Carbon ion irradiation abrogates Lin28B-induced X-ray resistance in melanoma cells

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ABSTRACT

The Lin28/let-7 axis plays an important role in tumor initiation and developmental processes. Lin28B is upregulated in a variety of cancers, and its overexpression enhances cancer cell proliferation and radioresistance through the suppression of let-7 micro RNA expression. In this study, we investigated the role of the Lin28/let-7 axis as a target for radiosensitization of melanoma cancer cells. The overexpression of Lin28B reduced mature let-7 microRNA expression in melanoma cell lines, and enhanced the sphere-forming ability of melanoma cell lines, which is a characteristic of cancer stem cell (CSC) populations. Interestingly, Lin28B-overexpressed melanoma cells were more resistant to X-ray irradiation than control cells, and Lin28B-induced radioresistance was abolished after carbon ion irradiation. Consistent with these results, Lin28B overexpression reduced the numbers of γH2A.X foci after X-ray irradiation, whereas carbon ion irradiation had no such effect. Our results suggest that a carbon ion beam is more effective than an X-ray beam in terms of killing cancer cells, possibly due to elimination of CSC populations.

KEYWORDS: carbon-ion beam, Lin28/let-7 axis, cancer stem cell, radioresistance

INTRODUCTION

The cancer stem cell (CSC) hypothesis posits that tumors are maintained by a subpopulation of cancer cells that are able to proliferate and self-renew. Indeed, numerous studies have demonstrated that CSCs play a pivotal role in cancer initiation, progression and recurrence [1, 2]. Consequently, research has focused on the development of novel therapies that are cytotoxic to CSCs to achieve cures for various cancers. One of the major obstacles to cancer treatment is resistance to radiotherapy. It has been demonstrated that CSC populations are highly resistant to irradiation compared with non-stem cells [3–6]. Thus, targeting CSCs might enable improvement of the radiosensitivity of cancer cells.

Radiotherapy is one of the most common methods for cancer treatment. Most cancer patients receive radiation therapy, either alone or in combination with surgery and chemotherapy, to shrink the tumor mass or kill cancer cells [7]. Conventionally, X-ray beams are used for radiotherapy, but charged particles such as protons and carbon ions (C-ions) have attracted much interest because of their superior dose distribution and high biological effectiveness [8–10]. Although protons comprise the majority of cancer treatments involving charged particle therapy so far, the number of cancer patients treated with C-ion therapy has gradually increased since the National Institute of Radiological Sciences (NIRS) began clinical trials in 1994 [11, 12]. To date, >12 000 patients have been treated with C-ions [12], and the number of heavy ion facilities worldwide is constantly increasing [9].

MicroRNAs (miRNAs) are small non-coding RNAs that repress gene expression by binding to a 3’ untranslated region (UTR) of
target messenger RNAs (mRNAs) [13]. One of these miRNAs, let-7, is well-known as a tumor suppressor. Members of the let-7 family are generally downregulated in various types of cancers, and play important roles in diverse biological processes, including cell proliferation, differentiation and apoptosis, through the regulation of multiple oncogenes such as Lin28, high-mobility group AT-hook 2 (HMGA2) and MYC [14, 15]. The RNA binding protein, Lin28B, is an inhibitor of let-7 microRNAs. This protein binds to the terminal loops of the precursors of let-7 and sabotages their post-transcriptional processing [16]. Several studies have suggested that the Lin28/let-7 axis plays a critical role in the regulation of embryonic and induced pluripotent stem cells [17, 18]. In addition, Cheng et al. suggested that Lin28B is a putative CSC marker for the recurrence of hepatocellular carcinoma [19].

Previously, our group suggested that the macroH2A1/Lin28B/let-7 regulatory network regulates the cancer stem–like properties of bladder cancer cells [20]. A previous study revealed that Lin28B-overexpressing bladder cancer cells are more resistant to γ-ray irradiation compared with control cells. Based on this finding, in this study we investigated the effect of X-ray or C-ion irradiation on the survival of control and Lin28B-overexpressing melanoma cells to clarify the role of the Lin28B/let-7 axis in radiosensitization.

