Long non-coding RNA PGM5-AS1 modulates epithelial-mesenchymal transition and invasion and metastasis of osteosarcoma cells by impairing miR-140-5p-mediated FBN1 inhibition

Wei Liu

The First Hospital of Jilin University

Pengcheng Liu

The First Hospital of Jilin University

Hang Gao

The First Hospital of Jilin University

Xu Wang

The First Hospital of Jilin University

Ming Yan (✉ yanmingdr@163.com)

The First Hospital of Jilin University <https://orcid.org/0000-0002-9762-4471>

Research article

Keywords: Osteosarcoma, Long non-coding RNA PGM5-AS1, microRNA-140-5p, Fibrillin-1

Posted Date: December 31st, 2019

DOI: <https://doi.org/10.21203/rs.2.19800/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background Osteosarcoma represents a uncommon tumor occurring in bone possessing elevated rate of incidence and reduced rate of healing. Hence, developing effective therapeutic modalities to treat osteosarcoma is essential. Various long non-coding RNAs (lncRNAs) are engaged in prognostic and diagnostic processes of diseases. Thus, we identified the molecular mechanism of lncRNA PGM5-AS1 in progression of osteosarcoma.

Methods Microarray-based analysis was employed to screen the osteosarcoma-related differentially expressed lncRNA. PGM5-AS1 level in osteosarcoma tissues and cells as well as the levels of microRNA-140-5p (miR-140-5p) and fibrillin-1 (FBN1) in osteosarcoma tissues were determined. Dual-luciferase reporter gene assay, RNA pull-down assay, and RNA immunoprecipitation assay were done to find out the relation among PGM5-AS1, miR-140-5p, and FBN1. shRNA, mimic, or inhibitor was applied to alter the levels of PGM5-AS1, miR-140-5p, and FBN1 in osteosarcoma cells to investigate how they regulated migration, invasion, and epithelial-mesenchymal transition (EMT) of osteosarcoma cells in vitro. The nude mice were injected with osteosarcoma cell line with depleted PGM5-AS1 to detect the role of PGM5-AS1 in tumorigenesis in vivo.

Results PGM5-AS1 possessing elevated level in osteosarcoma was identified as the differentially expressed lncRNA in osteosarcoma. Osteosarcoma tissues exhibited low miR-140-5p expression and high FBN1 expression. Results confirmed that PGM5-AS competitively bound to miR-140-5p to upregulate FBN1. Also, hindering PGM5-AS1 and FBN1 or enforcing miR-140-5p dampened migration, invasion, and EMT of osteosarcoma cells in vitro. Furthermore, silencing PGM5-AS1 markedly inhibited tumorigenesis in vivo.

Conclusion All in all, PGM5-AS1 depletion causes FBN1 reduction to retard osteosarcoma processes by negatively modulating miR-140-5p, highlighting PGM5-AS1 silencing as a biomarker for osteosarcoma management.

Introduction

Osteosarcoma is a rare malignant bone tumor of mesenchymal origin with presenting osteoblastic differentiation, malignant osteoid formation and accompanied by local swelling and pain [1]. It is a neoplasm with high prevalence in children and adults, while the highest incidence rate was observed among people aged > 65 years [2]. There have been ongoing improvements in the treatment options of osteosarcoma, such as amputation, intricate limb-sparing surgeries, and multiagent chemotherapy [3]. However, the biology of osteosarcoma is still not well understood. In spite of the current progress in osteosarcoma treatment, osteosarcoma patients still diagnosed with metastasis and relapse accompanied by low survival rate [4]. Therefore, developing novel ways to deal with osteosarcoma is in significant importance to raise the survival rate and reduced the death rate of osteosarcoma patients.

Long non-coding RNAs (lncRNAs) refer to small transcripts that do not encode proteins; it is extensively accepted that lncRNAs involve in a variety of processes of diseases, such as osteosarcoma [5]. A study proved that Taurine Upregulated gene 1 expression was high in osteosarcoma tissues and its decreased level resulted in attenuated proliferation of osteosarcoma cells [6]. PGM5 is a phosphotransferase that correlated well with the conversion between glucose-1-phosphate and glucose-6-phosphate [7]. PGM5-AS1 was also demonstrated to be expressed in a high level in anaplastic glioma [8], suggesting that PGM5-AS1 may involve in the progression of tumors. In the present study, PGM5-AS1 was identified to competitively bind to miR-140-5p. MicroRNAs (miRNAs), the small noncoding RNAs with a length of about 18–25 nucleotides, were also involved in diverse processes of tumors including osteosarcoma [9]. The miR-140-5p was found to participate in the survival and chemoresistance of osteosarcoma. Specifically, the elevated miR-140-5p increased autophagy and suppressed the cell proliferation induced by anticancer drugs [10, 11]. Moreover, the current study determined that miR-140-5p could directly target fibrillin-1 (FBN1). FBN1 is a common extracellular matrix molecule underlying complexes in sequestering of growth factors [12]. Osteosarcoma cells displayed elevated FBN1 expression and FBN1 level was linked to system of bone and skeletal [13]. On the basis of these published studies, it is assumed that PGM5-AS1 may interact with miR-140-5p and FBN1 to influence osteosarcoma processes, which was verified by both in vitro and in vivo assays.

Materials And Methods

Ethics statement

All procedures strictly conformed to the Ethics Committee and Experimental Animal Ethics Committee of the First Hospital of Jilin University. Written informed consents were obtained from all participants before the study. The animal experiments were strictly conformed to the principle of minimizing the pain, suffering and discomfort to the experimental animals.

Microarray-based analysis

The Gene Expression Omnibus (GEO) database was applied in retrieving microarray data related to osteosarcoma. The “limma” package was employed for the differential analysis and $|\log FC| > 2$ and $p\text{-value} < 0.05$ were selected as the threshold for screening differentially expressed genes. The expression location of PGM5-AS1 was predicted using the lncATLAS database (<http://lncatlas.crg.eu/>) and the downstream miRNA activity of PGM5-AS1 was predicted using the RAID database (<https://www.baidu.com/baidu?&ie=utf-8&word=ncbi%20geo>). The TargetScan database (http://www.targetscan.org/vert_71/), miRDI database (<http://ophid.utoronto.ca/mirDIP/index.jsp#r>), miRDB database (<http://mirdb.org/miRDB/index.html>), and miRmap database (<https://mirmap.ezlab.org/>) were used to predict the downstream target gene of miR-140-5p.

Tissue specimen samples

The osteosarcoma tissue samples were collected from 73 patients with detailed clinical data who were pathologically diagnosed as osteosarcoma and underwent surgical resection in the First Hospital of Jilin University from January 2013 to December 2015. The patients did not receive chemoradiotherapy before the surgery. There were 41 males and 32 females and the age of the patients ranged from 9 to 53 years with an average age of 26.68 ± 13.50 . Among these patients, 33 patients were aged ≤ 20 years old and 40 patients were aged > 20 years old; 38 patients' tumor diameter was ≤ 5 cm while 35 patients were > 5 cm. Besides, the adjacent normal bone tissues that locate 4 cm away from the osteosarcoma tissues were also collected from these patients [14, 15].

