FLASH proton radiotherapy spares normal epithelial and mesenchymal tissues while preserving sarcoma response

Anastasia Velalopoulou¹, Ilias V. Karagounis¹, Gwendolyn M. Cramer¹, Michele M. Kim¹, Giorgos Skoufos²,³, Denisa Goia⁴, Sarah Hagan¹, Ioannis I. Verginadis¹, Khayrullo Shoniyozov¹, June Chiango⁴, Michelle Cerullo⁴, Kelley Varner⁴, Lutian Yao⁵, Ling Qin⁵, Artemis G. Hatzigeorgiou²,³,⁶, Andy J. Minn¹, Mary Putt⁷, Matthew Lanza⁸, Charles-Antoine Assenmacher⁸, Enrico Radaelli⁸, Jennifer Huck⁴, Eric Diffenderfer¹, Lei Dong¹, James Metz¹, Constantinos Koumenis¹, Keith A. Cengel¹*, Amit Maity¹*, Theresa M. Busch¹*

1. Department of Radiation Oncology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA
2. Department of Electrical & Computer Engineering, Univ. of Thessaly, Volos 38221, Greece
3. Hellenic Pasteur Institute, Athens 11521, Greece
4. Department of Clinical Studies and Advanced Medicine, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA
5. Department of Orthopedic Surgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA
6. DIANA-Lab, Department of Computer Science and Biomedical Informatics, Univ. of Thessaly, Lamia 351 31, Greece
7. Department of Biostatistics, Epidemiology & Informatics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA
8. Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

*These individuals share senior authorship

Running title
FLASH PRT spares normal tissue & preserves sarcoma response

Keywords
RADIATION ONCOLOGY/Normal tissue response to radiation, SARCOMAS/Soft-tissue sarcoma, RADIOTHERAPY/FLASH proton radiotherapy, normal tissue toxicity, sarcoma
Financial support. The Department of Radiation Oncology and the Translational Center of Excellence (TCE) of the Abramson Cancer Center at UPenn are acknowledged for financial support. Additional funding (for G.S.) was provided (Greece and European Social Fund-ESF) through the Operational Programme "Human Resources Development, Education and Lifelong Learning" in the context of the project "Strengthening Human Resources Research Potential via Doctorate Research" [MIS-5000432], implemented by the State Scholarships Foundation (IKY), in the form of a PhD Scholarship.

Corresponding author
Theresa M. Busch
Radiation Biology Division
Department of Radiation Oncology
University of Pennsylvania
Smilow Center for Translational Research, Room 8-126
3400 Civic Center Blvd., Bldg. 421
Philadelphia, PA 19104-5156
Office: 215-573-3168
Email: theresa.busch@pennmedicine.upenn.edu

Disclosure of Potential Conflicts of Interest. Lei Dong is a member of Varian Speaker’s Bureau. James M. Metz is a consultant for IBA and Varian. All other authors declare no potential conflicts of interest.
FLASH proton radiotherapy spares normal epithelial and mesenchymal tissues while preserving sarcoma response

ABSTRACT

In studies of electron and proton radiotherapy, ultrahigh dose rates of FLASH radiation therapy appear to produce fewer toxicities than standard dose rates while maintaining local tumor control. FLASH-proton radiotherapy (F-PRT) brings the spatial advantages of PRT to FLASH dose rates (>40 Gy/sec), making it important to understand if and how F-PRT spares normal tissues while providing anti-tumor efficacy that is equivalent to standard-proton radiotherapy (S-PRT). Here we studied PRT damage to skin and mesenchymal tissues of muscle and bone and found that F-PRT of the C57BL/6 murine hind leg produced fewer severe toxicities leading to death or requiring euthanasia than S-PRT of the same dose. RNAseq analyses of murine skin and bone revealed pathways upregulated by S-PRT yet unaltered by F-PRT, such as apoptosis signaling and keratinocyte differentiation in skin, as well as osteoclast differentiation and chondrocyte development in bone. Corroborating these findings, F-PRT reduced skin injury, stem cell depletion, and inflammation, mitigated late effects including lymphedema, and decreased histopathologically detected myofiber atrophy, bone resorption, hair follicle atrophy, and epidermal hyperplasia. F-PRT was equipotent to S-PRT in control of two murine sarcoma models, including at an orthotopic intramuscular site, thereby establishing its relevance to mesenchymal cancers. Finally, S-PRT produced greater increases in TGF-β1 in murine skin and the skin of canines enrolled in a phase 1 study of F-PRT versus S-PRT. Collectively, these data provide novel insights into F-PRT-mediated tissue sparing and support its ongoing investigation in applications that would benefit from this sparing of skin and mesenchymal tissues.

Significance

These findings will spur investigation of FLASH radiotherapy in sarcoma and additional cancers where mesenchymal tissues are at risk, including head and neck cancer, breast cancer, and pelvic malignancies.
INTRODUCTION

Radiotherapy (RT) can be curative as a cancer treatment, but is also associated with acute and late toxicities. Hence, there is need for approaches to limit radiation-induced toxicity. FLASH RT, in which radiation is delivered at ultrahigh dose rates (>40 Gy/sec), has been reported to produce fewer toxicities than standard RT without compromising local tumor control. Most FLASH studies have utilized electrons (1, 2), but proton FLASH RT provides deeper tissue penetration than electrons, as well as the favorable spatial characteristics of protons (much less penumbra than electrons and no exit dose in contrast to X-rays) (3, 4). We recently published that, compared to standard dose rate, proton FLASH RT reduces long-term intestinal injury (5). In the current report, we study the effects of FLASH proton RT on skin and the mesenchymal tissues of muscle and bone. Toxicities in these regions may be dose limiting in a variety of cancers.

High dose radiation, often in combination with surgery, is used to treat cancers of the head and neck, breast, pelvic region, and sarcomas, commonly producing toxicities to skin, bone and/or soft tissue (6). Skin toxicities acutely manifest as erythema, desquamation, and alopecia or skin atrophy, while fibrosis and ulcer formation develop as late effects (7). Other late effects of RT include osteoradionecrosis, fracture, impaired wound healing and muscle atrophy, as well as lymphedema (8-10). Importantly, sarcoma is the archetypal mesenchymal cancer in which high dose RT is often used despite the high risk of toxicity. In an NCI Canada (NCIC) randomized trial for patients with soft tissue sarcoma (STS) undergoing surgery with either pre-op or post-op radiation, > grade 2 fibrosis was seen in 49% of patients, edema in 19%, and joint stiffness in 20% (11). Thus, injury to mesenchymal normal tissues remains a significant contributor to morbidity following STS treatment, as well as post radiation for a variety of epithelial cancers.

