miR-218 suppresses the proliferation of osteosarcoma through downregulation of E2F2

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Abstract. Osteosarcoma is the most common primary malignant bone tumor type in children and adolescents under 20 years of age. Biological characteristics include invasiveness, metastasis, abnormal differentiation and loss of contact inhibition. microRNAs (miRNAs) are involved in the transcriptional and post-transcriptional regulation of target mRNAs. Previous studies have demonstrated that miR-218 inhibits tumor formation and progression in glioma, colon cancer and renal cell carcinoma; however, the mechanism of action of miR-218 in osteosarcoma has not been completely determined. In the present study, it was demonstrated that miR-218 exhibited low expression and targeted E2F2 in osteosarcoma cells. Additionally, overexpression of miR-218 inhibited osteosarcoma cell proliferation, with the opposite result occurring following the knockdown of miR-218. Furthermore, it was determined that miR-218 inhibited tumor formation and reduced the expression of E2F2 and proliferating cell nuclear antigen in nude mice. Collectively, the present data demonstrated that miR-218 serves an important role in suppressing the proliferation of osteosarcoma cells, potentially regulated by E2F2, which may provide a novel protein marker for the treatment of osteosarcoma.

Introduction

Of all pediatric bone malignant tumor types, osteosarcoma has the highest incidence (1). Although surgical techniques and chemotherapy have improved, the treatment effects remain unchanged in detectable metastatic osteosarcoma (2,3). The rapid growth of metastases and chemotherapy resistance are the primary causes of not achieving complete remission (4); thus, it is vital to investigate an early detection marker and molecular target in order to achieve complete remission of osteosarcoma.

MicroRNAs (miRNAs) regulate protein expression through complementary base pairing to specific mRNAs that act in the 3'-untranslated regions (3'-UTRs) of target mRNAs (5). miRNAs serve an irreplaceable role in a variety of biological processes in cancer development and progression, including differentiation, proliferation metastasis and apoptosis (6-9). Previous studies have demonstrated that miR-218 is associated with the proliferation, migration and invasion of osteosarcoma (10,11); however, there is limited knowledge regarding the role that miR-218 serves in the proliferation of osteosarcoma. Therefore, the molecular mechanism underlying the action of miR-218 in osteosarcoma was investigated.

E2F2 is affiliated with a family of transcription factors that includes E2F1-8 (12). Studies have demonstrated that E2F factors are, not only involved in the expression of cell cycle-associated genes, but also in the expression of differentiation-, apoptosis-, autophagy- and metabolism-associated genes (13-15). Previously, E2F2 was determined to maintain G_0 arrest by regulating cell cycle-associated genes (13). In the present study, whether E2F2 was essential for osteosarcoma tumorigenesis was investigated.

In brief, the present results demonstrated that miR-218 inhibits the proliferation of human osteosarcoma cells through E2F2, and that E2F2 promotes osteosarcoma tumorigenesis.

Materials and methods

Antibodies. Anti-E2F2 (dilution, 1:500; cat. no. sc-633) antibodies and β-actin (dilution, 1:1,000; cat. no. sc-58673) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Secondary antibodies anti-mouse IgG, (dilution, 1:2,000; cat. no. 7076) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. 293, Human osteoblast hFOB1.19 and human osteosarcoma MG63 cell lines were obtained from the

Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and maintained at 37°C in an atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium, D-MEM/F-12 medium and Minimum Eagle's medium, respectively, and with 10% heat-inactivated fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA).

