European Review for Medical and Pharmacological Sciences 2020; 24: 535-548

The reduced lncRNA NKILA inhibited proliferation and promoted apoptosis of chondrocytes *via* miR-145/SP1/NF-κB signaling in human osteoarthritis

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Abstract. – OBJECTIVE: Growing evidence has shown that long non-coding RNAs (lncRNAs) play some roles in the progression of osteoarthritis. In this study, we investigated the functions and mechanisms of lncRNA NKILA (NKILA) of chondrocytes in human osteoarthritis (OA).

PATIENTS AND METHODS: RT-PCR was used to detect the expressions of NKILA and miR-145 in OA tissues. After transfection of NKILA overexpression lentivirus (LV-NKILA) and NKILA downregulation lentivirus (LV-shNKILA) into primary chondrocytes, MTT assay was carried out to measure the cell proliferation of chondrocytes. The expressions of SP1, Bcl-2, Bax, cleaved caspase-3 and NF-κB signaling factors were detected by Western blot. Moreover, luciferase assay was performed to explore the binding site of NKILA and miR-145, miR-145 and SP1. Finally, JSH, a NF-κB signaling inhibitor, was added into chondrocytes transfected with LV-shNKILA or miR-145 mimic to detect that NKILA functions via miR-145/SP1/NF-κB signaling pathway.

RESULTS: We found that NKILA and SP1 were significantly reduced, miR-145 was increased in cartilage tissues of OA patients. After LV-NKILA transfection, the proliferation ability of chondrocytes was improved and cell apoptosis was inhibited; however, the proliferation ability of chondrocytes was repressed, and cell apoptosis was increased in LV-sh NKILA group. MiR-145 was predicted to be a potential target of NKILA and luciferase gene reporter assay confirmed that NKILA could directly bind with miR-145. Furthermore, SP1 was predicted to be a target gene of miR-145 and luciferase gene reporter assay proved that miR-145 could directly bind with SP1. Finally, we added JSH, a NF-κB signaling inhibitor, into chondrocytes with LV-shNKILA or miR-145 mimic. Results showed that the repressed SP1 was reversed after the addition of JSH in both LV-shNKILA and miR-145 mimic group. Further, the repressed proliferation capacities and promoted cell apoptosis were also reversed after the addition of JSH.

CONCLUSIONS: According to the results, this study uncovers NKILA is reduced in human osteoarthritic cartilage tissues. Furthermore, we firstly uncover that the reduced NKILA could function as a ceRNA to improve miR-145, which inhibited SP1 expression and regulated NF-κB signaling pathway, thereby promoting tissue inflammation, and inhibiting proliferation and promoting apoptosis of chondrocytes. Thus, it may be used as a promising prognostic marker and a potential target for osteoarthritis.

Key Words:
LncRNA NKILA, Osteoarthritis, MiR-145, SP1, NF-κB signaling.

Introduction

Osteoarthritis (OA) is a common chronic joint disease that may cause local inflammatory responses, cartilage damage, and changes in cartilage structures[1-5]. At present, about 15% of the population in the world suffers from OA, especially middle-aged and elderly patients[6]. OA is characterized by articular cartilage degeneration, loss of extracellular matrix (ECM), extensive fibrosis and fissures, resulting in complete loss of cartilage surface[7]. Non-steroidal anti-inflammatory drugs (NSAIDs) or cyclooxygenase (COX)-2 inhibitors are commonly used to relieve symptoms in patients with OA[8,9]. While the advanced patients need surgical intervention, including arthroscopic surgery and artificial joint replacement[10, 11]. Some researchers reported that autologous chondrocyte transplantation may become a new treatment strategy for arthritis and bone defects[12,13]. However, due to the unclear pathogenesis of OA, there is no effective treatment and symptomatic solution.

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More and more evidence showed that OA may be associated with abnormal lncRNAs expressions in chondrocytes[14-17]. Long non-coding RNAs (lncRNAs) are a class of RNAs, which are more than 200 nucleotides in length, but do not have protein-coding functions. It has been revealed that lncRNAs could function as a competing endogenous RNA (ceRNA) to interact with microRNAs (miRNAs), which will regulate different functions of various diseases, including OA[18-21]. For example, Li et al[19] reported that lnc-CIR promotes the degradation of extracellular matrix of osteoarthritic chondrocytes through miR-27b. It has also been reported that lnc-ZFAS1 plays an important role in proliferation, apoptosis and migration of chondrocyte in osteoarthritis[20]; moreover, the lnc-XIST/miR-211 axis regulates proliferation and apoptosis of osteoarthritic chondrocytes via chemokine receptor CXCR4 and MAPK signaling[21]. Although many lncRNAs are associated with OA, evidence for NKILA and OA has not been found in current literature. A series of studies have confirmed that

