RESEARCH ARTICLE

LncRNA TM1-3P Regulates Proliferation, Apoptosis and Inflammation of Fibroblasts in Osteoarthritis through miR-144-3p/ONECUT2 Axis

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Objective: This study explores LncRNA TM1-3P effects on the proliferation, apoptosis, and inflammatory response of fibroblasts in osteoarthritis (OA) and its underlying mechanism.

Methods: Bioinformatics was performed to analyze OA disease-related genes, miRNA profiles, and function. The targeted regulation of LncRNA TM1-3P and miR-144-3p, ONECUT2 and miR-144-3p were analyzed by dual luciferase reporter gene assay, RNA Binding Protein Immunoprecipitation (RIP), and RNA pull down. Histopathological morphology of the knee joint was observed by hematoxylin–eosin (HE) and Annona Red O/Fast Green. The expressions of mRNAs and proteins were detected by RT-qPCR, Western blot, and immunohistochemistry. Unpaired T test was used between groups, and the one-way analysis of variance of repeated measurement data was applied for multi-group comparison, following Tukey's post-test.

Results: ONECUT2 and Smurf2 genes were significantly elevated in the osteoarthritis group compared with the normal group ($P < 0.001$, $P < 0.001$). Expressions of ONECUT2 and LncRNA TM1-3P were increased, and expression of miR-144-3p was decreased in interleukin (IL)-1β-induced human fibroblast synovial cells (hFSCs) (mRNA: 1.06 ± 0.24 vs. 3.29 ± 0.73, proteins: 0.22 ± 0.03 vs. 0.46 ± 0.22, 1.23 ± 0.22 vs. 3.76 ± 0.73, 1.06 ± 0.25 vs. 0.37 ± 0.13, $P < 0.01$, $P < 0.001$, $P < 0.01$, $P < 0.05$). Overexpression of miR-144-3p down-regulated the ONECUT2 expression, reduced cell proliferation, promoted apoptosis in hFSCs induced by IL-1β (mRNA: 0.89 ± 0.14 vs. 0.15 ± 0.01, $P < 0.05$; proteins: 0.46 ± 0.01 vs. 0.23 ± 0.01, $P < 0.001$; CCK8: 1.88 ± 0.07 vs. 1.65 ± 0.07; $P < 0.05$; EDU: 55.82 ± 1.44 *vs* 40.57 ± 2.24, $P < 0.05$; apoptosis: 10.57 ± 0.79 *vs* 16.36 ± 0.35, $P < 0.0001$). Overexpression of LncRNA TM1-3P up-regulated the expression of ONECUT2, promoted cell proliferation, and inhibited apoptosis (mRNA: 0.9 ± 0.09 *vs* 1.94 ± 0.12, $P < 0.05$; proteins: 0.61 ± 0.05 *vs* 0.76 ± 0.03, $P > 0.05$; CCK8: 2.07 ± 0.05 *vs* 2.47 ± 0.06; $P < 0.01$; EDU: 52.67 ± 1.17 *vs* 60.06 ± 3.24, $P < 0.05$; apoptosis: 10.57 ± 0.79 *vs* 16.36 ± 0.35, $P < 0.001$), which were reversed by the overexpression of miR-144-3p treatment (mRNA: 1.82 ± 0.07 *vs* 0.31 ± 0.07, $P < 0.0001$; proteins: 0.74 ± 0.02 *vs* 0.35 ± 0.01, $P < 0.01$; CCK8: 2.41 ± 0.01 *vs* 1.67 ± 0.02; $P < 0.0001$; EDU: 66.85 ± 2.86 *vs* 44.68 ± 1.97, $P < 0.0001$; apoptosis: 7.19 ± 0.19 *vs* 13.36 ± 0.53, $P < 0.0001$). Silencing LncRNA TM1-3P attenuated the injury of knee joint tissue, down-regulated the expression of ONECUT2, Smurf2, IL-1β, IL-6, TNF-α, and improved the expression of Rap1 in rats (0.71 ± 0.04 *vs* 0.48 ± 0.02, 0.68 ± 0.06 *vs* 0.36 ± 0.02, 0.74 ± 0.03 *vs* 0.49 ± 0.04, 0.78 ± 0.01 *vs* 0.54 ± 0.03, 0.68 ± 0.02 *vs* 0.4 ± 0.04, 0.24 ± 0.01 *vs* 0.4 ± 0.03, $P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.01$, $P < 0.05$).

Conclusion: LncRNA TM1-3P improved inflammation and damage of knee joints in OA rats through miR-144-3p/ONECUT2 axis, providing a new theoretical basis for gene therapy of OA.

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Introduction

Osteoarthritis (OA) is the most common degenerative disease, with degeneration of cartilage and synovial inflammation.[1] Cartilage breakdown products lead to synovitis in OA by stimulating synovial fibroblast cells (FSCs) to produce inflammatory mediators.[2] FSCs are heterogeneous groups of synovial cells and the main effector cells of synovial fibrosis in OA.[3] MicroRNAs (miRNAs) can inhibit the translation of target mRNA or promote mRNA degradation, thereby regulating pathology in the synovium.[4,5] miRNAs have been previously reported to play a role in the biological functions of FSCs in OA, including cell viability, apoptosis, inflammatory response, and ECM degradation. For example, miR-337-3p has a protective effect on OA by inactivating mitogen-activated protein kinases (MAPK) pathways *via* targeting SKP2 in FSCs.[6] miR-26a-5p overexpression reduced the injury of FSCs and alleviated OA injury *in vivo*.[4] Therefore, exploring the targeted interaction of miRNA-mRNA might provide a new therapeutic strategy for the clinical treatment of OA.

OA is also a disease characterized by joint hyperplasia, progressive degeneration, joint space narrowing and extracellular matrix (ECM) metabolism.[7] Once activated, FSCs are suggested to acquire a myofibroblast-like phenotype, driving fibrosis through excessive deposition of ECM components and enhanced systolic function in OA.[8] miR-103a-3p alleviates OA by inhibiting ECM degradation *via* targeting FGF18.[9] miR-144-3p dysregulation is involved in peroxisome dysfunction and interleukin (IL)-1β expression during the pathogenesis of OA.[10–12] A rat anterior cruciate ligament transection model demonstrated that administration of miR-144-3p mimics could improve OA progression and reduce IL-1β positive cells in synovial tissue.[13] The above studies have proved that miR-144-3p plays an important role in OA, and in-depth exploration of the mechanism of action of its targeted mRNA might contribute to the treatment of OA.

Long non-coding RNA (lncRNA) is an RNA molecule with a transcription length over 200 nt and lacking protein-coding ability.[14] LncRNA regulates gene expression by interacting with protein, RNA, and DNA.[15,16] It has been reported that LncRNA can act as sponges for miRNAs and play an important role in the regulation of OA.[17] For example, LncRNA NEAT1 competed with OPN to bind to miR-181c and knocking down NEAT1 inhibited proliferation, inflammation levels and ECM degradation of FSCs in OA, which are partially reversed by miR-181c inhibitors.[18] LncRNA MEG3 inhibits synovial cell proliferation and promotes apoptosis in OA rats by regulating Phosphatase and tensin homolog (PTEN).[19] ONECUT2, as a transcription factor, consists of two DNA-binding domains, a homeobox domain and a cleaved domain; MAPK was thought to be highly expressed in various diseases, including lung and prostate cancer.[20] As previously described, LncRNA LINC00671 exacerbates OA by promoting ONECUT2-mediated Smurf2 expression and ECM degradation in chondrocytes.[21] Circ_0084615 promotes colon cancer cell proliferation, migration, and invasion through miR-599/ONECUT2 axis.[22] Previous studies suggest that ONECUT2 might play an important role in the progression of OA. Currently, the mechanism of competitive endogenous RNAs (ceRNA) targeting ONECUT2 in hFSCs of OA is still unclear.

This study aims to investigate the underlying molecular mechanism of ONECUT2 in OA: (i) to predict the differentially expressed genes (DEGs) in OA by bioinformatics database; (ii) to assess the targeting relationship by the dual luciferase reporter gene assay, RNA Binding Protein Immunoprecipitation (RIP), and RNA pull down and explore the mechanism of targeted miRNAs and lncRNAs in IL-1β-induced human fibroblast synovial cells (hFSCs) *in vitro*; and (iii) to elucidate the function of targeted lncRNAs in OA rats. Our findings might provide new targets for clinical treatment of OA.

