

# lncRNA SNHG1 negatively regulates miRNA-101-3p to enhance the expression of ROCK1 and promote cell proliferation, migration and invasion in osteosarcoma

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**Abstract.** Osteosarcoma (OS) is a rare malignant bone tumor that commonly occurs in children and adolescents and causes pain and swelling of the long bones of the legs and arms. Long non-coding RNA (lncRNA) and micro (mi)RNA-101 are important in the initialization and progression of OS. However, the mechanism underlying the role of the lncRNA and miRNA-101 in OS remains to be fully elucidated. In the present study, through reverse transcription-quantitative polymerase chain reaction analysis, it was first found that the lncRNA SNHG1 was upregulated and miRNA-101-3p was downregulated in OS tissues and cell lines. Second, the knockdown of lncRNA SNHG1 induced cell apoptosis and maintained the cell cycle at the G0/G1 phase, which decreased the overall cell viability. Furthermore, according to a dual-luciferase assay and western blot analysis, miRNA-101-3p was found to be a target of the lncRNA SNHG1 in OS, which further regulated the expression of Rho-associated coiled-coil-containing protein kinase 1 (ROCK1). It was found that the phosphoinositide 3-kinase/ATK pathway was inactivated and that epithelial-mesenchymal transition was activated in OS cell lines with overexpression of the lncRNA SNHG1. Taken together, in OS cell lines, the lncRNA SNHG1 acted as an oncogene, and miRNA-101-3p was considered a tumor suppressor. The lncRNA SNHG1 promoted OS cell proliferation, migration and invasion by downregulating the expression of miRNA-101-3p, which enhanced the expression of ROCK1.

## Introduction

Osteosarcoma (OS) is a rare malignant bone tumor that commonly occurs in children and adolescents. OS usually causes pain and swelling of the long bones of the legs and

arms (1-3). The 5-year survival rate of young-onset OS is 61.6% globally due to advances in therapeutic strategies, including combinatorial chemotherapy and radiotherapy, over the last decade (4). Only a few oncogenes and tumor suppressors have been identified in the disease, limiting the development of potential novel therapeutic targets for OS treatment (5,6). Therefore, investigations on the mechanisms underlying the initialization, progression, and metastasis of OS are required to improve the therapeutic efficiency of the treatments. MicroRNAs (miRNAs) act as an important type of regulatory molecules in eukaryotic cells and consist of ~22 nucleotides (7,8). miRNAs have been widely reported to exhibit ectopic expression in various types of cancer, including stomach, colorectal, lung, squamous, and pancreatic cancer, and OS (9-15). In a previous study, a total of 268 miRNAs were identified to be either upregulated or downregulated in human OS cell lines. For example, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis has demonstrated that miRNA-9, miRNA-99, miRNA-195, miRNA-148a and miRNA-181a are upregulated, whereas miRNA-143, miRNA-145, miRNA-335 and miRNA-539 are downregulated in the MG-63 OS cell line (16). Different miRNAs can have different effects on cancer. Certain miRNAs promote the progression of tumors, whereas others inhibit the development of tumors (17,18).

miRNA-101-3p has been shown to be downregulated in several human malignancies (19-21), including OS (22). This miRNA acts as a tumor suppressor. Chang *et al* (23) suggested that miRNA-101 inhibits the autophagy of OS cells, thereby promoting chemotherapeutic efficiency. Furthermore, miRNA-101 was shown to inhibit OS metastasis by regulating enhancer of zeste 2 polycomb repressive complex 2 subunit (24). In another report, mammalian target of rapamycin (mTOR) was shown to be a target of miRNA-101 in OS cells; mTOR induced cell apoptosis and inhibited cell proliferation (25). Overall, miRNA-101 is considered to be a tumor suppressor in OS. However, the mechanism underlying the regulation of OS cell proliferation, migration, invasion and apoptosis by miRNA-101 remains to be fully elucidated.

In addition, the expression of miRNA-101-3p is regulated by several other factors in cancer cells (26). Long non-coding RNA (lncRNA), which consists of >200 nucleotides, is a class of non-coding RNA that is important in the development of human cancer (27,28). The dysregulation of lncRNA has been

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identified in several types of cancer, including renal cell carcinoma, melanoma, glioma cells, non-small cell lung cancer, and OS cells (29,30). In non-small cell lung cancer, Cui *et al* (19) found that the lncRNA SNHG1 was upregulated and inhibited the expression of miRNA-101-3p, which significantly promoted the progression of cancer. However, the mechanism underlying the regulation of the expression of miRNA-101 by lncRNAs and the effect of lncRNAs on cancer development in OS remain to be elucidated.

Rho-associated coiled-coil-containing protein kinase 1 (ROCK1) is considered to be a potential target unit of miRNA-101-3p in different types of cancer, including OS (31-34). ROCK1 is one of the four kinases whose inhibition induces cell apoptosis and poor cell viability in OS cells (22). The knockdown of ROCK1 in OS cells has been reported to decrease cell proliferation and promote cell death in OS cell lines. By contrast, the overexpression of ROCK1 in patients with OS is usually associated with poor prognosis, suggesting that it may be used as a prognosis marker and therapeutic target.

miRNA-101 has been shown to inhibit the progression of several types of cancer by targeting ROCK1 (22). However, the regulation of miRNA-101-3p by the lncRNA SNHG1; the subsequent association between the regulated miRNA-101-3p and its potential target, ROCK1; and the resulting OS development have not been fully investigated. Therefore, in the present study, the associations among the lncRNA SNHG1, miRNA-101-3p, and ROCK1 were investigated in OS cell lines. Furthermore, the molecular mechanism of this lncRNA SNHG1-miRNA-101-3p-ROCK1 pathway in regulating the proliferation, migration, invasion, and apoptosis of OS cells was examined, which may provide a potential prognostic marker and therapeutic target for OS treatment.

## Materials and methods

**Patients and specimens.** The present study was approved by the Research Ethics Committee of Nanfang Hospital, Southern Medical University (Guangzhou, China). Written informed consent was obtained from all participants. A total of 43 OS samples were obtained from patients who had surgical resection between 2015 and 2016 at Nanfang Hospital, Southern Medical University. Normal osteoblast samples were obtained from 12 individuals who succumbed to mortality in traffic accidents between 2015 and 2016 at Nanfang Hospital, Southern Medical University.

**Cell culture and reagents.** The MG63, U2OS, and Saos-2 human OS cell lines and the hFOB1.19 human osteoblast cell line were purchased from the Shanghai Institutes for Biological Sciences Cell Resource Center (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) with high glucose which supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for the cell culture. The humidified incubator for cell culture was set to 37°C with 5% CO<sub>2</sub>.

