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Original Article

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LncRNA SNHG5/miR-26a/SOX2 signal axis enhances proliferation of chondrocyte in osteoarthritis

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Abstract

Chondrocyte is involved in the destruction of joints in osteoarthritis (OA) patients. The aim of this study was to explore the expression level of small nucleolar RNA host gene 5 (SNHG5) and evaluate its function in chondrocyte. In our current study, the expression levels of SNHG5, miR-26a, and SOX2 in 17 pairs of articular cartilage tissues and in the non-OA group were assessed by real-time quantitative reverse-transcription polymerase chain reaction. Results showed that the levels of SNHG5 and SOX2 were significantly downregulated in OA tissues, while the level of miR-26a was upregulated. MTT, colony formation and cell transwell assays were performed to assess the function of SNHG5 on the cell viability, growth ability, and migration capacity in CHON-001 cells. It was found that SNHG5 could promote chondrocyte cell proliferation and migration. The relationship between SNHG5 and miR-26a was confirmed by RIP and the luciferase reporter assays. SOX2 was identified as a target gene of miR-26a by the luciferase reporter assay. Rescue assay was applied to verify the relationship among SNHG5, miR-26a, and SOX2. Our current study demonstrated that SNHG5 is involved in the mechanism of OA through functioning as a ceRNA to competitively sponge miR-26a, therefore, regulating the expression of SOX2.

Key words: lncRNA SNHG5, miR-26a, SOX2, osteoarthritis

Introduction

Osteoarthritis (OA) has been characterized by cartilage degradation, subcondral bone remodeling and synovitis [1]. OA is one of the two major causes for disability and chronic pain, and its incidence has been increasing among older persons and younger adults in recent years. Current studies are focused on the symptom control rather than the pathogeny and integration mechanisms, major developments in mechanics related to OA are critical for exploring novel treatments. Accumulating documents have identified that chondrocyte survival is related to the destruction of joints in OA patients [2–4]. Despite certain studies have been made, the molecular mechanisms associated with chondrocyte survival in OA still need to be further investigated.

Long non-coding RNAs (lncRNAs), with more than 200 nt in length, have been implicated to coordinate gene expression through gene imprinting, dosage compensation, and control of transcription or post-transcriptional processing, which play critical roles in multiple biological processes in various cancers, including OA [4]. However, the pathological contributions of lncRNAs to OA are still very few. In recent years, a novel level of post-transcriptional regulation has been explored where lncRNAs interact with miRNAs via functioning as competing endogenous RNAs

(ceRNAs), competitively sponging miRNAs to regulate the target mRNAs [5–7].

Small nucleolar RNA (snoRNA) host gene 5 (SNHG5), firstly reported to be located at the chromosomal translocation breakpoint involved in B-cell lymphoma, is a member of both the multiple non-protein-coding snoRNAs host gene family and the 59-terminal oligopyrimidine class of genes [8,9]. It has been previously reported that SNHG5 can directly bind with miR-32 and effectively act as a sponge for miR-32 to modulate the suppression of Krüppel-like factor (KLF)-4 [10].

In the present study, we measured the expression of SNHG5 in cartilage samples from OA patients and healthy subjects, and then investigated the role of SNHG5 in OA chondrocyte. Our findings revealed that the SNHG5/miR-26a/SOX2 axis functions as an important player in OA.

Materials and Methods

Articular cartilage tissues

The OA cartilage was isolated from the knee joints of 17 patients undergoing total knee arthroplasty from the department of Hematology and Rheumatology, the Fourth Affiliated Hospital of Harbin Medical University. And the normal articular cartilages were obtained from 17 patients without OA but with femoral neck fracture or rheumatic arthritis who underwent total hip replacement surgery. The detailed information of the patients is listed in **Table 1**. And the informed consents were obtained from all patients. The study was approved by the Human Ethics Committee of Department of Hematology and Rheumatology, the Fourth Affiliated Hospital of Harbin Medical University.

SNHG5 overexpression vector construction

The entire SNHG5 genomic locus was amplified by PCR (HiFi Platinum Taq; Invitrogen, Carlsbad, USA) using the following primers: SNHG5 forward: 5′-TACTGGCTGCGCACTTCG-3′, and SNHG5 reverse: 5′-TACCCTGCACAAACCCGAAA-3′. The cDNA was extracted from gel and cloned in pGEM T-Easy vector system (Promega, Madison, USA) and further subcloned into pcDNA3.1(+) resulting in pcDNA3.1/SNHG5 (Invitrogen).

Cell lines

Human chondrocyte cell line CHON-001, derived from normal human articular cartilage and HEK-293 cell line (ATCC, Manassas, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, USA) supplemented with 0.1 mg/ml G-418 (Gibco) and 10% fetal bovine serum (FBS; Gibco). The cells were incubated in humidified atmosphere with 5% CO_2 at 37°C and passaged at a ratio of 1:5.

