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RESEARCH ARTICLE

LncRNA DLX6-AS1 regulates osteosarcoma progression via the miR-200a-3p/GPM6P axis

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Abstract

LncRNA DLX6-AS1 takes part in the progression of various cancers. However, it is not elaborated clearly in osteosarcoma (OS) development. Therefore, we aimed to explore the impacts and specific mechanisms of DLX6-AS1 on the progression of OS. We estimated the pattern of DLX6-AS1 expression in Ost tissues and cells via quantitative reverse transcription polymerase chain reaction. A number of biochemical assays were carried out to assess the effects of DLX6-AS1. Target genes were predicted by bioinformatics methods. Then we used the transfection of si-RNA, miRNA inhibitor, and miRNA mimics to explore the underlying mechanisms and built tumor xenograft models for the *in vivo* experiments. A higher expression of DLX6-AS1 was found in OS tissues and cell lines, while knockdown of DXL6-AS1 suppressed OS cell metastasis and proliferation *in vitro* and *in vivo*. Mechanistically, it was revealed that DXL6-AS1 sponged miR-200a-3p, thus positively regulating the downstream GPM6B. In summary, DLX6-AS1 knockdown would inhibit OS cell migration, cell invasion, and cell proliferation, in which the DXL6-AS1/ miR-200a-3p/ GPM6B axis played a critical role.

Keywords: *LncRNA DLX6-AS1*; *osteosarcoma*; *miR-200a-3p*; *GPM6B*

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Received: 7 November 2021; Revised: 3 December 2021; Accepted: 3 December 2021; Published: 7 January 2022

Unfortunately, despite various treatments (e.g. surgical resection and systemic chemo-radiotherapy), the 5-year survival rate of osteosarcoma (OS) is still not optimistic (1, 2). There are many studies investigating the mechanisms underlying OS progression, including studies on long non-coding RNAs (lncRNAs) (3). An increasing number of reports reveal that lncRNAs affect the progression of multiple cancers functionally (4–7). For example, as an oncogenic lncRNA, distal-less homeobox 6 antisense 1 (DLX6-AS1) is located in chromosomal region 7q21.3. It was reported functioning in various types of cancers, including liver (8), gastric (9), lung (10), pancreatic 11), and ovarian cancers (12). However, DLX6-AS1 in OS development has not been elaborated clearly.

Non-coding RNAs of 22 nt in length are named microRNAs (miRNAs). The most common process of its function is to bind with the 3'-UTR of target mRNAs, thus downregulating the target gene expression (13). Meanwhile, lncRNAs could play the role of competing endogenous RNAs (ceRNAs) to sponge miRNAs to modulate their downstream target genes. Xie et al. reported that DLX6-AS1 sponged miR-16-5p and upregulated ARPP19 to accelerate the progression of cervical cancer (14). Furthermore, Zhao et al. revealed that DLX6-AS1 regulated the miR-505-3p/RUNX2 axis and promoted breast cancer cell proliferation (15). It also showed laryngeal cancer development driven by DLX6-AS1 via targeting miR-26a and TRPC3. However, whether DLX6-AS1 interacts with miRNAs in OS has not been elaborated clearly.

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Materials and methods

Clinical specimens

There were 40 pairs of OS tissues and adjacent normal tissues gathered from OS patients in our hospital from 2017 to 2019. Table 1 presents the detailed clinicopathological characteristics of OS patients. The current protocols were reviewed and approved by Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Informed written consent was obtained from every patient whose tissues and information were involved in this study.

Table 1. Relationship of lncRNA DLX6-AS1 expression and clinical variables among 40 OS patients

Characteristics	Number (Total *n* = 40)	DLX6-AS1 expression		*P*
		High (*n* = 28)	Low (*n* = 12)	
Age (years)				
≥ 50	24	17	7	0.789
< 50	16	11	5	
Gender				
Male	22	12	10	0.701
Female	18	10	8	
Tumor node metastasis stage				
I–II	22	13	9	0.019*
II–IV	18	15	3	
Lymph-node metastasis				
Negative	17	8	9	0.025*
Positive	23	20	3	
Tumor size (cm)				
> 3	24	20	4	0.022*
≤ 3	16	8	8	
Tumor differentiation				
Well	6	4	2	0.018*
Moderate	15	10	5	
Poor	18	14	4	

*$P < 0.05$, the difference is significant.

Table 2. Primers of qRT-PCR

Gene		Primers
DLX6-AS1	Forward	5'-CCAAATGCTACCATCCAGCC-3'
	Reverse	5'-TCTGGCTTCCCTTAACCAA-3'
miR-200a-3p	Stem-loop primer	5'-GTCGTATCCAGTGCAGGGTC CGAGGTATTCGCACTGGATACGA CACATCG-3'
	Forward	5'-TAACACTGTCTGGTAA-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
GPM6B	Forward	5'-ATGGCCTTAGCTTAGGCT -3'
	Reverse	5'-TTGGCAATGCCGTATTAGC-3'
β-Actin	Forward	5'-CTCCATCCTGGCCTCGCTGT-3'
	Reverse	5'-GCTGTCACCTTCACCGTTCC-3'

Cell culture

We purchased all cell lines from the Cell Bank of Chinese Academy of Science (Shanghai, China). The cells were maintained in the DMEM medium (Invitrogen) containing 10% fetal bovine serum (FBS, GiOSo) at 37°C with 5% CO_2.