**MATERIALS AND METHODS**

**Cell culture**

Human melanoma cells (G361, SK-MEL5, A375s2, A375 and A2058) were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured at 37°C under conditions of 20% O2 and 5% CO2 in Dulbecco’s modified Eagle medium (Wako, Osaka, Japan) containing 10% (v/v) fetal bovine serum, 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, penicillin (50 units/ml) and streptomycin (50 μg/ml).

**Western blot analysis**

Cells were lysed by RIPA lysis buffer (Millipore, Billerica, MA, USA) containing phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). The protein concentrations were determined using Pierce 660 nm protein assay kit (Thermo Scientific, Rockford, IL, USA). The cell extracts were boiled in SDS sample buffer (Bio-Rad, Hercules, CA, USA) and equal amounts of the proteins were loaded onto SDS-PAGE and transferred to PVDF (Millipore). Protein bands were detected with ECL solution (Amersham, GE Healthcare, NJ, USA) and visualized using a ChemiDoc XRS (Bio-Rad). The following primary antibodies were used: Lin28B (Abcam, Cambridge, MA, USA) and Actin (Santa Cruz, CA, USA).

**Cell line generation**

pCMV6-AC-GFP vectors with or without Lin28B ORF were purchased from ORIGENE (Rockville, MD, USA). Cells were transfected with these plasmids using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). Transfected cells were selected with 500 μg/ml of Geneticin (Thermo Scientific).

**Quantitative real-time PCR**

Total RNA containing microRNA was extracted using a miRNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s protocol. The quantity of isolated RNA was measured using a NanoPhotometer (IMPLEN, München, Germany), and 1 μg of RNA was reverse-transcribed using a miScript II RT kit (QIAGEN). The miScript universal primer (QIAGEN) was used as the antisense primer. SNORD61 and SNORD68 (QIAGEN) were used as the internal controls. The following primers were used: hsa-let-7a-5p TGA GGT AGG TTG TAT AGT T, hsa-let-7b-5p TGA GGT AGG TTG TTG TGT GGT T, hsa-let-7c-5p TGA GGT AGG TTG TAT AGT T, hsa-let-7d-5p AAG GGT AGT AGG TTG TGT GGT T, hsa-let-7e-5p TGA GGT AGG TTG TAT AGT T, hsa-let-7f-5p TGA GGT AGG TTG TAT AGT T, hsa-let-7g-5p TGA GGT AGG TTG TAT AGT T, hsa-let-7i-5p TGA GGT AGG TTG TAT AGT T and hsa-let-7i-5p TGA GGT AGG TTG TAT AGT T.

**Sphere-forming assay**

Cells were seeded on ultra-low attachment 24-well plates (Coming, Lowell, MA, USA) in serum-free Dulbecco’s modified Eagle medium/nutrient mixture F-12 (Invitrogen) supplemented with B27 (Invitrogen), N2 (Invitrogen), epidermal growth factor (20 ng/ml; Peprotech, London, UK) and basic fibroblast growth factor (10 ng/ml; Peprotech). After 1 week, spheres were counted and visualized using a phase-contrast Olympus microscope (Tokyo, Japan).

**Irradiations**

X-rays were irradiated using the 200-kVp X-ray generator (MultiRad225S, Faxitron Biopptics, LLC, Tucson, AZ, USA) with a total filtration of 0.5 mm aluminum and 0.5 mm copper. The dose rate of X-rays was 1.11 Gy/min. C-ion beam irradiation was performed at the Gunma University Heavy Ion Medical Center [21]. The conditions of irradiation were: energy of 290 MeV/nucleon and linear energy transfer of 50 keV/μm at the center of the 6 cm spread-out Bragg peak. The radiation dose (Gy) required for 10% survival rates (D10) were calculated using linear regression analysis.

