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LncRNA SND1-IT1 accelerates the proliferation and migration of osteosarcoma *via* sponging miRNA-665 to upregulate POU2F1

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Abstract. – OBJECTIVE: To clarify the role of long non-coding RNA (lncRNA) SND1-IT1 in accelerating the proliferative and migratory abilities of osteosarcoma (OS) *via* sponging miRNA-665 to upregulate POU2F1.

PATIENTS AND METHODS: The relative level of SND1-IT1 in OS tissues was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The target gene of SND1-IT1 was predicted by bioinformatics and verified by Dual-Luciferase reporter gene assay. Similarly, the target gene of miRNA-665 was identified. Correlation among SND1-IT1, miRNA-665 and POU2F1 was evaluated through linear regression test. Regulatory effects of SND1-IT1/miRNA-665/POU2F1 on cellular behaviors of MG63 and U2OS cells were evaluated.

RESULTS: SND1-IT1 was upregulated in OS, knockdown of which attenuated proliferative and migratory abilities of OS cells. MiRNA-665 was the target gene of SND1-IT1, which was negatively correlated to SND1-IT1 in OS. POU2F1 was the target gene of miRNA-665. Its level was negatively regulated by miRNA-665 and positively regulated by SND1-IT1. Inhibited proliferative and migratory abilities of OS cells with SND1-IT1 knockdown were partially elevated by transfection of miRNA-665 inhibitor, and further downregulated by POU2F1 knockdown.

CONCLUSIONS: LncRNA SND1-IT1 accelerates proliferative and migratory abilities of OS via sponging miRNA-665 to upregulate POU2F1, thus stimulating the progression of OS.

Key Words:
Osteosarcoma, LncRNA SND1-IT1, MiRNA-665, POU2F, Proliferation, Migration.

Introduction

Osteosarcoma (OS) is a type of bone malignant tumor with high invasiveness that mainly involves limbs[1]. Its morbidity and mortality remain high in adolescents[2]. A relevant report proposed that the survival of local OS is about 65%, which is only 20% in metastatic and recurrent OS[3]. The molecular pathogenesis of OS has been explored in recent years. Nevertheless, mechanisms underlying drug-resistance and metastasis of OS remain unclear[4].

Long non-coding RNA (lncRNA) is a non-coding RNA of 200 nt in length, exerting a vital regulatory role in tumor cell behaviors[5]. Recently Hu et al[6] have illustrated the involvement of lncRNAs in malignant phenotypes of OS cells. LncRNA SND1-IT1 is a newly discovered non-coding RNA that regulates the promoter of oncogenes[7]. This work mainly explored the potential function of SND1-IT1 in the progression of OS.

MiRNAs are small, endogenous, non-coding RNAs of 18 to 25 nucleotides long. They participate in multiple cellular behaviors[8]. Abnormal activation of miRNAs influences the occurrence and progression of malignancies, including OS[9]. LncRNAs are proved to serve as ceRNAs, which sponges a certain miRNA to regulate its target gene, thus altering the post-transcriptional regulation[10]. MiRNA-665 is closely related to the function of the central nervous system, showing a protective effect on the apoptosis of hippocampal neurons[11]. Currently, studies on miRNA-665 and lncRNAs in OS progression are rarely reported.

POU2F1 is located on chromosome 1q24, also known as OCT-1[12]. It is a ubiquitous transcription factor that regulates transcription of genes involved in inflammation and cell cycle by binding to cis-acting octamer elements. POU2F1 mediates cell differentiation and proliferation by regulating housekeeping genes, such as H2B and snRNA. It participates in immunity and inflammation by regulating the expressions of tissue-specific target genes[13]. POU2F1 is reported to be upregulated in OS[14]. In this experiment, we investigated whether lncRNA SND1-IT1 could mediate POU2F1 expression by adsorbing miRNA-665, thereby ac-

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celerating the proliferative and invasive abilities of OS. Our results aim to provide novel ideas for clinical diagnosis and treatment of OS.

Patients and Methods

Expression Patterns of SND1-IT1 and POU2F1

This study was approved by the Ethics Committee of Gongli Hospital, the Second Military Medical University. Signed informed consents were obtained from all participants before the study. The OS tissues and normal bone tissues were collected in our hospital from 2015 to 2018. The Microarray GSE12885 containing OS tissue data were downregulated from the GEO database (www.ncbi.nlm.nih.gov/geo). The expressions of SND1-IT1 and POU2F1 in normal tissues and OS tissues were analyzed by limma R package.

Cell Culture and Transfection

OS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin in a 5% CO_2 and 37°C incubator. Until 70% of confluence, cells were transfected with relative plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 6 hours, the fresh medium was replaced for another 24 h incubation.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), chloroform and isopropanol. The extracted RNA was quantified and reversely transcribed into complementary deoxyribose nucleic acid (cDNA), followed by Polymerase Chain Reaction (PCR) using the SYBR Green method. Primer sequences were as follows: SND1-IT1: forward: 5'-CCTGAGCGGCAGATCAACC-3', reverse: 5'- AGGTAGATCATGCCATACTCTCG-3'; POU2F1: forward: 5'-ACTGTGGACCTCAGGTTGGACT-3', reverse: 5'-GCACCAGGGTCTCCGATTTG-3'; MiRNA-665: forward: 5'-ACCAGGAGGCTGAGG-3', reverse: 5'-GAGCAGGCTGGAGAA-3'; GADPH: forward: 5'-CCCAGCCTCAAGATCATCAGCAATG-3', reverse: 5'-ATGGACTGTGGTCATGAGTCCTT-3'; U6: forward: 5'-CGCTTCGGCAGCACATATACTAAAATTGGAAC-3', reverse: 5'-GCTTCACGAATTTGCGTGTCATCCTTGC-3'.

