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Radiologic Differences between Bone Marrow Stromal and Hematopoietic Progenitor Cell Lines from Fanconi Anemia (*Fancd2*^{-/-}) Mice

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Abstract

Fancd2 plays a central role in the human Fanconi anemia DNA damage response (DDR) pathway. *Fancd2*^{-/-} mice exhibit many features of human Fanconi anemia including cellular DNA repair defects. Whether the DNA repair defect in *Fancd2*^{-/-} mice results in radiologic changes in all cell lineages is unknown. We measured stress of hematopoiesis in long-term marrow cultures and radiosensitivity in clonogenic survival curves, as well as comet tail intensity, total antioxidant stores and radiation-induced gene expression in hematopoietic progenitor compared to bone marrow stromal cell lines. We further evaluated radioprotection by a mitochondrial-targeted antioxidant GS-nitroxide, JP4-039. Hematopoiesis longevity in *Fancd2*^{-/-} mouse long-term marrow cultures was diminished and bone marrow stromal cell lines were radiosensitive compared to *Fancd2*^{+/+} stromal cells (*Fancd2*^{-/-} D₀ = 1.4 ± 0.1 Gy, $\bar{n}$ = 5.0 ± 0.6 vs. *Fancd2*^{+/+} D₀ = 1.6 ± 0.1 Gy, $\bar{n}$ = 6.7 ± 1.6), P = 0.0124 for D₀ and P = 0.0023 for $\bar{n}$, respectively). In contrast, *Fancd2*^{-/-} IL-3-dependent hematopoietic progenitor cells were radioresistant (D₀ = 1.71 ± 0.04 Gy and $\bar{n}$ = 5.07 ± 0.52) compared to *Fancd2*^{+/+} (D₀ = 1.39 ± 0.09 Gy and $\bar{n}$ = 2.31 ± 0.85, P = 0.001 for D₀). CFU-GM from freshly explanted *Fancd2*^{-/-} marrow was also radioresistant. Consistent with radiosensitivity, irradiated *Fancd2*^{-/-} stromal cells had higher DNA damage by comet tail intensity assay compared to *Fancd2*^{+/+} cells (P < 0.0001), slower DNA damage recovery, lower baseline total antioxidant capacity, enhanced radiation-induced depletion of antioxidants, and increased CDKN1A-p21 gene transcripts and protein. Consistent with radioresistance, *Fancd2*^{-/-} IL-3-dependent hematopoietic cells had higher baseline and post irradiation total antioxidant capacity. While, there was no detectable alteration of radiation-induced cell cycle arrest with *Fancd2*^{-/-} stromal cells, hematopoietic progenitor cells showed reduced G₂/M cell cycle arrest. The absence of the mouse *Fancd2* gene product confers radiosensitivity to bone marrow stromal but not hematopoietic progenitor cells.

INTRODUCTION

Fanconi anemia (FA) is an autosomal recessive syndrome associated with a biallelic mutation in one or more of the 15 FA pathway gene products leading to bone marrow

failure, defective DNA damage response and predisposition to cancer (1). Fanconi anemia consists of defects in one or more of 15 complementation groups (A, B, C, D1, D2, E, F and G). FancA, FancC, FancF and FancG, proteins interact to form a nuclear complex, which is required for the downstream activation of the human *FancD1* (BRCA2) protein. Activation of human *FancD2* results in the assembly of *FancD2*/BRCA1 foci, which participate in DNA double-strand break repair (1). A review of the FA pathway and interaction of the multiple gene products with other elements of the DNA repair process has recently been published (2). It is unknown whether inactivation or absence of any one FA pathway gene alters ionizing radiation sensitivity uniformly in all cell lineages.

The defect in DNA damage response in some FA patients makes them sensitive to ionizing radiation, limiting effective radiotherapy for solid tumors as well as total-body irradiation for marrow transplantation (3). The radiosensitivity of human *FancD2* cell lines has been shown to be greater than that of cell lines from patients with the *FancG*^{-/-}, *FancA*^{-/-} or the *FancB*^{-/-} genotype (4). The radiosensitivity of *Fancd2*^{-/-} mice is consistent with the radiosensitivity of *FancD2*^{-/-} patient cell lines (5, 6). Radiosensitivity is not a universal feature of FA patient-derived cells (7, 8).

In the studies presented here, we evaluated the longevity of hematopoiesis in *Fancd2*^{-/-} mouse long-term marrow cultures. We also compared the radiosensitivity of hematopoietic progenitor cell lines to stromal cells (mesenchymal stem cells) and analyzed stromal cells and hematopoietic progenitor cell lines for radiation induced alteration in cell cycle distribution. Furthermore, we investigated DNA damage response by comet tail intensity, induction of pro-inflammatory, oxidative stress and cell cycle regulating gene products, irradiation effects on total antioxidant stores and the effect on radiosensitivity of the mitochondrial-targeted reactive oxygen species (ROS) scavenger JP4-039 (9).

METHODS

Mice

Fancd2^{-/-}, *Fancd2*^{+/-} and *Fancd2*^{+/+} (C57BL/6J background) (10) were generously provided by the Dana Farber Cancer Institute. Mice were housed 4/cage according to Institutional IACUC regulations and fed standard Purina laboratory chow.

Long-Term Bone Marrow Cultures

Long-term bone marrow cultures (LTBMC) were established from the femur and tibia marrow of *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} mice as described previously (11–13). 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% CO₂. After 4 weeks, the horse serum was replaced with 25% FBS (Gibco, Gaithersburg, MD) (14). The cultures were observed weekly for hematopoietic cell production and cobblestone island formation. Cobblestone islands of greater than or equal to 50 cells were scored weekly in each flask 12–14). A two-sided two-sample *t* test was used to compare the number of cobblestone islands between *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} cultures each week. *P* values less than 0.05 were regarded as significant.

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

Non-adherent cells were harvested from *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} mouse LTBMC at week 4 and cultured in six-well tissue culture plates in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal calf serum (FCS) 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 using published methods (14, 15).

Clonal cell sublines were established from each of the *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 of IL-3. Cells were then replated in methylcellulose, colonies selected at 14 days and cultured as above, to establish subcloned lines. Confirmation of genotype after repeated subcloning was carried out for each cell line.

