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Original Article

Molecular mechanisms underlying the enhancement of carbon ion beam radiosensitivity of osteosarcoma cells by miR-29b

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Abstract: Carbon ion radiotherapy (CIRT) is more effective than conventional photon beam radiotherapy in treating osteosarcoma (OSA); however, the outcomes of CIRT alone are still unsatisfactory. In this study, we aimed to investigate whether *miR-29b* acts as a radiosensitizer for CIRT. The OSA cell lines U2OS and KHOS were treated with carbon ion beam alone, γ-ray irradiation alone, or in combination with an *miR-29b* mimic. OSA cell death as well as invasive and migratory abilities were analyzed through viability, colony formation, Transwell, and apoptosis assays. *miR-29* expression was downregulated in OSA tissues compared to that in normal tissues and was associated with metastasis and relapse in patients with OSA. Further, *miR-29b* was found to directly target the transcription factor Sp1 and suppress the activation of the phosphatase and tensin homolog (PTEN)-AKT pathway. Conversely, Sp1 was found to attenuate the inhibitory effects of *miR-29b* in OSA cells. When used in combination with *miR-29b* mimic, carbon ion beam markedly inhibited invasion, migration, and proliferation of OSA cells and promoted apoptosis by inhibiting AKT phosphorylation in a Sp1/PTEN-mediated manner. Taken together, *miR-29b* mimic improved the radiosensitivity of OSA cells via the PTEN-AKT-Sp1 signaling pathway, presenting a novel strategy for the development of carbon ion beam combination therapy.

Keywords: miR-29b, carbon ion, radiosensitivity, osteosarcoma cells, AKT, PTEN, Sp1

Introduction

Osteosarcoma (OSA) is the most common malignant primary neoplasm of the bone in children and young adults [1, 2]. As OSA cells are not readily killed by photon beams, conventional radiation therapy does not play a major role in the treatment of OSA [3, 4]. High linear transfer energy (LET) carbon ion radiotherapy (CIRT) is an innovative method in cancer treatment resulting in improved quality of life owing to its high radiocurability, which is attributable to its strong biological impact and more desirable dose distribution than that of proton beam and photon beam radiation therapies [5, 6]. The physical and biological advantages of CIRT make it ideal for targeting radioresistant tumors or tumors located near sensitive organs [7, 8]. Over the past 25 years, CIRT has been predominantly applied in the treatment of several cancers including OSA. According to our clinical data, CIRT is an effective and safe modality to treat unresectable OSA of the trunk; therefore, it offers appreciable long-term functional results and a decent survival advantage without accompanying morbidities [9-13]. We previously explored how and why CIRT alone or in combination with DNA-damaging drugs such as gemcitabine and cisplatin is more effective in killing radioresistant cancer stem cells (CSCs) than photon beam therapy [14-20]. However, limitations exist in the application of CIRT alone

or in combination with existing anticancer drugs for the treatment of more advanced and aggressive cancers [21]. As the overall survival of patients with OSA is still poor, an effective novel molecular targeted combination therapy is warranted for expanding the therapeutic effects of CIRT.

MicroRNAs (miRNAs) are endogenously expressed on coding RNA molecules that regulate gene expression negatively at the post-transcriptional level by base-pairing with the untranslated region (UTR) of the target mRNAs [22, 23]. A single miRNA binds and regulates nearly 100 different transcripts [24, 25]. Therefore, miRNAs are deemed the master regulators of gene expression, influencing majority of cell events and activities, metabolism, infection, as well as cancer [26-28]. The anti-tumor effects of miRNAs have been demonstrated by targeting the miRNA network, indicating a great therapeutic potential of miRNAs in cancer treatment [29, 30]. Thus, there is a need for developing combination therapy involving carbon ion beam and new potential molecular targeted drugs such as miRNA mimics/antagomirs. Recently, several studies have shown that *miR-29b*, a miR-29 family member, is altered in several human cancers and serves as a critical tumor suppressor [31]. The enforced expression of *miR-29b* in cancer cells impedes extracellular matrix remodeling [32], tumor-suppressor promoter methylation [30], and antiapoptotic signaling [33, 34]. In addition, the expression of *miR-29b* and *miR-29a* is downregulated via activation of survival-promoting and multiple growth signaling pathways such as the ones that involve c-myc, Hedgehog, and NF-kB [35]. *miR-29b* overexpression induce apoptosis *in vitro* and anti-tumor effects *in vivo* in acute myeloid leukemia and rhabdomyosarcoma [36-46]. We previously reported that downregulation of *miR-29c* expression resulted in enhanced expression of KLF4, a transcription factor that maintains breast CSCs, leading to the inhibition of CSC production. This finding suggested that *miR-29c* negatively regulates breast CSCs [47]. Recently, we have demonstrated that zoledronic acid (ZOL), one of the bisphosphonates, is a drug used to treat osteoporosis and bone metastasis, effectively enhanced carbon ion beam radiosensitivity accompanied with upregulation of miR-29b expression in OSA cells [48].

In this study, we aimed to elucidate the molecular mechanisms underlying miR-29b-induced carbon ion beam radiosensitization of OSA cells.

Materials and methods

Cell culture

U2OS and KHOS/NP OSA cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle medium (DMEM) [before being supplemented with fetal bovine serum (FBS; WelGene), 1% (v/v) penicillin-streptomycin, and 10% FBS (Gibco®; Thermo Fisher Scientific, Waltham, MA)] in a humidified incubator at 37°C and 5% CO_2. OSA tissues and matched non-tumor tissues were derived after obtaining informed consent from 14 patients who were operated at the Korea Institute of Radiological and Medical Sciences (Institutional Review Board Approval Number K-1603-001-001). Primary cell cultures were established from this tissue. Simply put, the tissue was finely chopped into a slurry with a blade, washed with phosphate buffered saline (PBS), and centrifuged at 1000 rpm for 3 minutes. The supernatant is then discarded and the pellet resuspended in serum-free Dulbecco-modified Eagle's medium (DMEM, WelGene, Daegu, South Korea) containing 0.05-0.1% (w/v) Type I collagenase (Gibco®, Life Technologies). After 2 h, cells were washed clean with PBS and maintained in DMEM containing 20% (v/v) FBS.

Reagents

Anti-p21 (sc-397), anti-β-actin (sc-81178), anti-Slug (sc-166476), and anti-Snail (sc-10432) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-cleaved poly-ADP ribose polymerase (PARP) (#9541), CDK6 (#3136), MCL-1 (#4572), Sp1 (#9389), PTEN (#9559) p-AKT (Ser473) (#4060), total AKT (#9272), p-4EBP1 (S65) (#9451) and p-GSK-3b (Ser9) (#9336) were purchased from Cell Signaling Technology (Danvers, MA).

Irradiation

The cells were irradiated with carbon ion beams accelerated by the heavy ion medical accelerator in Chiba at the National Institute of Radiological Sciences. The details regarding the beam characteristics of carbon ion beams, biological irradiation procedures, and dosimetry are described elsewhere [30]. Briefly, we used 290 MeV/nucleon carbon ion beams with dose

average LET of 50 KeV/μm at the center of spread-out Bragg peak. As a reference, we irradiated cells with Cs-137 γ-rays (Atomic Energy of Canada, Ltd., Ontario, Canada) or X-rays (Titan-320, GE Co., USA) at a dose rate of 2.45 or 3.81 Gy/min, respectively, at the Korea Institute of Radiological and Medical Sciences and/or NRIS. The cells were irradiated with γ-rays (2, 4, or 6 Gy) or carbon ion beams (1, 2, or 3 Gy).

miRNA and transient transfection

miR-29b mimic and control mimic were purchased from Bioneer (Daejeon, South Korea). The cells were transfected with 60 nM control or miR-29b mimic for 24 h by using G-fectin miRNA Transfection Reagent. Then, the cells were transfected with si-SP1 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Cell viability assay

The cells were seeded in 96-well plates at a density of 5000 cells/well and incubated for the time points indicated therein. To quantify cell viability, a culture medium containing an equal amount of EZ-Cytox reagent was added to the cells and incubated for 4 h. Then, the cells were treated with miR-29b mimic for 24 h and seeded and irradiated. Cell viability was measured after 48 h using a Multiskan EX instrument (Thermo Fisher Scientific) at 450 nm.

Colony formation assay

At 24 h post-transfection with miR-29b mimic, the cells were reseeded and incubated for 7-9 days. The colonies were then fixed with methanol, stained with 0.4% crystal violet (Sigma-Aldrich, St. Louis, MO), and counted.