Constructs and production of lentivirus. The miR-218 complementary DNA (cDNA) sequences were inserted between the *Bam*HI/*Mlu*I restriction sites in the pGLV3/H1 vector (0.03 pmol) (Shanghai GenePharma Co., Ltd., Shanghai, China). The miR-218 short-hairpin RNA (shRNA) oligomer (target sequence: 5'-GATCCACATGGTTAGATCAAGCAC AACGATACATGGTTAGATCAAGCACAAACCGGTACA TGGTTAGATCAAGCACAATCACACATGGTTAGATCA AGCACAATTTTTTG-3') was subcloned into the pGLV3/H1 construct between the *Bam*HI-*Mlu*I restriction sites. MG63 cells were constructed using the three-plasmid lentivirus system, where the second-generation lentiviral packaging, pspax2, and viral envelope, pMD2G (Shanghai GenePharma Co., Ltd.) were co-transfected in 293 cells using PolyJet (SignaGen Laboratories, Rockville, MD, USA), according to the manufacturer's protocols, to package the viruses. The above three plasmids (pspax2: pMD2G: pGLV3/H1-miR-218=1 ug: 1 ug: 2 ug), opti-MEM (500 ul) and PolyJet (12 ul) were mixed and allowed to stand for 20 min. It was subsequently added to a six-well plate with MG63 cell density of 70%. After 12 h, the medium was changed, and the supernatant, containing lentivirus, was collected for 72 h.

Establishment of stable cell lines. For overexpression of miR-218, the MG63 cells were infected by green fluorescent protein (GFP) positive miR-218 viruses. Following 48 h of infection, the cells were cultured in Minimum Eagle's Medium containing 2.5 lg/ml puromycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The surviving cells were cultured into cell lines stably expressing miR-218. The control, expressing GFP, and miR-218-sponge, expressing GFP, groups were obtained according to the methods described above.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). cDNA was obtained using mRNA RT (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. qPCR was performed using an ABI 7300 real-time PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) with FastStart Universal SYBR® Green Mix (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocols. The primers for the amplification of E2F2 and β-actin were as follows: E2F2 forward, 5'-AGCTGG ATCTGGAGGGGATT-3' and reverse, 5'-AGGACCCCATCC TCTGACTC-3'; and β-actin forward, 5'-CATGTACGTTGC TATCCAGGC-3' and reverse, 5'-CGCTCGGTGAGGATC TTCATG-3'. The molecular weights were 196 and 195 bp, respectively. Thermocycling protocol included pre-denatured at 95°C for 3 min, denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and extension at 72°C for 1 min for 40 cycles. Expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (16).

EdU experiment. The EdU incorporation assay (5-ethynyl-2'-deoxyuridine; Guangzhou RiboBio Co., Ltd., Guangzhou, China) was used to evaluate the number of proliferating cells. A total of $1x10^4$ cells, MG63/control, MG63/miR-218 and MG63/miR-218-sponge cells, were plated in 96-well plates and cultured for an additional 24 h. Cells were treated with 50 μM EdU for 2 h at 37°C and then fixed with 4% formaldehyde for 30 min at room temperature. Subsequently, 2 mg/ml glycine antagonized formaldehyde and 0.5% Triton X-100 was added and incubated for 15 min at room temperature. Following this, 100 μl 1X Apollo reaction cocktail (Guangzhou RiboBio Co., Ltd.) and DAPI dye were added to the cells and incubated for 30 min at room temperature, respectively. Images were obtained using the fluorescent Olympus IX-71 inverted microscope (magnification, x200; Olympus Corporation, Tokyo, Japan).

Flow cytometry. Cells ($1x10^5$) were plated in 6-well plates and cultured for 24 h at 37°C. The cells were digested using trypsin and single suspension cells were collected in 1.5 ml Eppendorf tubes. Subsequently, the cells were treated with 70% ethanol for 15 min at 0°C and then incubated with RNase for 30 min at 37°C. Following this, the cells were dyed with propidium iodide (10 ug/ml; Thermo Fisher Scientific, Inc.) and protected from light for 30 min at room temperature. Cells were then subjected to flow cytometry with a flow cytometer and the results were analyzed through CellQuest Pro 4.0.2 software (BD Biosciences, Franklin Lakes, NJ, USA).

Plate colony formation. A total of $2x10^2$ cells, MG63/control, MG63/miR-218 and MG63/miR-218-sponge cells, were plated in 6-cm dishes and cultured for 14 days at 37°C. Cells were fixed with 4% formaldehyde for 30 min and then incubated with 0.05% crystal violet for 30 min at room temperature. Images were captured using a Canon 70D camera (Canon, Inc., Tokyo, Japan). Colonies with >50 cells were counted.

Bioinformatics prediction. The miR-218 target prediction was performed using bioinformatics algorithms from TargetScan 6.2 (http://www.targetscan.org/) and PicTar (http://pictar.mdc-berlin.de/).

