Screening of novel long non-coding RNA in the progression of osteoarthritis

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Screening of novel long non-coding RNA in the progression of osteoarthritis

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ABSTRACT

Screening of novel long-noncoding RNA in the progression of osteoarthritis

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Osteoarthritis (OA) is a common degenerative disease that is characterized by joint damage, inflammation, and persistent pain—primarily attributed to cartilage deterioration. Despite multiple therapeutic approaches, a definitive cure remains elusive, thereby prompting the need for comprehensive research to uncover the fundamental causes and develop effective treatments. Recent insights highlight the critical role of long non-coding RNAs (lncRNAs) in various developmental and epigenetic regulatory processes, which are relevant to the management of OA. This study focused on examining the differential expression of lncRNAs in primary human chondrocytes and elucidating their potential regulatory role in modulating the inflammatory response within chondrocytes. The transcriptomic differences were examined by RNA-seq analysis and any differentially expressed lncRNAs between human intact and damaged tissue-derived chondrocytes were identified. 26 lncRNAs exhibited upregulation by more than 6-fold in chondrocytes derived from damaged tissue, whereas 15 lncRNAs showed an upregulation of more than 2-fold in an in vitro inflammatory

environment induced by TNF-α. After transfecting 15 siRNAs specific to each target lncRNA into TC28a2 cells, seven siRNAs were observed to exhibit a knockdown efficiency of more than 40%. Seven siRNAs were transfected into TC28a2 cells and an inflammatory environment was induced by using TNF-α. The expression of type II collagen (COL2A1) was assessed through Western blot analysis and revealed that LOC107985352 and LOC107985369 silencing by siRNA resulted in an elevated level of COL2A1. Furthermore, the downregulation of LOC107985352 and LOC107985369 resulted in a decreased expression of catabolic and inflammation-related genes. These findings suggest that the downregulation of LOC107985352 and LOC107985369 in osteoarthritic chondrocytes may impact chondrocyte homeostasis by regulating the inflammatory response; furthermore, they indicate that these lncRNAs may serve as important regulators and promising therapeutic targets in the progression of OA.

Keywords: osteoarthritis, long non-coding RNA, inflammatory response, RNA-sequencing, chondrocyte

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I. INTRODUCTION

Osteoarthritis (OA) is a prevalent degenerative disease, which is often found in the elderly. It is characterized by irreversible joint damage, which is caused by abnormalities within or around the joints. OA is typified by the progressive degradation of articular cartilage and the emergence of synovial inflammation, which leads to persistent inflammation and chronic pain. OA affects multiple joints in the body, with cartilage damage being its primary concern.[1] In recent years, a variety of therapeutic approaches have been utilized to manage OA, including non-steroidal anti-inflammatory drugs (NSAIDs), anti-inflammatory agents, and treatments involving hyaluronic acid.[2] Existing treatments predominantly prioritize pain mitigation by modulating inflammatory responses. However, despite ongoing therapeutic efforts, a definitive cure for OA remains elusive. Consequently, significant challenges persist in effectively managing the development of OA. To overcome these

obstacles, it is imperative that comprehensive investigations aimed at elucidating the fundamental etiological factors of OA are performed using a multifaceted research approach.

Inflammatory reactions play a crucial role in OA, including the production of inflammatory cytokines, chemokines, and other mediators from the synovium and chondrocytes. Additionally, the secretion of cytokines, such as IL-1β (interleukin 1 beta), TNF (tumour necrosis factor), and IL-6 (interleukin 6), further contributes to disease progression.[3,4] During the initial phases of OA, there is a collapse in the pericellular matrix that surrounds cartilage cells, which leads to the exposure of cell surface receptors. This process is accompanied by a rapid decrease in proteoglycan levels, which is driven by the expression of genes, such as ADAMTS5, MMP13, and aggrecanases, ultimately, causing the collagen network to breakdown.[5] Moreover, the release of cytokines associated with inflammatory responses, including IL-1β, TNF, and IL-6, along with the activation of the MAP mitogen-activated protein kinase (MAPK) responsible for catabolic processes, have been identified as highly significant factors that contribute to OA progression.[6] These signalling systems work in conjunction with other signalling systems, such as bone morphogenetic proteins (BMPs) and Wnt, to interfere with the anabolic activities of the cartilage cells and generate various matrix-degrading proteins.[7] During the late stages of OA, there is an upregulation in genes and proteins associated with hypertrophy. Additionally, factors linked to angiogenesis rise and cause vascular invasion and cartilage expansions, thereby accelerating the deterioration of knee cartilage.[8,9] Initially considered to be driven primarily by mechanical factors, OA has revealed itself to be a far more complex disease involving the release of inflammatory mediators by cartilage, bone, and synovium.[10-12]

Most transcripts are non-coding RNAs (ncRNAs), whereby approximately 90% of DNA is transcribed, yet only 2% is translated into proteins.[13] Among them, lncRNA typically refers to RNA transcripts that are longer than 200 base pairs and do not encode proteins. Similar to mRNA, lncRNA is transcribed by RNA polymerase II (Pol II), and undergoes the addition of the 5' cap, splicing, and polyadenylation; however, it distinguishes itself significantly in terms of its structural and functional attributes, revealing a notably intricate regulatory profile.[14] LncRNAs are known to participate in diverse biological processes, including chromosome structure alterations, transcription, translation, RNA biogenesis, and cellular stability, which is achieved through interactions with other RNAs, DNA, and proteins.[15] Moreover, lncRNA could potentially be used as therapeutic targets due to their high tissue-specificity and regulation of specific facets of cellular networks, thereby indicating that lncRNAs are superior to proteins in terms of potential and the undesired toxic effects associated with their targeting. Additionally, the lack of translation, rapid degradation, and low expression levels may facilitate quicker effects with lower doses.[16]

Representative lncRNAs, such as H19, GAS5, MALAT1, XIST, and HOTAIR, have been identified as key players in the development of OA, exerting their regulatory influence through interactions with target miRNAs or proteins.[17] Notably, H19 levels increase in osteoarthritic tissues, and MiR-675, derived from H19, is recognized to enhance cartilage cell substrate production via the transcriptional activity of type II collagen (COL2A1), while simultaneously inhibiting the proliferation and apoptosis of cartilage cells by modulating miR-106a-5p.[18,19] MALAT1 plays a role in the development of OA by enhancing cell growth and facilitating cartilage formation, while also suppressing cell apoptosis and preventing extracellular matrix (ECM) degradation. The underlying mechanism concerning the function of MALAT1 is as a sponge for miR-150-5p, meaning

it also indirectly modulates AKT3.[20] Furthermore, substantial extracellular lncRNAs are detectable in the serum and synovial fluid of OA patients, thereby indicating the potential significance of lncRNAs in the development of OA and their potential utility as diagnostic markers for the disease.[17]

Thus, investigating novel lncRNAs and identifying mechanisms underlying the interaction between lncRNAs and their target miRNAs or proteins during the progression of OA is especially important. Furthermore, ongoing research is essential to discover and characterize new lncRNAs for the potential treatment and prevention of OA.

In this study, RNA-sequencing (RNA-seq) was conducted to identify novel lncRNAs potentially involved in the progression of OA. Our findings suggest that modulating the expression of these lncRNAs could potentially impede the development of OA.

II. MATERIALS AND METHODS

1. Isolation of primary human chondrocytes

Following approval from the Institutional Review Board of Yonsei University College of Medicine under the reference number 2019-1374-002, cartilage samples were acquired from six individuals diagnosed with osteoarthritis (OA), who had undergone total knee arthroplasty. The human cartilage tissues were divided into two groups: one comprising intact tissues without any signs of abrasion, and the other consisting of damaged tissues, characterized by fibrosis and a relatively reddish appearance. To isolate human primary chondrocytes, cartilage tissues were aseptically extracted from the articular surface and finely chopped into small fragments. The minced tissues were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM-HG, Gibco, USA) supplemented with 0.1% proteases for 30 minutes, followed by incubation with 0.1% collagenase for 4 hours, both at 37 °C and 5% CO_2. After incubation, the cell suspensions were filtered through a 40 μm cell strainer to remove debris, then, the cells were washed with phosphate-buffered saline (PBS). Subsequently, the cells were cultured in DMEM-HG with 20% foetal bovine serum (FBS, Gibco, USA) and 1% antibiotic–antimycotic solution (Gibco, USA), and cultured in the incubator at 37 °C and containing 5% CO_2.

2. Cell line and cell culture

The TC28a2 cell line (Sigma-Aldrich, St. Louis, MO, USA) was used in this study. The TC28a2 cell line is commonly employed to investigate the mechanisms of normal and pathological cartilage repair related to chondrocyte biology and physiology. TC28a2 cells

were cultured in high-glucose DMEM (Gibco, CA, USA) supplemented with 10% foetal bovine serum (Gibco, CA, USA) and 1% antibiotic–antimycotic solution (Gibco, CA, USA), while being maintained in an incubator at 37 °C and with 5% CO_2. Upon reaching 80% confluency, the cells were harvested using 0.05% trypsin–EDTA (Gibco, CA, USA), washed, centrifuged, resuspended, and then, seeded into new plates. Fresh medium was replaced every 2–3 days.