Hematopoietic Cell Colony-Forming Assays

A total of 5×10^4 non-adherent cells from the *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} long-term bone marrow cultures were removed from the LTBMCM flasks and 1.67×10^3 cells/dish were plated in triplicate in semi-solid medium consisting of methylcellulose in IMDM, FBS, 10% bovine serum albumin (BSA), WEHI-3 cell line conditioned medium (15) (as a source of IL-3), L-glutamine, 3 U/mL erythropoietin and 2-mercaptoethanol. Colony forming unit granulocyte-macrophage (CFU-GM) of 50 cells or greater were counted on days 7 and 14 after plating. A two-sided two-sample *t* test was used as described above. Fresh marrow colonies were subdivided for GFU-GM, burst forming unit erythroid (BFU-E) and granulocyte-erythroid-megakaryocyte-macrophage. (CFU-GEMM)

Bone Marrow Stromal Cell Lines and Clonal Cell Sublines

Adherent cell layers from one 4-week-old LTBMCM from *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} mice were trypsinized and expanded by passage into DMEM + 10% FBS to establish bone marrow stromal cell lines according to published methods (12). Cells were passaged for 10 weeks to establish cell lines. Cultures were incubated at 37°C in 5% CO₂.

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

Fancd2^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} bone marrow stromal cells were irradiated in suspension with doses between 0–8 Gy at 70 cGy/min using a Shepherd Mark 1 ¹³⁷Cs 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% CO₂ for 9–11 days, stained with crystal violet and colonies of 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, 17).

Irradiation Survival Curves for *Fancd2*^{-/-} Mouse IL-3-Dependent Cell Lines and *Fancd2*^{-/-} Mouse Whole Bone Marrow

Fancd2 IL-3-dependent non-adherent cells or whole bone marrow from *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} mice were irradiated in suspension with doses between 0–8 Gy as described above. Cells were plated in triplicate in methylcellulose medium containing recombinant mouse stem cell factor (SCF), IL-3, IL-6 and recombinant human erythropoietin (EPO) (Stem Cell Technologies, Vancouver, BC). CFU-GM were scored on day 7–9 for the *Fancd2* IL-3-dependent cell lines and CFU-GM, BFU-E and CFU-GEMM were scored between days 10–13 for the *Fancd2*^{+/+}, *Fancd2*^{+/-} or *Fancd2*^{-/-} whole bone marrow. In some experiments, colonies growing in plates containing cells that were

irradiated with 6 or 8 Gy were removed and expanded. Data were analyzed with single-hit multitarget models according to published methods (16, 17).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Unirradiated cells from each stromal and hematopoietic progenitor cell lines, *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} as well as cells exposed to 5 Gy of irradiation, were harvested at 24 h after exposure. Real time polymerase chain reaction (RT-PCR) was used to analyze radiation inducible transcripts for transcription factors Nfkb and Nrf2, and cytokines including TGFB1 for the oxidative stress response enzyme MnSOD (Sod2), and for irradiation response related transcripts p21 and p53, as well as for cell cycle checkpoint kinase Chk1 using published methods (11). The results were presented as fold increase in gene expression above baseline level, which was adjusted to that for nonirradiated C57BL/6NHsd wild-type mouse bone marrow stromal or hematopoietic progenitor cells. The magnitude of elevation in RNA attributable to irradiation was quantitated for each cell line.

RT-PCR Reaction Conditions

Total RNA was extracted from cell pellets using Trizol reagent (Invitrogen). A total of 2 µg of RNA was used to synthesize cDNA in a 20 µL reaction system, according to the instructions of High Capacity cDNA Reverse Transcription Kit (A&B Applied Systems, no. 4368814) with the reaction conditions of 40 cycles of 95°C (denaturation) for 15 s and 60°C (annealing and elongation) for 1 min using the Eppendorf RealPlex2 Mastercycler®. For RT-PCR, reaction conditions were as follows: 96-well plates were prepared with 10 µl of Taqman Gene Expression Master mix; 5 µl of RNase-free water; 1 µL of the corresponding Taqman Gene Expression probe; and 4 µL of cDNA (totaling 2 µg of cDNA) using the Eppendorf epMotion® 5070 automated pipetting system (Eppendorf, Westbury, NY). PCR amplification of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the housekeeping gene (Gen-Bank: NM_008084). Expression of our RNAs of interest included: Nrf2 (Gen-bank: NM_010902.3), Sod2 (Gen-Bank: NM_013671.3), Nfkb (Gen-Bank: NM_008689.2), TGFB1 (Gen-Bank: NM_011577.1), Chk1 (Gen-Bank: NM_007691), p21 (Gen-Bank: NM_001111099) and p53 (Gen-Bank: NM_001168250).

Flow Cytometric Apoptosis Analysis

Fancd2^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} mouse stromal cell lines and IL-3-dependent hematopoietic progenitor cell lines were harvested at baseline (unirradiated, control) and at 4 or 24 h after irradiation with 3 or 5 Gy. Cells were collected, washed with ice-cold phosphate buffered saline (PBS) and re-suspended in binding buffer at a cell density of 1×10^6 /mL. Apoptosis analysis was performed using an Annexin V-FITC apoptosis detection kit (Abcam, Cambridge, MA) according to manufacturer's instructions. Cells were stained with 5 µL of Annexin V-FITC and 10 µL of propidium iodide (PI) (20 µg/mL), then incubated in the dark at 25°C for 15 min. Samples were acquired on an Accuri™ C6 flow cytometer (BD sciences, Franklin Lakes, NJ) and 30,000 cells/experimental group were analyzed using Cellquest software. Details of methods for cell cycle distributions are as follows: Cells from *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} mouse stromal and IL-3-dependent cell lines were exposure to 0, 3 or 5 Gy of irradiation. At 4 and 24 h after irradiation, the cells were washed three times in PBS, fixed in 70% ethanol and stored for 24 h at -20°C. The cells were then stained with 0.1 µg/ml of propidium iodide and cell cycle analysis was performed by flow cytometry on 1×10^7 cells from each cell line. Experiments were carried out in triplicate.

Measurement of Irradiation Induced DNA Damage by Comet Tail Intensity Assay

Measurement of DNA damage after irradiation was performed as described previously (18). Cells from each mouse *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} cell line were irradiated with either 0 or 5 Gy and incubated at 37°C for 10 min, 1, 6 and 24 h, at which time the cells were rapidly chilled to 4°C to stop DNA repair. The zero time point samples used as reference are taken from unirradiated cells. The cells were mixed in low melt agarose, and 500 cells were placed on the sample area of a CometSlide (CometAssay® 4250-050-K, Trevigen, Inc., Gaithersburg, MD). The slides were rapidly chilled to 4°C and kept in the dark to prevent DNA repair. The slides were then placed in prechilled lysis solution and kept at 4°C for 60 min followed by washing in neutral electrophoresis buffer for 30 min at 4°C and electrophoresis at 21 V for 1 h at 4°C, then immersed in DNA precipitation solution for 30 min at room temperature followed by 70% ethanol for 30 min, and dried at 45°C for 15 min. The cells were then stained with SYBR Green 1 and examined with a fluorescence microscope. The comet tails for each of 150 cells were quantified using the Comet Assay IV software (Trevigen, Inc.).