Western blot analysis. Cell protein was extracted from the osteosarcoma cell lines by bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's protocols. Equivalent amounts (40 ug) of protein were added to 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA) using a wet transfer method, where the PAGE gel and PVDF membranes are placed in a thermostat covered with ice with a constant current of 225 mA for 2.5 h at 0°C. Following blocking with 5% skimmed milk powder overnight at 4°C, the primary antibodies (E2F2 and β-actin) were added and incubated at 4°C overnight. Subsequently, the secondary antibody was added and incubated for 2 h at room temperature. The Pierce enhanced chemiluminescent Plus Western Blotting Substrate (Thermo Fisher Scientific, Inc.) was added to PVDF membranes and images were obtained using Image Lab 5.2 (Bio-Rad, Laboratories, Inc. Hercules, CA, USA). Results were
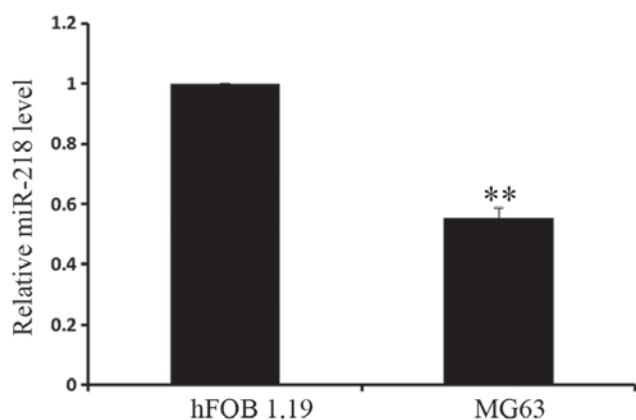
Figure 1. Expression level of miR-218 in human osteosarcoma cells. Total complementary DNA from osteoblastic hFOB1.19 and osteosarcoma MG63 cell lines were analyzed for the expression of miR-218 using reverse transcription-quantitative polymerase chain reaction. **P<0.01 compared with hFOB1.19. miR, microRNA.

analyzed using ImageJ 1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

Luciferase reporter experiments. E2F2 3'UTR was subcloned between the *Sac*I/*Xbo*I restriction sites in the pmirGLO vector (Shanghai GenePharma Co., Ltd.). The pmirGLO-MT-BMI1 3'UTR plasmid was constructed by removing the binding site for miR-218 (UUCGUGUU). PmirGLO-WT-E2F2 3'UTR/pmirGLO-MT-E2F2 3'UTR and phRL-TK were co-transfected into MG63 cells using Polyjet. Luciferase activity was assessed using the Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA) 36 h after transfection. Luciferase activity was normalized to *Renilla* luciferase activity.

Animal experiments. Female BALB/c nude mice (n=6; ~20 g), 4-5 weeks of age, were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA). The mice were housed in a pathogen-free plastic cage with a sealed air filter, at room temperature (26-28°C) and relative humidity of 40-60%. Mice had access to drinking water and fed with an autoclaved diet and were under a 10/14 h light/dark cycle, respectively. Following corresponding lentivirus infection of MG63 cells, the corresponding cell lines, MG63/control, MG63/miR-218 and MG63/miR-218-sponge cells, was obtained. A total of $2x10^6$ cells/0.1 ml MG63/control, MG63/miR-218 and MG63/miR-218-sponge cells in phosphate saline were injected subcutaneously into the lower back of nude mice. After eight weeks, nude mice were sacrificed by intraperitoneal injection of pentobarbital sodium (150 to 200 mg/kg), in accordance with the guidelines for laboratory animals established by and approved by the Xuzhou Children's Hospital Animal Care and Use Committee. The tumors were measured by mean as follows: Volume=(length x width 2)/2).

