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RBE and Particle Therapy Biology

# The In Vitro Response of Tissue Stem Cells to Irradiation With Different Linear Energy Transfers



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## Summary

The difference in response of normal tissue to different radiation qualities is poorly understood. Recently, we developed a method to culture/expand salivary gland stem cells to allow the assessment of the in vitro response of tissue stem cells to different radiation qualities. Our results indicate differences in the response of stem cells to photons and carbon ions at different linear energy transfers and a relative resistance to particle irradiation of salivary gland stem cells compared with

**Purpose:** A reduction in the dose, irradiated volume, and sensitivity of, in particular, normal tissue stem cells is needed to advance radiation therapy. This could be obtained with the use of particles for radiation therapy. However, the radiation response of normal tissue stem cells is still an enigma. Therefore, in the present study, we developed a model to investigate the in vitro response of stem cells to particle irradiation.

**Methods and Materials:** We used the immortalized human salivary gland (HSG) cell line resembling salivary gland (SG) cells to translate the radiation response in 2-dimensional (2D) to 3-dimensional (3D) conditions. This response was subsequently translated to the response of SG stem cells (SGSCs). Dispersed single cells were irradiated with photons or carbon ions at different linear energy transfers (LETs;  $48.76 \pm 2.16$ ,  $149.9 \pm 10.8$ , and  $189 \pm 15$  keV/ $\mu\text{m}$ ). Subsequently, 2D or 3D clonogenicity was determined by counting the colonies or secondary stem cell-derived spheres in Matrigel.  $\gamma\text{H2AX}$  immunostaining was used to assess DNA double strand break repair.

**Results:** The 2D response of HSG cells showed a similar increase in dose response to increasing higher LET irradiation as other cell lines. The 3D response of HSG cells to increasing LET irradiation was reduced compared with the 2D response. Finally, the response of mouse SGSCs to photons was similar to the 3D response of HSG cells. The response to higher LET irradiation was reduced in the stem cells.

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immortalized human salivary gland cells.

**Conclusions:** Mouse SGSC radiosensitivity seems reduced at higher LET radiation compared with transformed HSG cells. The developed model to assess the radiation response of SGSCs offers novel possibilities to study the radiation response of normal tissue in vitro. © 2016 Elsevier Inc. All rights reserved.

## Introduction

Radiation therapy with photons (with or without surgery or chemotherapy) is an effective cancer treatment but can result in side effects such as radiation-induced fibrosis (1), xerostomia (2), cardiopulmonary disease (3), and radiation-induced liver damage (4). The risk of these side effects is related, not only to the dose and volume of the normal tissue that is co-irradiated, but also to the location of the stem cells (5). Therefore, reducing the dose–volume parameters and assessing the localization and radiosensitivity of stem cells are currently the focus of advancing radiation therapy.

It is important to understand the radiation response of tissue/adult stem cells (ASCs), because these are for a major part responsible for the long-term regeneration of tissue (5). However, it is complicated to study ASCs after irradiation in vivo, and these cells cannot be cultured in 2-dimensional (2D) systems. Recently, it has become possible to culture ASC types in 3-dimensional (3D) culture systems, such as stem cells isolated from intestine (6), liver (7), and salivary glands (8). The use of 3D culture systems would allow for in vitro studies of ASCs in response to radiation. Recently, our group developed a 3D culture system of normal tissue, as spheres (a cluster of cells growing in all directions >50  $\mu\text{m}$  in diameter) and as organoids, containing stem cells (8). This system comprises multiple cell types (including stem cells) and represents an unprecedented opportunity to obtain knowledge about the mechanisms of the normal tissue response to irradiation.

Although a large number of normal tissue cell types (eg, fibroblasts [9], adipose [10], keratinocytes [11], and hematopoietic stem cells [12–14]) have been used in in vitro radiation studies, no studies have used stem cells involved in solid tissue regeneration after irradiation. A significant development in the in vitro cell culturing studies was the development of 3D culture systems, which have been used for studies of cancer models and treatment (15–17), drug discovery (18), and radiation therapy (19). 3D cell culture models are more physiologically relevant and offer a more realistic environment. Cells grown in 2D cultures are generally flat; however, in 3D cultures, the cells are rounder and divide to form spheroid structures consisting of multiple cells originating from single cells. In 3D cell systems, different cell–cell and cell–matrix interactions exist, and the components of the extracellular matrix of the 3D membrane itself also play a role in radioresistance compared with 2D culture systems (15, 18, 19).

Further potential improvements of radiation therapy include charged particle therapy, allowing improved tumor targeting, and dose–volume reduction, sparing normal tissue (2, 20, 21). However, the limited use of particle therapy has limited our current knowledge of its biologic effects. Although in vitro studies found important differences with photon treatment (22), most of these studies have considered tumorigenic cell lines in a 2D environment. Very little is known about the effects of particle therapy on normal tissue stem cells.