Western blotting

OSA cells were treated with miR-29b or irradiation and incubated for 24 or 48 h. The cells were then lysed with RIPA buffer. Next, the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% (v/v) skim milk in PBS and 0.1% Tween 20 and then incubated with the indicated antibodies (1:1,000), followed by secondary antibodies (1:1,000). Finally, the blots were developed using the Pierce Enhanced Chemiluminescence Western Blotting Substrate (Thermo Fisher Scientific) and scanned.

Detection of apoptotic cells by Annexin V staining

The cells were treated with miR-29b and incubated for 48 h. Then, the cells were washed with ice-cold PBS, trypsinized, and suspended in 1 × binding buffer at a density of 1×10^6 cells/mL. Cell suspension aliquots (100 μL) were mixed with 5 μL annexin V FITC and 10 μL propidium iodide stock solution (50 μg/mL in PBS) through gentle vortexing and incubated for 15 min at room temperature. To each sample, 1 × buffer (400 μL) was added and analyzed using FACScan flow cytometer. At least 10000 cells were counted for all samples, and data analysis was performed using CellQuest software.

Transwell assays

The invasive and migratory abilities of OSA cells were determined using Transwell chambers (Millipore) according to the manufacturer's instructions. The cells were placed on the upper chamber of Transwell containing 150 μL of medium that was either untreated or treated with irradiation, miR-29b, or miR-29b along with irradiation for 24 h. The cells that migrated through the Matrigel/gelatin-coated membrane were stained using a cell stain solution provided with the Transwell chamber assay kit (Chemicon, Millipore).

DNA constructs

A DNA fragment of the Sp1 3'-UTR that contained the putative binding site of miR-29b was amplified with PCR by using the following primers: 5'-GTGTCTAGAGATTAGGGGAGGGTTGGAG-3' (forward) and 5'-GTGGAATTCGTCCAAAAGG-CATCAGGG-3' (reverse), before cloning into the pGL3luc vector. For creating the mutant reporter, four nucleotide mutations were induced in the putative miR-29b binding site using EZchange™ Multi Site-directed Mutagenesis Core Kit (Enzynomics, Daejeon, South Korea). The primer sequences used were 5'-GGT-TCTTCTGGGGacgaAATCAGGCCCCTG-3' (forward) and 5'-CAGGGGCCTGATTtcgtCCCCAGAA-GAACC-3' (reverse). The vectors were confirmed by sequencing (Bioneer).

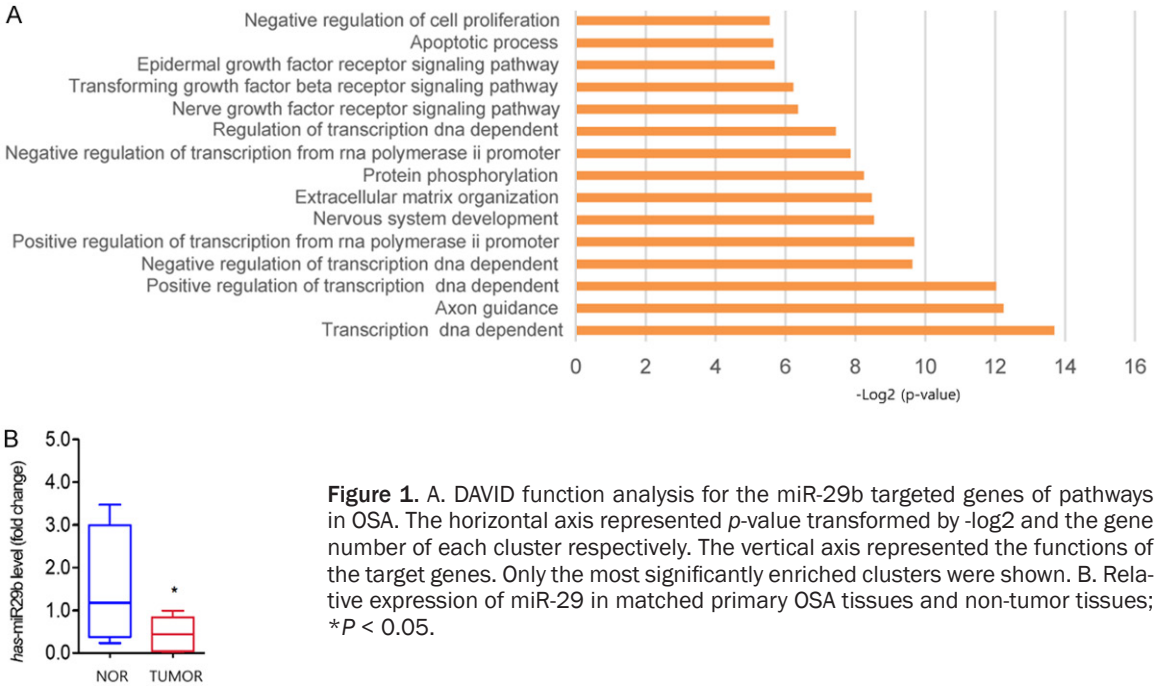


Figure 1. A. DAVID function analysis for the miR-29b targeted genes of pathways in OSA. The horizontal axis represented *p*-value transformed by -log2 and the gene number of each cluster respectively. The vertical axis represented the functions of the target genes. Only the most significantly enriched clusters were shown. B. Relative expression of miR-29 in matched primary OSA tissues and non-tumor tissues; $*P < 0.05$.

Luciferase assay

miR target prediction sites were used to predict Sp1 as a direct target gene of miR-29b. According to these prediction sites, Sp1 was included as the potential target. The cells were seeded in a 24-well plate. Then, Sp1 3'-UTR mutant plasmids or reporter constructs were co-transfected with miR-29b as well as pRL-CMV-Renilla internal control plasmid using Lipofectamine 2000. The Dual-GloTM Luciferase Assay System was used to determine the luciferase activity. The relative activities were normalized to Renilla luciferase activity.

Plasimd transient transfection

Prior to transfection, cells were plated on 6-well plates. After reaching 80% confluence, it was transfected with 2 μg of plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's procedure.

RNA extraction and quantitative reverse-transcription PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). Quantitative reverse-transcription PCR was performed using KAPA Biosystems' KAPA SYBR FAST qPCR kit according to the manufacturer's instructions.

Caspase activity assay

Caspase activity was measured using the caspase family activity assay kits, according to the manufacturer's instructions.

Statistical analysis

Experiments involving cell culture were performed in triplicate. The data were expressed as values of mean ± standard deviation. The statistical differences between different groups were analyzed using ANOVA and Student's *t*-test (two-tailed). The following *P*-values were considered significant: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Results

miR-29b expression is downregulated in OS specimens and cell lines

To understand the signatures of differentially expressed genes (DEGs), we performed *miR-29b* gene ontology analysis. Accordingly, these DEGs were significantly enriched in the processes of cell death and negative regulation of cell proliferation (**Figure 1A**). qRT-PCR analysis revealed that miR-29b expression in primary tumor tissues was significantly enhanced when compared to that in matched non-tumor tissues (IRB: No. K-1603-001-001) (**Figure 1B**).

miR-29b inhibits OSA cell proliferation, survival, migration, and invasion via Sp1 suppression

miR-29b was transfected into KHOS and U2OS cells, which was confirmed by qRT-PCR analysis after 24 h (**Figure 2A**). To determine whether *miR-29b* suppresses the oncogenic phenotype of OSA cells, we performed gain-of-function assays in OSA cells using *miR-29*b mimics. As shown in **Figure 2B**, *miR-29* mimic significantly inhibited OSA cell growth from 48 h after transfection. We also found that *miR-29b* mimic significantly inhibited colony formation and cell proliferation in KHOS and U2OS cells (**Figure 2C**, **2D**). Interestingly, growth inhibition was associated to upregulation of the cell-cycle inhibitor p21 and downregulation of pro-survival factors CDK-6 and MCL-1, both observed 24 h after cell transfection with miR-29b. Importantly, miR-29b-induced apoptosis was found to be associated with PARP activation, as evidenced by increased cleavage using Western blotting (**Figure 2E**). And transfection after 48 h resulted in a substantial accumulation of cell populations in G1 and a decrease in cell cycle S phase populations (**Figure 2F**). Furthermore, we found that *miR-29b* mimic suppressed the migratory and invasive ability of KHOS and U2OS cells (**Figure 2G**). Data regarding the circRNA/miRNA/mRNA interaction network of *miR-29b*, obtained using Cytoscape, were integrated to construct a cancer-related signaling network that includes *miR-29b* target genes. Most target genes in the network were considered closely associated with cell proliferation and tumor growth (**Figure 2H**). Thus, these findings indicate that *miR-29b* significantly inhibits proliferation, survival, migration, and invasion of OSA cells.

