A role for homologous recombination and abnormal cell cycle progression in radioresistance of glioma initiating cells

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Abbreviations list: DSB: Double strand break, GBM: Glioblastoma multiforme, GIC: Glioma initiating cell, HR: Homologous recombination, NHEJ: Non-homologous end joining, NPC: Neural progenitor cell.

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common form of brain tumour with a poor prognosis and resistance to radiotherapy. Recent evidence suggests glioma initiating cells (GICs) play a central role in radioresistance through DNA damage checkpoint activation and enhanced DNA repair. To investigate this in more detail, we compared the DNA damage response in non-tumour forming neural progenitor cells (NPCs) and GICs isolated from GBM patient specimens. As observed for GBM tumours, initial characterization demonstrated that GICs have long-term self-renewal capacity. They express markers identical to NPCs and have the ability to form tumours in an animal model. In addition, these cells are radioresistant to varying degrees, which could not be explained by enhanced non-homologous end joining (NHEJ). Indeed, NHEJ in GICs was equivalent or in some cases reduced as compared to NPCs. However, there was evidence for more efficient homologous recombination (HR) repair in GICs. We did not observe a prolonged cell-cycle nor enhanced basal activation of checkpoint proteins as reported previously. Rather, cell-cycle defects in the G1/S and S-phase checkpoints were observed by determining entry into S-phase and radioresistant DNA synthesis following irradiation. These data suggest that HR and cell-cycle checkpoint abnormalities may contribute to the radioresistance of GIC and that both processes may be suitable targets for therapy.
INTRODUCTION

Gliomas are the most common adult brain cancers, comprising 80% of diagnosed cases (1).

Anaplastic astrocytoma (grade III) and GBM (grade IV) tumours are highly lethal (1).

Standard therapy is combined local aggressive cytoreductive surgery followed by radiotherapy. Unfortunately, GBM are particularly resistant to conventional treatment leading to recurrence of brain tumours. A pool of GICs (2), similar to NPCs (3), can survive exogenous damage, such as the lethal double strand breaks (DSBs) to repopulate tumour cells following treatment.

DSBs are highly detrimental to the structural integrity of chromosomes. To counteract this damage, normal and cancer cells initiate the DNA-damage response. DSBs are sensed primarily by the MRN complex; Mre11, Rad50 and Nbs1 proteins, that rapidly relocates to the site of damage and recruits ATM (Ataxia-telangiectasia mutated) kinase, resulting in downstream phosphorylation cascades that initiate cell-cycle arrest and DNA repair (4). There are two major DSB pathways engaged during DNA repair. NHEJ is initiated predominantly during the G1-phase of the cell-cycle and since the majority of cells are in G1-phase, NHEJ is considered the major pathway for DSB repair. The second repair mechanism occurs during late S- and G2-phases. HR is an error-free method of repair utilizing sister chromatids as templates to replace damaged DNA (5). In contrast, NHEJ relies on ligases and excision repair enzymes to adhere broken DNA ends which can introduce spontaneous mutations (6). Depending on the severity of insult, cells with a high mutational burden may initiate apoptosis to minimize damage to the genome and prevent mutations from being maintained through subsequent cell divisions.

Compared to normal cells, cancer cells have enhanced DNA repair pathways, which confer greater survival. Investigations characterizing sub-populations of aberrant stem cells have
shown efficient DSB repair through various DNA damage markers, but no specific pathways have been identified to explain the increased survival (7). Recent reports also provided evidence for differences in radiosensitivity between sub-populations of GICs (8-10), suggesting disparity in DNA repair efficiency. Whether DNA repair is a major mechanism of radioresistance in GICs remains controversial. To further understand these DNA repair pathways, we compared the DNA damage response of ionizing radiation (IR) in non-tumour forming NPCs (11, 12) and GICs isolated from GBM tumours. The significance of this comparison is the potential for identifying key proteins or pathways unique to GICs. In our study, increased survival of GICs following IR was observed. Although, GICs had a higher proportion of surviving cells, attenuated checkpoint kinase activation suggested inadequate cell-cycle arrest at G1/S, allowing a portion of G1 cells to enter S-phase. NHEJ activity was reduced in several GIC lines, but these differences did not account for altered survival. Interestingly, GICs appeared to favour the HR repair pathway. These data indicate that GICs have an ineffective G1/S checkpoint allowing damaged cells to enter S-phase and utilize HR to repair the genome.
**MATERIAL AND METHODS**