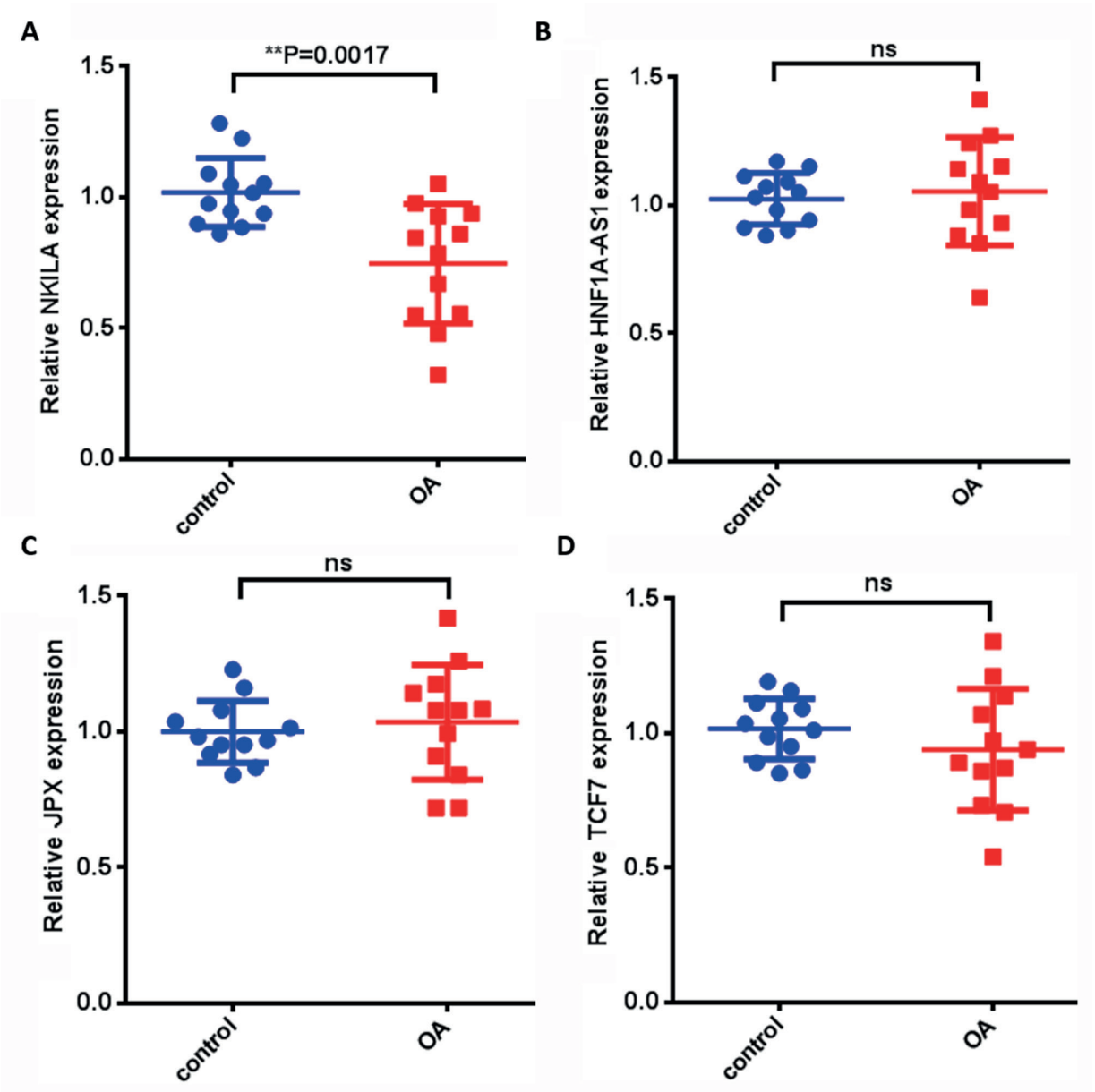

Figure 1. NKILA was reduced in cartilage tissue of human osteoarthritis. **(A)** The mRNA level of NKILA in osteoarthritis patients and control patients was detected by RT-PCR. **(B-D)** The mRNA level of lnc-HNF1A-AS1, lnc-JPX and lnc-TCF7 was also detected by RT-PCR. Data are shown as mean ± SD based on at least three independent experiments, $^{**}p<0.01$.

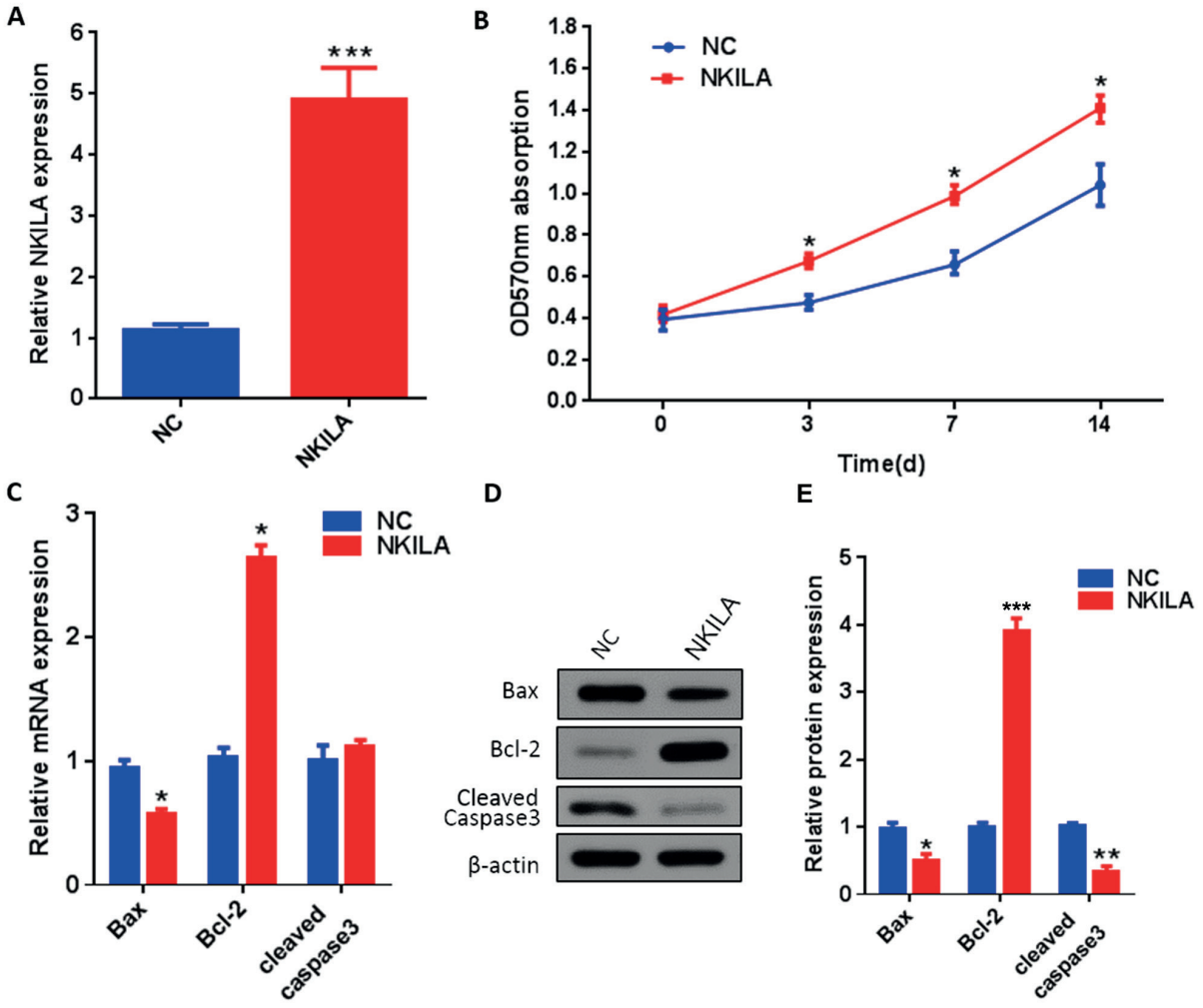

Figure 2. Upregulation of NKILA promotes proliferation of chondrocytes. **(A)** The NKILA expression was detected after LV- NKILA infection by RT-PCR. **(B)** The proliferation rate of chondrocytes was analyzed by MTT assay. **(C-E)** The mRNA and protein levels of apoptotic and anti-apoptotic genes were detected by RT-PCR and WB (magnifications x 1.2). Data are shown as mean ± SD based on at least three independent experiments, *p<0.05; **p<0.01; ***p<0.001.

NKILA is a regulator of cancer cell proliferation, migration and invasion in many cancers, such as osteosarcoma, hepatocellular carcinoma, small cell lung cancer, etc.[22-25], but its potential function in the development of OA remains unclear.

