Long non-coding RNA LINC00152 promotes tumorigenesis via sponging miR-193b-3p in osteosarcoma

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Abstract. The majority of the human genome has been revealed to be non-protein-coding, which are transcribed into noncoding RNAs (ncRNA), RNAs which are not translated into protein. Long non-coding RNAs (lncRNAs), including LINC00152, may be associated with the pathogenesis of different types of cancer. LINC00152 serves as an endogenous sponge by binding to micro-RNAs (miRNAs) and inhibiting their activity. The current study revealed that LINC00152 is overexpressed in osteosarcoma cells, leading to increased cell proliferation, and decreased G0/G1 cell cycle arrest and apoptosis. The binding of miR-193b-3p to LINC00152 was demonstrated by dual-luciferase assay, and led to miR-193b-3p downregulation in osteosarcoma cells. Knockdown of LINC00152 revealed an antitumorigenic effect by reducing cell proliferation and increasing G0/G1 arrest and apoptosis. Inhibiting miR-193b-3p reversed the effects of LINC00152 knockdown. These results suggested that LINC00152 binds to miR-193b-3p and reduces its expression level, leading to increased cell proliferation and decreased G0/G1 cell cycle arrest and apoptosis in osteosarcoma cells.

Introduction

Osteosarcoma is the most common primary malignant bone tumor with the majority of cases occurring in the lower long bones (1). It is predominantly diagnosed in children and adolescents aged between 10 and 25 years (2,3). Patients with osteosarcoma are likely to experience pain and are susceptible to bone fracture due to weakening of the affected bone. Radiotherapy and chemotherapy alone may not be sufficient to treat osteosarcoma, and complete cure of the disease often requires surgical resection. To date, the most common treatment for osteosarcoma is by limb-salvage, which is the removal of tumor without amputation. However, due to the high local recurrence after the initial treatment, it is often combined with chemotherapy, or further amputation if necessary (4).

Previous studies have revealed that the amount of non-coding DNA in genomes increased consistently as eukaryotic cell complexity increased (5,6). Non-coding DNA accounts for the majority of the human genome, and this is transcribed into non-coding RNA (ncRNA). Among the ncRNAs, long non-coding RNA (lncRNA), micro RNA (miRNA/miR) and small interfering RNA (siRNA) serve regulatory roles in cells. ncRNAs often demonstrate plasticity in expression (7). Dysregulation of ncRNA expression and function is associated with a number of different diseases, including gastric cancer (8), prostate cancer (9), preeclampsia (10), and neurodegenerative diseases (11). lncRNAs consist of >200 nucleotides and serve important roles in cell development and differentiation (12-14). Furthermore, alteration of lncRNA expression is associated with different types of cancer (15-18). Thus, lncRNAs may serve as potential biomarkers for cancer (19). The lncRNA LINC00152 has been implicated in several types of cancer including hepatocellular carcinoma, gastric cancer and gallbladder cancer, by affecting cell proliferation, cell cycle arrest and apoptosis (20-23). LINC00152 interacts with multiple signaling pathways such as the mTOR signaling pathway by binding onto promoters through cis-regulation and attenuating the expression of certain proteins, or by binding directly onto the proteins (20). Furthermore, LINC00152 functions as an endogenous sponge by binding to a number of miRNAs, including miR-138, miR-4647, miR-103a-3p, miR-4775 and miR-139-5p, and inhibiting their functions (24-29).

The aim of the present study was to elucidate the biological functions of LINC00152 in osteosarcoma, as well as the underlying molecular mechanisms involved. The expression level of LINC00152 in osteosarcoma cell lines was analyzed and compared with that in normal cells. LINC00152 knockdown was used to analyze the effect of LINC00152 on cell proliferation and to demonstrate whether LINC00152 exhibits tumorigenic effects similar to those reported in other studies (30-33). The current study also aimed to reveal potential miRNA candidates for LINC00152 binding. Sequence-specific binding between miR-193b-3p and LINC00152 was validated by a dual-luciferase assay. The overall effects of the differential expression of miR-193b-3p and LINC00152 was further investigated by performing loss-of-function experiments. The

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results obtained in the current study increase the understanding of the role of LINC00152 in osteosarcoma and may aid the development of treatment strategies which result in decreased recurrence and an increased survival rate, as well as decreased risk and costs associated with surgical resection.