Table 1. Clinical characteristics of the study objects

Characteristics	Normal	OA
Gender		
Male (*n*)	9	10
Female (*n*)	8	7
Ages (years)		
Mean	56.7 ± 6.4	60.5 ± 6.4
Range	48–66	52–70

Cell transfection

Cells were transiently transfected with plasmid or siRNA oligonucleotide against SNHG5 using RNAiMax and Lipofectamine® LTX with PlusTM Reagent (Thermo Scientific, Waltham, USA) according to the manufacturer's protocols. Cells were plates in 6-well plates at a density of 1×10^4 cells/well and incubated for 12 h at 37°C. The culture medium was replaced with fresh DMEM and transfected with specific construct. Cells were collected at 72 h for experiment.

MTT assay

The cell growth and viability of chondrocytes were measured by MTT assay. In brief, cells were plated on a 96-well plate at a density of 1×10^5 cells/well. After the indicated treatments, 20 μl of MTT stock solution (5 mg/ml; Sigma, St Louis, USA) was added to each well and incubated at 37°C. After 4 h, the medium was discarded, and formazan crystals were dissolved by adding dimethyl sulfoxide (DMSO; Sigma) at 200 μl/well. After gentle shaking for 15 min, the absorbance at 490 nm was measured by using a microplate reader (Bio-Tek Instruments, Winooski, USA).

Colony formation assay

At 48 h after transfection, cells were treated with 0.3% soft agar and incubated in 6-well plates at 500 cells/well in DMEM with 10% FBS at 37°C. Later, the medium was replaced with unsalted DMEM every 2 days. Two weeks later, the cells were fixed with methanol under the condition of −20°C for 5 min, and then were stained with 0.1% crystal violet. The number of visible colonies was counted manually by means of an IX71 microscope (Olympus, Tokyo, Japan). The assay was performed in triplicate.

Cell migration assay

Cell migration capacity was measured by designing the transwell chamber (8-μm pore size; Corning, Cambridge, USA). At 48 h after transfection, cells in serum-free media were placed into the upper chamber. The medium containing 10% FBS was added into the lower chamber. After 48 h of incubation, cells remained in the upper membrane were wiped off, while cells migrated were fixed with methanol, then stained with 0.1% crystal violet and finally counted under the IX71 microscope. Three independent experiments were carried out.

Real-time quantitative reverse-transcription polymerase chain reaction

Total RNAs from tissues and cells were isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with PrimeScript RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's protocol. Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed with SYBR PrimeScript RT-PCR Kits (Takara) based on the manufacturer's instructions. The SNHG5, SOX2, and miR-26a levels were calculated with the $2^{-\Delta\Delta Ct}$ method, which was normalized to GAPDH mRNA or U6 rRNA, respectively. All assays were performed in triplicate. The primers used in this research were as follows: *GAPDH* forward: 5′-ATGGGGAAGGTGAAGGTCG-3′, and reverse: 5′-GGGTCATT GATGGCAACAATATC-3′; *SNHG5* forward: 5′-CGCTTGGTT AAAACCTGACACT-3′, and reverse: 5′-CCAAGACAATCTGGCC TCTATC-3′; *miR-26a* forward: 5′-GCGGCGGTTCAAGTAATCC AGG-3′, and reverse: 5′-ATCCAGTGCAGGGTCCGAGG-3′; *SOX2*

forward: 5′-GGGAAATGGGAGGGGTGCAAAAGAGG-3′, and reverse: 5′-TTGCGTGAGTGTGGATGGGATTGGTGT-3′; *U6* forward: 5′-CTCGCTTCGGCAGCACA-3′, and reverse: 5′-AACGCTTCAGGAATTTGCGT-3′.

Dual luciferase reporter assay

In order to figure out how SNHG5 comes into play a role in OA, we predicted the target genes of SNHG5 by means of a bioinformatics prediction (StarBase v2.0) and the sequence analysis. On the basis of the predicted results and initial assays, the following pairs of recombinant plasmids were constructed: pmirGLO-SNHG5-wt and pmirGLO-SNHG5-mut, pmirGLO-SOX2-wt and pmirGLO-SOX2-mut. PCR was used to get the wild-type SNHG5 sequence and wild-type SOX2 mRNA. Mutant SNHG5 without miR-26a binding sites and mutant SOX2 were acquired through the overlap extension PCR with the mutant primers. For the luciferase reporter analysis, pmirGLO-SNHG5-wt or pmirGLO-SNHG5-mut was co-transfected with miR-26a mimics or miRNA NC into HEK-293 cells by Lipofectamine-mediated gene transfer. The relative luciferase activity was normalized to Renilla luciferase activity 48 h after transfection. The data were relative to the fold change of the corresponding control groups defined as 1.0.

Western blot analysis

Cells were lysed by using Radio Immunoprecipitation Assay (RIPA) buffer (Thermo Scientific) containing a protease inhibitor cocktail (P8340; Sigma), and 30 μg of protein was then separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein loading was estimated by using mouse anti-GAPDH monoclonal antibody. The membranes were blocked with 10% non-fat milk in 1× TBST (TBS+Tween) for 2 h at room temperature, washed and then probed with the rabbit anti-human SOX2 (1:2000 dilution) and GAPDH (1:3000 dilution) overnight at 4°C, followed by treatment with secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. The proteins were detected using the enhanced chemiluminescence system and exposed to X-ray films. All antibodies were purchased from Abcam (Cambridge, USA).

