

SPHK2 Knockdown Inhibits the Proliferation and Migration of Fibroblast-Like Synoviocytes Through the IL-17 Signaling Pathway in Osteoarthritis

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Objective: Synovial inflammation is vital for the progression of osteoarthritis (OA). The objective of this study was to explore the effects and potential molecular mechanisms of sphingosine kinase 2 (SPHK2) on the proliferation and migration of fibroblast-like synoviocytes (FLS).

Methods: A TNF- α -stimulated FLS model and a papain-induced OA rat model were constructed. The functions of SPHK2 knockdown in OA were explored by a series of in vivo and in vitro assays. Downstream target genes of SPHK2 were investigated using transcriptome sequencing and validated by reverse transcription quantitative PCR (RT-qPCR). The effects of the SPHK2/IL-17 signaling pathway on inflammation, proliferation, and migration of OA-FLS were investigated using the IL-17 pathway inhibitor (secukinumab) and the activator (rhIL-17A).

Results: TNF- α stimulation promoted SPHK2 expression at mRNA and protein levels in OA-FLS. SPHK2 knockdown reduced IL-1 β , IL-6, MMP-2, MMP-9, cyclinD1, and PCNA levels and suppressed proliferation and migration of OA-FLS. SPHK2 knockdown alleviated cartilage damage and synovial inflammation in the OA rat model. LRRIQ3, H4C8, CXCL1, CABP4, COL23A1, and PROK2 expression levels were regulated by SPHK2. SPHK2 knockdown inhibited the protein levels of IL-17A, IL-17RA, and Act1. The IL-17 pathway inhibitor secukinumab enhanced the inhibitory effect of SPHK2 knockdown on the proliferation and migration of OA-FLS, while the IL-17 pathway activator rhIL-17A exerted the opposite effect.

Conclusion: SPHK2 knockdown inhibits proliferation and migration of OA-FLS by blocking the IL-17 pathway, which provides a novel approach to the OA treatment.

Keywords: sphingosine kinase 2, fibroblast-like synoviocyte, IL-17 signaling pathway, proliferation, migration

Introduction

Osteoarthritis (OA) is a whole-joint disease that leads to disability and reduces quality of life among older adults.¹ The pathologic features of OA involve loss of articular cartilage, subchondral bone dysfunction, and synovial inflammation.² Current OA treatment strategies primarily aim to alleviate pain and mitigate symptoms due to the lack of effective medications.³ Therefore, it is imperative to further explore the pathogenic mechanisms and potential therapeutic targets for OA.

Increasing studies suggest that fibroblast-like synoviocytes (FLS), which are mesenchymal cells present in the synovium, have a key role in the development of OA.⁴ FLS are involved in the chronic inflammatory response and actively contribute to the production of numerous inflammatory cytokines, chemokines, and matrix-degrading molecules associated with cartilage degradation, thereby fostering the development of OA.⁵ Activated FLS in rheumatoid arthritis (RA) pathogenesis demonstrates tumor cell-like traits, such as enhanced migration and invasion, leading to joint

destruction.⁶ Recent findings have revealed that the biology of FLS in OA closely resembles that of RA and exhibits distinct pro-inflammatory features.⁷ In addition, FLS mediates epigenetic modifications in OA, such as DNA methylation, histone modification, and chromosomal remodeling, which in turn affect OA progression.⁸ Hence, targeting the inflammation and hyperproliferation of FLS could potentially lead to improved treatment strategies for OA.

Sphingolipids are mediators of inflammation and sphingosine kinase (SPHKs) affects inflammatory pathways in FLS. SPHK1 and SPHK2 are the two isoforms of SPHK. Genetically suppressing SPHK1 activity in TNF α -stimulated mouse FLS inhibits the inflammatory mediator levels and inflammatory pathways activation.⁹ SPHK2 mediates hypoxia-triggered chemokine release in normal FLS and RA-FLS.¹⁰ Synovial fibroblasts from RA patients show more higher SPHK2 expression than that in the THP-1 human macrophage cell lines and human skin fibroblasts, and SPHK2 may regulate the autonomous proliferation of synovial fibroblasts.¹¹ Moreover, a previous bioinformatics analysis has indicated that SPHK2 is highly expressed in synovial tissues of OA patients.¹² Fan et al found that DANCER upregulates SPHK2 expression in OA chondrocytes, thereby promoting cell proliferation and inhibiting apoptosis.¹³ However, the roles and the molecular mechanisms of SPHK2 in FLS of OA remain unclear.

IL-17 is a 20–30 kDa glycosylated homodimeric cytokine that is predominantly produced by activated CD4⁺ memory (CD45RO⁺) T cells.¹⁴ IL-17 promotes the expression of TNF- α , IL-1 β , IL-8, IL-6, NF- κ B, and G-CSF, which in turn affects cell activation, growth, and proliferation[10]. Additionally, IL-17 upregulates Toll-like receptor 3 expression and stimulates proliferation in RA-FLS.^{15,16} In OA, IL-17 is engaged in inflammatory responses, hypoxic responses, complement production, and angiogenesis in chondrocytes and synovial fibroblasts.¹⁷ Moreover, silencing lipid-mediated S1PR4, a member of the sphingolipid SIP receptors, decreases proliferation, migration, and pro-inflammatory responses, and promotes apoptosis of RA-FLS, partly by inhibiting IL-17.¹⁸ However, the correlation between IL-17 and SPHK in OA is unclear.

In this study, OA-FLS were treated with TNF- α (0 ng/mL, 1 ng/mL, 5 ng/mL, and 10 ng/mL), and the expression level of SPHK2 was then measured. Inflammatory factor levels and cell migration were assessed after SPHK2 knock-down. Transcriptome sequencing revealed a significant correlation between SPHK2 and IL-17 signaling pathways. Further investigation revealed that SPHK2 knockdown inhibited the protein levels of IL-17RA and Act1, which suppressed the proliferation and migration of FLS. Our investigation offers a novel approach for treating OA.

Materials and Methods

Cell Culture and Processing

Human osteoarthritis fibroblast-like synoviocytes (OA-FLS) were acquired from Icellbioscience Biotechnology Co., Ltd., (Shanghai, China). The cells were grown in DMEM medium (11965092; Thermo Fisher Scientific, Massachusetts, USA) enriched with 10% fetal bovine serum (Thermo Fisher Scientific), 100 units/mL penicillin, and 100 μ g/mL streptomycin (C0222; Beyotime, Shanghai, China) in an incubator maintained at 37°C with 5% CO₂. For TNF- α treatment, cells were exposed to varying concentrations of TNF- α (1 ng/mL, 5 ng/mL, 10 ng/mL; 210-TA; R&D Systems, Minneapolis, MN, USA) for 24 h. The LV-shSPHK2 vector, along with the corresponding control empty LV-shNC (Shanghai GenePharma Co., Ltd, Shanghai, China) were constructed to silence SPHK2, and subsequently transfected into OA-FLS. LV-shSPHK2 was designed and synthesized as follows: 5'- GTTGCTCAACTGCTCACTGTT-3'. To assess the impact of SPHK2 silencing on the IL-17 signaling pathway, OA-FLS were pre-incubated with 100 μ g/L recombinant human interleukin 17A (rhIL17A; IL-17 signaling pathway activator; Cell Signaling Technology, Danvers, Massachusetts, USA), or 50 μ g/mL secukinumab (IL-17 signaling pathway inhibitor; MB2757-1; MeilunBio, Dalian, China) for 24 h before TNF- α stimulation.

Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA from OA-FLS was extracted using the TRIzol reagent (15596026CN; Thermo Fisher Scientific), followed by the synthesis of complementary DNA (cDNA) from 1 μ g of the isolated RNA. The RT-qPCR analysis of cDNA was performed using SYBR Premix Ex Taq (RR420A; Takara, Dalian, China) according to the manufacturer's protocol. The target gene expression levels were normalized to GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method. The primer sequences are detailed in [Supplementary Table 1](#).

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IL-17A and interleukin 8 (IL-8) in cell supernatant, interleukin 6 (IL-6), interleukin-1 β (IL-1 β), and matrix metalloproteinase-2 (MMP-2) in cell supernatant and rat serum, and TNF- α in rat serum were assessed utilizing corresponding ELISA kits (Esebio Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's guidelines.

Wound Migration Assay

After OA-FLS cultures reached 90% confluence, cells were cultured in serum-free medium for 5 h. OA-FLS monolayers were mechanically wounded using pipette tips and administered with TNF- α for 1 h. Using an Olympus inverted microscope (Olympus, Tokyo, Japan), cell migration rate was observed and recorded at 0 and 24 h. The migrated cells were quantified by the Image J software.

Western Blot Assay

Cells were harvested from OA-FLS and rat cartilage tissues and subsequently lysed in RIPA buffer (R0010; Solarbio, Beijing, China) that added with 100x phenylmethylsulfonyl fluoride. A total of 50 μ g of proteins were separated by electrophoresis using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to the polyvinylidene difluoride membranes (PVDF, Roche, Basel, Switzerland). The PVDF membranes were blocked with 5% nonfat milk for 1 h and then incubated with the primary antibodies at 4°C overnight. After rinsing with TBST, the blot was incubated with an HRP-labeled secondary antibody for 2 h. The specific proteins were examined utilizing ECL reagents (RPN2106; Amersham, Little Chalfont, UK) and were quantified using Image J software. Primary antibodies included SPHK2 (ab320741; 1:2000; Abcam, Cambridge, UK), IL-17A (ab318150; 1:2000; Abcam), IL-17RA (ab263908; 1:2000; Abcam), Act1(ab137395; 1:2000; Abcam), and GAPDH (ab9485; 1:2000; Abcam).

Transcriptome Sequencing

Total RNA was extracted from OA-FLS in the SPHK2 knockdown and its negative control groups using the TRIzol reagent (15596026CN; Thermo Fisher Scientific) and then polyadenylated mRNA was purified using Oligo(dT) magnetic beads. The RNA was fragmented to about 300 bp in length using ion interruption. Reverse transcription of RNA was performed using 6-base random primers and reverse transcriptase to synthesize cDNA. The library was subjected to PCR amplification to produce fragments of approximately 450 bp. Subsequently, the libraries underwent quality control using the Agilent 2100 Bioanalyzer. Next-generation sequencing was performed, and the libraries were subjected to paired-end sequencing utilizing the Illumina sequencing platform. Each aligned gene's expression level underwent normalization and was denoted in fragments per kilobase of transcript per million mapped reads (FPKM).

Bioinformatics Analysis

The DESeq package was employed for the differential analysis of gene expression. The differentially expressed genes (DEGs) were screened based on $|\log_2\text{FoldChange}| > 1$ and $P < 0.05$. A volcano plot illustrating DEGs was created using the ggplot2 package in R software (version 4.2.3). Furthermore, the concatenated DEGs from all comparison groups and samples underwent bi-directional cluster analysis using the pheatmap package. Distances were assessed with the Euclidean method, and hierarchical clustering with the longest distance was performed. Additionally, Gene Ontology (GO) annotation analysis was conducted using the topGO package, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed in the KAAS database (<http://www.genome.jp/tools/kaas/>).

5-ethynyl-2'-deoxyuridine (EdU)

The evaluation of cell proliferation was performed employing EdU incorporation assay with an EdU assay kit (C0071S; Beyotime), following the manufacturer's instructions. Briefly, cells were cultivated in 96-well plates and treated with 10 μ M EdU at 37°C for 2 h. Subsequently, cells were fixed in 4% formaldehyde for 15 min at room temperature and permeabilized in 0.3% Triton X-100 for 10 min. Cells were then treated with Click Additive Solution for 30 min and nuclei were stained using Hoechst 33342. Cell proliferation capacity was analyzed by a fluorescent microscope

(Olympus). The percentage of EdU-positive cells was assessed by Image J software in five randomly selected fields using the formula $\text{EDU-positive cell count/total cell count} \times 100\%$.

Construction of OA Rat Model

Sprague-Dawley rats (6 weeks old, weighing 200–220 g) were procured from SPF Biotechnology Co., Ltd. (Beijing, China). Rats were housed at 24°C under a 12- light-dark cycle with food and water provided ad libitum. The rats were randomly divided into four groups, including the control, OA, OA + LV-shNC, and OA + LV- shSPHK2 groups, with six rats in each group. An animal model of OA was induced by injecting papain into the joints of rats based on methods described in previous research.¹⁹ Briefly, on days 1, 4, and 7 of the experiment, 20 μL of 4% papain (10 units/mg; P4762; Sigma–Aldrich, St Louis, MO) and 20 μL of 0.03 mol/L L-cysteine were injected into the cavity of the right knee joint of the mice at a 1:1 ratio, respectively. The control group was injected with an equal amount of saline. After six weeks of model induction, the rats were deeply anesthetized with 2% isoflurane administered through an inhalation anesthesia machine for 3 min. Then, articular cartilage tissues and serum were collected from each group of rats for subsequent experiments. SPHK2 knockdown in the OA + LV-shSPHK2 group was carried out via local the lentiviral particles of SPHK2-targeted shRNA (Hanbio Tech, Shanghai, China) injection into the joint cavity of rats following the last papain and L-cysteine injection. Meanwhile, rats in the OA + LV-shNC group were injected with lentiviral particles of control shRNA. All procedures adhered to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Histological observation

After fixation in 4% paraformaldehyde for 24 h and decalcification in 10% EDTA, the cartilage tissues of rats were dehydrated, embedded in paraffin, dehydrated with ethanol in conventionally gradient, and then cut into 4- μm sections. For hematoxylin-eosin (HE) staining, tissue sections were stained with hematoxylin staining solution (C0107; Beyotime) for 5 min, washed in 0.5% hydrochloric acid in alcohol for 10s, and then stained in eosin for 30s. After graded ethanol dehydration and xylene transparency, the sections were sealed with neutral gum. Sections were visualized under a microscope (Olympus), and the nuclei of chondrocytes showed blue color and the cytoplasm was pink. For safranin O-fast green staining, sections were treated with freshly prepared Weigert's hematoxylin staining solution (G1371; Solarbio) for 5 min to stain the nuclei, followed by a quick immersion in acidic differentiation solution for 15s. After washing in distilled water for 10 min to remove excess stain, the sections were immersed in solid green staining solution for 5 min. Then, a brief wash in a weak acid solution for 15s was performed and the sections were immersed in safranin O solution for 5 min. Anhydrous ethanol was rapidly dehydrated three times, as well as xylene clear three times for 5 min each, and sections were sealed with neutral gum. The normal cartilage appeared red and the background appeared green. The Osteoarthritis Research Society International (OARSI) scoring system was used to determine the extent of cartilage deterioration.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 8 software, and continuous variables are expressed as mean $\pm$ SD from three independent experiments. Differences between two groups were assessed using an unpaired Student's *t*-test. For comparisons involving multiple means, one-way ANOVA followed by Tukey's test was employed. A $P < 0.05$ was considered statistically significant.