Materials and methods

Cell culture. The human osteosarcoma cell lines U2OS, Saos-2, MG63 and MNNG/HOS, as well as the human osteoblast cell line HFOB 1.19, were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The HFOB 1.19 cells were cultured in Ham's F-12 nutrient mixture containing 10% fetal bovine serum (FBS); and the MNNG/HOS cell line was cultured in Eagle's minimum essential medium. All other cell lines were cultured in RPMI medium 1640 containing 10% FBS. All culture media, as well as FBS, were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). All cell lines were incubated at 37˚C and with 5% CO_2 in an incubator.

Cell transfection. Transfections were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The miR-193b-3p inhibitor and two siRNAs targeting LINC00152 were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). The following sequences were used: i) miR-193b-3p inhibitor, 5'-AGCGGGACUUUGAGGGCCAGUU-3'; ii) miR-negative control (NC), 5'-CAGUACUUUUGUGUAGUACAA-3'; iii) si-LINC00152-1, 5'-UGAUCGAAUAUGACAGACACCGAAA-3'; iv) si-LINC00152-2, 5'-CAGGGAAUCUUUCAGCUGGAUUCCG-3'; and v) si-NC, 5'-UUCUCCGAACGUGUCACGUdTdT-3'. The concentration of miR-193b-3p inhibitor and miR-NC was 150 nM. The concentration of si-LINC00152-1, si-LINC00152-2 and si-NC was 40 nM. Subsequent experiments were performed 48 h after transfection. The human osteosarcoma cell lines U2OS, Saos-2, MG63, MNNG/HOS and the human osteoblast cell line HFOB 1.19 were used for transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from clinical tissues and cultured cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. First-strand cDNA was synthesized using a Reverse Transcription it (Takara, Bio, Inc., Otsu, Japan). The reaction was carried out at 42˚C for 60 min and was terminated by heating to 70˚C for 5 min. qPCR was subsequently performed using the SYBR® Green PCR Master mix (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and an ABI Prism 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primer pairs were used for the qPCR: LINC00152, forward 5'-AAAATCACGACTCAGCCCCC-3' and reverse, 5'-AATGGGAAACCGACCAGACC-3'; GAPDH forward, 5'-GGGAGCCAAAAGGGTCAT-3' and reverse, 5'-GAGTCCTTCCACGATACCAA-3'; miR-193b-3p forward, 5'-AACTGGCCCTCAAAGTCCC-3' and reverse, 5'-ATACCTCGGACCCTGCACTG-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The PCR thermocycling conditions were: Pre-denaturation at 94˚C for 3 min; followed by 35 cycles of 94˚C for 50 sec, 37˚C for 1 min, 72˚C for 1.5 min; with a final extension of at 72˚C for 7 min. U6 small nuclear RNA (snRNA) was used as a normalization control for the detection of miR-193b-3p and GAPDH was used as a normalization control for LINC00152. mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the controls (U6 snRNA or GAPDH) (34). RT-qPCR was used to determine expression in U2OS, Saos-2, MG63, MNNG/HOS and HFOB 1.19 cells.

Cell proliferation assay. The U2OS transfected cells were adjusted to a cell density of $2x10^3$ cells/well, then seeded into 96-well plates and cultured for 0, 24, 48 and 72 h. A total of 10 μl Cell Counting Kit-8 reagent (CCK-8; Beyotime Institute of Biotechnology, Haiman, China) was added to each well. The 96-well plate was incubated at 37˚C with 5% CO_2 for 2 h, and absorbance was measured at a wavelength of 450 nm using a microplate reader.

Colony formation assay. The U2OS cells transfected with si-LINC00152 or with si-NC were trypsinized into single-cell suspensions 48 h following transfection and seeded into six-well plates. The cells were incubated for ~2 weeks and then fixed with 4% paraformaldehyde for 15 min at room temperature. Colonies were photographed using a light microscope and counted from three randomly selected fields (magnification x4).

Flow cytometry analysis. The apoptosis levels of each the U2OS cells sample was determined 48 h after transfection. U2OS cells were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) using an annexin V-FITC/PI apoptosis detection kit (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For cell cycle analysis, cells were analyzed using a Cycletest Plus DNA Reagent kit (Becton, Dickinson and Company). The kits were used according to the manufacturer's protocol. Following 15 min of incubation with Cycletest Plus DNA Reagent kit, cells were examined using FACSCalibur flow cytometry system (FACScan; BD Biosciences, Franklin Lakes, NJ, USA).