Materials and Methods

Cell Experiment and Grouping

hFSCs (CP-H094, Procell, Wuhan, China) were purchased from Procell. Cell suspensions were inoculated in DMEM (D5796, Sigma, Saint Louis, MO, USA) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2. Adherent fibroblastic synovial cells with 70%–80% degrees of fusion were randomly divided into the Control and IL-1β groups. Control group cells were cultured normally without treatment. The OA model of hFSCs in the IL-1β group was constructed with 10 ng/ml IL-1β for 72 h.[23] FSCs were randomly divided into the NC group, miR-144-3p mimic group, miR-144-3p mimic + oe-NC group and miR-144-3p mimic + oe-ONECUT2 group. Hsa-miRNA NC, hsa-miR-144-3p (GenePharma, Shanghai, China), oe-NC, and oe-ONECUT2 plasmid (Honorgene, Changsha, China) were transfected with Lip2000 (11,668,019, Invitrogen, Carlsbad, CA, USA) to construct NC group, miR-144-3p mimic group, miR-144-3p mimic + oe-NC group and miR-144-3p mimic + oe-ONECUT2 group.

Animal Experiment and Grouping

OA model in rats was constructed by an unstable medial meniscus model.[24] Thirty-two male Sprague–Dawley rats (200–250 g) were purchased from Hunan Slyke Jingda Animal Research Center and randomly divided into four groups with eight rats in each group, including the Sham group, OA

group, LV-sh-LncRNA NC group, and LV-sh-LncRNA TM1-3p group. In the OA model, after the right knee joint was exposed through the medial articular capsule incision, the extensor muscle was gently moved, the medial meniscus was cut, and then the medial articular capsule incision and skin were sutured. The sham group opened the joint capsule, but the medial meniscus tibial ligament remained intact. One week after surgery, TM1-3P downregulated lentivirus (LV-sh-LncRNA TM1-3p) and LV-sh-LncRNA NC were injected into the knee joints of OA rats twice a week. Sham group and OA group were given equal volume medium for 4 weeks. The Biomedical Research Ethics Committee of Hunan Normal University (2021. NO. 309) approved the animal studies.

Bioinformatics Analysis

This study chose GSE55457 data sets, using R language (limma package) analysis of different genes in OA and normal. The cutoff was $|\log FC| > 1$ & $q < 0.05$. The R clusterProfiler package and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/) were applied for gene functional enrichment analysis. Finally, a multiMiR package was adopted to map the miRNA prediction of differential genes.

Dual Luciferase Reporter Gene Assay

The targeted interactions between LncRNA TM1-3P and hsa-miR-144-3p, ONECUT2 and hsa-miR-144-3p were analyzed using a dual luciferase assay kit (E1910, Promega Corporation, Madison, WI, USA). Human double luciferase reporter plasmids pHG-MirTarget-TM1-3P (wild type), pHG-MirTarget-TM1-3P-Mut (mutant), pHG-MirTarget-ONECUT2-3U (wild type), pHG-MirTarget-ONECUT2-3U-Mut (mutant), miRNA NC, and hsa-miR-144-3p mimics were purchased from HonorGene. In short, pHG-MirTarget-TM1-3P, pHG-MirTarget-TM1-3P-Mut, pHG-MirTarget-ONECUT2-3U and pHG-MirTarget-ONECUT2-3U-Mut were transfected into the plasmid (DP117, Tiangen, Beijing, China). 293A cells (HG-NC071, HonorGene, Changsha, China) were cultured with 24-well plates and transfected with each plasmid and miRNA. The detection groups of LncRNA TM1-3P and hsa-miR-144-3p were as follows: WT + miR-NC group (293A cells co-transfected with pHG-MirTarget-TM1-3P plasmid and miRNA NC), WT + miR-144-3p group (293A cells co-transfected with pHG-MirTarget-TM1-3P plasmid and hsa-miR-144-3p mimics), Mut + miR-NC group (293A cells co-transfected with pHG-MirTarget-TM1-3P-Mut plasmid and miRNA NC), and Mut + miR-144-3p group (293A cells co-transfected with pHG-MirTarget-TM1-3P-Mut and hsa-miR-144-3p mimics). The detection groups of ONECUT2 and hsa-miR-144-3p were as follows: WT + miR-NC group (293A cells co-transfected with pHG-MirTarget-ONECUT2-3U plasmid and miRNA NC), WT + miR-144-3p group (293A cells co-transfected with pHG-MirTarget-ONECUT2-3U plasmid and hsa-miR-144-3p mimics), Mut + miR-NC group (293A cells co-transfected with pHG-MirTarget-ONECUT2-3U-Mut plasmid and miRNA NC), and Mut + miR-144-3p group (293A cells co-transfected with pHG-MirTarget-ONECUT2-3U-Mut and hsa-miR-144-3p mimics). 100 μL 1 × PLB lysate was added into each well to lysate cells. The cell lysates were added with LAR II assay solution and Stop&Glo assay solution. The fluorescein activity of firefly and sea kidneys was detected by a chemiluminescence detector (GloMax 20/20, Promega Corporation, Madison, WI, USA).

Cell Counting Kit (CCK)-8

The cells were digested and inoculated at a density of 5×10^3 cells/well, 100 μL per well. Each group was set up with five compound holes for culture adhesion. Then the drug-containing medium was removed, and 100 μL CCK8 medium (1:10, NU679, DOJINDO, Kumamoto, Japan) was added to each well. Cells were incubated at 37°C with 5% CO_2 for 4 h. Bio-Tek microplate analyzer (MB-530, hui song, Shenzhen, China) was applied to analyze the absorbance at 450 nm.

Flow Cytometry

The cells were digested and collected with trypsin without EDTA, washed with PBS, and centrifuged at 2000 rpm for 5 min. About 3.2×10^5 cells were collected. Then a 500 μL binding buffer was added to suspend the cells. Annexin V-FITC 5 μL (KGA108, keyGEN Bio TECH, Nanjing, China) and 5 μL Propidium Iodide (MA1037, Meilunbio, Dalian, China) were added and reacted for 10 min in the dark. Finally, flow cytometry (A00-1-1102, Beckman, Brea, CA, USA) was used for detection.

EDU

The cells were digested and inoculated into 96-well plates for culture, and corresponding intervention treatments were performed according to experimental groups after 24 h. The cell in each well was added 50 μL EDU (50 Mm, C10310-1, RIBBIO, Guangzhou, China) and incubated for 24 h. The cells in each well were incubated with 100 μL 4% paraformaldehyde, 100 μL glycine, 100 μL PBS, and 100 μL penetrant. The cells in each well were added 100 μL of 1 × Apollo® staining reaction and incubated in a decolorizing shaker for Apollo staining. The cells in each well were added 100 μL Hoechst 33342 (C1025, Beyotime, Shanghai, China) reaction solution and incubated in a decolorizing shaker at room temperature. The cells were cleaned with PBS and observed by a biological microscope (DSZ2000X, Beijing Zhongxian Hengye Instrument, Beijing, China).

Hematoxylin and Eosin (HE) and Annona Red O/Fast Green Staining

Synovial tissue specimens of rats were fixed by formaldehyde decalcification, dehydrated and embedded in paraffin (P3558, Sigma, Saint Louis, MO, USA). The tissue was cut into 5 μm slices with a slicer (YD-315, Zhejiang Jinhua Yidi Experimental equipment, Jinhua, China). The sections were dewaxed to water using gradient alcohol (75%–100%).

The histopathological observation was performed with hematoxylin and eosin staining, respectively. Annona Red O/Fast Green staining was performed with an Annona Red O/Fast Green staining kit (Abiowell, Changsha, China). Afterward, the sections were dehydrated with gradient alcohol (95%–100%) for 5 min each. The sections were placed in xylene for 10 min, sealed with neutral gum and observed under a microscope (BA410T, Motic, Xiamen, China).

Immunohistochemistry

The sections were immersed in 0.01M citrate buffer (pH6.0) for the thermal repair of antigen. The sections were added with 1% periodate acid. The sections dropped with appropriate dilution of anti-CollagenII (ab34712, 1:100, Abcam, Cambridge, UK), anti-Aggrecan (13880-1-AP, 1:200, Proteintech, Rosemont, IL, USA), anti-matrix metallopeptidase (MMP)13 (18165-1-AP, 1:100, Proteintech, Rosemont, IL, USA), anti-ADAMTS5 (DF13268, 1:100, Affinity, Sterling, VA, USA), 4°C overnight. The sections were added with 50–100 μL DAB working solution (ZSGB-BIO, Beijing, China) and incubated. The sections were stained with hematoxylin and dehydrated in a gradient of alcohol (60%–100%). The sections were observed under a microscope (BA410T, Motic, Xiamen, China).

Quantitative Reverse Transcription-polymerase Chain Reaction (qRT-qPCR)

TRIzol reagent (15,596,026, Thermofisher, Waltham, MA, USA) was applied to extract RNA from samples. cDNA was synthesized using an mRNA reverse transcription kit (CW2569, CWBIO, Beijing, China) and miRNA reverse transcription kit (CW2141, CWBIO, Beijing, China). The sequences of target genes were searched on NCBI, and primer5 software was performed to design primers. The expression levels of target genes were analyzed by UltraSYBR Mixture (CW2601, CWBIO, Beijing, China) and $2^{-\Delta\Delta CT}$. Primer sequences are shown in Table 1.