Result

SPHK2 Knockdown Inhibits Inflammation and Cell Migration in TNF- α -Induced OA -FLS

OA-FLS were treated with TNF- α at various concentrations (0, 1, 5, and 10 ng/mL). RT-qPCR and Western blot demonstrated that the SPHK2 expression at mRNA and protein levels were gradually elevated with increasing concentrations of TNF- α , and its expression was significantly higher in the 5 ng/mL TNF- α and 10 ng/mL TNF- α treatment groups than that in the non-TNF- α treated group (Figure 1A and B). Then, 10 ng/mL of TNF- α was chosen for the subsequent experiments. In OA-FLS, we knocked down SPHK2 and confirmed the knockdown efficiency by assessing both mRNA and protein levels through RT-qPCR and Western blot analyses, respectively (Figure 1C and D).

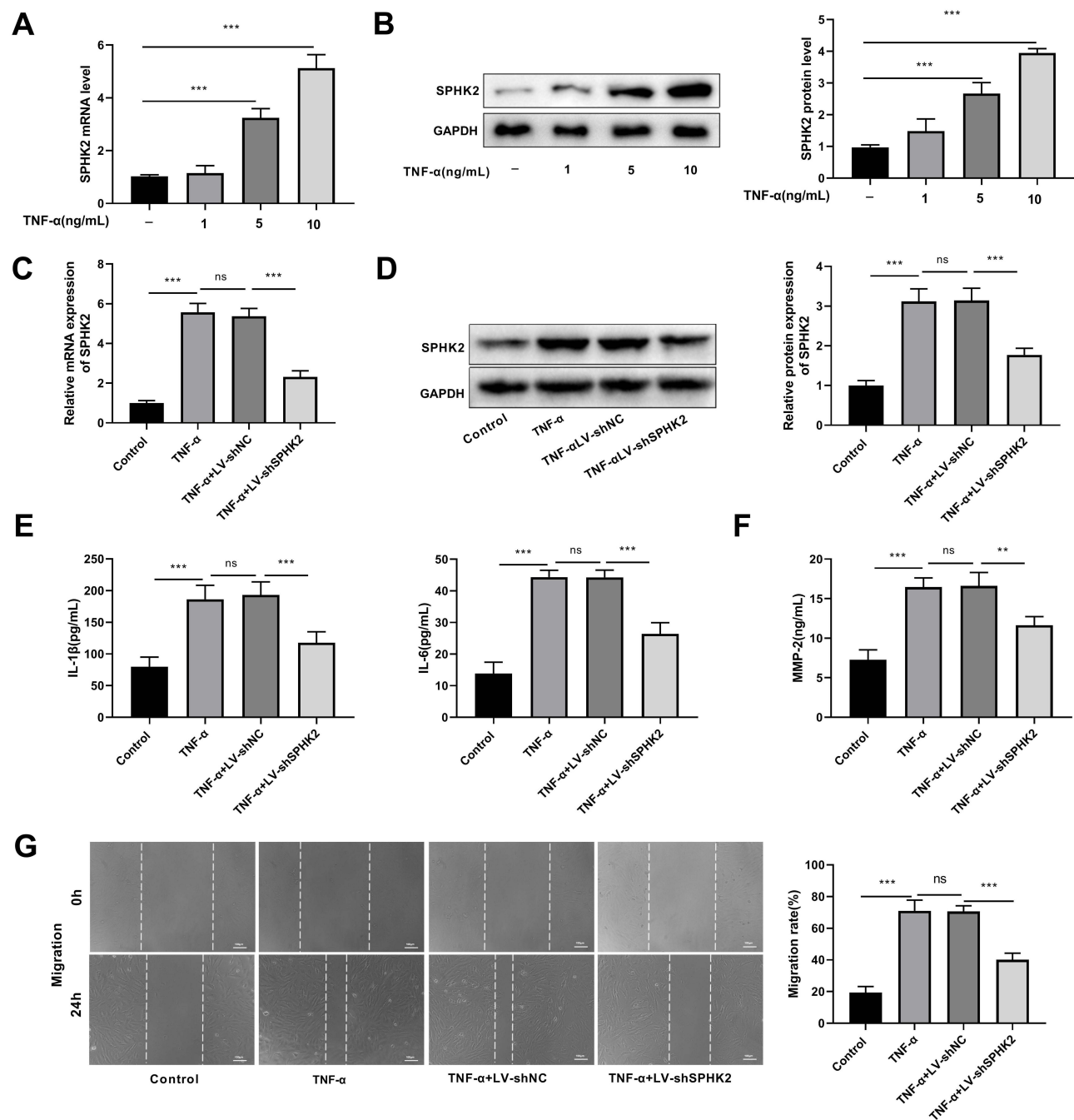


Figure 1 SPHK2 expression was elevated in response to TNF-α stimulation and SPHK2 knocked down inhibited inflammatory response and cell migration in OA-FLS. (**A** and **B**). RT-qPCR and Western blot were used to detect the mRNA and protein expression levels of SPHK2 under the TNF-α treatment at the concentrations of 0, 1, 5, and 10 ng/mL, respectively. (**C** and **D**). RT-qPCR and Western blot were used to detect the knockdown efficiency of SPHK2. (**E** and **F**). Levels of inflammatory factors (IL-1β and IL-6) and matrix metalloproteinase (MMP-2) in the control, TNF-α, TNF-α + LV-shNC, and TNF-α + LV-sh SPHK2 groups were measured by ELISA assay. (**G**). Effect of knockdown of SPHK2 on OA-FLS migration was assessed by wound healing assay. Scale: 100 μm. ***P* < 0.01, ****P* < 0.001 vs control group or TNF-α + LV-shNC group.

Inflammatory factor levels were examined using ELISA assay. The findings indicated that the levels of IL-1β and IL-6 were significantly increased in the TNF-α-treated group in comparison to that in the control group. Conversely, IL-1β and IL-6 concentrations were significantly lower in the TNF-α + LV-shSPHK2 group than that in the TNF-α + LV-shNC group (Figure 1E). In addition, the level of MMP-2 in the TNF-α-treated group was significantly higher than that in the control group. Compared to the TNF-α + LV-shNC group, MMP-2 level was notably reduced in the TNF-α + LV-shSPHK2 group (Figure 1F). The impact of SPHK2 on the migration of OA-FLS cells was evaluated by wound healing

assay. The results showed that TNF- α stimulation significantly promoted the migration of OA-FLS, which was counteracted by SPHK2 knockdown (Figure 1G).

