GPNMB silencing suppresses the proliferation and metastasis of osteosarcoma cells by blocking the PI3K/Akt/mTOR signaling pathway

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Abstract. Glycoprotein non-metastatic melanoma protein B (GPNMB) is a glycoprotein that is highly expressed in various types of cancer, including osteosarcoma. However, its cellular functions and related mechanisms in osteosarcoma remain unclear. In the present study, a higher GPNMB mRNA level was observed in osteosarcoma tissues, than in adjacent non-cancerous tissues. In addition, upregulation of the GPNMB mRNA and protein level was detected in the osteosarcoma cells SaOS2, 143B, MG63 and U2OS using western blot analysis and qPCR. Following transfection with GPNMB siRNA, the proliferation, migration and invasion of MG63 and U2OS cells were assessed using MTT and Transwell assays. The knockdown of GPNMB markedly inhibited the proliferation and metastasis of MG63 and U2OS cells. GPNMB silencing inhibited the activation of PI3K/Akt/mTOR signaling in MG63 and U2OS cells. PI3K/AKT activator insulin-like growth factor-1 (IGF-1) significantly activated the PI3K/Akt/mTOR signaling and reversed the suppressive effects of GPNMB silencing. IGF-1 counteracted the inhibitory effects of GPNMB silencing on the proliferation and metastasis of the MG63 and U2OS cells. In conclusion, we provided evidence that GPNMB silencing regulated the proliferation and metastasis of osteosarcoma cells by suppressing the PI3K/Akt/mTOR signaling pathway. Thus, GPNMB may be a potential therapeutic target for osteosarcoma treatment.

Introduction

Osteosarcoma is the most common malignant bone tumor affecting bone growth, particularly in children and adolescents (1). Osteosarcoma is closely associated with lung metastasis, causing death in ~15-25% of osteosarcoma patients (2). The survival rate for patients with metastatic disease or with tumor recurrence is <20% (3). Despite developments in osteosarcoma therapy, the relative 5-year survival rate of osteosarcoma patients remains only 60-70% due to high malignancy, invasion and metastasis (4). Therefore, it is of great importance to clarify the mechanism of osteosarcoma and develop new therapeutic strategies to prevent metastasis in osteosarcoma and improve patient prognosis.

Glycoprotein non-metastatic melanoma protein B (GPNMB), also known as osteoactivin, encodes the type I transmembrane proteins of 572 amino acids (5). GPNMB contains an extracellular domain, a transmembrane region and a cytoplasmic domain (6,7). GPNMB is expressed in numerous normal tissues, such as bone, the hematopoietic system and skin and it correlates with many biological processes, such as tissue regeneration, inflammation, cell proliferation, adhesion and migration (8). Several studies have reported that GPNMB is also expressed in malignant tissues and influences the metastasis of tumor cells (6,9-11). Furthermore, GPNMB level is reportedly elevated in several malignant cancers, such as uveal melanoma (12), prostate (13) and lung cancer (14). Halim *et al* (15) reported that GPNMB overexpression is prevalent in osteosarcoma. However, the potential impact of GPNMB on the progression of osteosarcoma remains unclear.

The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway is a major signaling cascade. The PI3K/Akt/mammalian target of the mTOR signaling pathway regulates downstream of the receptor tyrosine kinases, including insulin-like growth factor-1 receptor (IGF-1R) (16). This pathway plays a pivotal role in a variety of biological activities that regulate cell growth, survival and migration (17). In addition, researchers have confirmed that abnormalities in the PI3K/Akt/mTOR signaling pathway are involved in the carcinogenesis of various cancers, including osteosarcoma (18,19). This pathway is reportedly activated in osteosarcoma and its suppression could inhibit the proliferation and invasion of osteosarcoma cells (19). Ono *et al* (20) demonstrated that the extracellular fragment of GPNMB has neuroprotective effects and activates PI3K/Akt pathway. However, whether the PI3K/Akt/mTOR

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pathway is involved in the effects of GPNMB on osteosarcoma remains unclear. The present study was conducted to clarify the role of GPNMB in osteosarcoma. To understand the possible mechanisms involved, the effect of GPNMB on the PI3K/Akt/mTOR signaling pathway was explored. The results of the present study provided a prospective therapeutic target for osteosarcoma.