Assay for Intracellular Antioxidant Levels

Fancd2^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} mouse stromal and IL-3-dependent hematopoietic progenitor cell lines were irradiated with 0, 5 or 10 Gy. Cells were harvested at 24 h after irradiation and snap-frozen in liquid nitrogen. Cell pellets were then thawed and mechanically homogenized in cold phosphate buffer solution. Protein concentrations were standardized by Bradford assay to 1 mg/mL of protein sample and antioxidant reductive capacity (antioxidant status) was measured using a commercial kit (Northwest Life Science Specialties, Vancouver, WA). This assay measures the antioxidant capacity of cells based on the ability of cellular antioxidants to reduce Cu⁺⁺ to Cu⁺, which reacts with bathocuproine to form a color complex absorbing at 480–490 nm. The antioxidant activity was compared to a standard curve generated using trolox units and all data was, therefore, expressed as millimolar equivalents of trolox units.

Western Analysis for p21, p53 and MnSOD Protein Expression

Cells from each *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} mouse stromal and IL-3-dependent hematopoietic progenitor cell line were pelleted and lysed in NP-400 buffer [50 mM Tris, pH 7.8, 10 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1 mM 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. Primary anti-MnSOD antibody (1:2000 dilution, no. ab13533,) and anti p53 antibody (1:10 dilution, mAB PAB421, no. OP03L) were obtained from Abcam. Primary antibody against p21 was obtained from Santa Cruz Chemical Laboratories, Santa Cruz, CA (1:200 dilution, no. SC-397). As a control gene product, GAPDH (Sigma Aldrich, St. Louis, MO) antibody was used. Subsequently, horseradish peroxidase anti-rabbit or anti-mouse secondary antibody (Promega, Pittsburgh, PA) was applied and membranes developed with SuperSignal West Dura ECL (Thermo Scientific, Rockford, IL). Western blots were carried out in triplicate and total protein density tabulated using LabWorks Image Acquisition and Analysis Software (UVP Imaging System, Upland, CA).

Statistical Methods

The *in vitro* radiation survival curves were analyzed with the single-hit multi-target model and were compared using D₀ (final slope representing multiple-event killing) and $\bar{n}$ (extrapolation number measuring width of the shoulder on the radiation survival curve) (16,17). Results for D₀ and $\bar{n}$ were presented as the mean $\pm$ standard error (SD) from

multiple measurements and compared with the two-sided two-sample *t* test. For the other continuous endpoints, comparisons were also performed using a *t* test if they were normally distributed or with Wilcoxon rank sum test otherwise. For the comet assay, data were log transformed so that their distribution was close to normal, and a linear regression model was built on the log transformed data in all 5 Gy irradiated groups, using cell line and time of measurement and their interaction term as explanatory variables. The F test in this model was used to compare slopes between any two of the 3 cell lines and a significant *P* value indicated a significant difference in the change of tail length with time after irradiation. In all these tests, a *P* value of less than 0.05 was regarded as significant. As an exploratory analysis, we did not adjust *P* values for multiple comparisons.

For LTBM data, weekly cobblestone island numbers; non-adherent cell numbers ($\times 10^5$); percentage confluence of adherent cells; day 7 colony forming cells at each week 14 harvest; and day 14 colony forming cell count at each weekly harvest were counted as described (11–13). Data are summarized as mean $\pm$ standard deviation, *n* is the number of mice used and *P* values were calculated with the two-sided two-sample *t* test. Significant *P* values are shown in red. A two-sided two-sample *t* test was used to analyze gene expression.

For Western blots data were analyzed by densitometry and summarized with mean $\pm$ standard deviation in each group. *P* values for the comparison between any two groups were calculated with the two-sided two-sample *t* test. *n* is the sample size. P1 is for the comparison with the corresponding 0 Gy group in the same column; P2 is for the comparison with the corresponding *Fancd2*^{+/+} mouse group in the same row.

RESULTS

Continuous Bone Marrow Cultures from *Fancd2*^{-/-} Mice have Reduced Longevity of Hematopoiesis

To determine if the longevity of hematopoiesis in long term culture was diminished in the bone marrow of *Fancd2*^{-/-} mice, several parameters of long-term hematopoiesis in continuous bone marrow cultures were quantitated comparing *Fancd2*^{-/-} with *Fancd2*^{+/+} and *Fancd2*^{+/-} explanted mouse bone marrow. As shown in Fig. 1A, cultures reached confluence of the adherent layer at different times, indicating slower proliferation of stromal cells from *Fancd2*^{-/-} marrow. Cobblestone island formation, representative of hematopoietic cell generating foci on the adherent layer, was detected in all marrow culture groups at around four weeks (Fig. 1B), however, increased numbers of these cobblestone islands were seen in *Fancd2*^{+/+} compared to *Fancd2*^{+/-} and *Fancd2*^{-/-} long-term bone marrow cultures. Cumulative production of cobblestone islands was higher in *Fancd2*^{+/+} and *Fancd2*^{+/-} marrow cultures compared to *Fancd2*^{-/-} marrow cultures, reflecting diminished hematopoietic activity in *Fancd2*^{-/-} cultures *in vitro* (Fig. 1C).

The production of non-adherent cells followed weekly cell cycling parameters as previously published for long-term bone marrow cultures (12, 13) and was observed in all culture groups. Figure 1D demonstrates the continuous robust production of hematopoietic cells in *Fancd2*^{+/+} and *Fancd2*^{+/-} long-term bone marrow cultures compared to the earlier decrease in production of hematopoietic cells in *Fancd2*^{-/-} long-term bone marrow cultures. Cumulative production of non-adherent cells reached a plateau earlier in *Fancd2*^{-/-} cultures compared to other groups (Fig. 1E).