Luciferase reporter assay. U2OS cells were seeded into a 96-well plate for 24 h and subsequently co-transfected with: pmirGLO-LINC00152-wild-type (WT) and miR-NC; pmirGLO-LINC00152-WT and miR-193b-3p; pmirGLO-LINC00152-mutant (MT) and miR-NC; or pmirGLO-LINC00152-MT and miR-193b-3p (Promega Corporation, Madison, WI, USA), using Lipofectamine® 2000, respectively. Following 48 h at 37˚C, Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter assay system (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis. SPSS software (version 17; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis throughout the study. StarBase software (version 2.0; http://starbase.sysu.edu.cn/) was used for bioinformatics analyses in the study. All

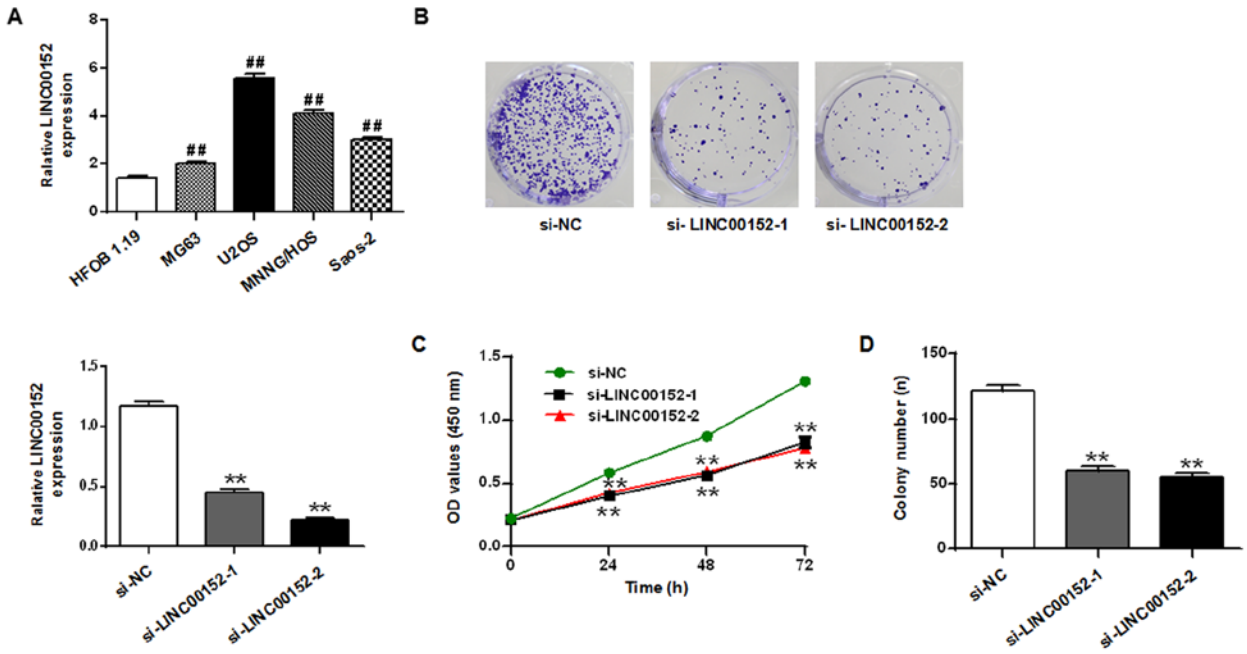

Figure 1. Knockdown of LINC00152 inhibits cell proliferation and colony formation, and induces G0/G1 cell cycle arrest in osteosarcoma cells. (A) Expression levels of LINC00152 in various osteosarcoma cell lines were determined by reverse transcriptase-quantitative polymerase chain reaction with GAPDH as an internal control. ##P<0.01 vs. HFOB 1.19. (B) Analysis of LINC00152 expression after transfecting si-NC, si-LINC00152-1 and si-LINC00152-2 into U2OS cells. LINC00152 expression levels were normalized to GAPDH. **P<0.01 vs. si-NC. (C) Cell proliferation in U2OS cells was measured at the indicated time points using a Cell Counting Kit-8 assay. (D) A colony formation assay was used to determine the number of colonies formed by U2OS cells. **P<0.01 vs. si-NC. All tests were performed at least three times. The data are presented as the mean ± standard deviation. si, short interfering; NC, negative control; OD, optical density.