Therefore, we developed a 3D culture model for the study of the radiation response of ASCs useable for both photon and particle irradiation. We used a human submandibular salivary gland cell line (HSG) (23), derived from a patient who had received external irradiation, that still had differentiation potential (24), to optimize our 3D culture system for carbon ion irradiation. We compared the radiation effects that culturing in 3D had after carbon ion irradiation with those in 2D culturing, in both the plateau and the spread out Bragg peak regions of the beam. Finally, using our salivary gland culture system, we are the first to show the survival of tissue-specific stem cells in response to radiation treatment, using both photon and carbon ions. This could represent a novel model for studying normal tissue response to irradiation.

## Methods and Materials

### Cell line culturing

The HSG cell line was cultured in Dulbecco's modified Eagle medium/F12 (1:1) (Gibco, Life Technologies, Norwalk, CT), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

For the 3D cultures, the cells were detached using Trypsin-EDTA 0.05% for 5 minutes at 37°C and counted. The number of cells was adjusted to 200,000 cells/mL. Suspension was added to basement membrane Matrigel (model no. 354234; BD Biosciences, Franklin Lakes, NJ) at a ratio of 1:2 (25  $\mu\text{L}$  of cell suspension to 50  $\mu\text{L}$  of Matrigel) and seeded in 12-well tissue culture plates. One week after seeding, the gels were incubated with dispase (1 mg/mL in culturing media; Gibco, Life Technologies) for 45 to 60 minutes at 37°C to dissolve the Matrigel. Next, the spheres were counted, processed into single cells using Trypsin-EDTA 0.05%, and reseeded for irradiation treatment.

## Isolation of salivary gland cells

SGs from 8- to 12-week-old female C57BL/6 mice (Harlan, The Netherlands) were dissected. SG cells were isolated and cultured to form spheres as described previously (8, 25, 26). For details, see the [Supplemental Materials and Methods](#) section (available online at [www.redjournal.org](http://www.redjournal.org)).

## Irradiation treatment

Photon irradiation was performed with a  $^{137}\text{Ce}$  source (IBL 637 Cesium-137  $\gamma$ -ray machine) with a dose rate of 0.59 Gy/min or with x-rays using the XStrahl 200 X-ray Therapy System with a dose rate of 0.52 Gy/min. The method of carbon ion irradiation has been previously described (22, 27) ([supplemental data](#); available online at [www.redjournal.org](http://www.redjournal.org)). For the irradiation sessions in Matrigel, a 3.5-mm-long spread out Bragg peak with a diameter of 30 mm was developed, which resulted in a dose averaged linear energy transfer (LET) of  $149.9 \pm 10$  keV/ $\mu\text{m}$  at the center of the Matrigel samples; the plus/minus data represent the LET at the anterior and posterior of the gel. Photon irradiation of 2D HSG was performed in 60-mm dishes, and all other irradiation sessions were performed in 12-well culture plates to accommodate the physical constraints of the carbon ion beam. All 2D cultures were irradiated on plates with 70% to 80% confluency, and all irradiation of 3D cultures was performed on single cells. Further specifics regarding the cell densities and the time of irradiation after seeding are mentioned in specific assays.

## Clonogenic survival assays

Clonogenic survival assays were performed similar to the method described by Chiu et al. (28). (For details, see the [supplemental data](#); available online at [www.redjournal.com](http://www.redjournal.com).)

To determine the sensitivity of the cells cultured in 3D after irradiation, a modified 3D survival assay was performed. Cells were seeded as single cells in Matrigel as described in previous sections 2 hours before irradiation with 0 to 8 Gy. For HSG cells irradiated with 0 to 2 Gy were seeded at  $5 \times 10^3$  cells per well, and cells irradiated with 4 or 8 Gy were seeded at  $1 \times 10^4$  cells per well but with equal volumes of Matrigel per well. Mouse SGSCs (mSGSCs) were irradiated and replated at a density of  $2 \times 10^4$  cells per well (0-2 Gy) or  $6 \times 10^4$  cells per well (4-8 Gy). At 1 week after irradiation, the spheres and cells were counted. Survival was calculated as follows:

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

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

## Immunofluorescent microscopy

Two days before irradiation for immunofluorescent microscopy of the 2D cultures, the cells were seeded in glass-bottomed, 12-well, tissue culture plates (p12-1.5H-N; In Vitro Scientific [now Cellvis], Mountain View, CA). At specified time points after irradiation, the cells were fixed for 15 minutes in 2% paraformaldehyde and permeabilized in 0.2% Triton X-100 for 10 minutes.

For the 3D cultures,  $4 \times 10^4$  single cells were seeded in Matrigel 2 hours before irradiation. Dispace was added 30 minutes before the stated time points. The cells were spun for 30 seconds and washed with phosphate-buffered saline. The cells were resuspended in 2% paraformaldehyde, placed on Adhesion slides (Marienfeld-Superior), fixed for 15 minutes, and permeabilized for 10 minutes.