Immunohistochemistry. Immunohistochemistry experiments were performed using the S-P immunohistochemistry kit (OriGene Technologies, Inc., Rockville, MD, USA). The 4% paraformaldehyde fixed paraffin sections (4 micron) were deparaffinized in xylene for 5 min and rehydrated in

graded ethanol (100, 95, 85 and 75%) at room temperature for 5 min per step. Subsequently, antigen retrieval was conducted by heating sections in citric buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a pressure cooker at 120°C. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide at room temperature for 30 min. Sections were incubated with 2.5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) to reduce non-specific binding at room temperature for 10 min. Anti-proliferating cell nuclear antigen antibodies at 1:100 dilution in 2.5% BSA (PBS as control) were incubated at room temperature for 1 h and then incubated with biotin-conjugated goat anti-rabbit IgG (cat. no. ZB-2010; Zhongshan Goldenbridge Biotech Co.) and horseradish peroxidase-conjugated streptavidin (cat. no. AR100017; OriGene Technologies, Inc.) at room temperature for 20 min. Staining was performed by incubating sections for 2-10 min with the chromogenic reagent 3,3'-diaminobenzidine (DAB) followed by quenching of the reaction with water. Sections were analyzed according to the degree of color, as longer periods of incubation resulted to darker background color for analysis. Hematoxylin was used as the counterstain for cell nuclei for 2 min and then sections were dehydrated using graded ethanol (100, 95, 85 and 75%) and xylene at room temperature. Images were captured using an fluorescent Olympus IX-71 inverted microscope (magnification, x400).

Statistical analysis. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analyses. Results are shown as the mean ± standard error of the mean. Statistical significance was analyzed using the Student's t-test (two groups) and one-way analysis of variance (multiple groups) and Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-218 expression is attenuated in osteosarcoma cells. To determine the expression level of miR-218, it was examined in the osteoblastic cell line hFOB1.19 and the osteosarcoma cell line MG63 using RT-qPCR. It was determined that miR-218 had significantly increased expression in osteoblasts, compared with osteosarcoma cells (Fig. 1). The present results demonstrated that miR-218 serves an important role in osteosarcoma tumorigenesis, which provides preliminary evidence for future research.