FLASH RT reduces toxicities in animal models, including early demonstration in mouse lung that electron FLASH produced less fibrosis than the same dose delivered at standard (<1 Gy/s) dose rate (1). Sparing of other normal tissues by FLASH electron RT has also been demonstrated, including in brain (12, 13) and the gastrointestinal tract (2). The mechanisms for sparing by FLASH are incompletely elucidated; dose-rate dependent differences in hypoxia response/tissue microenvironment/ redox chemistry (12, 14-17), stem cell proliferation (5, 18) and inflammatory signaling (1, 18-20) have been implicated in early studies.
F-PRT provides equivalent control of epithelial-derived cancers in murine models compared with standard proton RT (S-PRT), including pancreatic adenocarcinomas and head and neck squamous cell carcinomas (5, 19), but its effect on mesenchymal cancers has not been determined. In the current studies, we demonstrate equivalent F-PRT control of murine sarcomas compared with S-PRT, accompanied by less damage to relevant normal tissues including skin, lymphatics, bone and muscle. Studies are informed by RNAseq analyses that identify differential activation of specific gene pathways by F-PRT versus S-PRT. Lastly, work is extended into tissues from an ongoing canine trial of F-PRT at our institution. Our studies clearly show reduction in mesenchymal tissue injury following F-PRT and indicate differential activation of specific gene pathways that generate mechanistic hypotheses for these effects.

MATERIALS AND METHODS

Mice

Eight to 10-week-old female C57BL/6 and C3H/HeJ mice (The Jackson Laboratory) were maintained in AALAC-accredited facilities and all procedures approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania (Philadelphia, PA). To propagate fibrosarcoma syngeneic tumors, 5x10^5 cells from a sarcoma line established from the LSL-Kras^{G12D/Wt; p53^{FL/FL}} GEMM model (C57Bl/6 background) at the 5th passage or 3x10^5 cells of the established RIF mouse sarcoma cell line (C3H/HeJ background, RRID:IMSR_JAX:000659) at the 9th passage were injected subcutaneously or intramuscularly in the right thigh. Cells from the LSL-Kras^{G12D/Wt; p53^{FL/FL}} GEMM model were recently isolated for the purpose of this research. The established RIF cell line has been maintained in the laboratory of Dr. Busch for more 20 years and originated from cells of the same line at Roswell Park Cancer Institute in Buffalo, NY. Both cell lines were authenticated and tested mycoplasma negative by IDEXX BioAnalytics (CellCheck 19 plus) in 2021. Tumors were irradiated upon reaching 100 mm^3 in volume. Tumor size was measured utilizing calipers and volume calculated as: (Length x Width^2)/2.

Proton irradiation
Irradiation with FLASH (69-124 Gy/sec) and standard (0.39–0.65 Gy/sec) dose rates was carried out using a proton beam with energy 230 MeV (range ~32 g/cm²), delivered via a horizontal beam line in a dedicated research room with an IBA Proteus Plus (Louvain-La-Neuve, Belgium) C230 Cyclotron. Mice were irradiated with the entrance (plateau) region of the beam with a field size of 2 x 2 cm (square collimator) per mouse for normal tissue studies and 1 cm diameter circular collimator for tumor studies. A double scattered system was used to create a uniform field at the target with additional details on the irradiation set-up and dosimetry in the Supplementary Materials and Methods. Total dose (without any weighting for relative biological effectiveness) is as described for each study.

RNA sequencing

Briefly, raw RNA-Seq datasets were quality checked and pre-processed, then alignment, gene-level quantification and differential expression analysis was conducted using STAR [STAR, RRID:SCR_004463] (21), RSEM (22) and EBSeq (23) respectively. This was followed by pathway enrichment analysis with details provided in the Supplementary Materials and Methods. RNA-Seq datasets can be obtained under accession number GSE173944 in the Gene Expression Omnibus (GEO) database at NCBI.

Skin reaction and lymphedema scoring

Skin reaction was recorded using a published system of 10 grades, ranging from 0.5 to 3.5 (24). Lymphedema was scored as a function of its associated swelling using a system of 4 grades ranging from 0-4. Each grade represented the additional thickness in mm of swelling for the irradiated versus the non-irradiated foot.

Bioluminescence imaging of inflammation

Bioluminescence imaging of myeloperoxidase (MPO) activity is based on the systemic administration of luminol, allowing the quantification of phagocyte-mediated inflammation in the context of acute dermatitis (25). As described elsewhere (26), mice were intraperitoneally (i.p) injected with luminol (300 mg/kg; luminol sodium salt, Sigma A465-5G) at indicated days post-radiation. Bioluminescent images were acquired on an IVIS Spectrum imager (Perkin Elmer, Waltham, MA) from anesthetized (isoflurane) mice at 10 minutes after
luminol injection when chemiluminescence signal reached maximum values, using a 5-minute exposure. Equally-sized regions of interest (ROIs) were drawn around the irradiated area of the right hind leg of each mouse and the average radiance (photons/sec/cm$^2$/sr) quantified using Living Image Software 4 7.3 (Perkin Elmer).

**Histopathology**

Histopathology of skin and bone tissue were performed followed standard protocol (described in Supplementary Materials and Methods) of the Comparative Pathology Core at the University of Pennsylvania School of Veterinary Medicine.

**Fluorescence microscopy and image analysis**

Fluorescence microscopy of 10µm skin sections was performed to assess apoptosis by the TUNEL assay and Lgr6 and TGF-β1 expression by immunofluorescence. Quantification of immunofluorescence is presented as integrated density normalized to total nuclei area. Details on the staining process and image analysis are available in Supplementary Materials and Methods.

**Canine sample collection**

A trial of F-PRT vs S-PRT for dogs with clinical/radiographic diagnosis of extremity osteosarcoma was conducted with the approval of the IACUC at the University of Pennsylvania and the Privately Owned Animal Protocol (POAP) committee and in collaboration with the Penn Veterinary Clinical Investigations Center (VCIC) at the University of Pennsylvania School of Veterinary Medicine. Dogs were randomly assigned to F-PRT vs S-PRT, delivered under general anesthesia to separate 2.6 cm diameter circular areas of tumor-containing bone and normal bone. Five days following therapy, standard of care whole limb amputation surgery was performed and samples of irradiated and unirradiated tissues were collected in 10% buffered formalin for immunohistochemical analysis, as well as snap frozen for protein analysis. Details are provided in the Supplementary Materials and Methods.
ELISA

Mouse and dog skin sections (~20 mg) were lysed in 1x RIPA containing protease and phosphatase inhibitors followed by homogenization and sonication. Samples (in duplicate) were quantified using the Pierce™ BCA Protein Assay (23225). Total TGF-β1 quantification was performed using the R&D Systems Mouse/Rat/Porcine/Canine TGF-β1 Quantikine ELISA Kit (MB100B). 40 µl of each sample was assayed in duplicate after activation (1 N HCl), neutralization (1.2 N NaOH/0.5 M HEPES), and dilution with 50 µl of calibrator diluent. TGF-β1 levels were normalized by protein content.

Chromogenic Immunohistochemistry

For immunohistochemistry, 5 µm thick paraffin sections were mounted on ProbeOn™ slides (Thermo Fisher Scientific). The immunostaining procedure was performed using a Leica BOND RXm automated platform combined with the Bond Polymer Refine Detection kit (Leica #DS9800). Staining and analysis of TGF–β1 was performed as described in the Supplementary Materials and Methods, on slides that were counterstained in hematoxylin.

