Silencing Ubc9 expression suppresses osteosarcoma tumorigenesis and enhances chemosensitivity to HSV-TK/GCV by regulating connexin 43 SUMOylation

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Abstract. The ability of herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) systems to kill tumor cells is partially dependent on the integrity of gap junction intercellular communication (GJIC) of targeted tumor cells. Recent studies have suggested that connexin 43 (Cx43), which serves a role in gap junction-mediated intercellular communication, is regulated by small ubiquitin-like modifiers (SUMOs). However, the roles of these post-translational modifications remain to be elucidated. The present study demonstrated overexpression of SUMO-conjugating enzyme Ubc9 (Ubc9) protein in osteosarcoma. Silencing Ubc9 by siRNA inhibited osteosarcoma cell proliferation and migration, and significantly increased the sensitivity of cells to HSV-TK/GCV systems both *in vitro* and *in vivo*. Further experimentation demonstrated that silencing Ubc9 induced decoupling of SUMO1 from Cx43, generating increased free Cx43 levels, which is important for reconstructing GJIC and recovering cellular functions. In conclusion, the present study revealed a novel method for the effective restoration of GJIC in osteosarcoma cells, which may increase their sensitivity to conventional chemotherapy.

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Introduction

Osteosarcoma is a rare type of cancer (1,2). However, its incidence has been reported to have increased yearly in developed and developing countries, particularly in China (3). Rapidly changing ecological environments and living habits are thought to have contributed to this increase (4). Unfortunately there are no general solutions to address the increasing incidence. Generally speaking, surgical resection is the primary treatment mode of osteosarcoma, and hormone therapy, radiotherapy and chemotherapy serve auxiliary therapeutic roles (5,6). With the current treatment options, patient prognosis is relatively poor (1,2,5,7).

Herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) systems have been widely applied in suicide cancer gene therapy (8,9). Theoretically, HSV-TK phosphorylates GCV to GCV-monophosphate, which is then converted to GCV-triphosphate by endogenous cellular nucleoside kinases (10). GCV-triphosphate acts as a DNA chain terminator due to the lack of a functional 3'-OH group, terminating DNA replication and causing apoptosis (11).

An important feature of the HSV-TK/GCV suicide gene system is that its ability to kill tumor cells is largely dependent on the integrity of gap junction intercellular communication (GJIC) (12). Connexin 43 (Cx43), a member of the connexin family, is a component of gap junctions. These are intercellular channels that connect adjacent cells, permitting the exchange of low molecular weight molecules, including ions and secondary messengers to regulate cell death, proliferation and differentiation (13-15). Unfortunately, numerous types of cancer, including glioma, gastric cancer, hepatocellular carcinoma, breast cancer, prostate cancer and ovarian cancer, frequently lose Cx43 expression (16-19), which leads to defects in GJIC and decreases the effectiveness of HSV-TK/GCV systems (16-19).

Small ubiquitin-like modifier (SUMO) conjugation is a post-translational regulatory process which functions in all eukaryotes, mediated by SUMO activating enzyme, SUMO

conjugating enzyme and SUMO ligase, which attach SUMO to target proteins (20-22). Ubc9, the only SUMO E2 conjugating enzyme, is often overexpressed in tumors (23-25), suggesting that it may be involved in molecular events required during cancer development (21,24,25). Recently, Kjenseth *et al* (26) reported that Cx43 is covalently modified and regulated by SUMOylation in HeLa cells. However, the role of this process in osteosarcoma remains poorly understood. Therefore, the present study investigated Cx43 SUMOylation in osteosarcoma, and assessed whether this process positively or negatively influences the integrity of GJIC function, and whether it may be used to enhance the efficacy of HSV-TK/GCV systems.

Materials and methods

Tissue specimens. Fresh surgical specimens were collected from 16 osteosarcoma patients diagnosed at the Department of Bone and Soft Tissue Tumors (Tianjin Medical University Cancer Institute and Hospital, Tianjin, China) between January 2016 and December 2016. The diagnosis was made by a senior pathologist and confirmed by another experienced pathologist (Department of Pathology, The Fifth Central Hospital of Tianjin). The present study was approved by the ethics committee of Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) and written informed consent was obtained from all patients.

Immunohistochemistry. Paraffin-embedded tissues were cut into 5-μm-thick slices, which were then dewaxed in xylene, hydrated in order of 100, 90, 70 and 50% ethanol and microwaved at 80 kPa, 117˚C for 3 min for antigen retrieval. This was followed by 3% hydrogen peroxide treatment (OriGene Technologies, Inc., Beijing, China) to remove endogenous peroxidase, and blocking with goat serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at room temperature for 30 min. Next, samples were incubated with a rabbit polyclonal connexin 43/GJA1 primary antibody (dilution, 1:2,000; cat. no. ab11370; Abcam, Cambridge, UK) overnight at 4˚C. A goat anti-rabbit IgG H&L hoseradish peroxidase-conjugated secondary antibody (dilution, 1:5,000; cat. no. ab205718; Abcam) was then applied at 37˚C for 1 h. The sections were stained with hematoxylin (cat. no. G1140; Soulebao Technology Co., Ltd.; Beijing, China) at the stock concentration at room temperature for 8 min, and mounted onto cover slips.

