MicroRNA-145-5p inhibits osteosarcoma cell proliferation by targeting E2F transcription factor 3

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Abstract. Osteosarcoma is a common type of bone tumor that primarily occurs in children and young adults. MicroRNA (miRNA/miR) dysregulation is associated with the progression of osteosarcoma; therefore, the aim of the present study was to investigate the biological functions and molecular mechanisms of miR-145-5p in osteosarcoma. The expression of miR-145-5p in osteosarcoma tissues and cell lines was quantified using reverse transcription-quantitative PCR (RT-qPCR). The effect of miR-145-5p on the proliferation of osteosarcoma cells was detected using Cell Counting Kit-8 and colony formation assays, as well as cell cycle distribution analysis. The effect of miR-145-5p on tumor growth was further investigated *in vivo* using a subcutaneous tumor model in nude mice. The interaction between miR-145-5p and E2F transcription factor 3 (E2F3) was determined using bioinformatics analysis, a luciferase assay, RT-qPCR and western blotting. The results revealed that miR-145-5p expression was decreased in osteosarcoma cell lines and tissues compared with the corresponding normal controls. Increased miR-145-5p expression inhibited the proliferation and colony formation ability of osteosarcoma cells, and induced G_1 phase arrest. Furthermore, mice injected with tumor cells overexpressing miR-145-5p exhibited smaller tumors than those in the control group. Further investigation revealed that miR-145-5p binds to and decreases the expression of E2F3. In addition, the mRNA levels of E2F3 were negatively associated with miR-145-5p in osteosarcoma tissues, and increasing E2F3 expression abrogated the inhibitory effects of miR-145-5p on osteosarcoma cells. Collectively, the results obtained in the present study suggest that miR-145-5p may suppress the progression of osteosarcoma, and may serve as a useful biomarker for the diagnosis of osteosarcoma, as well as a therapeutic target.

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Introduction

Osteosarcoma is a primary bone malignancy that predominantly occurs in children and adolescents, and accounts for 60% of bone tumors cases (1,2). In the past decade, great progress has been made in the treatment of osteosarcoma, and current therapeutic strategies include surgical resection, chemotherapy and radiotherapy. However, due to a high degree of metastasis and recurrence, the outcome of patients with osteosarcoma remains unsatisfactory (2). Although the molecular mechanisms of osteosarcoma have attracted widespread attention, those involved in disease progression have not been fully elucidated (3). Therefore, investigating the molecular mechanism underlying the development of osteosarcoma may aid the identification of novel biomarkers and potential targets for the diagnosis and treatment of the disease (4).

MicroRNAs (miRNA/miR) are non-coding RNAs that have been widely reported to inhibit the expression of their target genes, and play key roles in cellular processes including proliferation, differentiation and metastasis (5). Various studies have demonstrated that miRNA dysregulation is one of the main factors leading to the development of osteosarcoma (6). Wang *et al* (7) revealed that miR-193a is involved in the chemoresistance of osteosarcoma cells and that upregulating the expression of miR-193a promotes chemosensitivity. Liu *et al* (8) demonstrated that miR-377 may serve as a tumor-suppressive miRNA by inducing apoptosis in osteosarcoma cells. Hu *et al* (9) revealed that miR-1285-3p exerts a tumor suppressor effect in osteosarcoma and may serve as a novel biomarker for the diagnosis of the disease. In addition, Chu *et al* (10) found that miR-136 expression was low in a collection of osteosarcoma tissues compared with adjacent normal tissues, and that this was negatively associated with prognosis. Furthermore, Wang *et al* (11) revealed that miR-628-5p increased the proliferation and migration of osteosarcoma cells by decreasing the expression of interferon

induced protein 44 like. Therefore, a clear understanding of the effects and relative molecular mechanisms of miRNAs may facilitate the identification of novel biomarkers for the diagnosis of osteosarcoma, as well as potential therapeutic targets (12).

The present study investigated the biological functions and the latent mechanism of miR-145-5p in osteosarcoma using a series of molecular biological experiments including CCK-8, colony formation, cell cycle distribution analysis, western blot, RT-qPCR and luciferase assays. The results obtained in the present study suggest that miR-145-5p may serve as a diagnostic biomarker and therapeutic target in patients with osteosarcoma, which has potential to inhibit the proliferation of osteosarcoma via targeting E2F transcription factor 3 (E2F3).

Materials and methods

Patient samples. A total of 20 patients were enrolled in the present study, who were pathologically diagnosed with osteosarcoma and had received surgical resection at Guizhou Orthopedics Hospital (Guizhou, China) between March 2014 and May 2019. The patients included 14 males and 6 females with a mean age of 17.15±15.17 years (range 7-45 years); 11 patients were diagnosed in the early stage, while 9 patients were diagnosed in the advanced stage of disease. The inclusion criteria were as follows: i) The tissues were obtained during surgery and osteosarcoma was diagnosed by two pathologists; ii) the patients were diagnosed and treated for the first time; and iii) the patients were willing to participate. The exclusion criteria were as follows: i) Patients with other malignancies; ii) patients with other systemic diseases; iii) patients who had received treatment prior to admission; and iv) patients (and/or their families) who refused to participate. All 20 patients provided osteosarcoma tissue samples, while only 10 patients also provided adjacent normal tissues. The present study was approved by the Committee of Guizhou Orthopedics Hospital and was performed in accordance with the Declaration of Helsinki. All patients provided written informed consent.

