Downregulation of IDH2 exacerbates the malignant progression of osteosarcoma cells via increased NF-κB and MMP-9 activation

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Abstract. Isocitrate dehydrogenase 2 (IDH2) is a mitochondrial NADP-dependent isocitrate dehydrogenase. It is considered to be a novel tumor suppressor in several types of tumors. However, the role and related mechanism of IDH2 in osteosarcoma remain unknown. The expression and significance of IDH2 were investigated by immunohistochemistry in formalin-fixed paraffin sections from 44 osteosarcoma patients. IDH2 was downregulated via lentiviral vector-mediated RNA interference (RNAi) in the Saos-2 and MG-63 human osteosarcoma cell lines. The effect of IDH2 downregulation on human osteosarcoma was studied *in vitro* by MTT, flow cytometry and invasion assays. Nuclear factor-κB (NF-κB) and matrix metalloproteinase-9 (MMP-9) assays were also used to study the likely molecular mechanism of IDH2 downregulation on the malignant progression of osteosarcoma cells. The results revealed that the expression of IDH2 was inversely correlated with pathological grade and metastasis in osteosarcoma. IDH2 downregulation promoted a pro-proliferative effect on the Saos-2 and MG-63 osteosarcoma cell lines. IDH2 downregulation accelerated cell cycle progression from S to G2/M phase. The pro-proliferative effect induced by IDH2 downregulation may be ascribed to increased NF-κB activity via IκBα phosphorylation. The invasive activity of osteosarcoma cells was also significantly promoted by IDH2 downregulation and may result from elevated MMP-9 activity. In conclusion, IDH2 downregulation may exacerbate malignant progression via increased NF-κB and MMP-9 activity and may implicate the potential biological importance of IDH2 targeting in osteosarcoma cells. Downregulation of IDH2 exacerbates the malignant progression of osteosarcoma cells via increased NF-κB and MMP-9 activation.

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Introduction

Osteosarcoma is the most prevalent primary malignant bone tumor in children and young adults, and is characterized by aggressive invasion, early metastasis and resistance to existing chemotherapeutics (1). In recent years, the survival rate of osteosarcoma patients has improved due to advances in aggressive systemic chemotherapy. However, the survival rate of osteosarcoma patients is still low for those with primary metastases and relapse compared to patients with localized disease (2,3). Moreover, multidrug combination chemotherapy for osteosarcoma leads to ototoxicity, cardiac toxicity and secondary malignancies (2). Thus, it is vital to identify novel approaches for both diagnosis and treatment that are more efficient than the currently available methods to resolve these problems and improve the prognosis of osteosarcoma (4). An understanding of the molecular events that drive the progression and metastasis of osteosarcoma would facilitate better diagnosis and treatment strategies.

Recently, NADP^{+}-dependent isocitrate dehydrogenases (IDHs), including IDH1 and IDH2, were found to be downregulated in glioma, melanoma and bladder cancer (5-8). By providing NADPH, IDHs play an important role in controlling the mitochondrial redox balance and mitigating cellular oxidative damage (9,10). In our previous study, IDH1 was shown to be a tumor-suppressor gene in osteosarcoma and inhibited the malignant progression of osteosarcoma (11,12). However, the role of IDH2 in osteosarcoma remains unknown.

IDH2, a mitochondrial NADP-dependent enzyme, catalyzes oxidative decarboxylation and produces CO_2, NADPH and α-ketoglutarate from isocitrate in the mitochondria (13). NADPH is a vital cofactor for many enzymatic reactions, including fat and cholesterol biosynthesis and glutathione metabolism (14). It was demonstrated that mitochondrial NADP^{+}-dependent isocitrate dehydrogenase plays an important role in cellular defense against oxidative damage by providing NADPH, which is needed to regenerate the glutathione levels in mitochondria (15-17). Reactive oxygen

species (ROS), which are produced in mitochondria as a natural by-product of normal energy metabolism, are involved in over 150 human disorders (18). Increased ROS levels promote cellular oxidative stress that contributes to various processes in malignant tumors, including carcinogenesis, aberrant growth, angiogenesis and metastasis (19,20).

Tumor cell growth and invasion are important in malignant progression and are regulated by many biological regulators. One such regulator is NF-κB that regulates the expression of various genes involved in immunity, stress responses, inflammation and inhibition of apoptosis, thus providing appropriate conditions for tumor cell progression (21,22). NF-κB is constitutively activated in tumor cells, including osteosarcoma cells, and contributes to maintain the highly proliferative malignant phenotype as well as cellular invasion (23,24). NF-κB regulates several metastasis-related matrix metalloproteinases (MMPs), such as MMP1, MMP3 and MMP-9 (25-29). MMP-9 is recognized as a classic invasion- or metastasis-related NF-κB target gene and is described as an important biomarker that is directly associated with the metastatic processes in osteosarcoma (11,30,31).

In the present study, we first investigated the expression and significance of IDH2 in osteosarcoma biopsies *in vivo*. Next, we studied the role of IDH2 downregulation *in vitro* in the Saos-2 and MG-63 human osteosarcoma cell lines. Furthermore, we studied the related biological mechanisms that were induced by IDH2 downregulation in osteosarcoma cells.

