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## **Title: Lack of DNA damage response at low radiation doses in adult stem cells contributes to organ dysfunction**

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**Statement of translational relevance:**

Radiotherapy for cancer treatment may result in serious side effects due to co-irradiation of normal tissue. In the case of head and neck cancers, a major complication is xerostomia caused by damage to the salivary glands. Optimizing radiotherapy planning using modern radiotherapy techniques results in increasing the volume of the salivary glands receiving relatively low doses per fraction. We show that salivary glands over respond to low doses with a strong reduction in secretory potential. Using a 3D organoid model we show that this is due to low dose radiation sensitivity of murine and patient stem cells. Moreover, the data suggest that fractionated doses of less than 1 Gy may induce clinically relevant normal tissue effects.

This study indicates the importance of sparing glandular tissue stem cells during radiotherapy even from relative low radiation doses.

**Abstract:**

**Purpose:** Radiotherapy for head and neck cancer may result in serious side effects, such as hyposalivation, impairing the patient's quality of life. Modern radiotherapy techniques attempt to reduce the dose to salivary glands (SG), which however, results in low-dose irradiation of the tissue stem cells. Here we assess the low-dose sensitivity of tissue stem cells and the consequences for tissue function.

**Experimental Design:** Post-irradiation rat SG secretory function was determined after pilocarpine induction. Murine and patient-derived SG and thyroid gland organoids were irradiated and clonogenic survival was assessed. The DNA damage response (DDR) was analyzed in organoids and modulated using different radiation modalities, chemical inhibition and genetic modification.

**Results:** Relative low-dose irradiation to the high-density stem cell region of rat SG disproportionally impaired function. Hyper-radiosensitivity at doses  $<1$  Gy, followed by relative radioresistance at doses  $\geq 1$  Gy, was observed in SG and thyroid gland organoid cultures. DDR modulation resulted in diminished, or even abrogated, relative radioresistance. Furthermore, inhibition of the DDR protein ATM impaired DNA repair after 1 Gy, but not 0.25 Gy. Irradiation of patient-derived SG organoid cells showed similar responses, while a single 1 Gy dose to SG-derived stem cells resulted in greater survival than clinically relevant fractionated doses of  $4 \times 0.25$  Gy.

**Conclusions:** We show that murine and human glandular tissue stem cells exhibit a dose-threshold in DDR activation, resulting in low dose hyper-radiosensitivity, with clinical implications in radiotherapy treatment planning. Furthermore, our results from patient-derived organoids highlight the potential of organoids to study normal tissue responses to radiation.

## Introduction

Currently, more than half of all cancer patients are treated with radiotherapy. Unfortunately, radiotherapy is often accompanied by severe side effects induced by unavoidable co-irradiation of surrounding normal tissues (1, 2). Even modern radiotherapy of head and neck cancer (HNC) patients still results in functional impairment of co-irradiated salivary glands (SG), consequential xerostomia (dry mouth syndrome), alterations in speech and taste, difficulties with mastication and deglutition, dental caries and oral infections (3, 4), which have a major impact on patient-reported quality of life (5, 6). Currently no effective treatment exists for radiation-induced side effects in HNC patients. Radiation-induced normal tissue damage critically depends on the stem cell response, which is essential for the preservation of homeostasis and function of normal tissue after irradiation (7-9). High-precision techniques, such as intensity modulated radiotherapy (IMRT) and particle therapy (2), could be used to deliver the radiation dose to the tumor target while sparing the high-density stem cell region (8). However, these technologies differ greatly in the level to which dose to the high-density stem cell region can be reduced. Although IMRT can significantly reduce the mean dose to the gland, large volumes still receive a dose below 1 Gy per fraction.

Interestingly, there are several indications that cells can be disproportionately sensitive to radiation doses below 1 Gy, and that such low doses to specific regions of tissues may have a large impact on function and survival. Precisely targeted proton irradiation experiments on the rat cervical spinal cord indicated that besides the size of the irradiated volume, also the dose distribution determines the chance of paralysis of the fore and hind limbs (10-12). Importantly, very low doses to a region adjacent to a high dose volume were shown to markedly reduce the tolerance of the spinal cord (10-12). Furthermore, a dose of only 1 Gy to the cranial region of the rat parotid gland had a greater effect on secretory function than higher doses of 2-5 Gy to the same region (13), indicating a non-monotonous response with increasing radiation doses. This deviates from the expected monotonously decreasing dose response based on classic cell survival responses (14, 15).

Increased sensitivity to relatively low doses of radiation has been observed in *in vitro* survival assays of several, but not all, tumor cell lines, while the dose at which this disproportional sensitivity has been observed can vary depending on cell type, culture conditions and endpoint

studied (16, 17). Although the exact mechanism is not clear, some studies suggested the presence of a dose-threshold in initiation of the DNA damage response (DDR) (18). So far, comparable observations for normal tissue cells are primarily limited to 2D *in vitro* survival curves of keratinocytes and fibroblasts (19). However, these cells are not stem cells responsible for long-term homeostasis and regeneration after radiation damage (7-9, 20). Knowledge of the effects of low dose irradiation on normal tissue stem cells is currently restricted to the hematopoietic system, where *Rodrigues-Moreira et al.* showed that low dose irradiation induces persistent oxidative stress and results in long-term detrimental effects in hematopoietic stem cells (21). However, whether other normal tissue stem cells show high sensitivity to low doses is currently unknown.

Recent developments in the culture of organoids, three-dimensional tissue resembling structures derived from adult stem cells (9, 22), have opened unique opportunities to study normal tissue developments and pathological responses (23). These structures contain highly potent self-renewing stem cells that differentiate to all tissue lineages and are able to regenerate many tissues (9, 24, 25). Moreover, they enable the *in vitro* study of normal tissue stem cell responses to radiation (26). In this study, we use organoids to investigate the effects of low dose irradiation on normal tissue stem cells. First, we show that the impact of a low dose of radiation on rat parotid gland saliva secretion may be greater than that of higher doses. We demonstrate that this response is mimicked by, but not limited to, murine SG stem cells in 3D organoid cultures (9, 26-28) and is due to insufficient DDR activation at lower doses. Next, we show that also human SG stem cells isolated from patient biopsies show this response. Moreover, we demonstrate that low-dose radiation sensitivity is not limited to a single fraction, but accumulates after several fractions resulting in a pronounced additive response compared to that of an equal single fraction of a higher dose. Our data suggest that it may be important to take low-dose sensitivity of stem cells into account in the selection of radiotherapy technology and treatment plan optimization.

## Materials and Methods

### Isolation of murine salivary and TG cells

Murine SGs were dissected from 8-12 week-old female C57BL/6 mice (Harlan, The Netherlands). For experiments with cells from *Atm*<sup>-/-</sup> mice, SGs dissected from 12-16 week-old *Atm*<sup>-/-</sup> (129/SV x C57BL/6) mice were used. Generation and phenotyping of *Atm*<sup>-/-</sup> mice were previously described (29, 30). SG cells were isolated and cultured to form organoids as described previously (27, 28, 31).