Follow-up

We followed up the patients suffering from osteosarcoma via phone or return visit till December 2018 and also observed the overall survival rate of these patients. As of December 2018, 7 patients were lost to follow-up, the follow-up rate was 90.41%, and the follow-up duration was 5–36 months.

RNA in situ hybridization

The 463 bp DNA fragment (corresponding to nucleotide position 142–585 bp of PGM5-AS1) was cloned into the pSPT19 vector (10999644001, Sigma, Shanghai, China) and the antisense RNA probe was synthesized using the DIG RNA Labeling Kit (SP6/T7; 11175025910, Sigma, Shanghai, China). The DNA fragment sequence of PGM5-AS1-ISH was as follows: F, GATCGGAATTCCGGTAAGAGAATGTCCGAAAG; R, GATGCAAGCTTCACCATGCTGTGCTGTAGAT. The double DIG-labeled LNA microRNA probe (QIAGEN, Shanghai, China) for miR-140-5p was incubated with the paraffin-embedded osteosarcoma tissue sections. Briefly, after being deparaffinized, the tissue sections were treated with proteinase K (Roche, Basel, Switzerland), hybridized with 300 ng/mL probe at 60°C for 16 h, then incubated with anti-digoxigenin-AP (AP-conjugated Fab fragments) (11093274910, Roche, Basel, Switzerland) at 25°C for 1 h, and lastly added with BM Purple (11442074001, Roche, Basel, Switzerland) for color reaction [16–18].

Cell culture and treatment

A normal cell line hFOB 1.19 (CBP60724; Culture Medium: Dulbecco's modified eagle's medium [DMEM]: F12 + 0.3 mg/mL G418 + 10% fetal bovine serum [FBS]) and five cell lines related to osteosarcoma: U2OS (CBP60238; Culture Medium: McCoy's 5a + 10% FBS), SaOS-2 (CBP60742; Culture Medium: McCoy's 5a + 15% FBS), MG63 (CBP60233; Culture Medium: MEM + 10% FBS), HOS (CBP60787; Culture Medium: RPMI-1640 Medium + 10% FBS), and SJSA1 (CBP60236; Culture Medium: RPMI-1640 + 10% FBS) were purchased from Cobioer Biotechnology Co., Ltd. (Nanjing, China).

Osteosarcoma cells were plated into a 6-well plate with 3×10^5 cells in each well. Upon cell confluence was approximately 50%, sh-negative control (NC), sh-PGM5-AS1-1, sh-PGM5-AS1-2, inhibitor NC, miR-140-5p inhibitor, mimic NC, miR-140-5p mimic, or sh-FBN1 were delivered into the cells in accordance with the protocols of Lipofectamine 2000 (11668-019, Invitrogen, New York, California, USA). The mimic NC, miR-140-5p mimic, inhibitor NC, and miR-140-5p inhibitor were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Table 1 displays the sequences of shRNAs.

Table 1
shRNA sequences

Item	Sequence	Company
sh-PGM5-AS1-1	GGGTAAGAGAATGTCCGAAAG	Thermo Fisher
sh-PGM5-AS1-2	GGTAAGAGAATGTCCGAAAGA	Thermo Fisher
sh-FBN1	GCATGCACTTACGGATTTACT	Thermo Fisher

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The Trizol kit (16096020, Thermo Fisher Scientific, California, USA) was employed to extract the total RNA. Five µg RNA was reversely transcribed into complementary DNA (cDNA) in conformity to the protocols of the cDNA kit (K1622, Fermentas Inc., Ontario, CA, USA). RT-qPCR was carried out with the TaqMan MicroRNA Assay and TaqMan® Universal PCR Master Mix on the basis of the guidelines of TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) [19]. U6 was selected as the internal control for miR-140-5p, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected for PGM5-AS1 and FBN1. Table 2 shows the sequences of primers. The $2^{-\Delta\Delta C_t}$ method was employed to calculate the relative expression of the target genes.

Table 2
Primer sequences used in reverse transcription
quantitative polymerase chain reaction

Gene	Primer sequence (5' – 3')
PGM5-AS1	F: GCCCTGCCTTCAGCCTCCTCA
	R: GAAACGTCGGCCTGGCCTTGT
FBN1	F: GGGTCAAAGATCACGTGCACAG
	R: TTGTCAAAGCAGACGGAGGTC
miR-140-5p	F: TGCGGCAGTGGTTTTACCCTATG
	R: CCAGTGCAGGGTCCGAGGT
U6	F: GCTTCGGCAGCACATATACTAAAAT
	R: CGCTTCACGAATTTGCGTGTCAT
GAPDH	F: ATGGAGAAGGCTGGGGCTC
	R: AAGTTGTCATGGATGACCTTG

Western blot assay

The bicinchoninic acid protein assay kit (Thermo Fisher Scientific, California, USA) was used to extract the total protein, followed by measurement of protein concentration. Totally, after polyacrylamide gel electrophoresis, 30 µg proteins were transferred onto the polyvinylidene fluoride membrane (Amersham Healthcare, Chicago, Illinois, United States). After that, the membrane was sealed by 5% non-fat milk at room temperature for 1 h, followed by cultivation at 4 °C overnight in rabbit polyclonal antibodies against FBN1 (ab231094, 1 : 1000), E-cadherin (ab15148, 1 : 500), N-cadherin (ab76057, 1 : 1000), Vimentin (ab137321, 1 : 2000), and GAPDH (ab9485, 1 : 2500). All antibodies were provided by the Abcam Inc. (Cambridge, UK). The membrane was then incubated with secondary antibody to goat anti-rabbit immunoglobulin G (IgG) H&L (ab6721, 1 : 3000, Abcam Inc., Cambridge, UK) marked by horseradish peroxidase (HRP) at room temperature for 1 h. After being scanned and developed under the chemiluminescence instrument (Gel Company, San Francisco, CA, USA). The relative protein expression was analyzed using Image Pro Plus 6.0 (Media Cybernetics, MD, USA).

Transwell assay

Migration detection: Osteosarcoma cells in logarithmic growth stage were starved for 24 h until the final concentration of cells reached 2×10^5 cells/mL. Subsequently, the apical chamber was added with 0.2 mL of cell suspension and the basolateral chamber was added with 700 µL pre-cooled DMEM supplemented with 10% FBS. And then, the chambers were cultivated at 37°C with 5% CO₂ for 24 h. After being fixed with methanol for 30 min, the cells were subjected to 0.1% crystal violet staining for 20 min, and were observed under the inverted microscope. Five visual fields were randomly selected and the number of transmembrane cells was counted.

Invasion detection: The extracellular matrix (ECM) gel was diluted using serum-free culture medium at 1 : 9 till the final concentration was 1 mg/mL. The polycarbonate membrane in the 24-well apical chamber was supplemented with 40 µL ECM gel and then further cultivated at 37°C with 5% CO₂ for 5 h till the ECM gel was polymerized. An amount of 70 µL of pure DMEM was then added to the ECM gel and further incubated at 37°C for 0.5 h to hydrate the ECM gel. The starved osteosarcoma cells in serum for 24 h were resuspended with FBS-free DMEM until the final concentration reached 2.5×10^5 cells/mL. Then, the hydrated basement membrane of apical chamber was added with 0.2 mL of cell suspension and the basolateral chamber was added with 700 µL of pre-cooled DMEM conjugated with 10% FBS. The chambers were then cultivated under the conditions of 37°C with 5%CO₂ for 24 h. After being fixed with methanol for 30 min, the cells were subjected to 0.1% crystal violet staining for 20 min, and observation under the inverted microscope. We randomly selected 5 visual fields to count the number of cells that passed through the membranes.

