

Notch Promotes Radioresistance of Glioma Stem Cells

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ABSTRACT

Radiotherapy represents the most effective nonsurgical treatments for gliomas. However, gliomas are highly radioresistant and recurrence is nearly universal. Results from our laboratory and other groups suggest that cancer stem cells contribute to radioresistance in gliomas and breast cancers. The Notch pathway is critically implicated in stem cell fate determination and cancer. In this study, we show that inhibition of Notch pathway with γ -secretase inhibitors (GSIs) renders the glioma stem cells more sensitive to radiation at clinically relevant doses. GSIs enhance radiation-induced cell death and impair clonogenic survival of glioma stem cells but not non-stem glioma cells.

Expression of the constitutively active intracellular domains of Notch1 or Notch2 protect glioma stem cells against radiation. Notch inhibition with GSIs does not alter the DNA damage response of glioma stem cells after radiation but rather reduces Akt activity and Mcl-1 levels. Finally, knockdown of Notch1 or Notch2 sensitizes glioma stem cells to radiation and impairs xenograft tumor formation. Taken together, our results suggest a critical role of Notch signaling to regulate radioresistance of glioma stem cells. Inhibition of Notch signaling holds promise to improve the efficiency of current radiotherapy in glioma treatment. *STEM CELLS* 2010;28:17–28

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Malignant gliomas are among the most devastating malignancies. Despite recent advances in therapy, treatment of malignant gliomas remains palliative [1–3]. Maximal surgical resection of the tumor mass followed by radiotherapy and chemotherapy is the standard of care. Gliomas often respond to radiotherapy, but subsequent recurrence is almost inevitable, suggesting insufficient killing of tumorigenic cells [2, 4]. Recently, cancer cells with stem cell-like properties have been described in a wide range of human tumors. The cancer stem cell model suggests a hierarchical organization of tumors that a subpopulation of tumor cells at the apex drives and maintains human tumors [5]. Cancer stem cells of brain tumors have been prospectively enriched by selection of the CD133 (Prominin-1) cell surface marker [6–11], although some brain tumors may not express CD133, and hence CD133-negative cells may still have characteristics of cancer stem cells [12, 13]. Despite expression of certain neural and other stem cell markers (such as CD133, Musashi-1, Nestin, Sox2, and Olig2) by brain tumor stem cells, the definition of cancer stem cells

remains functional, requiring sustained self-renewal and tumor propagation. Our laboratory previously demonstrated that glioma stem cells were more resistant to radiation than matched non-stem glioma cells due to preferential activation of the DNA damage response pathway [7]. Other groups also reported that the cancer stem cells of breast cancers were relatively resistant to radiation, potentially due to lower levels of reactive oxygen species found in cancer stem cells [14].

The emerging role of cancer stem cells in tumor response to radiotherapy urges investigation on molecular mechanisms underlying radioresistance of these cells. One of these candidates is the Notch pathway. Notch mediates short-range cellular communication through interaction with ligands presented on neighboring cells [15]. The instrumental roles of Notch in regulation of self-renewal and cell fate determination in normal stem cells have been well established [16]. In mammals, Notch signals through four Notch receptors (Notch 1–4) and five ligands (Jagged-1 and -2, and Delta-like-1, -3, and -4) [17]. Activation of Notch involves sequential proteolytic cleavages that eventually lead to release and nuclear translocation of the intracellular domains of Notch receptors (NICDs) and subsequent activation of Notch-dependent transcription. The

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γ -secretase complex, which mediates the last proteolytic step for release of the NICDs, is essentially required for Notch activation [18, 19]. Inhibitors of γ -secretase (GSIs) are widely used to block Notch signaling *in vitro* and *in vivo*.

The Notch signaling pathway is essentially involved in the maintenance of a variety of adult stem cells, such as breast [20, 21], intestinal [22, 23], and neural stem cells [24–26], through promoting self-renewal and repressing differentiation. Aberrant Notch activity is found in a wide range of human tumors, such as leukemia [27], breast cancer [28, 29], and glioma [30]. Taking into account the instrumental functions of Notch in both stem cells and cancer biology, it is not surprising to find that Notch plays an important role in cancer stem cells [31]. Notch inhibition impairs the self-renewal capacity of mammosphere-initiating cells derived from *in situ* ductal carcinoma [32] and reduces colony formation by leukemic stem cells [33]. Activation of Notch through expression of NICD1 promotes growth and neurosphere formation of the SHG-44 glioma cell line [34]. Notch inhibition by GSI-18 induces apoptosis and differentiation in CD133+ stem-like cells enriched from medulloblastoma cell lines and impairs the tumorigenic capacity of these cells [35]. In this study, we questioned the potential role of Notch in regulating glioma stem cell radioresistance using pharmacological and genetic targeting strategies with an additional focus on potential molecular mechanisms and present data, which suggest that integrated Notch signaling is critically involved in radioresistance of glioma stem cells.

MATERIALS AND METHODS

Cell Culture and Enrichment of Glioma Stem Cells

Matched cultures enriched or depleted for glioma stem cells were isolated from human surgical specimens (T3359, T3691, T4105, T4302, and T4597) that were immediately implanted in immunocompromised mice (athymic BALB/c nu/nu) according to a method that has been described in our previous studies and those of others to preserve cancer stem cells in glioma models [7, 36–40]. Briefly, tumors were dissected, washed in Earle's balanced salt solution, digested with papain (Worthington Biochemical, Lakewood, NJ, <http://www.worthington-biochem.com>) and filtered through 70- μ m cell strainers to remove tissue pieces. Red blood cells were lysed in diluted phosphate-buffered saline solution (0.25 \times). Dissociated cells were then cultured overnight in stem cell media (neurobasal media supplemented with B27, epidermal growth factor, and basic fibroblast growth factor at 20 ng/ml; Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) before cell sorting for recovery of cellular surface antigens. The CD133-negative and CD133+ fractions were separated by magnetic sorting using the CD133 Microbead kit (Miltenyi Biotec, Bergisch Gladbach, Germany, <http://www.miltenyibiotec.com>). CD133-negative cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) but were cultured in stem cell media at least 24 hours prior to experiments to control differences in cell media. The cancer stem cell properties of the CD133+ cells were confirmed with fluorescent *in situ* hybridization, serial neurosphere assays, and xenotransplantation assays, but cultures depleted of cancer stem cells did not initiate tumors ([7, 36–40] and data not shown).

Antibodies

The antibodies purchased from Cell Signaling Technology (Beverly, MA, <http://www.cellsignal.com>) include phosphor-S296-Chk1 (#2349), total Chk1 (#2345), phosphor-T68-Chk2 (#2661), total Chk2 (#2662), phospho-S473 Akt (#9271), total Akt (#2920), PTEN (#9556), phospho-S15-p53 (#9286), Bcl-2 (#2870), Bcl-xL

(#2764), Mcl-1 (#4572), and Puma (#4976). Other antibodies used in this study are p53 antibody (#sc-126, Santa Cruz Biotechnology Inc., Santa Cruz, CA, <http://www.scbt.com>) and actin antibody (Millipore, Billerica, MA, <http://www.millipore.com>).

Radiation

Cells were irradiated as a single-cell suspension for CD133+ cells or as monolayer culture for CD133-negative cells using an AGFA X-RAD 320 irradiation system at 0.85 Gy/min.

Growth Curve, Caspase 3/7 Assay, and Clonogenic Survival Assay

Cells were aliquoted into a 96-well plate at 3,000 cells per well in triplicate immediately after treatments. Cell number was measured daily for 5 days using the CellTiter-Glo assay kit (Promega, Madison, WI, <http://www.promega.com>). Averaged cell titer of the sham-treated control group on day 1 was assigned a value of 1. All other relative cell titers were normalized accordingly.

