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## RESEARCH ARTICLE

# Most DNA repair defects do not modify the relationship between relative biological effectiveness and linear energy transfer in CRISPR-edited cells

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**Abstract**

**Background:** Cancer is a highly heterogeneous disease, driven by frequent genetic alterations which have significant effects on radiosensitivity. However, radiotherapy for a given cancer type is typically given with a standard dose determined from population-level trials. As a result, a proportion of patients are under- or over-dosed, reducing the clinical benefit of radiotherapy. Biological optimization would not only allow individual dose prescription but also a more efficient allocation of limited resources, such as proton and carbon ion therapy.

Proton and ion radiotherapy offer an advantage over photons due to their elevated Relative Biological Effectiveness (RBE) resulting from their elevated Linear Energy Transfer (LET). Despite significant interest in optimizing LET by tailoring radiotherapy plans, RBE's genetic dependence remains unclear.

**Purpose:** The aim of this study is to better define the RBE/LET relationship in a panel of cell lines with different defects in DSB repair pathways, but otherwise identical biological features and genetic background to isolate these effects.

**Methods:** Normal human cells (RPE1), genetically modified to introduce defects in DNA double-strand break (DSB) repair genes, ATM, BRCA1, DCLRE1C, LIG4, PRKDC and TP53, were used to map the RBE-LET relationship. Cell survival was measured with clonogenic assays after exposure to photons, protons (LET 1 and 12 keV/μm) and alpha particles (129 keV/μm). Gene knockout sensitizer enhancement ratio (SER) values were calculated as the ratio of the mean inactivation dose (MID) of wild-type cells to repair-deficient cells, and RBE values were calculated as the ratio of the MID of X-ray and particle irradiated cells. 53BP1 foci were used to quantify radiation-induced DSBs and their repair following irradiation.

**Results:** Deletion of NHEJ genes had the greatest impact on photon sensitivity (ATM<sup>-/-</sup> SER = 2.0 and Lig4<sup>-/-</sup> SER = 1.8), with genes associated with HR having smaller effects (BRCA1<sup>-/-</sup> SER = 1.2). Wild-type cells showed RBEs of 1.1, 1.3, 5.0 for low- and high-LET protons and alpha particles respectively. SERs for different genes were independent of LET, apart from NHEJ knockouts which proved to be markedly hypersensitive across all tested LETs. Due to this hypersensitivity, the impact of high LET was reduced in cell models lacking the NHEJ repair pathway. HR-defective cells had moderately increased sensitivity across all tested LETs, but, notably, the contribution of HR pathway to survival appeared independent of LET. Analysis of 53BP1 foci shows that NHEJ-defective cells had the least DSB repair capacity after low LET exposure, and no visible repair

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after high LET exposure. HR-defective cells also had slower repair kinetics, but the impact of HR defects is not as severe as NHEJ defects.

**Conclusions:** DSB repair defects, particularly in NHEJ, conferred significant radiosensitivity across all LETs. This sensitization appeared independent of LET, suggesting that the contribution of different DNA repair pathways to survival does not depend on radiation quality.

#### KEYWORDS

CRISPR—Cas9, DNA damage, DNA repair, high LET, homologous recombination, non-homologous end joining, proton therapy, radiation, radiosensitivity, RBE

## 1 | INTRODUCTION

Currently, decisions for most tumors regarding the radiation type (e.g., photons, protons, carbon ions) and dose fractionation regimens are determined from clinical experience and population-level clinical trials. However, cancer is a highly heterogeneous disease, driven by numerous genetic alterations which significantly affects radiosensitivity. This genetic variability is reflected in estimates which suggest that cancers at the same site which receive the same treatment may vary in radiosensitivity by 25% or more.<sup>1,2</sup> As a result, a significant number of patients are certainly under- or over-dosed, reducing the clinical benefit of radiotherapy.

To date, biological optimization is not considered in treatment planning. This would not only allow individual dose prescription but also a more efficient allocation of limited resources, such proton and carbon ion therapy. As an example, in the Netherlands a model-based approach is used to identify patients with head and neck cancer suitable for proton therapy based on dosimetric parameters, but with a lack of biological personalisation.<sup>3</sup> However, particle therapy offers not only dosimetric benefits, but also induces greater levels of complex damage than X-rays, due to their higher Linear Energy Transfer (LET), giving them a higher Relative Biological Effectiveness (RBE).<sup>4</sup>

It is well established that complex DNA damage is more difficult for the cellular machinery to repair than individual lesions.<sup>5,6</sup> Un- or mis-repaired DNA Double Strand Breaks (DSBs), either simple or complex, are the lesions that result in the cytotoxic, mutagenic and carcinogenic effects of radiation. Thus, cellular ability or inability to repair DSBs may be an effective indicator of the RBE of any form of radiation. Measurement of the rate of repair of DSBs by visualization of  $\gamma$ H2AX foci showed that both the half-times of re-joining and the fraction of residual DNA breaks increased with the quality of the incident radiation.<sup>7,8</sup>

