA and RA-FLSs. Moreover, FOXC1 was also significantly overexpressed in RA-FLSs. TRIM22 knockdown significantly reduced cell proliferation, migration, invasion and the inflammatory response, whereas cell apoptosis was significantly increased. Furthermore, the results demonstrated that FOXC1 may have positively mediated TRIM22 expression via binding to the TRIM22 promoter. Moreover, FOXC1 overexpression significantly reversed the outcome of TRIM22 knockdown on the proliferation, apoptosis, migration, invasion and inflammation of RA-FLSs. FOXC1 overexpression also significantly reversed the inactivation of the NF-κB signaling pathway caused by TRIM22 knockdown. In summary, the present study demonstrated that TRIM22 was potentially activated via FOXC1, which contributed to the progression of RA via the NF-κB signaling pathway.

Correspondence to: Dr Liping Dai, Department of Rheumatology, Shenzhen Futian Hospital for Rheumatic Diseases, 22 Nonglin Road, Shenzhen, Guangdong 518040, P.R. China
E-mail: lipinglili21@163.com

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that results in cartilage and bone damage, as well as disability (1). It is estimated that the prevalence of this disease is ~1% and women are at an increased risk of developing RA compared with men (2). RA is primarily characterized by warm, swollen and painful joints, predominantly the synovial joints (3,4). Moreover, RA may impact other organs, including the lungs, skin and eyes (5), which makes patients with RA more prone to suffer from cancer, cardiovascular disease, infectious disease, respiratory disease and osteoporosis (6). Increasing evidence supports the fact that genetic and environmental factors are implicated in the occurrence of RA, such as sex, tobacco smoking and dietary patterns (7). It is widely recognized that prompt and continuous treatment may ameliorate joint damage to impede irreversible disability when patients are diagnosed in the early stages (8,9). However, in spite of the substantially improved clinical outcomes that have resulted from the development of biological and targeted therapies in the past two decades, the etiopathogenesis of RA remains elusive and the therapeutic outcomes remain unsatisfactory (10,11).

Tripartite motif-containing (TRIM)22 is a member of the TRIM family, an evolutionarily conserved gene family (12). The TRIM family participates in numerous biological processes via E3 ubiquitin ligase activities (13). There is also increasing evidence that TRIM22 may serve a critical role in human diseases. For instance, Liu *et al* (14) reported

that TRIM22 contributes to the proliferation and invasion of colon cancer cells. Furthermore, Li *et al* (15) demonstrated that TRIM22 knockdown inactivates the PI3K/Akt/mTOR signaling pathway to prevent chronic myeloid leukemia. Moreover, TRIM22 has also been reported to predict the poor prognosis of non-small cell lung cancer and promote the epithelial-mesenchymal transition process (16). However, whether TRIM22 exerts regulatory functions in RA needs further exploration.

Forkhead box C1 (FOXC1) is a member of the forkhead family of transcription factors and is indispensable for tumor development and metastasis (17). Previous studies have highlighted the promoting role of FOXC1 in the progression of non-small cell lung cancer (18), glioma (19), triple-negative breast cancer (20) and pancreatic cancer (21). Moreover, FOXC1 activates the PI3K/AKT signaling pathway to increase fibroblast-like synoviocyte (FLS) proliferation in RA (22). However, the interaction between FOXC1 and TRIM22 in RA remains to be elucidated.

The NF-κB signaling pathway is composed of canonical signaling pathways activated via various stimuli with a rapid but transient transcriptional activity, as well as non-canonical signaling pathways stimulated via TNF receptor superfamily members (23). Previous studies have verified the pivotal role of NF-κB signaling in tumors and diseases (24,25). Furthermore, the NF-κB signaling pathway promotes RA (26,27).

The aim of the present study was to test the hypothesis that TRIM22 may be modulated via FOXC1 and influence the FLS phenotype in RA via NF-κB signaling.

Materials and methods

Cell culture. The human rheumatoid FLS MH7A cell line (RA-FLS) was purchased from the BeNa Culture Collection (Beijing Beina Chunglian Institute of Biotechnology) and the immortalized normal FLS cell line was purchased from the NTCC Type Culture Collection (BioVector Science Lab, Inc.). The culture medium for both RA-FLSs and normal-FLSs was DMEM (Hyclone; Cytiva) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). The cells were incubated at 37˚C with 5% CO_2.

Cell transfection. Small interfering RNAs (siRNAs/si) targeting TRIM22 (si-TRIM22#1/2; cat. no. siG000010346A-1-5/siG1072101848-1-5) were constructed by Guangzhou RiboBio Co., Ltd., with an siRNA negative control (NC; si-NC; non-targeting sequence; cat. no. siB06525141922-1-5). The pcDNA3.1 overexpression vector containing the FOXC1 gene (Ov-FOXC1) and NC overexpression vector (Ov-NC) were purchased from Shanghai GenePharma Co., Ltd. For TRIM22 silencing, RA-FLSs were transfected with 50 nM si-TRIM22#1/2 and si-NC. For FOXC1 overexpression, RA-FLSs were transfected with 2 μg Ov-FOXC1 and Ov-NC. Untransfected cells were regarded as the Control group. Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect the plasmids into RA-FLSs, which were seeded in 6-well plates at a density of $1x10^6$ for 48 h at 37˚C according to the manufacturer's instructions. The cells were collected for subsequent experiments 48 h following transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells in 6-well plates at a density of $2x10^5$ cells/well using the EasyPure® RNA Kit (TransGen Biotech, Co., Ltd.) according to the manufacturer's instructions. Complementary DNA was synthesized using a RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed using the SYBR Green Premix PCR Master Mix [Roche Diagnostics (Shanghai) Co., Ltd.] and the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR conditions were 95˚C for 10 min for initial denaturation, 40 cycles of denaturation 15 sec at 95˚C, annealing 30 sec at 60˚C, elongation 30 sec at 72˚C, and final extension for 5 min at 72˚C. The $2^{-\Delta\Delta Cq}$ method (28) was used to quantify relative mRNA expression levels, with GAPDH used as the endogenous control. The qPCR primer sequences were as follows: TRIM22 forward (F), 5'-GAGGTCAAGATGAGCCCACAG-3' and reverse (R), 5'-GCTTTTCCTGACATTCCTTGACC-3'; FOXC1 F, 5'-TAG CTACATCGCGCTCATCA-3' and R, 5'-ACCTTGACGAAG CACTCGTT-3'; TNF-α F, 5'-CTGGGCAGGTCTACTTTG GG-3' and R, 5'-CTGGAGGCCCCAGTTTGAAT-3'; IL-1β F, 5'-CCAAACCTCTTCGAGGCACA-3' and R, 5'-AGCCAT CATTTCACTGGCGA-3'; IL-6 F, 5'-GTCCAGTTGCCTTCT CCCTGG-3' and R, 5'-CCCATGCTACATTTGCCGAAG-3' and GAPDH F, 5'-AATGGACAACTGGTCGTGGAC-3' and R 5'-CCCTCCAGGGGATCTGTTTG-3'.