**Colony-forming assay**

Cells were seeded into T-25 flasks and incubated at 37°C overnight. Ten days after irradiation, colonies were fixed with methanol and stained with 2% Giemsa solution. The colonies containing at least 50 cells were scored.

**Immunocytochemistry**

Cells were fixed with 3.7% formaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 30 min. Then, cells were blocked with PBS containing 0.1% Triton X-100 and 3% bovine serum albumin at room temperature for 30 min, and stained with yH2AX antibody (Millipore). After staining, cells were incubated with Alexa Fluor 555 antibody (Life Technologies, Grand Island, NY, USA) at room temperature for 45 min in the dark. At least 100 cells in each group were counted and visualized using an Olympus microscope.
Statistical analysis
The data presented are representative of at least three independent experiments. Statistical analyses were performed using GraphPad Prism 7.0 Software (USACO Co., Tokyo, Japan). The Student’s t test was used for statistical comparisons. P-values of <0.05 were considered statistically significant.

RESULTS
Repression of mature let-7 miRNA production by Lin28B overexpression
To establish Lin28B-overexpressing cells, we assessed the level of Lin28B expression in five melanoma cell lines. The G361, A375s2 and SK-MEL5 cell lines showed reduced Lin28B expression (Fig. 1a). Therefore, we generated Lin28B-overexpressing cells using these three cell lines (Fig. 1b). Since Lin28B is an inhibitor of let-7 miRNAs, we next determined whether its overexpression reduced the levels of mature let-7 miRNAs. As shown in Fig. 1c, the expression of mature let-7 miRNAs was reduced in Lin28B-overexpressing melanoma cell lines compared with the control cells, even though differential rates of reductions were observed according to the cell line and the type of miRNA involved. Especially, all the let-7 miRNAs we tested were significantly reduced in Lin28B-overexpressed SK-MEL5 cells, while Lin28B-overexpressed G361 and A375s2 cells showed relatively less reduction of let-7 expression (Fig. 1c). Among the members of the let-7 family, let-7b, g and i miRNAs were significantly reduced by Lin28B overexpression in all three cell lines tested (Fig. 1c). These results indicate that the upregulation of Lin28B suppresses mature let-7 miRNA expression in melanoma cells in a cell line–dependent manner.

Sphere-forming capability of control and Lin28B-overexpressing melanoma cells
We next determined the sphere-forming capability of control and Lin28B-overexpressing melanoma cells to elucidate the role of Lin28B in CSC expansion. Control and Lin28B-overexpressing melanoma cells were seeded onto 24-well low-adhesion plates, and after 1 week, spheres were counted and visualized (Fig. 2). Overexpression of Lin28B significantly increased the sphere-forming capability of SK-MEL5 (Fig. 2a), G361 (Fig. 2b) and A375s2 (Fig. 2c) cells. These results suggest that Lin28B plays an important role in melanoma CSC regulation, possibly due to repression of mature let-7 miRNA production by Lin28B.

![Fig. 1. Overexpression of Lin28B suppresses let-7 miRNA expression. (a) The levels of Lin28B expression were determined using western blot analysis. (b) The expression of Lin28B in melanoma cells transfected with the pCMV6-AC-GFP (Con) or pCMV6-Lin28B-GFP (Lin28B) vectors was evaluated using western blot analysis. Actin was used as an internal control. (c) The levels of mature let-7 miRNAs in Con or Lin28B cells were assessed using quantitative real-time PCR (qPCR). P-values were calculated using Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001.](https://academic.oup.com/jrr/article-abstract/58/6/765/3803482)
Survival of control and Lin28B-overexpressing melanoma cells after treatment with C-ion or X-ray beams

