Knockdown of Long Non-coding RNA LINC00473 Protects CHON-001 Cells against Interleukin-1β-Induced Cell Injury

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Osteoarthritis (OA) is a chronic joint disease with high prevalence. However, effective treatment options for OA are still lacking. It was previously reported that LINC00473 was upregulated in patients with OA and upregulated LINC00473 might be associated with the progression of OA; however, the role of LINC00473 in OA remains to be investigated. CHON-001 human chondrocyte cells stimulated with 10ng/mL interleukin (IL)-1β were utilized to mimic OA in vitro. Protein expression, cell apoptosis and cell proliferation of CHON-001 cells were investigated by Western blot, Annexin V and propidium iodide (PI) double staining, cell counting-8 kit assay and immunofluorescence staining respectively. The result indicated IL-1β triggered viability decrease and apoptosis in CHON-001 cells, which was alleviated by LINC00473 knockdown. Meanwhile, IL-1β-induced upregulation of cleaved caspase 3 and Bax were ameliorated by LINC00473 knockdown. Likewise, IL-1β-induced downregulation of X-linked inhibitor of apoptosis protein was alleviated by LINC00473 knockdown. In addition, LINC00473 knockdown protected CHON-001 cells against IL-1β by inhibiting the methylation of LIM mineralization protein (LMP)-1 gene. Moreover, c-Jun N-terminal kinase (JNK)/nuclear factor-kappaB (NF-κB) signaling pathway was proved to be involved in the cell protective effect of LINC00473 knockdown in IL-1β treated CHON-001 cells. Taken together, LINC00473 knockdown protected CHON-001 cells from IL-1β induced cell injury via inhibition of the methylation of LMP-1. Thus, LINC00473 might possibly act as a novel therapeutic target for OA.

Key words osteoarthritis; LINC00473; LIM mineralization protein-1; nuclear factor-kappaB (NF-κB)

INTRODUCTION

Osteoarthritis (OA) is a highly prevalent chronic joint disease. OA is characterized by durative degeneration of articular cartilage and the associated pathological changes to synovium, subchondral bone, and surrounding joint tissues.1,2) At present, OA is regarded as a global public health problem as well as a principle cause of pain and disability worldwide.3,4) It is reported that 240 million people suffer from OA, including about 10% of men and 18% of women over 60 years of age globally.5) Current treatment options for OA include weight loss, exercise, oral and topical non-steroidal anti-inflammatory drugs (NSAIDs), and intra-articular therapies.3) However, these treatment strategies are lack of effectiveness.2) Given the high and growing prevalence of OA, it is urgent to identify more effective treatments for patients with OA.5) Therefore, further research on the pathology of OA and identification of new therapeutic target may be of benefit in helping to discover more effective drugs for such patients.

Long noncoding RNA (lncRNA), a class of non-protein coding transcript greater than 200 nucleotides in length, which already emerged as important effectors in the regulation of OA.5) LncRNAs could regulate the expression level of downstream genes through sponging microRNAs (miRNAs).5) In addition, microRNAs repress the expression of target gene through binding to the 3′ untranslated region of the genes.6) LncRNAs competitively bind with miRNAs and repressed the combination of miRNA and downstream genes, which eventually increases downstream gene expression.2,5,6)

It is reported that IncRNA LINC00473 was notably up-regulated in patients with OA and was associated with severe pain.7) Nevertheless, the role of LINC00473 in the progression of OA is still waiting to be investigated. Therefore, this study aimed to explore the role of LINC00473 in OA using an in vitro cell model.

MATERIALS AND METHODS

Cell Culture CHON-001 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) medium (Thermo Fisher, Waltham, MA, U.S.A.) containing 100 mg/mL streptomycin, 10% fetal bovine serum and 100 U/mL penicillin at 37°C in a humidified atmosphere with 5% CO2. The cells were incubated with interleukin (IL)-1β (10ng/mL, Sigma-Aldrich, St. Louis, MO, U.S.A.) for 24 h to mimic OA status in vitro.