NF-κB signaling is involved in a variety of intracellular and extracellular stimulatory responses and plays an important role in regulating immune response, cellular stress response and cancer development[22-25]. SP1 is a member of SP family in transcription factors, which is ubiquitously expressed in mammalian cells and can bind to and act through GC boxes to regulate gene expressions, such as NF-κB signaling pathway[22-25]. The NF-κB pathway is the main inflammatory signal, which is regulated by RelA (p65), RelB, NF-κB1, and NF-κB2. The activation of this pathway is mainly regulated via phosphorylation and nuclear localization of p65, thereby regulating downstream target gene expressions[26-29]. Some studies have shown that NF-κB signaling plays an important role in regulating chondrocyte proliferation, and the inhibition of NF-κB signaling can alleviate osteoarthritis[27, 30]; however, the detailed molecular mechanism remains unclear.

Our previous study screened the differentially expressed lncRNAs in osteoarthritis and verified in human cartilage tissue samples, which preliminarily confirmed that NKILA was downregulated in human osteoarthritis tissue. Studies showed that NKILA could regulate NF-κB signals in osteosarcoma and other tumors[22,23]. However, the functions of NKILA and the relationship with

NF-κB signals in osteoarthritis remained unknown. In this study, we intended to investigate the role of NKILA among chondrocytes in human osteoarthritis. And we want to explore the relationship between NKILA and NF-κB signaling in the development of osteoarthritis.

Patients and Methods

Sample Collection

12 cartilage tissue samples were collected from patients with osteoarthritis by surgical resection in our hospital from August 2013 to August 2017. And 12 healthy control tissue samples were collected from patients with traumatic emergency amputation at the same period. Patients with rheumatoid arthritis and infectious arthritis were excluded from our study. All patients underwent excision of the full thickness of the femoral condyle and a small amount of subchondral bone before the tissue examination. All tissue samples were frozen in a liquid nitrogen at -80°C. Informed consent was obtained from each patient. This study was approved by the Faculty of Medicine's Ethics Committee of our hospital.

Primary Chondrocytes Isolation and Cell Culture

The isolation and culture methods of primary chondrocytes from tissue samples were used according to the method reported by Wu et al[31].

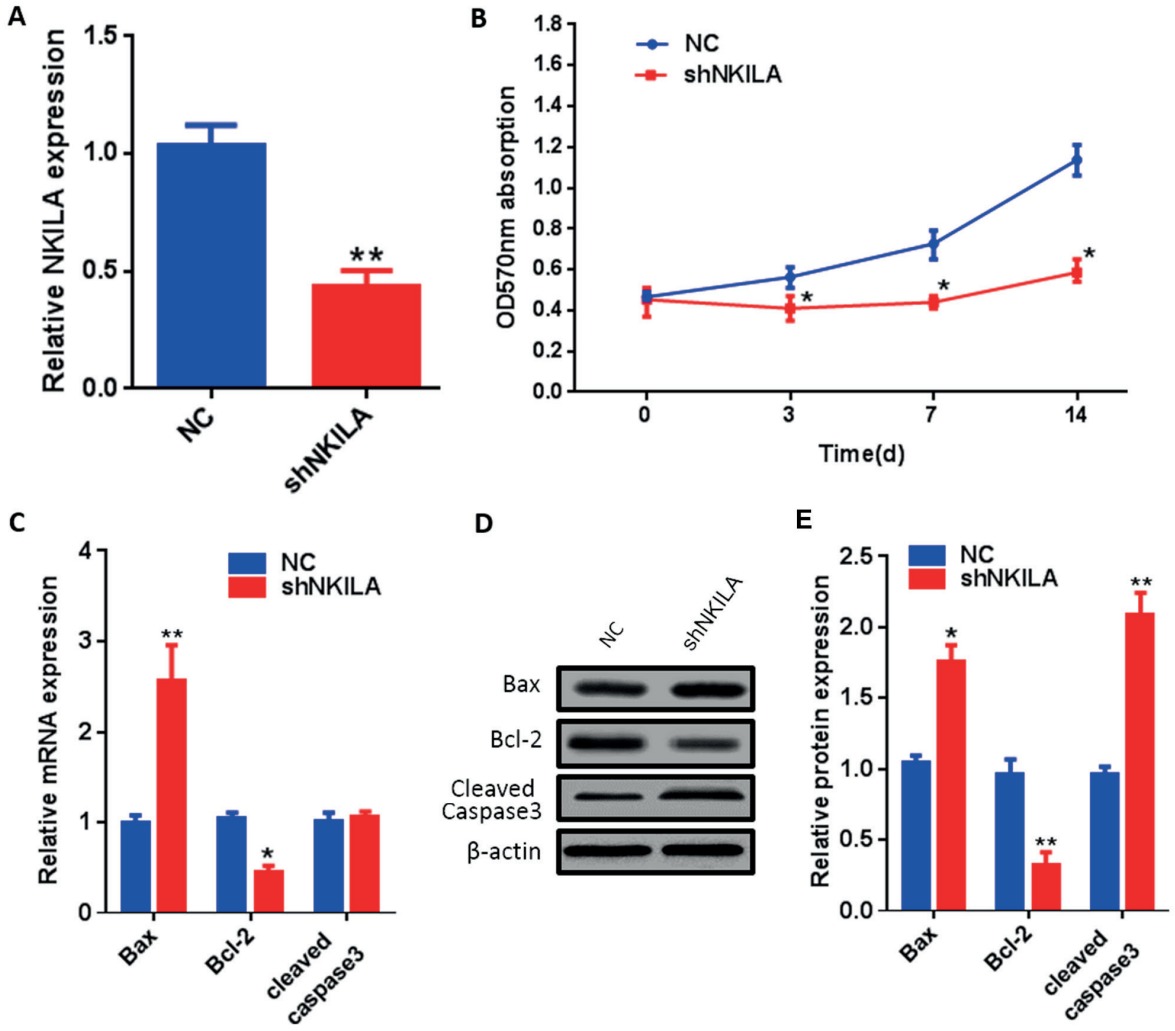

Figure 3. Downregulation of NKILA inhibited proliferation of chondrocytes. **(A)** The NKILA expression was detected after LV- shNKILA infection by RT-PCR. **(B)** The proliferation rate of chondrocytes was analyzed by MTT assay. **(C-E)** The mRNA and protein levels of apoptotic and anti-apoptotic genes were detected by RT-PCR and WB (magnifications x 1.2). Data are shown as mean ± SD based on at least three independent experiments, *p<0.05; **p<0.01.

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml), then cells were cultured at 37°C and 5% CO_2 in an incubator.

Construction of Lentivirus and Cell Transfection

The full length of human NKILA cDNA was synthesized and cloned into a lentivirus (Shanghai GenePharma Co, Ltd, Shanghai, China), which named LV-NKILA, resulting in NKILA overexpression. And the short hairpin NKILA (shNKILA) cDNA was synthesized and cloned into a lentivirus (Shanghai GenePharma Co, Ltd, Shanghai, China), which named LV-shNKILA, resulting in NKILA downregulation. LV-NKILA and LV-shNKILA and their negative control (NC) were respectively added into chondrocytes for 24 hours according to the manufacturer's protocol. Then the transfection efficiency was observed under an inverted fluorescent microscope. The stable cell lines of chondrocytes with NKILA overexpression or NKILA downregulation were constructed after 1 to 2 weeks. Cells were prepared and harvested for further study.