TABLE 1 Primer sequences

Gene ID		Primer sequences	Length (bp)
H-LncRNA TM1-3P	F	GGACCGGGAGATAGGAGTGT	120bp
	R	CACGGACTCCAGGTGATGAG	
H-ONECUT2	F	CATTTAGTCCAAGCCCCGGT	144bp
	R	CCGCTCTCATGCAGAGGTAG	
R-ONECUT2	F	AGAGGGTTCTATGCCGGTCT	166bp
	R	TATACTTGACCTGCCAGGCG	
H-miR-144-3p	F	GGCCCTGGCTGGGATATCAT	80bp
	R	GGTGCCCGGACTAGTACATC	
H-Smurf2	F	AGAGCTTGGTCCATTGCCTC	241bp
	R	CTGAACCAGGTCTCGCTTGT	
R-Smurf2	F	GTCCGGTCTCAGCGACATAG	201bp
	R	CAGAGGACCGAGCTCTTCAC	
H-IL1β	F	CCCTCTGTCATTCGCTCCC	185bp
	R	TAAAGAGAGCACACCAGTCCA	
R-IL1β	F	CAGCAGCATCTCGACAAGAG	123bp
	R	AAAGAAGGTGCTTGGGTCCT	
H-IL6	F	GCAATAACCACCCCTGACCCAA	154bp
	R	GCTACATTTGCCGAAGAGCC	
R-IL6	F	TCACTATGAGGTCTACTCGG	141bp
	R	CATATTGCCAGTTCTTCGTA	
H-TNFα	F	GAACCCCGAGTGACAAGCCT	120bp
	R	TATCTCTCAGCTCCACGCCAT	
R-TNFα	F	CCCCTCTATTTATAATTGCACCT	167bp
	R	CTGGTAGTTTAGCTCCGTTT	
H-Rap1	F	CCAGGCCCTTGGGTTAATACC	82bp
	R	TCGAACTTGGGTCTGCTTCC	
R-Rap1	F	ATGGAGGGGTGGACAAATCCT	80bp
	R	TGTCTGAGGGAAGCTGTGTTT	
H-Actin	F	ACCCTGAAGTACCCCATCGAG	224bp
	R	AGCACAGCCTGGATAGCAAC	
R-Actin	F	ACATCCGTAAAGACCTCTATGCC	223bp
	R	TACTCCTGCTTGCTGATCCAC	

Western Blot

RIPA lysate (Abiowell, Chinasha, China) was used to lysate cells or synovial tissues to extract total protein. The concentration of extracted protein was determined by BCA protein quantitative kit. Protein samples were separated by SDS-PAGE (Abiowell, Chinasha, China). The isolated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and incubated with anti-CollagenII (1:5000, ab188570, Abcam, Cambridge, UK), anti-Rap1 (12735-1-AP, 1:600, Proteintech, Rosemont, IL, USA), anti-Aggrecan (13880-1-AP, 1:2000, Proteintech, Rosemont, IL, USA), anti-ONECUT2 (21916-1-AP, 1:600, Proteintech, Rosemont, IL, USA), anti-Smurf2 (#12024, 1:1000, CST, USA), anti-MMP13 (18165-1-AP, 1:600, Proteintech, Rosemont, IL, USA), anti-ADAMTS5 (ab41037, 1:1000, Abcam, UK), anti-IL-1β (16806-1-AP, 1: 1000, Proteintech, Rosemont, IL, USA), anti-IL-6 (ab259341, 1:1000, Abcam, Cambridge, UK), anti-tumor necrosis factor-alpha (TNF-α) (17590-1-AP, 1: 1000, Proteintech, Rosemont, IL, USA), and anti-β-actin (66009-1-IG, 1:500, Proteintech, Rosemont, IL, USA) at 4°C overnight. The membrane was incubated with secondary anti-IgG (SA00001-1, 1:5000, Proteintech, Rosemont, IL, USA) and anti-IgG (SA00001-2, 1:6000, Proteintech, Rosemont, IL, USA) at 37°C for 90 min. Visualization was performed using chemiluminescence, and imaging analysis was performed using Quantity One professional gray analysis software.

RIP

RIP kit (RIP-12RXN, Sigma, Saint Louis, MO, USA) was performed to detect the interaction between miR-144-3p and ONECUT2 and between LncRNA TM1-3P and miR-144-3p. Briefly, the cell precipitate lysis was added with equal volume RIP lysis, repeated blow, incubated on ice for 5 min, and stored at −80°C for later use. Magnetic beads were vortexed with a vortex oscillator, then a 900 μL RIP immunoprecipitation buffer was added. The cell lysis products were thawed quickly, and 100 μL supernatant was taken and added into the centrifuge tube containing 900 μL RIP immunoprecipitation buffer and magnetic beads. The products were rotated overnight at 4°C. At the same time, 10 μL cracking products were taken as the input tube. 10 μL cracking products were mixed with 2 × SDS loading buffer and incubated at 95°C

for 5min. Then 500 μL RIP wash buffer was added, eddy briefly, and repeated cleaning six times. RNA was purified by proteinase K buffer. Finally, the expressions of miR-144-3p, ONECUT2, and LncRNA TM1-3P were detected by qRT-qPCR.

RNA Pull Down

RNA pull-down kit (20,164, Thermofisher, Waltham, MA, USA) was applied to detect the interaction between miR-144-3p and LncRNA TM1-3P. 50 μL streptavidin magnetic beads were added into a 1.5 ml microcentrifugation tube and cleaned with an equal volume of 20 mM Tris (pH 7.5). The magnetic beads were re-suspended by 1 × RNA capture buffer. 50 pM RNA probe/positive RNA/negative RNA was added to the mixture, mixed well, and incubated. The 1 × protein-RNA binding buffer was used to bind RNA to RNA. After washing, elution and purification of RNA–RNA complex, precipitation complex was obtained. Finally, the expression of miR-144-3p and LncRNA TM1-3P were detected by qRT-qPCR.

Statistics and Analysis

Graphpad Prism 8.0 statistical software was performed to analyze data in this study. All data were expressed as mean ± SD. Normality and homogeneity of variance tests were carried out. The data was of normal distribution and had homogeneity of variance. An unpaired *T*-test was applied between groups. The one-way analysis of variance of repeated measurement data was used for multi-group comparison, and Tukey's post-test was carried out. $P < 0.05$ indicated that the difference was statistically significant.

Results Bioinformatics Screening of Target mRNA in OA Synovial Tissue

The GSE55457 dataset was downloaded from the Gene Expression Omnibus (GEO) database. Bioinformatics analysis revealed that a total of 12,403 genes were detected in the OA and Normal groups, and 201 genes were different expressions between the groups (Fig. 1A). The heat map reflected the abundance of differentially expressed genes in the OA and Normal groups (Fig. 1B). Compared with the Normal group, ONECUT2 and Smurf2 genes were significantly up-regulated in the OA group (Fig. 1C,D). KEGG functional enrichment analysis indicated significant enrichment of Breast cancer ($P = 0.0050$) and MAPK Signaling Pathway (= 0.0064) (Fig. 1D). These results suggested that ONECUT2 and Smurf2 genes were up-regulated in OA, which might be related to the pathogenesis of OA.

The Expression of ONECUT2/Smurf2 in hFSCs Induced by IL-1β

HFSCs were stimulated with IL-1β *in vitro* and found ONECUT2 and Smurf2 gene expressions were elevated, and Rap1 gene expression was decreased (Fig. 2A). In addition, IL-1β induced the expressions of ONECUT2, Smurf2, MMP13, and ADAMTS5 proteins, while down-regulating CollagenII, Rap1, and Aggrecan proteins levels in hFSCs (Fig. 2B). IL-1β promoted the activity and proliferation, and reduced apoptosis of hFSCs (Fig. 2C–E). IL-1β treatment induced increased expression of IL-1β, IL-6 and TNF-α genes and proteins in hFSCs (Fig. 2F, G). Chen *et al.* have shown that ONECUT2 and Smurf2 were highly expressed in OA cartilage tissue.[21] Our results found that IL-1β induced ONECUT2/Smurf2 expression in hFSCs, which indicated ONECUT2 might also be closely related to the function of hFSCs in OA.

miR-144-3p Targeted the ONECUT2 Gene

Venn diagram exhibited the miRNA set of ONECUT2 targeted in diana_microt, targetscan, mirdb and pita databases, respectively (Fig. 3A). Bioinformatics prediction revealed that ONECUT2 was the binding site of miR-144-3p and indicated its mutated binding site (Fig. 3B). The dual luciferase reporter gene verified that miR-144-3p could directly bind to ONECUT2 (Fig. 3B). RIP assay confirmed the interaction between miR-144-3p and ONECUT2 protein with a strong binding ability (Fig. 3C). IL-1β treatment inhibited the expression of miR-144-3p in hFSCs (Fig. 3D). In summary, these findings suggested that miR-144-3p targeting ONECUT2 might be involved in the OA process of hFSCs induced by IL-1β *in vitro*.