Cell Counting Kit-8 (CCK-8)

Cells were seeded in the 96-well plate with 2×10^3 cells per well. Absorbance (A) at 450 nm was recorded at the appointed time points using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

Colony Formation Assay

Cells were seeded in the 6-well plate with 300 cells per well and cultured for 10-14 days. Subsequently, cells were subjected to 15-min fixation in 4% paraformaldehyde and 30-min staining in 0.1% violet crystal. After removing the staining solution, colonies were aired dried and observed under a microscope.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

Cells were labeled with 50 μmol/L EdU at 37°C for 2 h. Subsequently, cells were subjected to 30-min fixation in 4% paraformaldehyde and 20-min incubation in Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) containing 0.5% Triton-100. After washing with PBS containing 3% bovine serum albumin (BSA), 100 μL of dying solution was applied per well for 1 h incubation in the dark and cells were counter-stained with Hoechst33342 for 30 min. The ratio of EdU-positive cells was calculated.

Transwell Migration Assay

The dose of transfected cells was adjusted to 3.0×10^5/mL. 100 μL/well suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA) pre-coated with 40 μL of diluted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). In the bottom side, 600 μL of medium containing 20% FBS was applied. After 48 h of incubation, invasive cells were fixed in methanol for 10-15 min, dyed with 0.5% crystal violet for 20 min and counted using a microscope. Penetrating cells were counted in 5 randomly selected fields per sample.

Western Blot

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and quantified by bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA). The protein sample was loaded for electrophoresis. After transferring on a polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 hours, incubated with primary anti-

bodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by Image Software (Bethesda, MD, USA).

Dual-Luciferase Reporter Gene Assay

Wild-type and mutant-type Luciferase vectors were constructed. Cells were co-transfected with wild-type or mutant-type Luciferase vectors and transcription factor or NC for 24 h. Cells were then fully lysed, centrifuged at 10,000 g for 5 min, and 100 μL of supernatant was harvested for determining the Luciferase activity.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the *t*-test. The linear regression analysis was conducted for evaluating the relationship between the two genes. $p<0.05$ was considered statistically significant.

Results

SND1-IT1 Was Upregulated in OS

Heatmap showed differential expression of lncRNA SND1-IT1 in OS tissues and controls in the GSE12885 dataset (Figure 1A, 1B). Furthermore, qRT-PCR data also uncovered that SND1-IT1 was upregulated in OS tissues (Figure 1C).

Knockdown of SND1-IT1 Suppressed the Proliferative Ability of OS

Three plasmids of sh-SND1-IT1 (sh-SND1-IT1 1#, sh-SND1-IT1 2# and sh-SND1-IT1 3#) were constructed, and their transfection efficacy was verified in MG63 and U2OS cells (Figure 2A). Among them, transfection of sh-SND1-IT1 1# or sh-SND1-IT1 2# remarkably downregulated SND1-IT1 level in OS cells. The CCK-8 assay revealed the decreased viability in OS cells transfected with sh-SND1-IT1 1# or sh-SND1-IT1 2# at 48, 72 and 96 h (Figure 2B, 2C). Colony formation numbers markedly decreased in OS cells with SND1-IT1 knockdown (Figure 2D). Similarly, EdU assay showed a lower rate of EdU-positive cells in OS cells transfected with sh-SND1-IT1 1# or sh-SND1-IT1 2# relative to controls (Figure 2E). The above data all revealed that knockdown of SND1-IT1 suppressed the proliferative ability of OS cells.

Knockdown of SND1-IT1 Suppressed the Migratory Ability of OS

The transwell assay revealed fewer migratory cells in MG63 and U2OS cells transfected with sh-SND1-IT1 1# or sh-SND1-IT1 2#, suggesting the inhibited migratory ability (Figure 3).

SND1-IT1 Sponged MiRNA-665

Based on DIANA and miRDB analyses, miRNA-665 could bind to SND1-IT1 (Figure 4A). Their binding sequences were shown in Figure 4B. To further elucidate the biological role of miRNA-665, we constructed miRNA-665 mimic and inhibitor. Their transfection efficacy was ver-