Murine TGs were dissected from 8-12 week-old female C57BL/6 mice (Harlan, The Netherlands). TG tissue derived from 3 mice was collected in one tube in Hank's Balanced Salt Solution (HBSS) containing 1 % BSA and was mechanically digested using the GentleMACS dissociator (Miltenyi Biotech) followed by enzymatic digestion in HBSS/1 % BSA buffer containing collagenase I (100 U/ml; Gibco) and dispase (1,5 U/ml; Gibco). Cells were seeded into one well of a 12-well plate in DMEM-F12 medium (Gibco) containing 1 % penicillin/streptomycin (Gibco), glutamax (2 mM, Gibco), epidermal growth factor (20 ng/ml, Sigma Aldrich), fibroblast growth factor-2 (20 ng/ml, Peprotech) and 0.5 % B27 supplement (Gibco). After one day, primary organoids (passage 0) were disrupted using 0.05% trypsin-EDTA (Gibco) into single cells and reseeded at a density of  $2 \times 10^4$  cells/well in Matrigel (BD Biosciences) and medium supplemented with ROCK1-inhibitor (Y-27632; Sigma-Aldrich) was added to the gels.

All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

### Human SG isolation and culturing

This study was conducted in accordance with the Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects (adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, latest amendment 64th WMA General Assembly, Fortaleza, Brazil, October 2013). Methods for the collection of non-malignant human submandibular SG biopsies, isolation of SG cells and subsequent passaging have recently been developed (32).

In short, non-malignant submandibular tissue was collected (after informed consent and IRB approval) from donors undergoing elective tissue head and neck dissection for squamous cell

carcinoma of the oral cavity. Biopsies were collected after surgery in Hank's Balanced Salt Solution (HBSS) containing 1 % BSA. Biopsies were simultaneously mechanically digested using the GentleMACS dissociator (Miltenyi Biotech) and enzymatically digested in HBSS/1 % BSA buffer containing collagenase II and hyaluronidase. Cells were plated into 12-well plates at a concentration of  $4 \times 10^5$  cells per well in DMEM-F12 medium (Gibco) containing 1 % penicillin/streptomycin (Gibco), glutamax (2 mM, Gibco), epidermal growth factor (20 ng/ml, Sigma Aldrich), fibroblast growth factor-2 (20 ng/ml, Peprotech), 1 % N2 supplement (Gibco), insulin (10  $\mu\text{g ml}^{-1}$ , Sigma) and dexamethasone (1  $\mu\text{M}$ , Sigma). After 3-5 days, primary spheres (passage 0) were disrupted using 0.05 % trypsin-EDTA (Gibco) into single cells, counted and resuspended at  $4 \times 10^5$  cells/ml. 25  $\mu\text{l}$  of this solution was mixed with 50  $\mu\text{l}$  Basement Membrane Matrigel (BD Biosciences) and reseeded at the center of 12-well plates. 20 minutes after seeding, medium supplemented with ROCK1 inhibitor (Y-27632; Sigma-Aldrich) was added to the gels (passage 1) and subsequently incubated at 37 °C, 5 % CO<sub>2</sub>. One week after seeding, the media was replaced with dispase (1 mg/ml) and incubated for 30-60 minutes at 37 °C to dissolve the Matrigel and release the spheres. Spheres were processed to single cells using 0.05 % trypsin-EDTA, sphere and cell numbers were noted, and cells were reseeded in Matrigel.

Human SG organoid irradiations were performed with organoids from 4 different patients at all doses, except 4 Gy (for which N=3).

### **Irradiation treatment**

Irradiation with X-rays was performed using the Xstrahl 200 X-ray Therapy System at a voltage of 220 kV with a dose rate of 0.52 Gy/min (26). SOBP C-ion irradiations were performed with (high LET) 90 MeV/u carbon ions in a 3.5 mm long SOBP with a diameter of 30 mm, allowing accurate irradiation of the cells. This resulted in a dose-averaged LET of  $150 \pm 11$  keV/ $\mu\text{m}$  at the center of the Matrigel samples, while the  $\pm$  represents the LET at the anterior and posterior of the gel (26). Plateau C-ion irradiations were performed with an LET of  $49 \pm 2$  keV/ $\mu\text{m}$  (26).

Irradiation in an intense neutron field with a dose rate of 0.1 Gy/min were performed at the Physikalisch-Technische Bundesanstalt (Braunschweig, Germany). The neutrons were produced by the <sup>9</sup>Be+d reaction on a thick Be-target within a collimator at a deuteron energy of 13 MeV. The energy distribution is broad and extends from about 0.1 to 10 MeV (33). The 0-degree, "free-



in-air", tissue-kerma-averaged mean neutron energy is  $\langle E_n \rangle = 5.8$  MeV (34) and the dose-averaged LET in water is 56 keV/ $\mu\text{m}$  (35). Absorbed dose to tissue was determined using a calibrated tissue-equivalent ionisation chamber according to ICRU Report No.45 (36). The relative standard uncertainty for the total dose determination was 6 %.

The fraction of absorbed dose due to photon radiation was about 2.5 %. Specifics regarding cell densities and time of irradiation post-seeding are described in the specific assays.

### **Rat irradiations**

Previously described in *van Luijk et al.* (13). In brief, 9-10 week old male albino Wistar rats, weighing 230-250 g, were anesthetized and placed in a holder on a positioning rod by their incisors. Using the shoot-through technique, parotid glands were irradiated with 150 MeV protons at the Kernfysisch Versneller Instituut, Groningen. 45 mm thick brass collimators were used to give 100%, 50% cranial or 50% caudal irradiation. Stimulated saliva flow rate was determined 14 days prior to irradiation and up to 180 days post irradiation. Relative flow was calculated over 180 days and normalized to the area under the curve per dose to cranial (stem cell containing) region. All experiments were carried out in agreement with the Netherlands Experiments on Animals Act (1977) and the European Convention for the Protection of Vertebrates used for Experimental Purpose (Strasbourg, 18.III.1986).

### **3D clonogenic survival assay**

To determine sensitivity of SG and TG stem/progenitor cells following irradiation, a 3D organoid-based survival assay was performed, as previously described (26). Cells were seeded as single cells in Matrigel, as described above, 2 hours pre-irradiation with 0-4 Gy. Murine SG stem cells were irradiated and re-plated at a density of  $1 \times 10^4$  cells per well (0-2 Gy) or  $3 \times 10^4$  cells per well (4 Gy). For neutron irradiation, cells were irradiated in suspension and subsequently seeded in Matrigel due to the radiation set-up. One-week post-irradiation, spheres and cells were counted. Survival was calculated as follows:

$$\text{Organoid forming potential} = \frac{\text{Number of organoids harvested}}{\text{Cells seeded}} \times 100$$

$$\text{Surviving fraction} = \frac{\text{Organoid forming potential treated}}{\text{Organoid forming potential at 0 Gy}}$$

For combination treatments with ATMi (2.5  $\mu$ M; KU-55933; Axon Medchem, Netherlands) or PARPi (0.05  $\mu$ M; KU-0058948; Axon Medchem, Netherlands) media was changed 24 hours prior to passaging for irradiation and replaced with media supplemented with inhibitor (or DMSO). After 24 hours, organoids were released from Matrigel, processed and seeded as single cells (as above) and cultured with media supplemented with inhibitor (or DMSO control). Inhibitors were resuspended in DMSO and added to media at 1:1000.

