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Fractionated radiation-induced nitric oxide promotes expansion of glioma stem-like cells

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Glioblastoma remains an incurable brain disease due to the prevalence of its recurrence. Considerable evidence suggests that glioma stem-like cells are responsible for glioma relapse after treatment, which commonly involves ionizing radiation. Here, we found that fractionated ionizing radiation (2 Gy/day for 3 days) induced glioma stem-like cell expansion and resistance to anticancer treatment such as cisplatin (50 μM) or taxol (500 nM), or by ionizing radiation (10 Gy) in both glioma cell lines (U87, U373) and patient-derived glioma cells. Of note, concomitant increase of nitric oxide production occurred with the radiation-induced increase of the glioma stem-like cell population through upregulation of inducible nitric oxide synthase (iNOS). In line with this observation, downregulation of iNOS effectively reduced the glioma stem-like cell population and decreased resistance to anticancer treatment. Collectively, our results suggest that targeting iNOS in combination with ionizing radiation might increase the efficacy of radiotherapy for glioma treatment. (*Cancer Sci* 2013; 104: 1172–1177)

Glioblastoma is the most common and aggressive primary brain tumor. Despite the advent of modern surgical and medical treatments, glioblastoma remains an incurable brain disease.[1,2] Post-operative radiotherapy extends survival, but yields a median survival of less than a year; long-term survival is extremely rare.[3] This poor prognosis for glioblastoma patients reflects the prevalence of cancer recurrence after surgery, infiltration into other sites and intrinsic or acquired resistance to chemotherapy and radiotherapy.[4]

Ionizing radiation (IR) is widely used as a standard treatment for glioblastoma.[5,6] Clinically, because damage to normal tissues surrounding a tumor is unavoidable in radiotherapy, a high dose of IR is often fractionated to reduce the side-effects of radiotherapy. However, recent studies have reported that IR paradoxically promotes the malignant glioma cell phenotype, allowing relapse after treatment.[4,7] Notably, this effect is frequently observed after exposure to doses of fractionated radiation that are not sufficient to eradicate the primary tumor. Although the mechanisms underlying this effect are not well understood, it might reflect the selective expansion of intrinsically IR-resistant glioma cells or acquisition of radio resistance after exposure to IR.

In this context, considerable emerging evidence suggests that a subpopulation of glioma cells is highly tumorigenic and self-renewing, properties reminiscent of normal stem cells.[8–10] Because these cells constitute a small percentage of cells in a glioma and their gene expression profiles resemble those of normal neural stem cells, they have been called tumor-initiating cells or glioma stem-like cells (GSC). These GSC are thought to be responsible for glioma relapse after treatment and as such are regarded as good potential therapeutic targets. Moreover, the resistance of GSC to anticancer treatments such as radiotherapy and chemotherapy implicates these cells in IR-induced malignant progression in glioma. In line with the GSC hypothesis, exposure of glioma cells to IR leads to expansion of the GSC population; however, the molecular mechanisms by which IR promotes GSC are largely unknown.

Nitric oxide (NO) is a highly diffusible and short-lived pleiotropic regulator of many physiological processes, including vasodilatation, neurotransmission and macrophage-mediated immunity.[11] Nitric oxide is synthesized by three different NO synthases (NOS): neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), each encoded by a distinct gene.[12] Although NO exerts anticancer effects on tumor growth, it can also promote metastasis or inflammation depending on the cellular microenvironment, including the quantity of NO, redox status, cell type and cellular adaptation.[13–15] Thus, a variety of signaling pathways appear to be involved in NO-mediated cellular regulation.

Recent studies have suggested that NO is highly produced within gliomas and promotes GSC.[16,17] In line with these previous studies, we show that IR stimulates NO production in gliomas through induction of iNOS. Notably, downregulation of iNOS effectively inhibited IR-induced NO production, decreased the GSC population and abolished IR-induced resistance to radiotherapy and chemotherapy. Taken together, the results of our study demonstrate that radiotherapy for treatment of glioma could stimulate expansion of GSC via iNOS-mediated NO production, suggesting that combining radiotherapy with targeting iNOS might enhance the efficacy of brain tumor treatment.