**RT-qPCR analysis.** Total RNAs were collected from the patient specimens and cultured cells using TRIzol reagent

(Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA (2 µg) was reversed transcribed into cDNA using TaqMan™ Reverse Transcription reagents (Thermo Fisher Scientific, Inc.). The expression levels of miRNA-101-3p and the lncRNA SNHG1 were analyzed using the DyNAmo ColorFlash SYBR-Green qPCR kit (Thermo Fisher Scientific, Inc.) with the ABI Prism 7700 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). U6 and GAPDH were used as the internal controls. The sequences of the primers for miRNA-101-3p were as follows: Forward, 5'-GCGCGCATACAGTACTGTGATA-3' and reverse, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCAGT-3'. The sequences of the primer for the lncRNA SNHG1 were as follows: Forward, 5'-ACGTTGGAACCGAAGAGAGC-3' and reverse, 5'-GCAGCTGAATTCCCCAGGAT-3'. The sequences of the primers for U6 were as follows: Forward, CTCGCTTCGGCAGCACA and reverse, AACGCTTCACGAATTTGCGT. The sequences of the primers for GAPDH were as follows: Forward, CCAGGTGGTCTCCTCTGACTT and reverse, GTTGCTGTAGCCAATTTCGTTGT. The reaction conditions were: 95°C for 30 sec, followed by 40 cycles of 95°C for 3 sec and 60°C for 34 sec. The relative expression of target genes was calculated using the 2<sup>-ΔΔC<sub>q</sub></sup> method (35).

**miRNA transfection.** miRNA-101-3p mimic (5'-UACAGUACUGUGAUAACUGAACAGUUAUCACAGUACUGUAUU-3'), miRNA-101-3p mimic-inhibitor (5'-UUCAGUUAUACAGUACUGUA-3'), small interfering RNA (si)-SNHG1, si-ROCK1 and the negative control (5'-UUCUCCGAACGUGUCACGUTT-3') were obtained from GenePharma Co., Ltd. (Shanghai, China). Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) was used for miRNA and siRNA transfection. The sequences for si-SNHG1 were sense, 5'-CUUAAAGUGUUAGCAGACATT-3' and antisense, 5'-AAUGUCUGCUAACACUUUAAG-3'; the siROCK1 sequences were sense, 5'-GCACCAGTTGTACCCGATTTA-3' and antisense, 5'-TAAATCGGGTACAACCTGGTGC-3'.

**3-(4,5-Dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay.** In 96-well plates, following the transfection in different groups, the MG63 (2,500 cells/well) and U2OS (3,000 cells/well) were cultured in DMEM/F12 supplemented with 10% FBS for 24, 48, 72 and 96 h. Subsequently, 10 µl MTT was added into the culture medium for 60 min. The culture media was removed, and 100 µl DMSO was added into each well to dissolve the resulting crystals with agitation for 10 min. The optical density (OD) at 490 nm was measured using a microplate reader.

**Analysis of cell cycle and apoptosis via flow cytometry.** For the cell cycle analysis, the transfected cells were harvested and stained with propidium iodide using the Cell Cycle Analysis kit (Biyuntian; Jiangsu, China), followed by assessment using flow cytometry. Using FlowJo software 7.6 (Tree Star, Inc., Ashland OR, USA), the percentage of cells in different phases was counted. An FITC Annexin V Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze apoptosis. The transfected cells were harvested and re-suspended in binding buffer, and Annexin V-FITC and

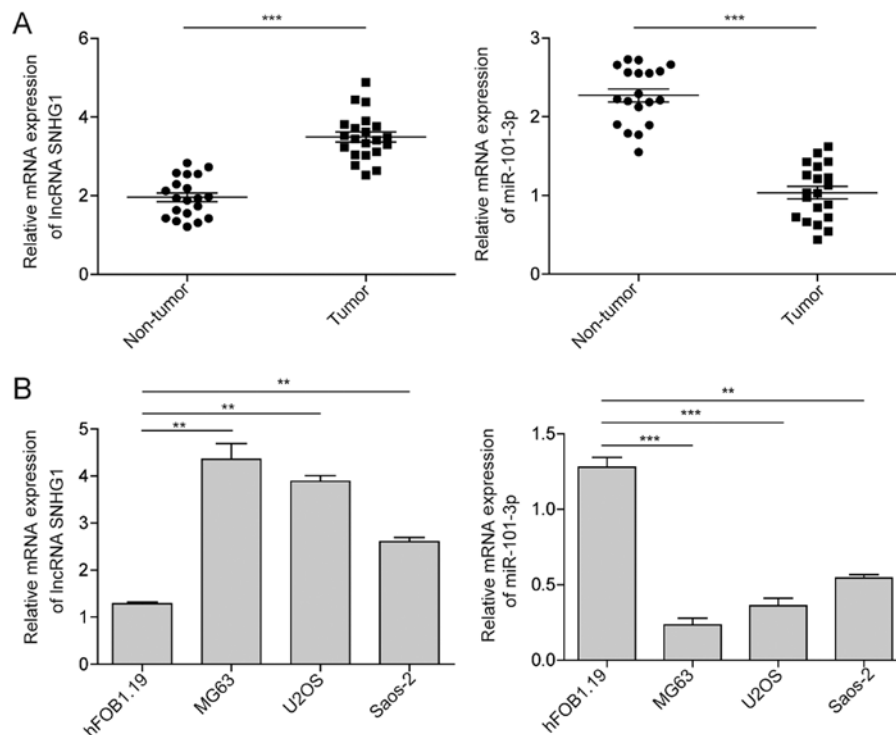


Figure 1. Overexpression of lncRNA SNHG1 and downregulation of miR-101-3p in OS cancer cells. (A) Expression levels of the lncRNA SNHG1 and miR-101-3p in non-tumor and tumor tissues. (B) Expression levels of lncRNA SNHG1 and miR-101-3p. Three OS cancer cell lines (MG63, U2OS, and Saos-2) and a normal cell line (hFOB1.19) were analyzed via reverse transcription-quantitative polymerase chain reaction analysis. The mean  $\pm$  standard deviation in the graph represents the relative levels from three replicated experiments. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . lncRNA, long non-coding RNA; miR, microRNA; OS, osteosarcoma.

propidium iodide were used to stain the cells. Flow cytometry was performed according to the manufacturer's protocol.