Wild-type murine SG irradiations were performed at the start of passage 2. All other irradiations (murine TG, murine *Atm*<sup>-/-</sup> and WT SG mutant, and human SG) were performed at the start of passage 3.

For fractionation experiments, spheres were counted and surviving fraction were calculated at both 7 days post seeding and 7 days post final irradiation of the fractionation schedule, similar as in previous experiments when seeding and single (and therefore final) irradiation were performed on the same day.

Since primary tissue-derived organoid cultures do not respond as reproducibly as cell lines, inhibition/irradiation experiments were performed using organoids isolated from the tissue of the same mouse with the same genetic background within one experiment. This may result in slightly different control curves between sets of experiments.

### **Immunofluorescent microscopy**

For immunofluorescent microscopy  $4 \times 10^4$  single cells were seeded in Matrigel 2 hours pre-irradiation. Dispace was added 30 minutes prior to stated time points. Cells were centrifuged and washed with PBS. Cells were then resuspended in 2 % paraformaldehyde, placed on Adhesion slides (Marienfeld), fixed for 15 minutes and permeabilized with 0.2 % Triton for 10 minutes.

All samples were incubated overnight at 4 °C with primary antibody (anti-phospho-Histone H2A.X (Ser139); 1:500; Cell Signaling, #2577) followed by incubation at room temperature with secondary antibody (Alexa-Fluor488; 1:800; Life Technologies, goat anti-rabbit, A11008) for 90 minutes. Nuclear staining was performed using Hoechst 33342 (Molecular Probes, Life

Technologies). Imaging was performed using TissueFAXs (Tissuegnostics), foci were analyzed and counted using ImageJ (NIH).

For ADP-ribosylation microscopy, mouse SG cells were seeded on growth factor reduced Matrigel coated immunofluorescent microscopy slides following 24 hours treatment with PARPi (or control). After 3 hours, cells were treated with 150  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 minutes, subsequently washed with PBS and fixed by incubating for 10 minutes in 4% PFA. After fixation cells were washed with PBS and then permeabilized by incubating with 0.3% Triton/2% BSA in PBS. Samples were incubated at room temperature for 1 hour with primary antibody (anti-PAR; 1:500; Trevigen, #4336-BPC-100) followed by incubation at room temperature with secondary antibody (Alexa-Fluor488; 1:1000; Life Technologies, goat anti-rabbit, A11008) for 60 minutes. Nuclear staining was performed using Hoechst 33342 (Molecular Probes, Life Technologies).

### **Data analysis**

All values are represented as mean  $\pm$  standard error of the mean (SEM) (\* $p \leq 0.05$ ) of at least three independent experiments. Student's t-test was used for testing statistical significance using GraphPad Prism (GraphPad software).

## Results

### *Glandular stem cells show a non-linear response at low radiation doses*

Previously, it was shown that irradiation of specific regions of the rat parotid gland with a low dose had a disproportionately high impact on the secretory function when compared to higher doses (13). We hypothesize that this is caused by the irradiation of the region that contains the glandular stem cells (8). Therefore, with this recently acquired knowledge, we re-analyzed previously obtained data to determine the *in vivo* effects of low doses of radiation on normal tissue stem cells. An ablative dose of 30 Gy of protons to the caudal 50% region of the rat SG was used to activate compensatory stem cell regeneration in the cranial 50% region (13) (Fig. 1A). Here, it was shown that subsequent irradiation of the cranial SG region containing a higher density of stem cells (8) with doses from 0 to 10 Gy immediately after the 30 Gy ablative dose to the caudal region, results in a further decrease in pilocarpine-induced salivary flow compared to ablation of the caudal region alone (Fig. 1B). Interestingly, this was not dose dependent, as a dose of 1 Gy led to an unexpectedly strong reduction in SG function, when compared to the 2-10 Gy doses (Supplementary Fig. 1), indicating that the *in vivo* function of SGs may indeed be excessively affected by low radiation doses to the regions with high stem cells densities.

To further determine whether stem cells may be affected disproportionately by low doses, we X-ray irradiated mouse SG organoid-derived cells, known to contain stem cells, using a recently described methodology (26). These cells have been shown to be able to extensively self-renew and differentiate to all cell types of the salivary gland, both *in vitro* and *in vivo* (9, 27, 28). In this culture system only stem-like cells can expand and form secondary *in vitro* 3D organoids (Fig. 2A) (9, 27, 28). The capability of a cell to form an organoid upon irradiation can, therefore, be used as a read-out of the radiation response of stem/progenitor cells (26). Indeed, irradiation of organoid-derived cells resulted in general in reduced levels of secondary 3D-organoid formation indicating loss of stem/progenitor cells, resulting in a conventional dose dependent radiation response with doses above 1 Gy (Fig. 2B and C). However, doses below 1 Gy showed a disproportionate reduction in 3D-organoid forming potential with a minimum SG stem cell survival at 0.25 Gy and an increasing SG stem cell survival for doses up to 1 Gy.

To show that this response was not specific for SG stem cells only, we irradiated thyroid gland (TG) derived stem cells, cultured as organoids in a similar manner to SG stem cells. Indeed,

the reduction in organoid forming potential of TG stem/progenitor cells was very similar, albeit not as pronounced, to that of SG stem cells with a minimum surviving fraction of TG stem cells also around 0.25 Gy (Fig. 2D). The observed response resembles previously observed low-dose hypersensitivity seen in cell lines (16, 18, 37), albeit with a much lower general radiation sensitivity at higher doses as observed in 3D culture systems of cell lines (26).

#### *DDR after low-dose IR*

A critical event determining the radiosensitivity of a cell is its ability to cope with DNA double-strand breaks (DSBs) after irradiation (38). It has been hypothesized that low-dose hypersensitivity originates from a lack of DNA repair activation at lower doses (39). To test this, SG organoid-derived cells were X-ray irradiated with 0.25 or 1 Gy. The resulting DSBs were quantified by determining the number of  $\gamma$ H2AX foci (40) at 30 minutes and 24 hours post-irradiation.

As expected, the initial (30 minutes) number of  $\gamma$ H2AX foci per cell increased with increasing doses (Fig. 2E and F). However, similar levels of residual damage were observed at 24 hours following both 0.25 and 1 Gy (Fig. 2E and F). Quantification of cells with 4 or more foci (to reflect an estimate of induced damage based on the majority of cells at 0 Gy having 0-3 foci) showed similar results (Supplementary figure 2), indicating that a much greater proportion of DSBs are cleared 24 hours after 1 Gy irradiation than after 0.25 Gy. Taken together this data suggest a less efficient activation of the DDR at 0.25 Gy than 1 Gy.