Statistical analysis

Data are summarized using means, with standard deviation (SD) as error bars unless otherwise noted. Parametric (ANOVA/T-tests) or rank-based tests (Kruskal-Wallis/Rank-sum) were chosen based on graphical inspection of the data. Multiple comparisons used either a Holm-Sidak approach (T-tests) or a Dunn approach (rank-based tests) to achieve the family-wise Type I error rate of 0.05. When the control lacked variation in an outcome, we compared only the F-PRT and S-PRT groups. All data are plotted and were analyzed in GraphPad Prism (version 8) or R (Version 3.6.1) [GraphPad Prism, RRID:SCR_002798]; details appear in Supplementary Materials and Methods.

RESULTS

F-PRT reduces severe morbidity compared to S-PRT. First, the sparing effect of F-PRT vs S-PRT was comprehensively investigated through survival studies, conducted following RT delivery to the murine hind leg.
with its associated tissues of skin, muscle and bone. At 30 Gy exposure (Fig 1A), F-PRT provided significant protection from mortality or severe morbidity (mandating euthanasia) compared to S-PRT over the ~250 days after irradiation. Morbidities included severe skin damage and soft tissue reaction assessed by ULAR veterinarians blinded to study groups. Median survival was 211 days after 30 Gy of S-PRT compared to >249 days for F-PRT (p=0.0406). Increasing the RT dose to 45 Gy (Fig 1B) decreased S-PRT median survival to 188 days, while the median survival exceeded 256 days for F-PRT. Notwithstanding, 60% of F-PRT animals did not succumb to severe morbidity within 256 days of 45 Gy treatment, a value statistically indistinguishable (p=0.1905) from the 30% of animals that survived S-PRT at this dose. Collectively, these data show that F-PRT caused fewer severe morbidities leading to death or requiring euthanasia than S-PRT in normal tissues of the murine leg, with a dose of 30 Gy achieving statistical significance.

Transcriptome analysis of skin reveals pathways upregulated by S-PRT, yet unaltered by F-PRT. RNAseq allowed insight into biological determinants of the differential effect of F-PRT vs S-PRT on murine skin. Full-thickness skin was collected 5 days after 30 Gy of F-PRT or S-PRT to the right hind leg in order to study transcriptome-level responses that could contribute to the survival-limiting morbidity presented above. Therefore, we focused on this intermediate time point so as to capture gene expression changes that occurred early as a result of differences between the F-PRT and S-PRT modalities, as well as the potentially further effects driven by each modality. The top 10 pathways upregulated by S-PRT and unaltered by F-PRT were identified (Fig 1C, with the involved genes provided in Supplementary Table 1). Pathways uniquely upregulated by S-PRT in murine skin included several associated with apoptosis and apoptotic signaling. Moreover, strong upregulation of pathways associated with keratin signaling, such as keratinocyte differentiation and cornification were uniquely identified in S-PRT vs F-PRT-treated skin.

We focused on genes and pathways upregulated by S-PRT and unaltered by F-PRT because of an interest in the types and mechanisms of tissue damage introduced by S-PRT while correspondingly spared by FLASH. However, some pathways were upregulated by F-PRT and unchanged by S-PRT (Supplementary Fig 1A); for example, F-PRT, but not S-PRT, promoted upregulation of pathways related to tissue and vascular repair in the skin, including anatomical structure morphogenesis, blood vessel morphogenesis, and vascular
development. These data suggest that F-PRT sparing of skin may result from less radiation-induced damage together with stronger expression of mechanisms for tissue repair. Other transcriptome-level differential effects of F-PRT vs S-PRT include pathways uniquely downregulated by each PRT modality (Supplementary Fig1B,C).

**F-PRT spares skin from radiation-induced damage.** Skin damage was a major driver of PRT-induced severe morbidity necessitating euthanasia (see Fig 1A,B); moreover, RNAseq of skin identified S-PRT to significantly upregulate apoptotic pathways that were unchanged by F-PRT. We evaluated PRT-induced skin damage based on a published scoring system (24) that assesses symptoms including erythema, dry/moist desquamation and partial/complete skin breakdown with loss of limb function. Skin reaction to PRT developed acutely (within weeks of irradiation), and mice treated with 30 Gy S-PRT to the hind leg developed a maximum skin reaction that was significantly (p=0.0397) more severe than that those treated with F-PRT (Fig 2A,B). Notably, the only mouse with a skin damage score of 3.5, indicating complete epidermal breakdown, was in the S-PRT-treated group.

In further interrogation of skin response, sections of treated skin were evaluated by immunofluorescent staining for stem cell markers. At both dose rates, PRT decreased the population of cells positive for Lgr6⁺, a marker of skin stem cells (Fig 2C, Supplementary Fig 2), which is consistent with the damaging effect of radiation. However, S-PRT produced greater Lgr6⁺ cell depletion than F-PRT (p=0.0246). Thus, these data suggest a mechanism by which the benefit of F-PRT could be propagated, i.e. due to a comparatively higher number of stem cells available after F-PRT to promote epidermal regeneration in comparison to S-PRT.

Finally, the pathology of F-PRT sparing relative to S-PRT was histologically evaluated in skin sections. Histopathology showed F-PRT to reduce the extent of epidermal necrosis compared to S-PRT (p=0.0459; Fig 2D,E). The increased presence of unhealed skin lesions after S-PRT vs F-PRT is consistent with both RNAseq evidence of greater induction of apoptosis after S-PRT and the significantly greater depletion of skin stem cells by this dose rate. Interestingly, F-PRT also significantly reduced hair follicle atrophy compared to S-PRT at 27 days after PRT (p<0.0001; Fig 2F,G). This atrophy is consistent with the presence of apoptotic cells in the hair follicle at 5 days after irradiation (Supplementary Figure 3). Collectively, findings align with RNAseq-revealed
upregulation of apoptosis, keratinocyte differentiation and keratinization by S-PRT (see Fig 1C) because many of the detected keratins are genes associated with the hair follicle and thus would be involved in its regeneration after damage.

F-PRT ameliorates skin inflammation, even for high radiation dose. Inflammation is characteristic of radiation-induced damage and an important component of wound repair. It is associated with the activation of myeloid cells, such as neutrophils, accompanied by erythema and edema that can manifest in epidermal hyperplasia during the healing process (27). By in vivo imaging, F-PRT vs S-PRT activation of myeloid cells was studied through a luminol-based chemiluminescent assay for myeloperoxidase. In mice treated with 30 Gy to their hind limb, less myeloid cell activation occurred after F-PRT vs S-PRT by day 18 (p=0.039) and 27 (p=0.064) (Fig 3A,B). Thus, F-PRT mitigated inflammatory response to PRT. Moreover, histopathological analyses (Fig 3C,D) showed F-PRT significantly reduced epidermal hyperplasia relative to S-PRT (p=0.0024). These data suggest that F-PRT favorably alters the pathology of wound healing compared to S-PRT.