**Dual luciferase activity assay.** The dual luciferase activity assay was used for miRNA target validation. The SNHG1 cDNA containing the putative miRNA-101-3p-binding site was amplified by PCR and was cloned into the luciferase reporter psiCHECK2 vector (Promega Corporation, Madison, WI, USA), termed SNHG1 wild-type (WT). The mutant (Mut) of SNHG1 was 5'-CTGTCATGCTGTCATGACTA-3'. Subsequently, the MG63 ( $1.5 \times 10^4$ ) and U2OS ( $1.8 \times 10^4$ ) cells were seeded in 24-well plates and were transfected with the SNHG1 WT or SNHG1 Mut and miRNA-101-3p or a scramble using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.). The luciferase activity in the transfected cells was analyzed using the dual-luciferase assay system (Promega Corporation), following the manufacturer's protocol.

**Invasion and migration assay.** A cell invasion assay was performed with Transwells coated with Matrigel (BD Biosciences). Briefly, MG63 ( $1 \times 10^5$ ) and U2OS ( $1.2 \times 10^5$ ) cells were re-suspended in a serum-free medium. Subsequently, 200  $\mu$ l of the cell suspension was added to the top chamber. To prepare the chemoattractant in the lower chamber, 600  $\mu$ l of medium containing 10% FBS was added into the lower chamber. The Transwell was incubated for 48 h at 37°C under 5% CO<sub>2</sub>. Subsequently, the cells in the top chamber were wiped off using a cotton swab, and the cells in the lower chamber were fixed in 4% formaldehyde and were stained with 0.1% crystal violet. Under a microscope (1X71; Olympus Corporation,

Tokyo, Japan), the cell numbers were counted. The average data were collected from three independent repeats.

A migration assay was performed using a two-chamber migration assay with a pore size of 8  $\mu$ m. The top chamber was seeded with 200  $\mu$ l of cells in serum-free medium, and 600  $\mu$ l of complete medium was added to the lower chamber. Following incubation at 37°C for 24 h, the traversed cells on the lower chamber were stained with crystal violet and were counted. The data were collected from three independent experiments.

**Western blot analysis.** The proteins were extracted using immunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) with protease inhibitors (Roche, Diagnostics GmbH, Mannheim, Germany). Protein concentration was determined with a bicinchoninic acid protein assay. Equal quantities of protein (10  $\mu$ g/lane) from cell lysates were separated via 8% SDS-polyacrylamide gel electrophoresis and were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The western blot analysis was performed using a Bio-Rad Bis-Tris Gel system according to the manufacturer's protocol. Briefly, primary antibodies (All purchased from Abcam, Cambridge, MA, USA) against ROCK1 (cat. no. ab45171; 1:2,000), anti-phosphoinositide 3-kinase (PI3K; cat. no. ab151549; 1:1,000), phosphorylated (p)-PI3K (cat. no. ab182651; 1:1,000), anti-protein kinase B (AKT; cat. no. ab8805; 1:1,000), anti-p-AKT (cat. no. ab38449; 1:1,000), anti-E-cadherin (cat. no. ab1416; 1:1,000), anti-N-cadherin (cat. no. ab76057; 1:1,000) were incubated with the membrane

Table I. Expression of lncRNA SNHG1 in relation to clinicopathologic features.

Characteristics	n	lncRNA SNHG1		P-value
		Negative	Positive	
Patients	43	13	30	
Age (years)				0.864
≤20	19	6	13	
>20	24	7	17	
Sex				0.370
Male	22	8	14	
Female	21	5	16	
Tissue size				0.002 <sup>a</sup>
D <8 cm	15	9	6	
D ≥8 cm	28	4	24	
Enneking stage				0.004 <sup>a</sup>
I	6	5	1	
II	24	7	17	
III	13	1	12	
Distant metastasis				0.034 <sup>a</sup>
Present	13	1	12	
Absent	30	12	18	

<sup>a</sup>P<0.05, considered statistically significant. lncRNA, long non-coding RNA; D, diameter.

at 4°C overnight with 5% blocking buffer. Horseradish peroxidase-conjugated secondary antibodies, including goat anti-rabbit (cat. no. ab7090; 1:5,000; Abcam), goat anti-mouse (cat. no. ab97040; 1:5,000; Abcam) were subsequently added for 1 h at room temperature, following washing the membrane three times with Tris buffered saline with 0.05% Tween-20. Following rinsing, the chemiluminescence signal was visualized following the addition of 200 µl Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA), and the bands were quantified using Image Lab™ 3.0 software (Bio-Rad Laboratories, Inc.).

**Statistical analysis.** All experiments were repeated at least three times, and data are expressed as the mean ± standard deviation. All the data were analyzed with SPSS 20.0 (IBM Corp., NY, Armonk, USA). The difference between two groups was compared using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**miRNA-101 is downregulated and lncRNA SNHG1 is upregulated in OS tissues and OS cell lines.** To determine the role of miRNA-101-3p and the lncRNA SNHG1 in OS, the present study examined the expression levels of miRNA-101-3p and the lncRNA SNHG1 via RT-qPCR analysis in OS tissues samples and OS cell lines. As shown in Fig. 1A, miRNA-101-3p was downregulated in the OS tissues compared with the expression in the adjacent non-tumor tissues. However, the lncRNA SNHG1 was upregulated in the OS tumor tissues compared

with its expression in the adjacent non-tumor tissues. The sample distribution and tumor stage in these patients were also analyzed (Table I). The results indicated that the expression of the lncRNA SNHG1 did not differ significantly with patient age or gender. However, patients with a large tumor size (>8 cm), later Enneking stage or distant metastasis exhibited a significantly higher expression level of lncRNA SNHG1. Furthermore, similar trends were observed in the OS tumor cell lines (Fig. 1B). All three OS cancer cell lines (MG63, U2OS, and Saos-2 cells), exhibited a significantly lower expression of miRNA-101-3p and higher expression of lncRNA SNHG1 compared with the expression levels in the normal hFOB1.19 cell line.

**lncRNA SNHG1 promotes cell proliferation.** To investigate the function of the lncRNA SNHG1 in OS tumorigenesis, si-lncRNA SNHG1 was transfected into two OS tumor cell lines. As shown in Fig. 2A successful transfection was achieved, which induced a lower expression of lncRNA SNHG1, as determined via RT-qPCR analysis. With these transfected cell lines, an MTT assay was performed to investigate the effect of the lncRNA SNHG1 on cell proliferation. As shown in Fig. 2B, the MTT OD values in the OS cell lines transfected with si-lncRNA SNHG1 at 48, 72 and 96 h were significantly lower than those of the control groups, demonstrating that inhibiting the lncRNA SNHG1 significantly decreased cell proliferation rates. These results indicated that the overexpression of lncRNA SNHG1 in OS tumor cell lines promoted OS cell proliferation *in vitro*.