Fluorescence in situ hybridization (FISH)

FISH was conducted in accordance with the protocols of Ribo™ IncRNA FISH probe Mix (Red) (Guangzhou RiboBio Co., Ltd., Guangzhou, Guangdong China) in order to identify the subcellular location of PGM5-AS1 in osteosarcoma cells. The osteosarcoma cells were plated onto the slide in a 6-well plate, followed by 1-h culture till the cell confluence was 80% or so. After being fixed with 1 mL 4%

paraformaldehyde, the cells were manipulated with protease K (2 µg/mL), glycine, and acetylation reagent, and then incubated with 250 µL pre-hybridization. After the pre-hybridization removal, the cells were hybridized overnight with 250 µL hybridization solution supplemented with 300 ng/mL probe. The cells were then observed under the fluorescence microscope (Olympus Optical, Tokyo, Japan).

Fractionation of nuclear/cytoplasmic RNA

The osteosarcoma cells were resuspended with Hypotonic Buffer A (10 mM HEPES [pH = 7.5], 0.5 mM DTT, 10 mM KCl, 1.5 mM MgCl₂) containing protease inhibitor and RNase inhibitor (N8080119, Thermo Fisher Scientific, Thermo Fisher Scientific, California, USA), followed by incubation on ice for 10 min and centrifugation under the conditions of 1000 g at 4°C for 10 min. The supernatant was collected and centrifuged at 15000 g for 15 min to collect the cytoplasm. The precipitate was washed with hypotonic buffer, and resuspended with Hypotonic Buffer B (10 mM HEPES [pH = 7.5], 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5% Nonidet P-40) and Radioimmunoprecipitation Assay Buffer (50 mM Tris HCl [pH = 7.5], 1500 mM KCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, pH = 8.0). The precipitate was centrifuged at 15000 g for 20 min to collect the supernatant, which contained the nucleus.

RNA pull-down assay

In conformity to the guidelines of the Lipofectamine 2000, the cells were manipulated with 100 pmol Bio-miR-140-5p or NC. After 48 of transduction, the cells were resuspended in 0.7 mL lysis buffer (5 mM MgCl₂, 100 mM KCl, 20 mM Tris [pH = 7.5], 0.3% NP-40, 50 U of RNase OUT [Invitrogen, Carlsbad, CA, USA], complete protease suppressor cocktail [Roche Applied Science, Indianapolis, IN, USA]). The cells were then centrifuged at 10000 g for 10 min to separate the cell lysate. RNA pull-down assay of miRNA was performed based on the previously published method [20, 21]. The expression of PGM5-AS1 in Bio-miR-140-5p or NC was detected using RT-qPCR.

RNA immunoprecipitation (RIP) assay

The interaction among miR-140-5p, PGM5-AS1 and FBN1 was identified using the RIP assay kit (Millipore Inc., Bedford, MA, USA). After being lysed with lysis buffer (P0013B, Beyotime Biotechnology Co., Shanghai, China), the cells were subjected to centrifugation at 35068 g at 4°C for 10 min to collect the supernatant. A big section of cell extraction was taken as the Input and the rest section was cultured with the antibody for co-precipitation as follows: 50 µL magnetic beads were resuspended in 100 µL RIP Wash Buffer and cultured with 5 µg antibody. The complex of bead and antibody was then resuspended using 900 µL RIP Wash Buffer, followed by incubation in 100 µL cell extraction overnight at 4°C. The samples were then placed on the magnetic base to collect the beads-protein complex. The antibodies used for the RIP assay were argonaute 2 (Ago2, ab32381, 1 : 50, Abcam Inc., Cambridge, UK) and IgG (1 : 100, ab109489, Abcam Inc., Cambridge, UK) was used as the NC.

Dual-luciferase reporter gene assay

The whole length of FBN1 3'UTR was amplified. The RT-qPCR products were cloned into the polyclonal sites of the downstream gene of pmirGLO (Promega, Madison, WI, USA) using the endonuclease sites Spe I and Hind III to construct the FBN1-wild type (WT) vector. The target gene prediction database including TargetScan, mirDIP, miRDB, and miRmap databases were used to predict the binding site between miR-140-5p and its target. FBN1-mutant type (MUT) vector was constructed via site-directed mutagenesis. Using renilla luciferase expression vector pRL-TK (TaKaRa Biotechnology Co. Ltd, Liaoning China) as the internal control, the plasmids were co-transfected with luciferase reporter vectors into HEK-293T cells. The Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was employed in measuring the activity of luciferase.

Xenograft tumor in nude mice

Twenty-four female BALB/c nude mice with an age of 3–4 weeks and a weight ranging from 14–18 g were housed under the conditions of constant temperature (25°C – 27°C) and constant humidity (45% – 50%). sh-NC or sh-PGM5-AS1 was delivered to osteosarcoma cell line U2OS and the nude mice were subcutaneously injected with 20 µL of cell suspension. The tumor volume was recorded every 6 days and calculated as follows: tumor volume = $a \times b^2 / 2$ (a, the maximum diameter; b, the minimum diameter). We also drew a curve of the tumor growth. Thirty days after injection, the nude mice were euthanized and the tumors were isolated from the mice and weighed. RT-qPCR and western blot analysis were carried out to detect the levels of PGM5-AS1, FBN1, miR-140-5p, and epithelial-mesenchymal transition (EMT)-related genes in tumor tissues.

Statistical analysis

The SPSS 21.0 software (IBM Corp. Armonk, NY, USA) was applied in data analysis. The measurement data were presented as mean ± standard deviation. The pair-designed data between two groups conforming to normal distribution and homogeneity of variance were analyzed using paired t-test. One-way analysis of variance (ANOVA), along with Tukey's post-hoc test was used for comparison of data among multiple groups. Repeated measurement ANOVA, together with Bonferroni's post-hoc test, was conducted to analyze the data at different time points. The Kaplan-Meier method was adopted to construct the overall survival curve and log-rank was used for analysis of the survival differences. Analysis of the enumeration data were performed using the Chi-square test. A value of $p < 0.05$ indicated that the difference was in statistical significance.

Results

Osteosarcoma tissues and cells display elevated PGM5-AS1 expression

Differentially expressed genes related to osteosarcoma were retrieved using microarray-based analysis. The GEO database was applied in retrieving the microarray data of GSE41445. lncRNA PGM5-AS1 expression was revealed to be increased in osteosarcoma samples through differential analysis of the

gene expression in 3 normal samples and 3 osteosarcoma samples, which were retrieved from GSE41445 (Fig. 1A).

