# CD133$^+$ Glioblastoma Stem-like Cells are Radiosensitive with a Defective DNA Damage Response Compared with Established Cell Lines

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**Abstract**

**Purpose:** CD133$^+$ glioblastoma tumor stem-like cells (TSC) have been defined as radioresistant. However, although previously classified relative to CD133$^-$ cells, the radiosensitivity of CD133$^+$ TSCs with respect to the standard glioblastoma model, established glioma cell lines, has not been determined. Therefore, to better understand the radioresponse of this cancer stem cell, we have used established cell lines as a framework for defining their *in vitro* radioresponse.

**Experimental Design:** The intrinsic radiosensitivity of CD133$^+$ TSC cultures and established glioma cell lines was determined by clonogenic assay. The TSCs and established cell lines were also compared in terms of DNA double-strand break (DSB) repair capacity and cell cycle checkpoint activation.

**Results:** Based on clonogenic analysis, each of the six TSC cultures evaluated was more sensitive to radiation than the established glioma cell lines. Consistent with increased radiosensitivity, the DSB repair capacity as defined by neutral comet assay and γH2AX and Rad51 foci was significantly reduced in TSCs compared with the cell lines. Although $G_2$ checkpoint activation was intact, in contrast to the cell lines, DNA synthesis was not inhibited in TSCs after irradiation, indicating the absence of the intra-S-phase checkpoint.

**Conclusions:** These data indicate that the mechanisms through which CD133$^+$ TSCs respond to radiation are significantly different from those of the traditional glioblastoma *in vitro* model, established glioma cell lines. If TSCs play a critical role in glioblastoma treatment response, then such differences are likely to be of consequence in the development and testing of radiosensitizing agents. (Clin Cancer Res 2009;15(16):5145–53)



Radiotherapy remains a primary treatment modality for glioblastomas significantly contributing to the prolongation of patient survival. However, although many glioblastomas initially respond, they essentially all recur; even in combination with surgery and chemotherapy, the median survival of patients with glioblastoma continues to be dismal with the vast majority succumbing to disease within 2 years of diagnosis (1). Toward improving the response of glioblastoma to radiotherapy, laboratory investigations aimed at defining the determinants of radiosensitivity and evaluating radiosensitizers have typically been done using long established glioma cell lines. However, recent evidence, consistent with the cancer stem cell model, suggests that human glioblastomas are driven and maintained by a subpopulation of clonogenic cells called tumor stem-like cells (TSC). Identification and isolation of glioblastoma TSCs has, for the most part, been based on the expression of stem cell-associated protein CD133 (2–4). CD133$^+$ cells isolated from glioblastoma surgical specimens have several properties in common with normal neural stem cells including continuous self renewal, expression of stem cell markers, and at least partial differentiation along neuronal and glial pathways (2–4). Moreover, as few as 100 CD133$^+$ cells have been reported to form invasive brain tumors in immunocompromised mice that simulate the original primary tumor histology (5).

Data implicating TSCs as critical to glioblastoma growth and maintenance suggest that they should also have a major role in determining treatment response (6, 7). With respect to radiotherapy, Bao et al. reported that CD133$^+$ glioblastoma cells were more resistant to radiation than CD133$^-$ cells isolated from the same tumor (6). In this study, compared with CD133$^-$, irradiation of CD133$^+$ cells resulted in a reduced level of apoptosis and an increased colony-forming efficiency. The increased survival was attributed to a more efficient activation of the radiation-induced DNA damage response, both DNA repair and activation of cell cycle checkpoints, in CD133$^+$ cells. Based on this comparison, it is now generally considered

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## Translational Relevance

CD133$^+$ glioblastoma tumor stem-like cells (TSC) are assumed to be radioresistant and thus likely to contribute to the resistance of glioblastoma *in situ*. However, this conclusion is based only on the comparison of these TSCs with CD133$^-$ cells. As shown here, compared with the traditional glioblastoma model of established cell lines, the TSCs were more radiosensitive with a defective DNA damage response. Because *in vitro* measures of radiosensitivity do not predict tumor response *in vivo*, the significance of the relative radiosensitivity of the TSCs to glioblastoma response *in situ* is unclear. However, these data indicate that the mechanisms mediating TSC radioresponse differ from those in the traditional model. Because glioma cell lines have long been used to define molecular determinants of radiosensitivity, if TSCs play a role in glioblastoma treatment response, then exploiting such differences may aid in the development of effective radiosensitizing agents.

that TSCs contribute to glioblastoma radioresistance, which then implicates this subpopulation as a critical target for improving therapeutic outcome (8, 9). These results also imply that CD133$^+$ cells provide an *in vitro* model system for studying mechanisms mediating glioblastoma radioresponse.

Although such an experimental model would be of considerable value with respect to fundamental studies of glioblastoma radioresponse as well as the identification of potential targets for radiosensitization, several critical questions remain. Quantitative clonogenic survival curves for CD133$^+$ and CD133$^-$ cells were not provided in the initial analysis (6), which prevents the comparison of their radiosensitivity to other tumor cells. In addition, although the levels of specific proteins associated with cell cycle checkpoints were evaluated, the actual activation of the checkpoints after irradiation was not determined. Finally, whether the radioresponse of CD133$^+$ TSCs differs from that of established glioma cell lines, which have long been the preclinical model used for developing potential glioblastoma therapies, was not addressed. The significance of such a comparison lies in the potential for identifying components/regulators "unique" to the radioresponse of TSCs and thus novel targets of glioblastoma radiosensitization and treatment, that is, if the response of TSCs and established glioma cell lines differ. However, if TSCs respond in a manner similar to that of established cell lines, although of interest, it is unlikely that further study will generate new information relevant to glioblastoma radiosensitivity and its modification. Therefore, to generate additional insight into the radiosensitivity of glioblastoma TSCs *in vitro*, we compared CD133$^+$ TSC lines to established glioma cell lines in terms of clonogenic survival and DNA damage response. As described here, CD133$^+$ glioblastoma TSCs are more radiosensitive *in vitro* than established glioblastoma cell lines, with a reduced capacity to repair DNA double-strand breaks (DSB), and although they have an intact $G_2$ checkpoint, they lack the intra-S-phase checkpoint.