Cell counting kit-8 (CCK-8) assay. Transfected RA-FLSs were seeded into 96-well plates ($3x10^4$ cells/well) and were then incubated for 12 h at 37˚C. Cells were then maintained for 24, 48 and 72 h respectively and 10 μl CCK-8 solution [Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.] was added for additional 2 h. The absorbance at 450 nm was assessed using a microplate reader (Perlong Medical Equipment Co., Ltd.).

Flow cytometry. Cell apoptosis was assessed using the Annexin V-FITC/PI Apoptosis kit (BD Biosciences). Following centrifugation at 1,000 x g for 5 min at 4˚C, transfected RA-FLSs in 6-well plates ($1x10^6$ cells/well) were rinsed in cold PBS. Subsequently, cells were re-suspended in 400 μl 1X binding buffer after the supernatant was removed, followed by the addition of 4 μl Annexin V-FITC at 4˚C for 15 min and 4 μl PI for 30 min in the dark at room temperature. Finally, the apoptotic rate of RA-FLSs was detected using a BD FACSCalibur flow cytometer (BD Biosciences) using BD Accuri C6 software (version no. 1.0.264.21; BD Biosciences).

Transwell assays. Transwell assays were performed using Transwell chambers (Corning, Inc.) with an 8.0 μm pore size. Briefly, $2x10^4$ transfected RA-FLSs resuspended in 400 μl serum-free DMEM medium were added to the upper chamber and 800 μl DMEM medium containing 15% FBS was added to the lower chamber. After 12 h at 37˚C, the migrating cells were fixed using 100% methanol for 10 min and were then stained with 0.1% crystal violet for 15 min at room temperature. Images were captured using a light microscope (magnification, x100; Olympus Corporation) and analyzed by ImageJ software (v1.8.0.112, National Institutes of Health). The Transwell invasion assays followed the same procedure

as the migration assays, except that the Transwell chambers in the invasion assays were precoated with Matrigel® (BD Biosciences) for 1 h at 37˚C.

ELISA. TNF-α (cat. no. ab181421), IL-1β (cat. no. ab214025) and IL-6 (cat. no. ab178013) levels were detected using human TNF-α ELISA kit, human IL-1β ELISA kit and human IL-6 ELISA kit purchased from Abcam. Briefly, transfected RA-FLSs were seeded into 96-well plates ($5x10^3$ cells/well). After centrifuging at 2,000 x g for 5 min at 4˚C, cell supernatant was collected to examine the secretion of inflammatory cytokines according to the instructions of the ELISA kits. The absorbance was read at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Chromatin immunoprecipitation (ChIP). The EZ-ChIP kit (cat. no. 9005; Cell Signaling Technology, Inc.) was used for the ChIP assays. RA-FLSs ($1x10^6$) were crosslinked by 1% formaldehyde (Sigma-Aldrich; Merck KGaA) by centrifugation at 300 x g for 3 min at 25˚C and washed in pre-cooled PBS for 10 min at 25˚C. The formaldehyde was quenched by the addition of glycine (Beijing Solarbio Science & Technology Co., Ltd.). Then a total of 300 µl SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl pH 8.0) was then used to lyse the cells, which were subsequently sonicated at 150 Hz and sheared with four sets of 10 sec pulses on wet ice using a high intensity ultrasonic processor to obtain chromatin fragments. An equal amount of chromatin (100 µl) was immunoprecipitated at 4˚C overnight, 5% of the supernatant was collected as input DNA. Immunoprecipitated products were collected after incubation with 100 ul Protein A/G magnetic beads coupled with 2 µg anti-IgG (cat. no. ab6715; 1 µg/µl; Abcam) or 2 µg anti-FOXC1 (cat. no. ab227977; 1 µg/µl; Abcam) for 90 min at 4˚C. The beads were washed using a magnetic separation rack and the bound chromatin was eluted from the beads with SDS buffer and subjected to RNase and proteinase K treatment (10 mg/ml) at 45˚C. Crosslinks were reversed by overnight incubation with 5 M NaCl at 65˚C, and Chip DNA was purified using spin columns. The recovered DNA fragments were subjected to PCR analysis using the SYBR Green Premix PCR Master Mix [Roche Diagnostics (Shanghai) Co., Ltd.] and the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR conditions were: 95˚C 10 min for initial denaturation, 40 cycles of denaturation 15 sec at 95˚C, annealing 30 sec at 60˚C, elongation 30 sec at 72˚C, and final extension for 5 min at 72˚C. PCR products were separated by 1% gel electrophoresis using agarose gels prestained with ethidium bromide. Bands were analyzed using ImageJ v1.5.1 (National Institutes of Health, Bethesda). The primer sequences used for ChIP were as follows: TRIM22 F, 5'-TGTGGTTGACACTCG GACTC-3' and R, 5'-TCACGCCTGTCTTAGTCAAG-3'.

Dual-luciferase reporter assay. The wild-type (WT; TRIM22-WT) and mutant FOXC1 binding sites in the TRIM22 promoter were cloned into the pGL3 vector (E1761; Promega Corporation). These reporter plasmids were co-transfected with 2 µg Ov-FOXC1 and Ov-NC into the cells seeded at $5x10^4$ cells per well in 24-well plates using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. After 48 h, luciferase activity was assessed using the Dual Luciferase Reporter Gene Assay Kit (Beyotime Institute of Biotechnology). Firefly luciferase activity was normalized to Renilla luciferase activity.

Western blotting. A RIPA lysis buffer (Nanjing Jiancheng Bioengineering Institute) was used to extract total protein from cells ($2x10^6$) and protein concentrations were then quantified using the BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific, Inc.). Subsequently, total protein (50 µg/lane) was separated using SDS-PAGE on a 10% gel (Wuhan Boster Biological Technology, Ltd.). Separated proteins were then transferred onto PVDF membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked using 5% skimmed milk for 30 min at room temperature and were then incubated with primary antibodies against the following targets: TRIM22 (1:1,000; cat. no. ab68071; Abcam), Bcl-2 (1:1,000; cat. no. 15071; Cell Signaling Technology, Inc.), Bax (1:1,000; cat. no. ab216494; Abcam), cleaved caspase-3 (1:1,000; cat. no. 9661; Cell Signaling Technology, Inc.), caspase-3 (1:1,000; cat. no. 9668; Cell Signaling Technology, Inc.), MMP2 (1:1,000; cat. no. ab92536; Abcam), MMP9 (1:1,000; cat. no. ab283575; Abcam), FOXC1 (1:1,000; cat. no. ab223850; Abcam), phosphorylated (p)-IκBα (1:1,000; cat. no. ab133462; Abcam), p-NF-κB (1:1,000; cat. no. ab76302; Abcam), IκBα (1:1,000; cat. no. ab76429; Abcam), NF-κB (1:1,000; cat. no. ab32536; Abcam) and GAPDH (1:1,000; cat. no. ab8245; Abcam) at 4˚C overnight. Following the primary antibody incubation, the membranes were washed three times with TBS +0.05% Tween-20 and were subsequently incubated with HRP-conjugated anti-rabbit (1:2,000; cat. no. ab6721; Abcam) or HRP-conjugated anti-mouse (1:2,000; cat. no. ab6789; Abcam) secondary antibodies at room temperature for 2 h. A Pierce ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.) was applied to visualize the protein bands and ImageJ (v1.8; National Institutes of Health) was adopted for analysis.

