Amelioration of Mucositis in Proton Therapy of Fanconi Anemia Fanca⁻/⁻ Mice by JP4-039

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Abstract. *Background/Aim: We tested JP4-039, a GS-nitroxide radiation damage mitigator in proton therapy of Fanconi anemia (FA) mice. Materials and Methods: Fanca⁻/⁻ and Fanca⁺/⁺ bone marrow stromal cells were pre-treated with JP4-039 and irradiated with either protons or photons (0-10 GyRBE) followed by clonogenic survival and β-Galactosidase senescence analysis. Fanca⁻/⁻ and Fanca⁺/⁺ mice were pretreated with JP4-039 for 10 min prior to oropharyngeal irradiation with either protons or photons (0 or 30 GyRBE) followed by sacrifice and measurement of oral cavity ulceration, distant hematopoietic suppression, and real-time polymerase chain reaction analysis. Results: JP4-039 reduced oral cavity ulceration in Fanca⁻/⁻ mice, transcripts Nfkb, Ap1, Sp1, and Nrf2, and proton therapy induced distant marrow suppression. Conclusion: JP4-039 protected Fanca⁻/⁻ and Fanca⁺/⁺ cells and mouse oral cavity from both proton and photon radiation.*

Fanconi anemia (FA) is an inherited disorder that is associated with anemia, congenital abnormalities and radiosensitivity. FA patients have an increased incidence of malignancy, in particular oral cavity squamous cell carcinomas. While the incidence of FA is low, an estimated 30 patients in the USA each year (1), survival of these patients has been prolonged due to advances in hematopoietic cell transplantation. Consequently, more FA

patients are developing malignancies, particularly squamous cell carcinomas of the head, neck, esophagus, and urogenital regions (2). Some FA patients are sensitive to radiation therapy due to a mutation in one or more of the 23 genes in the FA pathway (3). Radiation protective agents and normal tissue sparing proton therapy may help treat these patients safely. We tested the combination of JP4-039, a GS-nitroxide radiation damage mitigator, and proton therapy in a mouse model of FA.

Prior studies have demonstrated that intraoral administration of mitochondrial localized transgene protein manganese superoxide dismutase (SOD2) prior to daily fractionated RT decreased both acute and late oropharyngeal toxicity without affecting tumor control (4-6). The 4-Amino-Tempo (4-AT) demonstrated radioprotection; however, was limited by the need for high systemic doses (7, 8). A Gramicidin S (GS)-derived peptide isostere conjugated to 4-Amino-Tempo (4-AT; JP4-039) targets the nitroxide to the mitochondria, and, in several FA mouse models, allows for safe radioprotection (9-12). Mice exposed to a dose that is lethal to 50% of mice within 30 days (LD50/30) of total body irradiation (TBI) were protected by intraperitoneal (IP) injection of JP4-039 after 24 h of exposure (13, 14). In addition, in the radiosensitive Fancd2⁻/⁻, Fanca⁻/⁻ and Fancg⁻/⁻ mice, pre-treated with oral JP4-039, there was decreased oral cavity mucositis following single fraction or fractionated therapy (10, 14).

Proton therapy has been touted as a major therapeutic approach for a number of clinical scenarios, including: dose escalation, re-irradiation, and pediatric oncology. As protons traverse tissue, they slow-down and deliver the majority of their energy when they come to rest, a phenomenon referred to as the Bragg peak. Pencil beam scanning (PBS) is an advanced delivery method in which individual "pencil" beams, of varied energy, are used to "paint" dose on the desired target, layer by layer, allowing for complex treatment

volumes, and improved integral dose and novel approaches such as intensity modulated proton therapy (IMPT) to further improve treatment delivery while minimizing normal tissue exposure (15, 16).

In the present study, we sought to confirm the radioprotective effect of JP4-039 in FA mice, both *in vitro* and *in vivo*, using PBS proton therapy. Given the significant physical differences between protons and photons, we compared proton and photon therapy with respect to biological markers, including: distant bone marrow suppression, radiation induction of cell senescence and alterations in the level of RNA transcription for stress response and inflammation-related markers. To our knowledge, this is the first study to compare biological differences between photon and protons in FA *in vitro* and *in vivo* models.

Materials and Methods

Animal care. Fanca$^{-/-}$ mice (129/Sv) were generously provided by Dr. Markus Grompe (Oregon Health Sciences Center, Portland, OR, USA) and maintained four per cage according to Institutional Animal Use and Care Committee (IACUC) protocols and fed with a standard laboratory chow diet with deionized water. Briefly, Fanca$^{-/-}$ were generated by mutagenesis of the Fanca gene *via* replacement of exons 4-7 by the LacZ-Neo fusion marker in E14 ES cells, derived from the 129Ola strain, resulting in embryonic stem cell clones. Heterozygotes were inbred to generate homozygous mutant Fanca$^{-/-}$ offspring, as previously described in detail (17).

Bone marrow cultures. Bone marrow stromal cells (BMSC) were collected from the femora and tibiae of Fanca$^{-/-}$ and control Fanca$^{+/+}$ mice and cultured in T25 cm^2 plastic flasks (Corning® Inc., Corning, NY, USA) with McCoy's 5A media (Gibco, Gaithersburg, MD, USA) supplemented with 25% fetal calf serum (Cambrex, Rockland, ME, USA), 10^{-5} M hydrocortisone hemisuccinate, and 1% penicillin-streptomycin (Gibco). Cultures were maintained at 33°C in 7% CO_2, as described previously (8).