Sp1 is a direct target of miR-29 and miR-29 suppresses OSA growth and invasion through Sp1

The predicted binding site of *miR-29b* was identified in the 3'-UTR of *Sp1* mRNA. Therefore, we first constructed luciferase reporter plasmids containing the wild-type or mutant *miR-29b* target site in the *Sp1* 3'-UTR sequence (**Figure 3A**). KHOS and U2OS cells were co-transfected with *miR-29b* expression and reporter plasmids. *miR-29b* expression significantly decreased firefly luciferase activity but not mutant reporter activity, in the presence of wild-type 3'-UTR (**Figure 3B**). Furthermore, *miR-29b* overexpression for 48 h significantly decreased mRNA and protein levels of Sp1 compared with those in the control group (**Figure 3C**, **3D**), indicating that *miR-29b* suppressed *Sp1* expression by binding to its target site at the 3'-UTR of *Sp1*. We also found that *Sp1* overexpression was associated with reduced *miR-29b* levels (**Figure 3E**). And Sp1 silencing led to inhibition of OSA cell growth and cell proliferation, and Sp1 overexpression showed the opposite result (**Figure 3F-H**). These results demonstrate that *miR-29* suppressed the growth of OSA cells by directly downregulating *Sp1*. Thus, our findings show that *miR-29* forms an auto-regulatory loop with Sp1 to regulate OSA cell aggressiveness.

miR-29b activates the PTEN/AKT pathway by suppressing the Sp1 loop in OSA cells

miR-29b inhibits proliferation, migration, and invasion of tongue tumor cells via phosphatase and tensin homolog (PTEN)-AKT-SP1 pathway [30]. To examine whether *miR-29b* acts as a potential tumor suppressor in OSA cells, we performed pathway analysis of PTEN-AKT–SP1 and found that *miR-29b* significantly increased PTEN protein levels and downregulated phosphorylated AKT expression without affecting the total AKT levels (**Figure 4A**). We next transfected KHOS and U2OS cells with *miR-29b* expression plasmid and a luciferase reporter containing the wild-type or mutant *PTEN* promoter. A marked increase in the luciferase/*Renilla* ratio was found in cells transfected with the wild-type *PTEN* promoter reporter but not in those with the mutant reporter (**Figure 4B**). To ascertain whether *miR-29b* upregulates *PTEN* expression by targeting Sp1, we determined whether knockdown of endogenous Sp1 activity mimics the effects of exogenous *miR-29b* overexpression. There was a marked increase in the luciferase/renilla ratio in cells transfected with the wild-type PTEN promoter reporter, but not with the mutant reporter (**Figure 4C**). As expected, siRNA-mediated *Sp1* knockdown in OSA cells increased *PTEN* expression and consequently decreased AKT phosphorylation (**Figure 4D**). In addition, PI3K inhibition by LY294002 increased miR-29b and decreased Sp1 and p-AKT (S473) protein levels, so miR-29b was negatively regulated by PI3K/AKT (**Figure 4E**). Overexpression of AKT suppressed the miR-29b-dependent decrease in Sp1 mRNA levels (**Figure 4F**). Transfection of the synthetic

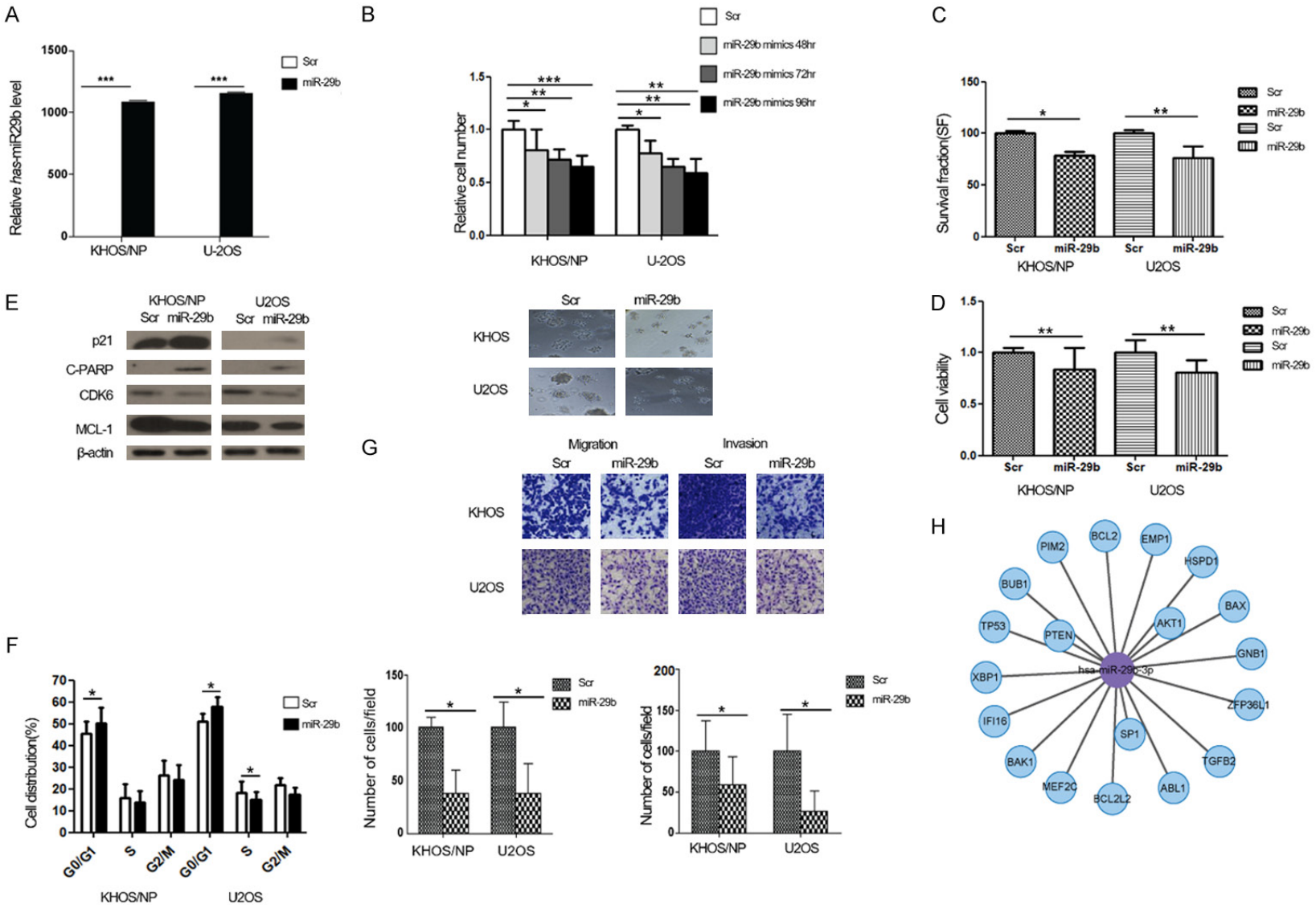


Figure 2. *miR-29b* inhibits OSA cell proliferation, survival, migration, and invasion. (A) qPCR analysis showing relative *miR29b* expression; ***$P < 0.001$. (B) Trypan blue assay of two OSA cell lines transfected with *miR-29b* or scrambled oligonucleotides (Scr); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (C, D) Colony formation assay (upper) and MTT assay (lower) using the two OSA cell lines after transfection with synthetic *miR-29b* or Scr; *$P < 0.05$, **$P < 0.01$. (E) Immunoblotting using the

indicated antibodies 48 h after transfecting the two OSA cell lines with synthetic *miR-29b* or Scr. (F) Inhibition of cell cycle progression by overexpression of miR-29b. Two OSA cell lines were transfected as in (A). Cells were stained with propidium iodide (PI) at 48 h post-transfection and analyzed with FACS. **P* < 0.05, (G) Migration and invasion of OSA cells decreased with *miR-29b* overexpression. Photo micrographs of Transwell migration and invasion of OSA cells transfected for 24 h; **P* < 0.05. (H) Establishment of a circRNA/miRNA/mRNA interaction network of pathways in cancer.