The caspase 3/7 activities at 48 or 72 hours after radiation were measured using the Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions. The caspase activities were normalized to the corresponding cell titers to generate the relative caspase 3/7 activities.

To measure clonogenic survival, CD133+ glioma cells were plated in 24-well plates at 50 cells per well for the sham-irradiated groups, 100 cells per well for cells irradiated at 1 Gy, or 500 cells per well for cells irradiated at 2 or 3 Gy. Eight wells were plated for each group. At 14 days after plating, neurospheres containing more than 50 cells were scored. Alternatively, CD133-negative glioma cells were plated in 6-well tissue culture plates in triplicates at 100 cells per well for the sham-irradiated groups, 200 cells per well for cells irradiated at 1 Gy, or 500 cells per well for cells irradiated at 2 or 3 Gy. At 21 days after radiation, cells were fixed and stained with 0.5% crystal violet. Colonies consisting of more than 50 cells were scored. To determine the surviving fractions, the number of neurospheres at each radiation dose was normalized to that of the corresponding sham-irradiated group.

RESULTS

GSIs Impair Cell Growth and Clonogenic Survival of Glioma Stem Cells in Combination with Radiation

Accumulating evidence suggests that the Notch pathway plays important roles in a wide range of human tumors as well as cancer stem cells [31, 41]. Because a recent study reported that radiation induced transient upregulation of Notch pathway components in endothelial cells [42], we investigated whether Notch activity is involved in radiation response of glioma stem cells. Short-term cultures enriched for glioma stem cells were isolated from surgical biopsy specimens and amplified because direct xenografts that have been demonstrated to maintain a cancer stem cell phenotype [39]. The stem cell-like properties of the CD133+ glioma cells specifically used in this study, including sustained self-renewal, expression of stem cell markers, multilineage differentiation, and the ability to recapitulate the original tumors in serial xenotransplantation assays, have been demonstrated in our previous publications [6, 7, 36–38, 40]. In T3359, T4105, and T4302 CD133+ glioma cells, radiation induced modest transcription activation from a Notch-responsive luciferase reporter (RBP-J κ reporter; SABiosciences, Frederick, MD, <http://www.sabiosciences.com>) but not from a control reporter (SABiosciences) (Fig. 1A, and supporting information Figure 1A–1C). Expression of Notch target genes, including Hes2 and, to a lesser degree, Hes4 and Hes5, was increased after

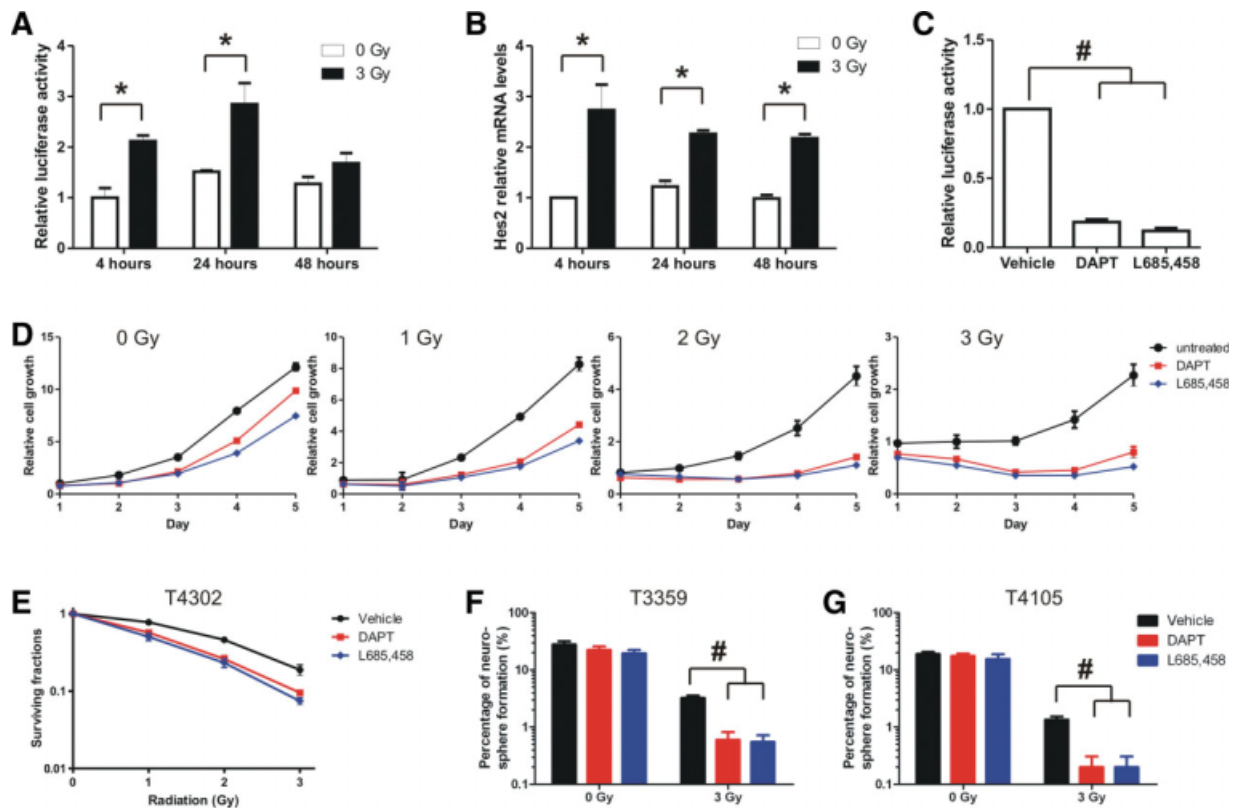


Figure 1. γ -Secretase inhibitors (GSIs) reduce cell growth and clonogenic survival of glioma stem cells after radiation. (A): T4302 CD133+ glioma stem cells were transfected with a Notch-responsive luciferase reporter (RBP-J κ reporter) premixed with the vector directing constitutive expression of *Renilla* luciferase. Cells were left unirradiated or irradiated at 3 Gy and were then collected to measure luciferase activities at the time points as indicated. In this and subsequent luciferase assays, firefly luciferase activity was normalized to the internal control *Renilla* luciferase activity, and the normalized firefly luciferase activity from the sham-irradiated control group was assigned a value of 1. Data are presented as the mean \pm standard error. (B): T4302 CD133+ glioma stem cells were left unirradiated or irradiated at 3 Gy. The relative mRNA levels of Hes2 were determined by quantitative real-time polymerase chain reaction at the time points as indicated. (C): T4302 CD133+ cells were transfected with the RBP-J κ reporter and treated with dimethyl sulfoxide (vehicle), 2 μ M DAPT, or 0.5 μ M L685,458 overnight, and cells were then collected for luciferase assays. (D): T4302 CD133+ cells were pretreated with 2 μ M DAPT or 0.5 μ M L685,458 for 4 hours and irradiated as indicated. Total viable cell numbers were then determined with a cell viability assay for the following 5 days. The averaged cell titers on day 1 of the control groups were assigned a value of 1. (E): T4302 CD133+ cells were pretreated and irradiated as described in (D). Cells were then plated to form neurospheres, and the surviving fractions were determined as described in Materials and Methods. The mean \pm standard error was derived from eight replicates within a representative experiment. (F,G): Neurosphere formation of T3359 and T4105 CD133+ cells treat with GSIs alone, 3 Gy radiation alone, or the combination was determined as described in (E). * P < .05 by Student's *t* test; # P < .001 by one-way ANOVA. Abbreviation: DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*l*-alanyl]-*S*-phenylglycine *t*-butyl ester.