RNA immunoprecipitation

RNA immunoprecipitation (RIP) was performed by using Thermo Fisher RIP kit (Thermo Scientific) based on the manufacturer's protocol. The Ago2 antibodies were purchased from Abcam. Normal mouse IgG (Abcam) was applied as negative control and anti-SNRNP70 (Abcam) was employed as positive control for the RIP procedure. Purified RNA was subject to qRT-PCR analysis to demonstrate the presence of the binding targets by using respective primers.

RNA interference

In order to analyze SNHG5, the specific si-RNA against SNHG5 (sh-SNHG5 group) and the negative control oligonucleotides (sh-RNA group) were acquired from Shanghai GenePharma Co., Ltd (Shanghai, China). The following primers were used for RNAi knockdown: control sense: 5′-GATCCGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAACTTTTTTG-3′, and control antisense: 5′-AATTCAAAAAAGTTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAACG-3′; SNHG5 sense: 5′-GAGGCCAGAUUGUCUUGGATT-3′, and SNHG5 antisense: 5′-UCCAAGACAAUCUGGCCUCTT-3′; SOX2 sense: 5′-CCGCGTTCTACCGGGTCCTCT-3′, and SOX2 antisense: 5′-GCCCATGCACCGCTACGACGTG-3′. To analyze the SNHG5-related miRNA, miR-26a mimics and inhibitor (for the miR-26a group) and scrambled oligonucleotides were also acquired from Shanghai GenePharma Co., Ltd.

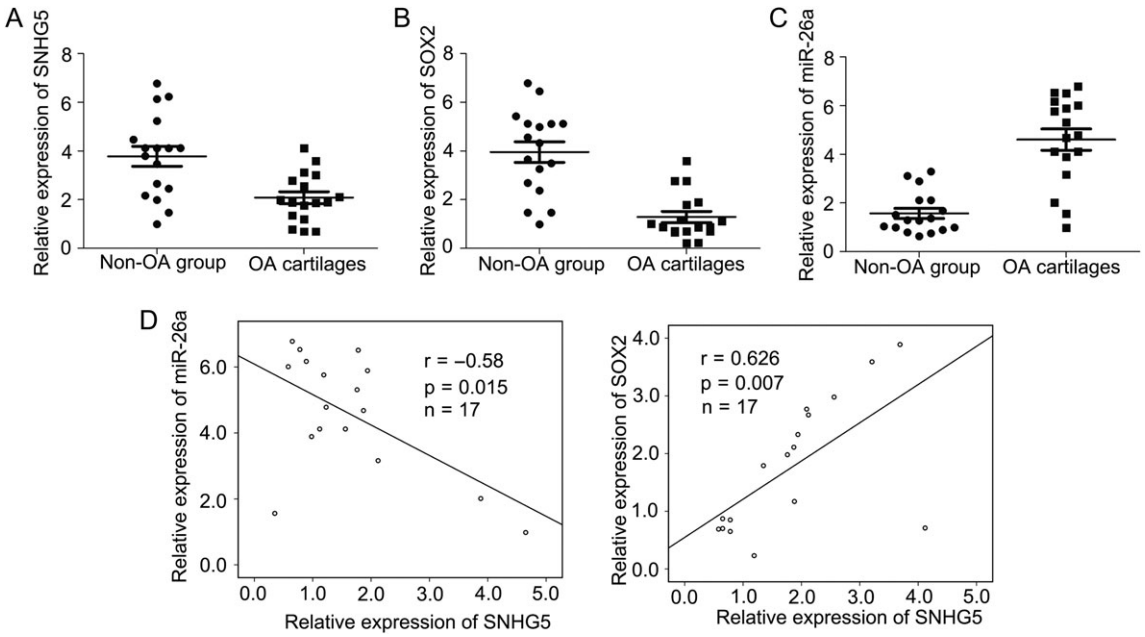

Figure 1. The SNHG5 and SOX2 expressions were downregulated and miR-26a was upregulated in OA cartilage (A–C) The expression levels of SNHG5, SOX2, and miR-26a were measured in OA cartilages and the non-OA group by qRT-PCR. (D) The correlations of SNHG5 with miR-26a and with SOX2 were analyzed by Spearman's correlation analysis. Error bars represented the mean ± SD of at least three independent experiments.

Statistical analysis

Data were shown as the mean ± SD of at least three independent experiments. The SPSS 17.0 software (SPSS Inc., Chicago, USA) was used for statistical analysis. Two group comparisons were performed with Student's *t*-test. Multiple group comparisons were analyzed with one-way ANOVA. Statistically, the significantly negative correlation between SNHG5 and miR-26a expression levels in 17 cases of OA samples was analyzed by Spearman's correlation analysis. And statistically, the significant positive correlation between SNHG5 and SOX2 expression levels in 17 cases of OA samples was analyzed by Spearman's correlation analysis. All tests performed were two-sided. $P < 0.05$ was considered statistically significant.