The diminished production of non-adherent cells in *Fancd2*^{-/-} cultures correlated with decreased weekly production (Fig. 1F) and cumulative production (Fig. 1G) of cells forming colonies at day 7 in semi-solid media. The diminished production of hematopoietic progenitors by *Fancd2*^{-/-} cultures were even more dramatic with the more primitive day 14

hematopoietic progenitor cell colonies that diminished in numbers sooner than in either *Fancd2*^{+/-} or *Fancd2*^{+/+} cultures (Fig. 1H). Cumulative day 14 colony forming progenitor production was reduced in *Fancd2*^{-/-} cultures compared to either *Fancd2*^{+/-} or *Fancd2*^{+/+} cultures (Fig. 1I). Results were analyzed as described in the statistical analysis of the Supplementary Table S1; <http://dx.doi.org/10.1667/RR13405.1.S1>. These data were consistent with decreased capacity of *Fancd2*^{-/-} long-term bone marrow cultures to withstand the oxidative stress of continuous culture in high humidity and high oxygen atmosphere (11, 12). These data confirm and extend other reports indicating that long-term bone marrow cultures from mice with genetically deficient capacity to handle oxidative stress show a more rapid decrease in hematopoiesis *in vitro* (11, 12). Several parameters of increased hematopoiesis were observed in marrow cultures from heterozygous *Fancd2*^{+/-} mouse compared to *Fancd2*^{+/+} mouse marrow, but not in cumulative non-adherent cell production (Fig. 1E), which showed the highest levels with *Fancd2*^{+/+} mouse marrow.

Radiosensitivity of *Fancd2*^{-/-} Mouse Marrow Stromal Cell Lines

We isolated and cloned adherent marrow stromal cell lines from *Fancd2*^{-/-}, *Fancd2*^{+/-} and *Fancd2*^{+/+} mouse marrow cultures as described in the Method section. In survival curve assays, *Fancd2*^{-/-} marrow stromal cells were radiosensitive ($D_0 = 1.4 \pm 0.1$ Gy, $\bar{n} = 5.0 \pm 0.6$) compared to *Fancd2*^{+/+} ($D_0 = 1.6 \pm 0.1$ Gy, $\bar{n} = 6.7 \pm 1.6$), $P = 0.0124$ and $P = 0.0023$, respectively. *Fancd2*^{+/-} stromal cells were also radiosensitive, by a smaller shoulder on the survival curve, $\bar{n}$ compared to *Fancd2*^{+/+} cells ($D_0 = 1.7 \pm 0.1$ Gy and $\bar{n} = 4.2 \pm 0.7$), $P = 0.0004$ for $\bar{n}$, respectively (Table 1, Fig. 2A).

Relative comet tail intensity was next analyzed in 5 Gy irradiated *Fancd2*^{-/-} stromal cell lines by comet assay. Comet tail intensity as a measure of maximal double-strand DNA break level was higher in *Fancd2*^{-/-} stromal cells 15.8 ± 0.5 compared to 8.7 ± 0.3 in *Fancd2*^{+/+} stromal cells ($P < 0.0001$) or *Fancd2*^{+/-} stromal cells (12.6 ± 0.4 , $P < 0.0001$) (Fig. 3). There was recovery of comet tail length indicative of partial repair of damaged DNA in *Fancd2*^{+/+} and *Fancd2*^{+/-} cells within 1 h and nearly complete repair by 6 h ($P = 0.0002$ and $P < 0.0001$, respectively). In contrast, the *Fancd2*^{-/-} stromal cells were slower to show recovered comet tail intensity as an indication of repair of DNA, with no detectable change at 10 min and 1 h after irradiation ($P = 0.0001$ at each time point). By 6 h, *Fancd2*^{-/-} bone marrow stromal cells detectably recovered comet tail intensity ($P = 0.0014$); however, this induction of DNA damage did not return to baseline levels by 24 h after irradiation ($P = 0.0001$). Relative cell cycle distributions were next analyzed and correlated with the radiation-induced changes in comet tail intensity.

Cell Cycle Distribution of *Fancd2*^{-/-} Mouse Bone Marrow Stromal Cells

We found no significant differences in G₂/M, S phase or G₁ arrest between irradiated *Fancd2*^{-/-} bone marrow stromal cells compared to *Fancd2*^{+/+} or *Fancd2*^{+/-} stromal cells (Supplementary Table S2; <http://dx.doi.org/10.1667/RR13405.1.S1>).

Antioxidant Capacity of *Fancd2*^{-/-} Bone Marrow Stromal Cell Lines

Fancd2^{-/-} marrow stromal cells had decreased baseline antioxidant levels (0.34 ± 0.04 trolox units) compared to *Fancd2*^{+/+} stromal cells (0.45 ± 0.11 trolox units) $P = 0.0363$. *Fancd2*^{-/-} stromal cells showed depletion of antioxidant stores as did *Fancd2*^{+/-} and *Fancd2*^{+/+} stromal cells at 24 h after 10 Gy irradiation (0.23 ± 0.01 trolox units compared to preirradiation level, $P = 0.0407$) (Fig. 4).

Radiation Resistance of *Fancd2*^{-/-} Mouse IL-3-Dependent Hematopoietic Progenitor Cell Lines and Fresh Marrow CFU-GM

In contrast to the data with *Fancd2*^{-/-} marrow stromal cells, *Fancd2*^{-/-} IL-3-dependent hematopoietic progenitor cells were radioresistant compared to *Fancd2*^{+/+} hematopoietic progenitor cells (Table 2). We confirmed this unexpected radioresistance of *Fancd2*^{-/-} IL-3-dependent hematopoietic progenitor cell lines with 8 different clonal sublines and all were radioresistant (Table 2), for clone 5 ($D_0 = 1.38 \pm 0.06$ Gy and $\bar{n} = 5.75 \pm 0.87$) and for clone 1 ($D_0 = 1.90 \pm 0.06$ Gy and $\bar{n} = 2.85 \pm 0.98$), all $P = 0.043$) (Fig. 2B, Table 2).

Because the radioresistance of *Fancd2*^{-/-} IL-3-dependent hematopoietic progenitor cells was unexpected, we tested for reversion mosaicism, which has been observed in peripheral blood lymphocytes (PBLs) and hematopoietic precursor cells of hematopoiesis in some FA patients, and which can reverse hematopoietic failure and normalize mitomycin resistance (17, 18). We performed copy number PCR analysis of the *FanCD2* gene in all our clonal cell lines (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR13405.1.S1>). All the *Fancd2*^{-/-} stromal and hematopoietic progenitor subclones tested showed a biallelic loss of the *FanCD2* gene while the heterozygote *Fancd2*^{+/-} stromal and hematopoietic progenitor cells had one *FanCD2* allele. The data confirm that *Fancd2*^{+/+} (wild-type), *Fancd2*^{+/-} and *Fancd2*^{-/-} stromal as well as hematopoietic progenitor cell lines retained appropriate copies of the mutant or wild type *FanCD2* allele relative to the starting original mouse genotype (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR13405.1.S1>).