Subsequently, flow cytometric analysis was used to evaluate the effect of the lncRNA SNHG1 on OS cell proliferation. As shown in Fig. 2C, the inhibition of lncRNA SNHG1 in OS

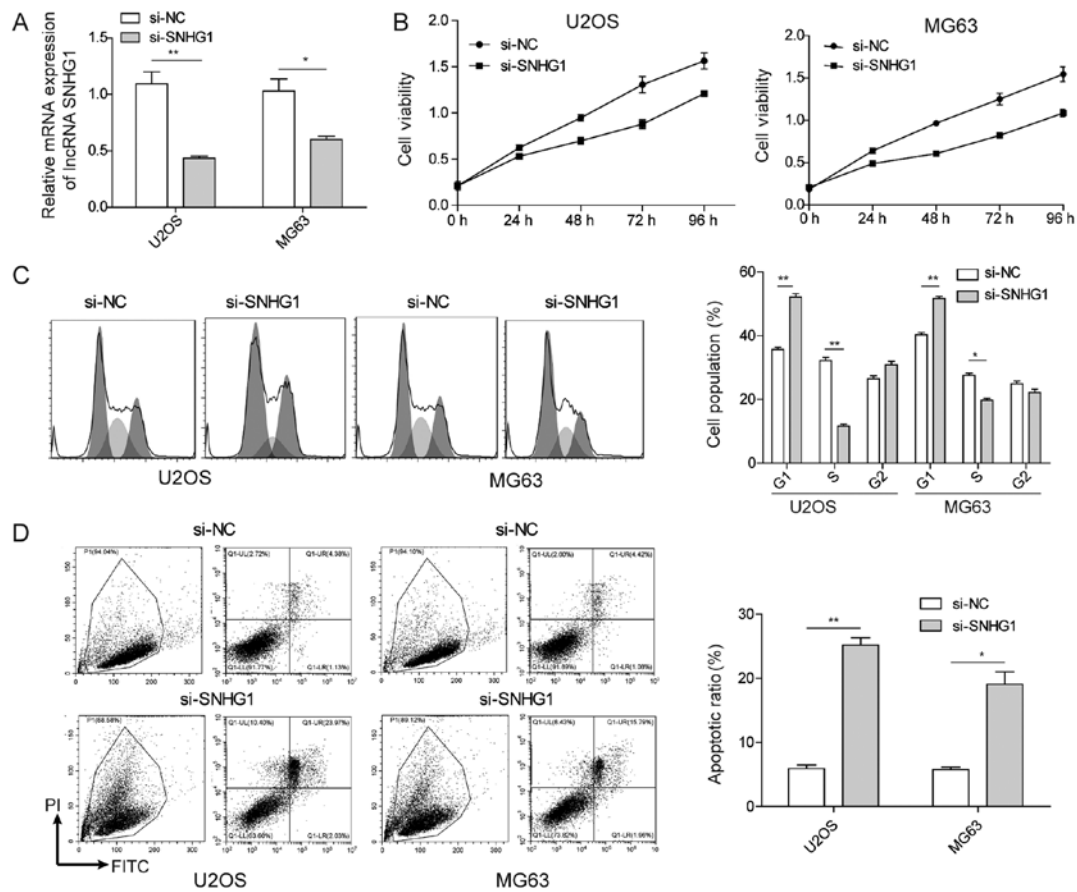


Figure 2. IncRNA SNHG1 promotes osteosarcoma cell proliferation. (A) IncRNA SNHG1 was downregulated following transfection with si-SNHG1 in the U2OS and MG-63 cell lines. (B) Cell viability was significantly reduced following transfection with si-SNHG1 in U2OS and MG-63 cells for different time scales. (C) Cell populations of the U2OS and MG63 cell lines at different cell-cycle stages were analyzed via flow cytometry following transfection with si-NC and si-SNHG1, respectively. (D) Cell apoptosis was enhanced following transfection with si-SNHG1 in U2OS and MG63 cancer cell lines, as evaluated via an apoptosis assay and flow cytometry. The mean  $\pm$  standard deviation in the graph represents the relative levels from three replicated experiments. \*P<0.05; \*\*P<0.01. IncRNA, long non-coding RNA; si-, small interfering RNA; NC, negative control; PI, propidium iodide.

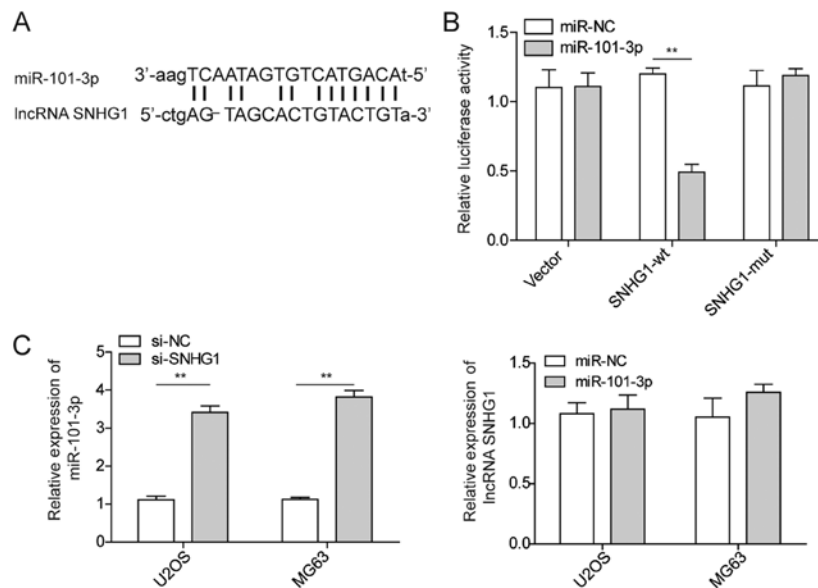


Figure 3. Regulation of miR-101-3p by the IncRNA SNHG1 in osteosarcoma cancer cells. (A) Prediction of miR-101-3p binding sites on the IncRNA SNHG1 transcript. (B) Luciferase activities in U2OS cells co-transfected with miR-101-3p or miR-NC and luciferase reporters containing empty vector, IncRNA SNHG1 WT, or IncRNA SNHG1 Mut. Data are presented as a relative ratio. (C) Relative expression levels of miR-101-3p in U2OS and MG63 cells transfected with si-SNHG1 or si-NC, as detected by RT-qPCR analysis. The relative expression levels of the IncRNA SNHG1 in U2OS and MG63 cells transfected with miR-101-3p or miR-NC were detected via RT-qPCR analysis. The mean  $\pm$  standard deviation in the graph represents the relative levels from three replicant experiments. \*\*P<0.01. IncRNA, long non-coding RNA; si-, small interfering RNA; miR, microRNA; NC, negative control; WT, wild-type; Mut, mutant; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

