miRNA-296-5p functions as a potential tumor suppressor in human osteosarcoma by targeting *SND1*

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Abstract

Background: The pathogenesis of osteosarcoma (OS) is still unclear, and it is still necessary to find new targets and drugs for anti-OS. This study aimed to investigate the role and mechanism of the anti-OS effects of miR-296-5p.

Methods: We measured the expression of miR-296-5p in human OS cell lines and tissues. The effect of miR-296-5p and its target gene staphylococcal nuclease and tudor domain containing 1 on proliferation, migration, and invasion of human OS lines was examined. The Student's *t* test was used for statistical analysis.

Results: We found that microRNA (miR)-296-5p was significantly downregulated in OS cell lines and tissues (control *vs*. OS, 1.802 ± 0.313 *vs*. 0.618 ± 0.235, $t = 6.402$, $P < 0.01$). Overexpression of miR-296-5p suppressed proliferation, migration, and invasion of OA cells. *SND1* was identified as a target of miR-296-5p by bioinformatic analysis and dual-luciferase reporter assay. Overexpression of *SND1* abrogated the effects induced by miR-296-5p upregulation (miRNA-296-5p *vs*. miRNA-296-5p + SND1, 0.294 ± 0.159 *vs*. 2.300 ± 0.277, $t = 12.68$, $P = 0.003$).

Conclusion: Our study indicates that miR-296-5p may function as a tumor suppressor by targeting *SND1* in OS.

Keywords: miR-296-5p; Osteosarcoma; SND1; Tumor suppressor; miRNA

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor, predominantly observed in children and adolescents. About 60% of the patients are between 10 and 20 years old.[1] Despite the use of neoadjuvant chemotherapy, the 5-year survival rate of patients with localized OS has increased from 20% in the early 1970s to 60%. However, the 5-year survival rate of OS patients with local invasion or metastasis at first diagnosis is still only 20%, and the average survival is only about 23 months.[2-4] The insensitivity of some patients to chemotherapy, and the recurrence and metastasis of tumors have always been challenging in the clinical setting. Although the incidence of OS is lower than that of other tumors, basic research and clinical treatment strategies have been the focus of ongoing studies owing to the high mortality and disability rate associated with OS.

MicroRNA (miRNA) is a regulatory non-coding RNA of about 18 to 25 nucleotides in length that is commonly detected in eukaryotes.[5,6] miRNAs bind to the 3′-untranslated region (3′-UTR) of a specific target protein, facilitating its post-transcriptional regulation. Since its discovery in nematodes in 1993, bioinformatics and biological studies have identified more than 2000 miRNAs involved in the regulation of more than one-third of gene transcription processes.[7-9] Some researchers believe that each miRNA can regulate the expression of > 100 genes.[10] Studies have shown that > 50% of genes encoding miRNAs are located on the "fragile sites" of chromosomes and are adjacent to deleted or amplified regions caused by human tumor mutations, revealing a direct relationship between miRNAs and tumorigenesis.[11-15]

The miRNA-296-5p, formerly known as miRNA-296, is closely linked to tumorigenesis. In the past 3 years, several

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studies have reported that miRNA-296-5p can inhibit various tumors. Vaira *et al*[16] first reported that miRNA-296-5p inhibits lung cancer metastasis by inhibiting the expression of Numb-like (Numbl) mRNA and protein. Xu *et al*[17] found that miRNA-296-5p can directly target and regulate Polo-like kinase-1 (*PLK1*) to inhibit cell viability in non-small cell lung cancer. Savi *et al*[18] also showed that miR-296 can inhibit the proliferation of breast cancer by targeting *SCRIB*. Lee *et al*[19] further described the role of miRNA-296-5p in prostate cancer as an inhibitor of tumor growth by targeting *Pin1*. Rong *et al*[20] found that overexpression of a miR-296-5p inhibitor could prevent the tumorigenesis of gastric cancer cells both *in vivo* and *in vitro*.

In this study, we investigated miRNA-296-5p expression in OS and analyzed the effects of its overexpression on cell proliferation, migration, and invasion. Furthermore, we identified the downstream target of miRNA-296-5p and investigated its role in regulating cell proliferation and invasion.

Methods

Ethical approval

All patients provided their informed consent, and this study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital (No. 2016KY124).

Cell culture

OS cell lines HOS, MG63, U-2OS, Saos-2, and human osteoblasts (HOB) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). OS cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Cat: 31800-022, Corning, New York, USA) supplemented with 10% fetal bovine serum (Cat: 10099, Gibco, Australia) at 37°C and 5% CO_2. HOB cells were cultured in the PromoCell medium at 37°C and 5% CO_2.

Human tissues

Human normal and primary OS tissues were obtained from 45 patients in the Zhejiang Provincial People's Hospital from August 2016 to January 2017.

Transfection

The recombinant miR-296-5p lentivirus and plasmid pCAGGS-GFP (Backbone: pCAGGS, insert: Green fluorescent protein [GFP]) were purchased from the Sangon Company (Shanghai, China). The plasmids were transfected into Saos-2 and HOS cells via Lipofectamine^{TM} 3000 (Invitrogen, Shanghai, China) and stably transfected cells were selected using puromycin (2 μg/mL, Beyotime, Shanghai, China). Successful transfection was confirmed through quantitative reverse transcription PCR (qRT-PCR) analysis.