Materials and methods

Tissue specimens and clinical data. Fifty-one formalin-fixed, paraffin-embedded osteosarcoma specimens (before the administration of neo-adjuvant chemotherapy) were collected according to the Chinese National Ethical Guidelines ('Code for Proper Secondary Use of Human Tissue', Chinese Federation of Medical Scientific Societies). Due to the limited available tumor material and follow-up information, only 44 of these osteosarcoma tumor samples were eligible for the present study. The mean age of the patients (mean ± SD) was 25.25±13.61 years (range 9-61; male/female ratio 32:12). Of the 44 eligible patients, 23 had non-metastatic tumors (53.3%). Osteoblastic osteosarcoma was the most common histopathological subtype and occurred in 29 patients (65.9%). The lower end of the femur was the most common site and was observed in 13 patients (29.5%). The patient distribution according to Rosen's histological grade (19,20) included stage I in 5 (11.3%), II in 16 (36.4%), III in 16 (36.4%) and stage IV in 7 patients (15.9%). Stages I and II were defined as low Rosen grade osteosarcoma specimens and stages III and IV were defined as high grade specimens. The patients were followed-up until death from the disease, or until their most recent clinical therapy at the end of the present study. The mean follow-up time (mean ± SD) was 4.26±1.99 years (range 0.5-9.0). All patients were diagnosed according to the osteosarcoma criteria defined by the World Health Organization. Written informed consent was obtained from each patient before he/she entered into the present study, and all study protocols were approved by the Ethics Committee for Clinical Research of Wuhan University, China.

Immunohistochemistry and specimen evaluation. The sections were cut from the formalin-fixed, paraffin-embedded osteosarcoma tissue and hydrated through graded alcohol solutions. For antigen unmasking, the sections were treated in a trypsin solution at 37°C for 10 min. The sections were then washed with deionized water and incubated with 3% H_2O_2 for 5 min. They were then incubated with the anti-IDH2 mAb at room temperature for 1 h, followed by a secondary antibody and the peroxidase-conjugated streptavidin-biotin complex (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37°C for 30 min. The immunoreactivity was visualized with 3,3'-diaminobenzidine (DAB) (Zymed, South San Francisco, CA, USA). The negative controls were obtained by omitting the primary antibody.

IDH2 staining was detected in the mitochondria and was scored by adding the number of cells displaying clear tumor cell labeling; the intensity of staining was scored between 0 and 6 (32,33). The proportion score was as follows: 0 indicates negative staining; +1 indicates ≤25% positive labeling in tumor cells; +2 indicates 25-50% positive tumor cells; and +3 indicates >50% positive tumor cells. The intensity score was as follows: 0 indicates no staining; +1 indicates weak staining; +2 indicates intermediate staining; and +3 indicates strong staining (32,33). For statistical analysis, the osteosarcoma patients were grouped as either the low expression group (scored 1-4) or the high expression group (scored 5-6). At least 5 separated neoplastic infiltration foci were analyzed in each specimen, followed by the evaluation of 10 slides/patient and 6 sections/slide. The immunostaining was assessed by three independent observers. The slides were scanned using a microscope (Carl Zeiss AG, Germany) by reviewing the entire spot and the images were recorded using a digital camera (DC500; Leica) and Leica FW4000 software. The images were processed using Adobe Photoshop.

Cell lines, culture and lentiviral infection. The Saos-2 and MG63 tumor cells (ATCC, LGC Promochem, Germany) were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, USA) with 10% fetal bovine serum (FBS) (Amresco, USA) and 0.1% penicillin/streptomycin and maintained in an atmosphere with 5% CO_2 at 37°C.

To downregulate IDH2, small interfering RNA (siRNA) sequences: LV-KD, 5'-GTGGACATCCAGCTAAAGTAT-3', were inserted into the pLL3.7 shRNA lentiviral vector (Genesil, Wuhan, China). The pLL3.7 lentiviral vector LV-mIκB (Genesil), which was aimed at overexpressing the mutant IκB (mIκB), was constructed as previously described to suppress NF-κB activity (13). siRNA sequences for MMP-9 downregulation were: 5'-ACCACAACAUCACCUAUUGTT-3' (34). The empty lentiviral vector (Genesil), LV-EV was used as a control. In some experiments, non-treated cells, named the NT cells, were used as another control. The lentiviral stocks were added to the Saos-2 and MG63 osteosarcoma cell lines.

The cells were infected with the lentivirus and selected with an 800 µg/ml G418 solution for the Saos-2 cells and a 500 µg/ml solution for the MG63 cells. The efficiency of the highest infection, as determined by G418 selection, was obtained at a multiplicity of infection (MOI) of 10 for the Saos-2 cells and 50 for the MG63 cells. The cells transfected with LV-KD or LV-EV were named the KD or EV cells,

respectively. After selection, the efficiency of infection was verified by western blotting. Polyclonal populations and clones displaying higher levels of IDH2 downregulation were chosen for the subsequent studies. After IDH2 downregulation, LV-mIκB or LV-siMMP-9 was transfected into the cells for the NF-κB and MMP-9 assays, respectively.

Protein isolation and western blot analysis. The cell lysates were prepared using lysis buffer from the Dual-Luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The lysates were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween (TBST) and washed 6 times with TBST. The IDH2 and MMP-9 proteins were detected using rabbit polyclonal primary antibodies (Protein Technology Group, USA). NF-κB, Bcl-2, JNK, p-JNK, ERK, p-ERK, IκBα and p-IκBα were detected by mouse monoclonal primary antibodies (Santa Cruz Biotechnology). The β-actin proteins were recognized by a monoclonal β-actin-specific mouse IgG (Santa Cruz Biotechnology) and used as the internal loading control. The antibodies were diluted according to the manufacturer's instructions and incubated overnight at 4˚C, followed by incubations with the peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulins (1:2,000; Santa Cruz Biotechnology) in TBST for 1 h. The signals were developed using an enhanced chemiluminescent reagent (Pierce Biotechnology, Rockford, IL, USA).