Next, to define dose dependencies of FLASH effect on skin, we considered a dose of 45 Gy. At this higher dose, F-PRT did not lessen the mean skin damage score as both S-PRT and F-PRT led to maximal (score 3.5) damage in several animals (Supplementary Figure 4). However, similar to that observed at 30 Gy, F-PRT at 45 Gy reduced the extent of myeloid cell activation (Fig 3E,F) and epidermal hyperplasia (Fig 3G) compared to S-PRT. Luminol chemiluminescence was significantly less at both day 18 (p=0.034) and day 27 (p=0.034) after 45 Gy of F-PRT compared to the same dose of S-PRT. In concert with FLASH mitigation of inflammation, Fig. 3G demonstrates significantly less epidermal hyperplasia (p=0.0124) after 45 Gy of F-PRT compared to S-PRT.

We further investigated if 45 Gy of F-PRT could alter the development of lymphedema as a long-term consequence of high dose S-PRT. At high dose, lymphedema, and its associated swelling, constitute a major RT-induced toxicity, one that is linked to chronic inflammation (28). Compared to S-PRT, F-PRT did not alter the incidence of lymphedema or its time course (Fig 4A). Lymphedema developed in ~40-50% of animals after both F-PRT and S-PRT, and the time of onset was similar. What distinguished F-PRT, however, was the reduced severity of the lymphedemic reaction (Fig 4B,C). Scored as a measure of foot thickness,
lymphedema was significantly more severe after S-PRT than F-PRT at times of peak response. Collectively, from the above data we glean that at high PRT dose, FLASH may not effectively mitigate acute cellular damage, manifested as skin ulceration; nevertheless, even at higher doses, F-PRT controls the development of inflammation and its consequences.

F-PRT reduces muscle and bone damage relative to S-PRT. We next considered F-PRT effect on mesenchymal tissues of muscle and bone. In histological sections of F-PRT vs S-PRT-treated (30 Gy) murine leg, F-PRT significantly (p=0.0089) decreased the extent of muscle atrophy compared to S-PRT (Fig 5A,B); S-PRT-treated tissue exhibited muscle fibers of smaller cross-sectional diameter that were accompanied by cytoplasmic vacuolation and internalization of nuclei. Similarly, when compared to F-PRT, bones treated with S-PRT showed more evidence of damage that included bone resorption and accompanying pockets of activated osteoclasts and osteoblasts (p=0.0065; Fig 5C,D). RNaseq was performed on bone from F-PRT vs S-PRT mice, revealing several pathways induced by S-PRT but unaltered by F-PRT, corroborating histopathological findings (Fig 5E, with the involved genes in Supplementary Table 2; also see Supplementary Fig 5A,B,C for alternative activation states). Pathways such as notochord development and joint development were upregulated by S-PRT, but not by F-PRT. Moreover, pathways related to bone remodeling such as osteoclast differentiation, endochondral bone morphogenesis, and chondrocyte development, were all upregulated by S-PRT, but unaffected by F-PRT. Thus, histological evidence of reduced (ameliorated) bone damage after F-PRT, compared to S-PRT-associated damage alongside evidence of bone remodeling at histological and genetic levels, suggests S-PRT imparted more damage on bone than F-PRT.

F-PRT is equipotent to S-PRT in controlling tumor growth. To be clinically impactful in oncology, F-PRT must not only spare normal tissues, but also provide anti-tumor efficacy equivalent to standard RT regimens. F-PRT sparing of mesenchymal tissues could be significant to numerous tumor types and anatomical sites, including soft tissue sarcomas. We evaluated the anti-tumor efficacy of F-PRT compared to S-PRT in two murine models of sarcoma. A sarcoma cell line established from GEMM model LSL-Kras\textsuperscript{G12D/wt, p53\textsuperscript{FL/FL}} (KP) was propagated subcutaneously in the thigh of C57BL/6 mice, and resulting tumors were treated with F-PRT or S-PRT. Tumor
response was indistinguishable between the dose rates, both in terms of the regrowth delay (Fig 6A) and the probability of tumor control (Fig 6B). Further evidence of the equivalent anti-tumor efficacy of F-PRT and S-PRT was found for fibrosarcoma (RIF) tumors propagated in C3H mice. The anti-tumor efficacy of F-PRT and S-PRT were indistinguishable in both subcutaneous (Fig 6C) and intramuscular orthotopic RIF tumors (Fig 6D), which importantly demonstrates F-PRT to be equivalent to S-PRT in providing tumor control over a range of conditions that could differ between these models such as their levels of oxygenation (29-31).

F-PRT induces less TGF-β1 compared to S-PRT in both murine and canine skin. Lastly, we evaluated TGF-β1, which is associated with dose rate-dependent RT effect on normal tissue and a key agent of RT-induced inflammatory reactions (1, 32). TGF-β1 expression was studied by immunofluorescence in sections of murine skin treated as controls or with F-PRT vs S-PRT (Fig 7A, Supplementary Fig 6A,B). Diffuse epidermal staining of TGF-β1 was noted at 18 days after skin exposure to S-PRT (30 Gy), aligning with inflammation at this timepoint (see Fig 3A,B). S-PRT significantly (p<0.0001) increased TGF-β1 relative to unirradiated tissue; in contrast, for F-PRT, increases were smaller and statistically indistinguishable from unirradiated tissue (p=0.3041) (Fig 7B). Overall, levels of TGF-β1 were higher after treatment with S-PRT than F-PRT (p<0.0001). ELISAs for TGF-β1 produced similar results (Fig 7C), with S-PRT elevating TGF-β1 compared to unirradiated controls (p=0.0023) whereas cytokine levels after F-PRT were similar to controls (p=0.3392).

Parallel to murine studies, we evaluated TGF-β1 in canine tissues collected as part of an ongoing canine clinical trial on the comparative safety of F-PRT vs S-PRT delivered to the extremities of patient animals with osteosarcoma. Patient characteristics among the dogs on this trial are described in Table 1. F-PRT or S-PRT at either 8 or 12 Gy was delivered to tumor and normal tissue of the canine leg, followed in 5 days by standard-of-care limb amputation and sample collection. Irradiated (either F-PRT or S-PRT) and unirradiated normal tissue was collected from each dog. The ratio of TGF-β1 (via ELISA) in irradiated versus unirradiated tissue of the same animal was determined. For the 8 Gy dose, TGF-β1 levels in the irradiated tissue generally differed by no more than 32% compared to the unirradiated tissue; the exception was one subject in the S-PRT group that had over a two-fold elevation. For the 12 Gy dose, differences in TGF-β1 expression were generally more
pronounced, and statistically significant for the S-PRT (p=0.0494) animals but not for the F-PRT group (p=0.1293) (Fig 7D). At the 12 Gy dose, we further evaluated TGF-β1 by immunohistochemical staining in the canine skin (Fig 7E,F, Supplementary Fig 7A,B). S-PRT resulted in strong increases in TGF-β1 that were especially noticeable in the hyperplastic epidermis. Staining levels in the irradiated skin of each dog were compared to unirradiated skin from the same animal and showed that S-PRT (p=0.047), but not F-PRT (p=0.378), elevated TGF-β1 protein.