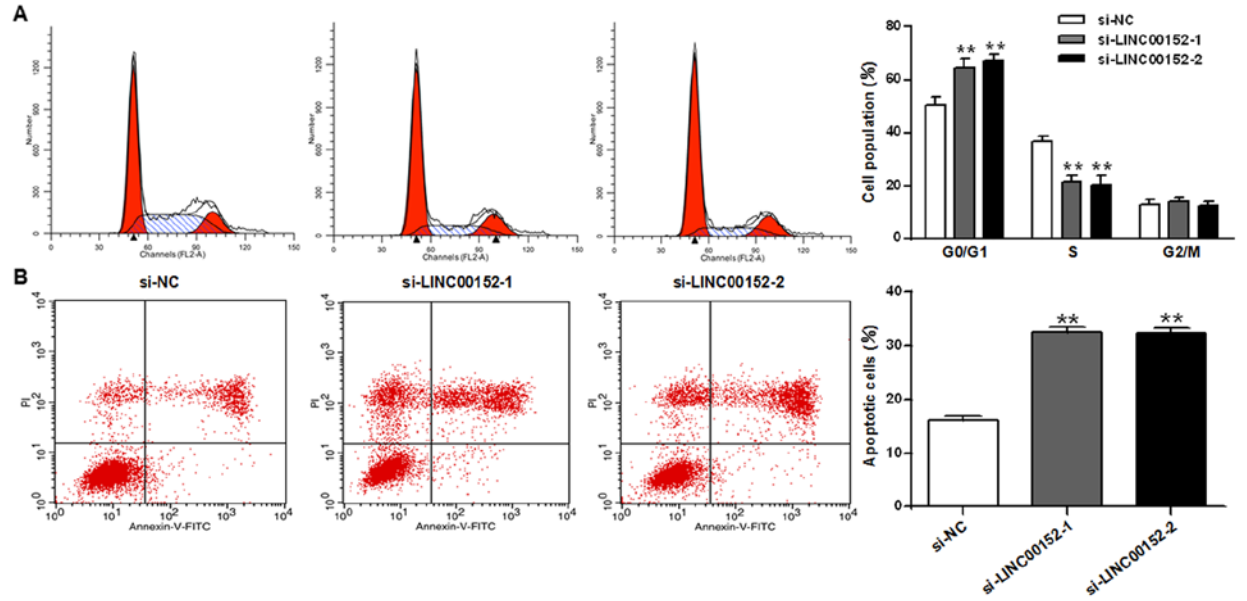

Figure 2. Knockdown of LINC00152 in U2OS cells significantly induces G0/G1 cell cycle arrest and apoptosis. (A) Flow cytometry was used to determine the cell cycle profile. A total of 48 h after transfection, cell numbers were counted based on DNA content consistent with G0/G1, S and G2/M phases. (B) Apoptosis of U2OS cells detected by flow cytometry. All tests were performed at least three times. All data are presented as the mean ± standard deviation. **P<0.01 vs. si-NC group. si, small interfering; NC, negative control, PI, propidium iodide; FITC, fluorescein isothiocyanate.

quantitative results are presented as the mean ± standard deviation. The independent-samples t-test was used for comparisons between two groups. Comparisons among multiple groups were performed by one-way analysis of variance followed by Bonferroni's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Knockdown of LINC00152 inhibits cell proliferation, and induces G0/G1 cell cycle arrest and apoptosis in osteosarcoma cells. The expression level of LINC00152 in the osteosarcoma cell lines U2OS, Saos-2, MG63 and MNNG/HOS was

