Research Paper

Small interfering RNA-mediated silencing of G-protein-coupled receptor 137 inhibits growth of osteosarcoma cells

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ABSTRACT

Purpose: Osteosarcoma is the most widespread primary carcinoma in bones. Osteosarcoma cells are highly metastatic and frequently develop resistance to chemotherapy making this disease harder to treat. This identifies an urgent need of novel therapeutic strategies for osteosarcoma. G-Protein-coupled receptor 137 (GPR137) is involved in several human cancers and may be a novel therapeutic target.
Methods: The expression of GPR137 was assessed in one osteoblast and three human osteosarcoma cell lines via the quantitative real-time polymerase chain reaction and western blot assays. Stable GPR137 knockdown cell lines were established using an RNA interference lentivirus system. Viability, colony formation, and flow cytometry assays were performed to measure the effects of GPR137 depletion on cell growth. The underlying molecular mechanism was determined using signaling array analysis and western blot assays.
Results: GPR137 expression was higher in the three human osteosarcoma cell lines, Saos-2, U2OS, and SW1353, than in osteoblast hFOB 1.19 cells. Lentivirus-mediated small interfering RNA targeting GPR137 successfully knocked down GPR137 mRNA and protein expression in both Saos-2 and U2OS cells. In the absence of GPR137, cell viability and colony formation ability were seriously impaired. The extent of apoptosis was also increased in both cell lines. Moreover, AMP-activated protein kinase α, proline-rich AKT substrate of 40 kDa, AKT, and extracellular signal-regulated kinase phosphorylation levels were down-regulated in GPR137 knockdown cells.
Conclusions: The results of this study highlight the crucial role of GPR137 in promoting osteosarcoma cell growth *in vitro*. GPR137 could serve as a potential therapeutic target against osteosarcoma.

1. Introduction

Osteosarcoma is the most common histological form of primary bone neoplasms and is often found in children and young adults. Although osteosarcoma is not a common cancer compared to others, more than 800 new osteosarcoma cases are diagnosed annually in the United States. Unfortunately, half of these are in children and teenagers [1]. Chemotherapy has been the primary treatment of high-grade malignant osteosarcoma and there has been substantial progress in improving the survival rate. However, the results continue to be unsatisfactory in the case of metastatic osteosarcomas and accompanying complex pathological conditions because the molecular cause of the disease remains unknown [2]. Current detailed investigations on the pathogenesis, progression, and prognosis of osteosarcoma have identified several molecules that might effectively modulate the initiation and progression of the disease. These include vascular endothelial growth factor (VEGF), mTOR, p53, Rb, APEX1, VEGFA, and c-myc [3]. Particularly, the importance and applicability of targeted therapy in osteosarcoma have been reviewed due to its high tendency to metastasize [4].

G-Protein-coupled receptors (GPRs) are the largest family of membrane-bound receptors and have been identified as key factors in many cancer types [5]. Cancer cells can evade immune detection and proliferate autonomously by hijacking the functions of GPRs [6]. Therefore, GPRs have become novel drug targets.

GPR family members play critical roles in the development and metastasis of osteosarcoma [7]. GPR137 belongs to a particular group of GPRs: orphan G protein-coupled receptors [8,9]. The GPR137 gene was discovered using homology screening and shares identity with a prostate-specific odorant-like orphan GPR that has been used as a diagnostic marker of prostate cancer [10]. Importantly, GPR137 is involved in several human cancers. Zong et al. [11] showed that GPR137 was highly expressed in high grade gliomas, and small interfering (si) RNA-mediated knockdown of GPR137 significantly suppressed their tumorigenic ability. A recent study revealed that GPR137 could modulate colon cancer cell proliferation and cell cycle progression [12].

However, the role of GPR137 in human osteosarcoma has not been well-studied.

Given the ability to silence any gene through RNA interference (RNAi), siRNA has received mounting attention in targeted therapy. Importantly, the potential of siRNA-mediated gene silencing in the treatment of osteosarcoma has been studied. Zhang et al. [13] demonstrated that siRNA-mediated knockdown of the β-catenin gene reduced the invasion ability of MG63 osteosarcoma cells.

