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Spatially Fractionated Microbeam Analysis of Tissue-sparing Effect for Spermatogenesis

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Spatially fractionated radiation therapy (SFRT) has been based on the delivery of a single high-dose fraction to a large treatment area that has been divided into several smaller fields, reducing the overall toxicity and adverse effects. Complementary microbeam studies have also shown an effective tissue-sparing effect (TSE) in various tissue types and species after spatially fractionated irradiation at the microscale level; however, the underlying biological mechanism remains elusive. In the current study, using the combination of an *ex vivo* mouse spermatogenesis model and high-precision X-ray microbeams, we revealed the significant TSE for maintaining spermatogenesis after spatially fractionated microbeam irradiation. We used the following ratios of the irradiated to nonirradiated areas: 50:50, 150:50 and 350:50 μm -slit, where approximately 50, 75 and 87.5% of the sample was irradiated (using center-to-center distances of 100, 200 and 400 μm , respectively). We found that the 50 and 75% micro-slit irradiated testicular tissues showed an almost unadulterated TSE for spermatogenesis, whereas the 87.5% micro-slit irradiated tissues showed an incomplete TSE. This suggests that the TSE efficiency for spermatogenesis is dependent on the size of the nonirradiated spermatogonial stem cell pool in the irradiated testicular tissues. In addition, there would be a spatiotemporal limitation of stem cell migration/competition, resulting in the insufficient TSE for 87.5% micro-slit irradiated tissues. These stem cell characteristics are essential for the accurate prediction of tissue-level responses during or after SFRT, indicating the clinical potential for achieving better outcomes while preventing adverse effects. © 2020 by Radiation Research Society

INTRODUCTION

In 1909, German radiologist Alban Köhler reported the first clinical observation of a tissue-sparing response after grid-radiotherapy (GRID) in which spatially fractionated irradiation was delivered using a grid-like pattern of beams (1, 2). In 1995, a notable tissue-sparing technique was reported in rat brain tissues after microbeam irradiation (3), which is based on the spatial fractionation of synchrotron-generated X-ray microbeams at the microscale level. This was performed at the Brookhaven National Laboratory (Upton, NY). Spatially fractionated radiation therapy (SFRT), such as GRID and microbeam radiation therapy (MRT), has shown an effective tissue-sparing effect (TSE), and microbeam studies have confirmed an effective TSE in a large variety of species and tissue types, although the underlying mechanism remains to be fully determined (2–9). The TSE of SFRT is the phenomenon by which normal tissues tolerate single exposures to narrow planes of synchrotron-generated X rays (micro-planar beams; microbeams) of up to several hundred Gy (10). In addition, recently published results from an ultra-high dose-rate (FLASH) radiation study have demonstrated a remarkable sparing of normal tissue after irradiation at ultra-high dose rate (>40 Gy/s), suggesting that the dose rate is involved in TSE (11).

Microbeams have undergone a renaissance since their introduction and early use in the mid-1960s, and the resurgence in the use of microbeams since the mid-1990s has coincided with major changes in our understanding of how radiation interacts with cells and tissues (12, 13). The development of single-particle microbeams, where a single cell and/or a subcellular compartment can be selectively irradiated with either one or multiple particles (14), has greatly facilitated our understanding of a variety of biological end points, including cytoplasmic effect, bystander effect and genomic instability (15). In particular, the evidence that non-targeted responses occur, so that cells not directly irradiated can respond to irradiated neighbors (16, 17), has brought about the evolution of new models of

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radiation response. Much of this has come from charged-particle microbeam studies, but, increasingly, X-ray and electron microbeams are starting to contribute quantitative and mechanistic information on non-targeted radiation responses. In addition, for tissue homeostasis, cell competition is essential as the cell fitness-sensing mechanism seen from insects to mammals that eliminates cells, which, although viable, are less fit than their neighbors (18). Damaged cells induced by non-uniform irradiation would be removed by the neighboring cells due to cell competition, resulting in the prevention of a pathological state, such as carcinogenesis (19). Given the use of high-precision microbeams, further radiobiological studies on such tissue-level responses in spatially fractionated radiation fields are also foreseen (20).