In mammalian cells, DNA DSBs are mainly repaired by two distinct pathways, Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR). These two pathways operate with different kinetics and are used differently throughout the cell cycle. HR is a relatively error-free repair pathway that utilizes the sister

chromatid as template, so it is only active through S- and G2 phases of the cell cycle. It relies on numerous proteins and protein complexes, for example, BRCA1/2 and RAD51.<sup>9</sup> In contrast to HR, NHEJ is available throughout the cell cycle and utilizes no homology to join DNA ends.<sup>10</sup> NHEJ is an error-prone repair pathway that is mediated by the KU70/80 heterodimer, DNA-PK and Lig4. NHEJ was also shown to be the predominant repair pathway for DSBs induced by photon radiation.<sup>9</sup> NHEJ-deficient cells are extremely sensitive to radiation and accumulate significant unrepaired DSBs as function of dose.<sup>11</sup> By contrast, HR-deficient cells exhibit moderate radiosensitivity and their radiosensitivity is enhanced when cells are synchronized in S-phase.<sup>12</sup>

Although evidence indicates that NHEJ is the major repair pathway for low-LET radiation induced DSBs, the contribution of different pathways to repair high-LET induced DSBs and complex damage is not clear. Previous studies have suggested that the NHEJ pathway plays a reduced role in the repair of complex DSBs induced by high-LET radiation, due to a smaller sensitizing effect in NHEJ-defective cells when exposed to protons or alpha-particles than the effect seen when exposed to X-rays.<sup>13–17</sup> Furthermore, some authors also suggest HR-defective cells have elevated sensitivity to high-LET radiations and thus much greater RBEs than HR-competent cells.<sup>16,18,19</sup> However, this is not consistent across the scientific literature.<sup>4,5</sup> Some authors reported a linear correlation between cellular photon sensitivity and high-LET radiosensitivity and this sensitivity is defined by NHEJ cellular capability.<sup>20–23</sup>

A better understanding of the RBE of different qualities of radiation as function of individual cellular genetic variations in genetic and transcriptional factors would enable us to develop more robust and individualized predictions of radiotherapy response.<sup>24,25</sup> Despite being widely accepted that genetic alterations affecting DNA damage repair machinery contribute to heterogeneity in cell radiosensitivity and causes variations in RBE values, there are currently very limited systematic data on RBE variation in human cancers to enable the development of effective predictive models.

The aim of this study was to better define the RBE/LET relationship in a panel of cell lines with different defects in DSB repair pathways, introduced with

CRISPR-Cas9, but with an otherwise identical biological features and genetic background to isolate these effects.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines

Immortalized retinal pigment epithelial RPE-1 cells were kindly provided by Dr. Kienan Savage (Queen's University Belfast, UK). Cells were grown in DMEM/F-12 with L-glutamine, containing 15 mM of HEPES, 10% FBS and 1% penicillin and streptomycin and maintained at 37°C in 5% CO<sub>2</sub>. RPE-1 cells were also used to perform CRISPR-Cas9 manipulation to the following genes: TP53, ATM, DCLRE1C (Artemis), BRCA1, LIG4 and PRKDC (DNA-PKcs). This CRISPR-Cas9 manipulation of these genes has been previously described and validated.<sup>26</sup>

### 2.2 | Irradiation setups

The X-ray irradiation were performed using an X-RAD 225 radiation source (Precision X-ray Inc., USA) at 225 kV, using a 2 mm copper filter at a current of 13.3 mA, resulting in a dose rate of 0.59 Gy/min a distance of 50 cm from the source. The beam characterization and determination of absorbed dose have been performed according to the Institute of Physics and Engineering in Medicine and Biology (IPEMB) code of practice (IPEMB1996), with an ionization chamber, Gafchromic film and Perspex phantom. Cells were irradiated at a distance of 50 cm from the source in 6-well plates with 2 mL of media per well (resulting in an average media thickness of 2.25 mm). Six-well plates were directly placed in the cabinet holder aligned with the beam center to ensure uniformity of the beam (with deviations of less than 5%). Cells were exposed to absorbed doses of 0.5, 1, 2, 4, 6 and 8 Gy. Absorbed doses to each sample were controlled by adjusting exposure time.

For external alpha irradiation, circular Mylar dishes with a thickness of 0.9 μm and surface area of 9.1 cm<sup>2</sup> were placed 2.9 mm from an uncollimated 50 × 50 mm<sup>2</sup> planar <sup>241</sup>Am alpha source, with a dose rate of 1.57 Gy/min. Incident average energy at the cell layer was 2.88±1.04 MeV with an LET of 129.3±15.2 keV/μm, as previously described.<sup>27</sup> Source irradiation uniformity was quantified using optical density measurements of exposed Gafchromic EBT3 dosimetry customized film (Ashland Specialty Ingredients, Parlin, NJ), where the protective polyester sheet was removed from one side of the film, allowing alpha particles to reach the active layer without being stopped in the protective layer. Dose distribution across the irradiated area is highly uniform with deviations of less than 5.5%. Cells were exposed to absorbed doses of 0.25, 0.5, 1, 1.5 and 2 Gy. Absorbed

doses to each sample were controlled by adjusting exposure time.