MTT assay. A total of $3.5x10^3$ cells were seeded in each test well in a 96-well plate to detect cell growth. After 1-6 days of culture, the cells were washed with phosphate-buffered saline (PBS). MTT (5 mg/ml) was then added to each well (including the control) and the mixture was incubated at 37˚C for 4 h. The culture medium was then replaced with an equal volume of dimethylsulfoxide (DMSO). After shaking the plate at room temperature for 10 min, the absorbance of each well was determined at 570 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell cycle analysis with flow cytometry. The cells were harvested by trypsinization, fixed with 70% pre-chilled alcohol and stored at 4˚C overnight. The alcohol was then removed by centrifugation at 1,000 rpm for 5 min, and the cells were treated with 0.1% Triton-X and DNase-free RNase (10 mg/ml) for 30 min (35). The cell DNA was stained with 1 mg/ml propidium iodide (PI) for 15 min in the dark and analyzed using a flow cytometer (FACScan; Becton-Dickinson, New York, NY, USA). The relative proportions of cells in the G0-G1, S and G2-M phases of the cell cycle were determined from the flow cytometry data.

Cell invasion assay. Cell invasion was determined using a two-chamber Transwell (Corning, New York, NY, USA). The upper surface of a polycarbonate membrane with 8-μm pores was coated with 1 mg/ml Matrigel (36). The cells (~$10x10^5$) were suspended in RPMI-1640 serum-free medium (Gibco, USA) and placed in the upper chamber; RPMI-1640 medium

containing 10% FBS was placed in the lower chamber. The cells were incubated at 37˚C for 48 h with 5% CO_2 in an incubator. At the end of the incubation, the cells on the upper surface of the membrane were completely removed by wiping with a cotton swab. Then, the membrane was fixed with methanol and stained with 0.1% crystal violet. The cells that invaded the Matrigel and reached the lower surface of the membrane were counted and photographed under a microscope.

NF-κB activity assay. An NF-κB reporter plasmid, NF-κB-Luc, was constructed by cloning five repeats of the NF-κB regulatory elements into the pLuc plasmid (Stratagene, La Jolla, CA, USA) to drive luciferase expression. The empty plasmid, Luc, containing a minimal TATA box, was used as a negative control. Approximately $2.0x10^6$ cells were transiently transfected with 10 μg of the NF-κB reporter plasmid by electroporation (37). Then, the cells were seeded and incubated in 24-well plates at 37˚C. After 24 h, the luciferase activity was analyzed using the Luciferase assay system (Promega) according to the manufacturer's instructions.

Intracellular ROS assay. To obtain dissociated Saos2 and MG63 cells for the ROS assay, the culture medium was first removed and the cells were washed two times with RPMI-1640 serum-free medium (Sigma-Aldrich). DCFH-DA, diluted to a final concentration of 10 μM with RPMI-1640 medium, was added to the medium and incubated for 20 min at 37˚C. The fluorescence was read at 488 nm for excitation and 530 nm for emission by flow cytometry (FACScan). The increase in the value compared to the control was viewed as the increase in intracellular ROS levels.

MMP-9 activity assay. The IDH2 and/or MMP-9 downregulated osteosarcoma cells were seeded in 6-well plates and incubated at 37˚C. After 24 h, the medium was removed. Then, the cells were washed and incubated in serum-free medium for 48 h (37). The MMP-9 activity in the medium and cell lysate was detected using the Fluorokine E Human MMP-9 Activity assay kit (R&D Systems) according to the manufacturer's protocol.

Statistical analysis. The statistical analyses were performed using the SPSS 13.0 software package for Windows (SPSS, Inc., Chicago, IL, USA). Comparisons between groups were analyzed by the t-test or Mann-Whitney U test. Associations were assessed by Pearson's or Spearman's correlation coefficients. Event-free survival was calculated from the start of treatment to relapse or metastasis. Overall survival was calculated from the beginning of treatment to the last follow-up or death of the patient. The Kaplan-Meier method was used for the survival analysis. $P<0.05$ was considered to indicate a statistically significant result. $P<0.01$ was considered to indicate a highly statistically significant result.