Quantitative PCR

Total RNA was isolated from tissue or cells using Trizol (Invitrogen). cDNA was synthesized from RNA by PrimeScript RT reagent (TaKaRa, Japan). Quantitative PCR (qPCR) was performed by SYBR Premix ExTaq reagent (TaKaRa, Japan) with cDNA as templates. The internal primers for miRNAs and mRNAs were U6 and GAPDH, respectively. The relative expression was analyzed by the 2-ΔΔCt method. Table 2 displays the information of all the primers.

Cell transfection

Si-RNA against DLX6A-AS1 (si-DLX6A-AS1), miR-200a-3p inhibitor and mimics, and also the negative controls were obtained from RiboBio (Nanjing, China). In addition, Lipo2000 (Invitrogen, USA) was used for transfection, according to the protocol.

CCK-8 assay

CCK-8 assay (Dojindo Molecular Technologies) determined cell viability following the protocols. In brief, we seeded transfected cells in 96-well plates (1×10^3/well), and then added CCK-8 solution every 24 h. A microplate reader detected the absorbance at 450 nm.

Colony assay

First, we seeded transfected cells in 12-well plates at 0.8×10^3/well. Two weeks later, we replaced the medium every 3 days. Then, cells were fixed and stained with methanol and 0.1% Crystal Violet (Promega, Nanjing, China), respectively. Finally, colonies were estimated using a microscope (Olympus, Japan).

Wound healing assay

The wound healing assay could help us to observe cell migration. A new peptide was used to create a scratch across the surface after transfected cells reached 90% confluence. After 24 h, the migration state was observed and graphed by an inverted microscope.

Transwell assay

The ability of cell invasion was determined by transwell chambers (BD Biosciences, USA). First, we placed cells in the upper Matrigel-covered chamber and filled the bottom chamber with medium containing 10% FBS. Then, after transfection, the cells on the upper chamber were washed away 24 h later. Next, cells were fixed and stained with

methanol and 0.1% Crystal Violet, respectively. Finally, invasion cells were counted under a microscope in five randomly chosen visual fields.

Western blot

Total proteins were extracted with radioimmunoprecipitation buffer (Beyotime). Proteins were separated by SDS-PAGE (10%), and then transferred onto the PVDF membranes (Millipore) and blocked with 5% skim milk. After that, primary antibodies against GPM6B (1:1,000, CST, Beijing) and β-actin (1:3,000, Sangon) were used to incubate with the membranes at 4°C. Ten hours later, the membranes were incubated in HRP-conjugated secondary antibodies at room temperature for 60 min. Electrochemiluminescence detection system (Beyotime, Shanghai, China) was used to visualize the protein signals.

Luciferase activity assay

Wt and mut sequences of target RNA were inserted into pGL3 empty vectors (Sangon, Shanghai, China). Using Lipo2000 (Invitrogen, USA), miR-200a-3p mimics were transfected with pGL3-mut or pGL3-wt into HEK-293T cells. After 48 h incubation, the relative luciferase activity was measured.

Immunohistochemistry assay

After being fixed with 10% formaldehyde, xenograft tumor tissues were sectioned into 5-μm thick slides. Then the samples had an incubation overnight at 4°C with primary antibody against GPM6B (1:1,100, Abcam) and Ki67 (1:1,100, Abcam), followed by another incubation with an HRP-conjugated antibody at room temperature for 2 h. The Pierce DAB substrate kit (Thermo Fisher Scientific) was used to illuminate the signals, and photographs from five randomly chosen fields were taken by microscopy.

RNA in situ hybridization (RNA ISH)

RNAscope® 2.0 HD detection kit (BROWN ACDBIO, Shanghai, China) and anti- DLX6A-AS/miR-200a-3p probe sets (BROWN ACDBIO, Shanghai, China) were used for RNA ISH experiments, according to their manufacturer's instruments. Photographs were captured by microscopy from five randomly chosen fields.

Animals

BALB/C nude mice (female, 4 weeks old) were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). Mice were injected with normal HOS cells (control group) or HOS cells transfected sh-DLX6A-AS1 stably. After that, tumor size was measured each week, and tumor weight was estimated 5 weeks later. The procedure of experiments was approved by Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

Statistics

Data were analyzed using SPSS software and were expressed as mean ± SD. The Student's *t*-test between two groups or one-way analysis of variance (ANOVA) following a Tukey's post hoc test among multiple groups was used to perform significant differences. The survival rate was calculated using the Kaplan–Meier method. Pearson correlation coefficient was used for statistical correlation. The *P*-value of < 0.05 was considered to be statistically significant. All experiments were repeated at least in triplicate.

Result

LncRNA DLX6-AS1 is highly expressed

To indicate the effect of DLX6-AS1 in OS, we first detected the expression pattern of DLX6-AS1 in OS tissues and adjacent normal tissues from 40 paired specimens. It showed that DLX6-AS1 was distinctly upregulated in OS tissues compared to normal tissues (Fig. 1a, b). Moreover, the expression levels of DLX6-AS1 in the OS cell lines (MG63, HOS, U2OS and Saos2) were higher than that in the normal human osteoblasts cell line (hFOB 1.19) (Fig. 1c). Furthermore, OS patients with higher DLX6-AS1 had a correlation with shorter overall survival (Fig. 1d).