MTT Assay

Chondrocytes were respectively transfected with LV-NC, LV-NKILA or LV-shNKILA for 48 h, then cells were digested and seeded on 96-well plates (5×10^3/well) and cultured in DMEM/F12 medium at a suitable condition for 0, 3, 7 and 14 d. Afterward, 10 μl 5g/l of MTT (Amresco, Solon, OH, USA) was added to each well and cultured for 4 h, and 150 μl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well for 30 mins. Then the proliferation rate of chondrocytes was measured by MTT assay. The absorbance (OD) value of each well was measured at 570 nm with microplate reader (Thermo Fisher, Waltham, MA, USA). Cell viability (P%)=OD (experimental group)/OD (control group)×100%. And every experiment was repeated for three times.

RNA Extraction and Quantitative Real-Time PCR

Total RNA of chondrocytes was extracted by using TRIzol (Invitrogen, Carlsbad, CA, USA) according to its protocol. Reverse transcription was performed by using PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the protocol. PCR primers were synthesized by Gene Pharma (ShangHai Gene Pharma, ShangHai, China). mRNA expressions were detected by SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan). GAPDH was used as a reference gene and $2^{\Delta\Delta CT}$ method was used to calculate the relative gene expressions. The gene specific primers were listed in Table I.

Table I. Sequences of primers for RT-PCR.

Genes	Primer sequences
Lnc-NKILA	Forward: 5'-AACCAAACCTACCCACAACG -3' Reverse: 5'- ACCACTAAGTCAATCCCAGGTG-3'
Lnc-MALAT1	Forward:5'- AGCGGAAGAACGAATGTAAC-3' Reverse: 5'- GAACAGAAGGAAGAGCCAAG-3'
Lnc-MEG3	Forward:5'- CTCAGGCAGGATCTGGCATA-3' Reverse: 5'- CCTGGAGTGCTGTTGGAGAA-3'
Lnc-p21	Forward:5'- CCTGTCCCACTCGCTTTC-3' Reverse: 5'- GGAACTGGAGACGGAATGTC-3'
miR-145	Forward:5'- GGCGTCCAGTTTTCCCAG-3' Reverse: 5'- CAGTGCTGGGTCCGAGTGA-3'
Bax	Forward:5'- GCGACTGATGTCCCTGTCTC-3' Reverse: 5'- AAAGATGGTCACGGTCTGCC-3'
Bcl-2	Forward:5'- CTCCCACAGACTCTGTAAG-3' Reverse: 5'- GCATTACCTGGGGCTGTAATT-3'
Caspase3	Forward:5'-ATTTGGAACCAAAGATCATACA-3' Reverse: 5'- CTGAGGTTTGCTGCATCGAC-3'
U6	Forward:5'- CGCTTCGGCAGCACATATACT -3' Forward:5'- CGCTTCACGAATTTGCGTGTC-3'
β-actin	Forward:5'- CCAAGGCCAACCGCGAGAAGAT -3' Forward:5'- -AGGGTACATGGTGGTGCCGCCA -3'

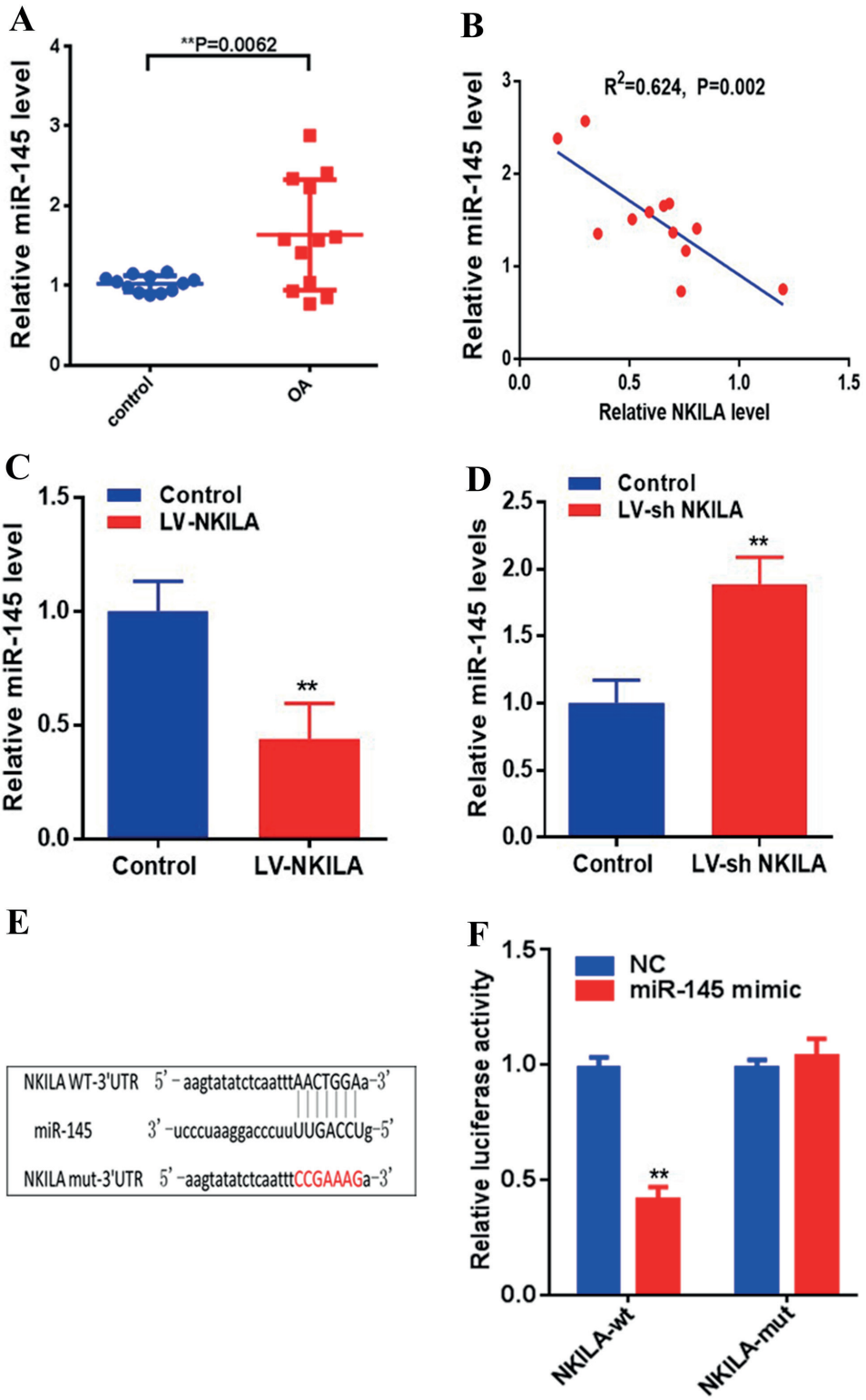

Figure 4. NKILA could directly bind with miR-145 in chondrocytes. **(A)** The levels of miR-145 were detected by RT-PCR in osteoarthritis patients and control patients. **(B)** The relationship between NKILA and miR-145 was analyzed by correlation analysis. **(C-D)** The levels of miR-145 were detected in chondrocytes infected with LV-NKILA and LV-sh NKILA. **(E)** Potential binding sites between NKILA and miR-145 were predicted by starBase v2.0 database and NKILA-wt and NKILA-mut sequences were constructed. **(F)** The luciferase reporter assay was performed to determine the binding site. Data are shown as mean ± SD based on at least three independent experiments, $^{**}p<0.01$.