miR-218 inhibits proliferation of osteosarcoma cells. To further investigate the molecular mechanism underlying miR-218 in osteosarcoma cells, a miR-218 overexpressing cell line was constructed using lentiviral infection and also miR-218 was knocked down in MG63 cells using shRNA specific for miR-218 (Fig. 2A). miR-218 expression was verified using RT-qPCR (Fig. 2B). To observe the results of altering the expression of miR-218 in MG63 cells, the cell cycle was analyzed using flow cytometry. Control cells had the following distribution of cell cycle phases: 46.47% in G_1; 39.02% in S and 14.50% in G_2/M. miR-218-overexpressing cells had the following distribution of cell cycle phases: 65.58% in G_1, 13.39% in S and 21.03% in G_2/M. miR-218-knockdown cells
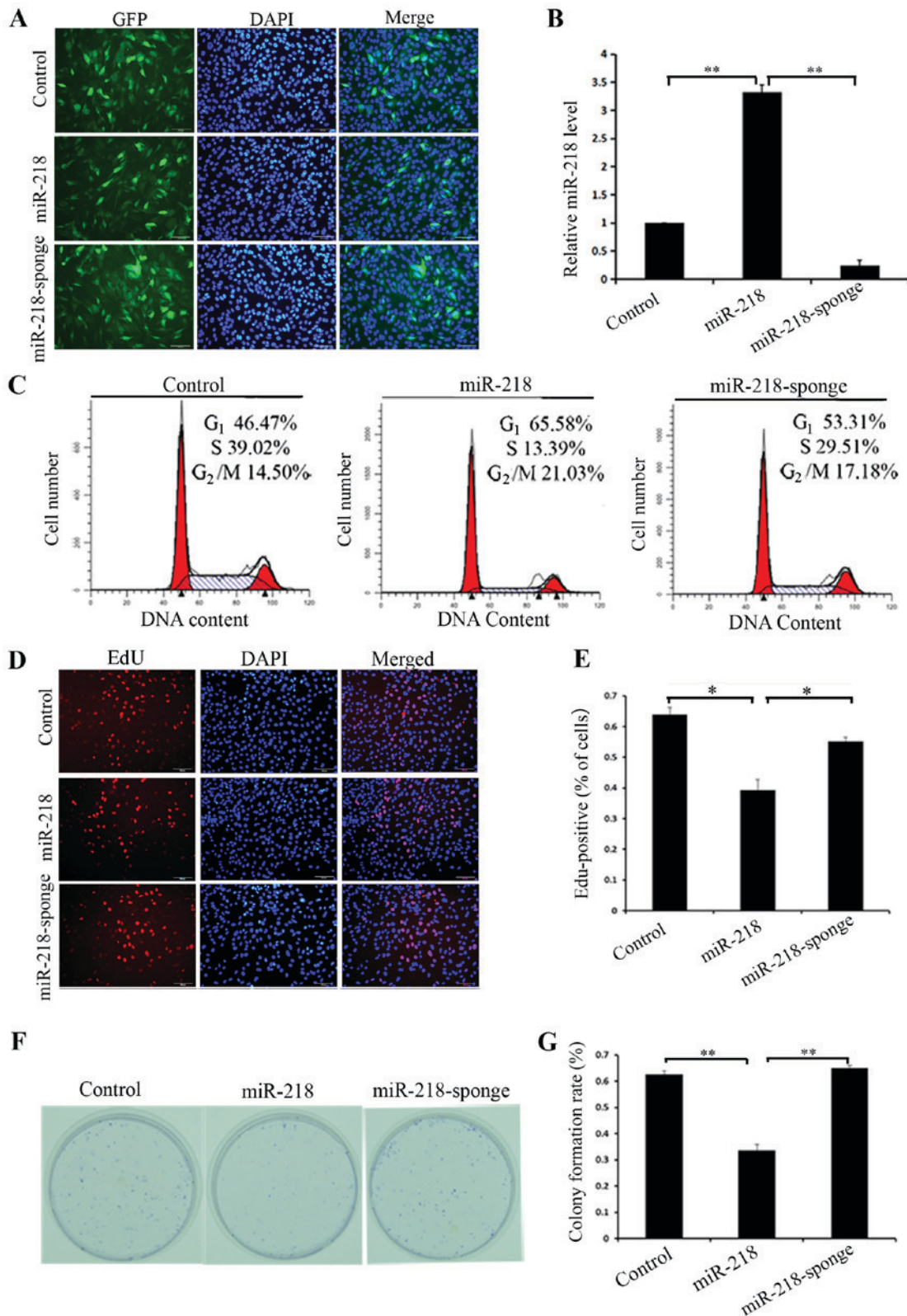
Figure 2. miR-218 significantly decreased the ability of osteosarcoma cells to proliferate. (A) GFP images displaying control, miR-218 and miR-218-sponge expressing stable MG63 cell lines. Scale bar, 100 μm; magnification, x200. (B) Expression of miR-218 was verified using reverse transcription-quantitative polymerase chain reaction. (C) The cell cycle was analyzed using flow cytometry. (D) The number of proliferating cells in the stable cell lines using the EdU proliferation assay. Scale bar, 100 μm; magnification, x200. (E) Statistical analysis of the EdU proliferation experiments. (F) Plate colony formation depicting the colony formation ability of the stable cell lines. (G) Statistical analysis of the colony formation experiments. GFP, green fluorescent protein; miR, microRNA. *P<0.05 and **P<0.01 compared with control and miR-218 group; and miR-218 and miR-218-sponge group.