Materials and methods

Cell lines and clinical specimens. The normal human fetal osteoplastic cell line (hFOB) and human osteosarcoma cell lines SaOS2, 143B, MG63 and U2OS were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific) at 37˚C in a humidified 5% CO_2 atmosphere. Twenty paired osteosarcoma and adjacent normal tissues were received from patients in the Second Affiliated Hospital of Xi'an Jiaotong University from January to March, 2017 (Xi'an, China). All patients provided informed written consent and the study was approved by the Institutional Research Ethics Committee of Xi'an Jiaotong University.

Cell transfection. MG63 and U2OS cells were seeded into 6-well plates ($1x10^5$ cells/well) and grown for 24 h. Subsequently, MG63 and U2OS cells were transfected with 50 μM of GPNMB siRNAs, siGPNMB-1 and siGPNMB-2 or negative control siRNAs, siNC-1 and siNC-2 (all from Thermo Fisher Scientific, Inc.) for 48 h using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific). The transfection efficiency was detected using qPCR and western blot analysis.

MTT assay. The effect of GPNMB on cell viability was assessed by an MTT assay, as previously reported (21). The cells were seeded into 96-well plates at $1x10^4$ cells/well. Subsequently, 20 μl of MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added at 24, 48 and 72 h and incubated for 4 h. The supernatant was aspirated. Dimethyl sulfoxide (DMSO; 200 μl; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) was added to dissolve formazan crystals. Absorbance at 490 nm was detected using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

5'-Bromo-2'-deoxyuridine assay. A 5'-bromo-2'-deoxyuridine (BrdU) assay was performed to assess the effect of GPNMB on cell proliferation, as previously described (22). Cells ($1x10^5$ cells/ml) grown on coverslips were incubated with BrdU (Sigma-Aldrich; Merck KGaA) for 40 min and stained with anti-BrdU antibody (1:200; cat. no. sc-70443; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. Images were captured under an MC 100 microscope (Carl Zeiss, Oberkochen, Germany), assessing the percentages of BrdU in six random fields.

Transwell assay. To prevent proliferation, the cells were cultured in 10 g/ml of mitomycin C (Sigma-Aldrich; Merck KGaA) for 2 h (23). Then, a Transwell invasion assay was performed using an invasion chamber coated with Matrigel (BD Biosciences, San Jose, CA, USA). The lower chamber was filled with 600 μl of medium. Cells ($1x10^5$ cells/well) were plated into the upper Transwell chambers in serum-free medium for 24 h at 37˚C. The cells on the surface of the upper chamber were wiped with a cotton swab. After fixing with methanol for 20 min and staining with 0.1% crystal violet for 30 min, the invasive cells on the surface of the bottom membrane were determined by counting five random 100X fields under an MC 100 microscope (Carl Zeiss). The migration assay was the same as the invasion assay, with the exception of the upper chamber which was not coated with Matrigel. Each experiment was performed in triplicate.

Quantitative real-time PCR (qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The synthesis of cDNA was performed with the PrimeScript™ RT reagent kit (Takara Biotechnology, Co., Ltd., Dalian, China). The qPCR was carried out using SYBR-Green Premix (Takara Biotechnology, Co., Ltd.). The Bio-Rad CFX96 Touch qPCR system (Bio-Rad Laboratories, Hercules, CA, USA) was used to analyze the signal. The primers for qRT-PCR were as follows: GPNMB forward, 5'-ACAAGGAATACAACCCAATA-3' and reverse, 5'-ATAGCCACTCCAGCACA-3'; GAPDH forward, 5'-GACTCATGACCACAGTCCATGC-3' and reverse, 5'-AGAGGCAGGGATGATGTTCTG-3'. The expression of mRNAs was normalized to GAPDH. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Proteins were resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then the separated proteins were transferred to polyvinylidene fluoride membranes (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, the membranes were blocked in 5% bovine serum albumin (BSA) for 2 h and probed with primary antibodies, including rabbit anti-GPNMB (1:1,000; cat. no. ab98856), rabbit anti-p-Akt (1:1,000; ab38449), rabbit anti-Akt (1:500; cat. no. ab8805), rabbit anti-p-mTOR (1:1,000; cat. no. ab109268) and rabbit anti-mTOR (1:2,000; cat. no. ab2732; all from Abcam, Cambridge, MA, USA) overnight at 4˚C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. ab6721; Abcam). The immunoblots were visualized with an enhanced chemiluminescence system (Pierce Biotechnology; Thermo Fisher Scientific, Inc.). Densitometry analysis was performed using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD, USA).