3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cultured human chondrocytes using the AccuPrep Universal RNA Extraction Kit (Bioneer, Korea). The isolated RNAs were reverse transcribed using AccuPower RT premix (Bioneer, Korea). The RNA concentration was assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). The primer sets employed in the study were obtained from Bioneer and tested. For real-time polymerase chain reaction analysis, the reverse transcription reaction mixture, along with the specific primers and SYBR green PCR master mix (PCR Biosystems, UK), was amplified by a StepOnePlus Real-Time PCR System device (Applied Biosystems, USA). The ΔΔCt method was employed to calculate relative gene expression, with β-actin and HPRT used as normalization factors for both mRNA and long non-coding RNA (lncRNA) expression levels. The primer sequences are listed in Table 1.

4. Western blotting

Cell pellets were rinsed in PBS and subjected to lysis using 50–100 μL of PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam, Korea), followed by

centrifugation at 13,000 rpm for 20 minutes. The protein concentrations from the extracted samples were assessed using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., USA). Approximately 20–30 μg of the protein samples were applied to an 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred onto polyvinylidene (PVDF) membranes. After being blocked in 5% skimmed milk (BD Biosciences, San Jose, CA, USA) at room temperature for one hour, the membranes were incubated with specific primary antibodies in 1% skimmed milk at 4 ℃ overnight. The used antibodies were β-actin (1:5000, sc-47778), COL2A1 (1:3000, sc-518017), and ACAN (1:3000, AB1031). All membranes were washed with TBST three times for 10 minutes each and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000, SA001-500 or SA002-500, GenDEPOT, Barker, TX, USA) at room temperature for one hour. Finally, all membranes were washed three times with TBST for 10 minutes and Western blot images were detected by Amersham ImageQuant™800 (Cytiva, MA, USA). β-actin was used as an internal normalization control.

5. RNA-sequencing analysis

Isolated RNAs were submitted to Macrogen Inc. (Korea) for total RNA-seq. The assessment of total RNA quality was conducted by spectrophotometry. To eliminate poor-quality reads and adapter sequences, the raw data was initially processed by the sequencer. Subsequently, the processed reads were aligned to the human genome (version hg19) using HISAT2 version 2.1.0. The reference genome sequence and annotation data for Homo sapiens were obtained from NCBI. Following this, known transcripts were

assembled using StringTie version 2.1.3b. The expression abundance of transcripts and genes was quantified in terms of read counts or FPKM values (fragments per kilobase of exon per million fragments mapped) per sample. These expression profiles formed the basis for subsequent analyses, including the identification of differentially expressed genes (DEGs). Gene relative abundances were measured in read counts using StringTie. Genes with non-zero read count values in the samples were retained, while those with zero or fewer were excluded. The filtered data underwent relative log expression (RLE) normalization, and the statistical significance of each differential expression was assessed using DESeq2. Hierarchical clustering analysis (using Euclidean distance and complete linkage) was performed based on the list of DEGs using normalized values. GO enrichment analysis was conducted using g: Profiler to identify each enrichment.

6. Transfection

TC28a2 cells, at 70–80% confluency, were plated in 6-well dishes and transfected with either the siRNA negative control (si-NC, Bioneer, South Korea) or siRNAs specific to selected lncRNAs (si-lncRNA, Bioneer, South Korea) using CalFectin (SignaGen Laboratories, USA). Briefly, 150 nM siRNA was mixed with 0.25 mL of serum-free high-glucose DMEM. The mixture was gently vortexed and then centrifuged to ensure any droplets settled at the bottom of the tube. For each well, 4 μL of CalFectin reagent was promptly combined with 0.25 mL of serum-free high-glucose DMEM. Then, the CalFectin solution was directly mixed with the 0.25 ml diluted siRNA solution. After incubating at room temperature for 15 min, each well was washed with PBS and then replenished with 0.5 mL of serum-free medium. Subsequently, the 0.5 mL

CalFectin/siRNA mixture was added dropwise to the medium in each well, and the mixture was evenly distributed by gently swirling the plate. After incubating for 4 hours in an incubator with 5% CO_2 at 37 ℃, 1 mL of DMEM, supplemented with 20% foetal bovine serum (Gibco, CA, USA) and without the antibiotic–antimycotic solution, was added to each well. Cells were harvested 24 hours after transfection to assess the siRNA knockdown efficiency or were exposed to TNF-α (10 ng/μL). The siRNA sequences are listed in Table 2.

7. Induction of in vitro inflammatory environment

TC28a2 cells were seeded in a 6-well culture plate at a confluency of 70–80% and exposed to TNF-α (R&D Systems Inc., USA) to induce an inflammatory environment. A TNF-α concentration of 10 ng/mL was administered for 5 consecutive days, with fresh TNF-α-containing medium exchanged daily.

8. Statistical analysis

The data are presented as the mean ± standard deviation (SD). Statistical significance between groups was determined using Student's t-tests for two-group comparisons or one-way ANOVA for comparisons involving more than two groups. Statistical analysis was conducted using Prism software version 8 (GraphPad Software, San Diego, CA, USA). In all figures and graphs, * indicates p-values < 0.05, ** indicates p-values < 0.01, and *** indicates p-values < 0.001.

Table 1. A list of primers used for qRT-PCR

Gene	F (5' → 3')	R (5' → 3')
β-actin	GTCCTCTCCCAAGTCCACACA	GGGCACGAAGGCTCATCATTC
HPRT	TGCAGACTTTGCTTTCCTTGGTCAGG	CCAACACTTCGTGGGGTCCTTTTCA
GAPDH	P267613 (Bioneer, South Korea)	
MMP13	P221090 (Bioneer, South Korea)	
ADMATS5	CAATGCCCACCGAACCATCT	GAACATCGACCAACTCTACTCC
IL6	P211161 (Bioneer, South Korea)	
IL1B	P314508 (Bioneer, South Korea)	
LOC105377043	CCATGAGACCACCCATTCT	AATGTGGCTTAGGCTAGCAG
LINC02202	TCCCAGGATGCTTTCTGTTG	TAGTCCTCAGGTATGGCCAG
LINC00578	TGGCACCCTTCATTCATTCC	TTGGGTCCTCTAGCAGTCTC
MGC27382	TCCACGTAGTCCTCTCCATC	AGTGCCCTTCATCAGGTAGA
LOC105374466	GACGGGTGATTTCTGCATTT	CAGTTCCAAAGCCCCTGTAG
LOC107985871	ACCCATTGCAGAACCAGAAG	TGTCAAAGGCCAGGATGTTC
LOC107985352	CGAGTGGTCATGTGGCAAAG	TCCTTGTGGTTGCAGAAGTG
SPRY4-IT1	TTATGGGAGCCTGTGAATGC	GCCTTGGAATCAGAAAGCAG
LOC107986606	TGGATAGGACATCGCAGTTG	TATCAAGGTCGGTGCTTCTG
LOC101928051	ACAAGCAACTCCATGAACCC	ATCCCAGCTCTGAAATTGGC
LINC00294	ACCCAATCAGTGAAGAAGCC	TGAGTCCAGTGTGGTGATCC
LINC02806	GGCCACTAACTGGATCTTGG	TCACTGAGGGAAGGAGTACG
LOC105375914	TTATTGGAAGCGGACGACAC	GACGGAGCAGAAAGAGACTG
LOC102723758	TGTCCTTTGTGGACTGTGTG	GTTGTTCCTTAGGTCCTGGC
LINC01503	ACATGACCGTGTGGAGAAAG	CTCAGGGTCTCTGGGTAGG
BVES-AS1	TCATGTGTTCTCACTTCCATCC	GCTTCATCCCTCTTGGACTG
LOC107985369	GCTTCGTCAAGAGAGGGAAG	TTCTTTGCACCTTGGTTTGC
LOC107985872	ATGGCATTGAAACTTTGGCG	TCCTCGCTGCATCATTTAGG
LINC01001	CAGCTCTTCTTCCTGGTTGG	AGGAGATGGGTAGGCACTG
LOC105370765	AAGTGCAGAAACCCACAGAG	ATAGGGAGGTGGAGGAATCG
ADAMTS9-AS2	GTACACACGCAGTCCTATCC	GCTCTGGAATCCTTCTGGTC
C1QTNF3-AMACR	ATTTATGCCAGGCTGAGTGG	TTCCTTCCACCTGACAAAGC
LOC101927741	CATTCCTTCAACACATCACTGAC	TCCCTTGTAAAGGAGCTTAATCC
LOC105371981	AATCCCATGCTTCCCTCTTG	CCCAAACAATGAACCTTTCCC

Table 2. A list of siRNAs used for transfection

Gene	Sequence (5' → 3')
LOC105377043	GACGGAAAAGACAGUCACU
LINC02202	AGACCAAGUCUUGCUCUGU
LINC00578	CAGCUAUUCAGUGGUACAA
MGC27382	CACGUAGUCCUCUCCAUCU
LOC107985871	GAAAUCUGGGUAGAACUAU
LOC107985352	GUGAUCUUCAAAAUGUGAU
LOC101928051	GUGGUAUGUACAAAGUGUA
LINC02806	CAGAGAAACAGAACCAACA
LOC105375914	GACUGGGAGCUAGAACCAUGAGU
LOC102723758	GAAAUCUGACAUGAGAACA
BVES-AS1	GACCUUCAAAUCAGUCAGU
LOC107985369	GUUUUCAGGAAUGGAACUU
LINC01001	GACCAUCCAGUUGCAGGAA
LOC105370765	CACACAGGAAGAUUCUCUA
ADAMTS9-AS2	CUCACUAACAUCUCCAUCA
LOC105377043-2	GACGGAAAAGACAGUCACU
LINC02202-2	AGACCAAGUCUUGCUCUGU
LINC00578-2	CAGCUAUUCAGUGGUACAA
MGC27382-2	CACGUAGUCCUCUCCAUCU
LINC02806-2	CAGAGAAACAGAACCAACA
LOC105370765-2	CACACAGGAAGAUUCUCUA
ADAMTS9-AS2-2	CUCACUAACAUCUCCAUCA