Effects of knockdown of DLX6-AS1

Wondering how DLX6-AS1 functioned during OS progression, we silenced the DLX6-AS1 expression in SW780 and T24 cell lines. As a result, the knockdown efficiency of si-DLX6-AS1 was remarkable (Fig. 2a). Subsequently, we investigated the impacts of DLX6-AS1 on cell proliferation and metastasis. It was predicted that knockdown of DLX6-AS1 inhibited OS cell viability and colony-forming ability (Fig. 2b, c). Moreover, OS cell invasion number and migration rate were attenuated upon DLX6-AS1 knockdown (Fig. 2d, e). Therefore, the results revealed that DLX6-AS1 might act as an oncogenic biomarker in OS development.

miR-200a-3p was a direct target of DLX6-AS1

As lncRNAs usually function as ceRNAs of miRNAs, miR-200a-3p was predicted as a target of DLX6-AS1 using StarBase v.2.0 bioinformatics software. The top 15 predicted target miRNAs are presented in Supplementary Table 1. The predicted binding sites are shown in Fig. 3a. Then, a luciferase reporter assay was used to confirm the prediction (Fig. 3b). The expression of miR-200a-3p was also upregulated significantly upon si-DLX6-AS1 transfection (Fig. 3c). Furthermore, the expression profile of miR-200a-3p in tissues was measured. miR-200a-3p had a lower expression level in OS tissues (Fig. 3d). Furthermore, miR-200a-3p had a negative correlation with DLX6-AS1 in OS tissues (Fig. 3e).

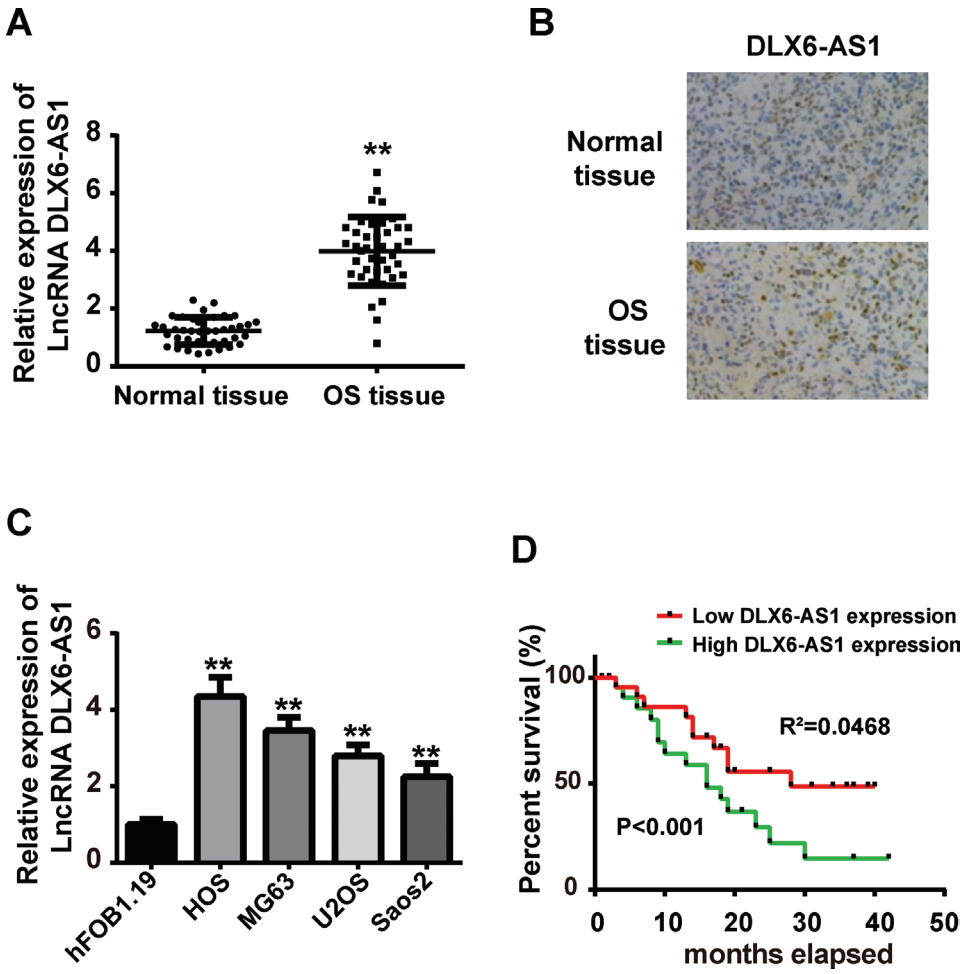

Fig. 1. Overexpression of LncRNA DLX6-AS1 in OS cell and tissue lines. (a) RT-qPCR measured DLX6-AS1 expression in OS tissues and adjacent normal tissues. (b) ISH detected DLX6-AS1 expression in OS tissue and adjacent normal tissue. (c) DLX6-AS1 expression in the normal human osteoblasts cell line (hFOB 1.19) and other OS cell lines (HOS, MG63, U2OS and Saos2) were measured by RT-qPCR. (d) High expression levels of DLX6-AS1 in OS patients predicted poor prognosis. $^{**}P < 0.01$, versus normal tissue or SV-HUC-1.

DLX6-AS1 regulated OS development through miR-200a-3p

Furthermore, we investigated whether miR-200a-3p was involved in DlX6-AS1-regulated OS progression. The miR-200a-3p inhibitor was transfected into OS cells with or without DLX6-AS1 knockdown. As a result, the miR-200a-3p inhibitor exerted a significant knockdown effect on miR-200a-3p (Fig. 4a). Subsequently, it was observed that miR-200a-3p inhibitor partially withdrawn the inhibition of cell proliferation (Fig. 4b, c), migration (Fig. 4d) and invasion (Fig. 4e) induced by si-DLX6-AS1. The quantitative analysis is shown in Fig. 4f.