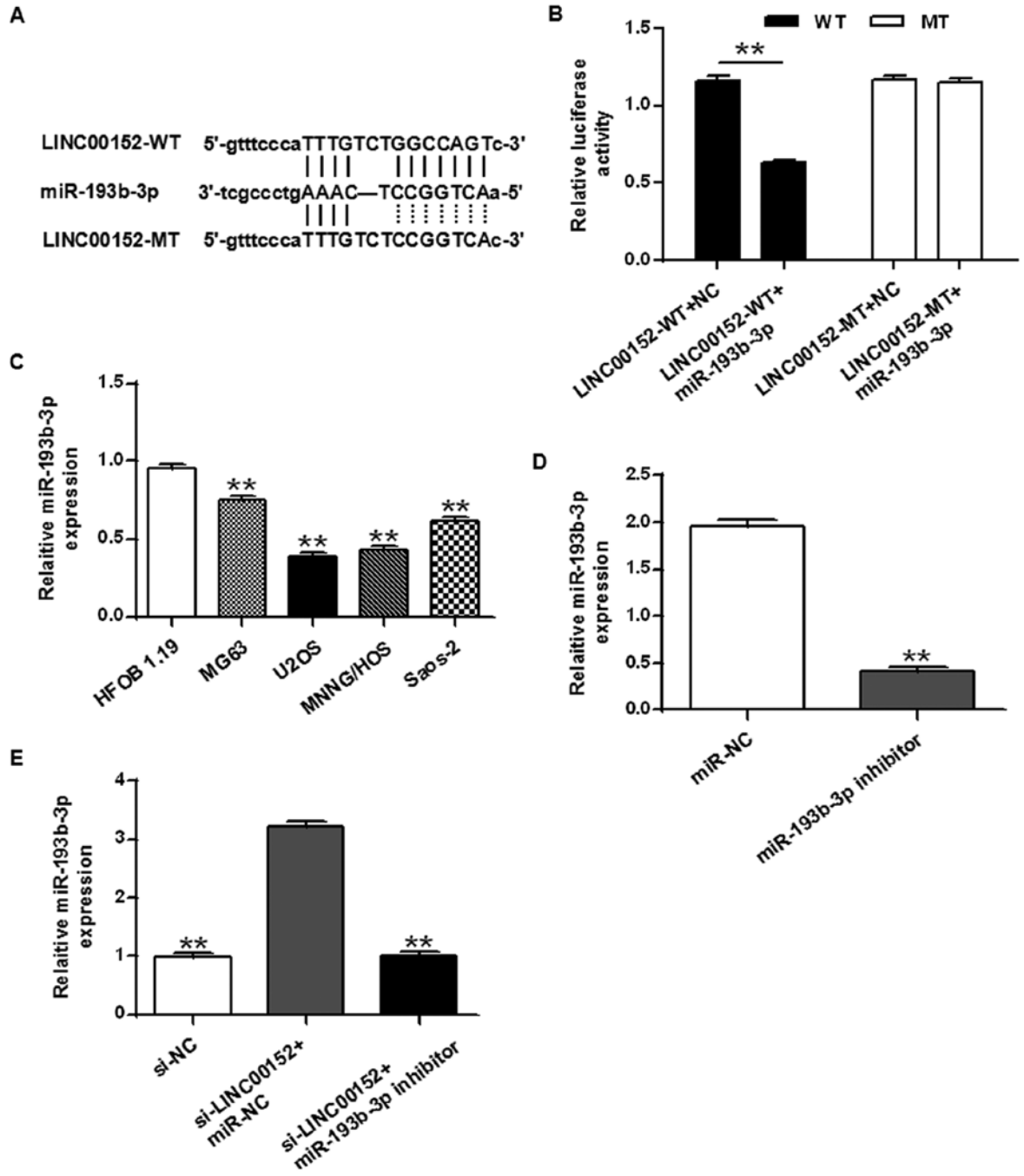

Figure 3. LINC00152 acts as a direct target of miR-193b-3p. (A) Complementary binding sites of LINC00152 and miR-193b-3p. (B) Dual-luciferase reporter assay revealed the putative complementary sites of miR-193b-3p with the 3'-untranslated region of LINC00152. (C) Expression levels of miR-193b-3p in the osteosarcoma cell lines were determined by reverse transcription-quantitative polymerase chain reaction using U6 small nuclear RNA as the internal control. (D) Expression levels of miR-193b-3p in U2OS decreased following transfection with miR-193b-3p inhibitor compared with the miR-NC. (E) U2OS cells were co-transfected with the miR-193b-3p inhibitor and si-LINC00152. All tests were performed at least three times. Data are presented as the mean ± standard deviation. The first **P<0.01 vs. si-NC group. The second **P<0.01 vs. si-LINC + miR-NC group. miR, microRNA; NC, negative control; si, small interfering; WT, wild type; MT, mutant.

quantified and compared with the expression level in the osteoblast cell line HFOB 1.19 (P<0.01; Fig. 1). The expression levels of LINC00152 were significantly upregulated in the four osteosarcoma cell lines compared with HFOB 1.19 cells, particularly in the U2OS cells. This was statistically significant compared to the other osteosarcoma cell line.