To determine if increased sensitivity below 0.5 Gy relates to an impairment in activation of the DDR (18, 39), the complexity of the DNA damage was modulated using high linear energy transfer (LET) particle radiation. Clustered damage induced by such densely ionizing particles is more difficult and slower to repair than damage induced by lower LET X-rays (41). Therefore, SG or TG-derived cells were irradiated with carbon ions of either  $49 \pm 2$  keV/ $\mu$ m (Intermediate LET) or  $150 \pm 11$  keV/ $\mu$ m (High LET), or neutrons (mixed LET) with a dose-averaged LET of 56 keV/ $\mu$ m (35). Irradiation with intermediate LET, high LET and mixed LET indeed resulted in a LET dependent exponential survival curve for both SG stem cells (Fig. 3A and B, and Supplementary figure 3A) and TG stem cells (Fig. 3C and D, and Supplementary figure 3B), without clear signs of an increased resistance at doses  $\geq 1$  Gy in SG stem cells or TG stem cells. Lower survival of stem cells observed in the  $\geq 1$  Gy dose range in response to irradiation with

increasing LET may be due to an incapability to repair more complex breaks (41), therefore, it is possible that the DDR is more efficiently activated at doses  $\geq 1$  Gy X-rays in contrast to doses  $< 1$  Gy in SG and TG stem cells.

The DDR includes signal transduction pathways that initiate DNA repair, but also cell cycle checkpoint arrest and cell death (42). The absence of DDR induction has previously been suggested to be the cause of increased sensitivity at lower doses of radiation in 2D-cultured cell lines (43). To further substantiate this in normal tissue stem cells, we next investigated the effect of inhibition of key DDR proteins, such as PARP-1 and ATM. PARP-1 is a key component of single-strand DNA break (SSB) repair, but is also known to be involved in DSB alternative end-joining (44). PARP-1 has been shown to play a role in the induction of DDR, possibly due to its delayed activation in response to DNA SSBs induced by ionizing radiation (45). Furthermore, PARP-1 is rapidly recruited to single strand breaks (45), and thus due to the large number of single strand breaks induced by ionizing radiation could potentially play a role in increased resistance in normal tissue stem cells.

Mouse SG organoid-derived cells were irradiated with X-rays in the presence or absence of a PARP-1 inhibitor (PARPi; KU-0058948), at a concentration which we show to significantly decrease the activity of PARP-1 (Supplementary figure 4A and B) without being too toxic to the cells (Supplementary figure 4C). PARP-1 inhibition resulted in a delayed initiation of DDR visible only at 0.5 Gy ( $p=0.0061$ ) (Fig. 4A). These data indicate that PARP-1 may play a role, although not essential, in the increased survival of normal tissue stem cells detected at doses  $\geq 0.5$  Gy and confirm the involvement of the DDR in SG stem cells.

To verify that the induction of the DDR is essential for induced resistance in normal tissue stem cells, the DDR was impeded by modulating the protein kinase ATM, an apical activator of the DDR to DSBs (46). Failure to activate the ATM-dependent G2/M checkpoint has been suggested to explain low-dose hypersensitivity in established cell lines (37, 43, 46). Therefore, due to its early and pivotal role in many DDR processes, ATM could potentially also contribute to the increased resistance of normal tissue stem cells. Indeed, treatment with ATM inhibitor (ATMi), KU-55933, at a sublethal concentration of the inhibitor (Supplementary figure 4D), did not influence the low-dose response of the SG stem cells (Fig. 4B); however, it did abolish the recovery resulting in a lower survival at doses  $> 0.5$  Gy, similar to that observed after particle irradiation.

To further confirm the role of ATM in the increased radioresistance in normal tissue stem cells, we irradiated SG stem cells isolated from *Atm*<sup>-/-</sup> mice. Here also the low-dose response was observed in both wild-type and *Atm*<sup>-/-</sup> SG stem cells, however, the induction of DDR appears to be completely absent in *Atm*<sup>-/-</sup> SG stem cells already at 0.5 Gy (Fig. 4C).

To investigate whether this was truly due to an impaired DDR, we next irradiated organoid-derived SG cells in the presence or absence of the ATMi and determined the number of  $\gamma$ H2AX foci at 30 minutes and 24 hours post-irradiation. No significant differences were observed between ATMi treated cells and DMSO controls at 30 minutes after both 0.25 and 1 Gy, indicating that the damage induction was not changed (Fig. 4D and E). After 24 hours, as expected, a pronounced reduction in  $\gamma$ H2AX foci was observed in DMSO-treated SG cells exposed to 1 Gy. However, no change in  $\gamma$ H2AX foci was seen after 0.25 Gy, indicating that DSB repair may not have been initiated at this lower dose. As expected, a significant increase in  $\gamma$ H2AX foci was observed at 24 hours after irradiation with 1 Gy in the presence of ATMi. Similar observations were made when the percentage of cells positive for  $\gamma$ H2AX (4 or more foci) were scored (Supplementary figure 5).

#### *Clinical relevance*

To test if the observed response in normal murine glandular tissue stem cells also occurs in human stem cells obtained from patient SG biopsies (32), we established a stem cell survival dose response curve. Indeed, low-dose hypersensitivity and increased radioresistance were also observed in human SG stem cells (Fig. 5A and B), indicative of a potential clinical relevance.

For radiotherapy, the dose is mostly delivered at (1.8 to 2) Gy daily fractions to the tumor, resulting in a lower dose per fraction to large parts of the normal surrounding tissue. It could be possible that the low-dose responses are only seen after the first fraction and that the DDR is activated in a second or following fraction, rendering the effect to be of minimal clinical relevance. Therefore, we next irradiated mouse SG organoid-derived cells with 4 daily fractionated doses of 0.25 Gy to mimic clinically relevant doses of radiation to the normal tissue and compared this to a single dose of 1 Gy. Organoids were counted and normalized at day 10 post seeding, being 7 days following the final fraction (Fig. 5C). In contrast to what would be expected in the case of DDR activation, we found that a single dose of 1 Gy consistently resulted in higher survival ( $92.1 \pm 3.0$  %) than 4 fractions of 0.25 Gy with a total dose of 1 Gy ( $74.6 \pm 0.5$  %) (Fig. 5D and E). This was also true if survival was calculated at 7 days post seeding (Supplementary figure 6), as per

previous experiments when seeding and single (and therefore final) irradiation were performed on the same day. These data indicate that multiple fractions of low radiation doses, received by normal tissue during fractionated radiotherapy, may be more detrimental to normal SG stem cell survival than a single equivalent higher dose.

Taken together, our data show that both murine and human normal tissue stem cells exhibit a low-dose hypersensitivity and delayed initiation of DDR following higher X-rays doses.