In recent clinical practice, radiation treatments for cancer have evolved to a high level of precision and accuracy; nevertheless, they may result in temporary, long-term or permanent gonadal toxicity (21–23). Damage to spermatogenesis can result from either direct irradiation of the testes or the scattered dose received during the radiation treatment of cancers, such as prostate, bladder, rectal and bone cancers (24). Also, genetic diversity in radiation sensitivity, such as hereditary DNA damage repair disorders, could be involved in the radiotherapy-related spermatogenic impairment (25). In addition, male reproductive potential continues to be adversely affected not only by clinical, but also environmental and occupational, radiation exposures (26, 27). During the process of spermatogenesis, sperm-forming cells can be affected by low-dose exposures through a number of potential mechanisms (28). With the above in mind, although the underlying mechanisms of TSE remain unclear, we hypothesized that the TSE of SFRT in the testes would be helpful for the preservation of male fertility while still delivering high doses to tumor.

In the current study, to approach our hypothesis, we investigated the TSE after spatially fractionated microbeam irradiation for preserving spermatogenesis in the testes. For these purposes, we used 5.35-keV monochromatic X-ray microbeams at the High Energy Accelerator Research Organization (KEK; Tsukuba, Japan) (29), and a unique *ex vivo* testes organ culture (30) that enabled us to monitor the progress of spermatogenesis and to assess radiation-induced effects easily. As previously reported, we have confirmed that this *ex vivo* model of spermatogenesis can reproduce the deterministic effects of radiation (e.g., temporary infertility and permanent sterility) after uniform exposure to conventional X rays (31) and 5.35-keV monochromatic X-ray microbeams (32). Furthermore, using 5.35-keV synchrotron-generated X-ray microbeams, approximately 50% of the tissue was irradiated (using center-to-center distances of 25, 100 and 400 μm , respectively), and we have shown, for the first time, an microbeam radiation-mediated TSE for the preservation of spermatogenesis. However, other ratios of the irradiated to nonirradiated areas remained to be investigated. Thus, in

this study, we use the following ratios: 50:50, 150:50 and 350:50 μm -slit, where approximately 50, 75 and 87.5% of the sample can be irradiated (using center-to-center distances of 100, 200 and 400 μm , respectively) to expand the applicability of this approach for the preservation of male fertility. Our technical combination of a unique *ex vivo* testes organ culture and high-precision X-ray microbeam will provide novel insights into the radiation-induced stochastic and deterministic effects on male reproduction as well as the clinical potential.

MATERIALS AND METHODS

Animals

Testes were obtained from 7 days postpartum (dpp) *Acr-GFP* transgenic mice from the RIKEN BRC (Tsukuba, Japan) through the National BioResource Project of MEXT, Japan. Female ICR, C57BL/6 (CLEA Japan) or ICR $\times$ C57BL/6F1 mice were mated with male transgenic mice to produce pups. These mice express the marker green fluorescent proteins (GFP) specific for meiosis, which are extremely useful for monitoring the progress of spermatogenesis (33, 34). All animal experiments in the current study conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (Animal Research Center of Yokohama City University, Yokohama, Japan).

Ex Vivo Testis Organ Culture

As described elsewhere (31), each testis sample was cut into 8–10 pieces approximately 1 mm³ in size when compacted. Each piece of tissue was immediately placed on a 1.5% agarose gel block immersed in 0.5 ml of α -MEM medium containing 10% knockout serum replacement (KSR; Thermo Fisher Scientific K.K., Yokohama, Japan), 1% antibiotic mixture solution (antibiotic-antimycotic; Thermo Fisher Scientific K.K.) and 0.2 mM NaHCO₃ in a 12-well culture dish. Samples were cultured in a humidified incubator at 34°C in an atmosphere of 95% air and 5% CO₂.

Live Tissue Imaging

As described elsewhere (31), the cultured samples were observed using fluorescence microscopy (Keyence Corp., Itasca, IL) with a 4 $\times$ magnification objective lens to capture GFP-fluorescent and bright-field images. After the observations at different time points, samples were returned to the incubator; images were acquired on alternate days. The exposure times were 1.5 s for the GFP-fluorescent images and 150 ms for the brightfield images. Although GFP expression was measured for 30 days in this study, Sato *et al.* (30) have previously shown that the explants can remain viable for up to 70 days.