The biological function of LINC00152 in osteosarcoma cells was subsequently investigated. Two siRNAs targeting LINC00152 were used in the knockdown experiments in U2OS cells. Transfecting with either one of the siRNAs suppressed the expression of LINC00152 compared with the si-NC. Compared to si-NC group, the relative LINC00152 expression of si-LINC00152-1 and si-LINC00152-2 groups were decreased (P<0.01; Fig. 1A). Results from the CCK-8 assay revealed that si-LINC00152-1 and si-LINC00152-2 significantly suppressed cell proliferation in U2OS cells compared with the si-NC group (P<0.01; Fig. 1C). Similarly, the colony formation assay revealed that U2OS cells transfected with

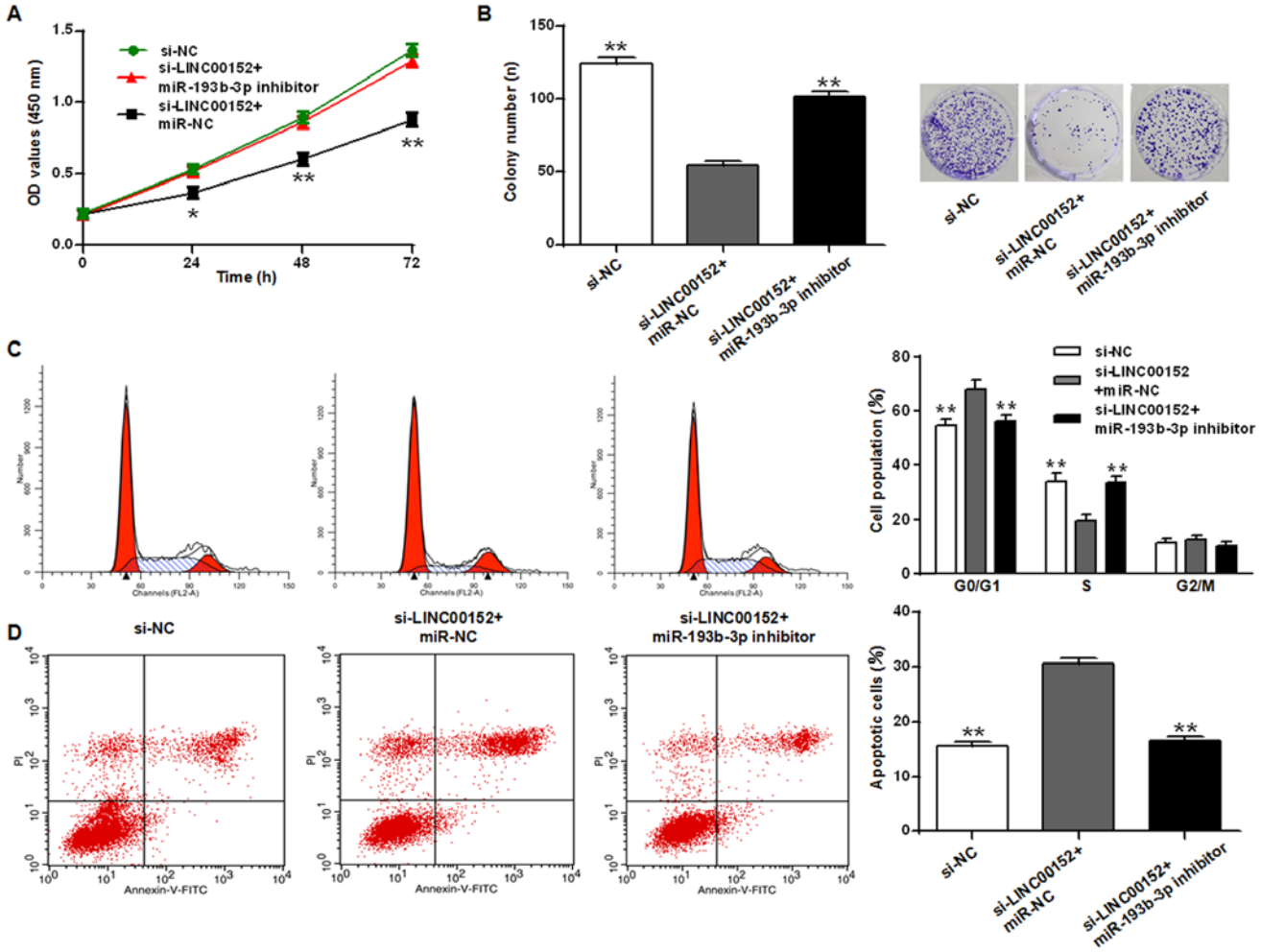

Figure 4. Effects of LINC00152 knockdown are reversed by miR-193b-3p in U2OS cells. (A) Cell proliferation was measured in the U2OS cells using the Cell Counting Kit-8 at the indicated time points. *P<0.05, **P<0.01 vs. si-NC or si-LINC+miR-193b-3p inhibitor group. (B) A colony formation assay was used to determine the number of colonies formed by U2OS cells. *P<0.01 vs. si-LINC+miR-NC group. (C) Flow cytometry was used to determine the cell cycle profile. A total of 48 h following transfection, cell numbers were counted based on DNA content of G0/G1, S and G2/M phases. **P<0.01 vs. si-LINC+miR-NC group. (D) Apoptosis of U2OS cells was detected by flow cytometry. **P<0.01 vs. si-LINC+miR-NC group. All tests were performed at least three times. Data are presented as the mean ± standard deviation. si, small interfering; NC, negative control, PI, propidium iodide; FITC, fluorescein isothiocyanate; miR, micro RNA.

si-LINC00152-1 and si-LINC00152-2 exhibited significantly fewer colonies compared with those transfected with si-NC (P<0.01; Fig. 1D). Flow cytometry analysis was performed to determine whether LINC00152 impacted the growth of osteosarcoma cells by affecting the cell cycle and apoptosis. The results revealed that the LINC00152 knockdown significantly induced G0/G1 cell cycle arrest (P<0.01; Fig. 2A) and increased apoptosis (P<0.01; Fig. 2B) in U2OS cells compared with cells transfected with si-NC.