All samples were incubated overnight at 4°C with primary antibodies (anti-phospho-Histone H2A.X [Ser139], clone JBW301 [1:500]; Millipore, 05-636, mouse; and anti-53BP1 [H-300]; 1:500; Santa Cruz Biotechnology, sc-22760, rabbit), followed by incubation at room temperature with secondary antibodies (Alexa Fluor 488; 1:800; Life Technologies; A11001; goat anti-mouse; and Alexa Fluor 594; 1:800; Life Technologies; A110012; goat anti-rabbit) for 90 minutes. Nuclear staining was performed using Hoechst 33342 (Molecular Probes; Life Technologies). Imaging was performed using TissueFAXs (Tissuegnostics), and foci were analyzed and counted using ImageJ.

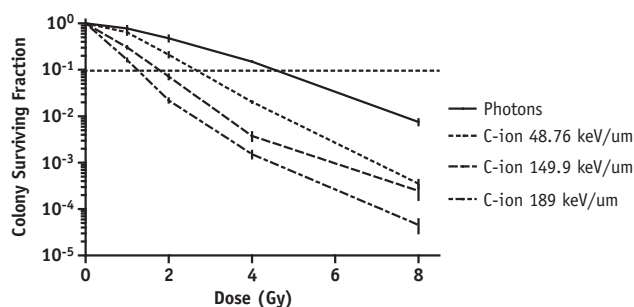
## Statistical analysis

All values are presented as the mean  $\pm$  standard error of the mean ( $P < .01$ ,  $P < .05$ ) of  $\geq 3$  independent experiments. Student's *t* test was used to test for statistical significance using GraphPad Prism (GraphPad software). If error bars are not visible, they were smaller than the data labels.

## Results

To study the in vitro response of SGSCs, we must assess this in 3D culture. However, to relate 2D models with a 3D model for SGSC irradiation, we first determined the 2D radiation response of HSG cells to photons or carbon ions of various LET amounts. As expected, increasing LET of radiation resulted in decreased survival of HSG cells at the same dose (Fig. 1). In line with the published data (20, 22), we observed an relative biologic effectiveness (RBE)<sub>10</sub> of approximately 1.72, 2.59, and 3.53 for a carbon ion LET of  $48.76 \pm 2.16$ ,  $149.9 \pm 10.8$ , and  $189 \pm 15$  keV/ $\mu\text{m}$  at 10% survival, respectively.

Next, we investigated whether culturing in 3D affects proliferation. No significant differences in population doublings in 2D culture ( $5.37 \pm 0.2$ ) compared with 3D culture ( $5.15 \pm 0.26$ ) were found at 7 days (Fig. 2A). This



**Fig. 1.** Survival of 2-dimensional cultured human salivary gland cells in response to photons, carbon ion (C-ion) 47.86 keV/μm, 149.9 keV/μm, and 189 keV/μm, as measured by clonogenic survival assays. Error bars represent standard error of mean;  $n \geq 3$ .

suggests that our 3D environment does not affect cell proliferation.

By performing a modified clonogenic assay, based on the efficiency of irradiated single cells to form spheres (Fig. 2C), we determined the survival of HSG cells cultured in 3D in response to high and low LET irradiation (Fig. 2D). Similar to 2D culturing, we observed that increasing LET at the same dose resulted in an increased radiosensitivity in HSG cells; however, in 3D culture, the HSG cells were more radioresistant than were the 2D HSG cells (Fig. 1), which has been demonstrated for other cell lines (19). The RBE<sub>10</sub> of HSG cells was 1.34 and 3.03 for carbon ions at  $48.76 \pm 2.16$  and  $149.9 \pm 10.8$  keV/μm, respectively. Owing to the physical nature of the beam, it was not possible to perform carbon ion irradiation using the highest LET ( $189 \pm 15$  keV/μm).

To exclude the role of intraspheroid oxygen diffusion as a factor for altered survival in 3D compared with 2D, we performed irradiation at various stages of HSG sphere development. The HSG cells were photon irradiated as single cells in media and subsequently seeded into Matrigel. Single cells already seeded in Matrigel or as 6-day-old spheres in Matrigel were dissociated and reseeded 2 days later in Matrigel. We found that the levels of survival were not affected (Figure E1; available online at [www.redjournal.org](http://www.redjournal.org)), indicating that the oxygen diffusion levels at irradiation were unlikely to have affected the HSG survival response to irradiation in the current setup. However, it must be acknowledged that other environmental effects could have been present that were not detected by our assay.

