

Original Article

MiR-218 suppresses osteosarcoma proliferation by down-regulating BMI1

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Abstract: Osteosarcoma is the most frequent cancer of primary bone tissue and is associated with a high metastatic potential and poor prognosis. However, the molecular mechanisms underlying osteosarcoma progression are not well defined. MicroRNAs play important roles in cancer development and could serve as therapeutic targets. Although miR-218 has been reported to inhibit the tumorigenesis of some cancers, its role in osteosarcoma remains poorly defined. Here, we investigated the effects of miR-218 overexpression in osteosarcoma cell lines, finding that it severely suppresses the proliferation and tumorigenesis of these human cancer derived cells. Moreover, miR-218 silencing promoted the proliferation and tumorigenesis of osteosarcoma cells lines. Furthermore, BMI1 was found to be a direct target of miR-218 in human osteosarcoma. Collectively, these findings suggest that exogenous over-expression of miR-218 may be an effective therapeutic strategy for osteosarcoma treatment.

Keywords: MicroRNAs, miR-218, proliferation, BMI1, osteosarcoma

Introduction

The mortality rates of children and adolescents with osteosarcoma are still very high, but early detection and timely treatment has increased the survival rate. Moreover, effective chemotherapy regimens have significantly improved outcomes, yielding better long-term relapse-free survival rates [1-3]. However, recurrence, lung metastasis and poor response to chemotherapy is still a difficulty in treating osteosarcoma [4]. Therefore, it is vitally important to discover an ideal early detection marker and molecular targets to cure osteosarcoma.

MicroRNAs (miRNA) are small, evolutionarily conserved, noncoding RNAs that regulate gene expression by complementary base pairing to specific mRNAs and play important roles in a wide variety of biological processes, including cell proliferation, invasion, migration, apoptosis, tumorigenesis, and senescence [5-7]. miRNAs affect gene expression transcriptionally and post-transcriptionally by binding the 3'-

untranslated regions (3'-UTRs) of target mRNAs, inhibiting protein translation and decreasing mRNA stability [8]. Many miRNAs have been discovered to regulate osteosarcoma development, including miR-143 [9], miR-193a [10], miR-497 [11] and miR-409 [12]. MiR-218-related effects on osteosarcoma have not been reported. Therefore, we investigated whether miR-218 suppresses proliferation in osteosarcoma cells.

BMI1, a member of the polycomb group family of transcriptional regulators, was originally regarded as an oncogenic partner of c-Myc in murine lymphomagenesis [13]. Subsequent studies have revealed that BMI-1 is abundantly expressed in osteosarcoma cells [14, 15]. Although BMI-1 has been inferred to be a proto-oncogene, very little is known about the precise role of BMI-1 in osteosarcoma occurrence and development [14]. Our data indicate that miR-218 inhibits the proliferation of osteosarcoma by downregulating BMI1 expression and could serve as a therapeutic target for human osteosarcoma.

Materials and methods

Antibodies and chemicals

Anti-BMI1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific for β -actin were obtained from Cell Signaling Technology (Danvers, MA, USA). Goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Millipore (Billerica, MA, USA).

Cell culture

The human osteosarcoma cell lines MG63 and U2OS and the human embryonic kidney cell line HEK293T were obtained from the cell bank of Shanghai Institutes of Chinese Academy of Sciences (Shanghai, China). Cells were grown at 37°C under 5% CO₂ in MEM or RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (Evergreen Biological Engineering, Hangzhou, China).

Constructs and lentiviral production

To overexpress miR-218, the miR-218 cDNA was cloned into pGLV3/H1 using BamH I and Mlu I cloning sites. To silence miR-218, a short-hairpin RNA (shRNA) oligomer (target sequence: 5'-GATCCACATGGTTAGATCAAGCACAACGATACATGGTTAGATCAAGCACAACCGGTACATGGTTAGATCAAGCACAATCACACATGGTTAGATCAAGCACAATTTTTTG) was inserted into pGLV3/H1 using BamH I and Mlu I cloning sites. Cells were transfected with Polyjet (SignaGen, Gaithersburg, MD, USA) according to the manufacturer's protocol. The corresponding plasmids and helper plasmids were co-transfected in HEK293T cells for propagating viruses.