**Glioma initiating cells extraction and maintenance.** GICs enrichment from tumours from three GBM patients was performed as described by Piccirillo *et al* (13), resulting lines were designated L1b, L2b and L3b. Briefly, tumours were dissociated into single cell suspensions before enriching for stem cells/neurospheres in serum-free media supplemented with 20ng/ml epidermal growth factor (EGF) and 10ng/ml basic fibroblast growth factor (bFGF). U251 and U87 neurosphere cultures were obtained from Day *et al* (14) and also underwent the GIC enrichment process. No authentication was performed by the authors. Neurosphere cultures were maintained at 37°C in 5% CO₂ with supplements (12) for 4 – 8 weeks before characterization as described in supplementary methods. The neurosphere media is a selective culture that enriches for progenitors and precursors but not differentiated cells. All primary and tumour cell lines grown in neurosphere media will contain a mixed population of progenitor and precursor cells. Throughout this paper we will refer to the entire population of cells within the cultures as GICs.

Non-tumourigenic NPCs derived from fetal brain tissue are commercially available (ReNcell, Millipore, USA), and were previously characterized by Reynolds *et al* (11, 12, 15). For experiments, neurosphere cultures were grown for five days, before trypsinizing with 0.25% trypsin-EDTA (Invitrogen, USA) in PBS into single cell suspensions prior to all indicated treatments (12).

**Survival assay.** Dissociated neurospheres were prepared as duplicates prior to IR. Protocol was previously described (16). Details are in supplementary methods.

**Non-homologous end joining assay.** The NHEJ reporter plasmid pEGFP-N1 (Clontech, USA) was digested with HindIII (New England Biolabs, USA) and purified by gel extraction kit (Qiagen, USA) and aliquots analysed by gel electrophoresis to confirm complete
digestion. Neurosphere lines were transfected using a Gene PulserII (Biorad, USA) at 220V, 960µF, infinite resistance. 1×10^6 dissociated cells were transfected with 1µg of linearized pEGFP-N1, and in parallel with 1µg circularized pEGFP-N1 as control for transfection efficiency. GFP expression was measured by FACScan (Becton Dickinson, USA) 48h later.

DNA pulse labelling. Iododeoxyuridine (IdU) analog (100µm) was added into neurosphere media. Cells were placed for 30min at 37°C before fixation and cytopinning onto Superfrost® plus slides (see supplementary methods). Slides were placed for 1min in lysis buffer (50mM Tris-HCl pH8.0, 1mM EDTA, 0.1% SDS) at room temperature. Slides were rinsed with PBS before incubating in 1M HCl, 5min on ice, followed by 2M HCl, 10min at room temperature and 10min at 37°C. Immunolabelling is described in supplementary methods.

Homologous recombination assay. Neurosphere lines were stably transfected as described above with 1µg of pDR-GFP and stable integrants selected with 1µg/ml – 8µg/ml puromycin (Sigma, USA) (17). Analysis was performed 48h after I-Sce I +/+ transfection as previously indicated (18).

Fibre assay for DNA replication. The DNA fibre assay was conducted as previously described (19). Total DNA fibres were scored (≥300) for each cell line. The percentage of new initiations was expressed as fold change [new initiations / (continuing + new initiations)].
RESULTS

Long-term growth potential of GIC and NPC. The gold standard to determine the presence of normal stem or progenitor cells is to evaluate their self-renewal and lineage differentiation (20, 21). Assays for GICs additionally also require to determine tumour formation in an animal model. We first examined whether GICs had stem cell markers and self-renewal capacity comparable to NPCs. The profile of stem cell markers differed between GIC cultures but U251 and L1b showed near identical marker expression to NPCs (Supplementary Fig. S1A). All cultures except U87 displayed CD49f expression (22). Two other stem cell markers, Sox2 and Nestin were detected in all GICs and NPCs with 80% – 95% and 78% – 93% positive cells respectively by IF (Supplementary Fig. S1A). When GIC and NPC were plated with 5% fetal calf serum, both cell types began to differentiate within 5 – 7 days (Supplementary Fig. S2A). Neural (β-III tubulin) and glial (GFAP and MAP) makers were analysed two weeks later. GICs expressed all lineage markers with percentage expression being 17% – 40% (β-III tubulin), 17% – 31% (MAP) and 18% – 41% (GFAP) across all GIC cultures. Lineage distribution in differentiated NPC was 31% (β-III tubulin), 18% (GFAP), and 17% (MAP) (Supplementary Fig. S2B and S2C). As surface marker expression is only one method to define stem cell populations, we also evaluated long-term self-renewal using serial dilution. Single cells were seeded from passage 1, and expansion between passages was measured (Supplementary Fig. S1B). All lines expanded constantly as demonstrated by an unchanged slope of a straight line.