radiation exposure (Fig. 1B, and supporting information Figure 1D–1G). Notch signaling in glioma stem cells was blocked by GSIs, *N*-[*N*-(3,5-difluorophenacetyl)-*l*-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), or L685,458, as shown by Notch-responsive luciferase reporter assays (Fig. 1C). Using CD133+ glioma cells derived from T4302 and other glioma sources, including T3359, T4105, and T4597 (Fig. 1D and data not shown), we showed that GSIs moderately reduced growth of CD133+ glioma cells in the absence of radiation (Fig. 1D, graph 1) but severely impaired growth of cells exposed to radiation at either 1, 2, or 3 Gy (Fig. 1D, graphs 2–4). On the fifth day after 2 Gy radiation, total cell numbers in the sham-treated groups were approximately four-fold more than that in GSI-treated groups. In contrast, without radiation treatment, sham-treated cells were only 25% (vs. DAPT-treated) or 60% (vs. L685,458-treated) more than GSI-treated cells 5 days after radiation.

Clonogenic survival assay is the standard assay to assess radiation responsiveness in vitro. In this study, we used neurosphere formation as a surrogate marker of the clonogenic survival of glioma stem cells. In combination with radiation,

GSIs significantly reduced clonogenic survival of the T4302 CD133+ glioma cells compared with the sham-treated groups for all radiation doses examined (Fig. 1E; P = .012 at 1 Gy and P < .001 at 2 and 3 Gy by one-way ANOVA). Similar results were demonstrated using CD133+ cells derived from other glioma sources, such as T3359, T4105, and T4597 (Fig. 1F, 1G, and supporting information Figure 2A, 2B). Taken together, these results strongly suggest that Notch inhibition with GSIs compromises radioresistance of glioma stem cells.

GSIs Enhance Radiation-Induced Cell Death in Glioma Stem Cells

Significantly greater radiation-induced cell death was observed in GSI-treated cells compared with sham-treated cells. Two days after 3 Gy radiation, sham-treated T4302 CD133+ cells contained approximately 30% dead cells, whereas 60% of cells were dead in the presence of DAPT or L685,458 (Fig. 2A). Conversely, GSIs did not have an obvious impact on the percentage of dead cells in the absence of radiation (Fig. 2A). The majority of GSI-treated cells died

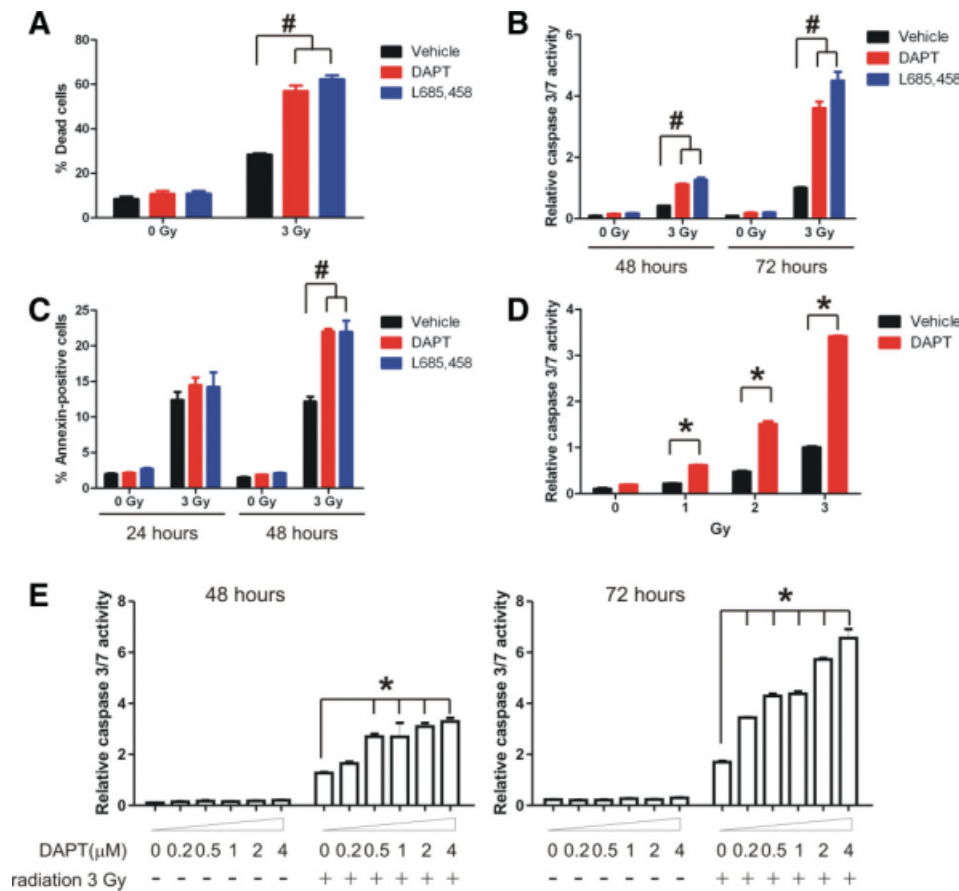


Figure 2. γ -Secretase inhibitors (GSIs) enhance radiation-induced cell death in glioma stem cells. (A): T4302 CD133+ cells were pretreated with DAPT or L685,458 for 4 hours and irradiated at 3 Gy. Percentage of dead cells was determined with trypan blue exclusion assay at 48 hours after radiation exposure. (B): T4302 CD133+ cells were treated with GSIs \pm radiation as indicated. Relative caspase 3/7 activities were determined by normalizing caspase activities to the corresponding cell titers. (C): T4302 CD133+ cells were treated as described in (A). At 24 or 48 hours after radiation exposure, cells were labeled with FITC-conjugated annexin V staining and analyzed with flow cytometry. (D): T4302 CD133+ cells were pretreated with 2 μ M DAPT \pm radiation as indicated. Relative caspase 3/7 activities were determined at 72 hours after radiation. (E) T4302 CD133+ cells were treated with DAPT at indicated concentrations for 4 hours and irradiated at 3 Gy. Relative caspase activities were determined at 48 or 72 hours after radiation. * $P < .01$ by Student's *t* test; # $P < .01$ by one-way ANOVA. Abbreviation: DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*l*-alaninyl]-5-phenylglycine *t*-butyl ester.

at 72 hours after 3 Gy of irradiation. However, an excessive amount of cell debris interfered with the trypan blue exclusion assay at this time point. Activation of caspase is a hallmark of apoptotic cell death and is critically involved in radioresponse [43–46]. Using luminescence-based caspase-3/7 assays, we examined how Notch inhibition affected radiation-induced caspase activation as a surrogate marker of cell death. GSIs alone did not significantly alter caspase activities in the sham-irradiated T4302 CD133+ cells (Fig. 2B; $P > .05$ by one-way ANOVA). In contrast, combining GSIs with 3 Gy radiation increased caspase activities by approximately twofold at 48 hours and by fourfold at 72 hours (Fig. 2B; $P < .01$ by one-way ANOVA). Similar results were detected using CD133+ glioma cells derived from several other glioma sources, including T3359, T3691, T4105, and T4597 (supporting information Figure 3 and data not shown). In validation studies, apoptotic cell death was assessed by flow cytometry analysis of annexin V, a cell surface marker for apoptosis. Both DAPT and L685,458 significantly increased annexin V-positive labeled cells at 48 hours after radiation (Fig. 2C; $P < .01$ by one-way ANOVA), although the effects were not obvious at 24 hours after radiation (Fig. 2C; $P > .05$ by one-way ANOVA). Additionally, DAPT enhanced radiation-induced

cell death at different radiation doses (Fig. 2D). The radiosensitizing effects of DAPT showed a concentration-dependent effect, with 200 nM DAPT (50% inhibition concentration as suggested by the manufacturer) still being effective (Fig. 2E). Collectively, these data demonstrate that Notch inhibition using GSIs sensitize glioma stem cells to radiation-induced cell death.