Bioinformatics tools. TRIM22 expression levels in synovial membrane samples from patients with RA and or healthy controls were predicted using the Gene Expression Omnibus (GEO) database (accession no. GSE12021; https//www.ncbi.nlm.nih.gov/geo/) (29) using GEO2R analysis of the GPL96 platform. The expression values of samples from patients with RA or healthy controls were determined and GraphPad Prism 8.0 (GraphPad Software, Inc.) was used to produce a scatter diagram. The potential binding sites between FOXC1 and the TRIM22 promoter were predicted using the JASPAR database (https://jaspar.genereg.net/).

Statistical analysis. All data were acquired from three independent experiments and are presented as the mean ± SD. The results were analyzed using SPSS 22.0 (IBM Corp.). The statistical differences between two groups were determined using an unpaired Student's t-test. The statistical differences between more than two groups were compared using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TRIM22 expression levels are increased in RA-FLSs. To determine the role of TRIM22 in RA, TRIM22 expression levels

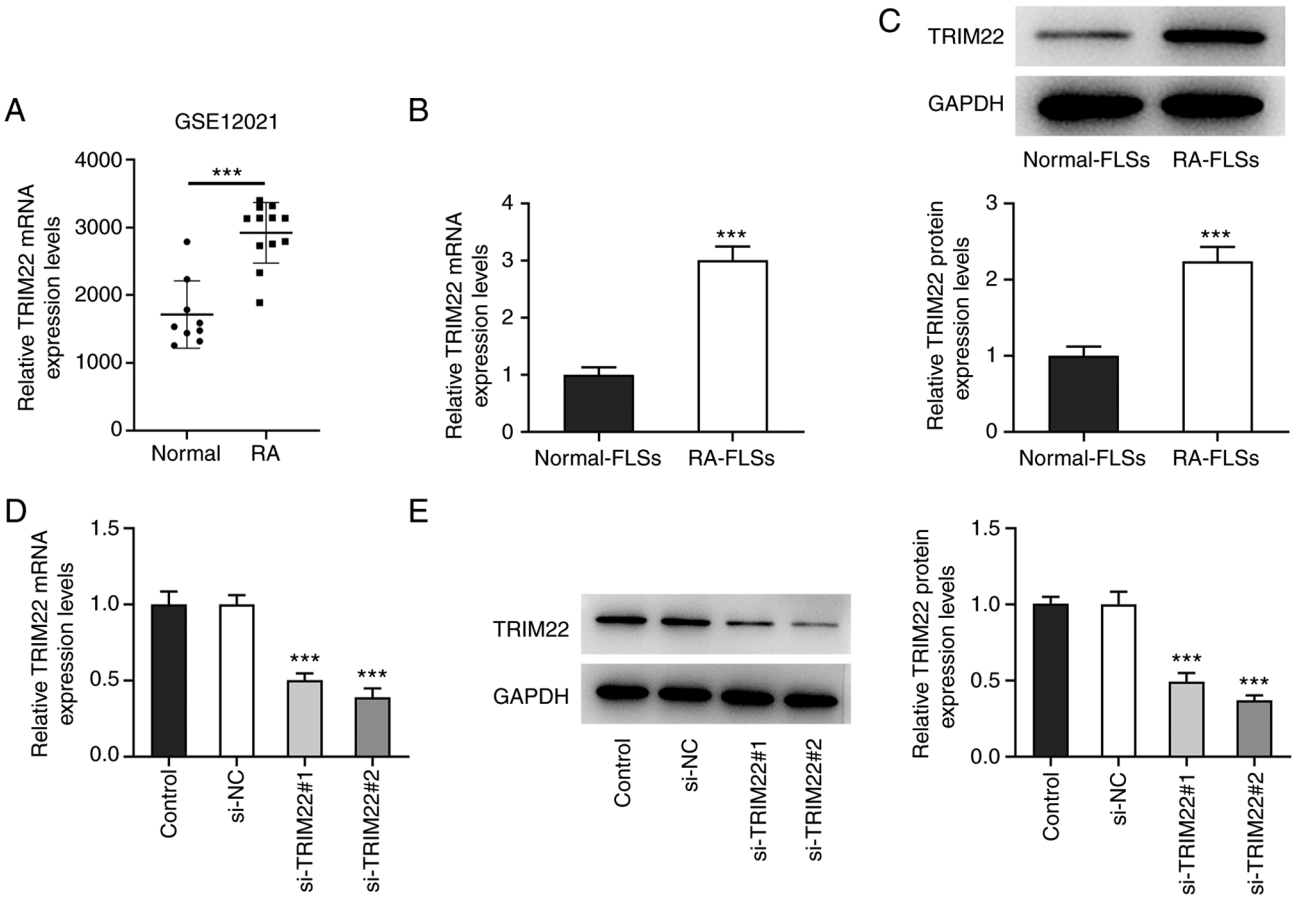

Figure 1. TRIM22 expression levels are increased in RA-FLSs. (A) TRIM22 gene expression levels were examined in synovial membrane samples of patients with RA and normal synovial membrane samples using the Gene Expression Omnibus database. TRIM22 mRNA and protein expression levels in RA-FLSs and normal-FLSs were determined using (B) RT-qPCR and (C) western blotting. ***P<0.001. si-TRIM22#1/2 transfection efficiency was analyzed using (D) RT-qPCR and (E) western blotting. ***P<0.001 vs. si-NC. TRIM22, tripartite motif-containing 22; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocyte; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering RNA; NC, negative control.

were detected in synovial membrane samples from patients with RA and healthy controls. The results demonstrated that TRIM22 mRNA expression levels in the synovial membrane samples of patients with RA was significantly higher compared with the samples from the healthy individuals (Fig. 1A). Furthermore, RT-qPCR and western blotting demonstrated that TRIM22 was also significantly upregulated in RA-FLSs compared with normal FLSs (Fig. 1B and C). Subsequently, TRIM22 expression was knocked down using siRNA transfection. Compared with the si-NC group, the mRNA and protein expression levels of TRIM22 were significantly reduced in the si-TRIM22#1 and #2 groups, especially in the latter (Fig. 1D and E). Therefore, si-TRIM22#2 was selected for the following functional experiments. These results suggested that TRIM22 may be highly expressed in RA-FLSs.

