Effect of lncRNA XLOC_005950 knockout by CRISPR/Cas9 gene editing on energy metabolism and proliferation in osteosarcoma MG63 cells mediated by hsa-miR-542-3p

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Abstract. Cancer cells use glucose via glycolysis to maintain tumor cell proliferation. However, the effect of long non-coding RNAs (lncRNAs) on glycolysis in osteosarcoma (OS) cells remains unclear. The present study aimed to investigate the involvement of the lncRNA XLOC_005950/hsa-microRNA (miR)-542-3p/phosphofructokinase, muscle (PFKM) axis in the regulation of glucose metabolism, cell proliferation and apoptosis in the progression of OS. lncRNA XLOC_005950, hsa-miR-542-3p and PFKM expression in OS tissues and cells was detected via reverse transcription-quantitative PCR analysis. CRISPR/Cas9 gene editing was used to knockout lncRNA XLOC_005950 expression in MG63 cells. Cell Counting Kit-8 assay, flow cytometry, PFKM activity, and glucose and lactic acid content determination were performed to assess the effects of lncRNA XLOC_005950 knockout and overexpression of hsa-miR-542-3p on the phenotypes of OS cells. The dual-luciferase reporter assay was performed to confirm the targeting associations between lncRNA XLOC_005950, hsa-miR-542-3p and PFKM. The results demonstrated that lncRNA XLOC_005950 expression was upregulated in OS tissues and cells. Functional experiments indicated that lncRNA XLOC_005950 knockout decreased PFKM activity, the intracellular glucose and lactic acid content, and cell proliferation, while increasing apoptosis of OS cells. Furthermore, lncRNA XLOC_005950 knockout upregulated hsa-miR-542-3p expression and downregulated PFKM expression. Overexpression of hsa-miR-542-3p suppressed PFKM expression. Furthermore, lncRNA XLOC_005950, as the molecular sponge of miR-542-3p in OS, modulated the downstream target gene, PFKM. Taken together, the results of the present study suggest that lncRNA XLOC_005950 knockout may inhibit the progression of OS via hsa-miR-542-3p-mediated regulation of PFKM expression.

Introduction

Osteosarcoma (OS), originating from osteoblasts, is the most common primary malignant bone tumor, arising frequently in the metaphysis of long bones (1). Recently, the focus of research on OS has been on biological macromolecules and genes, including activated oncogenes (2), tumor suppressor gene inactivation (3), the role of tumor microangiogenesis in promoting tumor development (4), imbalance of cell proliferation and apoptosis (5), abnormal expression of biological macromolecules (6,7) and other factors (8) that jointly promote the occurrence and development of OS. Findings on the biological functions of OS may eventually be applied to the clinic.

Long non-coding RNAs (lncRNAs), which are widely distributed in the human genome to modulate gene expression, have a variety of biological functions, such as signaling (9), acting as molecular decoys (10) and guiding (11). The molecular decoy function of lncRNA is also called the 'sponge' function, which can sequester small non-coding RNAs [microRNAs (miRNAs/miRs)] away from their targets and suppress their function (10). For example, lncRNA myocardin-induced smooth muscle lncRNA, inducer of differentiation is significantly upregulated in gastric cancer, and regulates the proliferation and apoptosis of gastric cancer by regulating the miR-29c-3p/MCL1 apoptosis regulatory, BCL2 family member axis (12). Furthermore, in OS, the Alu-mediated p21 transcriptional regulator, as an oncogenic lncRNA, can inhibit miR-132-3p expression and promote OS cell proliferation and migration (13). lncRNA XLOC_005950 is located on chromosome 6q27 (14), and to the best of our knowledge, there are

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currently no studies regarding its function in OS. Thus, the role of lncRNA XLOC_005950 in regulating cell metabolism in OS needs to be clarified.

miRNAs in eukaryotic cells, which are 20-24 nucleotides in length, are important and abundant components of gene regulatory networks, which contain lncRNA-regulated miRNAs (15), miRNAs that reverse-regulate lncRNA (16) and lncRNAs that regulate mRNA expression by targeting miRNAs (17). miR-542-3p expression is downregulated in colorectal cancer, non-small cell lung cancer and hepatocellular carcinoma (18-20). In OS, Luo *et al* (21) reported that lncRNA ADP dependent glucokinase-antisense RNA 1 regulates OS cell proliferation and apoptosis by modulating miR-542-3p expression. However, the regulation of glucose metabolism by miR-542-3p in OS still requires further investigation.

Aberrant metabolism is a hallmark of human cancer (22). Malignant tumor cells use glucose via glycolysis even when oxygen is sufficient (23), which is called aerobic glycolysis or more commonly, the Warburg effect (24). The Warburg effect is controlled by glycolytic enzymes, including glucose transporter, hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) (25). PFK is a key rate-limiting enzyme in glycolysis (26). It has been reported that PFK activity significantly increases in certain tumor cells and tissues (27-29). PFK, muscle (PFKM) is a known isoform of PFK in humans (26). A previous study demonstrated that reduced glycolysis induced by miR-21 is mediated through targeting of the PFKM isoform at the committed step of glycolysis (30). Thus, altering the metabolism by interfering with the aerobic glycolytic pathway of tumor cells is important for studying the biological behavior of tumors. Previous studies have reported that lncRNAs regulate glycolysis by sponging miRNAs (31,32).

The present study aimed to investigate the effect of the regulation of the lncRNA-miRNA-mRNA network on the energy metabolism pathway of aerobic glycolysis in OS cells, and investigate the effects of this regulatory axis on cell proliferation and apoptosis.

Materials and methods

Patients and tissue samples. Tumor tissues and corresponding adjacent normal tissues (3 cm away from the tumor tissues) were collected from 25 patients (age range, 7-65 years; mean age, 24.76 years; 14 men and 11 women) with OS at the Department of Orthopedic Surgery of The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) between September 2015 and March 2017. All tissue samples collected during tumor resection were immediately frozen in liquid nitrogen and stored at -80°C until subsequent experimentation. The present study was approved by the Medical Ethics Committee of Zhengzhou University (Zhengzhou, China; approval no. 2015-03) and patients provided written informed consent prior to the study start. Patient characteristics are listed in Table I. The mean lncRNA XLOC_005950 expression in 25 OS tissues was regarded as the cut-off point.

Cell culture and transfection. The human osteoblastic cell line, hFOB1.19, and three human OS cell lines, MG63, U2OS and Saos-2, were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. MG63,

U2OS and Saos-2 cells were maintained in DMEM (Biological Industries) supplemented with 10% fetal bovine serum (FBS, Biological Industries) and 1% penicillin/streptomycin (Beijing Solarbio Science & Technology Co., Ltd.), at 37°C with 5% CO_2. Human hFOB 1.19 cells were maintained in DMEM/F12 medium (Biological Industries) supplemented with 15% FBS (Gibco; Thermo Fisher Scientific, Inc.), at 33.5°C with 5% CO_2. Cells were harvested for subsequent experimentation after 48 h.