Results

The SNHG5 and SOX2 expressions were downregulated and miR-26a was upregulated in OA cartilage

As shown in **Fig. 1A,B**, the levels of SNHG5 and SOX2 were significantly downregulated in OA cartilages compared to that in the non-OA group ($P < 0.001$). The level of miR-26a was obviously upregulated in OA cartilages, compared with that in the non-OA group (**Fig. 1C**, $P < 0.001$). To further determine the relationship among the three molecules, a correlation analysis was performed. As shown in **Fig. 1D**, in the 17 OA samples, the level of SNHG5 was negatively correlated with the level of miR-26a, but was positively correlated with the level of SOX2. These results indicated that the function of SNHG5 might be associated with miR-26a and SOX2.

SNHG5 regulates the expression of SOX2 through competitively sponging miR-26a in CHON-001 cells

It has been demonstrated that lncRNAs interact with miRNAs via functioning as competing endogenous RNAs (ceRNAs), competitively sponging miRNAs and regulating the target mRNAs. And it has been previously reported that SNHG5 directly binds with miR-32 and effectively acts as a sponge for miR-32 to modulate the suppression of KLF-4 [10]. In order to notarize the function of SNHG5 as a ceRNA, we used the bioinformatics softwares starBase v2.0 (http://starbase.sysu.edu.cn) and miRandasites (http://www.microrna.org/microrna/home.do) to select the proper miRNA and mRNA independently. On the basis of the starBase v2.0, miR-26a was identified as the target

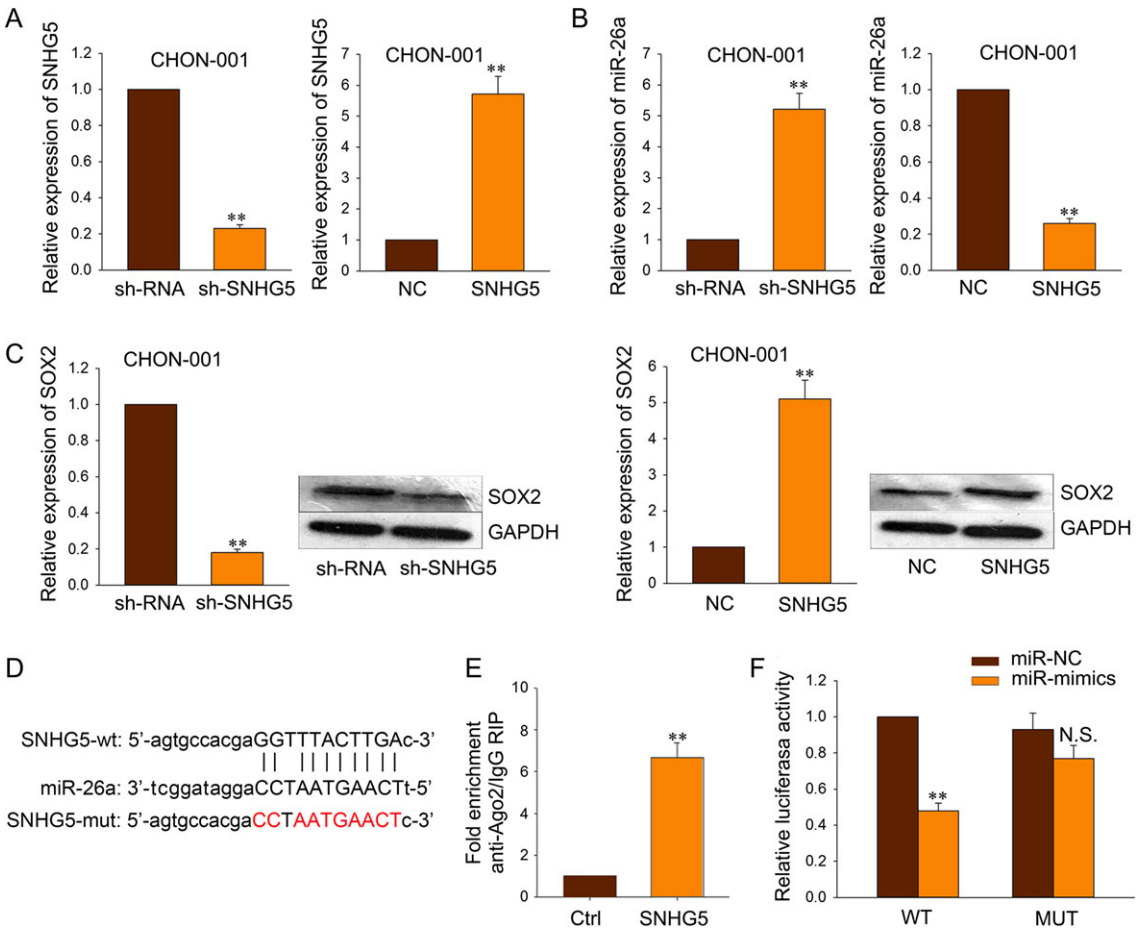

Figure 2. SNHG5 regulated the expression of SOX2 through competitively sponging miR-26a in CHON-001 cells (A) Satisfactory transfection efficiency was obtained at 48 h post-transfection as revealed by qRT-PCR. (B) The level of miR-26a was measured by qRT-PCR in response to the level of SNHG5. (C) Both the mRNA and protein levels of SOX2 in response to the level of SNHG5 were detected by qRT-PCR and western blot analysis respectively. (D) The binding sites between SNHG5 and miR-26a were predicted on the basis of starBase v2.0. (E,F) RIP and dual-luciferase reporter assays were performed to confirm the relationship between SNHG5 and miR-26a. Error bars represented the mean ± SD of at least three independent experiments. N.S.: no significant. $*P < 0.05$, $**P < 0.01$ vs. control group.