Immunohistochemistry (IHC)

IHC of OS tissues and adjacent normal tissues was performed. Tissues were fixed using formalin, embedded in paraffin, and then serially sectioned at 4 μm. Slides were then incubated with anti-SND1 antibody (ab65078, Abcam, Shanghai, China) overnight in a moist chamber at 4°C, washed in phosphate buffer saline (PBS), and then incubated with corresponding secondary antibodies. Slides were developed with diaminobenzidine and counterstained with hematoxylin.

qRT-PCR

The quantitative real-time polymerase chain reaction (qRT-PCR) analysis was conducted as previously described.[21] For mature miR-296-5p assays, total RNA was reverse transcribed using specific miRNA primers and reagents from the Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA obtained by PCR amplification of the primers was analyzed with Taqman miR-296-5p microRNA assay and measured using a prism 7900HT system (Applied Biosystems, Thermo Fisher, Waltham, MA, USA). U6 small nuclear RNA was used as an internal control. For the quantification of SND1 mRNA, qRT-PCR was performed using the rapid quantitative SYBR-Green RT-PCR kit (Takara, Japan). β-actin was used as an internal control. All reactions were performed in triplicate. The following primer sequences were used: miR-296-5p forward, 5′-TGC CTA ATT CAG AGG GTT GG-3′; miR-296-5p reverse, 5′-CTC CAC TCC TGG CAC ACA G-3′; SND1 forward, 5′-TGC AGC GGG GTA ATC CTG A-3′; SND1 reverse, 5′-CCT CCG ACA CTT CGT CAT CC-3′; U6 forward, 5′-CTC GCT TCG GCA CA-3′; U6 reverse 5′-AAC GCT TCA CGA ATT TGC GT-3′; GAPDH forward 5′-GGT CTC TGA CTT CAA CA-3′; GAPDH reverse, 5′-GTG AGG GTC TCT CTC TTC CT-3′.

Cell migration and invasion analysis

Cell migration and invasion assays were performed using 24-well transwell plates (Corning 8-mm pore size, New York, USA). For the transwell migration assay, 2.5×10^4 cells were incubated in the top chamber of the plate and lined with a non-coating film. For the invasion assay, 200 mg/mL Matrigel (Corning) was used to coat the chamber insert, and then 5×10^4 cells were plated in the top chamber. After 24 h of incubation, the cells were fixed with methanol, stained with crystal violet, and counted under the microscope. The results were calculated as the mean of three independent experiments.

Western blot

Cell lysates were resuspended in radio-immunoprecipitation assay dissolution buffer. The whole-cell lysates (30–60 μg in each well) were separated using 10% dodecylsulfate, sodium salt (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto a polyvinylidene fluoride membrane (Millipore, Danvers, MA, USA). The membrane was blocked with 5% skimmed milk and incubated overnight with primary antibodies against SND1 (ab65078) or β-actin (ab8227) at 4°C (Abcam, Cambridge, MA, USA). This was followed by the addition of a secondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The bands were

observed by using an enhanced chemiluminescence (ECL) system (Yeasen, China).

Luciferase reporter assay

OS cell lines HOB and Saos-2 were seeded in 24-well plates and then co-transfected with either miR-296-5p mimics or the negative control (NC), 50 ng of either pGL3-SND1-3′UTR-wt or pGL3-SND1-3′UTR-mut, and 10 ng of pRL-TK (Promega, Madison, WI, USA). Luciferase and Renilla signals were measured 48 h after transfection using the Luciferase Reporter Assay Kit (Promega). All experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Detected by Shapiro-Wilk test, the variables conform to normal distribution. Continuous variables were expressed as mean ± standard deviation (SD). Continuous variables between groups were analyzed using *t* test. The relationship between miR-296-5p and SND1 expression was analyzed by Spearman correlation analysis. *P* values of < 0.05 were considered statistically significant.

Results

miR-296-5p is downregulated in OS cell lines and clinical specimens

The expression levels of miR-296-5p in 45 cases of primary OS samples and paired adjacent normal tissues were analyzed by qRT-PCR. It was observed that the average expression level of miR-296-5p was lower in primary tumor samples compared to normal tissues (control *vs.* OS, 1.802 ± 0.313 *vs.* 0.618 ± 0.235, $t = 6.402$, $P < 0.01$) [Figure 1A]. Using the same technique, miR-296-5p expression in OS cell lines U2-OS, HOS, Saos-2, and MG-63 was analyzed and compared with the expression levels in normal HOB. As shown in Figure 1B, the expression level of miR-296-5p in the OS cell lines was lower than that in normal osteoblasts (all $P < 0.01$). These results suggest that miR-296-5p is downregulated in OS.

miR-296-5p suppresses OS cell proliferation, migration, and invasion in vitro

To investigate the function of miR-296-5p, HOS and Saos-2 cell lines were transfected with either miR-296-5p mimics or NC. The cells were then analyzed by qRT-PCR to confirm the overexpression (HOS: NC *vs.* miR-296-5p, 1.000 ± 0 *vs.* 7.900 ± 0.369, $t = 18.23$, $P < 0.01$; Saos-2: 1.000 ± 0 *vs.* 6.200 ± 0.322, $t = 16.18$, $P < 0.01$) [Figure 2A]. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, thiazolyl blue tetrazolium bromide (MTT) assay demonstrated that overexpression of miR-296-5p attenuated the growth rate of HOS (NC *vs.* miR-296-5p, 0.897 ± 0.032 *vs.* 0.587 ± 0.369, $t = 9.86$, $P < 0.01$) and SAOS-2 cells (Saos-2: 1.000 ± 0 *vs.* 6.200 ± 0.023, $t = 5.714$, $P < 0.01$) [Figures 2B and C]. Using the scratch test, we detected the effects of miR-296-5p on *in vitro* migration of HOS and Saos-2 cells transfected with miR-296-5p mimics or the NC. As shown in Figure 2D, miR-296-5p overexpression suppressed the migration of HOS and Saos-2 cells. Similarly, miR-296-5p overexpression suppressed the invasion of HOS and Saos-2 cells [Figure 2E]. These results suggest that miR-296-5p can suppress the migration and invasion ability of OS cells *in vitro*.