Statistical analysis. The data were analyzed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA) and are presented as the mean ± SD. Statistical analysis was performed using one-way ANOVA with subsequent SNK-q testing for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GPNMB expression is upregulated in osteosarcoma. The expression of GPNMB in human osteosarcoma tissues and

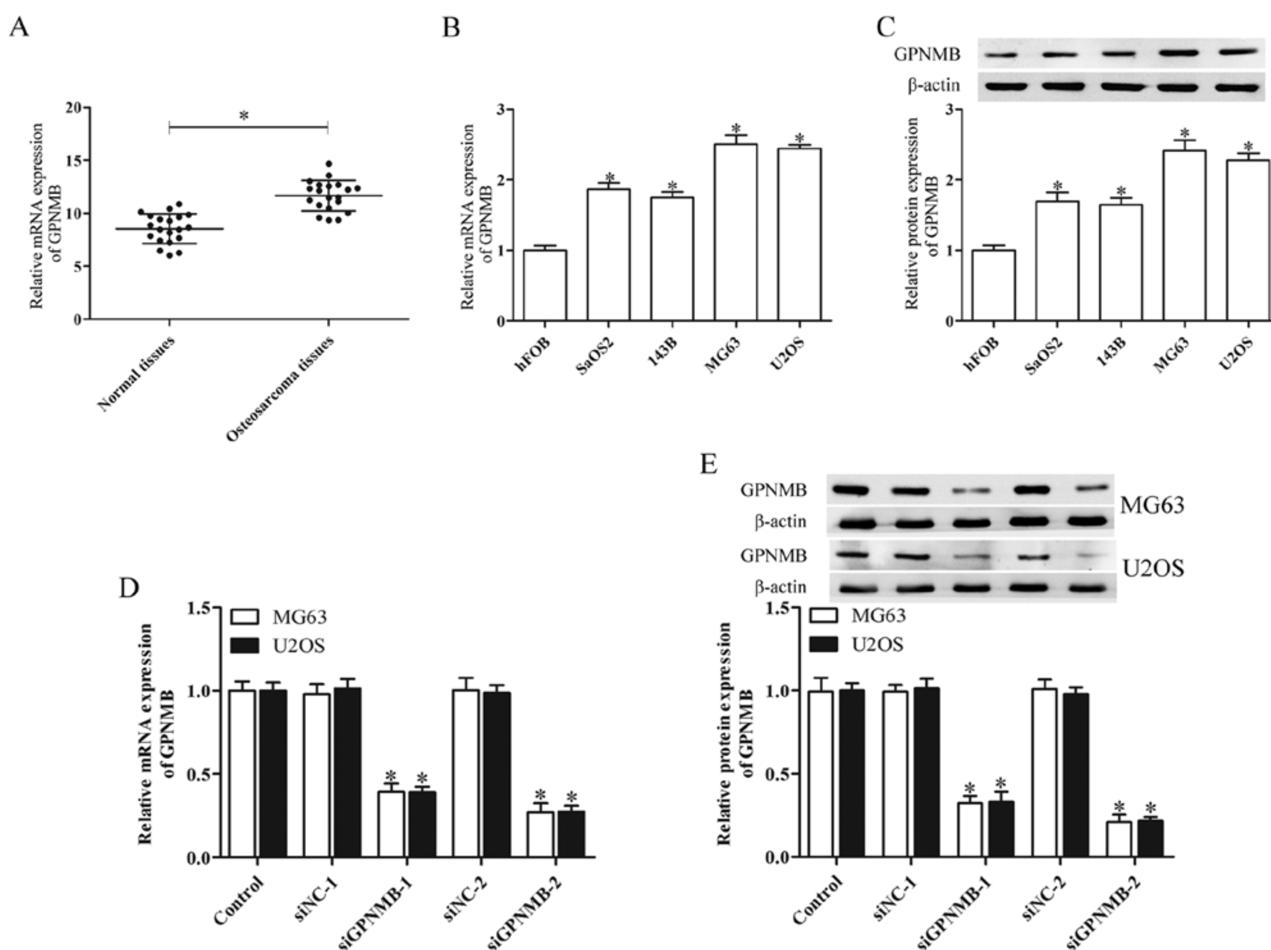

Figure 1. GPNMB is overexpressed in human osteosarcoma tissues and human osteosarcoma cell lines. (A) The mRNA expression of GPNMB in human osteosarcoma tissues and non-cancerous tissues was detected by qPCR. N=20, $^*P<0.05$ vs. adjacent non-cancerous tissues. (B) The mRNA and (C) protein levels of GPNMB in human osteosarcoma cell lines SaOS2, 143B, MG63 and U2OS were detected by qPCR and western blot analysis. N=3, $^*P<0.05$ vs. hFOB. (D and E) Interference efficiency was determined by qPCR and western blot analysis. siGPNMB-1 and siGPNMB-2 significantly downregulated the (D) mRNA and (E) protein expression of GPNMB in MG63 and U2OS cells. N=3, $^*P<0.05$ vs. the control. hFOB, human fetal osteoplastic cells.

human osteosarcoma cell lines was determined by qPCR and western blot analysis. The human osteosarcoma tissues derived from 20 patients demonstrated significantly higher GPNMB mRNA levels than the adjacent non-cancerous tissues (Fig. 1A). The mRNA and protein expression of GPNMB in the osteosarcoma cell lines SaOS2, 143B, MG63 and U2OS was upregulated in comparison with normal human fetal osteoplastic cells (hFOBs) (Fig. 1B and C). The MG63 and U2OS cells indicated higher GPNMB mRNA and protein expression than the SaOS2 and 143B cells. Therefore, these two cell lines were chosen for further study.