Cell culture and lentivirus transfection. In total, five osteosarcoma cell lines (U2OS, Saos2, MG63, SJSA-1 and 143B) and a normal osteoblast line (hFOB) were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Well5 cells (5) were purchased from the Chinese National Infrastructure of Cell Line Resource (cat. no. 3142C0001000000722; http://www.cellresource.cn/index.aspx). All cell lines were previously authenticated by short tandem repeat typing. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and maintained in a humidified incubator at 37˚C (5% CO_2). Lentiviruses containing miR-145-5p- and non-targeting miRNA DNA encoding plasmids (LV-miR-145-5p and LV-miR-NC, respectively) were purchased from Shanghai GeneChem Co., Ltd. Transfection was performed using Lipo2000 (Shanghai GeneChem Co., Ltd) according to the manufacturer's protocol (MOI=20 for 143B and Well5 cells). The miR-145-5p sequence used for lentiviral construction was 5'-GUCCAGUUUUCCCAGGAAUCCCU-3', and the LV-miR-NC sequence was 5'-UUCUCCGAACGUGUCACG UTT-3'. E2F3 (NM_001949.5) was used as a backbone and sub-cloned into the pcDNA3.1 vector to construct an E2F3 overexpression plasmid (Shanghai GeneChem Co., Ltd.), and an empty pcDNA3.1 vector was used as a negative control (NC).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the osteosarcoma tissues and cell lines using TRIzol® reagent, and subsequently reverse transcribed into cDNA using an mRNA First Strand cDNA Synthesis kit (both Yeasen Biotech Co., Ltd.) according to the manufacturer's protocols. miRNAs were reverse transcribed using the miRNA First Strand cDNA Synthesis kit, and qPCR was performed using the qPCR SYBR® Green Master Mix (both Yeasen Biotech Co., Ltd.). The following primer pairs were used: miR-145-5p forward, 5'-GTCCAG TTTTCCCAGGAATC-3' and reverse, 5'-AGAACAGTA TTTCCAGGAAT-3'; E2F3 forward, 5'-AGAAAGCGGTCA TCAGTACCT-3' and reverse, 5'-TGGACTTCGTAGTGC AGCTCT-3'; GAPDH forward, 5'-GGAGCGAGATCCCTC CAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCT CATGG-3'; and U6 forward, 5'-GCTTCGGCAGCACAT ATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTG CGTGTCAT-3'. Relative expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (13). GAPDH was used as the reference gene for E2F3, and U6 was used as the reference for miR-145-5p expression. The qPCR thermocycling conditions were as follows: 95˚C for 5 min, 40 cycles of 95˚C for 25 sec, annealing at 60˚C for 40 sec, and a final elongation step at 72˚C for 30 sec.

Cell Counting Kit-8 (CCK-8) assay. Following transfection with the miR-145-5p and NC lentiviruses (and subsequent incubation for 48 h), 143B and Well5 cells were seeded into 96-well plates at a density of $3x10^3$ cells/well, and cultured for 24, 48, 72 and 96 h. Subsequently, 10 μl CCK-8 solution (Wuhan Boster Biological Technology, Ltd.) was added to each well according to the manufacturers protocol, and the cells were cultured for an additional 2 h. The absorbance was measured at a wavelength of 450 nm.

Colony formation assay. At 48 h post-transfection with the miR-145-5p and NC lentiviruses, 143B and Well5 cells were seeded into a 6-well plate at a density of $1x10^3$ cells/well and cultured for 2 weeks. The cells were subsequently fixed with 4% polyoxymethylene (Wuhan Boster Biological Technology, Ltd.) for 30 min and stained with 1% crystal violet (Wuhan Boster Biological Technology, Ltd.) for 20 min, both at room temperature. The cells were photographed and the colonies were counted by eye.

Cell cycle distribution analysis. Osteosarcoma 143B and Well5 cells were harvested and fixed using 70% cold ethanol for 24 h at 4˚C. The cells were then stained with propidium iodide (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature in the dark. The cell cycle distribution was analyzed using a NovoCyte flow cytometer (Agilent

Technologies, Inc.,) and FlowJo software (FlowJo LLC, Version: 7.6.1).

Western blotting. Total protein was extracted from osteosarcoma tissues and 143B and Well5 cells using RIPA lysis buffer supplemented with protease inhibitor cocktail (1:100) and phenylmethylsulfonyl fluoride (1:100) (all Wuhan Boster Biological Technology, Ltd.). The protein concentration was determined using a bicinchoninic acid assay, and the protein samples (30 μg each) were separated via SDS-PAGE using a 10% gel. The separated proteins were transferred onto a PVDF membrane and blocked using 5% skim milk for 2 h at room temperature. The membrane was subsequently incubated with primary antibodies against cyclin D1 (cat. no. 19532-1-AP; 1:1,000), cyclin E1 (cat. no. 11554-1-AP; 1:1,000), cyclin dependent kinase (CDK) 2 (cat. no. 10122-1-AP; 1:1,000), CDK4 (cat. no. 11026-1-AP; 1:1,000), CDK6 (cat. no. 14052-1-AP; 1:1,000), E2F3 (cat. no. 27615-1-AP; 1:1,000) and GAPDH (cat. no. 60004-1-Ig; 1:1,000) (all ProteinTech, Inc.) for 14 h at 4˚C. The membrane was then washed using TBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse (cat. no. BA1050; 1:3,000) or goat anti-rabbit antibodies (cat. no. BA1054; 1:3,000) (both Wuhan Boster Biological Technology, Ltd.) for 2 h at room temperature. Finally, the protein bands were visualized using ECL reagent (Wuhan Boster Biological Technology, Ltd) and quantified using Image Lab v2 (Bio-Rad Laboratories Inc,). GAPDH served as the loading control.

Animal experiments. Female BALB/c nude mice (n=10; age, 4-6 weeks; weight, 15-18 g) were obtained from the Laboratory Animal Center of Guizhou Medical University (Guizhou, China). Mice were housed under specific pathogen-free conditions at 25˚C with a 12-h light/dark cycle and free access to food and water. A total of $1x10^7$ 143B cells overexpressing miR-145-5p or NC were injected into the subcutaneous tissues of the upper-right flank. The tumor volume was measured weekly as follows: Volume (mm^3) = (length x $width^2$)/2. The mice were sacrificed after 5 weeks and the tumor weights were measured. The animal experiments were approved by the Ethics Committee of Guizhou Medical University (Guizhou, China).

Immunohistochemical (IHC) staining. The tumor tissues acquired from the nude mice were dehydrated and embedded in paraffin (Wuhan Servicebio Technology Co., Ltd.) at room temperature. After being cut into 4-μm slices, the tissues were deparaffinized using xylene and rehydrated in a descending alcohol series at room temperature. After restoration with sodium citrate the samples were treated with 3% H_2O_2 to block endogenous peroxidase activity, and then blocked using 5% bovine serum albumin (BSA; Wuhan Servicebio Biotechnology Co., Ltd.) for 30 min at room temperature. The specimens were subsequently incubated with a primary anti-KI67 antibody (cat. no. 27309-1-AP; 1:400; ProteinTech) or anti-PCNA antibody (cat. no. 10205-2-AP; 1:400; ProteinTech) for 12 h at 4˚C, followed by a second incubation with HRP-conjugated secondary antibodies (cat. no. G1210-2-A-100; 1:200; Wuhan Servicebio Biotechnology Co., Ltd.) for 2 h at room temperature. After subsequent development using the Cell and Tissue Staining HRP-DAB kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol, images were captured with an orthophoto microscope (magnification, x400).