Cell lines and cell culture. The osteosarcoma cell lines, 143B, MG-63 and U-2OS, and the osteoblast cell line, hFOB1.19 were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA), at 37˚C in 5% CO_2.

Plasmids and transfection. The lentiviral plasmids pWPXLD-His-siR-Ubc9, pWPXLD-HA-Cx43 and pWPXLD-Flag-SUMO1 were synthesized by Biogot Technology Co., Ltd., (Nanjing, China), and were packaged in 293 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Then these viral plasmids were infected into U-2OS cells at 70% confluence at a concentration of 20 μl/ml, according to the manufacturer's protocol. Fory-eight hours after transfection, Ubc9 silencing was confirmed by western blotting. Proliferation, colony formation ability, migration capacity and apoptosis were detected by MTT assays (27,28), soft agar colony formation assays (27,29), wound healing assays (28), Transwell assays (27-29) and flow cytometry (28), as previously described. Ubc9 and Cx43 subcellular localizations were detected by immunocytochemistry as previously described (30). GJIC function was measured by the Lucifer Yellow dye transfer assay, as previously described (31). Briefly, cells were plated in the 35-mm dishes and grown to confluency. Scrape loading was performed using a sharp knife, and the monolayer cells were immersed in 0.05% of Lucifer Yellow (MW 457.2, Sigma-Aldrich Inc., Shanghai, China) for 3 min at room temperature, then the GJIC function was evaluated through transfer of Lucifer Yellow to neighboring cells from the border of scraped line. No dye transfer was evident in cells incompetent in GJIC.

Immunoprecipitation. Total protein was extracted from cells, and approximately 1 mg was diluted 10-fold with Triton X-100 lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 10 mM iodoacetamide and protease inhibitors), pre-treated with protein-agarose beads for 1 h at 4˚C, followed by the addition of the anti-HA tag antibody (dilution, 1:500; cat. no. ab18181; Abcam) or anti-Flag tag antibody (dilution, 1:50; cat. no. ab1162; Abcam). Following an incubation at 4˚C overnight, immunoprecipitates were washed three times with 1 ml Triton X-100 lysis buffer, then diluted in 2X SDS sample buffer. After heating for 10 min at 50˚C, the samples were evaluated by western blotting.

Western blotting. Total protein was extracted from fresh tissues or cells with lysis buffer (50 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4 and 1% Triton X-100, pH 7.4) and protein concentration was analyzed by BCA assay (Thermo Scientific Inc.).Then western blotting was performed by 4-15% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories), and blocked with 0.1% TBS-Tween and 5% skim milk powder for 1 h at room temperature. Next, the membranes were incubated with anti-Ubc9 (dilution, 1:2000; cat. no. ab75854), anti-SUMO1 (dilution, 1:2,000; cat. no. ab133352), anti-Cx43 (dilution, 1:2,000; cat. no. ab11370), anti-His (dilution, 1:1,000; cat. no. ab9108), anti-HA (dilution, 1:5,000; cat. no. ab9110) or anti-β-actin (dilution, 1:1,000; cat. no. ab8227) (all from Abcam) primary antibodies overnight at 4˚C. The membranes were then washed 5 times in 0.1% TBS-Tween and incubated for 1 h at room temperature with a chicken anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (dilution, 1:2,000; cat. no. sc-516087; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Labeled proteins were detected using a Super Signal protein detection kit (Pierce; Thermo Fisher Scientific, Inc.), and changes in protein levels were evaluated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