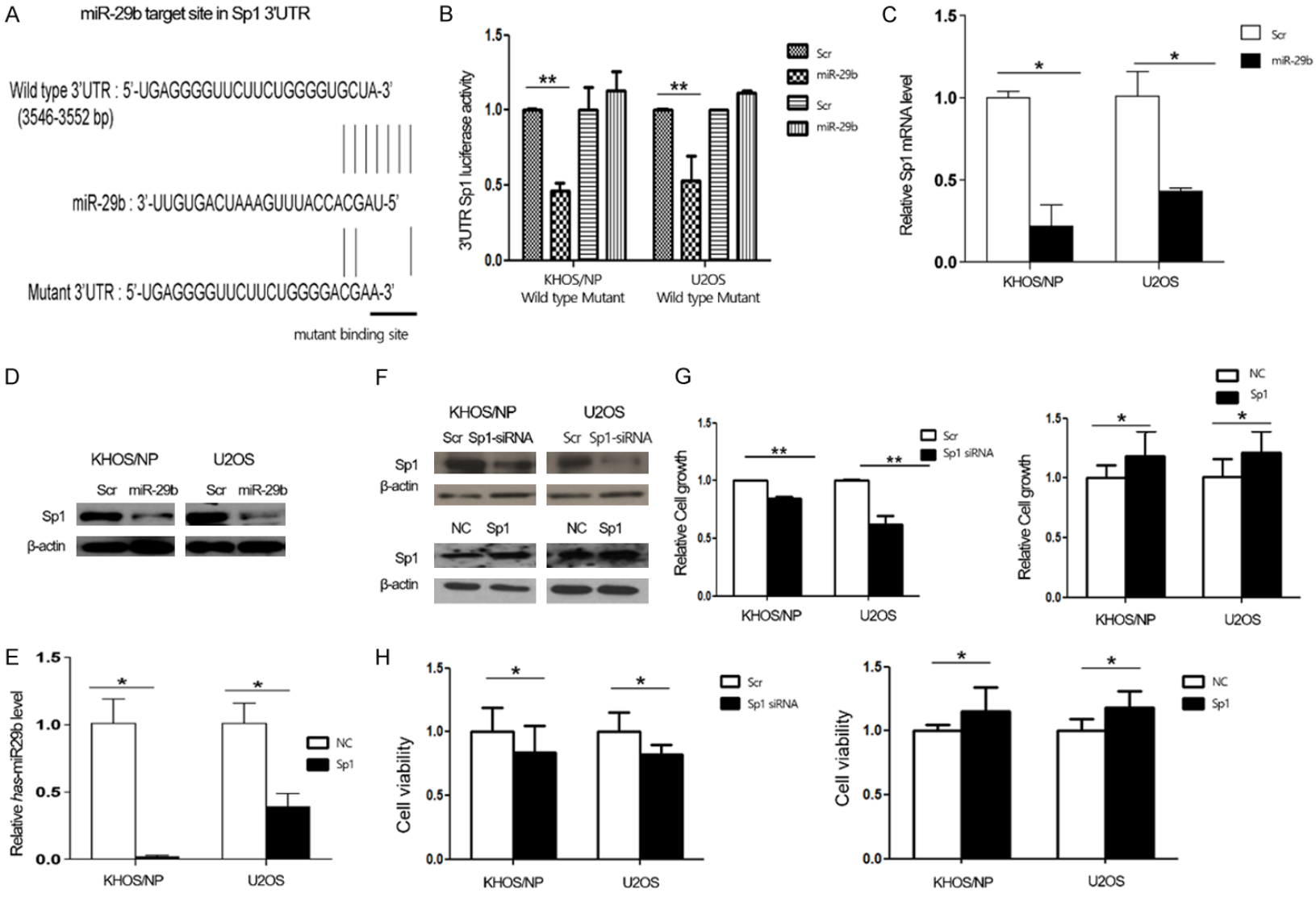


Figure 3. *miR-29b* inhibits Sp1 expression. A. Wild-type AKT (WT) and mutant (MUT) forms of putative miR-29 target sequences of the *Sp1* 3'-UTR. B. Analysis of the luciferase activity of psicheck-2-*Sp1* 3'-UTR WT and MUT vectors in OSA cells; $^{**}P < 0.01$. C. *Sp1* mRNA levels in the indicated cells were analyzed by qRT-PCR; $^{*}P < 0.05$. D. Sp1 protein levels in the indicated cells were examined by western blotting. E. *miR-29b* levels were analyzed by qRT-PCR in OSA cells transfected with *Sp1*; $^{*}P < 0.05$. F. Sp1 protein levels were detected by western blotting analysis after transfection of KHOS and U2OS cells with *si-Sp1 and Sp1*. G, H. Trypan blue assay and MTT assay were performed using KHOS and U2OS cells transfected with *si-Sp1 and Sp1*; $^{*}P < 0.05$.

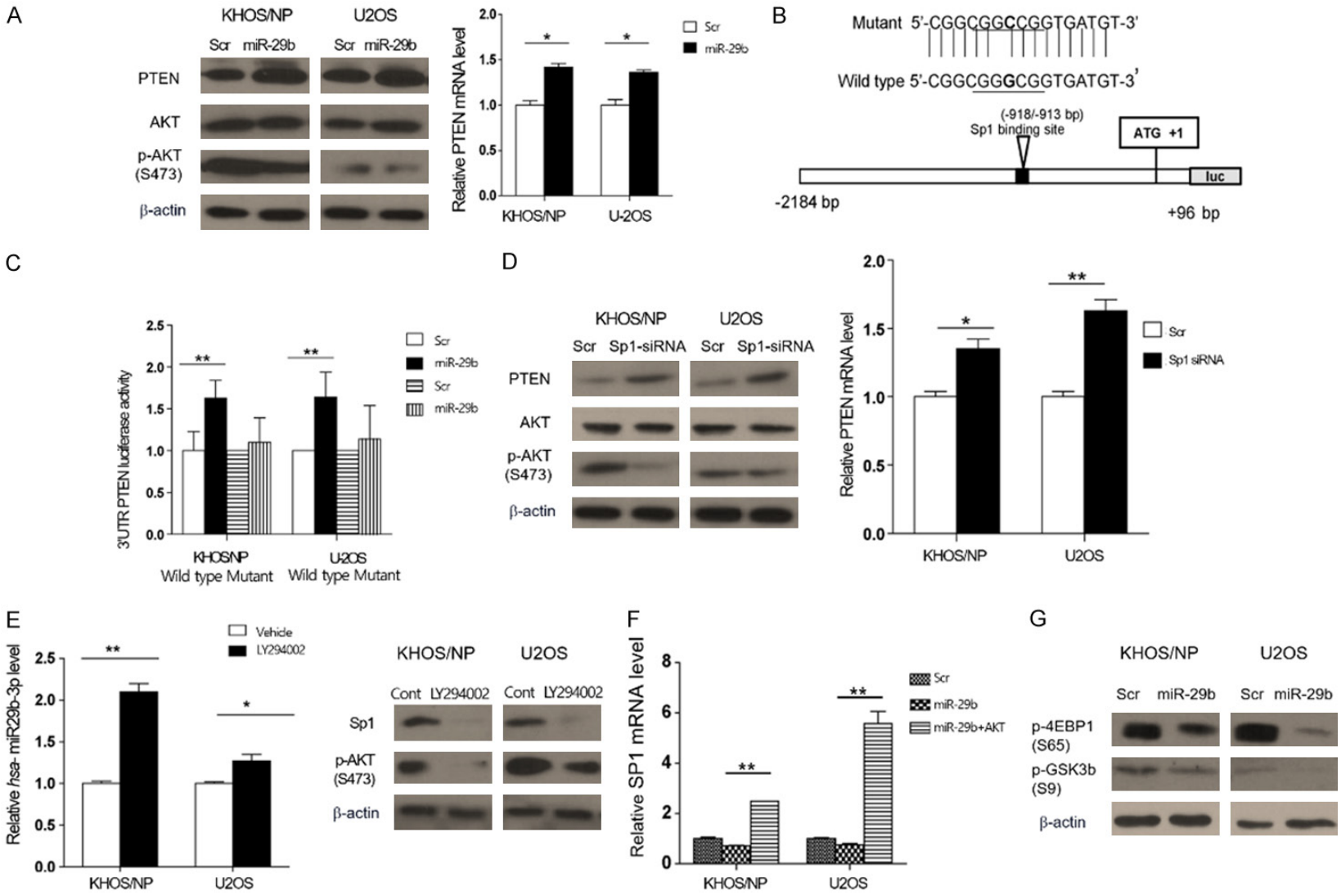


Figure 4. *miR-29b* activates the PTEN/AKT pathway via Sp1 suppression. A. PTEN, p-AKT, and AKT protein levels were analyzed by western blotting of two OSA cell lines transfected with *miR-29b*. *PTEN* gene expression in Scr- and miR-29b-transfected cells by qRT-PCR; $*P < 0.05$. B. The Sp1 binding site (middle) on the PTEN promoter (lower). Mutation of Sp1 binding site (upper). C. Relative luciferase activity in control or *miR-29b*-expressing OSA cell lines co-transfected with a wild-type or mutant *PTEN* promoter; $**P < 0.01$. D. PTEN, p-AKT, and AKT protein levels were analyzed by western blotting of two OSA cell lines transfected with si-*Sp1* RNA. PTEN gene expression in Scr- and si-Sp1-transfected cells by qRT-PCR; $*P < 0.05$; $**P < 0.01$. E. qRT-PCR of *miR-29b* in OSA cell lines treated with 20 mM LY294002 or vehicle (DMSO) for 48 h. Immunoblotting shows the protein levels of Sp1 and phosphorylated AKT at S473 in LY294002-treated cells; $*P < 0.05$, $**P < 0.01$. F. Cells were transfected with pcDNA.3.1-HA-myr-AKT dominant active construct or the empty vector pcDNA3.1 and analyzed after 48 h for Sp1 expression levels by qRT-PCR; $**P < 0.01$. G. The indicated antibodies were used for western blotting of OSA cells transfected with *miR-29b*.

miR-29b mimic also resulted in reduced phosphorylation of the AKT substrate glycogen synthase kinase-3b (p-GSK-3b (S9)) (**Figure 4G**). Moreover, we provided preliminary evidence that transfection of cells with *miR-29b* mimics inhibits mTOR-mediated signals, as evidenced by the reduced p-4EBP1 (S65) (**Figure 4G**). These findings demonstrate that *miR-29b* acts as a negative regulator of the PI3K/AKT pathway.