In the current study, we measured the expression of GRP13 in different osteosarcoma cells. With the aim to evaluate the roles of GRP13 played in osteosarcoma formation and development, we knocked it down in upregulated cells lines by using RNAi, determined the cell viability, colony formation and apoptosis change after GRP13 silence and discussed the mechanism.

2. Material and methods

2.1. Reagents and plasmids

Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum were purchased from Hyclone (Logan, UT, USA). Lipofectamine 2000 and TRIzol Reagent were obtained from Invitrogen (Carlsbad, CA, USA). M-MLV reverse transcriptase was purchased from Promega (Madison, WI, USA). The antibodies used were as follows: rabbit anti-GPR137 (Proteintech Group, Chicago, IL, USA), rabbit anti-phospho-AMP-activated protein kinase (AMPK)α (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-proline-rich AKT substrate of 40 kDa (PRAS40) (Cell Signaling Technology), rabbit anti-phospho-extracellular signal-regulated kinase (ERK)1/2 (Cell Signaling Technology), rabbit anti-AKT (Proteintech Group), rabbit anti-phospho-AKT (Cell Signaling Technology), rabbit anti-ERK (Proteintech Group), rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (Proteintech Group), and goat anti-rabbit horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA). Lentiviral vector pFH-L and the lentiviral packaging auxiliary carriers pVSVG-I and pCMV△R8.92 were purchased from Duruobio (Shanghai, China). SYBR Green Master Mix Kits and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The human embryonic kidney cell line 293 T, osteoblast hFOB 1.19 cells, and three human osteosarcoma cell lines (Saos-2, U2OS, and SW1353) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cell lines were cultured in DMEM supplemented with 10% heat inactivated foetal bovine serum and maintained in a humidified atmosphere of 5% CO_2.

2.3. Lentivirus vectors construction and infection

siRNA targeting GPR137 (NM_001170726.1) and non-silencing control siRNA were transformed into stem-loop-stem oligos and cloned into the pFH-L vector. The sequences used were: GPR137 siRNA (siGPR137): 5′-GACGCTTATGAACCTCTACTT-3′, and control siRNA (siCon): 5′-TTCTCCGAACGTGTCACGT-3′. The generated vectors were confirmed by DNA sequencing followed by transfection of the silencing and non-silencing siRNA-containing lentiviruses into 293 T cells. For the transfection, 293 T cells were cultured in 10 cm dishes at a density of 1×10^7 using standard protocols. Briefly, the medium was replaced 2 h prior to transfection with serum-free DMEM. The plasmids, including siRNA, packaging vector pHelper 1.0, and VSVG expression plasmid pHelper 2.0, were then added to 200 μL of Opti-MEM and 15 μL of Lipofectamine 2000. Following 48 h of transfection, the lentiviral particles were harvested by ultracentrifugation (80,000 g) at 4 °C for 30 min. Then, osteosarcoma cells (5×10^4 cells per well) were infected with the prepared lentiviruses (siGPR137 or siCon) in 6-well plates. Successful infections were confirmed after 96 h of infection by observing green fluorescent protein-expressing cells under a fluorescence microscope.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

GPR137 mRNA levels were measured by qRT-PCR analysis using the 2× SYBR Green Master Mix Kit with the DNA Engine Opticon™ System (MJ Research, Waltham, MA, USA). The PCR reaction mixture added to each vial contained 10 μL of 2× SYBR Green Master Mix, 0.8 μL of forward and reverse primers (2.5 μM), and 5 μL of cDNA (2 ng). The PCR experiment was carried out using the following protocol in a BioRad Connect RT-PCR platform: initial denaturation at 95 °C for 1 min, 40 cycles of denaturation at 95 °C for 5 s, and annealing extension at 60 °C for 20 s. Actin was used as the housekeeping mRNA reference. The primers (5′ to 3′) used for GPR137 expression analysis were: GPR137: forward (ACCTGGGGAACAAAGGCTAC) and reverse (TAGGACCGAGAGGCAAAGAC); actin: forward (GTGGACATCCGCAAAGAC) and reverse (AAAGGGTGTAACGCAACTA). Relative gene expression levels were quantified using the $2^{-\Delta\Delta CT}$ method.