Western Blot Total proteins in CHON-001 cells from each group were extracted with radio immunoprecipitation assay (RIPA) buffer (Beyotime, China). Bicinchoninic acid (BCA) protein assay kit (Beyotime, China) was utilized to quantify the total protein. Equal amounts of protein samples (20 µg) were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. Separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. Then, 5% skim milk was used to block PVDF membrane for 2h. Next, the PVDF membrane was incubated with primary antibodies overnight at 4°C. Primary antibodies utilized for Western blot assay were listed as blow: Anti-matrix metalloproteinase (MMP)1 (1 : 1000, Abcam, Cambridge, MA, U.S.A.), anti-MMP13 (1 : 1000, Abcam), anti-

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aggreccan (1:1000, Abcam), anti-cleaved caspase 3 (1:1000, Abcam), anti-X-linked inhibitor of apoptosis protein (XIAP) (1:1000, Abcam), anti-Bax (1:1000, Abcam), anti-XIAP (1:1000, Abcam), anti-LIM mineralization protein-1 (LMP-1) (1:1000, Abcam), anti-p-c-Jun N-terminal kinase (JNK) (1:1000, Abcam), anti-JNK (1:1000, Abcam), anti-p-inhibitor of kappaB (IkB) (1:1000, Abcam), anti-IkB (1:1000, Abcam), anti-p-p65 (1:1000, Abcam), anti-p65 (1:1000, Abcam), anti-β-actin (1:1000, Abcam). The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBST) for three times and then cultured with corresponding anti-rabbit secondary antibodies (1:2000, Abcam) for 1h at room temperature. Blots were visualized using chemiluminescence method with enhanced chemiluminescence (ECL) reagent (Santa Cruz, Dallas, TX, U.S.A.). Image J software (National Institute of Health, Bethesda, MD, U.S.A.) was used to perform grey value analysis of blots. Relative protein expression levels were normalized to β-actin and presented as fold changes.

**Knockdown of LINC00473 Using with Short-Hairpin RNA (shRNA)** LINC00473 shRNA1, LINC00473 shRNA2 and control shRNA were constructed in pcDNA3.1 by GenePharma. CHON-001 cells were transfected with pcDNA 3.1 vector containing LINC00473 shRNA sequence, control shRNA or empty pcDNA 3.1 vector using Lipofectamine 2000 kit (Thermo Fisher). The efficiency of LINC00473 knockdown was estimated by quantitative real-time RT-PCR (qRT-PCR) at 48h after transfection. Oligonucleotide sequences of shRNAs were: shRNA1, 5′-ACCCTTGCTGCAAGATCAGTTTATTTCAGAGATTAACCTGATTTTGCAAGACGGTTTTT-3′; shRNA2, 5′-GAAAGACAAAGCGGACACCTTAGAAATTCAAGAGATTATCTAGTGTCGCTGCTTCTTCTT-3′. Quantitative Real-Time RT-PCR (qRT-PCR) Total RNAs were extracted from CHON-001 cells in each group using TRIzol reagent. PrimeScript RT-PCR kit (TaKaRa, Japan) was applied to synthesize cDNA as per the manufacturer’s recommendation. Then, the cDNAs were subjected to quantitative real-time PCR using SYBR Green Premix Ex Taq (TaKaRa) to determine the expression of LINC00473 to quantitative real-time RT-PCR using SYBR Green PremixEx Taq (TaKaRa) to determine the expression of LINC00473 at an ABI Prism 7500 Sequence Detection System (PerkinElmer, Inc., Waltham, MA, U.S.A.). Relative expression level of genes was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results were analyzed using 2−ΔΔCt method. Primers sequences of LINC00473: forward, 5′-GGAACAATCATTCTCTCACTTGCC-3′; reverse, 5′-TTGTTGTCATTGTACCTCCTTGTC-3′. Primers sequences of ACTIN: forward, 5′-GTCCTACCCGCCCAAATGCTCTTCT-3′; reverse, 5′-TGCTGTCACCCCTTACCCGGCT-3′. Primers sequences of LMP-1: forward, 5′-CCCCTATCTGCCAGAGGAGTACTT-3′; reverse, 5′-GCAAGGACAGGAGGAGTTGTC-3′.