Materials and Methods

Chemical reagents and antibodies. Polyclonal antibodies to Nestin, Sox2 (SRY-box containing gene 2) and Musashi-1 were purchased from Millipore (Billerica, MA, USA). Polyclonal antibodies to iNOS and β-catenin antibodies were from BD Transduction Laboratories (Seoul, Korea), 4,6-diamidino-2-phenylindole (DAPI) and monoclonal antibodies to β-actin were from Sigma (St Louis, MO, USA) and Alexa Fluor 488-conjugated anti-mouse and anti-rabbit antibodies were from Invitrogen (Carlsbad, CA, USA). Phycoerythrin-conjugated CD133 (clone 293C3) and mIgG2b-PE were purchased from Miltenyi Biotec Ltd (Auburn, CA, USA).

Cell culture. U87MG and U373MG glioma cells were obtained from the Korean Cell Line Bank. The patient-derived GSC X01GB was established from acutely resected human tumor tissues obtained with written informed consent from a 68-year-old woman with a glioblastoma multiforme.[18] U87MG, U373MG and X01GB cells were cultured in

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Dulbecco's modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum.

Transfection. Small interfering RNA (siRNA) duplexes (40 nM) were introduced into cells using a Microporator-mini (Digital Bio Technology, Seoul, Korea) according to the procedure recommended by the manufacturer. Cells were harvested after 48 h for subsequent experiments. siRNA targeting iNOS (sense, 5′-GCACAGAAUGUUCCAGAAUUU-3′; antisense, 5′-AUUCUGGAACAUUCUGUGCUU-3′) and scrambled, negative control siRNA (sense, 5′-GUUCAGCGUGUCCGGCGA GTT-3′; antisense, 5′-CUCGCCGGACACGCUGAACTT-3′) were purchased from Samchully Pharmaceutical Co. Ltd (Seoul, Korea).

FACS analysis. For double staining, cells were stained with a polyclonal anti-rabbit Nestin antibody (Millipore, Billerica, MA, USA) and then with Alexa 488-conjugated anti-rabbit IgG and PE-conjugated CD133 antibody (Miltenyi Biotech, Auburn, CA, USA) in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and 2 mM ethylenediaminetetraacetic acid. As a control, cells were stained with isotype antibody (mIgG2b-PE; Miltenyi Biotech). Stained cells were then analyzed by fluorescence-activating cell sorting using a BD FACSCalibur system equipped with Cell Quest software (BD Biosciences, Seoul, Korea).

Quantification of cell death. Cell death was measured using FACS analysis of propidium iodide-stained cells. Cells were stained by incubating in propidium iodide (50 ng/mL) in PBS for 5 min at room temperature. A total of 10 000 per sample were analyzed by BD FACS Calibur using Cell Quest software (BD Bioscience, Seoul, Korea).

Western blot analysis. Cell lysates were prepared by incubating with lysis buffer (40 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.1% Nonidet-P40) supplemented with protease inhibitors. Proteins in whole-cell lysates were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL, USA). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline and incubated with primary antibodies overnight at 4°C. Blots were developed with a peroxidase-conjugated secondary antibody and proteins were visualized using enhanced chemiluminescence procedures (Amersham) according to the manufacturer's protocol.

Nitrite level determination. Nitric oxide production was determined by measuring the amount of nitrite using the Griess reagent, as described previously.[19] In brief, 50 μL Griess reagent I (1% sulfanilamide, 30% acetate) was added to medium collected from cultured cells or nitrite standards (100 μL), after which 50 μL Griess reagent II (0.1% N-1-naphtylethylenediamine, 60% acetate) was added. Nitrite was then quantified by measuring absorbance of the solution at 570 nm.

Irradiation. Cells in culture medium were plated in 60-mm dishes and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were exposed to radiation using a ^{137}Cs γ-ray source (Atomic Energy of Canada, Ltd, Mississauga, ON, Canada) at a dose rate of 3.81 Gy/min.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Following fixation, cells were incubated at 4°C overnight with rabbit polyclonal anti-human CD133 (1:200), anti-Nestin (1:200) and/or anti-Musashi-1 (1:200) primary antibody in PBS with 1% bovine serum albumin and 0.1% Triton X-100. Stained proteins were visualized using Alexa Fluor 488-conjugated anti-rabbit secondary antibodies (Molecular Probes, Seoul, Korea). Nuclei were counterstained with DAPI (Sigma). Stained cells were observed with an Olympus IX71 fluorescence microscope (Olympus, Seoul, Korea).

Statistical analysis. All experimental data are reported as means; error bars represent standard deviation (SD). Statistical analyses were performed using non-parametric Student's *t*-tests.