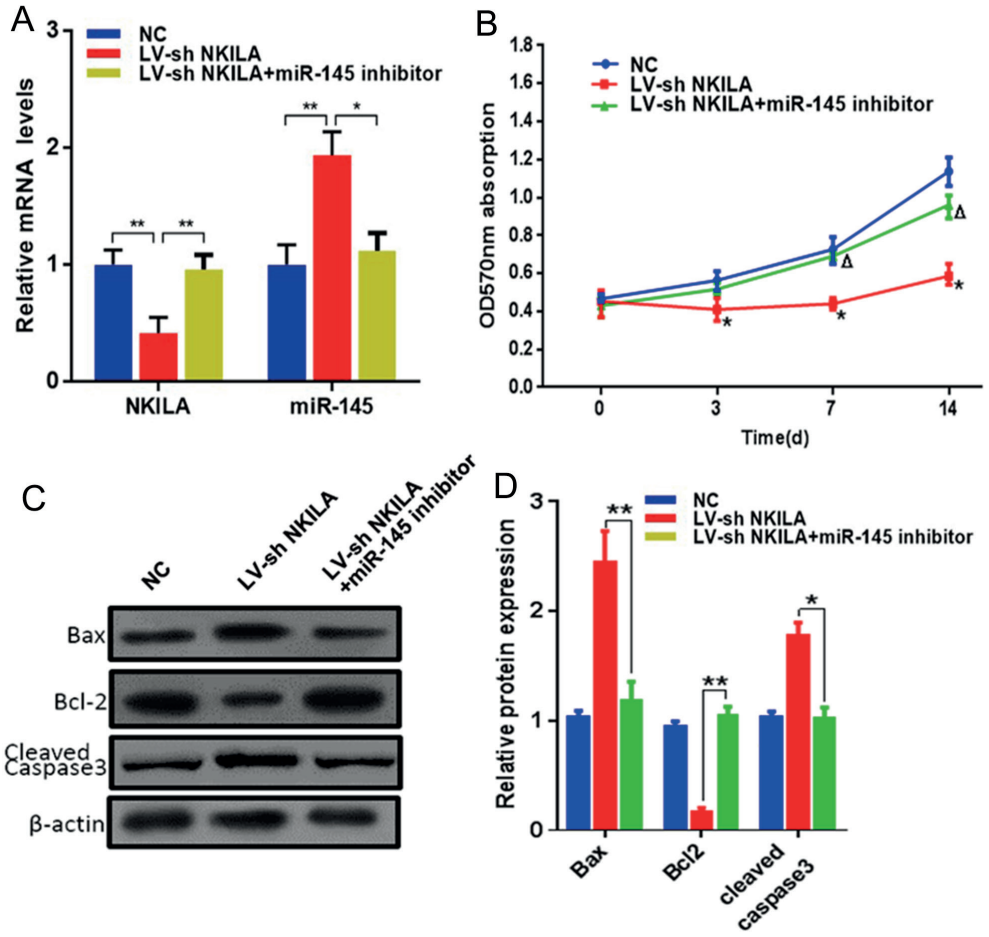

Figure 5. NKILA promoted proliferation of chondrocyte by regulating with miR-145. **(A)** The levels of NKILA and miR-145 were detected in LV-shNKILA or co-transfected with miR-145 inhibitor by RT-PCR. **(B)** The proliferation capacities were detected by MTT assay. **(C-D)** The protein levels of apoptotic genes were also detected by WB (magnifications x 1.2). Data are shown as mean ± SD based on at least three independent experiments. "Δ" means it was significantly different when compared with the LV-shNKILA group; *p<0.05; **p<0.01.

Protein Extraction and Western Blot

After the treatment of chondrocytes in each group for 48 h, the total protein was extracted from cells by using a RIPA lysis buffer (Biyuntian, Shanghai, China). The concentration was measured with BCA kit (Sigma-Aldrich). 50 ug proteins were added to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. After that, the membranes were blocked with 3%BSA (Amresco) at room temperature for 1 h. Then membranes were incubated with primary antibodies overnight at 4°C, all primary antibodies were bought from Cell Signaling Technology (CST), Bax (1:1000), Bcl-2 (1:1000), cleaved caspase-3 (1:1000), IkBα (1:1000), p-IkBα (1:1000), p65 (1:1000), β-actin (1:1000). Then they were subsequently incubated with matched secondary antibodies (1:5000) for 1 h. Protein bands were detected by Pierce ECL Western blot substrate (Thermo Fisher Scientific) with ECL detection system (Thermo Fisher Scientific).

Luciferase Assay

The potential binding sequence NKILA-wt with miR-145 and mutant sequence NKILA-mut were synthesized into pmiR-GLO (Promega, Madison, WI, USA). Chondrocytes were seeded into a 48-well plate and cultured for 12 h; after that, they were co-transfected with the NKILA-wt or NKILA-mut reporter gene plasmid and miR-145 mimic for 24 h. 200 ng plasmids were mixed with Lipofectamine 2000, Renilla luciferase plasmids and DMEM medium for 30 mins at room temperature, then the mixture was added into the co-transfected cells for 24 h. Finally, cells were lysed and the activities of Renilla