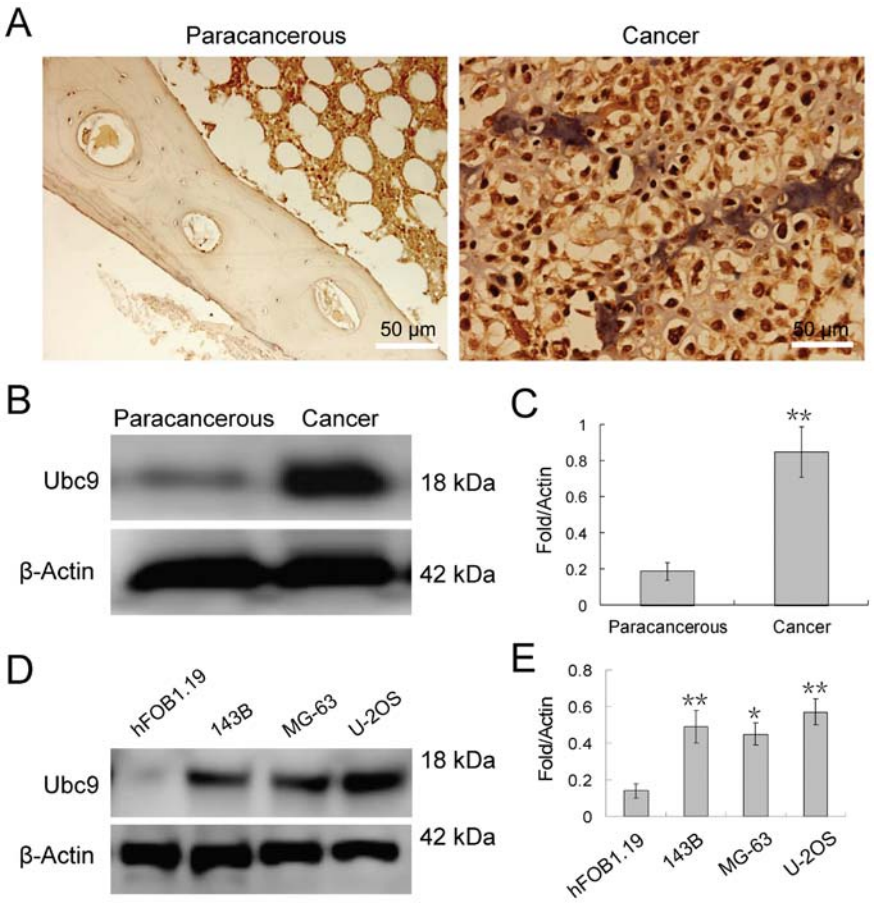

Figure 1. Ubc9 expression was significantly increased in osteosarcoma tissues and cell lines. (A) Ubc9 protein expression in osteosarcoma and adjacent tissues was detected by immunohistochemistry. (B) Ubc9 protein expression in osteosarcoma and adjacent tissues was detected by western blotting. (C) Quantitative analysis of the western blotting results, relative to β-actin. (D) Ubc9 protein expression in osteosarcoma cell lines was detected by western blotting. Quantitative analysis of the western blotting results, relative to β-actin. The graphs represent three independent experiments (mean ± standard deviation). *P<0.05 and **P<0.01 compared with control. Ubc9, SUMO-conjugating enzyme Ubc9.

In vitro HSV-TK/GCV treatment. The Ad-CMV-TK plasmid containing the HSV-TK gene was provided by the Institute of Life Science, Nankai University (Tianjin, China). HSV-TK mRNA expression was detected by reverse transcription-polymerase chain reaction (RT-PCR) analysis. The primer sequences used were as follows: HSV-TK, forward, 5'-CGATGACTTACTGGCAGGTG-3' and reverse, 3'-TGGGAGTAGAAGCTGGCG-5'; β-actin, forward, 5'-TCCCTGGAGAAGAGCTACGA-3' and reverse, 3'-GATCCACACGGAGTACTTGC-5'. Stably transfected cells were selected by G418 (1,000 mg/ml) and cultured in 24-well plates. When 50-80% confluency was reached, various concentrations of GCV ($1x10^{-3}$, $1x10^{-2}$, $1x10^{-1}$, 1x100, $1x10^{1}$ and $1x10^{2}$ mg/ml) were added to each well. After 48 h, Trypan blue (Sigma-Aldrich; Merck KGaA) staining was performed and the percentage of dead cells was calculated using a hemocytometer. In another group, a fixed concentration of GCV (10^{-1} mg/ml) was added to the stably HSV-TK-transfected cells, and 48 h later, lactate dehydrogenase (LDH) activity was measured using an LDH Activity Assay kit (BioVision, Inc., Milpitas, CA, USA), according to the manufacturer's instructions.

In vivo HSV-TK/GCV treatment. A total of 60 4-week-old female nude mice were purchased from the Animal Center of the Academy of Military Medical Sciences (Beijing, China) and housed at the Experimental Animal Center of The Fifth Central Hospital of Tianjin under controlled temperature conditions (22-24˚C), in a 12/12 h light/dark cycle. All experimental procedures were carried out according to the regulations and internal biosafety and bioethics guidelines of the Animal ethics committee of The Fifth Central Hospital of Tianjin (Tianjin, China). A tumor-bearing murine model was established as previously described (32). The 60 mice were randomly divided into 5 groups: i) Control, untransfected U-2OS cells were subcutaneously transplanted into the left shoulder, followed by treatment with PBS for 25 days; ii) HSV-TK, HSV-TK-transfected U-2OS cells were subcutaneously transplanted into the left shoulder of mice, followed by treatment with PBS every 2 days for 25 days; iii) HSV-TK/GCV, HSV-TK-transfected U-2OS cells were subcutaneously transplanted into the left shoulder, followed by treatment with 15 mg/kg GCV every 2 days for 25 days; iv) siR-neg/HSV-TK/GCV, HSV-TK- and siR-neg-co-transfected U-2OS cells were subcutaneously transplanted into the left shoulder, followed by treatment with 15 mg/kg GCV every 2 days for 25 days, and, v) siR-ubc9/HSV-TK/GCV, HSV-TK- and siR-ubc9-co-transfected U-2OS cells were subcutaneously transplanted into the left shoulder, followed by treatment with 15 mg/kg GCV every 2 days for 25 days. Tumor growth was measured using calipers every 5 days for 30 days. Tumor volume (V) was calculated

