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Abrogation of radioresistance in glioblastoma stem-like cells by inhibition of ATM kinase



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ABSTRACT

Resistance to radiotherapy in glioblastoma (GBM) is an important clinical problem and several authors have attributed this to a subpopulation of GBM cancer stem cells (CSCs) which may be responsible for tumour recurrence following treatment. It is hypothesised that GBM CSCs exhibit upregulated DNA damage responses and are resistant to radiation but the current literature is conflicting. We investigated radioresistance of primary GBM cells grown in stem cell conditions (CSC) compared to paired differentiated tumour cell populations and explored the radiosensitising effects of the ATM inhibitor KU-55933.

We report that GBM CSCs are radioresistant compared to paired differentiated tumour cells as measured by clonogenic assay. GBM CSC's display upregulated phosphorylated DNA damage response proteins and enhanced activation of the G2/M checkpoint following irradiation and repair DNA double strand breaks (DSBs) more efficiently than their differentiated tumour cell counterparts following radiation.

Inhibition of ATM kinase by KU-55933 produced potent radiosensitisation of GBM CSCs (sensitiser enhancement ratios 2.6–3.5) and effectively abrogated the enhanced DSB repair proficiency observed in GBM CSCs at 24 h post irradiation. G2/M checkpoint activation was reduced but not abolished by KU-55933 in GBM CSCs.

ATM kinase inhibition overcomes radioresistance of GBM CSCs and, in combination with conventional therapy, has potential to improve outcomes for patients with GBM.

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1. Introduction

Glioblastoma (GBM) is a highly radioresistant tumour with a propensity for local recurrence following therapy.

Radiotherapy is a key treatment modality for GBM and while clinical responses are observed, prognosis remains poor, even following triple modality therapy comprising surgery, chemotherapy and radiotherapy. Median survival rates for

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patients with good performance status are in the region of 12–16 months (Stupp et al., 2005). To improve the dismal survival figures, however, the mechanisms underlying radioresistance must be further elucidated and strategies developed to overcome them. Dominant clonal populations, which emerge as a result of genetic and epigenetic changes that confer a survival advantage, are thought to drive tumour growth (Bonavia et al., 2011; Nowell, 1976; Patel et al., 2014). The stochastic basis of this clonal evolution model has recently been challenged by the cancer stem cell (CSC) hypothesis (Reya et al., 2001). According to this hypothesis tumour growth is driven by a hierarchically organised population of tumourigenic cells that, similar to normal stem cells, self-renew and generate non-tumourigenic progeny. Although widely accepted, this hypothesis remains contentious. Data identifying the glycoprotein CD133 as a putative CSC marker in GBM (Bao et al., 2006; Galli et al., 2004; Singh et al., 2004) have been challenged (Beier et al., 2007; Joo et al., 2008; Kelly et al., 2009; Son et al., 2009) and the case for a cellular hierarchical organisation in GBM remains unproven.

Nevertheless, research has been facilitated by the identification of molecular markers that have been reported to identify tumour propagating/CSCs *in vitro*. CD133, Sox 2 and Nestin are commonly utilised, however it is likely that these markers identify GBM clonal populations which are heterogeneous in their tumorigenic potential. In keeping with this, a recent study has identified via single cell RNA sequencing a continuum of stemness-related expression states within single GBM tumours (Patel et al., 2014) suggesting that the cancer stem cell state displays significant plasticity. Functional assays of enhanced tumourigenicity remain the most useful indicator of stemness in GBM cell populations and the study of GBM CSCs relies upon the use of patient derived cell lines of low passage in order to prevent clonal drift.

The commonly observed clinical scenario of initial response to treatment followed by inevitable local recurrence may be explained by the failure of current therapies to sterilise CSCs, and several researchers have investigated whether CSCs are resistant to cytotoxic therapy. Chen et al. identified GBM initiating cells that were capable of repopulating GBM tumours *in vivo* following temozolomide treatment (Chen et al., 2012). Responses of GBM CSCs to radiotherapy have also been investigated, with conflicting results. Bao et al. demonstrated that CD133+ tumour cell populations were radioresistant compared to CD133– populations (Bao et al., 2006), a phenotype that was mediated by upregulation of the DNA damage response (DDR). Enhanced phosphorylation of cell cycle checkpoint proteins was demonstrated along with evidence of more efficient DNA repair, although the kinetics of DNA double strand break (DSB) repair were not examined in detail. In contrast, McCord et al. reported radiosensitivity and defective DDR in CD133+ tumour cell populations (McCord et al., 2009), but comparison of CD133+ populations with unrelated, established GBM cell lines may not be an optimal model. Ropolo et al. conducted a comparative analysis of DNA repair in stem and non-stem GBM cultures and found no evidence of enhanced DNA repair, although CD133+ cells had a prolonged cell doubling time along with enhanced cell cycle checkpoint protein activation (Ropolo et al., 2009).

Small molecule inhibitors of DDR proteins have been demonstrated to increase the radiosensitivity of tumour cells in pre-clinical studies. Many tumours are defective in DDR when compared to normal tissues (Bartkova et al., 2005), and targeting tumour-specific DDR deficiencies to enhance radiosensitivity of tumour cells is an attractive clinical strategy. Ataxia telangiectasia mutated (ATM) is a key DDR component, playing a central role in DSB repair and cell cycle checkpoints (Shiloh and Ziv, 2013). Deficiency of ATM function causes Ataxia Telangiectasia which is characterised by increased radiation sensitivity (Taylor et al., 1975) and inhibition of ATM kinase activity causes potent radiosensitisation of GBM cells (Biddlestone-Thorpe et al., 2013; Golding et al., 2012, 2009). The ATM-Chk2 pathway is known to have particular importance in the molecular pathogenesis of GBM. In the first study of endogenous DNA damage signalling in glioma, it was reported that whilst gamma H2AX was upregulated in all grades of glioma, robust activation of the ATM-Chk2 pathway was only observed in GBM (Bartkova et al., 2010). Hence inhibition of ATM has potential applications in the treatment of GBM, but detailed investigation of their radiosensitising effects in GBM CSC's and the underlying mechanisms has not yet been reported.

We characterised the radioresistant properties of primary GBM cell cultures propagated as paired cell lines either in serum free, stem cell promoting (“cancer stem cell”; CSC) conditions or in serum-containing, stem cell depleting differentiating culture conditions (“differentiated tumour cells”; diff) in order to compare GBM cancer stem cells (CSCs) with differentiated (or “non-stem”) GBM cells originating from the same parental tumour. Herein we report increased radioresistance of GBM CSC populations relative to differentiated cell populations that is associated with an enhanced DNA damage checkpoint response as well as an increased capacity for DSB repair. Consistent with published data we observed upregulation of ATM activity in GBM CSC's and report the novel observation that ATM inhibition potently radiosensitises GBM CSC's. A detailed analysis of the cell cycle checkpoint and DSB repair mechanisms underlying this effect is also presented.

