Journal of Orthopaedic Surgery and Research

RESEARCH ARTICLE Open Access

Ghrelin increases cis-platinum resistance and promotes aggressiveness of osteosarcoma by activating AKT and Wnt/β-catenin pathways

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Abstract

Osteosarcoma (OS) is the most common primary bone malignancy because of its extra high tendency of metastasis. In-depth research is needed to uncover the pathogenesis of patients with OS cells. We collected 74 tissue samples from patients with OS cells and measured the expression levels of ghrelin by immunohistochemistry. Ghrelin was added into OS cell lines in CCK8 assays, JC-1 staining and Western blot analysis were performed to explore its effect on the aggressiveness of OS cells and drug resistance. To determine its function, ghrelin was overexpressed or knocked down in OS cells and then detect cell proliferation in the xenograft mouse model and orthotopic model. Western blot analysis was performed to explore ghrelin-regulated signal pathways. In this work, we identified the relation between the level of ghrelin expression and poor prognosis of OS patients. As well as promoting proliferation, migration, and invation, ghrelin promotes the survival of OS in vitro as well as in vivo, and reduces the apoptosis of OS cells. What's more, ghrelin increases the resistance of cis-platinum by changing mitochondrial function and decreases the expression of MDR-1. Above all, these results demonstrated ghrelin exerts tumorigenic and metastatic effects and may be a potential therapeutic target.

Keywords Ghrelin, Osteosarcoma, Drug-resistence, Wnt/β-catenin pathway, AKT pathway

Introduction

As an acylated peptide, ghrelin contains 28 amino acids, an 8-carbon side chain linked to hydroxyl group of erine-3 residue. Ghrelin forms a protein precursor of 117 amino acids with a signal peptide [1]. It is mainly synthesized and released in the stomach, and works as an endogenous ligand of the growth hormone secretagogue receptor type 1a(GHS-R1a) and G-protein-coupled receptors (GPCRs) to regulate food intake and fat deposition, stimulating the growth hormone (GH) secretion [1]. As an orexigenic peptide hormone produced in peripheral organs, regulates appetite, nutrient sensing,

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and meal initiation, therefore identified as a key regulator of diabetes [3]. Deletion of ghrelin can improve glucose tolerance, reduce the blood glucose, enhance insulin secretion and decrease ATP production thereby improve the diabetic phenotype [6]. Besides, ghrelin can increase the activity of neurons expressing neuropeptide y (Npy) and the agouti-related protein (Agrp), meanwhile inhibite neurons that express proopiomelanocortin (Pomc), which resultes in stimulating food intake in mice [7]. What's more, ghrelin promotes gastric emptying, motility and acid secretion [10]. In addition, several sudies has indicted that ghrelin participated in regulating several processes involved in progression of cancer [12]. Ghrelin expression is increased in kidney cancer metastatic sites, including bones, lungs, adrenal glands and intestines [13]. Ghrelin can also promote oral tumor cell proliferation by regulating expression of GLUT1 [14].

The most common primary solid bone cancer is identified to be Osteosarcoma (OS), which has a high tendency to metastasize. The ratio of 5-year survival is below 25%. Osteosarcomas originate from primordial mesenchymal cells primarily in bones and is rarely found in soft tissue. The main treatment method currently is surgery. However, treatement with surgery alone can only save approximately 15–17% of osteosarcomas patients [15]. Patients with unresectable or relapsed osteosarcomas also face a dismal prognosis. Therefore, new approaches are required in urgency to improve the prognosis [16]. Genomic instability is a hallmark of osteosarcomas. Genomic alterations of TP53 inactivation, as well as RB inactivation are present in most OS cases [17]. Glutamate receptor and metabotropic 4 (GRM4) are expected to be related to bone tumorigenesis, as they play a role in cAMP signaling [18]. Moreover, parathyroid hormone can induce OS via cAMP signaling in Fischer 344 rats [20]. Thus, targeted therapy might be a management of the low overall survival problem.

In this study, patients with OS cells have a poor prognosis when their ghrelin levels are high, which promotes the proliferation, migration as well as invasion of osteosarcomas, and reducing the apoptosis of OS cells. Theoretically, ghrelin may activate AKT and Wnt/β-catenin pathways to promote OS progression. Meanwhile, ghrelin increases the resistance of cis-platinum by changing mitochondrial function and decreases the expression of multi-drug resistance protein 1 (MDR-1). Above all, results of this study demonstrated ghrelin exerts tumorigenic and metastatic effects and may be a potential therapeutic target.

Materials and methods

Patients and specimens

Histologically confirmed 74 tissue samples from OS patients were collected from Qilu Hospital of Shandong University, Jinan, China. Research protocols were completely approved by the Hospital Ethics Committee of Shandong University and written informed consent was obtained from every patients on the basis of the Declaration of Helsinki. Surgically resected tissue samples were reserved at − 80 °C after quick frozen in liquid nitrogen first.

Cell extraction and culture

The human K-HOS and MG-63 cell lines were obtained from the American Type Culture Collection (ATCC), and then kept properly in a sterile humidified incubator containing 5% CO2 using 10% FBS EMEM at 37 °C.

Mice experiments

For the animal experiments, we have got approvment from the Shandong University Animal Care and Use Committee and performed completely under the guidelines. For subcutaneous inoculation, OS cells were first obtained from cell culture plates and washed using PBS. 5 × 106 K-HOS cells was Suspended in 100ul PBS before injected subcutaneously into the 4-week-old nude mice(male). The volumes of tumor were measured and calculated as (large diameter × smaller diameter)2 / 2 every three days. K-HOS cells suspended in PBS at 1 × 106/ml and then injecte 0.1 ml solution into the mice left cardiac ventricles of 4-week-old male nude mice using 26G needles.

Establishment of ghrelin stable expressing and knockdown cell lines

cDNA sequences of Wide type ghrelin was constructed into PLVX-AcGFP-N1 vector before experiments. shRNA sequences of ghrelin gene were cloned into pLKO.1-puro vector. Plasmid overexpressing ghrelin were constructed and transfected into 293 T cells using E.coli strain Stbl3 as well as the silencing plasmid, combined with psPAX and PMD2.G with the application of Transfection Reagent Lipofectamine 3000 (Invitrogen). A 0.4 μm filter membrane was used to filter the supernatant after 72 h. Cells were plated as a density of 50–60% cells to be transfected by lentivirus particles, and this experiment is conducted in a 12-well plate. Establishment of stable cell lines can be estimated byRT-qPCR and Western blot after 1 week selecton by puromycin.

