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Amelioration of Radiation-Induced Oral Cavity Mucositis and Distant Bone Marrow Suppression in Fanconi Anemia *Fancd2*$^{-/-}$ (FVB/N) Mice by Intraoral GS-Nitroxide JP4-039

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The altered DNA damage response pathway in patients with Fanconi anemia (FA) may increase the toxicity of clinical radiotherapy. We quantitated oral cavity mucositis in irradiated Fanconi anemia *Fancd2*$^{-/-}$ mice, comparing this to *Fancd2*$^{+/-}$ and *Fancd2*$^{+/+}$ mice, and we measured distant bone marrow suppression and quantitated the effect of the intraoral radioprotector GS-nitroxide, JP4-039 in F15 emulsion. We found that FA mice were more susceptible to radiation injury and that protection from radiation injury by JP4-039/F15 was observed at all radiation doses. Adult 10–12-week-old mice, of FVB/N background *Fancd2*$^{-/-}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{+/+}$ were head and neck irradiated with 24, 26, 28 or 30 Gy (large fraction sizes typical of stereotactic radiosurgery treatments) and subgroups received intraoral JP4-039 (0.4 mg/mouse in 100 μL F15 liposome emulsion) preirradiation. On day 2 or 5 postirradiation, mice were sacrificed, tongue tissue and femur marrow were excised for quantitation of radiation-induced stress response, inflammatory and antioxidant gene transcripts, histopathology and assay for femur marrow colony-forming hematopoietic progenitor cells. *Fancd2*$^{-/-}$ mice had a significantly higher percentage of oral mucosal ulceration at day 5 after 26 Gy irradiation (59.4 ± 8.2%) compared to control *Fancd2*$^{+/+}$ mice (21.7 ± 2.9%, $P = 0.0063$). After 24 Gy irradiation, *Fancd2*$^{-/-}$ mice had a higher oral cavity percentage of tongue ulceration compared to *Fancd2*$^{+/+}$ mice irradiated with higher doses of 26 Gy ($P = 0.0123$). Baseline and postirradiation oral cavity gene transcripts were altered in *Fancd2*$^{-/-}$ mice compared to *Fancd2*$^{+/+}$ controls. *Fancd2*$^{-/-}$ mice had decreased baseline femur marrow CFU-GM, BFUe and CFU-GEMM, which further decreased after 24 or 26 Gy head and neck irradiation. These changes were not seen in head- and neck-irradiated *Fancd2*$^{+/+}$ mice. In radiosensitive *Fancd2*$^{-/-}$ mice, biomarkers of both local oral cavity and distant marrow radiation toxicity were ameliorated by intraoral JP4-039/F15. We propose that *Fancd2*$^{-/-}$ mice are a valuable radiosensitive animal model system, which can be used to evaluate potential radioprotective agents.

INTRODUCTION

Fanconi anemia (FA) is an autosomal recessive and x-linked inherited DNA repair deficiency syndrome associated with a biallelic mutation in one or more of the 16 FA pathway gene products that leads to congenital abnormalities, bone marrow (BM) failure, defective DNA repair and predisposition to cancer (*1–9*). Some FA patients experience progressive bone marrow failure during childhood, which frequently requires allogeneic hematopoietic stem cell transplantation (*3, 5*). The 16 currently reported FA proteins act as a signaling nexus and platform for actual biochemical repair of DNA by the processes of homologous recombination, nuclear excision repair and translesion DNA polymerases. The FA core complex known as FACC activates a second ID complex involving FancD2 and FancI. The core complex functions as a multi-subunit E3 ubiquitin ligase with one subunit (FancL) catalyzing the mono-ubiquitination of FancD2 and FancI after DNA damage (*10–12*). These mono-ubiquitinated subunits are recruited to DNA, where they participate in facilitating the DNA repair process (*13*).

Fanconi anemia patients are at increased risk for developing both leukemia and solid tumors, including head

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and neck squamous cell carcinoma (*1–2*), partly attributable to their underlying DNA damage response pathway alterations (*3–6*), which also contributes to their sensitivity to ionizing radiation and complicates chemoradiotherapy (*7, 8*).

Radiotherapy of head and neck cancer in FA patients presents a particular challenge due to the potential for severe oral mucositis, ulceration, pain, dysphagia and xerostomia (*7, 8*). *In vitro* studies with human cell lines from a *FancD2*$^{-/-}$ patient showed a radioprotective effect of the GS-nitroxide, JP4-039 (*14*). We evaluated radiation protection of the oral cavity and oropharynx in *Fancd2*$^{-/-}$ (FVB/N) mice by intraoral administration of the GS-nitroxide, JP4-039 delivered in a novel F15 liposomal formulation.

We also evaluated the effect of the *Fancd2*$^{-/-}$ genotype on radiation-induced head and neck RNA transcript biomarkers and suppression of distant femur marrow colony-forming progenitor cells. The results showed significant amelioration of both oral cavity toxicity and distant marrow suppression by intraoral administration of JP4-039 in *Fancd2*$^{-/-}$ mice, as well as heterozygote *Fancd2*$^{+/-}$ and control *Fancd2*$^{+/+}$ mice.

MATERIALS AND METHODS

Mice

Fancd2$^{-/-}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{+/+}$ mice (FVB/N background) (*15*) were derived from *Fancd2*$^{+/-}$ heterozygote breeding pairs generously provided by Dr. Paul Lambert of the University of Wisconsin (FVB/N background mice), Madison, WI. Mice were housed 5/cage according to institutional IACUC regulations, and mice were fed standard Purina laboratory chow. *Fancd2*$^{-/-}$ mice were derived from breeding pairs of *Fancd2*$^{+/-}$ mice. *Fancd2*$^{+/+}$ mice resulting from these breedings were used as control mice. All protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Veterinary care was provided by the Division of Laboratory Animal Resources of the University of Pittsburgh.

Long-Term Bone Marrow Cultures and Derivation of Cell Lines

Long-term bone marrow cultures (LTBMC) were established from the femur and tibia marrow of FVB/N background mice of genotypes, *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ as described by Berhane *et al.* (*16*). Briefly, the contents of a femur and tibia (N = 6/genotype) were flushed into McCoy's 5A medium (Gibco, Gaithersburg, MD) supplemented with 25% horse serum (Cambrex, Rockland, ME) and 10^{-5} *M* hydrocortisone sodium hemisuccinate. Cultures were incubated at 33°C in 7% CO_2. After 4 weeks, the horse serum was replaced with 25% FBS (Gibco) (*16*). The cultures were observed weekly for hematopoietic cell production and cobblestone island formation. Cobblestone islands, indicative of stromal cell adherent hematopoietic islands containing primitive stem cells greater than or equal to 50 cells, were scored weekly in each flask.

Establishment of IL-3-Dependent Hematopoietic Progenitor Cell Lines and Clonal Cell Sublines

Nonadherent cells were harvested from (FVB/N) *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mouse LTBMC at week 4 and cultured in six-well tissue culture plates in Iscove's modified Eagles medium (IMDM) supplemented with 20% fetal calf serum (FBS) and 1.0 ng/mL Interleukin 3 (IL-3) (Peprotech, Rocky Hill, NJ). The *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ cell lines were passaged weekly for 10 weeks to establish primary IL-3-dependent cell lines (*16*).

Clonal cell sublines were established from each of the (FVB/N) *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ parent lines by expansion of single colonies. Cells from primary IL-3-dependent cell lines were plated in 0.8% methylcellulose supplemented with 10% IMDM, 30% fetal bovine serum (FBS), 1% bovine serum albumin, 2 ng/mL IL-3 (Stemcell Technologies, Vancouver, Canada) at variable cell densities. At day 14, individual colonies were harvested and each cultured in a well of a 96-well plate in 0.2 mL of IMDM supplemented with 30% FBS and 1 ng/mL IL-3. Cells were then replated in methylcellulose-containing medium, colonies selected at day 14 and cultured as above to establish subcloned lines. Confirmation of genotype after repeated subcloning was performed for each cell line as published previously (*16*).

Establishment of Bone Marrow Stromal Cell Lines and Clonal Cell Sublines

Adherent cell layers from one 4-week-old LTBMC from each group of (FVB/N) *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice were trypsinized and expanded by passage into Dulbecco's modified Eagle medium (DMEM) + 10% FBS to establish bone marrow stromal cell lines according to published methods (*16*). Cells were passaged for 10 weeks to establish cell lines. Cultures were incubated at 37°C in 5% CO_2.

Clonogenic Radiation Survival Curves for Fancd2$^{-/-}$ *Mouse Bone Marrow Stromal Cell Lines*

(FVB/N) *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ bone marrow stromal cells were irradiated in suspension to doses between 0–8 Gy at 70 cGy/min using a Shepherd Mark 1 ^{137}Cs γ-ray source (J. L. Shepherd, San Fernando, CA). Cells were plated in quadruplicate in Linbro plates (Fisher Scientific, Pittsburgh, PA) and incubated at 37°C and 5% CO_2 for 9–11 days, stained with crystal violet and colonies of $\geq$50 cells were counted using a GelCount colony counter (Oxford Optronix, Oxford, UK). Data were analyzed with single-hit multitarget models according to published methods (*16*).

Radiation Survival Curves for Fancd2$^{-/-}$ *Mouse IL-3-Dependent Hematopoietic Cell Lines*

IL-3-dependent nonadherent cell lines derived from LTBMC from *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ or *Fancd2*$^{-/-}$ mice (FVB/N) were irradiated in suspension to doses between 0–8 Gy (*16*). Cells were plated in triplicate in methylcellulose medium containing recombinant mouse stem cell factor, IL-3, IL-6 and recombinant human erythropoietin (Stem Cell Technologies). Colony-forming unit granulocyte-macrophage (CFU-GM) numbers were scored on days 7–9 for the IL-3-dependent cell lines. Data were analyzed using the single hit, multitarget model.

Head and Neck Mouse Irradiation

Fancd2$^{-/-}$, *Fancd2*$^{+/-}$ and wild-type *Fancd2*$^{+/+}$ mice were head and neck irradiated with single fraction doses of 30 Gy, 28 Gy, 26 Gy or 24 Gy using a Varian Linear Accelerator, 6 MV, at a dose rate of 2 Gy/min. The mice were anesthetized using Nembutal and shielded appropriately, ensuring that only the head and neck region, above the cervical spine were irradiated (*17*). Ten-half-value layers of lead shielding was used around the irradiated area. TLD measurements were taken above, below and between the mice to ensure uniform target dose delivery.

Intraoral Administration of JP4-039 and the Vehicle F15

GS-nitroxide (JP4-039) mice were administered JP4-039 intraorally at 10 mg/kg in 100 μl F15 emulsion (0.4 mg JP4-039/mouse) 15 min prior to irradiation (*18*).