Results

In vivo tissues
IDH2 correlates with Rosen's histological grade and metastasis in osteosarcoma. IDH2 was mainly found in the
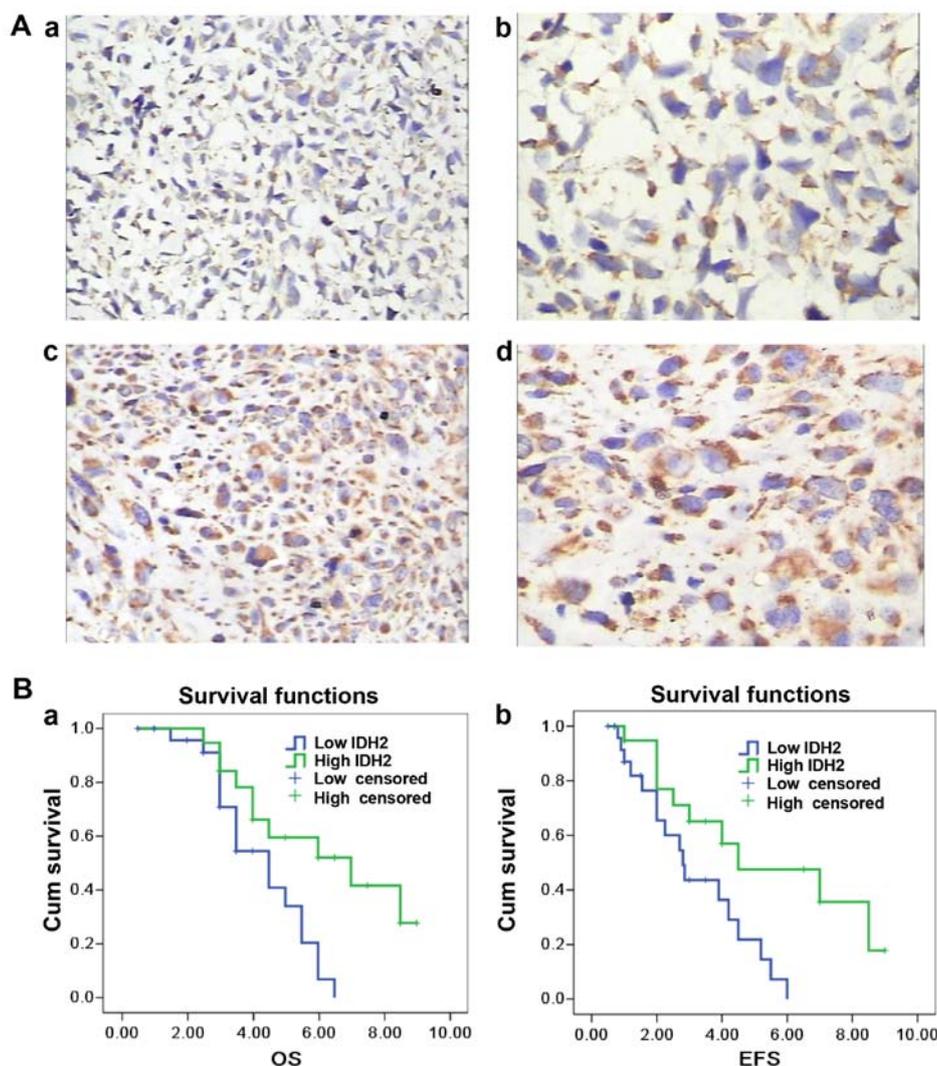
Figure 1. Expression and survival analyses of IDH2 in osteosarcoma biopsies. (A) IDH2 expression was specifically determined in the mitochondria by immunohistochemistry on paraffin-embedded tissue sections. Representative slides from Rosen's high grade osteosarcoma tissues (a and b) and low grade tissues (c and d). IDH2 immunoreactivity appears as strong brown staining in the low grade osteosarcoma biopsies. Magnification: x200 (a and c) and x400 (b and d). (B) The high IDH2 expression group represented the osteosarcoma patients with IDH2 expression scores >4. The patients with IDH2 expression scores ≤4 were recorded as the low IDH2 expression group. The survival time in the x-axis is given in years. (a) The patients with low IDH2-expressing tumors had no significant difference in overall survival (OS) compared to those with high IDH2-expressing tumors (P=0.063). (b) There was no significant trend toward event-free (relapse and metastasis included) survival (EFS) in the patients with IDH2 expression (P=0.074).

mitochondria (Fig. 1A). Of the 44 osteosarcoma specimens, 41 cases were IDH2-positive using immunohistochemistry (93.2%) and 19 exhibited high levels of staining (43.1%). The average IDH2 immunostaining score was 3.15 (SD, 1.46; range from 0 to 6). Lower IDH2 expression was observed in high Rosen's histological grade (38,39) osteosarcoma compared to the low grade specimens (P=0.005; r=-0.505) (Fig. 1A). IDH2 expression was inversely correlated with metastasis (P=0.026; r=-0.334). There was no significant correlation between IDH2 expression and overall survival (P=0.063) or event-free (relapse and metastasis included) survival (P=0.074) (Fig. 1B). IDH2 expression did not correlate with other clinical features, such as age, location of the primary tumor and histological type (P>0.05).

In vitro cell lines
IDH2 downregulation promotes cell proliferation. IDH2 downregulation was verified by western blotting. The IDH2 protein

was significantly decreased in the Saos-2 KD cells compared to the EV or NT cells (P<0.01) (Fig. 2A), respectively; similar results were obtained in the MG63 cells (Fig. 2A). There was no difference in IDH2 expression between the NT and EV cells, either in the Saos-2 or MG63 cells (P>0.05) (Fig. 2A).

IDH2 downregulation increased the cell growth rate in the Saos-2 KD cells by 1.7-fold and in the MG63 KD cells by 1.5-fold on day 6 compared to the Saos-2 EV or MG-63 EV cells (P<0.01) (Fig. 2B). IDH2 downregulation promoted the growth of osteosarcoma cells.