Synchrotron-Generated X-Ray Microbeams

To investigate the TSE for preserving spermatogenesis, we used a 5.35-keV monochromatic X-ray microbeam irradiator at the KEK Photon Factory synchrotron facility in Japan (29). As described elsewhere (32, 35), the beam width was variable using a remote-controlled four-dimensional slit. The exposure (photon fluence) was determined by measuring the X-ray beam intensity upstream of the slit system using a specially designed air-free ionization with parallel plate-collecting electrodes 30 mm in length with a 40-mm space between the electrodes (29). A silicon photodiode (International Radiation Detectors, Torrance, CA) was used as a second detector for the intensity measurement at both the incident beam position (upstream of the slit) and the microscope stage (downstream of the

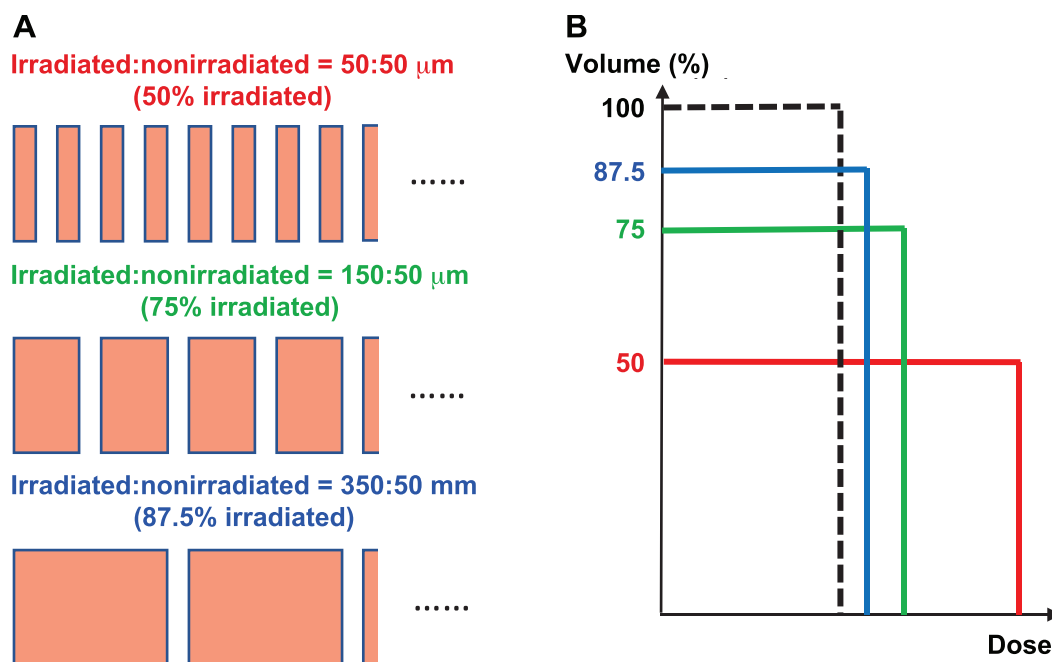


FIG. 1. Schematic of dose patterns used in experiments. Panel A: Different spatial patterns of irradiated to nonirradiated for 50, 75 and 87.5% exposures. Panel B: Dose volume histograms for 50, 75, 87.5 and 100% exposures.

slit). The exposure applied to the sample was 0.26 C/kg, which corresponds to an absorbed dose of approximately 5 Gy. The composition of soft tissue given by the International Commission on Radiation Units and Measurements (ICRU) (36) was used for the dose calculation.

Micro-slit Irradiation Settings

To perform spatially fractionated microbeam analyses, we used the following ratios of the irradiated to nonirradiated areas: 50:50, 150:50 and 350:50 μm -slit, where approximately 50, 75 and 87.5% of the sample was irradiated (using center-to-center distances of 100, 200 and 400 μm , respectively) (Fig. 1A). To observe the TSE for preserving spermatogenesis, the cultured samples were irradiated at 8 dpp with uniform exposure to 2.5 Gy ($n=3$), 50% micro-slit exposure to 5 Gy ($n=3$), 75% micro-slit exposure to 3.33 Gy ($n=4$), 87.5% micro-slit exposure to 2.85 Gy ($n=4$) or left nonirradiated ($n=3$) (Fig. 1B). Thus, we used the same dose of 2.5 Gy at the whole tissue level. A single sample from each mouse testis was randomly assigned to an experimental group. A minimum of three tissue samples each from donor mice were used for each experiment; contaminated samples were excluded.

As shown in Fig. 2A, the dose profiles were accurately calculated using a Monte Carlo particle transport simulation code, PHITS version 2.96 (37). The beam intensity was essentially flat within the beam width and the deviation of the dose was approximately $\pm 6\%$ of the averaged dose. Due to the very short range of secondary electrons (1.1 μm maximum) produced by the 5.35-keV X-ray irradiation, the doses delivered outside of the irradiated area was negligible ($<0.25\%$). Thus, the absorbed dose of 5 Gy was given by the X-ray energy deposition only in the slit-like exposed volume of the sample divided by the mass of this sample volume.