Clonogenic radiation survival curves. BMSCs derived from Fanca$^{-/-}$ and control Fanca$^{+/+}$ mice were maintained in complete media in T25 flasks and were stacked and irradiated to doses of 0-10 GyRBE *via* pencil beam scanning on an IBA Proteus® One proton therapy system (Ion Beam Applications SA, Louvain-La-Neuve, Belgium). Subgroups of cultures were pre-treated with 100 μM JP4-039 for 12 h prior to irradiation. Parallel cultures were also irradiated using a JL Shepherd Mark I Model 68A 137CS γ Irradiator (San Fernando, CA, USA) at 340 cGy per min along with subgroups treated with JP4-039 in a similar fashion.

The single-hit/multi-target model was used, and results are presented as the mean of at least three experiments, as previously described (18). The dose required to reduce the surviving fraction to 37% is defined as D0. The back extrapolation to the y-axis from the linear portion of the survival curve is defined as ñ. The survival curve is a log-to-linear plot with surviving fraction along the y axis (log) and radiation dose (GyRBE) along the x axis.

β-Galactosidase senescence assay. BMSCs from Fanca$^{-/-}$ and control Fanca$^{+/+}$ mice were irradiated with a dose of 10 GyRBE in T25 flasks using proton or photon radiation, respectively. Cells were plated in T25 flasks and evaluated after 5 days. β-galactosidase staining was completed as described in prior publications (18).

In vivo radiation. For proton therapy experiments, 8-10-week-old Fanca$^{-/-}$ and control Fanca$^{+/+}$ mice were packaged by the Division of Laboratory Animal Resources (DLAR) at UPMC and shipped from Pittsburgh, PA to Royal Oak, MI, USA *via* courier. Once arrived, the mice were given time to acclimate prior to irradiation. Mice were given 100 μl water alone or an additional 100 μl of JP4-039 (20 mg/kg), 4-AT/Miglyol (20 mg/kg), or Miglyol by oral gavage 10 min prior to irradiation. The mice were immobilized in modified 50 ml conical tubes. Once immobilized, the cast and mice were scanned in a Philips Big Bore helical CT scanner for proton planning. RayStation version 6 (RaySearch AB, Stockholm, Sweden) Treatment Planning System (TPS) was used. The wax material relative proton stopping power was used in the TPS directly. A uniform spread-out Bragg peak (SOBP) of 30 GyRBE was prescribed to the head and neck region to ensure homogenous dose coverage while sparing the remainder of the treated mice. Following RT, the mice were repackaged and shipped back to Pittsburgh. For photon therapy experiments, mice were treated with a dose of 30 GyRBE, as described previously (12).

Histopathologic assessment of oral cavity tissues. Mice treated with large doses of radiation become increasingly malnourished, due to radiation-induced oral mucositis, with associated mortality occurring at approximately 6 days post-irradiation. Moreover, the IACUC recommend that mice be euthanized if there is a >20% loss of body weight. Therefore, mice were sacrificed 5 days following irradiation.

Following sacrifice, the tongue was dissected and removed from the oropharynx and fixed in 2% PFA for 2 h and then stored in 30% sucrose. Next, blocks were sectioned in 5 μm slices and stained with hematoxylin and eosin (H&E). Slides were then scored, in a blinded fashion by two observers, for percentage ulceration, as described in prior publications (6, 12).

Abscopal hematopoietic colony-forming assays. Mice were sacrificed 5 days following head and neck irradiation and single cell suspensions of fresh bone marrow were obtained from non-irradiated tibiae and femora and plated in triplicate in 0.8% methylcellulose containing Iscove's medium supplemented with hematopoietic growth factors: recombinant murine stem cell factor, IL-3, IL-6, erythropoietin (Stemcell Technologies, Vancouver, Canada). The cells were assayed for multilineage colony-forming unit-granulocyte macrophage (CFU-GM) and burst-forming unit erythroid (BFU-e) at day 13. Colonies containing ≥50 cells were scored.