III. RESULTS

1. Characteristic changes in chondrocytes during the progression of osteoarthritis

To investigate any characteristic changes during the progression of osteoarthritis (OA), the phenotype of chondrocytes from cartilage tissues was determined. Human primary cartilage tissues were divided into two groups: intact and damaged. Divided tissues were subjected to isolate primary human chondrocytes. Intact tissue-derived chondrocytes exhibited a spherical or oval shape, while damaged tissue-derived chondrocytes displayed an elongated or irregular shape (Figure 1A), meaning that chondrocytes underwent morphological changes as OA progressed. Next, qRT-PCR analysis was performed to establish the expression pattern for inflammation marker genes from intact and damaged tissue-derived chondrocytes. The expressions of MMP13, PTGS2, and IL6 were significantly upregulated in the damaged tissue-derived chondrocytes compared to the intact tissue-derived chondrocytes (Figure 1B). The Western blot analysis indicates a decrease in type II collagen (COL2A1) in the damaged tissue-derived chondrocytes, compared to their intact counterparts. However, the expression patterns of AGGRECAN did not change in either the intact or damaged tissue-derived chondrocytes (Figure 1C). These results demonstrate that characteristic changes have occurred in human chondrocytes as OA progressed.

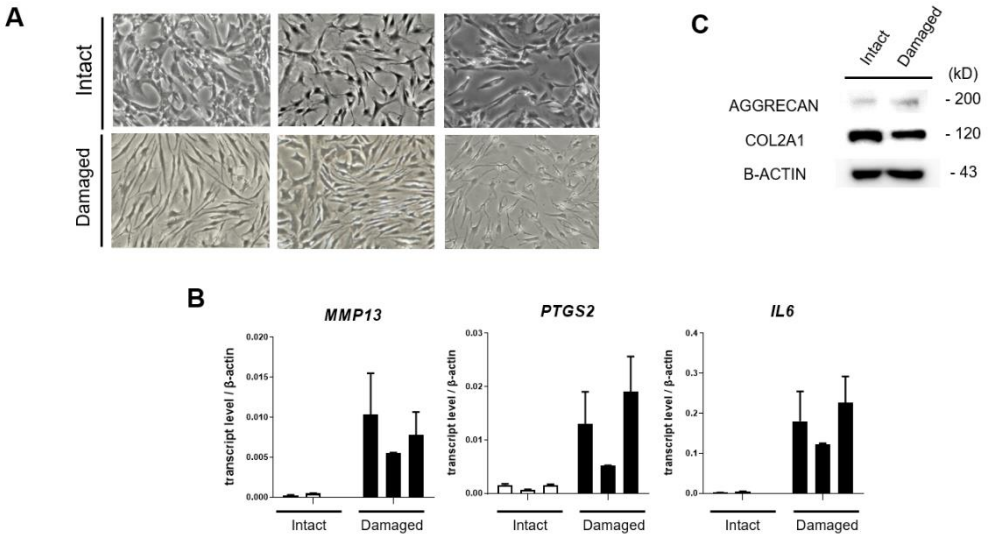


Figure 1. Observed distinctive alterations in human intact and damaged tissue-derived chondrocytes. (A) Light microscopy was used to observe the morphology of both intact and damaged tissue-derived human chondrocytes. (B) qRT-PCR was conducted to assess mRNA levels of involved in ECM degradation (MMP13) as well as inflammation-related genes (PTGS2 and IL6) (C) Protein levels of ECM markers were detected in both intact and damaged tissue-derived chondrocytes by Western blotting.

2. RNA-seq analysis reveals differential expression of lncRNAs and mRNAs

Next, total RNA-seq analysis was conducted to identify differentially expressed long non-coding RNAs (lncRNAs) in human intact and damaged tissue-derived chondrocytes. Total RNA was utilized for RNA-seq analysis and gene expression differences were compared between damaged (n = 3) and intact tissue-derived chondrocytes (n = 3). Hierarchical clustering revealed differential expression patterns by both lncRNAs and mRNAs between the damaged and intact groups (Figure 2A). The volcano plots illustrate the differences in lncRNA and mRNA expression levels between the intact and damaged groups (Figure 2B). A total of 1261 genes were upregulated (fold change > 2, raw p-value < 0.05), while 900 genes were downregulated in the damaged tissue-derived chondrocytes compared to the intact tissue-derived chondrocytes. Additionally, 228 differentially expressed lncRNAs were identified, with 184 lncRNAs being upregulated and 44 lncRNAs displaying downregulation (Figure 2C). To further understand the underlying mechanisms that may affect OA progression, all predicted genes were annotated according to GO analysis. GO analysis was conducted in each of the three categories: biological process (BP), molecular function (MF), and cellular component (CC) (Figure 3). A heatmap revealed that 26 lncRNAs were upregulated more than 6-fold during the progression of OA (Figure 4). Based on filtering, using a more than 6-fold change, it is postulated that these 26 lncRNAs might act as regulators in OA; thus, they were selected for further study. Further, RNA-seq analysis was conducted to identify differentially expressed lncRNAs and mRNAs in human chondrocytes. Transcriptomes showed significant alterations during the progression of OA, with lncRNAs playing a role in this process.

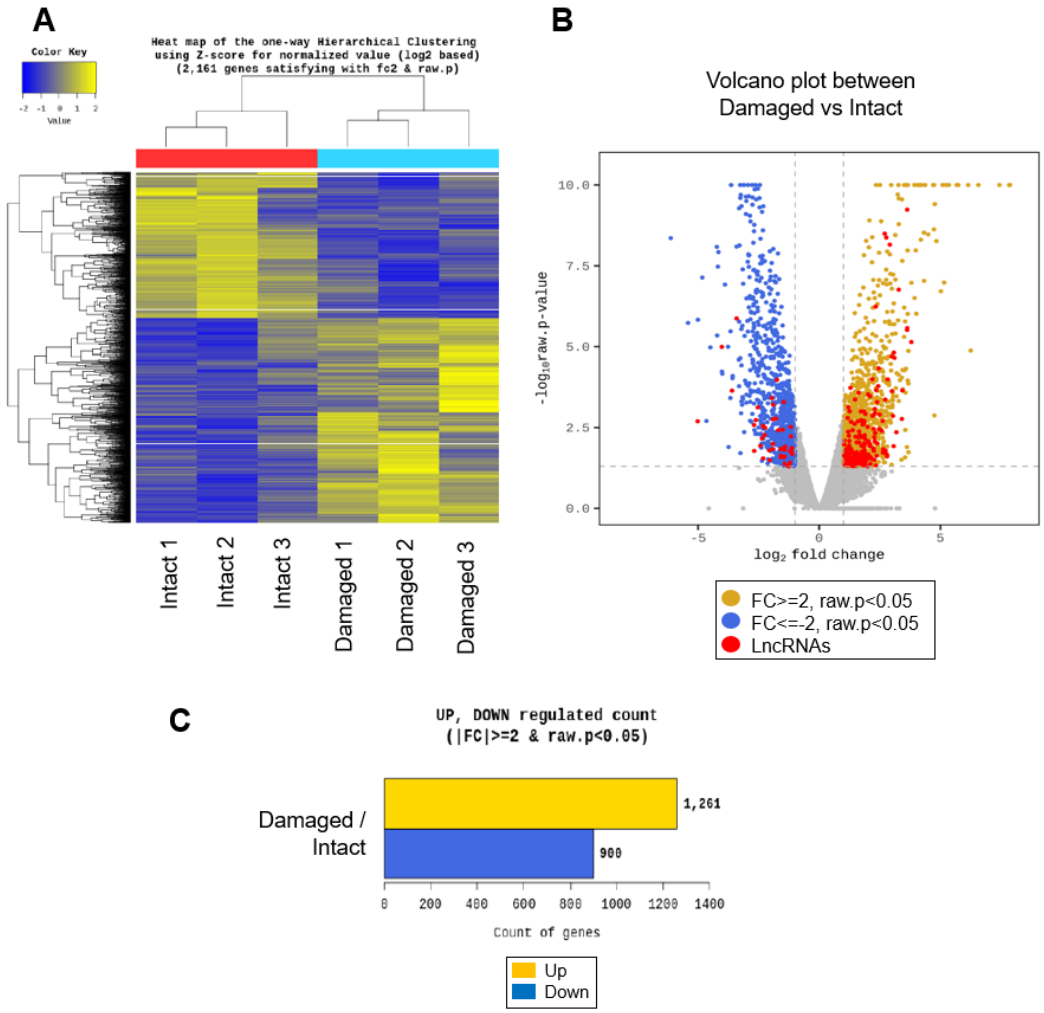
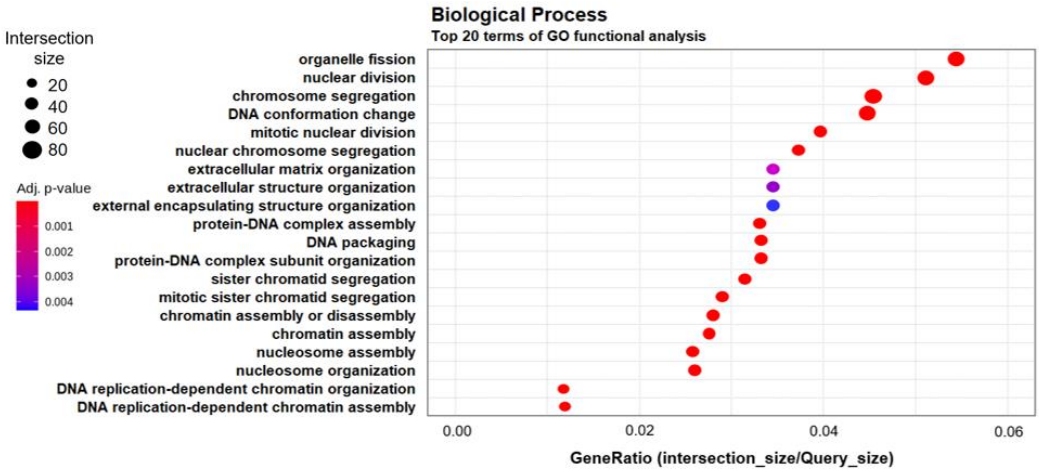
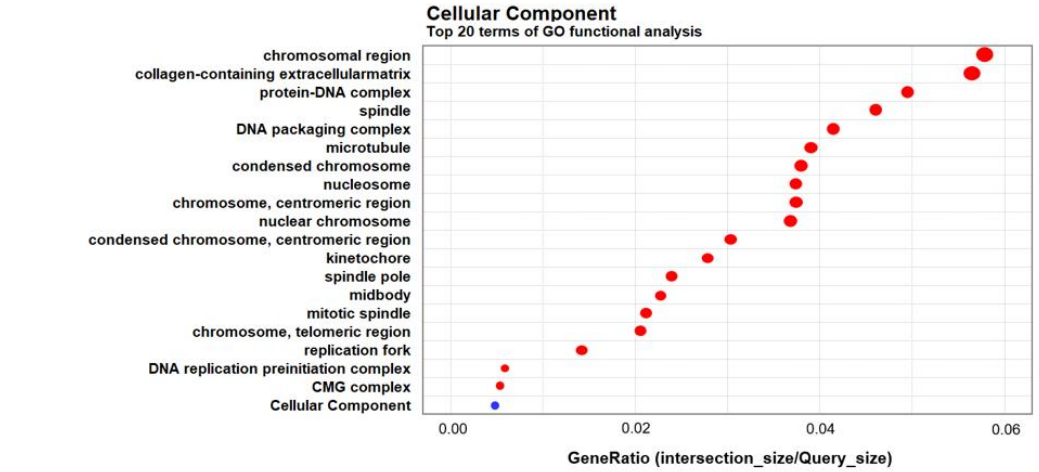