JP4-039 encapsulated in F15 liposomes was prepared as published (*18*). Briefly, JP4-039 (*18*) was formulated at final drug concentrations of 8 mg/ml in cationic multilamellar liposomes termed F-15. F-15 is a unique form of multilamellar liposome (N, N-dioleylamine amido-L-glutamate), which was utilized as a solvent for JP4-039 to facilitate adherence of the drug to the oral mucosa. The drug is entrapped between lipid bilayers and allows slow release over time from the liposome particles. F-15 was cationically charged to facilitate surface coating and retention for oral and oropharyngeal mucosa, and is composed of soy phosphatidylcholine (PC): Tween-80: N,N-di-oleylamine amido-L-glutamate (4:1:1 w/w ratio) with a final drug concentration of 8 mg/ml in PBS. F-15 has low toxicity to cultured mammalian cells (>0.5 mg/ml).

Soy PC and Lissamine™ rhodamine-phycoerythrin were obtained from Avanti Polar Lipids (Alabaster, AL); Tween-80, *tert-boc*-L-glutamic acid, oleylamine, dicyclohexylcarbodiimide, N-hydroxy-succinimide and trifluoroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's phosphate-buffered saline (DPBS) was obtained from Lonza (Walkersville, MD). A cationic lipid, L-glutamic acid-1,5,-dioleyl amide [NH_2-L-Glu($NHC_{18}H_{36}$)$_2$] was synthesized using a modified route as previously described (*18*), by coupling *tert-boc*-L-glutamic acid and oleylamine with dicyclo-hexylcarbodiimide and N-hydroxysuccinimide as the coupling agents, followed by use of trifluoroacetic acid as the deprotecting agent.

The lipid mixture (6 mg) and drug to be encapsulated (1 mg) were dissolved in 100 μl *tert*-butanol, frozen on dry ice and lyophilized overnight into a cake. The next day, a 62.5 μl DPBS was added to the lipid cake, which was allowed to hydrate for 24 h at room temperature. Cationic liposomes were prepared from the hydrated lipid suspension by manual homogenization using a pair of custom-made tight-fit tube and pestle until a homogeneous consistency was reached. Finally, the liposome suspension was removed from the tube and another 62.5 μl DPBS was used to rinse the tube and pestle and the wash solution was combined with the liposome suspension. Thus, 1 mg JP4-039 was formulated in 225 μl volumes. The final particle sizes were measured by a laser dynamic scattering method (NP-4 Particle Sizer, Beckman Coulter Inc., Brea, CA) and found to be in the range of 200–300 nm with a mean of ~255 nm in diameter. Each mouse received an intraoral injection of 100 μl of F15 formulation containing 400 μg JP4-039. To determine whether Tween-80 was required for effective uptake, an identical formulation (F14) without Tween-80 was tested (*18*) and was not effective.

Measurement of Radiation Toxicity: Histopathology of Oral Cavity

Tongue tissue from irradiated *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice and unirradiated control mice was harvested at days 2 and 5 postirradiation and fixed in 10% formalin. Five μm thick paraffin sections were stained with hematoxylin and eosin (H&E). Hematoxylin and eosin stained slides were scored by two blinded observers for percent ulceration. Ulceration was defined as loss of thickness of the epithelial layer of the tongue (*17*). For each genotype, at least three tongue samples per time point were scored per condition and approximately 270 individual sections were scored. Mucositis was quantitated as percent ulceration using LabWorks Image Acquisition and Analysis Software (UVP Bio-Imaging System, Upland, CA). Data is shown as mean percent ulceration ± standard deviation. Comparisons between groups were made with the two-sided two-sample *t* test. *P* values less than 0.05 were regarded as significant.

Femur Marrow Histopathology

Femurs from unirradiated control *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice and head- and neck-irradiated mice of each genotype were removed and fixed in 10% formalin. Bones were decalcified, paraffin embedded, sectioned and H&E stained for evaluation of bone marrow cellularity using light microscopy as reported previously (*19*).

Peripheral Blood Counts

Blood from individual control unirradiated *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice as well as mice from head- and neck-irradiated groups with or without JP4-039/F15 treatment was collected from the tail vein immediately prior to sacrifice. Tail blood (100 μL) was placed in coated ethylenediaminetetraacetic acid (EDTA) MiniCollect tubes (Greiner Bio-One, GmbH, Kremsmünster, Austria). Complete blood counts (CBC) and differential was performed within 24 h of collection by Marshfield Laboratories, Cleveland, OH.

Hematopoietic Cell Colony-Forming Assays

Femur bone marrow cells were plated in triplicate in methylcellulose containing medium supplemented with recombinant murine stem cell factor, recombinant murine IL-3, recombinant murine interleukin 6 (IL-6) and recombinant murine erythropoietin (Stem Cell Technologies Inc.) for assessment of hematopoietic cells within the marrow capable of forming colonies in semi-solid medium *in vitro*. Colonies were scored on day 13 for colony-forming unit-granulocyte macrophage (CFU-GM), burst-forming unit erythroid (BFUE), and multi-lineage colony-forming unit–granulocyte-erythroid-megakaryocyte-monocyte (CFU-GEMM) (*20*). Results are reported as mean ± standard deviation. The two-sided two-sample *t* test was used to compare treatment groups (*21*).

Real Time Polymerase Chain Reaction (RT-PCR) Analysis

Fancd2$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice that were unirradiated, head and neck irradiated only or given JP4-039/F15 and then irradiated had their tongue tissue and femur bone marrow excised and harvested at day 5 postirradiation. Tissue specimens were individually homogenized and RNA extracted using Trizol reagent (Life Technologies, Grand Island, NY). cDNA was synthesized with a total of 2 μg of RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, cat no. 4368814).

RT-PCR was used to analyze radiation-inducible or -suppressed transcripts for representative genes known to be involved in the response to ionizing radiation including: transcription factors AP-1, SP-1, NFkβ and Nrf2; cytokines IL-1α and TGFβ1; the oxidative stress response enzyme MnSOD (Sod2); and radiation response related transcripts p21 and p53 (*21*). The results are presented as fold increase or decrease in gene expression compared to baseline level, which was adjusted to either that of the unirradiated FVB/N wild-type *Fancd2*$^{+/+}$ mouse tongue tissue (or bone marrow cells) or in some experiments was adjusted to the baseline for that genotype (*Fancd2*$^{-/-}$ or *Fancd2*$^{+/-}$). The magnitude of change in RNA attributable to radiation exposure was quantitated for each sample.

RT-PCR reactions were performed using an Eppendorf Realplex2 Mastercycler and Eppendorf epMotion 5070 automated pipetting system (Eppendorf, Westbury, NY). PCR amplification of the GAPDH gene was used as the housekeeping gene (Gen-Bank: NM_008084). RNAs assayed: Nrf2 (Gen-bank: NM_010902.3), Sod2 (Gen-Bank: NM_013671.3), NFkβ (Gen-Bank: NM_008689.2), TGFβ1 (Gen-Bank: NM_011577.1), p21 (Gen-Bank:NM_001111099), p53 (Gen-Bank:NM_001168250), AP-1 (Gen-Bank:NM_010591.2), SP-1 (Gen-Bank: NM_013672.2), IL-1A (Gen-Bank:NM_010554.4) and GADD45 (Gen-Bank:NM_007836.1).

Western Blot Analysis for Nrf2, NFkβ and MnSOD Proteins

Tongue tissue from *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice that were unirradiated, irradiated or F15-JP4-039-treated then irradiated were cut into pieces (5–7 mm) on dry ice, homogenized and pelleted (irradiated tongue tissue with or without JP4-039 pretreatment were taken at day 5 postirradiation). The pellets were lysed in NP-400 buffer [50 m*M* Tris, pH 7.8, 10 m*M* EDTA, 150 m*M* NaC1, 1 m*M*

phenylmethylsulfonyl fluoride (PMSF), 1% NP-40 and a protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN). Protein samples were separated in 15% polyacrylamide gels by electrophoresis and transferred to nitrocellulose membranes (*22*). Primary anti-MnSOD antibody (cat no. 13533) and anti-Nrf2 (cat no. 31163) were obtained from Abcam (Cambridge, MA) and used at a 1:2,000 and 1:2,500 dilution, respectively. Anti-NFkβ (cat no. sc-114) was obtained from Santa Cruz Biotechnology, Santa Cruz, CA and used at a concentration of 1:100. As a control gene product, glycerol adelehyde-3-phosphate-dehydrogenase (GAPDH) (Sigma Aldrich) antibody was used. Horseradish peroxidase anti-rabbit or anti-mouse secondary antibody (Promega, Madison, WI) was used and membranes were developed with Super Signal West Dura ECL (Thermo Scientific, Rockford, IL). Western blots were prepared in triplicate and total protein density tabulated using LabWorks Image Acquisition and Analysis Software (UVP BioImaging System, Upland, CA).

Statistical Analysis

Histopathology of oral cavity tissue (tongue) was presented as percent ulceration. Each group was compared with the 0 Gy group, which was set at 0% ulceration, using the two-sided one-sample *t* test. At 26 and 28 Gy, each *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ group was compared to the corresponding group without JP4-039 for each genotype. In peripheral blood count experiments, CBC at 2 and 5 days after irradiation were compared to 0 Gy for each genotype. In the hematopoietic cell colony assays experiment, CFU-GM, BFU-E and CFU-GEMM data from each irradiated group were compared to the corresponding 0 Gy group. Comparisons were also made between genotypes. In all experiments, results were summarized as mean ± standard deviation for each group. *P* values for the comparison between groups were calculated using the two-sided two-sample *t* test.

In RT-PCR experiments, the data for gene expression were normalized by calculating the differences in threshold cycles (ΔCt) from the Ct-GAPDH and Ct-Target genes. The relative increase or decrease in expression was calculated by comparing the reference gene with the target gene (ΔΔCt) and using the formula for relative expression (=$2^{\Delta\Delta Ct}$). For each of the tissues (tongue or bone marrow), gene expression was compared between genotypes. For tongue tissue, we also compared the JP4-039-treated then irradiated group with the corresponding irradiation only group for each genotype. Pairwise comparison of the gene expression between any two groups was evaluated using the two-sided two-sample *t* test. Significance was indicated for a greater than twofold elevation or decrease in levels of each transcript at each set of conditions. In these exploratory studies, we did not adjust *P* values for multiple tests. *P* values less than 0.05 were regarded as significant (*23*).

Western blot data were analyzed as previously published (*16*) using densitometry. Results are summarized as mean ± standard deviation for each group. *P* values for the comparison between groups were calculated using the two-sided two-sample *t* test. *P* values less than 0.05 were regarded as significant (*16*).