2.5. Western blot analysis

After infection with lentivirus for five days, cells were washed, collected, and lysed with 2× sodium dodecyl sulphate (SDS) sample buffer containing 100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS, and 10% glycine on ice for 30 min. Then, the supernatant was collected by centrifuging (12,000×g for 15 min). The protein content was measured by the Lowry method and 20 μL of each sample containing 140 μg protein were electrophoresed on a 10% SDS-polyacrylamide gel, and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated with specific primary antibodies overnight at 4 °C, followed by secondary antibody treatment for another 2 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK) by exposing to X-ray film. Bands were quantified using an ImageQuant densitometry scanner (Molecular Dynamics, Sunnyvale, CA, USA).

2.6. 3-(4,5-Dimethylthiazol-2-yl) − 2,5-diphenyltetrazolium bromide (MTT) assay

Cells were cultured in 96-well plates at 2×10^3 cells/well and transfected with siRNA containing lentivirus for 1–5 days. At the end of each incubation period, 20 μL of MTT solution (5 mg/mL) were added to each well and incubated at 37 °C for 4 h. The formazan crystals were dissolved in 200 μL of acidic isopropanol (10% SDS, 5% isopropanol. and 0.01 M HCl). Finally, the optical density was measured using a microplate reader at 595 nm.

2.7. Colony formation assay

To further investigate the effect of GPR137 silencing on the proliferation of osteosarcoma cells, the colony formation assay was performed. Briefly, transfected cells (500 cells/well) were seeded into six-well plates and incubated for two weeks, replacing the medium every three days. After the incubation period, the cells were washed, fixed with 4% paraformaldehyde, and stained with freshly prepared diluted crystal violet dye for 20 min. Finally, the colonies were visualized and counted under a light/fluorescence microscope.

2.8. Cell apoptosis analysis

The influence of GPR137 depletion on cell apoptosis was analysed by flow cytometry. Lentivirus-transduced and control cells were seeded on 6 cm culture dishes at a density of 2×10^5 cells/dish and incubated

for 40 h. Then, the cells were collected and stained using an Annexin V-APC/7-AAD apoptosis detection kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's instructions. Finally, the cell fractions were analysed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA).

2.9. Antibody arrays

The PathScan® Intracellular Signaling Array Kit (Chemiluminescent Readout) was used for the antibody array assay according to the manufacturer's guidelines. Briefly, osteosarcoma cells were transfected with lentiviruses expressing siGPR137 or siCon and the cells were lysed after six days. Cell lysates were incubated on the pad slide provided in the kit, followed by adding a biotinylated detection antibody cocktail. The bound target antibodies, including AMPKα, PRAS40, and ERK1/2, were visualized by chemiluminescence with streptavidin-conjugated horseradish peroxidase and LumiGLO® reagent. An image of the slide was captured using standard chemiluminescent imaging and then analysed for spot intensities with array analysis software.

2.10. Statistical analysis

All data are expressed as means ± SD of three independent experiments. Data were analysed statistically using GraphPad Prism software and compared employing the T-test. Statistical significance was defined as a P value less than 0.05.

3. Results

3.1. Lentivirus-mediated silencing of GPR137 expression in osteosarcoma cells

We first determined differences in the expression of GPR137 mRNA and protein in osteoblast hFOB 1.19 cells and three human osteosarcoma cell lines (Saos-2, U2OS, and SW1353) using qRT-PCR and western blot assays, respectively. The three osteosarcoma cell lines all showed a higher expression of GPR137 than normal osteoblast hFOB 1.19 cells (Fig. 1A, B). Expression was highest in Saos-2 cells, followed by U2OS cells. Therefore, Saos-2 and U2OS cells were used for further experiments.