The expression of PGM5-AS1 in osteosarcoma tissues collected from 73 osteosarcoma patients was determined using RT-qPCR. It was found that osteosarcoma tissues presented higher PGM5-AS1 level than adjacent normal bone tissues (Fig. 1B). Besides, the situation of PGM5-AS1 in osteosarcoma tissues and adjacent normal bone tissues was determined by RNA in situ hybridization, whose results showed that PGM5-AS1 was found in the cytoplasm of osteosarcoma tissues with high expression (Fig. 1C-D). The osteosarcoma patients were assigned into patients with poorly expressed PGM5-AS1 and patients with highly expressed PGM5-AS1 to analyze the relationship between PGM5-AS1 expression and the clinical indicators of osteosarcoma patients. The expression of PGM5-AS1 was closely correlated to the tumor-node-metastasis stage, distant metastasis, tumor grade, and tumor size. However, PGM5-AS1 expression does not correlate to the age and gender of the patients (Table 3). The Kaplan-Meier method was used to analyze the correlation of PGM5-AS1 expression to overall survival in osteosarcoma patients and it was found that patients with low PGM5-AS1 level had longer overall survival time than the patients with high PGM5-AS1 level (log-rank $p < 0.05$) (Fig. 1E). Additionally, PGM5-AS1 expression in the normal cell line hFOB 1.19 and 5 osteosarcoma cell lines (U2OS, SaOS-2, MG63, HOS, and SJSA1) was tested by RT-qPCR. The results suggested that osteosarcoma cell lines presented higher level of PGM5-AS1 than normal cell line hFOB 1.19 ($p < 0.05$) (Fig. 1F). Therefore, we chose U2OS and HOS cells for following assays. In a word, PGM5-AS1 level was elevated in osteosarcoma tissues and cells.

Table 3
Relationship between PGM5-AS1 expression and the clinical indicators of
osteosarcoma patients

Clinicopathologic features	Cases (n)	PGM5-AS1 expression		p-value
		Low (n = 36)	High (n = 37)	
Age (years)				0.484
≤ 20	33	18	15	
> 20	40	18	22	
Gender				0.241
Male	41	23	18	
Female	32	13	19	
TNM stage				0.003
I + II	48	30	18	
III + IV	25	6	19	
Distant metastasis				0.013
Yes	25	7	18	
No	48	29	19	
Tumor grade				0.034
Low	41	25	16	
High	32	11	21	
Tumor size				0.001
≤ 5 cm	38	26	12	
> 5 cm	35	10	25	
Note: TNM, tumor-node-metastasis				

Knocking down PGM5-AS1 hinders osteosarcoma cell abilities of EMT, invasion and migration

With the expectation to dig out the mechanism by which PGM5-AS1 affects EMT, invasion, and migration of osteosarcoma cells, shRNA was used to alter the level of PGM5-AS1 in U2OS and HOS cells. According to RT-qPCR, in contrast to sh-NC treatment, sh-PGM5-AS1-1 treatment and sh-PGM5-AS1-2 treatment reduce PGM5-AS1 level in U2OS and HOS cells (Fig. 2A). As revealed by the results of RT-qPCR and

western blot analysis, PGM5-AS1 knockdown decreased the levels of N-cadherin and vimentin while elevated the level of E-cadherin in U2OS and HOS cells (Fig. 2B-C). Then, the abilities of invasion and migration in osteosarcoma cells were detected by conducting Transwell assay, whose results indicated that decreasing PGM5-AS1 resulted in reduced abilities of invasion and migration abilities in U2OS and HOS cells (Fig. 2D-E). Thus, depleting the expression of PGM5-AS1 dampens osteosarcoma cell EMT, invasion and migration.

PGM5-AS1 binds to miR-140-5p

Given that we have explored the role of PGM5-AS1 in osteosarcoma, we turned to investigate the relationship between PGM5-AS1 and miR-140-5p. As shown by RNA in situ hybridization, PGM5-AS1 expression was mostly found in the cytoplasm (Fig. 3A), which was further confirmed by FISH results (Fig. 3B) and fractionation of nuclear/cytoplasmic RNA (Fig. 3C). These findings suggested that PGM5-AS1 might have a regulatory function via the mechanism of ceRNA. Moreover, the downstream miRNA function of PGM5-AS1 was predicted using the RAID database. Expression of nine miRNAs was predicted to be affected by PGM5-AS1. Among these 9 miRNAs, miR-140-5p was the most significantly differentially expressed in osteosarcoma (Fig. 3D). Thus, it was implicated that PGM5-AS1 might influence the expression of miR-140-5p to be engaged in osteosarcoma progression. Many studies have demonstrated that miR-140-5p could be associated with the biological processes of cancers [22–24]. Therefore, we carried out RT-qPCR and western blot analysis to test the level of miR-140-5p in osteosarcoma tissues and adjacent normal bone tissues. It was suggested that miR-140-5p was poorly expressed in osteosarcoma tissues versus in the adjacent normal bone tissues (Fig. 3E). As indicated by RNA in situ hybridization, miR-140-5p expression was downregulated in the cytoplasm of osteosarcoma tissues (Fig. 3F). Furthermore, the results of RNA pull-down assay (Fig. 3G) and RIP assay (Fig. 3H) demonstrated that PGM5-AS1 could bind to miR-140-5p. Taken together, the results verified that PGM5-AS1 could competitively bind to miR-140-5p.

Depleted PGM5-AS1 expression contributes to repressed osteosarcoma progression by competitively binding to miR-140-5p

In order to identify the potential influence of PGM5-AS1 in the development of osteosarcoma, verification assays were performed. Initially, RT-qPCR was carried out to measure the levels of PGM5-AS1 and miR-140-5p in U2OS and HOS cells (Fig. 4A). The expression patterns of genes associated with EMT including E-cadherin, N-cadherin, and vimentin were detected by performing RT-qPCR and western blot analysis. As shown, knocking down miR-140-5p decreased E-cadherin expression, but increased N-cadherin and vimentin expression (Fig. 4B-C). The abilities of invasion and migration in osteosarcoma cells were assessed using Transwell assay, whose results showed promotion in abilities of migration and invasion ability in osteosarcoma cells (Fig. 4D-E). PGM5-AS1 reduction upregulated E-cadherin and downregulated

N-cadherin and vimentin (Fig. 4B-C), and attenuated abilities of migration and invasion in osteosarcoma cells (Fig. 4D-E). Co-treatment of silenced PGM5-AS1 and reduced miR-140-5p rescued the suppressive role of in osteosarcoma cell invasion and migration. Therefore, knock down of PGM5-AS1 upregulates miR-140-5p, thus inhibiting osteosarcoma development.