**Cell Viability Assay by Cell Counting Kit-8 (CCK-8)** CHON-001 cells from each group were collected, counted, and adjusted to 5×10^4/mL. Cells (100μL/well) were placed into 96 well plates and subjected to indicated treatments. Then, cell viability was measured with CCK-8 assay (Dojindo Laboratories, Japan) accordingly to manufacturer’s procedures. OD450 (absorbance at wavelength 450) was measured using a spectrophotometer.

**Immunofluorescence Staining for Ki67** Immunofluorescence staining for Ki67 was performed to estimate cell proliferation as well. After indicated treatment, CHON-001 cells were washed with PBS for three times and fixed with paraformaldehyde (4%) for 20min at room temperature. Then, Triton X-100 (0.2%) was added to permeabilize the cells for 10min. Next, the cells were blocked with 5% bovine serum albumin for 25min followed by incubation with primary antibody against Ki67 (1:500, Abcam) at 4°C for overnight. Next day, the cells were incubated with Alexa Fluor conjugated secondary antibody for 1h at room temperature. Cell nucleus was counter stained with 4′,6-diamidino-2-phenylindole (DAPI) (10mg/mL) for 15min at room temperature. Finally, five random fields of each group were selected and Ki67 positive cells were calculated.

**Apoptosis Assay** Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Thermo Fisher) was used for cell apoptosis assay. Briefly, the cells were harvested, washed with PBS for three times. Then, the cells were subjected to Annexin V-FITC and propidium iodide (PI) double staining for 20min followed by flow cytometry. Apoptosis rates were observed and analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

**Methylation Analysis of LIM Mineralization Protein-1 (LMP-1)** Methylation status of LMP-1 gene was detected by methylation-specific PCR (MS-PCR). Genomic DNA in CHON-001 cells was isolated from cells with Genomic DNA Purification Kit (Thermo Fisher). The extracted genome DNA was subjected to bisulfite modification with an EpiTect Bisulfite kit (Qiagen, Venlo, The Netherlands) in accordance with manufacturer’s procedures. Then, MS-PCR was employed to determine methylation status in the promoter region of LMP-1. Methylated- or unmethylated-specific primer pairs were designed to amplify the same region in the 5′-untranslated regions (UTR) of LMP-1 gene. The conditions of PCR were listed as below: hot-start at 95°C for 2min, denaturing at 94°C for 30s, annealing at 55°C for 30s, extension at 72°C for 30s, and a final elongation at 72°C for 10min. MS-PCR products were visualized by electrophoresis on 2% agarose gels. Primers specific for methylated DNA were LMP-1M forward, 5′-GTTAGGTTATCTGGCTTTTGCAAG-3′; reverse, 5′-CGAGAACGATCCGCTC-3′; Primers for unmethylated DNA were LMP-1U forward, 5′-GTTAGGTTATCTGGCTTTTGCAAG-3′; reverse, 5′-AAGAGAATCCCTCTCAAC-3′. Statistical Analysis Statistical analyses were tested using one-way ANOVA followed by Tukey’s test. p < 0.05 was considered statistically significant. All data were presented as mean ± standard deviation of mean (S.D.). GraphPad prism version 8.0 (Graphpad Software, San Diego, CA, U.S.A.) was used for statistical analysis. All experiments were repeated as least in triplicates.