Finally, to demonstrate that GICs could recapitulate tumours in vivo, we employed a Scid/Nod tumourigenesis model (23). Intracranially injected tumour neurospheres (U251) and primary glioma (L2b) produced glioblastoma in mice (Supplementary Fig. S1C). Survival curves are shown in Supplementary Figure S1D. Despite limited CD133 expression, they
were capable of forming tumours. This finding is consistent with recent publications showing that both CD133 positive and negative GICs can induce tumour development (24).

Histological analysis showed tumours with highly dense region of marked nuclear atypia, and pseudo palisades-like formation in surrounding tissue; all characteristic of GBM (Supplementary Fig. S1C)

**GIC cultures are radioresistant.** We next determined whether GIC cultures had radioresistant properties (25). Following 2Gy IR exposure, cell survival was determined by trypan blue assay (19). All GIC cultures had more viable cells over time compared to NPC (Fig. 1A) (p<0.05). Survival for GIC cultures ranged from 40% – 60% 96h after IR versus 20% for NPCs. GIC cultures also showed increased survival with doses from 2Gy – 10Gy (Fig. 1B). Alternate analysis using neurosphere size (23, 26) showed NPC after 2Gy had a 0.51 fold reduction in size (Supplementary Fig. S3A). In comparison, GICs had a 0.89 – 0.66 fold reduction, suggesting a lesser impact of IR-induced damage on GICs growth (p<0.01). At 5Gy dose, NPC reduced to 0.38 the original size, while the fold change in GICs size was 0.76 – 0.51 (p<0.01).

We next determined whether GIC survival was due to accelerated re-population or apoptosis resistance (27, 28). Radiation-induced DNA damage caused comparable cell death in GICs and NPCs at 24h and 48h post-IR (Fig. 1C and Supplementary Fig. S3B). We next employed CFSE labelling to measure proliferation. CFSE incorporates into the membrane and daughter cells inherit half of the dye after mitosis. Individual colours illustrate cell divisions (generation numbers) (Fig. 1D). All GICs proliferated at higher rates than NPC following irradiation indicating that continuous proliferation rather than apoptosis resistance in GICs accounted for increased survival. In detail, 96h post-2Gy IR showed reduced growth with NPC undergoing 5 – 6 divisions. U251, L2b and L3b showed little change in proliferation with U251 and L2b capable of 6 – 7 divisions and L3b 5 divisions. At 5Gy dose, U251
underwent 7 – 8 divisions, L2b and L3b 4 – 5 divisions whereas NPC proliferation reduced to 3 – 4 divisions. At high dose (10Gy), U251, L2b and L3b cells proliferated for 4 – 6 divisions versus 2 – 3 for NPCs.

For long-term proliferation after 2Gy irradiation, serial passage showed marked reduction in NPC during initial passages (1 – 2) before returning to normal growth from passage 3 (Supplementary Fig. S4A). After 5Gy reduction in growth was observed in GIC lines but with faster recovery. A reduction in growth was observed in NPC with recovery from passage 5 (Supplementary Fig. S4B). Conversely, L2b, U251 and U87 demonstrated recovery from passage 3, followed by L1b and L3b at passage 4.