hsa-miR-542-3p mimics negative control (NC), hsa-miR-542-3p mimics, hsa-miR-542-3p inhibitor NC and hsa-miR-542-3p inhibitor were purchased from Shanghai GenePharma Co., Ltd. The sequences were as follows: hsa-miR-542-3p inhibitor, 5'-UUCAGUUAUCAAUCUGUCACA-3'; hsa-miR-542-3p inhibitor NC, 5'-CAGUACUUUUGUGUAGUACAA-3'; hsa-miR-542-3p mimics, 5'-UGUGACAGAUUGAUAACUGAAA-3'; hsa-miR-542-3p mimics NC, 5'-UUCUCCGAACGUGUCACGUTT-3'. MG63 cells were transfected with 50 nM miR-542-3p mimics or miR-542-3p NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO_2. Following incubation for 6 h at 37°C, the medium was replaced with fresh complete DMEM and cultured for 36-48 h. Similarly, MG63 cells were transfected with 50 nM miR-542-3p inhibitor using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Transfected cells were immediately used for subsequent experiments.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was isolated from cells (hFOB1.19, MG63, U2OS and Saos-2 cells) and tissues using TransZol reagent (TransGen Biotech, Co., Ltd.). The RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). lncRNA and mRNA were reverse transcribed into cDNA using the PrimeScript™ RT Reagent kit with gDNA Eraser (Perfect Real Time) (Takara Biotechnology Co., Ltd.), according to the manufacturer's instructions. miRNA RT was performed with a stem-loop primer at 42°C for 60 min and 70°C for 5 min, using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). qPCR was subsequently performed on an ABI 7500 Fast system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR Premix Ex Taq II kit (Takara Biotechnology Co., Ltd.). The following thermocycling conditions were used for qPCR: Initial denaturation at 95°C for 30 sec; followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. The primer sequences used for qPCR were designed by Primer Premier 6.0 and synthesized by Sangon Biotech Co., Ltd. (Table II). Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (33). β-actin and U6 were used as endogenous controls for mRNA and miRNA expression levels, respectively. All experiments were performed in triplicate.

Plasmid construction. sgRNA was designed (http://crispr.mit.edu), synthesized (Sangon Biotech Co., Ltd.) and inserted into a PX459 vector (Addgene, Inc.) digested by BbsI. The sgRNA sequences directed against lncRNA XLOC_005950 were annealed and cloned into the PX459 vector. A total of two pairs of independent sgRNA sequences targeting lncRNA XLOC_005950 were designed: Forward oligo1,

Table I. Association between lncRNA XLOC_005950 expression and the clinicopathological characteristics of patients with osteosarcoma (n=25).

| | | lncRNA XLOC_005950 expression | | |
Characteristic	Number of cases	Low (n=15)	High (n=10)	P-value
Sex				0.7422
Male	14	8	6	
Female	11	7	4	
Age, years				0.6098
≤15	16	9	7	
>15	9	6	3	
Primary site				0.8702
Femur	13	8	5	
Tibia/Humerus	12	7	5	
Tumor size, cm				0.2936
≤4	8	6	2	
>4	17	9	8	
Clinical stage				0.0270[a]
I+II	9	8	1	
III	16	7	9	
Metastasis				0.0124[a]
Negative	10	9	1	
Positive	15	6	9	

[a]P<0.05. lncRNA, long non-coding RNA.

Table II. Primer sequences used for quantitative PCR.

Gene	Primer sequence (5'-3')
hsa-miR-542-3p	TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTTCAG
U6	AACGCTTCACGAATTTGCGT
lncRNA XLOC_005950	F: GTTCAGGACATAGGTGGATT
	R: CGACACTATCAGAGGCATT
PFKM	F: ACCCGTGGTTCTCGTCTC
	R: AAAGGCTGATGGCGTCCC
β-actin	F: CTCCATCCTGGCCTCGCTGT
	R: GCTGTCACCTTCACCGTTC

miR, microRNA; lncRNA, long non-coding RNA; PFKM, phosphofructokinase, muscle; F, forward primer; R, reverse primer.

5'-CACCGGGAGTGCGTTCGGTGTGCCG-3' and reverse oligo1, 5'-AAACCGGCACACCGAACGCACTCCC-3'; and forward oligo2, 5'-CACCGCAGGTGCATGCCAATATAC C-3' and reverse oligo2, 5'-AAACGGTATATTGGCATGCAC CTGC-3'. Successful insertion of each sgRNA was verified by sequencing.

CRISPR/Cas9 gene editing. A total of 4x10⁵ MG63 cells were seeded into 6-well plates and cultured for 24 h. After reaching 70-80% confluency, cells were transfected with PX459 plasmid vectors carrying sgRNAs using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 6 h post-incubation, the medium was replaced with fresh complete DMEM. After 48 h, puromycin (0.5 μg/μl) was added to the medium, and the transfected cells were subjected to puromycin selection. After 2 weeks of transfection, the surviving cells were expanded in the presence of 5 μg/ml puromycin. The expression of lncRNA XLOC_005950 in the cells was detected by genomic PCR analysis using the following primer sequences: Forward, 5'-CCTGCCAGTGTCTCCGCCGGT T-3' and reverse, 5'-GCCTGACCAACATGGTGAAGC-3'. The following thermocycling conditions were used for the PCR:

Initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; and a final step at 72°C for 10 min. The corresponding PCR-amplified fragments were ligated into pGEM-T vectors (pGEM-T Easy; Promega Corporation). The genome deletions were verified by sequencing prior to subsequent experimentation. CRISPR/Cas9 gene editing was used to knock out the genomic DNA fragments of lncRNA XLOC_005950 containing the binding sites of miR-542-3p in MG63 cells. The knockout MG63 cell line was labeled as MG63^{-/-} in the present study.

Glucose uptake and lactate production assay. Glucose, lactate and PFK assay kits were used to determine glucose content, lactic acid content and activities of PFK, respectively, following the manufacturer's instructions (cat. nos. BC2500; BC2230; BC0530; Beijing Solarbio Science & Technology Co., Ltd.). Briefly, the cultured cells (hFOB1.19, MG63 and MG63^{-/-} cells) were digested with 0.25% trypsin and centrifuged at 960 x g for 5 min at room temperature to obtain the supernatant. Subsequently, $5x10^6$ cells were lysed in 1 ml assay buffer for each kit, and the cells were sonicated by ultrasound and centrifuged to obtain the supernatant for detection. The detection reagents of the three kits were added to the supernatant sample of each respective group following the corresponding instructions. Following incubation at 37°C (for 15 min for glucose detection, 20 min for lactate detection and 10 min for PFK activity detection), the absorbance of the samples was measured using a spectrophotometer: The glucose content was measured at a wavelength of 505 nm; the lactic acid content at a wavelength of 570 nm and the PFK activity at a wavelength of 340 nm. The standard curve of lactic acid content detection was determined according to the standard product. The results in each group were normalized to the cell number, and independent experiments were performed in triplicate.

Cell proliferation assay. The Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc.) assay was performed to assess cell proliferation. Briefly, hFOB1.19, MG63 and knockout cells were seeded into 96-well plates (five replicate wells/group), with $2x10^3$ cells/well. MG63 cells were transfected with 40 nM miR-542-3p mimics (or miR-542-3p NC) and liposomes, and the medium was changed after 5 h. Subsequently, at 0, 24, 48 and 72 h, CCK-8 solution (10 μl/well) was added to each well. Following incubation for 1 h at 37°C with 5% CO_2, the absorbance of each well was measured at a wavelength of 450 nm, using a microplate reader (Bio-Rad Laboratories, Inc.).

Cell apoptosis assay. Annexin V-FITC/propidium iodide (PI) apoptosis assay reagent (cat. no. C1062L; Beyotime Institute of Biotechnology) was used to determine the apoptotic rate. Following transfection for 48 h, MG63 cells were transfected with 50 nM miR-542-3p mimics or miR-542-3p NC and washed twice with PBS at room temperature for 30 sec. The cells ($1x10^5$ cells/ml) were centrifuged at 1,000 x g for 5 min at room temperature to discard the supernatant and resuspended in 195 μl binding buffer (cat. no. C1062L-2; Beyotime Institute of Biotechnology). Subsequently, 5 μl Annexin V-FITC and 10 μl PI were added for double staining for 15 min at room temperature in the dark. Following incubation, centrifuge

tubes containing the stained cells were placed in an ice water bath, avoiding light. The rate of apoptosis was analyzed using a FACSCalibur flow cytometer (BD Biosciences). On the flow cytometry scattergrams, cells in the upper-left quadrant labeled as Annexin V-FITC[-]/PI[+] were designated as mechanically damaged cells and necrotic cells; cells in the upper-right quadrant tagged as Annexin V-FITC[+]/PI[+] were designated as late-apoptotic cells; cells in the lower-right quadrant labeled as Annexin V-FITC[+]/PI[-] were designated as early apoptotic cells; and cells in the lower-left quadrant labeled as Annexin V-FITC[-]/PI[-] were designated as viable cells.