Real-time polymerase chain reaction (RT-PCR) analysis of RNA transcripts. Mice were sacrificed 5 days following head and neck irradiation and tongue tissues from nonirradiated controls, radiation alone and from pretreatment JP4-039, 4-AT, and Miglyol were prepared for RT-PCR. Tissue from each mouse was prepared as previously described (12). RT-PCR analysis was performed to measure radiation-inducible transcripts of TGF-β, p53, p21, Rad51, SP-1, Sod1, Gadd45, Nfkb, IL-1α, AP-1, and Nrf2. The results were standardized with respect to glyceraldehyde phosphate dehydrogenase (GAPDH). Moreover, the results are presented as fold increase in gene transcript expression above the baseline level, which was defined as that of nonirradiated wild-type tongue tissue.
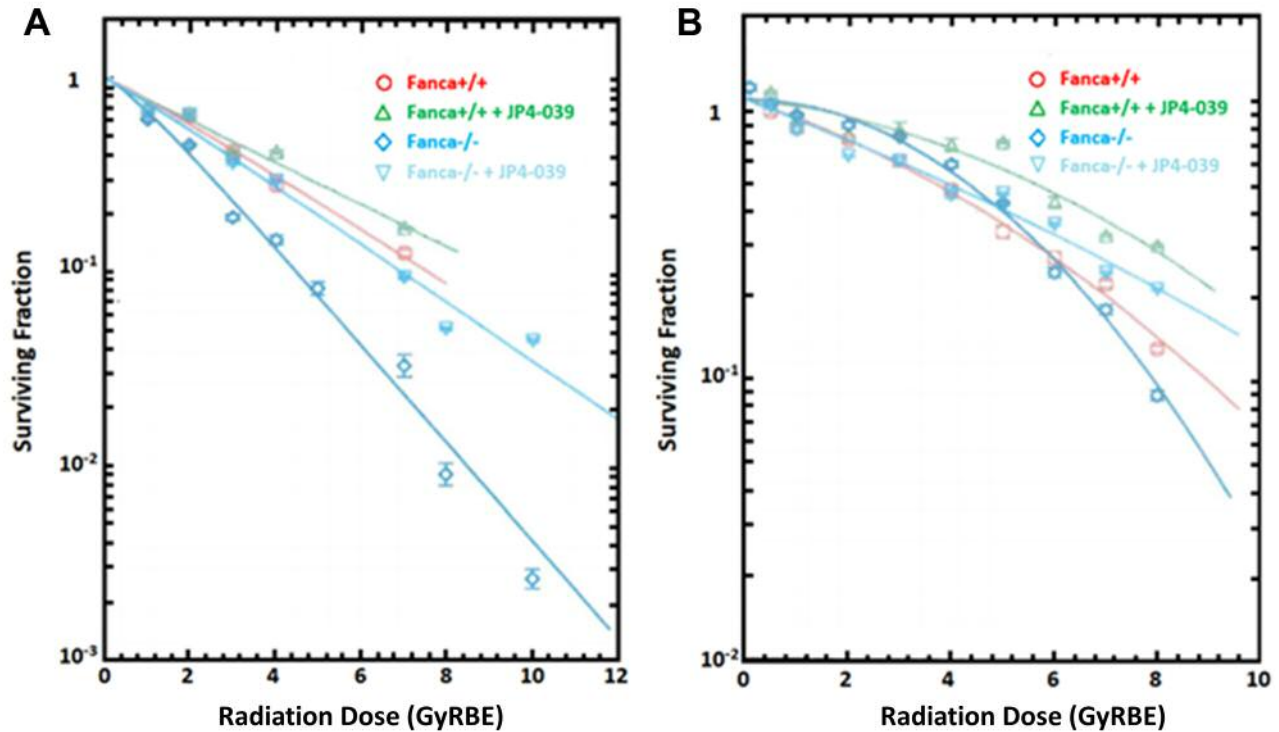
Figure 1. *Clonogenic survival curves for Fanca⁻/⁻ and Fanca⁺/⁺ BMSCs treated with either proton therapy (A) or photon therapy (B) in the presence or absence of JP4-039.*

Results

In vitro clonogenic radiation sensitivity assay. Clonogenic survival curves revealed that Fanca$^{-/-}$ bone marrow derived stromal cells were more radiosensitive to both proton (Figure 1A) and photon (Figure 1B) radiotherapy compared to wild-type Fanca$^{+/+}$ cells. The most pronounced difference observed was the dose required to reduce clonogenic survival by 37% (D0). Specifically, Fanca$^{-/-}$ cells treated with proton therapy had a D0=2.11±0.17 GyRBE; while Fanca$^{+/+}$ cells had a D0=3.28±0.10 GyRBE (p=0.0027). The shoulder (ñ) was significantly narrower in Fanca$^{-/-}$ cells treated with photon radiation compared to that in Fanca$^{+/+}$ cells (2.0±0.5 *vs.* 5.7±1.3, p=0.029).

Pre-treatment of bone marrow derived stromal cells with 10 μM JP4-039 for 12 h prior to irradiation provided significant radiation protection for Fanca$^{-/-}$ and Fanca$^{+/+}$ cell lines against both proton and photon radiation. For instance, Fanca$^{-/-}$ cells that were pre-treated with JP4-039 and then underwent proton radiation had a D0=3.01±0.19 GyRBE compared to D0=2.11±0.17 GyRBE for Fanca$^{-/-}$ cells treated with proton therapy alone (p=0.0125). The radioprotective effect of JP4-039 was observed in Fanca$^{-/-}$ cells treated with photon therapy as well.

In vitro β-galactosidase senescence assay. The percent of clonogenic cells stained for β-galactosidase was significantly increased following 10 GyRBE proton or photon radiation, a finding that was statistically significant compared to no radiation therapy among both Fanca$^{+/+}$ and Fanca$^{-/-}$ cells (Figure 2A). Interestingly, Fanca$^{-/-}$ cells that were treated with proton therapy were found to have statistically significant lower proportion of β-galactosidase positive clonogens compared to Fanca$^{+/+}$ cells treated at the same radiation dose (10% *vs.* 28%, p<0.05). Conversely, Fanca$^{-/-}$ cells that were treated with photon therapy had a statistically significantly higher proportion of β-galactosidase positive clonogens compared to Fanca$^{+/+}$ cells treated at the same radiation dose (17% *vs.* 7%, p<0.05).

We next tested Fanca$^{+/+}$ and Fanca$^{-/-}$ BMSCs (Figure 2B) for senescence after different doses of proton radiation and in the presence or absence of the radiation mitigator, JP4-039. The addition of JP4-039 did not affect the percent of cells stained positive for β-galactosidase at a dose level of 0, 5 or 10 GyRBE in either genotype. Interestingly, there was a statistically significant difference between Fanca$^{-/-}$ mice treated to 10 GyRBE with JP4-039 and Fanca$^{+/+}$ mice treated at the same radiation dose and JP4-039 concentration (p<0.04).