RESULTS

Radiosensitivity of Fancd2$^{-/-}$ *(FVB/N) Mouse Cell Lines and Oral Cavity Tissue*

We first compared the radiosensitivity of mesenchymal and hematopoietic cell lines derived from *Fancd2*$^{-/-}$ (FVB/N) mouse long-term bone marrow cultures, as described in Materials and Methods, by clonogenic radiation survival curves using clonal bone marrow stromal cell lines and IL-3-dependent hematopoietic progenitor cell lines. We used single-target, multi-hit clonogenic survival curve analysis. Marrow stromal cells from FVB/N *Fancd2*$^{-/-}$ mice were radiosensitive (Fig. 1A). In contrast, *Fancd2*$^{-/-}$ mouse IL-3-dependent hematopoietic cell lines were radioresistant (Fig. 1B) (Table 1). These results confirm and extend those in a previous publication with *Fancd2*$^{-/-}$ stromal and hematopoietic cell lines derived from a different background C57BL/6J mouse strain (*16*). In a prior study (*16*), the radiosensitivity of stromal cells was attributable to lower levels of antioxidant stores and prolonged time of repair of DNA strand breaks by comet assay. Also, consistent with the prior study (*16*), IL-3-dependent hematopoietic cell lines from FVB/N mice showed radioresistance.

Groups of *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ (FVB/N) mice (N = 3) were next head and neck irradiated with 24, 26, 28 or 30 Gy and the tissue excised after 2 or 5 days and evaluated. These radiation doses are consistent with stereotactic radiosurgery (SRS) and stereotactic body radiosurgery (SBRT) doses used in the treatment of defined target volumes in clinical radiotherapy of head and neck cancer. As shown in Fig. 2A, the day 2 time point was clearly too early to assess genotype-specific differences in histopathologic toxicity from head and neck irradiation, with little detectable change in any of the groups. In contrast, at day 5 after 24, 26, 28 or 30 Gy, there were significant increases in normal tissue damage measured as oral tissue mucosal breakdown (ulceration) in *Fancd2*$^{-/-}$ mice. Control *Fancd2*$^{+/+}$ mice showed no detectable toxicity after 24 Gy (Fig. 2B), but did show significant damage after 26, 28 or 30 Gy (Fig. 2B). In contrast, *Fancd2*$^{-/-}$ mice had a significantly greater percent ulceration compared to wild-type *Fancd2*$^{+/+}$ mice at day 5 after each of the four doses of radiation, including the lowest dose of 24, 26 (59.4 ± 8.23%, $P = 0.063$), 28 and 30 Gy (Fig. 2B). After 24 Gy, *Fancd2*$^{-/-}$ mice had a greater percent ulceration ($P = 0.0123$) than did the *Fancd2*$^{+/+}$ mice irradiated with the higher dose of 26 Gy. Control *Fancd2*$^{+/+}$ mice showed significantly increased ulceration after 26 ($P = 0.0059$), 28 ($P = 0.0007$) or 30 Gy ($P = 0.0019$) (Fig. 2B). Thus, both *Fancd2*$^{-/-}$ and heterozygote *Fancd2*$^{+/-}$ mice were sensitive to head and neck irradiation compared to *Fancd2*$^{+/+}$ mice measured as percent tongue ulceration at day 5. These data establish that the oral cavity tissue of *Fancd2*$^{-/-}$ FVB/N background strain mice was radiosensitive and that the radiosensitivity of the *Fancd2*$^{-/-}$ mouse oral cavity was consistent with the radiosensitivity of their bone marrow stromal cell lines *in vitro* (Fig. 1A).

Intraoral JP4-039/F15 Ameliorates Radiation-Induced Mucosal Ulceration in Fancd2$^{-/-}$ *Mice*

The above results established that the 28 Gy dose was detectably toxic to the oral cavity of all mouse genotypes when measured at day 5. We next irradiated *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice (6 mice/genotype) with a 28 Gy dose to the head and neck, and three mice per group received JP4-039/F15 (400 ug/mouse in 100 μl) intra-orally

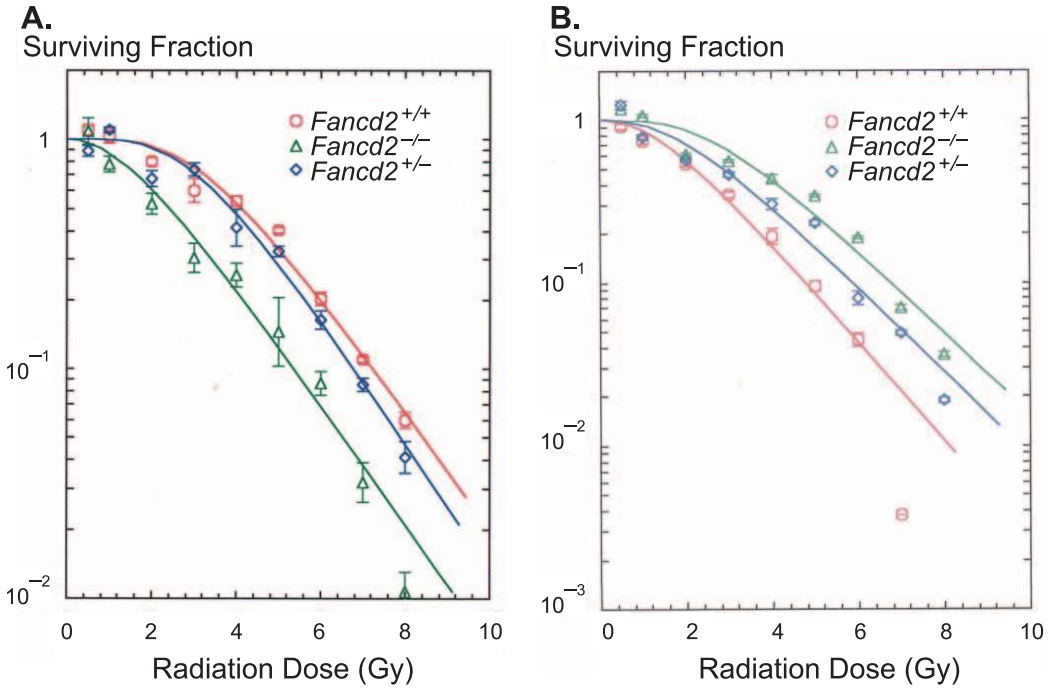

FIG. 1. Clonogenic radiation survival curves of *Fancd2*$^{-/-}$ (FVB/N) cell lines. Clonogenic radiation survival curves are shown for permanent bone marrow stromal (panel A) and interleukin 3 (IL-3)-dependent hematopoietic progenitor (panel B) cell lines derived from long-term bone marrow cultures established from whole bone marrow of 6–8-week-old [FVB/N (*Fancd2*$^{-/-}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{+/+}$)] mice according to the Materials and Methods section. Cell lines were passaged *in vitro* for two months and clonal sublines established. Clonogenic radiation survival curves were performed according to the Materials and Methods section, using the single hit, multi-target model for bone marrow stromal cell lines (panel A) and IL-3-dependent hematopoietic progenitor cell lines (panel B). Results are shown in linear quadratic presentation as mean and standard error of the mean (SEM) of three separate experiments. D_0 and ñ were compared for statistically significant differences according to published methods (*14*).

15 min before irradiation. There was a significant reduction in radiation-induced percent ulceration of oral cavity tissue at day 5 in JP4-039/F15 treated *Fancd2*$^{-/-}$, *Fancd2*$^{+/-}$, as well as wild-type *Fancd2*$^{+/+}$ mice (Fig. 2C and D). The groups that were irradiated with 28 Gy and treated with intraoral JP4-039/F15, had less than 25% tongue ulceration ($P < 0.005$) (Fig. 2C). Reduction in radiation-induced tongue ulceration by JP4-039/F15 treatment as a measure of oral cavity toxicity in *Fancd2*$^{-/-}$ mice was histologically comparable to the level of reduction observed in wild-type mice (Fig. 2D). All experiments were performed 3 times (Supplementary Table S1; http://dx.doi.org/10.1667/RR13633.1.S1).

Altered Baseline Gene Transcript Expression in the Oral Cavity and Femur Bone Marrow of Fancd2$^{-/-}$ *Mice*

We next determined whether biomarkers, including levels of gene transcripts for representative DNA damage associated genes, proinflammatory genes and oxidative stress response genes, were altered in unirradiated *Fancd2*$^{-/-}$ mice, and how these levels were affected by radiation and JP4-039/F15 treatment. *Fancd2*$^{-/-}$ mice (N = 5) showed elevated baseline levels compared to *Fancd2*$^{+/+}$ mice of RNA transcripts for Nrf2, p21 and p53 (Fig. 3A) ($P < 0.0001$ for Nrf2 and p21; $P = 0.0079$ for p53) (Fig. 3A). Unirradiated *Fancd2*$^{-/-}$ mouse oral cavity tissue had reduced levels of transcripts for other genes associated with the inflammatory response and DNA damage response repair

TABLE 1
FVB/N *Fancd2*$^{-/-}$ Stromal and IL-3-Dependent Hematopoietic Cell Line Clonogenic Radiation Survival Curves

Cell line	D_0 (Gy)	ñ
Stromal		
Fancd2$^{+/+}$	1.71 ± 0.03	8.2 ± 0.5
Fancd2$^{+/-}$	1.98 ± 0.41	5.3 ± 2.6
Fancd2$^{-/-}$	1.75 ± 0.09	2.5 ± 0.1
		$P = 0.0078$
Hematopoietic		
Fancd2$^{+/+}$	1.08 ± 0.06	2.07 ± 0.05
Fancd2$^{+/-}$	1.40 ± 0.18	3.61 ± 0.14
Fancd2$^{-/-}$	2.02 ± 0.11	3.73 ± 0.07
	$P = 0.0017$	**$P = 0.0027$**

Notes. Data are presented as the mean ± SEM of 3 experiments for the IL-3-dependent hematopoietic cell lines and 2 experiments for the stromal cell lines. All data were plotted using the single hit, multi-target model. Comparison between the *Fancd2*$^{+/+}$ and *Fancd2*$^{-/-}$ groups was made using the two-sided two-sample *t* test. *P* values less than 0.05 were regarded as significant and are shown in bold.