Expression of the Constitutively Active Intracellular Domains of Notch1 or Notch2 Attenuates the Radiosensitizing Effects of GSIs

Substrates of GSIs include more than 50 type I membrane proteins in addition to the Notch receptors [47]. The radiosensitizing effects of GSIs are not necessarily mediated through inhibition of Notch. We therefore validated the role of Notch in radioresponse of glioma stem cells by expression of the constitutively active intracellular domains of Notch1 or Notch2 (NICD1 or NICD2) that function downstream of GSIs. Activation of the Notch pathway by lentivirus-mediated expression of NICD1 or NICD2 was validated by upregulation of Notch target genes, including *Hes2*, *Hes4*, and *Hes5* (Fig. 3A), as well as increased luciferase activities generated

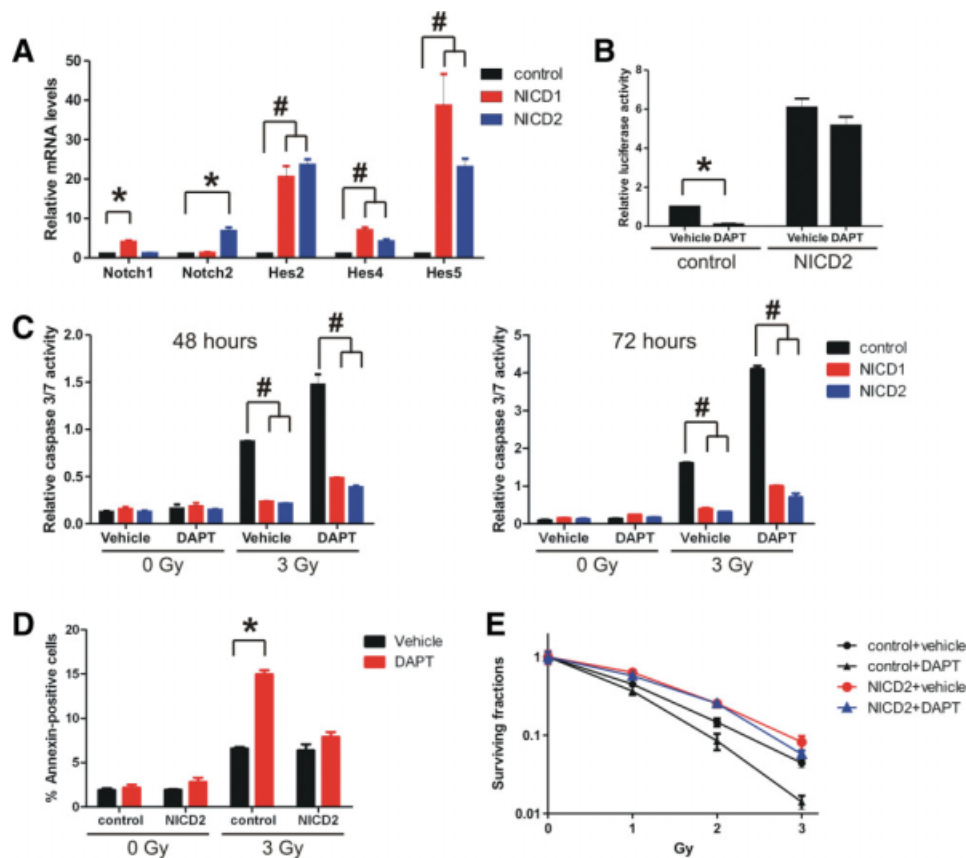


Figure 3. Expression of the intracellular domains of Notch1 or Notch2 attenuates the radiosensitizing effects of γ -secretase inhibitors (GSIs). T4302 CD133+ glioma cells were infected with control lentivirus or lentivirus directing expression of NICD1 or NICD2 and selected by puromycin. (A): Expression of Notch1, Notch2, Hes2, Hes4, and Hes5 were determined by quantitative real-time polymerase chain reaction. (B): Control cells or cells expressing NICD2 were transfected with Notch-dependent luciferase reporter and treated with dimethyl sulfoxide or 2 μ M DAPT overnight. Notch-dependent transcription activities were determined by luciferase reporter assays as described in Figure 1. (C): Control cells or cells expressing NICDs were treated with DAPT \pm radiation as indicated. Relative caspase 3/7 activities were determined at 48 or 72 hours after radiation. (D): Apoptotic cell death was determined with annexin V staining as described in Figure 2. (E): Surviving fractions were determined as described in Figure 1. * $P < .01$ by Student's t test; # $P < .001$ by one-way ANOVA. Abbreviations: DAPT, N -[N -(3,5-difluorophenacetyl)- l -alanyl]- S -phenylglycine t -butyl ester; NICD, Notch receptor intracellular domain.

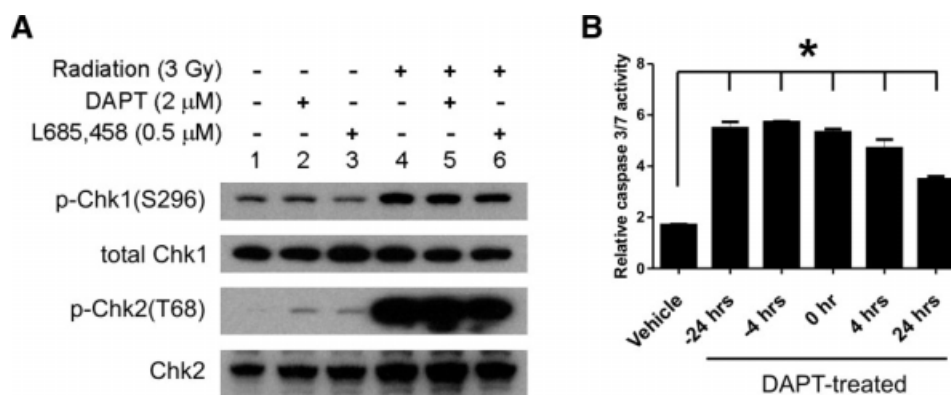


Figure 4. Notch pathway does not alter DNA damage response of glioma stem cells. (A): T4302 CD133+ cells were pretreated with DAPT or L685,458 for 24 hours before radiation. At 1 hour after radiation exposure, cells were harvested. The levels of activating phosphorylation of Chk1 and Chk2 were assessed by immunoblotting. (B): T4302 CD133+ cells were treated with 2 μ M DAPT at 4 and 24 hours before radiation (–24 hours or –4 hours) or at 0, 4, and 24 hours after radiation. Relative caspase 3/7 activities were measured 3 days after radiation exposure. * $P < .001$ by Student's t test. Abbreviation: DAPT, N -[N -(3,5-difluorophenacetyl)- l -alanyl]- S -phenylglycine t -butyl ester.

from the Notch-responsive reporter (Fig. 3B). Similar to the parental cells, cells infected with the control lentivirus were sensitive to DAPT when irradiated (Fig. 3C). In contrast, cells

expressing either NICD1 or NICD2 demonstrated significantly weaker radiation-induced cell death in comparison to control cells regardless of DAPT treatment (Fig. 3C). Additionally,