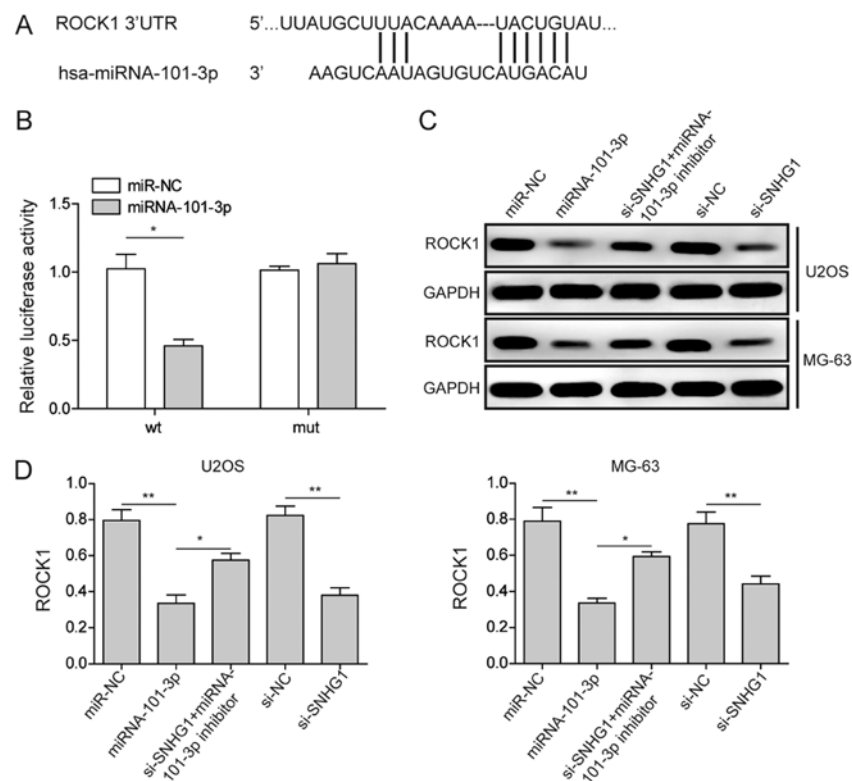


Figure 4. Regulation of ROCK1 via miR-101-3p in osteosarcoma cancer cells. (A) Prediction of miR-101-3p-binding sites on the ROCK1 transcript. (B) Luciferase activities in U2OS cells co-transfected with miR-101-3p or miR-NC and luciferase reporters containing ROCK1 WT or ROCK1 Mut. (C) Relative expression levels of ROCK1 in U2OS and MG63 cells transfected with miR-101-3p, miR-NC, si-SNHG1, si-NC, and si-SNHG1 + miR-101-3p inhibitor, as detected via western blot analysis. (D) Quantitative analysis of ROCK1 expression in U2OS and MG63 cells. The mean  $\pm$  standard deviation in the graph represents the relative levels from three replicate experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ . miR, microRNA; NC, negative control; WT, wild-type; Mut, mutant; si-, small interfering RNA; ROCK1, Rho-associated coiled-coil-containing protein kinase 1.

cancer cell lines decreased the percentages of cells at the G0/G1 and S phases, demonstrating that the lncRNA SNHG1 promoted cell cycle progression of the OS cancer cells. Furthermore, flow cytometric analysis was used to analyse cell apoptosis through Annexin V-FITC/propidium-iodide dual staining. The results showed that the proportion of apoptotic OS cells with si-lncRNA SNHG1 transfection was significantly enhanced compared with that in the control groups. Therefore, in the OS tumor cells, the high expression of lncRNA SNHG1 inhibited OS cell apoptosis.

**lncRNA SNHG1 inhibits the expression of miRNA-101-3p.** To determine the interaction between the lncRNA SNHG1 and miRNA-101-3p, the miRDB database was used to examine the potential complementary sequences between the lncRNA SNHG1 and miRNA-101-3p (Fig. 3A). Subsequently, dual-luciferase reporters containing either SNHG1 WT or SNHG1 Mut were constructed. First, the U2OS cells were co-transfected with miRNA-101-3p and SNHG1-WT or SNHG1-Mut and the luciferase intensity was measured. As shown in Fig. 3B, the SNHG1-WT and miRNA-101-3p-transfection groups demonstrated a significant decrease in luminescence intensity, which suggested that there may be a direct interaction between miRNA-101-3p and the lncRNA SNHG1. Subsequently, the expression of miRNA-101-3p was examined in OS cancer cell lines following knockdown of the lncRNA SNHG1 via siRNA (Fig. 3C). The results showed a significant enhancement of the expression of miRNA-101-3p in OS cancer cell lines with SNHG1 knockdown compared with that in the control groups.

A similar investigation was performed following manipulation of the expression of miRNA-101-3p. However, miRNA-101-3p transfection in OS cell lines did not affect the expression level of the lncRNA SNHG1. These results indicated that the lncRNA SNHG1 inhibited the expression of miRNA-101-3p in OS cancer cell lines.

**Identification of ROCK1 as a target of miRNA-101-3p.** The present study investigated the association between ROCK1 and miRNA-101-3p via a dual-luciferase assay. From the prediction of the miRDB database and the reported literature, ROCK1 was identified as a potential target of miRNA-101-3p (Fig. 4A). A ROCK1-WT and a ROCK1-Mut vector were constructed and were used to transfect U2OS cells with either miRNA-101-3p or miRNA-NC. The luciferase activity in the group transfected with ROCK1-WT and miRNA-101-3p was lower than the activity in the other control groups, indicating that ROCK1 was a target of miRNA-101-3p (Fig. 4B).