Luciferase reporter assay. TargetScan software (www.targetscan.org; Version 7.2) was used to predict the target genes of miR-145-5p. A dual-luciferase reporter assay was conducted to verify whether miR-145-5p binds to and regulates the expression of E2F3. The wild type (Wt) and mutant (Mut) 3' untranslated regions (UTR) of E2F3 were synthesized and sub-cloned into the psiCHECK-2 luciferase reporter vector (Promega Corporation). A total of $5x10^3$ 143B and Well5 cells were cultured for 24 h and co-transfected with the Wt/Mut E2F3 luciferase reporter vector, and the miR-145-5p mimic or NC, using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Finally, a dual-luciferase reporter assay system (Promega Corporation) was used to quantify luciferase activity 24 h post-transfection. Luciferase activity was normalized to that of *Renilla* luciferase.

Statistical analysis. Statistical analyses were performed using SPSS software (version 21.0; IBM Corp.). The paired t-test was used to compare two groups while one-way analysis of variance and Dunnett's post hoc test were used for the comparison of multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-145-5p expression is decreased in osteosarcoma tissues. The expression levels of miR-145-5p in osteosarcoma and normal adjacent tissues were quantified using RT-qPCR. miR-145-5p expression was significantly decreased in osteosarcoma tissues (n=20) compared with adjacent normal tissues (n=10; Fig. 1A). The expression level of miR-145-5p was also decreased in six osteosarcoma cell lines (U2OS, Saos2, MG63, SJSA-1, 143B and Well5) compared with the normal hFOB osteoblasts (Fig. 1B). Due to the evidence that the expression of miR-145-5p was decreased most significantly in 143B and Well5 cells, we then used these two cell lines to detect biology function of miR-145-5p in osteosarcoma.

miR-145-5p inhibits proliferation in vitro. In order to investigate the role of miR-145-5p in osteosarcoma, an osteosarcoma cell line that overexpressed miR-145-5p (LV- miR-145-5p) and a corresponding normal control cell line (LV-miR-NC) were successfully constructed (Fig. 2A). The results of the CCK-8 assay revealed that miR-145-5p overexpression significantly inhibited the proliferation of 143B and Well5 cells at 48, 72 and 96 h, compared with LV-miR-NC cells (Fig. 2B). Similarly, the results of the colony formation assay demonstrated that miR-145-5p overexpression significantly reduced colony formation in osteosarcoma cells compared with the LV-miR-NC cell line (Fig. 2C).

miR-145-5p induces G_1 phase arrest. The results of cell cycle distribution analysis revealed that miR-145-5p overexpression significantly increased the proportion of 143B and Well5 cells

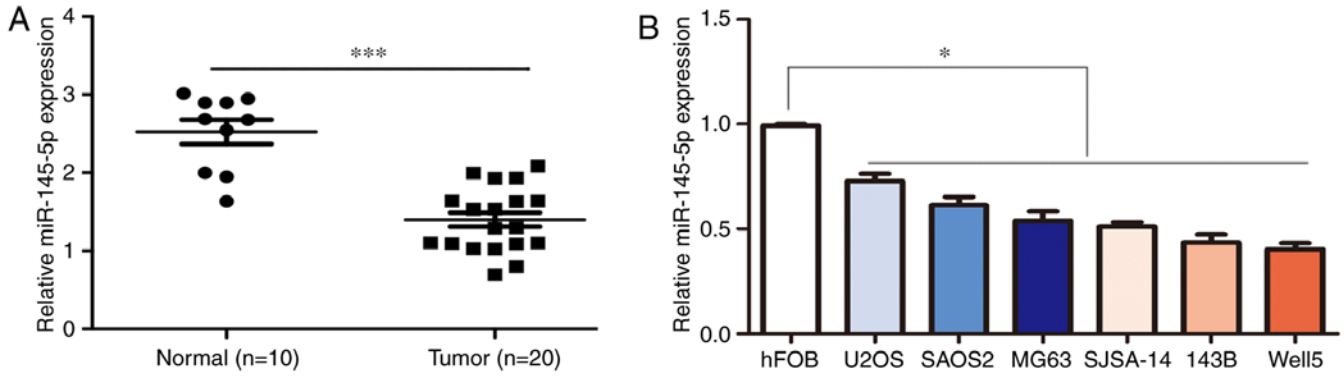

Figure 1. miR-145-5p is significantly downregulated in osteosarcoma. (A) miR-145-5p expression was detected in 20 osteosarcoma and 10 adjacent normal tissues using RT-qPCR. (B) The expression of miR-145-5p in the osteosarcoma cell lines U2OS, Saos2, MG63, SJSA-1, 143B and Well5, and the human normal osteoblast line hFOB was detected using RT-qPCR. *P<0.05 and ***P<0.01. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