Transcriptome Sequencing for Screening DEGs

Transcriptome sequencing was conducted to screen for downstream targets of SPHK2. The results showed that there were 401 DEGs between TNF- α + LV-shNC and TNF- α + LV-shSPHK2 groups, with 270 genes showing significant up-regulation and 131 genes showing significant down-regulation. The top 10 DEGs that were upregulated and downregulated are shown in [Supplementary Table 2](#). Furthermore, volcano plot of DEGs were drawn utilizing the ggplot2 package in R software to demonstrate the fold differences in expression of the genes (Figure 2A). Bidirectional clustering analysis of concatenated sets and samples from two sets of DEGs was then conducted using the Pheatmap package (Figure 2B).

Functional Enrichment Analysis for DEGs

The potential biological functions of DEGs were subsequently explored. The top 10 most significant GO annotations were shown in the histogram (Figure 3A). The results showed that in the BP term, DEGs were significantly correlated with the extracellular region, spanning component of membrane, and extracellular space. In CC, DEGs were mainly enriched in CXCR chemokine receptor binding, signaling receptor binding, and structural constituent of chromatin. In MF, DEGs were mainly involved in the antimicrobial humoral immune response, enzyme-linked receptor protein signaling pathway, and motor neuron apoptotic process. Based on the FDR values, the top 20 KEGG pathways were demonstrated in the bubble plot (Figure 3B). The findings demonstrated that the DEGs primarily participated in systemic lupus erythematosus, IL-17 signaling pathway, and protein digestion and absorption.

The Expressions of DEGs Were Validated by RT-qPCR

The expression levels of the top three DEGs that were upregulated and downregulated were confirmed by RT-qPCR. The data suggested that the expressions of CABP4, COL23A1, and PROK2 were greatly elevated, while LRRIQ3, H4C8, and CXCL1 expressions were notably reduced, which was consistent with the sequencing results (Figure 4).

SPHK2 Knockdown Inhibited the IL-17 Signaling Pathway

Previous studies have shown that the IL-17 pathway plays an important role in OA articular cartilage degeneration and synovial inflammation.^{20,21} To delve deeper into the regulatory role of SPHK2 on the IL-17 signaling pathway,

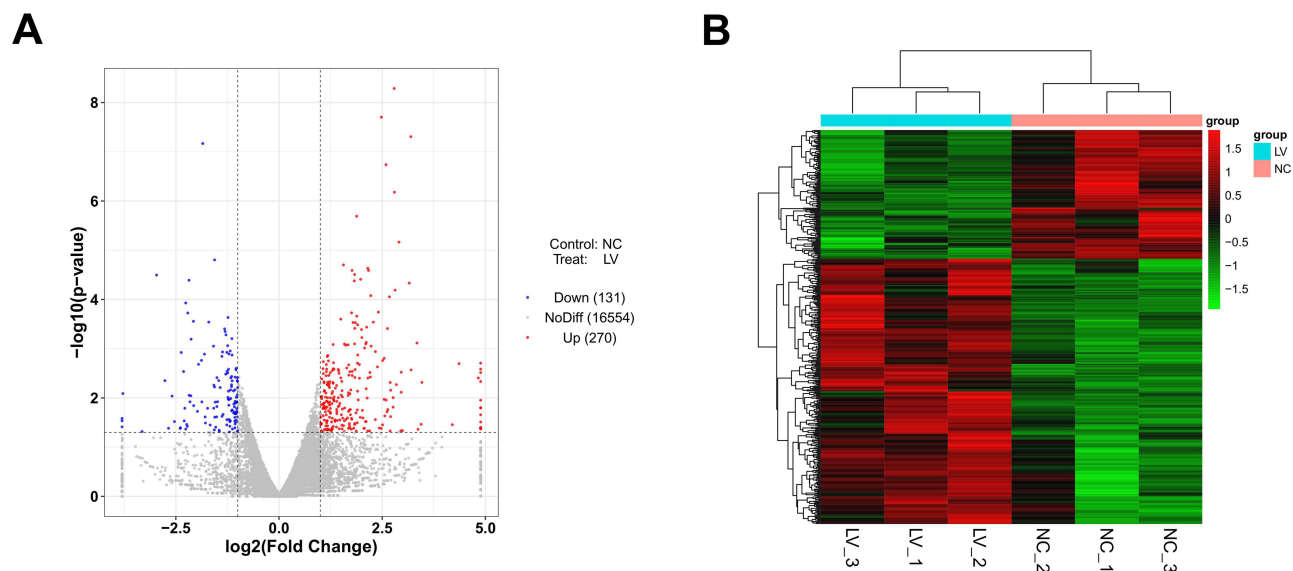


Figure 2 Volcano plot and heatmap of differentially expressed genes (DEGs) regulated by SPHK2 as determined by transcriptome sequencing. **(A)**, Volcano plot displaying DEGs, where red indicates up-regulated genes, blue indicates down-regulated genes, and gray indicates genes with no significant change in expression. The screening criteria for DEGs are $|\log_2\text{FoldChange}| > 1$ and $P < 0.05$. **(B)**, Heatmap for bidirectional cluster analysis of concatenated sets and samples from two sets of DEGs.

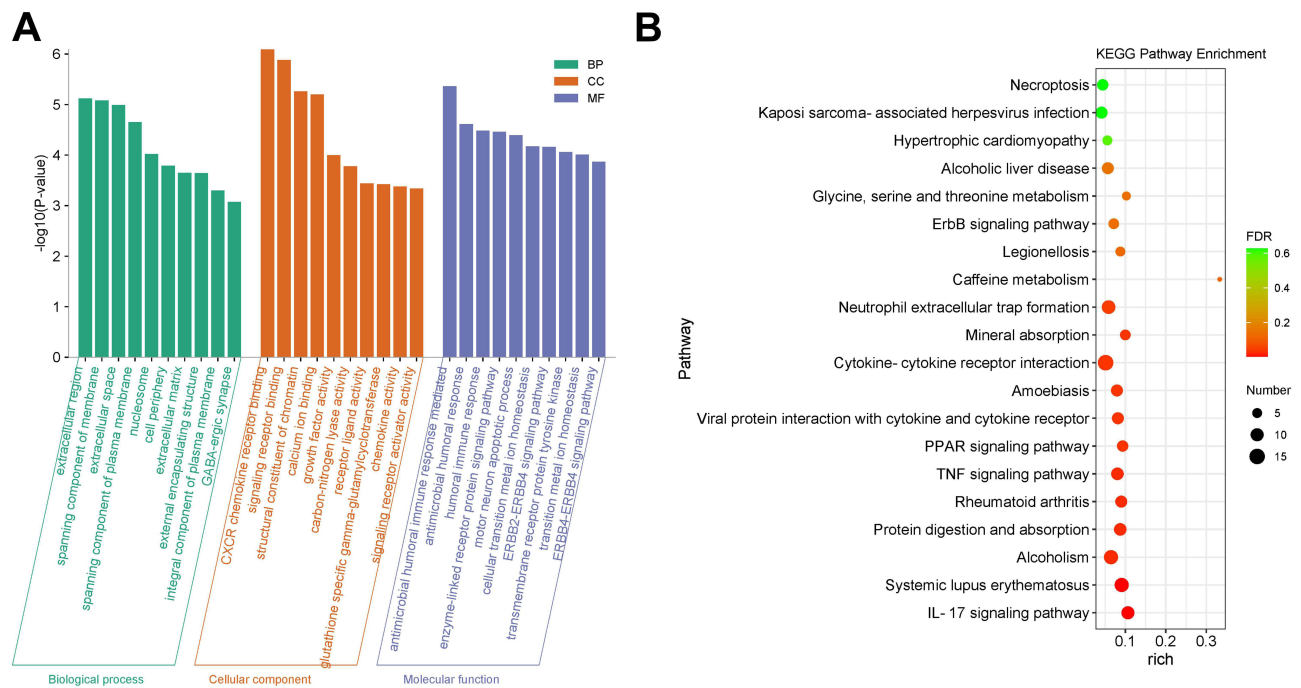


Figure 3 Enrichment analysis of DEGs. **(A)** Histogram of the top 10 Gene Ontology (GO) annotation categories, including cellular component (CC), molecular function (MF), and biological process (BP). **(B)** Bubble plot of the top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis results. A significance threshold of $P < 0.05$ was used to assess significant enrichment results.