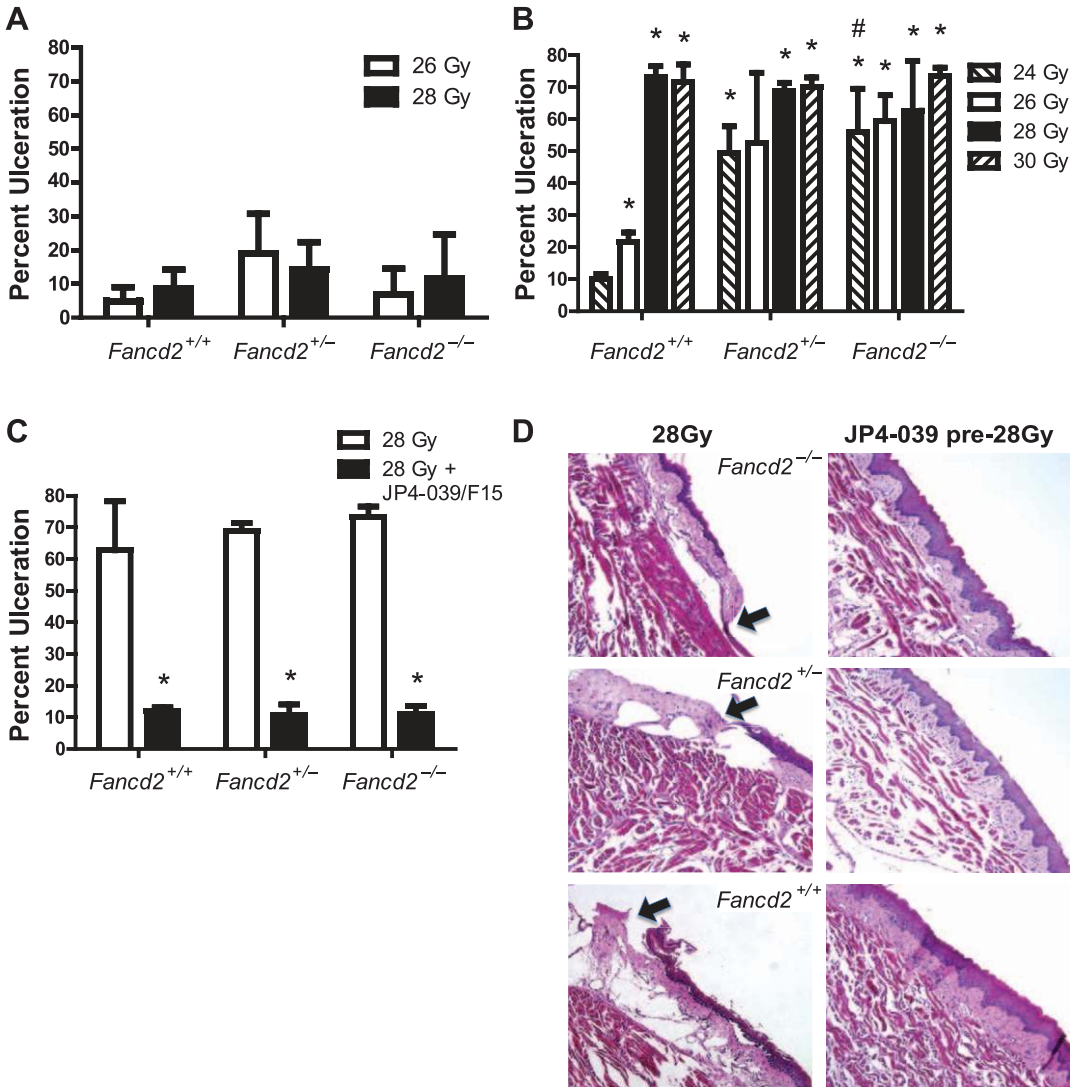

FIG. 2. JP4-039/F15 ameliorates radiosensitivity of the oral cavity of *Fancd2*$^{-/-}$ mice. The oral cavity of *Fancd2*$^{-/-}$, *Fancd2*$^{+/-}$ and wild-type *Fancd2*$^{+/+}$ mice were irradiated with doses of 30 Gy, 28 Gy, 26 Gy or 24 Gy using a Varian linear accelerator, at a dose of 2 Gy/min. Mice were sacrificed on day 2 (panel A) or day 5 (panels B, C and D) postirradiation To quantify mucositis after irradiation, ulceration of the tongue and oral cavity was measure in H&E stained tissue sections. Ulceration was defined as loss of thickness of the epithelial layer of tongue as described in the Materials and Methods sections. Panel A: Tongue tissue from *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice were 26 or 28 Gy irradiated and measured on day 2. For *Fancd2*$^{+/+}$ mice, *P* values were not significantly different from unirradiated controls of that genotype at day 2 ($P = 0.1758$ for 26 Gy and $P = 0.2659$ for 28 Gy). Similarly for *Fancd2*$^{+/-}$ mice, *P* values were not significantly different from unirradiated controls of the same genotype ($P = 0.1051$ for 26 Gy and $P = 0.0960$ for 28 Gy). For *Fancd2*$^{-/-}$ mice, *P* values were also not significantly different from their genotypic unirradiated controls ($P = 0.2526$ for 26 Gy and $P = 0.1177$ for 28 Gy). Panel B: Tissues were removed at day 5 after 24, 26, 28 or 30 Gy head and neck irradiation and a radiation dose response curve of ulceration was calculated. All radiation doses to *Fancd2*$^{-/-}$ or *Fancd2*$^{+/-}$ mice showed significantly increased ulceration compared to unirradiated mice of that genotype ($P \leq 0.0197$), *Fancd2*$^{+/-}$ mice at 26 Gy ($P = 0.0522$). Both 24 and 26 Gy radiation doses to *Fancd2*$^{-/-}$ mice cause significantly greater ulceration at day 5 than did the 26 Gy dose to *Fancd2*$^{+/+}$ mice ($P = 0.0123$ and 0.0017, respectively). *Significant ulceration compared to unirradiated mouse tongue of the same genotype. #Significantly increased ulceration after 24 Gy irradiation to the oral cavity of the *Fancd2*$^{-/-}$ mouse compared to 26 Gy irradiation to the oral cavity of *Fancd2*$^{+/+}$ mouse. Panel C: Histopathologic evidence of reduced radiation-induced ulceration by JP4-039/F15. Mice were 28 Gy irradiated with or without JP4-039/F15 treatment before exposure, and tissues were dissected and removed at 5 days postirradiation and ulceration scored. JP4-039/F15-treated mice were irradiated 15 min after the was drug administration. Significant **P* value for reduction of ulceration in the JP4-039-treated groups compared to irradiation control group. Administration of JP4-039 reduced percent ulceration significantly in *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ after 28 Gy exposure ($P \leq 0.0049$). Panel D shows representative tongue histopathology from each group at day 5 after 28 Gy irradiation only or after JP4-039 treatment before 28 Gy irradiation. Ulceration of the tongue was measured in H&E stained tissue sections, as described in the Materials and Methods section. Black arrows show boundary between full loss of epithelium and remaining epithelial layer. H&E staining was applied, as described in the Materials and Methods section. All images are 10× magnification.

pathways, NFkβ, TGFβ, IL-1α, SP-1, AP-1 and GADD45 (for all these genes P value was < 0.0001), (Fig. 3A) (Supplementary Table 2; http://dx.doi.org/10.1667/RR13633.1.S1). Thus, the oral cavity of *Fancd2*$^{-/-}$ and with some genes, heterozygote *Fancd2*$^{+/-}$ mice showed different levels of several RNA transcripts compared to the levels detected in the same oral cavity tissue from *Fancd2*$^{+/+}$ mice.

We next compared baseline levels of femur bone marrow expression of the same ten RNA transcripts between the three genotypes (Fig. 3B). Levels of each of 10 gene transcripts in *Fancd2*$^{-/-}$ or *Fancd2*$^{+/-}$ mice were not significantly different from those levels in *Fancd2*$^{+/+}$ mice (Fig. 3B) (Supplementary Table S2; http://dx.doi.org/10.1667/RR13633.1.S1). All results were compared to *Fancd2*$^{+/+}$ bone marrow. These results establish a lack of significant differences in 10 baseline gene transcript levels in the bone marrow compared to significant differences in oral cavity tissue of *Fancd2*$^{-/-}$ compared to *Fancd2*$^{+/+}$ mice (Supplementary Table 2; http://dx.doi.org/10.1667/RR13633.1.S1). In all studies, twofold increases or decreases were considered significant. It is unclear why some transcript levels were elevated in heterozygote mouse oral cavity (TGFβ) but not in homozygote deletion mice or lower in heterozygote but not in homozygous deletion mice (p21). These differences may reflect the time of tissue sampling, which may show peak elevations at different times or a true single copy gene influence on transcript levels.

The Effect of Head and Neck Irradiation on Levels of RNA Transcripts in the Oral Cavity and Bone Marrow of Fancd2$^{-/-}$ *Mice*

We next tested the effect of 28 Gy head and neck irradiation on both oral cavity and distant femur marrow expression of RNA transcripts in each of the three mouse genotypes. The proinflammatory cytokine IL-1α transcript levels were elevated in irradiated wild-type *Fancd2*$^{+/+}$ mouse oral tissue, confirming data in a prior publication (*24*) (Fig. 3C). In the oral cavity of *Fancd2*$^{-/-}$ mice, there was a significant increase in levels of NFkβ, IL-1α, Nrf2, p21 and p53 at day 5 after 28 Gy irradiation to the head and neck (Fig. 3C). Both heterozygote *Fancd2*$^{+/-}$ and wild-type *Fancd2*$^{+/+}$ mice also showed a decrease in TGFβ after head and neck irradiation (Supplementary Table S3; http://dx.doi.org/10.1667/RR13633.1.S1).

Femur bone marrow from each of the three mouse genotypes showed that head and neck irradiation had altered gene transcript levels. There were increased levels of AP-1 in *Fancd2*$^{-/-}$ mice (Fig. 3D). There was suppression of IL-1α in the bone marrow of *Fancd2*$^{+/-}$ mice after 28 Gy irradiation to the oral cavity. These results establish that irradiation to the head and neck region of each of 3 mouse genotypes altered the levels, although not uniformly, of representative gene transcripts in the bone marrow as well as the oral cavity (Supplementary Table S3; http://dx.doi.org/10.1667/RR13633.1.S1).

Modulation of Radiation-Induced Gene Transcript Responses in the Oral Cavity and Bone Marrow of Fancd2$^{-/-}$ *Mice by Intraoral Administration of JP4-039/F15*

Intraoral administration of JP4-039/F15 before 28 Gy irradiation to the head and neck altered the pattern of gene transcripts in the oral cavity (tongue) of *Fancd2*$^{-/-}$, as well as *Fancd2*$^{+/-}$ and *Fancd2*$^{+/+}$ mice (Fig. 3E) (Supplementary Table S4; http://dx.doi.org/10.1667/RR13633.1.S1). *Fancd2*$^{-/-}$ mice JP4-039/F15-treated then irradiated showed suppression of TGFβ, SP1, MnSOD and Nrf2 (all P values ≤ 0.0056). In *Fancd2*$^{-/-}$ mice, there was elevation of SP1 and GADD45. A decrease in Nrf2 and TFGβ was observed in all 3 genotypes (Fig. 3E).