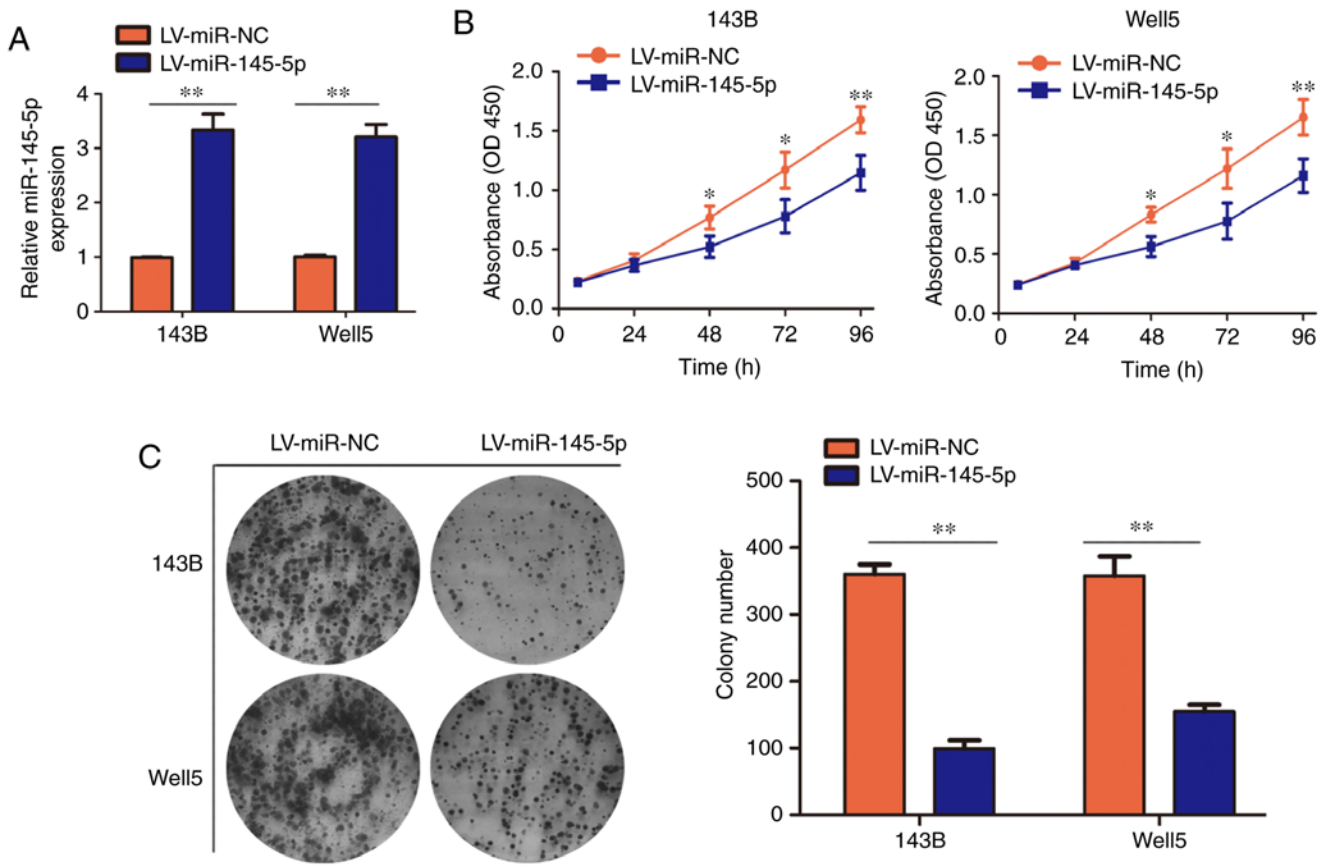

Figure 2. miR-145-5p decreases the proliferation of osteosarcoma cells *in vitro*. (A) Reverse transcription-quantitative PCR was used to determine transfection efficiency. The effect of miR-145-5p overexpression on the proliferation of osteosarcoma cells was detected using (B) Cell Counting Kit-8 and (C) colony formation assays. *P<0.05 and **P<0.01. miR, microRNA; LV, lentivirus; NC, negative control.

in the G_1 phase of the cell cycle, and decreased the proportion of cells in the S phase (Fig. 3A). Previous studies have revealed that cyclin D-CDK4/CDK6 and cyclin E/CDK2 are involved in cell cycle regulation at the G_1 phase, and that increased expression of these proteins is required for cells in G1 phase to pass the G1/S check point (14,15). In the present study, western blotting revealed that the protein expression levels of cyclin D1, cyclin E, CDK2, CDK4 and CDK6 were decreased in cells overexpressing miR-145-5p compared with the LV-miR-NC cells (Fig. 3B).

miR-145-5p inhibits osteosarcoma tumor growth in vivo. miR-145-5p overexpression inhibited the proliferation of osteosarcoma cells *in vitro*. Therefore, the effect of miR-145-5p overexpression *in vivo* was also investigated. The results revealed that mice injected with 143B cells overexpressing miR-145-5p exhibited slower tumor growth and lower tumor volumes compared with the LV-miR-NC group (Fig. 4A-C). Furthermore, the weight-loss rate of the mice injected with 143B cells overexpressing miR-145-5p was slower than that of the LV-miR-NC group (Fig. 4D). Moreover, the expression levels