Western blotting. Total protein was extracted from hFOB1.19 and MG63 cells using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Protein concentration was quantified using the BCA protein assay kit (Beijing Solarbio Science & Technology Co., Ltd.). The extracted proteins (50 μg/lane) were separated via SDS-PAGE (8%) and transferred onto PVDF membranes (EMD Millipore), using the wet transfer method. The membranes were blocked with 5% non-fat milk for 1.5 h at room temperature and then incubated with primary antibodies against PFKM, pyruvate kinase M1/2 (PKM2), HK2 (all diluted 1:200; cat nos. sc-166722; sc-365684; sc-374091, respectively) and β-actin (diluted 1:1,000; cat. no. sc-8432) (all from Santa Cruz Biotechnology, Inc.), overnight at 4°C. After washing with TBST, membranes were incubated with secondary antibodies (diluted 1:2,000; cat. no. sc-2031; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Protein bands were detected and visualized using an enhanced chemiluminescence reagent kit (Beyotime Institute of Biotechnology). β-actin was used as the loading control for normalization.

Bioinformatics predictions and luciferase reporter assay. Potential miRNAs interacting with lncRNA XLOC_005950 were predicted using DIANA tools (http://carolina.imis. athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2/ index). Putative binding sites of hsa-miR-542-3p and PFKM were predicted using TargetScan (http://www.targetscan.org/ vert_71), miRBase (http://www.mirbase.org) and miRWalk (http://mirwalk.umm.uni-heidelberg.de).

Dual-luciferase reporter assay. Genomic DNA was used as the template, and PCR was applied to obtain the PFKM 3'-untranslated region (UTR) and lncRNA XLOC_005950 wild-type (wt) sequences containing hsa-miR-542-3p binding sites to construct pGL3-promoter-PFKM-wt and pGL3-promoter-Lnc-wt vectors. pGL3-Promoter vector and pRL-TK vector were purchased from Promega Corporation. The primer sequences were as follows: PFKM forward, 5'-GCT CTACCTAATAAGTCCACATCTTCTC-3' and reverse, 5'-AAAGGCCGGCCCAGACAGCCAGCAAGTAG-3'; and lncRNA XLOC_005950 forward, 5'-GCTCTAGATGTCTCC GCCGGTTGAAA-3' and reverse, 5'-AAAGGCCGGCCC TGACCAACATGGTGAAGC-3'.

In addition, mutant (mut) segments of lncRNA XLOC_005950 and PFKM mRNA 3'-UTR were constructed by overlap-extension PCR, generating pGL3-promoter-Lnc-mut and pGL3-promoter-PFKM-mut vectors. MG63 cells were seeded into 24-well plates and co-transfected with
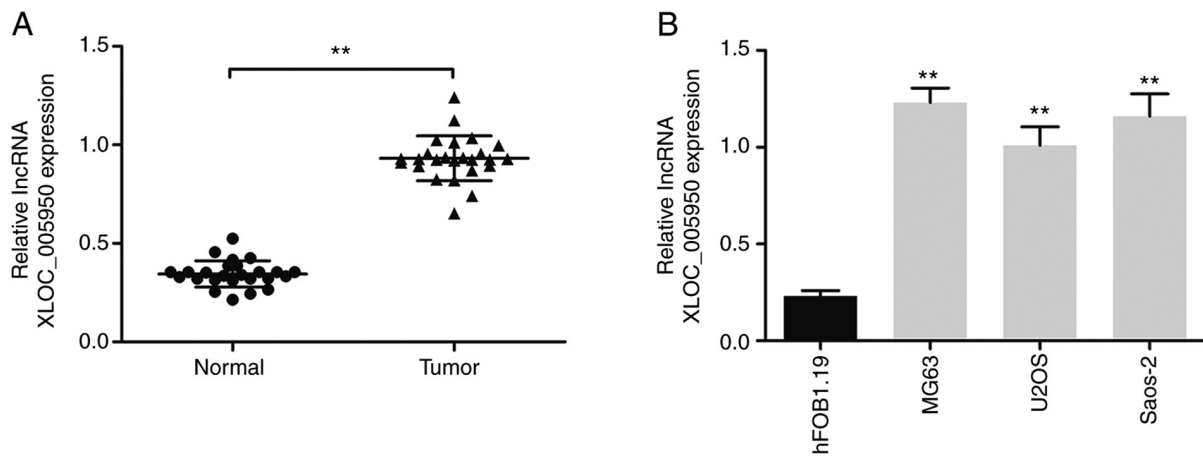
Figure 1. lncRNA XLOC_005950 expression is upregulated in OS tissues and cell lines. (A) RT-qPCR analysis was performed to detect lncRNA XLOC_005950 expression in 25 osteosarcoma tissues and paired normal tissues. (B) RT-qPCR analysis was performed to detect lncRNA XLOC_005950 expression in OS cell lines (MG63, U2OS and Saos-2) and human osteoblast hFOB1.19 cells. Data are presented as the mean ± standard deviation (n=3). **P<0.01. lncRNA, long non-coding RNA; OS, osteosarcoma; RT-qPCR, reverse transcription-quantitative PCR.

plasmids and hsa-miR-542-3p mimics or NC for 36 h using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 36 h, firefly luciferase and *Renilla* luciferase activities were measured on a microplate luminometer (Centro XS LB960; Titertek-Berthold) using a Dual Luciferase Reporter Assay kit (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis. Statistical analysis was performed using SPSS 19.0 software (IBM Corp.) and GraphPad Prism 5.0 software (GraphPad Software, Inc.). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. χ^2 and Fisher's exact tests were used to assess the association between lncRNA XLOC_005950 expression and the clinicopathological characteristics of patients with OS. Unpaired Student's t-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's post hoc test were used to compare differences between multiple groups. Pearson's correlation analysis was performed to determine the correlations between lncRNA XLOC_005950 and miR-542-3p, and miR-542-3p and PFKM. P<0.05 was considered to indicate a statistically significant difference.

Results

lncRNA XLOC_005950 expression is upregulated in OS tissues and cell lines. lncRNA XLOC_005950 expression was detected in 25 paired OS tissues and matched adjacent normal tissues via RT-qPCR analysis. The results demonstrated that lncRNA XLOC_005950 expression was upregulated in OS tissues and cell lines (MG63, U2OS and Saos-2) compared with adjacent normal tissues and human normal osteoblasts hFOB1.19, respectively (P<0.01; Fig. 1A and B). lncRNA XLOC_005950 expression was highest in MG63 cells compared with hFOB1.19 cells (Fig. 1B). Thus, MG63 cells were selected to construct lncRNA XLOC_005950 knockout cells. Furthermore, the clinical data indicated that lncRNA XLOC_005950 expression was significantly associated with

clinical stage and metastasis (Table I). Taken together, these results suggest that lncRNA XLOC_005950 expression is upregulated in OS tissues and MG63 cells, which may be involved in the progression of OS.

lncRNA XLOC_005950 knockdown triggers changes in aerobic glycolysis and inhibits PFKM expression in MG63 cells. To determine the potential biological significance of lncRNA XLOC_005950 in OS cells, a loss-of-function experiment was performed in MG63 cells. CRISPR/Cas9 gene editing was used to knock out lncRNA XLOC_005950 expression in MG63 cells. Sequencing demonstrated that the genomic DNA fragments of lncRNA XLOC_005950 containing the binding sites of miR-542-3p was knocked out in MG63 cells (Fig. 2A). Given that the experimental period required for using CRISPR technology to construct knockout cell lines was relatively long in the present study, only MG63 knockout cells were constructed to observe the effect of lncRNA XLOC_005950 knockout on the cell phenotype.