## Materials and Methods

***Neurosphere culture.*** A total of six neurosphere cultures were used in this study. Three cultures NSC11, NSC20, and NSC23, isolated from three human glioblastoma surgical specimens, were kindly provided by Dr. Frederick Lang (M. D. Anderson Cancer Center). Three additional neurosphere cultures (GBAM1, GBAM2, and GBMJ1) were generated at Moffitt Cancer Center from surgical specimens classified as glioblastoma according to WHO criteria (10) and were obtained following informed consent in accordance with the local institutional review board. The fresh glioblastoma specimens were dissociated mechanically, washed twice with ice-cold HBSS (Invitrogen), and then incubated at 37°C with trypsin (0.1%; Sigma) and DNase (0.4%; Sigma) for 2 to 4 h as described (4). For neurosphere formation, single-cell suspensions were added to standard tissue culture flasks containing DMEM/F-12 (Invitrogen), B27 supplement (Invitrogen), and human recombinant basic fibroblast growth factor and epidermal growth factor (50 ng/mL each; R&D Systems) as described (11). All cultures were maintained at 37°C in an atmosphere of 5% $CO_2$/95% air.

Each of the neurosphere cultures were initially composed of mixtures of CD133$^+$ and CD133$^-$ cells. To allow for the specific evaluation of the CD133$^+$ cells, fluorescence-activated cell sorting was used to sort CD133$^+$ cells from each culture. Neurospheres were disaggregated into single-cell suspensions by incubating in TryplE express (Sigma) at 37°C for 5 min followed by passaging through progressively smaller pipette tips. Single-cell suspensions were then labeled with an anti-CD133 antibody conjugated to phycoerythrin (AC133; Miltenyi Biotec) under sterile conditions. Cells were analyzed on a FACSvantage cell sorter (BD); cells positive for CD133 were collected, the purity of which was determined as >90% positive. For clonogenic analyses, the CD133$^-$ population was also collected from NSC11 and NSC20. The CD133$^+$ cells isolated from each culture were then placed back into neurosphere forming culture conditions and used for all described experiments. Each of the six CD133$^+$ cell cultures met the criteria for TSCs (4): continuous self-renewal, differentiation along glial and neuronal pathways on exposure to conditions in 10% serum as measured by glial fibrillary acidic protein and βIII tubulin expression, and tumor formation when injected intracerebrally into *nu/nu* mice (ref. 12; data not shown).

***Established cell lines.*** Three glioma cell lines were used in this study: U87 (American Type Culture Collection) and U251 and SF126 (Division of Cancer Treatment and Diagnosis Tumor Repository, National Cancer Institute-Frederick). The glioma cell lines were grown in monolayer in RPMI 1640 (Invitrogen) with 10% (U87 and SF126) or 5% (U251) fetal bovine serum. The A-T fibroblast cell line GM02052 was purchased from Coriell Institute for Medical Research and grown in MEM (Invitrogen) supplemented with 15% fetal bovine serum, sodium pyruvate, and nonessential amino acids (Invitrogen). Cultures were maintained at 37°C in an atmosphere of 5% $CO_2$/95% air.

***Irradiation.*** The established cell lines and the CD133$^+$ TSCs were irradiated as monolayer cultures using a using a XRad 160 X-ray source (Precision XRay) at a dose rate of 2.5 Gy/min.

***Clonogenic analysis.*** Cell survival was defined using a colony-forming efficiency assay. CD133$^+$ neurospheres were disaggregated into single-cell suspensions as described above. A specified number of cells were then seeded into poly-L-lysine–coated 6-well plates, which allows for adherent colony formation, containing the serum-free growth medium noted above. For established glioma lines, cultures were trypsinized to generate a single-cell suspension and a specified number of cells were seeded into each well of 6-well tissue culture plates. After allowing cells time to attach without doubling (6 h for glioma cell lines and 16 h for TSCs and CD133$^-$ cells), cultures were irradiated. Ten to 12 days after seeding (established glioma lines), or 14 to 21 days after seeding (CD133$^+$ TSCs), colonies were stained with crystal violet, and the number of colonies containing at least 25 cells was determined and the surviving fractions were calculated. The same procedure was done for

$CD133^-$ cells isolated from the initial unsorted NSC11 and NSC20 neurosphere cultures, which were seeded into the same stem cell growth medium as for $CD133^+$ cells. Data presented are the mean ± SE of three independent experiments.

***Immunofluorescent analyses of γH2AX and Rad51 foci.*** $CD133^+$ TSCs were seeded onto Lab-Tek CC2-treated tissue culture slides and glioma cell lines into Lab-Tek II standard tissue culture slides (Thermo Fisher) at least 24 h before use in an experiment. At specified times after irradiation cultures were fixed with 4% paraformaldehyde, permeabilized with 0.2% NP-40, and blocked with 1% bovine serum albumin in PBS containing 5% goat serum. The slides were incubated with primary antibodies (1:500 dilution) to phospho-H2AX (Upstate Biotechnology) or Rad51 (Santa Cruz Biotechnology) for 2 h at room temperature and with secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG at 1:500; Molecular Probes) and mounted with Anti-fade containing 4′,6-diamidino-2-phenylindole (Invitrogen). Cells were analyzed on a Zeiss upright fluorescent microscope. The number of foci was determined in 100 cells per condition and data were presented as foci number per cell ± 95% confidence intervals.

***Neutral comet assay.*** As a measure of DNA DSBs, the neutral comet assay was done using a commercially available kit according to the recommendations from the manufacturer (Trevigen) with slight modifications. Briefly, monolayer cultures were irradiated (10 Gy), and at specified times, single-cell suspensions were generated and washed with PBS. Cells were mixed with low melting agarose (1:10), and this mixture was pipetted onto the provided slides. Cell lysis was done at 4°C for 1 h. Cells were then subjected to electrophoresis for 20 min at room temperature and fixed with 70% ethanol and DNA was stained with SYBR Green. Digital fluorescent images were obtained using the IP Labs software (Signal Analytics). Data are expressed as % damage remaining, in which the Olive tail moment for the 0 time post-irradiation was set to 100% damage, with the remaining times post-irradiation normalized accordingly. Data represent the mean ± SE of three independent experiments.