IDH2 downregulation decreases the distribution of cells in the S phase and increases the distribution in the G2/M phase. The DNA of the cell populations was analyzed after stable transfection of IDH2 siRNAs into the Saos-2 and MG63 cells. IDH2 downregulation induced an increase in the G2/M population in the Saos-2 and MG-63 cell lines by 180.4 ± 3.5 and $60.3\pm2.2\%$ (P<0.05), respectively,
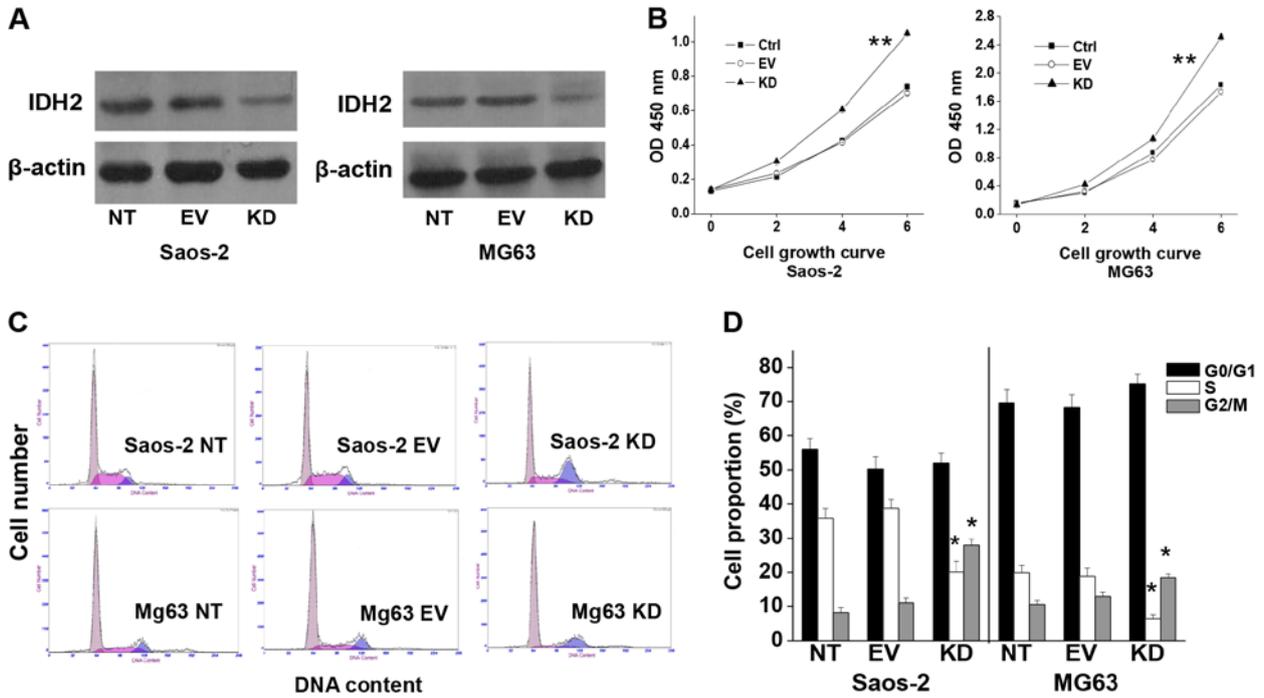
Figure 2. IDH2 depletion promotes cell proliferation, and alters the cell cycle distribution. (A) The IDH2 downregulation efficiency was verified at the protein level by western blot analysis in the KD cells with siIDH2 stable transfection compared to the empty vector (EV)-transfected cells and/or the non-treated (NT) cells. The IDH2 protein was significantly decreased in the Saos-2 KD ($P<0.05$) and MG63 KD cells ($P<0.05$). IDH2 expression was not different between the NT and EV cells in either the Saos-2 or MG63 cell line ($P>0.05$). β-actin expression was monitored as the control. A representative western blot result is shown. (B) Cell viability was evaluated by the MTT assay. IDH2 downregulation promoted cell proliferation in the Saos-2 KD and MG63 KD cells (**$P<0.01$) compared to the EV and NT cells. (C and D) The stable transfectants were harvested for DNA content analysis by flow cytometry. The empty vector and/or non-transfected cells were used as the controls. IDH2 downregulation induced an increase in the G2/M population accompanied by a reduction in the S phase population in the Saos-2 and MG-63 cell lines (*$P<0.05$). Representative results are shown.
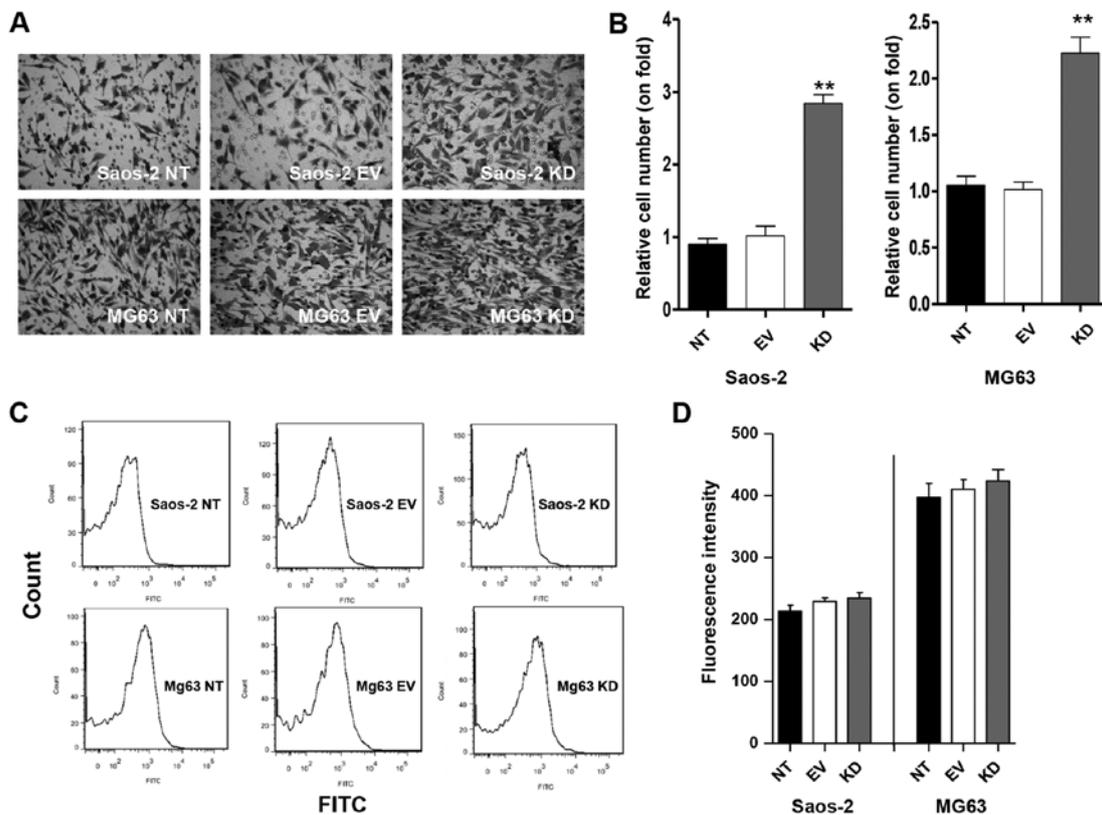