RNA isolation and real-time RT-qPCR

To perform the RT-qPCR assay, TRIzol Reagent was used. RevertAid First Strand cDNA Synthesis Kit was used for reverse transcription(Thermo, Waltham, MA, USA). ABI PRISM 7900HT Real-Time PCR detection system (Eppendorf, Germany) and SYBR Green PCR Master Mix (Thermo, USA) were used in the RT-qPCRassay.

Western blot analysis

A protease inhibitor cocktail (APExBIO, China) was added to RIPA lysis buffer (Beyotime Biotechnology, China) to obtain protein extracts. In this study, the concentration ofwas determined by a BCA protein assay kit. We mixed proteins with loading buffer (EpiZyme, China) and boiled them for 5 min for 4 min before running 10% SDS-PAGE (EpiZyme, China). After the proteins was transferred to a PVDF membrane (Millipore, Billerica, MA, USA), We blocked membranes with 5% BSA (Sangon Biotech, China) for 1 h, then incubated them with primary antibodies overnight in 3% BSA. After the 1 h incubation with secondary antibody and then being washed in TBST again, the protein bands were captured by photographic film (Kodak) with the help of chemiluminescence liquid (Millipore).

Immunohistochemistry

Paraffin sections were prepared using 10% formalin-fixed samples and 3% hydrogen peroxide-blocked samples. The antigen was retrieved at over 92 °C for 20 min in citrate buffer (10 mm, pH-6). Primary antibodies to ghrelin (1:50, Cell Signaling Technology) were first incubated on tissue sections for 16 h at 4 °C before the incubation with secondary antibodies (SP-9000, Sigma-Aldrich) for 30 min at 37 °C.

Cell viability and proliferation asssy

This study used Dojindo's Cell Counting Kit-8 (Kumamoto, Japan) to detect cell viability and proliferation. To get to know the cell viability, 3×103 cells were plated in a 96-well plate. Cell Counting Kit-8 solution was added to each well after 16 h of incubation at 37 °C. Then optical density (OD) values were tested at 450 nm using a multi-well scanning spectrophotometer (Multiskan MK3; Thermo Fisher Scientific). To get to know the cell proliferation, the OD values were measured accordingly at 72 h, 48 h, 24 h, and 12 h.

ATP test

K-HOS and MG-63 cells were collected from 6 wells cultured with MEM. And 1.5 ml tubes was used to collect the cell pellet. After washed with PBS thoroughly, and 200 µl lysis buffer was added and ultrasonically probed. The lysate was centrifuged at 4 °C at 12,000× g for 5 min. the ATP detection kit supplied by Beyotime (China) was used to test the ATP levels. Relative ATP level was calculated as ATP value / protein value. Multiscan MK3 by Bio-Rad (Finland) was used to measure the protein concentration at 562 nm.

Cell migration and invasion assay

Invasion assay was performed within chambers (BD, 3097) which was pre-coated with matrigel (BD, 354605) and kept at 37 °C for 30 min. A 10% FBS-EMEM medium was used in the lower chambers and the upper chambers were plated with 1×105 in 200 µl EMEM without FBS. To examine the cell migration, 3×104 cells wtih 200 µl EMEM serum-free were placed in the upper chamber without matrigel. The chambers were incubated at 37 °C for 20 h in a sterile humidified incubator containing 5% CO2. non-invading cells in the upper chamber were gently wiped off, while a 15-minute fixation in methanol at room temperature was followed by a 20-minute staining with 0.5% crystal violet.

Wound healing assay

We performed wounds in 60 mm dish after OS cells have been treated with Dlys and grown to 90–100% density. Olympus IX70 inverted microscope was applied to capture the images at 0 h and 24 h after washing the floated cells twice with PBS to remove them.

Apoptosis assay

Cells were harvested and washed twice in cold PBS before being resuspended in binding buffer at a density of 2 to 5×105/ml. 5 µl Annexin V-FITC (1% BSA, 100 mm NaCl, 50 mm TRIS, 0.02% Sodium Azide, pH 7.4) was added, the suspension was added to 190 µl binding buffer (1×) and 10 µl polyimide (PI) protected from light after at room temperature in 10 min. FACS-scan flow cytometers (Becton-Dickinson, San Jose, CA, USA) were used to analyze the status of cells, and Cell Quest analysis software (Becton-Dickinson) was used to determine cell apoptosis levels.

Immunofluorescent staining

We first fixed coverslips in 4% PFA for 15 min, then permeabilized the coverslips with 0.1% Triton X-100 at room temperature. The cells were then blocked 30 min with 5% BSA before reserved in the solution containing primary antibody at 4 °C. and then one hour incubation with secondary antibodies was performed. Hoechst 33,342 (1:1000 in PBS) was used in this assay to dye the cell nuclei.the slides were coated by anti-fade mounting medium (Beyotime, China). Further fluorescence microscopy was used to analyze microscopic images at magnifications of 200x and 630x.

5-ethynyl-2′-deoxyuridine proliferation assay

The 5-ethynyl-2′-deoxyuridine (EdU) assay was conducted by the application of EdU Kit (Byotime, China). placed on 24-well plates, Cells were primarily cultured with cis-platinum for 24 h with or without ghrelin, then add 10 µm EdU in and keep it for 2 h. Next, after fixed with 4% polyformaldehyde, cells were treated for 30 min with Apollo staining solution. the percentage of EdU-positive cells were counted using fluorescence

microscopy. the number of all cells and proliferating cells was recorded by Image J sofetware.

Bioluminescence imaging in vivo

After 28 d, bioluminescence imagingwas applied to monitore bone metastasis with the IVIS Imaging System (Caliper Life Sciences). Base Labelling Index (BLI) analysis was conducted with Living Image software.

Statistical analysis

All data in this study were recorded as mean values ± standard deviation (SD). For the analysis of the fold change between groups, Perseus had been used to test multiple hypotheses using FDR. Log-rank tests were used to compare the results of the Kaplan-Meier and log-rank survival analyses. Differences between two groups were analyzed by Student's t-test, and for the Differences among more than two groups, one-way ANOVA was performed. Statistical significance was determined by $P < 0.05$. To analyze the impact of clinical features on ghrelin expression, Pearson chi-squared and Fisher's exact tests were applied. Cox proportional hazard model is applied to identify the related factors of patients' survival rate. the GraphPad Prism Software was used in this study for All statistical analyses.