To investigate the functions of GPNMB in relation to osteosarcoma, we separately silenced its expression in the MG63 and U2OS cells via GPNMB siRNA transfection. A high inhibitory GPNMB mRNA and protein level was found in the MG63 and U2OS cells transfected with GPNMB siRNA ($P<0.05$; Fig. 1D and E). Of the two GPNMB siRNAs (siGPNMB-1 and siGPNMB-2), siGPNMB-2 was the most efficient and thus was chosen for further experiments.

GPNMB silencing inhibits proliferation and metastasis of osteosarcoma cells. Knockdown of GPNMB in the MG63 and U2OS cells resulted in reduced cell proliferation (Fig. 2A and B). The Transwell assay indicated that GPNMB silencing notably inhibited the migration (Fig. 2C and D) and invasion (Fig. 2E and F) of the MG63 and U2OS cells. These results indicated that GPNMB may promote the progression of osteosarcoma.

GPNMB silencing suppresses the activity of the PI3K/Akt/mTOR signaling pathway in osteosarcoma cells. The abnormal activation of the PI3K/Akt/mTOR signaling pathway plays a critical role in osteosarcoma pathogenesis (24,25). Therefore, we further evaluated whether GPNMB affected the PI3K/Akt/mTOR signaling pathway in osteosarcoma cells. The protein levels of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR were obviously upregulated in the MG63 and U2OS cells (Fig. 3A), whereas the protein levels were significantly downregulated by GPNMB siRNA in both MG63 (Fig. 3B) and U2OS cells (Fig. 3C). These results indicated that the GPNMB silencing inhibited the activation of the PI3K/Akt/mTOR signaling pathway in osteosarcoma cells.