FBN1 is the target of miR-140-5p

The target of miR-140-5p and their interaction were predicted and verified using dual-luciferase reporter gene assay, RNA pull-down assay and RIP assay. The predicted results and the upregulated genes retrieved from the microarray data GSE41445 showed that there were 3 genes in the intersection, which might be targeted and regulated by miR-140-5p (Fig. 5A). According to the results of quantification analysis, FBN1 was the most significantly expressed gene in osteosarcoma (Fig. 5B). miR-140-5p was then suggested to be linked to the processes of osteosarcoma by directly binding to FBN1. The level of FBN1 in osteosarcoma tissues and adjacent normal bone tissues was examined by RT-qPCR, showing that osteosarcoma tissues had elevated expression of FBN1 (Fig. 5C). The expression of FBN1 was measured with miR-140-5p expression in U2OS cells transfected with mimic or inhibitor. Enforced expression of miR-140-5p led to FBN1 depletion, while reduced expression of miR-140-5p caused FBN1 elevation (Fig. 5D-E). Knocking down PGM5-AS1 reduces FBN1 content and reduced PGM5-AS1 or suppressed miR-140-5p reversed the suppressive role of PGM5-AS1 silencing on FBN1 expression. As determined by dual-luciferase reporter gene assay, overexpressing miR-140-5p decreased the luciferase activity of FBN1-WT while suppressing miR-140-5p potentiated the luciferase activity of FBN1-WT without affecting luciferase activity of FBN1-MUT (Fig. 5F). Repressing both miR-140-5p and PGM5-AS1 rescued the downregulatory effect of miR-140-5p on luciferase activity (Fig. 5G). Furthermore, RIP assay revealed that miR-140-5p could bind to FBN1 (Fig. 5H) and PGM5-AS1 silencing enhanced the binding of miR-140-5p to FBN1 (Fig. 5I-J). These results provide evidence demonstrating that miR-140-5p specifically targets FBN1.

Restored expression of miR-140-5p reduces FBN1 to dampen processes of osteosarcoma

With the aim of finding out how miR-140-5p regulated osteosarcoma progression by modulating the expression of FBN1, RT-qPCR (Fig. 6A) and western blot analysis (Fig. 6B) were employed to test the levels of FBN1 and genes linked to EMT including E-cadherin, N-cadherin, and vimentin (Fig. 6C-D) and Transwell assay (Fig. 6E-F) was carried out to examine the abilities of invasion and migration in osteosarcoma cells. As the results revealed, depleting FBN1 downregulated FBN1, N-cadherin, and vimentin, upregulated E-cadherin, and attenuated the invasion and migration in osteosarcoma cells. While elevation in FBN1, N-cadherin, and vimentin, reduction in E-cadherin, and promotion in invasion and migration abilities of osteosarcoma cells were found in response to the knock down of miR-140-5p. Depleted FBN1 as well as depletion miR-140-5p rescued the suppressive role of FBN1 in invasion and

migration of osteosarcoma cells. In summary, an elevation of miR-140-5p expression downregulates FBN1 to inhibit the development of osteosarcoma.

PGM5-AS1 silencing inhibits tumorigenesis of osteosarcoma cells in vivo

The role of PGM5-AS1 in osteosarcoma in vivo was also assessed. Based on the tumor volume and weight, it was found that PGM5-AS1 silencing inhibited tumorigenesis of osteosarcoma cells (Fig. 7A), accompanied by downregulation of PGM5-AS1 and FBN1 expression, as well as upregulation of miR-140-5p expression (Fig. 7B). Moreover, RT-qPCR and western blot analysis were used to test the levels of genes correlated to EMT including E-cadherin, N-cadherin, vimentin was tested. Reducing PGM5-AS1 greatly elevated E-cadherin content and decreased the contents of N-cadherin and vimentin (Fig. 7C-D). In a word, PGM5-AS1 silencing results in repression on tumorigenesis in osteosarcoma cells in vivo.

Discussion

Osteosarcoma is a rare malignant mesenchymal tumor with a poor survival rate in spite of the improvements in recent diagnosis and management of osteosarcoma [25]. Hence, to find out effective biological markers to improve osteosarcoma management is in great importance. Recently, it is extensively accepted that a variety of lncRNAs and miRNAs act a critical part in influencing the progression of osteosarcoma [26]. However, there is no study elucidating the underlying mechanism of PGM5-AS1 in osteosarcoma. Thus, we performed the present study to reveal the specific mechanism of PGM5-AS1 in the network of lncRNA-miRNA-mRNA in osteosarcoma. Our results intriguingly proved that the silencing PGM5-AS1 elevates the expression of miR-140-5p to reduce FBN1 level, thus attenuating osteosarcoma cellular processes.

Osteosarcoma tissues were found to display elevated expression of PGM5-AS1 and FBN1 but reduced expression of miR-140-5p. Diverse lncRNAs have been widely reported to be upregulated in osteosarcoma. For instance, levels of both HULC and UCA1 were found to be elevated in osteosarcoma cells and tissues and the upregulation of these two lncRNAs were correlated to metastasis and clinical stage of osteosarcoma [27, 28]. Consistently, PGM5-AS1 as a gene related to the risk of anaplastic glioma, was suggested to be upregulated in anaplastic glioma [8]. Also, the upregulation of PGM5-AS1 was also found in colorectal cancer and the upregulated PGM5-AS1 expression was linked to the poor survival rate of colorectal cancer patients [7]. Besides, osteosarcoma cells were extensively demonstrated to display an increased expression of FBN1 [13, 29]. Likewise, tissues and cells in gastric cancer had increased levels of FBN1, whose reduction resulted in attenuating of the gastric cancer processes [30]. Moreover, Meng et al. found that the miR-140-5p level was linked to chemosensitivity in osteosarcoma, where the downregulated miR-140-5p expression in osteosarcoma tissues was observed following chemotherapy [10].

Besides, PGM5-AS1 was confirmed to competitively bind to miR-140-5p, thus increasing FBN1 expression. In consistence with our results, a formed study showed that lncRNA ODRUL upregulated MMP2 by directly downregulating miR-3182 to further induce the progression of osteosarcoma [31]. Also, the depleted SNHG12 upregulated miR-195-5p to downregulate Notch2, thus suppressing the progression of osteosarcoma [32]. Moreover, Ma et al. revealed that FBN1 was a target gene of miR-486-5p, whereby the elevated expression of miR-486-5p could target FBN1 to suppress the development of papillary thyroid carcinoma [33]. It was revealed that miR-140-5p negatively regulated IP3k2 to suppress autophagy in osteosarcoma after chemotherapy [11].

Furthermore, depleting the expression of PGM5-AS1 or FBN1, or enforcing the expression of miR-140-5p repressed osteosarcoma cell migration, invasion, and EMT of in vitro and attenuated tumorigenesis in vivo. In accordance with our results, a former study showed that reduced DANCR downregulated ALX to inhibit cell proliferation, invasion, and migration in vitro, as well as suppress tumor growth in vivo in osteosarcoma by specifically upregulating miR-33a-5p [34]. Similarly, the depletion of lncRNA prostate cancer-associated transcript 1 has led to the repressed cell invasion, migration, proliferation, and EMT of osteosarcoma cells [35]. Also, ovarian cancer migration and invasion were revealed to be attenuated following FBN1 silencing [36]. Consistently, elevating the expression of miR-140 negatively mediating the expression of histone deacetylase 4, leading to inhibited osteosarcoma cell proliferation and invasion in vitro and slowed tumor growth in vivo, thus highlighting overexpressed miR-140 as a tumor inhibitor in osteosarcoma [37]. Likewise, miR-140 was promoted as a suppressor in osteosarcoma owing to its role in repressing proliferation, migration, and tumor aggressiveness [38]. Furthermore, Zhang et al. confirmed that the depleting the expression of small nucleolar RNA host gene 16 led to increased miR-140-5p, thus downregulating ZEB1 and thereby dampening esophageal squamous cell carcinoma cell proliferation, migration, and EMT [39].