Results

Highly expressed ghrelin is associated with OS patients' poor prognosis

74 patients with OS cells were studied for ghrelin expression levels and metastasis-free survival. Ghrelin expression level was determined with immunohistochemistry. On the basis of the level of ghrelin expression in tumor tissues, we first divided the patients into two groups (Fig. 1A). According to the results (Fig. 1B), patients with high levels of ghrelin usually tends to have lower survival rate. Furthermore, we found much more expression of ghrelin in both tumor tissues and lung metastasis tissues by IHC in the nude mice with OS cells (Fig. 1C and D). All together. The data suggested that the high ghrelin level may promote the progression and invasion of OS and lead to poor clinical outcomes.

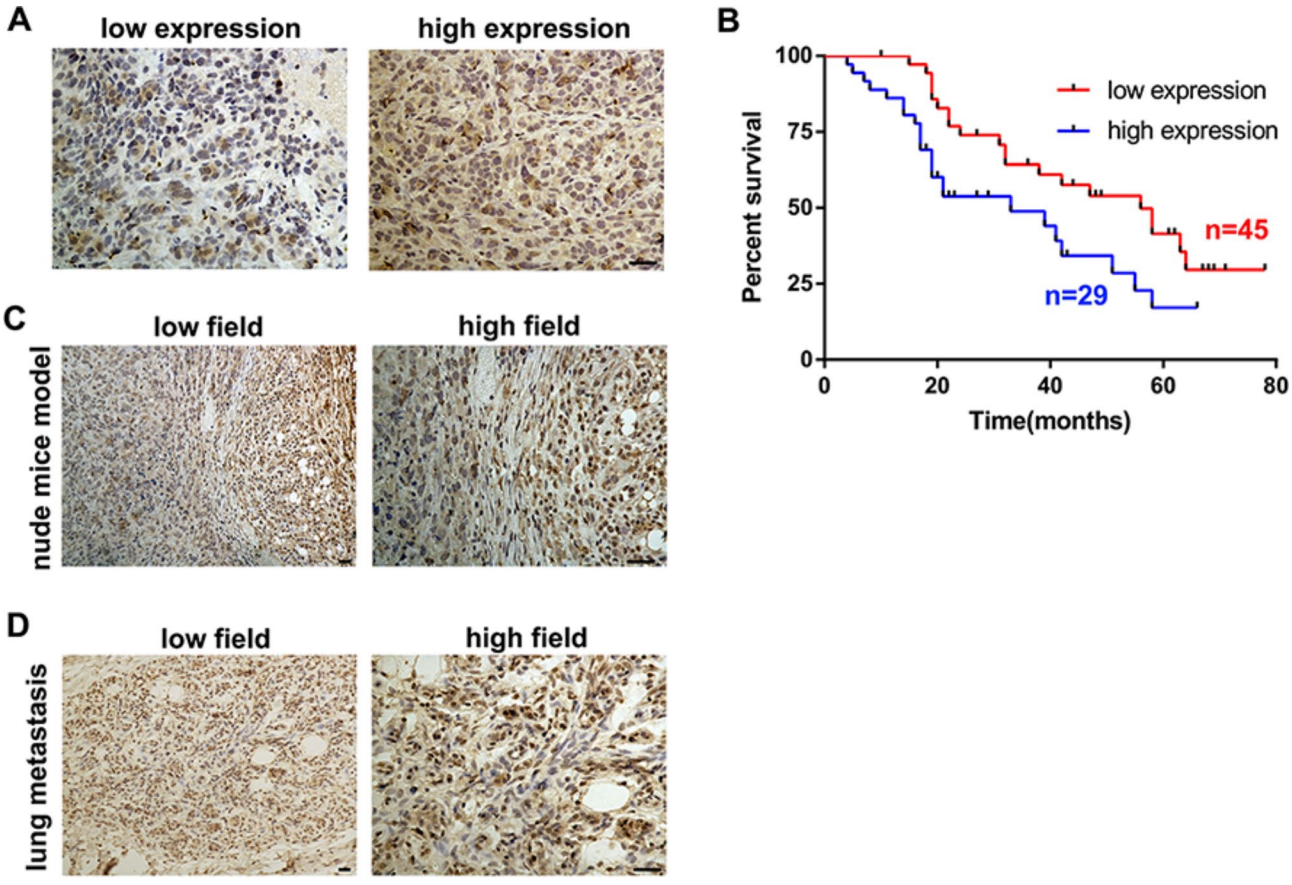

Fig. 1 High expression of ghrelin indicated to worse prognosis of OS patients. (**A**) Patients were separated into the low-expression group and the high-expression group by IHC. (**B**) Kaplan-Meier survival analysis was performed to show the survival rate of the low-expression group ($n = 45$) and the high-expression group ($n = 29$). (**C**) Expression of ghrelin in tumor tissue examined by IHC. (**D**):Expression of ghrelin in lung metastasis tissue examined by IHC. Data were in the form of mean ± standard deviation (SD). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