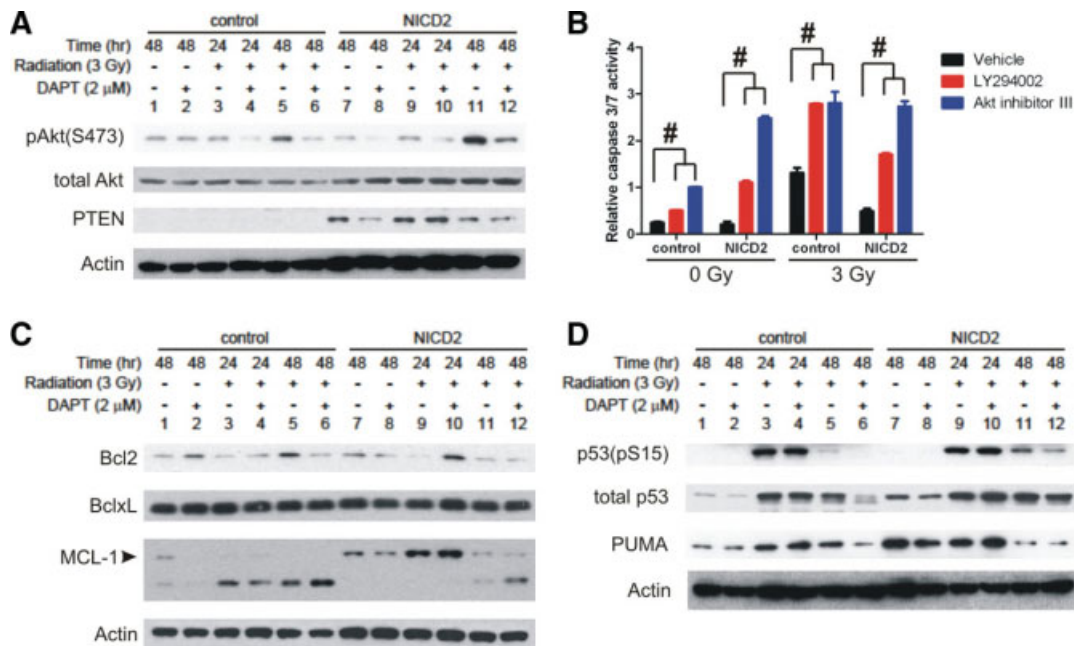


Figure 5. Notch pathway regulates radioresistance of glioma stem cells via the Akt pathway and Mcl-1. T4302 CD133+ cells were infected with control lentivirus or lentivirus directing expression of NICD2. Cells were pretreated with or without 2 μ M DAPT for 4 hours and exposed to 3 Gy radiation. Irradiated cells were collected at 24 or 48 hours after radiation, and sham-irradiated cells were collected at 48 hours after radiation. (A, C, D): Protein levels of phospho-Akt (S473), total Akt, PTEN, Bcl-2, Bcl-xL, Mcl-1, phospho-p53 (S15), total p53, and PUMA were assessed by immunoblotting. Actin was blotted as the loading control. (B): Control cells or NICD2-expressing cells were pretreated with 10 μ M PI3K inhibitor, 10 μ M LY294002, or 50 μ M Akt inhibitor III (SH-6), for 4 hours before radiation exposure. Relative caspase 3/7 activities were determined at 48 hours after radiation exposure. $^{\#}P < .001$ by one-way ANOVA. Abbreviations: DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*l*-alanyl]-*S*-phenylglycine *t*-butyl ester; NICD, Notch receptor intracellular domain.

DAPT treatment failed to increase the percentage of annexin V-positive cells in the presence of NICD2 expression (Fig. 3D). Finally, expression of NICD2 improved clonogenic survival of the T4302 glioma stem cells after radiation irrespective of DAPT treatment (Fig. 4E; $P = .004$, NICD2 vs. control \pm DAPT; $P < .01$, NICD2 + DAPT vs. control \pm DAPT, at 3 Gy radiation by one-way ANOVA), whereas DAPT treatment did not significantly alter clonogenic survival of irradiated NICD2-expressing cells (Fig. 4E; $P > .05$, NICD2 vs. NICD2+DAPT, by Student's *t* test). Additionally, expression of NICD1 or NICD2 protected glioma stem cells derived from T3359 and T4105 in a similar manner (supporting information Figure S4 and data not shown). Taken together, our data demonstrate that Notch activation by expression of NICDs attenuates the radiosensitizing effects of GSIs, suggesting that these activities of GSIs are crucially mediated through inhibition of Notch.

Notch Pathway Does Not Alter DNA Damage Response of Glioma Stem Cells

The DNA damage checkpoint response plays a critical role in cellular response to radiation [48, 49]. Our previous study showed that increased activation of the DNA damage response is implicated in radioresistance of the glioma stem cells [7]. To determine the mechanisms through which Notch promotes radioresistance of glioma stem cells, we first assessed whether the Notch pathway affected activation of the checkpoint kinases after radiation. Neither DAPT nor L685,458 significantly altered activating phosphorylation of Chk1 or Chk2 after radiation (Fig. 4A). Additionally, in either T4105 or T4302 CD133+ glioma cells, DAPT increased radiation-induced cell death even when it was added 24 hours after radiation (Fig. 4B, and supporting information Figure 5),

whereas the bulk DNA damage repair process is usually completed within a few hours after radiation exposure [50]. These results suggest that the DNA damage response pathway is not critically involved in the radioprotective activities of Notch.

Notch Promotes Radioresistance Through Regulation of the PI3K/Akt Pathway and Mcl-1

Notch inhibition renders glioma stem cells more sensitive to radiation-induced cell death. In a context-dependent manner, Notch can promote cellular survival through different mechanisms, such as activation of the PI3K/Akt pathway [51, 52] and upregulation of the prosurvival proteins Bcl-2 and Mcl-1 [53]. We compared alterations of these prosurvival factors between the NICD2-expressing glioma stem cells and the control cells treated with DAPT, radiation, or both. Activating phosphorylation at serine 473 of Akt was used as a surrogate marker of Akt activity. While radiation exposure induced Akt activation as shown by increased levels of phospho-S473 Akt, DAPT treatment reduced Akt activation (Fig. 5A). In contrast, expression of NICD2 increased Akt activities, which was partially attenuated but not abolished by DAPT (Fig. 5A). Similar results were found in other short-term glioma cultures, such as T3359 and T4105 (data not shown). Our laboratory recently reported that Akt activity was essential for survival of glioma stem cells [37]. In agreement with this finding, treatment of the PI3K inhibitor LY294002 or the Akt inhibitor III increased glioma stem cell death with or without radiation (Fig. 5B). Treatment with LY294002 or the Akt inhibitor reduced phosphorylation at S473 of Akt (supporting information Figure 6). Expression of NICD2 did not overcome the apoptotic effects of either LY294002 or the Akt inhibitor III,