Real-time PCR

RNA was extracted from stable lines and cDNA libraries were constructed using reverse transcription reagents (Roche, Basel, Switzerland) as described in the manufacturer's protocol. Real-time PCR was performed using an ABI 7300 real-time PCR instrument (Applied Biosystems, Carlsbad, CA, USA) using SYBR Green. Primers for the amplification of BMI1 and β -actin were as follows:

BMI1-F: 5'-GTGCTTTGTGGAGGGTACTTCAT, BMI1-R: 5'-TTGGACATCACAATAGGACAATACTT; β -

Actin-F: 5'-CATGTACGTTGCTATCCAGGC, β -Actin-R: 5'-CGCTCGGTGAGGATCTTCATG. The product sizes were 123 and 195 bp, respectively.

EdU assay

The function of miR-218 on the proliferation of MG63 and U2OS cells was evaluated by 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay using the EdU assay kit (Ribobio, Guangzhou, China) according to the manufacturer's protocol. Concisely, cells were seeded at a density of 4×10^3 cells per well and were incubated in triplicate in 96-well plates for 48 h, and then exposed to 50 μ M EdU for an additional 2 h at 37°C. The cells were then fixed with 4% formaldehyde for 30 min at room temperature, and afterwards 2 mg/ml glycine was added to neutralize the formaldehyde. After fixation, cells were permeabilized with 0.5% Triton X-100 for 15 min at room temperature, washed with PBS three times, treated with 100 μ l of 1 \times Apollo reaction cocktail for 30 min and finally, the DNA content was stained with DAPI for 30 min. Images were taken using an Olympus IX-71 inverted microscope (Olympus Corporation, Tokyo, Japan).

Cell counting kit-8 assay

To measure cell growth 5×10^3 cells in 100 μ l of media were seeded into 96-well plates and grown under normal conditions. After the cells had attached to the plate, 2- (2-methoxy-4-nitrophenyl) -3- (4-nitrophenyl) -5- (2,4-benzene disulfonate) -2H- tetrazolium monosodium salt (AAT Bioquest, Sunnyvale, CA, USA) was mixed into the media and incubated for 4 h. Living cells were counted every day by reading the absorbance at 450 nm using SynergyMx Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA).

Western blotting

Cells were lysed, and equal amounts of protein lysates were subjected to SDS-PAGE using a 10% polyacrylamide gel, and then blotted onto PVDF membranes (Millipore). After blocking with 5% bovine serum albumin, the membrane was probed with primary antibodies (BMI1, β -actin) at 4°C overnight and secondary antibodies at room temperature for 1.5 h. Bound antibodies were detected by the enhanced chemiluminescence Q6 system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Band den-