DISCUSSION

In these studies, we have focused on the effects of F-PRT on both epithelial and mesenchymal tissues –specifically soft tissues, bone, and muscle, and find that, compared to S-PRT, F-PRT can reduce skin damage and stem cell depletion; inflammation; late histopathologic changes (myofiber atrophy, bone resorption, hair follicle atrophy, epidermal hyperplasia) and lymphedema. Moreover, we found F-PRT has similar anti-tumor efficacy against sarcomas using two different models – tumors grown from sarcoma cells isolated from GEMM (LSL-KrasG12D/wt; p53FL/FL) mice and tumors grown from the established RIF murine sarcoma line. This is consistent with what we (5) and others have reported using other tumor models (1, 2, 5, 33). Given these findings, our studies offer strong pre-clinical rationale to evaluate FLASH-PRT in clinical situations where lymphedema and the integrity and/or development of the bone and muscle may be important issues, for example sarcomas, breast cancer, head and neck cancer, and pediatric malignancies. Although we studied single fractions in mice with doses up to 30-45 Gy, we do not think these specific doses would be used in patients. More likely, FLASH would be used in hypofractionated regimens, perhaps in as few as 3-5 fractions, such as those increasingly common for many malignancies including lung and prostate cancers (34). It was beyond the scope of the current study to test these hypofractionated regimens, but this is something that we plan to do in the future, both in our mouse models and in a canine trial.

Our studies are novel because we (i) incorporate the investigation of injuries to mesenchymal normal tissues that have been poorly studied to date, including bone; (ii) uniquely apply RNAseq technology to elucidate differences between proton FLASH and standard RT at the level of the transcriptome; (iii) include the
first ever molecular findings of FLASH effect in canines; and (iv) demonstrate the therapeutic equivalency of
FLASH and standard proton therapy in sarcoma tumor models, which are previously uninvestigated yet highly
relevant as a disease requiring high dose radiotherapy. We focus on protons RTs (5) because we believe that
this modality has the greatest likelihood of being translated widely into human clinical trials in the near future,
with a feasibility study recently initiated by Cincinnati Children’s and University of Cincinnati Medical Center
(35). Current clinical electron energies cannot penetrate deeply enough to treat most common cancers.
Likewise, units that can deliver X-rays at FLASH dose rates to treat human cancer are likely years away. In
contrast, current-day proton machines can be engineered to treat dogs and even some tumors in human
patients at FLASH dose rates. In this study, we have primary used higher energy “plateau” or early SOBP
protons that have a relatively low LET. Further investigations are ongoing on the effect of LET on the FLASH
mediated tumor: normal tissue differential response and will be critical in determining whether the spatial
advantages of proton RT will be optimally exploited in F-PRT.

Literature on proton FLASH in vivo is very limited, with some conflicting results. One group showed no
significant differences between zebrafish given proton FLASH-RT (100 Gy/sec) vs standard proton RT in most
parameters including embryonic survival and spinal curvature (36). In contrast, we have shown and others
confirmed that abdominal radiation of mice with proton FLASH-RT leads to less toxicity than standard dose
rate (4, 5). Yet, it remains important to investigate proton FLASH in sites other than the abdomen.
Reassuringly, a very recent report shows FLASH protons to clinically alter skin damage and leg contracture in
mouse (19). We similarly note FLASH sparing of clinical skin damage in the current report, but in studies that
delve into its underlying cause we further provide the first RNAseq data on response to proton FLASH (vs S-
PRT). Our studies provide first of its kind data on FLASH sparing of bone, accompanied by corroborating
histopathology that informs skin and bone responses to F-PRT vs S-PRT. Although TGF-β1 has been
investigated in the context of FLASH (19) we uniquely report on its levels in the normal skin of canine patients
treated with F-PRT vs S-PRT in studies that importantly demonstrate clinical translation of FLASH molecular
endpoints.

Compared to S-PRT, F-PRT reduces severe morbidity that would require euthanasia (Fig. 1A,B). Limb
injury may involve many compartments, but skin damage was a major driver of morbidity in this setting. Skin
toxicity was manifested acutely by erythema and dry/moist desquamation and in severe cases, partial/complete skin breakdown with loss of limb function (Fig 2A,B); evidence of complete epidermal breakdown (score 3.5) was only seen in the S-PRT arm. F-PRT also reduced the extent of epidermal necrosis (Fig. 2D, E) and spared of hair follicles (Fig 2F,G). Sparing of skin toxicity with electron FLASH RT has been reported in mini-pigs (28-34 Gy) and in cat patients (25-41 Gy) (37), and during revisions of this manuscript the feasibility and safety of electron FLASH in canine patients has also been demonstrated (38). Electron FLASH results in a lower incidence and severity of skin ulceration in mice (39), and proton FLASH reduced skin toxicity and leg contracture (19).

We employed RNAseq and immunohistochemical analyses to assess underlying mechanisms for the differential effects of FLASH relative to standard dose rate. Pathways related to apoptosis were upregulated with S-PRT but not F-PRT at 5 days post-radiation (30 Gy) in murine skin. By immunohistochemistry, S-PRT led to greater Lgr6+ cell depletion than F-PRT (Fig. 2C, Supplementary Fig 2). Taken together, our RNAseq and immunohistochemistry data suggest that F-PRT may lead to less severe injury through reduced apoptosis and reduced depletion of skin stem cells, allowing for greater epidermal regeneration in comparison to S-PRT. RNAseq also showed an upregulation in keratin-related pathways/genes with S-PRT (Fig. 1C), which is consistent with the epidermal hyperplasia and hyperkeratosis after S-PRT vs F-PRT (Fig. 3C,D). Conversely, F-PRT, but not S-PRT, upregulated pathways related to tissue and vascular repair in the skin, including anatomical structural morphogenesis, blood vessel morphogenesis, and vascular development. Hence, this suggests that F-PRT sparing of skin is through both a reduction in radiation-induced damage and stronger expression of mechanisms for tissue repair.