In contrast, bone marrow from each mouse genotype showed no significant changes in levels of RNA transcripts after intraoral JP4-039/F15 treatment prior to 28 Gy irradiation to the head and neck (Fig. 3F) (Supplementary Table S4; http://dx.doi.org/10.1667/RR13633.1.S1).

We next compared each mouse genotype response to the effect of intraoral JP4-039 treatment on radiation-induced RNA transcripts relative to its own baseline (Fig. 4A–C) (Supplementary Table S5A–C; http://dx.doi.org/10.1667/RR13633.1.S1). The response to JP4-039 was more prominent in the *Fancd2*$^{-/-}$ and *Fancd2*$^{+/-}$ mouse oral cavity relative to its own baseline, than that observed with *Fancd2*$^{+/+}$ mice, particularly with MnSOD.

We measured levels of representative proteins MnSOD, Nrf2 and NFkβ in the oral cavity in irradiated or JP4-039/F15-treated then irradiated mice (Fig. 5) (Supplementary Table S6; http://dx.doi.org/10.1667/RR13633.1.S1). Some protein levels in *Fancd2*$^{-/-}$ mice were concordant with levels of RNA transcripts after irradiation and/or JP4-039/F15 intraoral administration. *Fancd2*$^{-/-}$ mouse oral cavity tissue exhibited increased protein levels of MnSOD (Fig. 5) (Supplementary Table S6; http://dx.doi.org/10.1667/RR13633.1.S1) by Western blot, which correlated to increased levels of RNA transcripts (Fig. 4C). Nrf2 and NFkβ levels were decreased in *Fancd2*$^{+/-}$ mice by both protein and RNA levels after JP4-039/F15 pretreatment (Figs. 4B and 5) (Supplementary Table S6; http://dx.doi.org/10.1667/RR13633.1.S1). The lack of concordance between RNA and protein in other cases may reflect differing kinetics of change based on sampling time or feedback regulation.

Head and Neck Irradiation of Fancd2$^{-/-}$ *Mice Reduces Distant Femur Marrow Colony-Forming Hematopoietic Progenitor Cell Numbers*

The above results establish that there were changes in RNA transcripts in local tissue as well as distant femur bone marrow of *Fancd2*$^{-/-}$ mice after head and neck irradiation. We next quantitated the effects of radiation on head and

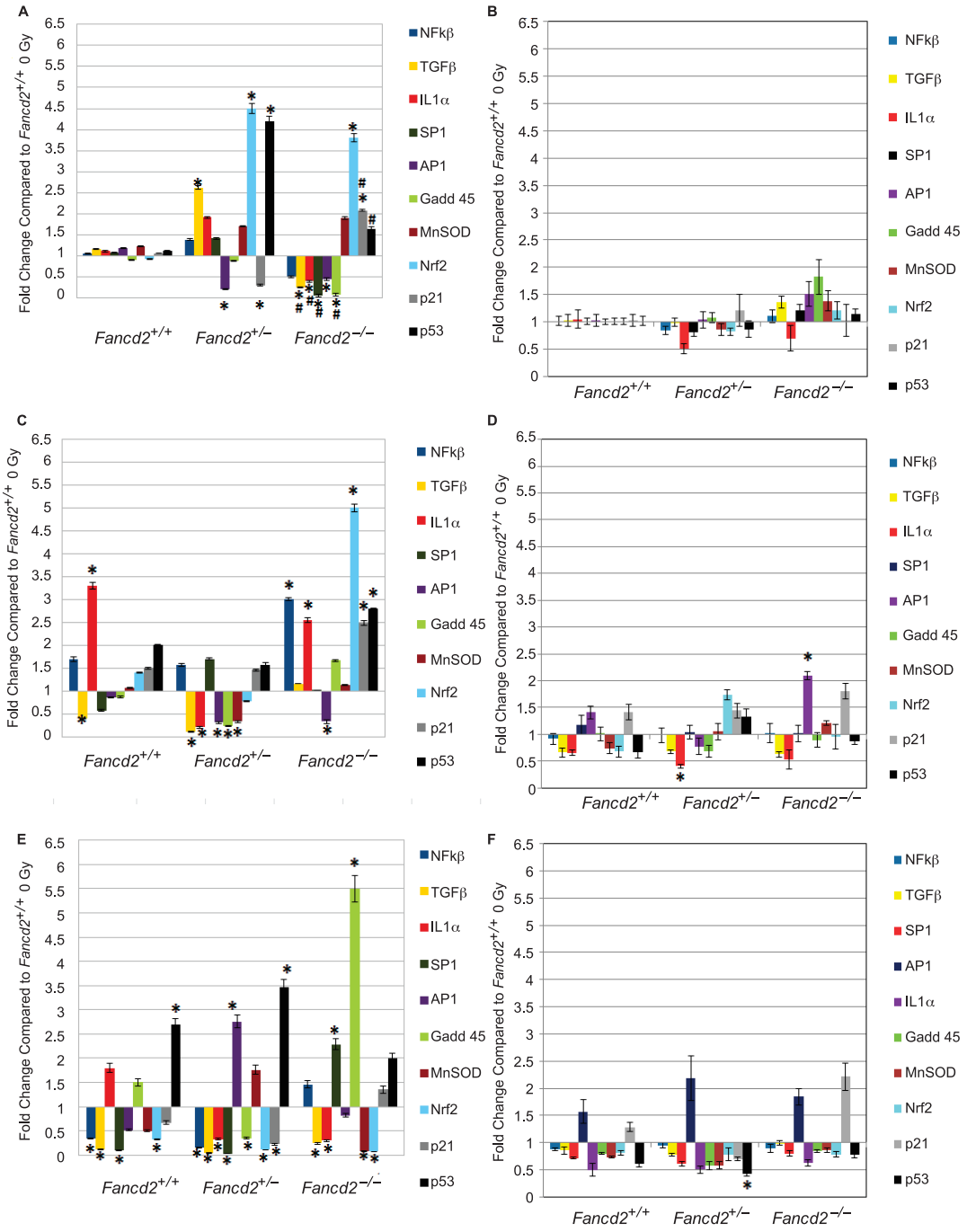

FIG. 3. Radiation-induced gene transcript levels in FVB/N mouse oral cavity and bone marrow are modulated by JP4-039/F15 administration to the oral cavity of *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice. Distinct patterns of oral cavity and femur bone marrow gene transcripts from *Fancd2*$^{-/-}$ mice are shown at baseline or after, head and neck postirradiation with or without intraoral JP4-039/F15 treatment before exposure. Panel A: Tongue tissue from unirradiated oral cavities of *Fancd2*$^{-/-}$ mice compared to *Fancd2*$^{+/-}$ and *Fancd2*$^{+/+}$ mice (n = 5 per genotype). RNA was extracted from tissues using Trizol. RT-PCR was performed, as described in the Materials and Methods section, using primers specific for promoters associated with: p21, p53, Gadd45a, MnSOD, Nrf2, NFkβ, TGFβ1, IL1α, SP1 and AP1. *Significant difference compared to *Fancd2*$^{+/+}$. #Significant difference compared to *Fancd2*$^{+/-}$ unirradiated tongue tissue. All the *P values in the *Fancd2*$^{-/-}$ group for gene transcript level were significant, $P \leq 0.0004$, compared to *Fancd2*$^{+/+}$. All the *P values in the *Fancd2*$^{+/-}$ group for gene transcript level were significant, $P < 0.0001$, compared to *Fancd2*$^{+/+}$. Results were standardized to irradiated *Fancd2*$^{+/+}$ mice. Panel B: Baseline femur bone marrow gene transcripts in *Fancd2*$^{-/-}$ compared to *Fancd2*$^{+/-}$ and *Fancd2*$^{+/+}$ mice. Panel C: Radiation-induced (28 Gy to head and neck) effect on oral cavity gene transcripts at day 5. Results compared to unirradiated *Fancd2*$^{+/+}$. All samples were taken day 5 after 28 Gy exposure to the head and neck. All values are significant, *$P \leq 0.03$, compared to oral cavity from unirradiated *Fancd2*$^{+/+}$ mice. Panel D: Radiation-induced effects (28 Gy to head and neck) on bone marrow gene transcripts. Results compared to unirradiated FVB/N *Fancd2*$^{+/+}$ mice. Statistically significant, *$P \leq 0.049$, compared to bone marrow from unirradiated *Fancd2*$^{+/+}$ mice. Panel E: Intraoral JP4-039/F15 amelioration of radiation-induced effects from 28 Gy exposure to the head and neck on oral cavity gene transcripts at day 5. All P values, in the *Fancd2*$^{-/-}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{+/+}$ groups treated with JP4-039 before exposure, for gene transcript levels were significant, $P \leq 0.0220$, when each was compared to irradiated controls. Results are from day 5