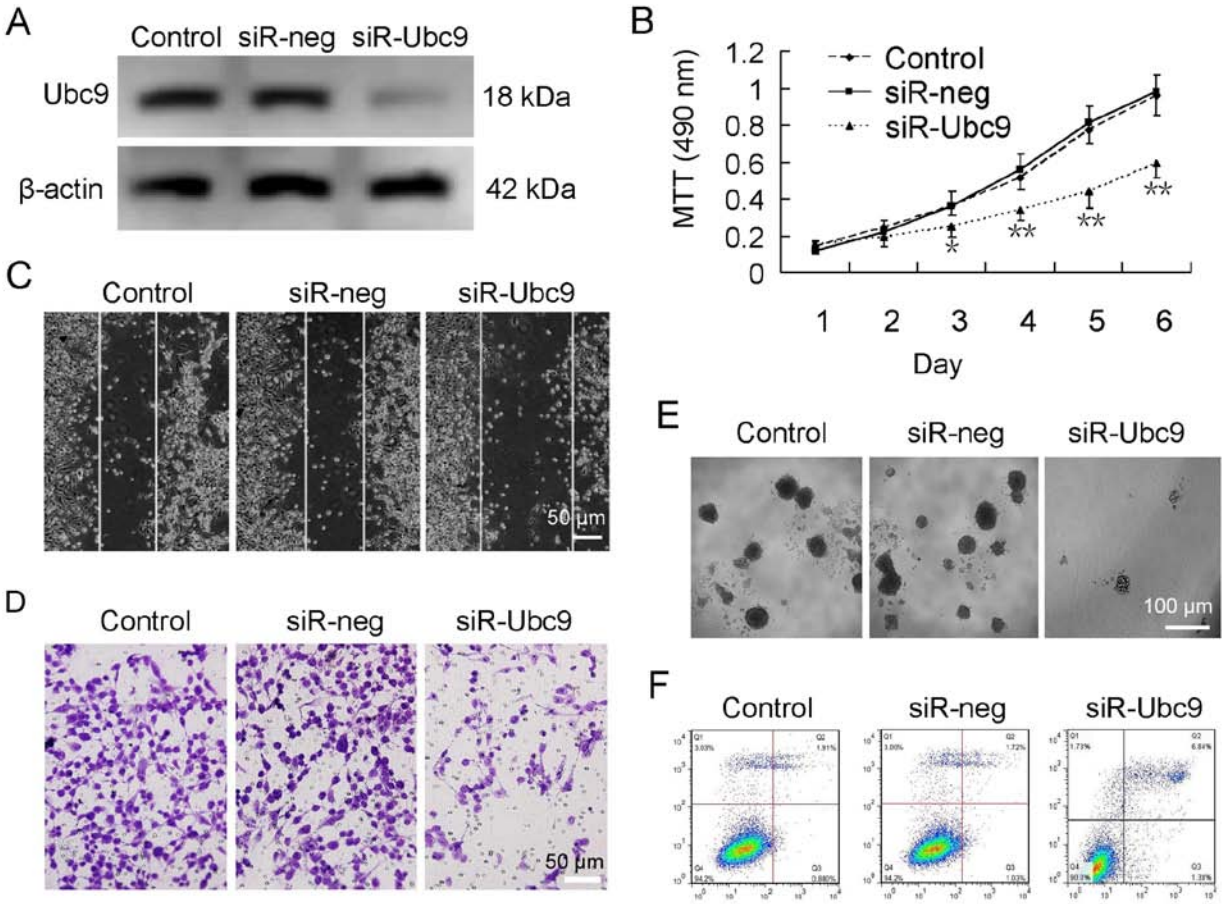

Figure 2. Ubc9-silencing inhibits the proliferation and migration of osteosarcoma cells and promotes apoptosis. (A) Ubc9 protein expression was detected by western blotting following siR-Ubc9 transfection. (B) Proliferation was detected using by MTT assay. (C) Cell migration ability was detected using wound healing assays. (D) Cell invasion ability was detected by Transwell assay (E) Cell colony formation ability was detected by soft agar colony formation assay. (F) Apoptosis was examined by flow cytometry. The percentage of apoptotic cells was 2.81±0.31, 2.79±0.25 and 8.23±0.67% in the control, siR-neg and siR-Ubc9 groups, respectively. The graphs represent three independent experiments (mean ± standard deviation). *P<0.05 and **P<0.01 compared with control. Ubc9, SUMO-conjugating enzyme Ubc9; siR, small interfering RNA; neg, negative control.

as follows: V = L x W^2 x 0.5 (L, length; W, width). The mice were sacrificed, and paraffin-embedded tissue sections were prepared for in situ apoptosis and immunohistochemical analyses. Apoptosis was detected by TUNEL staining using an in situ cell death kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Ki67, Cx43 and Ubc9 protein expression was detected by immunohistochemistry, as aforementioned, using the following primary antibodies: Ki67 (dilution, 1:250; cat. no. ab16667), Cx43 (dilution, 1:2,000; cat. no. ab11370) and Ubc9 (dilution, 1:4,000; cat. no. ab75854) (all from Abcam).

Statistical analysis. All experiments were repeated ≥3 times. All data are expressed as the mean ± standard error of the mean. All tests were two-tailed, and P<0.05 was considered to indicate a statistically significant difference. GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) was used for all statistical tests.