Proton irradiations were performed using a horizontal, passively-scattered beam line of 60 MeV maximal energy from the Douglas Cyclotron at Clatterbridge.<sup>28</sup> Cells in 35-mm dishes were positioned at the isocenter, 70 mm from a brass collimator (43 mm diameter). For high-energy protons, cells were irradiated directly by an approximately 1 keV/μm pristine beam of 58 MeV effective energy (dose rate ~ 5 Gy/min). Low-energy proton irradiations were performed using a modulator to generate a 27 mm spread-out Bragg peak and a 24.4 mm absorber was used to position the cells at the distal edge, corresponding to a mean proton energy of 11 MeV at a dose-average LET of 12 keV/μm (dose rate ~ 5 Gy/min).<sup>29,30</sup> The LET was estimated based on previous work utilizing the Clatterbridge beam.<sup>31</sup> Finally, proton absorbed doses were quantified using optical density measurements of exposed Gafchromic EBT3 film (Ashland Specialty Ingredients).<sup>32</sup> EBT3 film were attached to the bottom of each sample during exposure and then each film was scanned, and the red channel information was quantified against a standard curve produced with X-ray exposed films.

Due beam time restrictions two biological replicates were performed for proton response studies.

### 2.3 | Clonogenic survival assay

The colony formation assay was carried out according to published methods.<sup>33</sup> Cells were seeded into six-well plates with an optimized cell density according to the absorbed dose the day before irradiation. After irradiation, cells were incubated for 7 days. The colonies were stained with 4% crystal violet solution in ethanol and were manually counted, with a colony defined as consisting of at least 50 cells. From these counts, plating efficiency (PE) and survival fraction (SF) were calculated. Survival fraction was determined by the number of colonies formed after irradiation divided by the number of cells seeded, corrected for the PE of unirradiated cells. Data were fit to the linear quadratic equation ( $SF = e^{-(\alpha D + \beta D^2)}$ ) using non-linear regression.

Knockout sensitizer enhancement ratio (SER) values were calculated as the ratio of the mean inactivation dose (MID) of wild-type cells to repair-deficient cells and relative biological effectiveness (RBE) values were calculated using MID of X-ray and particle irradiated cells. MID is defined as the area under the dose response curve for a given condition.<sup>34</sup>

### 2.4 | DNA damage by immunofluorescence assay

Following 2 Gy irradiation, cells were fixed in 50:50 methanol-acetone solution and permeabilized

(0.5% Triton X-100 in PBS) at predetermined time points before being blocked in blocking buffer (5% FBS and 0.1% Triton X-100 in PBS) and stained with 53BP1 primary antibody (1:5000) (#NB100-304, Novus Biologicals, USA) and  $\gamma$ H2AX primary antibody (1:10000) (#05-636-I, Merks Chemicals, Germany) for 1 h. Cells were then washed three times in PBS and stained with Alexa Flour 568 goat anti-rabbit IgG secondary antibody (#A21429, Life Technologies) and Alexa Flour 488 goat anti-mouse IgG secondary antibody (#A21131, Life Technologies) (1:2000) in the dark for 1 h. Following staining, the cells were washed three times in PBS and mounted onto microscope slides using Prolong Gold anti-fade reagent with DAPI (#P36930, Invitrogen). Foci were manually counted from the whole nucleus of 50 randomly selected cells on each sample with a Nikon Eclipse Ti microscope (Nikon Corporation, Japan), using a 60 $\times$  objective.

Data are presented as the mean values of foci per cell and the respective standard deviation of three independent experiments. Data presented here is corrected for unirradiated background levels by subtracting the number of foci in unirradiated cells. For repair kinetic analysis, foci data were then fit with an exponential decay in GraphPad Prism 9,  $N = (N_0 - plateau) * e^{-kt} + plateau$ , where  $N_0$  represents the initial number of foci, plateau represents the residual damage and  $k$  is the rate of DSB repair.

## 2.5 | Statistical analysis

All experiments with X-ray and alpha-particle irradiations were performed in triplicate, however due to beam-time limitations, proton irradiations were performed with two biological replicates. Unpaired Student's t-test and one-way ANOVA were used for statistical evaluation. All statistics and graph plotting used GraphPad Prism 9.0 (GraphPad).

## 2.6 | Literature survey

Results for comparable published experiments were obtained to provide a benchmark for data in this work. A search was performed for studies investigating loss of DNA repair genes and their impact as a function of LET, which identified 13 relevant publications.<sup>14–18,20–23,35–38</sup> As the most commonly-reported endpoint, RBE for  $D_{10}$  (Dose that gives 10% survival) values were obtained from each paper, for both wild-type and knockout lines.