Delayed DSB repair in GICs during early recovery. The survival, serial passage and neurosphere formation data indicated that GICs might have enhanced DNA repair, allowing rapid growth post-IR. To examine DNA damage repair response, the levels of proteins central to DSB recognition and response were measured by immunoblotting. Interestingly, U87 and L3b had reduced protein expression compared to NPC (Fig. 2A). In U87, total ATM and Mre11 levels were reduced (29). Similarly, protein levels of MRN complex members in L3b were diminished. In contrast, L1b and L2b displayed increased MRN protein levels while no significant difference was identified between NPC and U251. Radiation did not cause any consistent change in levels of DNA damage response proteins that might account for changes in radioresistance. We next investigated whether differences in the efficiency of DNA DSB repair might explain radioresistance by following γH2AX foci formation and resolution (Fig. 2B). Since the maximum foci number at 1h differed between cultures, the rate of repair was expressed as fold change by normalizing to their own neurosphere lines. In NPC, efficient repair was identified by rapid reduction in γH2AX foci at 6h (reduced to 17%) (Fig. 2C). Conversely, GICs repair was slow at early timepoints (<6h) ranging from 33% – 73% (p<0.01). Repair of DNA DSB was not completed until 12h to 24h in GICs.
NHEJ function does not explain GIC radioresistance. To investigate repair mechanisms responsible for radioresistance, we measured NHEJ activity in vivo by recapitulating the joining of DNA ends with a linearized GFP-expression vector (Fig. 3A). NHEJ activity was detected in all neurosphere cultures (Fig. 3B), but was variable between GIC cultures. In U251 and U87, NHEJ efficiency was reduced to 23% (p<0.018) and 19% (p<0.024) compared to NPCs (46%). L2b had modestly reduced NHEJ activity, ~70% of NPC, while L3b was comparable to NPC. L1b had higher NHEJ activity (p<0.034) than NPC. As phosphorylation of DNA-PKcs correlates with NHEJ activation DNA-PKcs protein levels and its phosphorylation were measured by immunoblotting. Total DNA-PKcs levels did not change appreciably between GIC and NPC cultures (Fig. 3C). However, DNA-PKcs phosphorylation in U251 and U87 cultures was attenuated and consistent with the reduced levels of NHEJ in these cells.

When treated with DNA-PKcs inhibitor (DNA-PKi) to inhibit NHEJ activity prior to IR (Fig. 3D), U251 and L2b continued to show better survival (Fig. 3D). U251 at 24h demonstrated no significant difference in viable cells between DNA-PKi treated (76%) and IR only (79%). Even at 96h, viable cells were similar (47% versus 53%, respectively). Partial radiosensitivity occurred in L2b, with DNA-PKi treated (35%) and IR alone (46%) at 96h. In contrast, NPC showed increased radiosensitivity. At 24h, DNA-PKi treated NPC showed 35% viability versus IR alone (57%). Results at 96h were similar: DNA-PKi treated (11%) versus IR alone (21%) demonstrating that NPC are more dependent on NHEJ than GIC.

Increased HR repair in GICs. HR is the second major pathway involved in resolution of DSBs (30). Given the reduced levels of NHEJ for many GICs, we quantified HR using stably transfected pDR-GFP neurosphere lines containing a functional but interrupted GFP sequence with a single DSB site that is inducible only by I-Sce I expression, and a downstream complementary non-functional GFP sequence that is required for recombination (Fig. 4A)
(31). After I-Sce I expression to create a single DSB, GFP-positive cells were 0.74% for NPC but HR repair was approximately 4 fold higher in U251 (3.76%, p<0.0002) and L2b (3.94%, p<0.0005). L3b had a less efficient HR repair (1.18%, p<0.009). HR efficiency was confirmed by measuring Rad51 foci accumulation and disassembly (Fig. 4B and Supplementary Fig. S5) (32). After IR, maximal numbers of Rad51 positive cells (53% – 66%) were achieved by 6h for L2b and U251, and followed by rapid loss between 12h – 24h. In contrast, NPCs showed gradual increase with Rad51 positive cells (26%) at 12h and slow loss of foci (12%) at 36h, supporting slower HR activity. L3b was intermediate between NPC and, U251 and L2b (Fig. 4B). Brca1, a second marker for HR was also analysed (Supplementary Fig. S6A) (33, 34). Results showed that appearance and loss of Brca1 foci was similar to Rad51, again supporting rapid HR in GICs (Supplementary Fig. S6B).

Following treatment with Rad51 siRNA (Fig. 4C), both U251 and L2b showed a drastic decrease in cell viability post-IR (Fig. 4D). At 24h, Rad51 siRNA treated U251 showed ~21% viable cells versus control siRNA treated (76%). By 96h, Rad51 siRNA treated U251 had very low cell viability (~10%) versus control siRNA (55%). L2b behaved similarly; 24h, Rad51 siRNA L2b (26%) versus control siRNA (71%) and 96h, Rad51 siRNA L2b (9%) versus control siRNA (39%). These results contrasted with NPC, where Rad51 and control siRNA treated NPC demonstrated a similar percentage of viable cells (56% versus 55%, respectively at 24h, and 15% versus 23% at 96h). This clearly demonstrates the greater importance of HR for the survival of GIC compared to NPC.