LINC00152 acts as a direct target of miR-193b-3p. lncRNAs act as endogenous sponges for miRNAs and affect their expression (35). In order to elucidate the mechanisms underlying the effects of LINC00152 on osteosarcoma cells, bioinformatics analyses (Fig. 3) were performed to identify miRNAs which may bind to LINC00152. Bioinformatics analyses revealed that LINC00152 contains a binding site for miR-193b-3p (Fig. 3A). A dual-luciferase assay was performed to confirm the binding of miR-193b-3p to LINC00152. The results obtained revealed that reduced luciferase activity was observed only when both the LINC00152-WT reporter vector and miR-193b-3p were co-transfected (P<0.01 vs. LINC00152-WT reporter vector + NC; Fig. 3B). Co-transfecting the MT LINC00152 and miR-193-3p revealed no changes in luciferase activity compared with the LINC00152-MT + NC. The results obtained suggested that miR-193b-3p may exhibit sequence-specific binding to LINC00152.

The expression levels of miR-193b-3p in the osteosarcoma cell lines MG63, U2OS, MNNG/HOS and Saos-2, as well as in the osteoblast cell line HFOB 1.19 were measured. The expression levels of miR-193b-3p in all four osteosarcoma cell lines were significantly lower compared with that in HFOB 1.19 (P<0.01; Fig. 3C).

Loss-of-function experiments were performed and the expression level of miR-193b-3p was monitored. The transfection efficiency of the miR-193b-3p inhibitor was demonstrated by RT-qPCR (P<0.01 vs. miR-NC; Fig. 3D). Results revealed that knockdown of LINC00152 by transfecting with si-LINC00152 increased the expression of miR-193b-3p (P<0.01 vs. si-NC group); whereas co-transfecting si-LINC00152 and the miR-193b-3p inhibitor decreased the expression of miR-193b-3p compared with the

si-LINC00152 + miR-NC group (Fig. 3E; P<0.01). The results obtained suggested that LINC00152 decreased the expression of miR0193b-3p in osteosarcoma cells, by acting as a sponge for sequence-specific binding.