TRIM22 knockdown inhibits the proliferation and promotes the apoptosis of RA-FLSs. CCK-8 assays were used to assess the effect of TRIM22 knockdown on the proliferation of RA-FLSs. The results demonstrated that the knockdown of TRIM22 significantly reduced cell proliferation at 48 and 72 h compared with the si-NC group (Fig. 2A). Furthermore, the number of apoptotic cells in the si-TRIM22 group was significantly increased compared with the si-NC group, which suggested that TRIM22 knockdown potentially promoted the apoptosis of RA-FLSs (Fig. 2B). Moreover, compared with the si-NC group, western blotting indicated that TRIM22 knockdown significantly reduced the protein expression levels of the antiapoptotic protein Bcl-2, whereas those of the proapoptotic proteins Bax and cleaved caspase-3/caspase-3 were significantly increased (Fig. 2C). These results indicated that the knockdown of TRIM22 potentially inhibited the proliferation and promoted the apoptosis of RA-FLSs.

TRIM22 knockdown attenuates the migratory and invasive capacities of RA-FLSs. Cell migration and invasion were assessed using Transwell assays. The numbers of migrating cells and invading cells were significantly reduced following TRIM22 knockdown compared with the si-NC group (Fig. 3A and B). Furthermore, as MMP2 and MMP9 are involved in cell migration and invasion (30), western blotting was used to analyze the expression levels of these proteins. MMP2 and MMP9 protein levels were both significantly downregulated following TRIM22 knockdown compared with the si-NC group (Fig. 3C). Therefore, TRIM22 knockdown may have potentially inhibited the migration and invasion of RA-FLSs.

TRIM22 knockdown ameliorates inflammatory responses in RA-FLSs. TNF-α, IL-1β and IL-6 are well-characterized pro-inflammatory cytokines (31). Using ELISA, it was observed

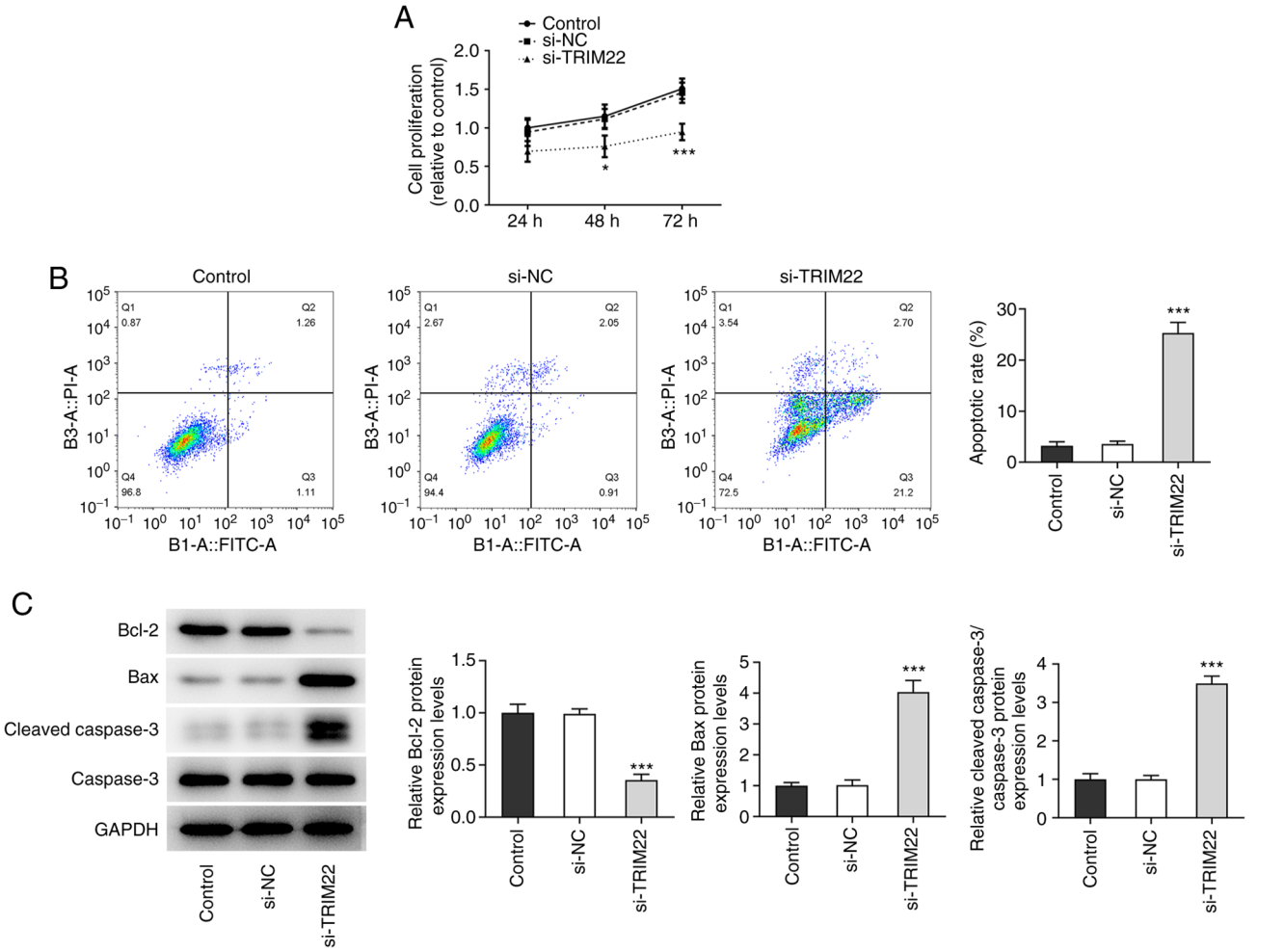

Figure 2. TRIM22 knockdown inhibits proliferation and promotes the apoptosis of RA-FLSs. (A) RA-FLS proliferation was detected using the Cell Counting Kit-8 assay. (B) Cell apoptosis was assessed using flow cytometry. (C) Western blotting was performed to assess the protein expression levels of apoptosis-related factors. ***P<0.001 vs. si-NC. TRIM22, tripartite motif-containing 22; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocyte; si, small interfering RNA; NC, negative control.

that TNF-α, IL-1β and IL-6 levels were significantly downregulated following TRIM22 knockdown compared with the si-NC group (Fig. 4A). Moreover, RT-qPCR further demonstrated that following TRIM22 knockdown, the mRNA expression levels of TNF-α, IL-1β and IL-6 were also significantly downregulated compared with the si-NC group (Fig. 4B). These results indicated that TRIM22 knockdown potentially reduced the production of pro-inflammatory cytokines by RA-FLSs.