Subsequently, the changes in aerobic glycolysis in OS cells following lncRNA XLOC_005950 knockdown were investigated. The results demonstrated that lncRNA XLOC_005950 knockdown via transfection with PX459 with sgRNA in MG63 cells markedly decreased glucose consumption and lactate production, and decreased PFKM activity (P<0.05 and P<0.01; Fig. 2B-D). Inhibition of lncRNA XLOC_005950 in the MG63^{-/-} group decreased PFKM rather than HK2 and PKM2 expression (Fig. 2E). Collectively, these results suggest that knockout of lncRNA XLOC_005950 can decrease glucose and lactic content, as well as decrease PFKM activity and protein expression, thus impairing aerobic glycolysis.

lncRNA XLOC_005950 knockdown inhibits MG63 cell proliferation and promotes cell apoptosis. Aerobic glycolysis, the main energy supply pattern of OS cells (24), was partially inhibited by knocking out lncRNA XLOC_005950 specific fragments. The present study investigated whether it could affect the proliferation and apoptotic abilities of
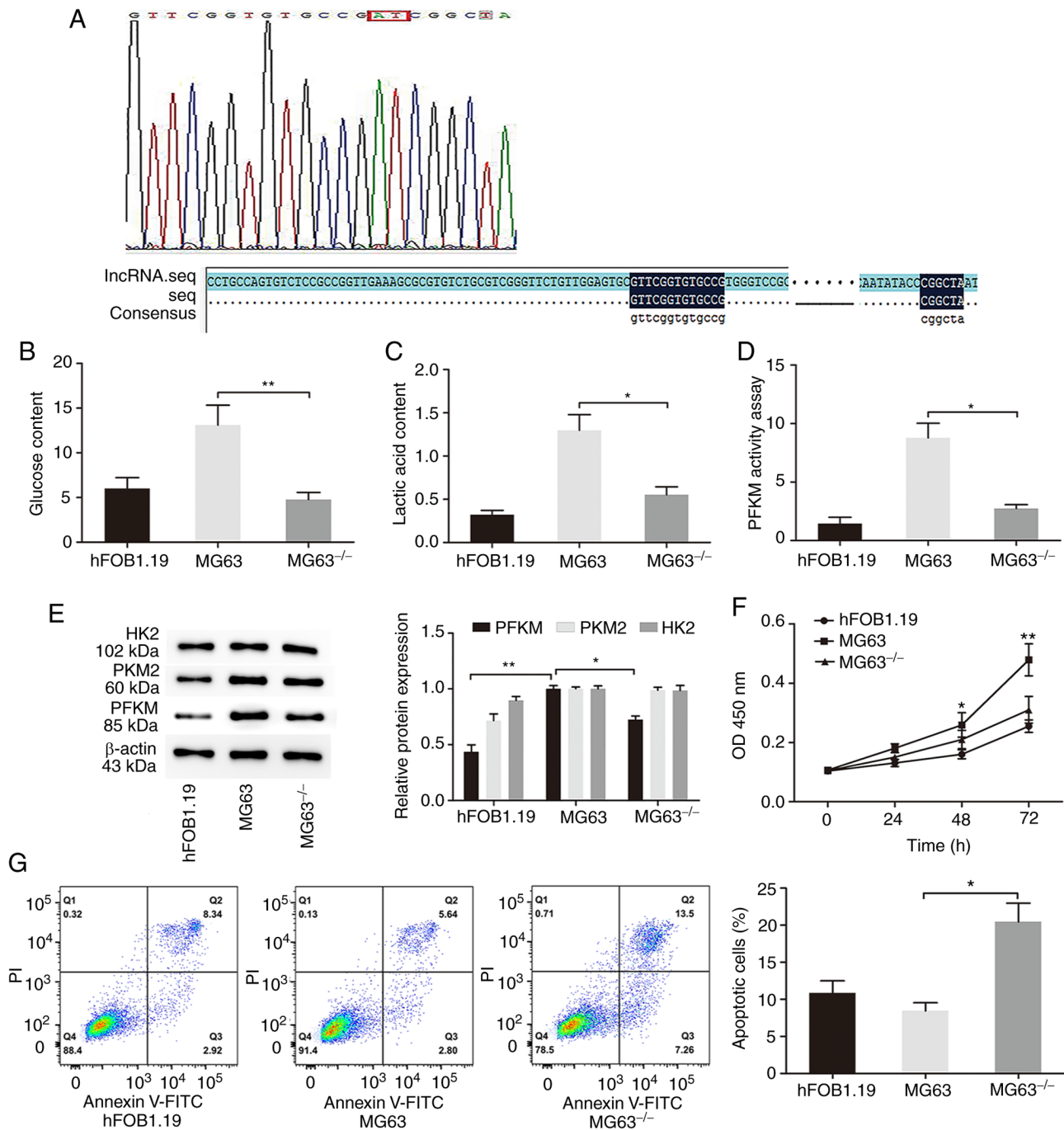
Figure 2. lncRNA XLOC_005950 knockdown triggers changes in osteosarcoma cells in terms of glycolysis and inhibits proliferation while promoting cell apoptosis. (A) Following CRISPR/Cas9 gene editing to knockout lncRNA XLOC_005950 expression in MG63 cells, sequencing demonstrated that the specific fragments of lncRNA XLOC_005950 had been knocked out. (B) Glucose consumption, (C) lactate production and (D) PFKM activity were detected in hFOB1.19, MG63 and knockout cells. (E) Western blot analysis was performed to detect the protein expression levels of hexokinase 2, PFKM and PKM2 in hFOB1.19, MG63 and knockout cells. β-actin was used as the internal control for normalization. (F) The Cell Counting Kit-8 assay was performed to assess cell proliferation. (G) Flow cytometry was performed to detect the apoptotic rates of cells. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01. MG63$^{-/-}$, MG63 cells with lncRNAXLOC_005950 knocked out. lncRNA, long non-coding RNA; PFKM, phosphofructokinase, muscle; PKM2, pyruvate kinase M1/2; OD, optical density.

OS cells. The results of the CCK-8 assay demonstrated that the proliferation of the MG63$^{-/-}$ group markedly decreased compared with that of the MG63 group (P<0.01; Fig. 2F). Furthermore, flow cytometry analysis was performed to detect cell apoptosis. The results demonstrated that the apoptotic rate of the MG63$^{-/-}$ group markedly increased compared with the MG63 group, and the difference was statistically significant (P<0.05; Fig. 2G). Taken together,

these results suggest that lncRNA XLOC_005950 knockdown suppresses OS cell proliferation and induces apoptosis *in vitro*.