gene of lncRNA SNHG5, and SOX2 was figured out to be the target of miR-26a in line with the miRandasites software. Therefore, we hypothesized that in OA, SNHG5 may regulate the expression of SOX2 through functioning as a ceRNA and competitively sponging miR-26a, thereby, regulating the expression of SOX2. To confirm the hypothesis, we first measured the levels of miR-26a and SOX2 in response to the level of SNHG5. CHON-001 cells were transfected with sh-SNHG5 or pcDNA3.1/SNHG5, which effectively induced suppression or promotion in the expression level of SNHG5, and satisfactory transfection efficiency was obtained 48 h later (**Fig. 2A**). As observed in **Fig. 2B**, the expression level of miR-26a was inhibited when SNHG5 was overexpressed, and was promoted when SNHG5 was inhibited. Additionally, both the mRNA and protein levels of SOX2 were consistent with the level of SNHG5 (**Fig. 2C**). To further determine the relationship between SNHG5 and miR-26a, we searched the binding sites between SNHG5 and miR-26a on the basis of the bioinformatics software starBase v2.0 (**Fig. 2D**). Furthermore, RIP and dual-luciferase reporter assays were performed to make a further confirmation. The result in the RIP assay showed that SNHG5 was enriched in beads which contain Ago2 antibody compared with the control group (IgG) (**Fig. 2E**). Furthermore, SNHG5-wt and SNHG5-mut were generated by using PCR and a mutagenesis kit. The fragments including the putative binding site were cloned into a pmirGLO vector as the pmirGLO-SNHG5-wt and pmirGLO-SNHG5-mut. And then the recombinant plasmids were co-transfected with miR-26a mimics and scrambled oligonucleotides into CHON-001 cells by using Lipofectamine™ 2000. Luciferase activity was tested at 24 h after transfection. It was found that miR-26a weakened the luciferase activity of pmirGLO-SNHG5-wt but not that of pmirGLO-SNHG5-mut (**Fig. 2F**). These results implied that SNHG5 regulated the expression of SOX2 through functioning as a ceRNA and competitively sponging miR-26a.

SNHG5 promotes CHON-001 cell proliferation and migration

Next we examined whether SNHG5 is involved in the regulation of chondrocyte proliferation and migration. Results from MTT and colony formation assays showed that the viability and proliferation ability of CHON-001 cells were significantly enhanced when SNHG5 was overexpressed and vice versa in the SNHG5 deletion cells ($P < 0.01$, **Fig. 3A,B**). Furthermore, the function of SNHG5 on CHON-001 cell migration was measured by the transwell assay. As shown in **Fig. 3C**, forced expression of SNHG5 could significantly increase the migration capacity, while deletion of SNHG5 led to an opposite result. These data together indicated that SNHG5 could promote cell viability, proliferation ability, and migration capacity of CHON-001 cells.