GPM6B was a direct target of miR-200a-3p

As miRNAs often function via downregulating target mRNAs, we predicted GPM6B to be the target gene of miR-200a-3p via TargetScan bioinformatics software. The top 30 target genes that are predicted are presented in Supplementary Table S2. Figure 5a shows the predicted binding sites. Then, we verified our prediction via a luciferase reporter assay (Fig. 5b). Moreover, the protein expression of GPM6B was downregulated upon miR-200a-3p mimics transfection (Fig. 5c). Furthermore, we detected the expression profile of GPM6B in tissues. The results revealed that GPM6B had much higher expression in OS tissues than in normal tissues (Fig. 5d). It revealed that GPM6B had a negative correlation with miR-200a-3p in OS tissues (Fig. 5e).

DLX6-AS1 positively regulated GPM6B via sponging miR-200a-3p

Next, we investigated the relationship among miR-200a-3p, DLX-AS1, and GPM6B. Western blot assays

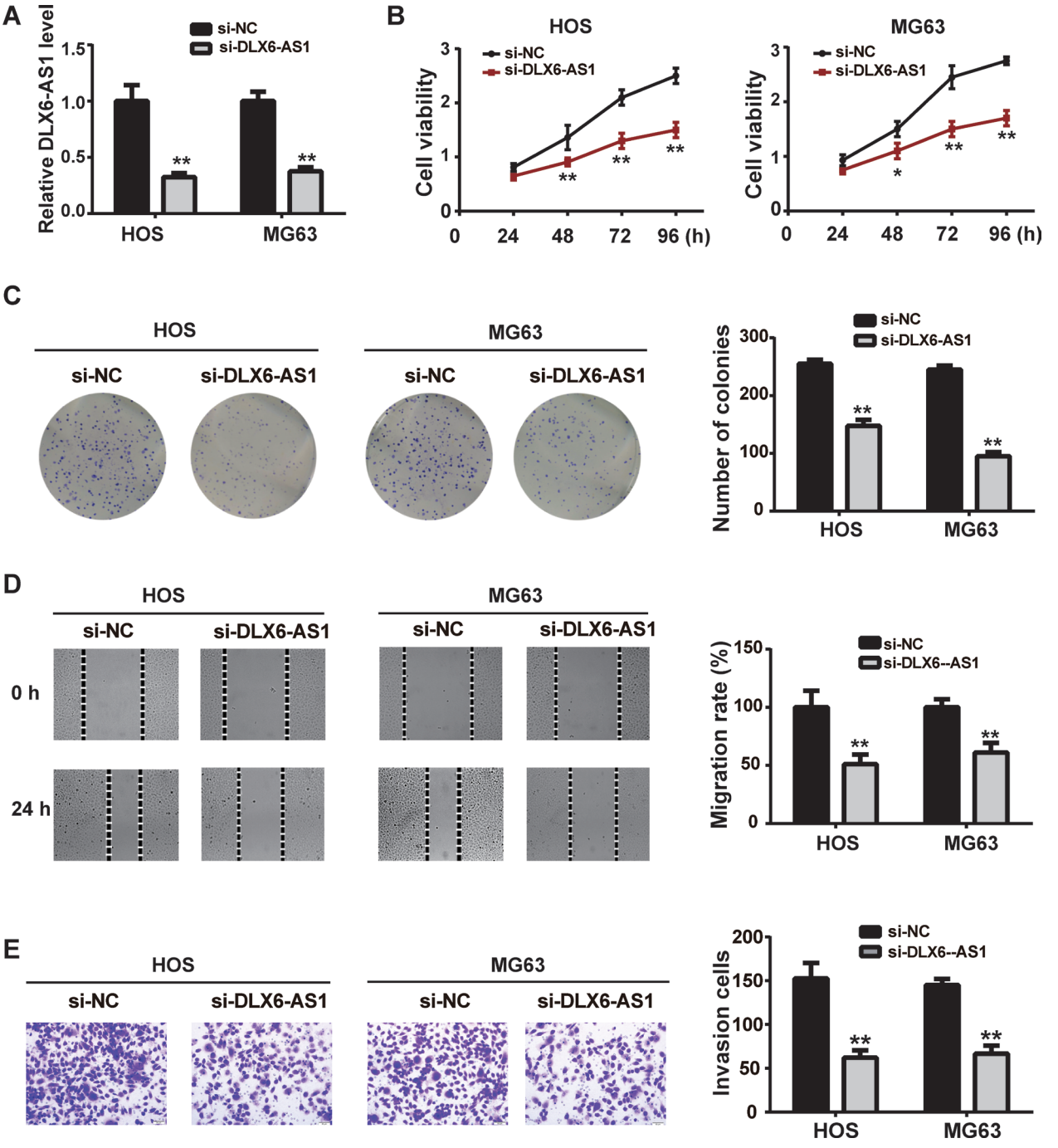

Fig. 2. Knockdown of DLX6-AS1 inhibited OS cells' proliferation, migration and invasion. (a) Knockdown efficiency was verified after si-DLX6-AS1 transfection by qPCR. (b, c) CCK-8 assay and colony formation assay was used to verify cell proliferation. (d) The cell migration ability was determined via wound healing assay. (e) Transwell assay was performed to detect the cell invasion ability. NC, negative control. $^{**}P < 0.01$, versus si-NC group.

and qPCR revealed that DLX6-AS1 knockdown suppressed the expression of GPM6B in both mRNA and protein, which were reversed partially by a miR-200a-3p inhibitor (Fig. 6a–c). In addition, a positive correlation exists between DLX6-AS1 and GPM6B (Fig. 6d).