FOXC1 induces the upregulation of TRIM22 in RA-FLSs. FOXC1 is considered to be an oncogenic transcription factor (15). The JASPAR database was used to predict the potential binding site between FOXC1 and the TRIM22 promoter (Fig. 5A). Furthermore, RT-qPCR and western blotting demonstrated that FOXC1 mRNA and protein expression levels, respectively, were significantly upregulated in RA-FLSs compared with normal-FLSs (Fig. 5B and C). Subsequently, FOXC1 mRNA and protein expression levels were significantly increased following transfection with the Ov-FOXC1 plasmid, compared with the Ov-NC group, and the overexpression efficiency was assessed (Fig. 5D and E). Moreover, the dual-luciferase reporter assay demonstrated that the luciferase activity of TRIM22-WT significantly increased in the Ov-FOXC1 group compared with the Ov-NC group while no obvious changes were observed in the luciferase activity of TRIM22-MUT in the Ov-FOXC1 group compared with the Ov-NC group (Fig. 5F). ChIP assays also demonstrated that the TRIM22 promoter was co-precipitated with the FOXC1 antibody, which confirmed the binding between the TRIM22 promoter and FOXC1 (Fig. 5G). Furthermore, FOXC1 overexpression significantly upregulated the mRNA levels and protein expression levels of TRIM22, which suggested that TRIM22 was potentially positively regulated via FOXC1 (Fig. 5H and I). Therefore, these results indicated that FOXC1 may be a transcriptional activator of TRIM22.

FOXC1 overexpression reverses the effects of TRIM22 knockdown on the proliferation, apoptosis, migration, invasion and inflammatory response of RA-FLSs. To confirm the interaction between FOXC1 and TRIM22 in RA-FLSs, rescue assays were performed. The mRNA and protein expression levels of TRIM22 were significantly downregulated in RA-FLSs transfected with si-TRIM22 compared with the control group. However, this was significantly reversed by FOXC1 overexpression compared with the si-TRIM22 + Ov-NC group (Fig. 6A and B). CCK-8 assays demonstrated that the significant reduction in the

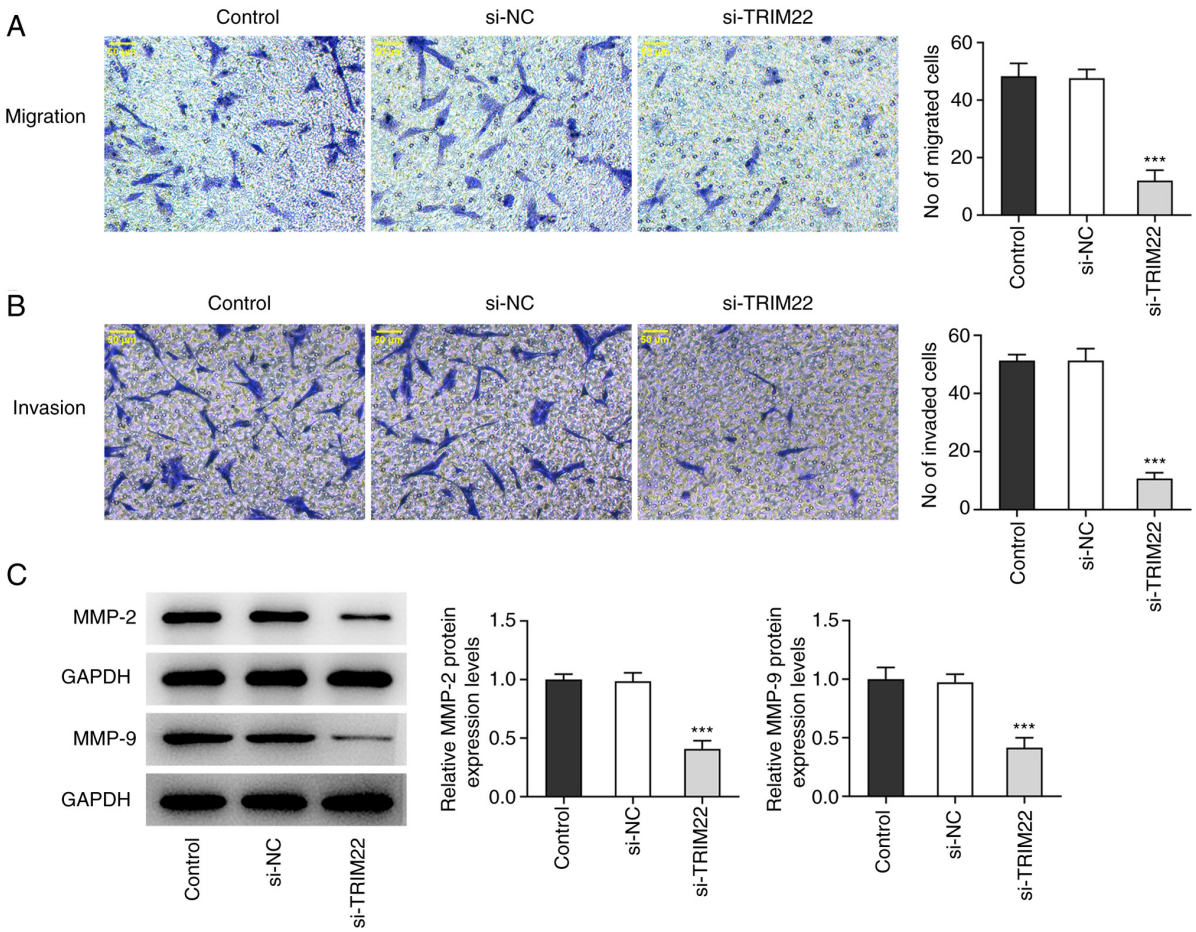

Figure 3. TRIM22 knockdown attenuates the migratory and invasive capacities of RA-FLSs. The (A) migration and (B) invasion of RA-FLSs were assessed via Transwell assays. Magnification, x200. (C) Western blotting was performed to analyze the protein expression levels of MMP2 and MMP9. ***P<0.001 vs. si-NC. TRIM22, tripartite motif-containing 22; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocyte; si, small interfering RNA; NC, negative control.