***Cell cycle phase analysis.*** Evaluation of cell cycle phase distribution was done using flow cytometry. The treatment protocols were essentially the same as in the clonogenic survival experiments, except that the cells were initially seeded into 10 cm dishes. All cultures were subconfluent at the time of collection. Cultures were collected for fixation, stained with propidium iodide, and analyzed by flow cytometry with cell cycle distributions determined using ModFit software (Verity Software).

***$G_2$ arrest.*** To evaluate the activation of the $G_2$ cell cycle checkpoint, mitotic cells were distinguished from $G_2$ cells and the mitotic index was determined according to the expression of phosphorylated histone H3 (Upstate Biotechnology) as detected in the 4N population using the flow cytometric method of Xu et al. (13). Data are expressed as mean ± SE of three independent experiments.

***Radioresistant DNA synthesis (intra-S-phase arrest).*** DNA synthesis was determined according to the incorporation of the thymidine analogue 5-ethynyl 2-deoxyuridine into genomic DNA and was done using the Click-IT EdU Alexa Fluor 488 kit for flow cytometry (Invitrogen). Cells were irradiated with 12 Gy; 30 min later, growth medium was replaced with medium containing 5-ethynyl 2-deoxyuridine (10 μmol/L). After 30 min, the medium was replaced with 5-ethynyl 2-deoxyuridine–free medium for 1 h, and cells were fixed and stained according to the manufacturer's protocol. In addition, cells were stained with propidium iodide to determine DNA content and cell cycle phase distribution. Cells were analyzed by flow cytometry using CellQuest software (BD) and data were analyzed using FlowJo software by gating on the S-phase population and then determining the % 5-ethynyl 2-deoxyuridine–positive cells in that population. Data are expressed as mean ± SE of three independent experiments.

## Results

To compare the intrinsic radiosensitivities of the $CD133^+$ TSCs and established glioma cell lines, it was necessary to perform clonogenic analyses under optimal growth conditions for each cell type. Toward this end, $CD133^+$ neurosphere cultures were disaggregated into single cells and seeded at specified numbers into poly-L-lysine–coated plates in neural basal medium containing epidermal growth factor and basic fibroblast growth factor, that is, stem cell growth medium. Under these conditions, the TSCs grow as adherent colonies and, in contrast to growth in medium containing FCS, maintain their CD133 expression for at least 3 weeks after seeding (Fig. 1A), which is consistent with CD133 serving as a marker for *in vitro*

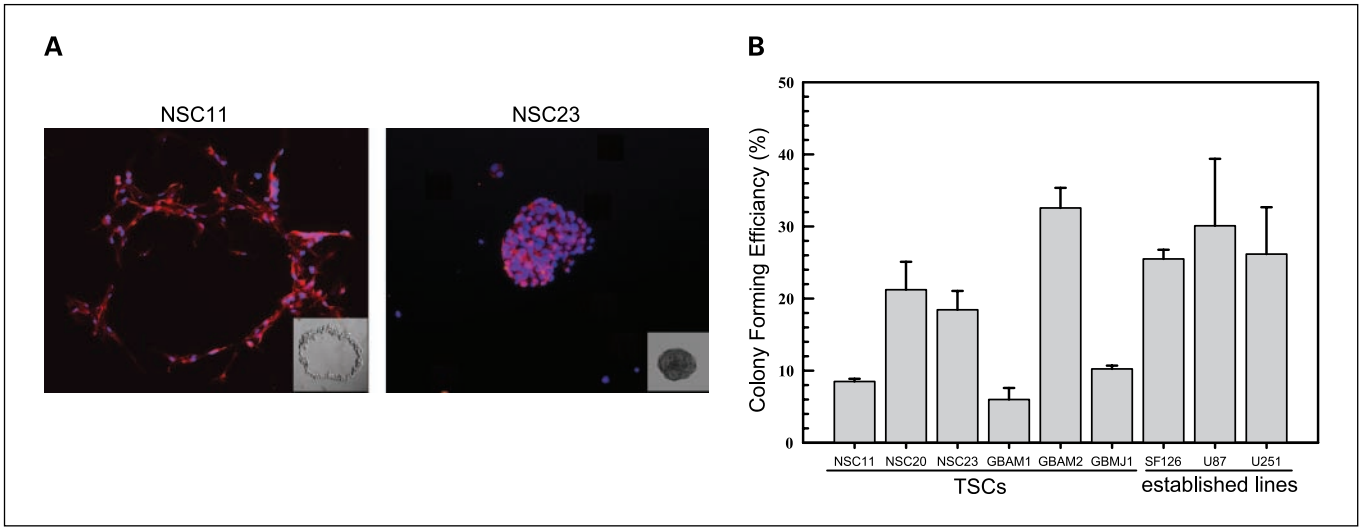



**Fig. 1.** Colony-forming efficiency. *A,* $CD133^+$ TSCs were plated as single cells onto poly-L-lysine–coated plates. Colonies were stained for CD133 (*red*) and counterstained with 4′,6-diamidino-2-phenylindole (*blue*) 21 d after seeding. *Left,* NSC11 colony (magnification, ×10) as an example of crater morphology; *right,* NSC23 colony as an example of spheroid morphology. *Insets,* phase-contrast photomicrographs of representative colonies (magnification, ×10). *B,* $CD133^+$ TSCs and established glioma cell lines were seeded as single cells and colony-forming efficiency was determined. Mean ± SE of three independent experiments.

clonogenicity (5) and the self-renewal characteristic of TSCs. Of note is the "crater" colony morphology of NSC11, which was the dominant morphology in half of the TSC cultures, and is similar to that reported for embryonic stem cells (14). The clonogenic analysis of the established glioma cell lines was also done under conditions of optimal growth (5-10% fetal bovine serum). The colony-forming efficiencies of untreated $CD133^+$ TSC cultures and established glioma cell lines are shown in Fig. 1B.