To knockdown the expression of GPR137 in osteosarcoma cells, lentivirus stably expressing a GPR137-targeting siRNA was transfected into the cells. qRT-PCR and western blot assays were performed to validate the successful infection. GPR137 mRNA levels were suppressed by 60% and 70% in Saos-2 and U2OS cells, respectively (Fig. 1C, $p < 0.01$, $p < 0.001$, respectively). Similarly, GPR137 protein expression was also inhibited in both Saos-2 and U2OS cells (Fig. 1D). Collectively, these results verified that the transfection of GPR137 siRNA into osteosarcoma cells efficiently knocked down GPR137 expression.

3.2. Knockdown of GPR137 decreased the survival of osteosarcoma cells

The effect of GPR137 silencing on the survival of osteosarcoma cells was analysed by the MTT and colony formation assays. As depicted in Fig. 2A, the proliferation of Saos-2 and U2OS cells in the siGPR137 groups was decreased significantly in comparison to the siCon groups ($p < 0.001$) at the fifth day of infection. Moreover, the size and number of colonies formed by both Saos-2 and U2OS cells were remarkably suppressed in the absence of GPR137 (Fig. 2B). Graphical representation of the colony numbers in GPR137 silenced and non-silenced cells showed that 45% and 80% of the colonies disappeared in the absence of GPR137 in Saos-2 and U2OS cells, respectively (Fig. 2B, $p < 0.05$, $p < 0.01$, respectively). The results clearly verified that GPR137 silencing strongly affected the survival of osteosarcoma cells.

3.3. GPR137 silencing increased apoptosis of osteosarcoma cells

Apoptosis of Saos-2 and U2OS cells was analysed by flow cytometry.
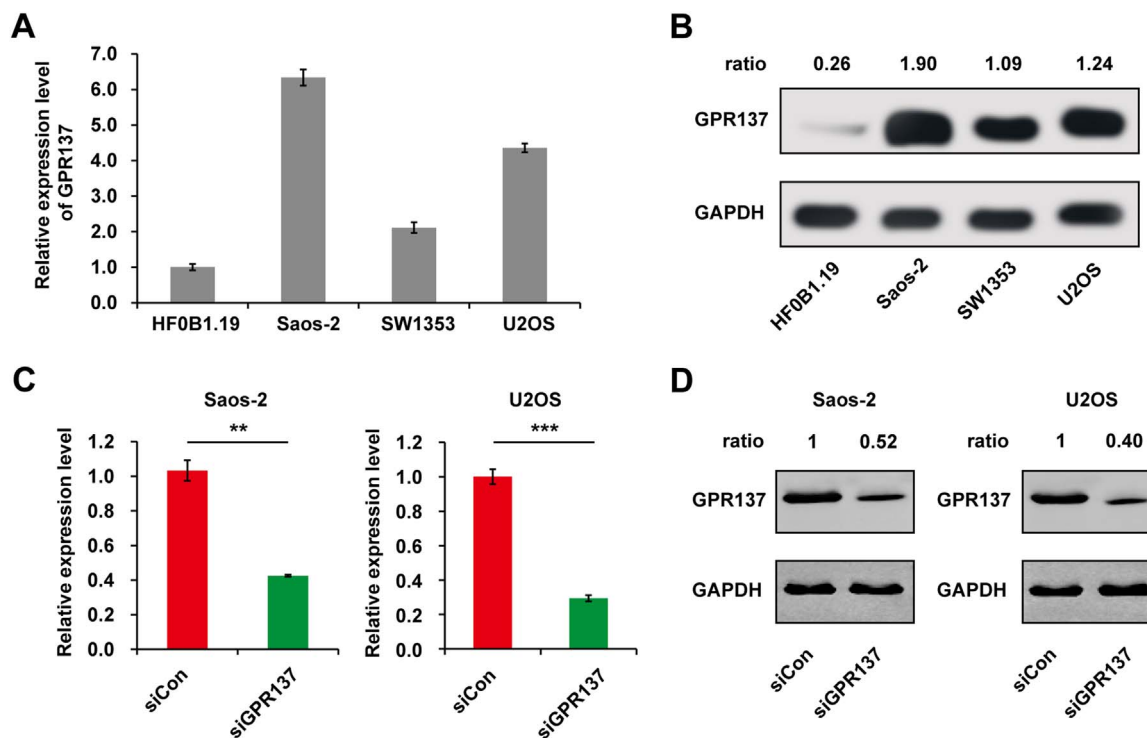