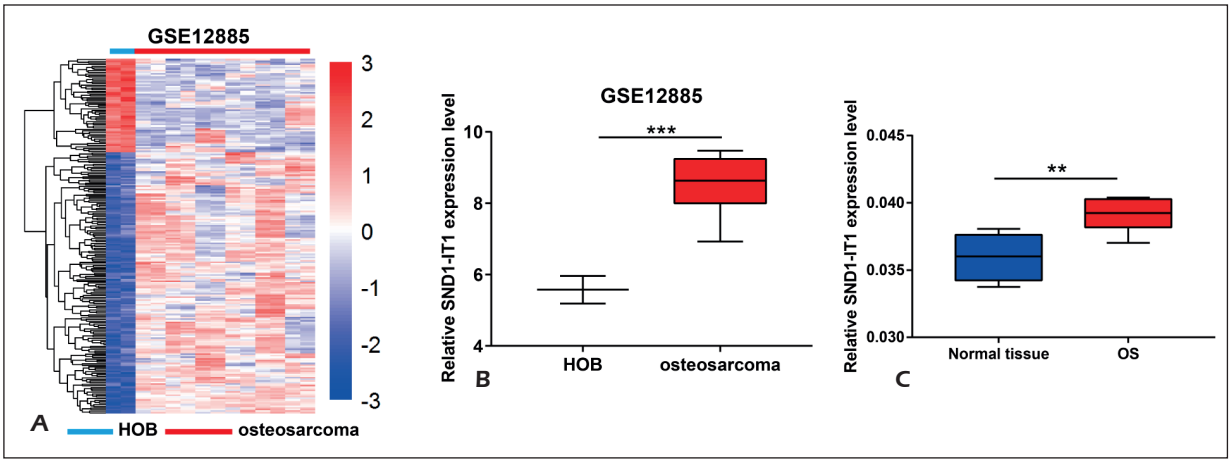

Figure 1. SND1-IT1 was upregulated in OS. **A**, Heatmap of differentially expressed lncRNAs in GSE12856 dataset. **B**, Relative level of SND1-IT1 in OS tissues of GSE12856 dataset. **C**, Relative level of SND1-IT1 in OS tissues and controls.