lncRNA XLOC_005950 is a target of hsa-miR-542-3p. To determine the potential molecular mechanism by which lncRNA XLOC_005950 is involved in OS progression, the correlations between lncRNA XLOC_005950 and miRNAs
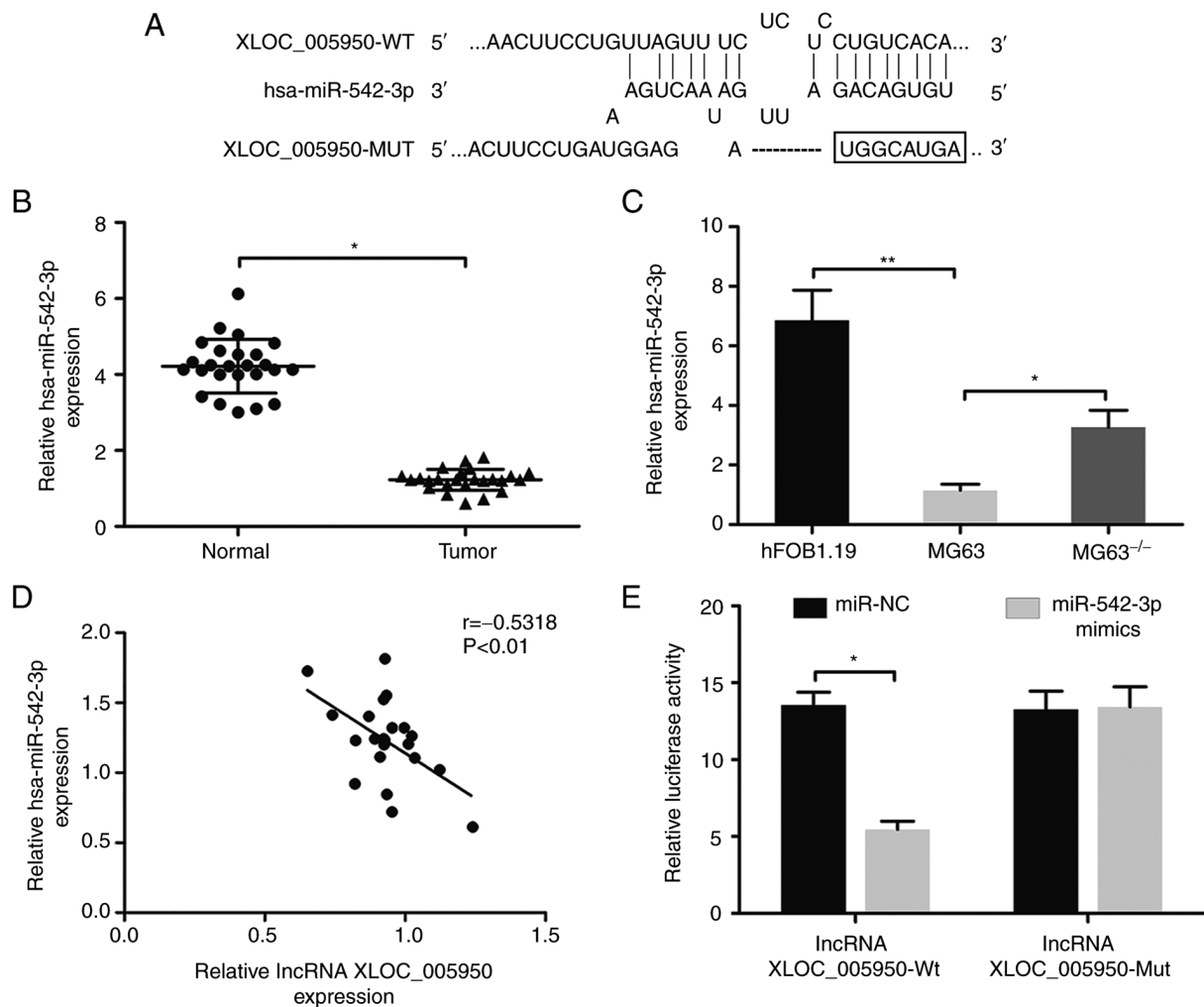
Figure 3. lncRNA XLOC_005950 is a target of hsa-miR-542-3p. (A) Binding sites of hsa-miR-542-3p and lncRNA XLOC_005950. (B) RT-qPCR analysis was performed to detect hsa-miR-542-3p expression in 25 paired OS tissues and adjacent normal tissues. (C) RT-qPCR analysis was performed to detect hsa-miR-542-3p expression in MG63, MG63$^{-/-}$ and human osteoblast hFOB1.19 cells. (D) Correlation analysis in OS specimens revealed a negative correlation between lncRNA XLOC_005950 and hsa-miR-542-3p expression. (E) MG63 cells were co-transfected with hsa-miR-542-3p mimics and pGL3-promoter-lnc-wt vector or pGL3-promoter-lnc-mut vector. The relative luciferase activity was detected via the dual-luciferase reporter assay. Data are presented as the mean ± standard deviation (n=3). *$P<0.05$, **$P<0.01$. MG63$^{-/-}$, MG63 cells with lncRNA XLOC_005950 knocked out. lncRNA, long non-coding RNA; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; OS, osteosarcoma; wt, wild-type; mut, mutant; NC, negative control.

were investigated. According to the results of DIANA tools, lncRNA XLOC_005950 contains binding sites for hsa-miR-542-3p (Fig. 3A). RT-qPCR analysis demonstrated that hsa-miR-542-3p expression was significantly downregulated in OS tissues compared with adjacent normal tissues ($P<0.05$; Fig. 3B). Similarly, the data indicated that hsa-miR-542-3p expression was downregulated in MG63 cells ($P<0.01$; Fig. 3C). Notably, lncRNA XLOC_005950 knockdown upregulated hsa-miR-542-3p expression ($P<0.05$; Fig. 3C). In addition, RT-qPCR analysis revealed a negative correlation between lncRNA XLOC_005950 and hsa-miR-542-3p expression (r=-0.5318, $P<0.01$; Fig. 3D). The dual-luciferase reporter assay was performed to verify the direct interaction between lncRNA XLOC_005950 and hsa-miR-542-3p. As presented in Fig. 3E, overexpression of hsa-miR-542-3p decreased the fluorescence intensity of the vector carrying pGL3-promoter-Lnc-wt. Collectively, these results suggest that recognition sites in hsa-miR-542-3p can directly bind to the lncRNA XLOC_005950 sequence.

hsa-miR-542-3p impairs glycolysis and suppresses PFKM expression in MG63 cells. The present study investigated the role of hsa-miR-542-3p in lncRNA XLOC_005950 knockout-induced glycolysis inhibition of OS cells. The results demonstrated that hsa-miR-542-3p expression was upregulated following transfection with hsa-miR-542-3p mimics in MG63 cells ($P<0.05$; Fig. 4A). Overexpression of hsa-miR-542-3p markedly decreased glucose and lactate content, as well as PFKM activity in MG63 cells ($P<0.05$; Fig. 4D-F).

Western blot analysis was performed to determine the regulatory mechanism of hsa-miR-542-3p on PFKM expression in OS cells. The results demonstrated that overexpression of hsa-miR-542-3p markedly decreased PFKM protein expression ($P<0.05$; Fig. 4B). Taken together, these results suggest that overexpression of hsa-miR-542-3p impairs glycolysis and suppresses PFKM protein expression in MG63 cells.