Figure 2. Differential expression of mRNAs and lncRNA in damaged tissue-derived chondrocytes. (A) Hierarchical clustering of RNA-seq data. Heatmaps showing osteoarthritis-associated lncRNAs and mRNAs. The relative expression of lncRNAs and mRNAs is depicted according to the color scale shown on the left. Yellow represents high relative expression, blue represents low relative expression, and -1, 0, and 1 are fold

changes in the corresponding spectrum. (B) A volcano plot was constructed to visualize differentially expressed genes between damaged and intact tissue-derived chondrocytes. Differentially expressed lncRNAs are indicated by red data points, while the X-axis indicates fold change (log 2), and the Y-axis presents P (-log 10) values. (C) Number of upregulated genes is depicted in yellow and downregulated genes are depicted in blue.





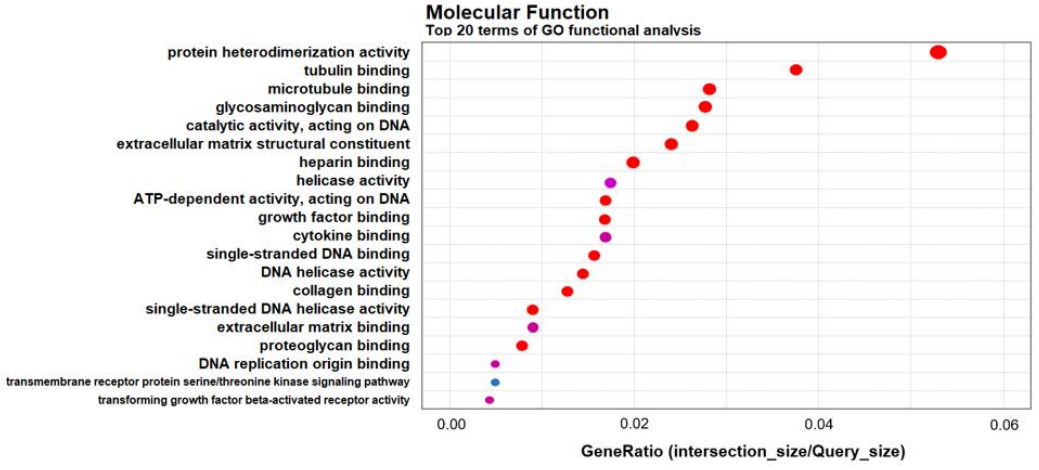


Figure 3. Top 20 terms from GO functional analysis in biological processes, molecular functions, and cellular components. Gene Ontology (GO) enrichment analysis was performed using g:Profiler, examining biological processes (BPs), molecular functions (MFs), and cellular components (CCs) according to the RNA-seq results. Term size filtering was applied (min = 10, max = 500) to prevent exaggeration of statistical significance. The metrics used for each analysis included intersection size (the count of unique DEGs associated with the term ID), gene ratio (intersection size/query size), and adjusted p-value (calibrated p-value derived from the Hypergeometric test with multiple testing corrections, specifically the false discovery rate (FDR)).

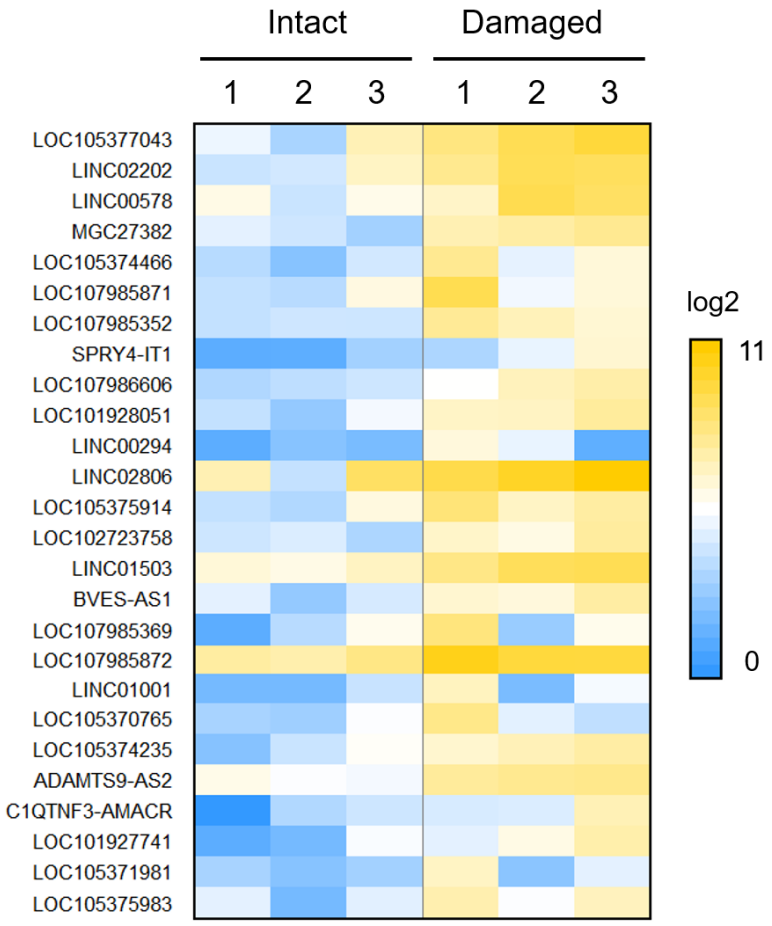


Figure 4. Heatmap of up-regulated lncRNAs in damaged tissue-derived human chondrocytes. The heatmap illustrates the lncRNAs upregulated by 6-fold in damaged tissue-derived chondrocytes, compared to intact tissue-derived chondrocytes. P-value filtering was set with a threshold of 0.05 to identify significant differences. Each column represents chondrocytes from a respective donor. The scale bar indicates that yellow corresponds to relatively high expression, while blue corresponds to relatively low expression. Normalized count data were used to draw a heatmap.