Carbon ion beam irradiation in combination with miR-29b mimic decreases OSA cell viability and increases apoptosis induction

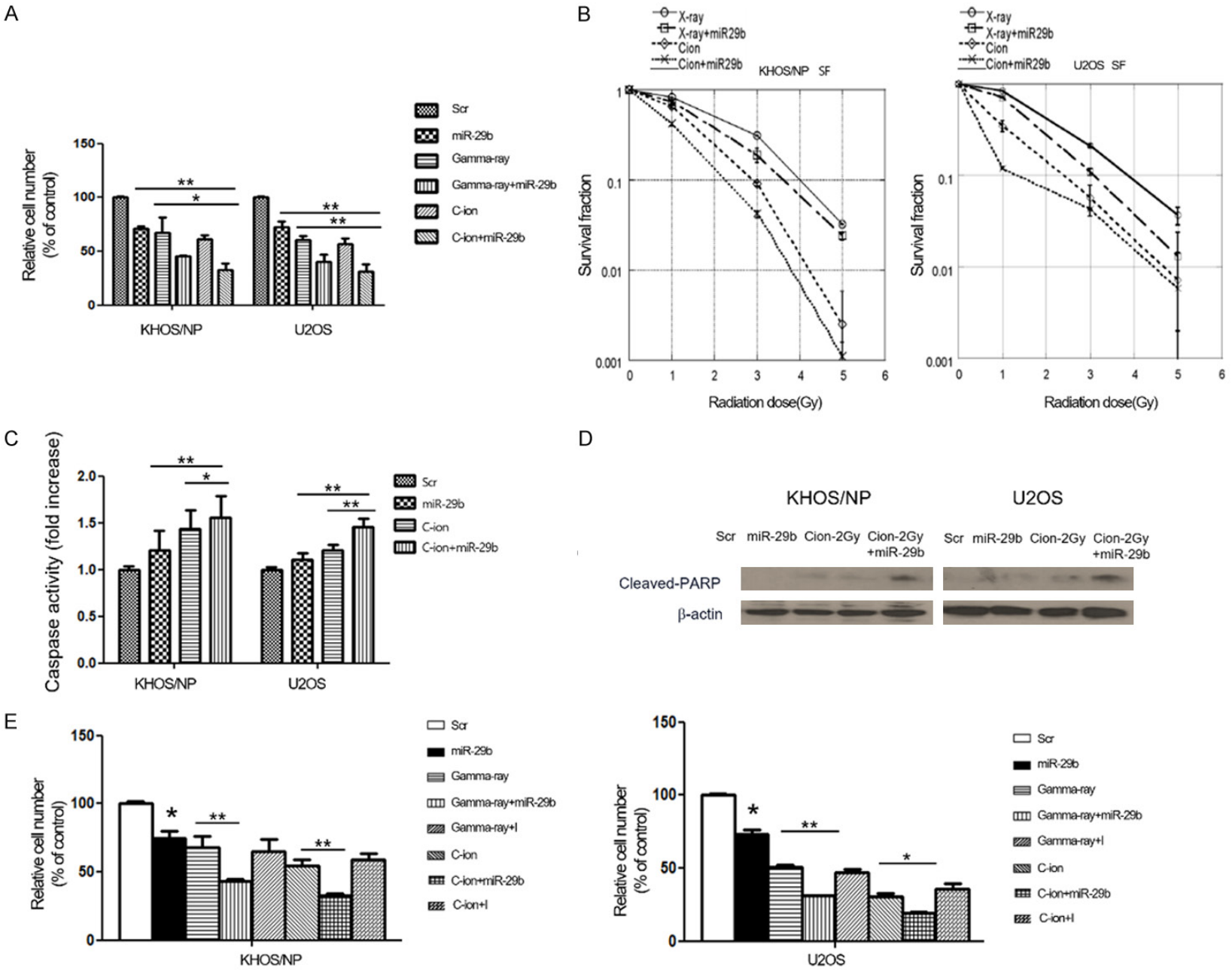
To evaluate the effects of *miR-29b* on the radiosensitivity of OSA cells, cell viability assays were performed as mentioned in Methods. OSA cells were first treated with carbon ion beam alone, γ-ray alone, or in combination with *miR-29b* pretreatment for 24 h. *miR-29b* pretreatment significantly reduced the cell growth of OSA cells following 72 h of irradiation (**Figure 5A**), indicating that *miR-29b* markedly enhances the radiosensitivity of OSA cells. In addition, carbon-ion beam + *miR-29b* mimic reduced the survival fraction of OSA cells significantly more than carbon ion beam irradiation alone (**Figure 5B**). We also found that apoptotic events induced by combination treatment of carbon ion beam irradiation and *miR-29b* mimic were associated with increased caspase-3 activation and activated PARP (**Figure 5C**, **5D**). Consistent with our assumption, antagomiR-29b-expressing cells showed proliferative characteristics and were less susceptible to carbon ion beam-induced growth inhibition (**Figure 5E**).

Carbon ion beam irradiation combined with the miR-29b mimic enhances radiosensitivity via Sp1 suppression in OSA cells

To investigate the molecular mechanism underlying the enhanced carbon ion radiosensitivity of OSA cells after treatment with the *miR-29b* mimic, we examined the changes in *Sp1* mRNA and protein levels in OSA cells. Compared with γ-ray irradiation, carbon ion beam irradiation dramatically downregulated Sp1 expression in KHOS cells and U2OS cells (**Figure 6A**). In addition, carbon ion beam irradiation significantly upregulated *miR-29b* expression compared with γ-ray irradiation in both KHOS and U2OS cells (**Figure 6B**). As shown in **Figure 6C**, *miR-29b* overexpression along with siSP1 treatment caused a significant inhibition in cell growth and invasive ability of both OSA cell lines starting at 48 h after transfection (**Figure 6D**). Western blotting results further confirmed that transcription factors associated with epithelial-mesenchymal transition proteins were dramatically decreased in carbon ion beam + *miR-29b*-treated cells (**Figure 6E**). Next, we examined the effect of mithramycin A, a Sp1-specific inhibitor, on the carbon ion radiosensitivity of OSA cells (**Figure 6F**). Mithramycin A significantly reduced the growth of OSA cells after a carbon ion beam. Furthermore, carbon ion irradiation showed a significant inhibition of cell viability than gamma-ray irradiation. These findings indicate that Sp1 is essential for the radioresistance of OSA cells and that decreased Sp1 expression may be involved in *miR-29b*-induced carbon ion radiosensitivity.