**RESULTS**

The Stimulation of CHON-001 Cells with IL-1β to Mimic OA in Vitro It has been proved that IL-1β plays a critical role in the pathogenesis of OA through driving a cascade of inflammatory and destructive responses within the joint. It has been proved that IL-1β plays a critical role in the pathogenesis of OA through driving a cascade of inflammatory and destructive responses within the joint. In the present study, 10ng/mL IL-1β was applied to stimulate CHON-001 cells to induce OA status in vitro. After 24h of incubation with IL-1β, the levels of MMP1, MMP3 and aggrecan in CHON-001 cells were evaluated by Western blot (Fig. 1A). The results illustrated that IL-1β
notably induced elevated expression of MMP1 and MMP13 in CHON-001 cells (Figs. 1B, C). Meanwhile, the level of aggregcan was diminished by IL-1β (Fig. 1D). However, these phenomena indicated OA phenotype in CHON-001 cells. These data suggested in vitro OA cell model was established.

**LINC00473 Knockdown Alleviated IL-1β-Induced Cell Injury**

To knockdown LINC00473 in CHON-001 cells, LINC00473 shRNA1 and LINC00473 shRNA2 were used. The level of LINC00473 was evaluated by qRT-PCR at 48 h after transfection. The result of qRT-PCR showed that LINC00473 shRNA2 achieved better inhibitory effect (Fig. 2A). Hereafter, LINC00473 shRNA2 was used to knockdown LINC00473 in the following experiments. Next, the level of LINC00473 in IL-1β-stimulated CHON-001 cells was estimated. As shown in Fig. 2B, the level of LINC00473 in cells was remarkably increased by IL-1β. Moreover, IL-1β-induced upregulation of LINC00473 was inhibited by LINC00473 shRNA2 (Fig. 2B). In order to investigate the effect of LINC00473 shRNA2 on cell proliferation and apoptosis of CHON-001 cells, CCK-8 assay, Ki67 staining and apoptosis assay were performed, respectively. The results indicated that IL-1β significantly decreased the cell viability in CHON-001 cells (Fig. 2C), while IL-1β induced cell injury was alleviated by LINC00473 knockdown. Consistently, IL-1β induced proliferation decline of CHON-001 cells was ameliorated by LINC00473 knockdown. These results demonstrated that LINC00473 knockdown was able to alleviate IL-1β-induced cell injury in CHON-001 cells.

**IL-1β-Induced Dysregulations of Aggrecan, MMP13, Cleaved Caspase 3, XIAP and Bax Were Revered by LINC00473 Knockdown**

Next, the expression of aggrecan, cleaved caspase 3, MMP13, XIAP and Bax were measured by Western blot assay (Fig. 3A). As shown in Figs. 3B and
C, IL-1β induced downregulation of aggrecan and upregulation of MMP13 were both reversed by LINC00473 shRNA2. Additionally, IL-1β upregulated the levels of cleaved caspase 3 and Bax and downregulated the expression of XIAP in CHON-001 cells (Figs. 3D–F). Consistently, IL-1β-induced dysregulations in cleaved caspase 3, Bax and XIAP were all inhibited by LINC00473 shRNA2 (Figs. 3D–F). Taken these results together, IL-1β-induced dysregulations of aggrecan, MMP13, cleaved caspase 3, XIAP and Bax were reversed by LINC00473 knockdown.

LINC00473 Knockdown Decreased the Methylation of LMP-1 in IL-1β-Treated Cells  LMP-1 is a protein facilitates cartilage, bone, and tendon repair.10 The methylation status in the promoter region of LMP-1 was evaluated using MS-PCR. As illustrated in Fig. 4A, we found IL-1β induced the methylation of LMP-1 promoter, while LINC00473 shRNA2 decreased methylation of LMP-1 promoter. Meanwhile, LINC00473 knockdown decreased the methylation...
of LMP-1 in IL-1β-treated cells (Fig. 4A). Next, the level of LMP-1 in CHON-001 cells from each group was measured by qRT-PCR (Fig. 4B). As expected, the result of qRT-PCR demonstrated that IL-1β resulted in downregulation of LMP-1 gene. In contrast, LINC00473 knockdown leaded to upregulation of LMP-1. Furthermore, IL-1β-induced downregulation of LMP-1 was reversed by LINC00473 shRNA2 (Fig. 4B). These findings demonstrated that LINC00473 knockdown could alleviate IL-1β-induced methylation of LMP-1.