## Discussion

In this study, we demonstrate a major impact of low radiation doses on *in vivo* SG function, which may be explained by a lack of DDR at low-doses resulting in hypersensitivity of normal tissue stem cells. Using an *in vitro* radiation survival assay of murine SG and TG organoid derived stem cells, we show similar effects after 0.25 and 1 Gy of X-rays resulting from a lack or presence of DDR activation, respectively. Increasing the complexity of DSBs using high LET irradiation and inhibition of key DDR proteins did not affect the hypersensitivity at low doses but resulted in delayed or abolished resistance at higher doses. Importantly, we show the presence of this phenomenon also in patient biopsy-derived submandibular SG organoids. Finally, we demonstrate that multiple clinically relevant fractions of low doses had a far greater impact on survival of SG stem cells than a single fraction of an equivalent dose. Taken together, our findings show the potential clinical relevance of low-dose hypersensitivity to X-rays in adult stem cells that may result in an unexpected functional decline of normal tissues receiving low radiation doses during radiotherapy treatment.

The recent discovery of a region within the SG containing a high density of stem cells which should and can be spared from irradiation (8) further stresses the importance of stem cells in the radiation response of tissues. Indeed, sparing of the high-density stem cell region was suggested to result in an improved normal tissue functioning after irradiation, however this didn't account for a possible low-dose hypersensitivity parameter. Our *in vivo* rat data show that indeed normal tissue stem cells are hypersensitive to low doses of radiation (1 Gy), albeit this may only be a temporary increased dysfunction compared to 2 or 3 Gy as they displayed the same level of impairment after 180 days. It could be that in the long run a lower number of stem cells could eventually regenerate the tissue. However, the impact on quality of life of earlier salivary gland dysfunction and related xerostomia seems to remain persistent (5).

Using a recently developed technique that allows the culture of tissue-specific organoids from single cells (9, 28, 32), we were able to assess responses of tissue-derived stem cells *in vitro*. These organoids closely resemble organ tissues, containing tissue-specific stem cells and differentiated cell types, and are more physiologically relevant than traditional 2D monolayer culture systems (23). Moreover, these organoid 3D culture systems allow the expansion of genetically and phenotypically stable adult, organ-specific stem cells (9, 47). Passaging of

organoid-derived single cells results in the formation of secondary organoids from stem cells only, as shown by their ability to form all SG lineages and rescue *in vivo* SG functionality after radiation damage in transplantation studies (9, 28). As such, the radiation response of stem cells may be reflected by the organoid forming potential of the cells seeded as single cells (26). So far, only 2D cultures of fibroblast and keratinocytes have been studied as representative models of low-dose radiation effects in normal tissue (19). However, normal tissue organoid responses, in combination with the observation that 3D culture systems can give rise to responses more similar to patients than 2D cultures (23), provided us with the unique opportunity to assess stem cell responses *in vitro*.

The dependency for the occurrence of induced resistance on efficient activation of the DDR is indicated by our DDR inhibition experiments. Our data indicates that PARP-1 may play a non-essential role in the induced resistance, as PARP-1 inhibition simply increased the dose at which increased resistance was observed but did not remove this effect. The role of PARP-1 in normal tissue stem cells has not been previously investigated at lower doses, but *Chalmers et al.* (45) suggested a role for PARP-1 in increased resistance of tumor cells. If true, this could have consequences for the therapeutic ratio of such a treatment. However, our data suggests that ATM plays an important role in induced resistance in normal SG stem cells since inhibition or knock-out of ATM abrogates this response, through impairment of the DDR. This might be due to a G2-phase checkpoint, as suggested by *Krueger et al.* to occur in 2D cultured fibroblast and tumor cell lines (39). *Rodrigues-Moreira et al.* demonstrated that low radiation doses induce persistent oxidative stress, which activates Nrf2 in stem cells resulting in long-term side effects (21). We cannot exclude the possibility of Nrf2 and oxidative stress activation in our system, however, it should be noted that the work of *Rodrigues-Moreira et al.* investigated much lower doses (20 mGy) (21). Cells of the hematopoietic system are known to be more radiosensitive than cells originating from other tissue (48), therefore it is possible that the radiation response of hematopoietic stem cells differs from solid tissue stem cells. Furthermore, differences in culturing conditions (2D vs 3D) have been shown to influence radiosensitivity (26, 49), and therefore could potentially influence the doses at which increased sensitivity is observed.

Currently radiation treatment planning considers the consequence of radiation to increase monotonously with dose, while stem cell sparing techniques are being implemented in order to reduce the dose to normal tissue stem cells (8). These techniques do not take into consideration

that normal tissue stem cells may be more sensitive to lower doses. However, based on our data one could conclude that low-dose hypersensitivity may have a significant impact on the normal tissue stem cells and, consequently, on the results of treatment planning. *Honore and Bentzen* (50) used a modelling study to investigate the role of low-dose hypersensitivity in treatment planning and suggested that much of the benefit of treatment planning may be offset if low-dose hypersensitivity is indeed present. Next to this, it has recently been shown that reducing dose to the high-density stem cell region of the SG during radiotherapy may improve post-therapy SG function following treatment of HNC (8). However, together with the current data, the low-dose response may be an important factor that needs to be considered in treatment planning. Minimizing the dose to normal tissue stem cells may only be optimal if the low-dose hypersensitivity dose range can be avoided in structures within an organ/tissue containing a high stem cell density. Proton therapy could potentially reduce the dose in low-dose regions even further, which may help to avoid the low-dose response of normal tissue stem cells (51).

One proposed mechanism behind late normal tissue complications of radiotherapy is that the cells remaining in the tissue have been compromised with a potential accumulation of sub-lethal damage. However, it has previously been shown that organ dysfunction and lack of regeneration of the salivary gland is accentuated by ablation of the high density stem cell region (8). We cannot rule out that the effect that we see is due to sub-lethal damage to the stem cells. Our *in vitro* clonogenic survival assay is based on the ability to form an organoid, therefore we cannot directly determine if the stem cells have undergone cell death or have survived but are unable to form an organoid.

Although we find a potentially important hypersensitive low-dose response in normal tissue stem cells, it should be noted that our model has some drawbacks. Although organoids resemble the tissue of origin, the radiation response of normal tissue is more complex than solely the stem cell response. Normal tissue effects, next to the response of stem cells, encompass cytokine, inflammatory, vasculature and connective tissue responses (1). Interestingly, healthy normal tissue stem cells may modulate the irradiated surrounding by inducing expression of compensatory anti-inflammatory, anti-fibrotic and pro-proliferation genes (33). Furthermore, we are aware that in organoid models, stem cells are pushed to proliferate (28) potentially increasing their intrinsic radiosensitivity. Under normal *in vivo* conditions, stem cells are largely quiescent and remain so until they are stimulated to divide by external factors, such as tissue damage (52).

Despite this distinction, our *in vivo* rat data closely resemble the *in vitro* observed low-dose hypersensitivity, albeit at a slightly higher dose. Furthermore, while organoids have previously been used to model diseases and for drug screening (23), we show the potential of this system to study *in vivo* normal tissue responses to radiation, potentially offering a means of patient response prediction in the future.

In conclusion, low-dose hypersensitivity might play a role in the normal tissue response away from the tumor where large volumes are irradiated with low fraction doses upon e.g. IMRT. Reducing the volume and the dose to these regions using particle therapy, such as proton therapy, may more accurately spare designated regions and result in further optimization of radiation therapy for cancer treatment.