Results

Nitric oxide production is involved in the fractionated radiation-induced expansion of GSC. To determine whether IR induces NO production in glioma cells, we analyzed NO levels by measuring the accumulation of nitrite in U87 or U373 glioma cell-cultured medium 48 h after exposure to a single dose (6 Gy) or fractionated dose (2 Gy × 3; 2 Gy/day for 3 days) of radiation. Notably, NO levels were markedly elevated in IR-exposed glioma cells, especially in those exposed to fractionated IR, compared with non-irradiated cells (Fig. 1a). In parallel, we also tested the expression levels of the three NOS isoforms, iNOS, eNOS and nNOS, in U87 and U373 glioma cells after IR exposure. Importantly, exposure to a single dose or fractionated IR significantly induced the expression of iNOS in both U87 and U373 glioma cells (Fig. 1b), suggesting that NO production is increased through upregulation of iNOS after IR in glioma cells. In agreement with this result, pretreatment with siRNA targeting iNOS blocked IR-induced NO production in U87 glioma cells (Fig. 1c).

We then studied whether elevation of NO levels is involved in IR-induced GSC expansion. To this end, we quantified the GSC population expressing CD133 and Nestin by FACS after irradiation of U87 and U373 glioma cells previously transfected with either siRNA targeting iNOS or scrambled control siRNA. As predicted, the $CD133^+/Nestin^+$ cell population was markedly increased after irradiation of U87 or U373 glioma cells. Notably, this increase was effectively blocked by pretreatment with siRNA targeting iNOS (Fig. 1d). In line with these results, pretreatment with siRNA targeting iNOS also attenuated the expression of Musashi-1, another stemness marker that was induced after fractionated irradiation (2 Gy × 3; Fig. 1e,f). Taken together, these results suggest that IR induces expansion of the GSC population through iNOS-mediated NO production.

Downregulation of iNOS attenuates the expression of radiation-induced stemness regulators. To further investigate the involvement of NO in radiation-induced GSC expansion, we examined the expression levels of the stemness regulators Sox2, Notch-1, Notch-2 and β-catenin. To this end, we exposed U87 and U373 glioma cells to fractionated radiation after treatment with siRNA targeting iNOS or scrambled control siRNA. In agreement with the above data, the expression of Sox2, Notch-2 and β-catenin, but not Notch-1, was markedly upregulated at the protein level in U87 glioma cells exposed to fractionated radiation compared with non-irradiated cells (Fig. 2a). Importantly, pretreatment with siRNA targeting iNOS effectively blocked the increase in Sox2, Notch-2 and β-catenin expression in irradiated U87 glioma cells. Moreover, an immunocytochemical analysis confirmed that siRNA-mediated downregulation of iNOS attenuated the IR-induced expression of Sox2 and β-catenin in U87 glioma cells (Fig. 2b). These results further support the conclusion that iNOS-mediated NO production is involved in IR-induced expansion of GSC.

Depletion of NO abolishes IR-induced adaptive resistance to chemotherapy and radiotherapy in glioma cells. Because GSC are reported to be resistant to chemotherapy and radiotherapy,[20,21] we then determined whether depletion of NO abolished the IR-induced adaptive resistance of GSC to anticancer treatment. To this end, we pretreated U87 glioma cells with siRNA targeting iNOS or scrambled control siRNA and then exposed them to fractionated radiation to induce adaptive resistance. Consistent with previous studies, $CD133^+$ cells were not affected and maintained their population after treatment with the anticancer agents such as cisplatin (50 μM), Taxol