GPNMB silencing suppresses the proliferation and metastasis of osteosarcoma cells by blocking the PI3K/Akt/mTOR

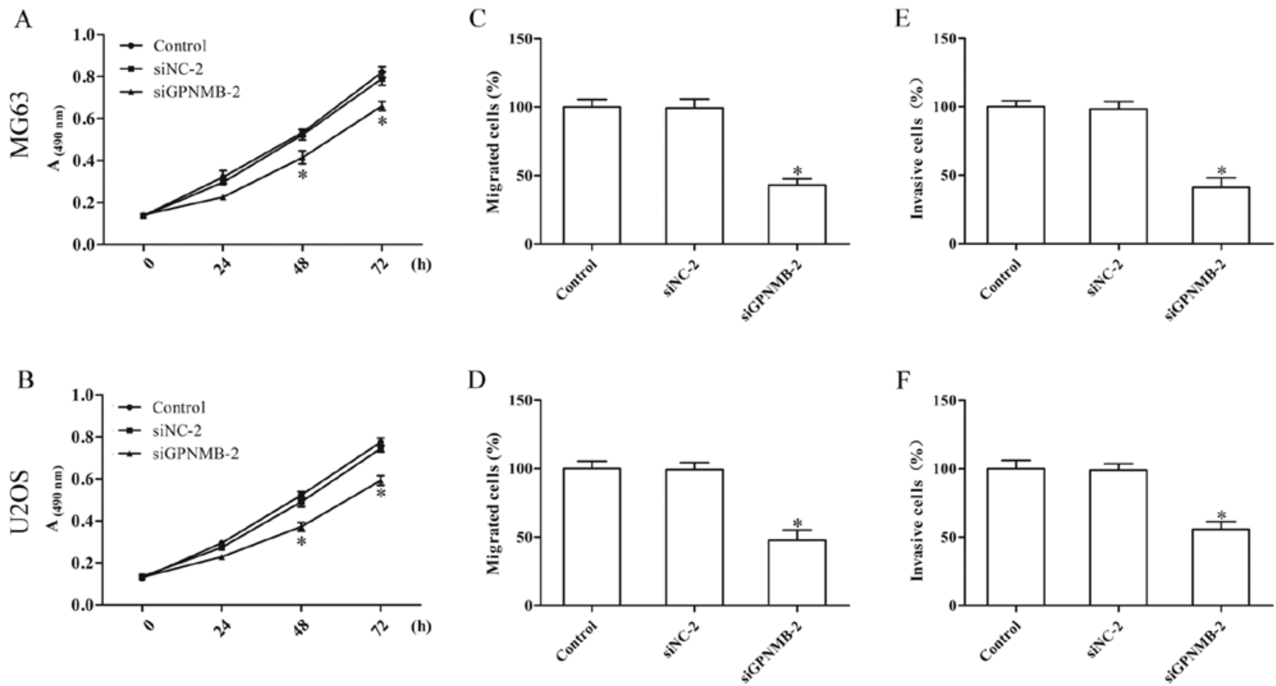

Figure 2. GPNMB silencing inhibits the proliferation, migration and invasion of osteosarcoma cells. The proliferation of (A) MG63 and (B) U2OS cells was detected by an MTT assay on three consecutive days. The siGPNMB-2-transfected cells led to a significant decrease in the proliferation of MG63 and U2OS cells. (C and E) Cell migration and invasion of MG63 and (D and F) U2OS cells were detected by Transwell assay. siGPNMB-2 significantly inhibited cell migration and invasion in MG63 and U2OS cells. N=3, *P<0.05 vs. the control.