Knockouts were grouped into HR and NHEJ genes. To enable comparisons to be made accounting for cell lines and experimental conditions, relative RBEs were calculated, as  $RBE_{Rel} = \frac{RBE_{KO}}{RBE_{WT}}$ , where  $RBE_{KO}$  and  $RBE_{WT}$  are the knock-out and wild-type RBEs respec-

tively for a given irradiation condition. In this case, values greater than 1 indicate an elevated sensitivity to high-LET radiations in the knockout line. Note that this is mathematically equivalent to the relative SER,  $SER_{Rel} = \frac{SER_{KO}}{SER_{WT}}$ , for comparison with other data in this work.

Results were separated into four groups according to LET—low proton LET ( $\sim 2.5$  keV/ $\mu$ m, mid-SOBP), high proton LET ( $\sim 10$  keV/ $\mu$ m, distal end), low ion LET ( $\sim 13$  keV/ $\mu$ m, mid-SOBP for heavier ions), and high ion LET ( $> 90$  keV/ $\mu$ m, distal end of ion peak or alpha particles). Mean relative sensitization and confidence intervals were calculated for each pathway and irradiation condition.

## 3 | RESULTS

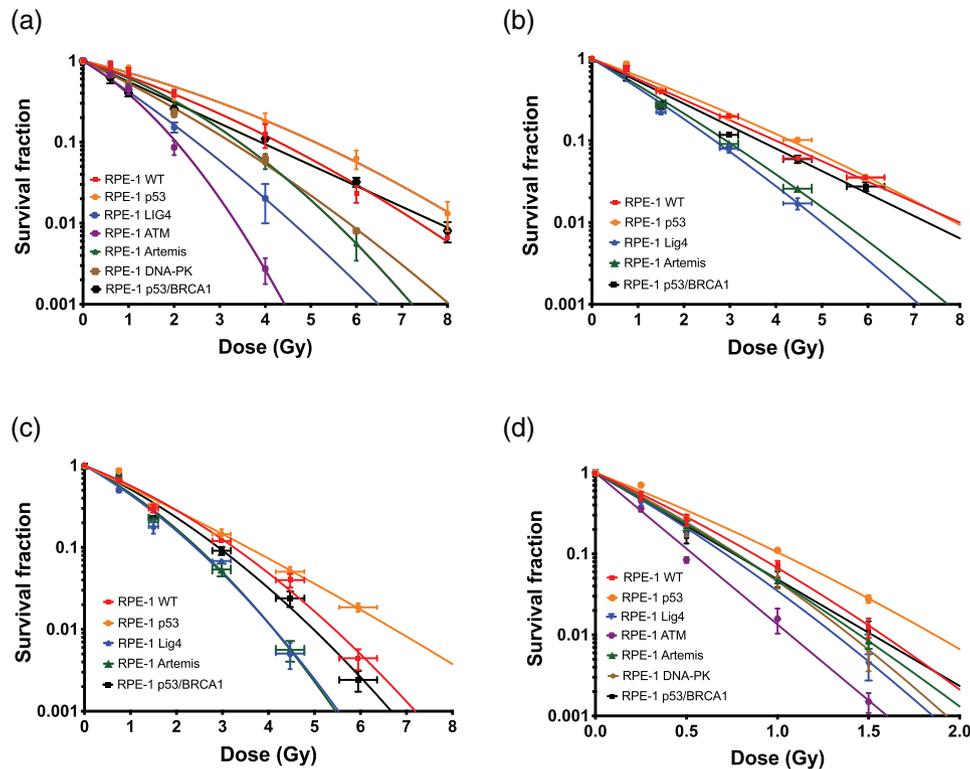
### 3.1 | Clonogenic survival and determination of RBE and SER

The survival curves for X-rays (A), proton at high (B) and low-energy (C), and alpha-particle (D) irradiated cells are shown in Figure 1. Across all irradiation conditions, cellular radiosensitivity followed the same trend, in descending order of sensitivity: ATM<sup>-/-</sup>, LIG4<sup>-/-</sup>, DNA-PK<sup>-/-</sup>, Artemis<sup>-/-</sup>, BRCA1<sup>-/-</sup>, wild-type, p53<sup>-/-</sup> cells. In other words, survival clearly depends on the NHEJ and HR pathways statuses, with a predominant role of NHEJ.

As expected, when LET increases, the survival curve demonstrates a steeper dose-response relationship highlighting the correlation between radiation quality and cytotoxic effect. We then calculated RBE values from these survival curves.

Wild type cells showed RBEs of 1.13, 1.29, 5.05 for low- and high-LET protons and alpha particles, respectively (Figure 2a). This trend of increasing RBE values is followed for all gene knockouts with the exception of Lig4<sup>-/-</sup>, where RBEs are 0.94, 0.99 and 3.49 for low- and high-LET protons and alpha particles respectively. Figure 2c shows the correlation between the LET and RBE for each clone. There was a positive correlation between the two variables for all knockouts ( $R^2 = 0.99$ ).