To determine the effect of Lin28B overexpression on the radiosensitivity of melanoma cells after X-ray or C-ion beam irradiation, clonogenic assays were performed. Control or Lin28B-overexpressing SK-MEL5, G361 and A375s2 cells (1 × 10^3) were seeded, and after 20 h, exponentially growing cells were exposed to X-ray or C-ion beams. Following X-ray irradiation, overexpression of Lin28B significantly increased radioresistance in all three of the cell lines we tested (Fig. 3). However, X-ray irradiation–induced radioresistance was completely abolished by irradiation with C-ion beams (Fig. 3). After X-ray irradiation, the D_{10} values of Lin28B-overexpressing SK-MEL5, G361 and A375s2 cells were greater than those of control cells (control: 5.40 ± 0.29, Lin28B: 6.10 ± 0.31, control: 4.49 ± 0.03, Lin28B: 5.02 ± 0.05 and control: 4.85 ± 0.09, Lin28B: 5.30 ± 0.35, respectively), but no significant changes were observed after C-ion irradiation (control: 3.30 ± 0.08, Lin28B: 3.40 ± 0.09, control: 2.44 ± 0.07, Lin28B: 2.44 ± 0.14 and control: 3.28 ± 0.05, Lin28B: 3.17 ± 0.09, respectively). These results suggest that a C-ion beam could eliminate melanoma cells that are resistant to X-ray irradiation.

Fig. 2. Overexpression of Lin28B enhances sphere-forming capability. (a–c) Con and Lin28B melanoma cells were incubated with DMEM/F12 supplemented with B27, N2, EGF and bFGF. The spheres in SK-MEL5 (a), G361 (b) and A375s2 (c) cells were counted and visualized after 1 week. P-values were calculated using Student’s t test.

Fig. 3. The C-ion beam abolishes Lin28B-induced X-ray resistance. (a–c) Con and Lin28B melanoma cells were left untreated or were treated with X-ray (2–8 Gy) or C-ion (1–4 Gy) irradiation, and the survival rates of SK-MEL5 (a), G361 (b) and A375s2 (c) cells were determined using the colony-forming assay. Square: X-ray; circle: C-ion; open: Con; closed: Lin28B. P-values were calculated using Student’s t test.
γH2AX foci formation by control and Lin28B-overexpressing melanoma cells after irradiation with C-ion or X-ray beams

To further investigate the effect of Lin28B overexpression on DNA damage, we next examined γH2AX foci formation by control and Lin28B-overexpressing SK-MEL5 and G361 cells after C-ion or X-ray irradiation. Consistent with the cell survival results, fewer γH2AX foci were formed by Lin28B-overexpressing cells than in control cells after X-ray irradiation. However, the overexpression of Lin28B did not affect foci formation after C-ion beam irradiation (Fig. 4a and b). Moreover, at 24 h after X-ray irradiation, only 14% of γH2AX foci remained in the Lin28B-overexpressing SK-MEL5 cells, compared with 23% in control cells (Fig. 4c). Furthermore, the number of foci remained higher in C-ion-irradiated Lin28B-overexpressing cells than X-ray-irradiated Lin28B-overexpressing cells. These results suggest that C-ion irradiation blocked the Lin28B-induced X-ray resistance through regulation of the DNA damage response.

**DISCUSSION**

Melanoma is considered a relatively radio-insensitive tumor type due to its poor prognosis and high recurrence rates after photon radiotherapy [22]. As such, surgery is generally preferred for the treatment of malignant melanoma, despite the high local recurrence rate and low overall survival rate [23, 24]. Nonetheless, recent studies have demonstrated that C-ion therapy has significant advantages over conventional radiotherapy in terms of improving the overall survival of melanoma patients [25–28]. Recently, several studies have reported radiobiological benefits of a C-ion beam for CSC regulation over a photon beam in a variety of cancer cells. In these studies, the proportions of putative colon or pancreatic CSCs were increased after X-ray irradiation, but a few or no changes were observed after C-ion treatment [29, 30]. Furthermore, Sai et al. showed that a C-ion beam combined with cisplatin effectively disrupts triple negative breast CSCs [31]. Although C-ion irradiation could attenuate CSC-induced radioresistance, the effect of C-ion beam treatment on radioresistant melanoma cells, which could be regarded as CSCs, has not yet been fully elucidated. In the present study, we therefore aimed to evaluate the advantages of C-ion beam over conventional radiotherapy in terms of killing of melanoma stem-like cells.