Evaluation of GFP Expression

As described elsewhere (32), the Acr-GFP expressions in the culture tissue showed the progression of spermatogenesis, and in the

nonirradiated cultures, the peak expression occurred at approximately 18–22 dpp. The observation of GFP expression along the tubules was designated as a sign of spermatogenesis. The expression was classified into six grades, 0–5, based on the expression area: 0, –20, –40, –60, –80 and –100%, respectively (38). The central area was omitted from the evaluation because, in many cases, this area lacked GFP expression due to spatial and nutrient flow restrictions (30). This is one of the technical limitations of *ex vivo* testes organ cultures for monitoring the process of spermatogenesis (31, 32).

Immunohistochemical Analysis

As described elsewhere (31), for immunofluorescence staining, cultured samples (from three donor mice) fixed with 4% (w/v%) paraformaldehyde in phosphate buffered saline (PBS) at 4°C for 6 h or overnight were cryo-embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan) and cut into 7- μm -thick sections. Sections were washed once with PBS for 5 min and in 0.2% PBT four times for 10 min each. Then, sections were blocked with Image-iT FX Signal Enhancer (Thermo Fisher Scientific Inc., Waltham, MA) for 30 min and incubated overnight at 4°C with the following first antibodies: rabbit anti- γ -H2AX antibody (1:1,000; Abcam, Cambridge, UK), rat anti-GENA antibody and mouse clone TRA98 (1:1,000; Osaka, Japan). The anti- γ -H2AX was used to detect DNA double-strand breaks in the nucleus (39). The anti-GENA was used to detect germ cells (40). Nuclei were counterstained with Hoechst 33342 (1 $\mu\text{g}/\text{ml}$; bisBenzimide H 33342) (41). The secondary antibodies were goat anti-rabbit IgG, goat anti-rat IgG and donkey anti-goat IgG, conjugated to Alexa Fluor® 488, 555 or 647 (1:200; Thermo Fisher Scientific, Inc.). The immunologically stained samples were mounted on slides in ProLong™ Diamond Antifade Mountant (Thermo Fisher Scientific, Inc.).

Periodic Acid-Schiff Staining

Periodic acid-Schiff (PAS) staining is commonly used to visualize the changes of a developing acrosome during acrosome-genesis (42)