Amelioration of radiation induced mucositis with JP4-039. Fanca$^{-/-}$ and Fanca$^{+/+}$ mice underwent oral cavity proton radiation therapy to 30 GyRBE and degree of mucositis was assessed 5 days later (Figure 3A). The percent oral mucositis was significantly elevated compared to no radiation in both the Fanca$^{+/+}$ (23.5% *vs.* 0.7%, $p<0.05$) and Fanca$^{-/-}$ mice (26.5% *vs.* 2.9%, $p<0.05$) (Figure 3B). However, the degree of oral mucositis among the Fanca$^{-/-}$ mice pre-treated with JP4-039 was lower compared to the mice treated with 30 GyRBE proton therapy alone (6.5% *vs.* 26.5%, $p<0.0001$). Notably, pre-treatment with the vehicle (Miglyol) or non-mitochondrial targeted 4-AT/Miglyol did not provide any significant reduction in oral mucositis.

Proton induced changes in oral mucosae transcripts. Proton irradiation of the oral cavity of Fanca$^{+/+}$ and Fanca$^{-/-}$ mice resulted in robust and statistically significant increase in Nfkb, Ap1, Sp1, and Nrf2 transcripts, relative to unirradiated oral cavity mucosa (Figure 4A-D). This pattern was recapitulated in both mice genotypes. In addition, pre-treatment with JP4-039 resulted in a statistically significant reduction in transcript levels compared to proton alone irradiated oral mucosa in Fanca$^{+/+}$ mice, but not among Fanca$^{-/-}$ mice. Similarly, both cytokine transcripts (TGF-β and IL-1α) and the oxidative stress response transcript Sod2 were robustly increased in proton irradiated oral mucosa of both Fanca$^{+/+}$ and Fanca$^{-/-}$ mice. Pre-treatment with JP4-039 reduced TGF-β and Sod1 transcript levels compared to 30 GyRBE alone in Fanca$^{+/+}$ mice but not in Fanca$^{-/-}$ mice. In addition, pre-treatment with JP4-039 reduced IL-1α transcript levels in Fanca$^{-/-}$ mice but not in Fanca$^{+/+}$ mice.

Finally, the radiation response-related transcripts Gadd45, Rad51, p21 and p53 were increased following proton irradiation of Fanca$^{+/+}$ and Fanca$^{-/-}$ oral mucosa, except p53 in Fanca$^{+/+}$ mice (Figure 5A-D). Moreover, pre-treatment with JP4-039 did not significantly reduce the levels of these transcripts compared to levels seen in proton irradiated oral mucosa, except for Gadd45 in Fanca$^{-/-}$ mice. Of note, JP4-039 modulated Rad51 and p53 transcript levels to a level similar to that of the nonirradiated oral mucosa of Fanca$^{+/+}$ mice.