SER values were also calculated for knockout of each gene for each radiation quality. Knockout of p53, a master regulator of essential cellular processes, was the only gene in our panel that increased the resistance of cells to radiation with SER around 0.89 for all tested radiation qualities. Key NHEJ genes had the greatest impact on photon sensitivity (Lig4<sup>-/-</sup> SER = 1.77 and DNA-PK<sup>-/-</sup> SER = 1.34), with genes associated with HR and complex damage having smaller effects (BRCA1<sup>-/-</sup> SER = 1.16 and Artemis<sup>-/-</sup> SER = 1.19) (Figure 2b). Moreover, Figure 2d shows that SER values associated with NHEJ associated genes (LIG4 and DNA-PK) and ATM decreases gradually with the LET (slope  $\sim -0.003$ ). However, SER values



**FIGURE 1** Cell survival curves after exposure with different qualities of radiation (a) X-rays, (b) low-LET protons, (c) high-LET proton, (d) alpha-particles in cells proficient or deficient in DSBs repair. The curves were fitted to the linear-quadratic model, and the presented results show the mean and standard deviation of three independent experiments. Dosimetric uncertainty is also presented for the proton irradiations.

associated with genes believed to be involved in the repair of more complex damage (Artemis and BRCA1, resection-dependent NHEJ and HR respectively) and p53 were almost constant (slope  $\sim 0$ ).

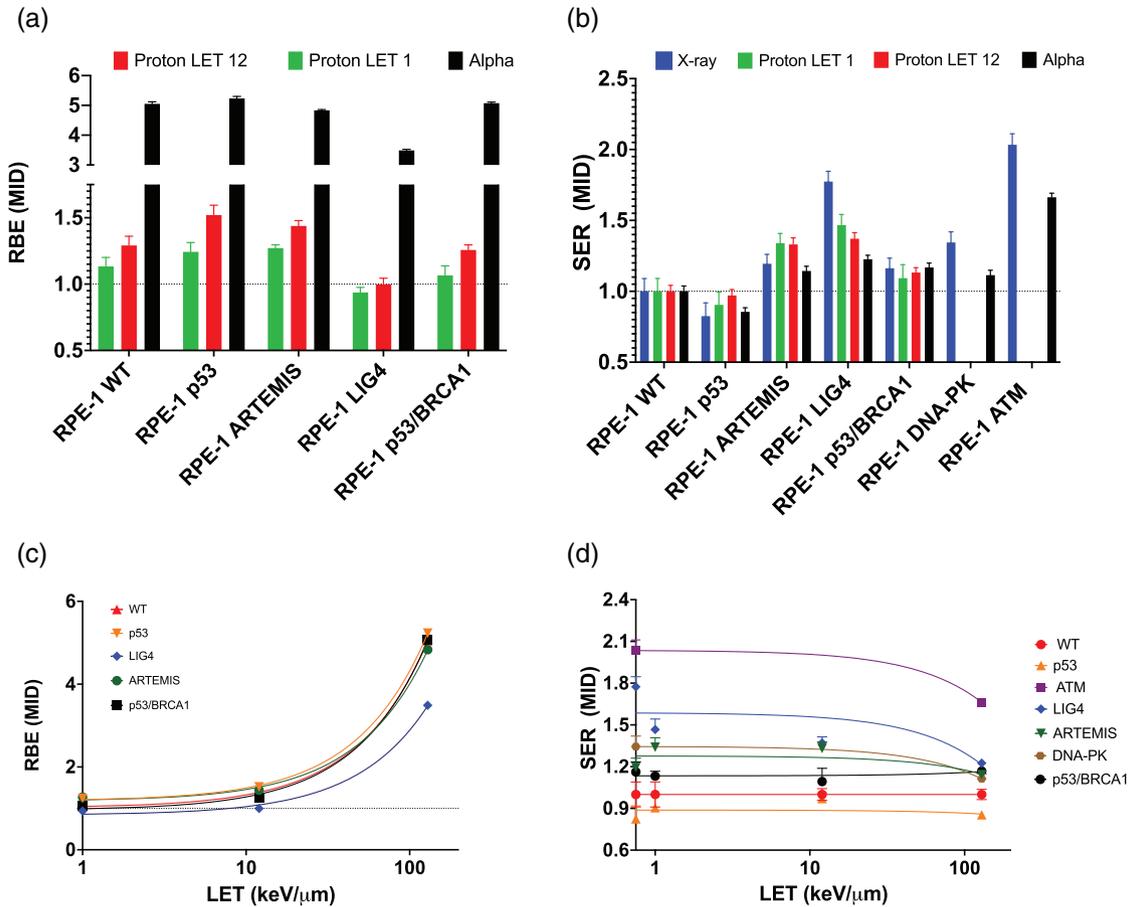
### 3.2 | Differences in DSB repair kinetics

To investigate if these differences in survival could be correlated with the yield of DSBs or their repair kinetics, we measured the kinetics of the DSB marker 53BP1 after 2 Gy exposure. Figure 3a shows the number of 53BP1 foci per cell in un-irradiated cells, where it is easily observed that removing key genes of cellular DNA repair machinery substantially increases the background level of DSBs in the cells. For example, a double knockout of p53 and BRCA1 increases the background level of DSBs approximately four-fold.