Knockdown of DLX6-AS1 suppressed OS progression in vivo

Xenograft models were built by injecting DLX6-AS1-depleted T24 cells or negative control T24 cells into the flank of the nude mice. We detected the tumor size each week and the tumor weight 5 weeks later. DLX6-AS1 knockdown

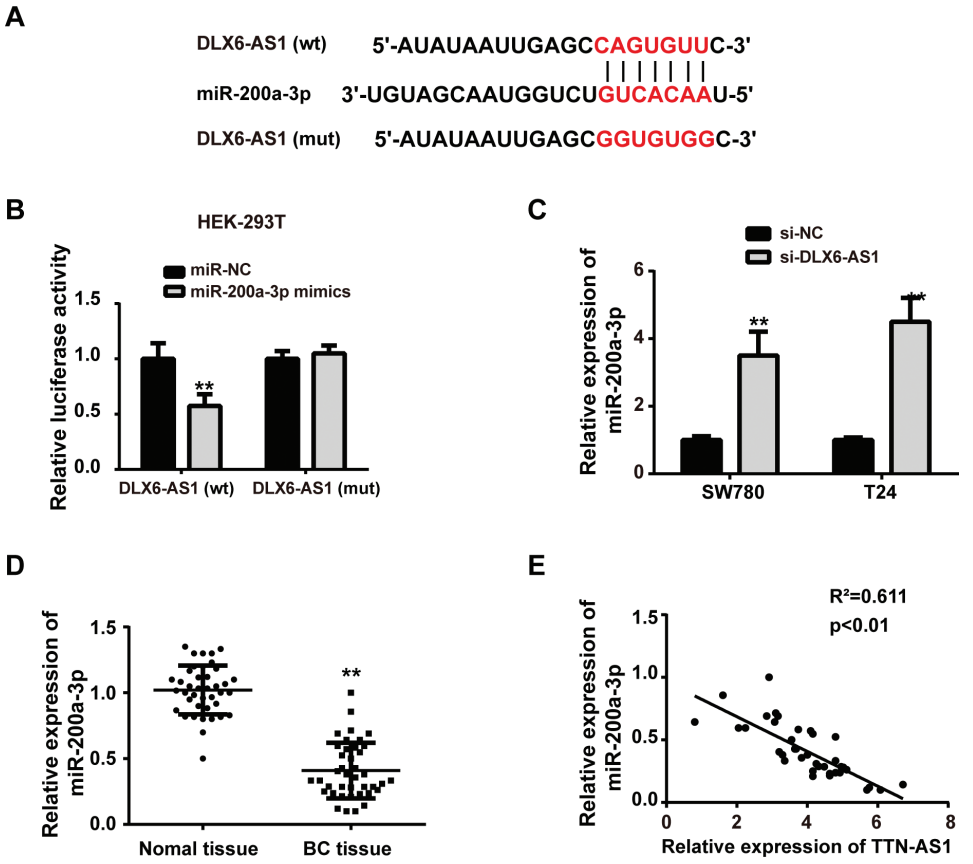

Fig. 3. DLX6-AS1 interacted with miR-200a-3p directly. (a) The binding sites between miR-200a-3p and DLX6-AS1 (StarBase v.2.0.) (b) The interaction between DLX6-AS1 and miR-200a-3p was confirmed via the luciferase assay. (c) miR-200a-3p was up-regulated after transfection of si-DLX6-AS1. (d) RT-qPCR measured the expression of miR-200a-3p in OS tissues and adjacent normal tissues. (e) DLX6-AS1 was negatively correlated with miR-200a-3p. NC, negative control. $^{**}P < 0.01$, versus miR-NC, si-NC or normal tissues.

attenuated the size and weight of tumors (Fig. 7a, b). In addition, the expression of GPM6B and Ki67 (a cell proliferation marker) was detected by ICH, showing that the expression levels of GPM6B and Ki67 were both downregulated in DLX6-AS1-depleted mice (Fig. 7c).

Discussion

LncRNAs attract increasing attention for their regulatory roles in multiple biological and pathological processes, including cancer development (16–19). On this basis, we paid attention to lncRNA-associated cancer therapy. LncRNA DLX6-AS1 is involved in the DLX gene family. It was observed that DLX genes have an abnormal expression in human solid tumor tissues, showing biological effects of DLX on cancer progression (20, 21). Previous reports also revealed the oncogenic role of DLX6-AS1 in various diseases. For example, DLX6-AS1 aggravated the development of ovarian (12, colorectal (22), pancreatic (4) and cervical cancers (23) by promoting cell proliferation and metastasis. However, the role of DLX6-AS1 during OS is still not clear. This research study revealed that DLX6-AS1 overexpressed in OS cells and tissues, which led us to suppose that DLX6-AS1 might have an oncogenic effect on OS progression. Subsequently, we found that DLX6-AS1 knockdown could inhibit OS cell migration, cell invasion and cell proliferation *in vitro* and inhibit tumor growth *in vivo*, confirming our supposition.