hsa-miR-542-3p suppresses proliferation and induces apoptosis in MG63 cells. Given that hsa-miR-542-3p inhibited
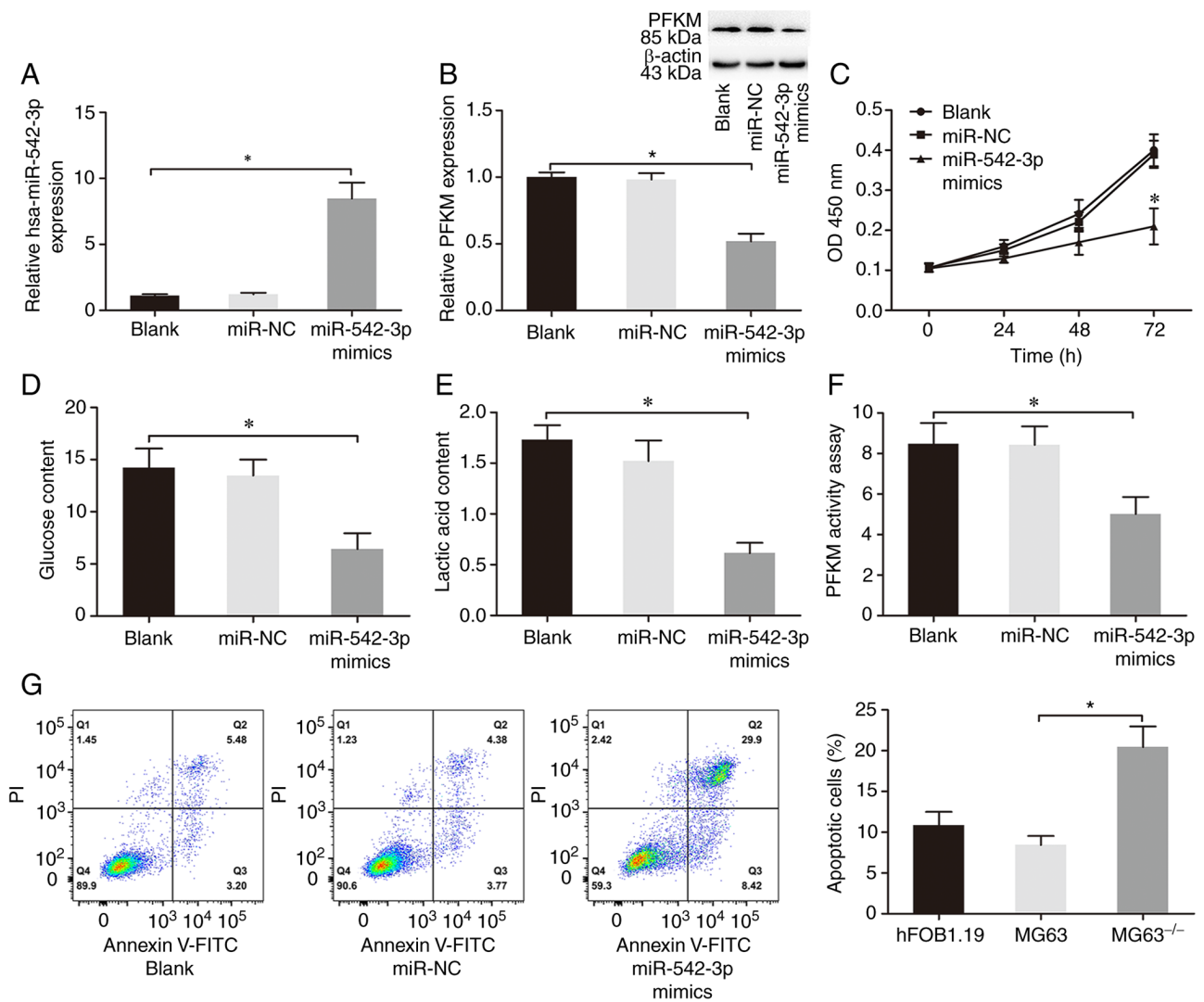
Figure 4. hsa-miR-542-3p impairs glycolysis, suppresses proliferation and induces apoptosis in OS cells. (A) MG63 cells were transfected with miR-NC or hsa-miR-542-3p mimics, and reverse transcription-quantitative PCR analysis was performed to detect hsa-miR-542-3p expression. (B) Western blot analysis was performed to detect PFKM protein expression in MG63 cells transfected with miR-NC or hsa-miR-542-3p mimics. β-actin was used as the internal control for normalization. (C) The Cell Counting Kit-8 assay was performed to assess cell proliferation. (D) Glucose and (E) lactic acid content, and (F) PFKM activity were determined in the blank, miR-NC and miR-542-3p groups. (G) Flow cytometric analysis was performed to detect the apoptotic rates of cells. Data are presented as the mean ± standard deviation. *P<0.05. Blank, MG63 cells transfected with Lipofectamine® 2000; miR-NC, MG63 cells transfected with hsa-miR-542-3p NC and liposomes; miR-542-3p mimics (hsa-miR-542-3p mimics), MG63 cells transfected with hsa-miR-542-3p mimics and liposomes. miR, microRNA; OS, osteosarcoma; NC, negative control; PFKM, phosphofructokinase, muscle; OD, optical density.

glycolysis in MG63 cells, the roles of hsa-miR-542-3p in the proliferation and apoptosis of OS cells were investigated. The results of the CCK-8 assay demonstrated that over-expression of hsa-miR-542-3p significantly suppressed cell proliferation (P<0.05; Fig. 4C). In addition, overexpression of hsa-miR-542-3p significantly induced MG63 cell apoptosis compared with the control and blank groups (P<0.05; Fig. 4G). Collectively, these results suggest that hsa-miR-542-3p, as a tumor suppressor in OS (18-20), can arrest cell proliferation and promote apoptosis in MG63 cells.

hsa-miR-542-3p mediates the effect of lncRNA XLOC_005950 on the metabolism and proliferation of MG63 cells. To determine whether hsa-miR-542-3p plays an important role in the development of lncRNA XLOC_005950-mediated OS, a hsa-miR-542-3p inhibitor was transfected into lncRNA XLOC_005950-knockout MG63 cells. The upregulation of

miR-542-3p mediated by lncRNA XLOC_005950 knockout was reversed following transfection with miR-542-3p inhibitor (P<0.01; Fig. 5A). In addition, rescue experiments indicated that the inhibitory effect caused by lncRNA XLOC_005950 knockout on cell glucose and lactate content, as well as on PFKM activity, were reversed following transfection with miR-542-3p inhibitor (P<0.05 and P<0.01; Fig. 5B-D). Taken together, these results suggest that lncRNA XLOC_005950 regulates MG63 cell metabolism and proliferation by targeting hsa-miR-542-3p.