had the following distribution of the cell cycle phases: 53.31% in G_1, 29.51% in S and 17.18% in G_2/M (Fig. 2C). Similar results were obtained using EdU proliferation (P=0.016) and colony formation assays (P=0.001), and demonstrated
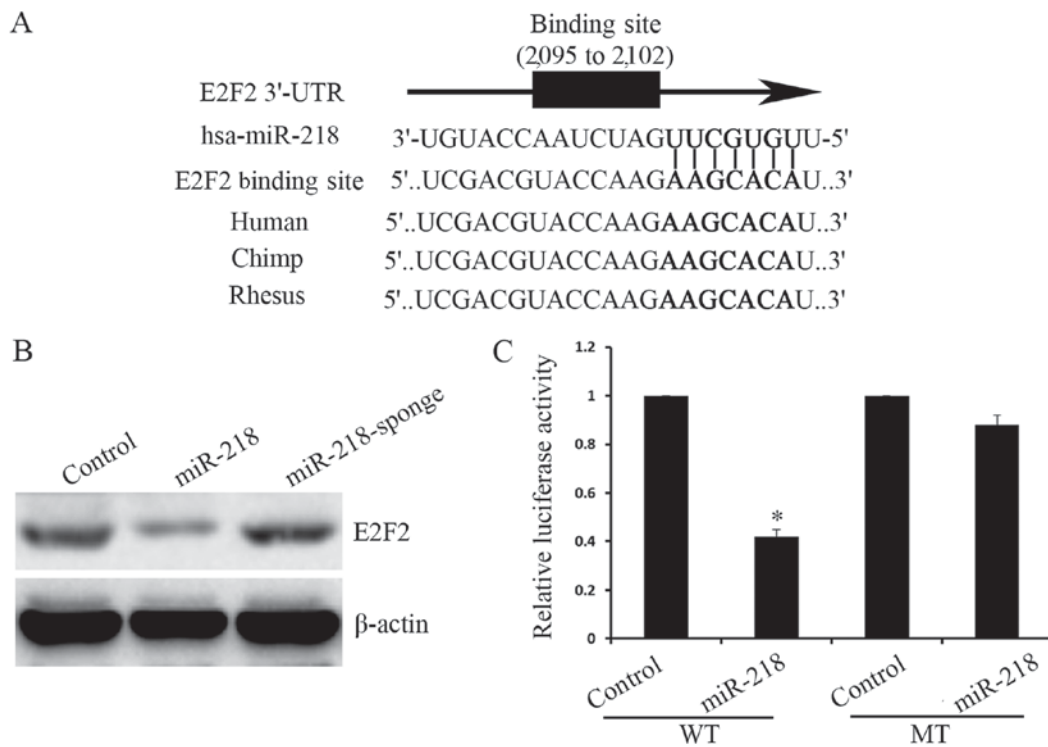
Figure 3. E2F2 is the target of miR-218 in human osteosarcoma cells. (A) Putative binding sites of miR-218 within the E2F2 3'UTR, as predicted using TargetScan and Pictar algorithm software. Bold letters represent the site of action. (B) Expression of E2F2 was verified using western blotting of protein lysates from the stable cell lines. (C) miR-218 decreased luciferase levels in MG63 cells expressing WT E2F2 3'UTR compared with the control group; however, luciferase levels increased in MG63 cells expressing MT E2F2 3'UTR compared with the control group. miR, microRNA; UTR, untranslated region; WT, wild-type; MT, mutant. *P<0.05 vs. control group and miR-218 group.

that overexpression of miR-218 inhibited proliferation and silencing of miR-218 promoted proliferation (Fig. 2D-G).

E2F2 is a direct target of miR-218. To study the mechanism underlying miR-218 inhibition of osteosarcoma proliferation, a bioinformatics software was used to demonstrate that miR-218 directly targeted the E2F2 3'UTR (Fig. 3A). The expression of E2F2 was significantly increased following overexpression of miR-218, as indicated by western blot analysis, and expression of E2F2 was increased following silencing of miR-218 (Fig. 3B). Furthermore, pmirGLO-WT-E2F2 3'UTR and pmirGLO-MT-E2F2 3'UTR plasmids were constructed and the luciferase activity in the pmirGLO-WT-E2F2 3'UTR group was determined to be significantly decreased, compared with the control group, while there was no difference between the control and the pmirGLO-MT-E2F2 3'UTR groups (Fig. 3C).

miR-218 inhibits tumor formation in vivo. Finally, the *in vivo* role miR-218 serves in osteosarcoma tumorigenicity in nude mice was investigated. After eight weeks, nude mice were sacrificed and their tumors were measured. Tumor sizes of mice injected with MG63 cells expressing miR-218 were reduced (P=0.001) and had a reduced growth rate, compared with mice injected with control or miR-218 sponge cells (P=0.001) (Fig. 4A and B). Immunohistochemistry also confirmed that overexpression of miR-218 inhibited proliferation and silencing of miR-218 promoted proliferation (P=0.001) (Fig. 4C and D). Western blot analysis of tumor tissues derived from mice receiving different treatments also confirmed that E2F2 was significantly downregulated in the

miR-218 overexpressing group, compared with the control group, and that E2F2 was significantly upregulated in the miR-218 knockdown group, compared with the miR-218 overexpressing group (P=0.02) (Fig. 4E and F).