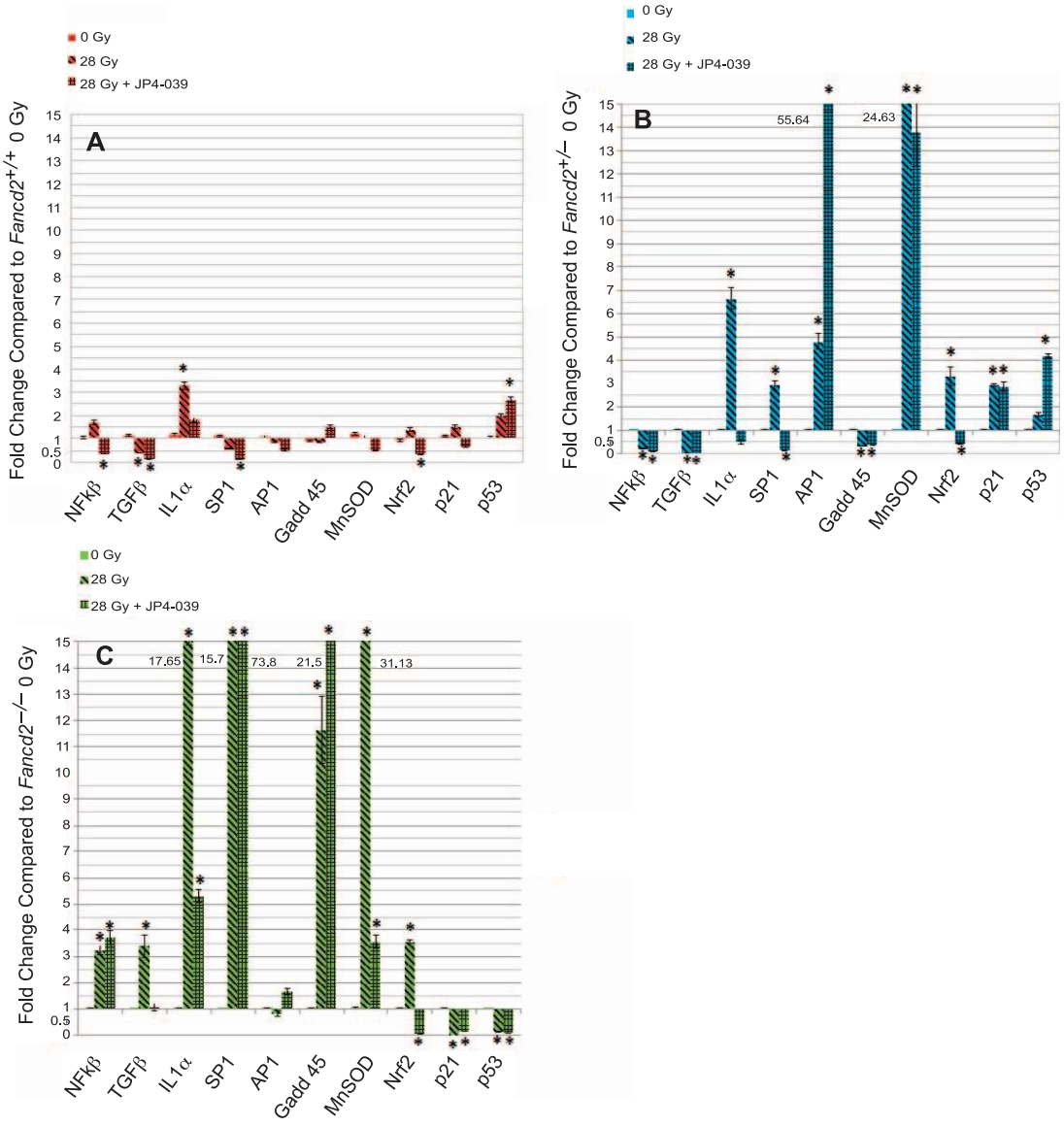

FIG. 4. Effects of intraoral JP4-039 treatment on 28 Gy irradiation-induced oral cavity RNA transcripts in *Fancd2*$^{+/+}$ (panel A), *Fancd2*$^{+/-}$ (panel B) and *Fancd2*$^{-/-}$ (panel C) mice relative to each genotype. RNA was extracted from tongue tissue of mice sacrificed at day 5 after 0, 28 Gy or JP4-039/F15 treatment before 28 Gy exposure to the head and neck. RT-PCR was performed, as described in the Materials and Methods section, using primers for p21, p53, Gadd45a MnSOD, Nrf2, NFkβ, TGFβ1, IL-1α, SP1 and AP1. Gene transcripts for 0, 28 Gy or JP4-039/F15 treatment with 28 Gy exposure in mice of each genotype: *Fancd2*$^{+/+}$ (panel A), *Fancd2*$^{+/-}$ (panel B) or *Fancd2*$^{-/-}$ (panel C) mice. Comparisons are shown for 28 Gy and JP4-039/F15 treatment with 28 Gy exposure compared to 0 Gy for each respective genotype. *Significant difference compared to 0 Gy. (Supplementary Table S5A, B and C; http://dx.doi.org/10.1667/RR13633.1.S1). Values were considered significant if there was at least a twofold increase or decrease (fold change of $\leq$ 0.5) in gene expression.

neck biologic parameters of hematopoiesis in the blood and bone marrow in *Fancd2*$^{-/-}$ mice. Blood from unirradiated *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice as well as from mice at day 2 or day 5 after head and neck irradiation was collected from each group. Red blood cell, white blood cell, lymphocyte and neutrophil counts in the irradiated group were not significantly reduced compared to unirradiated controls for *Fancd2*$^{+/+}$ or *Fancd2*$^{+/-}$ mice at the times tested (Supplementary Table S7; http://dx.doi.org/10.1667/RR13633.1.S1). There was a change in platelets, white

←
after head and neck irradiation and standardized to unirradiated *Fancd2*$^{+/+}$ mice. Panel F: JP4-039/F15 amelioration of radiation-induced effects from 28 Gy exposure on femur bone marrow transcripts. Results are standardized to unirradiated *Fancd2*$^{+/+}$ mouse marrow. *Statistically significant compared to unirradiated *Fancd2*$^{+/+}$. Values for all panels were considered significant if there was at least a twofold increase or decrease (fold change of $\leq$0.5) in gene expression. Statistical evaluation can be found in Supplementary Tables S2–S4 (http://dx.doi.org/10.1667/RR13633.1.S1).

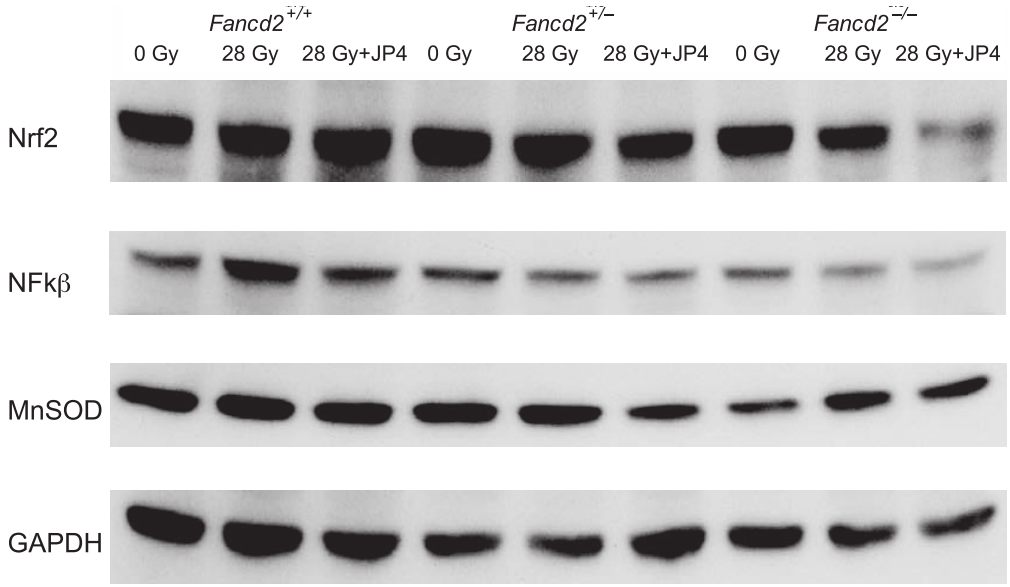

FIG. 5. Protein levels in oral cavity tissue at day 5 after 28 Gy irradiation to the head and neck of *Fancd2*$^{-/-}$ (FVB/N) mice. Tongue tissue was removed from unirradiated mice or day 5 after head and neck irradiation with 28 Gy or JP4-039/F15 treatment with 28 Gy exposure of *Fancd2*$^{+/+}$, *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ mice. Western analysis was performed using antibodies to Nrf2, NFkβ, MnSOD or GAPDH, as described in the Materials and Methods section. Protein level was quantitated using LabWorks Image Acquisition and Analysis Software (Supplementary Table S6; http://dx.doi.org/10.1667/RR13633.1.S1).

blood cells and lymphocyte levels in *Fancd2*$^{-/-}$ mice (Supplementary Table S7; http://dx.doi.org/10.1667/RR13633.1.S1), but there was no detectable difference in marrow cellularity between genotypes at day 5 after 28 Gy head and neck irradiation (Supplementary Fig. S1; http://dx.doi.org/10.1667/RR13633.1.S1).

We next compared baseline and postirradiation head and neck levels of bone marrow colony-forming progenitor cells in femur marrow from each genotype. There were reduced baseline numbers of CFU-GM in femur bone marrow from unirradiated *Fancd2*$^{-/-}$ mice compared to *Fancd2*$^{+/+}$ mice, $P = 0.0333$ (Table 2), although there was no detectable difference in baseline BFU-E or CFU-GEMM numbers (Table 2).

Hematopoietic colony assays were next performed with femur bone marrow harvested from the irradiated mice groups at day 2 or 5 after 24, 26, 28 or 30 Gy exposure to the head and neck. *Fancd2*$^{-/-}$ mice showed a significant reduction in CFU-GM, BFU-E and CFU-GEMM colony-forming cells at both days 2 and 5 after irradiation to the head and neck at each radiation dose (Table 2) (26 Gy, day 2, $P = 0.0166$; 28 Gy, day 2, $P = 0.0281$; 24 Gy, day 5, $P = 0.0124$; and 26 Gy, day 5, $P = 0.0338$). Decreased CFU-GM and CFU-GEMM numbers were also detected in *Fancd2*$^{+/-}$ mice at days 2 and 5 after 26 Gy or 24 Gy irradiation to the head and neck (Table 2). CFU-GM were significantly reduced at day 2 after 26 Gy irradiation in *Fancd2*$^{+/-}$ mice compared to unirradiated controls ($P = 0.0007$). *Fancd2*$^{-/-}$ mice had decreased numbers of BFU-E at day 5 after 24 or 26 Gy ($P = 0.0085$ or 0.0313) and reduced numbers of CFU-GEMM after 24 or 28 Gy ($P = 0.0004$ or 0.0413, respectively). Both *Fancd2*$^{-/-}$ and *Fancd2*$^{+/-}$ mice, at day 5 after 24 Gy irradiation, had fewer CFU-GM ($P = 0.0124$ and $P = 0.0011$, respectively) and CFU-GEMM ($P = 0.0144$ and $P = 0.0004$, respectively) compared to unirradiated controls. There was no reduction in numbers of CFU-GM, BFU-E or CFU-GEMM in wild-type *Fancd2*$^{+/+}$ mice after 28 or 30 Gy irradiation to the head and neck. At days 2 and 5 after 26 Gy irradiation, there was an increased number of BFU-E in irradiated *Fancd2*$^{+/+}$ mice compared to unirradiated mice, but reduced CFU-GM at day 5 after 26 Gy irradiation (Table 2). The data establish a prominent distant ''bystander'' or ''abscopal'' bone marrow suppression effect from irradiation of the head and neck in femur bone marrow of *Fancd2*$^{-/-}$ mice.