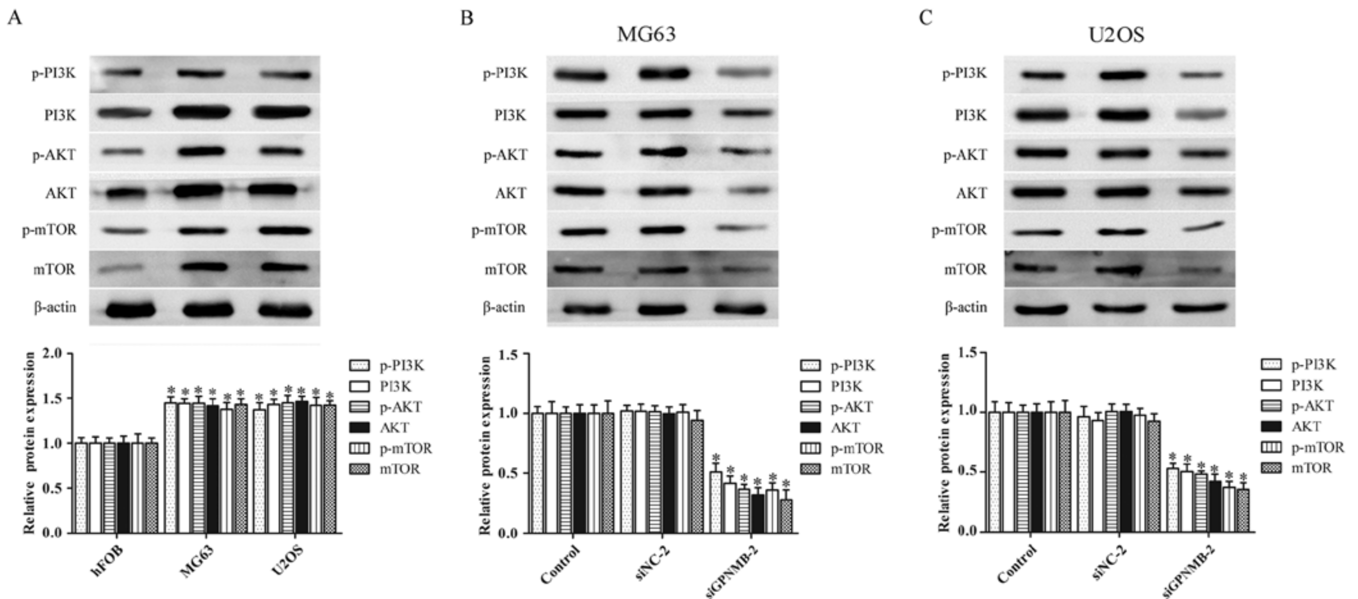

Figure 3. GPNMB silencing suppresses the activation of the PI3K/Akt/mTOR signaling pathway in osteosarcoma cells. (A) The protein expression of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR was upregulated in MG63 and U2OS cells. N=3, *P<0.05 vs. hFOB cells. (B and C) GPNMB silencing inhibited the PI3K/Akt/mTOR signaling pathway-related proteins in (B) MG63 and (C) U2OS cells. N=3, *P<0.05 vs. control.

signaling pathway. To identify whether the effect of GPNMB silencing on osteosarcoma cells was achieved by regulating the PI3K/Akt/mTOR signaling pathway, the MG63 and U2OS cells were cultured with IGF-1 (3 ng/ml) (26), an agonist of PI3K. IGF-1 activated the PI3K/Akt/mTOR signaling and abolished the inhibition of PI3K/Akt/mTOR signaling induced by siGPNMB-2, as evidenced by the upregulation of the protein expression of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR (Fig. 4A and B).

Furthermore, the inhibitory effect of GPNMB silencing on the proliferation (Fig. 4C and F), migration (Fig. 4D and G) and invasion (Fig. 4E and H) of the MG63 and U2OS cells was significantly reversed by IGF-1. These results demonstrated that GPNMB silencing inhibited the progression of