Radiation survival curves based on clonogenic analysis were generated for each of the $CD133^+$ TSC cultures and established glioma cell lines. In these experiments, TSCs and established cell lines were plated 16 or 6 h before irradiation, respectively, time periods that allowed for cell attachment and yet no division. Following this protocol, single cells, and not microcolonies, are subjected to irradiation, which eliminates the confounding parameter of multiplicity and its effects on apparent radiosensitivity. As shown in Fig. 2A, although there was variability among the TSC cultures with respect to radiosensitivity, they were all considerably more radiosensitive than the traditional glioma cell lines. For example, after 4 Gy each of the stem cell lines had surviving fractions of <0.12, the surviving fractions for the established cell lines were ~0.33. These data thus indicate that $CD133^+$ glioblastoma TSCs are more radiosensitive than the traditional glioma cell lines. We also compared the $CD133^+$ cells to $CD133^-$ cells isolated from the initial unsorted NSC11 and NSC20 neurosphere cultures (Fig. 2B). $CD133^-$ cells were grown in stem cell growth medium. It should be noted that $CD133^-$ cells isolated from the other neurosphere cultures did not proliferate sufficiently to form colonies when grown in stem cell growth medium or serum-containing medium. For NSC11 neurospheres, the $CD133^+$ TSCs were more resistant than $CD133^-$ cells, consistent with the previous results (6). However, there was essentially no difference in the radiation survival curves generated for the $CD133^+$ and $CD133^-$ cells isolated from NSC20 neurosphere cultures. These results suggest that the radioresistance of $CD133^+$ TSCs versus $CD133^-$ may be dependent on the tumor from which they were isolated.

Given the consistently greater radiosensitivity of the six $CD133^+$ TSCs compared with the established glioma cell lines, subsequent studies addressed the fundamental processes responsible. For these experiments, we focused on two TSC cultures (NSC11 and NSC20) and two established lines (U87 and SF126). In these studies, $CD133^+$ TSCs were seeded onto poly-L-lysine–coated tissue culture plates and established glioma cell lines onto standard tissue culture plastic at least 24 h before analysis and thus evaluated under the same monolayer growth conditions used to generate the cell survival curves (Fig. 2A).

Distribution through the cell cycle could account for differences in radiosensitivity with cells in S phase considered to be more radioresistant. However, as shown in Fig. 3, the percentage of S-phase cells in U87, SF126, and NSC20 cultures were essentially the same; NSC11 cultures had a higher S-phase percentage. These results indicate that the differences between the radiosensitivities of the TSCs and established glioma cell lines cannot be simply attributed to cell cycle phase distribution. The redistribution of cells after exposure to 2 Gy is also shown in Fig. 3. At 24 h after irradiation, the TSC cultures had a higher percentage of cells in $G_2$-M compared with the established cell lines. In other cell types, such an accumulation in

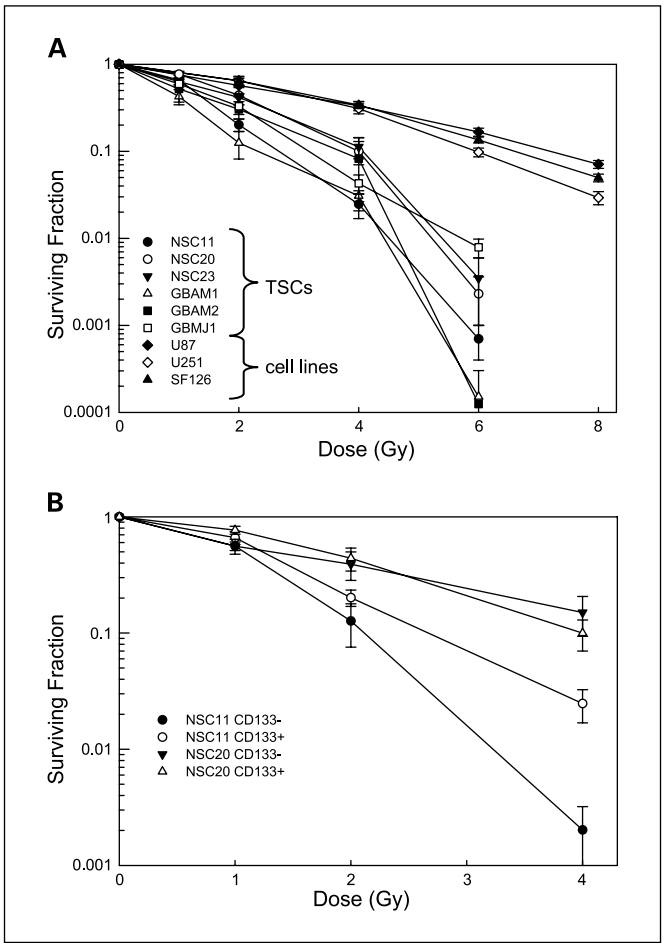



**Fig. 2.** Radiosensitivity of $CD133^+$ TSCs and established glioma lines. Cells were plated, allowed to attach, and exposed to graded doses of X rays. *A*, colony-forming efficiency was determined 10 to 14 (glioma cell lines) or 14 to 21 ($CD133^+$ TSCs) days later and survival curves were generated. *B*, survival curves corresponding to $CD133^+$ TSCs isolated from NSC11 and NSC20 neurosphere cultures were replotted with those generated for $CD133^-$ cells isolated from the same initial neurosphere cultures. Colonies of $CD133^-$ cells were determined at 18 d after irradiation. Mean ± SE of three independent experiments.

$G_2$-M has been attributed to cells that were irradiated in S phase, which then failed to undergo S-phase arrest (15). It should be noted that, in these cell cycle analyses, radiation was not found to induce a significant sub-$G_1$ population in any of the cultures or cell lines evaluated, including when the dose was increased to 6 Gy (data not shown). Thus, consistent with solid tumor cell lines in general, these data indicate that apoptotic death is an infrequent event after irradiation of TSCs and moreover does not account for the increased radiosensitivity of TSCs.