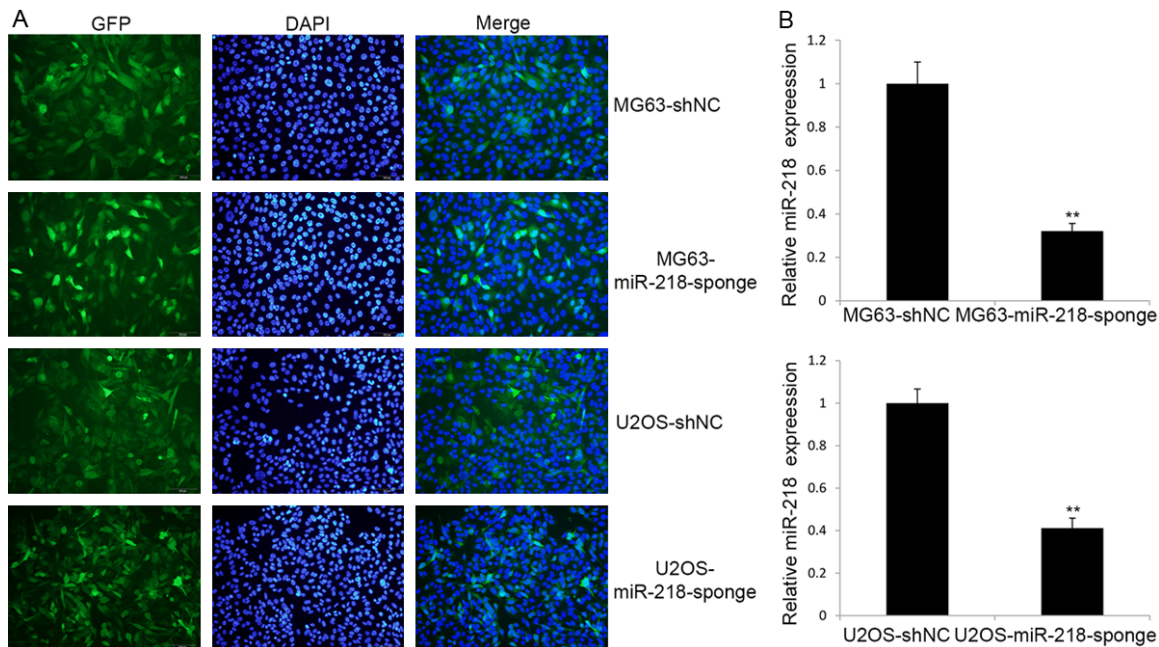


Figure 1. miR-218 silencing in MG63 and U2OS cells. A. shNC and miR-218-sponge expressing lentiviruses were packaged in HEK293T cells and used to infect the osteosarcoma cell lines MG63 and U2OS. Infected cells were stained with DAPI to detect the infection rate. The scale bar is 100 μ m. B. miR-218 expression was verified by RT-PCR. * $P < 0.05$, ** $P < 0.01$.

sities were measured using ImageJ (Wayne Rasband, National Institutes of Health, MD). All examined protein expression levels were calculated by normalizing the densitometry value of interest to that of the loading control.

Colony formation assay

For colony formation assays, 200 cells were plated in 6-cm dishes and incubated under normal conditions for 10 d. The cells were fixed with methanol and stained with 0.05% crystal violet to assess colony formation. After repeated washing with PBS, images were taken with a camera. Colonies containing more than 50 cells were counted.

Luciferase reporter assay

To construct the BMI1 3'UTR-Luc reporter, the BMI1 3'UTR was inserted into pmirGLO (GenePharma, Shanghai, China) using Sac I and Xbo I cloning sites. The mutated reporter, pmirGLO-MT-BMI1 3'UTR, was generated by deleting the binding site for miR-218 (UUCGUGUU) from the parent vector. PmirGLO-WT-BMI1 3'UTR, pmirGLO-MT-BMI1 3'UTR and phRL-TK were co-transfected into cells, and luciferase activity was detected using the Dual-Luciferase

Reporter Assay System (Promega, Madison, WI, USA).

Statistical analysis

SPSS software (SPSS Inc., Chicago, IL, USA) was used to perform statistical analyses. The results are presented as mean \pm SEM. Statistical significance was analyzed using Student's t-test, with $P < 0.05$ considered statistically significant.

Results

miR-218 down-regulation promotes osteosarcoma cell proliferation

Several studies have demonstrated that miR-218 has tumor-suppressive functions in other tumors [16-18]. However, the specific role of miR-218 in osteosarcoma has not been investigated in depth. Therefore, we explored the effects of blocking miR-218 activity on proliferation in the osteosarcoma cell lines MG63 and U2OS. miR-218 expression was downregulated by a miRNA sponge specific for miR-218 (miR-218 sponge). Stable lines expressing miR-218 sponge were established in MG63 and U2OS cells by lentiviral infection (Figure 1A), and