miR-144-3p Targeted ONECUT2 to Regulate hFSCs Proliferation, Apoptosis, and Inflammation

Compared with the miR-NC group, the expressions of miR-144-3p were highly elevated, and the expressions of ONECUT2 and Smurf2 were significantly down-regulated in the miR-144-3p group (Fig. 4A). Compared with the miR-144-3p + oe-NC group, ONECUT2 and Smurf2 levels were highly improved in the miR-144-3p + oe-ONECUT2 group (Fig. 4A). Overexpression of miR-144-3p induced the expressions of CollagenII, Aggrecan, and Rap1, while down-regulating the expressions of ONECUT2, Smurf2, MMP13, and ADAMTS5 in hFSCs induced by IL-1β (Fig. 4B, Fig. S1A). When ONECUT2 was overexpressed simultaneously, the results in the miR-144-3p group were reversed (Fig. 4B, Fig. S1A). Compared with the miR-NC group, the cell viability and proliferation were decreased, and the apoptosis was improved in the miR-144-3p group, which were reversed in miR-144-3p + oe-ONECUT2 group (Fig. 4C–E, Fig. S1B,D). Overexpression of miR-144-3p reduced IL-1β, IL-6, and TNF-α levels in hFSCs induced by IL-1β (Fig. 4F, G, Fig. S1C). Compared with the miR-144-3p + oe-NC group, the expression of IL-1β, IL-6, and TNF-α were significantly up-regulated in miR-144-3p + oe-ONECUT2 group (Fig. 4F, G, Fig. S1C). These findings revealed that miR-144-3p affected the proliferation, apoptosis, and inflammation in hFSCs by targeting ONECUT2.

TM1-3P Targeted miR-144-3p

Bioinformatics analysis revealed that TM1-3P could target miR-144-3p (Fig. 5A). Luciferase reporter gene assay confirmed that TM1-3P could directly bind to miR-144-3p

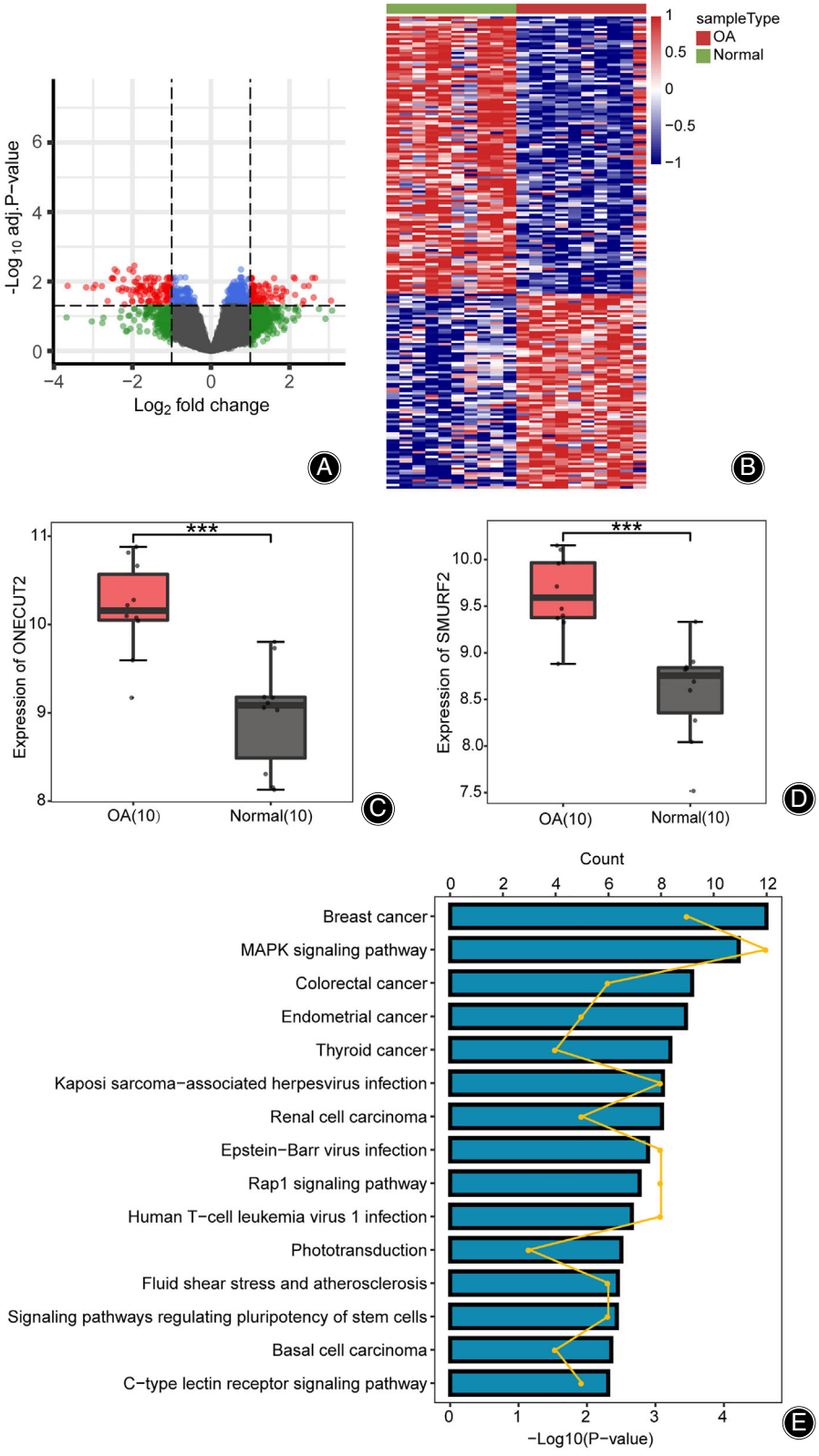

Fig. 1 Bioinformatics was applied to screen mRNA expression profile and function in OA. (A) The volcanic map revealed differentially expressed mRNA in OA. (B) The heat map exhibited the abundance of differentially expressed mRNA in OA. (C,D) A boxplot reflected the ONECUT2 and Smurf2 gene expressions. (D) KEGG functional enrichment analysis of differential mRNA. *$P < 0.05$ versus, Normal group.

(Fig. 5B). RIP assay confirmed the interaction between TM1-3P and miR-144-3p with a strong binding ability (Fig. 5C, D). IL-1β treatment promoted the expression of TM1-3P in hFSCs (Fig. 5E). In short, these results suggested that TM1-3P targeted the expression of miR-144-3p, which might be involved in IL-1β induced OA process in hFSCs.