2. Methods

2.1. Patient-derived GBM cell derivation and culture

R10, S2, E2 and G7 primary glioblastoma cell lines were derived from freshly resected GBM specimens as previously described (Faal Al-Mayhani et al., 2009). Tissue collection protocols were compliant with the UK Human Tissue Act 2004 (HTA Licence ref 12315) and approved by the local regional Ethics Committee (LREC ref 04/Q0108/60). Informed consent was obtained from each patient before surgery. Briefly, anonymised patient resection specimens were homogenised and seeded in serum free (SF) media to form spheroid aggregates which were then collected and plated onto extracellular matrix coated flasks (ECM 1:10 dilution, Sigma). Cells were allowed to form a primary monolayer then passaged in SF medium. Each cell line was subsequently cultured as paired populations grown either in stem cell enriching conditions (CSC) or in

differentiating conditions (diff) by passaging in SF media or differentiating media (DM) respectively. SF media consisted of Adv DMEM F12 medium (Gibco) supplemented with 1% B27 (Invitrogen), 0.5% N2 (Invitrogen), 4 µg/ml heparin, 20 ng/ml fibroblast growth factor 2 (bFGF, Sigma), 20 ng/ml epidermal growth factor (EGF, Sigma) and 1% L-Glutamine. DM consisted of MEM (Gibco) supplemented with 10% foetal bovine serum (FBS) Sigma, 1% L-glutamine and 1% sodium pyruvate. CSC cultures were grown as monolayers on Matrigel™ coated flasks in SF media. Neurosphere cultures were grown in identical media in 96 well plates without Matrigel™. Differentiated tumour cell cultures were grown as adherent monolayers in uncoated flasks in FCS containing media. All cell cultures were maintained at 37 °C, 5% CO₂ and routinely passaged every 3–4 days. For all experiments, low passage number cells were used (maximum 20 but more commonly 5 to 15 passages).

Cells cultured in neurobasal SF conditions exhibited up-regulated expression of the cancer stem cell markers CD133, Nestin and Sox2 compared with differentiated tumour cultures. CSC enriched cultures of G7 and E2 cells were tumorigenic after intracranial injection in CD1 nude mice. Injection of 1×10^5 E2 cells generated highly infiltrative tumours that resemble gliomatosis cerebri; tumour burden was significantly greater after injection of CSC enriched populations than differentiated tumour cell populations. CSC enriched cultures of G7 cells generated intracranial tumours that recapitulated key histological features of GBM and demonstrated an invasive phenotype whereas differentiated tumour cells generated well-demarcated, non-invasive tumours (Mannino et al., 2014).

2.2. Irradiation

Cultures were irradiated in tissue culture flasks at room temperature with 195 kV X-rays at a dose rate of 1.6 Gy/min in an Xstrahl RS225 cabinet.

2.3. ATM inhibitor

The ATM inhibitor KU-55933 (Tocris Bioscience) was solubilised in 100% DMSO to produce a 100 mM stock. In all experiments cells were exposed to 10 µM KU-55933 for 1 h prior to irradiation. Controls were exposed to media containing 0.01% DMSO.

2.4. Clonogenic assays

GBM CSC and differentiated tumour cell cultures were seeded at a density of 250 cells per well in Matrigel-coated 6 well dishes. CSC and differentiated tumour cultures were maintained in SF or DM respectively for the duration of the assay. Cells were incubated for 24 h then exposed to fresh media containing either 10 µM KU-55933 or 0.01% DMSO for 1 h before irradiation (1–5 Gy) or sham irradiation. Cells were then incubated for a further 24 h before aspiration of drug containing media and replacement with fresh media. G7 cells were incubated for 2 weeks and R10 and E2 cells for 3 weeks prior to fixation and staining. Colonies of diameter 50 cells or greater were counted using an automated colony counter (GelCount™, Oxford Optronix).

2.5. Neurosphere assays

GBM CSC were plated at a density of 10 cells per 100 µL medium per well in 96 well plates in SF media containing 10 µM KU-55933 or 0.01% DMSO and irradiated (2 Gy) or sham irradiated. Cultures were incubated for a further 48 h and a further 100 µL of fresh media was added. Neurospheres were counted manually under 5× magnification after 4 weeks and 3 weeks for E2 and G7 cells respectively.

2.6. Gamma H2AX foci quantification

E2 GBM CSC and differentiated tumour cells were plated on coverslips and exposed to 10 µM KU-55933 or an identical concentration of vehicle and treated with 1 Gy or sham irradiated. Cultures were fixed in 4% formaldehyde/PBS then permeabilised, blocked with 5% FCS, 0.5% BSA in 0.1% Triton-PBS and incubated with anti-gamma H2AX (Millipore, 1:100) and CENPF (Abcam, 1:250) antibodies overnight at 4 °C then with appropriate Alexa Fluor 568 or 488 secondary antibodies (Invitrogen). Nuclei were counterstained with DAPI.

Z-stacks were obtained at 63× magnification on a Zeiss 710 confocal microscope. 30 Z-stacks at each time point/condition were acquired for analysis. Volocity software was used to identify and count foci in each nucleus. Nuclei were categorised into “moderate/strongly positive for CENPF” and “weak/negative for CENPF” by the operator. Nuclei which exhibited confluent staining for gamma H2AX were assumed to be in S phase and were excluded from the analysis. The number of nuclei analysed for each data point ranged from 65 to 307 (CENPF positive) and 300 to 460 (CENPF negative).

2.7. FACS analysis

G7, S2 and E2 CSC and differentiated tumour cultures were irradiated with 5 Gy ± KU-55933. Cultures were disaggregated with Accutase and fixed in 70% ethanol.

Fixed cells were pelleted and incubated with anti-phosphorylated Histone H3 serine 10 antibody Alexa Fluor 488 conjugate 1:50 (Cell Signaling). Cells were resuspended in 1 mg/ml propidium iodide solution with RNAase A. FACS analysis was carried out on a FACSCalibur and results analysed using FlowJo 7.6.5.

2.8. Western blotting

Cultures were exposed to 10 µM KU-55933 or vehicle and irradiated with 5 Gy. Total cell lysates were prepared using SDS lysis buffer. Lysates were blotted onto membrane and probed with antibodies specific for phosphorylated ATM serine 1981 (Novus Biologicals), phosphorylated Chk2 threonine 68 (Cell Signaling) (Supplementary Figure 2C).

2.9. Cell proliferation assay

Cells were seeded onto 96 well plates. A range of concentrations of KU-55933 was added to each well. An ATP based cell viability assay was then carried out at 24 h and 6 days following addition of drug according to manufacturer's instructions (CellTiter-Glo, Promega).

2.10. Statistical analyses

All experiments were repeated 3 times except where stated and data points reported as mean \pm SEM. The unpaired t-test or one sample t test were used to generate *p* values. Gamma H2AX foci data was analysed using median foci counts per nucleus derived from 3 independent experiments. Mann Whitney tests were used to test significance because foci counts were not normally distributed, exhibiting significant positive skew when plotted as a histogram. A linear quadratic fit was applied to clonogenic data, and $SER_{0.37}$ values were calculated from fitted data. The ANOVA test was used to analyse differences in clonogenic survival curves. Minitab and GraphPad Prism were used for analyses.

3. Results

3.1. GBM CSC cultures are radioresistant in comparison to GBM differentiated tumour cell cultures

To evaluate radiation sensitivity, clonogenic survival assays were performed using R10, E2 and G7 cell lines. To minimise the impact of the different culture conditions, all experiments were conducted using Matrigel-coated plates. Under these conditions plating efficiency of CSC and differentiated tumour populations were similar, although subtle differences in colony morphology between CSC and differentiated tumour cell cultures were noted (Figure 1A). While both CSC and tumour differentiated cultures exhibited radioresistance, GBM CSC enriched cultures were significantly more resistant to radiation (1–5 Gy) than differentiated tumour cell cultures (Figure 1A). Although radioresistance of GBM CSC has been reported previously (Bao et al., 2006; Venere et al., 2014), to our knowledge this is the first demonstration of this phenotype in paired cell lines using clonogenic survival assays. Curves were fitted with the linear quadratic equation and dose modifying factors at a surviving fraction of 37% calculated (Table 1A). These showed that the dose of radiation required to produce an equivalent decrease in surviving fraction was 1.5 fold greater for R10 CSC ($p < 0.0001$), 1.3 fold greater for E2 CSC ($p < 0.001$) and 1.5 fold greater for G7 CSC ($p = 0.015$) than for the corresponding differentiated tumour cell cultures. Surviving fraction at 4 Gy (SF_{4Gy}) values were also statistically significantly higher in R10, E2 and G7 CSC populations than in corresponding differentiated tumour cell populations (Table 1B) confirming the radioresistant phenotype of these stem cell enriched primary glioblastoma cell populations. Mean SF_{4Gy} for E2 CSC and tumour differentiated cells were 0.62 and 0.46 respectively ($p = 0.001$), and 0.63 compared to 0.46 for G7 CSC and differentiated tumour cell populations respectively ($p = 0.05$). A similar significant difference was seen between CSC and differentiated tumour cells derived from primary R10 GBM cell cultures. SF_{4Gy} values have been cited rather than the more conventional SF_{2Gy} values because the inherent radioresistance of the cell populations rendered calculations based on the latter parameter less meaningful. Since radiotherapy is usually delivered in daily 2 Gy fractions, however, SF_{2Gy} is a more clinically relevant parameter and it is of