MIR-218 inhibits osteosarcoma cells proliferation by regulating BMI1

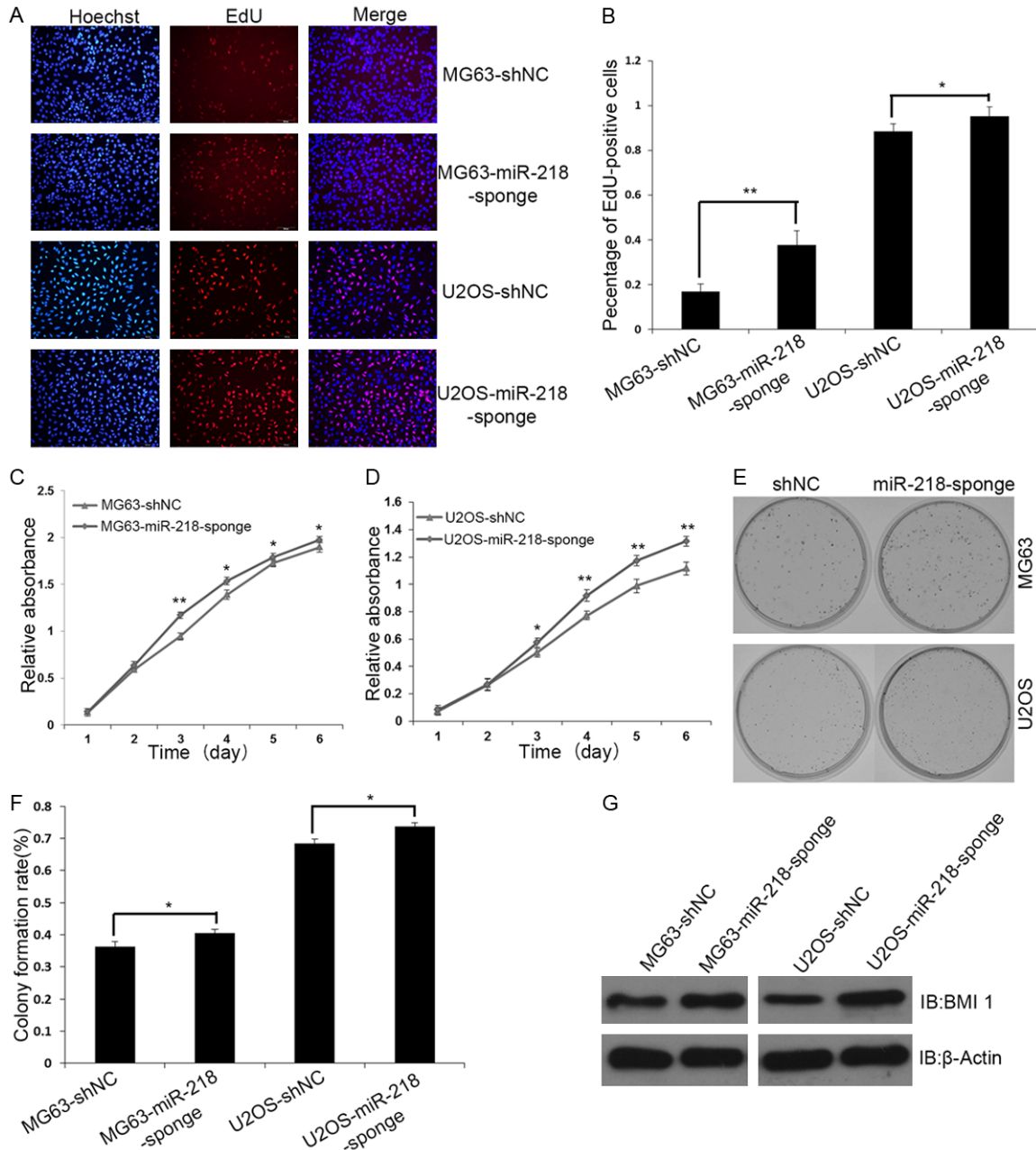


Figure 2. miR-218 silencing increases the proliferation rates of MG63 and U2OS cells. (A) EdU assay showing proliferating MG63 and U2OS cells. (B) Quantitation of the EdU experiments in MG63 and U2OS. (C, D) CCK8 assays demonstrating the cell viability of MG63 (C) and U2OS (D) cells. (E) Colony formation assays demonstrating the ability of cells to proliferate in soft agar. (F) Quantitation of the colony formation experiments. (G) BMI1 expression in control and miR-218-silenced cells by western blot. Experiments were repeated in triplicate; *P < 0.05, **P < 0.01.

decreased miR-218 expression was verified by real-time PCR (Figure 1B).