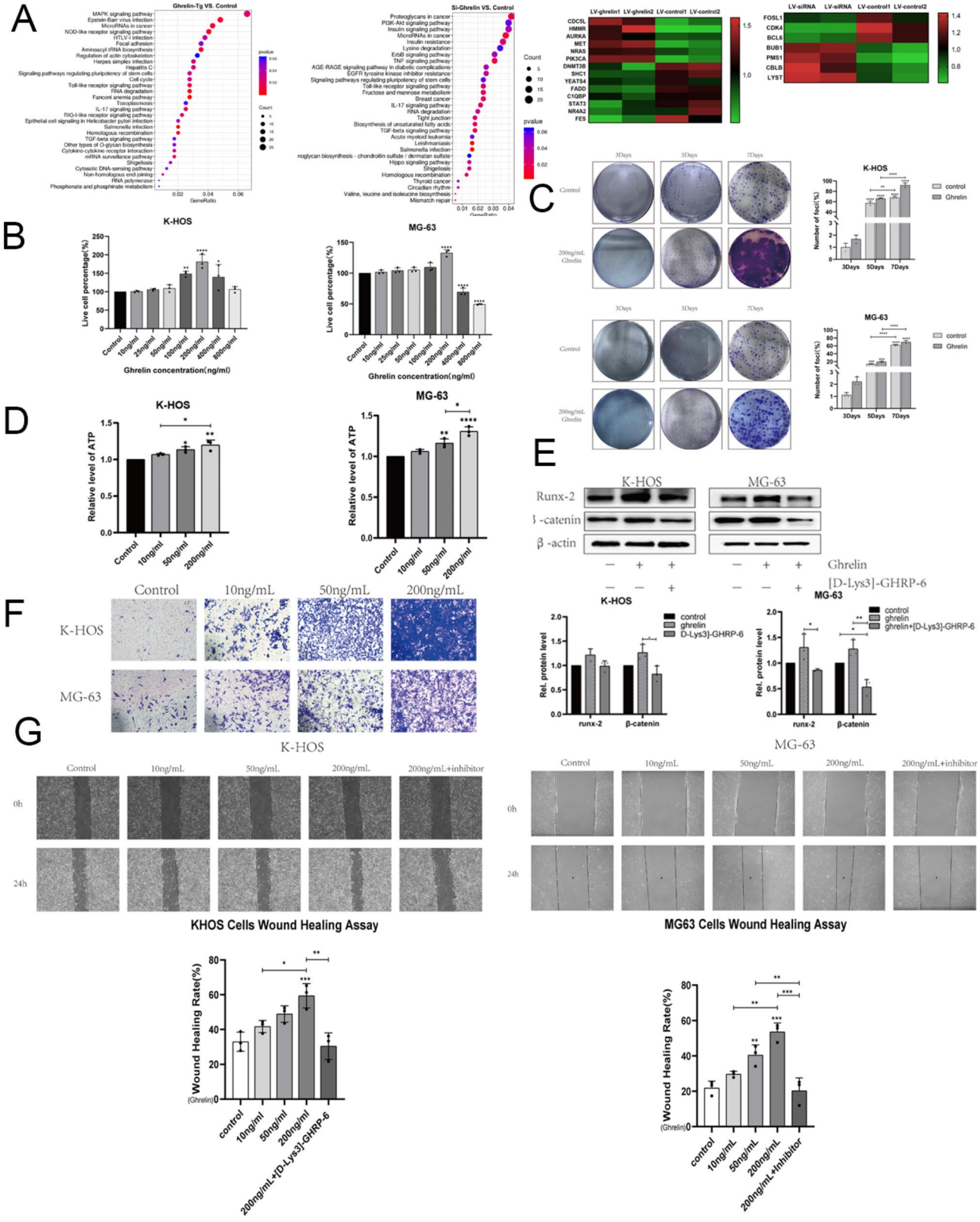

Fig. 2 (See legend on next page.)

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Fig. 2 Ghrelin promotes OS cells proliferation and migration capacity. (**A**) RNA-Seq data analysis of K-HOS and MG-63 cells with ghrelin overexpression or with ghrelin knockdown. (**B**) CCK8 assays of K-HOS and MG-63 cells were performed after adding ghrelin at the concentration of 15 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml, 400 ng/ml and 800 ng/ml to calculate the cell proliferation rate. (**C**) Plate colony assays of K-HOS and MG-63 cells were performed with adding ghrelin in the following 3, 5 and 7 d. (**D**) ATP test of K-HOS and MG-63 cells were performed with adding ghrelin at the concentration of 10 ng/ml, 50 ng/ml and 200 ng/ml to observe the cell biological activity. (**E**) Expression of Runx-2 and β-catenin in K-HOS and MG-63 cells adding ghrelin and adding ghrelin with Dlys was observed respectively by Western blot. **F**: Transwell assays of K-HOS and MG-63 cells were performed with adding ghrelin at the concentration of 10 ng/ml, 50 ng/ml and 200 ng/ml to test the invasion capacity. (**G**) Wound healing assay of K-HOS and MG-63 cells were performed with adding ghrelin at the concentration of 10 ng/ml, 50 ng/ml and 200 ng/ml and adding Dlys with ghrelin at the concentration of 200 ng/ml to test the migration capacity. Data were in the form of mean ± standard deviation (SD). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Ghrelin promotes proliferating and migrating capacity of OS cell

To further explore the role of ghrelin in human OS cells, the RNA-Seq data analysis was conducted based on the test in K-HOS and MG-63 cells with or without ghrelin knockdown. As shown in Fig. 2A, ghrelin increased the expression of cancer-promoting factors, whereas it had the opposite effect on cancer-suppressing factors. Conversely, when ghrelin was knocked down, cancer-suppressing factors were protected while cancer-promoting factors were suppressed, which precisely demonstrated ghrelin had a positive effect on cancer development. Cell Counting Kit-8 (CCK8) assays and plate colony assays analysis proved that ghrelin significantly promoted cell proliferation compared with control cells. When we added ghrelin at 200 ng/ml, the effect of promoting proliferation of OS cells was significantly increased. But these changes were continuously weakened when we added ghrelin at 400 ng/ml and 800 ng/ml (Fig. 2B). Meanwhile, when ghrelin was added at 200 ng/ml, the proliferation of OS cells was significantly increased at the 7d (Fig. 2C). Similar results could be found in the relative levels of ATP of OS cells (Fig. 2D). Runx-2 is a pivotal indicator in the process of bone formation or osteogenesis as well as in the development of OS, and the expression and genetic transcription of Runx-2 were regulated by Wnt/β-catenin signaling. It is also well known that growth hormone secretagogue receptor (GHSR) 1a, as the receptor of ghrelin, plays an important role in ghrelin biological action. Therefore, we used GHSR 1a inhibitor Dlys to investigate whether GHSR 1a was capable to influence the effects of ghrelin in OS cells. As shown in Fig. 2E, ghrelin increased the Runx-2 and β-catenin expression in K-HOS and MG-63 cells. When the Dlys was also added, the expression of Runx-2 and β-catenin decreased dramatically compared with adding ghrelin alone using Western blot assay (Fig. 2E). Similar results for the ability of migration and invasion of OS cells can be found by Transwell assays and the wound healing assay. Adding ghrelin dramatically increased the migratory capacity of K-HOS and MG-63 cells, as well as the matrigel invasion. And these effects depended on the concentration. Adding Dlys decreased the migratory capacity compared with adding ghrelin alone at the concentration of 200 ng/ml (Fig. 2F and G). Taken together, these results suggest that ghrelin regulates OS cell proliferation and migration capacity.

Ghrelin promotes tumor development in orthotopic and xenograft mouse model

The effects of ghrelin on tumor growth were assessed by injecting K-HOS cells in situ and under the skin of nude mice with or without silencing or over-expression of ghrelin. Control cells transformed into tumors within 28 d after the injection, whereas in K-HOS cells silenced ghrelin significantly suppressed tumor formation. Correspondingly, overexpression of ghrelin rapidly caused the formation of tumors within 28 d both in orthotopic model and xenograft model (Fig. 3A-E). Additionally, H&E staining revealed that the ghrelin overexpression group exhibited an increase in the proliferation of cells and the ghrelin silencing group decreased on the contrary (Fig. 3F and H). As a measure of apoptosis in tumor cells, we examined caspase 3 expression using IHC staining, the results suggested that tumor cells derived from ghrelin overexpressing cells had a significantly lower fraction of caspase 3 positive cells than control, whereas ghrelin silencing K-HOS cells had a much higher fraction (Fig. 3H). Together, these results suggest that ghrelin promotes development of tumor in orthotopic and xenograft mouse model.