Intraoral JP4-039/F15 Effects on Distant Bone Marrow Suppression in Head and Neck Irradiated Fancd2$^{-/-}$*Mice*

We tested whether irradiation of the head and neck induced suppression of bone marrow CFU-GM in *Fancd2*$^{-/-}$ mice were ameliorated by the administration of intraoral JP4-039/F15 prior to exposure. On day 5 the 26 Gy irradiated control *Fancd2*$^{+/+}$ mice, there was a significant decrease in CFU-GM compared to the 0 Gy control mice ($P = 0.0172$) (Table 3), and JP4-039/F15 treatment did ameliorate the difference compared to radiation treatment alone ($P = 0.0107$), where CFU-GM colony-forming progenitor numbers returned to control levels by JP4-039/F15 treatment (Table 3). However, JP4-039/F15 treatment produced no detectable increase in numbers of BFU-E or CFU-GEMM in the bone marrow of *Fancd2*$^{+/+}$ mice after exposure to 26 or 28 Gy. In *Fancd2*$^{+/-}$ mice, JP4-039/F15

TABLE 2
Effect of Head and Neck Irradiation on Marrow Granulocyte-Macrophage Hematopoietic Colony Forming Cells (CFU-GM) in *Fancd2*$^{-/-}$Mice

		Day 2		Day 5			
	0 Gy	26 Gy	28 Gy	24 Gy	26 Gy	28 Gy	30 Gy
A.							
CFU-GM							
FancD2$^{-/-}$	44.0 ± 8.1	29.2 ± 1.6	33.7 ± 2.3	31.8 ± 6.1	34.2 ± 2.8	37.6 ± 6.2	N.T.
	$P_{+/+}$ = 0.0333	P_0 = 0.0166	P_0 = 0.0281	P_0 = 0.0124	P_0 = 0.0338	P_0 = 0.1457	
FancD2$^{+/-}$	47.4 ± 5.7	33.0 ± 3.9	39.9 ± 7.2	33.7 ± 4.7	42.9 ± 8.9	50.2 ± 4.8	N.T.
	$P_{+/+}$ = 0.0891	P_0 = 0.0007	P_0 = 0.2016	P_0 = 0.0011	P_0 = 0.3026	P_0 = 0.4724	
FancD2$^{+/+}$	54.8 ± 7.1	47.8 ± 14.3	61.0 ± 0.3	N.T.	35.7 ± 4.5	56.0 ± 9.6	50.4 ± 1.9
		P_0 = 0.2163	P_0 = 0.1519		$P_0 < 0.0001$	P_0 = 0.7740	P_0 = 0.1658
B.							
BFU-E							
FancD2$^{-/-}$	34.6 ± 11.7	24.5 ± 9.7	35.7 ± 9.8	22.1 ± 2.0	24.9 ± 6.6	28.6 ± 4.9	28.3 ± 3.7
	$P_{+/+}$ = 0.0893	P_0 = 0.0785	P_0 = 0.8181	P_0 = 0.0085	P_0 = 0.0313	P_0 = 0.1588	P_0 = 0.6679
FancD2$^{+/-}$	30.2 ± 4.4	33.0 ± 5.8	32.2 ± 8.0	35.3 ± 9.3	32.7 ± 7.7	32.7 ± 3.8	N.T.
	$P_{+/+}$ = 0.4050	P_0 = 0.4745	P_0 = 0.3133	P_0 = 0.3133	P_0 = 0.5782	P_0 = 0.4944	
FancD2$^{+/+}$	27.1 ± 4.6	60.6 ± 13.3	36.6 ± 8.8	N.T.	42.1 ± 2.8	35.0 ± 3.3	28.3 ± 3.7
		$P_0 < 0.0001$	**P_0 = 0.0127**		**$P_0 < 0.0001$**	**P_0 = 0.0064**	P_0 = 0.6679
C.							
CFU-GEMM							
Fancd2$^{-/-}$	2.7 ± 0.9	1.8 ± 0.2	2.4 ± 0.4	1.1 ± 0.4	2.1 ± 0.8	1.8 ± 0.7	N.T.
	$P_{+/+}$ = 0.2837	P_0 = 0.0482	P_0 = 0.5201	P_0 = 0.0004	P_0 = 0.2395	P_0 = 0.0413	
Fancd2$^{+/-}$	2.2 ± 0.8	2.7 ± 0.3	2.3 ± 0.3	1.3 ± 0.3	2.1 ± 0.5	2.2 ± 0.2	N.T.
	$P_{+/+}$ = 1.0000	P_0 = 0.2401	P_0 = 0.5789	P_0 = 0.0144	(n = 3)	(n = 3)	
					P_0 = 0.7498	P_0 = 1.0000	
Fancd2$^{+/+}$	2.2 ± 0.5	4.3 ± 0.6	2.9 ± 0.7	N.T.	1.8 ± 0.7	2.4 ± 0.5	2.6 ± 0.4
					(n = 3)	(n = 3)	(n = 3)
		$P_0 < 0.0001$	P_0 = 0.1561		P_0 = 0.2746	P_0 = 0.5086	P_0 = 0.3256

Notes. Colony data are presented as mean ± standard deviation (n = 3/group). Comparison between groups was made using the two-sided two-sample *t* test. *P* values less than 0.05 were regarded as significant. Significant decrease in mean colony count is represented by underlined values. *P* values in bold represent significant increase in mean colony count. P_0 is the *P* value for the comparison with the corresponding 0 Gy group. $P_{+/+}$ is the *P* value for the comparison with the corresponding *Fancd2*$^{+/+}$ cell group. N.T. = not tested.

treatment showed no significant effect on CFU-GM, BFU-E or CFU-GEMM numbers compared to 26 or 28 Gy irradiated controls (Table 3). In contrast, with *Fancd2*$^{-/-}$ mice, intraoral JP4-039/F15 treatment prior to 26 Gy head and neck irradiation modulated the decrease in CFU-GM numbers compared to the level seen in 26 Gy irradiated mice ($P = 0.0090$), but there was less amelioration of the magnitude of femur bone marrow suppression of CFU-GEMM (Table 3).

DISCUSSION

The current results establish that the toxicity of head and neck irradiation using high-dose single fractions typical of those for SRS or SBRT is increased in *Fancd2*$^{-/-}$ (FVB/N) mice as measured by local radiation-induced mucositis, distant bone marrow suppression and biomarker gene transcription responses in both organs. Both *Fancd2*$^{-/-}$ and heterozygote *Fancd2*$^{+/-}$ mice were radiosensitive to head and neck irradiation compared to control *Fancd2*$^{+/+}$ mice measured as oral cavity tissue ulceration at day 5 postirradiation. These results confirm and extend previous publications showing radiosensitivity of total-body-irradiated *Fancd2*$^{-/-}$ mice and the critical role of the Fancd2 protein in the response to the radiation injury (*9–13, 16, 25–28*). Intraoral JP4-039/F15 protected normal tissues in head and neck irradiated *Fancd2*$^{-/-}$ mice as well as control *Fancd2*$^{+/+}$ mice. These data confirm prior studies showing that GS-nitroxides, including JP4-039, work at the level of the mitochondria to inhibit apoptosis, and extend these results to tissues and cells from experimental animals with a defective DNA damage response pathway (*14, 29–32*).

Baseline levels of gene transcripts for several categories of oxidative stress response pathways in unirradiated *Fancd2*$^{-/-}$ and *Fancd2*$^{+/-}$ heterozygote mouse oral cavity and bone marrow were different from those in wild-type *Fancd2*$^{+/+}$ mice. The data suggest compensatory baseline gene expression responses to the intrinsic DNA damage response defect in *Fancd2*$^{-/-}$ mice that are reflected as levels of expression of proinflammatory, oxidative stress and apoptosis related genes (*32*).

The elevated baseline levels of Nrf2 and p21 in *Fancd2*$^{-/-}$ mouse oral cavity tissues may indicate a compensatory oxidative stress response mechanism for Fancd2 protein

TABLE 3
Effect of Intraoral JP4-039 Treatment on Hematopoietic Colony Forming Cells in *Fancd2*$^{-/-}$ Mouse Femur Bone Marrow on Day 5 after 26 Gy Head and Neck Irradiation

Genotype	Experimental condition	CFU-GM	BFU-E	CFU-GEMM
Fancd2$^{-/-}$	0 Gy exposure	44.0 ± 8.1	34.6 ± 11.7	2.7 ± 0.9
		$P_{+/+} = 0.1584$	$P_{+/+} = 0.3622$	$P_{+/+} = 0.4918$
	0 Gy exposure with JP4-039 treatment	54.2 ± 7.0	33.1 ± 5.9	2.3 ± 0.3
		$P0 = 0.1352$	$P0 = 0.8334$	$P0 = 0.4122$
		$P_{+/+} = 0.6033$	$P_{+/+} = 0.7252$	$P_{+/+} = 0.3599$
	26 Gy exposure	34.2 ± 2.8	24.9 ± 6.6	2.1 ± 0.8
		$P0 = 0.1188$	$P0 = 0.2802$	$P0 = 0.4734$
		$P_{+/+} = 0.6612$	$\mathbf{P_{+/+} = 0.0140}$	$P_{+/+} = 0.6240$
	26 Gy exposure with JP4-039 treatment	50.9 ± 5.4	35.2 ± 4.7	1.6 ± 0.2
		$P0 = 0.2876$	$P0 = 0.9314$	$P0 = 0.1000$
		$\mathbf{P1 = 0.0090}$	$P1 = 0.0917$	$P1 = 0.3262$
		$P_{+/+} = 0.6129$	$P_{+/+} = 0.7054$	$\mathbf{P_{+/+} = 0.0241}$
	28 Gy exposure	37.6 ± 6.2	28.6 ± 4.9	1.8 ± 0.7
		$P0 = 0.3348$	$P0 = 0.4578$	$P0 = 0.2420$
		$\mathbf{P_{+/+} = 0.0495}$	$P_{+/+} = 0.1310$	$P_{+/+} = 0.2508$
	28 Gy exposure with JP4-039 treatment	43.5 ± 1.6	32.0 ± 1.9	1.7 ± 0.0
		$P0 = 0.9398$	$P0 = 0.7898$	$P0 = 0.1885$
		$P2 = 0.2951$	$P2 = 0.4266$	$P2 = 0.8075$
		$P_{+/+} = 0.0850$	$P_{+/+} = 0.9897$	$\mathbf{P_{+/+} = 0.0305}$
Fancd2$^{+/-}$	0 Gy exposure	47.4 ± 5.7	30.2 ± 4.4	2.2 ± 0.8
	0 Gy exposure with JP4-039 treatment	53.5 ± 2.1	38.8 ± 7.3	2.0 ± 0.0
		$P0 = 0.2606$	$P0 = 0.1857$	$P0 = 0.6667$
	26 Gy exposure	42.9 ± 8.9	32.7±7.7	2.1 ± 0.5
		$P0 = 0.4949$	$P0 = 0.6568$	$P0 = 0.8450$
	26 Gy exposure with JP4-039 treatment	49.1 ± 4.6	33.4 ± 5.3	2.1 ± 0. 2
		$P0 = 0.7119$	$P0 = 0.4611$	$P0 = 0.8203$
		$P1 = 0.3398$	P1 = 0.8922	$P1 = 1.0000$
	28 Gy exposure	50.2 ± 4.8	32.7 ± 3.8	2.2 ± 0.2
		$P0 = 0.5540$	$P0 = 0.5062$	$P0 = 1.0000$
	28 Gy exposure with JP4-039 treatment	54.6 ± 9.2	50.2 ± 12.1	2.7 ± 0.0
		$P0 = 0.3181$	$P0 = 0.0547$	$P0 = 0.4266$
		$P2 = 0.5101$	$P2 = 0.0751$	$P2 = 0.0572$
Fancd2$^{+/+}$	0 Gy exposure	54.8 ± 7.1	27.1 ± 4.6	2.2 ± 0.5
	0 Gy exposure with JP4-039 treatment	50.8 ± 9.3	34.4 ± 2.2	2.0 ± 0.3
		$P0 = 0.5853$	$P0 = 0.0661$	$P0 = 0.5614$
	26 Gy exposure	35.7 ± 4.5	42.1 ± 2.8	1.8 ± 0.7
		$\mathbf{P0 = 0.0172}$	$\mathbf{P0 = 0.0082}$	$P0 = 0.4216$
	26 Gy exposure with JP4-039 treatment	53.2 ± 5.0	33.6 ± 5.3	2.1 ± 0.2
		$P0 = 0.7725$	$P0 = 0.1859$	$P0 = 0.7415$
		$\mathbf{P1 = 0.0107}$	$P1 = 0.0692$	$P1 = 0.4676$
	28 Gy exposure	56.0 ± 9.6	35.0 ± 3.3	2.4 ± 0.5
		$P0 = 0.8685$	$P0 = 0.0726$	$P0 = 0.6213$
	28 Gy exposure with JP4-039 treatment	57.6 ± 7.3	32.1±10.6	2.8 ± 0.4
		$P0 = 0.6628$	$P0 = 0.4944$	$P0 = 0.2062$
		$P2 = 0.8350$	$P2 = 0.6757$	$P2 = 0.4169$