Figure 3. IDH2 depletion increases cell invasion, but does not change the intracellular ROS levels. (A and B) The invasive activity of the Saos-2 KD and MG63 KD cells was increased (**$P<0.01$) compared to the EV cells. Representative results under magnification x200 are shown. (C and D) There was no significant difference in the ROS levels in the Saos-2 KD or MG63 KD cells ($P>0.05$) compared to the EV and NT cells. Representative results are shown.
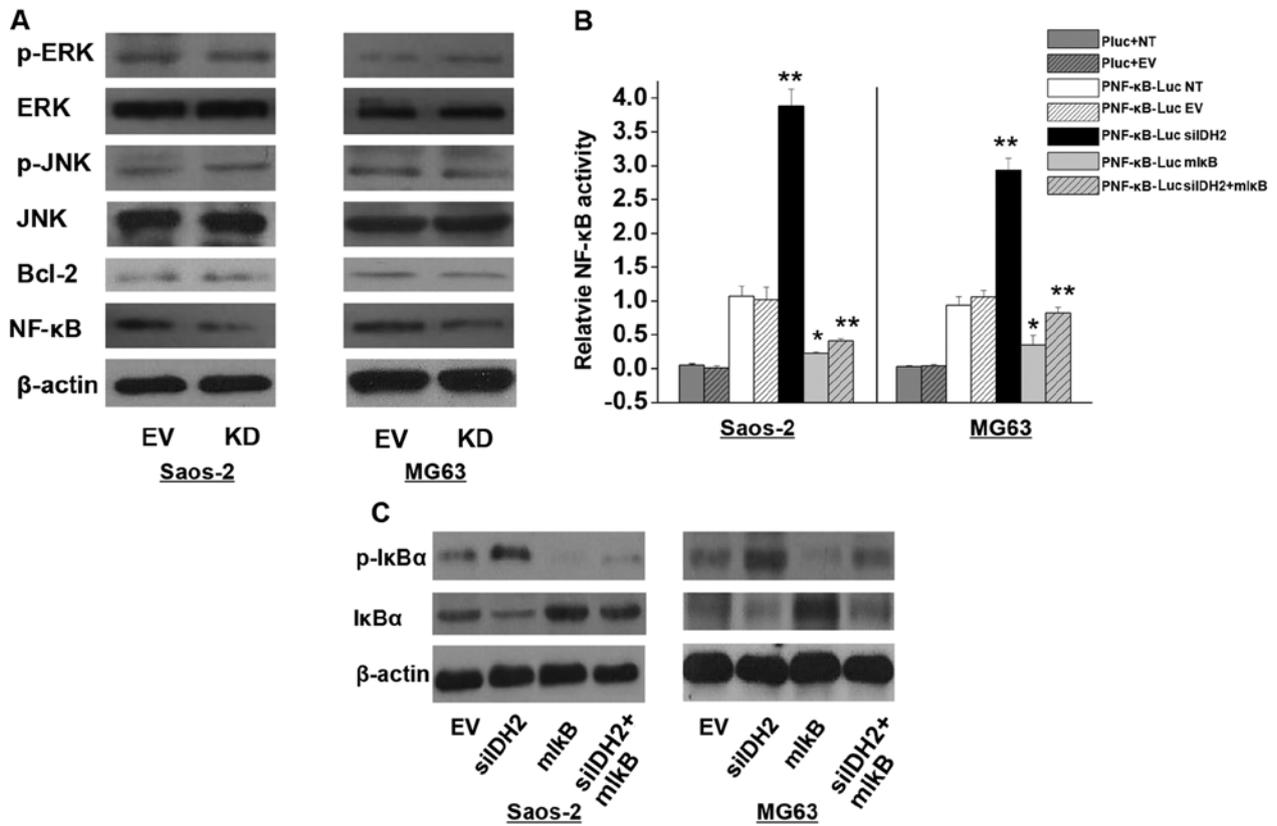
Figure 4. IDH2 downregulation elevates NF-κB transcriptional activity and IκB phosphorylation. (A) In both the Saos-2 and MG63 cells, the degradation of inactivated NF-κB was promoted by IDH2 downregulation compared to the EV cells (P<0.05). However, there was no significant difference in other proteins, such as Bcl-2, phosphorylated and total ERK, and phosphorylated and total JNK (P>0.05). A representative western blot result is shown. (B) Increased NF-κB transcriptional activity was detected in the siIDH2-transfected Saos-2 or MG63 cells (Saos-2 pNF-κB-Luc siIDH2 or MG63 pNF-κB-Luc siIDH2 cells) (P<0.05) compared to the EV cells (pNF-κB-Luc EV cells) using the NF-κB-Luc reporter. The NF-κB activity was significantly reduced by mIκB overexpression (pNF-κB-Luc mIκB cells). When the siIDH2-transfected Saos-2 and MG63 cells were transfected with mIκB (pNF-κB-Luc siIDH2 + mIκB cells), the NF-κB activity was markedly decreased (n=3; P<0.01). Transfection of the empty luc reporter in the NT cells (p-Luc NT cells) and EV cells (p-Luc EV cells) showed minimal background luciferase activity. The NT cells transfected with the NF-κB-Luc reporter (pNF-κB-Luc NT cells) did not show a significant change in luciferase activity; *P<0.05 and **P<0.01. Columns, means; bars, SE (standard deviation). (C) The Saos-2 and MG63 cells transfected with siIDH2 (siIDH2 cells) exhibited suppression of the expression of the NF-κB inhibitor IκBα and promoted the activation of a subset of p-IκBα compared to the EV cells (P<0.05). mIκB suppressed the IDH2 downregulation-induced IκBα phosphorylation (P<0.05). Representative results are shown.