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**Figure legends:**

**Fig. 1. *In vivo* low-dose proton radiation effects on rat SG secretory function.** (A) Schematic representation of the rat SGs of which the stem cell containing cranial region (grey) of rat SGs were irradiated with increasing proton doses following an ablative (30 Gy) dose to the caudal region (striped). (B) The saliva flow as % relative to baseline over time compared to an ablative dose to the caudal region alone was diminished to a greater extent following 1 Gy than higher doses of 2, 3 or 5 Gy. N = 6 for 1, 2 and 10 Gy, N = 7 for 3 Gy and N = 8 for 0 and 5 Gy. Error bars represent SEM.

**Fig. 2. SG and TG stem cells exhibit low-dose hypersensitivity in response to X-rays.** (A) Schematic representation of mouse SG isolation and culture. SG stem cells can be serially passaged or alternatively irradiated at passage 2. (B) Representative images of SG organoids 7 days following irradiation. Scale bars represent 50  $\mu\text{m}$ . After irradiation, organoids were counted and survival was calculated resulting in (C) the SG stem cell or (D) TG stem cell survival response to increasing doses of X-rays. (E) Representative images of  $\gamma\text{H2AX}$  foci (green) in the nucleus (blue) of mouse SG organoid-derived cells at 30 minutes and 24 hours after irradiation. (F) Quantification of average number of  $\gamma\text{H2AX}$  foci per cell at 30 minutes and 24 hours after irradiation. N  $\geq$  3, error bars represent SEM.

**Fig. 3 Effects of changing LET on low-dose hypersensitivity.** (A and B) Survival response to increasing doses of particle irradiation of SG or (C and D) TG stem cells. Intermediate LET =  $49 \pm 2 \text{ keV}/\mu\text{m}$ , High LET =  $150 \pm 11 \text{ keV}/\mu\text{m}$ , Mixed LET = neutron irradiation with a dose-averaged  $\langle\text{LET}\rangle$  of  $56 \text{ keV}/\mu\text{m}$ . N  $\geq$  3, error bars represent SEM.

**Fig. 4 Role of PARP-1 and ATM in induced resistance after low-dose hypersensitivity of normal tissue stem cells.** (A) Following inhibition of PARP-1, SG organoids derived cells were irradiated and cultured for 7 days, the number of organoids formed were counted and survival was calculated. Survival of irradiated cells with (B) inhibition or (C) knock-out of ATM. (D and E) ATM inhibition increases the persistent damage at 24 hours. (D) Quantification of the average number of induced foci per cell. N  $\geq$  3, error bars represent SEM. (E) Representative images of  $\gamma\text{H2AX}$  foci (green) in the nucleus (blue) of mouse SG organoid-derived cells at 30 minutes and 24 hours after irradiation. \* represents  $p < 0.05$ .

**Fig. 5 Clinical translation.** (A) The response of human SG stem cells to X-rays showing the presence of low-dose hyper-radiosensitivity. N=4 (except 4 Gy; N=3). (B) Representative images of human SG-derived organoids 7 days post irradiation. (C) Schematic representation of the irradiation plan for the fractionation experiment - upper panel represents a single 1 Gy dose, lower panel represents 4 x 0.25 Gy fractionation schedule. (D) Representative images of mouse SG-derived organoids following fractionated irradiation. (E) Survival response following fractionated irradiation. Scale bars represent 50  $\mu\text{m}$ . N  $\geq$  3, error bars represent SEM.