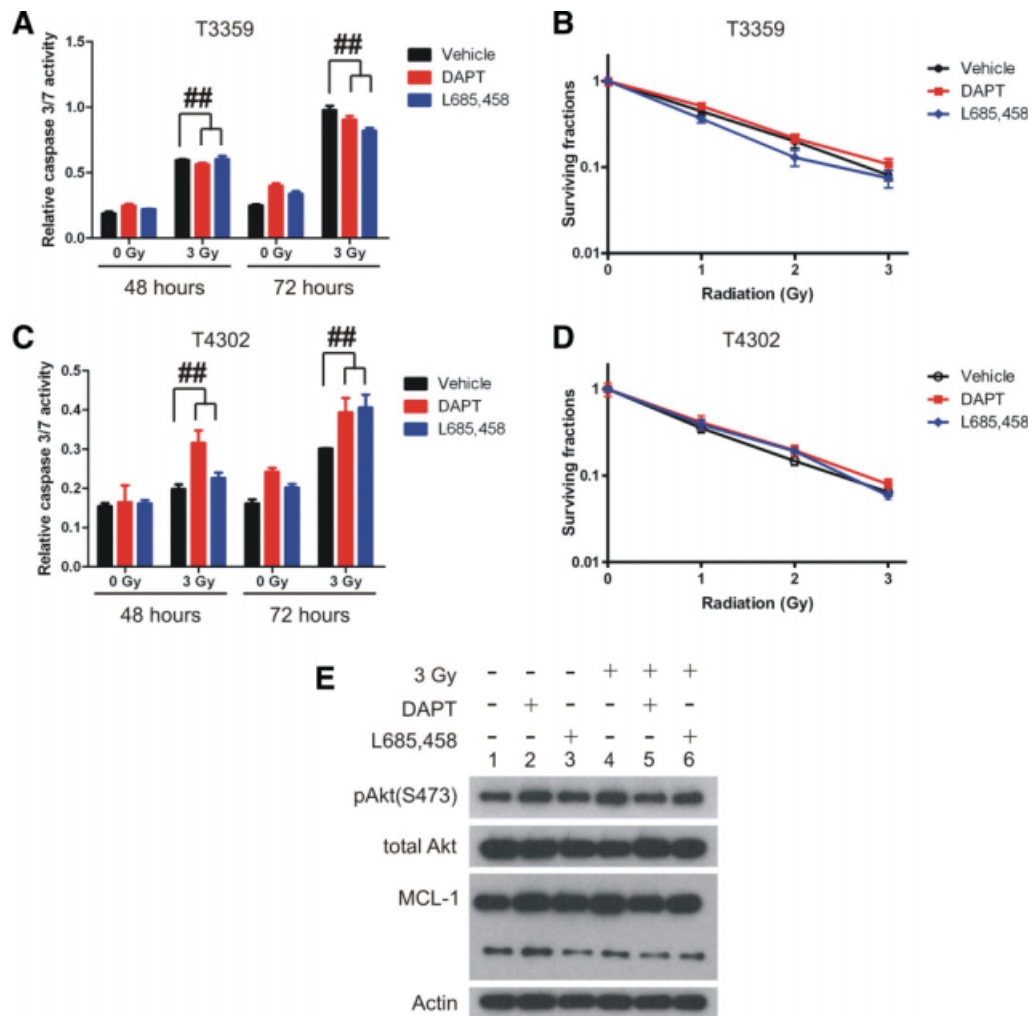


Figure 6. Notch inhibition by γ -secretase inhibitors (GSIs) does not significantly alter radiation response of CD133-negative glioma cells. T3359 CD133-negative cells were pretreated with 2 μ M GSI or 0.5 μ M L685,458 for 4 hours and irradiated at 3 Gy or as indicated. (A): Relative caspase 3/7 activities were determined at 48 or 72 hours after radiation as described in Figure 2. (B): After radiation, cells in 6-well plates were cultured for 3 weeks and then fixed and stained to count colonies. Surviving fractions were determined as described in Materials and Methods. (C, D): The effects of GSIs on cell death and clonogenic survival of T4302 CD133-negative cells were determined as described above. $^{###}P > .05$ by one-way ANOVA. (E): T4302 CD133-negative cells were treated with GSIs \pm radiation as described above. Levels of phospho-Akt (S473), total Akt, Mcl-1, and actin were determined by immunoblotting.

suggesting that the PI3K/Akt pathway functions downstream of Notch (Fig. 5B).

The anti-apoptotic Bcl-2 proteins, such as Bcl-2, Bcl-xL, and Mcl-1, are important promoters for cell survival [54]. The levels of Bcl-2 and Bcl-xL were not apparently altered by either radiation or Notch alteration (Fig. 5C). Conversely, radiation exposure or DAPT treatment decreased the levels of Mcl-1, and expression of NICD2 increased Mcl-1 levels even in the presence of DAPT (Fig. 5C). Interestingly, radiation or DAPT increased the levels of Mcl-1 proteins of a smaller size (Fig. 5C). These bands could represent either proteolytically cleaved Mcl-1 proteins generated during apoptosis or a truncated isoform of Mcl-1 (Mcl-1_S), which is pro-apoptotic due to loss of BH1 and BH2 domains [55].

The functional interaction between Notch and p53 is intriguing, as Notch can activate p53 [56, 57] or inhibit p53 [58] in a cell type-specific manner. As a crucial regulator of cellular radioresponse, p53 activity may be involved in the radioprotective function of Notch. We saw that expression of NICD2 increased p53 levels in the absence of radiation and

further enhanced p53 activation induced by radiation (Fig. 5D). Additionally, expression of the p53 target gene PUMA was upregulated by NICD2 (Fig. 5D). These results are consistent with a previous study showing that Notch1 upregulates p53 in gliomas [57] and suggest that the radioprotective functions of Notch are not mediated by inhibition of p53.

CD133-Negative Glioma Cells Do Not Respond to GSIs

We further examined whether Notch inhibition affected radioresponse of the CD133-negative fraction of gliomas. After similar procedures, cell death and clonogenic survival after radiation exposure were determined in the T3359 and T4302 CD133-negative cells with and without GSI treatment. In contrast to glioma stem cells, caspase activation or clonogenic survival of the CD133-negative glioma cells were not significantly altered by Notch inhibition using GSIs irrespective of radiation exposure (Fig. 6A–6D). Additionally, GSI treatment did not alter Akt activities or Mcl-1 levels in the CD133-