To determine the effect of miR-218 knockdown in MG63 and U2OS cells, cell proliferation was measured using the EdU (Figure 2A, 2B) and CCK-8 (Figure 2C, 2D) assays. The data showed that in both osteosarcoma cell lines miR-218 silencing increased proliferation rates. To as-

sess the effect of miR-218 knockdown on the transformation status of human osteosarcoma cells, we performed in vitro colony formation assays. The data showed that miR-218 inhibition increased colony formation (Figure 2E, 2F). Further experiments into the molecular mechanism of this activity revealed that miR-218 silencing was associated with increased BMI1 protein levels by western blot (Figure 2G).

MIR-218 inhibits osteosarcoma cells proliferation by regulating BMI1

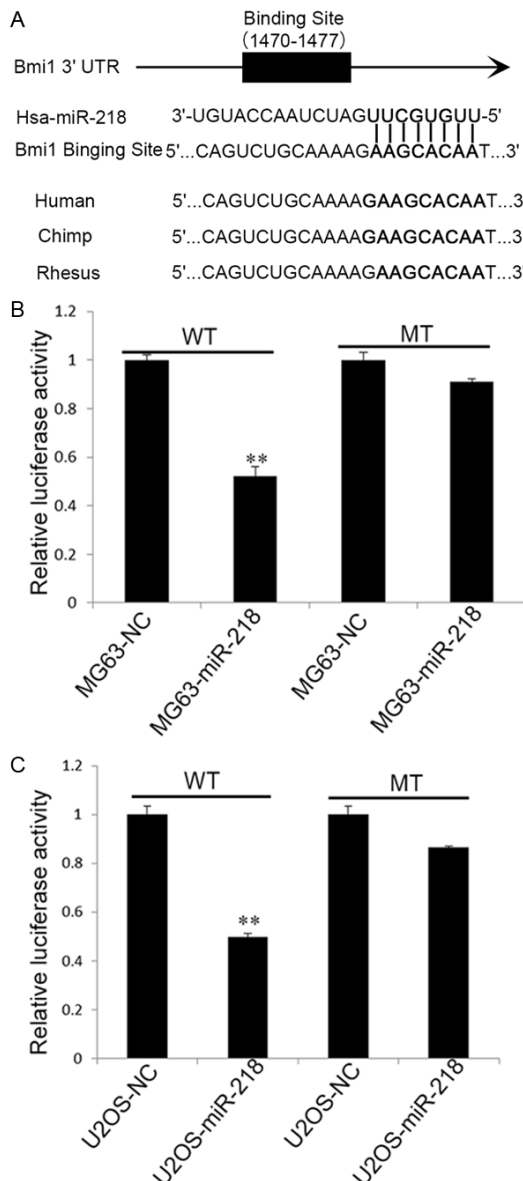


Figure 3. BMI1 is a target of miR-218. (A) Putative miR-218 binding sites within the BMI1 3'UTR, as predicted by the TargetScan and PicTar algorithms. (B and C) miR-218 expression decreased luciferase activity from the construct containing the wild-type BMI1 3'UTR, but did not change luciferase activity from the construct containing a mutated BMI1 3'UTR in MG63 (B) and U2OS (C) cells. * $P < 0.05$, ** $P < 0.01$.

These data suggest down-regulating miR-218 in osteosarcoma cells promotes cellular proliferation, and this effect may be partly mediated by increased BMI1 expression.