3. Verification of lncRNA expression within an in vitro inflammatory environment simulated by TNF-a

To validate the lncRNA expressions from the RNA-seq data in an in vitro inflammatory environment, inflammation was induced in TC28a2 cells by the addition of TNF-α. To investigate alterations in lncRNA expression, cells were harvested at each time point on days 0, 2, and 5 after TNF-α treatment (Figure 5A). Following the induction of an in vitro inflammatory environment, the expressions of COL2A1 and AGGRECAN, which are extracellular matrix markers (ECM), decreased in a time-dependent manner, as determined by Western blot analysis (Figure 5B). Since there are no alignments between transcript variants in LOC105375983 and LOC105374235, qRT-PCR primers could not be designed. However, the expression levels of the remaining 24 lncRNAs were successfully validated using qRT-PCR and the results indicate that the majority of the lncRNAs increased in a time-dependent manner in response to TNF-α treatment. However, the expression of LOC107985372 decreased, which was not consistent with the RNA-seq results (Figure 5C). To identify the potential lncRNA regulators for OA, candidates were selected based on the qRT-PCR results. The filtering process criteria involved a fold change greater than 2 and a p-value less than 0.05. The filtered lncRNAs were selected as candidates for target lncRNAs associated with OA progression (Figure 6A). As a result, 15 lncRNAs were selected as candidates, and the figure displays the outcomes of three individual experiments, with the fold changes and p-values evaluated for each (Figure 6B). These results correlated with the RNA-seq analysis and indicate that the majority of lncRNAs increased in the in vitro inflammatory environment.

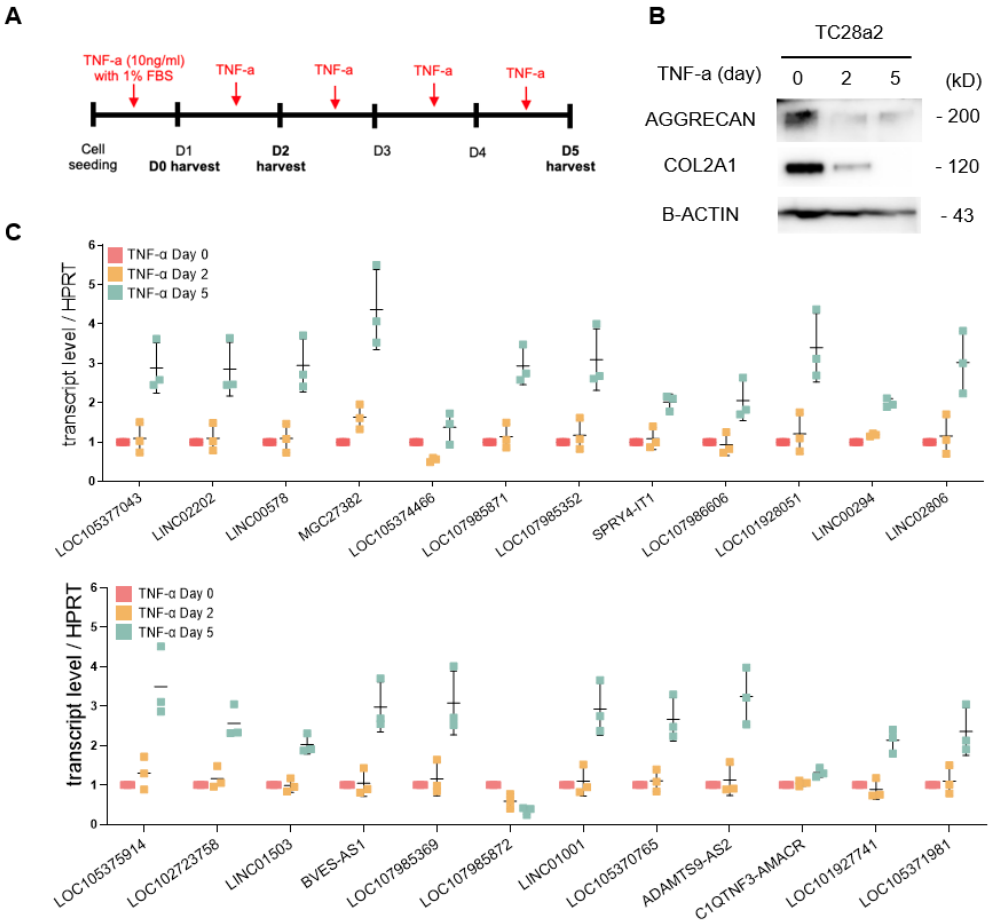
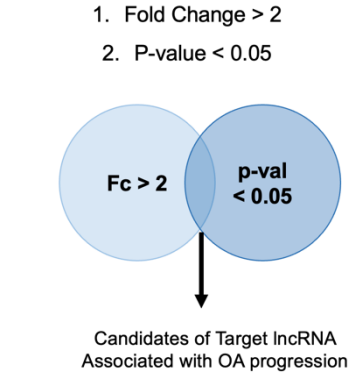


Figure 5. Validation of lncRNA expression following TNF-α induction in an in vitro inflammatory environment. (A) Schematic illustration depicting the induction of an in vitro inflammatory environment. TC28a2 cells were exposed to TNF-α (10 ng/mL) for five days to induce inflammation. Cells were harvested at designated time points. (B) Examination of changes in the expression of ECM marker proteins during the inflammation induction process. (C) The expression of 24 lncRNAs was identified by qRT-PCR analysis.

Total RNA was extracted from chondrocytes subjected to inflammation induction for 0, 2, and 5 days, with normalization using HPRT. Each dot represents an individual experiment.

A



B

No.	lncRNA	1 st		2 nd		3 rd	
		Fc	p-value	Fc	p-value	Fc	p-value
1	LOC105377043	2.454	6.23E-06	2.584	1.13E-04	3.623	7.81E-06
2	LINC02202	2.455	5.34E-05	2.465	7.06E-05	3.648	7.17E-06
3	LINC00578	2.413	9.10E-05	2.721	2.83E-05	3.714	3.99E-05
4	MGC27382	3.533	9.51E-05	4.068	3.76E-05	5.502	3.08E-07
5	LOC107985871	2.584	1.10E-06	2.749	6.04E-05	3.482	2.92E-04
6	LOC107985352	2.610	3.20E-04	2.681	6.84E-04	4.004	5.41E-05
7	LOC101928051	3.121	4.36E-05	2.696	2.36E-04	4.377	2.74E-05
8	LINC02806	3.008	5.95E-07	2.233	1.42E-04	3.826	1.32E-04
9	LOC105375914	3.106	6.82E-04	2.865	1.47E-04	4.511	6.43E-07
10	LOC102723758	2.315	7.70E-05	2.334	2.19E-04	3.046	1.67E-03
11	BVES-AS1	2.545	4.21E-07	2.689	2.62E-05	3.702	3.13E-05
12	LOC107985369	2.524	4.36E-07	2.708	9.98E-05	4.010	9.81E-06
13	LINC01001	2.745	4.02E-07	2.369	3.01E-05	3.655	1.72E-05
14	LOC105370765	2.472	3.08E-06	2.236	2.12E-04	3.293	2.10E-04
15	ADAMTS9-AS2	3.215	1.58E-04	2.537	2.78E-05	3.981	2.12E-05

Figure 6. Selection of lncRNA candidates involved in OA progression. (A) Based on previous qRT-PCR results, candidates for lncRNAs potentially involved in OA progression were identified using criteria of a fold change greater than 2 and a p-value less than 0.05. (B) The table shows that the fold change and p-value vary for each experiment (n = 3).

4. Identifying the preventive effect of COL2A1 degradation via siRNA-mediated knockdown of lncRNAs

To explore the functions of the 15 selected lncRNAs in the inflammatory environment, siRNA sequences were designed to silence each candidate, and the knockdown efficiency of the lncRNAs was validated through qRT-PCR, using GAPDH as the positive control. Seven lncRNAs (LOC107985871, LOC107985352, LOC101928051, LOC105375914, LOC102723758, BVES-AS1, and LOC107985369) were selected as candidates for the functional studies since they exhibited a fold change lower than 0.6, compared to si-NC (Figure 7A). However, lncRNAs with an initially lower transfection efficiency still exhibited reduced transfection efficiency when a different siRNA sequence was used (Figure 7B).

To explore the role of the seven selected lncRNAs in an inflammatory environment, siRNA-mediated knockdown was conducted in TC28a2 cells. Subsequently, inflammation was induced by TNF-α for two days (Figure 8A). Western blotting was conducted to investigate the preventive effect on COL2A1 degradation levels during inflammation following lncRNA silencing. The COL2A1 protein level decreased following the induction of inflammation, although cells transfected with si-LOC1079852 and si-LOC107985369 significantly rescued COL2A1 levels. Furthermore, LOC101985351, LOC102723758, and BVES-AS1 exhibited a reduced preventive effect on TNF-α-induced COL2A1 degradation (Figure 8B). These results demonstrate that silencing lncRNAs, especially LOC1079852 and LOC107985369, effectively suppressed COL2A1 degradation compared to the negative control.