In addition to studies of epithelial damage in the form of skin injury, we also examined injury to mesenchymal structures. By histopathology, F-PRT (30 Gy) led to less long-term muscle atrophy than F-PRT (Fig. 5A,B). F-PRT also produced less damage to the bone with less bone resorption and accompanying pockets of activated osteoclasts and osteoblasts, resulting in less bone remodeling (Fig. 5C,D). This is in keeping with our RNAseq data that showed S-PRT upregulation of pathways related to bone/chondrocyte development/morphogenesis (Fig. 5E). F-PRT also had a sparing effect on lymphedema; in mice that developed lymphedema after 45 Gy, the severity was much less with F-PRT than S-PRT (Fig. 4).
In all, the sparing effects of F-PRT included both acute and late toxicities that were induced by S-PRT. In the simplest terms, the acute phase of radiation toxicity involves cell death and inflammatory changes to normal tissues that may resolve, while late radiation effects involve chronic, unresolved inflammation, microenvironmental changes and fibrosis that lead to overall organ dysfunction. F-PRT sparing of both acute and late toxicities to skin, bone and soft tissues was mirrored by changes in inflammatory markers. Luminol chemiluminescence of myeloperoxidase activity showed that with 45 Gy there was less inflammation after F-PRT than S-PRT (Fig. 3E). Similar trends in reducing inflammation were seen with 30 Gy (Fig. 3A). Thus, even with a dose as high as 45 Gy, inflammation is reduced after FLASH, although it may lose its effect in mitigating more severe damage that would require euthanasia (Fig. 1B). This difference in inflammation can account for changes in edema and erythema between F-PRT and S-PRT, but the question remains as to whether it explains the sparing of other late toxicities. The relationship between acute and late toxicities following radiation is a complicated one, but TGF-β1 is thought to be a key mediator of both acute inflammation and late fibrosis. Indeed, previous investigations show less TGF-β1 secretion from cells or tissues following FLASH than standard RT (1, 20). Here, we observed that F-PRT induces less acute TGF-β1 secretion in irradiated murine and canine skin than S-PRT (Fig. 7). This could also explain the sparing effects of FLASH on lymphedema since in rodent models, RT has been shown to cause lymphatic vessel leakiness with apoptosis of lymphatic endothelial cells and subsequent fibrosis mediated by TGF-β1 (40, 41).

The sparing effects of F-PRT could perhaps involve stem cells. High dose RT may produce severe toxicity through destruction of stem cells that would survive lower doses and repopulate normal cells killed by radiation. This has been shown for satellite cells in muscle (42), keratin 15 (Krt15)+ basal cells in the esophageal epithelium (43), and Lgr5+ intestinal crypt stem cells (5). Groups including our own have shown that FLASH irradiation of the GI tract can spare proliferating cells in the crypt, in particular Lgr5+ stem cells (2, 5). Here, we find the Lgr6+ stem cell population in the skin to be spared by F-PRT compared to S-PRT. Reasons for cell sparing by FLASH are currently unknown, but it has been hypothesized that the generation of hypoxia during radiotherapy may play a role (17). Although it is a controversial area (44), mathematical modeling suggests that under initial conditions of intermediate O2 tension, FLASH-RT may deplete oxygen to levels that alter radiosensitivity (14, 45). Many stem cell compartments (mesenchymal, neural, hematopoietic)
exhibit pO$_2$ ranging from <1% - 8% in order to maintain their undifferentiated state (35), leading to the hypothesis that FLASH irradiation may spare these stem cell niches as a consequence of their hypoxia (46). In support of this idea, the hypoxia marker EF5 shows the epidermis to be modestly hypoxic and hair follicles moderately-to-severely hypoxic (47). Thus, the hair follicles could be more resistant to killing by FLASH-PRT as a result of a FLASH-mediated decrease in the pO$_2$ of their microenvironment. As hair follicles serve as one source of Lgr6$^+$ cells (48), Lgr6$^+$ cell sparing by FLASH also aligns with its sparing of hair follicles.

In addition to the above-studied mechanisms, our RNAseq analyses provide a wealth of novel data to continue to be explored for its revelations about FLASH mechanism. For example, RNAseq of bones reveals F-PRT, but not S-PRT, is associated with the upregulation of an integrated cluster of immune pathways. Among these, the activation of innate immune response, as well as response to type 1 interferon could potentially alter healing (49) and/or tumor response (50) to F-PRT compared to S-PRT and could serve to inform new avenues of investigation.

In summary, we demonstrate FLASH-proton RT to spare murine skin, muscle and bone compared to standard-proton RT. F-PRT minimally changes TGF-$\beta$1 levels in the skin of both mice and dogs while RNAseq data and immunohistochemical and pathological assays suggest proton FLASH to decrease radiation damage and favor mechanisms of tissue repair. The equipotent effect of FLASH- and standard-proton RT in controlling sarcoma growth are demonstrated here for the first time and, together with the above findings, support further investigation of FLASH toward clinical applications that would benefit from its sparing effects on skin and mesenchymal tissues.

**ACKNOWLEDGEMENTS.** We recognize services provided through the Small Animal Imaging Facility and the Cell and Animal Irradiation Core at the University of Pennsylvania. We thank Dazheng Zhang for his contribution to initial statistical analyses.
WORKS CITED


Table 1. Characteristics of canine patients and treatment parameters for FLASH or Standard proton radiotherapy

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Breed</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>Dose rate (Gy/s)</th>
<th>Dose (Gy)</th>
<th>FLASH or Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Black Lab</td>
<td>11.0</td>
<td>F</td>
<td>33.1</td>
<td>76.2</td>
<td>8.1</td>
<td>F</td>
</tr>
<tr>
<td>2</td>
<td>St Bernard</td>
<td>4.5</td>
<td>M</td>
<td>61.4</td>
<td>61.0</td>
<td>4.0</td>
<td>F</td>
</tr>
<tr>
<td>3</td>
<td>Mastiff</td>
<td>6.0</td>
<td>F</td>
<td>57.0</td>
<td>103.0</td>
<td>8.1</td>
<td>F</td>
</tr>
<tr>
<td>4</td>
<td>Mastiff</td>
<td>6.5</td>
<td>M</td>
<td>59.6</td>
<td>0.5</td>
<td>8.0</td>
<td>S</td>
</tr>
<tr>
<td>5</td>
<td>Rottweiler</td>
<td>8.0</td>
<td>M</td>
<td>55.2</td>
<td>111.5</td>
<td>8.0</td>
<td>F</td>
</tr>
<tr>
<td>6</td>
<td>Mixed Breed</td>
<td>8.0</td>
<td>M</td>
<td>25.2</td>
<td>0.5</td>
<td>8.1</td>
<td>S</td>
</tr>
<tr>
<td>7</td>
<td>Doberman/ Great Dane Mix</td>
<td>5.5</td>
<td>F</td>
<td>45.9</td>
<td>0.5</td>
<td>8.1</td>
<td>S</td>
</tr>
<tr>
<td>8</td>
<td>Mixed Breed</td>
<td>7.0</td>
<td>M</td>
<td>29.8</td>
<td>111.5</td>
<td>7.6</td>
<td>F</td>
</tr>
<tr>
<td>9</td>
<td>Golden Retriever</td>
<td>9.0</td>
<td>M</td>
<td>40.2</td>
<td>114.8</td>
<td>8.0</td>
<td>F</td>
</tr>
<tr>
<td>10</td>
<td>Shepherd Mix</td>
<td>9.0</td>
<td>M</td>
<td>35.7</td>
<td>0.5</td>
<td>8.0</td>
<td>S</td>
</tr>
<tr>
<td>11</td>
<td>Great Pyrenees</td>
<td>9.0</td>
<td>M</td>
<td>42.9</td>
<td>101.9</td>
<td>12.1</td>
<td>F</td>
</tr>
<tr>
<td>12</td>
<td>American Bulldog</td>
<td>10.0</td>
<td>M</td>
<td>45.4</td>
<td>0.5</td>
<td>12.0</td>
<td>S</td>
</tr>
<tr>
<td>13</td>
<td>Mixed Breed</td>
<td>8.0</td>
<td>F</td>
<td>36.0</td>
<td>0.1</td>
<td>12.0</td>
<td>S</td>
</tr>
<tr>
<td>14</td>
<td>Doberman</td>
<td>8.0</td>
<td>M</td>
<td>36.5</td>
<td>95.5</td>
<td>12.0</td>
<td>F</td>
</tr>
<tr>
<td>15</td>
<td>Labradoodle Mix</td>
<td>10.0</td>
<td>M</td>
<td>42.5</td>
<td>122.3</td>
<td>11.9</td>
<td>F</td>
</tr>
<tr>
<td>16</td>
<td>Great Dane</td>
<td>5.0</td>
<td>F</td>
<td>71.0</td>
<td>0.5</td>
<td>12.0</td>
<td>S</td>
</tr>
<tr>
<td>17</td>
<td>Rottweiler</td>
<td>6.0</td>
<td>F</td>
<td>34.4</td>
<td>0.5</td>
<td>11.9</td>
<td>S</td>
</tr>
<tr>
<td>18</td>
<td>Rottweiler/ Husky</td>
<td>10.0</td>
<td>M</td>
<td>46.3</td>
<td>128.2</td>
<td>12.0</td>
<td>F</td>
</tr>
<tr>
<td>19</td>
<td>Greyhound</td>
<td>1.5</td>
<td>M</td>
<td>28.0</td>
<td>0.6</td>
<td>11.9</td>
<td>S</td>
</tr>
</tbody>
</table>