Ghrelin promotes the proliferation of OS cells through AKT and Wnt/β-catenin pathways

We then explored the possible pathways through which ghrelin has an impact on the proliferation of OS cells. We found the AKT pathway was significantly higher after ghrelin was added in K-HOS and MG-63 cells for 45 min in comparison with the control group of Western blot analysis, while this effect can be blocked by the inhibitor of AKT (Fig. 4A). As showed in Fig. 4B, after adding different concentrations of ghrelin into K-HOS and MG-63 cells, the Runx-2 and β-catenin expression increased as the concentration got higher, while adding the inhibitor of β-catenin significantly suppressed Runx-2 and β-catenin expression (Fig. 4C). These revealed that ghrelin may promote the proliferation of OS cells by activating AKT and Wnt/β-catenin pathways.

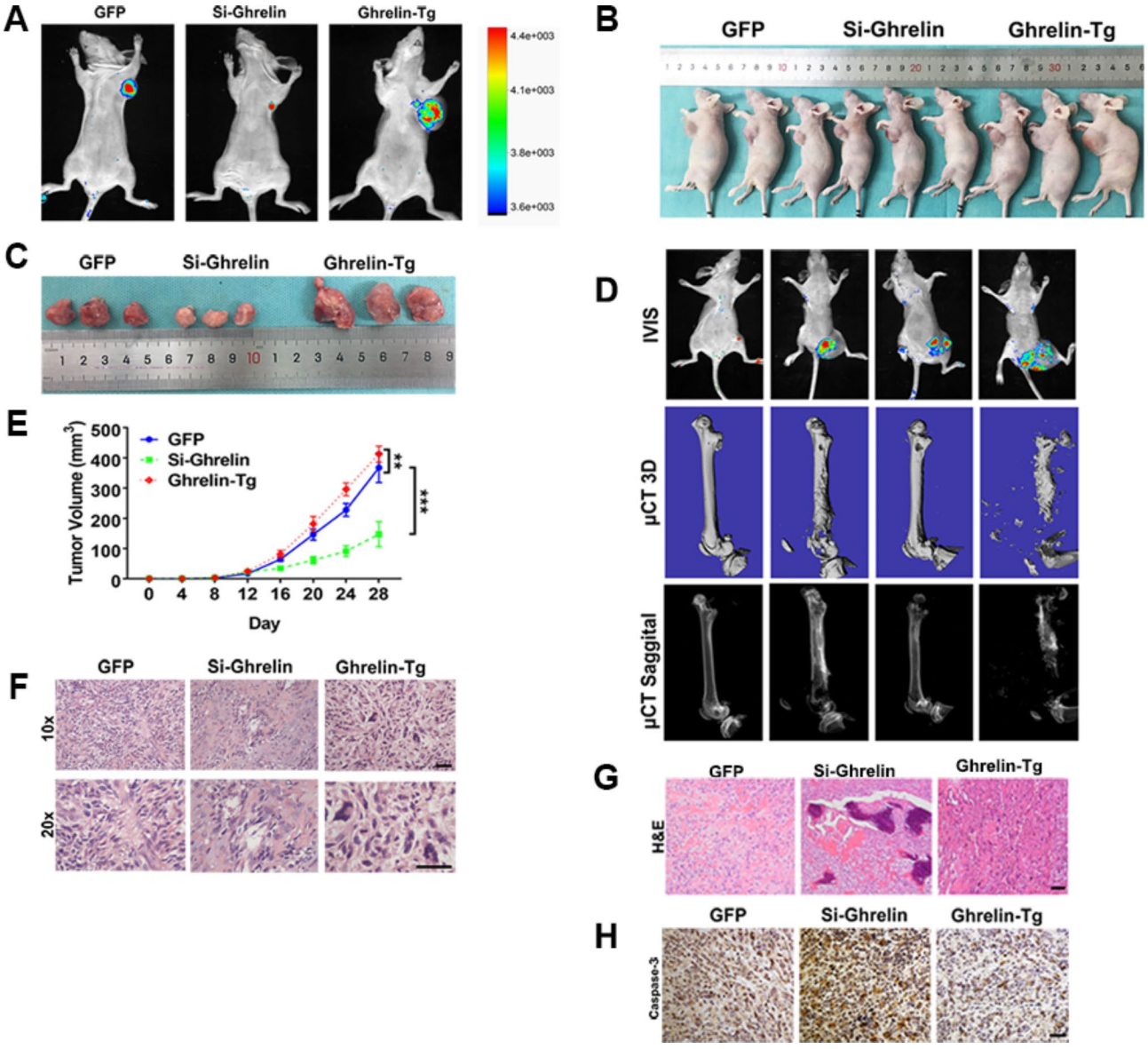

Fig. 3 Ghrelin promotes tumor development in xenograft and orthotopic model. Tumor growth in experimental BALB/c nude mice after injection of K-HOS cells transfected with control vector, constructed ghrelin overexpression vector and silencing plasmids. (**A**) Bioluminescence imaging demonstrated K-HOS cells colonized in subcutaneous after injection of K-HOS cells with control vector, constructed ghrelin overexpression vector and silencing plasmids in subcutaneous tissue. (**B**-**C**) Tumor volume was quantified after the injection in the following 4d to 28 d. (**D**) Bioluminescence imaging demonstrated K-HOS cells colonized mainly in bones based on GFP signals, and bone lesions were identified in the tibias by micro-computed tomography (μCT) in 3- dimensional reconstruction image and sagittal reconstruction image. (**E**) Tumor volume was quantified after injection for 4d to 28 d. (**F**) Representative images of H&E staining of tumors in xenograft model (scale bar, 50 μm). (**H**) Representative images of H&E staining of tumors in orthotopic model (scale bar, 50 μm). (**G**) Expression of caspase3 was determined by IHC staining. Data were in the form of mean ± standard deviation (SD). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Ghrelin increases resistance of cis-platinum by changing mitochondrial function

Cis-platinum is the most common anti-OS drug. But the resistance of cis-platinum remains a challenge for the treatment of OS patients. As shown in Fig. 5A, cis-platinum decreased the expression of ghrelin in K-HOS and MG-63 cells. While after adding ghrelin at the concentration of 10 ng/ml, 50 ng/ml and 200 ng/ml, the anti-tumor effect of cis-platinum in the proliferation of OS cells was significantly suppressed by EdU assays and flow cytometry (Fig. 5B and C). Additionally, Ki-67 staining confirmed that adding ghrelin exhibited an increase in the proliferation of cells (Fig. 5D). Through tunnel assays, we found cis-platinum increased the apoptosis of K-HOS and MG-63 cells while ghrelin reversed the apoptotic effect of cis-platinum (Fig. 6B). Compared