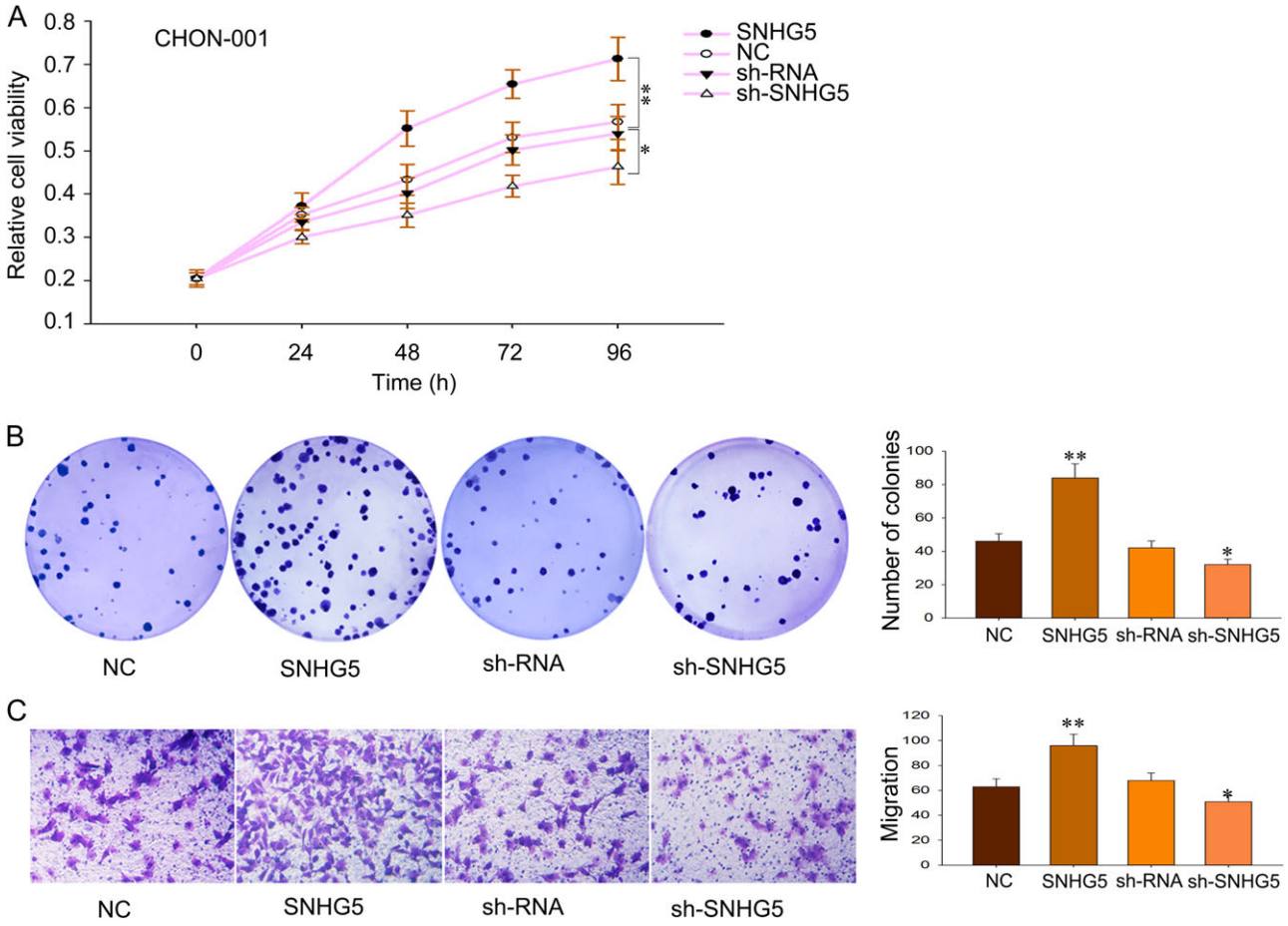

Figure 3. SNHG5 promoted CHON-001 cell proliferation and migration (A,B) MTT and colony formation assays were performed to detect the viability and proliferation ability of CHON-001 when SNHG5 was overexpressed or downregulated. (C) Transwell assay was employed to determine the migration capacity of CHON-001 after SNHG5 was overexpressed or downregulated. Error bars represented the mean ± SD of at least three independent experiments. N.S.: no significant. *$P < 0.05$, **$P < 0.01$ vs. control group.

The function of miR-26a is opposite to that of SNHG5

To explore the function of miR-26a in CHON-001 cells, we transfected the CHON-001 cells with miR-26a mimics or inhibitor to effectively induce promotion or suppression of the miR-26a level (**Fig. 4A**). MTT and colony formation assays demonstrated that the cell viability and proliferation ability of CHON-001 cells were significantly weakened after the introduction of miR-26a mimics and opposite results were obtained in cells transfected with miR-26a inhibitor (**Fig. 4B,C**). Additionally, we detected the effect of miR-26a on CHON-001 cell migration. As presented in **Fig. 4D**, miR-26a mimics could significantly decrease the migration capacity, while opposite result was obtained in cells transfected with miR-26a inhibitor. These data suggested that the function of miR-26a was opposite to the function of SNHG5, indicating that SNHG5 competitively regulates miR-26a.

The function of SNHG5 relies on the regulation of SOX2

The results obtained above demonstrated that SNHG5 could competitively sponge miR-26a, thereby exerting pro-proliferation and pro-migration function. However, miR-26a was not the final functional molecule. We have revealed that the level of SOX2 is inversely correlated with the level of miR-26a. In order to confirm that SOX2 is a target of miR-26a and the final functional molecule in the signal pathway of SNHG5/miR-26a, we took advantage of the bioinformatics website MicroRNA.org (http://34.236.212.39/microrna/home.do) to search for the binding sites between miR-26a and SOX2 (**Fig. 5A**). Then the dual-luciferase assay was applied to verify their binding, and results showed that the activity of pmirGLO-SOX-wt rather than that of pmirGLO-SOX-mut were lessened by miR-26a mimics (**Fig. 5B**). To further confirm that, rescue assay was performed. As shown in **Fig. 5C,D**, miR-26a could abolish the effect of SNHG5 on cell viability and proliferation ability, but this activity could be partially reversed by introduction of SOX2. Additionally, miR-26a could abrogate the effect of SNHG5 on cell migration capacity, but this activity could be in part rescued by co-transfection with SOX2 (**Fig. 5E**). These findings revealed that SNHG5/miR-26a/SOX2 signal axis could enhance the proliferation ability and migration capacity of chondrocytes in OA.