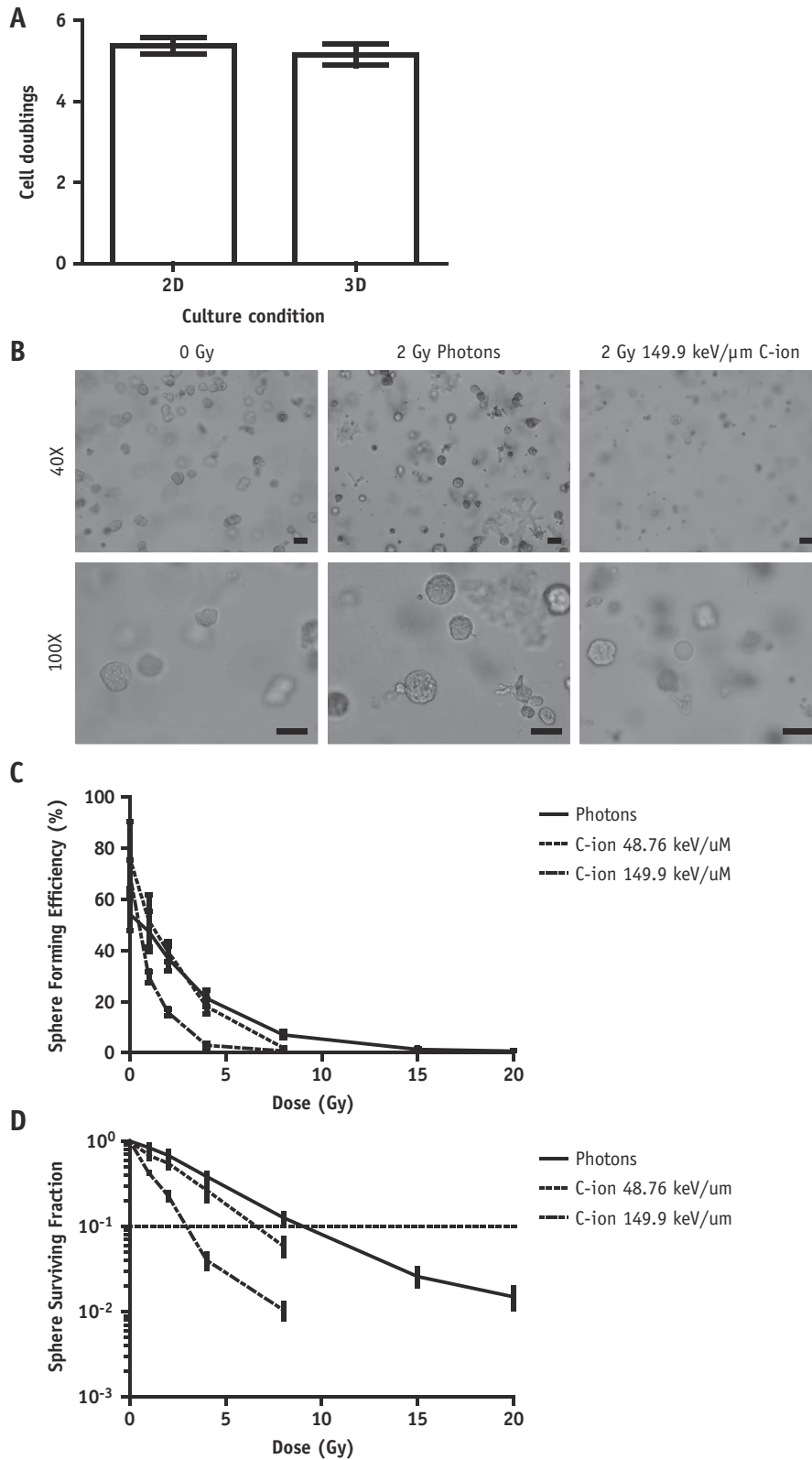
Next, we assessed the levels of DNA double strand breaks (DSBs) (29-31) in HSG cells after irradiation with 1 Gy and subsequent repair using immunofluorescent microscopy for γH2AX (Fig. 3). At 24 hours after 1-Gy photon irradiation,  $33.6\% \pm 3.4\%$  of the cells were still positive for γH2AX ( $\geq 3$  foci). In contrast, significantly more positive cells were found after  $149.9 \pm 10.8$  and  $189 \pm 15$  keV/μm of 1 Gy carbon ions;  $41.4\% \pm 3.4\%$  ( $P = .0486$ ) and  $49.1\% \pm 6.2\%$  ( $P = .0055$ ), respectively. Irradiation at  $48.76$  keV/μm showed no difference from

photons for residual DSBs, consistent with the survival data at 1 Gy. In line with previous studies, a significantly greater proportion of cells remained positive for DSBs after higher LET irradiation, indicating persistent DNA damage 24 hours after irradiation, a key factor in determining cell survival (30).