Discussion

In this study, we aimed to elucidate the molecular mechanism underlying miR-29b-induced carbon ion beam radiosensitization of OSA cells. We found that carbon ion beam irradiation combined with *miR-29b* mimic significantly increased caspase-3 activation compared with that of carbon ion beam irradiation alone, suggesting that the enhanced radiosensitization of *miR-29b* may be partially due to an increased induction of apoptosis. Regarding the molecular mechanisms underlying the radiosensitizing


A
Relative cell number (% of control)
150
100
50
0
KHOS/NP
U2OS
Scr
miR-29b
Gamma-ray
Gamma-ray+miR-29b
C-ion
C-ion+miR-29b
B
X-ray
X-ray+miR29b
Cion
Cion+miR29b
KHOS/NP SF
U2OS SF
Survival fraction
1
0.1
0.01
0.001
0 1 2 3 4 5 6
Radiation dose(Gy)
C
Caspase activity (fold increase)
2.0
1.5
1.0
0.5
0.0
KHOS/NP
U2OS
Scr
miR-29b
C-ion
C-ion+miR-29b
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KHOS/NP
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Scr miR-29b Cion-2Gy Cion-2Gy +miR-29b
Cleaved-PARP
β-actin
E
Relative cell number (% of control)
150
100
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KHOS/NP
U2OS
Scr
miR-29b
Gamma-ray
Gamma-ray+miR-29b
Gamma-ray+I
C-ion
C-ion+miR-29b
C-ion+I

Figure 5. Carbon ion beam irradiation combined with *miR-29b* mimic decreased OSA cell survival and enhanced apoptosis induction. A. Trypan blue assay of OSA cells after transfection with synthetic *miR-29b* or carbon ion or X-ray irradiation + *miR-29b* mimic combination treatment; $*P < 0.05$, $**P < 0.01$. B. Colony formation assay of OSA cells after transfection with synthetic *miR-29b* or carbon ion or X-ray irradiation + *miR-29b* mimic combination treatment. C. Analysis of caspase activity in both OSA cell lines 48 h after carbon ion beam treatment alone or in combination with *miR-29b*. Data were collected using Multiskan EX at 405 nm (upper); $*P < 0.05$, $**P < 0.01$. D. Cleaved-PARP protein level was analyzed by western blotting of two OSA cell lines with the indicated treatment (lower). E. Two OSA cells were transduced with the empty vector or *miR-29b* mimic or antagomiR-29b and then treated with irradiation for 48 h. Viable cells were determined using trypan blue assay; $**P < 0.01$.

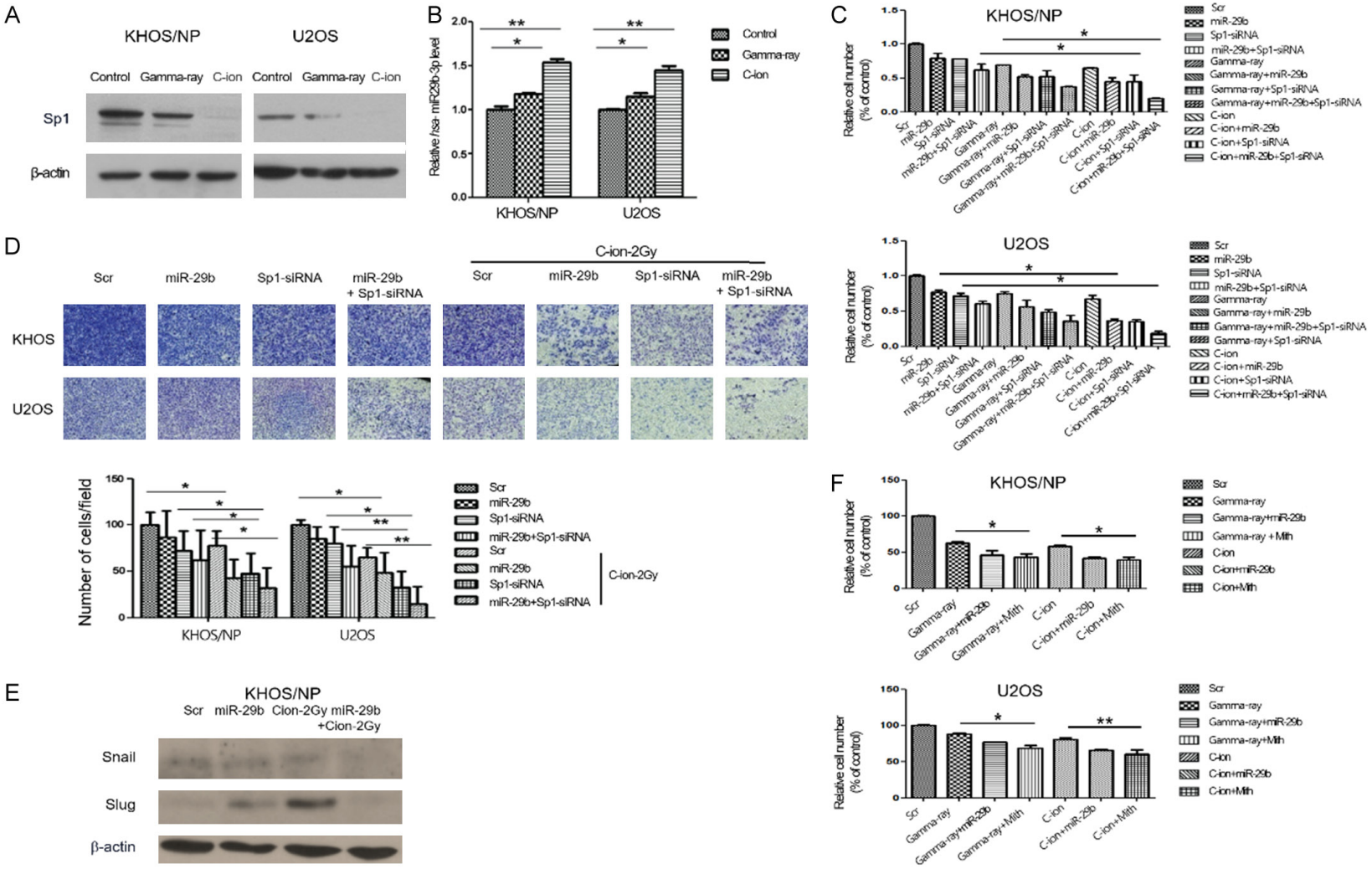

Figure 6. Carbon ion beam combined with *miR-29b* mimic enhanced radiosensitivity in OSA cells via Sp1 suppression. A. Immunoblotting using the indicated antibodies for both OSA cell lines treated for 24 h with each radiation. B. *miR-29b* levels after γ-ray or carbon ion beam irradiation were analyzed by qRT-PCR; $*P < 0.05$, $**P < 0.01$. C. Trypan blue assay of both OSA cell lines 48 h after treatment with the indicated treatment ; $*P < 0.05$. D. Transwell migration and invasion assays were performed using the two OSA cell lines transfected with *miR-29b* or si*SP1*; $*P < 0.05$, $**P < 0.01$. E. Immunoblotting of the indicated antibodies 48 h after synthetic *miR-29b* transfection of KHOS/NP cells combined with carbon ion beam treatment. F. Trypan blue assay of miR-29b or carbon ion or Gamma-ray or irradiation + *miR-29b* mimic combination treatment in both OSA cells treated with mithramycin-A (100 nmol/L) or vehicle (DMSO) for 15 h; $**P < 0.01$ Mith decreased the cell number of OSA cells treated with Carbon ion; $*P < 0.05$, $**P < 0.01$.

effects of *miR-29b*, the *miR-29b*-Sp1 loop has been shown to play a major role in this process.

First, we analyzed *miR-29b* expression in primary OSA and non-tumors from 14 patients and found that *miR-29b* expression was lower in tumor tissues than in the corresponding non-tumor tissues. Our recent study involving miRNA microarray profiling from four paired samples of OSA versus matched nonmalignant tissues also showed the same results. This finding is also consistent with previous reports demonstrating that *miR-29b* expression is decreased in various tumors, such as multiple myeloma, lung tumors, and gastric cancer, and acts as a tumor suppressor gene [28, 35, 38-46]. Then, we confirmed that treatment with *miR-29b* mimic alone significantly inhibited cell proliferation and survival and suppressed cell migration and invasion, accompanied by enhanced p21 expression and decreased CDK6 and MCL1 expression in KHOS and U2OS cells. This finding is consistent with previous reports demonstrating that *miR-29b* acts as a tumor suppressor in various cancers, such as prostate, breast, glioblastoma, and lung cancers [40-43]. Moreover, the *miR-29b* mimic significantly reduced Sp1 expression by binding to its target site at the 3'-UTR of *Sp1*. In contrast, *Sp1* overexpression reduced *miR-29b* expression, whereas silencing of *Sp1* increased *miR-29b* levels, consequently suppressing the proliferation, migration, and invasion of OSA cells. These results demonstrate that *miR-29b* inhibits the growth and invasion of OSA cells, at least in part, by downregulating *Sp1*. Therefore, we speculate that a *miR-29b*-Sp1 regulatory feedback loop occurs in OSA cells and radiation-treated OSA cells, and it could be a potential therapeutic target. This finding is in agreement with previous findings where *miR-29b* was found to be negatively correlated with Sp1 expression [44-46].