Results

Ubc9 is highly expressed in osteosarcoma tissues and cell lines. Recent studies have demonstrated that Ubc9 protein levels are overexpressed in various types of tumor, including colorectal, prostate, lung, breast and pancreatic cancer (33-35). Furthermore, upregulation of Ubc9 expression has been suggested to be accompanied by protein SUMOylation events (36). Therefore, in the present study, the expression of Ubc9 protein was investigated in osteosarcoma and non-tumor tissues. Immunohistochemical staining revealed that Ubc9 protein was highly expressed in osteosarcoma tissue compared with normal adjacent tissues and localized to the nucleus of osteosarcoma cells (Fig. 1A). Furthermore, western blotting analysis also demonstrated that Ubc9 expression in osteosarcoma tissue was approximately 4-fold of that in adjacent tissues (Fig. 1B and C). Similar results were achieved in the osteosarcoma cell lines (Fig. 1D and E). Seeing as the protein expression level of Ubc9 in U-2OS cells was the highest of the 3 osteosarcoma cell lines tested, knockdown of Ubc9 may lead to more pronounced effects in this cell line. Therefore, U-2OS cells were selected for subsequent experiments.

Silencing Ubc9 inhibits proliferation and migration, and promotes apoptosis of osteosarcoma cells. To analyze the role of Ubc9 in osteosarcoma and to determine whether silencing of Ubc9 may inhibit carcinogenesis, Ubc9 expression was

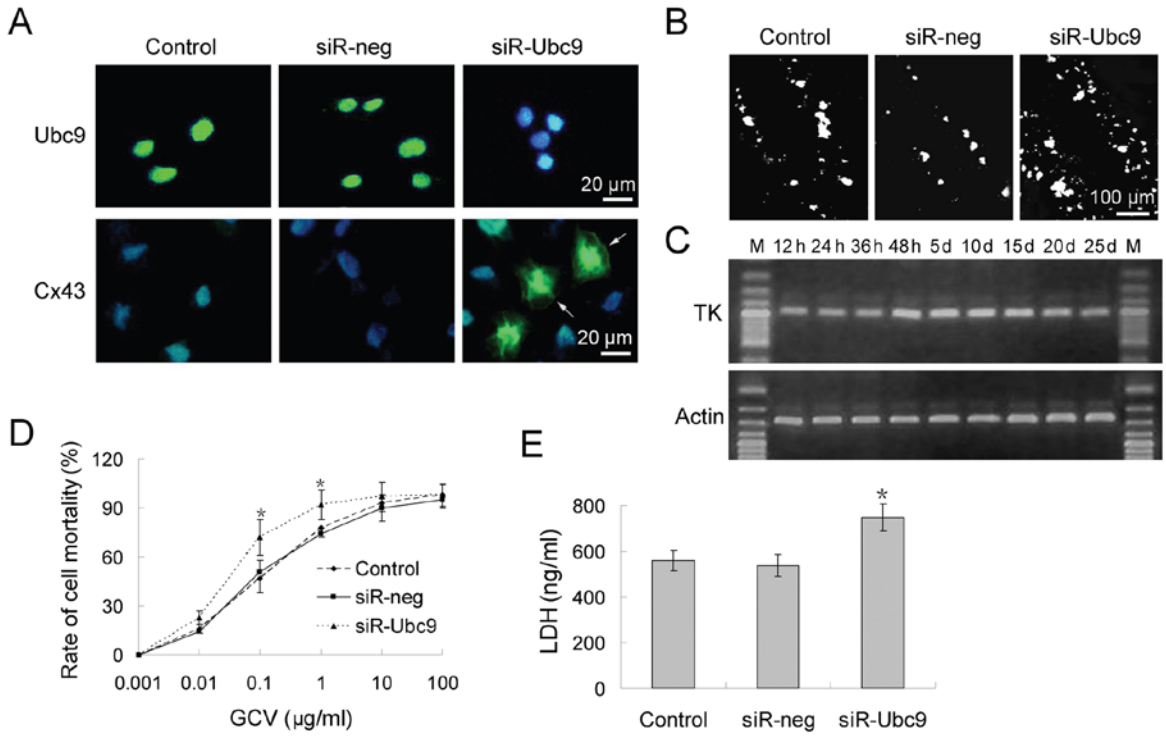

Figure 3. Ubc9-silencing improves GJIC function between osteosarcoma cells and increases their sensitivity to HSV-TK/GCV *in vitro*. (A) Ubc9 and Cx43 protein expression was detected by immunocytochemistry. (B) GJIC function was measured by Lucifer Yellow dye transfer assay. (C) HSV-TK mRNA expression was detected by reverse transcription-quantitative polymerase chain reaction. (D) The percentage of dead cells was counted using a hemocytometer and trypan blue staining following exposure to different concentrations of GCV. (E) LDH content was measured by enzyme-linked immunosorbent assay when cells had been exposed to 10^{-1} mg/ml GCV for 48 h. The graphs represent three independent experiments (mean ± standard deviation). *P<0.05 and **P<0.01 compared with control. Ubc9, SUMO-conjugating enzyme Ubc9; GJIC, gap junction intercellular communication; HSV-TK, herpes simplex virus thymidine kinase; GCV, ganciclovir; LDH, lactate dehydrogenase; siR, small interfering RNA; neg, negative control; Cx43, connexin 43.

silenced in U-2OS cells using siRNA (Fig. 2A). Further experimentation demonstrated that the proliferation, migration and colony forming abilities of U-2OS cells were significantly decreased following Ubc9 silencing (Fig. 2B-E). Furthermore, there was an increase in the apoptotic rate from ~2% in untreated cells to ~7% in Ubc9-knockdown cells (Fig. 2F).