interest to note that this value was between 83% and 90% in the three GBM CSC populations tested. Analysis of the CSC and differentiated tumour cell survival curves by ANOVA demonstrated a statistically significant difference in all three cell lines ($p < 0.001$).

3.2. Glioblastoma CSC enriched cultures exhibit an enhanced DNA damage response following exposure to ionising radiation

To investigate the mechanisms underlying the radiation resistance of GBM CSC and examine the hypothesis that the DNA damage response contributes to this phenotype as previously reported, we interrogated a panel of DNA damage response proteins. ATM represents a key hub of the DNA damage response having roles in both cell cycle control and DNA repair. As a biochemical readout we measured phosphorylation of ATM at serine 1981 and Chk2 at threonine 68 in cell cultures following exposure to radiation. Serine 1981 is an autophosphorylation site of ATM which correlates with activation of DDR while Chk2 is a major phosphorylation target of ATM and an important mediator of cell cycle checkpoints following ionising radiation.

Western blot analysis revealed an upregulated DDR in CSC enriched cultures compared to differentiated tumour cell cultures. Ionising radiation (5 Gy) induced a robust pATM s1981 and pChk2 thr68 response in E2 CSC cultures at 1, 3 and 6 h, whereas this response was less prominent in E2 differentiated tumour cell cultures (Figure 1B). A similar pattern in upregulation of pATM and pChk2 was seen in R10 CSCs when compared to R10 differentiated tumour cells 1 h after radiation. In the absence of radiation, upregulation of pATM s1981 and pChk2 thr68 was also evident in G7 CSC cultures compared to G7 differentiated tumour cell cultures. Indeed, the predominant effect in G7 CSC appeared to be upregulation of baseline levels of pATM rather than additional upregulation of the pATM response following radiation.

We hypothesised that these different DDR responses would translate into different cell cycle checkpoint activation kinetics following IR. Neither E2 nor G7 cells exhibited a G1/S checkpoint response to ionising radiation (Supp Figure 1), so we quantified radiation induced activation of the G2/M checkpoint by flow cytometric analysis of phosphorylated histone H3 at serine 10, a specific marker of mitotic cells. E2, G7 and S2 GBM CSC cultures all displayed enhanced activation of the G2/M checkpoint at 3 and 6 h following radiation compared with differentiated tumour cell populations; this was manifested by a rapid fall in the percentage of mitotic cells in the CSC populations (Figure 2A). These data demonstrate for the first time that the cell cycle checkpoint phosphoprotein changes in GBM CSCs described here and published previously (Bao et al., 2006) result in enhanced cell cycle checkpoint activation in GBM CSCs. This phenomenon is likely to contribute to the radioresistant phenotype of GBM CSCs.

3.3. Resolution of gamma H2AX foci induced by ionising radiation is enhanced in glioblastoma CSC populations

Alongside cell cycle checkpoint activation, repair of radiation induced DNA breaks is a key determinant of radiation

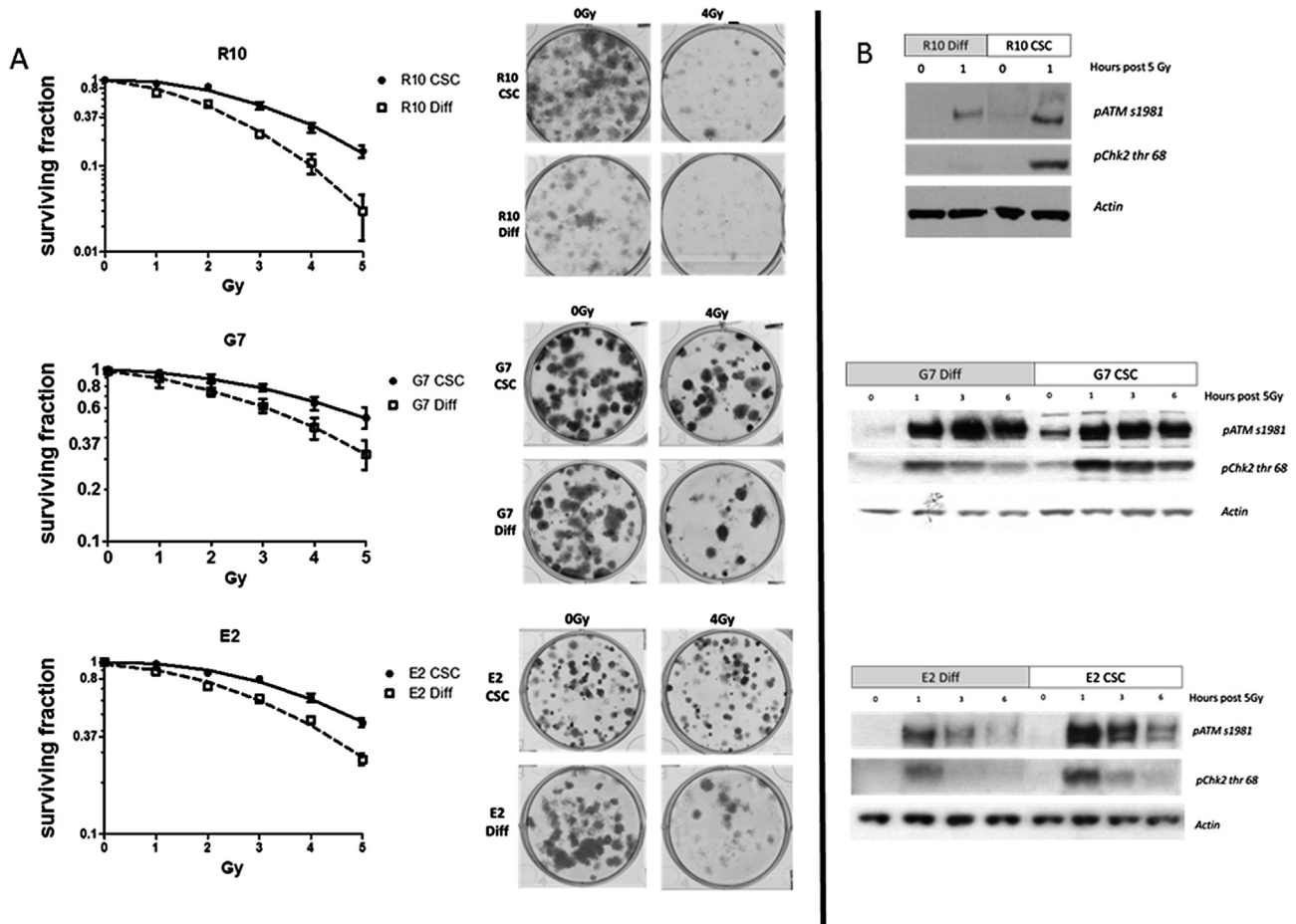


Figure 1 – A, Clonogenic survival curves showing response of CSC and differentiated tumour cell cultures to ionising radiation in R10, E2 and G7 primary cell cultures. Mean surviving fraction plus SEM of 9 independent experiments is shown for E2 and G7, whilst means of 3 independent experiments are shown for R10 fitted to a linear quadratic model. Curves are significantly different by ANOVA (E2 CSC vs differentiated tumour cells $p < 0.001$, G7 CSC vs differentiated tumour cells $p < 0.001$, R10 CSC vs differentiated tumour cells $p < 0.0001$). Representative images of colony formation at 0 and 4 Gy in each population are shown. B, Western blots comparing levels of pATM s1981 and pChk2 thr 68 at 0, 1, 3 and 6 h following 5 Gy of ionising radiation in E2 and G7 CSC and differentiated tumour cell populations. Levels of pATM and pChk2 are shown at 1 h post 5 Gy in the R10 cell line.