**miRNA-101-3p controlled by lncRNA SNHG1 inhibits cell proliferation, migration and invasion by inhibiting ROCK1.** From the previous results, it was shown that the lncRNA SNHG1 significantly inhibited the expression of miRNA-101-3p and that ROCK1 was a target of miRNA-101-3p. Therefore, the association among the lncRNA SNHG1, miRNA-101-3p and ROCK1 was investigated using western blot analysis. As shown in Fig. 4C, U2OS and MG63 cells were transfected with miRNA-NC, miRNA-101-3p, si-SNHG1 with miRNA-101-3p



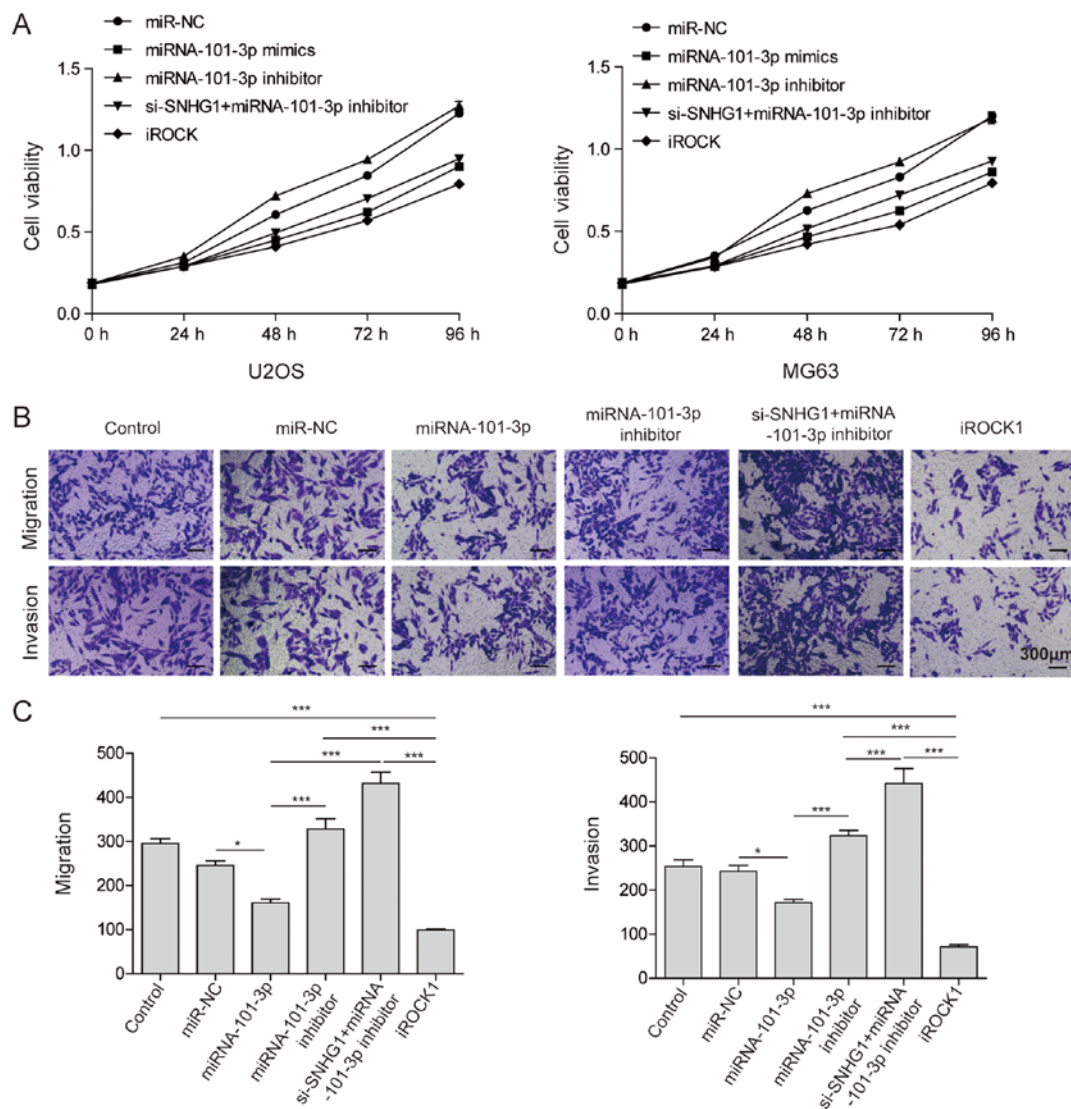


Figure 5. lncRNA SNHG1 promotes osteosarcoma cell proliferation, invasion and migration. (A) U2OS and MG63 cells were transfected with miR-NC, miR-101-3p, miR-101-3p inhibitor, si-lncRNA SNHG1 + miR-101-3p inhibitor, and iROCK1. An MTT assay was performed to determine the cell viability. (B) Migration and invasion abilities of U2OS cells following no transfection or transfection with miR-NC, miR-101-3p, miR-101-3p inhibitor, si-lncRNA SNHG1 + miR-101-3p inhibitor, and iROCK1 were investigated via a Transwell assay. Magnification, x100. (C) Cell numbers in the bottom chambers for migration and invasion assays were determined in the groups. The mean  $\pm$  standard deviation in the graphs represent the relative levels from three replicate experiments. \* $P < 0.05$ ; \*\*\* $P < 0.001$ . lncRNA, long non-coding RNA; miR, microRNA; NC, negative control; WT, wild-type; Mut, mutant; si-, small interfering RNA; ROCK1, Rho-associated coiled-coil-containing protein kinase 1; iROCK1, si-ROCK1.

inhibitor, si-NC and si-SNHG1. Compared with its expression following miRNA-NC transfection, ROCK1 was significantly decreased in the group treated with miRNA-101-3p, which was consistent with the results of the dual-luciferase assay. Furthermore, by inhibiting the lncRNA SNHG1 and miRNA-101-3p or inhibiting the lncRNA SNHG1 only, ROCK1 was significantly inhibited. Combined with the previous results, it was concluded that the lncRNA SNHG1 inhibited the expression of miRNA-101-3p, which further enhanced the expression of ROCK1 in OS cancer cell lines.

The present study also investigated the associations among the lncRNA SNHG1, miRNA-101-3p and ROCK1, and their effect on cell viability, migration and invasion. The U2OS and MG-63 cells were transfected with miRNA-NC, miRNA-101-3p, miRNA-101-3p inhibitor, si-SNHG1 with miRNA-101-3p inhibitor, and si-ROCK1. As shown in Fig. 5A, the MTT assay results indicated that miRNA-101-3p and

si-ROCK1 treatments decreased the cell viability of OS cancer cells, which suggested that any pathways that inhibited ROCK1 inhibit the cell viability of OS cancer cells. Additionally, a Transwell assay was used to investigate their effect on OS cell migration and invasion. As shown in Fig. 5B and 5C, transfection with miRNA-101-3p mimics and si-ROCK1 inhibited cell migration and invasion; however, the miRNA-101-3p inhibitor and si-SNHG1 with miRNA-101-3p inhibitor enhanced cell migration. All of the above results suggested that the lncRNA SNHG1 inhibited the expression of miRNA-101-3p, leading to the activation of ROCK1 in OS cancer cells, which significantly enhanced cell proliferation, migration and invasion ability.