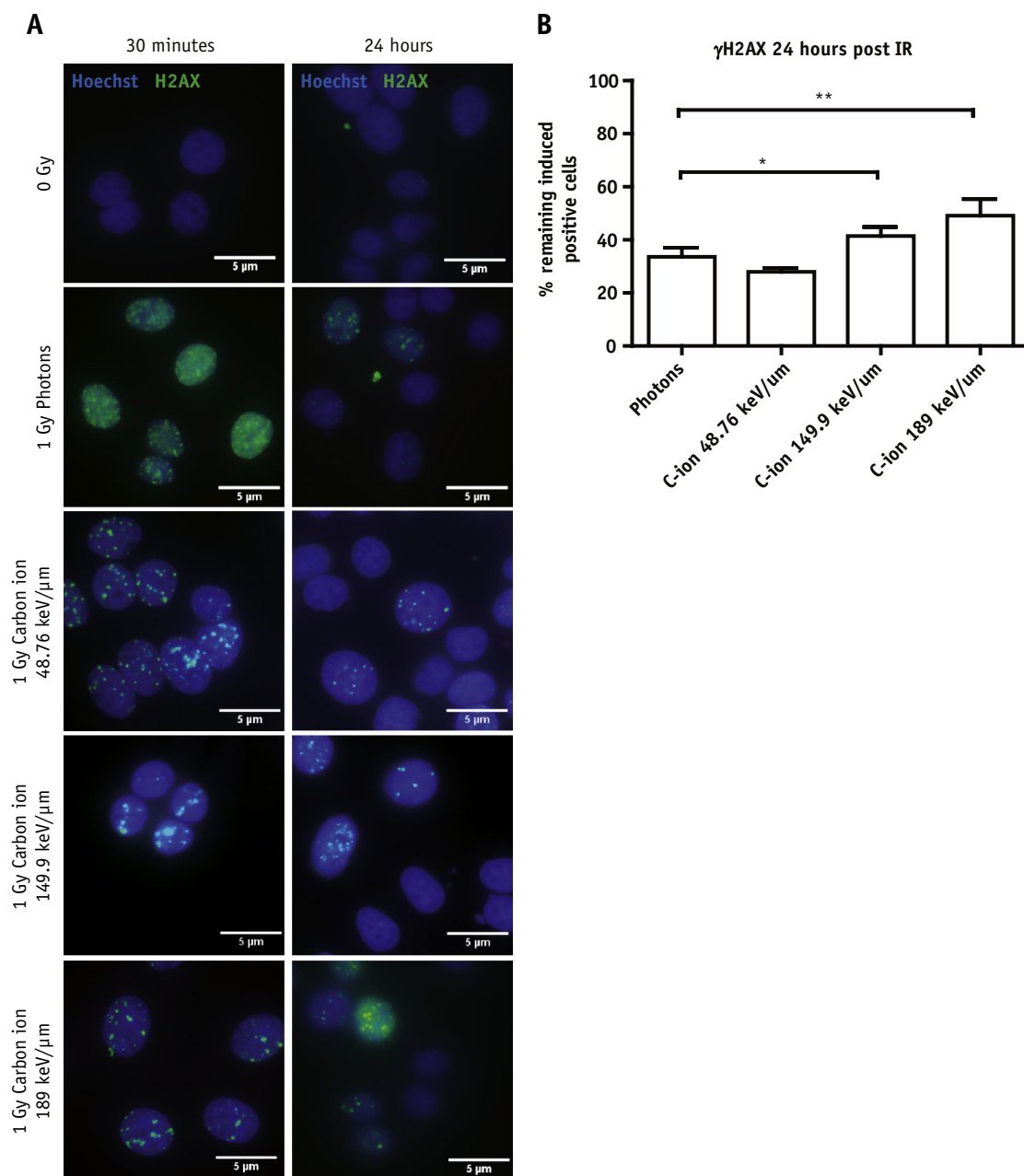
Next, the levels of DSBs in 3D cultured cells after 1 Gy of irradiation were assessed. Residual γH2AX after  $149.9 \pm 10.8$  keV/μm was significantly greater than those after photons ( $62.9\% \pm 4.6\%$  to  $37.9\% \pm 1.5\%$ ;  $P = .0067$ ). However, no difference was found between photons and  $48.76 \pm 2.16$  keV/μm ( $43\% \pm 5.7\%$ ; Fig. 4). This was not surprising, because in terms of survival, 1 Gy of photons was comparable to  $48.76 \pm 2.16$  keV/μm carbon ions, and survival after  $149.9 \pm 10.8$  keV/μm carbon ions was significantly decreased.