Radiation induced bone marrow suppression and mitigation of the suppression by JP4-039. Fanca$^{+/+}$ and Fanca$^{-/-}$ mice that received 30 GyRBE to the oral cavity developed distal bone marrow suppression when evaluated with CFU-GM (Figure 6A) and BFU-e (Figure 6B) colony forming assays. The absolute difference in CFU-GM cells between no radiation and 30 GyRBE was similar for both Fanca$^{+/+}$ and Fanca$^{-/-}$ mice. In contrast, the magnitude of distal marrow suppression, reflected by BFU-e cells, was much higher in the Fanca$^{-/-}$ mice, while the difference was not statistically significant in Fanca$^{+/+}$ mice.
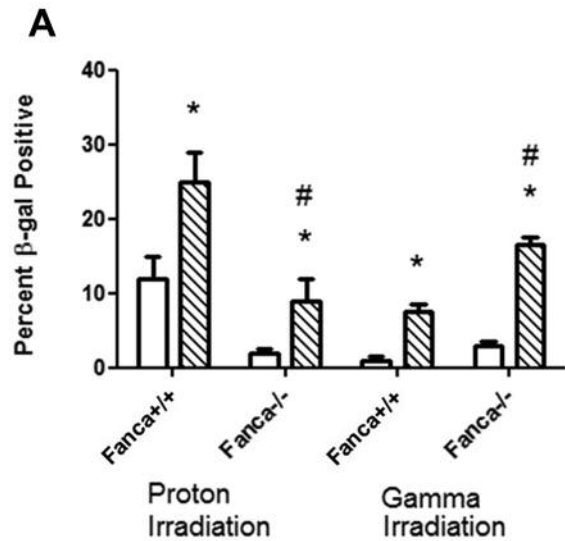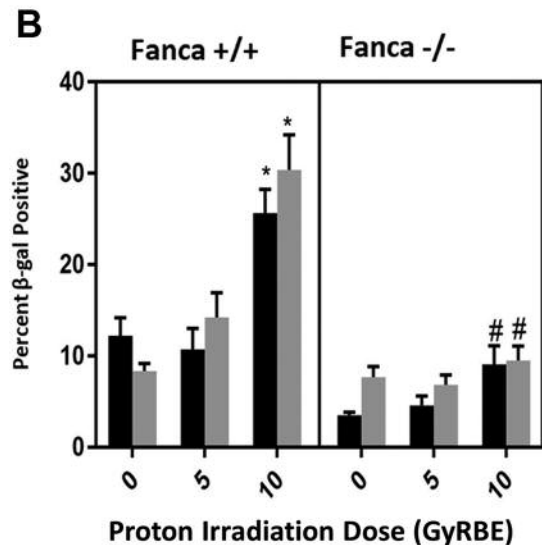
Figure 2. *β-Galactosidase assay for Fanca$^{+/+}$ or Fanca$^{-/-}$ BMSCs following (A) proton or photon radiation therapy. Effect of (B) JP4-039 on proton-induced senescence in both FA models.*
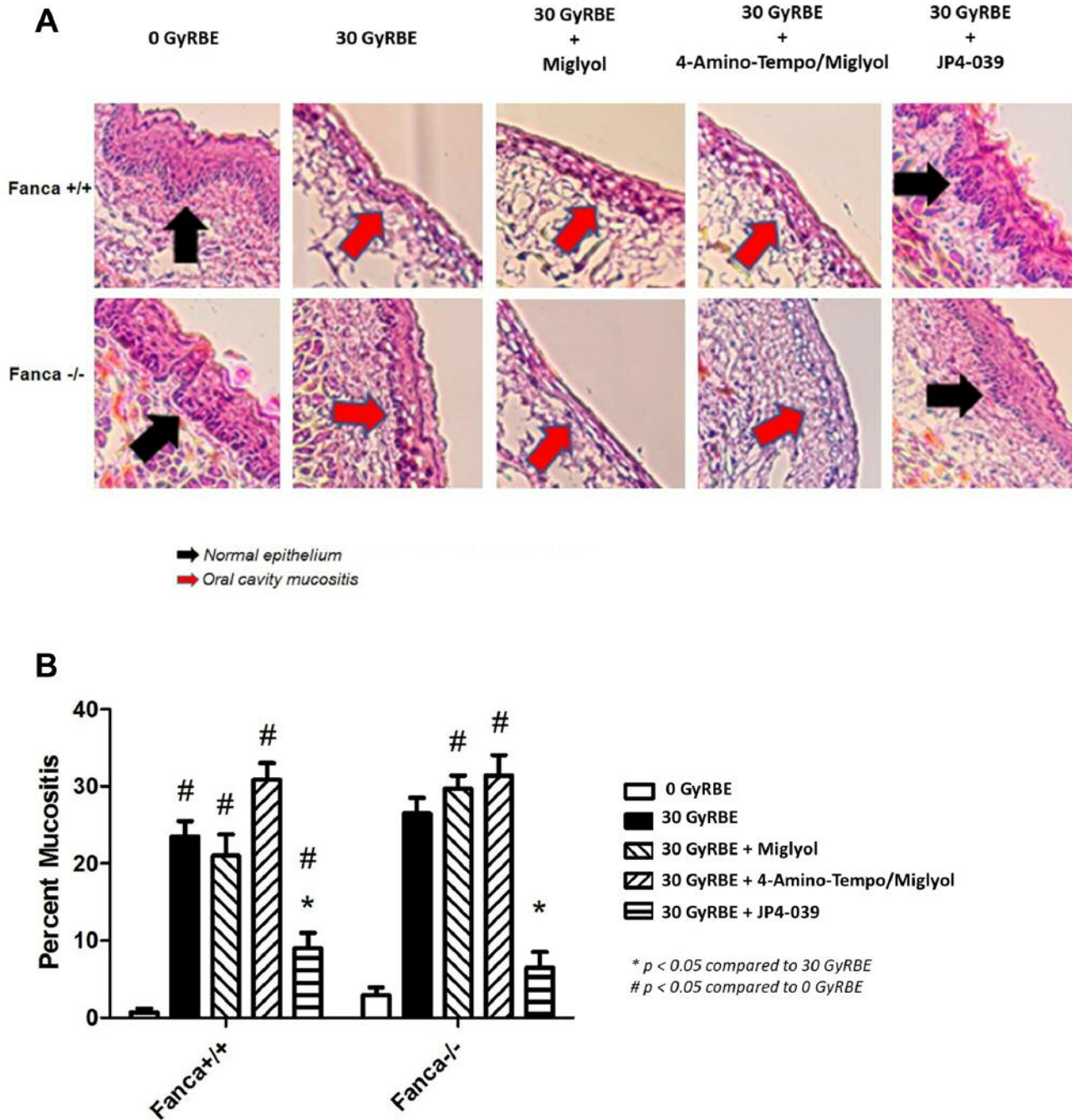
Figure 3. *Fanca^+/+^ and Fanca^−/−^ mice were irradiated with 30 GyRBE to the oral cavity, in the presence or absence of vehicle, non-targeted 4-AT or JP4-039, and the percent of mucositis was assessed (A/B).*