Fig. 1. Knockdown of GPR137 by siRNA-expressing lentivirus in osteosarcoma cells. GPR137 mRNA (A) and protein (B) levels were measured by the quantitative real time polymerase chain reaction (qRT-PCR) and western blot assays, respectively, in normal osteoblast hFOB 1.19 cells and three osteosarcoma cell lines (Saos-2, U2OS, and SW1353). GPR137 mRNA (C) and protein (D) levels in Saos-2 and U2OS cells infected with siRNA against GPR137 (siGPR137) or control siRNA (siCon) were measured by qRT-PCR and western blot assays, respectively. Experiments were performed in triplicate and the significance level was calculated at **$p < 0.01$ or ***$p < 0.001$.
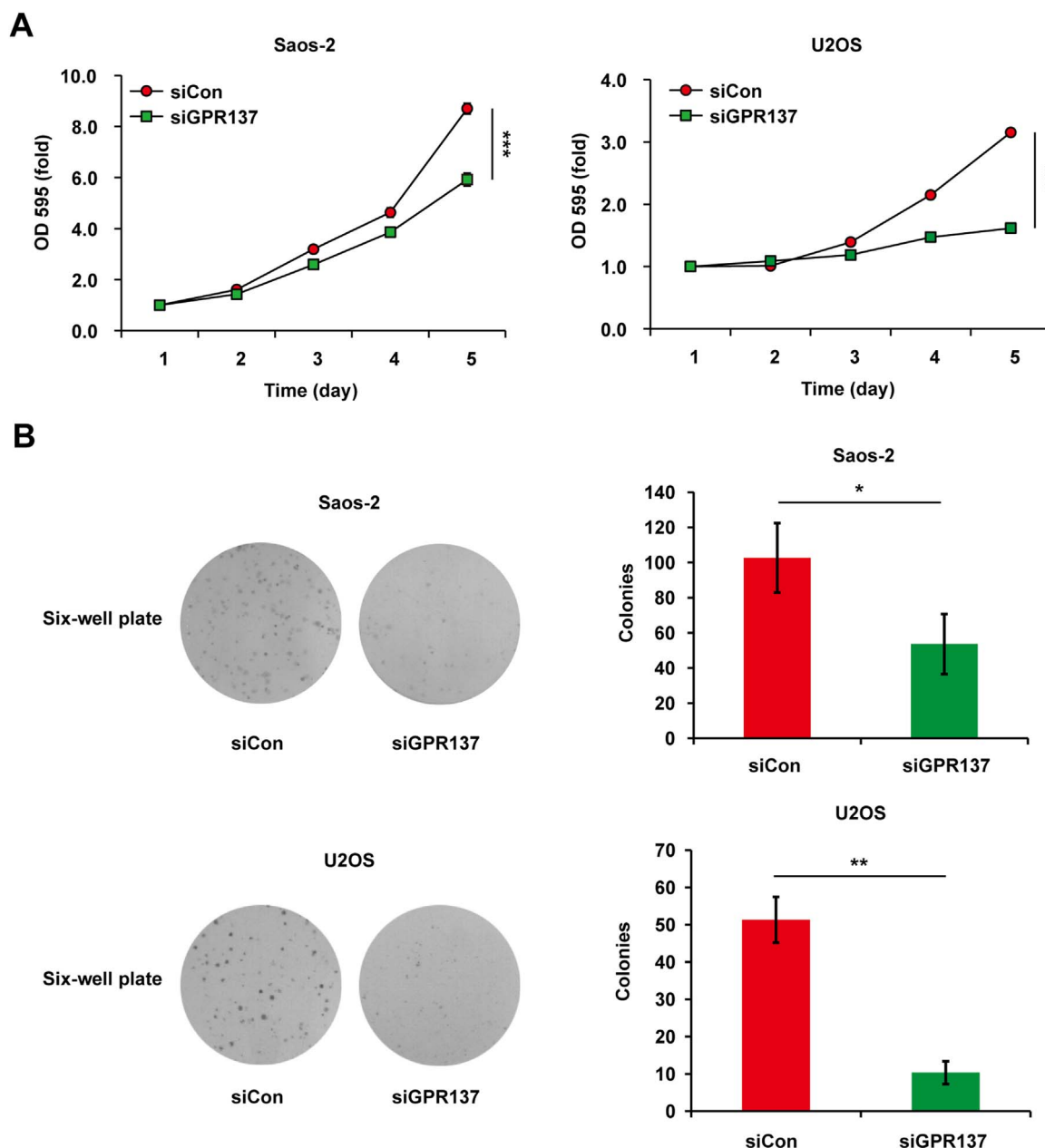
Fig. 2. GPR137 silencing affected the proliferation of osteosarcoma cells. (A) Growth curves of Saos-2 and U2OS cells infected with siRNA against GPR137 (siGPR137) or control siRNA (siCon) were analysed by the MTT assay over a period of five days. (B) The size and number of colonies formed by Saos-2 and U2OS cells infected with siGPR137 and siCon. Experiments were performed in triplicate and the significance level was calculated at *$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$.