Conclusions

Collectively, our findings provide new evidence demonstrating that the depleted PGM5-AS1 competitively binds to miR-140-5p, resulting in the decreased expression of FBN1 and suppressed osteosarcoma progression (Fig. 8). Our novel results promote effective biological markers to better manage osteosarcoma and lay theoretical foundation for elucidating the specific mechanism of lncRNA in osteosarcoma. However, more in-depth investigations are still needed in the assessment and implication of the PGM5-AS1/miR-140-5p/FBN1 axis on clinical practice.

Abbreviations

lncRNAs: long non-coding RNAs, miR-140-5p: microRNA-140-5p, FBN1: fibrillin-1, EMT: epithelial-mesenchymal transition, GEO: Gene Expression Omnibus, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, IgG: immunoglobulin G, HRP: horseradish peroxidase, ECM: extracellular matrix, WT: wild type, MUT: mutant type, EMT: epithelial-mesenchymal transition, ANOVA: analysis of variance.

Declarations

Acknowledgements

We give our sincere gratitude to the reviewers for their valuable suggestions.

Funding

This study was supported by National Natural Science Foundation of China (No. 81802077).

Availability of data and materials

The primary data for this study is available from the authors on direct request.

Authors' contributions

Wei Liu, Pengcheng Liu designed the study. Hang Gao , Xu Wang and Ming Yan collated the data, carried out data analyses and produced the initial draft of the manuscript. Wei Liu, Pengcheng Liu and Ming Yan contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.

Ethics approval and consent to participate

All procedures strictly conformed to the Ethics Committee and Experimental Animal Ethics Committee of the First Hospital of Jilin University. Written informed consents were obtained from all participants before the study. The animal experiments were strictly conformed to the principle of minimizing the pain, suffering and discomfort to the experimental animals.

Consent for publication

Not applicable.

Competing interest

The author declares no competing interest exists.

References

1. Yang J, N Wang. Analysis of the molecular mechanism of osteosarcoma using a bioinformatics approach. *Oncol Lett.* 2016;12(5):3075-80.
2. Lindsey BA, JE Markel, ES Kleinerman. Osteosarcoma Overview. *Rheumatol Ther.* 2017;4(1):25-43.
3. Isakoff MS, SS Bielack, P Meltzer, R Gorlick. Osteosarcoma: Current Treatment and a Collaborative Pathway to Success. *J Clin Oncol.* 2015;33(27):3029-35.
4. Luetke A, PA Meyers, I Lewis, H Juergens. Osteosarcoma treatment - where do we stand? A state of the art review. *Cancer Treat Rev.* 2014;40(4):523-32.
5. Yang Z, X Li, Y Yang, Z He, X Qu, Y Zhang. Long noncoding RNAs in the progression, metastasis, and prognosis of osteosarcoma. *Cell Death Dis.* 2016;7(9):e2389.
6. Zhang Q, PL Geng, P Yin, XL Wang, JP Jia, J Yao. Down-regulation of long non-coding RNA TUG1 inhibits osteosarcoma cell proliferation and promotes apoptosis. *Asian Pac J Cancer Prev.* 2013;14(4):2311-5.
7. Zhu H, J Yu, H Zhu, Y Guo, S Feng. Identification of key lncRNAs in colorectal cancer progression based on associated protein-protein interaction analysis. *World J Surg Oncol.* 2017;15(1):153.
8. Wang W, Z Zhao, F Yang, H Wang, F Wu, T Liang, X Yan, J Li, Q Lan, J Wang, et al. An immune-related lncRNA signature for patients with anaplastic gliomas. *J Neurooncol.* 2018;136(2):263-71.
9. Kobayashi E, R Satow, M Ono, M Masuda, K Honda, T Sakuma, A Kawai, H Morioka, Y Toyama, T Yamada. MicroRNA expression and functional profiles of osteosarcoma. *Oncology.* 2014;86(2):94-103.
10. Meng Y, R Gao, J Ma, J Zhao, E Xu, C Wang, X Zhou. MicroRNA-140-5p regulates osteosarcoma chemoresistance by targeting HMGN5 and autophagy. *Sci Rep.* 2017;7(1):416.
11. Wei R, G Cao, Z Deng, J Su, L Cai. miR-140-5p attenuates chemotherapeutic drug-induced cell death by regulating autophagy through inositol 1,4,5-trisphosphate kinase 2 (IP3k2) in human osteosarcoma cells. *Biosci Rep.* 2016;36(5):
12. Sengle G, K Tsutsui, DR Keene, SF Tufa, EJ Carlson, NL Charbonneau, RN Ono, T Sasaki, MK Wirtz, JR Samples, et al. Microenvironmental regulation by fibrillin-1. *PLoS Genet.* 2012;8(1):e1002425.
13. Han JA, JY Kim, JI Kim. Analysis of gene expression in cyclooxygenase-2-overexpressed human osteosarcoma cell lines. *Genomics Inform.* 2014;12(4):247-53.
14. Hu X, Y Liu, C Qin, Z Pan, J Luo, A Yu, Z Cheng. Up-regulated isocitrate dehydrogenase 1 suppresses proliferation, migration and invasion in osteosarcoma: in vitro and in vivo. *Cancer Lett.* 2014;346(1):114-21.
15. Yuan G, Y Zhao, D Wu, C Gao. Mir-150 Up-Regulates Glut1 and Increases Glycolysis in Osteosarcoma Cells. *Asian Pac J Cancer Prev.* 2017;18(4):1127-31.
16. Sun Y, B Qin. Long noncoding RNA MALAT1 regulates HDAC4-mediated proliferation and apoptosis via decoying of miR-140-5p in osteosarcoma cells. *Cancer Med.* 2018;7(9):4584-97.

