Published in final edited form as:

Radiat Res. 2019 February ; 191(2): 139–153. doi:10.1667/RR15199.1.

Continuous One Year Oral Administration of the Radiation Mitigator, MMS350, after Total-Body Irradiation, Restores Bone Marrow Stromal Cell Proliferative Capacity and Reduces Senescence in Fanconi Anemia (Fanca^{-/-}) Mice

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Abstract

We quantitated age-related accumulation of senescent cells in irradiated Fanconi anemia (FA) (Fanca^{-/-}) mouse cell lines *in vitro* and monitored the effect of continuous administration (via drinking water) of the water-soluble radiation mitigator, MMS350, on tissues *in vivo* over one year after 7.5 Gy total-body irradiation (TBI). Irradiated Fanca^{-/-} mouse bone marrow stromal cell lines showed increased numbers of beta-galactosidase- and p21-positive senescent cells compared to Fanca^{+/+} cell lines, which was reduced by MMS350. One week after 7.5 Gy TBI, Fanca^{-/-} mice showed increased numbers of senescent cells in spleen compared to Fanca^{+/+} controls, decreased bone marrow cellularity, failure of explanted bone marrow to proliferate *in vitro* to form a hematopoietic microenvironment and no detectable single stromal cell cloning capacity. There was no detectable amelioration by MMS350 administration at one week. In contrast, one year post-TBI, Fanca^{-/-} mice demonstrated fewer senescent cells in brain and spleen compared to Fanca^{+/+} controls. While Fanca^{-/-} mouse bone marrow stromal cells explanted one year post-TBI still failed to proliferate *in vitro*, continuous oral administration of 400 μ M, MMS350 in drinking water restored explanted stromal cell proliferation. The data indicate that continuous administration of MMS350 modulated several properties of TBI-accelerated aging in Fanca^{-/-} mice as well as control mice, and support further study of MMS350 as a modulator of radiation late effects.

INTRODUCTION

Recovery from sublethal total-body irradiation (TBI) is associated with multiple delayed effects, including acceleration of age-related markers of aging (1–10), reduction of stem cell populations in multiple organs (1, 3–5), tissue and organ failure (9–10) and carcinogenesis (11–12). Previously reported studies have demonstrated reduction in proliferative capacity of

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Editor's note. The online version of this article (DOI: 10.1667/RR15199.1) contains supplementary information that is available to all authorized users.

explanted stromal cells from the marrow of TBI mice at 3 months postirradiation (12). It is well known that proliferating, as well as quiescent stem cells in hematopoietic organs of TBI mice require an intact and functioning hematopoietic microenvironment (6, 13). Irradiated marrow stromal cells continue to function if maintained in cell-contact and proliferation-inhibited monolayers *in vitro* (13) or if left undisturbed after TBI *in situ*; however, the cells fail to proliferate if placed in growth-stimulating conditions in tissue culture (12). Furthermore, intact functioning tissues, in patients at months to years after local irradiation, often demonstrate late connective tissue and vascular changes, and reduced healing capacity after irradiation (14).

Preirradiation administration of antioxidant drugs, including manganese superoxide dismutase-plasmid liposomes (9), as well as continuous postirradiation antioxidant/chemopreventive diets (10), modulate some of the effects of TBI on aging. Sublethal-TBI mice also demonstrate life shortening compared to nonirradiated controls, suggesting a role for continuous release of abscopal factors from irradiated tissues (15). The continuous postirradiation administration of a water-soluble small molecule radiation mitigator (MMS350) (16, 17) has been shown to reduce radiation-induced late-effects pulmonary fibrosis (18), suggesting that it may be therapeutic for other radiation-induced late effects.

Fanconi anemia (FA) animal models display a defective scaffold for DNA repair enzymes, and some FA patients demonstrate both age-related depletion of hematopoietic stem cells and radiosensitivity (19–24). The goal of the current study was to determine whether Fanca^{-/-} mice, followed for one year after sublethal 7.5 Gy TBI, demonstrated accelerated senescence in a proliferating tissue (spleen) compared to a slowly or nonproliferating tissue (brain), and whether there was a detectable late effect on cells of the hematopoietic microenvironment. Testing was also performed to determine whether continuous administration of MMS350 in irradiated mice (16) modulated biomarkers of aging, as well as late-stage effects. The results demonstrate increased acute and late effects of TBI on the bone marrow of Fanca^{-/-} compared to control mice receiving 7.5 Gy TBI. Furthermore, continuous oral administration of MMS350 over one year period ameliorated radiation-induced late effects in both Fanca^{-/-}, as well as control mice. The data support further investigations into MMS350 administration for mitigating late-stage effects of TBI.