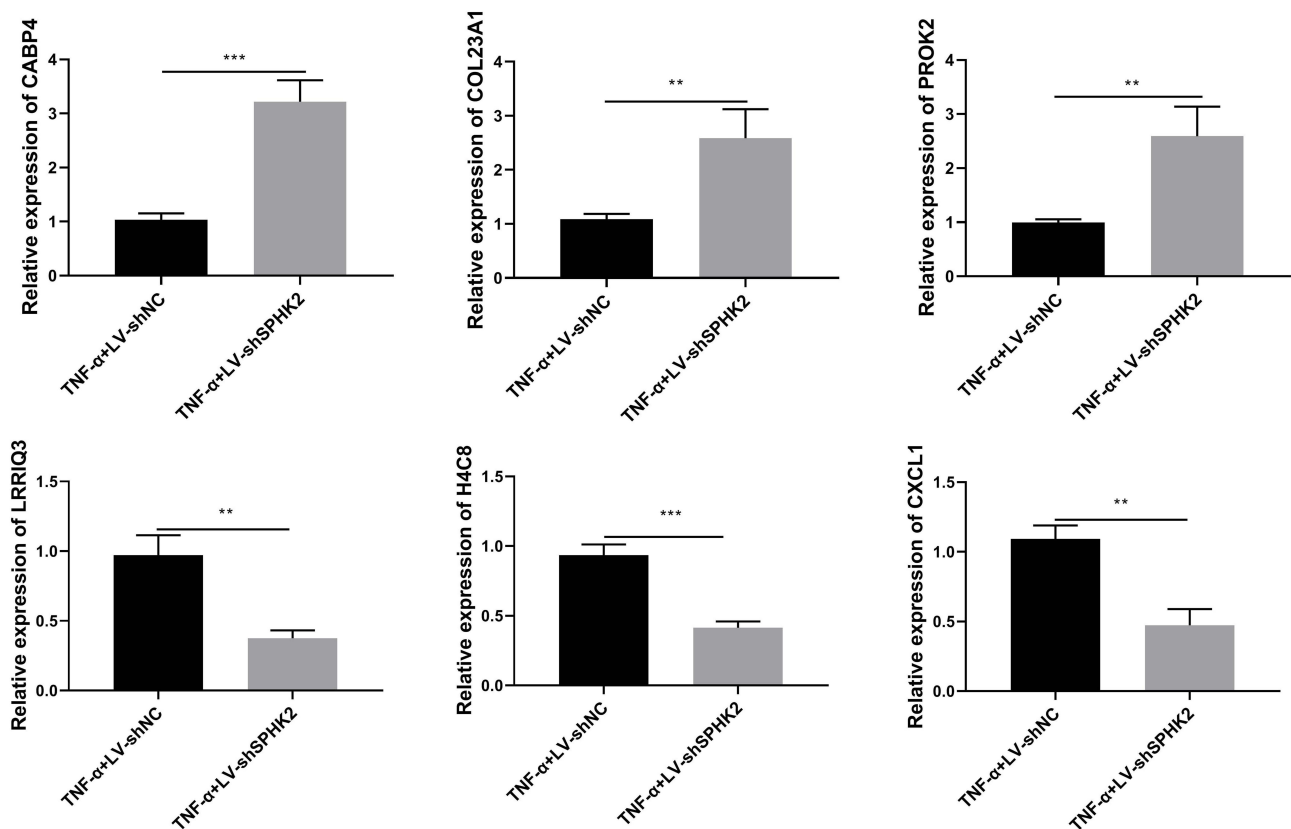


Figure 4 RT-qPCR was employed to evaluate the regulatory effects of SPHK2 on the target genes. After knockdown of SPHK2, the top three up-regulated DEGs (CABP4, COL23A1, and PROK2) and the top three down-regulated DEGs (LRRIQ3, H4C8, and CXCL1) were verified by RT-qPCR. ** $P < 0.01$, *** $P < 0.001$ vs TNF- α + LV-shNC group.

protein levels associated with the IL-17 signaling pathway were examined by Western blot. The outcomes revealed that the protein levels of IL-17A, IL-17RA, and Act1 were substantially increased in the TNF- α -treated group in relation to the control group. Simultaneously, the protein expressions of IL-17A, IL-17RA, and Act1 were substantially reduced following SPHK2 knockdown relative to the TNF- α + Lv-shNC group (Figure 5A). Furthermore, TNF- α treatment promoted the levels of inflammatory factors IL-17A and IL-8. Conversely, SPHK2 knockdown suppressed IL-17A and IL-8 levels compared with the TNF- α + Lv-shNC group (Figure 5B).

SPHK2 knockdown inhibited cell proliferation and migration by suppressing the IL-17 signaling pathway in TNF- α -induced OA-FLS

Subsequently, we explored whether SPHK2 knockdown influenced the OA-FLS function by modulating the IL-17 signaling pathway. The results showed that rhIL-17A treatment (IL-17 signaling pathway activator) reversed reduced IL-6, IL-1 β , and MMP-2 concentrations induced by silencing SPHK2. Additionally, in contrast with the TNF- α + LV-sh SPHK2 group, secukinumab treatment, an IL-17 pathway inhibitor, further suppressed IL-6, IL-1 β , and MMP-2 expression levels (Figure 6A and B). The proliferation of OA-FLS cells was assessed by CCK-8 and EdU assays. The analysis indicated that cell proliferation was markedly reduced in the TNF- α + LV-shSPHK2 group in relation to that in the TNF- α + LV-shNC group. Compared with the TNF- α + LV-shSPHK2 group, the capacity of cell proliferation was significantly enhanced in the TNF- α + LV-shSPHK2 + rhIL-17A group, while substantially lowered in the TNF- α + LV-shSPHK2 + secukinumab group (Figure 6C and D). Wound healing assay demonstrated that rhIL-17A significantly reversed the reduction in the migration levels caused by silencing SPHK2, whereas secukinumab treatment further inhibited cell migration capacity (Figure 6E). Additionally, we found that mRNA expression levels of the proliferation-associated genes (PCNA and cyclinD1) and the migration-associated gene (MMP-9) were promoted by treatment with TNF- α . Knockdown of SPHK2 reduced the mRNA levels of these genes, an effect that was further strengthened by secukinumab and significantly attenuated by rhIL-17A (Figure 6F).