We measured radiation-induced DNA damage in *Fancd2*^{-/-} IL-3-dependent hematopoietic progenitor cells using the comet assay; double-strand DNA break level by comet tail intensity was 13.5 ± 0.4 in *Fancd2*^{-/-} after irradiation compared to 12.8 ± 0.4 in *Fancd2*^{+/+} or *Fancd2*^{+/-} (10.1 ± 0.3) hematopoietic progenitor cells. As shown in Fig. 3B, *Fancd2*^{-/-} hematopoietic progenitor cells were faster at repairing DNA double-strand breaks by 1 h. This result was clearly different from that of *Fancd2*^{-/-} stromal cells, which had delayed repair detected at 1 h after irradiation (Fig. 3A).

Radioresistance of Fresh Marrow CFU-GM from *Fancd2*^{-/-} Mice

To confirm that clonogenic *Fancd2*^{-/-} mouse IL-3-dependent hematopoietic progenitor cells were radioresistant, we next performed CFU-GM assays using *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} fresh whole bone marrow. CFU-GM, BFU-E and CFU-GEMM were scored between days 10 and 13. Radioresistance was detected with *Fancd2*^{-/-} marrow CFU-GM measured by the shoulder on the survival curve ($\bar{n} = 3.02 \pm 0.10$, $P = 0.001$ compared to *Fancd2*^{+/+}) and ($P = 0.0001$ compared to *Fancd2*^{+/-}). Radioresistance was not detected with heterozygote *Fancd2*^{+/-} relative to *Fancd2*^{+/+} marrow CFU-GM ($\bar{n} = 1.24 \pm 0.18$ compared to $\bar{n} = 1.35 \pm 0.18$, respectively). There was no significant difference observed in the BFU-E or CFU-GEMM among the three genotypes (*Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-}) (Table 3). Therefore, as with *Fancd2*^{-/-} IL-3-dependent cell lines, fresh marrow CFU-GM from *Fancd2*^{-/-} mice was radioresistant.

Irradiation Alters the Cell Cycle Distribution of *Fancd2*^{-/-} Mouse IL-3-Dependent Cell Lines

The cell cycle distribution of *Fancd2*^{-/-} IL-3-dependent hematopoietic progenitor cells showed a significantly reduced G₂/M pileup at 4 h after 5 Gy irradiation ($P = 0.0079$), and at 4 and 24 h after 3 Gy compared to *Fancd2*^{+/+} IL-3-dependent cells ($P = 0.0432$ and $P = 0.0079$, respectively) and this alteration was not normalized by 24 h after 3 Gy (Supplemental Table S3; <http://dx.doi.org/10.1667/RR13405.1.S1>). These results were in contrast to the lack of cell cycle effect of irradiation on stromal cell lines (Supplemental Table S2; <http://dx.doi.org/10.1667/RR13405.1.S1>).

Antioxidant Levels in IL-3-Dependent *Fancd2*^{-/-} Mouse Hematopoietic Progenitor Cells

We hypothesized that the different radiation responses of stromal cells versus hematopoietic cells from *Fancd2*^{-/-} mice might be attributable to cell phenotype dependent differences in the oxidative stress response. We next assayed for baseline and post-irradiation total antioxidant reduction capacity and antioxidant enzyme gene expression in stromal cells compared to hematopoietic progenitor cells from each genotype. Baseline and post-irradiation antioxidant reduction capacity, measured by millimolar trolox equivalents was higher in *Fancd2*^{-/-} IL-3-dependent hematopoietic progenitor cells compared to bone marrow stromal cells (Fig. 4). Furthermore, *Fancd2*^{-/-} hematopoietic progenitor cells maintained total antioxidant stores after 5 Gy and showed less depletion after 10 Gy ($P = 0.031$) compared to *Fancd2*^{+/+} or heterozygote *Fancd2*^{+/-} hematopoietic cells (Fig. 4B). Thus, the baseline and post-irradiation antioxidant levels in irradiated *Fancd2*^{-/-} hematopoietic cell were very different from those in stromal cells. *Fancd2*^{-/-} marrow stromal cells showed decreased baseline and further radiation-induced depletion of antioxidant levels.

Radiation Resistance of *Fancd2*^{-/-} Mouse Hematopoietic Cell Lines Correlates with Different Baseline and Radiation-induced Levels of Stress Response Related RNA Transcripts and Proteins

We next tested the hypothesis that the different irradiation response of *Fancd2*^{-/-} mouse stromal compared to hematopoietic cells might be supported by differences in levels of radiation-induced RNA transcripts (Fig. 5) and proteins (Fig. 6). We analyzed levels of transcripts for representative cell cycle regulator and oxidative stress response genes.

Fancd2^{-/-} stromal but not IL-3-dependent hematopoietic progenitor cells showed irradiation-increased levels of CHK1 transcripts (Fig. 5C, F).

Levels of baseline and radiation-induced transcripts for the oxidative stress induced transcription factor Nrf2 (nuclear-erythroid related factor 2) and MnSOD (manganese superoxide dismutase) were first compared. *Fancd2*^{-/-} hematopoietic and stromal cells had higher baseline MnSOD and Nrf2 transcript levels compared to *Fancd2*^{+/+} and *Fancd2*^{+/-} stromal and hematopoietic cells (Fig. 5C, F). After 5 Gy irradiation, *Fancd2*^{-/-} stromal but not hematopoietic cells showed increases in both MnSOD and NRF2 ($P = 0.0001$ and $P = 0.0001$, respectively). However, MnSOD gene transcript levels in *Fancd2*^{-/-} stromal cells did not correlate with protein levels (Fig. 6C, Supplementary Table S4C; <http://dx.doi.org/10.1667/RR13405.1.S1>).

There were elevated baseline and radiation-induced levels of p53 gene transcripts in *Fancd2*^{-/-} IL-3-dependent hematopoietic but not stromal cells (Fig. 5C, F). As with MnSOD and Nrf2, p53 protein levels in irradiated *Fancd2*^{-/-} hematopoietic or stromal cells did not correlate with the RT-PCR elevated transcripts and was not statistically significantly elevated ($P = 0.466$) (Fig. 6D, Supplementary Table S4A and D; <http://dx.doi.org/10.1667/RR13405.1.S1>).

A concordance of elevated RNA transcript levels with elevated protein was detected with p21 in irradiated *Fancd2*^{-/-} as well as in *Fancd2*^{+/-} and *Fancd2*^{+/+} stromal cells (Fig. 5C, Fig. 6B) (Supplementary Table S4B; <http://dx.doi.org/10.1667/RR13405.1.S1>). While p21 transcript levels were not elevated in irradiated *Fancd2*^{-/-} IL-3-dependent cell lines (Fig. 5F), there was elevated protein (Fig. 6E, Supplementary Table S4E; <http://dx.doi.org/10.1667/RR13405.1.S1>).