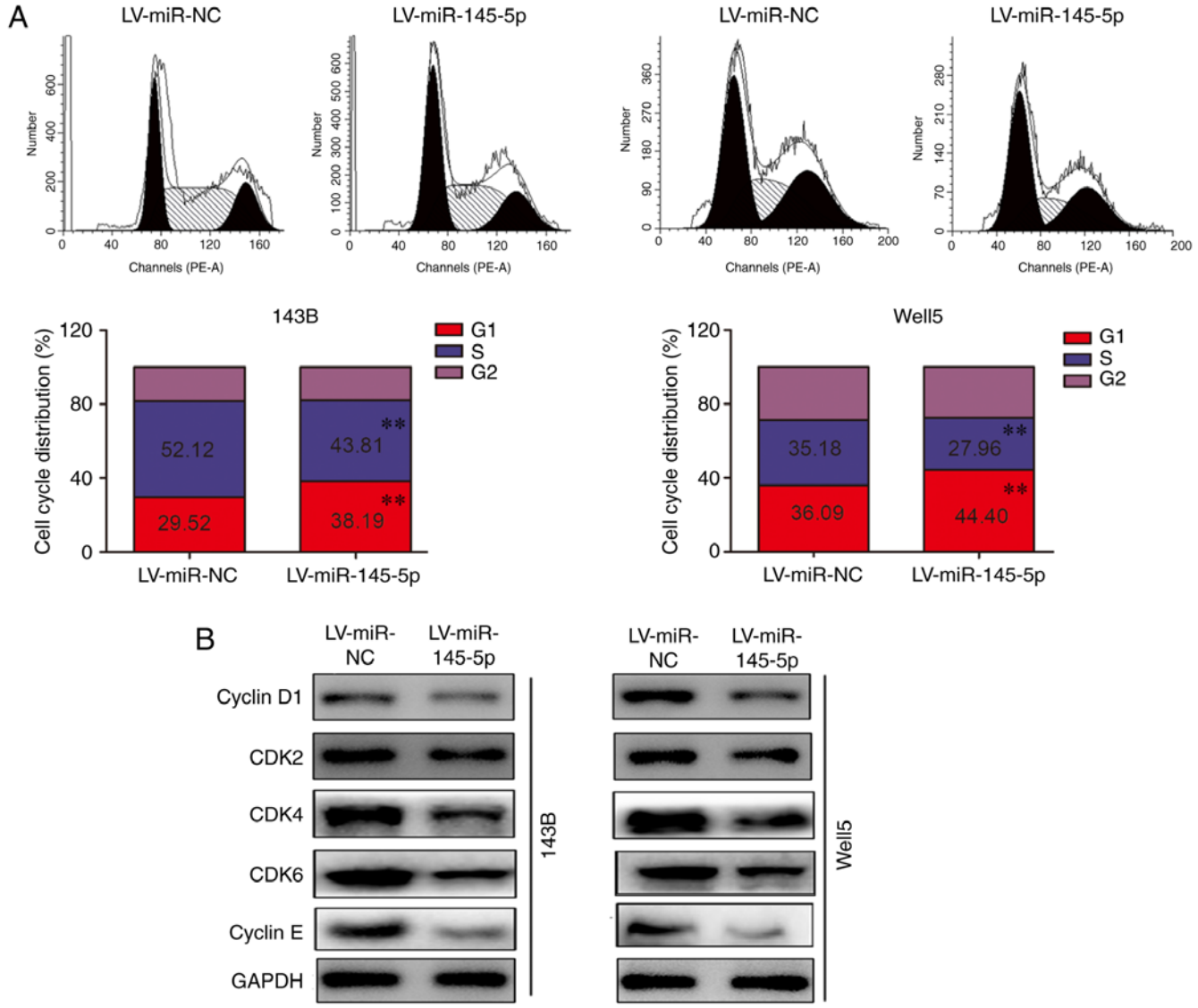

Figure 3. miR-145-5p induces G_1 phase arrest in osteosarcoma cells. (A) Cell cycle distribution analysis in 143B and Well5 cells overexpressing miR-145-5p. (B) Western blotting was used to detect the expression of CDK2, CDK4, CDK6, cyclin E and cyclin D1 in cells overexpressing miR-145-5p. GAPDH was used as loading control. **P<0.01. miR, microRNA; CDK, cyclin dependent kinase.

of KI67 and PCNA were decreased in the miR-145-5p-overexpression group compared with the LV-miR-NC group (Fig. 4E). Collectively, these results suggest that increasing the expression level of miR-145-5p inhibits tumor growth *in vivo*.

E2F3 is directly regulated by miR-145-5p. Using TargetScan, E2F3 (which has conservative binding sites for miR-145-5p; Fig. 5A) was identified as one of the downstream targets of miR-145-5p. Previous studies showed that miR-145-5p was indicated as a novel biomarker in osteosarcoma (16), and therefore, E2F3 was hypothesized to be a key target of miR-145-5p in osteosarcoma. A luciferase reporter assay was subsequently used to validate this result. Co-transfection of miR-145-5p mimics in 143B and Well5 cells significantly inhibited the luciferase activity of the WT E2F3 3'-UTR sequence, but failed to inhibit that of the MUT E2F3 3'-UTR (Fig. 5B). The mRNA and protein expression levels of E2F3 were also significantly decreased in cells overexpressing miR-145-5p compared with the LV-miR-NC cells (Fig. 5C and D). Furthermore, the mRNA expression levels of E2F3 were negatively associated with miR-145-5p in osteosarcoma tissues (Fig. 6A), and E2F3 expression was increased in osteosarcoma tissues (Fig. 6B) and cell lines (Fig. 6C and D).

Restoration of E2F3 decreases the inhibitory effect induced by miR-145-5p. To investigate whether E2F3 was involved in the miR-145-5p-induced inhibitory effect on proliferation, the expression of E2F3 in miR-145-5p-overexpressing cells was restored (Fig. 7A). CCK-8 and colony formation assays revealed that restoring E2F3 expression decreased the inhibitory effect of miR-145-5p on the proliferation of osteosarcoma cells (Fig. 7B and C). Furthermore, cell cycle distribution analysis revealed that co-transfection with lentiviruses delivering miR-145-5p and E2F3 significantly decreased the proportion of osteosarcoma cells in the G_1 phase (Fig. 7D). Additionally, co-transfection increased the expression of cyclin D1, cyclin E, CDK2, CDK4 and CDK6 (Fig. 7E).