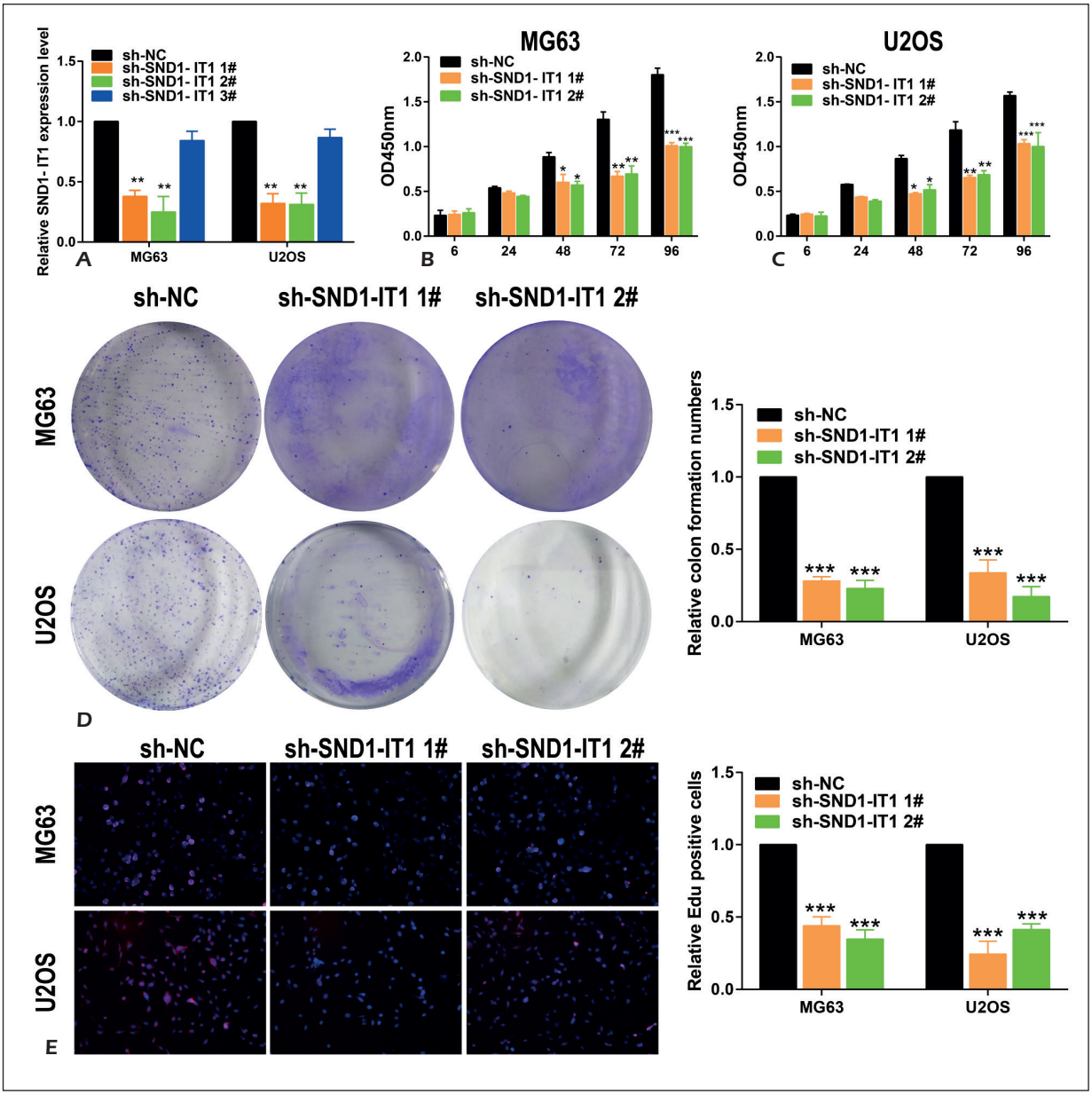

Figure 2. Knockdown of SND1-IT1 suppressed the proliferative ability of OS. **A**, Transfection efficacy of sh-SND1-IT1 1#, sh-SND1-IT1 2# and sh-SND1-IT1 3# in MG63 and U2OS cells. **B**, CCK-8 assay showed viability in MG63 cells transfected with sh-NC, sh-SND1-IT1 1# or sh-SND1-IT1 2# at 6, 24, 48, 72 and 96 h. **C**, CCK-8 assay showed viability in U2OS cells transfected with sh-NC, sh-SND1-IT1 1# or sh-SND1-IT1 2# at 6, 24, 48, 72 and 96 h. **D**, Colony formation assay showed the formed colonies in MG63 and U2OS cells transfected with sh-NC, sh-SND1-IT1 1# or sh-SND1-IT1 2# (magnification: 10×). E, EdU assay showed EdU-positive cells in MG63 and U2OS cells transfected with sh-NC, sh-SND1-IT1 1# or sh-SND1-IT1 2# (magnification: 40×).

ified in OS cells (Figure 4C). Overexpression of miRNA-665 downregulated SND1-IT1 level, and conversely, knockdown of miRNA-665 upregulated its level (Figure 4D). Meanwhile, knockdown of SND1-IT1 greatly upregulated miRNA-665 level in OS cells (Figure 4E). A negative correlation between SND1-IT1 and miRNA-665 levels in OS was identified ($p<0.001$, $R^2=0.827$, Figure 4H). Subsequently, the Dual-Luciferase reporter gene assay illustrated that the Luciferase activity decreased in OS cells co-transfected with SND1-IT1-WT and miRNA-665 mimic (Figure 4F, 4G). It is believed that SND1-IT1 negatively regulated miRNA-665 level by binding to it.

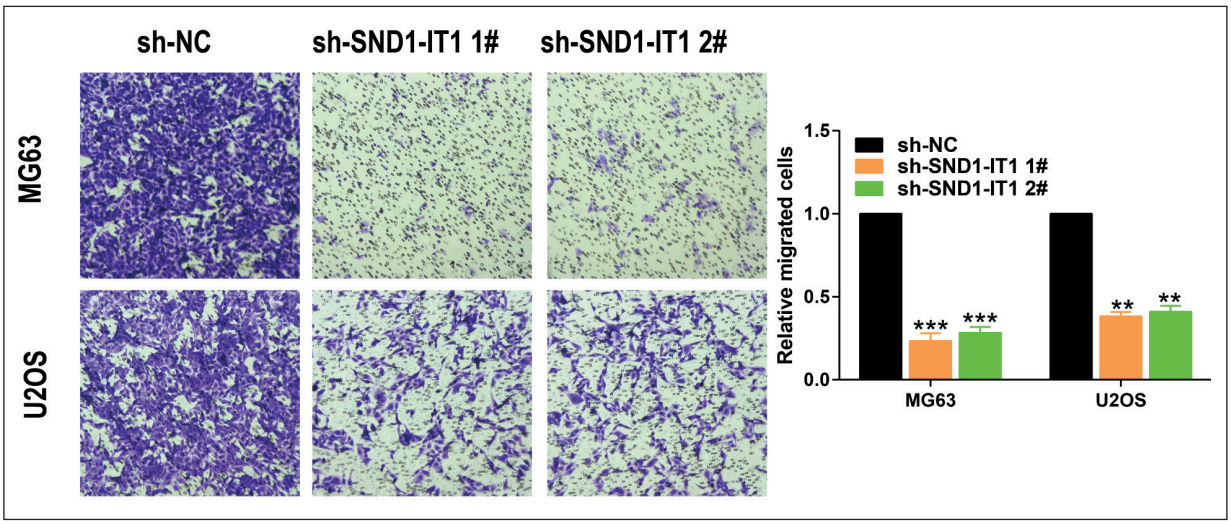

Figure 3. Knockdown of SND1-IT1 suppressed the migratory ability of OS. The transwell assay showed migratory cells in MG63 and U2OS cells transfected with sh-NC, sh-SND1-IT1 1# or sh-SND1-IT1 2# (magnification: 40×).