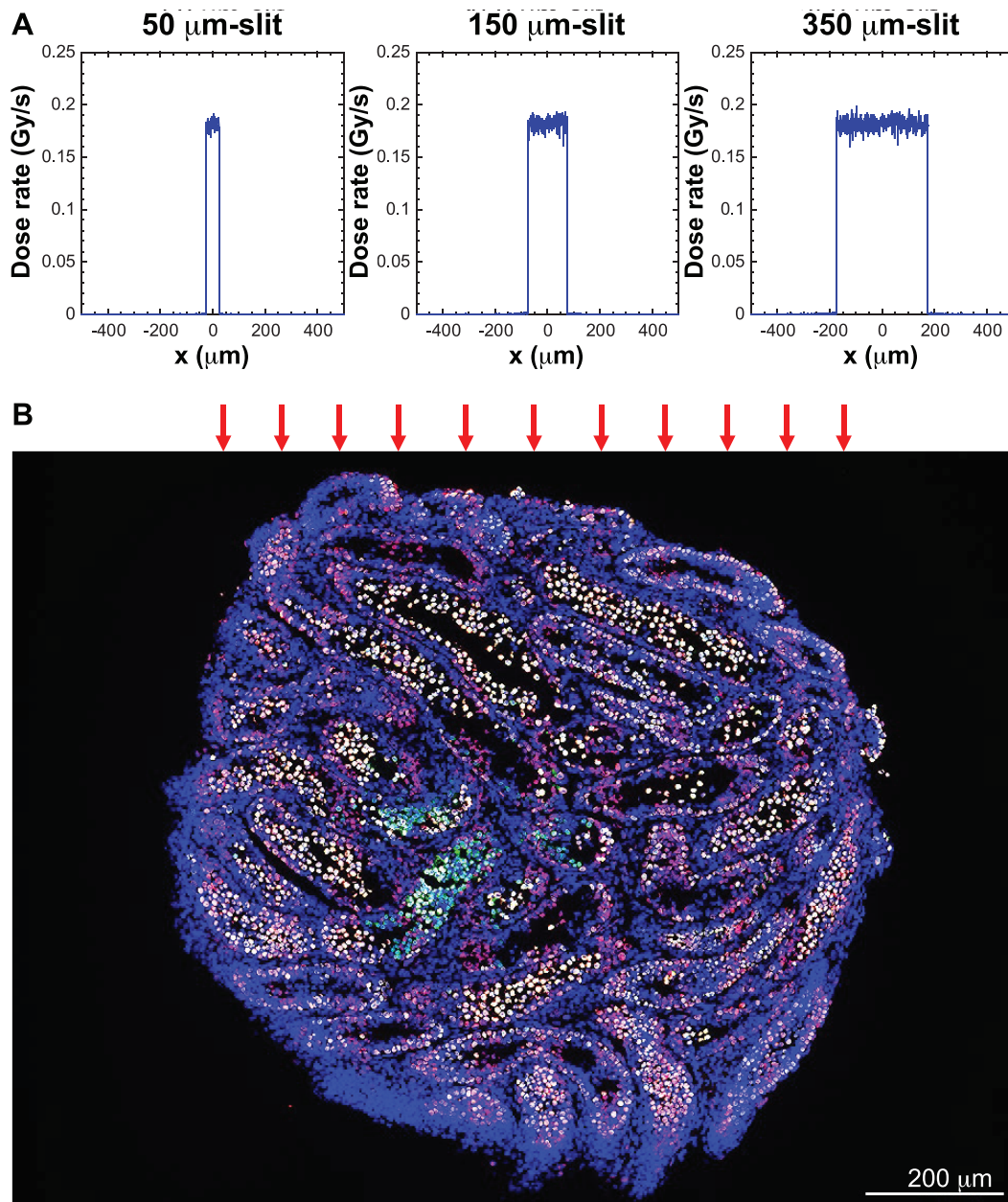


FIG. 2. Dose profile of spatially fractionated microbeams in the culture tissues. Panel A: Calculated dose profiles of 50, 150 and 350 μm -slit beams at the KEK Photon Factory. Panel B: Immunohistochemical images of *ex vivo* testicular culture tissues after micro-slit X-ray microbeam irradiation. Immunohistochemical images of *ex vivo* testicular culture tissues 1 h after 10 Gy 50–50 μm -slit X-ray microbeam irradiation. Staining for γ -H2AX, anti-germ cell-specific antigen antibody (anti-GENA), and Hoechst (for DNA) is shown as red, green and blue, respectively. Red arrows indicate the micro-slit irradiated areas. Scale bar = 200 μm .

and can reveal the presence of round or elongating spermatids. Tissues were fixed with Bouin's fixative (Muto Pure Chemicals, Tokyo, Japan) and embedded in paraffin. One section showing the largest cut surface was selected for each specimen and stained with PAS-hematoxylin.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to assess differences in the GFP expression grade. Values with $P < 0.05$ were considered to indicate a significant difference.

RESULTS AND DISCUSSION

Using immunohistochemical staining, we detected the dose distribution of micro-slit X-ray microbeams in *ex vivo* mouse spermatogenesis models. To observe radiation-induced effects on spermatogenesis, the cultured samples were irradiated at approximately 8 dpp. The staining of γ -H2AX was used to confirm the 50% 50 μm -slit irradiated areas in the cultured tissue. As shown in Fig. 2B, the distribution of immune-stained γ -H2AX in the sample was