BMI1 is a direct target of miR-218

Bioinformatic analysis using the TargetScan and PicTar algorithms showed that BMI1 is a

potential target of miR-218 (**Figure 3A**). To test for a direct interaction between miR-218 and the BMI1 3'UTR, we constructed plasmids containing wild-type and mutated BMI1 3'UTRs (pmirGLO-WT-BMI1 3'UTR and pmirGLO-MUT-BMI1 3'UTR, respectively). Luciferase reporter experiments demonstrated that upregulating miR-218 led to a significant decrease in luciferase activity from the pmirGLO-WT- BMI1 3'UTR in MG63 and U2OS cells, miR-218 overexpression did not cause any significant change in luciferase activity from pmirGLO-MUT- BMI1 3'UTR (**Figure 3B, 3C**).

MiR-218 overexpression inhibits osteosarcoma cell proliferation

To further explore the role of miR-218 in osteosarcoma cells, miR-218 was overexpressed by lentiviral infection (**Figure 4A**). The expression of miR-218 was verified by RT-PCR (**Figure 4B**). Cell proliferation was assayed using EdU labeling and showed that overexpressed miR-218 significantly decreased proliferation rates (**Figure 5A, 5B**). CCK-8 and colony formation experiments also showed that overexpression of miR-218 reduced the ability of osteosarcoma cell to proliferate (**Figure 5C-F**). Moreover, we found that miR-218 overexpression was accompanied by a reduction in BMI1 expression (**Figure 5G**). Therefore, we conclude that miR-218 overexpression in osteosarcoma cells inhibits proliferation, and this effect may be mediated by BMI1 downregulation. In conclusion, BMI1 is a direct target of miR-218, and miR-218 suppresses the proliferation of human osteosarcoma cells by regulating BMI1 expression.

Discussion

Our study demonstrated that BMI1 is a direct target of miR-218 and promotes the proliferation of osteosarcoma cells. To explore the exact role of miR-218 in osteosarcoma cells, we created viral-mediated miR-218-overexpressing and miR-218-silenced human osteosarcoma cell lines from MG63 and U2OS cells. The results showed that silencing miR-218 significantly increases cell proliferation by upregulating BMI1 expression, whereas the opposite results were found in osteosarcoma cells that overexpress miR-218. These data demonstrate the post-transcriptional regulation of BMI1 by miR-218 and revealed that BMI1 is an important mediator of miR-218 activity in osteosarcomas.