MATERIALS AND METHODS

Mice and Animal Facilities

129/Sv Fanca^{+/+} and 129/Sv Fanca^{-/-} mice were derived from Fanca^{-/-} heterozygote breeding pairs (25). Female mice were housed 5 per cage and male mice housed 4 per cage according to Institutional Animal Care and Use Committee (IACUC) regulations. Mice were fed standard Purina® laboratory chow. Fanca^{-/-} mice were derived from breeding pairs using Fanca^{+/+} mice. Fanca^{+/+} mice resulting from these breeding pairs were used as control mice. All protocols were approved by the University of Pittsburgh IACUC (Pittsburgh, PA). Veterinary care was provided by Division of Laboratory Animal Resources at University of Pittsburgh. 129/Sv Fanca^{+/+} and 129/Sv Fanca^{-/-} mice were sham irradiated or 7.5 Gy TBI using a Shepherd Mark I ¹³⁷Cs gamma-ray source (JL Shepherd & Assoc.), according to methods published elsewhere (26). Subgroups were given 400 μM MMS350 in drinking

water or regular water (18). Subgroups of mice were sacrificed at one week or one year postirradiation.

Preparation of Radiation Mitigator Drug MMS350

The syntheses of GS-nitroxide JP4-039 (29) and the bis-oxetanyl sulfoxide MMS350 (16) have been described elsewhere. Metformin hydrochloride (Tocris Bioscience, Bristol, UK). MMS350 was administered to cells *in vitro* dissolved directly in tissue culture media. JP4-039 administered to cells *in vitro* was first dissolved in DMSO. MMS350 was administered to mice *in vivo* in a solution in the drinking water of mice at 400 μ M concentration (18).

Long-Term Bone Marrow Cultures

Long-term bone marrow cultures (LTBMCs) were established according to published methods (27) from the femur and tibia marrow of 129/Sv Fanca^{+/+} and 129/Sv Fanca^{-/-} mice at one week or one year after 7.5 Gy TBI with or without continuous 400 μ M MMS350 in drinking water, as described elsewhere (18). Briefly, the contents of a femur and tibia were flushed into McCoy's 5A medium (Thermo Fischer Scientific™, Waltham, MA) supplemented with 25% horse serum (Cambrex, Rockland, ME) and 10⁻⁵ M hydrocortisone sodium hemisuccinate (Thermo Fisher Scientific). For each group, 2 mice were used to establish LTBM and two flasks were created per mouse. Cultures were incubated at 33°C in 7% CO₂. Media was changed weekly. After 4 weeks, the horse serum was replaced with 25% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA) (25, 27). The cultures were observed weekly for hematopoietic cell production and adherent cell layer confluence. Nonadherent cell production data are expressed as mean $\pm$ standard error (SEM) of weekly nonadherent cell number and cumulative nonadherent cell production. Confluence data are expressed as mean $\pm$ SEM of percentage adherent cell layer confluence.

Hematopoietic Colony-Forming Cell Assay

Each week after explant and establishment of LTBM, 1 $\times$ 10⁵ nonadherent cells were plated in triplicate in methylcellulose-containing media plates, as described elsewhere (27). Cells were incubated in 37°C in 5% CO₂. At days 7 and 14 after plating, colonies of 50 cells were counted. The plates scored at day 7 were returned to the incubator for scoring at day 14. Data for days 7 and 14 were presented as mean $\pm$ SEM of weekly colony-forming cells and cumulative colony-forming cells (27).

Establishment of Bone Marrow Stromal Cell Lines

Adherent cell layers from one LTBM per group were trypsinized and expanded by passage into Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS to establish bone marrow stromal cell lines according to published methods (27). Established criteria were used for authentication of cell lines (25). Genotyping, which we performed for all bone marrow stromal cell lines, established and confirmed that they were indeed from the Fanca^{+/+} or Fanca^{-/-} genotype. The established lines maintained stromal cell features including differentiation capacity to osteoblast, and support of hematopoietic cells in co-culture. We documented cell surface phenotype, biology and absence of capacity to differentiate to

hematopoietic cells. These cell lines were characterized as bone marrow stromal cells or mesenchymal stem cells (25, 27). Cell lines were incubated at 37°C in 5% CO₂ and passaged for 10 weeks to establish cell lines.