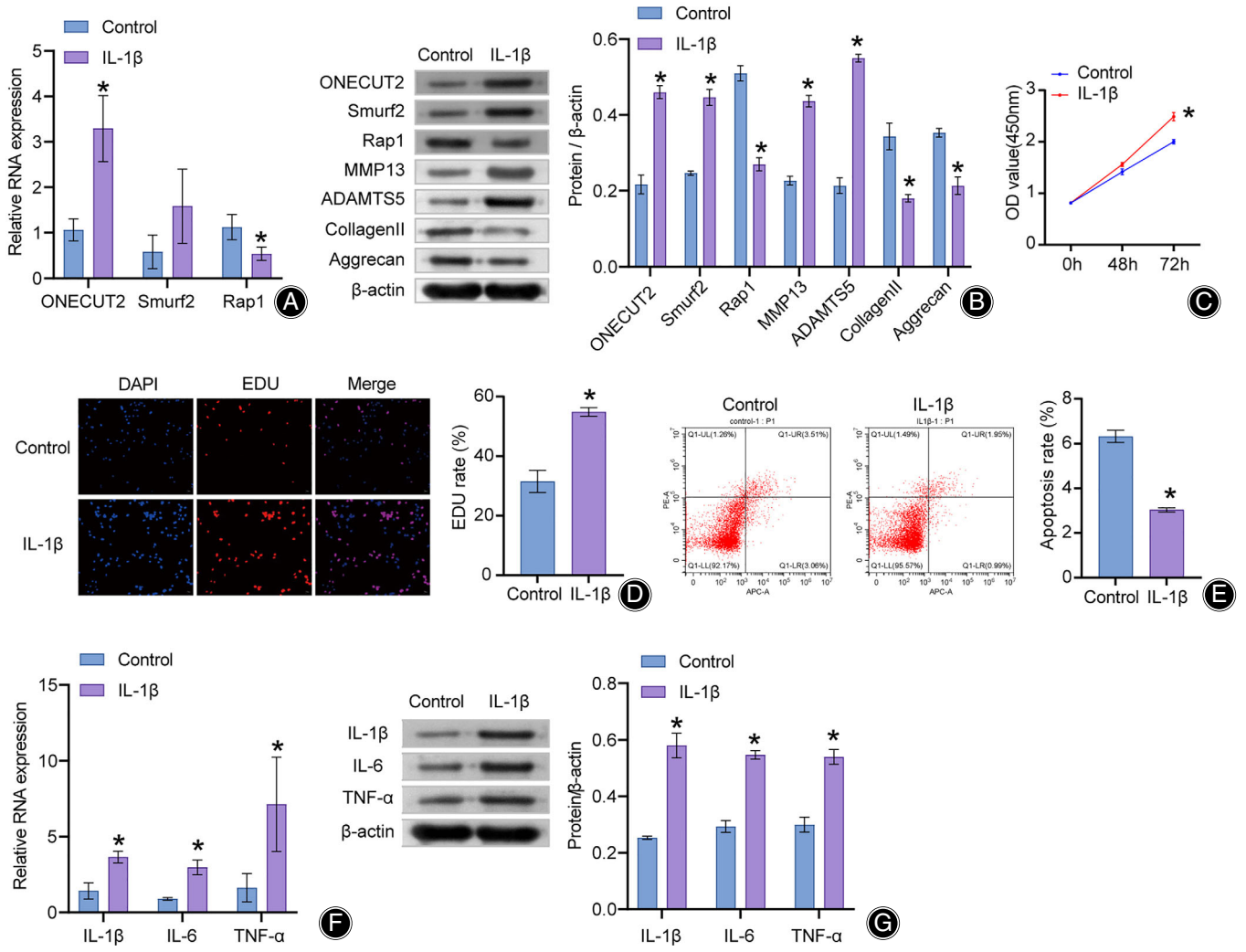

Fig. 2 IL-1β induced ONECUT2/Smurf2 expression in hFSCs. (A) The expressions of ONECUT2, Smurf2, and Rap1 were tested by qRT-PCR. (B) ONECUT2, Smurf2, Rap1, MMP13, ADAMTS5, CollagenII, and Aggrecan levels were calculated by western blot. (C) CCK8 was performed to detect cell viability. (D) Cell proliferation was tested by EDU. (E) Cell apoptosis was examined by flow cytometry. (F,G) The expressions of IL-1β, IL-6, and TNF-α were measured by qRT-PCR and western blot. *$P < 0.05$ *vs* Control group.

TM1-3P Targeted miR-144-3p to Regulate hFSCs Proliferation, Apoptosis, and Inflammation

Compared with the LV-LncRNA NC group, the expressions of TM1-3P, ONECUT2 and Smurf2 were improved, while miR-144-3p and Rap1 were down-regulated in the LV-Lnc TM1-3P group (Fig. 6A). Compared with LV-Lnc TM1-3P + miR-NC group, ONECUT2 and Smurf2 expressions were decreased, while miR-144-3p and Rap1 expressions were elevated in LV-Lnc TM1-3P + miR-144-3p group (Fig. 6A). Overexpression of TM1-3P promoted ONECUT2, Smurf2, MMP13 and ADAMTS5 expressions while down-regulating CollagenII, Aggrecan, and Rap1 expressions (Fig. 6B, Fig. S1F). These results were reversed in the LV-TM1-3P + miR-144-3p group (Fig. 6B, Fig. S1F). Overexpression of TM1-3P promoted cell viability and proliferation while inhibiting apoptosis of hFSCs, which were reversed in the LV-TM1-3P + miR-144-3p group (Fig. 6C–E, Fig. S1E, G). Compared with the LV-LncRNA NC group, the expressions of IL-1β, IL-6, and TNF-α were improved in the LV-Lnc TM1-3P group (Fig. 6F, G, Fig. S1H). Compared with the LV-Lnc TM1-3P + miR-NC group, the expressions of IL-1β, IL-6, and TNF-α were down-regulated in LV-Lnc TM1-3P + miR-144-3p group (Fig. 6F, G, Fig. S1H). In summary, TM1-3P could target miR-144-3p to regulate IL-1β-induced hFSCs proliferation, apoptosis, and inflammation.

Inhibition of TM1-3P Alleviated OA in Rats

HE staining exhibited that the morphology of cartilage and synovial membrane in the Sham group was normal, and the

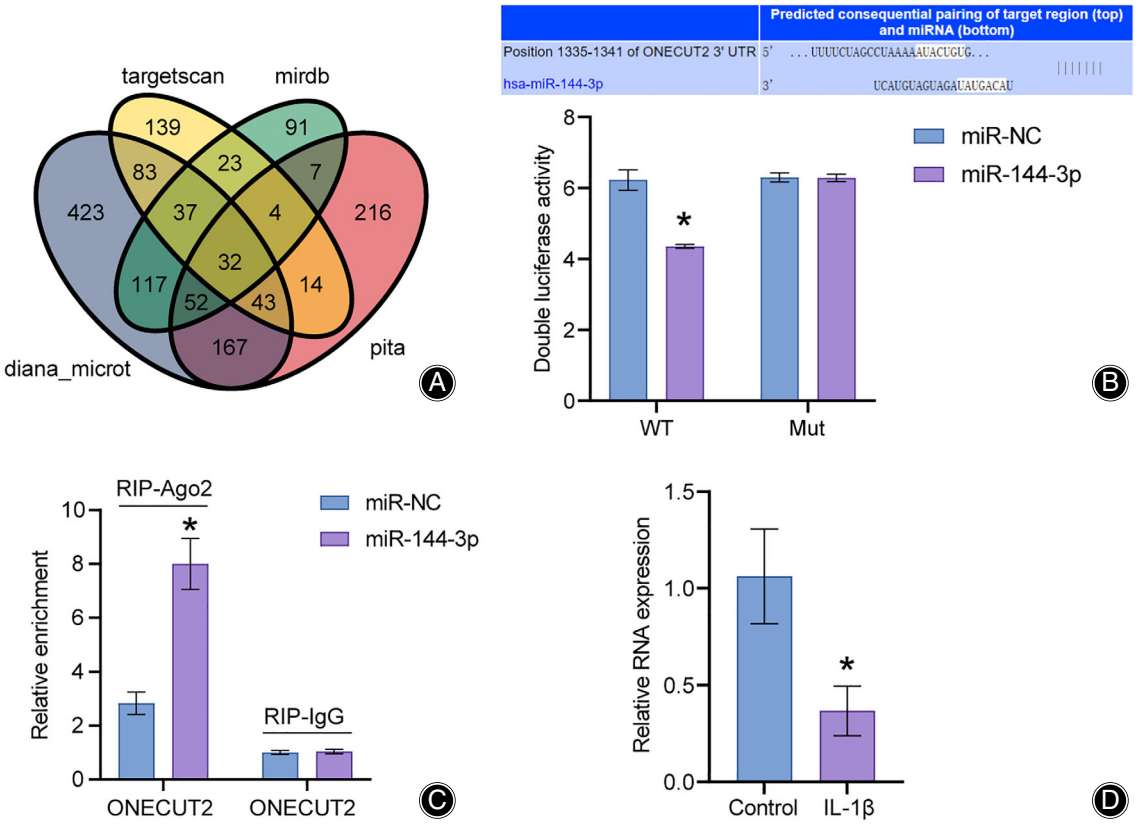

Fig. 3 miR-144-3p targeted the ONECUT2 gene. (A) Venn diagram demonstrated ONECUT2-targeted miRNAs. (B) Bioinformatics and dual luciferase reporter gene assay analyzed the binding sites of ONECUT2 to miR-144-3p. (C) RIP was used to assess the effects of miR-144-3p mimic on ONECUT2 enrichment in Anti-IgG and Anti-Ago2. (D) The expression of miR-144-3p was calculated by qRT-PCR. *$P < 0.05$ *vs* Control group.

cartilage tissue in the OA and LV-sh-NC groups was seriously damaged (Fig. 7A). LV-sh-TM1-3P treatment relieved cartilage damage in OA rats (Fig. 7A). Annona Red O/Fast Green staining found LV-sh-TM1-3P treatment alleviated cartilage damage in OA rats (Fig. 7B). Compared with the Sham group, MMP13 and ADAMTS5 expressions in synovial tissues were elevated, and the expressions of CollagenII and Aggrecan were decreased in the OA group (Fig. 7C, D). The LV-sh-TM1-3P intervention reversed the results (Fig. 7C, D). In addition, compared with the Sham group, the expressions of Rap1 in synovial tissues were down-regulated, while the expressions of ONECUT2 and Smurf2 were increased in the OA group (Fig. 7E). The LV-sh-TM1-3P intervention reduced ONECUT2 and Smurf2 levels, and promoted the expression of Rap1 in synovial tissues of OA model rats (Fig. 7E). Compared with the Sham group, the expressions of IL-1β, IL-6, and TNF-α in synovial tissues of the OA group were elevated, which was reversed in the LV-sh-TM1-3P group (Fig. 7F). Altogether, these findings suggested that inhibition of TM1-3P could effectively relieve knee joint injury and inflammation in rats with OA.