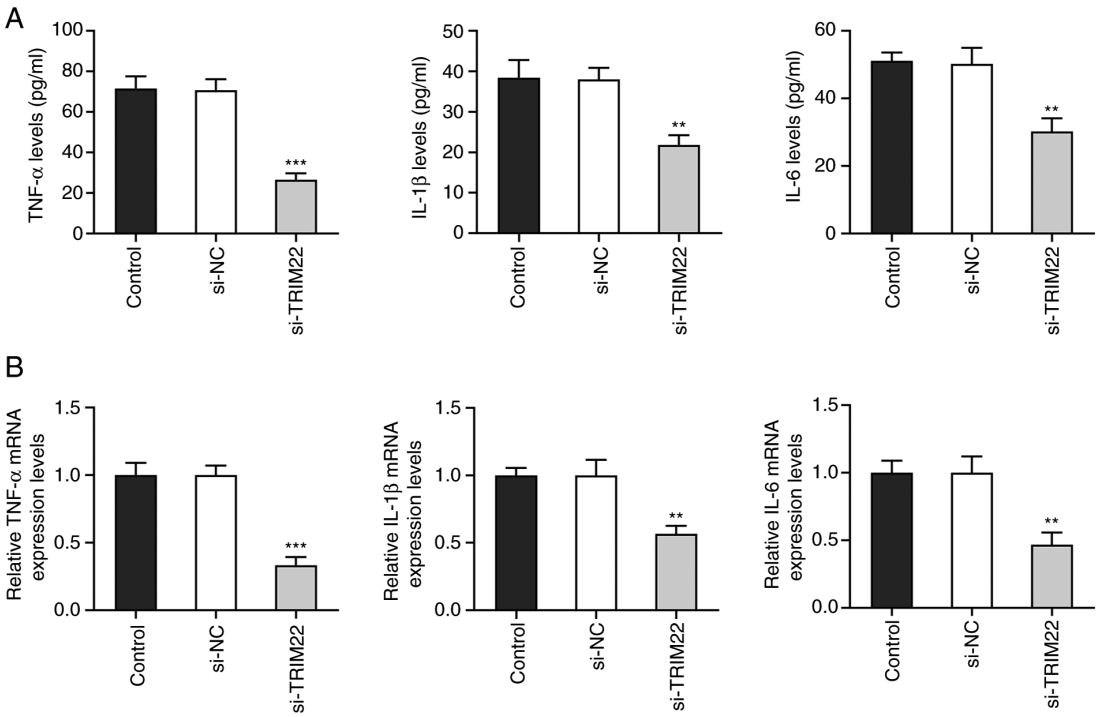

Figure 4. TRIM22 insufficiency ameliorates inflammatory responses in rheumatoid arthritis-fibroblast-like synoviocytes. (A) Levels of TNF-α, IL-1β and IL-6 were assessed using ELISA. (B) Reverse transcription-quantitative PCR was performed to determine the mRNA expression levels of TNF-α, IL-1β and IL-6. **P<0.01, ***P<0.001 vs. si-NC. TRIM22, tripartite motif-containing 22; si, small interfering RNA; NC, negative control.

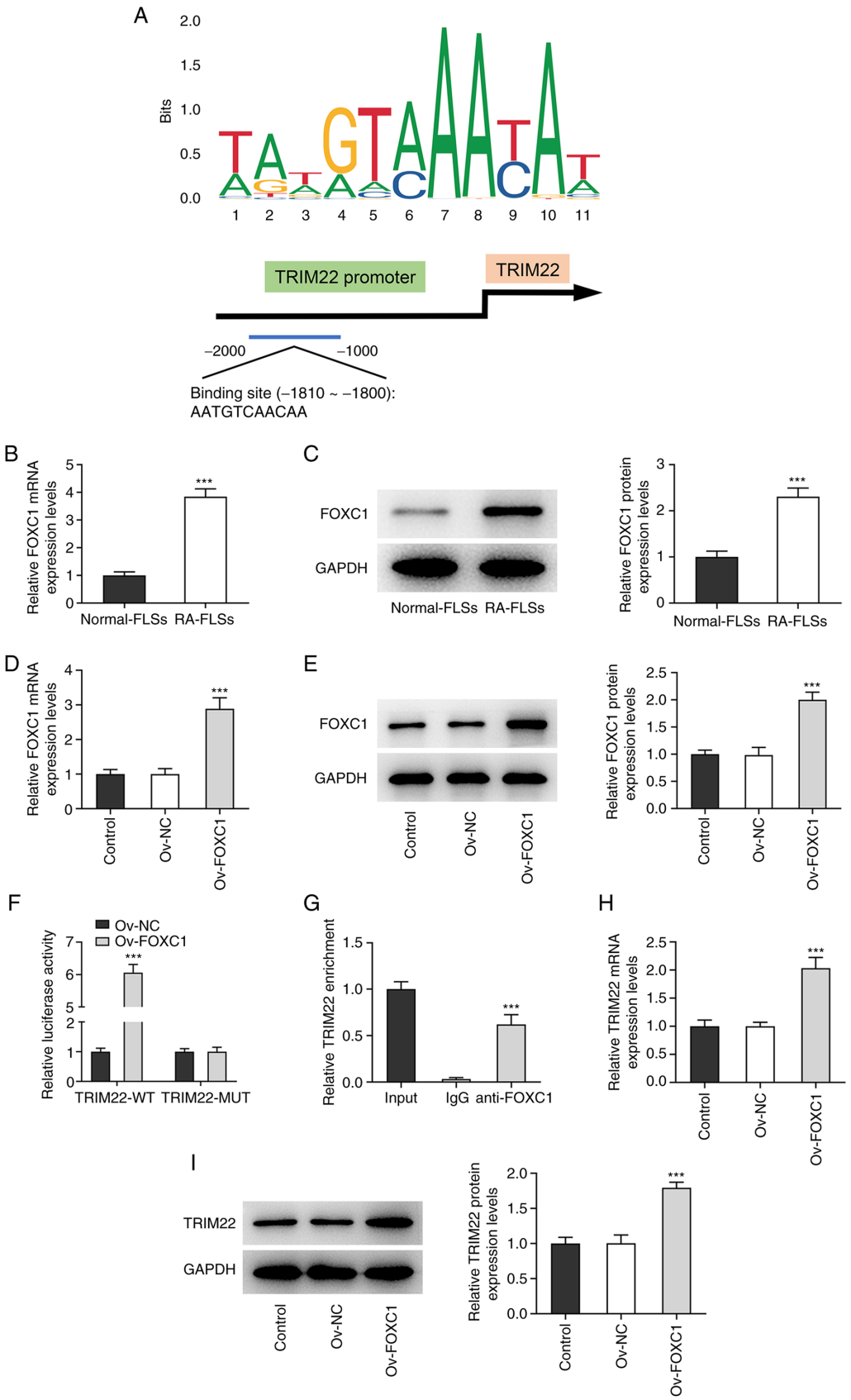

Figure 5. FOXC1 induces the upregulation of TRIM22 in RA-FLSs. (A) The possible binding site between FOXC1 and the TRIM22 promoter was predicted using the JASPAR database. FOXC1 mRNA and protein expression levels in RA-FLSs and normal-FLSs was determined using (B) RT-qPCR and (C) western blotting. ***P<0.001. Ov-FOXC1 transfection efficiency was analyzed using (D) RT-qPCR and (E) western blotting. ***P<0.001 vs. Ov-NC. (F) Dual-luciferase reporter assays were performed to assess the luciferase activity of TRIM22-WT and TRIM22-MUT following transfection with Ov-FOXC1. ***P<0.001 vs. Ov-NC. (G) Chromatin immunoprecipitation confirmed the abundance of the TRIM22 promoter in the FOXC1 antibody fraction. Input was the positive control. ***P<0.001 vs. IgG. TRIM22 mRNA and protein expression was assessed using (H) RT-qPCR and (I) western blotting, respectively, following FOXC1 overexpression. ***P<0.001 vs. Ov-NC. FOXC1, Forkhead box C1; TRIM22, tripartite motif-containing 22; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocyte; RT-qPCR, reverse transcription-quantitative PCR; Ov, overexpression; NC, negative control; WT, wild-type; MUT, mutant.