A major determinant of cellular radiosensitivity is the repair of DNA DSBs, the DNA lesion primarily responsible for radiation-induced cell death. As an initial approach to defining the DSB repair capacity, we applied the neutral comet assay. In these experiments, cells grown in monolayer were irradiated with 10 Gy and collected for analysis immediately after irradiation or at times out to 24 h. As expected, there was no difference among the culture types in the initial level of radiation-induced DSBs as measured by the Olive tail moment (data not shown). Typical of established cell lines, within 6 h after 10 Gy, the percentage of

DNA damage remaining in the SF126 and U87 cell lines returned to control levels (Fig. 4A). In contrast, there was a significant percentage of DNA damage remaining in the TSCs at 6 h, which remained at essentially the same levels out to at least 24 h post-irradiation. These data suggest that TSCs have a reduced capacity to repair radiation-induced DSBs compared with the established glioma cell lines. Of note, in their analysis of DNA repair in $CD133^+$ TSCs, Bao et al. used the alkaline comet assay done after 3 Gy (6), a method that measures the induction and repair of DNA single-strand breaks (16), which are of little significance in radiation-induced cell death.

As an additional measure of DSB repair, the dispersal of phosphorylated histone H2AX (γH2AX) nuclear foci, which has been established as a sensitive indicator of DSBs, was determined. In these experiments, cells were exposed to 2 Gy and the number γH2AX foci/cell was determined out to 24 h (Fig. 4B). The number of foci/cell in each line decreased between 1 and 6 h. However, although foci numbers returned to control levels by 24 h in the established glioma cell lines, a significant number of residual γH2AX foci remained in the TSCs. Thus, the analysis of γH2AX, which is a chromatin level manifestation of DSBs, is consistent with the neutral comet data, indicating that the DSB repair capacity of TSCs is significantly reduced compared with established glioma cell lines.

DSBs are repaired in mammalian cells via nonhomologous end-joining and homologous recombination repair (HRR). Whereas γH2AX is a marker of DSB in general (17), Rad51 foci are considered indicators of those DSBs subject to HRR (18). As shown in Fig. 4B, exposure of each of the cell types to 2 Gy resulted in detectable Rad51 foci by 1 h. In the established cell lines, as for γH2AX, the number of Rad51 foci began to decrease after 1 h, returning to control levels by 24 h post-irradiation. In contrast, Rad51 foci in the TSCs changed little after 1 h and remained significantly above control levels out to at least 24 h. Because γH2AX levels in the TSCs decreased dramatically between 1 and 6 h post-irradiation, although Rad51 foci remained at essentially the same levels from 1 to 24 h, these results suggest that the DSB repair defect in the TSC lines may involve some aspect of HRR.

In addition to DSB repair, the DNA damage response includes the activation of cell cycle checkpoints. To compare the radiation-induced activation of the $G_2$ checkpoint in TSCs and established glioma cell lines, the method of Xu et al. was used, which distinguishes between $G_2$ and mitotic cells (15). This assay determines the percentage of mitotic cells in the 4N population (mitotic index) according to the flow cytometric analysis of phosphorylated histone H3, which is specifically expressed in mitotic cells. As shown in Fig. 5, irradiation of both cell types resulted in the decrease in mitotic cells by 1 h reaching a maximum reduction by ~4 h, consistent with the rapid onset of $G_2$ arrest and with previously published results (15, 19). These results suggest that TSCs effectively activate the $G_2$ checkpoint in response to radiation. However, whereas the mitotic index of the TSC cultures returned to control levels by 16 h, the mitotic index of established lines was increased relative to control cells

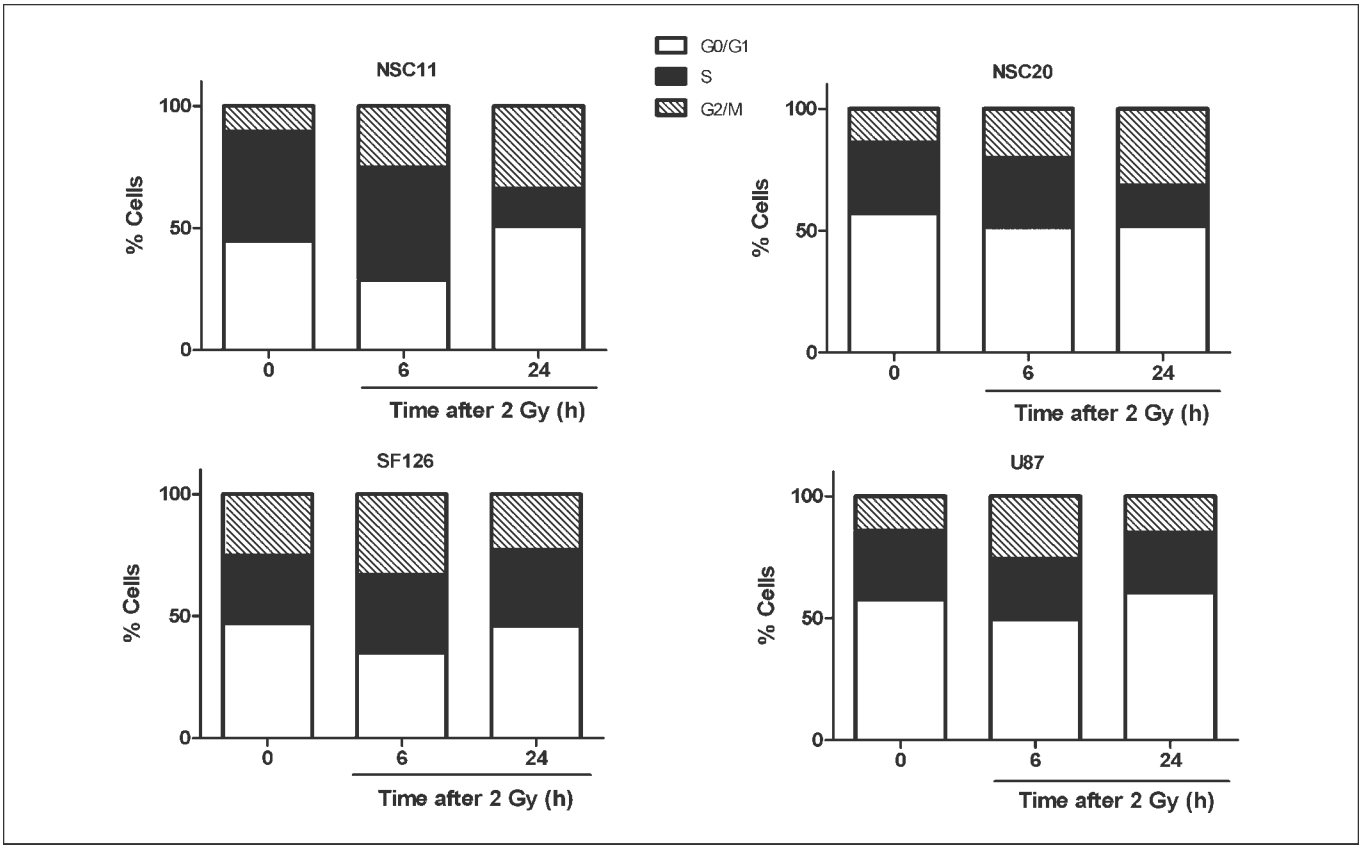
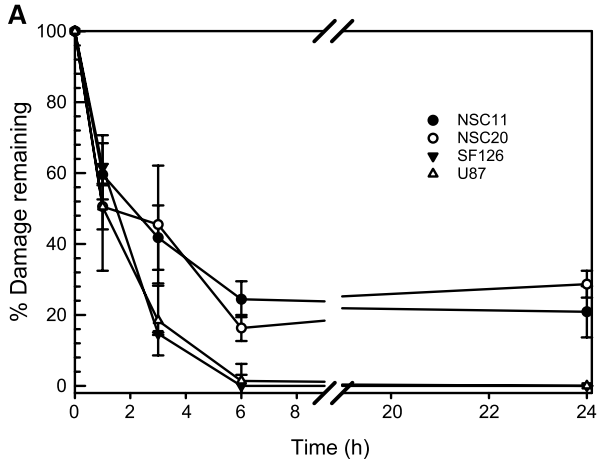