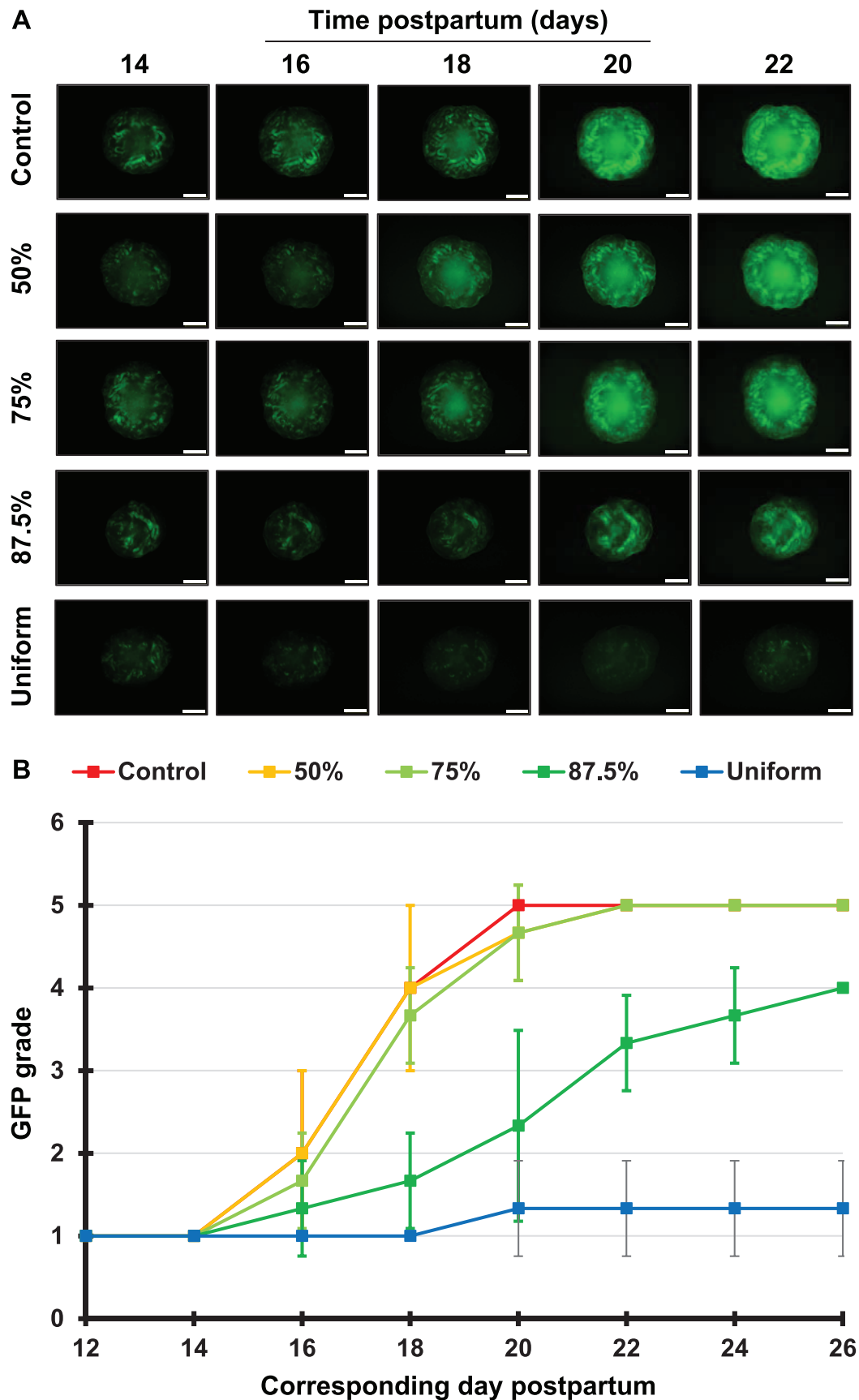


FIG. 3. Chronological GFP expression changes after spatially fractionated microbeam irradiation. Panel A: Representative images show GFP expression changes in single cultures after uniform irradiation with 2.5 Gy,