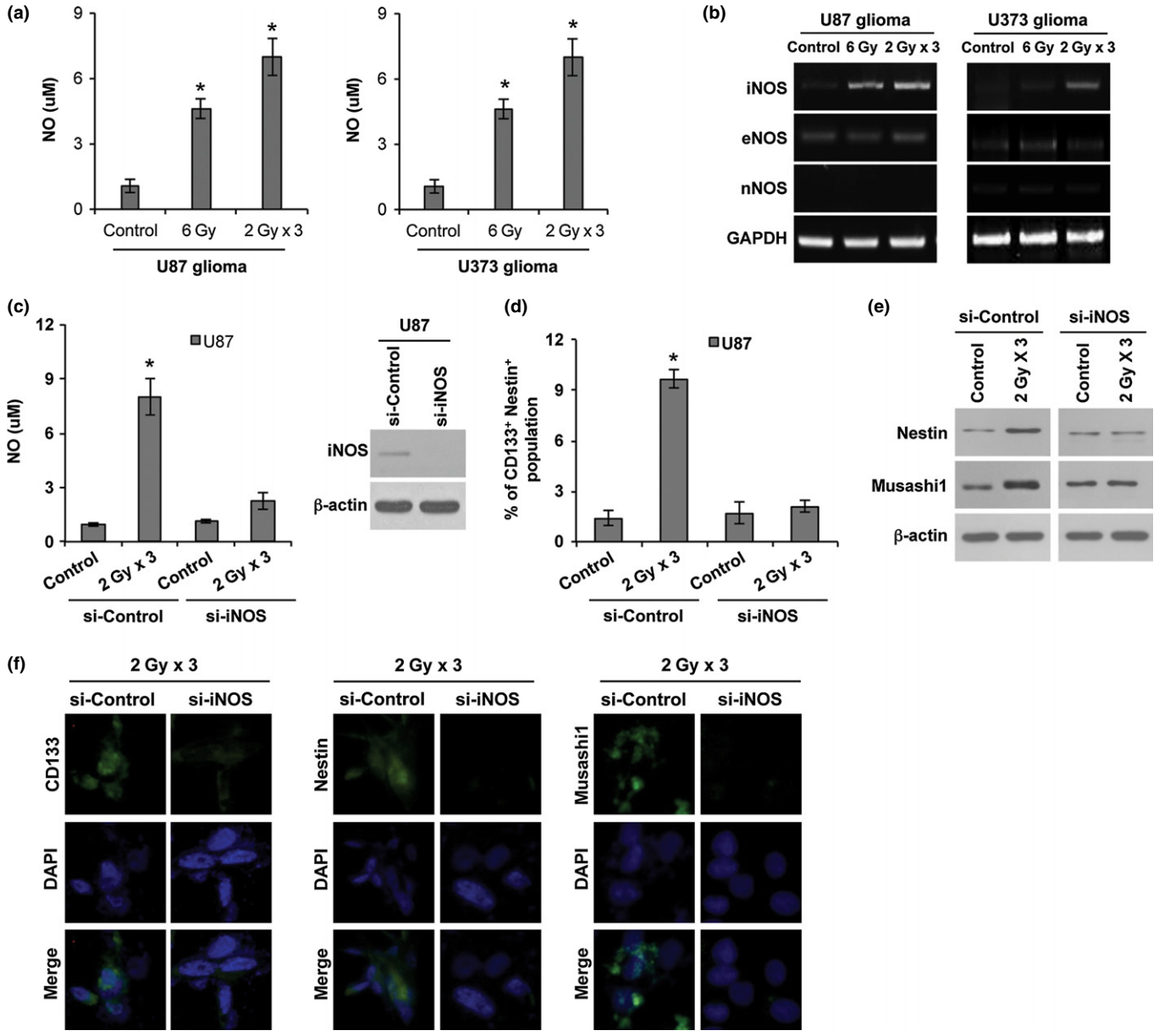

Fig. 1. Single-dose ionizing radiation and fractionated radiation promote glioma stem-like cells through nitric oxide (NO) production. (a) Quantification of NO by measuring the concentration of nitrites in U87 and U373 glioma cell cultures after single-dose radiation (6 Gy) or fractionated radiation (2 Gy/day × 3 days). (b) Western blot analysis for iNOS, eNOS and nNOS after single-dose radiation (6 Gy) or fractionated radiation (2 Gy × 3). (c) Quantification of NO by measuring the concentration of nitrites in U87 glioma cells transfected with siRNA targeting iNOS or scrambled control siRNA and then exposed to fractionated radiation (2 Gy × 3). (d) FACS-based quantification of the $CD133^+/Nestin^+$ cell population in U87 glioma cells transfected with siRNA targeting iNOS or scrambled control siRNA and then exposed to fractionated radiation (2 Gy × 3). (e) Western blot analysis for Nestin and Musashi-1 in U87 glioma cells treated with siRNA targeting iNOS or scrambled control siRNA and then exposed to fractionated radiation (2 Gy × 3). (f) Immunocytochemistry for CD133, Nestin and Musashi-1 in U87 glioma cells treated with siRNA targeting iNOS or scrambled control siRNA and then exposed to fractionated radiation (2 Gy x 3). β-actin was used as the loading control. Data are presented as means ± SD (error bars) of triplicate samples. $*P < 0.01$. eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; NOS, nitric oxide synthase.