In the present study, *miR-29b* mimic significantly increased PTEN expression and reduced phosphorylated AKT levels without affecting total AKT levels. *Sp1* knockdown increased *PTEN* expression and subsequently decreased AKT phosphorylation in OSA cells. Moreover, inhibition of PI3K by LY294002 decreased Sp1 protein expression and increased *miR-29b* levels as further proof for the negative regulation exerted by PI3K/AKT on *miR-29b* expression. These findings demonstrate that *miR-29b* acts as a negative regulator of the PI3K/AKT pathway. *miR-29b* inhibits AKT phosphorylation by suppressing PI3K and increasing *PTEN* expression [42, 43]. Carbon ion beam irradiation effectively inhibited Sp1 expression. Carbon ion beam irradiation in combination with miR-29b mimic showed stronger cell killing effects and suppression of OSA cell migration and invasion than γ-ray irradiation. These results present novel evidence on the major molecular mechanisms underlying the enhancement of carbon ion beam radiosensitivity of OSA cells by *miR-29b*.

In summary, carbon ion beam irradiation in combination with *miR-29b* mimic efficiently suppressed the invasion, migration, and proliferation of OSA cells and promoted apoptosis by inhibiting AKT phosphorylation via Sp1/PTEN-AKT pathway (**Figure 7**). The *miR-29b* mimic enhanced radiosensitivity of OSA cells to carbon ion beam irradiation via PTEN-AKT-Sp1 pathway, suggesting a strategy for the development of novel combination CIRT.

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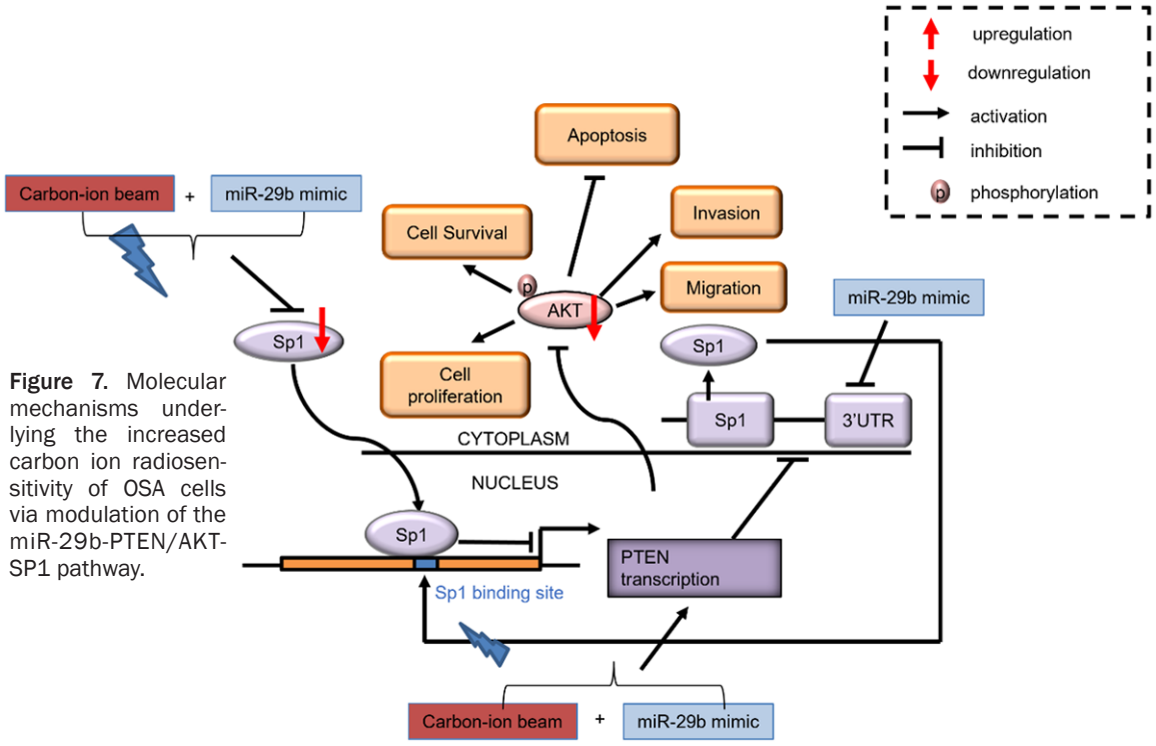

Figure 7. Molecular mechanisms underlying the increased carbon ion radiosensitivity of OSA cells via modulation of the miR-29b-PTEN/AKT-SP1 pathway.

Disclosure of conflict of interest

None.

Abbreviations

CIRT, carbon-ion radiotherapy; CSC, cancer stem cell; LET, linear transfer energy; DMEM, *Dulbecco's modified Eagle's medium*; FBS, fetal bovine serum; GSK-3b, glycogen synthase kinase-3b; HIMAC, heavy ion medical accelerator in Chiba; KIRAMS, Korea Institute of Radiological and Medical Sciences; miRNAs, microRNAs; NIRS, National Institute of Radiological Sciences; OSA, *Osteosarcoma*; QST, National Institutes for Quantum and Radiological Science and Technology; UTR, untranslated region; PTEN, phosphatase and tensin homolog.

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References

[1] Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, Zoubek A, Jürgens H and Winkler K. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 2002; 20: 776-790.

[2] Jaffe N, Puri A and Gelderblom H. Osteosarcoma: evolution of treatment paradigms. Sarcoma 2013; 203531.

[3] Schwarz R, Bruland O, Cassoni A, Schomberg P and Bielack S. The role of radiotherapy in osteosarcoma. Cancer Treat Res 2009; 152: 147-164.

[4] DeLaney TF, Park L, Goldberg SI, Hug EB, Liebsch NJ, Munzenrider JE and Suit HD. Radiotherapy for local control of osteosarcoma. Int J Radiat Oncol Biol Phys 2005; 61: 492-498.

[5] Kanai T, Endo M, Minohara S, Miyahara N, Koyama-ito H, Tomura H, Matsufuji N, Futami Y, Fukumura A, Hiraoka T, Furusawa Y, Ando K, Suzuki M, Soga F and Kawachi K. Biophysical characteristics of HIMAC clinical irradiation system for heavy-ion radiation therapy. Int J Radiat Oncol Biol Phys 1999; 44: 201-210.