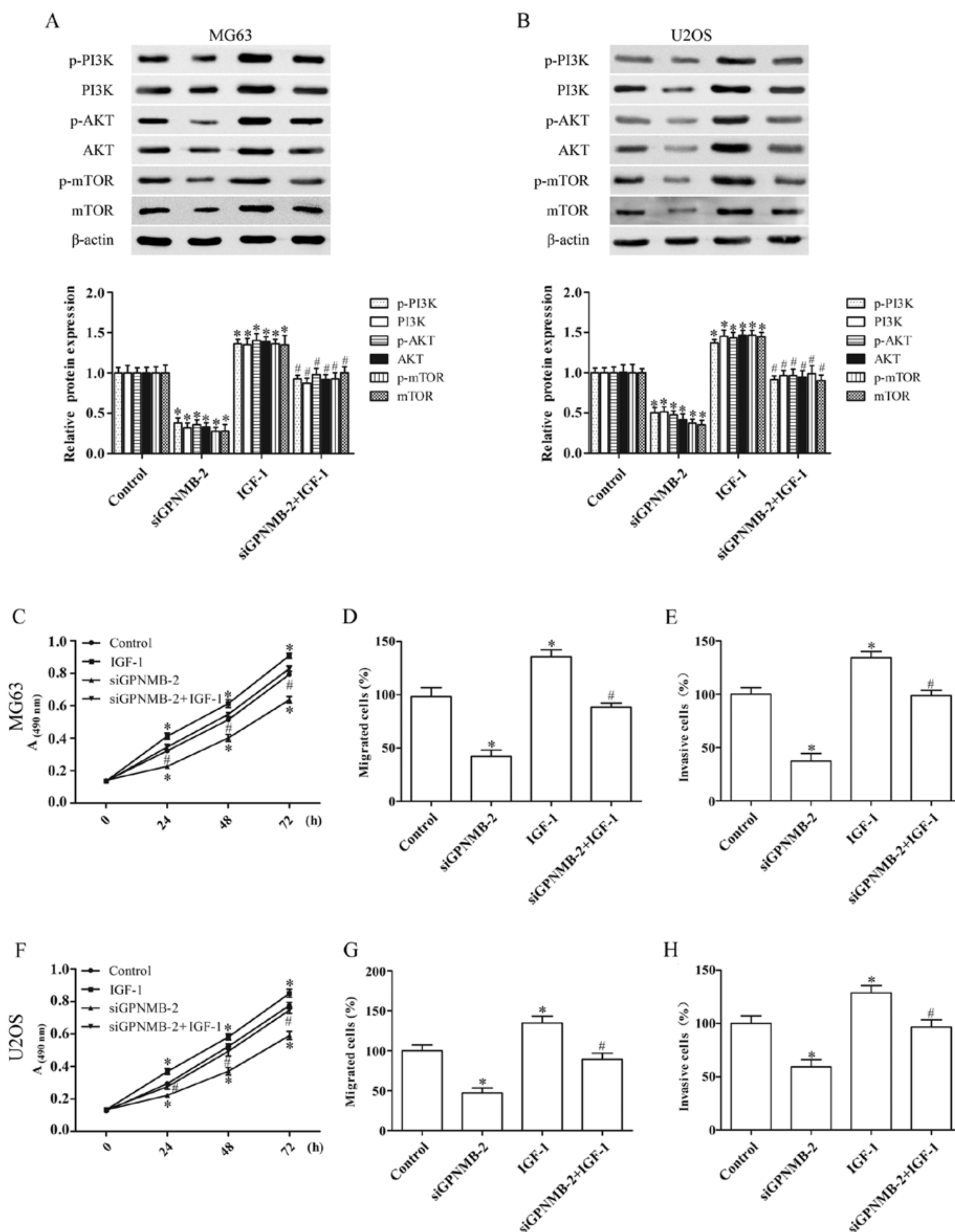

Figure 4. GPNMB silencing inhibits osteosarcoma tumorigenesis by suppressing the PI3K/Akt/mTOR pathway. The cells were treated with IGF-1 (3 ng/ml) to stimulate the PI3K/Akt/mTOR signaling pathway. (C-H) IGF-1 efficiently reversed the inhibitory effects of GPNMB siRNA on the PI3K/Akt/mTOR pathway in (A) MG63 and (B) U2OS cells. IGF-1 counteracted the effects of siGPNMB-2 on the (C and F) proliferation, (D and G) migration and (E and H) invasion of the MG63 and U2OS cells. N=3, *P<0.05 vs. the control; #P<0.05 vs. siGPNMB-2.

osteosarcoma cells by blocking the PI3K/Akt/mTOR signaling pathway.