Discussion

OA is the commonest joint disease and typically begins with an aging-related disruption of the articular cartilage surface. Multiple factors are related to the occurrence of OA, including aging, abnormal mechanical loading, trauma, and genetic predisposition. Accumulating documents have identified that chondrocyte survival is involved in the destruction of joints in OA patients. Recently, miRNAs have been reported to play essential roles in the regulation of OA. But little has been reported about the function of lncRNAs in OA. The lncRNAs are RNA molecules over 200 nt in length with little protein-coding potential [11]. Accumulating evidences have shown that lncRNAs play pivotal role in tumorigenesis in a wide repertoire of biological processes [12–14].

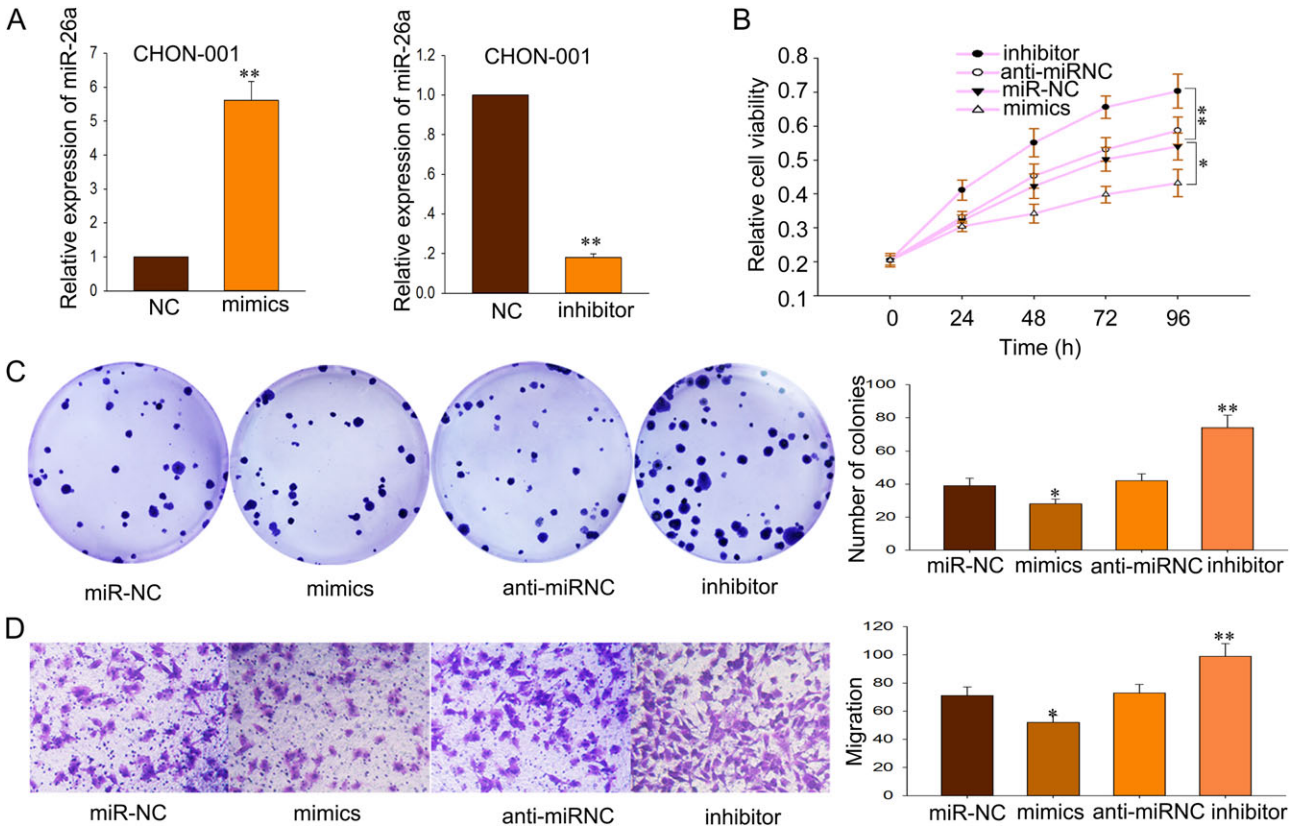

Figure 4. The function of miR-26a was opposite to that of SNHG5 (A) Satisfactory transfection efficiency was obtained after 48 h as revealed by qRT-PCR. (B, C) MTT and colony formation assays were performed to detect the viability and proliferation ability of CHON-001 after the treatment with miR-26a mimics or miR-26a inhibitor. (D) Transwell assay was employed to determine the migration capacity of CHON-001 after being treated with miR-26a mimics or miR-26a inhibitor. Error bars represented the mean ± SD of at least three independent experiments. N.S.: no significant. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