**LINC00473 Regulated JNK/Nuclear Factor-kappaB (NF-κB) Signaling Pathway in CHON-001 Cells**

Next, Western blot assay was performed to investigate the mechanism by which LINC00473 shRNA2 regulated CHON-001 cells viability. The expression of LMP-1, p-JNK, JNK, p-IκB, IκB, p-p65 and p65 were detected (Fig. 5A). As illustrated in Fig. 5B, IL-1β suppressed the protein expression of LMP-1. In the opposite, LINC00473 shRNA2 remarkably increased the protein expression of LMP-1. Moreover, IL-1β-induced downregulation of LMP-1 was reversed by LINC00473 shRNA2 (Fig. 5B). In addition, IL-1β resulted in the increase of p-JNK, p-IκB and p-p65, which were all ameliorated by LINC00473 shRNA2 (Figs. 5C–E). These findings suggest that LINC00473 regulated the viability of CHON-001 cells via regulating JNK/NF-κB signaling pathway.

**DISCUSSION**

OA is a highly prevalent chronic joint disease without satisfactory treatment options. Identification of new therapeutic target may be of benefit in helping to discover effective drugs. Our findings demonstrated that LINC00473 knockdown alleviated IL-1β-induced cell apoptosis and viability decline, contributing to identify new targets for the treatment of OA. To the best of our knowledge, most of current researches on LINC00473 are limited to cancer related therapeutic areas. The findings of this study got new insights of regulation effect of LINC00473 on the viability of human chondrocytes, revealing its potentials role in OA. At the same time, the present study may inspire extensive research of LINC00473 in other therapeutic areas.

As we know, LINC00473 plays an important role in many types of cancers. A previous study of Mo et al. reported that LINC00473 promoted hepatocellular carcinoma progression via increasing the expression of HMGA2. Furthermore, Xing
et al. reported that LINC00473 promoted the proliferation of prostate cancer cells via Janus kinase–signal transducer and activator of transcription 3 (JAK–STAT3) signaling pathway by regulating miR-195-5p/SEPT2 axis. In another study of Bai et al., LINC00473 accelerated the invasion, proliferation and migration in breast cancer cells. In the contrast, our findings demonstrated that knockdown of LINC00473 promoted the proliferation of IL-1β-treated CHON-001 cells. The reason of this difference may be because LINC00473 regulates cell proliferation via distinct mechanisms in normal and cancer cells.

In the present study, we illustrated that LINC00473 knockdown promoted the cell viability of CHON-001 cells through regulating methylation of LMP-1 gene via JNK/NF-κB signaling pathway. These findings are of benefit to further elucidate the protective effect of LINC00473 knockdown in OA. LMP-1 was regarded as an intracellular protein that has been proved to induce bone formation in vitro and in vivo. The association between LMP-1 and JNK signaling pathway were found in another previous study of Liu et al. They reported that when LMP-1 exerted its anti-inflammatory role in lipopolysaccharide (LPS)-stimulated pre-osteoclasts, the phosphorylation of LMP-1 exerted its anti-inflammatory role in lipopolysaccharide (LPS)-stimulated pre-osteoclasts, the phosphorylation of JNK was attenuated. Our findings of LMP-1 regulated JNK/NF-κB signaling pathway were in line with the findings of Liu et al. However, the detailed mechanism underlying the protective effect of LINC00473 knockdown on IL-1β-treated CHON-001 cells was not fully revealed yet. The key molecules by which LMP-1 mediated the regulation of JNK/NF-κB were waiting to be investigated.

CONCLUSION

LINC00473 knockdown protected CHON-001 cells from IL-1β-induced apoptosis and cell viability decline via decreasing methylation of LMP-1. Furthermore, JNK/NF-κB signaling pathway was involved in the protective effect of LINC00473 knockdown. Thereby, LINC00473 may inspire the development of novel targeted therapy for OA.

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Conflict of Interest The authors declare no conflict of interest.

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