Finally, we tested the response of mSGSCs in our 3D culturing model. In salispheres (spheres formed from SG-derived cells [25]) cultured from dispersed mouse SG cells, only the stem/progenitor cells can form secondary spheres; all other cells will die off even without irradiation (8). We irradiated single salisphere cells and determined the sphere-forming efficiencies of the cells after passaging (Fig. 5A) as a representative of the surviving number of mSGSCs 7 days after irradiation. Sphere-forming efficiencies were normalized to 0 Gy to calculate the surviving fraction (Fig. 5B). After photon irradiation, the isolated mSGSCs displayed a clear dose–response relationship, with survival decreasing from  $68.3\% \pm 12.6\%$  for 1 Gy of photons to  $15.5\% \pm 1.8\%$  survival at 8 Gy. Irradiating with carbon ions (both  $48.76 \pm 2.16$  keV/μm and  $149.9 \pm 10.8$  keV/μm) also resulted in a clear dose–response curve, albeit with a stronger response to carbon ions, with survival of  $45.3\% \pm 4.2\%$  after 1 Gy of  $149.9 \pm 10.8$  keV/μm carbon ions and only  $2.4\% \pm 0.2\%$  survival after 8 Gy. It was necessary to extrapolate the photon survival curve to determine an RBE<sub>10</sub> for isolated mSGSCs. The calculated RBE<sub>10</sub> values were 1.72 for  $48.76 \pm 2.16$  keV/μm and 2.90 for  $149.9 \pm 10.8$  keV/μm, similar to HSG cells irradiated in 3D cultures. These data suggest that, similar to cell lines, the radiosensitivity of SGSCs increases with increasing LETs.

When we compared the response of HSG cells in 3D cultures with that of mSGSCs (Fig. 5C-E), we found that HSG cells and mSGSCs showed a similar pattern of radiosensitivity to photons. These results suggest that HSG cells might be more similar to a normal stem cell-containing population than previously thought (23), in particular, in response to irradiation. However, when we compared the response of the same cells to carbon ion irradiation, we found that the mSGSCs were less radiosensitive to both  $48.76 \pm 2.16$  keV/μm ( $P = .0314$  at 4 Gy, and  $P = .0415$  at 8 Gy) and  $149.9 \pm 10.8$  keV/μm ( $P = .0149$  at 4 Gy, and  $P = .0057$  at 8 Gy) than HSG cells. This could suggest that the stem cells themselves might be a key factor to a potential increased radioresistance to higher LET irradiation.



**Fig. 2.** (A) Cell number doublings of nonirradiated human salivary gland (HSG) cells cultured in 2-dimensional versus 3-dimensional conditions. (B) Representative images of (non-)irradiated HSG spheres. (C) Sphere-forming efficiency of HSG cells in Matrigel after irradiation, on which (D) survival of nonirradiated HSG cells is based. Error bars represent standard error of mean;  $n \geq 3$ . Scale bar = 100  $\mu\text{m}$ .