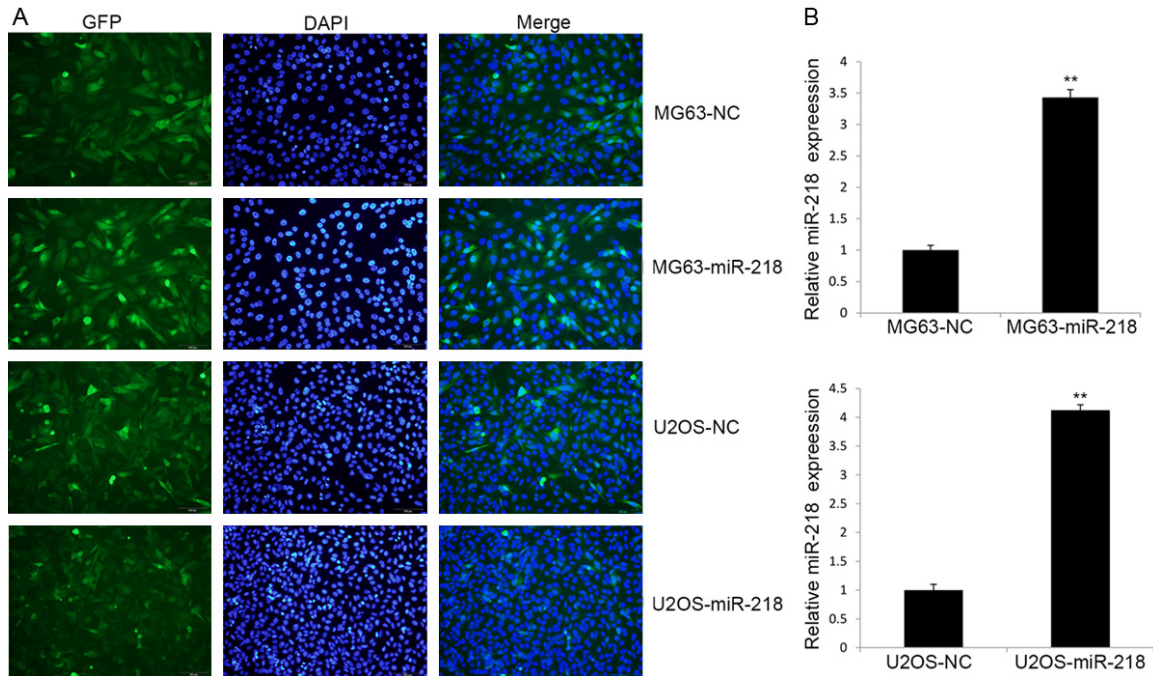


Figure 4. miR-218 overexpression in MG63 and U2OS cells. A. Empty vector and miR-218 expressing lentiviruses were packaged in HEK293T cells used to infect the osteosarcoma cell lines MG63 and U2OS. Infected cells were stained with DAPI to detect the infection rate; miR-218 is shown in green, and nuclei are shown in blue. The scale bar is 100 μ m. B. miR-218 expression was verified by RT-PCR. * $P < 0.05$, ** $P < 0.01$.

A significant downregulation of miR-218 has been reported in human gliomas compared with normal brain tissue [18-20], and in this context miR-218 was shown to directly target IKK-beta [18], RTK [21] and CDK6 [22, 23], preventing the proliferation, migration and invasion glioma cells and cells of other cancer types. To identify additional miR-218 targets in osteosarcoma cells, we used a combination of bioinformatic analyses and Luciferase reporter assays, which revealed that miR-218 regulates osteosarcoma cell proliferation by modulating BMI1 expression by directly targeting the BMI1 mRNA.

BMI1, a member of the polycomb group complex, has been considered a potential prognostic biomarker and therapeutic target, and is essential for the stem cell self-renewal [24-26]. The role of BMI1 in cancer is complex, as it regulates expression of many genes with known roles in cancer development and progression such as PTEN [27], telomerase [28], the Akt/GSK3 β /Snail pathway [29] and E-cadherin [30]. Bmi1 can regulate self-renewal and proliferation of cancer stem cells from other types of tumors such as prostate cancer, hepatocellular carcinoma, and pancreatic cancer [31-33]. Our study suggests that BMI1 downregulation could

inhibit the proliferation and colony-forming ability of human osteosarcoma cells. Together, our results demonstrate that BMI1 might be a growth promoting factor in osteosarcoma, and therefore, could also serve as a potential target for therapy.

In conclusion, our work shows that miR-218 overexpression inhibits the proliferation of osteosarcoma cells through a novel mechanism that involves direct miR-218-mediated suppression of BMI1, whereas miR-218 silencing increases the proliferation of osteosarcoma cells. This is the first study to focus on the role of miR-218 in osteosarcomas cells. Our study reveals the function of miR-218 in osteosarcomas cells and demonstrates that miR-218 might be a potential biomarker to measure the effectiveness of osteosarcoma treatment. However, a complete understanding of the molecular mechanisms of miR-218 activity in osteosarcoma is necessary before it can be validated as a viable drug target.