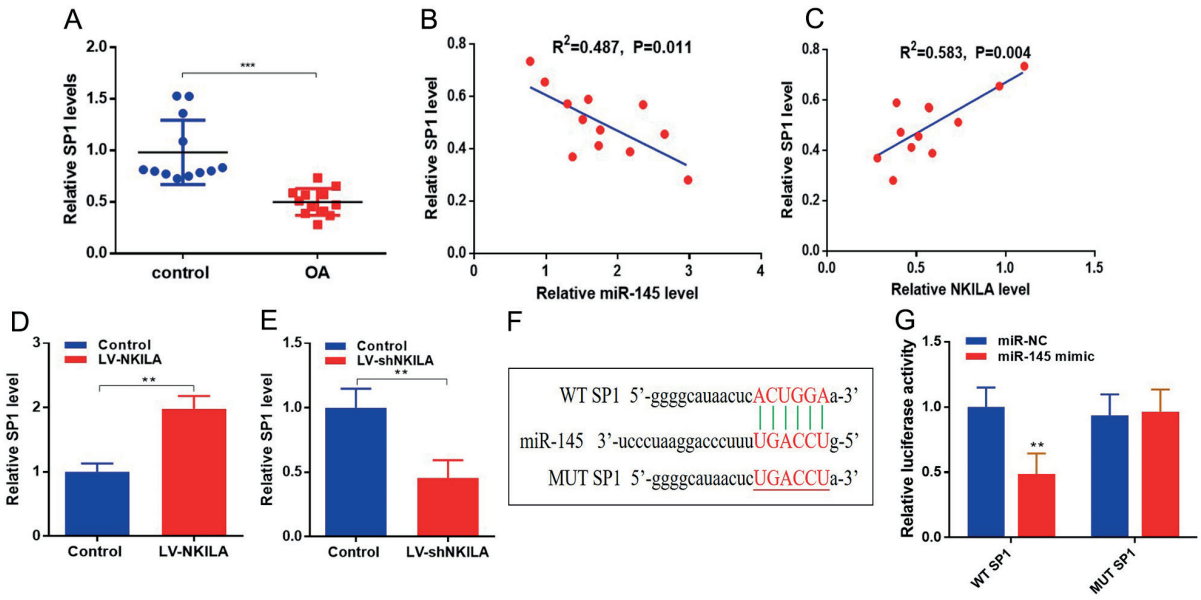

Figure 6. MiR-145 could directly bind with SP1 in chondrocytes. **(A)** The protein levels of SP1 were detected by WB in OA patients and control. **(B-C)** The relationships between SP1 and miR-145, SP1 and NKILA were analyzed by correlation analysis. **(D-E)** The protein levels of SP1 were detected in chondrocytes infected with LV-NKILA and LV-sh NKILA. **(F)** Potential binding sites between SP1 and miR-145 were predicted and WT-SP1 and MUT-SP1 sequences were constructed. **(G)** The luciferase reporter assay was performed to determine the binding site. Data are shown as mean ± SD based on at least three independent experiments, $^{**}p<0.01$, $^{***}p<0.001$.

luciferase and Firefly luciferase were measured by using Promega Luciferase Assay (Promega, Madison, WI, USA). Data were normalized against the activity of the Renilla luciferase and the ratio of them revealed the relative activity of luciferase.

Statistical Analysis

All data were analyzed by SPSS 19.0 (IBM Corp. Released 2010; IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). The data were expressed as the mean±SD. Every assay was applied at least three independent experiments or replicates. The significance between two groups was analyzed by Student's *t*-test and multiple comparison between the groups was performed by SNK method after ANOVA analysis. If *p*-value <0.05, it was considered statistically significant.

Results

NKILA was Reduced in Cartilage Tissue of Human Osteoarthritis

The total RNA of cartilage specimens with osteoarthritis and normal cartilage specimens were extracted and four lncRNAs were detected by RT-PCR. Reports showed that lnc-HNF1A-AS1, lnc-JPX and lnc-TCF7 participated in the proliferation or the apoptosis of some cancers[31-34], and we also detected those gene expressions in our samples. Results showed that NKILA was significantly reduced in cartilage tissue of human osteoarthritis (Figure 1A) ($p<0.05$), while no differences had been found in other three lncRNAs (Figure 1B-D) ($p>0.05$), which indicated that NKILA might play some important roles in osteoarthritis.

Upregulation of NKILA Promoted Proliferation of Chondrocytes

To explore the functions of NKILA in chondrocytes of osteoarthritis, the LV-NKILA was constructed, which resulted in NKILA overexpression. After LV-NKILA infection to human primary chondrocytes, the NKILA level was significantly increased (Figure 2A) ($p<0.001$). The MTT assay was performed to evaluate whether it could regulate the proliferation of chondrocytes. The results showed that the overexpression of NKILA significantly promoted chondrocytes proliferation at 3 d, 7 d and 14 d, compared with the control group (Figure 2B) ($p<0.05$). In addition, we also detected the apoptotic gene expressions to evaluate wheth-