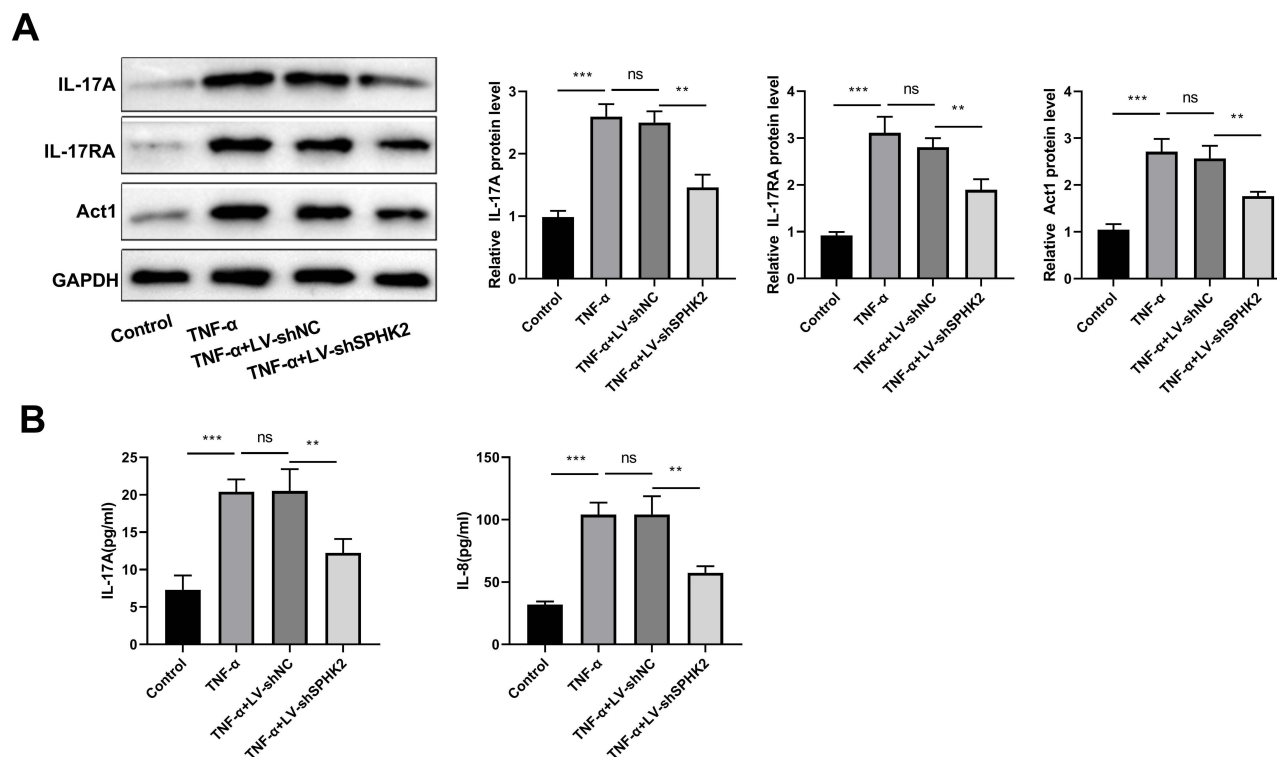


Figure 5 SPHK2 knockdown suppressed the IL-17 signaling pathway. (A). Protein expression levels of IL-17A, IL-17RA, and Act1 in the control, TNF- α + LV-shNC, and TNF- α + LV-sh SPHK2 groups were evaluated by Western blot. (B). Levels of inflammatory factors IL-17A and IL-8 were measured by ELISA. ** p < 0.01, *** p < 0.001 vs control group or TNF- α + LV-shNC group.