*Phosphoinositide 3-kinase (PI3K)/AKT pathway is activated and EMT is induced.* To clarify the signaling pathways involved in the function of the lncRNA SNHG1, miRNA-101-3p, and ROCK1 in OS cancer cells, the expression of the components of

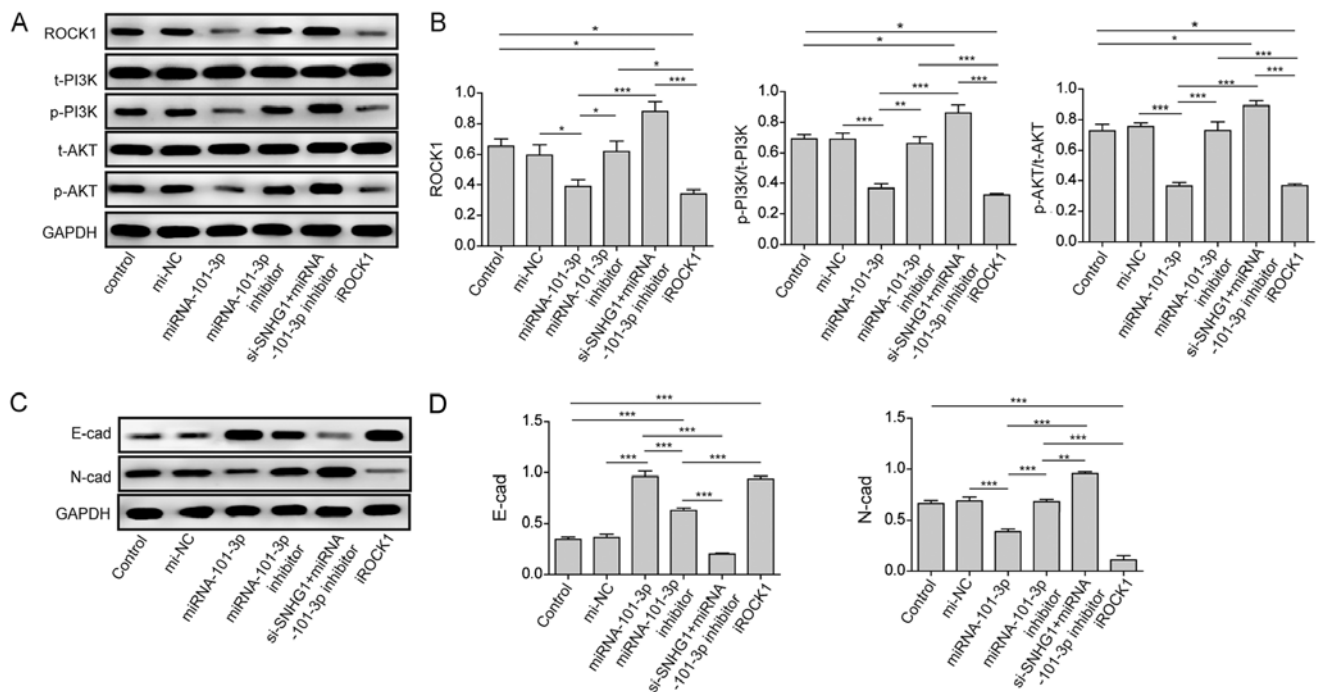


Figure 6. PI3K/AKT pathway and EMT activation. (A) Overexpression of miR-101-3p inhibited the PI3K/AKT pathway via the downregulation of ROCK1. Western blot analysis was used to analyse the expressions of related components in U2OS cells transfected with nothing, miR-NC, miR-101-3p, miR-101-3p inhibitor, si-lncRNA SNHG1 + miR-101-3p inhibitor, and iROCK1. (B) Expression levels of ROCK1, p-PI3K/t-PI3K, and p-AKT/t-AKT in the different groups. (C) Overexpression of miR-101-3p inhibited the activation of EMT by promoting the expression of E-cad and inhibiting N-cad. Western blot analysis was used to analyse the expressions of N-cad and E-cad in U2OS cells transfected with nothing, miR-NC, miR-101-3p, miR-101-3p inhibitor, si-lncRNA SNHG1 + miR-101-3p inhibitor, and iROCK1. (D) Expression levels of E-cad in the different groups. The mean  $\pm$  standard deviation in the graph represents the relative levels from three replicate experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . lncRNA, long non-coding RNA; miR, microRNA; NC, negative control; WT, wild-type; Mut, mutant; si-, small interfering RNA; ROCK1, Rho-associated coiled-coil-containing protein kinase 1; iROCK1, si-ROCK1; PI3K, phosphoinositide 3-kinase; p-, phosphorylated; t-, total; EMT, epithelial-mesenchymal transition; E-cad, E-cadherin; N-cad, N-cadherin.

the PI3K/AKT and EMT pathways were measured via western blot analysis. As shown in Fig. 6A and B, the overexpression of miRNA-101-3p in the group transfected with miRNA-101-3p mimics resulted in a downregulation in the expression of ROCK1, p-PI3K, and p-AKT. However, the knockdown of miRNA-101-3p by the miRNA-101-3p inhibitor alone significantly enhanced the expression of ROCK1, p-PI3K, and p-AKT. In addition, the co-transfection of si-SNHG1 and the miRNA-101-3p inhibitor enhanced the expression of these three proteins compared with miRNA-101-3p-mimics transfection. These results indicated that the PI3K/AKT pathway was activated in OS cancer cells.

Usually, activation of the PI3K/AKT pathway induces EMT in cancer cells. Therefore, the present study also investigated the EMT pathway. As shown in Fig. 6C and D, miRNA-101-3p mimics and si-ROCK1 promoted the expression of E-cadherin and inhibited the expression of N-cadherin. By contrast, treatment with the miRNA-101-3p inhibitor alone or si-SNHG1 with the miRNA-101-3p inhibitor inhibited the expression of E-cadherin and promoted the expression of N-cadherin. These results demonstrated that EMT was also activated in OS cancer cells, and that the inhibition of lncRNA SNHG1 or overexpression of miRNA-101-3p were able to inhibit activation of the EMT process in OS cells.

## Discussion

Osteosarcoma (OS) is a common aggressive mesenchyme-derived bone tumor that primarily affects children and

adolescents (3). The main strategies for OS treatment include surgical resection, chemotherapy, and radiotherapy (8). Despite the efforts of clinicians and researchers, the overall survival rate of patients with OS remains poor, particularly for those who are at advanced clinical stages (6). Therefore, the identification a novel diagnostic biomarker and the mechanism involved in OS tumorigenesis and metastasis, which may offer a potential therapeutic target, is crucial. Previously, the dysregulation of lncRNAs has been reported during tumorigenesis and progression in several types of malignant tumors. For example, Wang *et al* (36) reported that the lncRNA AK093407 promoted OS cell proliferation and inhibited its apoptosis by activating signal transducer and activator of transcription 3. Therefore, the present study focused on the abnormal expression of the lncRNA SNHG1 in OS and its underlying molecular mechanism in regulating OS tumorigenesis.