SND1 is highly expressed in OS

Tissue samples of seven patients with OS and three healthy controls were collected to detect SND1 expression through western blotting [Figure 3A]. The expression of the SND1 protein in OS samples was higher than that in the control. Similarly, immunohistochemistry (IHC) analysis showed that SND1 expression in OS was higher than that in normal

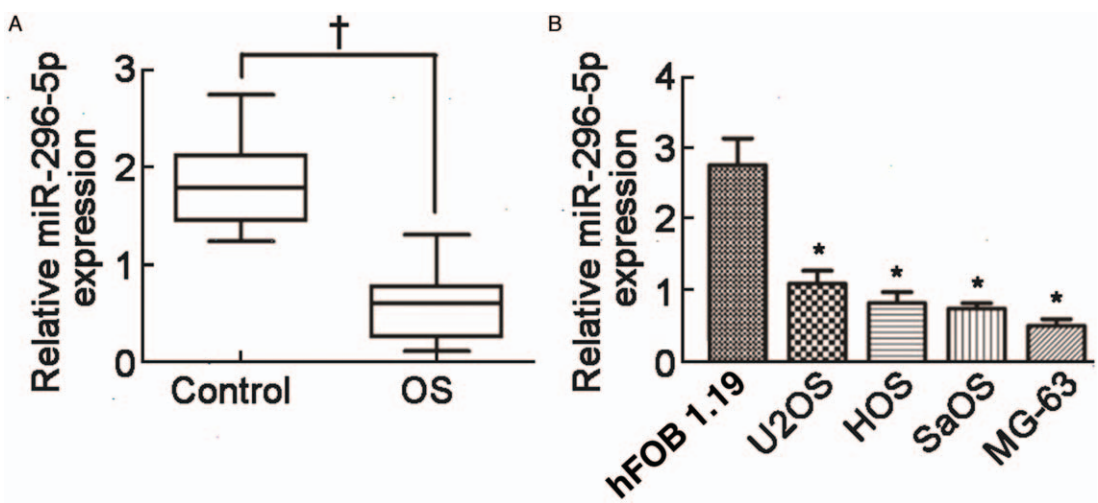

Figure 1: Levels of mature miR-296-5p expression in osteosarcoma cell lines and tissues. (A) Expression levels of miR-296-5p were measured by qRT-PCR in osteoblasts. (B) Expression levels of miR-296-5p were measured by qRT-PCR in five osteosarcoma cell lines. qRT-PCR: quantitative reverse transcription polymerase chain reaction; OS: osteosarcoma; hFOB 1.19: a normal osteoblast cell line; $^{*}P < 0.05$, compared to hFOB 1.19; $^{\dagger}P < 0.01$, compare to Control.