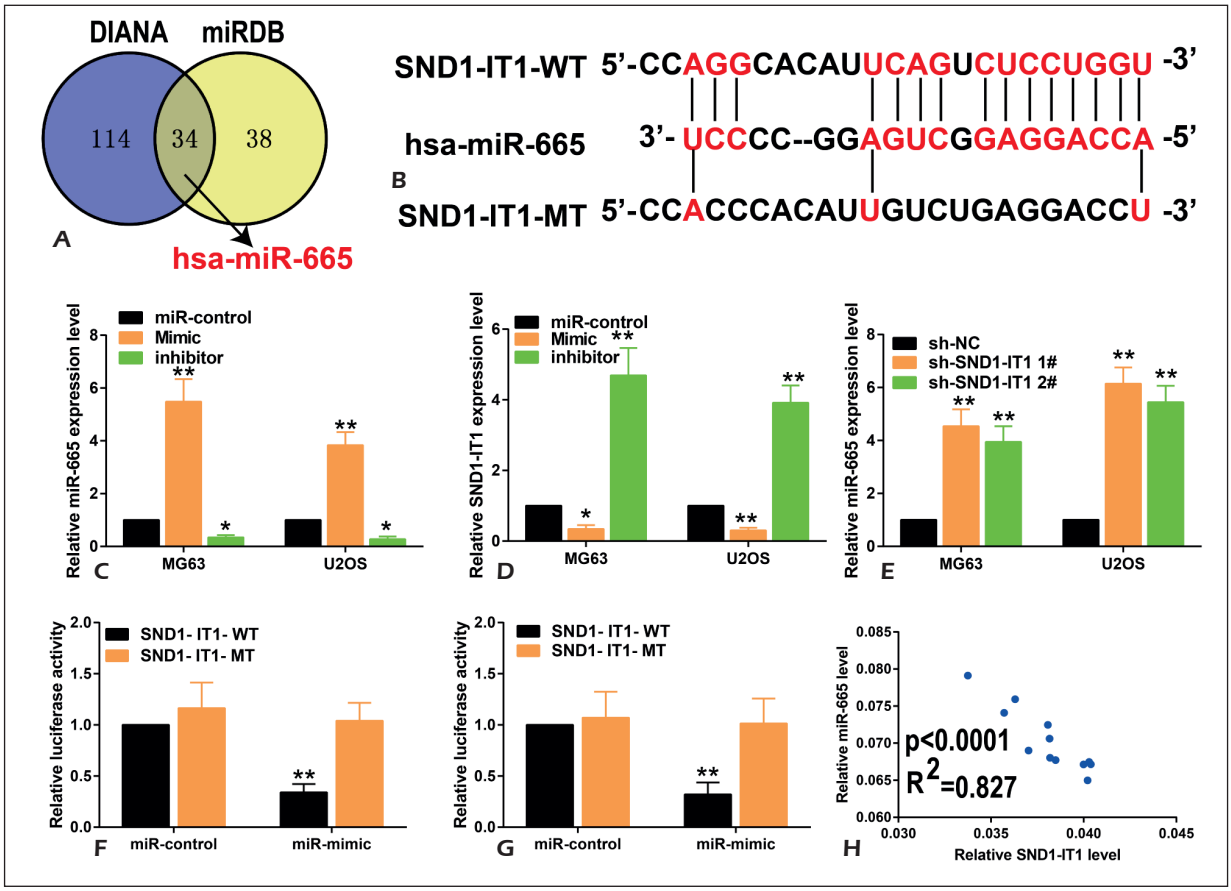

Figure 4. SND1-IT1 sponged miRNA-665. **A**, DIANA and miRDB predicted binding miRNAs to SND1-IT1. **B**, Potential binding sequences between SND1-IT1 and miRNA-665. **C**, Transfection efficacy of miRNA-665 mimic and inhibitor in MG63 and U2OS cells. **D**, Relative level of SND1-IT1 in MG63 and U2OS cells transfected with miR-control, miRNA-665 mimic or miRNA-665 inhibitor. **E**, Relative level of miRNA-665 in MG63 and U2OS cells transfected with sh-NC, sh-SND1-IT1 1# or sh-SND1-IT1 2#. **F**, Relative luciferase activity in MG63 cells co-transfected with SND1-IT1-WT/SND1-IT1-MT and miR-control/miRNA-665 mimic. **G**, Relative Luciferase activity in U2OS cells co-transfected with SND1-IT1-WT/SND1-IT1-MT and miR-control/miRNA-665 mimic. **H**, A negative correlation between miRNA-665 and SND1-IT1 levels.