Graphical representation of the flow cytometry data clearly indicated that the number of early apoptotic cells in both siGPR137 Saos-2 and U2OS cells was significantly increased compared with that in the siCon groups. However, late apoptosis was increased in Sao-2 cell and decreased in U2OS cells. These different phenomena need a further mechanism study (Fig. 3A, B). These results indicated that GPR137 silencing induced early apoptosis in osteosarcoma cells.

3.4. GPR137 silencing activated intracellular signaling pathways in osteosarcoma cells

Because GPR137 silencing affected cell survival, the PathScan® Intracellular Signaling Array Kit was used to examine which intracellular signaling pathways were activated by GPR137. As shown in Fig. 4A, the level of phosphorylated ERK1/2 were decreased significantly, while AMPKα, and PRAS40 stayed the same, in the absence

of GPR137 in USOS cells. Similarly, the western blot assay revealed that the level of phosphorylated ERK was also reduced in siGRP137-infected cells compared with that in the siCon group (Fig. 4B). These data suggested that ERK, AMPKα, and AKT signaling pathways were activated in GRP137-silenced osteosarcoma cells.

4. Discussion

Osteosarcoma is most frequent in adolescents between 10 and 20 years of age. This cancer also occurs more often in males than females [14]. High-grade osteosarcoma commonly occurs juxtaposed to the knee joint involving the distal femur or proximal tibia [15]. When diagnosed with osteosarcoma, the cancer has commonly metastasized into other tissues, mainly the lungs. The standard curative method for osteosarcoma is surgery combined with chemotherapy [1]. The survival rate post-surgery is prolonged by the use of chemotherapy. However,
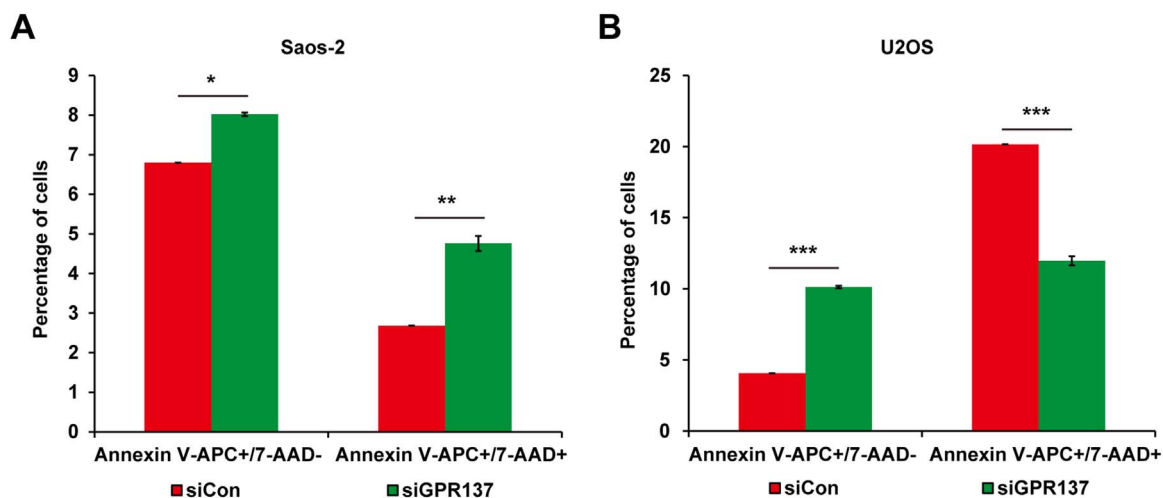
Fig. 3. Effect of GPR137 silencing on apoptosis of osteosarcoma cells. Graphical representation of the proportion of apoptotic Saos-2 (A) and U2OS (B) cells infected with siRNA against GPR137 (siGPR137) or control siRNA (siCon). Experiments were performed in triplicate and the significance level was calculated at *p < 0.05, **p < 0.01, or ***p < 0.001.
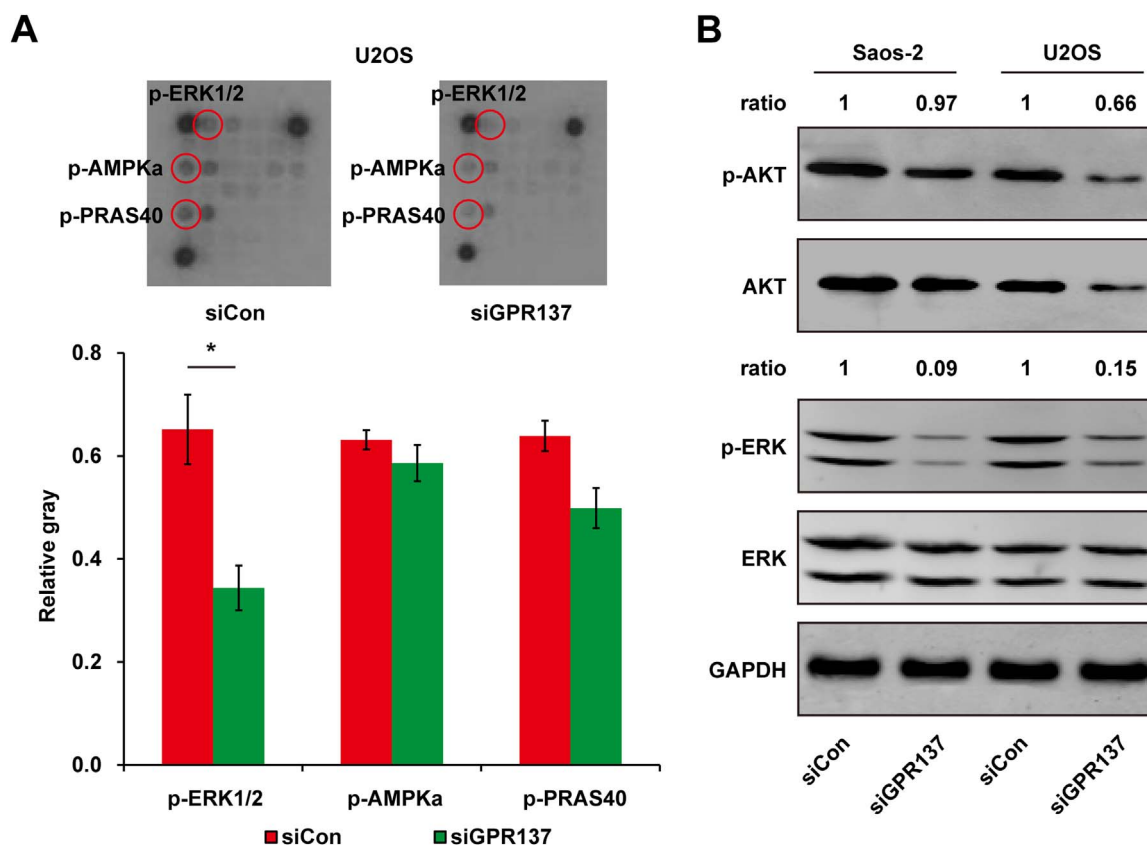