1. ELISA not run on these samples; 2. irradiated with SOBP protons; 3. irradiated with photons. See methods for complete details.
Figure legends

**Figure 1.** F-PRT protects from morbidities and attenuates upregulation of pathways involved in keratinization and apoptosis, compared to S-PRT. Kaplan-Meier plots of survival following (A) 30 Gy and (B) 45 Gy of proton radiation, delivered to the mouse hind leg; n=10, statistical analysis by Log-rank test. Events record mortality or mandated euthanasia due to morbidity. (C) Gene Ontology (GO) enrichment analysis of the differentially expressed genes (upregulated) in the skin of S-PRT-treated mice compared to F-PRT-treated mice; n=4 per group.

**Figure 2.** F-PRT alleviates skin damage compared to S-PRT. (A) Skin reaction of the irradiated leg after exposure to 30 Gy of F-PRT or S-PRT. Maximum skin score for each mouse is presented over an observation period of 8 months. NR, n=5; F-PRT and S-PRT, n=10; statistical analysis by Welch’s t-test. (B) Pictures of irradiated skin damage scored as 1.5 or 3.5 compared to untreated skin (score 0); score of 1.5 indicates localized breakdown of the skin with a scaly/crusty appearance, while 3.5 corresponds to full-thickness skin barrier loss. (C) Quantification of immunofluorescent detection of skin populations of Lgr6+ stem cells at 18 days following 30 Gy of F-PRT vs S-PRT. Lgr6+ cells were quantified on 3-4 skin sections per mouse (n=5 mice, statistical analysis by Kruskal-Wallis test followed by Wilcoxon Rank Sum test.) (D) Blinded evaluation of epidermal necrosis in skin H&E-stained slides, at 27 days after 30 Gy of proton RT. NR, n=3; F-PRT and S-PRT, n=5; statistical analysis by Welch’s t-test. (E) Representative H&E images of mild and severe ulceration. On the mild ulcer, black arrow points to ulcerated epidermis and an asterisk indicates a crust. On the severe ulcer, necrosis and inflammation are shown in the epidermis (black arrow), which extends through the dermis (red arrow) and subcutis (yellow arrow). 100x magnification. Scale bar: 300 µm. (F) Blinded evaluation of hair follicle atrophy in skin H&E-stained slides at 27 days after 30 Gy of PRT. NR, n=3; S-PRT and F-PRT, n=6. Statistical analysis by Welch’s t test. (G) Representative images of H&E-stained non-irradiated normal hair follicles and hair follicle atrophy of irradiated skin. Upper: normal (non-irradiated) skin with normal hair follicle units (black arrows); middle: irradiated skin with hair follicle units (black arrows) that are reduced in number and size; lower: Hair follicles are completely absent. H&E, 200x magnification. Scale bar: 200 µm. For (A), n=3; F-PRT and S-PRT, n=6; statistical analysis by Welch’s t test. Values in (A), (C), (D) and (F) represent mean ± SD.

**Figure 3.** F-PRT reduces inflammation in the irradiated leg compared to S-PRT. For 30 Gy of proton irradiation: (A) quantification of luminol chemiluminescence as a reporter of inflammation, (B) accompanied by representative images of inflammatory signal at Day 18 [NR, n=5; F-PRT and S-PRT, n=10; statistical analysis by mixed-effects model]; (C) blinded histopathological evaluation of hyperplasia at 27 days post PRT [NR, n=3; F-PRT and S-PRT, n=6; statistical analysis by Welch’s t-test]; and (D) representative H&E-stained images of skin hyperplasia [Left: normal epidermal layer (black arrow); Center: moderate chronic damage including a
thickened hyperplastic epidermis (black arrow); right: severe chronic damage, including a severely thickened and hyperplastic epidermis (black arrow) with thick layers of orthokeratotic keratin (red arrow) and long rete pegs (yellow arrow); H&E, 200x magnification; scale bar: 200 µm. For 45 Gy of proton irradiation: (E) quantification of luminol chemiluminescence, (F) accompanied by representative images of inflammatory signal at Day 18 [NR, n=5; F-PRT and S-PRT, n=10; statistical analysis by mixed-effects model]; (G) blinded histopathological evaluation of hyperplasia at 27 days post PRT. N=10 per group with statistical analysis by Welch’s t-test.

**Figure 4.** F-PRT reduces the severity of lymphedema. (A) Kaplan-Meier curve of lymphedema incidence in mice that received PRT to the hind leg (45 Gy). NR, n=5; F-PRT and S-PRT, n=10; statistical analysis by Log-rank test. (B) Pictures of lymphedema representing scores of 0 (swelling < 1mm) and 3 (swelling = 3 mm); swelling ≥3 mm mandates euthanasia of the mouse. (C) Time course of severity among mice that developed lymphedema. F-PRT, n=4; S-PRT, n=3; statistical analysis by multiple cross-sectional T-tests with false discovery rate (FDR) of 0.05, values represent mean ± SEM, *p<0.05.