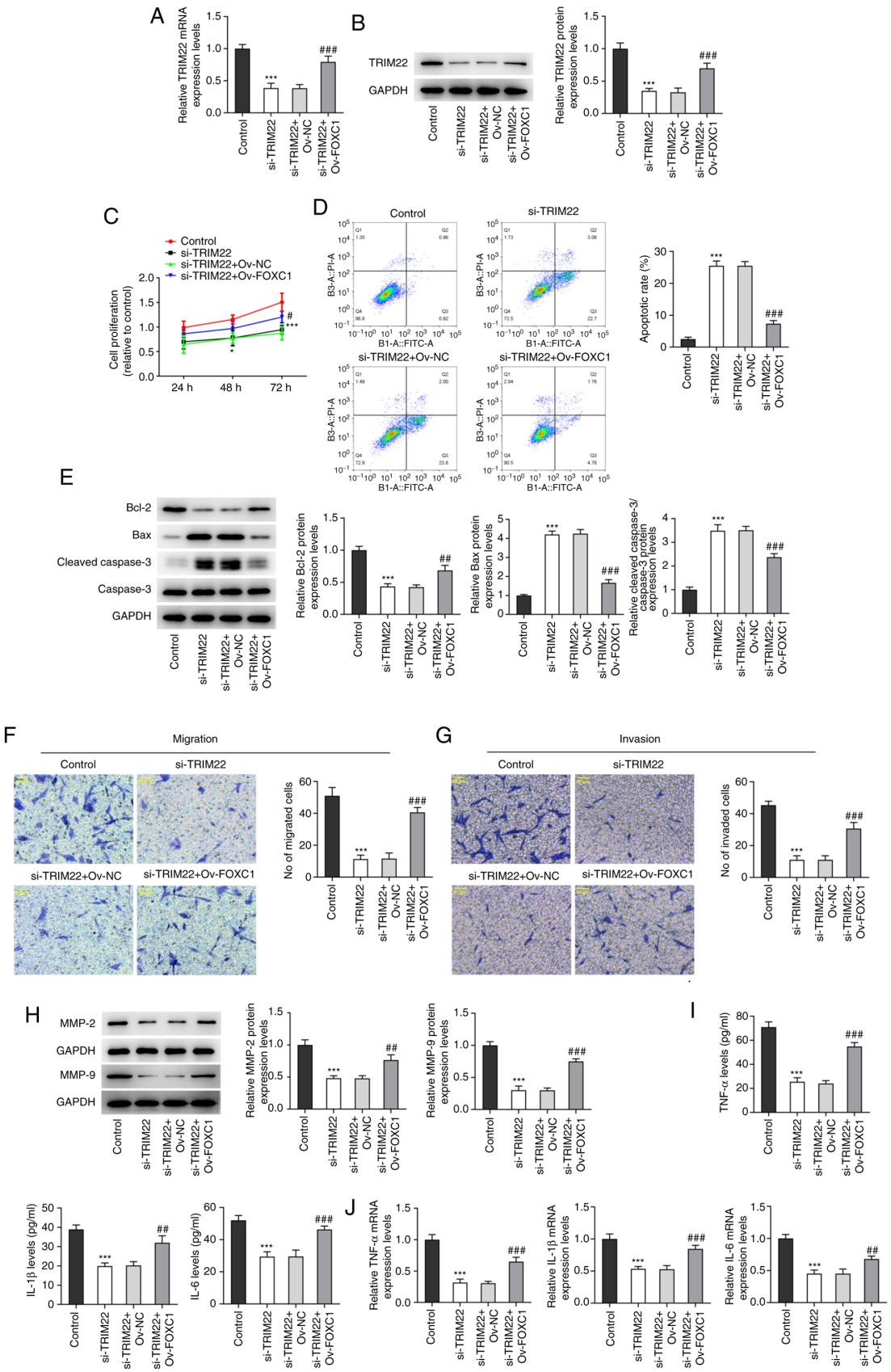

Figure 6. FOXC1 overexpression counteracts the impacts of TRIM22 reduction on the proliferation, apoptosis, migration, invasion and inflammatory response of RA-FLSs. mRNA and protein expression levels of TRIM22 in RA-FLSs transfected with si-TRIM22 and Ov-FOXC1 were detected via (A) RT-qPCR and (B) western blotting. (C) RA-FLS cell proliferation was detected using the Cell Counting Kit-8 assay. (D) Cell apoptosis was assessed using flow cytometry. (E) Western blotting was performed to analyze the protein expression levels of apoptosis-related proteins. The (F) migration and (G) invasion of RA-FLSs were assessed via Transwell assays. Magnification, x200. (H) Western blotting was performed to analyze the protein expression levels of MMP2 and MMP9. (I) Levels of TNF-α, IL-1β and IL-6 were assessed using ELISA. (J) RT-qPCR determined the mRNA expression levels of TNF-α, IL-1β and IL-6. *P<0.05 and ***P<0.001 vs. si-NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. si-TRIM22 + Ov-NC. FOXC1, Forkhead box C1; TRIM22, tripartite motif-containing 22; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocyte; si, small interfering RNA; Ov, overexpression; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

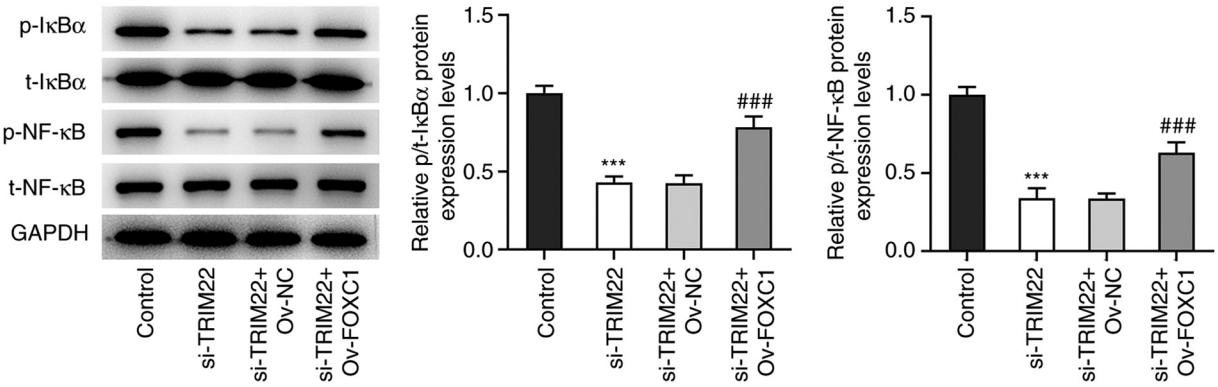

Figure 7. FOXC1-induced TRIM22 activates the NF-κB signaling pathway. Western blotting determined the protein expression levels of p/t-IκBα and p/t-NF-κB. ***P<0.001 vs. si-NC; ###P<0.001 vs. si-TRIM22 + Ov-NC. FOXC1, Forkhead box C1; TRIM22, tripartite motif-containing 22; p, phosphorylated; t, total; si, small interfering RNA; Ov, overexpression; NC, negative control.