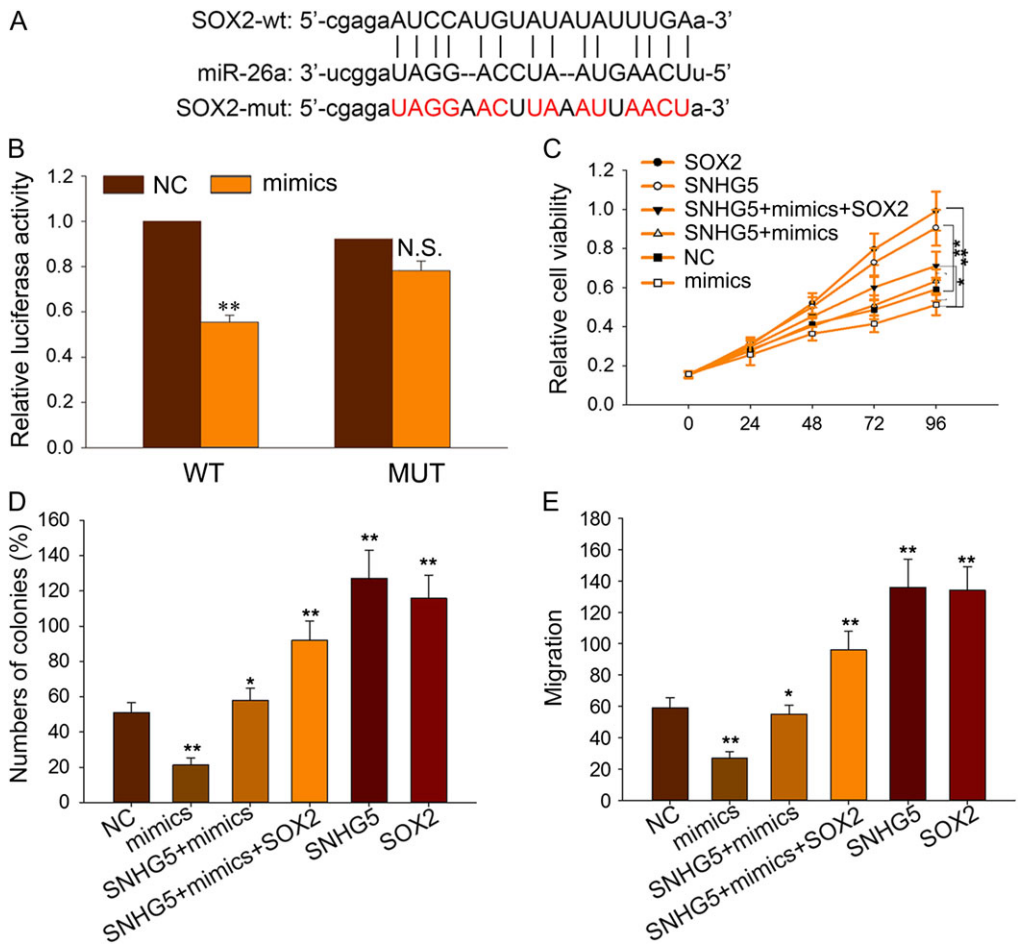

Figure 5. The function of SNHG5 relies on the regulation of SOX2 (A) The binding sites between miR-26a and SOX2 were predicted with miRandasites. (B) Dual-luciferase assay was performed to confirm the regulation relationship between miR-26a and SOX2. (C,D) MTT and colony formation assays were performed to detect the viability and proliferation ability of CHON-001 cells when the cells were co-transfected with miR-26a, SNHG5, and SOX2. (E) Transwell assay was employed to determine the migration capacity of CHON-001 cells when the cells were co-transfected with miR-26a, SNHG5, and SOX2. Error bars represented the mean ± SD of at least three independent experiments. N.S.: no significant. $*P < 0.05$, $**P < 0.01$ vs. control group.

SNHG5, firstly reported to be located at the chromosomal translocation breakpoint involved in B-cell lymphoma, is a member of both the multiple non-protein-coding snoRNAs host gene family and the 59-terminal oligopyrimidine class of genes [8,9]. The abnormal expression of SNHG5 has been reported in cancers. Damas *et al.* [15] reported that SNHG5 could promote colorectal cancer cell survival by counteracting STAU1-mediated mRNA destabilization. And Zhao *et al.* [16] also demonstrated that long non-coding RNA SNHG5 could suppress gastric cancer progression by trapping MTA2 in the cytosol. Currently, a novel level of post-transcriptional regulation has been explored where lncRNAs interact with miRNAs via functioning as competing endogenous RNAs (ceRNAs), competitively sponging miRNAs to regulate the target mRNAs [17–23]. SNHG5 has also been identified as a ceRNA, regulating the proliferation and migration of gastric cancer cells [10]. Despite the implications of SNHG5 in the regulation of a variety of transcription factors and physiological processes, the role of SNHG5 in OA remains to be explored. In present study, we first detected the levels of SNHG5, miR-26a and SOX2 in OA tissues and non-OA-tissues. Our findings demonstrated that SNHG5 and SOX2 were significantly downregulated in OA tissues, while the level of miR-26a was upregulated. Then, gain-of-function and loss-of-function experiments revealed that SNHG5 could promote chondrocyte cell proliferation and migration, and it was also identified that miR-26a/SOX2 signal pathway mediated the function of SNHG5 in OA.

In conclusion, our study was the first to investigate the role of SNHG5 in OA. The results indicated that SNHG5 was significantly downregulated in OA, and forced expression of SNHG5 could promote the proliferation and migration of chondrocyte cells. MiR-26a is a potential target gene of SNHG5, and miR-26a/SXO2 signal pathway mediates the function of SNHG5 in chondrocyte cells, implicating the potential application of SNHG5 in the treatment of OA.

Funding

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