sensitivity, with DNA double strand breaks (DSB) being the critical lesions. To investigate whether DSB repair contributes to the relative radioresistance of our GBM CSC populations we conducted a detailed, quantitative analysis of induction and resolution of nuclear gamma H2AX foci in E2 CSC and differentiated tumour cells following exposure to ionising radiation (1 Gy, [Figure 2](#)). Since cells in G2 phase have increased DNA content and therefore exhibit approximately twice as many DSB per Gy compared to cells in G1, separate analyses were conducted for cells staining positively and negatively for CENPF, which is a marker of cells in G2 phase. S phase cells exhibit diffuse gamma H2AX staining and were excluded from the analysis. This approach enabled us to exclude any influence of cell cycle phase on foci counts.

No difference in induction of gamma H2AX foci between E2 CSC and differentiated tumour cell cultures was observed at 1 h, and the early or ‘fast’ kinetics of foci resolution did not differ between populations when measured at the 3 h time point ([Figure 2B](#)). However, E2 GBM CSC cultures displayed a

significantly enhanced ability to resolve DNA DSBs at 24 h compared to E2 differentiated tumour cell cultures. This enhanced repair of a subset of DSBs was evident in both CENPF negative and CENPF positive populations of cells: median number of gamma H2AX foci at 24 h was 3 in differentiated tumour cells versus 1 in CSC in CENPF negative cells ($p < 0.001$) and 11 in differentiated tumour cells versus 7 in CSC in CENPF positive cells ($p = 0.0001$). Unirradiated CENPF positive E2 CSCs exhibited a small but statistically significant increase in median number of foci compared to unirradiated differentiated tumour cell cultures (median gamma H2AX foci 6 in CSC versus 3 in differentiated tumour cells, $p = 0.004$, [Figure 2B](#)). This difference was not evident in CENPF negative cells.

These data provide the most detailed evidence to date of a modest but significant increase in the ability of GBM CSC to execute repair of a subset of DSB. That this enhanced repair capacity was observed only at the 24 h time point indicates firstly that it may be of clinical significance (since the level

Table 1 – A, Dose modifying factors at 37% clonogenic survival ($DMF_{0.37}$) with 95% confidence intervals for CSC and differentiated tumour cell cultures (diff) of R10, G7 and E2 indicating that CSC cultures are significantly more radioresistant than differentiated tumour cell cultures in both cell lines. $DMF_{0.37}$ values were calculated from clonogenic survival data fitted to a linear quadratic model as shown in **Figure 1**. **B**, Mean surviving fractions at 4 Gy (SF_{4Gy}) with 95% confidence intervals for CSC and differentiated tumour cell E2, G7 and R10 cultures. Means of 9 independent experiments each performed in triplicate for E2 and G7, and means of 3 independent experiments in triplicate for the R10 cell line. *P* values for 2 sample *t* test of mean SF_{4Gy} are also shown.

A						
	DMF 0.37 (95% CI)					
E2 CSC vs diff	1.30 (1.16, 1.44) (<i>p</i> < 0.001)					
G7 CSC vs diff	1.52 (1.10, 1.93) (<i>p</i> = 0.015)					
R10 CSC vs diff	1.47 (1.41, 1.54) (<i>p</i> < 0.0001)					
B						
	E2 CSC	E2 diff	G7 CSC	G7 diff	R10 CSC	R10 diff
Mean SF 4 Gy (95% CI)	0.62 (0.56, 0.69)	0.46 (0.41, 0.51)	0.63 (0.52, 0.75)	0.46 (0.33, 0.58)	0.28 (0.21, 0.35)	0.11 (0.053, 0.16)
T test of means	<i>p</i> = 0.001		<i>p</i> = 0.05		<i>p</i> = 0.018	