Fig. 4. Effect of GPR137 knockdown on intracellular cell survival signaling pathways. (A) Signaling pathways were assessed with the Intracellular Signaling Array Kit in U2OS cells infected with siRNA against GPR137 (siGPR137) or control siRNA (siCon). (B) The expression levels of phosphorylated AKT and ERK were analysed by the western blot assay in Saos-2 and U2OS cells infected with siGPR137 or siCon. Experiments were performed in triplicate and the significance level was calculated at *p < 0.05.

the success rates are far from ideal in patients with recurrent or metastatic osteosarcomas [16]. Therefore, finding new strategies to treat osteosarcoma remains an urgent clinical need.

Despite osteosarcoma being the most common form of bone cancer, the aetiology of the disease is poorly elucidated [17]. There is growing knowledge of its association with specific genetic changes. GPR family proteins are highly expressed in osteosarcoma, and around 40% of the drugs used for this disease are designed to target these proteins. However, there are large numbers of GPR family proteins that have not been studied in detail, and their involvement in cancer progression is still unknown. GPR137 belongs to the orphan GPR group and is involved in cell growth in various human cancers including prostate [18], pancreatic [19], urinary bladder [20], and colon cancers [21]. In the current study, we found that GPR137 was overexpressed in osteosarcoma cells compared with normal osteoblasts.

RNAi technology can be used to silence the expression of any disease-related transcribed genomic sequence in a selective and sequence-dependent manner [22]. Recently, several important studies have focused on the impact of siRNAs on tumorigenesis and the progression of osteosarcoma [23–25]. For the current study, we used in vitro cellular

models to investigate our hypothesis. As clearly shown by the results, lentivirus-mediated GPR137 silencing significantly reduced the proliferation and increased the apoptosis of both Saos-2 and U2OS cells. This indicated that GPR137 may be associated with the survival of osteosarcoma cells.

AMPK is a heterometric protein that functions as a cellular energy sensor [26]. In addition, it is well-documented that AMPK is involved in energy regulation of bone tissues by supplying energy through glucose and lipid oxidation [27,28]. Furthermore, AKT, as a parameter of tumours, could enhance tumour progression, invasiveness, and inhibit apoptosis of tumour cells [29,30]. PRAS40 is a significant substrate of AKT3 kinase, which regulates the apoptotic sensitivity of cancer cells and thereby promotes tumorigenesis such as melanoma [31]. Additionally, activation of ERK1/2 is directly involved in the proliferation and metastasis of osteosarcoma cells [32]. In our study, we found that GPR137 silencing down-regulated the phosphorylation levels of ERK1/2 in both Saos-2 and U2OS cells. However, p-Akt was only down-regulated in U2OS and stayed the same in Saos-2 cells. This different changing tendency need a further study. In conclusion, knockdown of GPR137 may suppress osteosarcoma cell proliferation partly via regulating the expression of ERK1/2.

5. Conclusions

Our results indicate that GPR137 may be involved in regulating the growth of osteosarcoma cells. RNAi-mediated GPR137 depletion could potently suppress the proliferation of osteosarcoma cells *in vitro*. Additional studies are required to validate its oncogenic functions, as this is the first report on the direct involvement of GPR137 in osteosarcoma.

Funding

Conflict of interest statement

None.

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