**DNA synthesis inhibition after IR is deficient in GICs.** As GICs had a higher percentage of cells undergoing HR, we hypothesized GICs had unperturbed entry into S-phase post-irradiation. To examine this, we employed IdU pulse labelling to measure DNA synthesis (Fig. 5A), Three GICs were selected (U251, L2B and L3b), because they showed different efficiencies of HR. NPC, U251, L2b and L3b had 25%, 30%, 29% and 31% IdU positive cells
respectively (Fig. 5B). Following IR, IdU positive cells were reduced 2 fold for NPC but in
GICs no reduction occurred (30 – 32% IdU positive) indicating cells continued to enter S-
phase after IR (p<0.01). This is supported by asynchronous cell-cycle analysis showing
unperturbed entry into S-phase in GIC (p<0.05) after 5Gy, indicating an attenuated S-phase
checkpoint (Fig. 5C).

**DNA initiations remain relatively unchanged in GICs after irradiation.** We next
examined GICs’ capacity to initiate DNA synthesis following IR. Cells were pulse-labelled
with CldU prior to irradiation to visualize ongoing DNA synthesis followed by a pulse with
IdU post-IR to detect new initiations (Fig. 6A). Since initiations differed between neurosphere
lines, we normalized to unirradiated cells and compared fold change after DNA damage. In
NPCs, new initiations reduced by 66% (Fig. 6B). Interestingly, U87, L1b and L2b presented
with relatively unaltered new initiation post-IR (p<0.0003). Partial inhibition of new
initiations occurred in U251 (42% reduction, p<0.023) and L3b (32% reduction, p<0.008).
But, all GICs maintained a higher level of new DNA replication after IR compared to NPCs.
Thus GICs exhibit radioresistant DNA synthesis indicating attenuation of the intra-S-phase
checkpoint (35).

To investigate further, we examined ATM activation, and phosphorylation of downstream
substrates (SMC1, Chk1 and Chk2) (36). ATM activity was attenuated in U87 with remaining
GICs similar to NPC (Fig. 6C). U87 also had reduced levels of SMC1, Chk1 and Chk2
proteins and radiation-induced phosphorylation of these proteins was defective (Fig. 6C).
U251 and L1b, which had normal levels of ATM and SMC1 showed reduced radiation-
induced phosphorylation of these proteins, including Chk2. Activation of p53 was also
measured. Basal p53 level was comparable to NPC in GICs, except U87 and L1b where levels
were low. p53 phosphorylation was detected in NPC, U251, L2b and L3b with a stronger
response in U251 (Fig. 6C). No phosphorylation was detected for U87 or L1b, which might be explained by low p53 protein.

Overall, our data suggest that defective checkpoint activation in GICs allows unperturbed S-phase entry resulting in preferential use of the HR pathway.
DISCUSSION

The mechanism for radioresistance in glioma has remained unclear because previous studies compared between sub-populations of aberrant glioma stem cells without utilizing normal neuronal stem cells (8, 9). Human neural stem cells are an ideal control, given their normal repair profile and non-tumour forming nature (37). We utilized the recently developed immortalized human NPC as a control in this study to allow for comparison between populations with ‘normal’ (NPC) and aberrant (GIC) radiosensitivity. The NPCs were similar to neural stem cells as shown by their extensive self-renewal capacity and multipotency allowing formation of different lineages. NPC sustain self-renewal and proliferation, without rapidly developing chromosomal abnormalities (11). Given the capacity to compare to NPCs it seemed relevant to focus on the overall GIC population, while incorporating NPC as a benchmark to determine the efficiency of DNA damage responses.