The ROS Scavenger JP4-039 Increases Radioresistance of *Fancd2*^{-/-} Marrow Stromal Cell Lines

The effect of the mitochondrial-targeted nitroxide radio-protector and radiation mitigating drug JP4-039 (9, 19) on radiosensitivity was tested with bone marrow stromal and IL-3-dependent hematopoietic cell lines derived from *Fancd2*^{-/-} compared to *Fancd2*^{+/+} mice. As shown in Table 4, *Fancd2*^{-/-} and *Fancd2*^{+/+} marrow stromal and IL-3-dependent hematopoietic progenitor cell lines were treated with 10 μ M JP4-039 for 1 h preirradiation. Both *Fancd2*^{+/+} marrow stromal and IL-3-dependent hematopoietic progenitor cell lines were radioprotected by JP4-039 treatment (Table 4). *Fancd2*^{-/-} bone marrow stromal cells were also protected by JP4-039 in the clonogenic assay. In contrast, there was no detectable protective effect of JP4-039 on *Fancd2*^{-/-} and *Fancd2*^{+/+} IL-3-dependent hematopoietic progenitor cells (Table 4).

DISCUSSION

Deletion of the human *Fancd2* gene has been implicated in altered genome stability, apoptosis, cell cycle control, and sensitivity to DNA cross-linking agents (5, 6, 21–23). Recent evidence indicates a link between the FA pathway and that of the oxidative stress response suggesting a role for FA proteins in the cellular antioxidant defense system (5). Significant molecular evidence suggests that the function of the FA pathway is to serve as a platform for multiple elements of the DNA repair pathway. This subject has recently been reviewed in detail (1, 2).

The present studies demonstrate reduced duration of hematopoiesis in *Fancd2*^{-/-} mouse LTBMCS consistent with reduced capacity of marrow to handle stress of long term culture (11, 12). An explanation for the greater levels of several parameters of hematopoiesis in *Fancd2*^{+/+} marrow cultures is not yet known. Studies with freshly removed tissues from *Fancd2*^{-/-} compared to *Fancd2*^{+/+} and *Fancd2*^{+/-} mice are in progress. While *Fancd2*^{-/-} mouse marrow stromal cell lines were radiosensitive, there was an unexpected radioresistance of IL-3-dependent hematopoietic progenitor cell lines and fresh marrow CFU-GM (granulocyte macrophage progenitor cells). Radioresistance of hematopoietic cells was confirmed with clonogenic radiation survival curves in each of eight *Fancd2*^{-/-} hematopoietic cell clonal lines including two that were derived from 6 and 8 Gy irradiation surviving colonies in semi-solid medium plates (17).

Fancd2^{-/-} radiosensitive stromal cells showed diminished recovery of comet tail intensity as a measure of DNA repair after 5 Gy irradiation and had lower antioxidant stores. In contrast, *Fancd2*^{-/-} radioresistant IL-3-dependent hematopoietic progenitor cells showed rapid recovery of comet tail intensity as a measure of repaired DNA within 6 h after 5 Gy irradiation, and had higher baseline antioxidant stores and less antioxidant depletion after irradiation. The data indicate an intrinsic radioresistance of *Fancd2*^{-/-} hematopoietic progenitor but not stromal cells and correlate with differences in clonogenic survival, comet tail intensity recovery as a measure of DNA repair capacity and oxidative stress response.

There was concordance between radiation-induced p21-CDKN1A transcripts and protein with *Fancd2*^{-/-} stromal cells, but not with MnSOD or p53. The lack of concordance with MnSOD and p53 may be explained by timing of the sample collection for the proteins relative to the RNA.

We confirmed through genotype analysis of all our cell lines that the heterogeneity between bone marrow stromal and hematopoietic cells radiosensitivity was not attributable to revertant mosaicism, which has been reported in cells of some FA patients (24). In our current studies, *Fancd2* gene copy number analysis showed that all cell lines studied

maintained a biallelic loss of *Fancd2* gene and the heterozygote *Fancd2*^{+/-} cells had one *Fancd2* allele, but gene mutations were not studied.

Cell cycle regulation and activation of cell cycle checkpoints is another pathway implicated in cellular responses to ionizing irradiation (23–25), and differed between radiosensitive *Fancd2*^{-/-} stromal and radioresistant IL-3-dependent hematopoietic cells. There was no detectable cell cycle alteration in irradiated *Fancd2*^{-/-} stromal cells; however, IL-3-dependent *Fancd2*^{-/-} cells showed a significantly reduced G₂/M pileup at 4 h after 5 Gy irradiation and at 4 and 24 h after 3 Gy irradiation compared to *Fancd2*^{+/+} IL-3 cells. The cell cycle alterations in irradiated IL-3-dependent *Fancd2*^{-/-} cell lines were associated with low levels of Chk1 at baseline and after 5 Gy. Whether there was a survival advantage by down-regulating Chk1 during clonal cell line selection of *Fancd2*^{-/-} IL-3-dependent cell lines is not known. Prior reports showed that Chk1 was overexpressed in FA hematopoietic stem cells, T-cells and erythroid progenitors (23, 26–27). In addition to the differential total antioxidant capacity, it is possible that difference in cell cycle population might have accounted for the distinctive radiosensitivity of *Fancd2*^{-/-} mouse stromal cells compared to hematopoietic progenitor cells (25). Since human *FanCD2* function in DSB repair is tightly associated with homologous recombination in S/G₂ phase and a higher G₁ population was found in mouse *Fancd2*^{-/-} hematopoietic progenitors, but not in mouse *Fancd2*^{-/-} stromal cells, we plan to pursue this possible explanation in future studies.

Because the FA pathway has been linked to that of the oxidative stress response (5, 6, 27–30), we tested the effect of the small molecule mitochondrial-targeted nitroxide JP4-039, which is a ROS scavenger and has been shown to be effective in the radioprotection of normal murine tissues *in vivo* (9, 19). Furthermore, JP4-039 ameliorates radiosensitivity *in vitro* of fibroblast cell lines derived from human Fanconi Anemia patient's cell lines including *FanCD2*^{-/-} (4). The current studies showed radioprotection of mouse *Fancd2*^{-/-} stromal, but not IL-3-dependent hematopoietic progenitor cells by JP4-039. Whether the lack of effect of JP4-039 on *Fancd2*^{-/-} hematopoietic cell radioresistance reflects a saturated maximum antioxidant defense or a different radiation induced death pathway in *Fancd2*^{-/-} cells will require further studies.