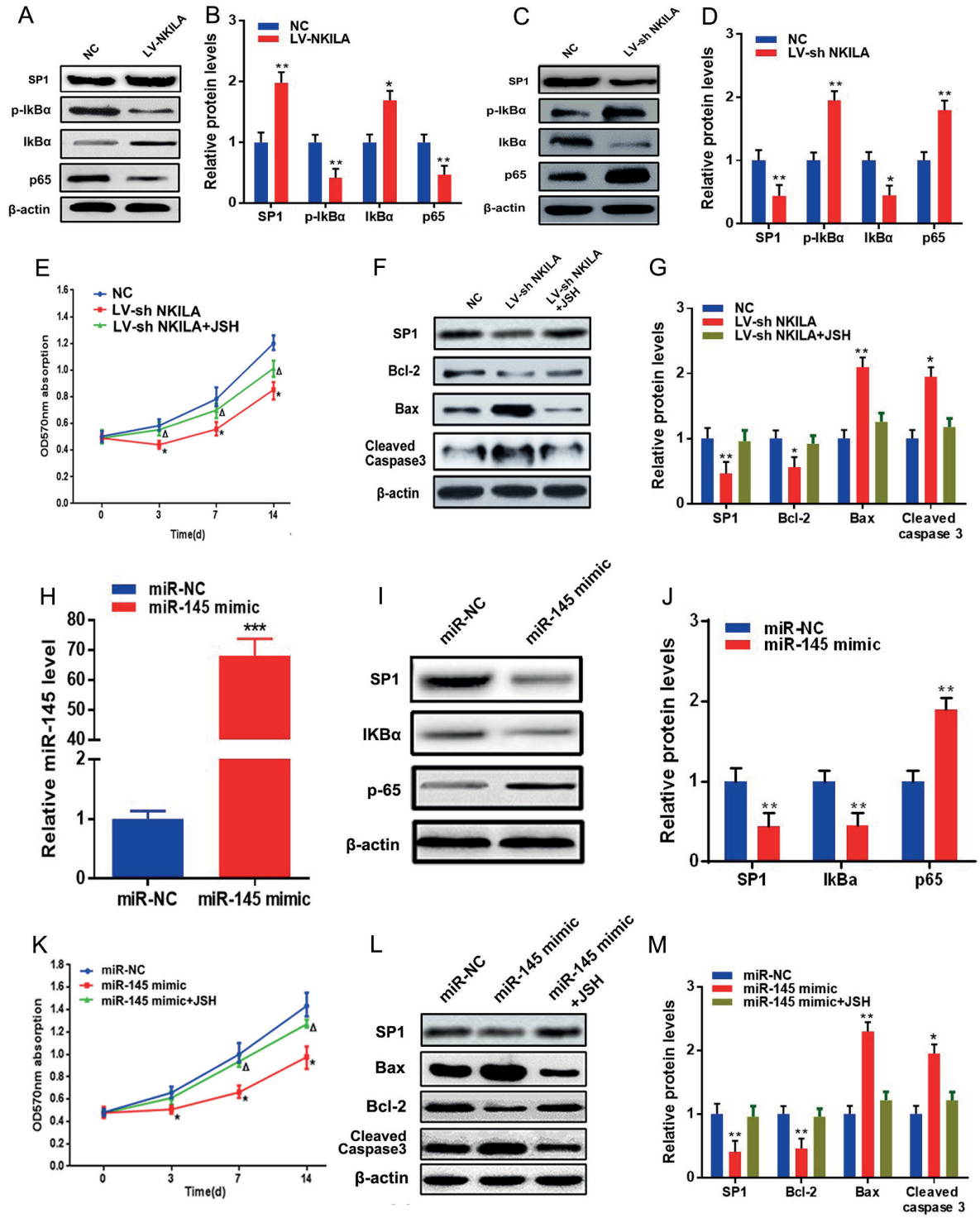

Figure 7. NKILA promoted proliferation and inhibited apoptosis via miR-145/SP1/NF-κB signaling pathway in chondrocytes. **(A-D)** The protein levels of SP1, p-IkBα, IkBα and p65 were detected in chondrocytes transfected with LV-NKILA and LV-shNKILA by WB (magnifications x 1.2). **(E)** The cell capacities were measured in chondrocytes with LV-shNKILA or added with JSH by MTT assay. **(F-G)** The protein levels of SP1, apoptotic and anti-apoptotic genes were also detected by WB (magnifications x 1.2). **(H)** The miR-145 levels were detected after miR-145 mimic transfection into chondrocytes by RT-PCR. **(I-J)** The protein levels of SP1, IkBα and p65 were detected by WB (magnifications x 1.2). **(K)** The proliferation capacities were detected by MTT assay. **(L-M)** The protein levels of SP1 and apoptotic genes were also detected by WB (magnifications x 1.2). Data are shown as mean ± SD based on at least three independent experiments. "Δ" means it was significantly different when compared with the miR-145 mimic group; *p<0.05; **p<0.01, ***p<0.001.

er it could regulate the apoptosis of chondrocytes. Results showed that the apoptotic genes, Bax and cleaved caspase-3, were significantly decreased, while the anti-apoptotic gene Bcl-2 was significantly increased in LV-NKILA group (Figure 2C-E) ($p<0.05$), compared to the control. These results indicated that the upregulation of NKILA promoted proliferation of chondrocytes.

Downregulation of NKILA Inhibited Proliferation of Chondrocytes

To further prove the functions of NKILA in chondrocytes of osteoarthritis, the LV-shNKILA was constructed, which resulted in NKILA downregulation. After LV-shNKILA infection to human primary chondrocytes, the NKILA level was significantly repressed (Figure 3A) ($p<0.01$). The MTT assay showed that the downregulation of NKILA significantly repressed chondrocytes proliferation at 3 d, 7 d and 14 d, compared with the control group (Figure 3B) ($p<0.05$). Furthermore, the apoptotic genes, Bax and cleaved caspase-3, were significantly increased, while the anti-apoptotic gene Bcl-2 was significantly repressed in LV-NKILA group (Figure 3C-E) ($p<0.05$), compared to the control, which further indicated that the downregulation of NKILA repressed the proliferation of chondrocytes. Above all, these results indicated that NKILA could promote proliferation and inhibit apoptosis of chondrocytes.

NKILA Could Directly Bind with miR-145 in Chondrocytes

To explore the detailed mechanism of how NKILA promoted cell proliferation and inhibited apoptosis of chondrocytes in osteoarthritis, we used starBase v2.0 database and miR-145 was identified as a potential targeting miRNA that contained potential binding sites with NKILA. Then we detected miR-145 expressions in osteoarthritis patients by RT-PCR. Results showed that miR-145 was significantly increased in osteoarthritis patients (Figure 4A) ($p<0.001$). Moreover, the expression of miR-145 was negatively correlated with NKILA in osteoarthritis patients (Figure 4B) ($p<0.01$). In addition, the level of miR-145 was significantly decreased in chondrocytes infected with LV-NKILA (Figure 4C) ($p<0.01$), while it was increased in LV-sh NKILA chondrocytes (Figure 4D) ($p<0.01$). These results suggested that NKILA negatively interacted with miR-145, which was predicted to be a target of NKILA (Figure 4E). To further confirm that NKILA could function as a ceRNA to competitively bind with miR-145, NKILA-wt and NKILA-mut sequences were constructed and the luciferase gene reporter assay was performed. Results showed that the relative luciferase activity in chondrocytes co-transfected with NKILA-wt and miR-145 mimic was significantly repressed compared with that in the cells transfected with miR-145 NC. However, the relative luciferase activity in chondrocytes co-transfected with NKILA-mut and miR-145 mimic was reversed (Figure 4F) ($p<0.01$). These results indicated that NKILA could function as a ceRNA to competitively bind with miR-145 in chondrocytes.

NKILA Promoted Proliferation of Chondrocyte by Regulating with miR-145

To further investigate the function of miR-145 in the regulation of chondrocyte proliferation by NKILA, the miR-145 inhibitor was transfected into chondrocytes with LV-sh NKILA. Then we detected the mRNA levels of NKILA and miR-145. The results showed that NKILA level was decreased in LV-sh NKILA, while it was reversed after transfecting with miR-145 inhibitor (Figure 5A) ($p<0.01$). The miR-145 level was increased in LV-sh NKILA, while it was reversed after transfecting with miR-145 inhibitor (Figure 5A) ($p<0.05$). These results suggested that miR-145 inhibitor was successfully transfected into chondrocytes and it could competitively bind with miR-145, thereby upregulating NKILA levels. Furthermore, we detected the proliferation capacity by MTT assay and western blot. Results showed that the cell proliferation was reduced in LV-shNKILA group, while it was significantly increased after transfecting with miR-145 inhibitor (Figure 5B) ($p<0.05$). Western blot results revealed that Bcl-2 was reduced while Bax and cleaved Caspase3 were increased in LV-shNKILA group; however, they were reversed after transfecting with miR-145 inhibitor (Figure 5C,D) ($p<0.05$). These above results indicated that NKILA promoted cell proliferation and inhibited apoptosis by binding with miR-145 in chondrocytes.

MiR-145 Could Directly Bind with SP1 in Chondrocytes

To further explore the role of miR-145 in chondrocytes, TargetScan database was performed to evaluate target genes of miR-145 and SP1 was predicted as a target gene of miR-145, which was associated with cell proliferation via NF-κB signaling pathway[24,25]. We found that the protein level of

SP1 was also repressed in OA patients (Figure 6A) ($p<0.01$), which was negatively correlated with miR-145 and positively correlated with NKILA (Figure 6B,C) ($p<0.01$). Furthermore, we detected the protein levels of SP1 in chondrocytes with LV-NKILA and LV-sh NKILA. Results showed that SP1 was increased following with NKILA overexpression (Figure 6D) ($p<0.01$), while it was repressed following with NKILA downregulation (Figure 6E) ($p<0.01$). These results suggested that SP1 negatively interacted with miR-145, which was positively correlated with NKILA. To further confirm that miR-145 could target bind with SP1, WT-SP1 and MUT-SP1 sequences were constructed and the luciferase gene reporter assay was performed. Results showed that the relative luciferase activity in chondrocytes co-transfected with WT-SP1 and miR-145 mimic was significantly repressed compared with that in the cells transfected with miR-145 NC. However, the relative luciferase activity in chondrocytes co-transfected with MUT-SP1 and miR-145 mimic was reversed (Figure 6F) ($p<0.01$). These results indicated that miR-145 might directly bind with SP1 in chondrocytes and regulated the development of OA.