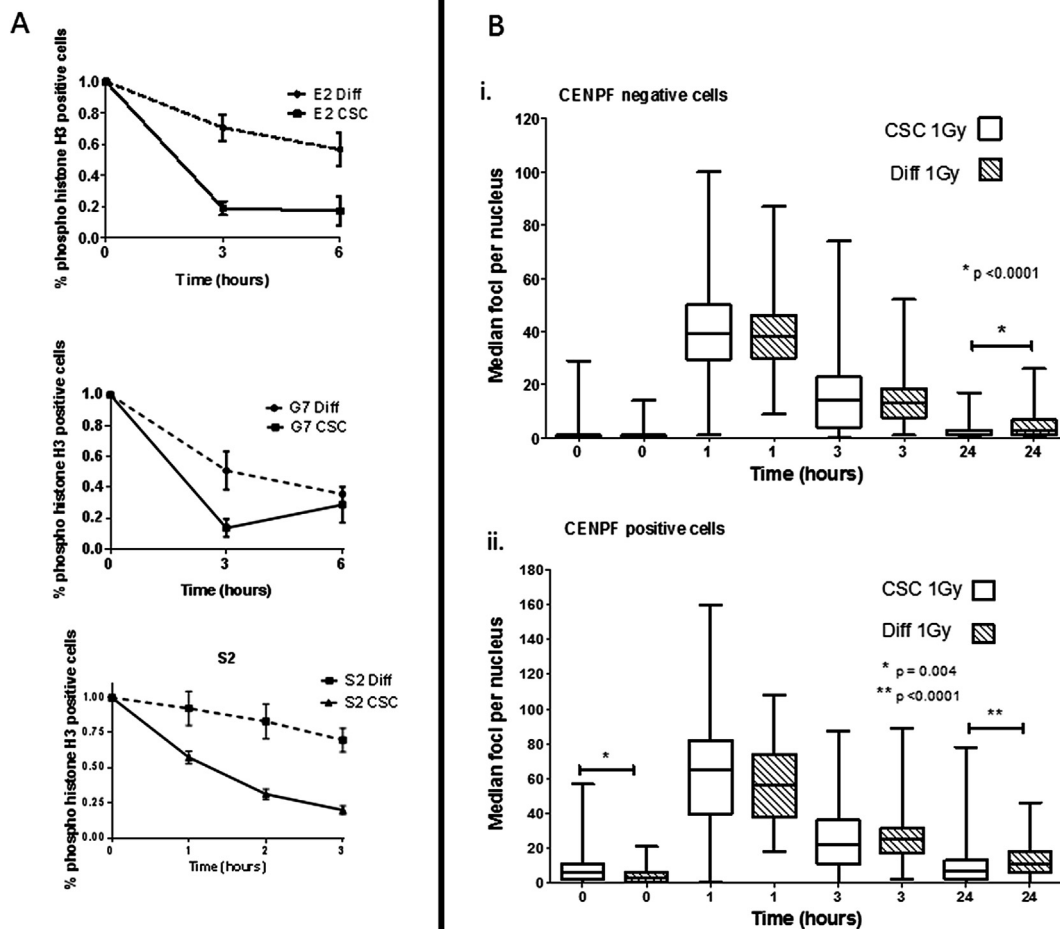


Figure 2 – A Interrogation of the G2/M checkpoint in E2, G7 and S2 CSC and differentiated tumour cell populations by FACS analysis of mitotic cells detected by immunostaining for phosphorylated Histone H3 at 0, 3 and 6 h following 5 Gy irradiation in E2 and G7, and 1, 2 and 3 h following 5 Gy irradiation in S2. Mean values plus SEM of 3 independent experiments are shown after normalisation to unirradiated control values. **B** Quantification of gamma H2AX foci detected by immunofluorescence in E2 CSC and differentiated tumour cells following irradiation. Cells were fixed at time points of 0, 1, 3 and 24 h following 1 Gy and then stained for the DNA double strand break marker gamma H2AX. Nuclei were stained for CENPF, a G2 cell cycle marker, and foci quantified separately in **i.** CENPF negative and **ii.** CENPF positive cells to allow exclusion of cell cycle effects on foci number. gamma H2AX foci were identified and quantified using Volocity software. Box and whisker plots represent results of 3 independent experiments. *P* values calculated by Mann Whitney test.

of unresolved DSB at 24 h correlates with radiation sensitivity both *in vitro* and *in vivo*, Banath et al., 2004), and secondly that the previously noted upregulation of ATM signalling may contribute to the specific DNA repair phenotype observed in our GBM CSC populations, since ATM has a specific role in 'slow' repair of DSB (Goodarzi et al., 2010; Kuhne et al., 2004).

3.4. Upregulated DDR in GBM CSCs can be effectively abrogated by ATM kinase inhibition

Given that GBM CSCs have an upregulated ATM response following ionising radiation, we hypothesised that inhibition of ATM kinase activity by the commercially available and well characterised inhibitor KU-55933 would result in modification of their radioresistant phenotype. Pre-treatment with 10 μ M KU-55933 for 1 h resulted in complete inhibition of ATM mediated phosphorylation of Chk2 at threonine 68 in both E2 and G7 CSC and differentiated tumour cell cultures in response to irradiation (5 Gy, Figure 3A and B). Radiation

induced autophosphorylation of ATM at serine 1981 was also markedly reduced by KU-55933 in all four populations (Figure 3A and B). The effects of ATM kinase inhibition on viability and proliferation of GBM CSC and differentiated tumour cell cultures were investigated using a CellTiter-Glo™ cell proliferation assay (Supplementary Figure 2A and B). This demonstrated that 10 μ M KU-55933 had no significant cytotoxic effect on CSC or differentiated tumour cell populations when cells were treated for 24 h or 6 days. Next we investigated the effect of ATM inhibition on radiation induced G2/M arrest (Figure 3C and D). In E2 CSC enriched cultures the enhanced G2/M checkpoint activation observed previously was only partially abrogated by 10 μ M KU-55933 whereas in E2 differentiated tumour cell cultures more pronounced inhibition was demonstrated at all time points following radiation treatment. A similar trend was seen in the G7 cell populations. Hence G2/M arrest after radiation in CSC cultures appears to be only partly sensitive to ATM kinase inhibition.

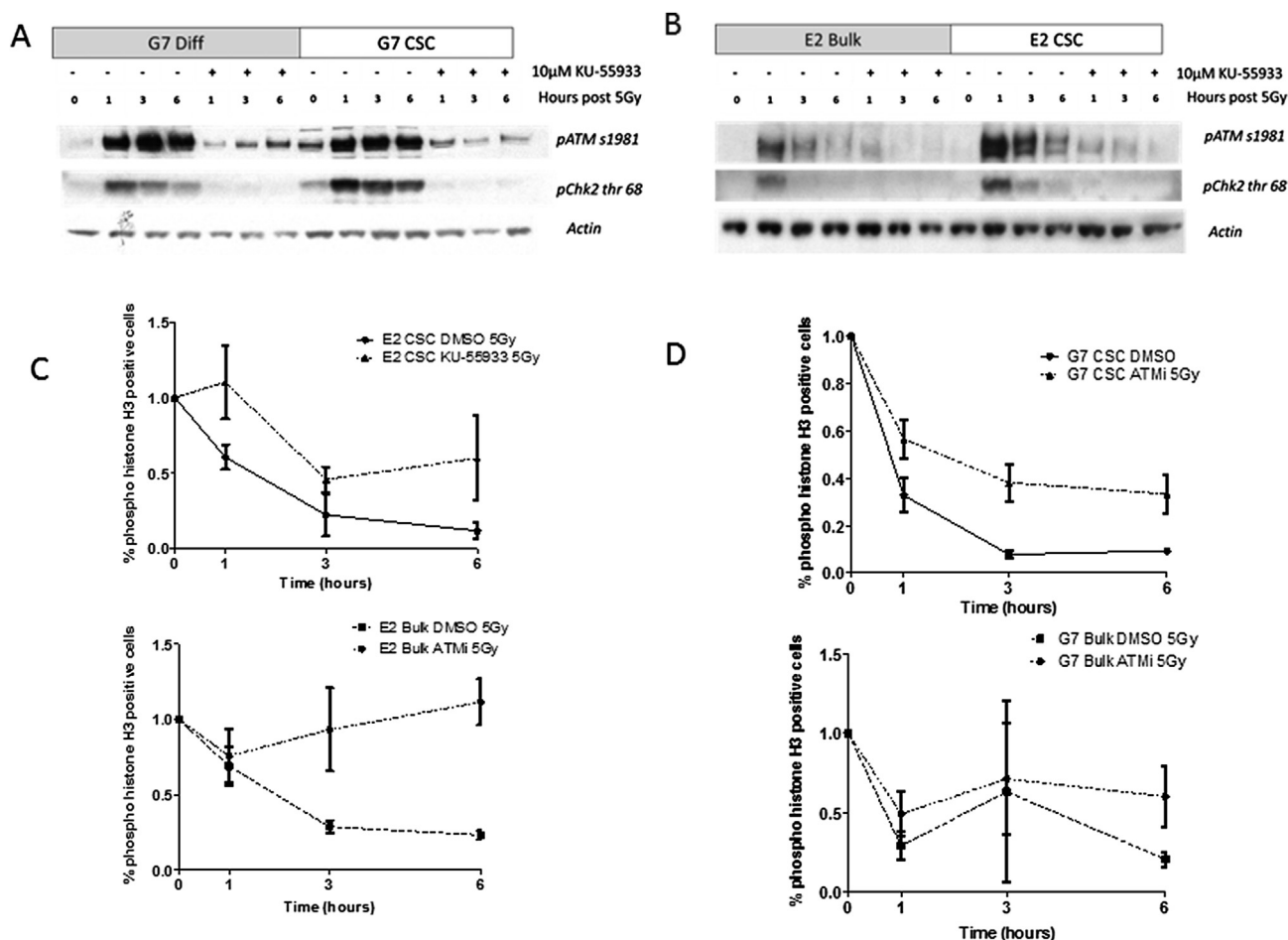


Figure 3 – Effect of KU-55933 on ATM and Chk2 phosphorylation and the G2/M checkpoint following ionising radiation. Extensions of the Western blots previously shown in Figure 2A are shown in A. G7 and B. E2, CSC and differentiated tumour cell cultures treated with 10 μ M KU-55933 for 1 h prior to irradiation (5 Gy) and lysed at the time points shown following irradiation. Interrogation of the G2/M checkpoint by FACS analysis of mitotic cells identified by positive immunostaining of phosphorylated Histone H3 following treatment with 10 μ M KU-55933 for 1 h prior to irradiation with 5 Gy in the C. E2 and D. G7 CSC and differentiated tumour cell cultures. Data points represent mean and SEM of 3 independent experiments.