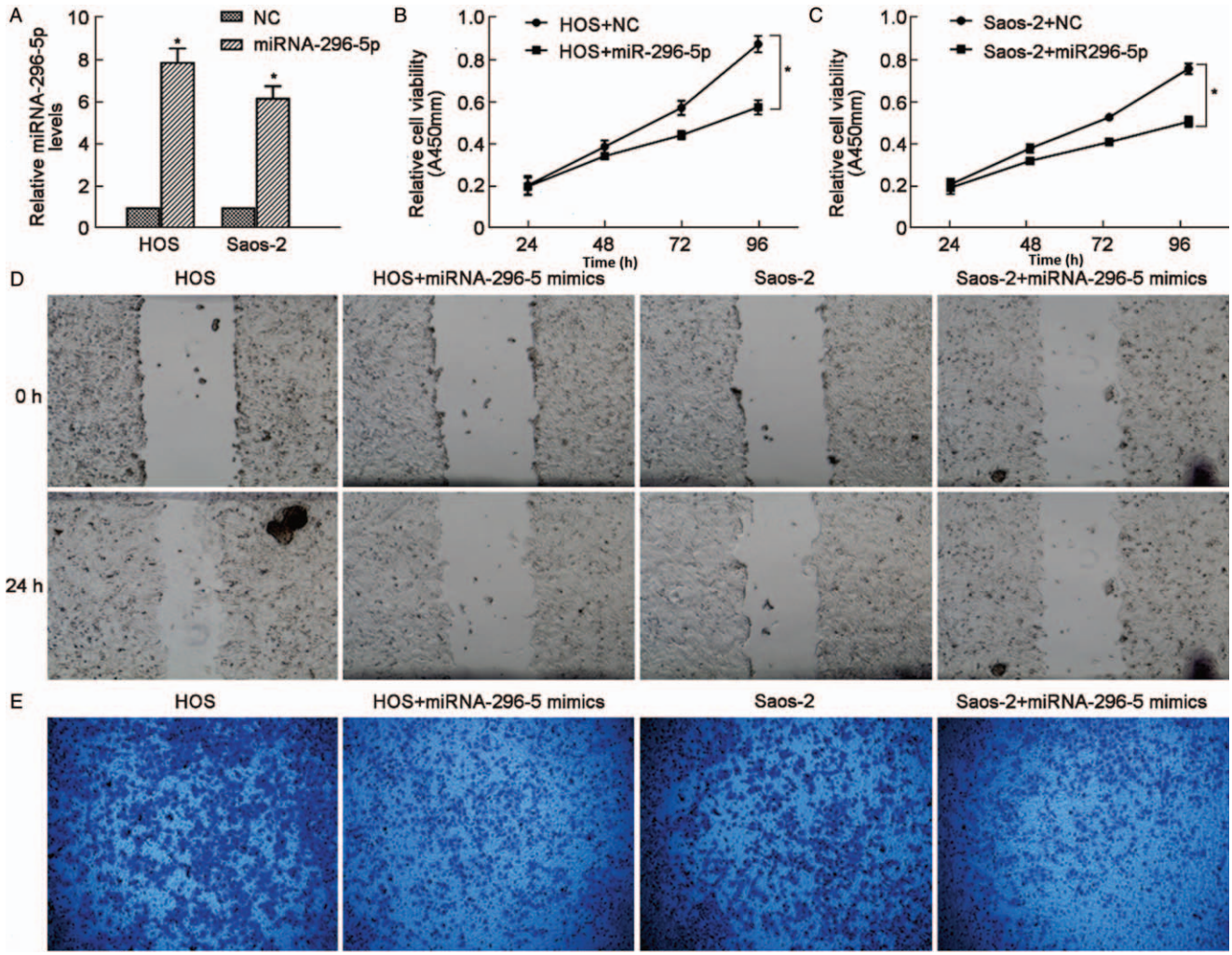

Figure 2: Effects of miR-296-5p on the proliferation, migration, and invasion of osteosarcoma cells *in vitro*. (A) HOS and Saos-2 cells were transfected with either miR-296-5p mimics or the negative control (NC). Twenty-four hours later, cells were assessed by qRT-PCR. (B and C) MTT assay. (D and E) Wound healing assay (bright field; original magnification 10×) and transwell migration and invasion assays (crystal violet staining; original magnification 10×) of HOS and Saos-2 cells transfected with miR-296-5p mimics or NC. $^{*}P < 0.01$. qRT-PCR: quantitative reverse transcription polymerase chain reaction.

bone tissue [Figure 3B]. Subsequently, we detected SND1 relative mRNA expression in four OS cell lines (U2OS, HOS, Saos-2, and MG-63), and a normal osteoblast cell line (hFOB 1.19) using qRT-PCR ($P < 0.01$) [Figure 3C]. SND1 expression in U2OS, HOS, SAOS-2, and MG-63 was higher than that in hFOB 1.19 cells. The western blot analysis yielded the same result [Figure 3D]. These findings suggest that SND1 is strongly expressed in the OS.

Knocking down SND1 inhibits proliferation, migration, and invasion of OS cell lines

To investigate the role of SND1 in OS cell lines, we knocked down *SND1* in HOS and SAOS-2 cells using short hairpin RNA (shRNA). First, we used western blotting to detect *SND1* knockdown efficiency in HOS and SAOS-2. SND1 expression decreased significantly after the transfection of shRNA in HOS and SAOS-2 cells [Figure 4A]. MTT assay showed that HOS and SAOS-2 transfected with shSND1 resulted in decreased proliferation. Additionally, we detected the effects of SND1 knockdown on migration and invasion of HOS and SAOS-2 cells *in vitro* (NC *vs.* SND1 Knockdown, 0.897 ± 0.030 *vs.* 0.447 ± 0.025, $t = 9.86$, $P < 0.01$) [Figure 4B]. As shown in Figure 4C, SND1 knockdown significantly suppressed the migration of HOS and SAOS-2 cells ($P < 0.05$). Similarly, SND1 knockdown suppressed the invasion of HOS and SAOS-2 cells [Figure 4D]. These results suggest that SND1 facilitates the migration and invasion of OS cells *in vitro*.

SND1 is a direct downstream target of miR-296-5p

To investigate the molecular mechanism of miR-296-5p-mediated oncogenic effects, we determined the downstream targets using TargetScan and detected *SND1* as one of the target genes of miR-296-5p. miRNA alignment analysis revealed that the 3′-UTR of SND1 harbors two putative binding sites for miR-296-5p [Figure 5A]. To further explore this observation in human primary tumors, the correlation between miR-296-5p expression and SND1 was analyzed in 12 OS tissues. We found that there was a negative correlation between the expression of miR-296-5p and SND1 in tumor tissues [Figure 5B and C]. Dual-luciferase assays revealed that miR-296-5p overexpression