The reduced longevity of *Fancd2*^{-/-} mouse long marrow cultures and radiosensitivity of marrow stromal cells, while hematopoietic cells were radioresistant suggests that the *in vivo* radiosensitivity of hematopoiesis in *Fancd2*^{-/-} mice (10) may involve an indirect mechanism of radiation effect on stromal cells transmitted to hematopoietic cells. *In vitro* co-culture studies and selective marrow stromal cell transplantation studies may be required to test this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Reduced hematopoietic longevity in *Fancd2*^{-/-} mouse long-term bone marrow cultures. Eight marrow cultures per group were established as described in the Methods section and cultured as described. Groups were tested weekly for (panel A) percentage confluence of adherent layer; (panel B) cobblestone islands per flasks scored weekly; (panel C) cumulative cobblestone islands; (panel D) non-adherent cells/flask; (panel E) cumulative non-adherent cells/flask; (panel F) weekly day 7 colony forming progenitor cell; (panel G) cumulative day 7 non-adherent colony forming cells; (panel H) weekly non-adherent day 14 colony forming cells; and (panel I) cumulative day 14 colony forming cells. (●) *Fancd2*^{+/+}; (▲) *Fancd2*^{+/-}; and (■) *Fancd2*^{-/-}. (Statistical analysis is shown in Supplementary Table S1; <http://dx.doi.org/10.1667/RR13405.1.S1>).

FIG. 2.

Radiosensitivity of *Fancd2*^{-/-} bone marrow stromal cells and radioresistance of *Fancd2*^{-/-} IL-3-dependent hematopoietic progenitor cells. Cells from each bone marrow stromal (panel A) or hematopoietic progenitor (panel B) cell line were irradiated and assayed for colony formation as described in the Methods section (plating efficiencies were 40–70% and plating densities were 100–400 cells/well. Results are the mean $\pm$ SEM of at least 3 experiments comparing cell lines of each phenotype plotted, as single-hit, multi-target models (16, 17).

FIG. 3. Different rates of repair of ionizing-radiation-induced DNA strand breaks in *Fancd2*^{-/-} bone marrow stromal compared to IL-3-dependent hematopoietic progenitor cell lines. Comet assays were carried out as described in the Methods section comparing: (panel A) Bone marrow stromal cell lines and (panel B) IL-3-dependent hematopoietic progenitor cell lines from each genotype. *Fancd2*^{-/-} stromal cells had higher DNA damage post-irradiation (**P* < 0.0001) and had delayed recovery compared to *Fancd2*^{+/+} (**P* < 0.0001) or *Fancd2*^{+/-} (**P* < 0.0001) stromal cells. *Indicates a significant difference compared to *Fancd2*^{+/+} cells.

A. *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} Stromal Cell Lines

*P = 0.0109 compared to *Fancd2*^{+/+} at 0 Gy

[#]P < 0.005 compared to 0 Gy for the same cell line

B. *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} IL-3 Dependent Hematopoietic Progenitor Cell Lines

*P < 0.01 compared to *Fancd2*^{+/+} at 0 Gy

[#]P = 0.0461 compared to *Fancd2*^{+/+} at 0 Gy

[&]P = 0.0121 compared to *Fancd2*^{+/-} at 0 Gy

FIG. 4.

Different levels of baseline and radiation-induced antioxidant capacity between *Fancd2*^{-/-} bone marrow stromal and IL-3-dependent hematopoietic progenitor cell lines. Cell lines from *Fancd2*^{-/-} mouse marrow stromal (panel A) and hematopoietic progenitor (panel B) cells were exposed to 5 or 10 Gy irradiation. Cells were harvested at 24 h after irradiation. Protein concentrations were standardized to 1 mg/mL and total antioxidant reductive capacity (antioxidant status) was measured as described in the Methods section. The antioxidant activity was compared to a standard curve generated using trolox units and all data was, therefore, expressed as millimolar (mM) equivalents of trolox units. *Significant difference compared to *Fancd2*^{+/+} cell lines.

FIG. 5.

Different levels of gene transcripts in baseline and radiation-induced *Fancd2*^{-/-} marrow stromal cells compared to IL-3-dependent hematopoietic progenitor cell lines. Marrow stromal cells (panels A–C) and IL-3-dependent hematopoietic progenitor cell lines (panels D–F) were compared. RNA was extracted from cell pellets from each cell line using trizol. RT-PCR was performed as described in the Methods section using primers specific for promoters associated with: p21, Chk1, p53, MnSOD, Nrf2, Nfkb and TGFB1. *Significant difference compared to *Fancd2*^{+/+} cell lines. *P < 0.05 compared to *Fancd2*^{+/+} at 0 Gy; #P < 0.05 compared to *Fancd2*^{+/+} at 5 Gy.

FIG. 6.

Different levels of baseline and radiation-induced p21, p53 and MnSOD protein in *Fancd2*^{-/-} bone marrow stromal and IL-3-dependent hematopoietic progenitor cell lines. Protein level in each cell lines was analyzed by Western analyses as described in the Methods section: at 0 Gy (baseline), at 24 or 48 h after 5 Gy irradiation. Panel A: p53 in stromal cells; panel B: p21 in *Fancd2*^{-/-} and control *Fancd2*^{+/+} and *Fancd2*^{+/-} stromal cells; panel C: MnSOD in stromal cells; panel D: p53 in IL-3-dependent cells; panel E: p21 in IL-3-dependent hematopoietic progenitor cells; and panel F: MnSOD in IL-3-dependent hematopoietic progenitor cells. Molecular size for these proteins as follows: MnSOD is 25 kDa; GAPDH is 37 kDa; p21 is 21 kDa and p53 is 53 kDa. See Supplemental Tables 4A–C) for p53, p21 and MnSOD protein expression in *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} stromal cells. (For p53, p21 and MnSOD protein expression in *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} IL-3-dependent cells see Supplementary Table S4D–F; <http://dx.doi.org/10.1667/RR13405.1.S1>.)

TABLE 1Radiosensitivity of *Fancd2*^{-/-} Bone Marrow Stromal Cells

Bone marrow stromal cell lines	D ₀ (Gy)	$\bar{n}$	Plating efficiency
<i>Fancd2</i> ^{-/-}	1.4 ± 0.1 P = 0.0124	5.0 ± 0.6 P = 0.0023	26.5 ± 2.3
<i>Fancd2</i> ^{+/-}	1.7 ± 0.1 P = 0.0906	4.2 ± 0.7 P = 0.0004	38.2 ± 14.3
<i>Fancd2</i> ^{+/+}	1.6 ± 0.1	6.7 ± 1.6	40.4 ± 7.6

Notes. Cells from *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} bone marrow stromal cell lines were irradiated and assayed for colony formation as described in the Methods section. Plating densities were 100–400 cells/well. Results are the mean ± SEM of at least 3 experiments. Significant *P* values compared to *Fancd2*^{+/+} stromal cells are **bold**.