(500 nM) or exposure to IR (10 Gy), indicating that $CD133^+$ cells are resistant to anticancer treatment (Fig. 3a). However, pretreatment with siRNA targeting iNOS attenuated the IR-induced increase in the $CD133^+$ cell population that are resistant to anticancer treatment. In agreement with these results, U87 glioma cells exposed to fractionated radiation escaped cell death induced by the anticancer agents cisplatin, Taxol or IR (10 Gy), indicating that these cells had acquired resistance to anticancer treatment after fractionated radiation (Fig. 3b–d). Notably, however, pretreatment with siRNA targeting iNOS effectively prevented glioma cells from acquiring IR-induced adaptive resistance to cisplatin, Taxol and IR. Taken together, these results suggest that iNOS-mediated NO production is necessary for the acquisition of IR-induced adaptive resistance to chemotherapy and radiotherapy in glioma cells.

Depletion of NO blocks IR-induced expansion of GSC and acquisition of resistance to anticancer treatment in patient-derived glioma cells. To extend these observations, we examined whether irradiation also increased GSC via NO

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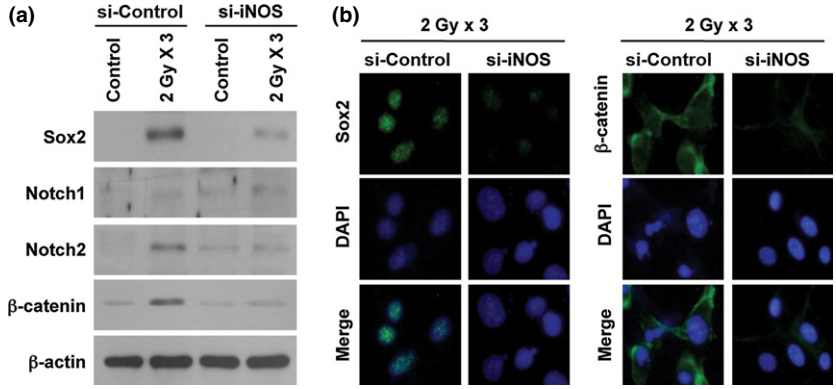

Fig. 2. Downregulation of inducible nitric oxide synthase (iNOS) attenuates the expression of ionizing radiation-induced stemness regulators. (a) Western blot analysis for Sox2, Notch-1, Notch-2 and β-catenin in U87 glioma cells treated with siRNA targeting iNOS or scrambled control siRNA and then exposed to fractionated radiation (2 Gy × 3). (b) Immunocytochemistry for Sox2 and β-catenin in U87 glioma cells treated with siRNA targeting iNOS or scrambled control siRNA and then exposed to fractionated radiation (2 Gy × 3). β-actin was used as the loading control.