Furthermore, we investigated the possible mechanisms in DLX6-AS1-regulated OS development. It is well known that lncRNAs could serve as ceRNAs via competitive binding to target miRNAs in different cancer types. For example, lncRNA UCA1 was revealed to aggravate bladder cancer by targeting miR-195 (24). Zhou et al. demonstrated that DLX6-AS1 aggravated colorectal cancer by functioning as ceRNA for miR-577 (25). Furthermore, it was reported that DLX6-AS1 played a role of ceRNA of miR-181 in promoting pancreatic cancer (11). Yang et al. showed that DLX6-AS1 regulated the Wnt/β-catenin pathway via sponging miR-497-5p in

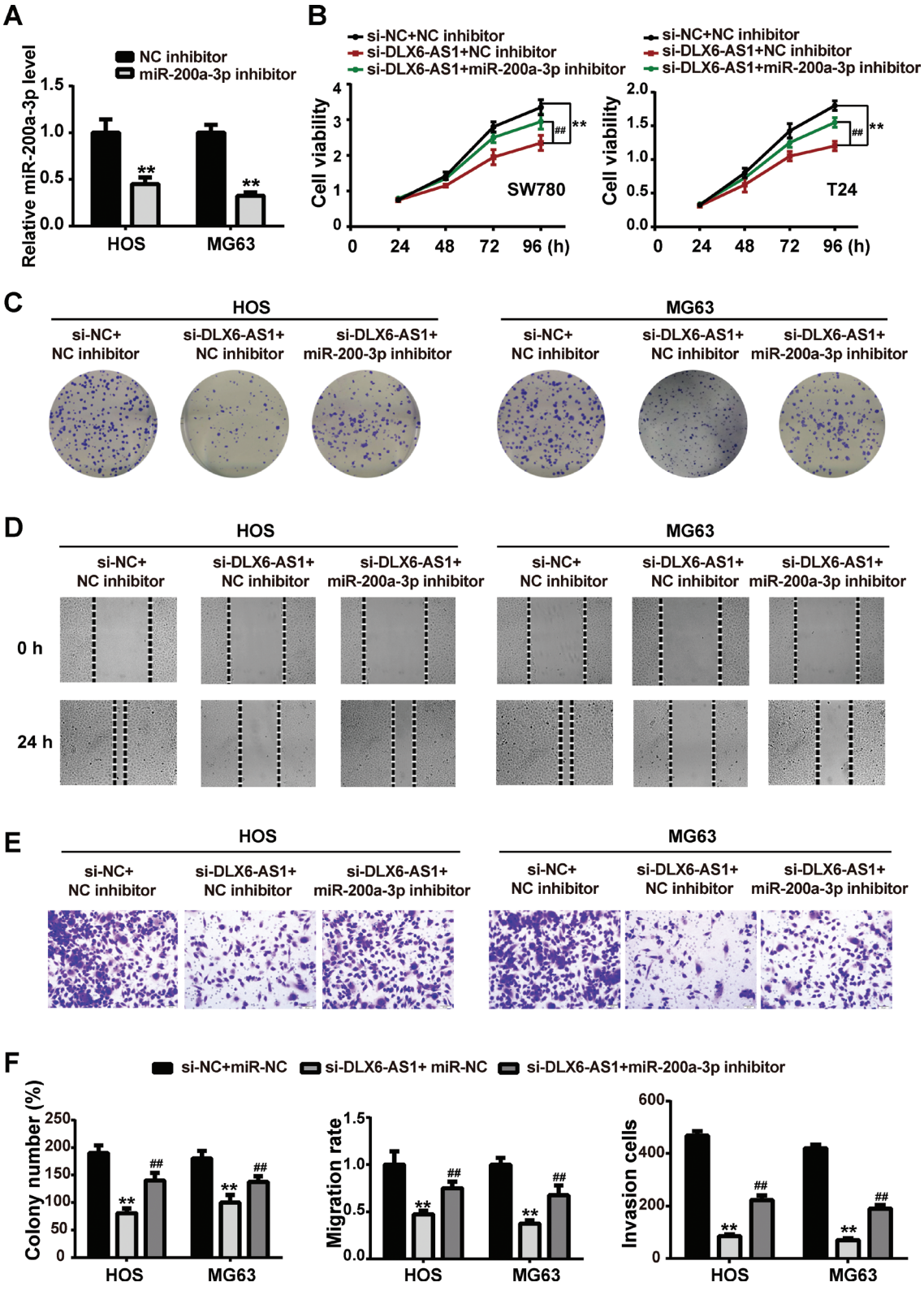

Fig. 4. DLX6-AS1 regulated OS progression through miR-200a-3p. (a) Relative expression levels of miR-200a-3p in OS cells after miR-200a-3p inhibitor transfection. (b, c) Colony formation assay and CCK-8 assay were performed to estimate cell proliferation with si-DLX6A transfection under miR-200a-3p inhibitor or NC inhibitor. (d, e) Cell migration and invasion were investigated through the wound healing assay and transwell assay with miR-200a-3p inhibitor or NC inhibitor. (f) Quantitative analysis was presented as bar graphs. NC, negative control. $^{**}P < 0.01$, versus NC inhibitor or si-NC+NC inhibitor; ##<0.01, versus si-DLX6-AS1+NC inhibitor.