whereas the S phase population was reduced by 53.2±5.8 and 69.6±2.7% (P<0.05), respectively, compared to the empty vector control (Fig. 2C and D). The population in the G0/G1 phase was not significantly changed in the present study (P>0.05) (Fig. 2C and D). The EV cells did not show significant changes in the cell cycle distribution in the Saos-2 and MG63 cells compared to the NT cells (P>0.05) (Fig. 2C and D). IDH2 downregulation in the osteosarcoma cells induced cell cycle progression from the S to the G2/M phase.

IDH2 downregulation exacerbates cell invasion. Next, the effect of IDH2 on cell invasion was investigated. As shown in Fig. 3A and B, IDH2 downregulation promoted the cell invasive activity of the Saos-2 KD cells by 2.8-fold and the MG63 KD cells by 2.2-fold compared to the Saos-2 EV or MG-63 EV cells (P<0.01). The EV cells did not show significant changes in invasion activity in the Saos-2 and MG63 cells compared to the NT cells (P>0.05) (Fig. 3A and B). IDH2 downregulation promoted the invasion of osteosarcoma cells.

IDH2 downregulation does not change the intracellular ROS levels. The intracellular ROS levels in the Saos-2 and MG63 cell lines were also investigated. IDH2 downregulation did not induce a significant difference in the ROS levels in the Saos-2 KD or MG63 KD cells (P>0.05), respectively, compared to the EV and NT cells (Fig. 3C and D).

IDH2 downregulation increases NF-κB activation and IkBα phosphorylation. In both the Saos-2 and MG63 cells, the degradation of inactivated NF-κB was increased by IDH2 downregulation compared to the EV cells (Fig. 4A). However, there was no significant difference in the protein expression of other regulators, such as Bcl-2, JNK and ERK (Fig. 4A). NF-κB transcriptional activity was analyzed by comparing the luciferase activity of the pNF-κB-Luc transfected EV cells to the basal activity. We investigated the overexpression of mIκB (a constitutive NF-κB inhibitor) on the cells with IDH2 downregulation. NF-κB activity was promoted in the Saos-2 and MG63 KD cells with stable IDH2 downregulation compared to the respective EV and/or NT cells (P<0.01). The increased NF-κB activity induced by IDH2 downregulation was inhibited by mIκB overexpression (P<0.01) (Fig. 4B). In addition, Fig. 4C shows that IDH2 downregulation increased the expression of p-IκBα (P<0.01). The significant p-IκBα
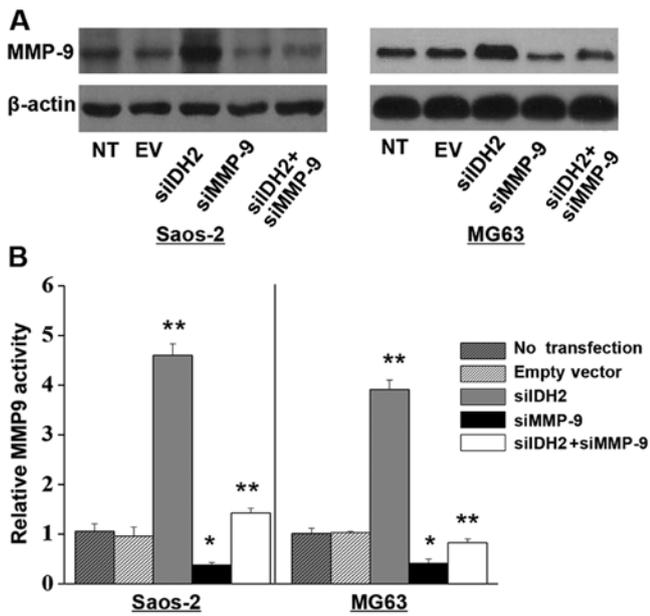
Figure 5. Downregulation of IDH2 increases MMP-9 activation. (A and B) MMP-9 expression was upregulated by IDH2 siRNA transfection (P<0.01) and downregulated by MMP-9 siRNA transfection (P<0.05). MMP-9 downregulation suppressed the increased MMP-9 expression following IDH2 downregulation (P<0.01). Representative results are shown. β-actin served as the loading control; **P<0.01, *P<0.05.

upregulation induced by IDH2 downregulation was inhibited by mIκB overexpression (Fig. 4C).

IDH2 downregulation elevates MMP-9 activation. Western blot analysis and an MMP-9 activity assay were conducted. We found that the MMP-9 protein levels were markedly increased in the IDH2 downregulated Saos-2 or MG63 cells (Fig. 5A and B). In addition, there was a 3.5- and 2.7-fold increase in the MMP-9 activity (Fig. 5A and B) in these cells, respectively. Furthermore, the increased protein expression and activity of MMP-9 induced by IDH2 downregulation were significantly inhibited by MMP-9 downregulation (Fig. 5A and B).