Pre-treatment with JP4-039 reduced proton-induced distal marrow suppression in both mice models. Moreover, there was a statistically significant increase in CFU-GM cells compared to the all subgroups for both Fanca^+/+^ and Fanca^−/−^ mice ($p<0.05$). There was a reduction in CFU-GM cells in response to 30 GyRBE relative to non-irradiated controls, indicative of abscopal bone marrow suppression. Pre-treatment of proton-treated Fanca^+/+^ (Figure 6C) and Fanca^−/−^ (Figure 6D) mice with JP4-039 increased marrow recovery relatively to proton therapy alone ($p<0.0001$) and compared to the photon treatment + JP4-039 group ($p=0.0014$).
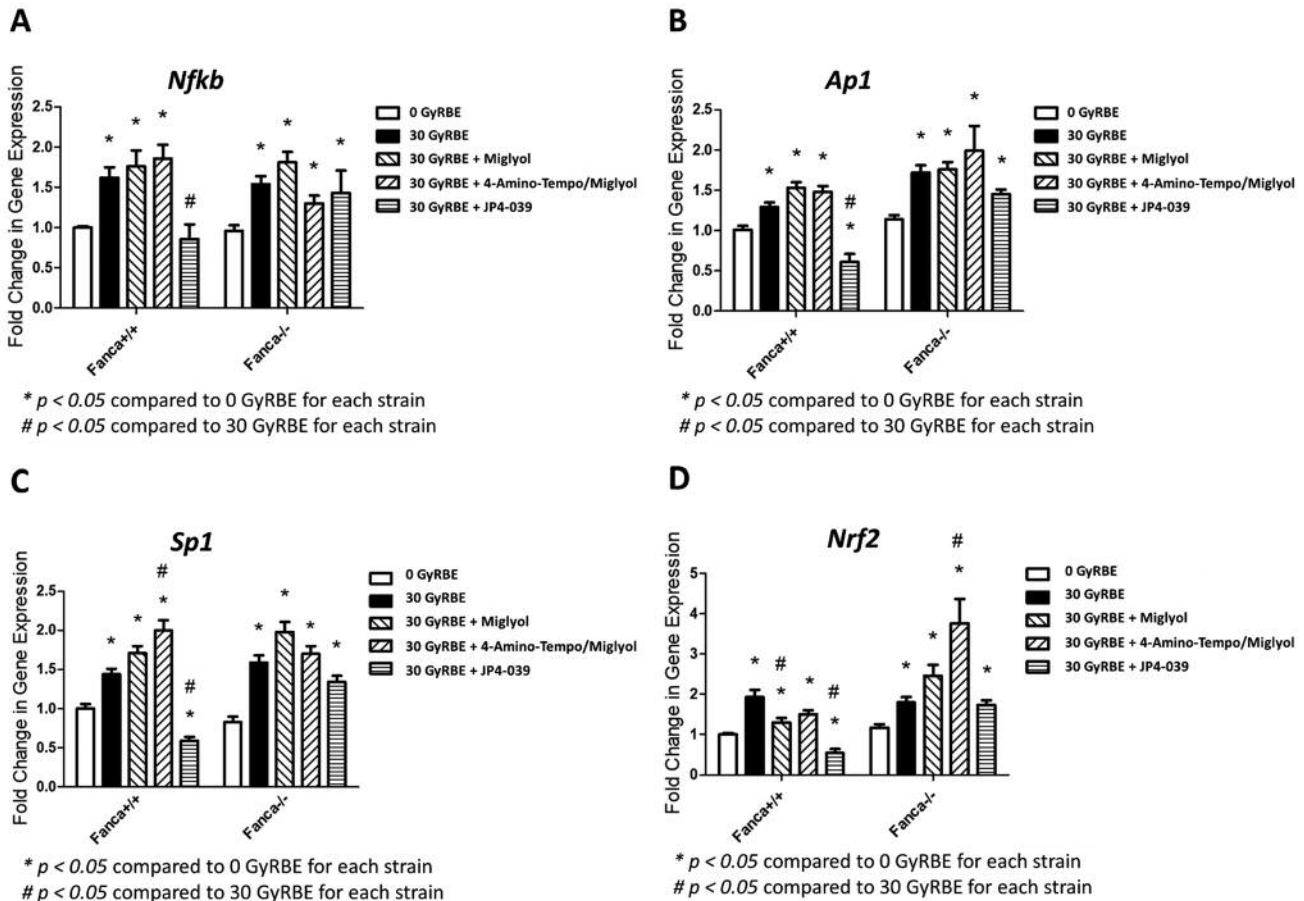
Figure 4. *Proton-induced changes in the pro-inflammatory transcription factor transcripts (A) Nfkb, (B) Ap1, (C) Sp1 and (D) Nrf2 are mitigated by oral administration of JP4-039 in both FA models.*

Discussion

FA patients are at high risk of developing squamous cell carcinoma of the head and neck. With the advent of stem cell transplantation and the increased life expectancy more FA patients are at risk. FA patients with locally advanced oral cavity cancer often require a combination of surgery, radiation therapy and chemotherapy. There are subsets of FA patients that are highly radiosensitive, and there is no established biomarker to identify these patients. Therefore, novel interventions, which spare and/or protect normal tissue are needed. To that end, we have extended the preclinical efficacy of JP4-039 in photon irradiation (10-12) by evaluating its utility in Fanca$^{-/-}$ and Fanca$^{+/+}$ mice models irradiated with a high single fraction of proton radiation.

The International Commission on Radiation Units (ICRU)-78 report assumes a relative biological effectiveness (RBE) of 1.1 for protons, while heavier particles are reported to have a variable RBE (19). However, a fixed RBE for protons is based on assumptions of macroscopic and homogeneous dose in a volume of interest, which do not remain valid at the microscopic level (20). Therefore, the biological consequences of proton therapy are possibly different at the microscopic level when compared to photon radiation (21). In the current study, we observed photon curves demonstrating a characteristic shoulder while the proton curve had a more linear shape (Figure 1A and B). These differences may reflect an inherent difference in the biological response to proton therapy.