Authentication of Bone Marrow Stromal Cell Lines

The stromal cell (mesenchymal stem cell) phenotype of Fanca^{+/+} and Fanca^{-/-} bone marrow stromal cell lines was validated before any experiment was started. Cells were passaged four times *in vitro* in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin and streptomycin, and then tested for a panel of biomarkers. The cloning efficiency of Fanca^{+/+} and Fanca^{-/-} bone marrow stromal cell lines after four passages *in vitro* was 305 out of 500 and 410 out of 600 single cells plated, respectively, in 96-well Lingbro plastic petri dishes, in 0.1 ml complete media. Cloning efficiency was defined as the capacity of single cells to grow to confluence in the 96-well plate, then continued passage *in vitro* after explant to larger culture dishes. In contrast, IL-3-dependent hematopoietic progenitor cell lines showed a cloning efficiency in the same media (in the absence of IL-3) of 0/850 single cells plated. Fresh bone marrow cells from each mouse genotype, subjected to cloning under the same conditions, revealed 0/850 single cells plated forming clonal lines. Bone marrow stromal cell lines and clonal sub-cell lines of Fanca^{+/+} and Fanca^{-/-} were positive for detection of mRNA, by rt-PCR, and protein by Luminex assay for collagen type I, collagen type II, collagen type III and collagen type IV, and secreted TGF-β measured by Western blots of serum-free media collected at 24 h after incubation. In contrast, IL-3-dependent cell lines, and fresh bone marrow from each mouse genotype, were negative by rt-PCR, as well as for collagen I-IV and TGF-β. Bone marrow stromal cell lines were negative for cell surface CD45, CD3e, CD5, CD8a, B220, GR-1, Terr119, CD48, CD41 and CD150, all markers of lympho-hematopoietic cells. Stromal cells formed no colonies per 10⁵ cells plated in semisolid media *in vitro*, in media supplemented with G-CSF, GM-CSF or IL-3. In contrast, fresh bone marrow demonstrated semisolid media cloning efficiency of over 10% in all three growth factors. IL-3-dependent cell lines from each genotype formed colonies in semisolid media only in the presence of IL-3 at 25%. Bone marrow stromal cell lines were positive for oil-red-O staining, when grown in the presence of 10⁻⁵ M hydrocortisone sodium hemi-succinate, while no positivity was detected with fresh bone marrow or IL-3-dependent cell lines. When grown in osteogenic tissue culture media supplemented with 10⁻⁵ M dexamethasone, beta-glycerophosphate and ascorbate, Fanca^{+/+} and Fanca^{-/-} bone marrow stromal cells synthesized osteoid, while there was no such detection with IL-3-dependent cell lines or sorted hematopoietic cells from Fanca^{+/+} or Fanca^{-/-} fresh marrow. Both Fanca^{+/+} and Fanca^{-/-} stromal cell lines were negative in histochemical staining for endothelin, leukocyte alkaline phosphatase, esterase and myeloperoxidase. Cytospin preparations of Fanca^{+/+} and Fanca^{-/-} bone marrow stromal cell lines stained with Wright's/Giemsa for analysis of hematopoietic cells showed no evidence of segmented nuclei cytoplasmic secretory granules, or electron microscopic evidence of secretory granules typical of macrophages.

Establishment of Single Cell Derived Clonal Marrow Stromal Cell Lines

Single cell cloning experiments were performed using fresh marrow, an adherent cell layer from a LTBMCM at week 13 after explant, and bone marrow stromal cell lines that were

established as described elsewhere (27). Flow cytometry was used to plate single cells into the wells of a 96-well plate. Cells were grown in DMEM supplemented with 20% FBS, 1% antibiotic-antimycotic solution and 1% L-glutamine. Plates were observed weekly for growth. Clonal lines were established from wells that showed growth after single cell plating.

Beta-Galactosidase Assay for Senescence of Mouse Tissues and Irradiated Bone Marrow Stromal Cell Lines

129/Sv Fanca^{+/+} and 129/Sv Fanca^{-/-} bone marrow stromal cell lines were plated in duplicate in six-well plates, incubated overnight to allow attachment, then irradiated to various doses using a Shephard Mark I ¹³⁷Cs gamma-ray source. Cells were kept at sub-confluence and grown in DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic solution and 1% L-glutamine. Cells were incubated at 37°C in 5% CO₂. Drugs were added to media 1 h prior to incubation and supplemented in media continuously afterwards.