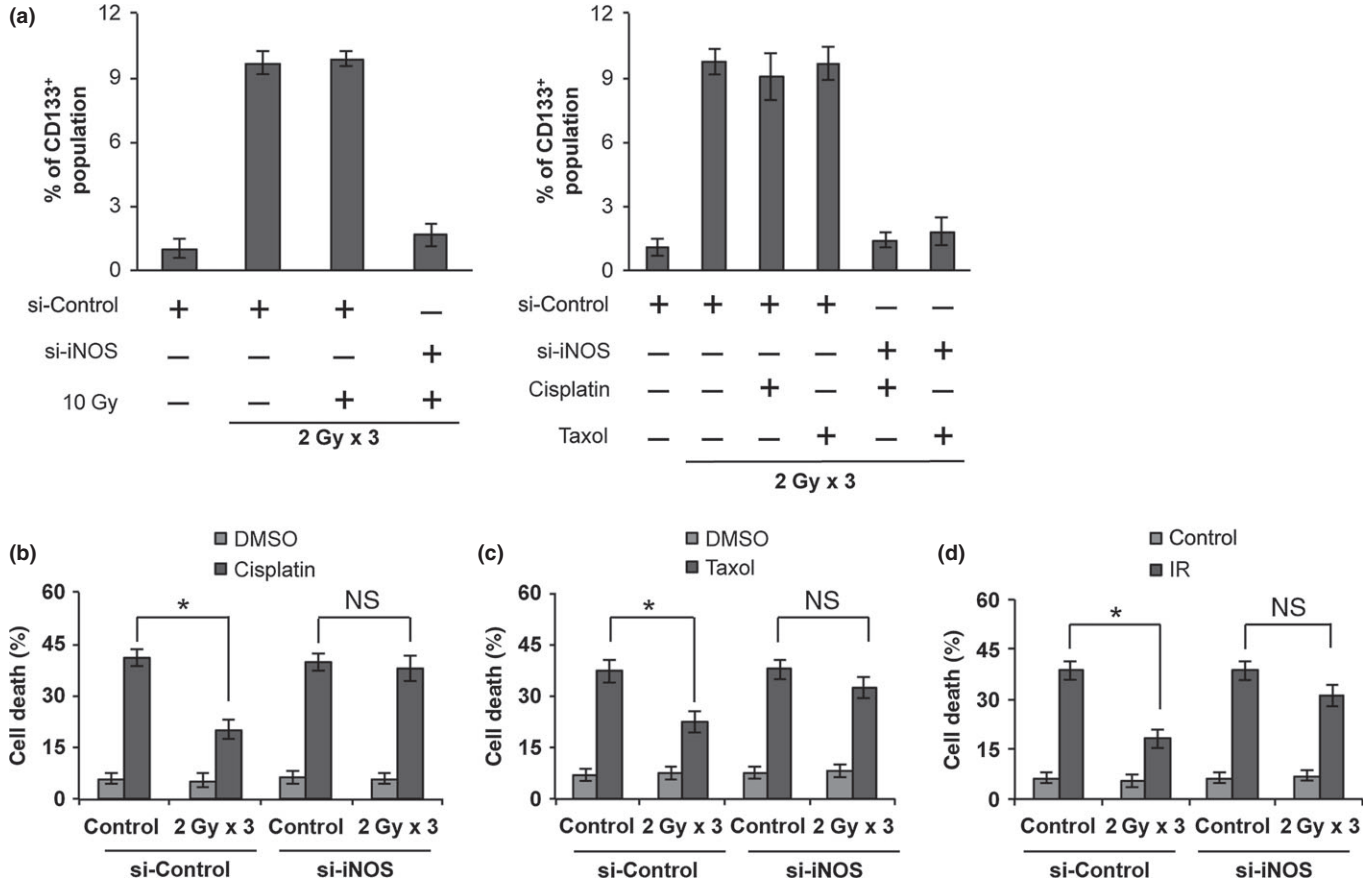

Fig. 3. Depletion of nitric oxide abolishes ionizing radiation (IR)-induced adaptive resistance to chemotherapy and radiotherapy in glioma cells. (a) FACS-based quantification of the $CD133^+$ cell population. U87 glioma cells transfected with siRNA targeting inducible nitric oxide synthase (iNOS) or scrambled control siRNA were exposed to fractionated radiation (2 Gy × 3) and/or treated with cisplatin (50 μM), Taxol (500 nM) or IR (10 Gy). (b–d) Quantification of cell death by FACS using propidium iodide staining. U87 glioma cells transfected with siRNA targeting iNOS or scrambled control siRNA were exposed to fractionated radiation (2 Gy × 3) and/or treated with cisplatin (50 μM; a), Taxol (500 nM; b) or IR (10 Gy; c). Data are presented as means ± SD (error bars) of triplicate samples. $^*P < 0.01$. NS, not significant.

production in patient-derived glioma cells and whether blocking NO production prevented this expansion. Previously, X01 glioma cells were derived from acutely resected human tumor tissues of a patient with glioblastoma multiforme.[18] Consistent with the results obtained from the U87 glioma cell line, exposure of patient-derived X01 glioma cells to fractionated radiation increased NO production compared with controls (Fig. 4a). In parallel with this result, a FACS analysis revealed that fractionated radiation led to an expansion of the $CD133^+$ cell population (Fig. 4b). Notably, pretreatment with siRNA