NKILA Promoted Proliferation and Inhibited Apoptosis via miR-145/SP1/NF-κB Signaling Pathway in Chondrocytes

The detailed mechanism of how NKILA promoted proliferation and inhibited apoptosis in chondrocytes remained unknown, and we found that NKILA and NF-κB signaling are mutually regulated in some cancers[22,23]. Our previous results indicated that miR-145 could directly bind with SP1, which could regulate NF-κB signaling pathway[24,25]. As a result, we assumed that NKILA could bind to miR-145, which targeted at SP1 and regulated NF-κB signaling pathway, thereby regulating cell proliferation and apoptosis in chondrocytes. To verify that assumption, firstly, we detected some NF-κB signaling factor after transfecting with LV-NKILA and LV-shNKILA. Results showed that the protein levels of p-IkBα and p65 were repressed, SP1 and IkBα were increased in the LV-NKILA group (Figure 7A,B) ($p<0.05$); however, these proteins were reversed in LV-shNKILA group (Figure 7C,D) ($p<0.05$). To further verify that NKILA promoted proliferation and inhibited apoptosis via SP1/NF-κB signaling pathway, JSH, a NF-κB signaling inhibitor, was added into chondrocytes infected with LV-sh NKILA. Results showed that the protein level of SP1 and the proliferation capacity was repressed, cell apoptosis was increased in LV-shNKILA group, while they were reversed after the addition of JSH. (Figure 7E-G) ($p<0.05$). These results indicated that NKILA inhibited SP1/NF-κB signaling pathway in chondrocytes.

Finally, we transfected miR-145 mimic into chondrocytes. Results showed that the levels of miR-145 were significantly increased after miR-145 mimic transfection (Figure 7H) ($p<0.01$). The protein levels of SP1 and IkBα were repressed, while p65 was increased in miR-145 mimic group (Figure 7I,J) ($p<0.01$). Then we added JSH into cells with miR-145 mimic to verify that miR-145 regulated cell proliferation via SP1/NF-κB signaling pathway. Results showed that the protein level of SP1 and the proliferation capacity was repressed, cell apoptosis was increased in miR-145 mimic group, while they were reversed after the addition of JSH. (Figure 7K-M) ($p<0.05$). Above all, these results indicated that NKILA might promote proliferation and inhibit apoptosis of chondrocytes through binding with miR-145, which targeted at binding with SP1 and then regulated NF-κB signaling pathway.

Discussion

Recent studies have found that lncRNAs are closely related to the development of OA. The abnormal expressions of many lncRNAs are involved in the extracellular matrix degradation, proliferation and apoptosis of chondrocytes[18-21]. Increasing evidence indicates that lncRNAs are biological regulators of OA and can be used as a biomarker for early prevention, clinical diagnosis and treatment of OA[14-17]. But the specific functions and detailed mechanisms of lncRNAs in the development of OA remain unclear. Our group found that NKILA was downregulated in osteoarthritic cartilage tissues, which might play some roles in the progression of OA.

To further investigate the roles of NKILA in OA, we constructed the LV- NKILA and LV-shNKILA, resulted in NKILA overexpression and downregulation, which were infected to chondrocytes. After that, the cell viability, apoptotic and antiapoptotic genes were detected. Results showed that the overexpression of NKILA promoted the proliferation of chondrocytes and inhibited apoptosis; on the contrary, the downregulation of NKILA resulted with opposite results, indicating that NKILA promoted proliferation and inhibited apoptosis of chondrocytes.

However, the detailed mechanism remained unknown. We used starBase v2.0 database and miR-145 was identified as a potential target miRNA that contained potential binding sites with NKILA. Next, we detected miR-145 expressions in OA patients. Results showed that miR-145 was increased in OA patients, which was negatively correlated with NKILA in OA patients. Luciferase gene reporter assay indicated that NKILA could function as a ceRNA to competitively bind with miR-145 in chondrocytes. Researchers[35-38] showed that miR-145 regulated a variety of processes in some cancers, including cell proliferation, differentiation and EMT. In our study, NKILA inhibited miR-145 and then promoted chondrocyte proliferation, which was consistent with the previous reports. To further investigate the function of miR-145 in the regulation of chondrocyte proliferation by NKILA, the miR-145 inhibitor was transfected into chondrocytes with LV-sh NKILA. We found that the cell proliferation capacity and anti-apoptotic genes were reduced, while the apoptotic genes were increased in LV-shNKILA group; however, they were reversed after transfecting with miR-145 inhibitor. These data indicated that NKILA promoted cell proliferation and inhibited apoptosis by binding with miR-145 in chondrocytes.

SP1 is a member of SP family in transcription factors, which is ubiquitously expressed in mammalian cells and can bind to and act through GC boxes to regulate gene expressions and cell proliferation, such as NF-κB signaling pathway[22-25]. And TargetScan database was used to predict target genes and SP1 was predicted as a potential target gene of miR-145. Then, we detected SP1 protein expressions in OA patients. Results showed that SP1 was repressed in OA patients, which was negatively correlated with miR-145 and positively correlated with NKILA. Furthermore, luciferase assay confirmed that miR-145 could directly bind with SP1. These findings indicated that miR-145 might directly bind with SP1 in chondrocytes and regulate the development of OA.

Collectively, we assumed that NKILA could bind to miR-145, which targeted at SP1 and regulated NF-κB signaling pathway, thereby regulating cell proliferation and apoptosis in chondrocytes. To prove our assumption, we found that the protein levels of p-IkBα and p65 were repressed, and SP1 and IkBα were increased in the LV-NKILA group. However, these proteins were reversed in LV-shNKILA group. To further verify that NKILA promoted proliferation and inhibited apoptosis via SP1/NF-κB signaling pathway, JSH, a NF-κB signaling inhibitor, was added into chondrocytes infected with LV-sh NKILA or miR-145 mimic. Data showed that the repressed SP1 were both reversed after the addition of JSH, and the repressed proliferation capacities and promoted cell apoptosis were also reversed after the addition of JSH. Above all, these results indicated that the reduced NKILA might inhibit proliferation and promote apoptosis of chondrocytes through binding with miR-145, which targeted at binding with SP1 and then regulated NF-κB signaling pathway.

Conclusions

This study reveals that lncRNA NKILA is reduced in human osteoarthritic cartilage tissues. Furthermore, we firstly uncover that the reduced NKILA could function as a ceRNA to improve miR-145, which inhibited SP1 expression and regulated NF-κB signaling pathway, thereby promoting tissue inflammation, inhibiting proliferation and promoting apoptosis of chondrocytes, which may be used as a promising prognostic marker and a potential target for osteoarthritis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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