Notes. Colony data are presented as mean ± standard deviation. Comparison between groups was made using the two-sided two-sample *t* test. *P* values less than 0.05 were regarded as significant and are shown in bold. *P*0 is the *P* value for the comparison with 0 Gy exposed group. *P*1 is the *P* value for the comparison with 26 Gy exposed only group and *P*2 is the *P* value for the comparison with 28 Gy exposed only group. $P_{+/+}$ is the *P* value for the comparison with the *Fancd2*$^{+/+}$ group under the same experimental condition.

deficiency. Radiation exposure increased oral cavity levels of p53, p21, Nrf2, IL-12 and NFkβ in *Fancd2*$^{-/-}$ mice, which were modulated by JP4-039/F15-treatment before irradiation; perhaps due to neutralization of proinflammatory and pro-apoptotic cellular responses. NFkβ is a regulator of radiation-induced genes involved in mucositis (*24*) known to induce production of IL-1 and TGFβ, and to regulate other parameters of inflammation (*24*). Chemotherapeutic agents, which upregulate NFkβ and subsequent proinflammatory cytokines (TGFβ, IL-6 and IL-1β), have also been shown to increase radiation mucositis (*24*). Since both *Fancd2*$^{-/-}$ and *Fancd2*$^{+/-}$ mice had significantly more histopathologic evidence of radiation-induced oral cavity damage, the lower levels of TGFβ may relate to the reduced protection of epithelial barrier function. The current data indicate that intraoral administration of JP4-039/F15

partially reverses ionizing radiation-induced tissue damage and modulates (although inconsistently) levels of damage-associated biomarkers in *Fancd2*$^{-/-}$, *Fancd2*$^{+/-}$ and control *Fancd2*$^{+/+}$ mice.

There was a significant effect of JP4-039 on the p53/p21 signaling pathway. There were significant increases in some DNA damage response transcripts (such as p53, p21 and Nrf2) in oral tissues from irradiated *Fancd2*$^{-/-}$ mice, and JP4-039/F15 treatment lowered levels of all 3 inhibitors (Fig. 3E). This pattern was not observed in the oral cavity of *Fancd2*$^{+/-}$ or *Fancd2*$^{+/+}$ mice (Fig. 3E), and may appear inconsistent with the observed inhibition of apoptosis as the proposed method of action of the drug. The observation that p21 levels were lower in *Fancd2*$^{+/-}$ and *Fancd2*$^{+/+}$ mice, while p53 levels were high (Fig. 3E), may be explained by time of sampling with regard to peak transcript levels in different genotypes, or nonuniform drug action on different components of the apoptosis signaling pathway in each genotype. This discrepancy may be resolved by future measurements of transcript levels for genes involved in distal steps in the apoptotic pathway, including caspase-3 and poly-ADP-ribosyl polymerase at multiple time points after irradiation and JP4-039/F15 drug treatment.

The current studies also document distant marrow suppression by head and neck irradiation in *Fancd2*$^{-/-}$ mice. The distant or ''abscopal'' effect was detectable at lower head and neck radiation doses of 24 or 26 Gy compared to the dose of 28 Gy required to produce comparable local oral mucosal damage in wild-type mice. Marrow suppression in *Fancd2*$^{-/-}$ mice by head and neck irradiation was accompanied by a modest reduction in peripheral blood counts and reduced CFU-GM, BFU-E and CFU-GEMM numbers, but had no effect on marrow cellularity. A higher level of muscle tissue damage by the 28 Gy dose in *Fancd2*$^{-/-}$ mice may have accelerated release of humoral mediators that suppressed distant marrow progenitor cell numbers. We did not analyze the effects of head and neck irradiation or JP4-039/F15 intraoral administration on femur marrow totipotential bone marrow hematopoietic stem cells that reconstitute lethally irradiated recipient mice. Previous publications have demonstrated that *Fancd2*$^{-/-}$ mice have reduced bone marrow stem cell numbers, quantitated by competitive repopulation assay (*33, 34*). These prior studies also documented the genotoxic consequences of endogenous aldehyde production on bone marrow stem cells and the protective effect of the Fancd2 protein (*33, 34*). Observations in the *Fancd2*$^{-/-}$ mouse model have been extended to clinical observations in Fanconi anemia patients with variant ALDH2 and bone marrow failure (*35*). Whether ionizing radiation induces aldehydes and/or other forms of DNA crosslinking molecules in *Fancd2*$^{-/-}$ mice is the subject of ongoing investigations.

In the current studies, suppression of CFU-GM, BFU-E and CFU-GEMM colony-forming progenitor cells by head and neck irradiation in *Fancd2*$^{-/-}$ mice indicates that distant marrow hematopoietic progenitor cells may be a sensitive marker for measuring levels of toxic mediator(s) released by irradiated tissue. *Fancd2*$^{-/-}$ mice may be a valuable model system in which to isolate these factor(s) released in irradiated tissues, which produce the distant ''bystander'' or ''abscopal'' effect. There was a modest, but clear amelioration of distant marrow suppression by JP4-039 intraoral treatment. Further studies will be required to elucidate the distant marrow suppression observed in head and neck irradiated *Fancd2*$^{-/-}$ mice, and the mechanism of local tissue radioprotection by intraoral JP4-039.

SUPPLEMENTARY INFORMATION

Supplementary Table S1. Statistical analysis of radiation dose dependent increased percent ulceration in the oral cavity of *Fancd2*$^{-/-}$ mice and amelioration by JP4-039/F15.

Supplementary Table S2. Statistical analysis of relative levels of baseline gene transcripts in the bone marrow and tongue of *Fancd2*$^{-/-}$ mice.

Supplementary Table S3. Statistical analysis of the relative levels of gene transcripts in the bone marrow and tongue from ten of the 28 Gy irradiated *Fancd2*$^{-/-}$ mice.

Supplementary Table S4. Statistical analysis of relative levels of gene transcripts in the bone marrow and tongue of the ten irradiated and JP4-039-treated *Fancd2*$^{-/-}$ mice standardized to *Fancd2*$^{+/+}$ or *Fancd2*$^{+/-}$ mice.

Supplementary Table S5A. Statistical analysis of relative levels of gene transcripts in the oral cavity tissue of the 10 irradiated and JP4-039-treated *Fancd2*$^{+/+}$ mice standardized to their own genotype baseline.

Supplementary Table S5B. Statistical analysis of relative levels of gene transcripts in the oral cavity tissue of the 10 irradiated and JP4-039-treated *Fancd2*$^{+/-}$ mice standardized to their own genotype baseline.

Supplementary Table S5C. Statistical analysis of relative levels gene transcripts in the oral cavity tissue of the 10 irradiated and JP4-039-treated *Fancd2*$^{-/-}$ mice standardized to their own genotype baseline.

Supplementary Table S6. Levels of expression of Nrf2, NFkβ and MnSOD protein of *Fancd2* $^{+/+}$, *Fancd2* $^{+/-}$ or *Fancd2*$^{-/-}$ mice treated with JP4-039/F15 before head and neck irradiation.

Supplementary Table S7. Complete blood counts (CBC) are not altered by head and neck irradiation of *Fancd2*$^{-/-}$ mice compared to *Fancd2*$^{+/-}$ and *Fancd2*$^{+/+}$ mice (each group is 5 mice).

Supplementary Table S7A. CBC of *Fancd2*$^{+/+}$ mice on day 0 compared to the CBC of *Fancd2*$^{+/-}$ and *Fancd2*$^{-/-}$ at day 0 baseline.

Supplementary Table S7B. CBC of *Fancd2*$^{+/+}$ mice at days 2 and 5 after irradiation compared to CBC of 0 Gy irradiated *Fancd2*$^{+/+}$ mice.

Supplementary Table S7C. CBC from *Fancd2*$^{+/-}$ mice at days 2 and 5 after irradiation compared to CBC from *Fancd2*$^{+/-}$ mice at day 0.

Supplementary Table S7D. CBC from *Fancd2*$^{-/-}$ mice at days 2 and 5 after irradiation compared to CBC from *Fancd2*$^{-/-}$ mice at day 0.

Supplementary Fig. S1A. Femur bone marrow cellularity at day 5 after head and neck irradiation of *Fancd2*$^{-/-}$ mice.

Supplementary Fig. S1B. Bone marrow cellularity in *Fancd2*$^{+/-}$ mice.

Supplementary Fig. S1C. Bone marrow cellularity in *Fancd2*$^{+/+}$ mice.

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