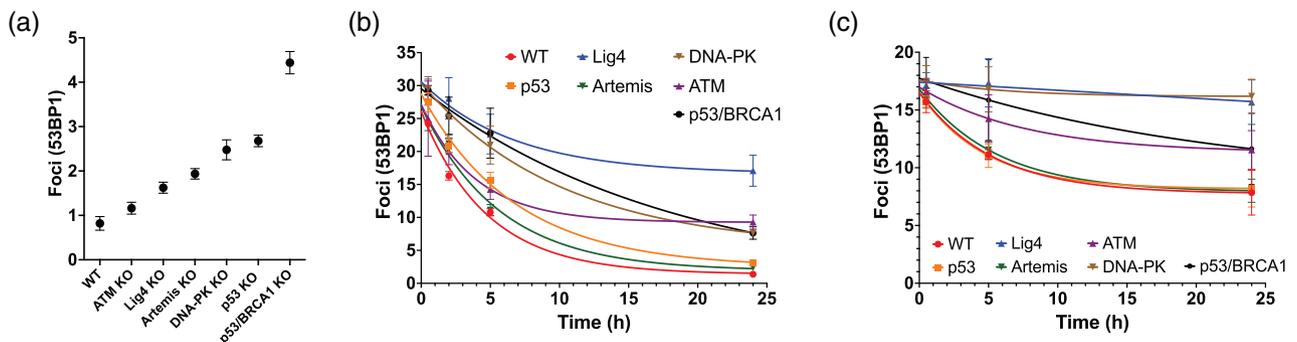
Evaluating foci kinetics, it was observed that X-rays induced significantly higher levels of DSBs compared to alpha-particles. However, the repair of DSBs was slower with alpha-particles, which correlates with the fact that alpha-particles cause more complex damage. This difference is particularly evident when considering the percentage of residual damage in wild-type cells, which was found to be  $10 \pm 5\%$  and  $50 \pm 11\%$  for photons and alpha-particles, respectively, 24 h after exposure. Addi-

tional information on the percentage of repair for the complete set of cells can be found in Supplementary Table 1. Moreover, alpha particle induced foci were on average larger and brighter than foci induced by X-rays (data not shown). Interestingly, deficiency in key genes of the DNA repair response system did not significantly alter the overall amount of radiation induced DSBs at 1 h after irradiation ( $p = 0.78$  X-ray exposure and  $p = 0.88$  alpha-particle exposure, 1-way ANOVA). However, despite similarities in the amount of initial damage, cells with genetic deficiencies in DNA repair proteins differed in their capacity and kinetics of repair of radiation induced damage. Foci analysis after photon exposure showed percentages of DSB repair of  $90 \pm 4\%$  for WT,  $81 \pm 3\%$  for p53<sup>-/-</sup>,  $83 \pm 1\%$  for Artemis<sup>-/-</sup>,  $69 \pm 4\%$  for BRCA1<sup>-/-</sup>,  $68 \pm 3\%$  for DNA-PK<sup>-/-</sup>,  $59 \pm 5\%$  for ATM<sup>-/-</sup> and  $39 \pm 4\%$  for Lig4<sup>-/-</sup>.

Overall, NHEJ-defective cells had the least DSB repair after low LET exposure, and no visible repair after high LET exposure. HR-defective cells also had slower repair kinetics, but the impact of HR deficiency was not as severe as NHEJ. This can be seen by the percentage of DSBs repaired 24 h after exposure to 2 Gy of alpha particles: wild type repair  $50 \pm 12\%$ , HR deficient cells repair  $34 \pm 17\%$  and NHEJ deficient cells repair  $7.9 \pm 10\%$ . Artemis and p53 deletion had only a slight or no impact on the DSB repair kinetics (Figure 3b,c).



**FIGURE 2** Relative biological effectiveness (RBE) and sensitizer enhancement ratio (SER) for each genetically modified clone. (a) RBE values for low-, high-LET protons and alpha-particles in each cell model, calculated as the ratio of X-ray MID and each particle-exposure MID. (b) SER values for X-rays, low-, high-LET protons and alpha-particles in each cell model calculated as the ratio of MID of wild type cells to repair-deficient cell models for each irradiation type. (c) Correlation between RBE and incident radiation LET. (d) SER variation with LET.