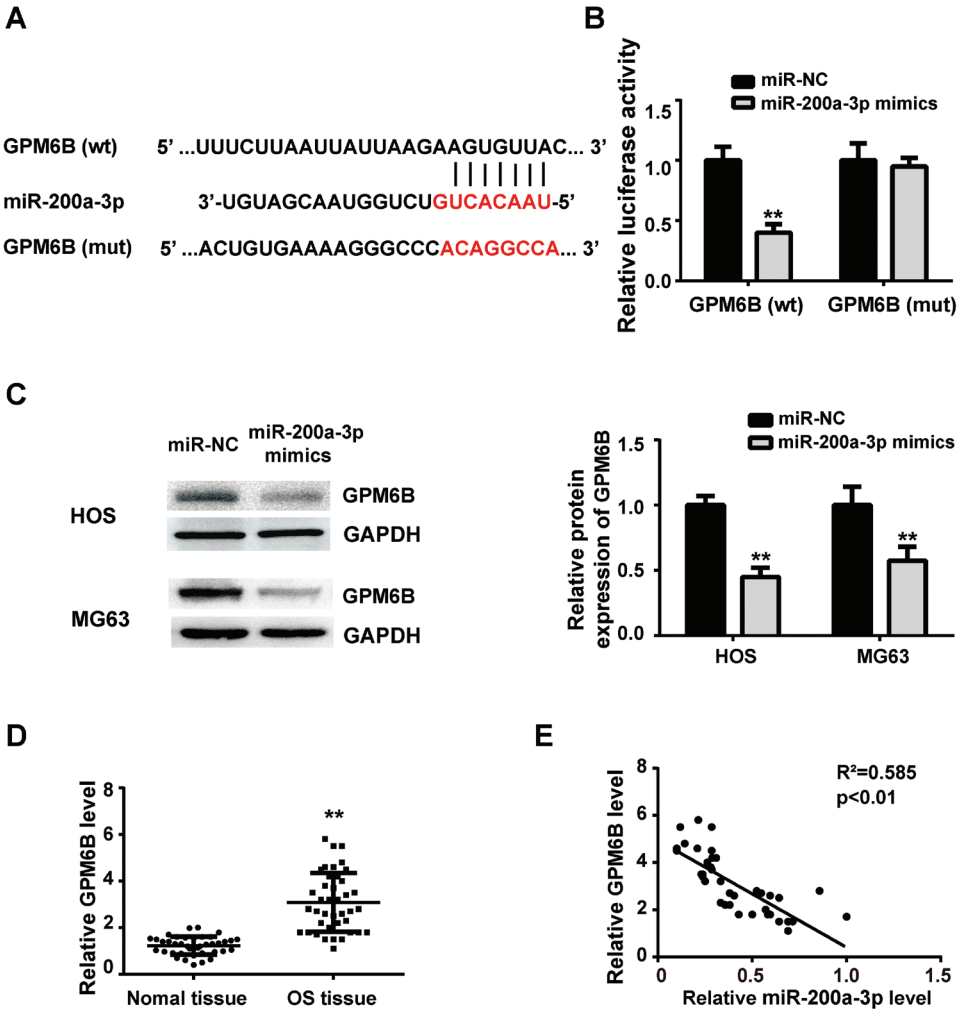

Fig. 5. miR-200a-3p targeted GPM6B directly. (a) Binding sites between DLX6-AS1 and miR-200a-3p via TargetScan. (b) The mutual effect between miR-200a-3p and GPM6B in HEK-293T cells was confirmed by a luciferase assay. (c) The protein expression of GPM6B was estimated with miR-200a-3p mimics transfection. (d) The mRNA expression of GPM6B in OS tissues and adjacent normal tissues was measured via RT-qPCR. (e) miR-200a-3p had a negative correlation with GPM6B. NC, negative control. $^{**}P < 0.01$, versus miR-NC or normal tissues.

pancreatic cancer (26). Thus, we predicted the targets of DLX6-AS1 and focused on miR-200a-3p as a potential target by bioinformatics analysis. MiR-200a-3p is known as a member of miR-200 having anti-tumor property. It played the role as a tumor inhibitor in different cancer types, such as esophageal (27), lung (28) and colorectal cancers (22). Of note, it was reported to act as the target of lncRNAs in the progression of multiple cancers. For example, Wu et al. showed that SNGH15 sponged miR-200a-3p to drive papillary thyroid carcinoma (29). Li et al. found that HULC targeted miR-200a-3p and promoted hepatocellular carcinoma (30). Wei et al. demonstrated that MALAT1 regulated miR-200a-3p, and then contributed to the progression of non-small-cell lung cancer (28). Whereas the mechanism of miR-200a-3p in OS and whether it is involved in the DLX6-AS1-regulated OS progression remains to be unknown. This study confirmed that DLX6-AS1 targeted miR-200a-3p by luciferase reporter assay and demonstrated that DLX6-AS1 sponged miR-200a-3p and modulated OS cell proliferation, cell invasion, and cell migration.

In addition, miRNAs were reported to modulate gene expression via binding to the target mRNAs 3′-UTR (31). To investigate the downstream mechanism of miR-200a-3p, we predicted glycoprotein M6B (GPM6B) as the potential target gene of miR-200a-3p. GPM6B functioned in several types of cancers. Cyndia et al. found that GPM6B was upregulated in human lymphoid leukemia tissues and exerted oncogenic roles in the progression of lymphoid leukemia (32). It also revealed that GPM6B promoted breast cancer progression by inhibiting cell apoptosis (33). This study identified GPM6B as the direct