**Figure 5.** Damage to the muscle and bone by proton RT is ameliorated by F-PRT as compared to S-PRT. (A) Blinded evaluation of myofiber atrophy of the gastrocnemius muscle of mice treated by 30 Gy of F-PRT vs S-PRT, at 27 days after RT. Statistical analysis by Welch’s t-test. (B) Representative images of H&E-stained non-irradiated normal myofibers and myofiber atrophy of irradiated tissue. Myofibers of the irradiated tissue show degenerative changes including decreased cross-sectional diameter (i.e., atrophy), vacuolation of the cytoplasm (black arrows), and internalization of nuclei (yellow arrows). H&E, 400x magnification. Scale bar: 60 µm. (C) Blinded evaluation of bone remodelling in mice treated by 30 Gy of F-PRT vs S-PRT, at 27 days after PRT. Statistical analysis by Welch’s t-test. (D) Representative images of H&E-stained bone sections. Left: normal (non-irradiated) bone with normal bone marrow; center: irradiated bone with resorption (black arrows) lined by activated osteoclasts and osteoblasts (yellow arrows); right: multiple large regions of bone resorption (black arrows) with numerous activated osteoclasts and osteoblasts (yellow arrows). H&E, 200x magnification. Scale bar: 200 µm. For (A) and (C), NR, n=3; F-PRT and S-PRT, n=6; statistical analysis by Welch’s t test. Values in (A) and (C) represent mean ± SD. (E) Gene Ontology (GO) enrichment analysis of the differentially expressed (upregulated) genes in the S-PRT-treated mouse leg bone; n=4 per group

**Figure 6.** F-PRT and S-PRT are equipotent in treatment of two models of murine sarcoma. (A) Growth curve of murine sarcoma established from sarcoma cells isolated from GEMM model LSL-KrasG12D/wt;p53 FL/FL and subcutaneously (s.c.) propagated in C67BL/6 mice followed by irradiation with 12 Gy of F-PRT or S-PRT. N=9, statistical analysis by Log-rank test. (B) Tumor control probability following F-PRT vs S-PRT of the sarcoma-bearing mice with tumors reaching a volume of < 500mm³. N=13, statistical analysis by Log-rank test. (C) Growth curve of RIF murine fibrosarcomas subcutaneously propagated in C3H/HeJ mice irradiated with 30 Gy
of F-PRT or S-PRT. N= 8 mice per group; statistical analysis by Log-rank test. (D) Growth curve of RIF tumor propagated orthotopically in the leg muscle (i.m.) and irradiated with 30 Gy of F-PRT or S-PRT. N=5 mice per group; statistical analysis by Log-rank test. All tumors were irradiated on Day 10 after tumor cell inoculation (black lightning symbol). Data of (A), (C) and (D) plot mean ± SD.

Figure 7. Lower levels of TGF-β1 are induced in F-PRT-treated mouse and canine skin compared to S-PRT. (A) Immunofluorescent evaluation of TGF-β1 in mouse skin irradiated with F-PRT or S-PRT (30 Gy) (Scale bar, 100μm; Magnification, 10x). (B) Quantification of TGF-β1 signal in skin; n=5, with 6 sections per mouse; statistical analysis by Kruskal-Wallis test. (C) ELISA of TGF-β1 in the skin of NR (n=9), F-PRT (n=8) and S-PRT (n=8) treated mice at 18 days post PRT (30 Gy); statistical analysis by Kruskal-Wallis test. (D) ELISA of TGF-β1 in the skin of canine patients at 5 days post PRT; values normalized to NR samples from the same animal; 8 Gy, n=5 (F-PRT) and n=4 (S-PRT); 12 Gy, n=3 (F-PRT) and n=4 (S-PRT). Statistical analysis by paired T-test (NR vs PRT by dog) on the log-transformed data. 8 Gy S-PRT, P=0.4364; 8 Gy F-PRT, P=0.8241; 12 Gy F-PRT, P=0.1293. (E) Immunohistochemical evaluation of TGF-β1 in canine skin irradiated with F-PRT or S-PRT (12 Gy) compared to the matched unirradiated area (Scale bar, 200μm; Magnification, 10x). (F) Quantification of TGFβ1 signal in canine skin; F-PRT, n=3; S-PRT, n=4, with 6 sections per dog; statistical analysis by mixed effects models. Values represent mean ± SD.
Figure 1

A. 30 Gy

B. 45 Gy

C. Top 10 most significant upregulated pathways unique in the Standard irradiated mouse skin samples

Regulation of cysteine type endopeptidase activity
Positive-regulation of cysteine type endopeptidase activity
Intrinsic apoptotic signaling pathway in response to ER stress
Apoptotic signaling pathway
Intrinsic apoptotic signaling pathway
Keratinocyte differentiation
Epidermis development
Cornification
Keratinization
Smoothed signaling pathway
Figure 2

A. 30 Gy

Skin Reaction Score

P = 0.0397

NR  S-PRT  F-PRT

B.

Skin score: 0  Skin score: 1.5  Skin score: 3.5

C. 30 Gy

Mouse LGR6 Integrated Density

P = 0.0013

P < 0.0001

P = 0.0246

NR  S-PRT  F-PRT

D. 30 Gy

Epidermal Necrosis Score

P = 0.0459

NR  S-PRT  F-PRT

E.

Score: 2

Mild ulcer

Score: 4

Severe ulcer

F. 30 Gy

Hair follicle atrophy Score

P < 0.0001

NR  S-PRT  F-PRT

G.

Score: 0

Normal hair follicle

Score: 3

Moderate hair follicle atrophy

Score: 4

Severe hair follicle atrophy
Figure 3

A. 30 Gy

B. NR S-PRT F-PRT

Day 18

C. 30 Gy

D. Normal epidermis Moderate hyperplasia Severe hyperplasia with hyperkeratosis

E. 45 Gy

F. NR S-PRT F-PRT

Day 18

G. 45 Gy

Hyperplasia Score

Downloaded from cancerres.aacrjournals.org on November 22, 2021. © 2021 American Association for Cancer Research.
Figure 5

A. 30 Gy

Myofiber Atrophy Score

NR S-PRT F-PRT

P=0.0089

B. Normal myofibers Myofiber atrophy

Score: 0 Score: 4

C. 30 Gy

Bone Remodeling Score

NR S-PRT F-PRT

P=0.0065

D. Normal bone Mild bone remodelling Severe bone remodelling

Score: 0 Score: 2 Score: 4

E. Top 10 most significant upregulated pathways unique in the Standard irradiated mouse bone samples

- Cellular response to acid chemical
- Response to acid chemical
- Multinuclear osteoclast differentiation
- Notochord development
- Intrinsic apoptotic signaling pathway in response to ER stress
- Chondrocyte development
- Cartilage development involved in endochondral bone morphogenesis
- Negative regulation of cellular component organization
- Endodermal cell differentiation
- Embryonic skeletal joint development
# FLASH proton radiotherapy spares normal epithelial and mesenchymal tissues while preserving sarcoma response

Anastasia Velalopoulou, Ilias V. Karagounis, Gwendolyn M. Cramer, et al.

*Cancer Res* Published OnlineFirst July 28, 2021.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-21-1500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2021/07/29/0008-5472.CAN-21-1500.DC1">http://cancerres.aacrjournals.org/content/suppl/2021/07/29/0008-5472.CAN-21-1500.DC1</a></td>
</tr>
<tr>
<td>Author Manuscript</td>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
</tr>
</tbody>
</table>

**E-mail alerts**  
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/early/2021/07/28/0008-5472.CAN-21-1500. Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.