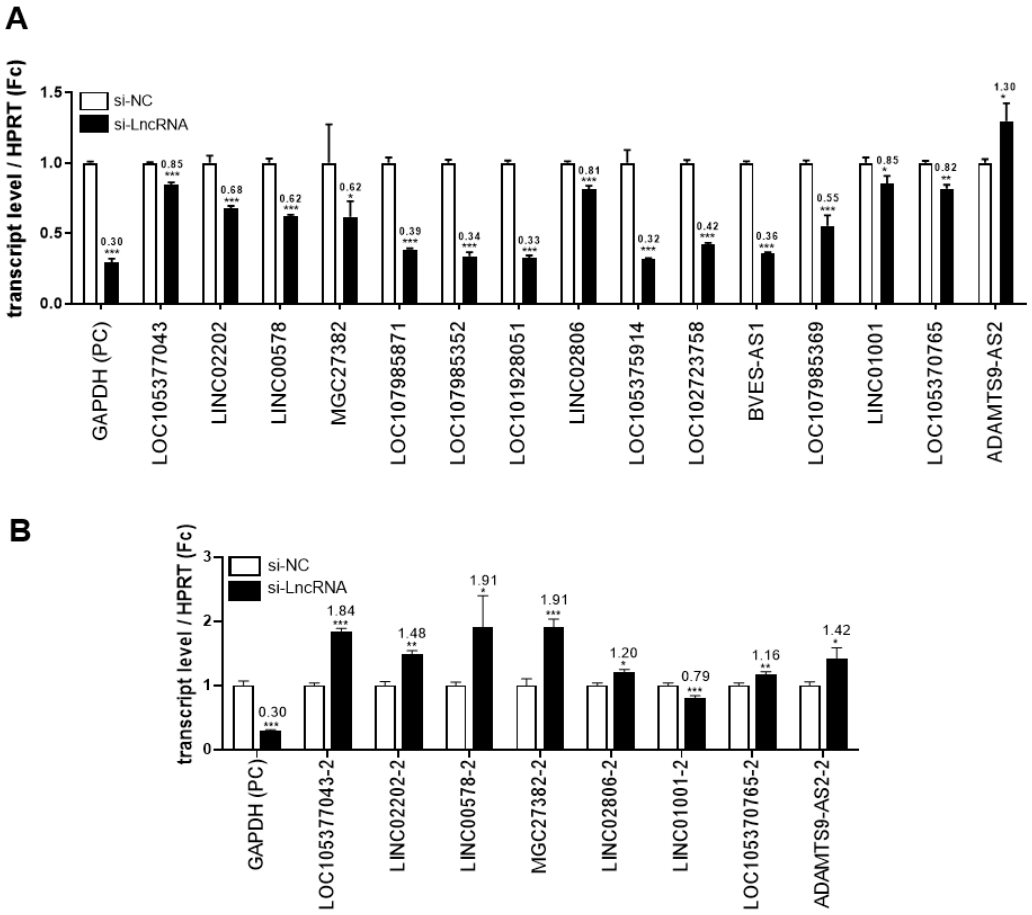


Figure 7. Confirmation of lncRNA siRNA knockdown efficiencies in the TC28a2 cell line. (A), (B) qRT-PCR was performed to verify the siRNA knockdown efficiencies for each targeted lncRNA in the TC28a2 cell line. si-GAPDH was used as a positive control.

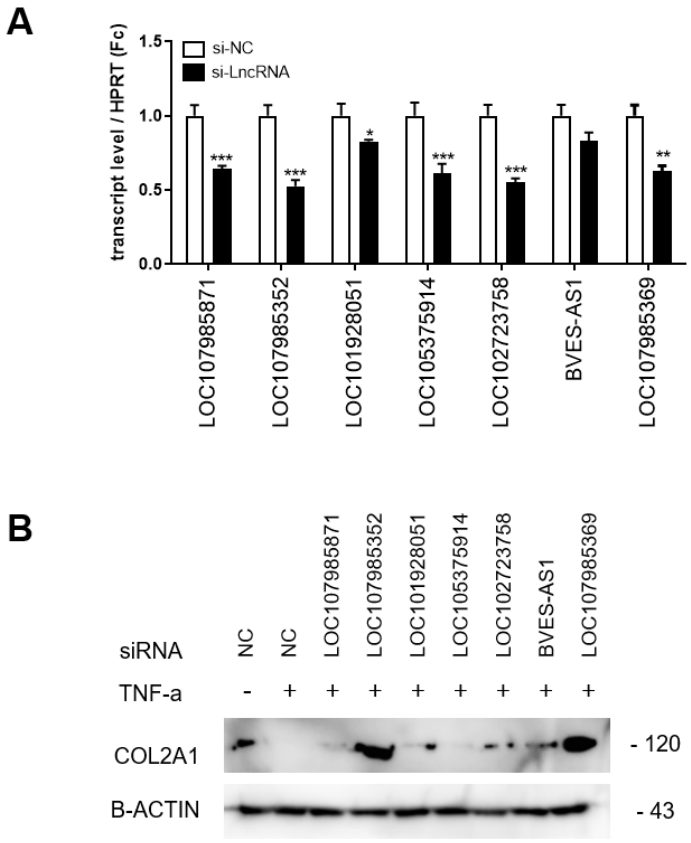


Figure 8. siRNA knockdown of lncRNAs prevents COL2A1 degradation in the TC28a2 cell line inflammation environment. (A) The inhibitory effect of silencing lncRNAs with siRNA was assessed using qRT-PCR. (B) The prevention effect of COL2A1 degradation after inducing inflammation, dependent on siRNA knockdown, was identified by Western blotting.

5. Effect of knockdown of LOC107985352 and LOC107985369 on COL2A1 degradation in a substantial inflammatory environment

To investigate the functional roles of LOC107985352 and LOC107985369 in inflammation, an inflammatory environment was induced in TC28a2 cells after siRNA transfection by adding TNF-α in a time-dependent manner. The cells were transfected with siRNA and incubated for 24 hours. Following a medium exchange, they were exposed to TNF-α. Cells were harvested at days 0, 1, 3, and 5 (Figure 9A) and qRT-PCR was performed to confirm each knockdown efficiency (Figure 9B). Western blot analysis revealed that cells transfected with si-LOC107985352 and si-LOC107985369 exhibited increased COL2A1 levels compared to cells transfected with si-NC. In the groups treated with si-LOC107985369 and si-LOC107985352, the COL2A1 levels increased at day 0 and remained elevated until day 3, whereas this effect was not observed in the si-NC group. The protein expression of AGGRECAN slightly increased in both the si-LOC107985369 and si-LOC107985369-transfected groups (Figure 9C). These results indicate that inhibiting LOC107985352 and LOC107985369 prevents COL2A1 degradation, a process that may be associated with the pathogenesis of OA.

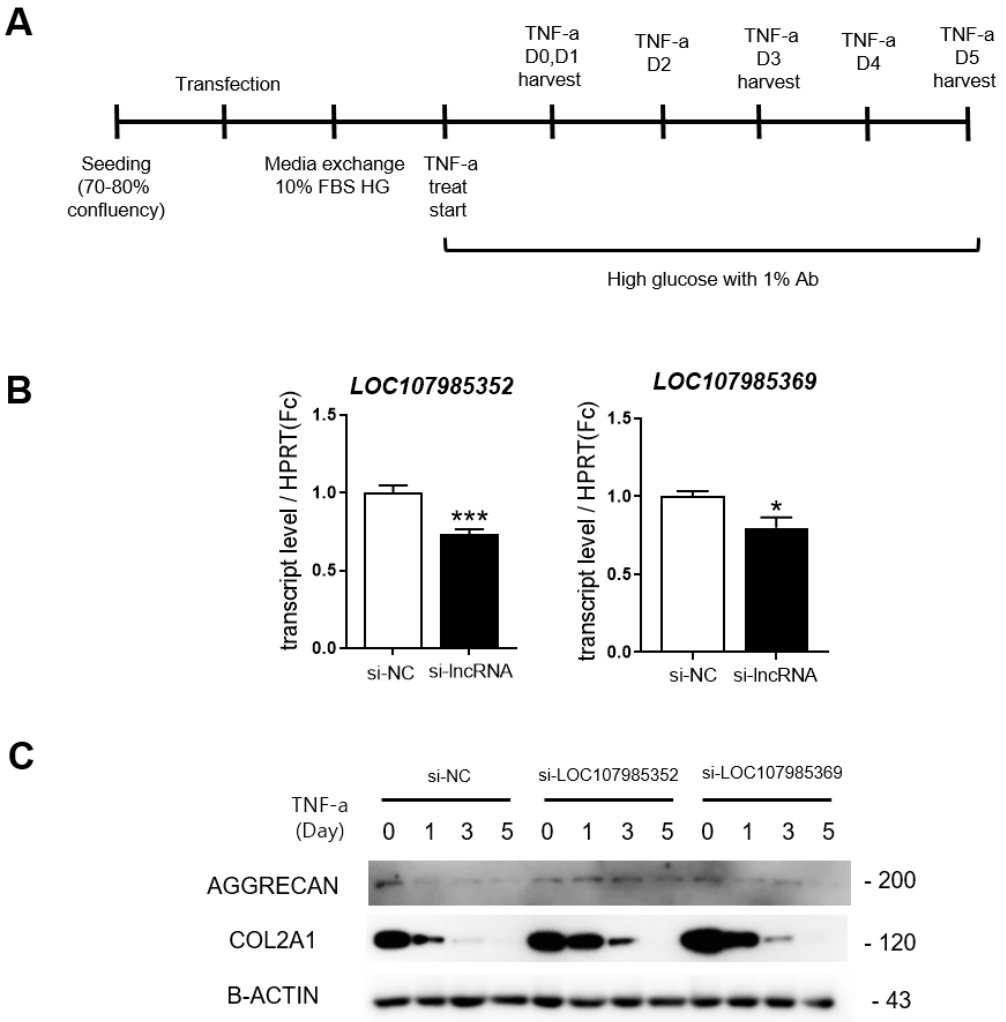


Figure 9. siRNA-mediated knockdown of LOC107985352 and LOC107985369 effectively enhances COL2A1 expression in TC28a2 cells. (A) Schematic illustration depicting the experimental conditions. TC28a2 cells were seeded at 70–80% confluency and transfected with the appropriate siRNA the following day. Inflammation was induced by exposing the cells to TNF-α (10 ng/mL) for five days in a high-glucose medium with 1% antibiotics but without serum. (B) Knockdown efficiency was confirmed by qRT-PCR

analysis. (C) Western blotting analysis was conducted to assess the expression of COL2A1 and AGGRECAN after the five-day inflammatory environment induction and following transfection with si-LOC107985352 and si-LOC107985369.