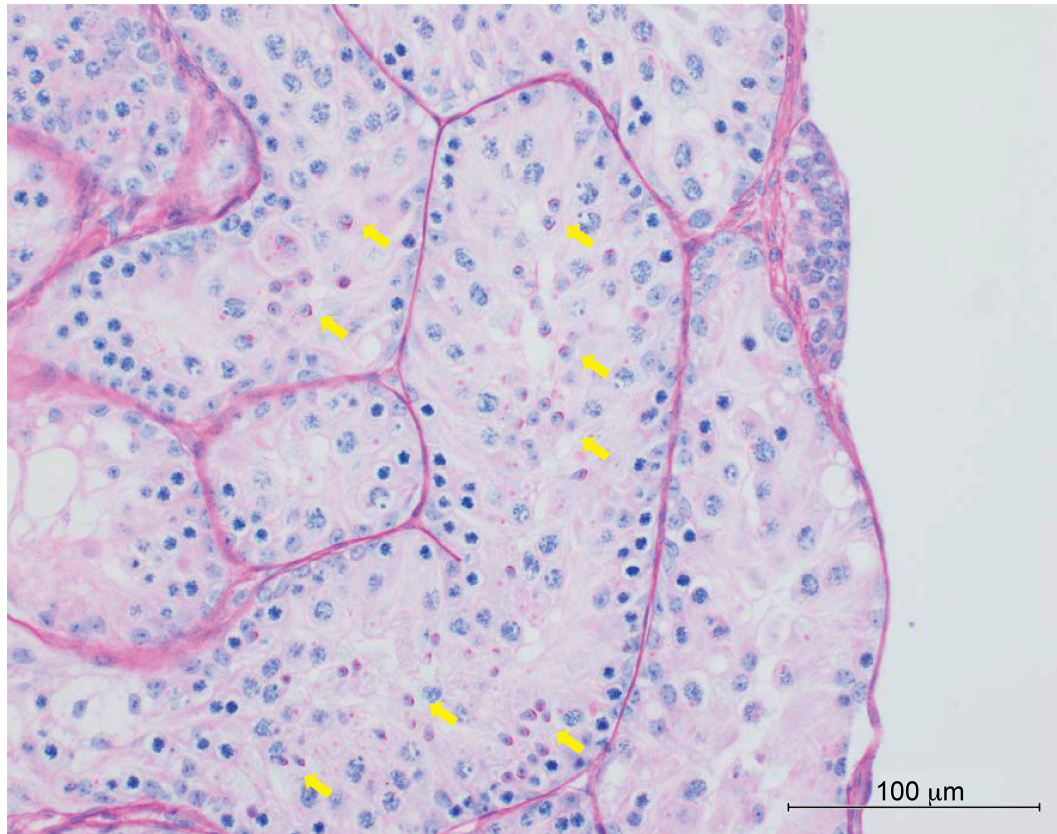


FIG. 4. Histological image with PAS staining of culture tissues. 87.5% micro-slit irradiated tissues, demonstrating round and elongating spermatids, are indicated by yellow arrows. After culturing for more than one month postirradiation, PAS staining was done to investigate the progress of spermatogenesis. Scale bar = 100 μm .

a good approximation of the shape of the microbeam irradiation patterns. There were clear border lines (with a linewidth of a single cellular level) of $\gamma\text{-H2AX}$ foci expression between the irradiated and nonirradiated areas, indicating that there were no doses delivered to the nonirradiated areas via high-energy secondary electrons, which have been known to cause frequently undesirable effects on nonirradiated areas when conventional radiation sources are used. As previously described elsewhere (31, 32), the centers and edges of samples showed tissue damage due to the technical limitations of the *ex vivo* testes organ culture method. In addition, due to the lack of vascular and matrix structure in the cultures, all aspects of the tissue microenvironment may not be accurately recapitulated. These are technical limitations associated with our method, and further technical development of organ or 3D culture methods is expected to overcome these limitations.

Next, we altered the volume of the irradiated tissue by changing the ratios of the irradiated to nonirradiated areas at the microscale level to a 50:50 μm -slit (50% irradiation), 150:50 μm -slit (75% irradiation) or 350:50 μm -slit (87.5% irradiation). As shown in Fig. 3, the 50 and 75% micro-slit irradiated tissues showed similar Acr-GFP expressions to those observed in the control (0% irradiation), indicating the occurrence of the TSE, whereas the 87.5% micro-slit irradiated tissues showed delayed and reduced Acr-GFP expression (Fig. 3A). These are significantly different from the uniform irradiated (100% irradiation) tissues, while received the same total dose, 2.5 Gy at the whole tissue level (Fig. 3B). These findings suggest that the TSE was more effective when more germ stem cells survived after irradiation of the cultured tissue. In addition, as shown in Fig. 4, via histological analysis using PAS staining, we detected round and elongating spermatids in the tissues after 87.5% micro-slit irradiation and incubation for approxi-

←
50–50 μm -slit (50%) 5 Gy irradiation, 150–50 μm -slit (75%) 3.33 Gy irradiation, 350–50 μm -slit (87.5%) 2.85 Gy irradiation, or left nonirradiated (control), respectively. Scale bars = 500 μm . Panel B: Quantification of chronological changes in GFP expression with the grade score. A single sample from each mouse testis was randomly assigned to an experimental group. A minimum of four tissue samples each from different donor mice were used for each experiment; however, contaminated samples were excluded. Data show the mean GFP expression $\pm$ SD from 12 to 26 dpp.

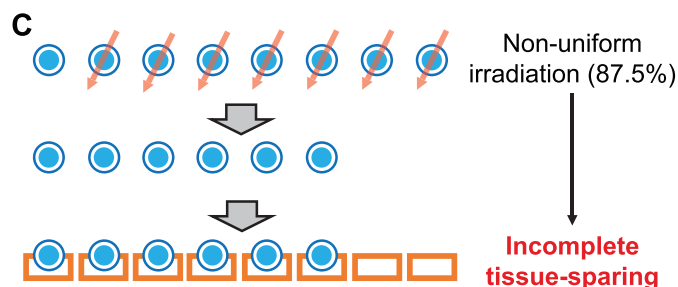
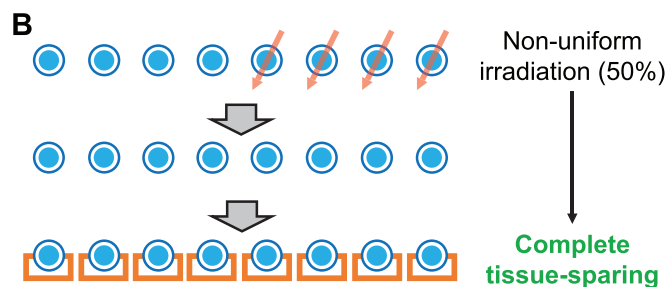
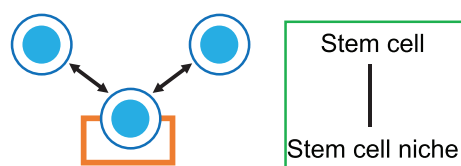
A Stem cell competition

