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# Long non-protein coding RNA DANCR functions as a competing endogenous RNA to regulate osteoarthritis progression via miR-577/SphK2 axis

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ABSTRACT

Long noncoding RNAs (lncRNAs) have been known to be involved in multiple diverse diseases, including osteoarthritis (OA). This study aimed to explore the role of differentiation antagonizing non-protein coding RNA (DANCR) in OA and identify the potential molecular mechanisms. The expression of DANCR in cartilage samples from patients with OA was detected using quantitative reverse transcription–polymerase chain reaction. The effects of DANCR on the viability of OA chondrocytes and apoptosis were explored using cell counting kit 8 assay and flow cytometry assay, respectively. Additionally, the interaction among DANCR, miR-577, and SphK2 was explored using dual-luciferase reporter and RIP assays. The present study found that DANCR was significantly upregulated in patients with OA. Functional assays demonstrated that DANCR inhibition suppressed the proliferation of OA chondrocytes and induced cell apoptosis. The study also showed that DANCR acted as a competitive endogenous RNA to sponge miR-577, which targeted the mRNA of SphK2 to regulate the survival of OA chondrocytes. In conclusion, the study revealed that lncRNA DANCR might promote the proliferation of OA chondrocytes and reduce apoptosis through the miR-577/SphK2 axis. Thus, lncRNA DANCR might be considered as a potential therapeutic target for OA treatment.



## 1. Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by deterioration in the integrity of cartilage, reduced cartilage cellularity, subchondral sclerosis, and synovial inflammation [1]. It is a leading cause of pain, disability, and shortening of adult working life around the world [2]. Multiple factors have been found to be implicated in the etiology of OA. However, no approved treatment is available that can modify the disease progression [3]. Chondrocyte is the only cell type in mature cartilage, which dominates the degenerative process of cartilage [4]. Evidence has suggested that the altered expression of genes in chondrocytes is associated with the synthesis and degradation of cartilage, which might provide novel therapeutic targets for OA.

Long noncoding RNA (lncRNA) is a set of RNAs (longer than 200 nucleotides in length) without the function of encoding proteins, but it regulates gene expression at the chromatin modification, transcriptional, or post-transcriptional level [5]. LncRNAs have been proven to regulate embryo development, immune response, cell maintenance, cancer occurrence, and so on [6,7]. Recently, the aberrant expression of lncRNAs in OA has been reported. For example, Su et al. showed that lncRNA MEG3 was downregulated and inversely associated with VEGF levels in OA [8]. Wang et al. showed that lncRNA UCA1 enhanced the expression of MMP-13 by inhibiting miR-204-5p in human chondrocytes [9]. Li et al. showed that lncRNA CIR promoted chondrocyte extracellular matrix degradation in OA by acting as a sponge for miR-27b [10]. Those studies indicated that lncRNAs played important functions in OA progression.

LncRNA differentiation antagonizing non-protein coding RNA (DANCR), a recently reported lncRNA, was downregulated during the differentiation of cultured human epidermal cells [11]. Recent

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studies showed the important role of DANCR in biological functions. For example, Tang et al. showed that lncRNA DANCR involved osteolysis after total hip arthroplasty by regulating the expression of FOXO1 to inhibit osteoblast differentiation [12]. Zhang et al. revealed that DANCR regulated the proliferation and osteogenic differentiation of human bone–derived marrow mesenchymal stem cells via the p38 MAPK pathway [13]. Xu et al. indicated that DANCR functioned as a competing endogenous RNA to regulate the expression of RAB1A by sponging miR-634 in glioma [14]. However, its function in OA progression remains unclear.

The present study investigated the biological role and underlying mechanism of lncRNA DANCR in OA. The data showed that lncRNA DANCR was upregulated in OA chondrocytes. Functional assays showed that DANCR inhibition suppressed the proliferation of OA chondrocytes and induced apoptosis. Moreover, the study suggested that DANCR promoted the progression of OA chondrocytes through the miR-577/SphK2 axis. Therefore, the study indicated that DANCR could act as a potential therapeutic target for treating OA.

## 2. Materials and methods

### 2.1. Patients and specimens

Patients diagnosed with OA and healthy subjects (with a normal joint) were included in this study. OA cartilage was isolated from the knee joints of 36 patients undergoing total knee arthroplasty. The normal articular cartilage was isolated from the knee joints of 13 patients with femoral neck fracture without OA or rheumatic arthritis who underwent total hip replacement surgery. Informed consent was obtained from all tissue donors included in the present study. This study was approved by the ethics committee of Jinling Hospital, Nanjing University.