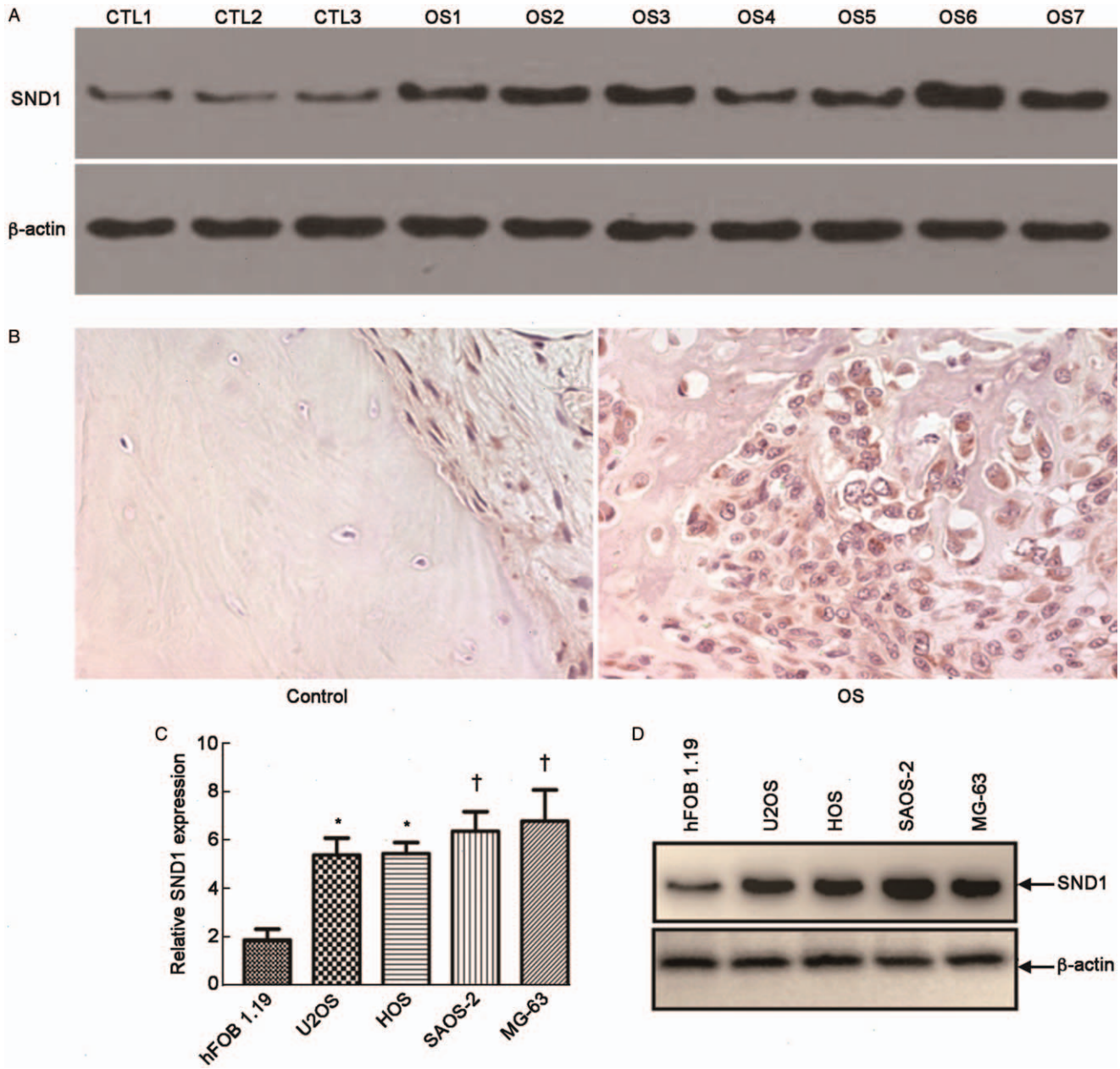

Figure 3: Levels of SND1 expression in osteosarcoma cell lines and tissues. (A and B) Expression levels of SND1 were measured by western blotting and IHC in normal and osteosarcoma tissues (original magnification, 40×). (C and D) The expression level of miR-296-5p was measured by qRT-PCR in osteoblasts and four osteosarcoma cell lines and humanosteoblasts (HOB) cells. CTRL: control; IHC: immunohistochemistry; OS: osteosarcoma; qRT-PCR: quantitative reverse transcription polymerase chain reaction. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, compared to hFOB 1.19.

reduced the SND1 3′-UTR luciferase reporter activity (HOS: NC *vs.* miR-296-5p, 1.000 ± 0 *vs.* 0.283 ± 0.044, $t = 16.25$, $P < 0.01$; Saos-2: 1.000 ± 0 *vs.* 0.390 ± 0.028, $t = 29.30$, $P < 0.01$) [Figure 5D]. These results suggest that miR-296-5p can directly target *SND1* in OS cells by interacting with its 3′-UTR.

Antitumor effects of miR-296-5p are mediated through the repression of SND1

Based on the above findings, we hypothesized that miR-296-5p might inhibit OS cell proliferation, migration, and invasion by repressing SND1 expression. To verify the direct targeting effect of miR-296-5p on *SND1*, we generated SND1-Flag plasmids and transfected them into HOS cell lines. Subsequently, miR-296-5p mimics were transfected into these cells. Western blot and qRT-PCR analyses revealed that miR-296-5p overexpression did not affect the mutated SND1-Flag expression (SND1 *vs.* miRNA-296-5p + SND1, 2.701 ± 0.402 *vs.* 2.300 ± 0.277, $t = 1.45$, $P = 0.22$) [Figures 6A and B]. Overexpression of SND1 abrogated the effects induced by miR-296-5p upregulation (miRNA-296-5p *vs.* miRNA-296-5p + SND1, 0.294 ± 0.159 *vs.* 2.300 ± 0.277, $t = 12.68$, $P = 0.003$). MTT assay showed that mutated SND1 facilitated HOS cell proliferation, despite the presence of miR-296-5p (miR-296-5p *vs.* SND1+miR-296-5p, mean = −0.593, 95%CI: −0.422 to −0.258, $P < 0.01$) [Figure 6C]. Similarly, in scratch and