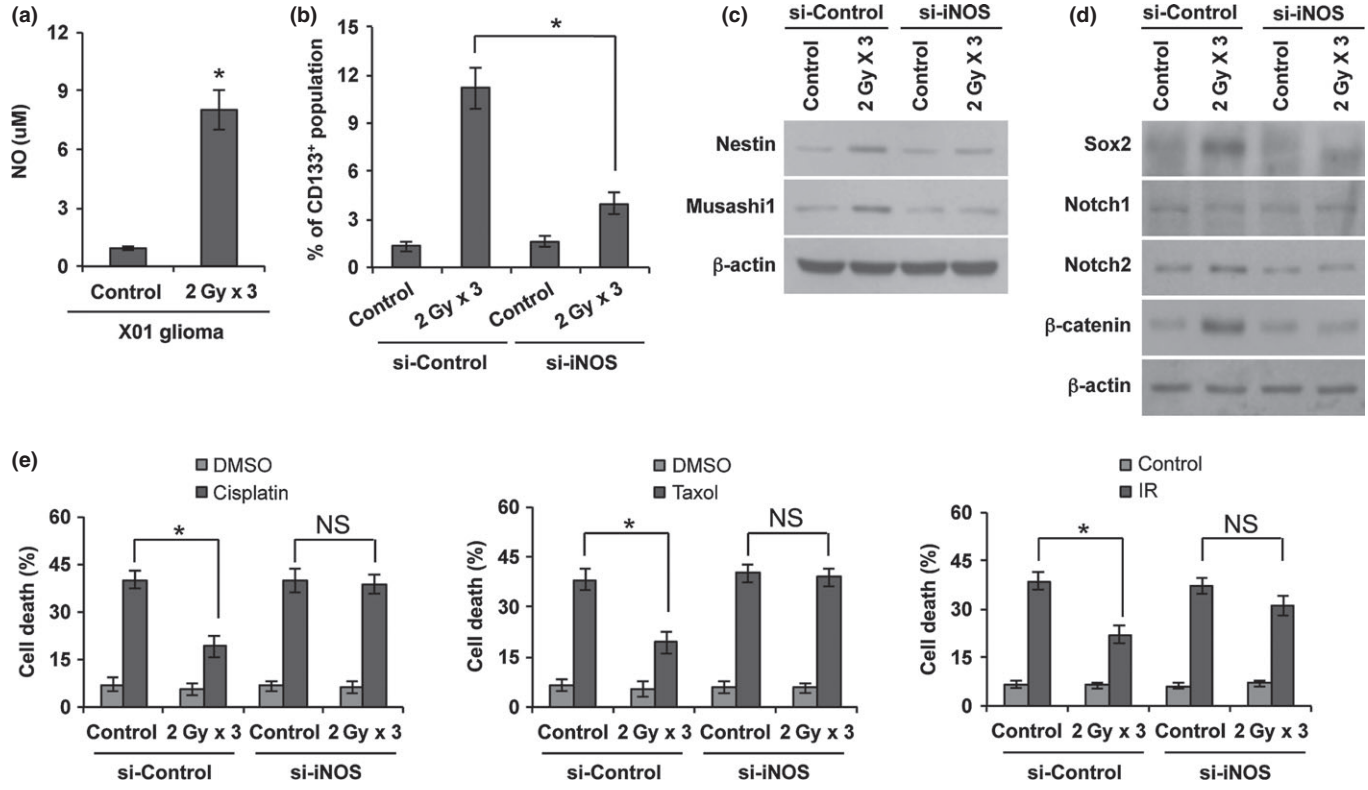

Fig. 4. Ionizing radiation (IR)-induced nitric oxide (NO) production promotes glioma stem-like cell expansion and acquisition of resistance to anticancer treatment in patient-derived glioma cells. (a) Quantification of NO by measuring the concentration of nitrites in patient-derived X01 glioma cells after fractionated radiation (2 Gy × 3). (b) FACS-based quantification of the $CD133^+$ cell population in patient-derived X01 glioma cells transfected with siRNA targeting inducible NO synthase (iNOS) or scrambled control siRNA and then exposed to fractionated radiation (2 Gy × 3). (c,d) Western blot analysis for the stemness markers Nestin and Musashi-1 (c) and stemness regulators Sox2, Notch-1, Notch-2 and β-catenin (d) in patient-derived X01 glioma cells transfected with siRNA targeting iNOS or scrambled control siRNA and then exposed to fractionated radiation (2 Gy × 3). (e) Quantification of cell death by FACS using propidium iodide staining. Patient-derived X01 glioma cells transfected with siRNA targeting iNOS or scrambled control siRNA were exposed to fractionated radiation (2 Gy × 3) and then treated with cisplatin (50 μM), Taxol (500 nM) or IR (10 Gy). β-actin was used as the loading control. Data are presented as means ± SD (error bars) of triplicate samples. $*P < 0.01$. NS, not significant.

targeting iNOS markedly attenuated the increase in the $CD133^+$ cell population compared with the treatment with scrambled control siRNA (Fig. 4b). In addition, IR-induced Nestin and Musashi-1 expression were also decreased by pretreatment with siRNA targeting iNOS (Fig. 4c). In agreement, the expression levels of the stemness regulators Sox2, Notch-2 and β-catenin were increased by irradiation in patient-derived X01 glioma cells and, in each case, these increases were attenuated by pretreatment with siRNA targeting iNOS (Fig. 4d).