In the present study, the lncRNA SNHG1 was found to be overexpressed in OS tumor tissues compared with its expression in adjacent non-tumor tissues. In addition, it was found that the expression of lncRNA SNHG1 in the OS tumor cell lines was significantly upregulated compared with the expression in the non-tumor cell line, which was consistent with the results reported by Jiang *et al* (37) previously. Wang *et al* (38) also found that the lncRNA SNHG1 promoted tumorigenesis in OS. In all OS tumor tissues and cell lines, the expression of miRNA-101-3p was significantly decreased compared with that in the control groups, which indicated a potential association between the lncRNA SNHG1 and miRNA-101-3p. Furthermore,



using an MTT assay, RT-qPCR analysis, and flow cytometric analysis, the present study determined the effect of the lncRNA SNHG1 on OS cell proliferation, cell cycle phase, and apoptosis. First, by silencing the expression of lncRNA SNHG1, all three OS tumor cell lines exhibited lower cell viability, and the flow cytometric analysis indicated that tumor cell apoptosis increased following transfection with the si-lncRNA SNHG1. In addition, the cell cycle phase was analyzed following lncRNA SNHG1 inhibition. The result revealed that the low OS cell proliferation was caused by G1/G0 phase arrest and cell apoptosis. These data suggested that the lncRNA SNHG1 may be considered as a tumor oncogene in OS progression.

Previous reports have indicated that the expression and activity of miRNA can also be regulated by lncRNAs. For example, Li *et al* (39) found that the lncRNA UCA1 promoted glutamine metabolism by targeting miRNA-16 in human bladder cancer. Cui *et al* (19) reported that the lncRNA SNHG1 contributed to the progression of non-small cell lung cancer by inhibiting miRNA-101-3p. Wang *et al* (38) reported that the lncRNA SNHG1 inhibited miRNA-326 and promoted tumorigenesis in OS. However, the association between the lncRNA SNHG1 and miRNA-101-3, and their effect on tumorigenesis and progression in OS remain to be elucidated and require documentation to provide an efficient therapeutic strategy. In the present study, it was found that miRNA-101-3p was an inhibitory target of the lncRNA SNHG1 in OS using sequence complementarity analysis and a dual-luciferase assay. In OS tissues and cell lines, the lncRNA SNHG1 and miRNA-101-3p showed a significant negative correlation. By inhibiting the expression of the lncRNA SNHG1 in OS cancer cells, the expression of miRNA-101-3p was significantly enhanced. However, miRNA-101-3p transfection did not affect the expression of the lncRNA SNHG1. These data indicated that miRNA-101-3p was a direct target of the lncRNA SNHG1. Furthermore, miRNA has been reported to inhibit the expression of ROCK1 in OS cancer cells to prevent cell proliferation, migration and invasion. Therefore, the present study also examined the associations among lncRNA SNHG1, miRNA-101-3p and ROCK1 in OS cancer cell lines using a dual-luciferase assay and western blot analysis. The results showed that ROCK1 was a direct target of miRNA-101-3p in OS cancer cells. In addition, by knocking down the expression of lncRNA SNHG1 using si-SNHG1 in U2OS and MG-63 cells, the expression of ROCK1 was significantly decreased, which showed a similar effect as for the overexpression of miRNA-101-3p. However, co-transfection with si-SNHG1 and miRNA-101-3p inhibitor promoted the expression of ROCK1 in the two cell lines. These results suggested that, in OS cancer cell lines, ROCK1 was a direct target of miRNA-101-3p and that ROCK1 can be directly controlled by the expression of lncRNA SNHG1.

Furthermore, the present study examined how the associations among the lncRNA SNHG1, miRNA-101-3p, and ROCK1 affect cell proliferation, migration and invasion. Using an MTT assay and Transwell assay, it was found that the overexpression of miRNA-101-3p in OS cancer cell lines inhibited cell proliferation, cell migration and invasion; this result was similar to that obtained when the cells were transfected with si-ROCK1. By contrast, treatment with the miRNA-101-3p inhibitor or co-transfection with si-SNHG1 and miRNA-101-3p inhibitor promoted cell proliferation, migration and invasion.

A previous report indicated that miRNA-101-3p inhibited OS tumor growth and migration by regulating the PI3K/AKT pathway (22). In OS tumors, the PI3K/AKT pathway is the most frequently activated signal transduction pathway and contributes to cancer initiation, development and metastasis. Therefore, the present study also investigated the mechanism underlying lncRNA SNHG1 regulation in OS cancer cell lines using western blot analysis. Through the transfection of miRNA-101-3p mimics or si-SNHG1 in U2OS cells, the expression levels of PI3K and AKT were significantly inhibited. By contrast, co-transfection with si-SNHG1 and miRNA-101-3p inhibitor, or transfection with miRNA-101-3p inhibitor alone, promoted the activation of the PI3K/AKT pathway in OS cells. Furthermore, it was found that EMT was inactivated by treatment with miRNA-101-3p or si-SNHG1. EMT can be divided into three general subtypes based on the phenotype of the output cells (40). Type 1 EMT involves primitive epithelial cell transition into mobile mesenchymal cells. Type 2 EMT transitions cells to resident tissue fibroblasts through involvement of the secondary epithelial or endothelial cells. Type 3 EMT occurs in epithelial carcinoma cells, generating secondary tumor nodules by transforming these cells to metastatic tumor cells that migrate to metastatic sites (40). Therefore, it was hypothesized that miRNA-101-3p or si-SNHG1 can be used to inhibit type 3 EMT during cancer development.

In conclusion, the present study indicated that the lncRNA SNHG1 acted as an oncogene in OS cancer; it downregulated the expression of miRNA-101-3p and promoted cancer cell proliferation, migration and invasion through activating the expression of ROCK1, the PI3K/AKT pathway and EMT. The results provided a possible molecular mechanism of tumorigenesis and development in OS cancer involving the lncRNA SNHG1 and miRNA-101-3p, which may be used for the development of a novel therapeutic strategy for OS.

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#### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

#### Authors' contributions

RD, JC and JZ conceived and designed the study. RD and JZ performed the experiments; acquired, analyzed and interpreted the data; and edited the manuscript. RD was involved in the clinical study and preparation of the manuscript. JC reviewed the manuscript.

#### Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Nanfang Hospital, Southern Medical University

(Guangzhou, China). Written informed consent was obtained from all participants.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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