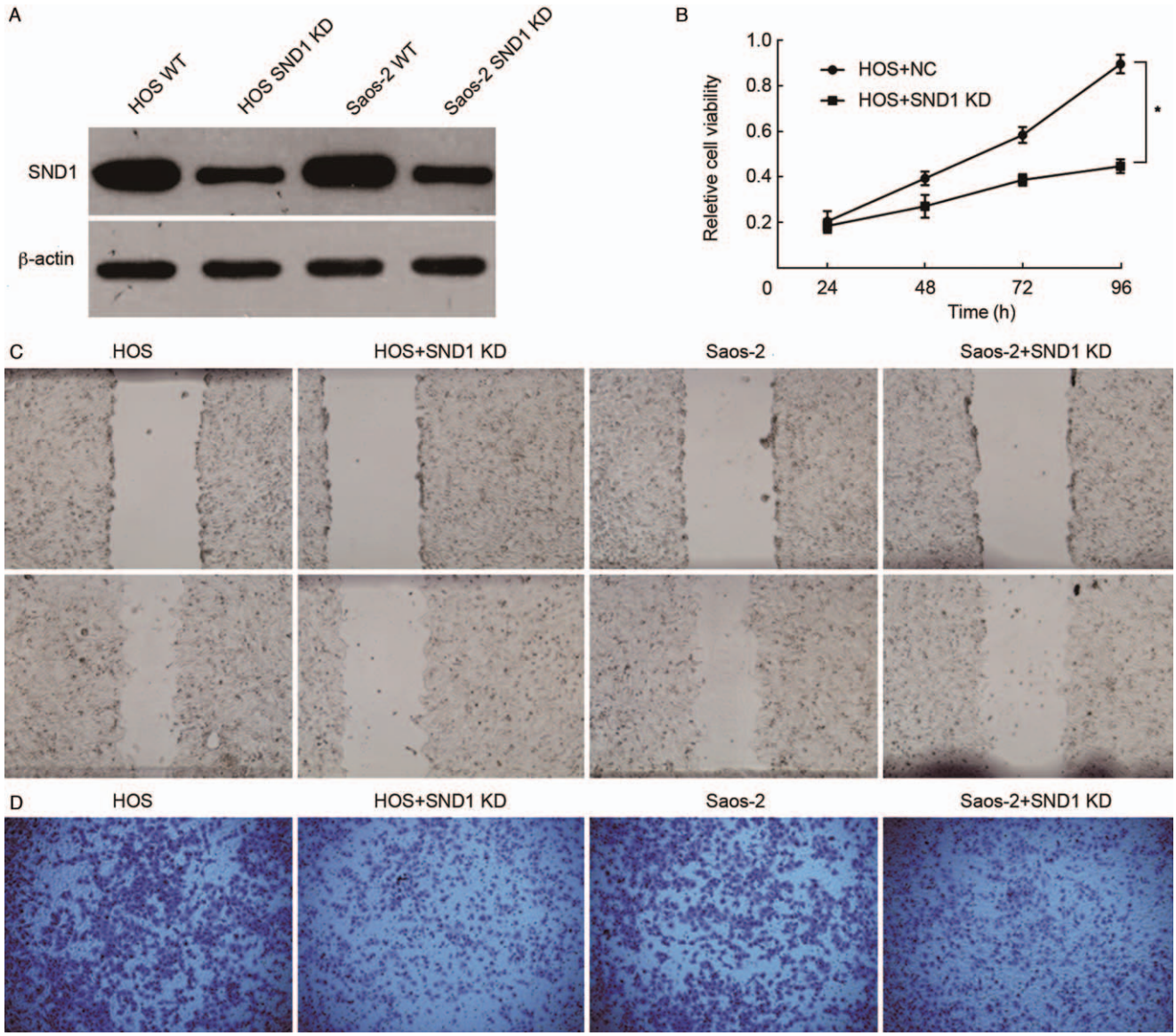

Figure 4: Effects of Staphylococcal Nuclease Domain-Containing Protein 1 (SND1) on the proliferation, migration, and invasion of osteosarcoma cells *in vitro*. (A) HOS and Saos-2 cells were transfected with SND1 shRNA or NC. Twenty-four hours later, cells were assessed by qRT-PCR. (B) MTT assay. (C and D) Wound healing assay (bright field; original magnification 10×) and transwell migration and invasion assays (crystal violet staining; original magnification 10×) of HOS and Saos-2 cells transfected with SND1 shRNA or NC. NC, negative control; qRT-PCR, quantitative reverse transcription polymerase chain reaction; shRNA, short hairpin RNA. *$P < 0.01$.

transwell assays, we observed that mutated SND1 facilitated HOS cell migration and invasion, even in the presence of miR-296-5p overexpression [Figures 6D and E].

Discussion

Mounting evidence has shown that miRNAs participate in the development of human cancers. Among these miRNAs, studies have found that miRNA-296-5p has a tumor-promoting effect. Maia *et al*[22] showed that the expression of miRNA-296-5p is also associated with radiotherapy resistance in early laryngeal carcinoma. Patients with early stage laryngeal carcinoma and high expression of miRNA-296-5p have higher radiotherapy resistance and shorter tumor-free survival.[22] Li *et al*[23] also revealed that miR-296-5p can promote the proliferation of gastric cancer by inhibiting Caudal-related homeobox 1 (CDX1). Although several studies have suggested that miR-296-5p plays an important regulatory role in the development of tumors, the exact role of miR-296-5p in OS has not yet been reported. According to our preliminary study results, the expression level of miR-296-5p in OS tissues was significantly lower than that in normal bone tissues, suggesting that it may affect OS inhibition.

In this study, we found that miR-296-5p was downregulated in all four OS cell lines compared to normal HOB, and the overall average expression levels were lower in primary samples compared to normal tissues. Overexpression of miR-296-5p in OS cells suppressed cell proliferation and inhibited cell migration and invasion. These findings support the view that miR-142-3p mainly functions as a tumor suppressor in OS.