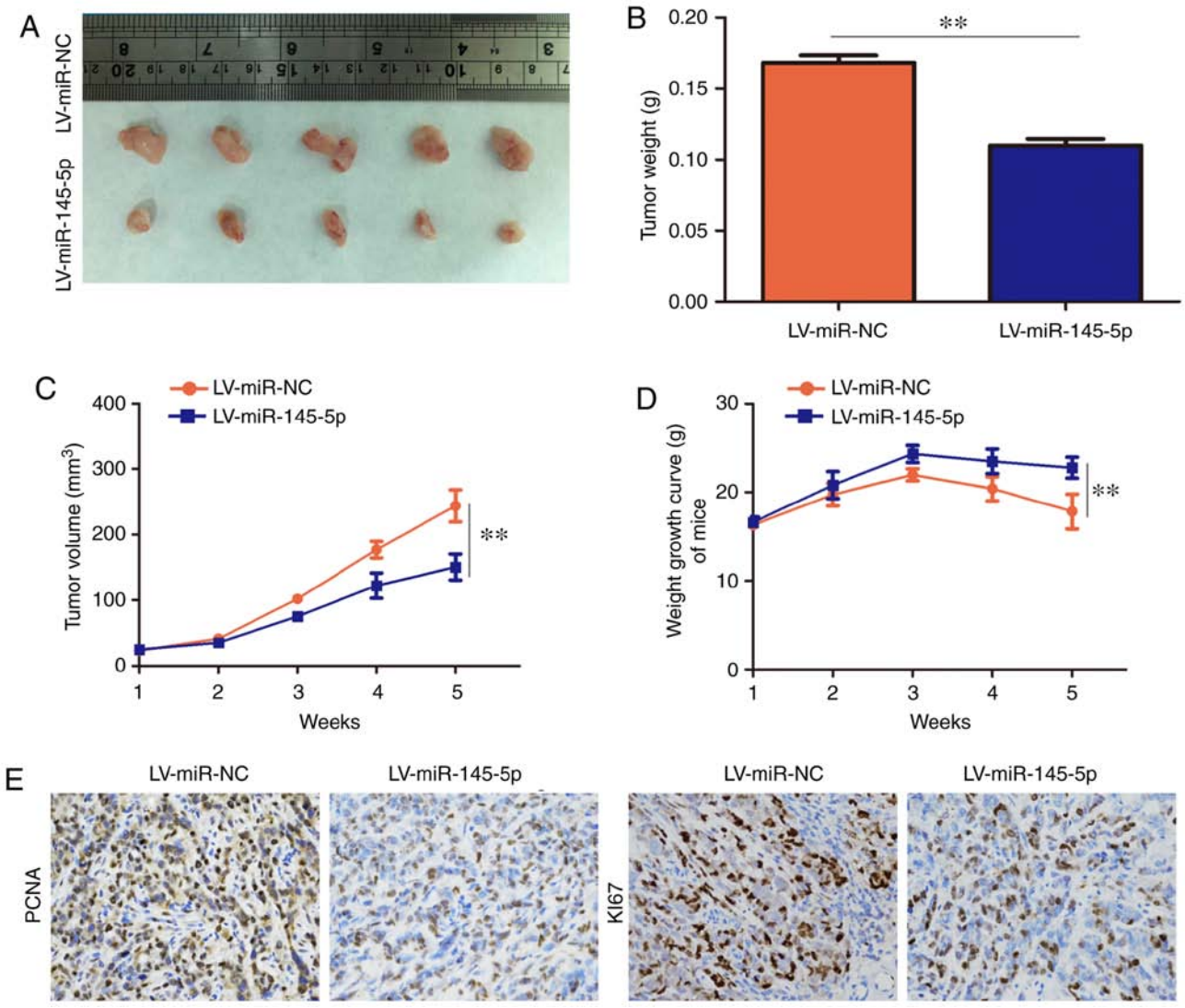

Figure 4. miR-145-5p inhibits tumor growth *in vivo*. (A) Representative images of subcutaneous tumors harvested from mice injected with 143B cells overexpressing miR-145-5p, or LV-miR-NC cells. (B) Tumor weights of mice injected with 143B cells overexpressing miR-145-5p, or the LV-miR-NC cells. (C) 143B cells overexpressing miR-145-5p or miR-NC were injected into the subcutaneous tissues of nude mice and tumor growth was monitored for 5 weeks. (D) The weight of the mice injected with 143B cells overexpressing miR-145-5p or the LV-miR-NC was monitored weekly. (E) Representative images of KI67 and PCNA expression in tissues obtained from mice injected with 143B cells overexpressing miR-145-5p and the LV-miR-NC group. Magnification, x400 and **P<0.01. LV, lentivirus; miR, microRNA; NC, negative control; PCNA, proliferating cell nuclear antigen.

Discussion

The biological function of miR-145-5p has been demonstrated in various cancer types, including bladder cancer (17), laryngocarcinoma (18) and gall bladder carcinoma (19). Wang *et al* (20) indicated that miR-145-5p was downregulated in hepatocellular carcinoma and served as a tumor suppressor. Tang *et al* (21) demonstrated that the expression level of miR-145-5p was decreased in breast cancer tissues compared with adjacent tissues, and was negatively associated with overall survival rate. Jian *et al* (22) revealed that miR-145-5p decreased the drug resistance of prolactinoma and increased sensitivity to chemotherapy. In addition, Zhou *et al* (23) showed that miR-145-5p affected the differentiation of gastric cancer cells by inhibiting the expression of Kruppel like factor 5, and Wu *et al* (24) demonstrated that downregulation of miR-145-5p increased the proliferation and migration of bladder cancer cells. In the present study, miR-145-5p was found to be downregulated in osteosarcoma tissues and cell lines compared with the corresponding normal controls. Overexpression of miR-145-5p decreased the proliferation of osteosarcoma cells, inhibited colony formation and induced G_1 phase arrest. Similarly, overexpression of miR-145-5p significantly decreased the expression of cyclin D, CDK4, CDK6, cyclin E and CDK2 in osteosarcoma, preventing the cells from passing the G_1 phase check point. To the best of our knowledge, the present study is the first to suggest that miR-145-5p serves as a tumor-suppressive miRNA in osteosarcoma.

It is widely reported that miRNAs regulate transcription by binding to their target genes (11). Bioinformatics analysis has revealed a number of potential target genes of miR-145-5p, including E2F3, an important member of the E2F family that participates in the cell cycle, metastasis and proliferation (25). The role of E2F3 in several types of cancer, including osteosarcoma, has been reported (26-28). E2F3 was indicated to be involved in the progression of melanoma, and to promote cellular proliferation (29). Downregulation of E2F3 also