3.5. ATM inhibition potently radiosensitises GBM CSC enriched cultures

The radiosensitising effects of KU-55933 were then investigated in GBM CSC and differentiated tumour cell populations using the clonogenic survival assay. Very marked radiosensitisation of GBM CSCs was observed in R10, E2 and G7 cell lines (Figure 4A, Table 2). Sensitiser enhancement ratios at 37% survival ($SE_{0.37}$) were 2.60 (1.72, 3.4) for E2 CSCs compared with 2.01 (1.27, 2.86) for E2 differentiated tumour cells and 3.17 (2.63, 3.71) for R10 CSCs compared with 2.23 (2.16, 2.30) for R10 differentiated tumour cells. Potent radiosensitisation was observed in both CSC and differentiated G7 cells with $SE_{0.37}$ values of 3.46 (1.75, 5.18) and 3.43 (1.99, 4.86) respectively. ATM inhibition in the absence of radiation had no effect on clonogenic survival of any of the six populations. SF_{4Gy} values were significantly reduced by KU-55933 in CSC and differentiated tumour cell cultures in R10, E2 and G7 cell lines (Table 2).

To confirm the effects of ATM inhibition and radiation on GBM CSC survival we conducted neurosphere formation assays in E2 and G7 CSC populations. The extent to which

radiation (2 Gy) inhibited neurosphere formation *in vitro* was significantly increased by treatment with 10 μ M KU-55933 in both E2 and G7 CSC populations (Figure 4B). ATM inhibition in the absence of radiation did not affect neurosphere formation, and neither KU-55933 nor radiation treatment affected neurosphere diameter or morphology.

3.6. The enhanced ability of E2 stem cultures to resolve gamma H2AX foci at 24 h post irradiation is effectively abrogated by inhibition of ATM

Since ATM inhibition had only a modest effect on G2/M checkpoint integrity in CSC we investigated whether the profound effects of KU-55933 on the radiation sensitivity of CSC could be explained by modulation of DNA DSB repair. As before, gamma H2AX foci in G1 and G2 phase populations of E2 cells were analysed separately and compared between GBM CSC and differentiated tumour cell cultures.

As expected, ATM inhibition caused a significant reduction in gamma H2AX foci in unirradiated E2 cells and at early time points after IR (up to 3 h) in all experiments (Figure 5A and B). This is consistent with ATM being the major phosphorylator

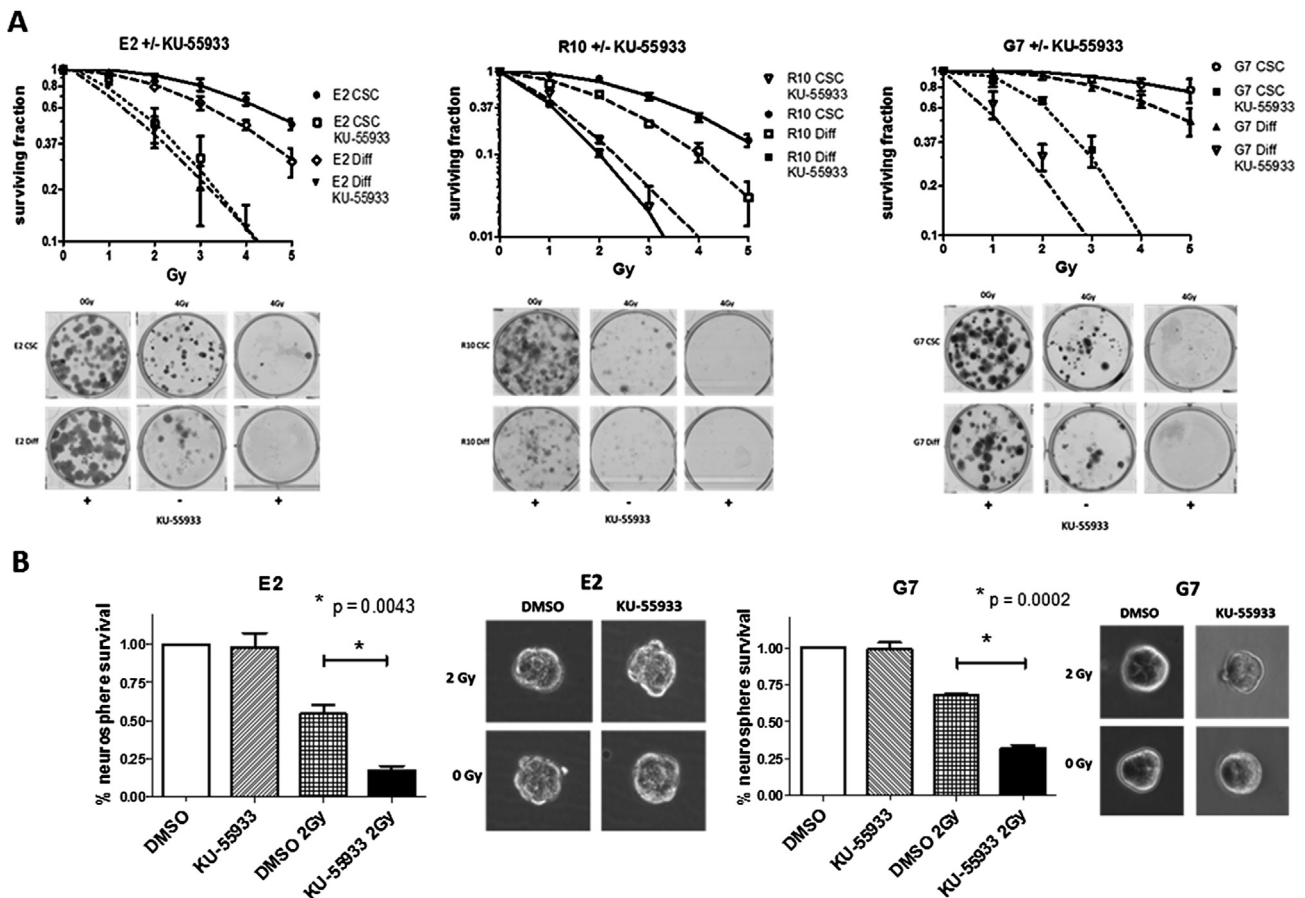


Figure 4 – Effects of KU-55933 on radiosensitivity of E2, R10 and G7 cell lines. A, Clonogenic survival curves comparing effects of KU-55933 plus radiation versus radiation alone on CSC and differentiated tumour cell cultures in E2, R10 and G7 cell lines. Representative images of colony formation at 0 and 4 Gy are also shown. B, Neurosphere formation assay in E2 and G7 CSC cultures. 10 cells per well were seeded into 96 well plates and treated with KU-55933 or DMSO prior to irradiation with 2 Gy. Neurospheres were quantified manually after 3 (G7) or 4 weeks (E2) under 5 \times magnification. Mean plus SEM of 3 independent experiments shown, normalised to control values. Neurosphere forming efficiency of controls: E2 = 18.56% (15.72, 21.40), G7 = 34.74% (28.50, 40.98). Representative images of neurospheres are shown.

Table 2 – Sensitiser enhancement ratios at 37% clonogenic survival ($SER_{0.37}$) and SF_{4Gy} values for KU-55933 treatment in combination with radiation. $SER_{0.37}$ values were calculated from clonogenic survival data fitted to a linear quadratic model as shown in Figure 4. Mean SF_{4Gy} values for CSC and differentiated tumour cell E2, R10 and G7 populations in the absence and presence of KU-55933 (plus 95% CI's; 3 independent experiments each in triplicate).