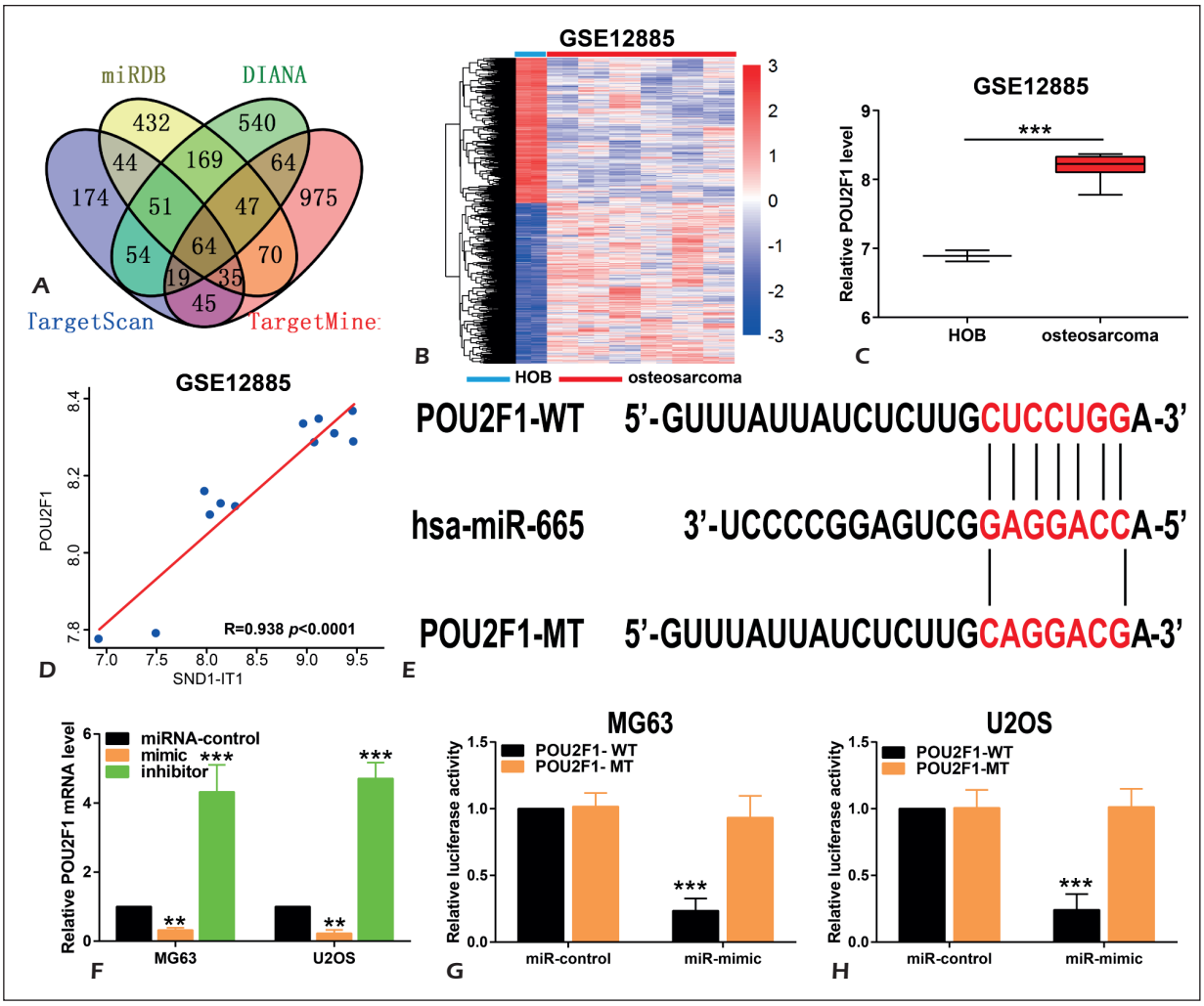

Figure 5. MiRNA-665 could bind to POU2F1. **A**, DIANA, miRDB, TargetScan and TargetMine predicted binding mRNAs to miRNA-665. **B**, Heatmap of differentially expressed mRNAs in GSE12885 dataset. **C**, Relative level of POU2F1 in OS tissues of GSE12885 dataset. **D**, A positive correlation between POU2F1 and SND1-IT1 levels. **E**, Potential binding sequences between POU2F1 and miRNA-665. **F**, Relative level of POU2F1 in MG63 and U2OS cells transfected with miR-control, miRNA-665 mimic or miRNA-665 inhibitor. **G**, Relative Luciferase activity in MG63 cells co-transfected with POU2F1-WT/ POU2F1-MT and miR-control/miRNA-665 mimic. **H**, Relative Luciferase activity in U2OS cells co-transfected with POU2F1-WT/ POU2F1-MT and miR-control/miRNA-665 mimic.

MiRNA-665 Could Bind to POU2F1

Potential binding mRNAs to miRNA-665 were predicted using miRDB, DIANA, TargetScan and TargetMine (Figure 5A). In the downloaded GSE12885 dataset, the heatmap further depicted differentially expressed mRNAs in OS (Figure 5B). Based on the comprehensive analysis, POU2F1 was identified to be upregulated in OS (Figure 5C). By analyzing the data provided in GSE12885, a positive correlation was observed between SND1-IT1 and POU2F1 (Figure 5D). Their potential binding sequences were shown in Figure 5E. Moreover, transfection of miRNA-665 mimic could downregulate POU2F1 level, and knockdown of miRNA-665 upregulated POU2F1 level in OS cells (Figure 5F). As the Dual-Luciferase reporter gene assay illustrated, POU2F1 could directly bind to miRNA-665 (Figure 5G, 5H).

MiRNA-665 Negatively Regulated POU2F1 Level

Transfection of miRNA-665 mimic in MG63 and U2OS cells downregulated the protein level of POU2F1, and transfection of miRNA-665 inhibitor achieved the opposite trend (Figure 6A). The mRNA level of POU2F1 was negatively regulated by miRNA-665 as well (Figure 6B). It is found that the protein level of POU2F1 was downregulated in OS cells transfected with sh-SND1-IT1 1# or sh-SND1-IT1 2# (Figure 6C, 6D). Through the linear