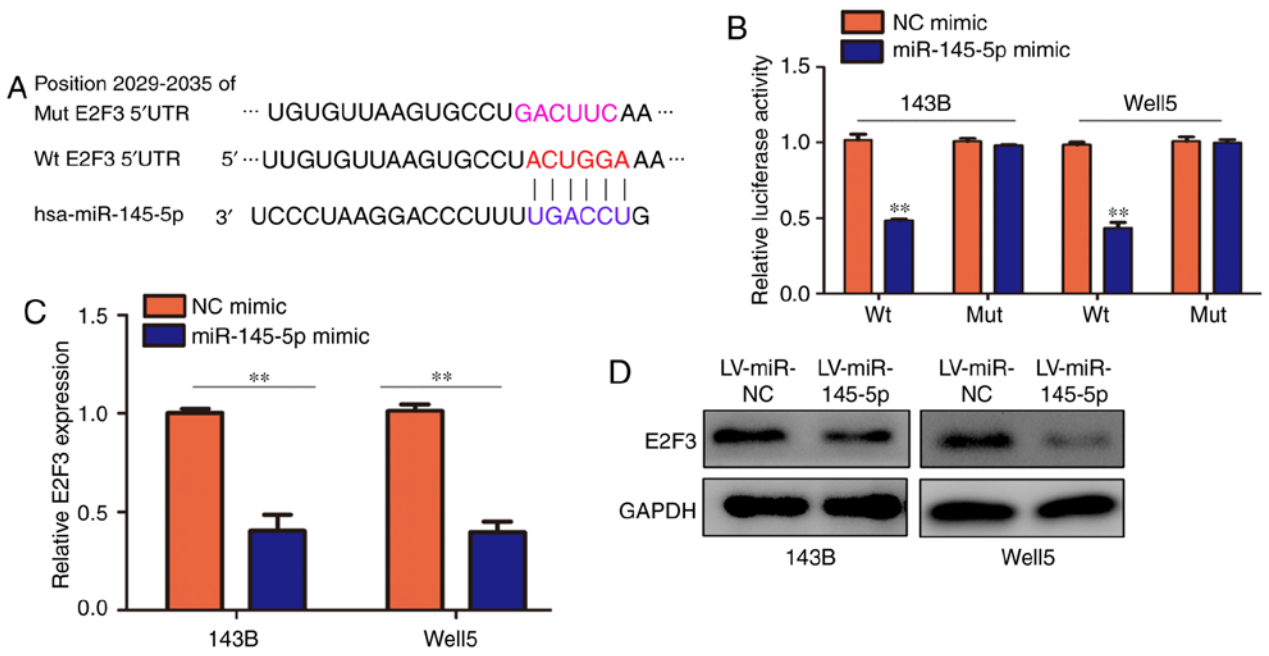

Figure 5. E2F3 is a target of miR-145-5p in osteosarcoma. (A) miR-145-5p binds to the 3'-UTR of E2F3. The sequence of the mutated site is highlighted. (B) The miR-145-5p mimic inhibited the luciferase activity of osteosarcoma cells, while the mutant 3'-UTR E2F3 abolished this effect. Overexpression of miR-145-5p decreased the (C) mRNA and (D) protein expression levels of E2F3 in osteosarcoma cells. **P<0.01. E2F3, E2F transcription factor 3; miR, microRNA; UTR, untranslated region; LV, lentivirus; miR, microRNA; NC, negative control; Wt, wild-type; Mut, mutant.

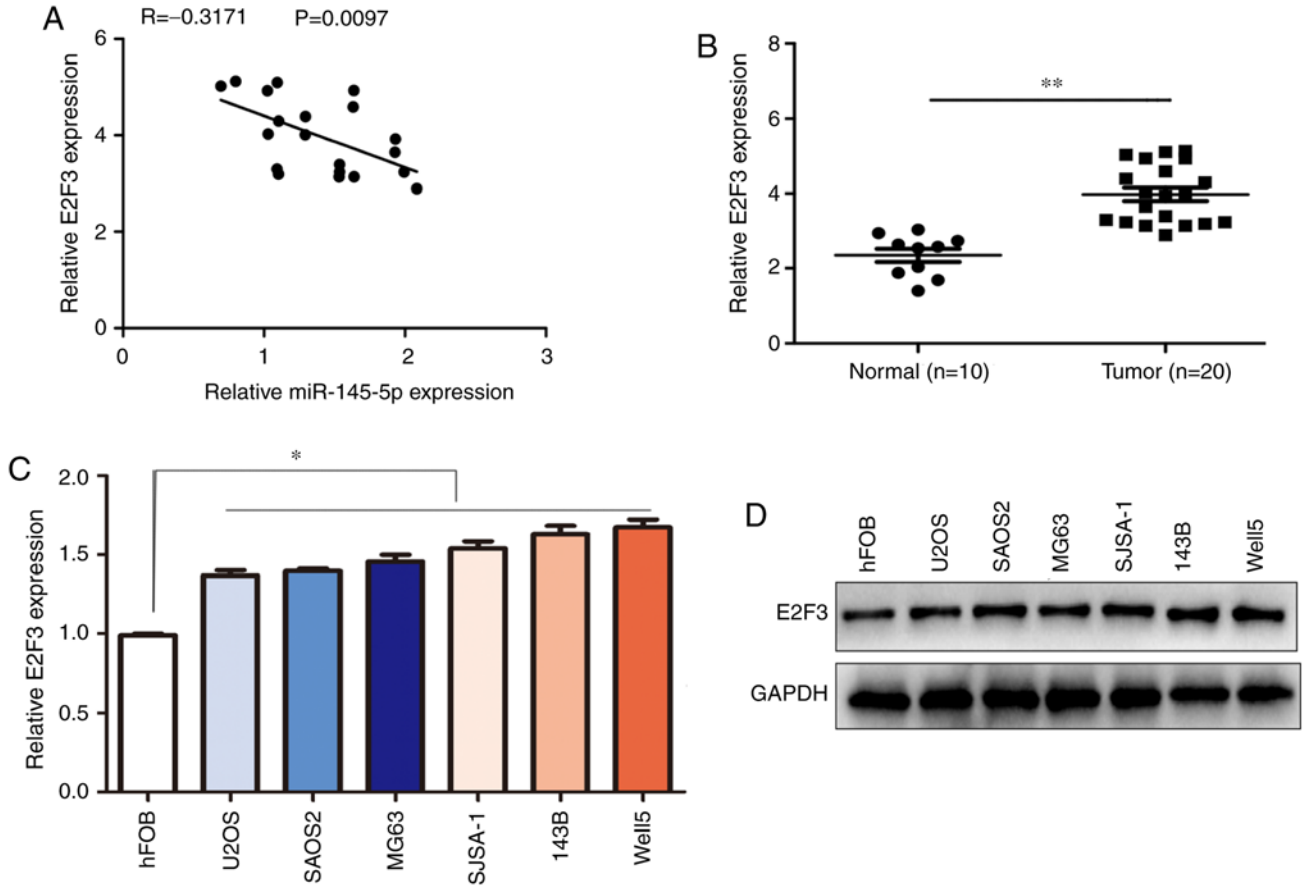

Figure 6. E2F3 is highly expressed in osteosarcoma tissues and cell lines. (A) miR-145-5p expression was inversely associated with that of E2F3 in osteosarcoma tissues. (B) The mRNA expression levels of E2F3 were detected in 20 osteosarcoma tissues and 10 normal adjacent tissues using RT-qPCR. (C) The mRNA expression levels of E2F3 in the U2OS, Saos2, MG63, SJSA-1, 143B and Well5 osteosarcoma cell lines, and in human normal osteoblast hFOB cells were detected using RT-qPCR. (D) The protein expression levels of E2F3 in U2OS, Saos2, MG63, SJSA-1, 143B and Well5 cells, and in the human normal osteoblasts (hFOB) were detected using RT-qPCR. *P<0.05 and **P<0.01. E2F3, E2F transcription factor 3; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