Cancer stem cells (CSCs), expressing CD133 have been implicated as the radioresistant population responsible for tumour development (2, 7). CD133+ glioma cells showed increased survived post-IR versus CD133− tumour cells (7). Chk2 kinase inhibition reversed this radioresistance. However, recent data cast doubt on these early findings. Under hypoxic conditions, CD133− CSCs expressed CD133+ (38, 39). Also, in vivo studies demonstrated that CD133− CSCs drive tumourigenesis in nude rats (24, 40). In our study, we demonstrated Sox2 and Nestin expression in all cultures, but noted differences in CD49f expression between various GIC lines (41, 42). Despite low CD133 expression, all GICs induced tumour formation when injected intracranially into mice, suggesting CD133 expression does not correlate with tumourigenic potential. Further studies also showed similar radioresistant properties between CD133+/− populations following DNA damage (8, 9); measurement of γH2AX foci, failed to distinguish significant differences between CD133+ and CD133− CSCs (24, 40). However, timepoints were limited to 1h (maximum foci), and 24h (background
levels). These timepoints would not detect differences in the rate of DNA DSB repair as seen here where the rate was slower in GICs though they were more radioresistant than NPC (Fig. 1A and 1B). Supporting data from self-renewal and neurosphere size comparison also indicates GICs have greater recovery than NPC after IR. Surprisingly, NHEJ was reduced 2 fold in U251 and U87, the more radioresistant neurosphere cultures. This was borne out in total repair of DNA DSBs as determined by loss of γH2AX foci. In these experiments, the rate of DSB repair was reduced at initial timepoints and more breaks remained at later times. Combined data indicates that DNA DSB repair, at least by NHEJ, does not explain radioresistance in GICs as demonstrated by DNA-PKcs inhibition having a lesser effect on GICs than NPCs (Fig. 3D). In contrast to HR repair, as determined by pDR-GFP expression and formation and resolution of Rad51 and Brca1 foci, was more efficient in the radioresistant cultures (U251 and L3b). GICs treated with Rad51 siRNA showed increased radiosensitivity versus control siRNA treated cells. Conversely, Rad51 knockdown had little effect on NPC radiosensitivity confirming the importance of HR particularly in GICs (Fig. 4D). Therefore increased dependence on HR coupled with defective cell-cycle checkpoints could explain the increased GIC radioresistance.

Cancer cells are characterized by abnormalities in cell-cycle control including prolonged activation of the mitotic checkpoint, constitutive activation of the G1/S checkpoint by p53 and activation by the ATM-53BP1-Chk2 pathway. Constitutive activation can cause selective pressure in tumours and subsequent inactivation of DNA damage responses (43, 44). Human embryonal carcinoma cells were shown to be defective for the G1/S checkpoint after DNA damage but exhibited both S-phase and G2/M delay (45). However, when these cells were differentiated with retinoic acid they failed to arrest in G1 and quickly exited S-phase arresting in G2/M. In our study, GICs (U251, L2b and L3b) continued to enter S-phase after IR at rates comparable to untreated cells, demonstrating failure of the G1/S checkpoint. GICs also
exhibited radioresistant DNA synthesis. In a normal cell, DNA damage accumulating from
cells passing through S-phase carrying DNA damage would increase cell death. In contrast,
tumour cells circumvent cell-cycle checkpoints and continue undergoing cell divisions with a
“DNA damage burden” without inducing cell death, thus resulting in increased survival.
Interestingly, this phenomenon has previously been associated with human genetic disorders
categorized by radiosensitivity and chromosomal instability and is a determinant of a
defective intra-S-phase checkpoint (36). It seems likely that the G₂ checkpoint is still intact in
GICs. This contrasts with data for embryonic carcinomas, which displayed prominent S- and
G₂-phase checkpoints (45). While enhanced basal activation of Chk1 and Chk2 was shown in
CD133⁺ glioma cells (9), we saw no evidence of this in GICs. These results are in keeping
with the cell-cycle data since we did not observe a delay at the G₁/S checkpoint, as reported
for CD133⁺ glioma cells (9). Bao et al (7) postulated that cell-cycle delay may represent a
general mechanism for genome protection in GICs. This pattern of radiation-induced
signaling in GICs and its relationship to radioresistance is difficult to explain, since IR-
induced activation of ATM kinase appears normal in all GICs except U87. Failure to observe
normal radiation signaling through SMC1, Chk1 and Chk2 in U87 can be explained by
reduced protein levels. However, reduced signaling through SMC1 in U251 and L1b is
intriguing given normal ATM activation and SMC1 protein levels. Phosphorylation of SMC1
is important for maintaining genome integrity and enhancing cell survival (46). The reduced
reliance on SMC1 phosphorylation in GICs may be compensated for enhanced HR and cell-
cycle checkpoint changes.