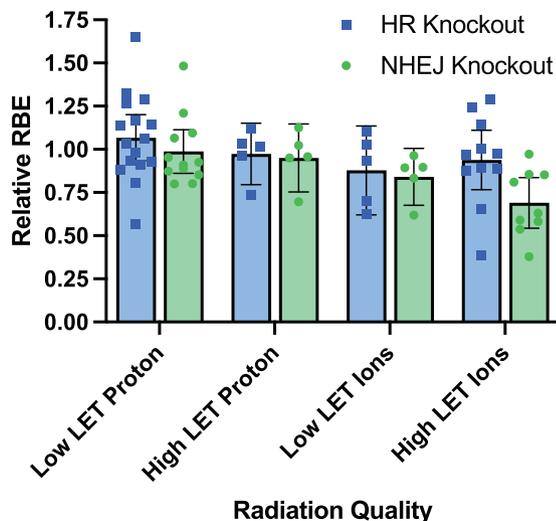


**FIGURE 3** 53BP1 foci, a marker for DSB damage and repair kinetics, as a function of genetic status and LET. (a) Background DSBs per cell for each cell model. (b) DSB repair kinetics after 2 Gy irradiation with X-rays. Data points represent mean and standard deviation of 3 independent repeats. (c) DSB repair kinetics after 2 Gy irradiation with alpha-particles. Data points represent mean and standard deviation of two independent repeats.

### 3.3 | Comparison to literature data

To benchmark this data, a compilation of published RBE values for cell models with known deficiencies in

key proteins involved in different DSB repair pathways was analyzed (Figure 4). The impact of HR and NHEJ knockout are summarized using data from a series of published papers.<sup>14–18,20,21,23,35–38</sup> While there is



**FIGURE 4** Compilation of published relative biological effectiveness (RBE) values. Relative RBE values were calculated for cells with loss of HR (blue) or NHEJ (green) genes, by taking the ratio of the RBE in knockout cells to equivalent wildtype cells. Results grouped into low and high LET protons ( $\sim 2.5$  and  $\sim 10$  keV/ $\mu\text{m}$ ) and low and high LET ions ( $\sim 13$  and  $> 50$  keV/ $\mu\text{m}$ ). Significant heterogeneity is seen, but no systematic evidence of greater sensitivity to protons or ions for either HR or NHEJ knockout. There is a trend towards lower relative RBEs at the highest LETs, but this is only significant for high-LET carbon exposure in NHEJ-defective cells, where significant overkilling was seen, and thus reduced RBE ( $p = 0.0013$ ).

significant heterogeneity in the results, there is no consistent evidence of HR or NHEJ knockouts leading to significantly higher RBEs compared to wild-type cells across the range of studies. There is a suggestion of a downwards trend in knockout sensitization with increasing LET, but this is only significant for NHEJ-defective lines exposed to high LETs, where the cells see significant overkilling ( $p = 0.001$ , one-sample t-test). These data are consistent with our results which showed no systematic impact on RBE (or difference in SER) across lower LETs in these models.

## 4 | DISCUSSION

We used immortalized human cells (RPE-1) genetically modified to be deficient in important components of different DSB repair pathways to assess the dependency of the RBE of different radiation qualities on cellular DSB repair capabilities.

Our analyses of cells deficient (or proficient, WT) in DSB repair, corroborate previous findings that variability in intrinsic radiosensitivity to photons strongly depends on NHEJ and HR statuses.<sup>17,20</sup> Our data also showed that intrinsic cellular radiosensitivity translated across all tested LETs. That is,  $ATM^{-/-}$  and  $Lig4^{-/-}$  cells were the most radiosensitive cells for all tested LETs and  $p53^{-/-}$  cells were the most radioresistant (Figure 1).

Previously, mutations in the p53 tumor suppressor gene have been associated with poorer prognosis than cancers with wild-type p53.<sup>39</sup>

Thus, data presented here support the conclusion that DNA repair capability is an important factor influencing intrinsic cellular radiosensitivity over a broad range of LETs. Moreover, an approximate linear correlation was observed between RBE and radiation LET (Figure 2). Therefore, if a particular cell is radiosensitive to photons, this linear correlation suggests it will be sensitive to particle irradiation, with the same relative sensitivity to other cells, until overkilling begins to occur. This implies that variations in DNA repair capabilities have a similar impact on the cellular response to radiation regardless of the LET. This linear correlation across repair capacities was first proposed by Suzuki et al.,<sup>22</sup> and was also successfully applied by Flint et al. in a predictive model of proton radiation response.<sup>36</sup>

On contrary, Liu et al. have linked relative proton hypersensitivity to defects in the HR or Fanconi Anemia (FA) pathway in lung cancer cell lines, which leads to accumulation of persistent DSBs after proton exposure compared with photon irradiated cells. In this particular study FANCD2 mutations increased RBE value from the standard 1.1 to 1.39.<sup>14</sup> In agreement with this idea, Fontana et al. demonstrated that an inefficient HR pathway rendered tumor cells more sensitive toward proton than photon irradiation (RBE 1.54 for HR-deficient cells vs. RBE 1.25 in WT-cells), while inhibition of DNA-PK or NHEJ-defects sensitized to both types of irradiation.<sup>15,16</sup> It is starting to be evident from the available published data that HR repair pathway variable dependence is cell and tumor specific.