Discussion

Main Findings

In this work, our results indicated that ONECUT2 and LncRNA TM1-3P were increased in IL-1β-induced hFSCs, while miR-144-3p was decreased. The bioinformatics database predicted that miR-144-3p targeted ONECUT2 and TM1-3p targeted miR-144-3p, and the targeting relationship was verified by the dual luciferase reporter gene assay, RNA Binding Protein Immunoprecipitation (RIP) and RNA pull down. TM1-3p competitively bound miR-144-3p to promote ONECUT2 expression, up-regulated Smurf2, IL-1β, IL-6, and TNF-α expressions, down-regulated Rap1 level, and promoted ECM degradation. In addition, knockdown of TM1-3p reduced ONECUT2, Smurf2, IL-1β, IL-6, and TNF-α expression in rats, promoted Rap1 expression, inhibited ECM degradation, and alleviated OA.

The DEGs in OA

To explore the underlying mechanism of OA, we first established an *in vitro* model of OA by IL-1β-induced hFSCs. Inflammatory response and ECM degradation are enhanced during the development of OA, and reducing the inflammatory response and

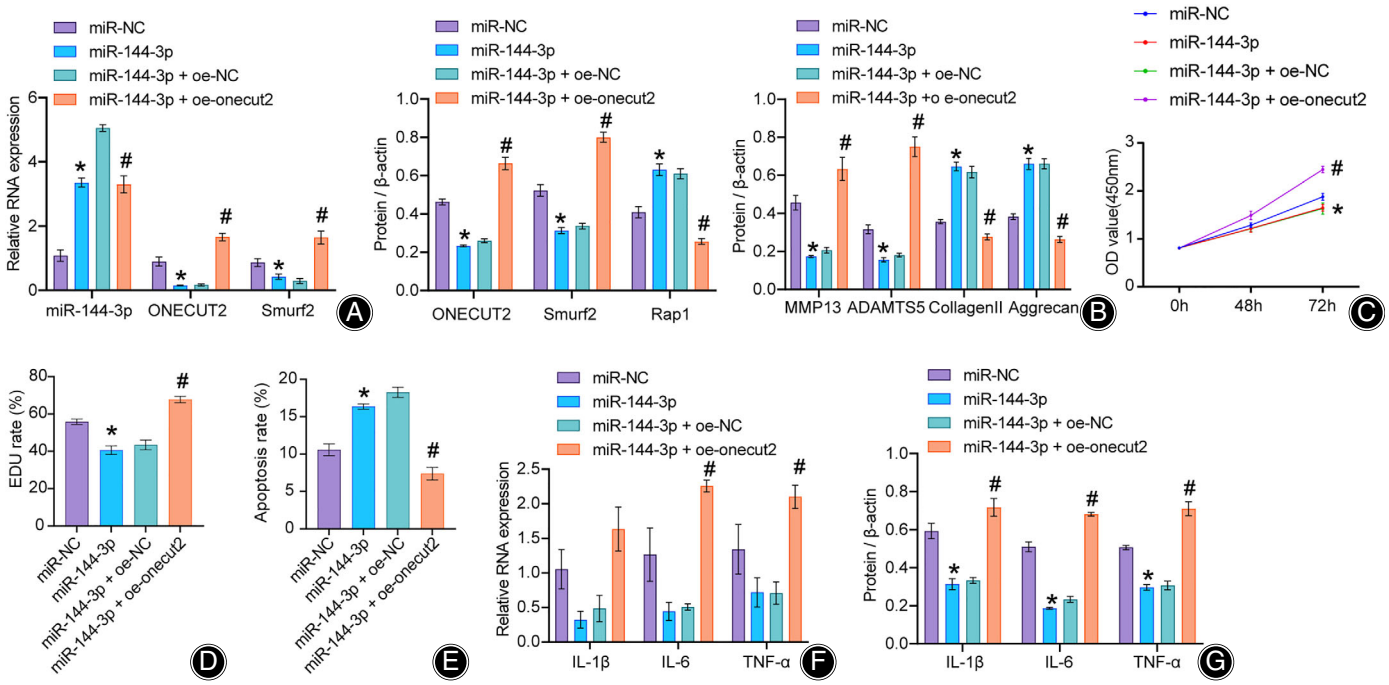

Fig. 4 miR-144-3p affected hFSCs proliferation, apoptosis, and inflammation by targeting ONECUT2. (A) The expressions of miR-144-3p, ONECUT2, and Smurf2 were tested by qRT-PCR. (B) The levels of ONECUT2, Smurf2, Rap1, MMP13, ADAMTS5, CollagenII, and Aggrecan were examined by western blot. (C–E) Cell viability, proliferation, and apoptosis were assessed by CCK8, EDU, and flow cytometry, respectively. (F,G) The expressions of IL-1β, IL-6, and TNF-α were measured by qRT-PCR and western blot. *$P < 0.05$ *vs* miR-NC group, #$P < 0.05$ *vs* miR-144-3p + oe-NC group.

ECM degradation could alleviate OA development.[25] The results revealed that IL-1β improved the expression of MMP13, ADAMTS5, IL-1β, IL-6, and TNF-α in hFSCs, while down-regulating Rap1, CollagenII, and Aggrecan levels. The results reflected that the OA cell model was established successfully.

Next, the DEGs in OA were identified using comprehensive bioinformatics analysis. It was found that these DEGs were mainly involved in the PI3K-Akt signaling pathway and Rap1 signaling pathway through gene ontology (GO) and KEGG.[26] Smurf2 in chondrocytes leads to spontaneous OA in mice.[27] Silencing Smurf2 could enhance the proliferation of OA chondrocytes and inhibit apoptosis and inflammation.[28] Smurf2-mediated up-regulation of β-catenin by inducing proteasome degradation of GSK-β in chondrocytes might activate articular chondrocyte maturation and associated gene expression changes, an early event of OA.[29] It has been confirmed that ONECUT2 could bind Smurf2.[21] Bioinformatics analysis demonstrated that ONECUT2 and Smurf2 genes were remarkably elevated in the OA group compared with the control group. KEGG pathway analysis exhibited that DEGs were involved in the Rap1 signaling pathway. In addition, IL-1β induced improved expression of ONECUT2 and Smurf2, while decreased expression of miR-144-3p, Rap1, CollagenII, and Aggrecan in hFSCs. The above studies proved that ONECUT2/Smurf2 was involved in the pathogenesis of OA, but the specific action process remains unknown.

The Potential Mechanism of ONECUT2 in IL-1β-induced hFSCs

ONECUT2, as a transcription factor, regulates a wide range of protein expressions related to cell proliferation, migration, adhesion, differentiation, and cell material metabolism.[30] ONECUT2 upregulation induces activation of the Wnt signaling pathway and cell cycle regulation pathway.[31] In OA, LINC00671 inhibits chondrocyte proliferation, while enhancing apoptosis and ECM degradation, which is easily reversed by silencing ONECUT2 or Smurf2.[21] Our study demonstrated that miR-144-3p could target binding and regulate onecut2 expression. miR-144-3p inhibited IL-1β-induced ONECUT2, Smurf2, IL-1β, IL-6, and TNF-α expressions in hFSCs, cell proliferation, and ECM degradation, while promoting apoptosis and Rap1 expression, which is partially reversed by ONECUT2 overexpression. Our results are consistent with previous studies. miR-144-3p was involved in inflammatory reactions of septic acute lung injury,[32] preeclampsia,[33] *Mycobacterium abscess* infection,[34] and peritonitis.[35] Therefore, the above studies confirmed that miR-144-3p/ONECUT2 could be a key process of OA inflammatory response.