Discussion

Osteosarcoma is the most common bone tumor with a high mortality rate. In the present study, we revealed that glycoprotein non-metastatic melanoma protein B (GPNMB) was aberrantly overexpressed in osteosarcoma tissue and osteosarcoma cells. The knockdown of the expression of GPNMB significantly inhibited the progression of osteosarcoma cells by inhibiting the PI3K/Akt/mTOR signaling pathway. Our findings indicated that GPNMB may be a promising therapeutic target for osteosarcoma. GPNMB is

a transmembrane glycoprotein involved in various pathological processes, including the development of cancer. It has been reported to increase cellular survival signals, thus promoting tumor growth (6). In addition, GPNMB is highly expressed in various types of cancer, including osteosarcoma (15). The results from a pediatric preclinical testing program of solid tumor xenografts revealed that GPNMB was primarily expressed in osteosarcoma xenografts (27). In a clinical trial, targeting GPNMB with the antibody-drug conjugate glembatumumab vedotin demonstrated the potential utility of targeting GPNMB for the treatment of osteosarcoma (28), however, its role in human osteosarcoma remains unclear.

In the present study, the expression of GPNMB was highly expressed in osteosarcoma tissue and osteosarcoma cells. To elucidate the biological function of GPNMB in osteosarcoma cells, we used GPNMB siRNA to knock down the expression of GPNMB. GPNMB silencing inhibited the proliferation, migration and invasion of the MG63 and U2OS cells. Collectively, these results indicated that GPNMB plays a vital role in the tumorigenicity and progression of osteosarcoma, which is consistent with the role of GPNMB in other cancers (14,29,30).

The PI3K/Akt/mTOR signaling pathway plays a critical role in many biological processes, such as the survival, migration and progression of various types of cancer (31,32). PI3K activates its downstream molecule Akt, the core component of the PI3K/AKT signaling pathway, which in turn results in the phosphorylation and activation of mTOR through a cascade of regulators and ultimately increases the proliferation and metastasis of the tumor cells (33). A previous study has revealed that the aberrant activation of the PI3K/Akt/mTOR signaling pathway was a pivotal event in the pathogenesis and progression of osteosarcoma cells (34). To investigate the mechanisms of GPNMB silencing in the progression of osteosarcoma cells, we assessed the effect of GPNMB silencing on the PI3K/Akt/mTOR signaling pathway-related proteins p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR. Consistent with previous studies (35,36), we revealed that the PI3K/Akt/mTOR signaling pathway was activated in osteosarcoma cells, however the GPNMB silencing inhibited the activation of the PI3K/Akt/mTOR signaling pathway. The present study also revealed that the inhibitory effect of GPNMB silencing on the proliferation, migration and invasion of the MG63 and U2OS cells was partly reversed by the PI3K/Akt agonist IGF-1. These results demonstrated that the GPNMB silencing exerted an inhibitory effect on the progression of osteosarcoma by suppressing the PI3K/Akt/mTOR signaling pathway. However, IGF-1 did not completely reverse the effect of GPNMB, indicating that other signaling pathways may be involved in the effect of GPNMB on osteosarcoma. Future research is warranted to further investigate these pathways.

In conclusion, the present study revealed that GPNMB was highly expressed in osteosarcoma and that GPNMB silencing inhibited the proliferation and metastasis of osteosarcoma cells by suppressing the PI3K/Akt/mTOR signaling pathway *in vitro*. Other possible effects of GPNMB should be investigated *in vivo*, as GPNMB may be a potential therapeutic target for the treatment of osteosarcoma.

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Availability of data and materials

The osteosarcoma and adjacent normal tissues were received from patients in the Second Affiliated Hospital of Xi'an Jiaotong University from January to March, 2017 (Xi'an, China).

Authors' contributions

RJ and YYJ conceived and designed the study. RJ, YYJ, YLT and HJY performed the experiments. YLT and ZL analyzed the data. RJ and XQZ wrote and reviewed the manuscript. All authors read and approved the 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

All patients provided informed written consent and the study was approved by the Institutional Research Ethics Committee of Xi'an Jiaotong University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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