**Fig. 3.** Cell cycle phase distribution. Cells were seeded 24 h before irradiation (2 Gy) and collected at 6 and 24 h for analysis of cell cycle phase distribution. Representative of three independent experiments.



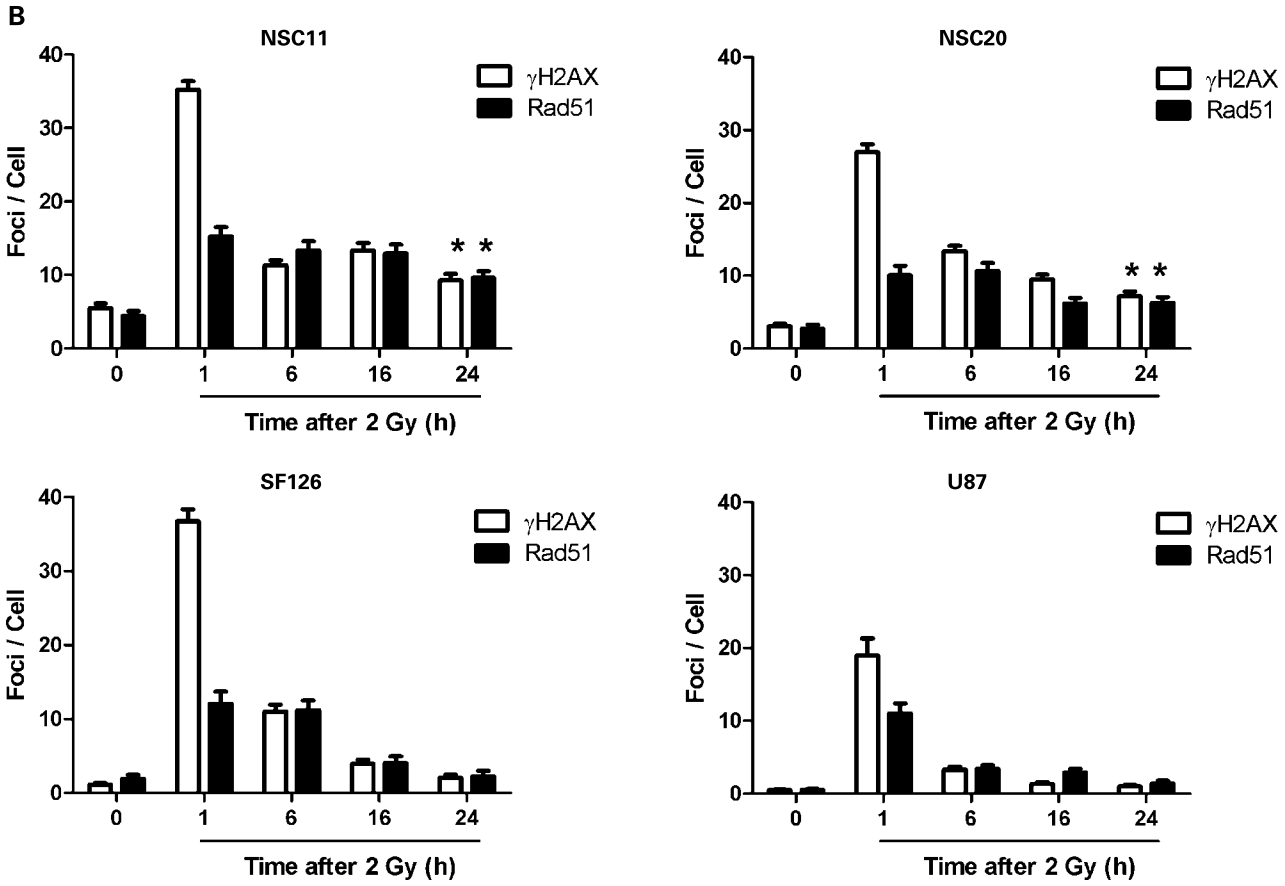




**Fig. 4.** DNA DSB repair. *A,* neutral comet assay. Cells were irradiated (10 Gy) and analyzed at times out to 24 h. Data are expressed as percent damage remaining in which the tail moment immediately after radiation corresponds to 100% damage. Mean ± SE of three independent experiments. *B,* induction and dispersal of γH2AX foci and Rad51 foci. Cells were irradiated (2 Gy) and analyzed at times out to 24 h. Mean ± 95% confidence intervals. *, $P < 0.01$ for control cells versus 24 h post-irradiation.

by 8 h (SF126) or 16 h (U87) after irradiation. Such an overshoot has been observed previously by Xu et al. and attributed to radiation-induced cell cycle synchronization (15).