6. Impact of downregulating LOC107985352 and LOC107985369 on the expression of catabolic genes and inflammation-related genes in an inflammation milieu

To investigate the role of lncRNA in an inflammatory environment, the expressions of catabolic genes (MMP13, ADAMTS5) and inflammatory-related genes (IL6, IL1B, PTGS2) were analysed by qRT-PCR following siRNA transfection. The downregulation of LOC107985352 was found to reduce the mRNA expression of MMP13, ADAMTS5, IL1B and PTGS2 within the inflammatory environment. Notably, IL6 mRNA expression did not decrease after LOC107985352 downregulation (Figure 10). Similarly, the downregulation of LOC107985369 resulted in reduced MMP13 and ADAMTS5 mRNA expressions in the inflammatory environment. Additionally, both IL6, IL1B and PTGS2 exhibited a significant decrease in mRNA expression (Figure 11). These results indicate that the downregulation of LOC107985352 can reduce MMP13 and ADAMTS5 expression, both of which are associated with ECM degradation. Furthermore, the downregulation of LOC107985369 effectively resulted in decreased expression of catabolic and inflammation-related genes.

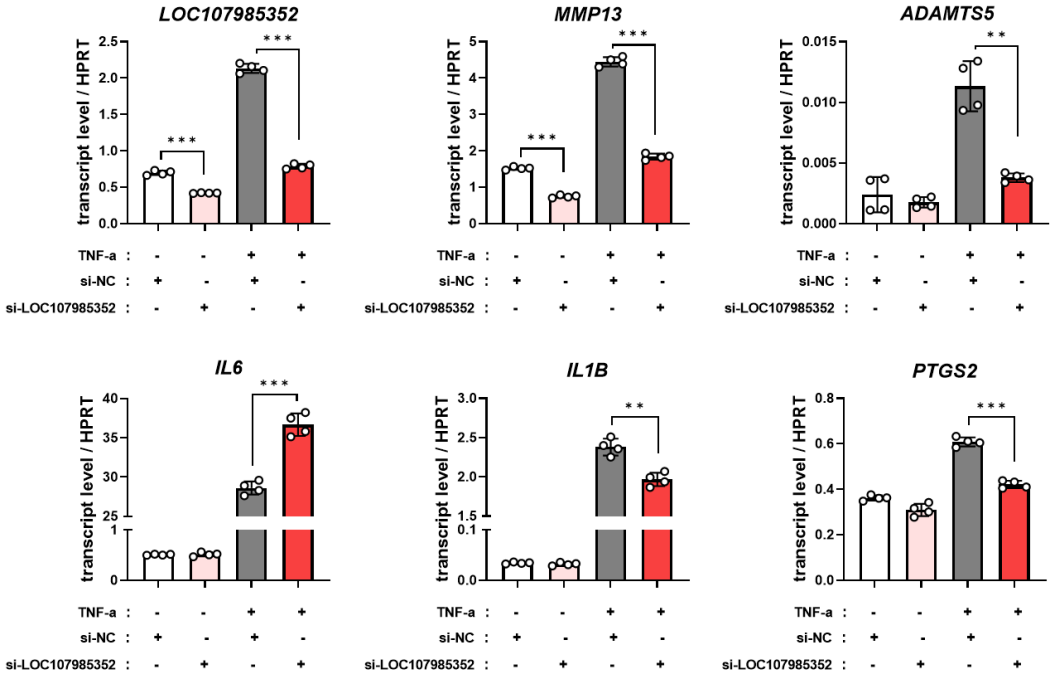


Figure 10. siRNA-mediated knockdown of LOC107985352 reduces catabolic gene expression in an inflammatory environment. The mRNA expression levels of catabolic genes (MMP13, ADAMTS5) and inflammatory-related genes (IL6, IL1B, PTGS2) were analysed by qRT-PCR after si-LOC107985352 transfection during the five-day TNF-α-induced inflammation period.

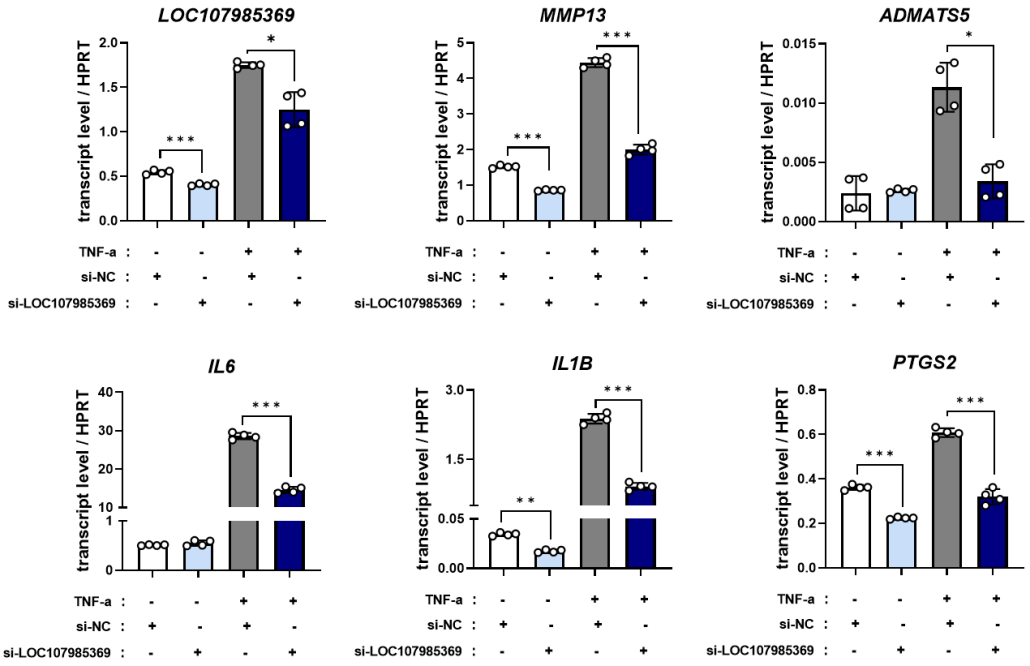


Figure 11. siRNA-mediated knockdown of LOC107985369 effectively reduces catabolic gene and inflammatory-related gene expressions in an inflammatory environment. The mRNA expression levels of catabolic genes (MMP13, ADAMTS5) and inflammatory-related genes (IL6, IL1B, PTGS2) were analysed by qRT-PCR after si-LOC107985369 transfection and during the five-day TNF-α-induced inflammation period.

IV. DISCUSSION

The cartilage extracellular matrix (ECM) predominantly consists of a type II collagen (COL2A1) network, which is interwoven with fibrous proteins, proteoglycans (PGs), and hyaluronic acid (HA).[21] Under normal circumstances, chondrocytes maintain a state of metabolic and physiological equilibrium. However, when the osteoarthritic environment experiences inflammation, it can disrupt their metabolic balance and cartilage remodelling functions. Anomalous chondrocyte metabolism is a reaction to alterations within the inflammatory microenvironment and might assume a significant function in both cartilage degeneration and OA progression.[22] Proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 contribute to the inflammatory milieu in synovial joints, while saturated fatty acids (SFAs) and polyunsaturated fatty acids (n-3 PUFAs) act as proinflammatory metabolites.[3,23,24] Inflammatory mediators produced by bone, cartilage, and synovial tissue trigger the activation of chondrocytes, thereby disrupting their homeostatic state. A process that is marked by excessive production of reactive oxygen species (ROS), mitochondrial dysfunction, and increased expression of genes related to catabolism and inflammation, which ultimately leads to chondrocyte apoptosis.[25-27] Therefore, it is crucial to understand the alterations in chondrocyte homeostasis that take place as OA progresses.

Long non-coding RNAs (lncRNAs) operate by controlling the epigenetic status of nearby and distant protein-coding genes through both cis- and trans-acting mechanisms. These lncRNAs influence gene expression during transcription, RNA processing, and translation primarily by interacting with chromatin-modifying complexes, serving as scaffold modifiers, or engaging with transcription factors as co-regulators in transcriptional processes.[28] However, the role of lncRNAs concerning OA remains largely unknown. In

this study, RNA-seq was conducted to analyse the entire transcriptome in both intact and damaged tissue-derived chondrocytes and identify novel lncRNAs associated with the development of OA. The phenotypes of the chondrocytes obtained from the intact and damaged human cartilage tissues were characterized prior to conducting RNA-seq analysis. The results showed that inflammation-related genes were upregulated in damaged tissue-derived chondrocytes. Additionally, the COL2A1 protein level was shown to decrease as OA progressed (Figure 1). COL2A1 constitutes the main structural component within cartilage tissue, thus, when COL2A1 undergoes denaturation, it results in reduced chondrocyte proliferation, proteoglycan loss, and increased collagen cleavage.[29]

The RNA-seq data revealed that 1261 genes were upregulated, while 900 genes were downregulated in response to OA progression. Moreover, 228 lncRNAs were detected with differential expressions, of which 184 were upregulated, and 44 were downregulated (Figure 2). After applying a fold change filter to our RNA-seq analysis, the expression of 26 lncRNAs was identified to exhibit a fold change higher than 6-fold during OA progression. Therefore, these lncRNAs were selected as candidates for further study (Figure 4). When conducting a Gene Ontology (GO) analysis for biological processes (BPs), ‘organelle fission’ emerged as the top-ranking GO term. In the cellular component (CC) category, ‘chromosomal region’ was the most related, and ‘protein hetero dimerization activity’ was the leading GO term for molecular function (MF). Organelle fission is a cellular process that pertains to the organization, arrangement, or breakdown of specialized cellular structures known as organelles. These organelles encompass various components within cells, including the nucleus, mitochondria, plastids, vacuoles, vesicles, ribosomes, and the cytoskeleton. Notably, organelle fission does not encompass alterations to the plasma membrane. Furthermore, the term 'chromosomal region' refers to segments of

chromosomes that encompass elements beyond chromatin. Additionally, 'protein heterodimerization activity' denotes the binding of a protein to a nonidentical partner to form a heterodimer.[30] Through this analysis, we identified that during OA pathogenesis some pathways were significantly altered. Gene Ontology (GO) analysis can serve as a valuable tool for identifying interactions between lncRNAs and mRNAs and to facilitate further studies.