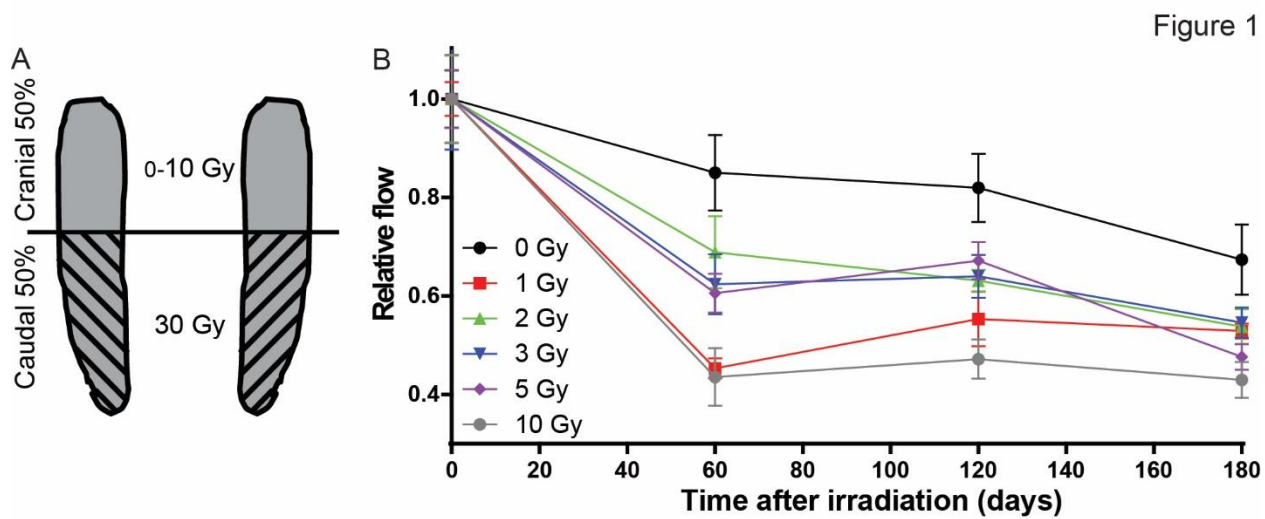


Figure 2

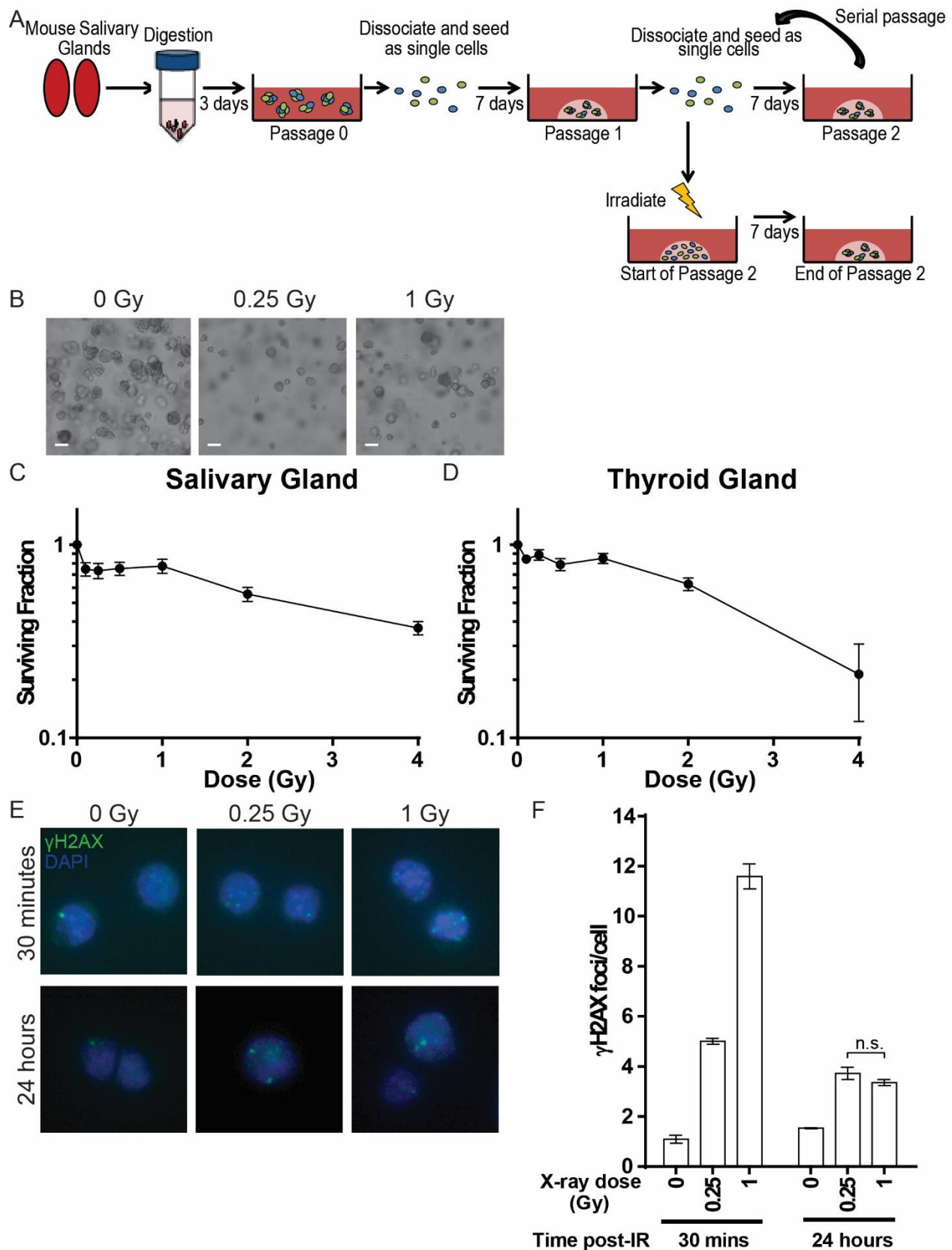


Figure 3

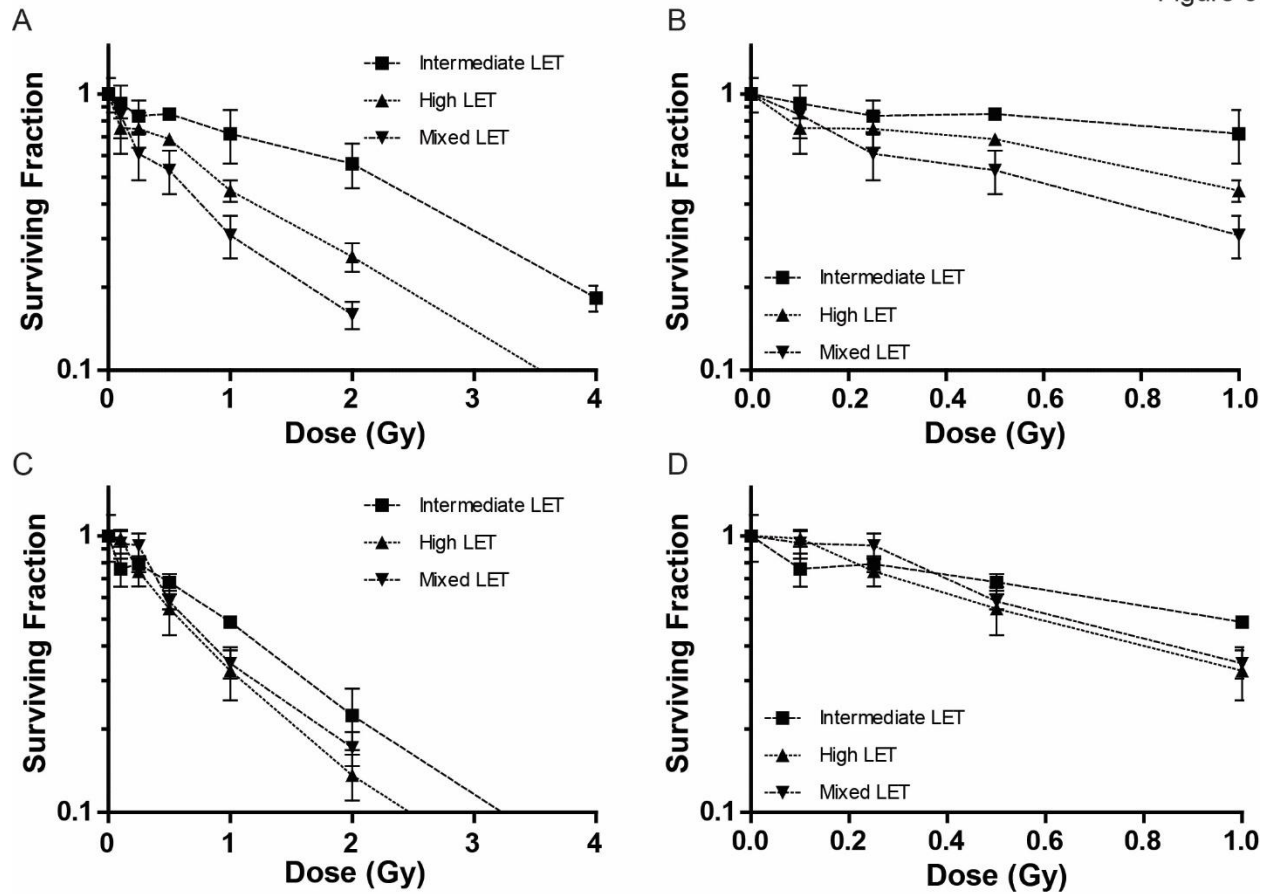




Figure 4

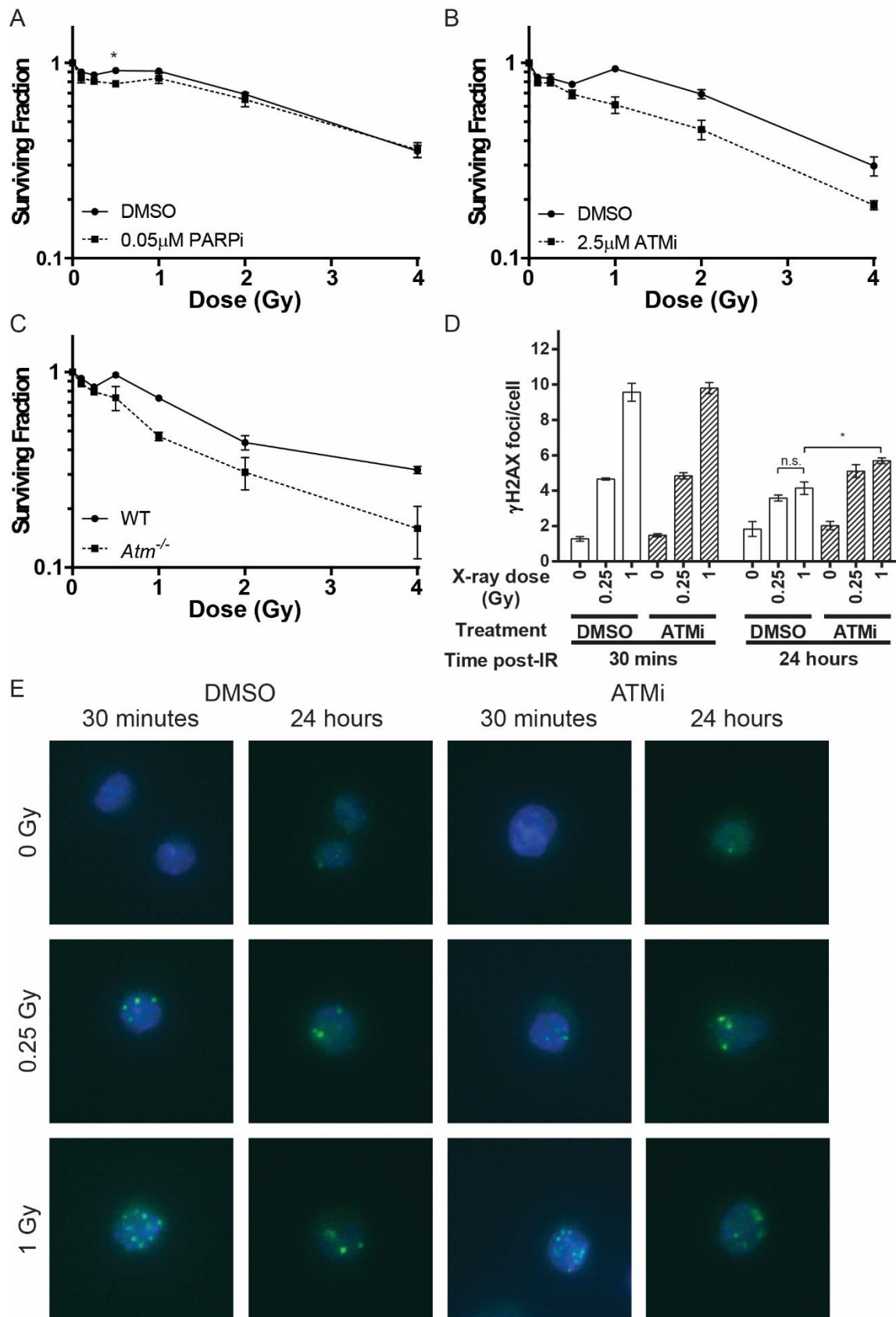