lncRNA XLOC_005950 controls the hsa-miR-542-3p target, PFKM. To further determine whether hsa-miR-542-3p is involved in lncRNA XLOC_005950-regulated PFKM expression, its potential targets were predicted using the TargetScan, miRBase and miRWalk databases. The results revealed that a hsa-miR-542-3p binding site existed in the
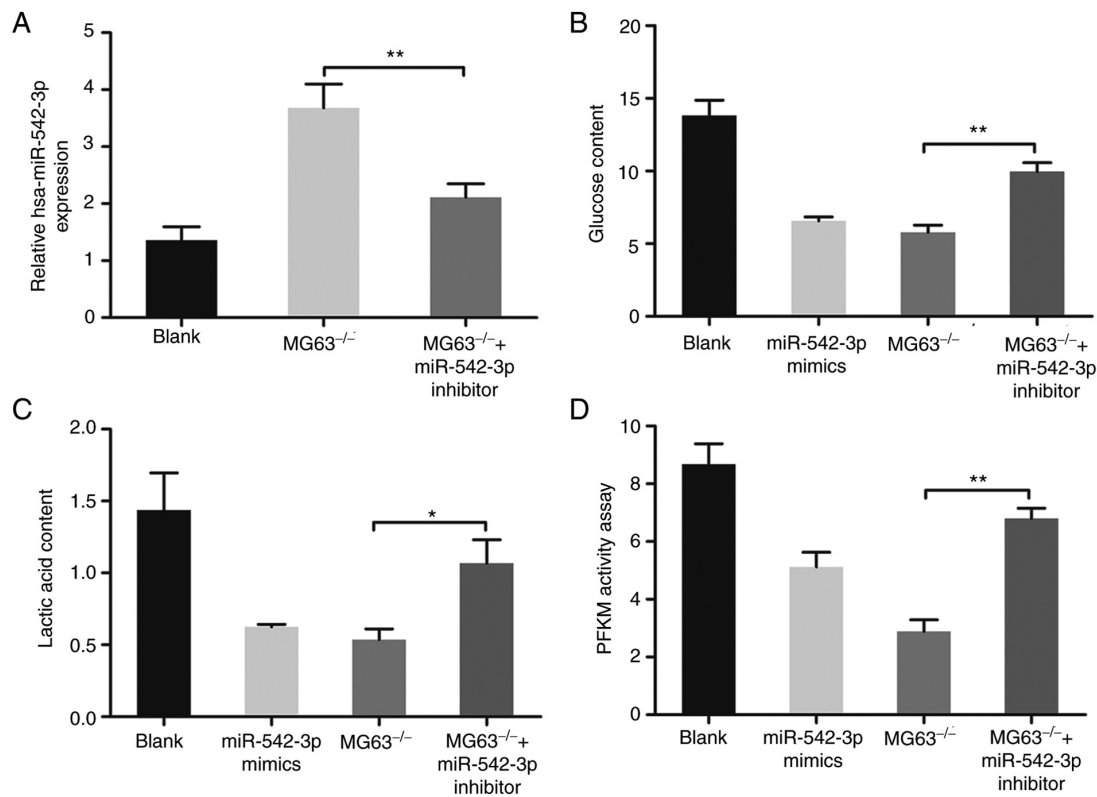
Figure 5. hsa-miR-542-3p mediates the effect of lncRNA XLOC_005950 on the metabolism and proliferation of MG63 cells. (A) Reverse transcription-quantitative PCR analysis was performed to detect miR-542-3p expression in MG63 cells, knockout MG63 cells and knockout MG63 cells transfected with miR-542-3p inhibitor. (B) Glucose consumption, (C) lactate production and (D) PFKM activity were detected in MG63 cells, knockout MG63 cells and knockout cells transfected with miR-542-3p inhibitor. $^*P<0.05$, $^{**}P<0.01$. Blank, MG63 cells transfected with Lipofectamine® 2000; miR-542-3p mimics (hsa-miR-542-3p mimics), MG63 cells transfected with hsa-miR-542-3p mimics and liposomes; MG63$^{-/-}$, MG63 cells with lncRNAXLOC_005950 knocked out; MG63$^{-/-}$ + miR-542-3p inhibitor, knocked out MG63 cells transfected with miR-542-3p inhibitor and liposomes. miR, microRNA; lncRNA, long non-coding RNA; PFKM, phosphofructokinase, muscle.

PFKM 3'-UTR (Fig. 6A). The luciferase activity of the pGL3-promoter-PFKM-wt reporter plasmid significantly decreased in the presence of hsa-miR-542-3p mimics (Fig. 6B). These results suggest that PFKM is a downstream target of hsa-miR-542-3p in OS.

RT-qPCR analysis demonstrated that PFKM expression was upregulated in OS tissues and MG63 cells compared with adjacent normal tissues and normal osteoblast hFOB1.19 cells, respectively (P<0.01; Fig. 6C and D). Furthermore, lncRNA XLOC_005950 knockdown inhibited PFKM protein expression, and lncRNA XLOC_005950 bound to hsa-miR-542-3p to inhibit hsa-miR-542-3p expression. To analyze the regulatory pathway, RT-qPCR analysis was performed, and the results demonstrated that lncRNA XLOC_005950 knockout or overexpression of hsa-miR-542-3p significantly decreased PFKM expression in MG63 cells (P<0.05; Fig. 6E and F). In addition, lncRNA XLOC_005950 and PFKM expression were highly expressed in OS tissues and cells (Figs. 1A and B, 6C and D), while hsa-miR-542-3p expression decreased (Fig. 3B and C). Correlation analysis revealed a negative correlation between hsa-miR-542-3p and PFKM expression in OS tissues (r=-0.5815, P<0.01; Fig. 6G). Notably, lncRNA XLOC_005950 knockdown increased hsa-miR-542-3p expression and suppressed PFKM expression, thus preventing glycolysis in OS cells, inhibiting the main energy supply of tumor cells and inhibiting the proliferation of OS cells (Fig. 7).

Collectively, these results suggest that lncRNA XLOC_005950 regulates PFKM expression via hsa-miR-542-3p, affecting the glycolytic pathway of MG63 cells, and regulating cell proliferation and apoptosis.