[6] Suzuki M, Kase Y, Yamaguchi H, Kanai T and Ando K. Relative biological effectiveness for cell-killing effect on various human cell lines irradiated with heavy-ion medical accelerator in Chiba (HIMAC) carbon-ion beams. Int J Radiat Oncol Biol Phys 2000; 48: 241-250.
[7] Kamada T, Tsujii H, Blakely EA, Debus J, De Neve W, Durante M, Jäkel O, Mayer R, Orecchia R, Pötter R, Vatnitsky S and Chu WT. Carbon ion radiotherapy in Japan: an assessment of 20 years of clinical experience. Lancet Oncol 2015; 16: e93-e100.
[8] Ebner DK and Kamada T. The emerging role of carbon-ion radiotherapy. Front Oncol 2016; 6: 140.
[9] Mohamad O, Imai R, Kamada T, Nitta Y and Araki N; Working Group for Bone and Soft Tissue Sarcoma. Carbon ion radiotherapy for inoperable pediatric osteosarcoma. Oncotarget 2018; 9: 22976-22985.
[10] Demizu Y, Jin D, Sulaiman NS, Nagano F, Terashima K, Tokumaru S, Akagi T, Fujii O, Daimon T, Sasaki R, Fuwa N and Okimoto T. Particle therapy using protons or carbon ions for unresectable or incompletely resected bone and soft tissue sarcomas of the pelvis. Int J Radiat Oncol Biol Phys 2017; 98: 367-374.
[11] Imai R, Kamada T, Tsuji H, Tsujii H, Tsuburai Y and Tatezaki S; Working Group for Bone and Soft Tissue Sarcomas. Cervical spine osteosarcoma treated with carbon-ion radiotherapy. Lancet Oncol 2006; 7: 1034-1035.
[12] Matsunobu A, Imai R, Kamada T, Imaizumi T, Tsuji H, Tsujii H, Shioyama Y, Honda H and Tatezaki S; Working Group for Bone and Soft Tissue Sarcomas. Impact of carbon ion radiotherapy for unresectable osteosarcoma of the trunk. Cancer 2012; 118: 4555-4563.
[13] Sugahara S, Kamada T, Imai R, Tsuji H, Kameda N, Okada T, Tsujii H and Tatezaki S; Working Group for the Bone and Soft Tissue Sarcomas. Carbon ion radiotherapy for localized primary sarcoma of the extremities: results of a phase I/II trial. Radiother Oncol 2012; 105: 226-231.
[14] Matoulkova E, Michalova E, Vojtesek B and Hrstka R. The role of the 3' untranslated region in post-transcriptional regulation of protein expression in mammalian cells. RNA Biol 2012; 9: 563-576.
[15] Cui X, Oonishi K, Tsujii H, Yasuda T, Matsumoto Y, Furusawa Y, Akashi M, Kamada T and Okayasu R. Effects of carbon ion beam on putative colon cancer stem cells and its comparison with X-rays. Cancer Res 2011; 71: 3676-3687.
[16] Oonishi K, Cui X, Hirakawa H, Fujimori A, Kamijo T, Yamada S, Yokosuka O and Kamada T. Different effects of carbon ion beams and X-rays on clonogenic survival and DNA repair in human pancreatic cancer stem-like cells. Radiother Oncol 2012; 105: 258-265.
[17] Sai S, Wakai T, Vares G, Yamada S, Kamijo T, Kamada T and Shirai T. Combination of carbon ion beam and gemcitabine causes irreparable DNA damage and death of radioresistant pancreatic cancer stem-like cells in vitro and in vivo. Oncotarget 2015; 6: 5517-5535.
[18] Sai S, Vares G, Kim EH, Karasawa K, Wang B, Nenoi M, Horimoto Y and Hayashi M. Carbon ion beam combined with cisplatin effectively disrupts triple negative breast cancer stem-like cells in vitro. Mol Cancer 2015; 14: 166.
[19] Sai S, Suzuki M, Kim EH, Hayashi M, Vares G, Yamamoto N and Miyamoto T. Effects of carbon ion beam alone or in combination with cisplatin on malignant mesothelioma cells in vitro. Oncotarget 2018; 9: 14849-14861.
[20] Kim EH, Kim MS, Furusawa Y, Uzawa A, Han S, Jung WG and Sai S. Metformin enhances the radiosensitivity of human liver cancer cells to gamma-rays and carbon ion beams. Oncotarget 2016; 7: 80568-80578.
[21] Tsuboi K. Advantages and limitations in the use of combination therapies with charged particle radiation therapy. Int J Part Ther 2018; 5: 122-132.
[22] Majoros WH and Ohler U. Spatial preferences of microRNA targets in 3' untranslated regions. BMC Genomics 2007; 8: 152.
[23] Rodriguez A, Griffiths-Jones S, Ashurst JL and Bradley A. Identification of mammalian microRNA host genes and transcription units. Genome Res 2004; 14: 1902-1910.
[24] Cullen BR. Transcription and processing of human microRNA precursors. Mol Cell 2004; 16: 861-865.
[25] Takahashi RU, Prieto-Vila M, Kohama I and Ochiya T. Development of miRNA-based therapeutic approaches for cancer patients. Cancer Sci 2019; 110: 1140-1147.
[26] Miao J, Wu S, Peng Z, Tania M and Zhang C. MicroRNAs in osteosarcoma: diagnostic and therapeutic aspects. Tumour Biol 2013; 34: 2093-2098.
[27] Kong YW, Ferland-McCollough D, Jackson TJ and Bushell M. MicroRNAs in cancer management. Lancet Oncol 2012; 13: e249-e258.
[28] Varshney J and Subramanian S. MicroRNAs as potential target in human bone and soft tissue sarcoma therapeutics. Front Mol Biosci 2015; 2: 31.
[29] Hayes J, Peruzzi PP and Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol Med 2014; 20: 460-469.
[30] Jia LF, Huang YP, Zheng YF, Lyu MY, Wei SB, Meng Z and Gan YH. miR-29b suppresses proliferation, migration, and invasion of tongue squamous cell carcinoma through PTEN-AKT signaling pathway by targeting Sp1. Oral Oncol 2014; 50: 1062-1071.

[31] Yan B, Guo Q, Fu FJ, Wang Z, Yin Z, Wei YB and Yang JR. The role of miR-29b in cancer: regulation, function, and signaling. Onco Targets Ther 2015; 8: 539-548.
[32] Mott JL, Kurita S, Cazanave SC, Bronk SF, Werneburg NW and Fernandez-Zapico ME. Transcriptional suppression of mir-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-kappaB. J Cell Biochem 2010; 110: 1155-1164.
[33] Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CE, Callegari E, Schwind S, Pang J, Yu J, Muthusamy N, Havelange V, Volinia S, Blum W, Rush LJ, Perrotti D, Andreeff M, Bloomfield CD, Byrd JC, Chan K, Wu LC, Croce CM and Marcucci G. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood 2009; 113: 6411-6418.
[34] Amodio N, Di Martino MT, Foresta U, Leone E, Lionetti M, Leotta M, Gullà AM, Pitari MR, Conforti F, Rossi M, Agosti V, Fulciniti M, Misso G, Morabito F, Ferrarini M, Neri A, Caraglia M, Munshi NC, Anderson KC, Tagliaferri P and Tassone P. miR-29b sensitizes multiple myeloma cells to bortezomib-induced apoptosis through the activation of a feedback loop with the transcription factor Sp1. Cell Death Dis 2012; 3: e436.
[35] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
[36] Liu S, Wu LC, Pang J, Santhanam R, Schwind S, Wu YZ, Hickey CJ, Yu J, Becker H, Maharry K, Radmacher MD, Li C, Whitman SP, Mishra A, Stauffer N, Eiring AM, Briesewitz R, Baiocchi RA, Chan KK, Paschka P, Caligiuri MA, Byrd JC, Croce CM, Bloomfield CD, Perrotti D, Garzon R and Marcucci G. Sp1/NFkappaB/HDAC/miR-29b regulatory network in KIT-driven myeloid leukemia. Cancer Cell 2010; 17: 333-347.
[37] Zhang K, Zhang C, Liu L and Zhou J. A key role of microRNA-29b in suppression of osteosarcoma cell proliferation and migration via modulation of VEGF. Int J Clin Exp Pathol 2014; 7: 5701-5708.
[38] Jones KB, Salah Z, Del Mare S, Galasso M, Gaudio E, Nuovo GJ, Lovat F, LeBlanc K, Palatini J, Randall RL, Volinia S, Stein GS, Croce CM, Lian JB and Aqeilan RI. miRNA signatures associate with pathogenesis and progression of osteosarcoma. Cancer Res 2012; 72: 1865-1877.
[39] Wang H, Tang M, Ou L, Hou M, Feng T, Huang YE, Jin Y, Zhang H and Zuo G. Biological analysis of cancer specific microRNAs on function modeling in osteosarcoma [sci rep: 5382]. Sci Rep 2017; 7: 5382.
[40] Zhang T, Xue X and Peng H. Therapeutic delivery of miR-29b enhances radiosensitivity in cervical cancer. Mol Ther 2019; 27: 1183-1194.
[41] Chung HJ, Choi YE, Kim ES, Han YH, Park MJ and Bae IH. miR-29b attenuates tumorigenicity and stemness maintenance in human glioblastoma multiforme by directly targeting BCL2L2. Oncotarget 2015; 6: 18429-18444.
[42] Li G, Zhao J, Peng X, Liang J, Deng X and Chen Y. The mechanism involved in the loss of PTEN expression in NSCLC tumor cells. Biochem Biophys Res Commun 2012; 418: 547-552.
[43] Hu H, Hu S, Xu S, Gao Y, Zeng F and Shui H. miR-29b regulates ang II-induced EMT of rat renal tubular epithelial cells via targeting PI3K/AKT signaling pathway. Int J Mol Med 2018; 42: 453-460.
[44] Wang B, Li W, Liu H, Yang L, Liao Q, Cui S, Wang H and Zhao L. miR-29b suppresses tumor growth and metastasis in colorectal cancer via downregulating Tiam1 expression and inhibiting epithelial-mesenchymal transition. Cell Death Dis 2014; 5: e1335.
[45] Wang J, Chu ES, Chen HY, Man K, Go MY, Huang XR, Lan HY, Sung JJ and Yu J. microRNA-29b prevents liver fibrosis by attenuating hepatic stellate cell activation and inducing apoptosis through targeting PI3K/AKT pathway. Oncotarget 2015; 6: 7325-7338.
[46] Yan B, Guo Q, Nan XX, Wang Z, Yin Z, Yi L, Wei YB, Gao YL, Zhou KQ and Yang JR. Micro-ribonucleic acid 29b inhibits cell proliferation and invasion and enhances cell apoptosis and chemotherapy effects of cisplatin via targeting of DNMT3b and AKT3 in prostate cancer. OncoTargets Ther 2015; 8: 557-565.
[47] Vares G, Sai S, Wang B, Fujimori A, Nenoi M and Nakajima T. Progesterone generates cancer stem cells through membrane progesterone receptor-triggered signaling in basal-like human mammary cells. Cancer Lett 2015; 362: 167-173.
[48] Kim EH, Kim MS, Takahashi A, Suzuki M, Vares G, Uzawa A, Fujimori A, Ohno T and Sai S. Carbon-Ion beam irradiation alone or in combination with zoledronic acid effectively kills osteosarcoma cells. Cancers 2020; 12; 698-712