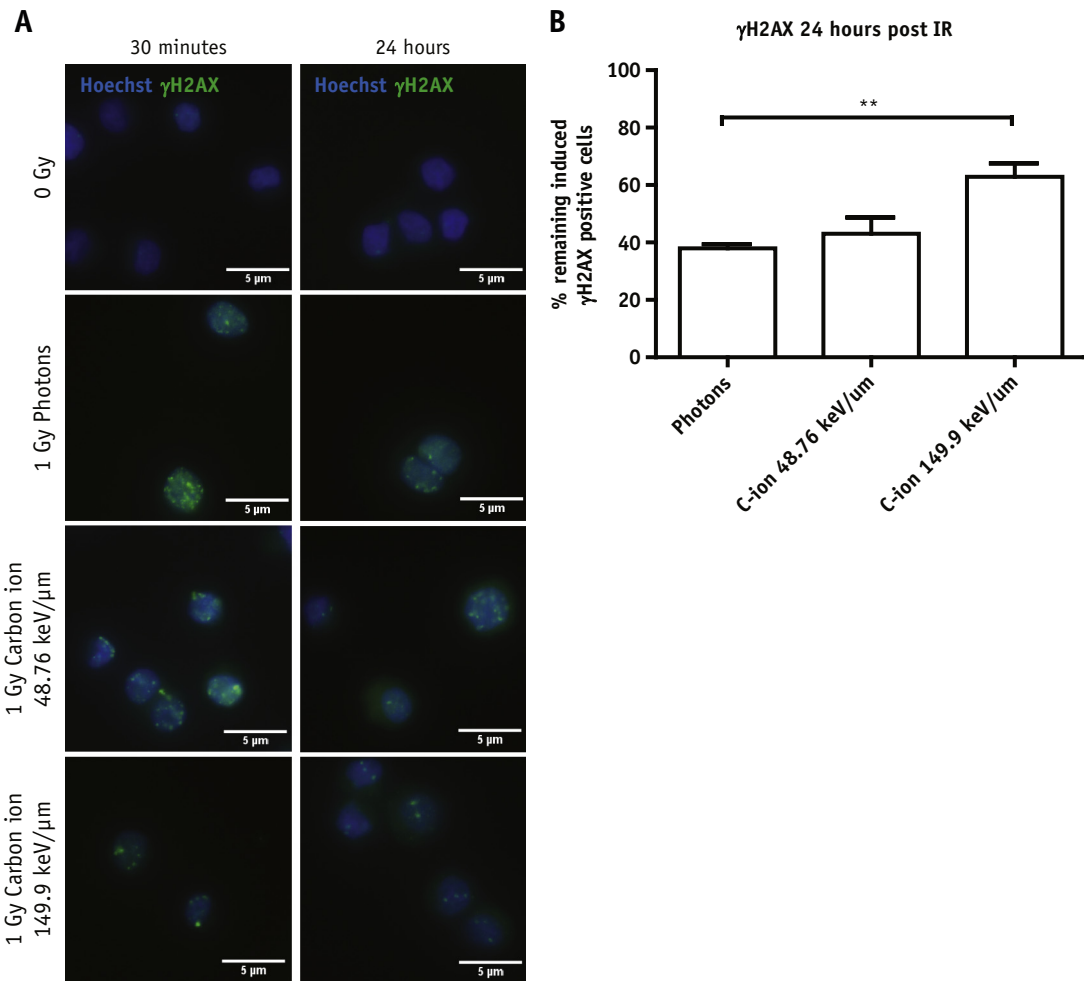


**Fig. 3.** Levels of double stranded DNA breaks studied at 30 minutes, 4 hours, and 24 hours after irradiation of 2-dimensional cultures by immunofluorescent microscopy for (A)  $\gamma$ H2AX. (B) The levels of persistent damage 24 hours after irradiation in 2-dimensional culture. Error bars represent standard error of mean;  $n \geq 3$ . \* $p < 0.05$ , \*\* $p < 0.01$ .

## Discussion

We developed a model derived from our previously established mSGSC cultures (8) to assess the normal tissue response after irradiation with modalities with differing LETs. We showed a similar radiosensitivity to photon irradiation of mSGSCs and 3D-cultured HSG cells. A potential reduced sensitivity of mSGSCs to higher LET carbon ions was observed. We believe this model might represent a novel method to obtain knowledge on normal tissue side effects.

Our mSGSC model is a 3D culture (8). Therefore, to optimize our model for irradiation, we used the transformed epithelial, non-neoplastic HSG cell line (23), which can be cultured in both 2D and 3D spheres (24). As expected, in 2D cultures, a clear dose–response relationship was seen with increasing doses of photon irradiation. After carbon ion irradiation, this dose–response effect was even more evident with increasing LETs. The RBE values were within the range found for other cell lines after carbon ion irradiation (20, 22). Owing to the more complex nature of DNA damage induced by particle therapy (32), this was not



**Fig. 4.** (A) Representative images of  $\gamma$ H2AX immunofluorescent microscopy after 3-dimensional irradiations. (B) Quantification of residual damage 24 hours after irradiation in Matrigel.

unexpected, because this damage is slower to repair and therefore more likely to remain unrepaired 24 hours after irradiation. It has been shown that persistent damage at 24 hours after irradiation is critical in cell lethality (30). The percentage of cells that remained positive for DNA DSBs 24 hours after irradiation was higher after carbon irradiation, which correlates with increased radiosensitivity with increasing LETs. The 2D survival data and the DNA residual damage showed that this is a strong model for furthering our studies toward 3D studies.