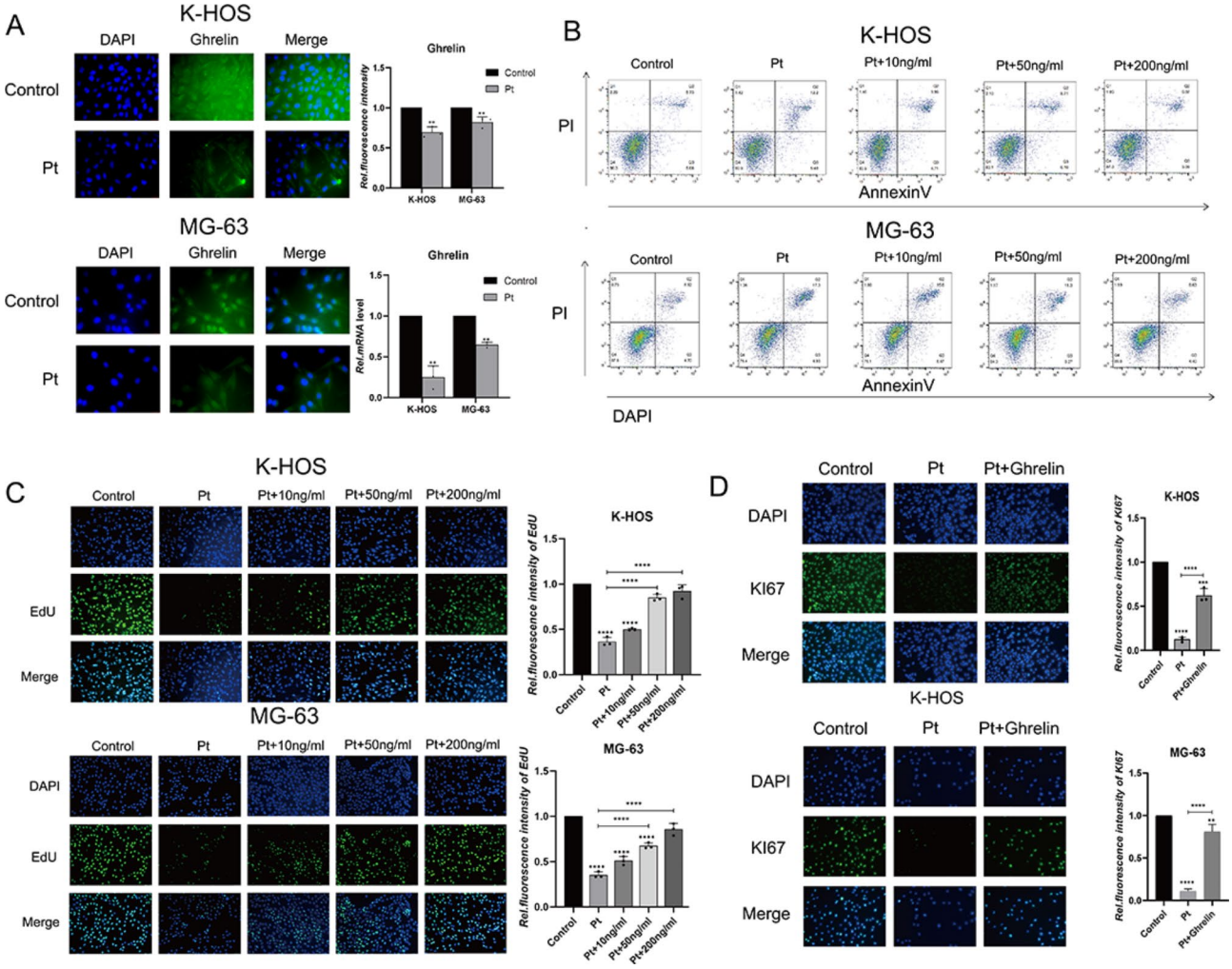

Fig. 4 Ghrelin promotes the proliferation of OS cells through AKT and Wnt/β-catenin pathways. (**A**) Expression of AKT and p-AKT of K-HOS and MG-63 cells after adding ghrelin and ghrelin with MK2260 was examined respectively by Western blot. (**B**) Expression of Runx-2 and β-catenin of K-HOS and MG-63 cells was examined respectively by adding ghrelin at the concentration of 10 ng/ml,50 ng/ml and 200ng/ml by Western blot. (**C**) Expression of Runx-2 and β-catenin of K-HOS and MG-63 cells after adding ghrelin and ghrelin with ICG-001 was examined respectively by Western blot. Data were in the form of mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

to adding cis-platinum alone, adding ghrelin dramatically decreased the RNA level and mRNA expression of caspase3 and Bax and increased the RNA level and mRNA expression of Bcl2, which indicated that ghrelin could reverse the pro-apoptotic effect of cis-platinum (Fig. 6C and D). As the vital function of mitochondrial dysfunction in drug resistance, we studied the interaction between ghrelin and mitochondrial homeostasis. to detect the membrane potential change of mitochondria, We used JC-1 staining method. After adding cis-platinum, the ratio of monomers of JC-1 increased, which indicates the mitochondrial damage. But these changes were continuously weakened when adding ghrelin at the concentration of 10 ng/ml, 50 ng/ml and 200 ng/ml. We noticed that when the concentration of ghrelin was up to 200ng/ml, the ratio of monomers was closed to that of control groups. These results indicated that the ghrelin might have drug resistance effect by suppressing the mitochondrial toxicity of cis-platinum (Fig. 6A). Multi-drug resistance protein 1 (MDR-1) belongs to the ATP-binding cassette (ABC) transporters family and plays roles as a molecular efflux pump. A reduction in xenobiotic molecule accumulation, which results in drug resistance, is achieved by removing drugs from OS cells. MDR-1 was found to be enriched in mitochondrial membrane potential and the increased MDR-1 has prevented the mitochondrial DNA damage from causing cell death. We found ghrelin promoted the expression of MDR-1 in K-HOS and MG-63 cells (Fig. 6E). In conclusion, ghrelin could increase the resistance of cis-platinum by reducing the damage of mitochondria and overexpression of MDR-1.