Silencing of Ubc9 partially restores GJIC function in osteosarcoma and enhances sensitivity to chemotherapy. Previous studies have reported that Cx43 is covalently modified and regulated by SUMOylation (26); however, the specific role of Cx43 SUMOylation remains unknown. In the present study, the effect of silencing Ubc9 on the function of GJIC was investigated in osteosarcoma, as well as whether this mechanism may be used for osteosarcoma treatment. Firstly, it was investigated whether Cx43 protein expression was restored by silencing Ubc9 in osteosarcoma cells (Fig. 3A). Scrape loading and dye transfer assays revealed that control the control group exhibited poor dye-coupling. This was indicative of GJIC inhibition. However, GJIC function was partially restored following transfection with Ubc9 siRNA. Lucifer Yellow was transmitted to neighboring cells from the loaded cells via the injured scraping border (Fig. 3B).

Subsequently, a conventional HSV-TK/GCV system was employed to detect whether Ubc9-silencing could increase chemotherapy sensitivity. RT-qPCR analysis revealed that the highest level of HSV-TK expression occurred 48 h after transfection, and that HSV-TK expression was maintained for ≥25 days (Fig. 3C). Cells stably expressing HSV-TK were incubated in medium containing 10^{-3}-10^{2} mg/ml GCV for 48 h. The cell viability of U-2OS cells was 50% at 10^{-1} mg/ml GCV in the control and siR-neg group. However, ≥70% cells died at this concentration in the siR-Ubc9 group (Fig. 3D). LDH experiments confirmed these results (Fig. 3E).

Ubc9-silencing reduces SUMOylated Cx43 and increases free Cx43 levels. To explore the association between Ubc9 silencing and Cx43 SUMOylation, the protein levels of SUMO1 and Cx43 were detected following Ubc9-silencing. It was revealed that Ubc9-silencing significantly reduced the levels of conjugated SUMO1, and increased the level of free SUMO1 protein. The level of free Cx43 protein was also increased (Fig. 4A and B). Exogenous HA-Cx43 and Flag-SUMO1 were co-transfected into U-2OS cells with or without His-siR-Ubc9. The results confirmed that silencing of Ubc9 inhibited the conjugation of SUMO-1 to its substrate proteins, and induced decoupling of SUMO1 from Cx43 (Fig. 4C).

Silencing of Ubc9 increases the sensitivity of osteosarcoma to HSV-TK/GCV in vivo. To verify whether Ubc9-silencing enhanced chemosensitivity *in vivo*, xenografts tumors were established in immunodeficient mice. The results demonstrated that transfection of U-2OS cells with HSV-TK alone had an insignificant effect on tumor growth. However, when GCV was intraperitoneally injected, there was a significant decrease in tumor volume and weight (Fig. 5A and B). Co-transfection