In conclusion, the present results indicated that miR-218 suppresses the proliferation of human osteosarcoma cells through E2F2 and E2F2 is a direct target of miR-218.

Discussion

Previous studies demonstrated that miR-218 suppressed the migration and invasion in osteosarcoma cells (11); however, research on the role of miR-218 in proliferation has not been reported for osteosarcoma cells. In the present study, it was determined that miR-218 exhibited reduced expression in osteosarcoma cells, compared with osteoblasts. miR-218 may serve a tumor suppressor role in osteosarcoma. To investigate the exact role of miR-218 in osteosarcoma cells, vectors used to upregulate and downregulate the expression of miR-218 in human osteosarcoma MG63 cells were constructed using the three-plasmid lentivirus system. The results demonstrated that miR-218 suppresses the proliferation of human osteosarcoma cells through E2F2 *in vitro* and *in vivo* and E2F2 is a direct target of miR-218.

Numerous studies have demonstrated that miR-218 can be regarded as a tumor suppressor in a number of cancer types, including esophageal carcinoma (17), hepatocellular carcinoma (18), lung carcinoma (19), pancreatic cancer (20), glioma (21) and prostate cancer (22). It has been reported that miR-218 can also be regarded as a tumor suppressor in osteosarcoma (11),
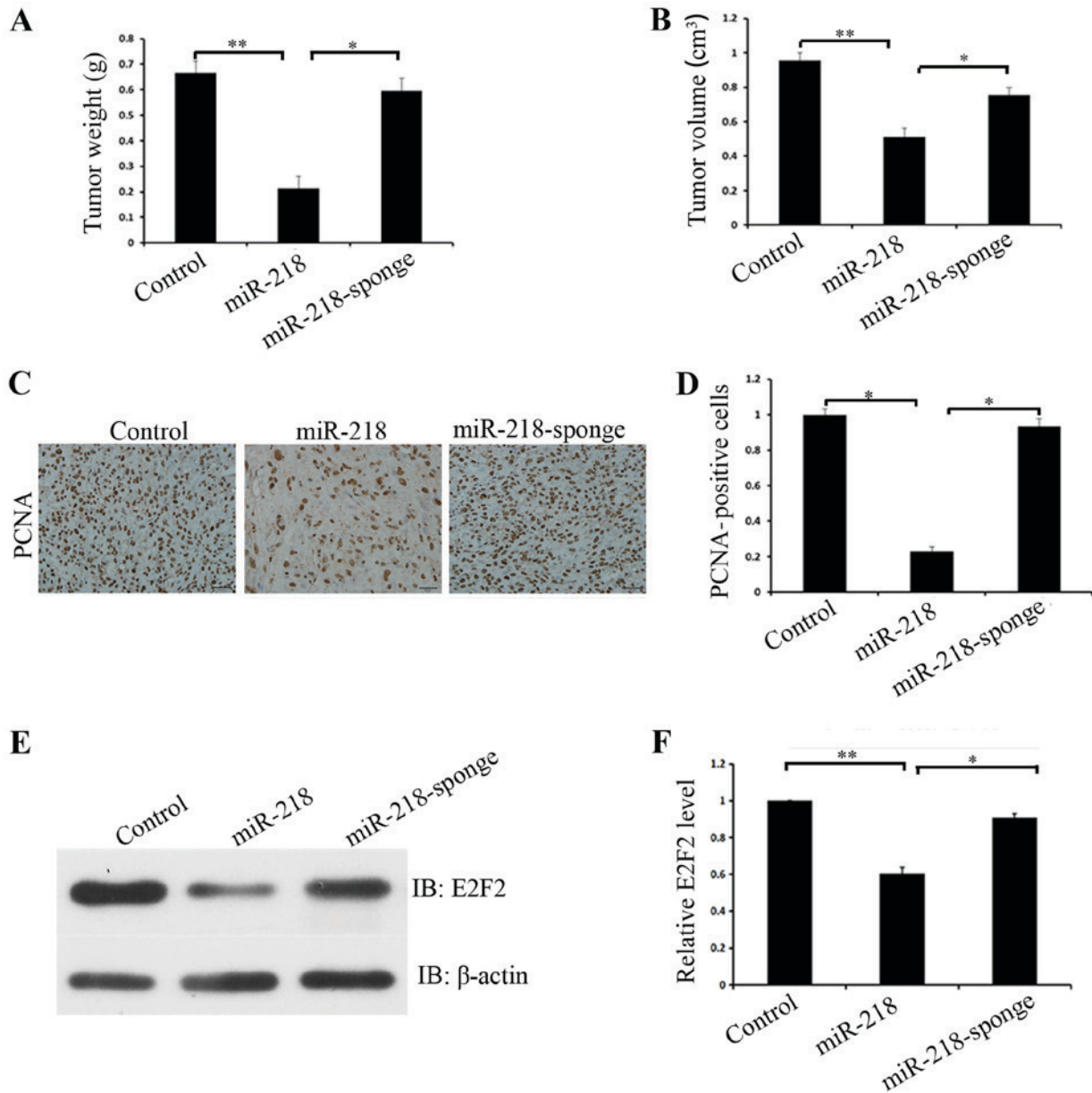
Figure 4. miR-218 inhibits the tumorigenicity of human osteosarcoma cells *in vivo*. (A) Statistical analysis of mean tumor weight calculated as follows: Weight=(weight1+weight2+weight3)/3. (B) Statistical analysis of tumor volume. (C) Representative images depicting PCNA-positive cells (scale bar, 20 μm; magnification, x400). (D) Statistical analysis of the PCNA index. (E) Expression of E2F2 was verified using western blotting of tumor tissues from nude mice. (F) Quantification of relative expression level of E2F2 in miR-218, miR-218-sponge and control groups. miR, microRNA; PCNA, proliferating cell nuclear antigen. $^{*}P<0.05$ and $^{**}P<0.01$ compared with control group and miR-218 group; miR-218 and miR-218-sponge group.