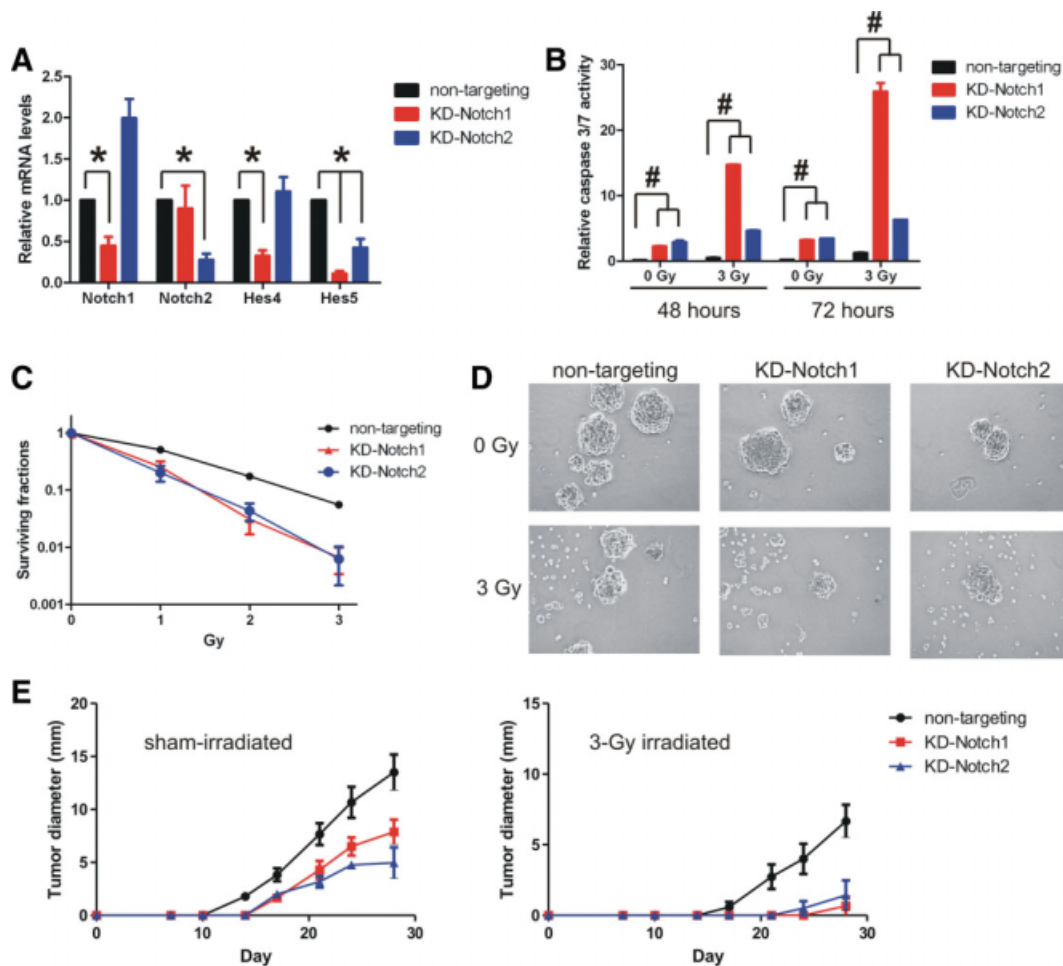


Figure 7. Knockdown of Notch1 or Notch2 increases radiosensitivity of glioma stem cells in vitro and in vivo. T4302 CD133+ cells were infected with lentivirus directing expression of nontargeting shRNA or shRNAs specific to Notch1 or Notch2 (KD-Notch1 or KD-Notch2) and selected with puromycin. (A): Relative mRNA levels of Notch1, Notch2, Hes4, or Hes5 were determined with quantitative real-time polymerase chain reaction. * $P < .05$ by Student's t test. (B): Relative caspase 3/7 activities were determined in control cells or cells with Notch knockdown treated with or without radiation. # $P < .001$ by one-way ANOVA. (C): Clonogenic survival was compared between the control group and cells with Notch knockdown as described in Figure 1. (D): Representative images ($\times 100$) of neurospheres formed by T4302 CD133+ cells with or without Notch knockdown 2 weeks after radiation. (E): After lentivirus infection and puromycin selection, T4302 CD133+ cells were left unirradiated or irradiated at 3 Gy, and they were immediately injected into both flanks of athymic nude mice at 250,000 cells per injection. Three mice were inoculated for a total of six injections per group. Tumor growth was monitored twice a week for 4 weeks until diameters of the largest tumors reached approximately 20 mm.

negative glioma cells (Fig. 6E). Taken together, our data suggest that the radiosensitizing effects of Notch inhibition are specific to the cancer stem cell fraction of gliomas.

Knockdown of Notch1 or Notch2 Sensitizes Glioma Stem Cells to Radiation and Impairs Xenograft Tumor Formation

Individual Notch receptors may have distinct biological functions in certain tumor types [59]. It has been demonstrated that expression of the constitutively intracellular regions of Notch1 or Notch2 appears to exert opposite effects in medulloblastomas, with Notch1 acting as a tumor suppressor and Notch2 acting as a tumor promoter [60]. In gliomas, knockdown of Notch1 impairs cell growth and survival [61]. By expression of NICD1 or NICD2, our results suggest that both Notch receptors promote radioresistance of glioma stem cells. To gain a better understanding of how individual Notch receptors are involved in radioresponse, we determined the effects of knockdown of Notch1 or Notch2 on radioresistance

through lentivirus-mediated expression of shRNA sequences targeting Notch1 or Notch2. The efficiency of downregulation of the corresponding Notch receptors and Notch target genes were determined by quantitative real-time polymerase chain reaction (Fig. 7A). Consistent with previous report, knockdown of Notch1 alone increased apoptotic cell death in T3359 and T4302 CD133+ cells. Interestingly, similar effects were achieved by knockdown of Notch2 (Fig. 7B, and supporting information Figure 7A). Cells with reduced Notch1 or Notch2 expression were highly sensitive to radiation-induced cell death (Fig. 7B, and supporting information Figure 7A). Consistently, the postradiation clonogenic survival of glioma stem cells was severely impaired by knockdown of either Notch1 or Notch2 (Fig. 7C, and supporting information Figure 7B). When exposed to 3 Gy radiation, glioma stem cells expressing shRNA specific to Notch1 or Notch2 rarely formed neurospheres (Fig. 7C, 7D). Additionally, knockdown of Notch1 or Notch2 alone reduced cell viability of the CD133-negative glioma cells but did not significantly increase radiation-induced cell death (supporting information Figure 7C; P

> .05, irradiated groups vs. corresponding sham-irradiated groups by Student's *t* test). These results further validate the specific role of Notch pathway in regulation of radioresistance and suggest that multiple Notch receptors may be involved in radiation response of glioma stem cells.

We examined how impaired Notch signaling affected radiation response of glioma stem cells *in vivo* using a flank xenograft model. Control T4302 CD133+ glioma cells or cells with reduced Notch1 or Notch2 expression were subcutaneously inoculated into both flanks of nude mice without radiation exposure or immediately after 3 Gy radiation exposure. The sham-irradiated control cells formed palpable tumors at all injection sites at day 14 after injection (Fig. 7E, and supporting information Table 1). Knockdown of either Notch1 or Notch2 alone modestly extended tumor latency (Fig. 7E). Tumors formed by irradiated control cells were first detected at day 17, and all became palpable at day 21 (Fig. 7E). In combination with radiation, knockdown of Notch1 or Notch2 not only extended the tumor latency (Fig. 7E) but also significantly decreased tumor incidence (1 of 6 for knockdown of Notch1, and 2 of 6 for knockdown of Notch2; supporting information Table 1). Taken together, our results suggest that integrated Notch signaling is critically implicated in radiation resistance of glioma stem cells *in vivo*.

DISCUSSION

Fundamental improvements in brain cancer treatment will require the development of new therapeutic paradigms. Temozolomide, an oral methylating chemotherapeutic agent, became the standard of care for newly diagnosed glioblastomas when used concurrently with external beam radiation followed by adjuvant therapy [3], suggesting that combining chemotherapies with radiation may shift the survival curve. Despite the benefit of temozolomide, glioblastomas continue to be highly resistant to radiation [4]. We recently demonstrated that the cancer stem cell fraction of gliomas was more resistant to radiation than non-stem glioma cells [7]. Given the high tumorigenic capacity of glioma stem cells, this paradigm suggests that disrupting radioresistance of glioma stem cells may augment the efficacy of radiotherapy. We have already shown that inhibitors of the checkpoint kinases, Chk1 and Chk2, sensitized glioma stem cells to radiation [7]. However, DNA damage checkpoint inhibitors may have limited therapeutic index due to shared dependence of normal cells on these molecules in radioresponse [62]. In this study, we described a novel radioprotective role of Notch signaling in glioma stem cells. Our results show that radiation induces Notch activation, and Notch inhibition by GSIs or Notch1/2-specific shRNA renders glioma stem cells more sensitive to radiation. In further support of these observations, activation of Notch via expression of NICD1 or NICD2 promotes radioresistance of glioma stem cells. Collectively, these pharmacological and genetic approaches show that Notch activity is critically implicated in radioresistance of glioma stem cells, suggesting that the Notch pathway may serve as a potential therapeutic target for improvement of radiotherapy against gliomas.