	$SER_{0.37}$ (95% CI)	SF_{4Gy} (-KU-55933) (95% CI)	SF_{4Gy} (+KU-55933) (95% CI)
E2 CSC	2.60 (1.72, 3.40)	0.67 (0.52, 0.83)	0.088 (0.037, 0.14)
E2 bulk	2.01 (1.27, 2.86)	0.48 (0.43, 0.53)	0.11 (0.030, 0.18)
G7 CSC	3.46 (1.75, 5.18)	0.83 (0.69, 0.97)	0.096 (0.074, 0.12)
G7 bulk	3.43 (1.99, 4.86)	0.66 (0.54, 0.77)	0.034 (0, 0.1)
R10 CSC	3.17 (2.63, 3.71)	0.28 (0.21, 0.35)	0.12 (0.099, 0.12)
R10 bulk	2.23 (2.16, 2.30)	0.11 (0.053, 0.16)	0

of histone H2AX at early time points and reflects reduced signalling at DSB rather than reduced numbers of DSB. Hence it was not possible to interrogate the impact of ATM inhibition on DSB induction or early repair dynamics using this technique. In an attempt to address this issue, neutral comet assays were undertaken but were not sufficiently sensitive to provide useful information (data not shown).

Distinct effects of ATM inhibition were observed at later time points, however. In the CENPF negative CSC population KU-55933 treatment was associated with a statistically

significant increase in unresolved gamma H2AX foci at 24 h, an effect that was not apparent in the differentiated tumour cell population (Figure 5A).

In CENPF positive populations, KU-55933 was associated with a statistically significant increase in unresolved gamma H2AX foci at 24 h in both CSC and differentiated tumour cell populations (Figure 5B). These data indicate that ATM inhibition significantly retards repair of the subset of radiation induced DSB that is resolved with slow kinetics, an effect that was particularly marked in CSC populations and only observed in G2 phase cells in differentiated tumour cell populations.

4. Discussion

The aim of this study was to present a detailed and clinically relevant analysis of radiation responses in GBM CSCs and to characterise the effects of chemical inhibition of ATM on these parameters. Investigation of primary GBM cell lines propagated as paired CSC and differentiated tumour cell cultures permitted direct comparison of the radiation responses of GBM CSC and non-CSC populations, in contrast to previous studies which have relied upon comparisons between cell lines from different parental tumours or comparison of primary CSC enriched populations to established cell lines (McCord et al., 2009; Ropolo et al., 2009).

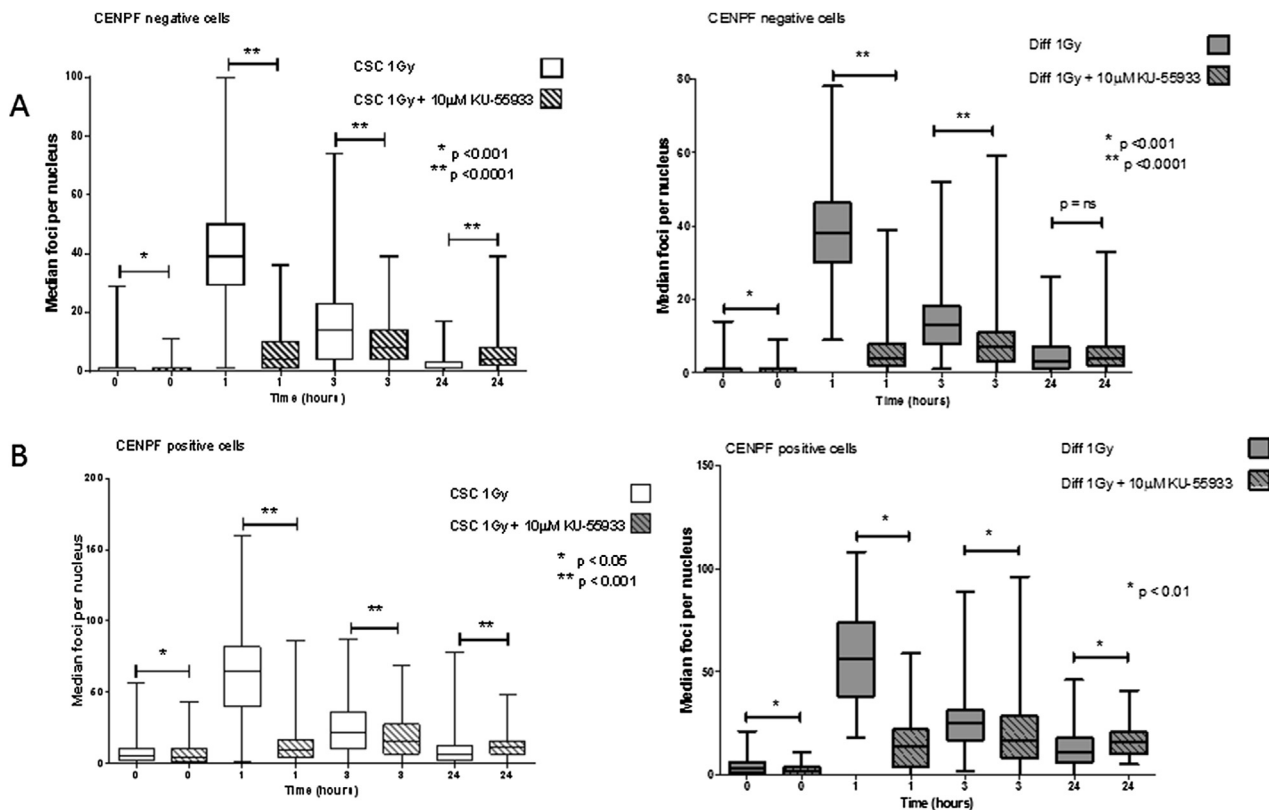


Figure 5 – Quantification of gamma H2AX foci in E2 CSC and differentiated tumour cells following treatment with 10 μM KU-55933 and 1 Gy radiation. Median number of foci per nucleus in A. CENPF negative CSC and differentiated tumour cells following irradiation ±10 μM KU-55933 and B. CENPF positive CSC and differentiated tumour cells following irradiation ±10 μM KU-55933. Box and whisker plots represent results from 3 independent experiments, p values calculated by Mann Whitney U test.

In keeping with the seminal observations of Bao and colleagues (Bao et al., 2006), direct comparison of clonogenic survival between paired CSC and differentiated tumour cell cultures indicated that GBM does indeed contain radioresistant tumour-propagating sub-clones. Clonogenic survival assays are the gold standard *in vitro* measurement of radiosensitivity, and have been shown to correlate with clinical outcome (Bjork-Eriksson et al., 2000, 1998; West et al., 1997). Our data support the widely stated yet still contentious theory that radioresistance of CSCs makes a significant contribution to treatment failure in GBM.