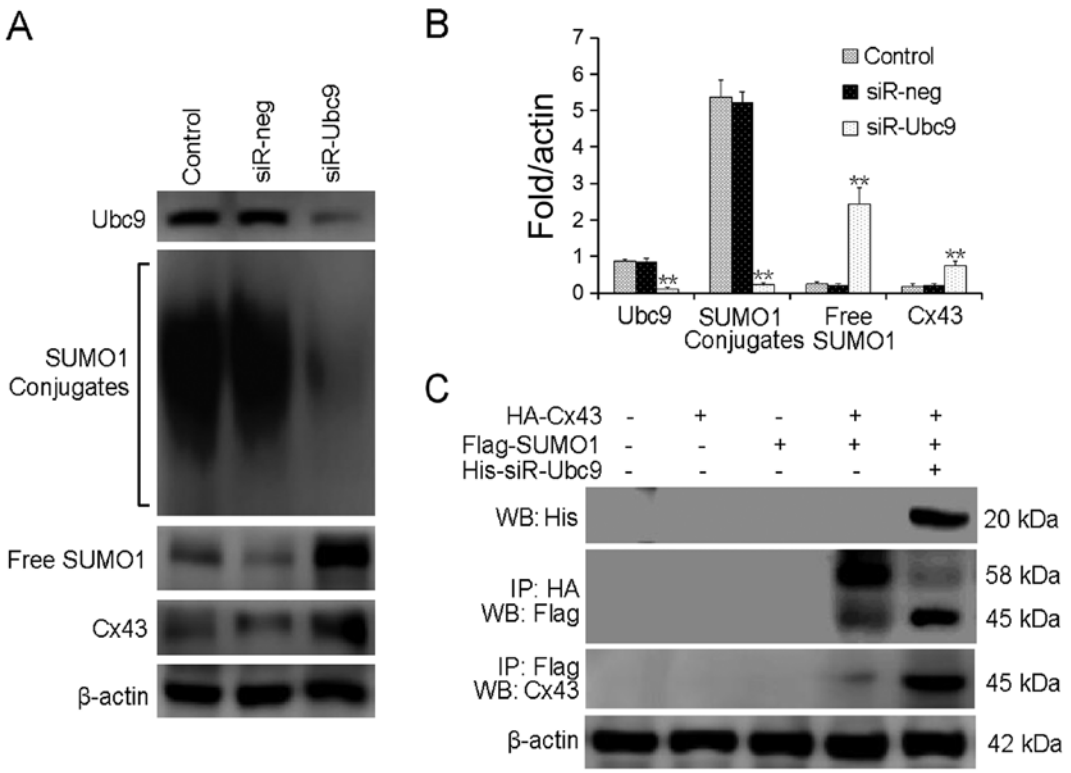

Figure 4. Ubc9-silencing reduces Cx43-SUMOylation, increasing free Cx43 levels. (A) SUMOylated Cx43 levels were detected by western blotting. (B) The bar chart presents the quantified SUMOylated Cx43 protein levels. (C) Exogenous HA-Cx43 and Flag-SUMO1 were co-transfected into U-2OS cells with or without His-siR-Ubc9. The cell lysates were subjected to an immunoprecipitation assay. **P<0.01, compared with control. Ubc9, SUMO-conjugating enzyme Ubc9; GJIC, gap junction intercellular communication; LDH, lactate dehydrogenase; siR, small interfering RNA; neg, negative control; Cx43, connexin 43; Ip, immunoprecipitation; WB, western blot.

of the siR-Ubc9 plasmid and HSV-TK with GCV administration further reduced tumor volume, and also induced apoptosis (Fig. 5A and C). *In situ* apoptosis detection demonstrated that the HSV-TK/GCV system induced apoptosis of a proportion of tumor cells, and that Ubc9-silencing further enhanced the therapeutic effect (Fig. 5C). Finally, the protein expression levels of Ki67, Cx43 and Ubc9 were detected in xenograft tumor tissues. The results demonstrated that Ubc9 silencing significantly inhibited the rate of proliferation, and restored GJIC function *in vivo* (Fig. 5D-F).

Discussion

Recent studies have reported that SUMOylation is frequently upregulated during malignant transformation in a range of tumors, including lung cancer, prostate cancer, gastric cancer, breast cancer and glioma (20-25,37). Ubc9, the only SUMO-E2-conjugating enzyme, is has been demonstrated to be overexpressed in various types of cancer cells (33-36). Therefore, in the present study, Ubc9 expression was analyzed in osteosarcoma tissues and in three osteosarcoma cell lines. The results revealed that Ubc9 protein expression was significantly increased in osteosarcoma tissues and cell lines. However, it was not determined whether the level of Ubc9 protein was associated with the malignancy of osteosarcoma due to the limited number of tissue samples.

To further analyze the role of Cx43 SUMOylation in maintaining the integrity and function of the GJIC between cancer cells, a lentiviral plasmid that induced Ubc9 silencing was constructed. The majority of substrate proteins, which were originally bound to SUMO1, underwent deSUMOylation following Ubc9-silencing. The levels of free Cx43 were also significantly increased. Immunocytochemistry and Lucifer Yellow dye transfer experiments confirmed that Ubc9-silencing partially restored the structure and function of GJIC, which was likely mediated by free Cx43.

SUMO1 competes with ubiquitin for the same lysine binding sites on a substrate protein, preventing the target protein from being hydrolyzed (38). This may explain the increased free Cx43 protein levels. However, contrary to expectation, Cx43 deSUMOylation increased Cx43 levels via silencing Ubc9, which improved the GJIC function between cells. Proteins that perform different functions in different stress conditions are often modified by a variety of post-translational modifications, including phosphorylation, acetylation, methylation and ubiquitination (39). Unfortunately, the specific regulatory mechanism that underlies the relationship between Cx43 levels and decreased SUMOylation remains unclear.

Whether the recovery of GJIC triggered by Ubc9-silencing could be transformed and utilized to improve the sensitivity of chemotherapeutic drugs was a major focus of the present study. Silencing of Ubc9 improved the sensitivity of osteosarcoma cells to HSV-TK/GCV chemotherapy both *in vitro* and *in vivo*.

In addition to the above findings, the present study also examined the effect of Ubc9-silencing on proliferation, migration and apoptosis of osteosarcoma cells. It was demonstrated that inhibition of Ubc9 expression directly suppressed the