17. Taniue K, A Kurimoto, Y Takeda, T Nagashima, M Okada-Hatakeyama, Y Katou, K Shirahige, T Akiyama. ASBEL-TCF3 complex is required for the tumorigenicity of colorectal cancer cells. *Proc Natl Acad Sci U S A*. 2016;113(45):12739-44.
18. Fang Z, S Yin, R Sun, S Zhang, M Fu, Y Wu, T Zhang, J Khaliq, Y Li. miR-140-5p suppresses the proliferation, migration and invasion of gastric cancer by regulating YES1. *Mol Cancer*. 2017;16(1):139.
19. Xie M, T Ma, J Xue, H Ma, M Sun, Z Zhang, M Liu, Y Liu, S Ju, Z Wang, et al. The long intergenic non-protein coding RNA 707 promotes proliferation and metastasis of gastric cancer by interacting with mRNA stabilizing protein HuR. *Cancer Lett*. 2019;443(67-79).
20. Lal A, MP Thomas, G Altschuler, F Navarro, E O'Day, XL Li, C Concepcion, YC Han, J Thiery, DK Rajani, et al. Capture of microRNA-bound mRNAs identifies the tumor suppressor miR-34a as a regulator of growth factor signaling. *PLoS Genet*. 2011;7(11):e1002363.
21. Wang H, X Huo, XR Yang, J He, L Cheng, N Wang, X Deng, H Jin, N Wang, C Wang, et al. STAT3-mediated upregulation of lncRNA HOXD-AS1 as a ceRNA facilitates liver cancer metastasis by regulating SOX4. *Mol Cancer*. 2017;16(1):136.
22. Lan H, W Chen, G He, S Yang. miR-140-5p inhibits ovarian cancer growth partially by repression of PDGFRA. *Biomed Pharmacother*. 2015;75(117-22).
23. Gullu G, I Peker, A Haholu, F Eren, Z Kucukodaci, B Gulec, H Baloglu, C Erzik, A Ozer, M Akkiprik. Clinical significance of miR-140-5p and miR-193b expression in patients with breast cancer and relationship to IGFBP5. *Genet Mol Biol*. 2015;38(1):21-9.
24. Wang M, M Du, L Ma, H Chu, Q Lv, D Ye, J Guo, C Gu, G Xia, Y Zhu, et al. A functional variant in TP63 at 3q28 associated with bladder cancer risk by creating an miR-140-5p binding site. *Int J Cancer*. 2016;139(1):65-74.
25. Geller DS, R Gorlick. Osteosarcoma: a review of diagnosis, management, and treatment strategies. *Clin Adv Hematol Oncol*. 2010;8(10):705-18.
26. Wang C, J Jing, L Cheng. Emerging roles of non-coding RNAs in the pathogenesis, diagnosis and prognosis of osteosarcoma. *Invest New Drugs*. 2018;36(6):1116-32.
27. Sun XH, LB Yang, XL Geng, R Wang, ZC Zhang. Increased expression of lncRNA HULC indicates a poor prognosis and promotes cell metastasis in osteosarcoma. *Int J Clin Exp Pathol*. 2015;8(3):2994-3000.
28. Li W, P Xie, WH Ruan. Overexpression of lncRNA UCA1 promotes osteosarcoma progression and correlates with poor prognosis. *J Bone Oncol*. 2016;5(2):80-5.
29. Summers KM, NJ Bokil, JM Baisden, MJ West, MJ Sweet, LJ Raggatt, DA Hume. Experimental and bioinformatic characterisation of the promoter region of the Marfan syndrome gene, FBN1. *Genomics*. 2009;94(4):233-40.
30. Yang D, D Zhao, X Chen. MiR-133b inhibits proliferation and invasion of gastric cancer cells by up-regulating FBN1 expression. *Cancer Biomark*. 2017;19(4):425-36.

31. Zhu KP, XL Ma, CL Zhang. LncRNA ODRUL Contributes to Osteosarcoma Progression through the miR-3182/MMP2 Axis. *Mol Ther.* 2017;25(10):2383-93.
32. Zhou S, L Yu, M Xiong, G Dai. LncRNA SNHG12 promotes tumorigenesis and metastasis in osteosarcoma by upregulating Notch2 by sponging miR-195-5p. *Biochem Biophys Res Commun.* 2018;495(2):1822-32.
33. Ma X, J Wei, L Zhang, D Deng, L Liu, X Mei, X He, J Tian. miR-486-5p inhibits cell growth of papillary thyroid carcinoma by targeting fibrillin-1. *Biomed Pharmacother.* 2016;80(220-26.
34. Jiang N, X Wang, X Xie, Y Liao, N Liu, J Liu, N Miao, J Shen, T Peng. LncRNA DANCR promotes tumor progression and cancer stemness features in osteosarcoma by upregulating AXL via miR-33a-5p inhibition. *Cancer Lett.* 2017;405(46-55.
35. Zhang X, Y Zhang, Y Mao, X Ma. The lncRNA PCAT1 is correlated with poor prognosis and promotes cell proliferation, invasion, migration and EMT in osteosarcoma. *Onco Targets Ther.* 2018;11(629-38.
36. Wang Z, Y Liu, L Lu, L Yang, S Yin, Y Wang, Z Qi, J Meng, R Zang, G Yang. Fibrillin-1, induced by Aurora-A but inhibited by BRCA2, promotes ovarian cancer metastasis. *Oncotarget.* 2015;6(9):6670-83.
37. Xiao Q, L Huang, Z Zhang, X Chen, J Luo, Z Zhang, S Chen, Y Shu, Z Han, K Cao. Overexpression of miR-140 Inhibits Proliferation of Osteosarcoma Cells via Suppression of Histone Deacetylase 4. *Oncol Res.* 2017;25(2):267-75.
38. Gu R, YF Sun, MF Wu, JB Liu, JL Jiang, SH Wang, XL Wang, Q Guo. Biological roles of microRNA-140 in tumor growth, migration, and metastasis of osteosarcoma in vivo and in vitro. *Tumour Biol.* 2016;37(1):353-60.
39. Zhang K, J Chen, H Song, LB Chen. SNHG16/miR-140-5p axis promotes esophagus cancer cell proliferation, migration and EMT formation through regulating ZEB1. *Oncotarget.* 2018;9(1):1028-40.

Figures

Figure 1

Osteosarcoma tissues and cells exhibited elevated PGM5-AS1. A, Expression of PGM5-AS1 in normal samples and osteosarcoma samples retrieved from the microarray data GSE41445; the abscissa represents sample type, the ordinate represents the expression of PGM5-AS1, and the number in the upper left represents the p-value. B, PGM5-AS1 level in adjacent normal bone tissues and osteosarcoma tissues from 73 osteosarcoma patients measured by RT-qPCR. C-D, Expression location of PGM5-AS1 in adjacent

normal bone tissues and osteosarcoma tissues from 73 osteosarcoma patients identified using RNA in situ hybridization (n = 73). E, The relationship between PGM5-AS1 expression and overall survival time of osteosarcoma patients analyzed using the Kaplan-Meier method. F, Expression of PGM5-AS1 in the normal cell line hFOB 1.19 and osteosarcoma cell lines (U2OS, SaOS-2, MG63, HOS, and SJSA1), as determined by RT-qPCR. * p < 0.05, vs. normal samples or hFOB 1.19 cell line. The measurement data were expressed as mean ± standard deviation. Data analysis in B were conducted by paired t-test, data analysis in D by an unpaired t-test, and data analysis in F by one-way ANOVA, together with Tukey's post-hoc test. Each reaction was run in triplicate.

Figure 2

Depleting PGM5-AS1 attenuated osteosarcoma cell EMT, invasion, and migration. sh-NC, sh-PGM5-AS1-1 or sh-PGM5-AS1-2 were delivered into U2OS and HOS cells. A, Expression of PGM5-AS1 in U2OS and HOS cells, as measured using RT-qPCR. B, Relative expression of EMT-related genes in U2OS and HOS cells detected by RT-qPCR. C, Protein expression of EMT-related genes in U2OS and HOS cells detected by western blot analysis. D, Migration ability of U2OS and HOS cells detected by Transwell assay (200 ×; scale bar = 50 μm). E, Invasion ability of U2OS and HOS cells detected by Transwell assay (200 ×; scale bar = 50 μm). * p < 0.05, vs. U2OS and HOS cells manipulated with sh-NC. The measurement data were presented as mean ± standard deviation. One-way ANOVA along with Tukey's post-hoc test was conducted to analyze the data among multiple groups. The experiment was repeated three times.