As described above, several reports have shown that as LET increases, the relative importance of HR seems to increase relative to NHEJ, and thus inhibition of key elements of HR was sometimes suggested to synergize with high-LET radiation. In all these studies, NHEJ-defective cells proved to be markedly hypersensitive. Due to this hypersensitivity, the impact of NHEJ knockout on survival decreases with increasing incident radiation LET due to overkilling effects. This occurs when individual tracks have a significant chance of causing lethality, making it difficult to further enhance cell killing. Comparing previously published papers (Figure 4), there was no statistically significant difference in RBE for HR or NHEJ knockouts, except at the highest LETs where overkill was significant. However, there was significant heterogeneity in the literature, with different cell backgrounds, irradiation conditions, and gene knockout methods used across these studies. While there are some trends among conditions (e.g., repair defects led to higher RBEs in rodent cell lines than humans, mutations and siRNA led to higher RBEs than inhibitors), none were statistically significant. Further work is needed to identify if these observations are simply the result of statistical

uncertainties between studies, or if they are reflective of a more complex, conditional dependence on these pathways which could be exploited to improve therapeutic outcomes.

Interestingly, when considering how best to allocate proton and carbon ion beam therapy, these results suggest that, rather than focusing on sensitizing mutants such as HR and NHEJ defects, it would be more important to select patients with radio-resistant tumors who will benefit the most from elevated LETs and associated RBE.

Corroborating our survival data, we observed elevated levels of residual damage, indicative of unrepaired DNA DSBs, after high-LET irradiation in comparison with photons, which supports the notion that high-LET irradiation results in more complex DNA damage. Moreover, NHEJ-defective cells had almost no DSB repair and HR-defective cells also had slower DSB repair kinetics but which was not as severe as NHEJ deficiency. NHEJ was shown to be indispensable to ensure cell survival upon irradiation regardless of its quality, corresponding to the reported involvement of NHEJ in the repair of approximately 80% of all DSBs induced by radiation.<sup>19,40</sup>

It was previously demonstrated that at clinically relevant doses (2 Gy) the contribution of the HR pathway in the repair of radiation-induced DSBs in G2 cells ranged between 10% and 20%.<sup>9,40,41</sup> Recently, Mladenov et al. demonstrated that the HR contribution was dose dependent, and that with decreasing doses, its contribution would steadily increase and reach values around 10% at 2 Gy, 30% at 1 Gy and 50% at 0.5 Gy.<sup>42</sup> Our DNA repair experiments were performed with 2 Gy exposures, where HR contribution has theoretically a smaller role in DSB repair, and our results are in good agreement with this model.

Additionally, Artemis and p53 showed a limited impact on DSBs repair kinetics. It has previously been reported that a subset of DSBs induced by radiation (~10%) require Artemis nuclease activity.<sup>43</sup> However, genetic mutations in Artemis increase radiosensitivity through pronounced G2/M arrest but generally do not grossly affect overall DSB repair.<sup>44</sup> This reduced contribution of Artemis in DSB repair and limitations in resolving complex DSBs via microscopy—particularly for alpha-particle exposures—can obscure possible biological impacts of the Artemis protein in DSB repair that are seen in the survival response.<sup>45</sup>

Despite HR and NHEJ repair pathways being the major players for DSBs repair, efforts should also be done to elucidate the possible impact of alternative repair pathways such as alternative End Joining (altEJ) as well as deficiencies in key genes of SSBs repair pathways, as many involved genes (such as ERCC1, PARP1, MLH1, XPC, MSH2, APEX1, XRCC1) are commonly mutated in most human cancers.

## 5 | CONCLUSION

Results presented here support that DNA repair capability is an important factor influencing intrinsic cellular radiosensitivity. An approximately linear correlation between RBE and LET was also shown for all cell lines. This implies that cells which are more sensitive to photons will also be similarly more sensitive to particle therapy. Moreover, it suggests the major DNA repair pathway is NHEJ, independent of LET and, in contrast to some published data, these genetically modified cell lines show no increased dependence on HR with increasing LET. These data suggest that rather than targeting DNA repair defects, the greatest benefit for allocating limited proton and carbon ion beams may be towards patients with radioresistant tumors who will benefit the most from these radiation types.

### AUTHOR CONTRIBUTIONS

Conceptualization, Francisco D. C. Guerra Liberal and Stephen J. McMahon; methodology, Francisco D. C. Guerra Liberal and Stephen J. McMahon; validation, Francisco D. C. Guerra Liberal, Jason L. Parsons and Stephen J. McMahon; formal analysis, Stephen J. McMahon; investigation, Francisco D. C. Guerra Liberal; data curation, Francisco D. C. Guerra Liberal; writing—original draft preparation, Francisco D. C. Guerra Liberal; writing—review and editing, Francisco D. C. Guerra Liberal, Jason L. Parsons and Stephen J. McMahon; supervision, Jason L. Parsons and Stephen J. McMahon; funding acquisition, Stephen J. McMahon. All authors have read and agreed to the published version of the manuscript.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

### CONSENT FOR PUBLICATION

Not applicable.

### DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and its supplementary information file.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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