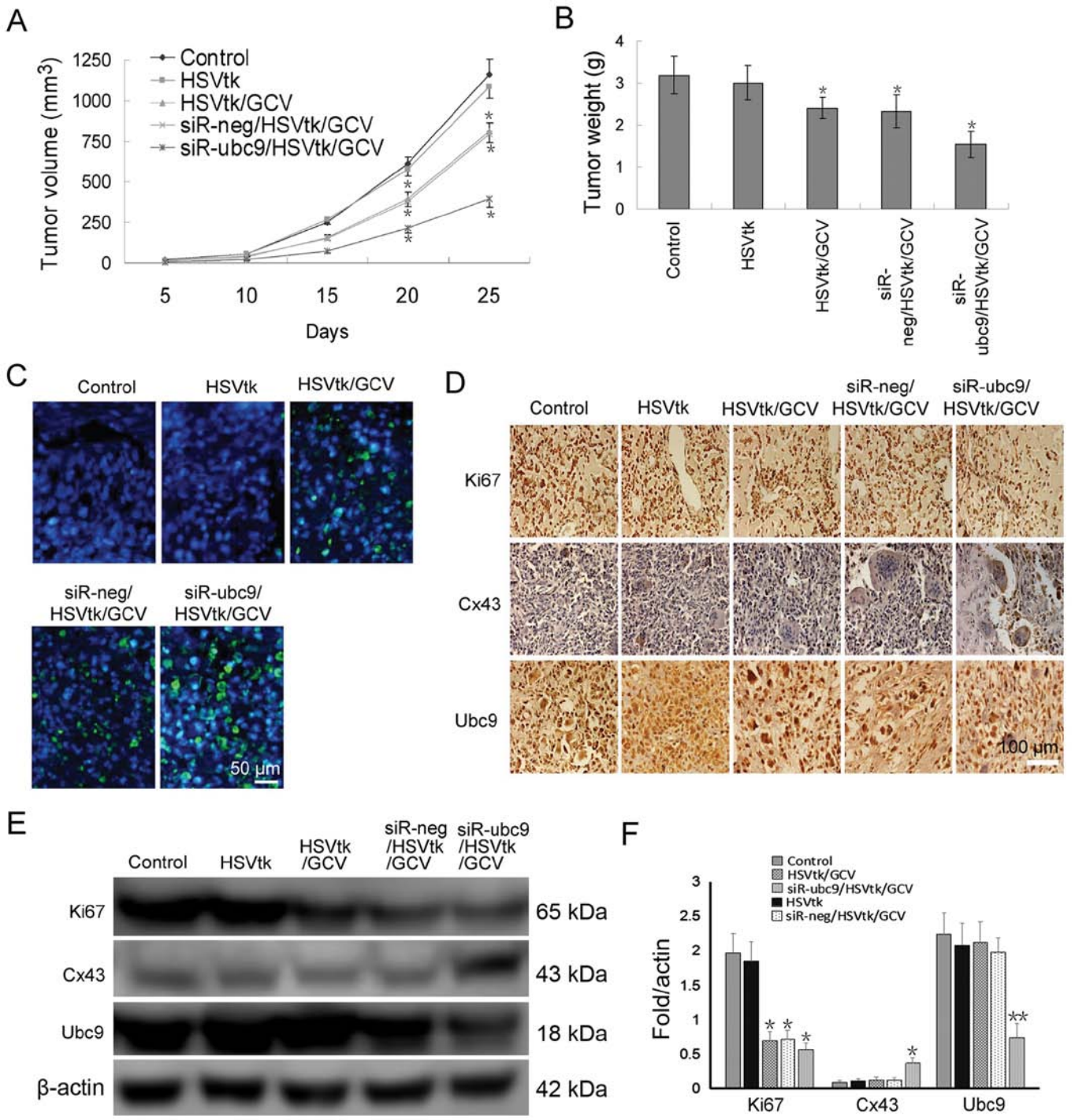

Figure 5. Ubc9-silencing significantly increases the sensitivity of osteosarcoma cells to HSV-TK/GCV *in vivo*. (A) Tumor volumes were monitored over 25 days. (B) Tumor weight was measured at the end of the total treatment time. (C) Apoptosis in xenograft tumor sections was detected by TUNEL assay. Ki67, Cx43 and Ubc9 protein expression in xenograft tumor sections was detected by (D) immunohistochemistry, and (E) western blotting. (F) Bar chart presenting quantified protein expression detected by western blotting. The graphs represent three independent experiments (mean ± standard deviation). *P<0.05 and **P<0.01 compared with control. Ubc9, SUMO-conjugating enzyme Ubc9; HSV-TK, herpes simplex virus thymidine kinase; Cx43, connexin 43; GCV, ganciclovir; siR, small interfering RNA.

proliferation and migration of osteosarcoma cells, and induced apoptosis. However, the apoptotic rate only increased from 2~7% following Ubc9 silencing.

Recent studies have demonstrated that osteosarcoma cells maintain their proliferation and migration capabilities via the PI3K/Akt pathway (40-42). Other studies confirmed that Akt SUMOylation regulates proliferation, tumorigenesis and the cell cycle (43,44). In addition, Akt-SUMOylation regulates global SUMOylation, including that of Akt and Ubc9, STAT1 and CREB (45). Due to the important role of Akt-SUMOylation in tumorigenesis, this mechanism may also be involved in osteosarcoma formation.

In conclusion, the present study indicates that Cx43-SUMOylation occurs in osteosarcoma tissues and is involved in regulating Cx43 gap junctions. However, the underlying molecular mechanism remains unclear. Importantly,

the present study provides a novel strategy to improve the chemotherapy sensitivity of osteosarcoma by inducing deSUMOylation of Cx43. This gives us an important indication that there will be a broad space for development in this field in the future.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XZL and WHW conceived and designed the study. DYZ, KY, ZY, YXL, XFM, XYB, FTL and LLL, performed the experiments. DYZ and KY wrote the paper. XZL and WHW reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of Tianjin Medical University Cancer Institute and Hospital (Tianjin, China).

Patient consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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