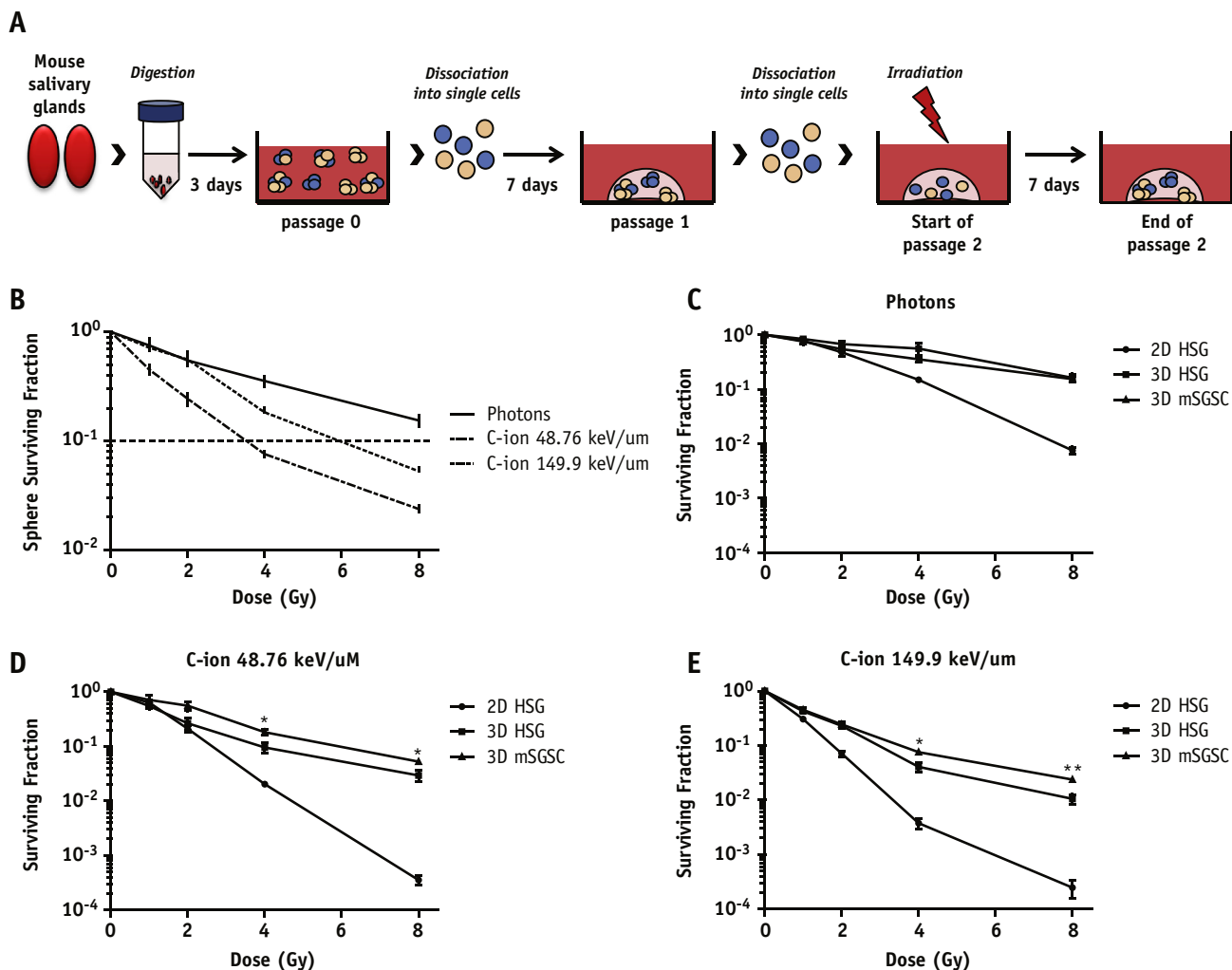
Next, we determined the effects that culturing these cells under 3D conditions would have after irradiation. We showed similar radiosensitivity reduction under 3D culture conditions for HSG cells compared with other cell lines (19, 33). Furthermore, we found that the 3D cultured cells showed a similar phenotypic response to differing LET irradiation as in 2D conditions. Again, in line with previous studies (30) and similar to our 2D findings, a significantly greater level of  $\gamma$ H2AX with increasing LETs correlated with increased radiosensitivity. The levels of residual damage were perhaps greater than expected compared with

the levels of survival at 1 Gy, which might suggest that measuring at a later time point after irradiation might be more insightful in terms of final clonogenic survival.

Finally, we irradiated mSGSCs that had been cultured in our recently developed culture system for normal tissue (8). Because this system consists of several cell types, including stem cells, we believe it will be an ideal model for studying normal tissue side effects after irradiation. We found that our mSGSCs showed a similar radiosensitivity to HSG cells in response to photons. This might indicate that HSG cells are a strong model for studying the normal tissue response to photons. In response to increasing LETs, the mSGSCs showed an increased radiosensitivity compared with HSG cells. This suggests that the stem cells might be more radioresistant to increasing LETs than to photons.

This is the first in vitro study to irradiate mSGSCs also using high LET carbon ions. Our model had some limitations. First, in a true in vivo environment, ASCs reside in a quiescent state and only divide in response to certain cues, for example, to regenerate damaged tissue (34). However, in our system, SGSCs are driven toward proliferation (8),





**Fig. 5.** (A) Schematic overview of isolation, expansion, and irradiation of mouse salivary gland stem cells (mSGSCs). (B) Surviving fraction of isolated mSGSCs after photon and carbon ion (48.76 keV/ $\mu$ m and 149.9 keV/ $\mu$ m) irradiation. (C-E) Comparison of responses of human salivary gland cells grown in 2-dimensional versus 3-dimensional versus mSGSCs in response to (B) photons, (C) carbon ions at 48.76 keV/ $\mu$ m, and (D) carbon ions at 149.9 keV/ $\mu$ m. Error bars represent standard error of mean;  $n \geq 3$ . \* $p < 0.05$ , \*\* $p < 0.01$ .

thereby possibly altering the response to irradiation. It is also important to note that environmental factors, such as inflammatory processes, macrophages, and secreted cytokines (eg, transforming growth factor- $\beta$  [35]) in response to radiation, are not present in our system; the addition of these factors might alter the response.

## Conclusions

However, our model still represents a breakthrough method for studying normal tissue response in vitro, showing important differences in the response of ASCs to high and low LET radiation modalities. We believe that the use of ASCs gives a better understanding of the normal tissue response than other 3D models currently available. A recent study has been performed to investigate the response of adipose-derived stem cells irradiated under 3D conditions

(10), in which the investigators also observed an increased resistance under 3D conditions and differences between LET irradiation modalities. However, these are not tissue-specific stem cells; therefore, we believe our model might be an improved alternative to study normal tissue damage after irradiation. In the future, the use of patient-derived ASCs could determine differences in individual patient-specific responses and contribute to patient-specific treatment planning.

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