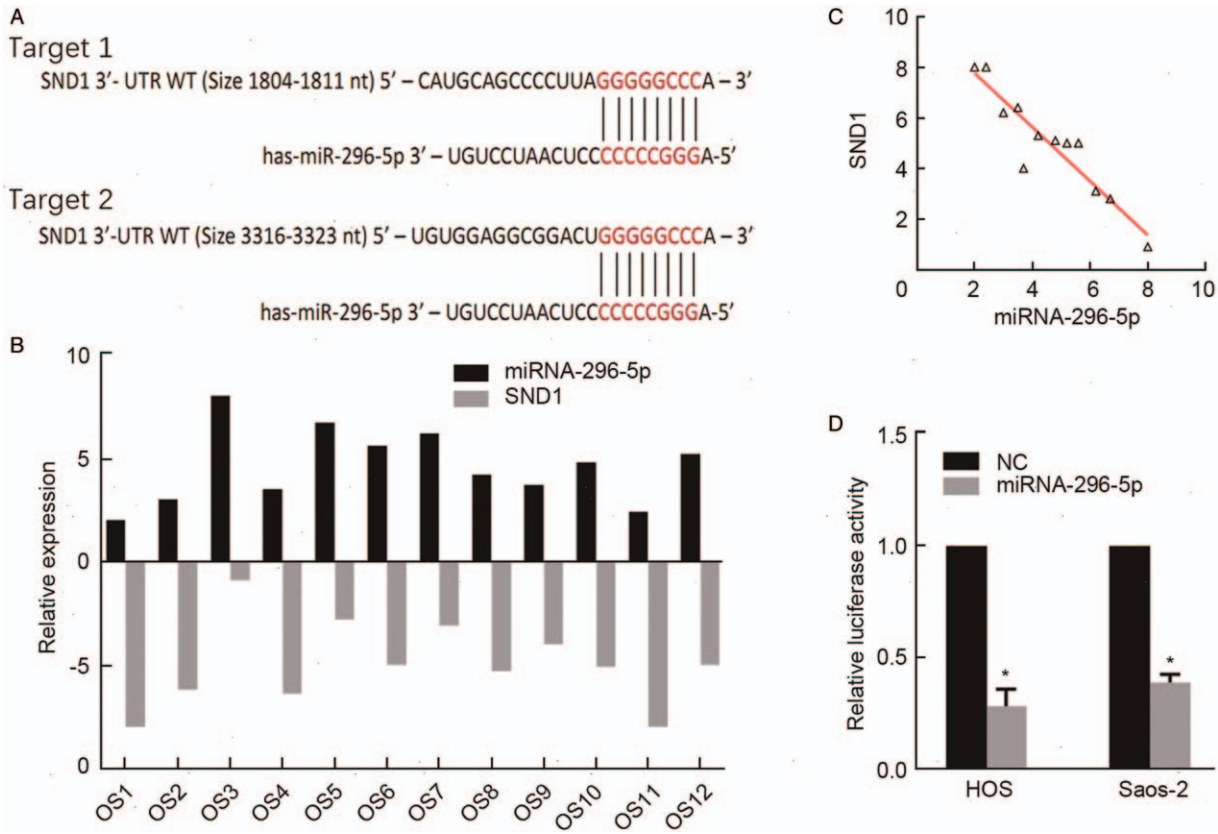

Figure 5: *SND1* is a direct target of miR-296-5p. (A) miR-296-5p and its putative binding sequences in the 3′-UTR of SND1. Mutations were generated in the complementary site that binds to the seed region of miR-296-5p. (B) The qRT-PCR analysis of miR-296-5p and SND1 expression in 12 human osteosarcoma tissues. (C) Spearman correlation analyses between relative miR-296-5p expression and relative SND1 mRNA expression in 12 osteosarcoma tissues. (D) Luciferase reporter assay in HOS and Saos-2 cells revealed that miR-296-5p overexpression suppressed the activity of the firefly luciferase carrying the wild-type (wt) 3′-UTR of SND1. $^{*}P < 0.01$. OS: osteosarcoma; qRT-PCR: quantitative reverse transcription polymerase chain reaction.

Tudor staphylococcal nuclease (SND1) is an important component of the RNA-induced silencing complex (RISC) and is a core component of the RNA interference (RNAi) mechanism.[24,25] Recent studies have shown that SND1 is a highly conserved component of the programmed cell death degradation group,[26,27] revealing its important role in the process of programmed cell death. Because SND1 plays an important role as a major component of RISC, its role in the tumor has become a topic of interest in recent years. Several studies have shown that SND1 plays an important regulatory role in the occurrence, development, and metastasis of breast cancer, liver cancer, prostate cancer, lung cancer, malignant glioma, gastric cancer, cervical cancer, and other malignant tumors.[28-38] As SND1 is a core component of RISC, most of the previous studies have focused on its role in miRNA regulation and related diseases, while studies on the regulation of its own expression and related mechanisms are relatively rare. In 2015, relevant research began to explore the regulation of miRNA on SND1 and its related mechanisms.[36-38] Emdad *et al*[36] found that inhibition of miRNA-184 in malignant glioma cells can upregulate the expression of SND1 and promote tumor invasion. Other studies have also found that miRNA-184 can inhibit the proliferation and invasion of human glioma and breast cancer cells by inhibiting the expression of genes, including SND1 and MMP-2/9.[37] Ma *et al*[38] found that miRNA-361-5p can inhibit the growth and metastasis of colorectal and gastric cancers by targeting *SND1*. However, the role of SND1 in OS was unclear. A recent study analyzed 72 single-nucleotide polymorphisms in 21 miRNA processing genes, in 99 patients with OS and 387 healthy controls. Single-nucleotide polymorphisms were found to be associated with the susceptibility of OS and were located in the genes involved in the major components of RISC (*CNOT1*, *CNOT4*, and *SND1*), revealing that SND1 may play an important role in the occurrence, development, and metastasis of OS.[39]