Figure 3

PGM5-AS1 binds to miR-140-5p in osteosarcoma. A, The expression location of PGM5-AS1 analyzed using RNA in situ hybridization. PGM5-AS1 expression in the cytoplasm was shown above the 0 line and PGM5-AS1 expression in the nucleus was shown under the 0 line. B, The expression location of PGM5-AS1 in U2OS cells detected using FISH (200 ×; scale bar = 50 μm). C, The expression location of PGM5-AS1 in U2OS and HOS cells analyzed by fractionation of nuclear/cytoplasmic RNA. D, Regulatory miRNA of PGM5-AS1 predicted using the RAID database. E, miR-140-5p level in osteosarcoma tissues and adjacent normal bone tissues from 73 osteosarcoma patients determined by RT-qPCR. F, Expression of miR-140-5p in osteosarcoma tissues from 73 osteosarcoma patients, as measured using RNA in situ hybridization (n = 73; 400 ×; scale bar = 25 μm). G, The binding between miR-140-5p and PGM5-AS1 detected by RNA pull-down assay. H, The binding between miR-140-5p and PGM5-AS1 detected by RIP assay. * p < 0.05, vs. U2OS and HOS cells treated with sh-NC, adjacent normal bone tissues, Bio-NC, or IgG. The data were measurement data and expressed as mean ± standard deviation. Data in E were analyzed using t-test. Unpaired t-test was used to analyze the data between the two groups and one-way ANOVA together with Tukey's post-hoc test was applied in data analysis among multiple groups. The experiment was repeated three times.

Figure 4

Silencing of PGM5-AS1 downregulates miR-140-5p to suppress the development of osteosarcoma. The si-PGM5-AS1-1 and miR-140-5p inhibitor, as well as the corresponding NC, were delivered into the U2OS and HOS cells. A, Expression of PGM5-AS1, miR-140-5p and FBN1 in U2OS and HOS cells detected by RT-qPCR. B, RT-qPCR results of EMT-related gene expression (E-cadherin, N-cadherin, and vimentin) in U2OS and HOS cells. C, Western blot analysis of EMT-related gene protein expression (E-cadherin, N-cadherin, and vimentin) in U2OS and HOS cells. D, Migration ability of U2OS and HOS cells assessed by Transwell assay (200 ×; scale bar = 50 μm). E, Invasion ability of U2OS and HOS cells assessed by Transwell assay (200 ×; scale bar = 50 μm). * p < 0.05, vs. U2OS and HOS cells treated with both si-NC and inhibitor NC; # p < 0.05, vs. U2OS and HOS cells treated with both si-PGM5-AS1-1 and inhibitor NC. The data were measurement data and expressed as mean ± standard deviation. One-way ANOVA as well as Tukey's post-hoc test was used for data analysis among multiple groups. The experiment was repeated three times.

Figure 5

miR-140-5p negatively modulated FBN1. Mimic or inhibitor or shRNA was used to interfere the levels of miR-140-5p and PGM5-AS1 in U2OS cells. A, Prediction results of the gene targeted by miR-140-5p. The ovals represent the predicted results and the upregulated genes in the microarray data GSE41445. The overlapped section refers to the intersection of the five ovals. B, Expression of MMP, FBN1, and KLF9 in U2OS cells with altered PGM5-AS1 expression detected by RT-qPCR. C, FBN1 level in osteosarcoma tissues and adjacent normal bone tissues from 73 osteosarcoma patients tested using RT-qPCR. D, Relative expression of FBN1 in U2OS cells with altered miR-140-5p expression detected by RT-qPCR. E, Protein expression of FBN1 in U2OS cells with altered miR-140-5p expression detected by western blot analysis. F, The binding of miR-140-5p and FBN1 confirmed by dual-luciferase reporter gene assay. G-H, Luciferase activity of FBN1 measured by dual-luciferase reporter gene assay. I, Interaction between miR-140-5p and FBN1 in U2OS cells as detected by RIP assay. J, Interaction between miR-140-5p and FBN1 in U2OS cells with altered PGM5-AS1 expression as measured using RIP assay. * p < 0.05, vs. U2OS cells treated with sh-NC, adjacent normal bone tissues, IgG treatment, or U2OS cells treated with both sh-NC and inhibitor NC; # p < 0.05, vs. U2OS cells administered with inhibitor NC or AGO2 enrichment in sh-NC. The measurement data were expressed as mean $\pm$ standard deviation. The data in C were analyzed using paired t-test. Unpaired t-test was employed for data analysis between two groups and one-way ANOVA, as well as Tukey's post-hoc test was used for comparison of data among multiple groups. The experiment was repeated three times.

Figure 6

miR-140-5p upregulation hindered the expression of FBN1 to dampen osteosarcoma processes. The expression of miR-140-5p in U2OS and HOS cells was altered with inhibitor and the expression of FBN1 was altered with shRNA. A, Relative expression of FBN1 in U2OS and HOS cells measured using RT-qPCR. B, Protein expression of FBN1 in U2OS and HOS cells detected by western blot analysis. C, Expression of genes related to EMT including E-cadherin, N-cadherin, and vimentin in U2OS and HOS cells measured using RT-qPCR. D, Protein levels of EMT-related genes (E-cadherin, N-cadherin, and vimentin) in U2OS and HOS cells detected by western blot analysis. E, Migration ability of U2OS and HOS cells measured by Transwell assay (200 ×; scale bar = 50 μm). F, Invasion ability of U2OS and HOS cells measured by Transwell assay (200 ×; scale bar = 50 μm). * $p < 0.05$, vs. U2OS cells treated with combined sh-NC and inhibitor NC; # $p < 0.05$, vs. U2OS cells treated with combined sh-FBN1 and sh-NC. The data were measurement data and expressed as mean $\pm$ standard deviation. Data among multiple groups were analyzed using one-way ANOVA, followed by Tukey's post-hoc test. The experiment was repeated three times.

Figure 7

Tumorigenesis of osteosarcoma cells in vivo was suppressed resulting from the downregulation of PGM5-AS1. A, Tumor volume and tumor weight of the nude mice. B, Expression of PGM5-AS1, FBN1, and miR-140-5p in developed tumors detected by RT-qPCR. C, Expression patterns of genes related to EMT including E-cadherin, N-cadherin, and vimentin tested using RT-qPCR. D, Protein expression of EMT-related genes (E-cadherin, N-cadherin, and vimentin) as measured by western blot analysis. * $p < 0.05$, vs. the nude mice injected with sh-NC-treated U2OS cells. The data were measurement data and expressed as mean $\pm$ standard deviation. Unpaired t-test was performed to compared the data between two groups and repeated measurement ANOVA along with Bonferroni's post-hoc test was conducted for analysis of data at different time points. $n = 12$.

PGM5-AS1

Figure 8

Modulatory role of PGM5-AS1 in the cellular processes of osteosarcoma by interacting with miR-140-5p and FBN1. Knocking down the expression of PGM5-AS1 specifically binds to miR-140-5p to silence the level of FBN1 and thereby attenuate EMT, invasion, and migration of osteosarcoma in vitro as well as tumorigenesis in vivo.