The oncogenic role of Notch is highlighted by the presence of mutations of the Notch pathway components in a variety of human tumors, in particular leukemia and breast cancer [63]. Upregulation of Notch pathway components also presents in glioblastoma multiforme cell lines and surgical biopsy specimens [30]. A critical role of the Notch pathway in the maintenance of brain tumor stem cells has recently

emerged [41]. Our results suggest that the radioprotective functions of Notch are specific to the CD133+ glioma stem cells. Neither cell death nor clonogenic survival of the non-stem glioma cells are significantly altered by GSI treatment irrespective of radiation exposure. Knockdown of Notch1 or Notch2 modestly impaired cell viability in the non-stem glioma cells, but did not alter radiation-induced cell death. These studies suggest that stem cell-specific pathways, such as Notch, can serve as potential therapeutic targets that may not be readily appreciated by studies using whole tumors.

The Notch pathway can promote or repress tumorigenesis in a context-dependent manner. For example, loss of Notch1 or expression of a dominant negative MAML1 mutant in mouse epidermis induces hyperplasia and subsequent skin tumors [64, 65]. In medulloblastomas, expression of NICD2 promotes tumor growth, whereas NICD1 is tumor suppressive [60]. Conversely, knockdown of Notch1 impaired proliferation and survival of glioma cell lines [61]. Using lentivirus-mediated expression of NICD1/2 or multiple Notch1/2-specific shRNAs, our data suggest that both Notch1 and Notch2 promote radioresistance in glioma stem cells, although the relative contribution of each Notch receptor is undefined. It remains to be elucidated why knockdown of individual Notch receptors leads to moderate loss of cell viability in glioma stem cells in the absence of radiation, whereas blockage of Notch signaling using GSIs only reduces cell growth. These effects may represent sustained inhibition of Notch by stable expression of shRNAs, off-target activities of the shRNAs, or oversaturation of the microRNA/shRNA pathways [66]. Alternatively, membrane-bound Notch receptors may have important biological functions in gliomas that have not been fully recognized, as a recent report suggests that membrane-anchored Notch1 activates Akt in a transcription-independent manner [67]. The specific role of each individual Notch receptor in regulation of radioresistance of glioma stem cells is beyond the scope of this study, but there is substantial interest with regard to the therapeutic potential of Notch inhibition because specific Notch neutralizing antibodies are under clinical development for systemic cancer treatment and may have distinct adverse effects in a therapeutic setting. Notch1 is a key regulator of intestinal stem cell fate determination and gut homeostasis [22, 23]. Clinical trials using GSIs for treatment of Alzheimer's disease are primarily associated with gastrointestinal toxicity [68, 69], which is likely due to inhibition of Notch1 by GSIs. If Notch receptors other than Notch1 are also essentially required for radioresistance of glioma stem cells, small molecules or antibodies that specifically target these receptors while sparing Notch1 may achieve similar radiosensitizing effects in gliomas with reduced gut toxicity.

Our results demonstrate that Notch inhibition in irradiated glioma stem cells dramatically increased cell death within 3 days after radiation, suggesting that Notch is crucially involved in postradiation survival of glioma stem cells. In accordance with these observations, Notch upregulates the pro-survival factors Akt and Mcl-1 in glioma stem cells. It has been reported that Mcl-1 is a critical regulator of survival in neural precursor cells during development and in response to DNA damage [53, 70]. Akt is known to upregulate Mcl-1 levels [71, 72]. Therefore, the regulation of Mcl-1 by Notch can be potentially mediated through Akt. The PI3K/Akt pathway plays a central role in the radioprotective functions of Notch, as shown by our results that inhibitors of PI3K or Akt abolish the radioprotective activities of NICD2. This pathway has profound functions in many aspects of cell growth, proliferation, and survival [73]. We previously described an important link between the PI3K/Akt pathway with growth and survival

of glioma stem cells [37]. Confirmation of these findings came from studies of a mouse medulloblastoma model in which the PI3K/Akt pathway is activated in medulloblastoma stem cells after radiation and is essentially required for postradiation cell survival [74]. In addition, inhibition of Akt sensitizes the medulloblastoma stem cells to radiation [74]. The mechanisms through which Notch activates the PI3K/Akt pathway in glioma stem cells remain to be elucidated. Although Notch can activate Akt through negatively regulated PTEN in T-cell acute lymphoblastic leukemia [51], our results show that expression of NICD2 upregulated PTEN irrespective of radiation exposure, suggesting that Notch does not activate the PI3K/Akt pathway by repressing PTEN. Alternatively, the Src-related tyrosine kinase p56lck has been reported to mediate Notch-induced Akt activation [52]. Our results show that neither a p56lck-specific inhibitor nor a pan-Src family kinase inhibitor altered radiation response of glioma stem cells (data not shown), suggesting that Src family tyrosine kinases are not implicated in radiation response of glioma stem cells. Interestingly, a recent study reports a non-canonical transcription-independent Notch function that membrane-anchored Notch1 is sufficient to activate Akt [67]. This novel mechanism may be implicated in Akt activation by Notch in glioma stem cells, but details of this functional interaction remain to be determined. Our data show that NICD2 upregulated PTEN and p53 in glioma stem cells instead of repressing these two tumor suppressors, suggesting that these two proteins may not be directly involved in the radioprotective functions of Notch. However, postradiation survival of medulloblastoma stem cells can be promoted by cell cycle arrest mediated through PTEN and p53 [74]. Therefore, it is possible that, in glioma stem cells, Notch-induced PTEN and p53 indirectly contribute to reduced cell death after radiation by promoting cell cycle arrest. Taken together, our study suggests a Notch-regulated signaling pathway that is critically involved in radiation response of glioma stem cells. This pathway provides multiple potential therapeutic targets at several levels, including the Notch receptors, the kinases (PI3K, Akt), and Mcl-1, a molecule that directly regulates the apoptosis cascade.

The last proteolytic step that generates the active intracellular domains of Notch receptors is dependent on the γ -secretase complex [18, 19]. A substantial number of GSIs have been developed for treatment of Alzheimer's disease because the γ -secretase complex mediates production of β -amyloid peptides, the precursor of amyloid plaques found in Alzheimer's disease [75]. Given their activities of inhibiting Notch, GSIs have also been actively examined for their anti-cancer efficacy in a variety of tumor types, such as T-cell acute lym-

phoblastic leukemia [76], breast cancer [77], Kaposi's sarcoma [78], medulloblastoma [79], intestinal adenoma [23], etc. Clinical trials with the GSI MK0752 (Merck & Co., Whitehouse Station, NY, <http://www.merck.com>) are underway in patients with breast cancer and pediatric central nervous system malignancies (<http://clinicaltrials.gov>). Our study suggest that GSIs alone only display limited growth inhibitory effects in glioma stem cells; in combination with radiotherapy, however, GSIs may provide significant therapeutic benefits by promoting the radiosensitivity of glioma stem cells. Therefore, additional efforts will be required to evaluate the clinical benefits of GSIs in combination with radiation as the monotherapy trials of GSIs may not show significant antitumor efficacy in tumors such as gliomas. Taking account into the prevalent role of Notch in regulation of stem cell behavior and cancer biology, it is reasonable to speculate that the radiosensitizing effects of Notch inhibition are not limited to gliomas and may be a broadly useful treatment paradigm.

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DISCLOSURES OF POTENTIAL FINANCIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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