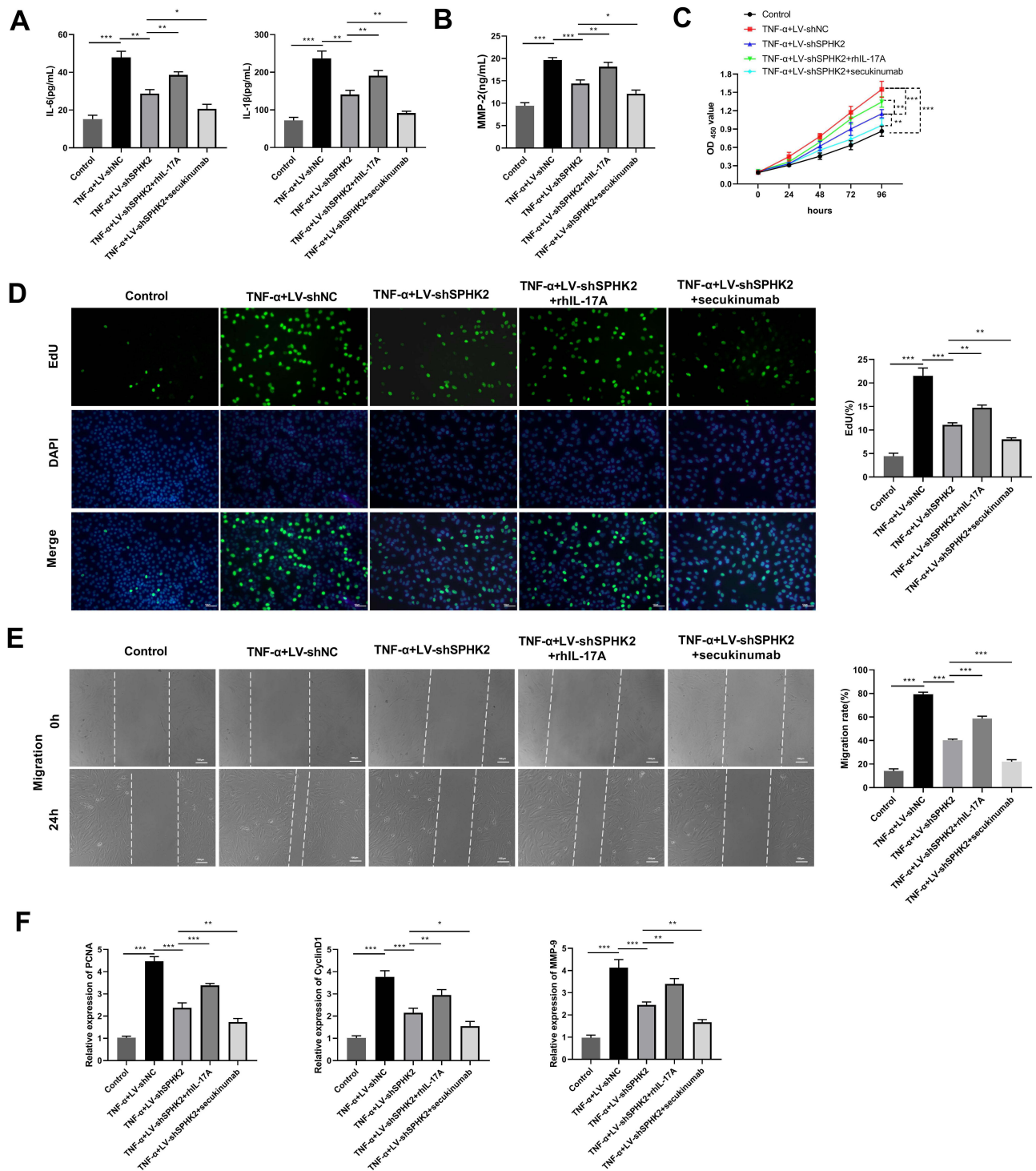


Figure 6 SPHK2 knockdown suppressed OA-FLS proliferation and migration via inhibiting the IL-17 signaling pathway. (**A** and **B**). ELISA was used to measure the effects of the IL-17-pathway inhibitor (secukinumab) and activator (rhIL-17A) on the levels of IL-1 β , IL-6, and MMP-2. (**C** and **D**). Cell proliferation was estimated in the control, TNF- α + LV-shNC, TNF- α + LV-sh SPHK2, TNF- α + LV-shSPHK2 + rhIL-17A, and TNF- α + LV-shSPHK2 + secukinumab groups by CCK-8 and EdU assays. Scale: 100 μ m. (**E**). Representative images of wound healing at 0 and 24 h in the four groups. Scale: 100 μ m. (**F**). RT-qPCR was used to detect the expression levels of the proliferation-associated genes PCNA, cyclinD1, and the migration-associated gene MMP-9 in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs TNF- α + LV-shNC or TNF- α + LV-shSPHK2 group.

SPHK2 Knockdown Ameliorated Cartilage Tissue Injury and Synovial Inflammation in OA Rats

Subsequently, we explored the role of SPHK2 in the OA rat model. Western blot results showed that SPHK2 protein expression was significantly higher in the OA model group than that in the control group. Moreover, protein expression of SPHK2 was significantly lower in the OA + LV-shSPHK2 group compared to the OA + LV-shNC group, indicating that SPHK2 expression was effectively knocked down (Figure 7A). HE staining of cartilage tissues revealed that the cartilage membrane in the knee joints of rats in the control group was smooth, with a normal distribution of chondrocytes. In contrast, the OA group and OA + LV-NC group showed a rough cartilage surface, disorganized arrangement of chondrocytes, and evident chondrocyte depletion. After SPHK2 knockdown, the cartilage tissue damage in the rats was significantly reduced (Figure 7B). Safranin O-fast green staining results demonstrated that, compared with the control group, the surface layer of cartilage in the OA and OA + LV-NC groups was irregular, with uneven staining of chondrocytes and partial destaining. The OA + LV-shSPHK2 group showed reduced chondrocyte damage compared to the OA + LV-shNC group (Figure 7C). Consistent with staining, the OARSI score was higher in the OA group compared with the control group. SPHK2 knockdown significantly declined the OARSI score (Figure 7D). Moreover, we observed high levels of inflammatory factors, including TNF- α , IL-1 β , and IL-6, in the OA rat model. SPHK2 knockdown reduced the levels of these inflammatory factors (Figure 7E).

Discussion

OA is defined by cartilage destruction, subchondral bone remodeling, and synovial inflammation. The invasion of FLS into the synovium is recognized as a crucial element in the pathogenesis of OA.^{22,23} In this investigation, FLS were induced with TNF- α , and it was observed that the expression level of SPHK2 was increased. Knockdown of SPHK2 inhibited IL-1 β , IL-6, MMP-2, PCNA, cyclinD1, and MMP-9 levels as well as the proliferation and migration of OA-FLS. Knockdown of SPHK2 alleviated cartilage tissue damage and synovial inflammation in OA rats. Transcriptome sequencing revealed that SPHK2 knockdown facilitated the upregulation of CABP4, COL23A1, and PROK2 while decreasing the LRRIQ3, H4C8, and CXCL1 expressions, which was confirmed in vitro by RT-qPCR. Moreover, SPHK2 knockdown suppressed the protein levels of IL-17A, IL-17RA, and Act1, which were the key mediators in the IL-17 pathway. Furthermore, we found that the IL-17 pathway inhibitor secukinumab augmented the suppressive impact of SPHK2 knockdown on the proliferation and migration of OA-FLS, while the IL-17 pathway activator exerted a contrasting effect.

There are two main cell types in the synovium, including FLS and macrophage-like synoviocytes.²⁴ TNF- α generated by macrophages is one of the key elements in the early activation of FLS[17]. In the present investigation, we stimulated FLS with TNF- α at concentrations of 1 ng/mL, 5 ng/mL, and 10 ng/mL, and found that the mRNA level and protein level of SPHK2 were considerably elevated in a TNF- α dose-dependent manner. Proinflammatory cytokines, including IL-6 and IL-1 β are produced during synovitis.²⁵ IL-6, TNF- α , and IL-1 β are secreted into the joint space, where they act as mediators that attract immune cells, leading to joint damage and pain.²⁶ IL-6, TNF- α , and IL-1 β can enhance the invasive characteristics of FLS.²⁷ In the present investigation, it was found that knockdown of SPHK2 inhibited the IL-6 and IL-1 β levels. Furthermore, we observed that knockdown of SPHK2 led to mitigated cartilage tissue integrity and decreased synovial inflammation in OA rat model.

Unrestricted “cancer-like” proliferation of synovial FLS contributes to the destruction of multiple joints, osteoporosis, systemic inflammation, and other vital organ involvement.²⁸ FLS and macrophage-like synoviocytes expand and the migration of FLS to cartilage and bone promotes joint erosion in OA and RA.²⁴ Furthermore, heightened proliferation and migration abilities of FLS facilitate their attachment to articular cartilage, secretion of matrix-degrading enzymes, and subsequent cartilage invasion and degradation.²⁹ Research has elucidated the crucial function of sphingosine phosphate and ceramide phosphate in regulating essential cellular activities like proliferation, invasion, and apoptosis. SPHK is the major limiting enzyme for sphingosine-based phosphates in cells.³⁰ SPHK1 knockdown hampers FLS migration and invasion through inhibition of PI3K/AKT pathway and MMP-2/9 expressions.³¹ MiR-577 suppresses the proliferation, migration, and invasion of papillary thyroid cancer cells via its interaction with SPHK2.³² By