miR-193b-3p inhibitor rescues the growth inhibition induced by LINC0012 knockdown in U2OS cells. The CCK-8 assay (Fig. 4A) presented different significance levels at 24, 48 and 72 h, and colony formation assay (Fig. 4B) revealed that the miR-193b-3p inhibitor reversed the growth inhibition resulting from LINC00152 knockdown in osteosarcoma cells (P<0.05 and P<0.01 vs. si-NC group, respectively). Similarly, cell cycle analysis revealed that LINC00152 knockdown induced cycle arrest at the G0/G1 phase and promoted apoptosis, while the miR-193b-3p inhibitor reduced these effects (Fig. 4C and D; P<0.01). The miR-193b-3p inhibitor may reverse the growth inhibition effect of LINC00152 knockdown in osteosarcoma cell lines, indicating the antagonistic effects of LINC00152 and miR-193b-3p.

Discussion

The expression level of LINC00152 in osteosarcoma cell lines was investigated, and significantly higher expression levels of LINC00152 were observed in the osteosarcoma cell lines compared with an osteoblast cell line. Two siRNAs targeting LINC00152 were demonstrated to inhibit the expression of LINC00152 in U2OS cells. Knockdown of LINC00152 resulted in growth inhibition, demonstrated by a reduction in cell proliferation and colony formation. Knockdown of LINC00152 in U2OS cells significantly induced G0/G1 cell cycle arrest and apoptosis. These results suggested a positive association between LINC00152 and osteosarcoma. LINC00152 was previously associated with colon carcinomas, prostate cancer, leukemias, bladder and kidney cancer (15-18).

The current study aimed to explore the mechanism underlying the tumorigenic effect of LINC00152. LINC00152 acts as an endogenous sponge by binding several miRNAs and inhibiting their activity (35). The bioinformatics analyses performed in the current study revealed that LINC00152 contains a putative binding site for miR-193b-3p. Previous studies revealed that the miR-193b cluster demonstrated tumor-suppressive effects by reducing cell proliferation, as well as by reducing the total number of cells in the S-phase (36-38). Furthermore, miR-193b acts as a tumor suppressor and is epigenetically silenced in prostate cancer cells (37), and may serve a similar role in osteosarcoma cells. miR-193b-3p was therefore selected as a potential target of LINC00152 in the current study. Sequence-specific binding of miR-193b-3p to LINC00152 was validated by the dual-luciferase assay. Furthermore, the expression of miR-193b-3p was significantly lower in the four osteosarcoma cell lines, particularly in U2OS, compared with the osteoblast cell line. LINC00152 may downregulate miR-193b-3p in osteosarcoma cells. The expression of miR-193b-3p in osteosarcoma cells with LINC00152 knockdown was investigated. The results obtained indicated a significantly increased miR-193b-3p expression resulting from LINC00152 knockdown compared with si-NC, while co-transfecting with a miR-193b-3p inhibitor reversed this effect.

A recent study performed a comprehensive deep sequencing analysis on osteosarcoma cell samples to identify differentially expressed RNAs. The authors revealed that 65 miRNAs, 233 lncRNAs and 1,405 mRNAs were differentially expressed in osteosarcoma cells compared with normal cells, suggesting that dysregulation of RNA signaling may be involved in the progression of osteosarcoma (39). The present study revealed a novel miRNA-lncRNA target pair that is dysregulated in osteosarcoma cell lines. The overexpression of LINC00152 downregulated miR-193b-3p in osteosarcoma cells, potentially by acting as a competing endogenous RNA that binds to miR-193b-3p. Cell cycle analysis revealed that this dysregulation reduced G0/G1 arrest and promoted cell cycle progression, which resulted in increased cell proliferation and decreased apoptosis. A previous study demonstrated that LINC00152 may be potentially downregulated by miR-376c-3p (40), suggesting that ncRNAs form a complex signaling network that is highly associated with a number of different diseases, such as gastric and hepatocellular carcinoma.

The current study revealed that the dysregulation of LINC00152 and miR-193b-3p may contribute to osteosarcoma; however, further experiments using clinical samples are required to verify these results. This may improve the understanding of epigenetic regulation via LINC00152 and miR-193b-3p in osteosarcoma and may provide insights for potential therapeutic treatment strategies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PL, WH, YL and YW performed the experiments. PL analyzed the data and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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