In summary, we demonstrated that GICs are more radioresistant than NPC but the extent of
resistance is variable. Based on these results we suggest that treatment of patients with drugs
(i.e.: ATM or ATR inhibitor) specifically targeting the HR pathway or blocking S-phase
transition may be novel therapeutic approaches. This approach may be more effective on
GBM than surrounding tissues as HR is less utilized in NPCs.
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FIGURE LEGEND

FIGURE 1: Survival in glioma initiating cells depends on proliferation. The difference in cell count at each timepoint is normalized back to its own individual unirradiated sample and viability is expressed as a percentage. A-B, Time (A) and dose (B) dependent survival of non-tumourigenic and tumourigenic cultures after irradiation. C, GICs total apoptosis (percentage at 24h and 48h) after 2Gy irradiation. Columns represent two independent experiments combined. D, Proliferation rate of neurosphere cultures determined by CFSE staining. Single cells were untreated (0Gy) or treated with indicated doses of IR, and analysed by flow cytometry 96h later. Colour with the respective numeric symbol represents the number of generations each line has undergone.

FIGURE 2: Ionizing radiation induced DNA damage resolves slowly in glioma initiating cells. A, Cells were treated with and without 2Gy IR as indicated (+ or -) and harvested after 1h. DNA damage response protein levels were determined by immunoblotting. GAPDH is a loading control. B, Cells were irradiated (2Gy) and harvested at indicated times. Representative images of GIC and NPC with γH2AX foci (green), Hoechst nucleus stain (blue) and merged. C, For each timepoint, the average foci number was scored and expressed as fold change against each individual neurosphere line at maximum foci (1h).

FIGURE 3: Glioma initiating cells do not show enhanced non-homologous end joining. A, NHEJ events were quantified within the gated region (box) after 48h. Total GFP fluorescence events from damaged (linearized DNA) plasmid transfected cells were scored against intact (circular) plasmid transfected cells for each line to account for transfection efficiency. B, Graph quantifies the efficiency of NHEJ. C, Phosphorylated and total DNA-PKcs protein levels determined by immunoblotting 1h after IR. GAPDH is a loading control. D, Efficiency of NHEJ with (DNA-PKi) and without (DMSO) DNA-PKcs inhibitor.
Neurosphere cultures were treated with DNA-PK i or DMSO 1h prior to IR treatment. Cell viability was measured by the trypan blue exclusion assay.

**FIGURE 4: Increased homologous recombination repair in glioma initiating cells.**

A, Stable clones of NPC, U251 and L2b cultures with integrated pDR-GFP vector were either mock transfected or with inducible I-Sce I<sup>+/−</sup> vector. HR efficiency was measured 48h later by flow cytometry to detect GFP-positive cells (above diagonal line). Graph quantifies the efficiency of HR. B, Cells treated with 5Gy IR and immunolabelled to detect Rad51 foci at the indicated timepoints. Representative images show Rad51 foci (green), Hoechst stain (blue) and merged. Graph quantifies the number of cells with Rad51 foci. C, Cells were irradiated after treatment with either Rad51 or negative control siRNA and harvested 48h later. Levels of Rad51 and GAPDH (loading control) were determined by immunoblotting. D, Cells were treated with negative control or Rad51 siRNA, after 48h cells received IR and viable cells measured by trypan blue exclusion assay.

**FIGURE 5: Ionizing radiation does not inhibit DNA synthesis in glioma initiating cells.**

Cells were incubated with 10µM IdU immediately prior to either mock or 5Gy treatment before fixation at 1h. Columns represent percentage of cells undergoing DNA synthesis. A, IF images showing IdU incorporation foci (red), Hoechst stain (blue) and merged. B, Data quantification representing the average percentage of IdU-labelled nuclei. C, Cells were either mock or 5Gy IR treated and cell-cycle stage determined 1h after exposure. Cells stained with propidium iodide were quantified by flow cytometry.

**FIGURE 6: Glioma initiating cells continue to initiate replication after ionizing radiation via defective cell-cycle checkpoint.**

A, Diagram illustrating the use of two different nucleoside analogues to study DNA replication after IR-induced damage. Representative images of replication signals from DNA fibres; ongoing forks, unidirectional
and bidirectional illustrated by continuous and dashed arrows (left and middle panel), and
new initiation as dash arrows only (right panel). B, New initiations after DNA damage. To
quantify the frequency of firing of origins of replication, the number of red fibres (dashed
arrows) was divided by the total sum of red and green-red signals (continuous and dashed
arrows). Data in graph are normalized to the individual neurosphere line and average new-
initiations shown as fold change. C, Immunoblot of phosphorylated and total protein (ATM,
SMC1, Chk1, Chk2 and p53) following mock and 2Gy.
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