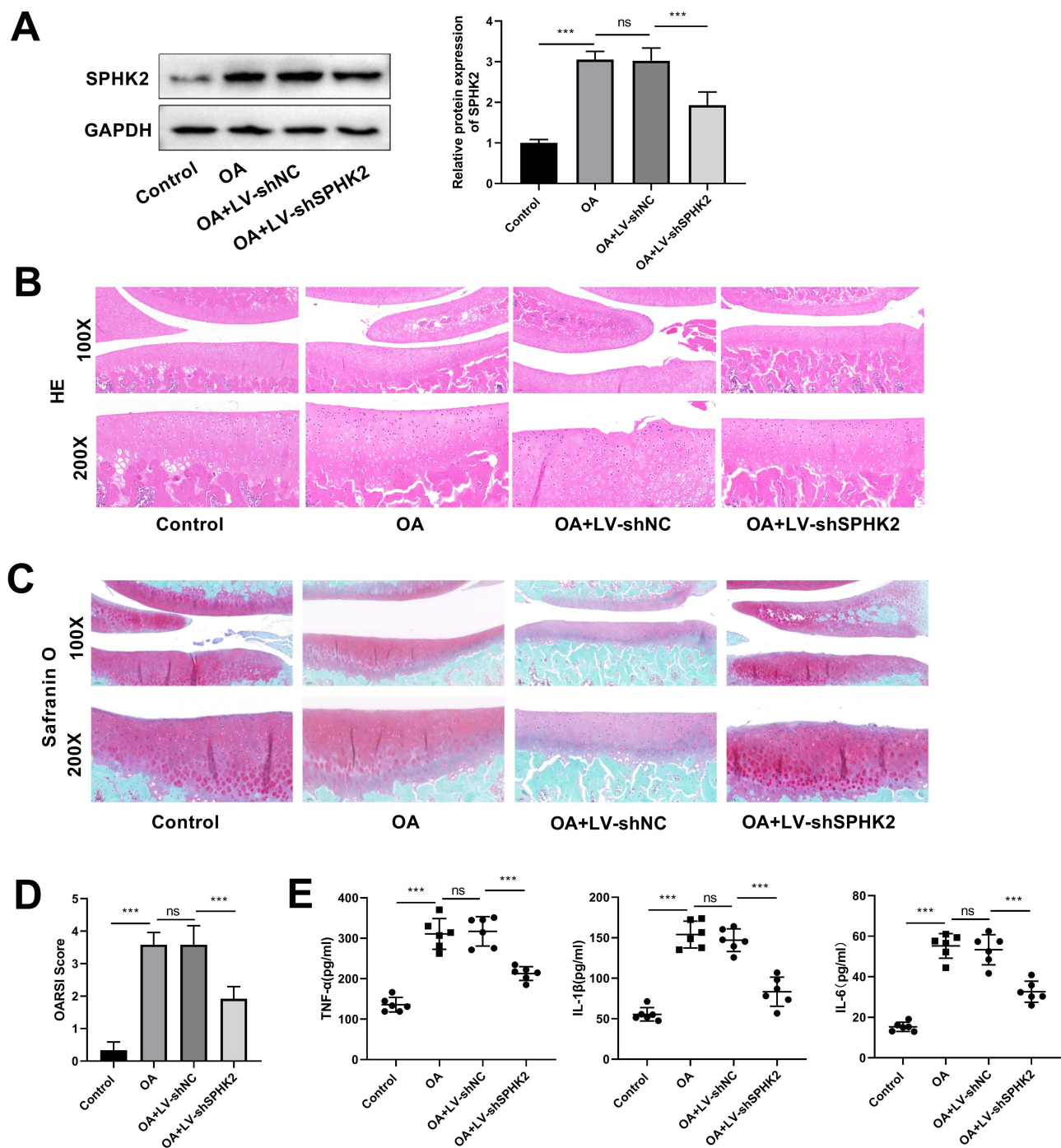


Figure 7 SPHK2 knockdown alleviated OA injury, synovial inflammation in the OA rat model. **(A)** Western blot analysis was performed to detect SPHK2 protein level in the OA models and the SPHK2 knockdown OA models. **(B and C)** The morphological structure of cartilage tissue was analyzed using hematoxylin and eosin (HE) and Safranin O-fast green staining. HE staining: magnification 100x, scale bar 100 μ m; Safranin O-fast green staining: magnification 200x, scale bar 50 μ m. **(D)** Osteoarthritis Research Society International (OARSI) scores of cartilage tissues in each group. **(E)** ELISA is used for measuring inflammatory factor levels (TNF- α , IL-1 β , and IL-6). N = 6. *** p < 0.001 vs control group or OA + LV-shSPHK2 group.

downregulating SIX4 and SPHK2, resveratrol inhibits the non-small cell lung cancer cell proliferation, invasion, and migration by regulating the Wnt/ β -catenin pathway.³³ In this investigation, SPHK2 knockdown suppressed the OA-FLS proliferation and migration. Furthermore, we found that SPHK2 silencing suppressed the expression level of MMP-2, MMP-9, Cyclin D1, and PCNA. MMP-2 and MMP-9 play an important role in FLS metastasis and invasiveness.^{34,35} Cyclin D1 and PCNA are key proteins involved in the regulation of cell cycle and DNA replication, which are key

contributors to cell proliferation.³⁶ Our study demonstrated the roles and potential molecular mechanisms of SPHK2 for OA-FLS proliferation and migration.

IL-17 can increase the expression and/or cooperate with local inflammation factors, including TNF- α and IL-1 β to exacerbate synovitis in OA.³⁷ Another study has shown that IL-17 is highly expressed in the synovial tissues of individuals with OA, triggering the release of IL-6, IL-23, and TGF- β 1, and promoting MMP9 expression, which in turn leads to synovial inflammation and cartilage matrix degradation.³⁷ The E3 ubiquitin ligase-active multifunctional signaling protein Act1 interacts with the IL-17-inducible receptor SEFIR structural domain. Act1 is essential in autoimmune and inflammatory illnesses that are dependent on the IL-17 pathway, and Act1 deficiency inhibits the expression of genes associated with inflammation when IL-17 is triggered.³⁸ In the current study, SPHK2 knockdown reduced the protein expression of IL-17RA and Act1. The IL-17 pathway is implicated in multiple biological processes in FLS. By inhibiting IL-17A cytokine signaling in RA, cyclidin reduces the hyperproliferative potential of FLS.³⁹ The migration of FLS is bolstered by the IL-17-dependent upregulation of LAT1 via the mTOR/4E-BP1 pathway.⁴⁰ Our investigation revealed that the IL-17 pathway inhibitor secukinumab amplified the inhibitory impact of SPHK2 knockdown on the proliferation and migration of OA-FLS. Conversely, the IL-17 pathway activator rhIL-17A promoted proliferation and migration of these cells. Studies have revealed that the IL-17 signaling pathway is regulated in OA by various target genes, such as lncRNA CASC2, CCN2, and STAT3.^{15,41,42} In our investigation, SPHK2 was found to be engaged in the modulation of the IL-17 pathway. Targeting the gene transcription and translation pathway is anticipated to emerge as a new approach for OA treatment. Moreover, Secukinumab, an IL-17 signaling pathway inhibitor, is approved for clinical use in the treatment of ankylosing spondylitis and psoriatic arthritis.^{43,44} Anti-IL-17 treatment shows promise as a therapeutic strategy for patients with OA. Thus, the present study found that SPHK2 knockdown impeded the IL-17 signaling pathway, which provided new insights into the treatment of OA.

However, there are some limitations to this study. First, the precise mechanism by which SPHK2 interacts with the IL-17 signaling pathway, including the potential involvement of third-party molecules, remains to be clarified. Second, the findings obtained in this study are based on the TNF- α -stimulated OA-FLS and the papain-induced OA rat model, so the role of SPHK2 in other OA animal models and its potential clinical applications require further investigation.

Conclusion

Our findings indicate that SPHK2 is highly expressed in TNF- α -induced OA-FLS and OA rat model. SPHK2 knockdown inhibits inflammatory factor release and migration in OA-FLS. Knockdown of SPHK2 alleviates cartilage injury and synovial inflammation in rats with OA. Mechanistically, knockdown of SPHK2 inhibits the IL-17 signaling pathway, which in turn hinders the development of OA. Our investigation provides new insights into the treatment of OA.

Data Sharing Statement

The data and materials supporting the findings of this study are available from the corresponding authors upon request.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflict of interest.

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