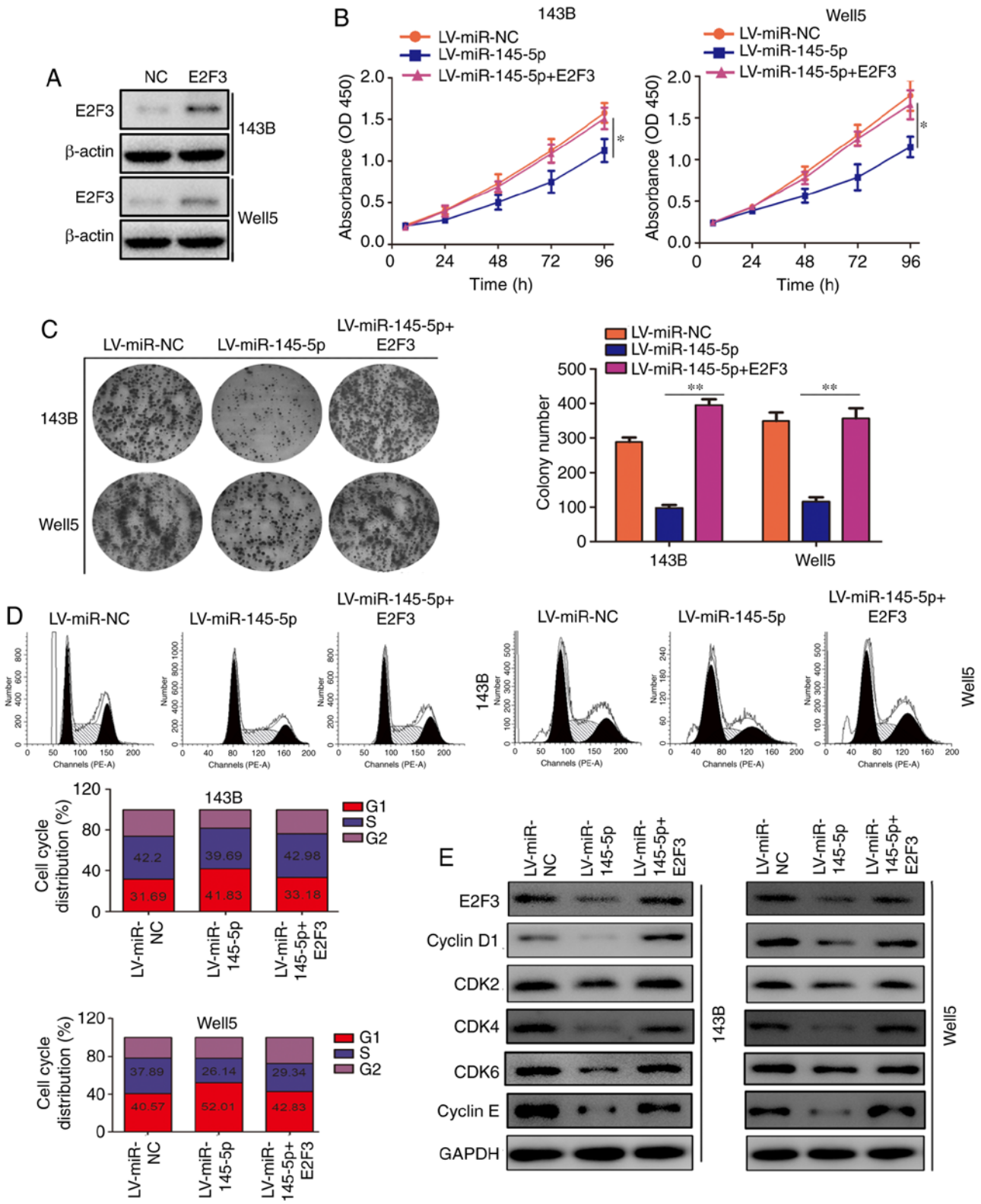

Figure 7. Restoration of E2F3 reverses the inhibitory effects of miR-145-5p. (A) The efficiency of the E2F3 overexpression plasmid was detected using western blotting. 143B and Well5 cells were divided into 3 groups and transfected with LV, LV-miR-145-5p or LV-miR-145-5p + E2F3 plasmids. (B) Cell Counting Kit-8 and (C) colony formation assays were used to detect proliferation in each group. (D) Cell distribution analysis was also performed. (E) Western blotting was used to detect the expression levels of E2F3, CDK2, CDK4, CDK6, cyclin E and cyclin D1 in each group. *P<0.05 and **P<0.01. E2F3, E2F transcription factor 3; LV, lentivirus; miR, microRNA; NC, negative control; LV, lentivirus; CDK, cyclin depen dent kinase.

decreased the proliferation and migration of hepatocellular carcinoma cells (30) and inhibited the progression of pancreatic cancer (31,32). Furthermore, various studies have indicated that the downregulation of E2F3 may suppress the proliferation and metastasis of osteosarcoma (26), and that E2F3 may serve as a biomarker for this disease (16).

The present study revealed that E2F3 is directly and negatively regulated by miR-145-5p. In addition, overexpression of miR-145-5p inhibited the expression of E2F3. E2F3 was highly expressed in osteosarcoma tissues and cell lines, and its expression was negatively associated with that of miR-145-5p. Restoration of E2F3 reversed the inhibitory effects on proliferation induced by miR-145-5p. To the best of our knowledge, the present study is the first to demonstrate the potential link between miR-145-5p and E2F3 in osteosarcoma.

In conclusion, the present study demonstrated that miR-145-5p may act as a tumor-suppressive miRNA in osteosarcoma. miR-145-5p suppressed osteosarcoma cell proliferation by targeting E2F3; therefore, miR-145-5p and E2F3 may serve as novel diagnostic biomarkers and therapeutic targets for osteosarcoma.

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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

HL, RP, QL and CR performed the experiments; CR also collected the samples. JS and HW contributed to the data analysis and presentation. JW and HC designed the experiment and wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics Committee of Guizhou Orthopedics Hospital approved. Written informed consent was obtained from the patients who provided the specimens. The present study was performed in accordance with the principles outlined in the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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