In OS cell lines (HOS, U2OS, Saos-2, and MG-63), we found that the expression of miR-296-5p was significantly lower than that of HOB (hFOB 1.19), but the expression of SND1 in OS cell lines and tissues was significantly higher than that in hFOB 1.19 and normal bone tissues, which were consistent with those in OS tissue specimens. We predicted that SND1 is one of the potential targets of miR-296-5p. Based on the identification of conserved binding sites, we found that the expression of SND1 was negatively correlated with the expression of miR-296-5p in OS tissues and cell lines. Subsequently, we found that luciferase activity was significantly reduced after the co-transfection of miR-296-5p mimics and SND1 3′-UTR vector harboring the miR-296-5p target sequence, in a dual-luciferase assay. Next, we overexpressed SND1 in miR-296-5p mimic-overexpressed HOS cell lines and found that SND1 can subvert the repression of proliferation, migration, and invasion by miR-296-5p mimics, which further suggests that miR-296-5p might regulate the proliferation and

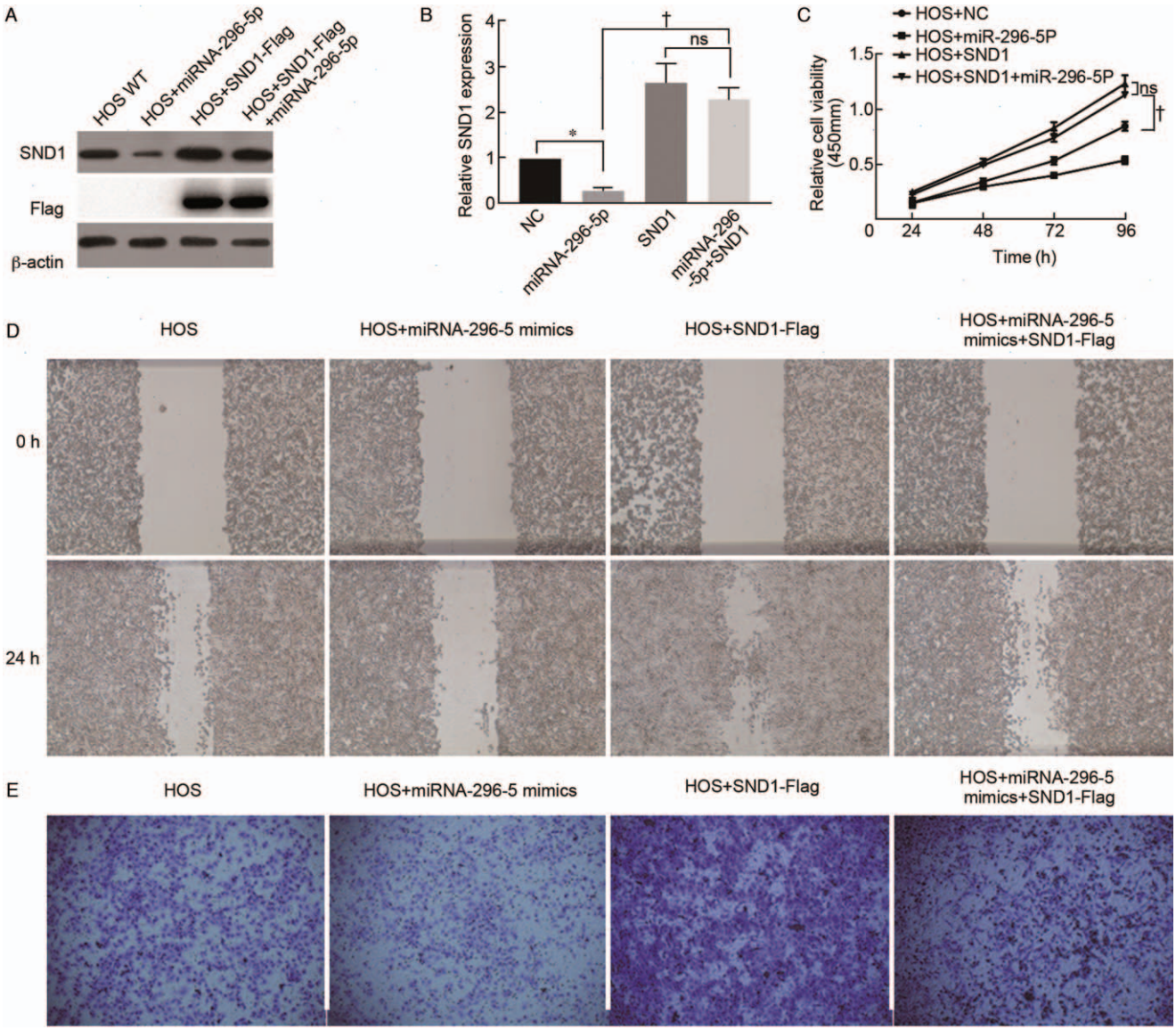

Figure 6: SND1 overexpression rescues the phenotype of osteosarcoma cells with miR-296-5p overexpression. (A and B) Western blot and qRT-PCR analyses of SND1-flag expression in HOS cells transfected with miR-296-5p mimics, SND-flag, or NC. (C) MTT assay. (D and E) Wound healing assay (bright field; original magnification, 10×) and transwell assay (crystal violet staining; original magnification 10×) of HOS and Saos-2 cells transfected with the SND1-flag, miR-296-5p mimics, or NC. NC, negative control; qRT-PCR, quantitative reverse transcription polymerase chain reaction. $^{*}P < 0.05$, $^{\dagger}P < 0.01$. ns: no significance.

metastasis of OS by targeting *SND1*. Indubitably, this study had a few limitations such as the limited number of clinical samples. Moreover, the relevant mechanisms need to be explored further.

Our results indicate that miR-296-5p is downregulated in OS and exerts tumor-suppressive effects in OS cells by targeting *SND1*. Supplementation of miR-296-5p may serve as an effective strategy for the treatment of OS.

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Conflicts of interest

None.

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