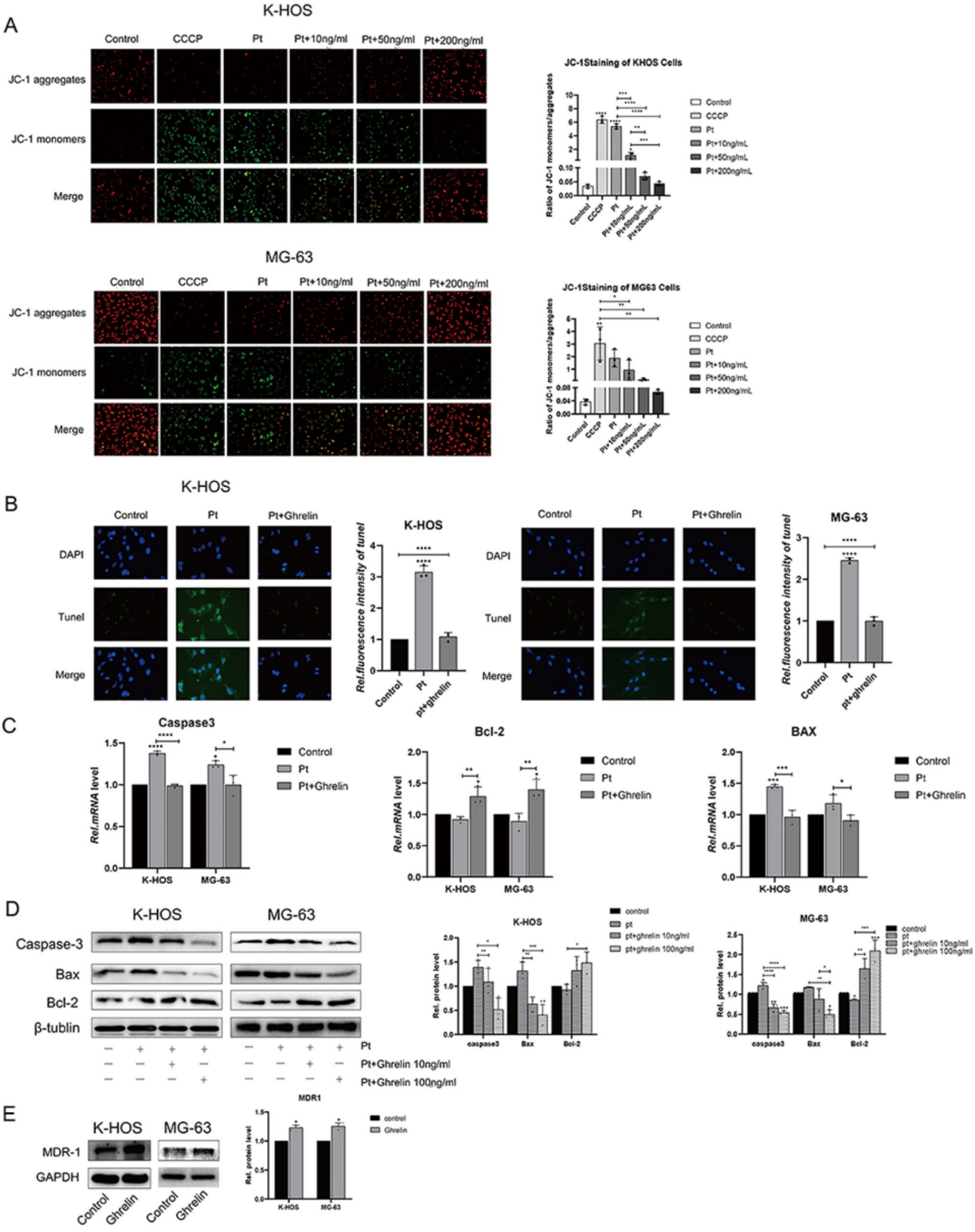

Fig. 5 Ghrelin increased the resistance of cis-platinum on the proliferation of OS cells. (**A**) Expression of ghrelin of K-HOS and MG-63 cells decreased after adding cis-platinum. (**B**-**C**) Flow cytometry assays and EdU tests were used to analyze the effects of cis-platinum and ghrelin at the concentration of 10ng/ml, 50ng/ml and 200 ng/ml on the proliferation of K-HOS and MG-63 cells. (**D**) Ki67 assays were used to test the proliferation of K-HOS and MG-63 cells after adding cis-platinum with or without ghrelin. Data were in the form of mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