The aberrant upregulation of Lin28A and its homologue Lin28B is present in a variety of cancers, and high levels of these proteins are associated with malignancy [20, 32–34]. The let-7 miRNAs are
the main downstream targets of Lin28A and B, and their oncogenic roles are mediated primarily by suppression of mature let-7 production [35]. For example, knockdown of Lin28B in hepatocellular carcinoma cells (HCCs) inhibited proliferation in vitro and reduced in vivo tumor growth in SCID mice. In contrast, overexpression of Lin28B suppressed the expression of let-7 family members, hence enhancing the tumorigenicity of HCCs [36]. The Lin28/let-7 axis not only regulates cancer initiation and progression, but also has a role in regulating the radiosensitivity of cancer cells. Knockdown of Lin28B increased let-7g levels, which decreased the expression of K-RAS, resulting in enhanced radiosensitivity in lung cancer cells [37]. Similarly, the induction of Lin28B expression by knockdown of a histone variant, macroH2A1, leads to suppression of let-7 miRNAs, and enhanced radioresistance in bladder cancer cells [20]. In the present study, we also found that Lin28B-overexpressing melanoma cells exhibit suppression of the expression of mature let-7 miRNAs (Fig. 1C), supporting the notion that Lin28B is a key regulator of let-7 miRNAs.

Although limited data on the role of Lin28B in radiosensitivity have been reported, the importance of the Lin28/let-7 axis in CSC regulation is established in various types of cancer [16, 35]. In this study, we have shown that Lin28B overexpression significantly enhances the sphere-forming capability of melanoma cells (Fig. 2). Sphere-forming assays have been widely used to identify CSCs in vitro. Many studies suggest that the subpopulation of cancer cells that comprise spheres exhibits increased potential for self-renewal and tumorigenicity [38, 39]. Thus, our results suggest that the enhanced expression of Lin28B in melanoma cells may contribute to CSC expansion. A large body of evidence suggests the importance of CSCs in radioresistance [40]. In the present study, we have shown that Lin28B-overexpressing melanoma cells are more resistant to X-ray irradiation compared with control cells, and that Lin28B-induced X-ray resistance was completely abolished by C-ion irradiation, suggesting that the expansion of stem-like melanoma cells due to Lin28B overexpression could be prevented by C-ion treatment (Fig. 3).

The phosphorylation of histone H2AX on Ser-139 (which is termed γH2AX) is a marker for the DNA double-strand breaks and occurs rapidly (within minutes) after γ-ray irradiation, and dephosphorylation occurs after the repair of DNA [40–42]. Because of its characteristics, the ability to form γH2AX foci after ionizing radiation has been used to evaluate the radiosensitivity of cancer cells. In this study, we demonstrated that the overexpression of Lin28B significantly reduced γH2AX foci formation in SK-MEL5 and G361 cells after 2 Gy of X-ray irradiation, indicating that overexpression of Lin28B attenuates the DNA damage of melanoma cells (Fig. 4). Since the relative biological effectiveness values of the C-ion beam were ~2 in both SK-MEL5 and G361 cells (1.83 and 1.88, respectively), we used 1 Gy of C-ion irradiation to evaluate the effect of Lin28B overexpression on the DNA damage. Interestingly, we found that Lin28B overexpression did not affect the formation of γH2AX foci after C-ion irradiation. One possible explanation for this observation is that elimination of CSCs is enhanced by Lin28B overexpression after C-ion beam irradiation. However, precise downstream mechanisms should be determined to clarify this possibility. In conclusion, the results of the present study suggest that the Lin28B/let-7 axis may play an important role in melanoma CSC expansion, thereby regulating X-ray sensitivity in melanoma cells. Moreover, we provide evidence that C-ion beams could be used to eliminate X-ray–resistant melanoma cells, supporting the notion that C-ion therapy has an advantage over conventional radiotherapy in terms of improving the survival of cancer patients.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

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