To elucidate the factors underlying this radioresistance we performed a detailed quantification of gamma H2AX foci resolution in CSC and differentiated tumour cell populations originating from the same parental tumour. Gamma H2AX foci formation is observed following DSB generation after ionising radiation exposure and is a sensitive and specific indicator of DSBs (Sedelnikova et al., 2002). Quantification of residual foci 24 h after irradiation has been shown to correlate with other measures of radiosensitivity including SF_{2Gy} (Banath et al., 2004). We demonstrated a small but significant enhancement of DSB repair 24 h post irradiation in CSCs compared to differentiated tumour cells. Other investigators have used alkaline comet assay to measure DNA repair in CSC's, which quantifies SSBs and other non-lethal DNA damage. Ropolo et al. conducted an examination of gamma H2AX foci in sorted CD133 positive and CD133 negative cells and found no evidence of enhanced repair in the CD133 population (Ropolo et al., 2009). However the investigators did not quantify the number of foci per nucleus, instead relying upon percentage of cells with foci as a surrogate measure. This may explain why our detailed analysis has detected subtle differences between CSC and differentiated tumour cell populations. The differences in foci resolution at 24 h are small but are likely to have radiobiological significance. It is clear that a single unrepaired DSB can be lethal to a cancer cell (Schwager, 1982) and the marked radiosensitivity of ATM or Artemis deficient cells is associated with failure to repair only about 10% of radiation induced DSB (Riballo et al., 2004). The number of excess unresolved DSBs we observed in E2 differentiated tumour cells compared with CSC equates to approximately 5% of the induced DSB burden.

Biochemical interrogation of DDR proteins showed that CSC populations exhibited upregulation of phosphorylated ATM and Chk2 either at baseline or in response to radiation, which is in keeping with the published literature (Bao et al., 2006). We also observed significant differences in G2/M checkpoint kinetics following radiation: CSC populations activated the G2/M checkpoint more proficiently than differentiated tumour cell populations over a 6 h period post irradiation, which would allow additional time for DSB repair in CSCs and contribute to their radioresistant phenotype.

Since radioresistance of CSC populations was associated with enhanced DSB repair and augmented G2/M checkpoint activation, ATM represents a very promising target. ATM is a major hub of the DDR, modulating activity of many proteins in response to DSBs (Shiloh and Ziv, 2013). ATM has a clearly defined function in initiating early G2/M checkpoint arrest following ionising radiation (Xu et al., 2002) and also has important roles in the repair of a subset of DNA lesions

representing 10–15% of radiation-induced DSB. This subset is thought to comprise heterochromatic (Goodarzi et al., 2008) and/or other complex DSBs (Alvarez-Quilon et al., 2014; Riballo et al., 2004). Defects in ATM function manifest as a deficit in the slow phase of DSB repair, which is most evident 24 h after radiation (Goodarzi et al., 2010; Kuhne et al., 2004).

As well as abrogation of radiation induced ATM autophosphorylation at serine 1981, we observed complete inhibition of phosphorylation of Chk2 at threonine 68 (a major phosphorylation target of ATM) in the presence of the ATM inhibitor KU-55933. These effects were sustained up to 6 h following radiation. Consistent with these observations the G2/M checkpoint was almost completely abrogated in both E2 and G7 differentiated tumour cell populations. In contrast, only partial inhibition of the G2/M checkpoint was observed in CSC populations. This unexpected observation indicates that CSCs possess ATM independent mechanisms for activation and maintenance of the G2/M checkpoint, whereas differentiated tumour cell populations appear to be more reliant on ATM function for G2/M checkpoint integrity. Ataxia telangiectasia related protein (ATR) is an obvious candidate for the backup role (Fokas et al., 2014) but as yet no data is available to verify this proposed function.

ATM inhibition was extremely effective in overcoming the inherent radioresistance of GBM CSCs. SER_{0.37} values were in the range of 2–3.5, which is significantly greater than the enhancement achieved by radiosensitisers in current clinical use. Golding et al. (Golding et al., 2012) reported similar radiosensitisation in established GBM cell lines using inhibitors of ATM, however our study represents the first analysis of ATM inhibition in CSCs by clonogenic assay. Raso et al. (Raso et al., 2012) investigated the sensitising effects of ATM on GBM CSC and differentiated tumour cells using an MTT assay; this showed ATM inhibition to have a protective effect on survival of differentiated tumour cells whereas CSCs were sensitised. We have identified a profound radiosensitising effect of ATM inhibition on differentiated tumour cells as well as CSC populations. Clonogenic survival assays are the gold standard for measuring radiobiological effects and the differing results may reflect use of a more robust assay, or be cell line dependent. However it is difficult to relate the profound effects of ATM inhibition on the DDR to a radioprotective effect. Interestingly in E2 cells, ATM inhibition seemed to completely abrogate the relative radioresistance of CSC's, with the survival curve of the KU-55933 treated CSCs being superimposed on that of the KU-55933 sensitised E2 differentiated tumour cells. This finding indicates that ATM function is the dominant component of CSC radioresistance in this cell line.

The influence of ATM inhibition on DNA repair was investigated in the E2 cell line by quantification of gamma H2AX foci. ATM is known to be a key phosphorylator of histone H2AX at early time points following irradiation, however DNAPK and ATR can contribute to this function (Burma et al., 2001). Correspondingly inhibition of foci formation was observed up to 3 h post irradiation in KU-55933 treated cells, which is likely to represent inhibition of signalling pathways responsible for phosphorylation of H2AX rather than reduced DSB formation. The assessment of DNA repair kinetics via analysis of gamma H2AX foci formation and resolution relies upon post translational modifications and is

therefore a surrogate marker of DNA DSB repair rather than a direct assessment of religation of broken DNA ends. Resolution and formation of gamma H2AX foci can be altered by manipulation of phosphatases without necessarily reflecting repair of the underlying DNA DSB (Goldstein et al., 2013; Kinner et al., 2008). This supports our view that the reduction in foci formation under conditions of ATM inhibition at early timepoints following radiation reflects reduced gamma H2AX formation rather than reduced DNA DSB induction. At the 24 h time point we observed a significant increase in residual gamma H2AX foci in CSCs in both G1 and G2 cell populations, which is highly likely to make a major contribution to the radiosensitising effects of KU-55933. In differentiated tumour cells this increase was only evident in the G2 population. We have therefore identified a likely mechanism for the enhanced radiosensitisation of CSCs in the E2 cell line.

Biddlestone-Thorpe and colleagues (Biddlestone-Thorpe et al., 2013) demonstrated ATM inhibition in an orthotopic xenograft glioma model *in vivo* using the ATM inhibitor KU-60019 delivered via an intracranial pump. They found that survival was prolonged 2–3 fold by ATM inhibition and radiotherapy versus controls. This study also showed that radiosensitisation was influenced by p53 status. This may have important implications for tumour specificity, since surrounding normal tissue will be p53 wild type and thus expected to experience less radiosensitisation. Other investigators have also shown that astrocytes in the brain (a critical normal supporting tissue) exhibit downregulated ATM (Gosink et al., 1999; Schneider et al., 2012) and are not radiosensitised by ATM deficiency. More work is required to investigate the possible toxic effects of radiation and ATM inhibition; however these studies suggest that GBM may be preferentially radiosensitised in comparison to normal tissue.

A recent study (Venere et al., 2014) describes a key role of the SSBR protein PARP-1 in the constitutive DDR activation and relative radioresistance of GBM CSCs. In conjunction with our findings this paper reinforces the importance of the DDR in GBM CSC treatment resistance and underlines the potential of selective DDR kinase inhibitors to improve the efficacy of radiotherapy in this setting.

These findings have considerable implications for the management of GBM in patients. We have illustrated the radioresistance of GBM CSCs and confirmed that enhanced DDR plays a large part in this radioresistance. Furthermore our investigations confirm that GBM CSCs have enhanced checkpoint activation and DSB repair which is ATM dependent. Our results add to the growing body of evidence that CSC resistance plays an important part in the failure of current therapies to achieve local control in GBM. Most importantly we demonstrate that potent radiosensitising effects can be achieved in GBM CSCs by chemical inhibition of ATM. In conclusion, DDR modulation is a promising therapeutic strategy for the treatment of radioresistant solid tumours, particularly GBM.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.molonc.2014.08.003>.

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