An evaluation of lncRNA expression was conducted within an in vitro inflammatory environment using qRT-PCR. However, while an attempt was made to design primers based on consensus regions among transcript variants, it was not feasible for LOC105375983 and LOC105374235 due to a lack of consensus; thus, they were excluded from the study. RNA-seq data initially yielded 26 lncRNAs with a fold change higher than 6-fold, however, 15 lncRNAs were identified in TC28a2 cells exposed to TNF-α for 2 days, which exhibited a fold change higher than 2-fold. The variation in fold changes can be attributed to disparities between primary chondrocytes derived from patient tissues and the in vitro inflammatory responses in cell lines. These 15 lncRNAs were chosen as candidate target lncRNAs since they were associated with OA progression. Moreover, they were subsequently employed in further investigations (Figures 5 and 6).

To investigate the inhibitory effect of silencing 15 lncRNAs, siRNAs were designed that targeted each specific lncRNA, and their effectiveness was confirmed. However, it should be noted that knockdown efficiencies over 40% were only exhibited by seven siRNAs. Therefore, different siRNAs featuring distinct sequences were used in the transfections of the eight remaining lncRNAs, although they still exhibited lower knockdown efficiencies (Figure 7). This could be attributed to the localization of lncRNAs, as siRNAs primarily function in the cytoplasm.[31] Nevertheless, studies have indicated that a significant portion

of lncRNAs are predominantly located in the nucleus. Furthermore, most lincRNAs notably also tend to be primarily localized in the nucleus.[32] Our study has certain limitations, particularly in that it did not investigate the remaining eight lncRNAs. However, it is essential to identify their localization, meaning it may be necessary to consider employing other gene modification techniques, such as CRISPR–CAS9, to elucidate these gene functions.

To investigate the potential preventive role of lncRNAs in the degradation of COL2A1 within an inflammatory environment, TC28a2 cells were transfected with siRNAs and exposed to TNF-α. As a result, the knockdown of LOC107985352 and LOC107985369 significantly rescued COL2A1 levels in the inflammatory environment. Thus, it is hypothesized that these two lncRNAs may exert a significant regulatory influence on OA progression (Figure 8).

The role of LOC107985352 and LOC107985369 in COL2A1 degradation was assessed under sustained inflammatory conditions. These results suggest that the suppression of LOC107985352 and LOC107985369 could increase COL2A1 expression (Figure 9), an important component of ECM molecules and a crucial extracellular signalling molecule that regulates chondrocyte proliferation, differentiation, and metabolism.[33] Furthermore, qRT-PCR analysis was conducted to investigate the roles of LOC107985352 and LOC107985369 in an in vitro inflammatory environment. The results showed that the downregulation of LOC107985352 led to a reduction in mRNA expression of MMP13 and ADAMTS5, compared to the si-NC transfected group. This suggests that LOC107985352 may play a role in preventing cartilage degradation by inhibiting catabolic gene expressions. However, it is noteworthy that the downregulation of LOC107985352 also reduced the mRNA expression of IL1B but not IL6. This observation implies that LOC107985352

might interact with different upstream pathway in response to IL1B, while IL6 expression remains unaffected. Specifically, the upregulation of IL6 mRNA expression is typically associated with the activation of the NF-κB pathway.[34] Therefore, it is conceivable that LOC107985352 plays a role in the NF-κB signaling pathway and potentially interacts with molecules such as enhancers, silencers, or transcription factors that modulate the mRNA expression of IL6. However, further study is needed to identify the exact molecules that may interact with LOC107985352 (Figure 10). Meanwhile, the downregulation of LOC107985369 led to a decrease in mRNA expression of both the catabolic genes and inflammation-related genes, thereby suggesting that LOC107985369 may serve as a significant regulator in OA pathogenesis (Figure 11).

Since LOC107985352 and LOC107985369 knockdown resulted in an increase in COL2A1 levels, it is hypothesised that these lncRNAs may have a direct interaction with the COL2A1 promoter or an indirect interaction with the transcription factors that activate the COL2A1 promoter. Furthermore, considering that many lncRNAs function by interacting with microRNAs as sponges, it is plausible that LOC107985352 and LOC107985369 may also interact with microRNAs, thereby indirectly affecting the degradation of target mRNAs. While numerous studies have explored the roles of lncRNAs in various diseases, there is currently no existing literature on LOC107985352 and LOC107985369. Nevertheless, our findings underscore the significant roles played by LOC107985352 and LOC107985369 in upregulating COL2A1 expression, while also downregulating catabolic and inflammation-related genes in osteoarthritic chondrocytes. Further mechanistic investigations are required to elucidate the specific mechanisms through which these lncRNAs regulate the inflammatory response in chondrocytes, thereby maintaining chondrocyte homeostasis and contributing to the development of OA.

V. CONCLUSION

In this study, RNA-seq analysis was conducted using human primary chondrocytes obtained from both intact and damaged tissues. Subsequently, 15 long non-coding RNAs (lncRNAs) were identified in TC28a2 cells that exhibited increased expression in an in vitro inflammatory environment, which had been induced by TNF-α treatment. Western blot analysis revealed that LOC107985352 and LOC107985369 knockdowns led to an increase in type II collagen (COL2A1) expression. Additionally, the downregulation of LOC107985352 and LOC107985369 effectively reduced the expression of both catabolic and inflammation-related genes. The results of this study highlight two potential therapeutic lncRNAs: LOC107985352 and LOC107985369. These findings underscore the promise of disease-associated lncRNAs and emphasize the importance of further investigations into their biological roles and underlying mechanisms because they offer great potential for gene therapy, which is aimed at halting the progression of OA.

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ABSTRACT (IN KOREAN)

골관절염 진행에서 신규 긴 비암호화 RNA의 탐색

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골관절염은 관절 손상, 염증, 그리고 지속적인 통증을 특징으로 하는 빈도 높게 발생하는 퇴행성 질환이다. 현재까지도 병의 진행을 완전히 막아주는 근본적인 치료법이 없기 때문에, 병의 발병과 진행 과정에 대한 근본적인 원인을 밝히는 것과, 효과적인 치료법 개발이 필요하다. 최근 연구들에서 골관절염에서 긴 비암호화 RNA가 다양한 발달 및 후성유전학적 조절 과정에서 중요한 역할을 한다고 보고되었다. 본 연구의 목적은 인간의 정상 및 손상 연골조직 유래 세포에서 발현 차이를 나타내는 긴 비암호화 RNA를 식별하고, 이러한 긴 비암호화 RNA의 조절을 통해 연골세포의 염증 반응이

조절되는지 확인하는 것이다. RNA-시퀀싱 분석을 통해 정상 및 손상 조직 유래 연골세포간의 전사체학적 차이를 조사하고 발현 차이를 나타내는 긴 비암호화 RNA를 스크리닝 함으로서, 결과적으로 26개의 긴 비암호화 RNA가 인간 손상 연골세포에서 6배 이상 증가하며, TNF-α에 의해 유발된 체외 염증 환경에서는 15개의 긴 비암호화 RNA가 2배 이상 증가하는 것을 확인하였다. 15개의 긴 비암호화 RNA에 대한 타겟 짧은 간섭 RNA를 처리하였을 때, 7개의 짧은 간섭 RNA가 40% 이상의 유전자 발현 억제 효율을 나타내는 것을 확인하였다. 체외 염증 모사 환경에서 짧은 간섭 RNA를 사용하여 7개의 긴 비암호화 RNA를 억제 시킨 뒤 COL2A1의 단백질 발현을 확인 한 결과, LOC107985352와 LOC107985369를 억제 시킨 경우 COL2A1 발현이 가장 유의미하게 증가하고, 이화작용과 염증 반응에 관여하는 유전자 발현이 감소됨을 확인하였다. 이러한 연구 결과는 골관절염 연골세포에서 LOC107985352와 LOC107985369의 하향 조절이 연골세포의 염증 반응을 조절 함으로서, 연골세포 항상성 유지에 영향을 미칠 수 있음을 의미한다. 따라서 LOC107985352와 LOC107985369은 골관절염 진행에 중요한 조절 인자가 될 수 있으며, 이러한 긴 비암호화 RNA는 골관절염 치료를 위한 효과적인 타겟이 될 수 있을 것으로 사료된다.

핵심되는 말 : 골관절염, 긴 비암호화 RNA, 염증 반응, RNA-시퀀싱, 연골세포