TABLE 2

Radioresistance of *Fancd2*^{-/-} Hematopoietic Progenitor Cell Lines

Cell line	D ₀	$\bar{n}$	Plating efficiency
<i>Fancd2</i> ^{-/-}	1.71 ± 0.04 P = 0.001	5.07 ± 0.52	0.27 ± 0.02
<i>Fancd2</i> ^{+/-}	1.64 ± 0.08 P = 0.0004	4.40 ± 0.99 P = 0.002	0.21 ± 0.09
<i>Fancd2</i> ^{+/+}	1.39 ± 0.09	2.31 ± 0.85	0.81 ± 0.18
<i>Fancd2</i> ^{-/-} clones			
clone 1	1.90 ± 0.06 P = 0.004	2.85 ± 0.98	0.24 ± 0.15
clone 2	1.63 ± 0.08 P = 0.007	5.34 ± 2.56	0.18 ± 0.006
clone 3	1.56 ± 0.02 P = 0.006	6.06 ± 0.30 P = 0.006	0.25 ± 0.0
clone 4	1.65 ± 0.09 P = 0.043	2.47 ± 0.37	0.17 ± 0.006
clone 5	1.38 ± 0.06 P = 0.013	5.75 ± 0.87 P = 0.013	0.15 ± 0.006
clone 6	1.44 ± 0.0	3.74 ± 0.11 P = 0.025	0.14 ± 0.04
clone 7	1.55 ± 0.04 P = 0.018	3.84 ± 0.20 P = 0.018	0.12 ± 0.006
clone 8	1.72 ± 0.04 P = 0.016	2.04 ± 0.18	0.12 ± 0.0

Notes. Cells from *Fancd2*^{+/+} IL-3-dependent cell line (32D cl3), *Fancd2*^{+/-} and *Fancd2*^{-/-} hematopoietic progenitor cell lines were irradiated and assayed for colony formation as described in the Methods section. Clones 7 and 8 were cell lines expanded from colonies growing in plates containing cells exposed to 6 and 8 Gy irradiation, respectively. Plating densities were 100–400 cells/well. Results are the mean ± SEM of at least 3 experiments. *P* values compared to *Fancd2*^{+/+} IL-3-dependent cell line, (32D cl 3) are **bold**.

Radioresistance of Hematopoietic Colony Forming Progenitor Cells from Fresh *Fancd2*^{-/-} Mouse Bone Marrow

TABLE 3

Fancd2	CFU-GM			BFU-E			CFU-GEMM		
	bone marrow	D ₀	$\bar{n}$	D ₀	$\bar{n}$	D ₀	D ₀	$\bar{n}$	$\bar{n}$
<i>Fancd2</i> ^{-/-}	1.62 ± 0.09	3.02 ± 0.10	1.67 ± 0.18	1.4 ± 0.2	2.46 ± 0.25	1.02 ± 0.02			
	<i>P</i> ₁ = 0.049 ^a	<i>P</i> ₁ = 0.001 ^a							
	<i>P</i> ₂ = 0.007 ^b	<i>P</i> ₂ = 0.0001 ^b							
<i>Fancd2</i> ^{+/-}	2.23 ± 0.07	1.24 ± 0.03	2.15 ± 0.11	1.64 ± 0.36	2.35 ± 0.18	1.00 ± 0.00			
<i>Fancd2</i> ^{+/+}	2.44 ± 0.27	1.35 ± 0.18	2.11 ± 0.43	1.66 ± 0.33	2.02 ± 0.11	2.11 ± 0.60			

Notes. Whole-femur bone marrow from *Fancd2*^{+/+}, *Fancd2*^{+/-} and *Fancd2*^{-/-} mice were irradiated in single cell suspension with doses between 0–8 Gy as described in the Methods section. CFU-GM, BFU-E and CFU-GEMM were scored when colonies reached 50 cells in days 10–13. Results are the mean ± SEM of three separate experiments.

^a *P*₁ = comparison between +/+ group and -/- group.

^b *P*₂ = comparison between heterozygote +/+ group and -/- group.

TABLE 4

Radiation Protection of *Fancd2*^{-/-} Marrow Stromal Cells by ROS-Scavenging Nitroxide, JP4-039

Stromal cell line	JP4-039	D ₀	$\bar{n}$
<i>Fancd2</i> ^{-/-}	0	1.45 ± 0.05 P1 = 0.0021	4.99 ± 0.65 P1 = 0.0040
<i>Fancd2</i> ^{-/-} + JP4-039	preirradiation	1.73 ± 0.10 P2 = 0.0237 P3 = 0.0458	5.10 ± 2.00
<i>Fancd2</i> ^{+/+}	0	1.80 ± 0.08	8.33 ± 0.73
<i>Fancd2</i> ^{+/+} + JP4-039	preirradiation	2.33 ± 0.23 P1 = 0.0177	5.37 ± 1.44
Hematopoietic cell line		D ₀	$\bar{n}$
<i>Fancd2</i> ^{-/-}	0	1.56 ± 0.25	3.46 ± 1.99
<i>Fancd2</i> ^{-/-} + JP4-039	preirradiation	1.44 ± 0.17	3.49 ± 1.10
<i>Fancd2</i> ^{+/-}	0	1.87 ± 0.21	3.85 ± 1.57
<i>Fancd2</i> ^{+/-} + JP4-039	preirradiation	2.09 ± 0.28	2.65 ± 0.57
<i>Fancd2</i> ^{+/+}	0	1.31 ± 0.10	1.00 ± 0.10
<i>Fancd2</i> ^{+/+} + JP4-039	preirradiation	2.3 ± 0.3 P = 0.043	1.00 ± 0.10

Notes. *In vitro* survival curves were performed with bone stromal and hematopoietic progenitor cells lines. Cells were incubated in 10 μ M JP4-039 for 1 h before exposure as described in the literature (9, 19), then irradiated with doses ranging from 0–8 Gy, plated in 4-well tissue culture plates, incubated for 7 days at 37°C, and stained with crystal violet. Colonies >50 cells were counted and analyzed using a linear-quadratic model or single-hit, multi-target model (9, 19). D₀ and $\bar{n}$ are expressed as mean ± SD of at least 3 experiments. *P* < 0.05 were significant and are **bold**. P1 = comparison with *Fancd2*^{+/+} control unirradiated; P2 = comparison with *Fancd2*^{-/-} unirradiated; and P3 = comparison with *Fancd2*^{+/+} JP4-039 (preirradiation).