Discussion

To date, the alteration of IDH2 expression levels has been identified in several types of carcinoma (5,41-44). In melanoma, IDH2 was found to be frequently downregulated, and an increase in tumor-free survival resulted from the overexpression of IDH2 (9). IDH2 was also reported to be downregulated in early phase colon carcinoma compared to peritumoral tissues (45). However, the expression and significance of IDH2 in osteosarcoma remain unknown. In our study, it was observed that IDH2 was expressed at a lower level in high-grade osteosarcoma, and 3 of the 44 osteosarcoma patients did not express IDH2. There was a similar trend toward increased metastasis in patients with low IDH2 expression, although no significant correlation was observed between IDH2 expression and survival. Our previous study indicated that patients with low IDH1 expression tended to have higher pathological grade tumors with increased metastatic potential (12). A higher 5-year survival rate was also found in the IDH1 high expres-

sion group vs. the IDH1 low expression group, although there was no significant correlation between IDH1 expression and overall survival (12). The significant similarity of IDH1 and IDH2 in osteosarcoma suggests that IDH1 and IDH2 could both be potential biomarkers for assessing malignant progression and predicting the risk of metastasis in osteosarcoma.

Next, cell proliferation and metastasis studies were performed to explore the biological significant of IDH2 downregulation in osteosarcoma. In our study, IDH2 downregulation increased cell growth in osteosarcoma. Furthermore, it decreased the proportion of cells in the S phase and increased the proportion in the G2/M phase, suggesting a pro-progression effect. We also found that IDH2 downregulation exacerbated cell invasion in the Saos-2 and MG63 osteosarcoma cell lines. In colon cancer, IDH2 downregulation has been reported to inhibit the growth of colon carcinoma cells (45). Kim *et al* demonstrated that the reduction in IDH2 levels in malignant melanoma cells has antitumorigenic effects (13). However, our previous study showed that IDH1 upregulation inhibited cell proliferation in the 143B and MG63 osteosarcoma cell lines, whereas IDH1 downregulation exacerbated cell proliferation (12). In the present study, we found a similar biological significance of IDH1 and IDH2 downregulation in osteosarcoma cells. Moreover, this suggested that, similar to IDH1, IDH2 downregulation exacerbated cell proliferation and metastasis in osteosarcoma cells.

The levels of reactive oxygen species (ROS) were analyzed in the osteosarcoma cell lines to explore the mechanism of IDH2 downregulation in cell proliferation and metastasis. Mitochondrial NADP$^+$-dependent isocitrate dehydrogenase was reported to have an important role in cellular defense against oxidative damage by supplying NADPH, which is needed to produce glutathione (15-17). Cellular oxidative stress arising from high levels of ROS contributes to the development and progression of malignant tumors, including carcinogenesis, aberrant growth, angiogenesis and metastasis (19). Thus, mitochondrial NADP$^+$-dependent isocitrate dehydrogenase is fundamentally important for the defense against ROS, which was detected in our study. The result showed that there was no significant difference in the intracellular ROS levels between the downregulated osteosarcoma cells and non-treated cells. ROS, which were involved in the effects of IDH2 downregulation in melanoma cells, were significantly elevated (19). In addition, this surprising difference may suggest different mechanisms of IDH2 in different tumor cells.

NF-κB is constitutively activated and is implicated in cellular proliferation and invasion in osteosarcoma cells (21,23). NF-κB activation occurs via phosphorylation of IκBα and IκBβ and IκBα phosphorylation is essential for the release of active NF-κB (46). Therefore, we examined whether and how IDH2 affected NF-κB activity. Notably, IDH2 downregulation increased NF-κB degradation in osteosarcoma cells, suggesting that NF-κB may be involved in the IDH2 downregulation-induced pro-proliferation effect. IDH2 downregulation increased NF-κB activity as well as IκBα phosphorylation in the Saos-2 and MG63 cells compared to the control cells. This result is supported by the finding that increased NF-κB activity and IκBα phosphorylation can be inhibited by overexpression of mIκB (a constitutive NF-κB inhibitor). IDH2 downregulation increased the activity of

NF-κB, which was likely due to IκBα phosphorylation, and, therefore, contributed to the IDH2 downregulation-induced pro-growth function in the osteosarcoma cell lines.

In our study, we also examined the effects of IDH2 downregulation on the invasion of osteosarcoma cells. We found that IDH2 downregulation promoted the invasion of osteosarcoma cells into Matrigel. MMPs are involved in the processes of tumor cell invasion and metastasis (25) and are directly associated with the metastatic processes in osteosarcoma (30,31). We found, for the first time, that IDH2 downregulation increased MMP-9 expression at the protein level and also activated MMP-9. This is supported by the finding that increased MMP-9 expression and activation can be inhibited by siMMP-9. These findings suggest that the potential pro-metastatic activities following IDH2 downregulation could be partially interpreted by the elevated MMP-9 activity. In addition, NF-κB activity was increased following IDH2 downregulation in our study. Based on our results, IDH2 downregulation likely promoted cell invasion, at least in part, through increased activation of NF-κB and its target genes: MMPs.

The limitation of the present study is that it is a retrospective study with limited samples. In addition, the lower IDH2 expression in the higher grade osteosarcoma samples may not indicate a mutation in this gene. Furthermore, IDH2 siRNA was used only in a small number of cell lines. Further studies are needed to confirm the precise molecular regulation of IDH2 and NF-κB and their interaction to elucidate the role of IDH2 in cell growth and invasion in animal models. However, it may still be valuable to study the role of IDH2 in osteosarcoma. In addition, the pro-proliferation and pro-invasion activities as well as the potential effects of IDH2 on cell immortalization and the inflammatory response in osteosarcoma remain to be elucidated in relevant models.

In conclusion, IDH2 downregulation may indicate high pathological grade and metastasis. IDH2 downregulation induced malignant progression via increased NF-κB and MMP-9 activity in osteosarcoma *in vitro*. IDH2 may be an effective target by which to develop new therapeutic strategies against osteosarcoma.

Acknowledgements

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