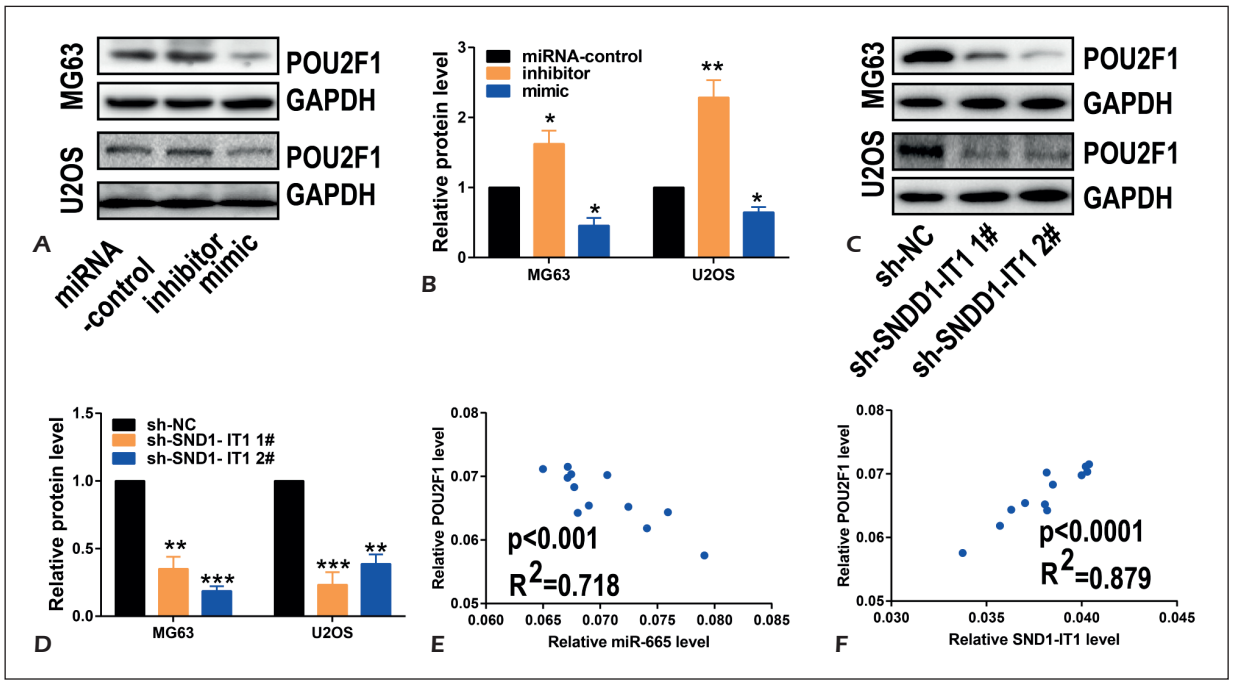

Figure 6. MiRNA-665 negatively regulated POU2F1 level. **A**, Western blot analysis of POU2F1 in MG63 and U2OS cells transfected with miR-control, miRNA-665 mimic or miRNA-665 inhibitor. **B**, Protein level of POU2F1 in MG63 and U2OS cells transfected with miR-control, miRNA-665 mimic or miRNA-665 inhibitor. **C**, Western blot analysis of POU2F1 in MG63 and U2OS cells transfected with sh-NC, sh-SND1-IT1 1# or sh-SND1-IT1 2#. **D**, Protein level of POU2F1 in MG63 and U2OS cells transfected with sh-NC, sh-SND1-IT1 1# or sh-SND1-IT1 2#. **E**, A negative correlation between miRNA-665 and POU2F1 levels. **F**, A positive correlation between SND1-IT1 and POU2F1 levels.

regression analyses, POU2F1 level was negatively correlated to miRNA-665 level (R^2=0.718, p<0.001, Figure 6E), but positively correlated to SND1-IT1 level (R^2=0.879, p<0.0001, Figure 6F).

Regulatory Role of SND1-IT1/MiRNA-665/POU2F1 Axis in OS

To elucidate the interaction among SND1-IT1, miRNA-665 and POU2F1 in OS, we constructed sh-POU2F1, which exerted a pronounced transfection efficacy in OS cells (Figure 7A). A series of rescue experiments were conducted to clarify the involvement of SND1-IT1/miRNA-665/POU2F1 axis in cellular behaviors of OS cells. Inhibited proliferative ability of OS cells with SND1-IT1 knockdown were partially elevated by transfection of miRNA-665 inhibitor, and further downregulated by POU2F1 knockdown (Figure 7B, 7C). The transwell assay yielded the same trends at their migratory ability (Figure 7D, E).

Discussion

OS is a common and highly invasive bone tumor. Its incidence and mortality remain the highest in primary bone tumors[15]. Malignant phenotypes of tumor cells are the leading causes of OS-related death[16]. Therefore, exploring new targets that block the malignant phenotypes of OS are well concerned.

Abnormal regulation of lncRNAs has an impact at the epigenetic level, causing dysregulated cell growth that leads to tumor development[17]. Effective control of cell growth and invasion is the key to alleviate tumor progression and further improves therapeutic efficacy[18]. Gao et al[19] demonstrated that lncRNA MALAT1 is upregulated in OS, which is correlated to tumor size, tumor stage and distant metastasis. Sun et al[20] concluded that the upregulated lncRNA FGFR3-AS1 is closely related to poor prognosis of OS. In this work, it is found that lncRNA SND1-IT1 was upregulated in OS.