proliferation of RA-FLSs caused by TRIM22 knockdown could be significantly reversed via FOXC1 overexpression compared with the si-TRIM22 + Ov-NC group (Fig. 6C). Furthermore, TRIM22 silencing-induced cell apoptosis was significantly suppressed following the overexpression of FOXC1 compared with the si-TRIM22 + Ov-NC group (Fig. 6D). This result was also confirmed by the significant downregulation of Bcl-2 protein expression levels and the significant upregulation of Bax and cleaved caspase-3/caspase-3 protein expression levels following TRIM22 knockdown compared with the control. However, these changes were significantly reversed via FOXC1 overexpression compared with the si-TRIM22 + Ov-NC group (Fig. 6E). Furthermore, knockdown of TRIM22 significantly reduced the migration and invasion of RA-FLSs compared with the control; however, this effect was significantly reversed by FOXC1 overexpression compared with the si-TRIM22 + Ov-NC group (Fig. 6F and G). Compared with the control the protein expression levels of MMP2 and MMP9 in the si-TRIM22 group were significantly decreased. However, they were significantly increased in the si-TRIM22 + Ov-FOXC1 group compared with the si-TRIM22 + Ov-NC group (Fig. 6H). Moreover, ELISA and RT-qPCR demonstrated that FOXC1 overexpression significantly reversed the reduced inflammatory response in RA-FLSs transfected with si-TRIM22 compared with the si-TRIM22 + Ov-NC group (Fig. 6I and J). Collectively, these results indicated that TRIM22 potentially contributes to the progression of RA via FOXC1.

FOXC1-induced TRIM22 activates the NF-κB signaling pathway. Western blotting demonstrated that TRIM22 knockdown significantly reduced the protein expression levels of p/total (t)-IκBα and p/t-NF-κB compared with the control. This effect was significantly reversed following FOXC1 overexpression compared with the si-TRIM22 + Ov-NC group. Therefore, these results indicated that TRIM22 may be activated via FOXC1 to stimulate the NF-κB signaling pathway (Fig. 7).

Discussion

RA is an autoimmune disease that is primarily characterized by the development of aggressive FLS phenotypes (32). FLSs are resident joint-lining mesenchymal cells and aberrant hyperplasia of these cells serves an important role in the pathogenesis of RA (32). Moreover, FLSs contribute to joint inflammation via inducing the release of pro-inflammatory cytokines (33). TRIM22 is an evolutionarily ancient protein that is involved in cellular differentiation and proliferation, which serves an integral role in certain types of cancer and autoimmune diseases (12). Previous studies have suggested that TRIM22 serves a role as a tumor suppressor in endometrial cancer and gastric cancer (34,35). However, numerous studies have also highlighted the oncogenic properties of TRIM22 in chronic myeloid leukemia (15), glioblastoma (36), non-small cell lung cancer (14) and colon cancer (14). Moreover, Kang *et al* (37) reported that TRIM22 knockdown exerts an anti-inflammatory effect over oxygen-glucose deprivation/reoxygenation-stimulated HCN-2 cells. Similar to these aforementioned studies, the present study highlighted the role of TRIM22 in the inflammatory phenotypes of FLSs in RA. The results of the present study demonstrated that TRIM22 was expressed at significantly higher levels in the synovial membrane samples of patients with RA and in RA-FLSs. TRIM22 knockdown significantly inhibited the proliferation, migration and invasion of RA-FLSs and promoted cell apoptosis.

TNF-α is a pro-inflammatory cytokine (38). It has previously been reported that TNF-α is abundant in the serum and arthritic synovium of patients with RA and that targeting TNF-α represents an efficacious method for RA therapy (39,40). IL-1β and IL-6 are also central regulators in the inflammatory response (31). In the present study, TRIM22 knockdown significantly reduced the secreted levels and the mRNA expression levels of TNF-α, IL-1β and IL-6. These results therefore suggested that TRIM22 may potentially drive the inflammatory response of RA-FLSs.

The forkhead family of transcription factors has been associated with diverse biological processes (41). For example, FOXC1 is an indispensable member of the forkhead family and its upregulation predicts poor survival outcomes in multiple types of carcinomas (42). FOXC1 also promotes aggressive tumor phenotypes in triple-negative breast cancer (43), pancreatic cancer (21), oral squamous cell carcinoma (44) and prostate cancer (45). Furthermore, Yu *et al* (22) demonstrated that FOXC1 exacerbates the

proliferation of RA-FLSs. Moreover, FOXC1 may elicit a potent activity in tumors via gene regulation. For example, glutathione peroxidase 8 expression is induced via FOXC1, which promotes the development of gastric cancer (46). Furthermore, lysyl oxidase is a downstream effector of FOXC1 in non-small cell lung cancer (47). Similarly, the present study suggested that the FOXC1 transcription factor may have a potential binding site on the TRIM22 promoter. The results demonstrated that FOXC1 was significantly highly expressed at both mRNA level and protein level in RA-FLSs and FOXC1 overexpression significantly upregulated the mRNA expression and protein expression of TRIM22 in RA-FLSs. The potent affinity of the TRIM22 promoter with FOXC1 was also verified using the dual-luciferase reporter and ChIP assays. Furthermore, rescue assays demonstrated that following FOXC1 overexpression the significantly reduced proliferation, migration, invasion, inflammation and increased apoptosis of RA-FLSs observed following TRIM22 silencing, were significantly reversed.

NF-κB is a ubiquitous transcription factor involved in fundamental cellular functions, including cell survival, inflammation and immune responses (48). Increasing evidence has suggested that the dysregulation of NF-κB signaling may influence the development of RA (26,27,49). Furthermore, TRIM22 has been regarded as an activator of the NF-κB signaling pathway (37,50). The role of FOXC1 in the proliferation and invasion of cancer cells may also be attributed to NF-κB modulation (51). Consistent with these previous findings, the present study also demonstrated that TRIM22 knockdown potentially inhibited the NF-κB signaling pathway via the downregulation of the protein expression levels of p/t-IκBα and p/t-NF-κB. However, this was significantly reversed via the overexpression of FOXC1. A previous study reported that FOXC1 activates the PI3K/AKT signaling pathway to increase FLS proliferation in RA (22). However, whether FOXC1 activates the PI3K/AKT signaling pathway to aggravate FLS proliferation in RA via the NF-κB signaling pathway should be studied further in the future.

In summary, the present study demonstrated that TRIM22 overexpression potentially promoted RA via significantly increasing the proliferation, migration, invasion, inflammation and inhibiting the apoptosis of FLSs. Furthermore, the results suggested that TRIM22 expression was potentially induced via the FOXC1 transcription factor and activated the NF-κB signaling pathway in RA. Overall, these findings demonstrated a potential novel role of TRIM22 in RA and highlighted the underlying regulatory mechanism. These results may have provided insight into potential strategies for targeted RA therapeutics.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LD performed the experiments; YW and XH analyzed the data; and LD, YM designed the experiments, interpreted the data and wrote the manuscript. All authors read and approved the final manuscript. LD and YW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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