Finally, we examined whether NO-mediated signaling is also necessary for IR-induced acquisition of resistance to anticancer treatment in patient-derived glioma cells. As expected, irradiated X01 glioma cells were more resistant to cisplatin, Taxol and IR (10 Gy) than unpretreated controls (Fig. 4e). Consistent with the results obtained with U87 glioma cells, this acquired resistance to anticancer therapy was prevented by downregulation of iNOS. Taken together, these results suggest that iNOS-mediated NO production is necessary for acquisition of IR-induced adaptive resistance to chemotherapy and radiotherapy as well as stemness in patient-derived glioma cells.

Discussion

Despite the fact that IR is widely used as a standard treatment for glioblastoma, recent studies have reported that irradiation paradoxically promotes the malignant glioma cell phenotype, causing relapse after treatment.[4,7] However, the mechanisms underlying this effect are not well understood.

In the present study, we observed that irradiation, especially fractionated radiation, increased the GSC population through induction of NO production. Previous studies have shown that GSC are identified by expression of CD133, Musashi-1 and Nestin, which are known markers of normal neural stem cells.[8,22] Singh *et al.*[8] showed that $CD133^+$ cells are self-renewing and tumorigenic compared with CD133-negative cells. Notably, injection of as few as 100 $CD133^+$ cells formed a brain tumor that could be serially transplanted and was a phenocopy of the patient's original tumor, whereas injection of as many as 10^5 CD133-negative cells failed to cause tumor formation. Moreover, Nestin and CD133 expression levels are correlated with glioma grade and the survival rate of patients.[23] Importantly, in the present study, we observed that irradiation of glioma cells caused an increase in the $CD133^+$/$Nestin^+$ cell population, indicative of the increase of the GSC population. Because glioma cells are a heterogeneous population, a subset of GSC that are intrinsically resistant to radiation might have been selected by radiation and evaluated as a higher percentage relative to non-GSC. However, this is unlikely because significant cell death did not occur in glioma cells after exposure to radiation (Figs 3,4e). This observation led to the alternative hypothesis that a subpopulation of glioma cells might be adapted to radiation and acquired radio-resistance in association with self-

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renewal and tumorigenic capacity through a mechanism distinct from irradiation-mediated selection. Importantly in this context, the GSC population was increased to a greater extent by fractionated radiation than by a single high dose of radiation in the present study. Because a higher single dose of radiation is predicted to more powerfully select for GSC than fractionated radiation, induction of a greater increase in GSC by fractionated radiation supports the hypothesis that IR-induced expansion of GSC is due to adaptation of glioma cells, in association with acquisition of stemness and resistance to anticancer treatment. This hypothesis is supported by previous studies showing that somatic cells can be reprogrammed by Yamanaka transcription factors.[24,25] Importantly, these findings challenge the concept that differentiation of stem cells or progenitors into functional somatic cells is an irreversible process. Although this dedifferentiation occurred artificially *in vitro* and has not yet been observed in living organisms, cancer cells appear to undergo reprogramming, exploiting the stemness machinery for their malignant progression. In line with this hypothesis, many oncogenes, including Notch, Nanog, β-catenin and c-Myc, are also expressed in normal stem cells.[26,27] Moreover, many normal neural stem cell markers are also markers of GSC.[8,22] Importantly, Kim *et al.*[28] found that non-stem cancer cells can be converted to cancer stem-like cells through the IL-6-JAK1-STAT3 signaling pathway, implying that dedifferentiation occurs in cancers. Yang *et al.*[29] also suggested that there is dynamic equilibrium between cancer stem cells and non-stem cancer cells in breast cancer cell populations. Taken together, these previous studies suggest that differentiation is not an irreversible process, at least in cancers, and support our hypothesis that IR stimulates dedifferentiation of glioma cells to GSC through NO production.

In conclusion, we found that irradiation of glioma cells promotes GSC through iNOS-mediated NO production. siRNA-mediated downregulation of iNOS not only effectively attenuated the IR-induced increase in GSC, but also diminished resistance of glioma cells to anticancer treatment. Our observations are consistent with previous studies reporting that NO is selectively produced in $CD133^+$ glioma cells in primary tumor specimens through iNOS and promotes GSC expansion.[16,30] Taken together with previous studies, our demonstration of IR-induced expansion of GSC might reflect dedifferentiation of glioma cells to GSC. These findings suggest that therapeutic targeting of iNOS in combination with IR might increase the efficacy of radiotherapy for glioma treatment.

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Disclosure Statement

The authors have no conflict of interest.

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