Plenty of lncRNAs could mediate cellular behaviors of OS cells through direct regulation or indirect regulation *via* targeting miRNAs or mRNAs[21]. Xie et al[22] identified that silence of POU2F1 could suppress the proliferative ability of OS cells, which also induces cell cycle arrest and apoptosis. As a target gene of miR-9-5P, POU2F1 level is inhibited by miR-9-5P. Furthermore, they illustrated that lncRNA TUG1 could sponge miR-9-5P

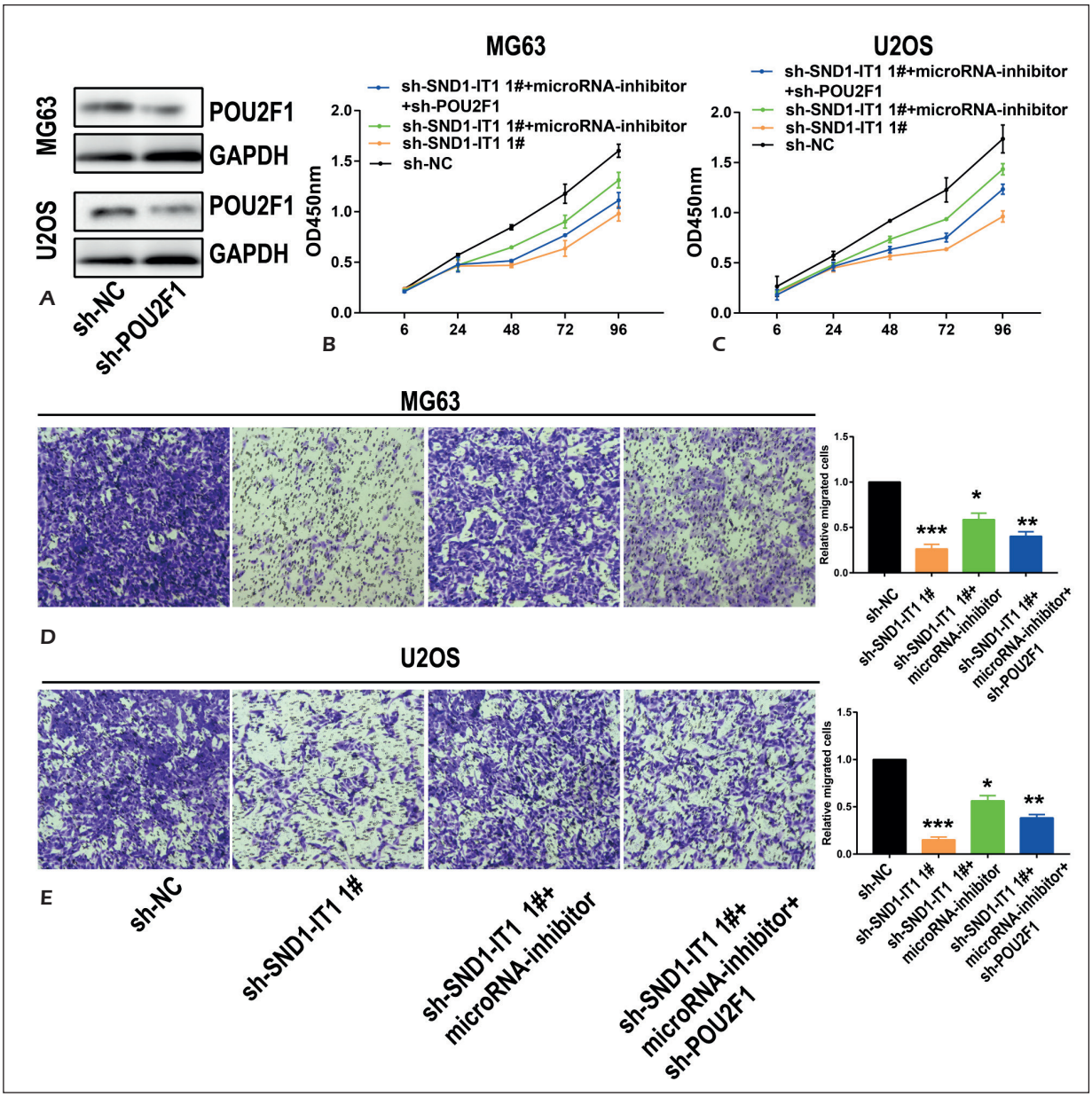

Figure 7. Regulatory role of SND1-IT1/miRNA-665/POU2F1 axis in OS. **A**, Transfection efficacy of sh-POU2F1 in MG63 and U2OS cells. MG63 and U2OS cells were transfected with sh-NC, sh-SND1-IT1 1#, sh-SND1-IT1 1# + miRNA-665 inhibitor, or sh-SND1-IT1 1# + miRNA-665 inhibitor + sh-POU2F1. **B-C**, CCK-8 assay showed viability in each group. **D-E**, The transwell assay showed migratory cells in each group (magnification: 40×).

to upregulate POU2F1, suggesting the potential of TUG1 as a therapeutic target for OS. This study revealed that lncRNA SND1-IT1 accelerated the proliferative and invasive abilities of OS by sponging miRNA-665. Meanwhile, miRNA-665 could regulate POU2F1 level in OS cells. The potential effect of SND1-IT1/miRNA-665/POU2F1 axis on influencing cellular behaviors of OS cells was identified. LncRNA SND1-IT1 accelerated the malignant progression of OS by sponging miRNA-665 to upregulate POU2F1. These key molecules could be utilized as therapeutic targets for OS.

Conclusions

In summary, lncRNA SND1-IT1 accelerates proliferative and migratory abilities of OS *via* sponging miRNA-665 to upregulate POU2F1, thus stimulating the progression of OS.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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