Recent publications suggest that senescence is one of multiple cellular responses to ionizing irradiation both *in vitro* and *in vivo*. Fanca$^{-/-}$ and Fanca$^{+/+}$ stromal cell lines were irradiated *in vitro* and serial cultures were then tested for senescence by β-galactosidase staining according to published methods (22). As shown in Figure 2, both Fanca$^{-/-}$ and Fanca$^{+/+}$ BMSCs demonstrated proton- and photon-induced increase in the percent of senescent cells quantitated by β-galactosidase staining 5 days post-irradiation. However, the relative increase in proton irradiated Fanca$^{-/-}$ cells was lower compared to photon irradiated Fanca$^{-/-}$ cells. The data suggest
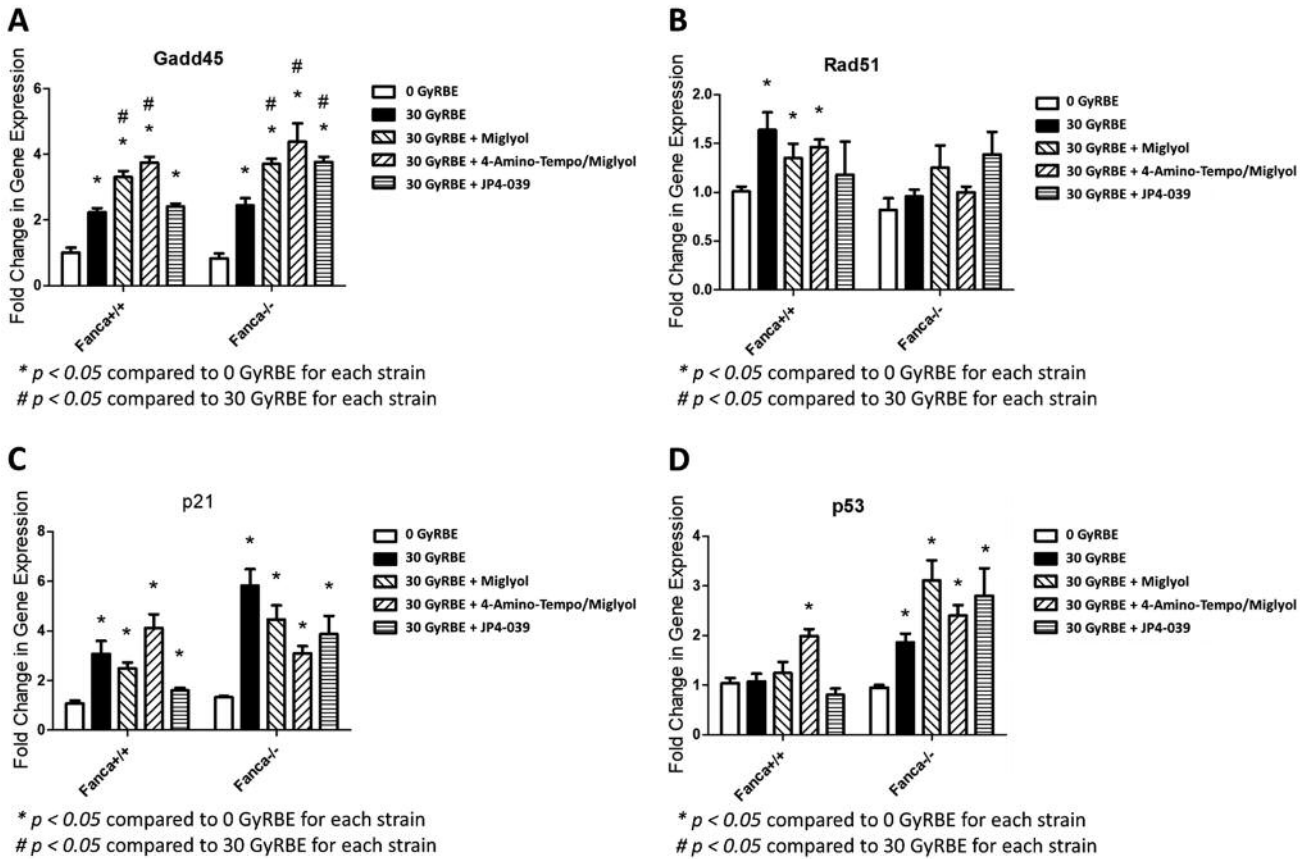
Figure 5. *Proton-induced changes in the radiation response-related transcripts (A) Gadd45, (B) Rad51, (C) p21, and (D) p53 in both FA models.*

putative biological differences in the response of cells to photon compared to proton irradiation.

While β-galactosidase is one of multiple markers of senescence, other studies measuring levels of p16, p21 and telomere length have also been used as biomarkers for quantitating senescence. Future studies will determine whether these three biomarkers follow the same pattern as that observed with β-galactosidase and may confirm the biological difference in the response between proton and photon irradiated Fanca$^{-/-}$ and Fanca$^{+/+}$ cell lines. Moreover, fractionated irradiation dose response curves may further elucidate potential differences in the molecular mechanisms of senescence pathways between protons and photons.

We have published extensively on the radioprotective effects of JP4-039 in cell culture and mouse models (12, 14, 19, 23-26). In the present report, we demonstrated that orally administered JP4-039, prior to head and neck proton irradiation, mitigates radiation-induced tongue ulceration in both Fanca$^{+/+}$ and Fanca$^{-/-}$ models. At the genetic level, pre-treatment with JP4-039 differentially modulated transcription levels, with reduction in TGF-β, Sod1, Nfkb, Ap1, Sp1 and

Nrf2 among Fanca$^{+/+}$ mice and IL-1α among Fanca$^{-/-}$ mice. In contrast, pre-treatment with JP4-039 did not significantly reduce the transcript levels in Fanca$^{-/-}$ mice. The magnitude of changes in proton irradiated oral mucosa was lower compared to photon radiation. These results further extend that of prior publications demonstrating a therapeutic effect of JP4-039 with respect to reduction of irradiation-induced transcripts of stress response genes and inflammatory cytokines (10-12).

We have previously demonstrated that single-fraction head and neck photon radiation results in distal bone marrow suppression in both Fancd2$^{+/+}$ and Fancd2$^{-/-}$ mice, and this is reduced with JP4-039 pretreatment (10-12). The current study further extended these findings and demonstrated that JP4-039 reduces proton induced distant bone marrow suppression as well. Additionally, JP4-039 with proton therapy resulted in a statistically significant relative increase in bone marrow activity when compared to JP4-039 plus photon radiation, supporting other possible biological differences between photon and proton irradiation.