To define the activation of the intra-S-phase checkpoint, DNA synthesis was determined according to 5-ethynyl 2-deoxyuridine (a thymidine analogue) incorporation (Fig. 6). As a control for cells that do not activate the intra-S-phase checkpoint after irradiation, an A-T fibroblast cell line was evaluated; these cells contain mutated ATM and are characterized by radioresistant DNA synthesis (20). Irradiation (12 Gy) of the established cell lines resulted in a decrease in DNA synthesis of ~70%, which corresponds to the activation of the intra-S-phase checkpoint and is consistent with previous results generated from other tumor and normal cell lines (21, 22). However, irradiated TSC cultures maintained the same level of DNA synthesis as in control cells, a response similar to that of the A-T cell line. These data indicate that, in contrast to established glioma and other human tumor cell lines, $CD133^+$ TSCs are unable to activate the intra-S-phase checkpoint in response to radiation.

## Discussion

It was initially reported that $CD133^+$ glioblastoma TSCs are radioresistant compared with $CD133^-$ tumor cells (6), a finding that has received considerable attention. More recently, a cancer stem cell population isolated from a mouse breast tumor model was also found to be radioresistant compared with tumor cells without a stem-like phenotype (23). The data presented here, however, indicate that not all $CD133^+$ TSCs are radioresistant compared with $CD133^-$ cells. Ropolo et al. (24) recently reported that, in contrast to the initial study (6), the DNA DSB repair capacity of $CD133^+$ glioblastoma cells was not significantly different from $CD133^-$ cells. Thus, it appears that there is at least some tumor type dependence regarding the relative radiosensitivities of glioblastoma TSCs and $CD133^-$ cells. However, regardless of whether there is a difference in the *in vitro* radiosensitivity of $CD133^+$ cells and non-stem-like tumor cells, their putative role in glioblastoma growth and maintenance implicates TSCs as a determinant of treatment response. Thus, although defining the radiosensitivity of $CD133^-$ cells is of interest in terms of the radiobiology of tumor subpopulations, with respect to the development of strategies that enhance glioblastoma response to radiotherapy, of potentially greater relevance is a detailed understanding of the specific processes mediating TSC radioresponse. Toward this end, the studies presented here focused on $CD133^+$ glioblastoma TSCs.

In that radiosensitivity/resistance is a relative term, the radioresponse of $CD133^+$ TSCs was compared with that of established glioma lines, the traditional *in vitro* glioblastoma model. Clonogenic survival curves revealed that each of the six $CD133^+$ TSC cultures was more radiosensitive than the established glioma lines. Although providing a reference point for characterizing the radioresponse of the TSCs, the significance of their relatively greater *in vitro* radiosensitivity in terms of glioblastoma resistance *in situ* is unclear. Results generated from a variety of established tumor cell lines (23–25) as well as primary cultures (26) have shown that *in vitro* radiosensitivity does not predict *in vivo* tumor radioresponse. Specifically, established glioma cell lines do not simulate glioblastoma radioresistance; their radiosensitivities are not different from that of cell lines initiated from other histologies that typically respond to radiotherapy (24). However, the consistent difference between TSCs and established glioma cell lines in terms of their radiation survival curves does imply that the mechanisms mediating their respective radiosensitivities are different. Established cell lines have long served as the experimental model for investigating glioblastoma radiosensitivity and its modification, that is, radiosensitization. The current targeted approach to radiosensitizer development depends on an understanding of the molecular determinants of radiosensitivity, which then provides a source of potential targets. The significance of the survival curves shown in Fig. 2A is the implication that the molecular determinants of TSC radiosensitivity, and thus potential targets for radiosensitization, differ from those in established cell lines.

Using established glioma cell lines as a benchmark for expected results, we have begun to define the components of the radiation-induced DNA damage response in $CD133^+$ TSC cultures. Because the repair of DNA DSBs is essential to the survival of an irradiated cell, the DSB repair capacity of $CD133^+$ TSCs was analyzed according to two parameters: γH2AX foci and neutral comet formation. At sites of radiation-induced DSBs, the histone H2AX is rapidly phosphorylated (γH2AX) forming readily visible nuclear foci (17, 25), which then disperse as a function of time. The number of γH2AX foci remaining at 24 h after irradiation has been shown to correlate with cellular radiosensitivity (26, 27). Whereas γH2AX reflects a chromatin level response to DSBs, the neutral comet assay provides a more direct measure of DNA strand breaks and, in contrast to the alkaline comet assay, selectively detects DSBs over single-stranded breaks (16). Thus, as illustrated by the different time courses and radiation dose requirements, γH2AX and neutral comet analyses quantify different manifestations of DSBs and thus different aspects of the repair process. Compared with the established glioma cell lines, both analyses indicated that $CD133^+$ TSCs have a reduced capacity to repair radiation-induced DSBs, which is likely to be a major contributor to their relatively greater degree of radiosensitivity. Moreover, based on the comparison to Rad51 foci formation and dispersal, the DSB repair defect in the TSCs appears to involve HRR. Although Rad51 foci provide an indication of HRR activity, functional analysis of this repair pathway in TSCs is required to confirm a defect. However, these results suggest that targeting HRR may be an effective therapy against $CD133^+$ TSCs. For example, poly(ADP-ribose) polymerase inhibitors have been shown to be extremely toxic to HRR-deficient cells (28, 29).