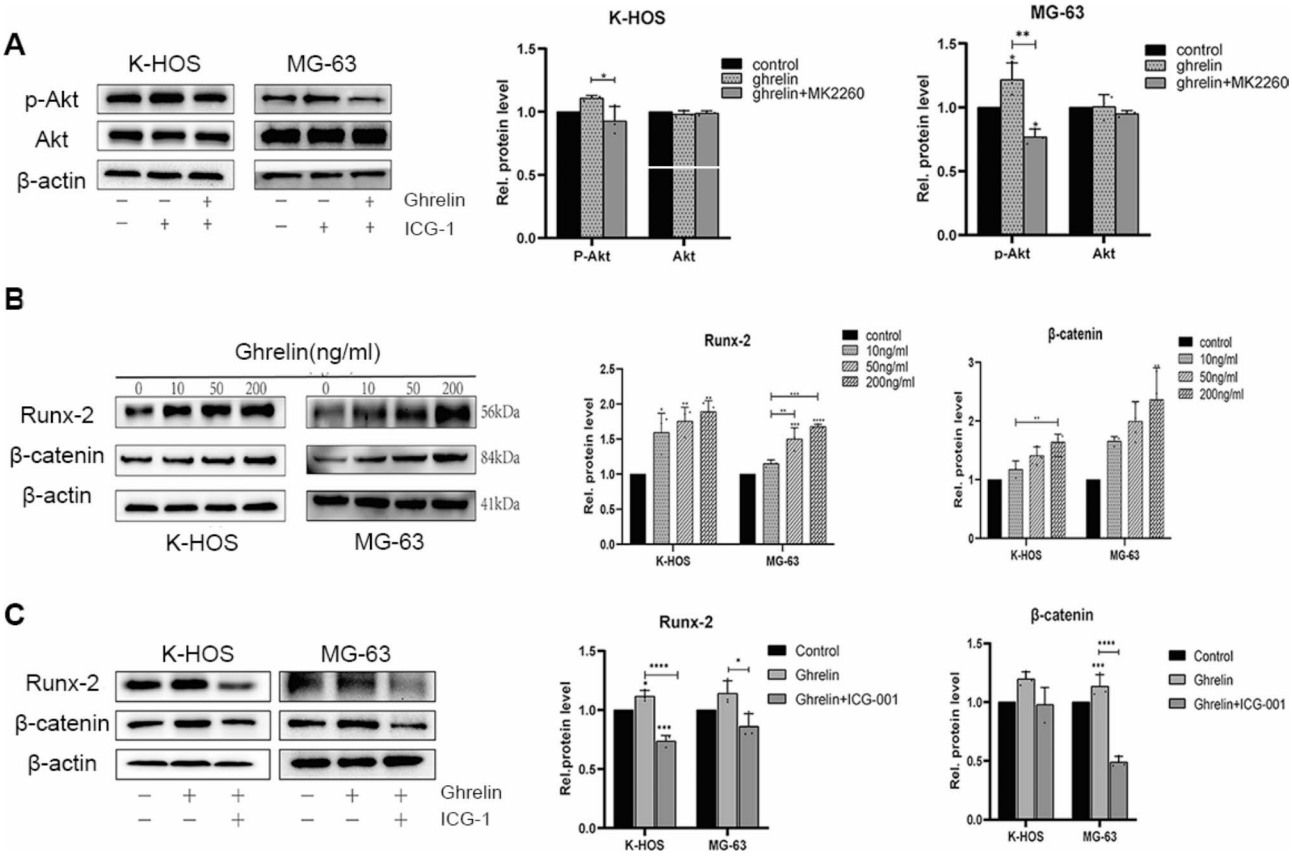

Fig. 6 Ghrelin increased the resistance of cis-platinum by changing the mitochondrial function and inducing the expression of MDR-1. (**A**) Mitochondrial membrane potential of KHOS and MG63 cells treated with cis-platinum after adding ghrelin at the concentration of 10ng/ml, 50ng/ml and 200ng/ml was determined by JC-1 staining. (**B**) Apoptosis of K-HOS and MG-63 cells adding cis-platinum with or without ghrelin was determined by Tunel assays. (**C**-**D**) Western Blot and RT-qPCR analysis were performed to determine the expression and RNA level of several apoptotic biomarkers such as caspase3, Bcl-2 and Bax of K-HOS and MG-63 cells after adding cis-platinum with or without ghrelin. (**E**) Expression of MDR-1 of KHOS and MG63 cells was determined by Western blot. Data were in the form of mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Discussion

In this study, we discovered that ghrelin/AKT/Wnt/β-catenin was involved in the OS development. A high level of ghrelin expression was associated with worse prognosis among OS patients. Furthermore, the over-expressed ghrelin aggravated the malignancy of OS and increased phosphorylation level of AKT and MAPK. In addition, ghrelin was also found to enhance cisplatin resistance. These clinical and mechanistic explorations strongly suggested that ghrelin/AKT/Wnt/β-catenin could promote the occurrence and development of OS.

As an endogenous peptide hormone, ghrelin functions in many physiological processes, including regulating energy balance in the body, stimulating the growth hormone secretion [9], as well as regulating fat metabolism, intestinal motility, gastric acid secretion and sleep [22]. These effects contribute to the involvement of ghrelin in tumoral development, and its local effects on various cancer cell lines are inconsistently reported in different studies. For example, In patients with aggressive breast cancer, tumor grade, tumor size, and Ki-67 expression are inversely related. And nonmalignant breast tissue doesn't express [23], or much lower ghrelin comparing with malignant tissue [24]. While it is obviously different in colorectal cancer, ghrelin expression was revealed in malignant human colon cell lines and also in normal cells. It is evident that ghrelin is more abundant in malignant tissues than in normal cells and in cancerous tissues than in adjacent normal tissues of cancer patients Immune responses to ghrelin were much stronger in advanced tumor. And Colorectal cancer cells expressed ghrelin at similar levels to tumor grade. Similarly, in our study, higher expression of ghrelin was associated with a worse prognosis in regarding of patients, and the expression of ghrelin was upregulated in cancerous tissue and lung metastasis tissue in the nude mice with OS cells. Additionally, we found that ghrelin played a critical role in impacting the migration, invasion and proliferation of OS cells.

Although ghrelin appears to be an endogenous ligand for GHSR, the effect of ghrelin on tumors didn't act through the ghrelin-growth hormone axis. Because of

GHSR/NF-kB pathway activation, ghrelin can promote migration in the gastric area [25], and in pancreatic adenocarcinoma via GHSR/PI3K/Akt signaling pathways [26]. Furthermore, Angiogenesis may be promoted by ghrelin through the ERK2 signaling pathway in human microvascular endothelial cells. we found that adding ghrelin leads to the stimulation of AKT and Wnt/β-catenin pathways, while adding inhibitors of AKT and β-catenin would cause the decrease of the proliferation of OS. Our results showed that ghrelin promoted the OS cells to proliferate through AKT and Wnt/β-catenin pathways.

In addition, cis-platinum, as a common anti-OS drug, significantly decreased the expression of ghrelin. And the anti-tumor effect of cis-platinum could be significantly suppressed when ghrelin was added at the concentration of 10 ng/ml and 50 ng/ml. Through JC-1 staining of K-HOS and MG-63 cells, with CCCP added as a positive control, we observed a significant attenuation in cisplatin-induced mitochondrial damage when the concentration of ghrelin reached 200 ng/ml. And ghrelin induced the overexpression of MDR-1 which might protect the mitochondrial DNA damage as well. These results showed ghrelin played an important role in drug resistance by protecting mitochondria from DNA damage.

Conclusion

In summary, our results demonstrated that ghrelin regulated the occurrence and development of OS through AKT and Wnt/β-catenin pathways and induced cis-platinum resistance by protecting mitochondria from DNA damage. These results showed manipulating ghrelin levels in clinical practice of treating OS maybe a novel therapeutic method.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13018-024-05261-2.

Supplementary Material 1: Expression of ghrelin in human osteosarcoma tissues and normal tissues.

Acknowledgements

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Author contributions

SL and TW defined the problem and helped in developing the methodology. FL, WZ, HW and XM helped to Imaging processing. XS, KL, GS and HH charged for data collation and analysis. MC and YF wrote the manuscript text. LZ, WL and XS substantively revised it. All authors reviewed the manuscript.

Funding

The work was completed with the support of the the National Natural Science Foundation of China (NO. 82073437, NO. 82002844).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This research protocol was approved by the Institutional Review Board of Qilu Hospital of Shandong University and The First Affiliated Hospital of Jinan University. The clinical procedures adhered to the principles of the declaration of Helsinki. Informed consent was obtained from all individual participants included in the study. This study confirming that all experiments were performed in accordance with relevant guidelines and regulations. This study confirmation of compliance with ARRIVE guidelines. (KYLL-2019(KS)-352).

Competing interests

The authors declare no competing interests.

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Received: 26 August 2024 / Accepted: 11 November 2024
Published online: 10 March 2025

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