Non-coding RNAs, such as miRNAs and LncRNAs, regulate varying levels of the gene in various immune diseases and serve as potential drug targets and biomarkers of disease progression.[36] LncRNAs exert biological functions by forming endogenous small interfering RNA (siRNA) and

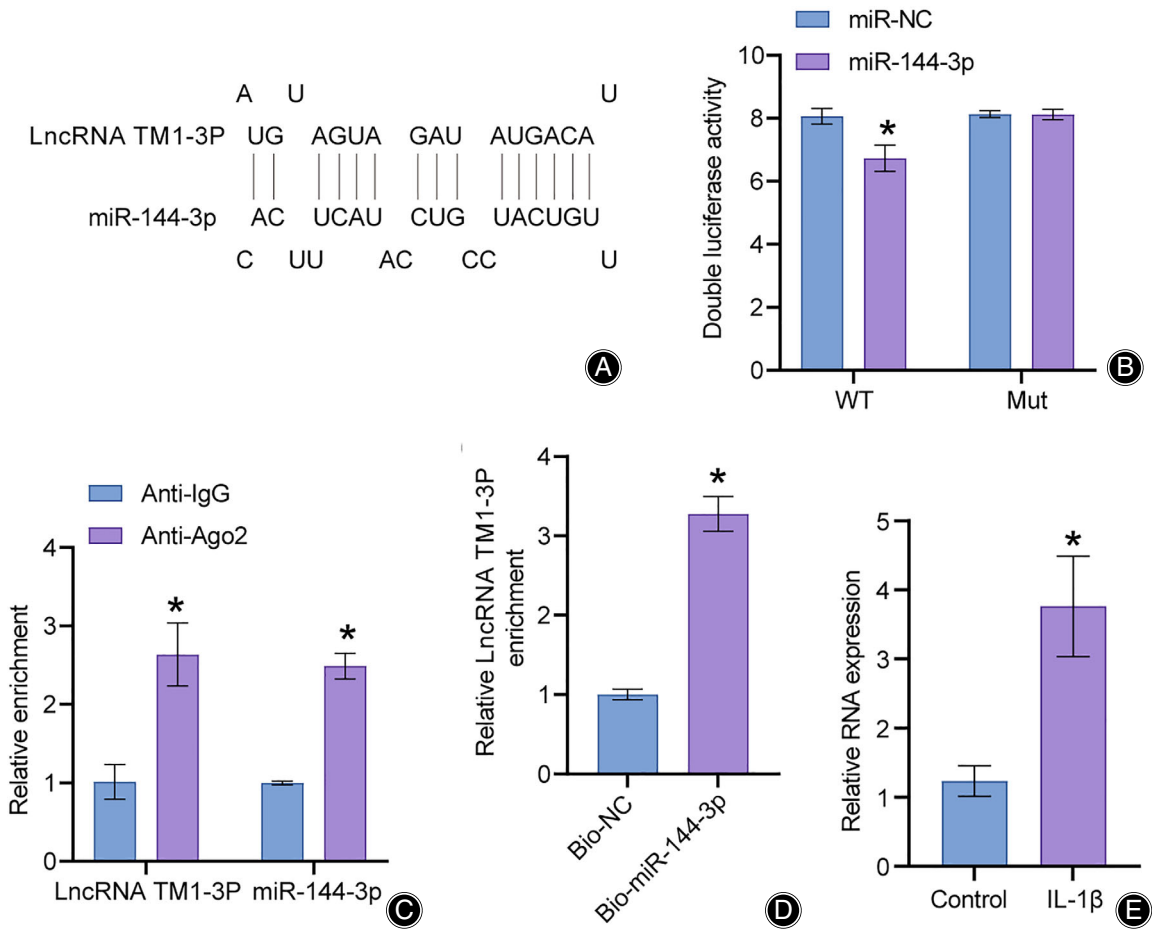

Fig. 5 TM1-3P targeted the miR-144-3p gene. (A) Bioinformatics predicted the binding sites of miR-144-3p and TM1-3P. (B) The binding of TM1-3P to miR-144-3p was detected by a dual luciferase reporter gene assay. (C) The enrichment of TM1-3P and miR-144-3p in Anti-IgG and Anti-Ago2 was evaluated by RIP. (D) RNA pull down assay was applied to test the enrichment of TM1-3P and miR-144-3p in Bio-NC and Bio-miR-144-3p. (E) The expression of TM1-3P was calculated by qRT-PCR. *$P < 0.05$ *vs* Control group.

natural miRNA sponges. LncRNAs are involved in several pathological processes during OA, including ECM degradation, inflammatory response, apoptosis, and angiogenesis.[37] Xu *et al.* reflected that LncRNA MCM3AP-AS1 aggravated the progression of OA by regulating the miR-149-5p/Notch1 signaling pathway.[38] LINC00623/miR-101/HRAS axis regulates OA chondrocyte apoptosis, senescence, and ECM degradation through MAPK signal transduction, which might play a key role in OA development.[39] Our study confirmed that TM1-3P promoted the expressions of ONECUT2, Smurf2, IL-1β, IL-6, and TNF-α in hFSCs, cell proliferation, and ECM degradation, while inhibiting apoptosis and Rap1 levels, while miR-144-3p reversed this result.

The Function of TM1-3P in vivo

Our results found that silencing TM1-3P improved articular synovial injury in OA rats by inhibiting inflammation and regulating miR-144-3p/ONECUT2 axis.

Previous studies have demonstrated the roles of miR-144-3p and ONECUT2 in OA, respectively, influencing the article's novelty. However, its underlying molecular mechanism remains poorly understood. In the present study, we further elucidated the effects and potential mechanisms of miR-144-3p and ONECUT2 on inflammatory factors (IL-1β, IL-6, and TNF-α), ECM degradation, and cell viability, proliferation, and apoptosis in OA. In addition, this work clarified the mechanism of TM1-3P in OA for the first time. Our results revealed that TM1-3P promoted the expression of ONECUT2 by sponging miR-144-3p, and knocking down TM1-3P alleviated OA in rats. In this study, we preliminarily explored the mechanism of TM1-3P in OA.

There is abundant evidence that non-coding RNA plays a crucial role in the proliferation and differentiation of osteoblasts and bone marrow stromal cells.[40] For example, LncRNA HOTTIP accelerates osteogenic differentiation by interacting with TAF15 to stabilize DLX2.[41] LncRNA KCNQ1OT1 promotes osteogenic differentiation through

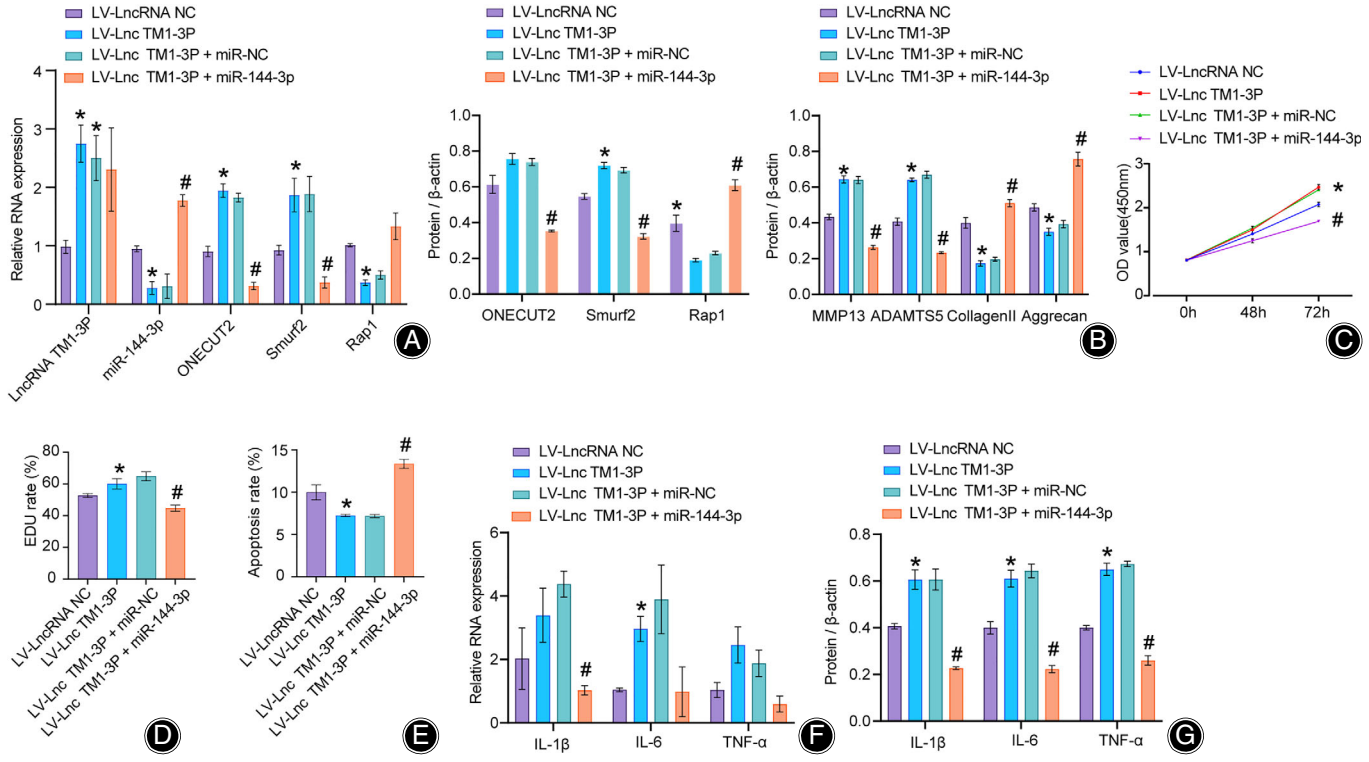

Fig. 6 TM1-3P targeted miR-144-3p and affected IL-1β-induced hFSCs proliferation, apoptosis, and inflammation. (A) TM1-3P, miR-144-3p, ONECUT2, Smurf2, and Rap1 levels were assessed by qRT-PCR. (B) The expressions of ONECUT2, Smurf2, MMP13, ADAMTS5, CollagenII, and Aggrecan were examined by western blot. (C) CCK8 was applied to evaluate cell viability. (D) Cell proliferation was detected by EDU. (E) Cell apoptosis was tested by flow cytometry. (F,G) IL-1β, IL-6, and TNF-α levels were detected by qRT-PCR and western blot. *$P < 0.05$ versus,*vs* LV-LncRNA NC group, #$P < 0.05$ *vs* LV-Lnc TM1-3P + miR-NC group.