which is consistent with the present data. In a previous study, expression of miR-218 was significantly reduced in osteosarcoma tissues, compared with non-tumorous tissues (10). In agreement with that report, it was determined that miR-218 was expressed at a reduced level in osteosarcoma, compared with osteoblast cells. Previous studies demonstrated that miR-218 may serve a role in tumor suppression through targeting BMI1 (17,23), RET (18), tumor protein D52 (22), KIT (24) and SH3 domain-containing growth factor receptor bound protein 2-like protein 1 (25). Furthermore, it was determined that miR-218 directly targeted the E2F2 3'UTR in osteosarcoma.

E2F2 is a member of the E2F transcription factor family, which serves a central role in the regulation of cell cycle progression (13,26,27). E2F2 may serve the role of a promoter of tumorigenesis. A number of recent studies support this point,

reporting that E2F2 is a pivotal member in promoting the proliferation of osteosarcoma (26), human glioblastoma (28) and hepatocellular carcinoma cells (29); however, in contrast to these data, E2F2 suppresses the proliferation of renal carcinoma (30) and colon carcinoma cells (31). In the present study, it was indicated that the downregulation of E2F2 suppressed the proliferation of human osteosarcoma cells by overexpressing miR-218 and that upregulation of E2F2 promoted the proliferation of human osteosarcoma cells by silencing miR-218. Thus, it was confirmed that E2F2 promotes osteosarcoma tumorigenesis.

In conclusion, it was determined that miR-218 had reduced expression in osteosarcoma cells, inhibiting the proliferation through targeting E2F2 directly, and E2F2 can promote osteosarcoma tumorigenesis. This result may be significant in the early detection of osteosarcoma, which could have treatment

implications and could be a valuable protein marker in the early detection of osteosarcoma in children.

Availability of data and materials

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

Authors' contributions

CX, MJ, and YG proposed the concept and design of the study. CX, YG, RH and QA performed experiments, prepared all figures, tables and manuscript. SX applied biology software predicts target genes for miRNAs. LW and YW performed the data and figure analysis.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Xuzhou Children's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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