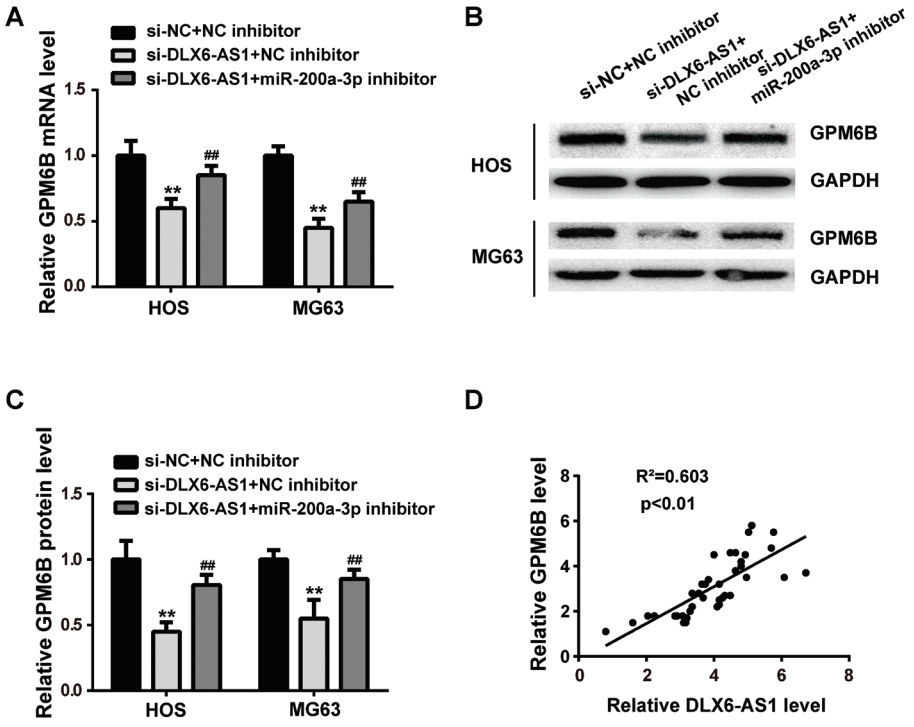

Fig. 6. DLX6-AS1 positively regulated GPM6B via sponging miR-200a. (a) Under si-DLX6-AS1 transfection with miR-200a-3p inhibitor or NC inhibitor, the mRNA expression of GPM6B was detected by qPCR. (b, c) Under si-DLX6-AS1 transfection with miR-200a-3p inhibitor or NC inhibitor, GPM6B protein expression was detected by qPCR. (d) DLX6-AS1 had a positive correlation with GPM6B. NC, negative control. $^{**}P < 0.01$, versus si-NC+NC inhibitor; ##<0.01, versus si-DLX6-AS1+NC inhibitor.

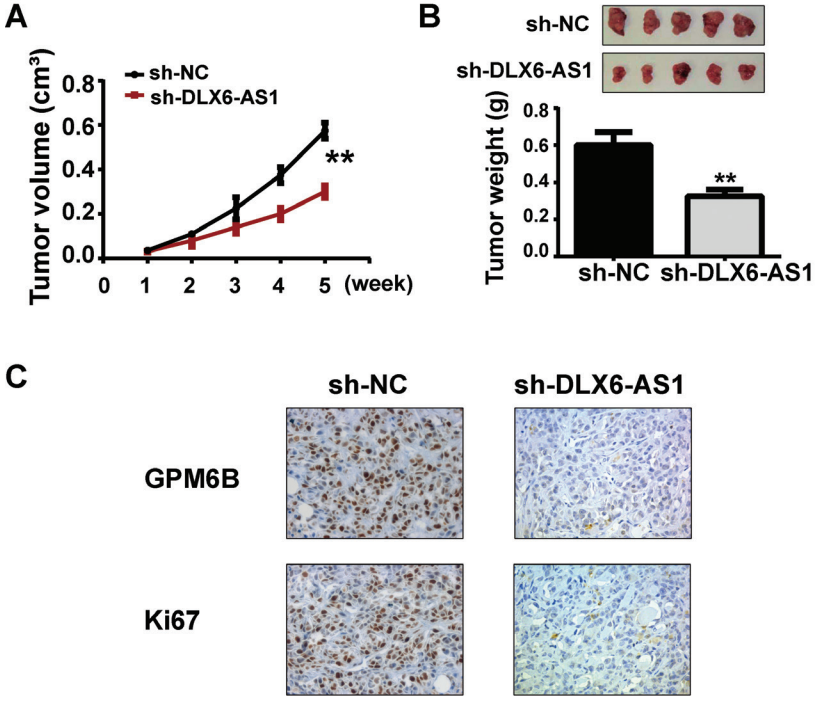

Fig. 7. DLX6-AS1 knockdown suppressed OS tumorigenesis in vivo. Mice were injected with DLX6-AS1-depleted or normal HOS cells, (a) tumor volume of each mouse was detected weekly; (b) tumor weight of each mouse was measured 5 weeks later; (c) immunohistochemistry staining was performed to detect the GPM6B and Ki67 protein expression levels. NC, negative control. $^{**}P < 0.01$, versus sh-NC group.

target of miR-200a-3p. Furthermore, it was revealed that DLX6-AS1 positively regulated GPM6B via sponging miR-200a-3p, indicating that DLX6-AS1 regulated OS progression through the miR-200a-3p/GPM6B axis.

Conclusions

Taken together, we discovered that DLX6-AS1 is highly expressed in OS tissues and cell lines, and accelerated cell migration, cell proliferation, and cell invasion via regulating the miR-200a-3p/GPM6B axis. It also suggested that DLX6-AS1 is a candidate target for OS diagnosis and treatment.

Conflict of interest and funding

None declared.

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