JP4-039 was able to protect Fanca$^{-/-}$ and Fanca$^{+/+}$ cells from both proton and photon radiation. This was also seen in
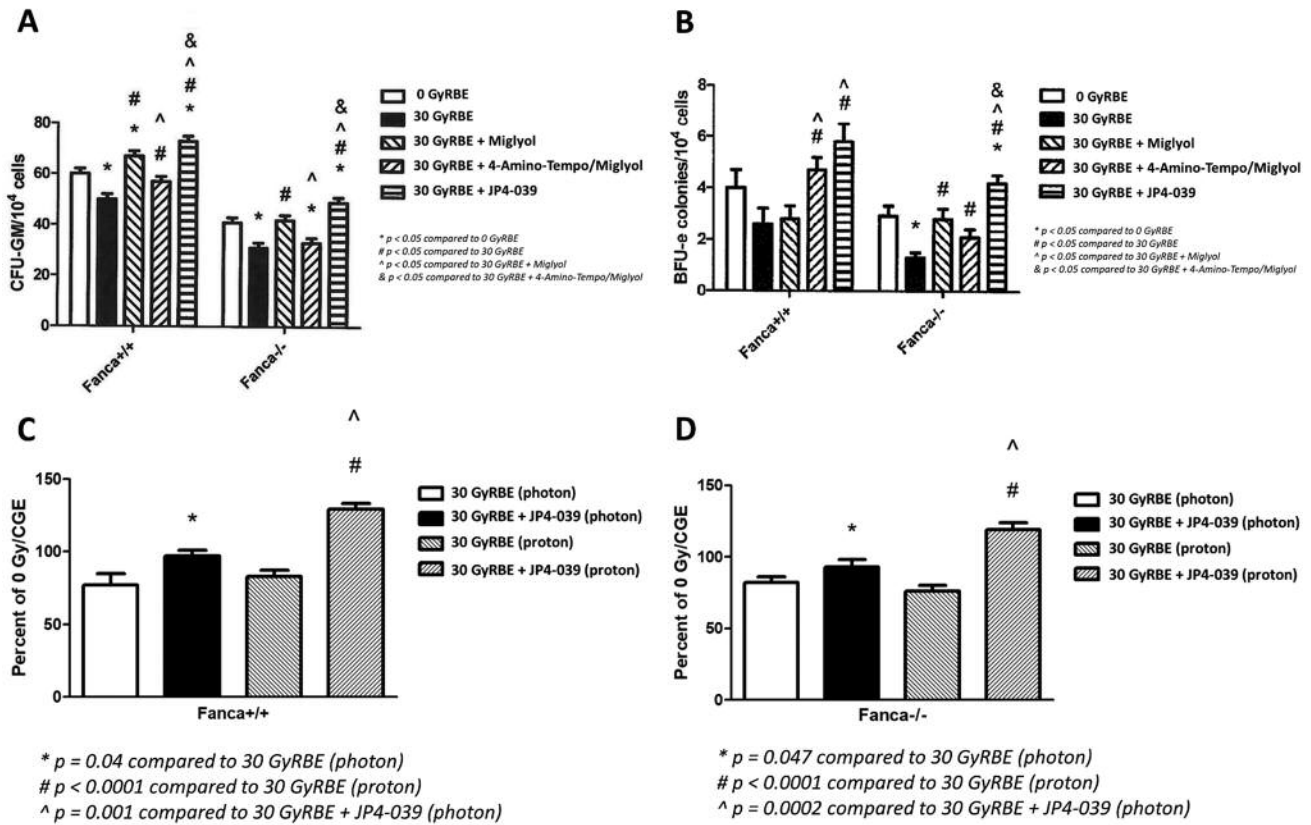
Figure 6. *Distant bone marrow suppression [(A) CFU-GM and (B) BFU-e] following oral cavity proton irradiation of Fanca^{+/+} and Fanca^{−/−} mice, relative to no radiation (C-D) in the presence or absence of JP4-039.*

our *in vivo* model, in which oral JP4-039 reduced the degree of oral mucositis following a large single fraction of 30 GyRBE to the head and neck region. These data support future clinical trials to evaluate the use of intraoral JP4-049 among FA patients receiving radiation therapy and indicate that more research is required to understand the differences between photon and proton irradiation.

Conflicts of Interest

Dr. Greenberger reports grants from NIH, during the conduct of the study; Dr. Wipf reports grants from NIH, during the conduct of the study; In addition, Dr. Wipf has a patent WO 2012068081 A1 20120524 issued; Dr. Epperly reports grants from NIH, during the conduct of the study. There are no other conflicts of interest to disclose.

Authors' Contribution

Conception and design: TJ Quinn, GD Wilson, MW Epperly, JS Greenberger, CW Stevens, P Kabolizadeh; Administrative support: X Ding, GD Wilson, MW Epperly, D Franciola, JS Greenberger, CW Stevens, P Kabolizadeh; Provision of study materials or patients: X Ding, X Li, GD Wilson, MW Epperly, P Wipf, JS Greenberger, CW Stevens, P Kabolizadeh; Collection and assembly of data: TJ Quinn, K Buelow, A Sivananthan, S Thermozier, A Henderson, M Epperly, JS Greenberger, CW Stevens, P Kabolizadeh; Data analysis and interpretation: TJ Quinn, GD Wilson, MW Epperly, P Wipf, JS Greenberger, CW Stevens, P Kabolizadeh; Manuscript writing: TJ Quinn, GD Wilson, MW Epperly, P Wipf, JS Greenberger, CW Stevens, P Kabolizadeh; Final approval of manuscript: TJ Quinn, X Ding, X Li, GD Wilson, K Buelow, A Sivananthan, S Thermozier, A Henderson, MW Epperly, D Franciola, P Wipf, JS Greeenberger, CW Stevens, P Kabolizadeh.

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