Discussion

Previous studies have reported a variety of complex roles for lncRNAs in the regulation of gene expression, involving several cellular processes (15,17,34). Aberrant expression of lncRNA is associated with the occurrence and development of several diseases, including cancer (35,36). Zheng and Min (37) demonstrated that HOX transcript antisense RNA (HOTAIR) is highly expressed in OS cell lines. HOTAIR knockdown (using small interfering RNA) decreased the proliferation rate of OS cells and promoted cell apoptosis. In a previous study, lncRNA XLOC_005950 was reported in gene chip analysis by Cabili *et al* (14). In the preliminary experiment of this research, based on analysis results by Cabili *et al*, RT-qPCR was performed to detect the expression of multiple lncRNAs in hFOB1.19 and MG63 cells. lncRNA XLOC_005950 was the highest expression in MG63 cells compared with normal osteoblast hFOB1.19, and was selected as the research target. The present study used CRISPR/Cas9-based genome editing system to analyze the effects of lncRNA XLOC_005950 deletion in MG63 cells. The results demonstrated that the efficiency
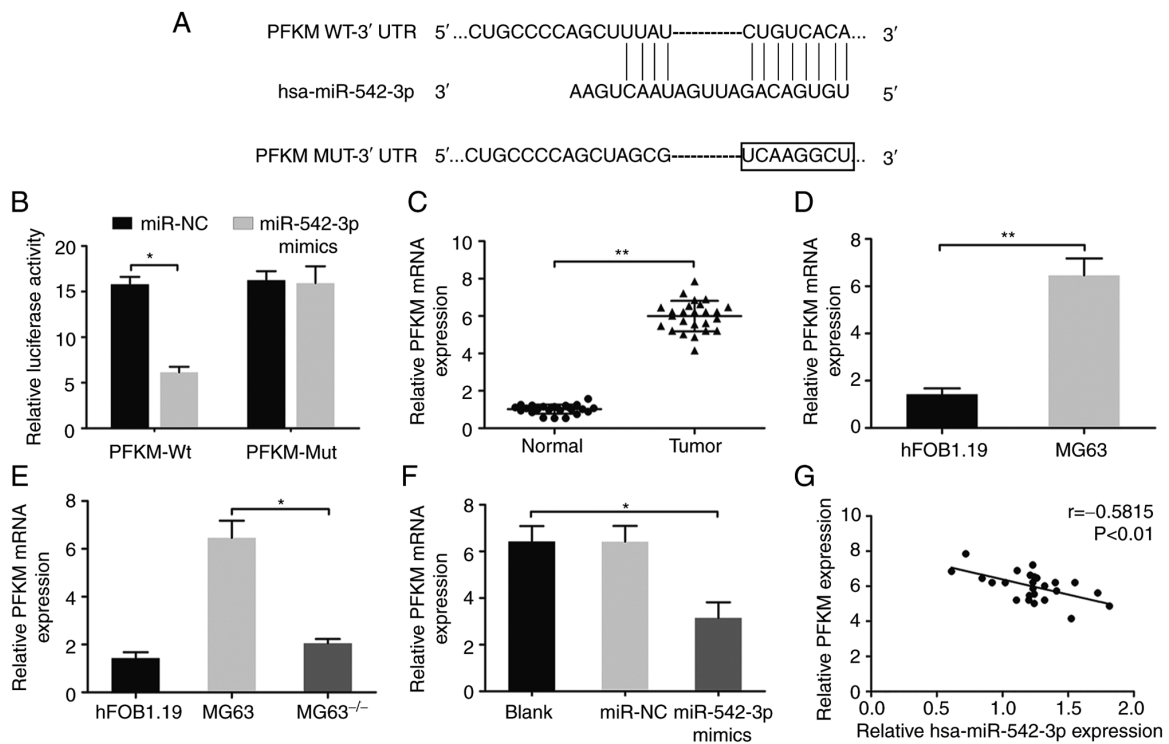
Figure 6. lncRNA XLOC_005950 regulates the expression of the hsa-miR-542-3p target, PFKM. (A) A predicted hsa-miR-542-3p binding site was identified to exist in the PFKM 3'-UTR. (B) MG63 cells were co-transfected with hsa-miR-542-3p mimics and PFKM 3'-UTR reporter construct. (C) Relative expression of PFKM in 25 paired OS tissues and adjacent normal tissues. (D) Relative expression of PFKM in MG63 cells and human osteoblast hFOB1.19 cells. Reverse transcription-quantitative PCR analysis was performed to detect PFKM mRNA expression in MG63 cells following (E) lncRNA XLOC_005950 knockdown or (F) overexpression of hsa-miR-542-3p. (G) A negative correlation was observed between hsa-miR-542-3p and PFKM expression in OS specimens. Data are presented as the mean ± standard deviation (n=3). $^*P<0.05$, $^{**}P<0.01$. Blank, MG63 cells transfected with Lipofectamine® 2000; miR-NC, MG63 cells transfected with hsa-miR-542-3p NC and liposomes; miR-542-3p mimics (hsa-miR-542-3p mimics), MG63 cells transfected with hsa-miR-542-3p mimics and liposomes. MG63$^{-/-}$, MG63 cells with lncRNA XLOC_005950 knocked out. lncRNA, long non-coding RNA; miR, microRNA; PFKM, phosphofructokinase, muscle; UTR, untranslated region; OS, osteosarcoma; NC, negative control; wt, wild-type; mut, mutant.
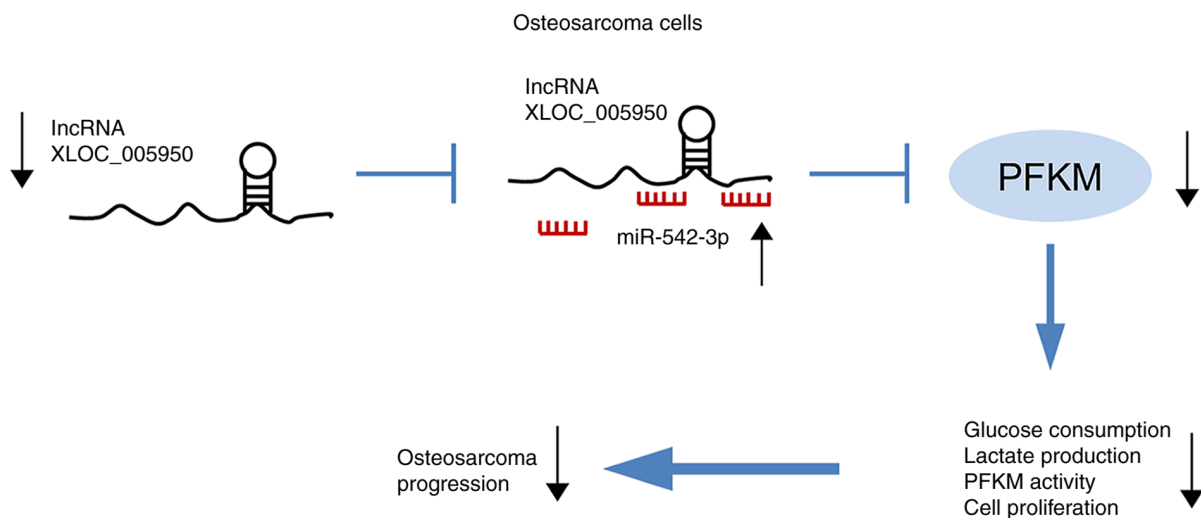

Figure 7. Schematic diagram of a latent mechanism of the lncRNA XLOC_005950/hsa-miR-542-3p/PFKM axis in regulating the glycolysis and proliferation of osteosarcoma cells. lncRNA, long non-coding RNA; miR, microRNA; PFKM, phosphofructokinase, muscle.

of glycolysis metabolism of knockout cells decreased, cell proliferation was inhibited and the apoptosis rate increased. These results demonstrated that CRISPR/Cas9-mediated knockout of lncRNA inhibited the metabolism and proliferation of MG63 cells *in vitro*. Furthermore, increased lncRNA XLOC_005950 expression promoted malignant proliferation

of OS cells, and clinicopathological analyses identified that patients with OS with high lncRNA XLOC_005950 expression exhibited a higher clinical stage compared with those with low expression. lncRNA XLOC_005950 may serve as an oncogene in OS progression, which may provide a new target for diagnosis and treatment of OS.

Several studies have suggested that miRNAs may exert functions by targeting mRNAs, and negatively regulating gene expression (38-40). miRNAs are abnormally expressed in OS. For example, miR-381 expression (41) is upregulated in OS, while the expression levels of miR-126 (42) and miR-143 (43) are downregulated. Zhong *et al* (44) reported that lncRNA small nucleolar RNA host gene 8 promotes the proliferation of OS cells by downregulating miR-542-3p expression. Based on bioinformatics analysis and the dual-luciferase reporter assay, hsa-miR-542-3p has a specific binding site for lncRNA XLOC_005950, and PFKM is a target gene of hsa-miR-542-3p. The present study demonstrated that hsa-miR-542-3p expression markedly decreased in OS cells and tissues. Overexpression of hsa-miR-542-3p inhibited the biological processes of MG63 cells, which was consistent with the effect of knocking out lncRNA XLOC_005950 in MG63 cells. Thus, lncRNA XLOC_005950 may act as a molecular sponge to repress the function of hsa-miR-542-3p.

Glycolysis provides energy for the rapid proliferation of tumor cells. It has been reported that metabolic reprogramming promotes tumor cell survival and tumor development, suggesting that tumor cells may depend on specific metabolic pathways (45). Zancan *et al* (46) reported that PFKM and PFKL are highly expressed in breast cancer cells. Ahsan *et al* (47) identified that PFKM gene region 12q13.11 as a breast cancer susceptibility locus, is associated with breast cancer risk. PFK activity is associated with changes in intracellular metabolism (27,29). The results of the present study demonstrated that PFKM was highly expressed in OS cells and tissues. After gene editing aimed to knockout lncRNA XLOC_005950 in MG63 cells, or after hsa-miR-542-3p mimics transfection, the activity and expression of PFKM decreased, and lactic acid and glucose content also decreased, which subsequently decreased cell proliferation compared with that of untreated MG63 cells. These results suggest that lncRNA XLOC_005950 may promote tumorigenesis and tumor development in OS, which may be caused by enhanced expression of PFKM and promotion of the aerobic glycolysis pathway in OS cells.

In conclusion, the results of the present study demonstrated that the loss of lncRNA XLOC_005950 or overexpression of hsa-miR-542-3p attenuated PFKM expression, PFKM activity, glucose and lactic content and cell proliferation by modulating the lncRNA XLOC_005950/hsa-miR-542-3p/PFKM axis, which potentially reveals the underlying molecular mechanism of OS. However, the present study is not without limitations. Prospective studies are required to validate these results in more cell lines. In addition, whether lncRNA XLOC_005950 can target other miRNAs, and whether other factors are involved in the regulatory network to influence OS progression require investigation.

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Availability of data and materials

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

Authors' contributions

YL, YDW and ZJ conceived and designed the present study. YZ and YSW and YDW collected the samples. ZJ, YDW, XS, XZ and SX performed the experiments. ZJ, YDW, XS, and YZ analyzed the data. ZJ drafted the initial manuscript. YDW, ZJ and YL confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Zhengzhou University (Zhengzhou, China; approval no. 2015-03). Written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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