In addition to DSB repair, a critical component of the radiation-induced DNA damage response is cell cycle checkpoint activation. In their investigation, Bao et al. reported that critical checkpoint regulatory proteins (ATM, Chk1, Chk2, and Rad17) were phosphorylated in $CD133^+$ glioblastoma cells after irradiation (6). However, whether the $CD133^+$ cells actually underwent arrest at the specified locations in the cell cycle after irradiation (checkpoint activation) was not determined. As shown here, although displaying an intact $G_2$ checkpoint, the $CD133^+$ TSC cultures were deficient in activating intra-S-phase arrest. Whether the abrogated S-phase checkpoint contributes to

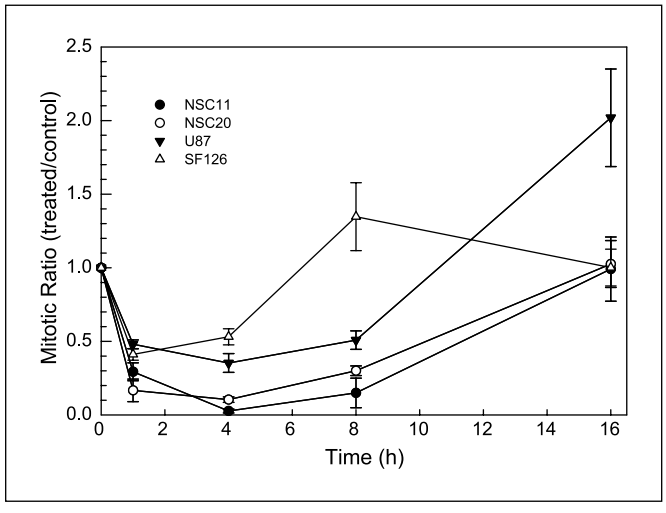



**Fig. 5.** $G_2$ checkpoint activation. Cells were irradiated (2 Gy) and phospho-H3 expression was analyzed in the 4N population at times out to 16 h. Data are expressed as mitotic ratio, the percentage of phospho-H3–positive cells in irradiated/unirradiated levels. Mean ± SE of three independent experiments.

the relative radiosensitivity of TSCs versus established cell lines is unclear. Xu et al. reported that the lack of a S-phase checkpoint alone does not enhance radiosensitivity (13). However, the S-phase checkpoint does play a critical role in genome maintenance (30). Thus, an abrogated S-phase arrest in the $CD133^+$ TSC is consistent with the proposed role of genomic instability of cancer stem cells as a driving force of tumor development and heterogeneity (31).

The defective DNA damage response of the $CD133^+$ TSCs may seem counterintuitive for cells supposedly critical to the glioblastoma initiation and maintenance. However, this defect is consistent with the recently proposed concept that the activation of the DNA damage response provides a critical barrier to cancer development (32, 33). Data now suggest that oncogene-induced DNA damage response inhibits cell proliferation in the early stages of tumorigenesis; in this situation, only tumor cells with mutated or epigenetically silenced repair and/or checkpoint genes continue to divide (34). The consequence of this selection procedure is that the cells that ultimately form a malignant tumor have a defective DNA damage response and exhibit a high degree of genomic instability (34). The role of the DNA damage response in preventing tumor development has for the most part been defined using human tissue specimens and *in vivo* model systems (32–34). Glioma cell lines, as well as the vast majority of established tumor cell lines, have an apparently intact DNA damage response and thus, as noted by Halazonetis et al. (35), may not accurately represent human tumor cells *in situ*. In contrast, the compromised DSB repair and checkpoint activation of $CD133^+$ TSCs suggest that they may better simulate those tumor cells that have escaped the DNA damage response barrier and may thus provide a more relevant *in vitro* model of human tumors.

Although the compromised DNA damage response of $CD133^+$ TSCs *in vitro* may be consistent with human tumor cells *in vivo*, in light of the radioresistance of glioblastoma, the enhanced radiosensitivity that accompanies such a defect seems paradoxical. This may suggest a critical role for the microenvironment as a determinant of radiosensitivity or, not mutually exclusive, that the putative genomic instability of the TSCs facilitates the generation and selection of resistant cells. Alternatively, the significance of *in vitro* radiosensitivity based solely on long established tumor cell lines may require further analysis. Clearly, the implication of the *in vitro* radioresponse of $CD133^+$ TSCs and the mechanisms involved require additional investigation. However, although several questions remain, the data presented here indicate that the processes regulating the radioresponse of the long-studied established glioma cell lines are substantially different from those operative in $CD133^+$ TSCs. If these TSCs play a critical role in glioblastoma treatment response, then such differences are likely to be of consequence in translational studies aimed at the development and testing of potential radiosensitizing agents.

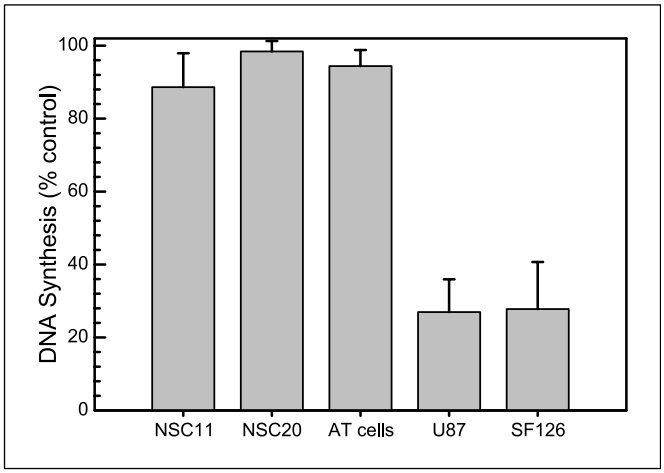



**Fig. 6.** Intra-S-phase checkpoint activation. Thirty minutes after irradiation (12 Gy), cells received a 30 min pulse of 5-ethynyl 2-deoxyuridine (10 μmol/L) followed by incubation for 1 h in 5-ethynyl 2-deoxyuridine–free growth medium before analysis. DNA synthesis was defined according to 5-ethynyl 2-deoxyuridine incorporation. Data are expressed as percent untreated control. Mean ± SE of three independent experiments.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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