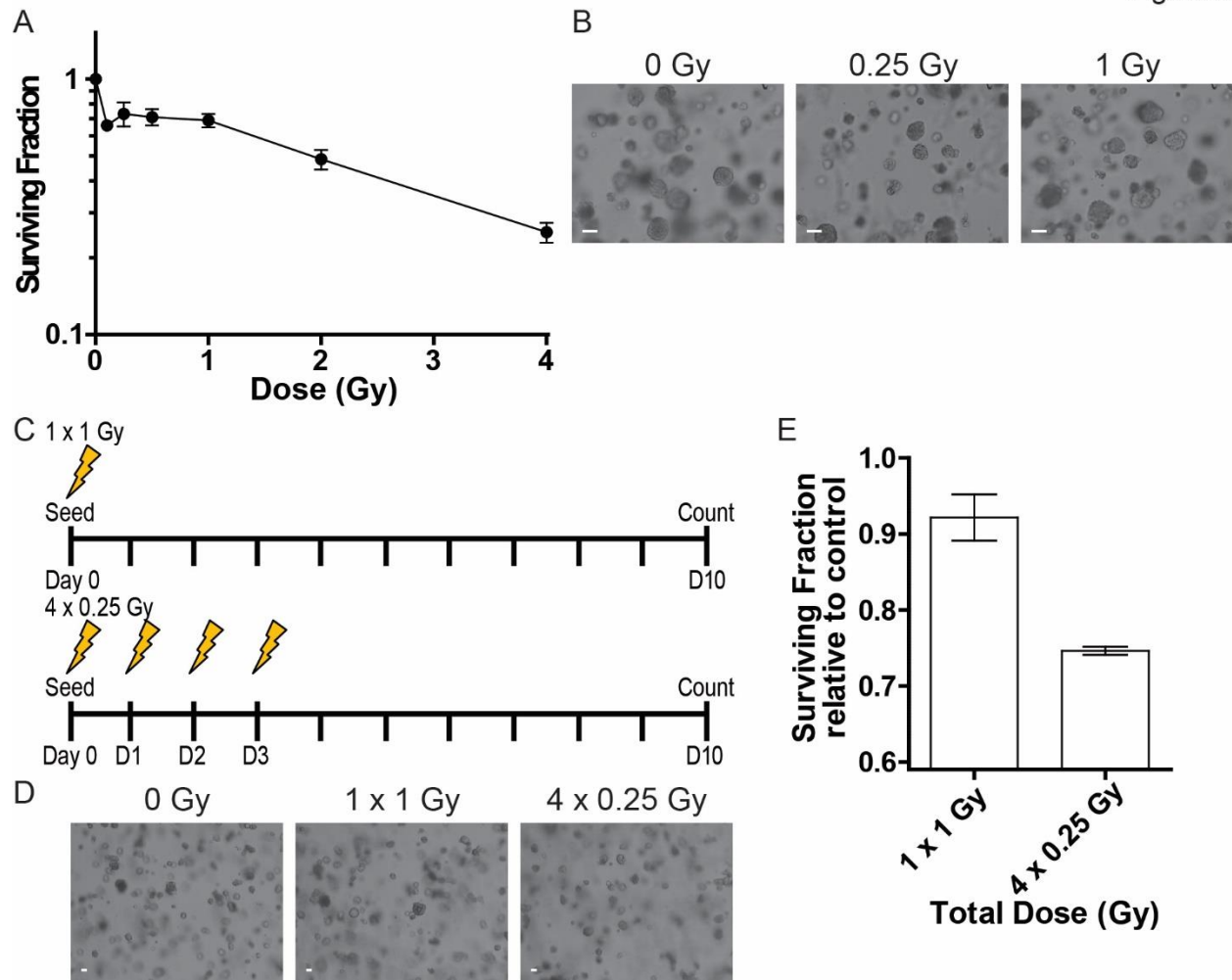




Figure 5



# Clinical Cancer Research

## Lack of DNA damage response at low radiation doses in adult stem cells contributes to organ dysfunction

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