### 2.2. Chondrocyte culture and transfection

The obtained articular cartilage was minced and digested with 0.2% collagenase II in Dulbecco's modified Eagle's medium (DMEM) to obtain chondrocytes. The chondrocytes were maintained in DMEM containing 10% fetal bovine serum (Gibco) for 24 h at 37 °C, filtered through a 0.075-mm cell strainer, and washed with sterile phosphate-buffered saline (PBS). After 2 weeks, the first-passage chondrocytes were obtained, which were incubated in a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C. The medium was changed every 2 days.

The short interfering RNAs (siRNAs) against DANCR were purchased from Riobo Company. siRNAs were transfected into cells using Lipofectamine 3000 according to the manufacturer's protocol (Invitrogen). For miRNA transfection, miR-577 mimics, miR-577 inhibitors, and negative control (RiboBio) were transfected into cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

### 2.3. Quantitative real-time PCR

The cells were washed with PBS twice before RNA purification. RNA was purified using TRIzol following the manufacturer's protocol. The concentration and purity were determined using NanoDrop 2000 spectrophotometer (Thermo Fisher). cDNA was synthesized using an RT2 First Strand Kit (Qiagen). Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) reaction was performed using GoTaq qPCR Master Mix (Promega) on ABI 7900 (Applied Bioscience). Relative gene expression was calculated using the $2^{-\triangle\triangle CT}$ method, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control.

### 2.4. Cell counting kit 8 assays

The cells were seeded in a 96-well plate at a density of $2 \times 10^3$ cells/well and incubated in a 5% $CO_2$ and 37 °C humidified incubator. The final volume in each well was 100 μL in triplicate. After incubation for 24, 48, 72, and 96 h, 10 μL of 5 mg/mL cell counting kit 8 (CCK-8) solution (Dojindo) was added to each well for a further 4-h incubation. The absorbance was measured at 450 nm using a plate reader (BioTek uQuant).

### 2.5. Bromodeoxyuridine incorporation assay

The cell proliferation was examined using the bromodeoxyuridine (BrdU) assay (Roche) according to the manufacturer's protocol. Briefly, the chondrocytes were seeded in 96-well plates at a density of $5 \times 10^3$ cells in a final volume of 100 μL per well and then treated with different concentrations of icariin for 3 days. After 4 h of BrdU (10 μM) treatment, the cells were incubated with peroxidase-conjugated anti-BrdU antibody for 90 min, and BrdU incorporation was detected by incubating cells with tetramethylbenzidine as a substrate. Color development was directly proportional to the amount of DNA synthesis and thereby to the number of proliferating cells. BrdU-positive cells were counted.

### 2.6. Cell apoptosis assays

The cells were harvested, washed twice with PBS, resuspended in 1 × binding buffer at a concentration of $1 \times 10^6$ cells/mL, and then incubated with Annexin V–PE and PI (BD) for 15 min at room temperature in the dark. After adding 400 μL of binding buffer and incubation for 1 h, the stained cells were analyzed by FACS flow cytometry (BD) within 1 h.

### 2.7. Luciferase reporter assay

The 3′-UTR region of DANCR was amplified in wild and mutant types using specific primers. The amplicons were purified and subsequently cloned into a psiCHECK-2 vector. hRluc was used as a fluorescent reporter, and hRluc was used as a fluorescent calibrator. The 3′-UTR sequence was cloned to the downstream of hRluc sequence. miRNA targets in the 3′-UTR region were used to affect the gene expression. Thus, the cells were co-transfected with miR-577 mimics and reporter vector. The interaction between miR-577 and target gene was determined depending on the comparative fluorescent value.

### 2.8. RNA immunoprecipitation analysis

The cells were lysed using an EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore) following the manufacturer's protocol. Then, 100 μL of whole cell lysate was incubated with anti-Argonaute2 (anti-Ago2) or anti-immunoglobulin G (IgG) (negative control). After 2 h, the immunoprecipitated RNA was isolated. The RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific), and the RNA quality was determined using a bioanalyzer (Agilent). Next, the purified RNA was further used in the RT-qPCR analysis.

### 2.9. Western blot analysis

Total proteins were extracted from cells using RIPA lysis buffer (Beyotime). The protein concentrations were measured using an Enhanced Bicinchoninic Acid Protein Assay Kit (Bio-Rad). Lysates of total protein were separated using a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Millipore). The

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