FIG. 5. Stem cell competition and tissue-sparing effect. Panel A: Stem cell competition for residence in the niche is shown. Panel B: Nonirradiated spermatogonial stem cell pool after 50% micro-slit irradiation and the complete tissue-sparing effect are shown. Panel C: Nonirradiated spermatogonial stem cell pool after 87.5% micro-slit irradiation and the incomplete tissue-sparing effect are shown. Red arrows indicate exposure to spatially fractionated radiation.

mately 35 days, which is long enough for the period of spermatogenesis. Offspring can be produced by sperm cells or round or elongating spermatids in *ex vivo* cultured tissues via intracytoplasmic sperm injection or round spermatid injection techniques (30). Thus, the differentiated spermatogonial cells produced in 350:50 μm -slit irradiated (87.5%) tissues, due to the tissue-sparing effects, could produce offspring, showing the preservation of male fertility.

Current knowledge on stem cell biology with respect to radiation-induced adverse effects has been summarized in the International Commission on Radiological Protection's (ICRP) Publication 131 (43). In homeostatic conditions, a tissue stem cell is considered to divide asymmetrically in the stem cell niche to produce a stem cell and a progenitor cell, and the stem cell niche usually provides a shelter from various genotoxic stresses. In addition, there is a competition of tissue stem cells for the occupancy of the niche with the retention of favorable cells and the elimination of unwanted cells (Fig. 5A). This tissue microenvironment-based selection is likely to function as a tissue-based quality control measure after irradiation that is independent of the molecular and cellular quality controls, such as DNA

damage repair and apoptosis. In the testes, mouse spermatogonial cell transplantation was first performed by Brinster and colleagues in 1994 and resulted in donor cell spermatogenesis in the recipient testes, demonstrating that the spermatogonial stem cell niche is in seminiferous tubules (44). Therefore, we noticed that our *ex vivo* spermatogenesis model is appropriate for investigating germ stem cell migration/competition for residence in the niche after physical or chemical stresses, including radiation.

Our experimental results showed that such tissue-level quality control has great benefit for maintaining tissue functions during or after SFRT, such as GRID and microbeam irradiation. In fact, our live-tissue imaging revealed that the 50 and 75% micro-slit irradiated testicular tissues showed an almost unadulterated TSE for spermatogenesis (Fig. 5B), whereas the 87.5% micro-slit irradiated tissues showed an incomplete TSE (Fig. 5C). Thus, the TSE efficiency for spermatogenesis would be dependent on the size of the nonirradiated spermatogonial stem cell pool in the irradiated testicular tissues, indicating that stem cell migration/competition is possibly involved in the underlying mechanisms of TSE. In addition, there could be a spatiotemporal limitation of stem cell migration/competition, resulting in the insufficient TSE for 87.5% micro-slit irradiated tissues. If other 87.5% space-fractionated irradiation patterns such as grid, square or triangular grating, and random patterns, were used, it would be possible to show whether a significant TSE for maintaining spermatogenesis had spatial dependency. Overall, this indicates that SFRT can restrict the high-dose regions occurring within tumors, while sparing surrounding testes for the preservation of male fertility.

One important further direction could be the investigation of which molecular signals of stem cell migration/competition are involved in the TSE process during the long process of spermatogenesis. An international joint research group recently reported that spermatogenic stem cell density is tightly regulated by the supply of fibroblast growth factors (FGFs) from lymphatic endothelial cells, and that the competition for the limited supply of FGFs regulates stem cell density homeostasis via positive and negative feedback, not chemo-attraction (45). Such feedback control is possibly involved in the TSE for maintaining spermatogenesis after spatially fractionated irradiation, and it may also occur in other systems. Further investigations on stem cell migration/competition in various tissues and species are immediately expected, and the findings on such biological aspects will provide a special cue for enhancing the benefits of radiation treatment while preventing its adverse effects.

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