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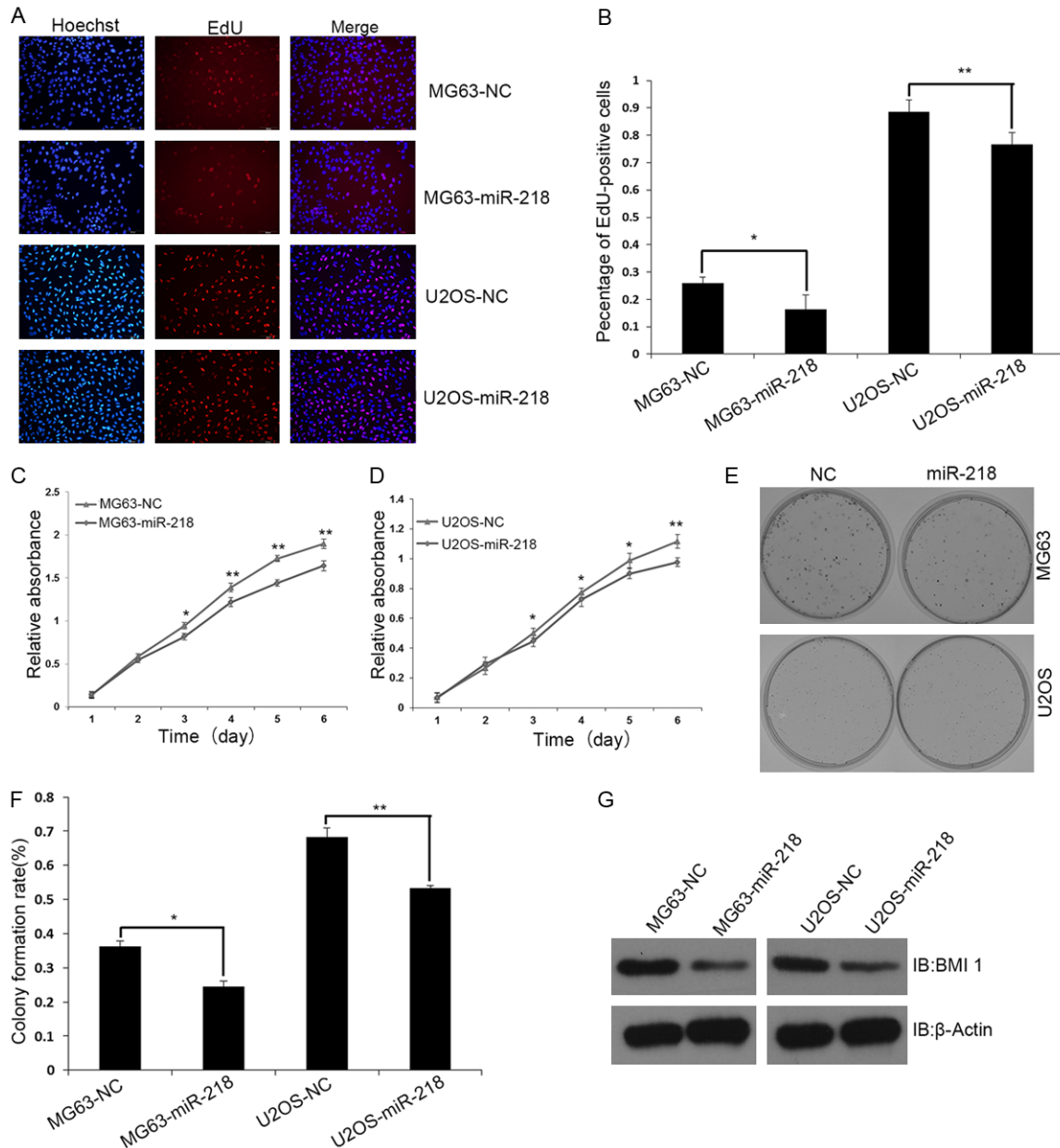


Figure 5. miR-218 overexpression caused a significant decrease in the proliferation rates of osteosarcoma cells. A. EdU assay showing proliferating MG63 and U2OS cells. B. Quantitation of the EdU experiments in MG63 and U2OS. C, D. CCK8 assays demonstrating the cell viability of MG63 (C) and U2OS (D) cells. E. Colony formation assays demonstrating the ability of cells to proliferate in soft agar. F. Quantitation of the colony formation experiments. G. BMI1 expression in control and miR-218-overexpressing cells by western blot. Experiments were repeated in triplicate; * $P < 0.05$, ** $P < 0.01$.

Disclosure of conflict of interest

None.

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