miR-205-5p/RICTOR axis.[42] LncRNA might be involved in OA disease progression. LncRNA plays a crucial role in regulating downstream gene expression and maintaining cell function and homeostasis, especially in chondrocytes, synovial cells, osteoblasts, osteoclasts, and skeletal muscle cells.[43] Several studies have confirmed that LncRNA could participate in the regulation of OA-related inflammation by targeting miRNA. For example, LncRNA BLACAT1 regulates the differentiation of bone marrow stromal stem cells by targeting miR-142-5p, which is beneficial to the treatment of OA.[44] LncRNA HCG18 knockdown promotes osteogenic differentiation of human BMSCS by targeting the miR-30a-5p/NOTCH1 axis.[45] It has been reported that ONECUT2 accelerates tumor proliferation by activating the expression of RHO-associated protein kinase 1 (ROCK1) in gastric cancer.[31] TNF-α enhances ROCK1 expression and reduces autophagy activation, and ROCK1 knockdown reduces endoplasmic reticulum stress and promotes autophagy, leading to the reversal of BMSCS osteogenic differentiation under inflammatory conditions.[46] SNHG7 inhibits apoptosis and autophagy by targeting SYVN1 to sponge miR-34a-5p, thereby alleviating OA.[47] Trehalose ameliorates oxidative stress-mediated mitochondrial dysfunction in OA through selective autophagy.[48] We reasonably and boldly speculated that TM1-3P might regulate autophagy, oxidative stress, or osteogenic differentiation through ONECUT2 to affect OA progression, which is our next project's research direction and focus.

Strengths and Limitations

For the first time, we clarified the function and possible mechanism of TM1-3P in OA. However, there are some limitations. First, the potential molecular mechanism of TM1-3P in OA needs to be further explored. Second, the TM1-3P effect on chondrocyte function in OA is not clearly defined. In the next project, we will explore other targets and pathways of TM1-3P in OA. Third, other underlying mechanisms of ONECUT in OA have not been elucidated. In future work, we will explore the mechanism of TM1-3P on chondrocyte function in OA. The role of ONECUT2-related ceRNA in OA is a future work direction.

Conclusion

The above studies proved that TM1-3P mediated the inflammatory response of OA through miR-144-3p/ONECUT2 axis, thus improving the damage to the knee joint and

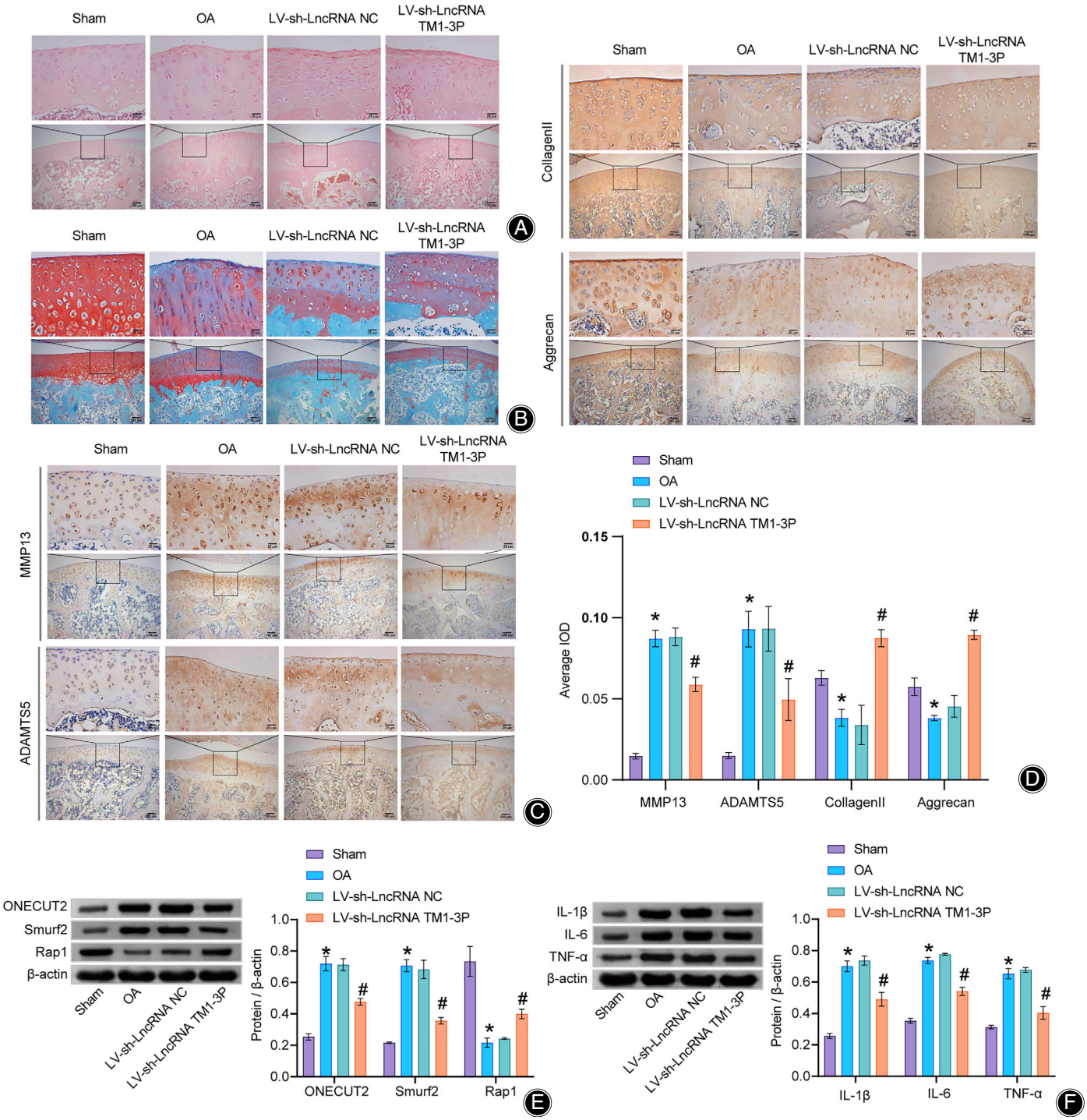

Fig. 7 TM1-3P alleviated synovial tissue damage and inflammation in OA rats. (A,B) Histopathological changes in knee joints were observed by HE and Annona Red O/Fast Green staining. (C,D) MMP13, ADAMTS5, CollagenII, and Aggrecan expressions in synovial tissue were examined by immunohistochemistry. (E) The expressions of ONECUT2, Smurf2, and Rap1 in synovial tissue were tested by western blot. (F) IL-1β, IL-6, and TNF-α levels in synovial tissue were evaluated by qRT-PCR and western blot. *$P < 0.05$ *vs* Sham group, #$P < 0.05$ *vs* LV-sh-LncRNA NC group.

providing a new strategy for clarifying the pathogenesis and treatment of OA. TM1-3P inhibitors might be a promising therapeutic agent for OA.

Author Contributions

All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Data curation, Yangfei Yi, Ningyin Yang, Zirui Yang and Xiaojun Tao; Formal analysis, Yangfei Yi, Ningyin Yang and Zirui Yang; Methodology, Yufei Li; Project administration, Yufei Li; Visualization, Yangfei Yi, Ningyin Yang and Zirui Yang; Writing-original draft, Yangfei Yi and Ningyin Yang; Writing-review and editing, Yufei Li and Xiaojun Tao.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

Supporting Information

Additional Supporting Information may be found in the online version of this article on the publisher's web-site:

Fig. S1. TM1-3P targeted miR-144-3p and affected IL-1β-induced hFSCs proliferation, apoptosis, and inflammation. (A) The expressions of ONECUT2, Smurf2, Rap1, MMP13, ADAMTS5, CollagenII, and Aggrecan were assessed by western blot. (B) Cell apoptosis was detected by flow cytometry. (C) The levels of IL-1β, IL-6, and TNF-α were calculated by western blot. (D,E) Cell proliferation was measured by EDU. (F) Western blot was used to examine the expressions of ONECUT2, Smurf2, Rap1, MMP13, ADAMTS5, CollagenII, and Aggrecan. (G) Cell apoptosis was tested by flow cytometry. (H) The levels of IL-1β, IL-6, and TNF-α were calculated by western blot.

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