At indicated time points, chromogenic beta-galactosidase assay (Cell Signaling Technology®, Inc., Danvers, MA) was performed according to the manufacturer's protocol. Briefly, cells were washed with phosphate buffered saline (PBS) then incubated 4 min with 1× fixative solution. Cells were washed with PBS twice, then beta-galactosidase solution was added. Beta-galactosidase solution was prepared fresh for each assay and titrated to pH 6.0 (28). Plates were wrapped with Parafilm® (Thermo Fisher Scientific) and incubated overnight at 37°C in 0% CO₂. Cells were washed with PBS twice, then with methanol once. Samples were plated in duplicate. The percentage of positive blue cells was counted using light microscopy at 20× magnification, ensuring a minimum of 500 cells counted per sample (28).

Real Time Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed as described elsewhere (18). Cells were trypsinized, washed with PBS twice, then resuspended in 1 ml TRIzol® reagent (Invitrogen™, Carlsbad, CA). Organs were harvested, chopped to fine pieces using a razor and placed in round-bottom tube with 1 ml of TRIzol reagent. Samples were homogenized for up to 10 s using Omni Mixer Homogenizer (Omni International, Kennesaw, GA).

After 5-min incubation, 200 µl of chloroform was added, followed by a 5-min incubation at room temperature. The sample was centrifuged at 12,000g for 15 min at 4°C. The aqueous phase was transferred to a new 1.5-ml Eppendorf tube and 0.5 ml isopropyl alcohol was added. After 10-min incubation at room temperature, the sample was centrifuged at 12,000g for 15 min at 4°C. Supernatant was removed and pellet was resuspended in 1 ml 70% ethanol. After 5 min, the sample was centrifuged at 12,000g for 15 min at 4°C. Supernatant was removed and RNA pellet was dried for 10 min before resuspending in water. Samples were stored at -80°C until quantitation.

RNA was quantified using a spectrophotometer. For each sample, 2 µg of total RNA was reverse transcribed to complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®, Foster City, CA), according to manufacturer's protocol.

Expression of specific RNA transcripts was quantitated by RT-PCR. Primer mix was created using 10 µl of iTaq™ 2X Universal Probes Supermix, 1 µl primer and 5 µl of nuclease-free water for each reaction. Of this primer mix, 16 µl was added to each well of a 96-well PCR plate. In addition, 4 µl of cDNA from each sample was added to a well of the 96-well plate, with each sample being run in triplicate. Samples were centrifuged at 1,500 rpm for 5 min at 8°C to ensure that the entire reaction mix was at bottom of well. RT-PCR reactions were run by the Eppendorf Realplex® Mastercycler (Eppendorf North America, Hauppauge, NY) using 45 cycles of 95°C for 15 s and 59°C for 30 s.

Data for each gene transcript were normalized by calculating ΔC_t , the difference between C_t of target genes and glyceraldehyde-phosphate dehydrogenase (GAPDH). Expression relative to control samples was determined by calculating $2^{-\Delta C_t}$, the difference between control and experimental C_t , and then $2^{-\Delta C_t}$. Data are presented as fold change $\pm$ SEM.

RT-PCR primers for p21 [Cdkn1a (assay ID Mm04205640_g1)], and Gapdh (assay ID Mm99999915_g1) were purchased from Thermo Fisher Scientific.

Immunofluorescence Assay for Detection of p21

Bone marrow stromal cell lines were plated in Nunc™ Lab-Tek™ II CC2™ Chamber Slide System (Thermo Fisher Scientific) and incubated overnight to allow attachment, then irradiated to various doses using a Shephard Mark I ¹³⁷Cs gamma-ray source. Cells were kept at sub-confluence and grown in DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic solution and 1% L-glutamine. Cells were incubated at 37°C in 5% CO₂. Drugs were added to media 1 h prior to incubation and supplemented in media chronically until fixation.

At indicated times after irradiation, cells were washed with PBS twice and fixed with 4% paraformaldehyde for 20 min. After washing with PBS twice, cells were permeabilized by adding 0.1% Triton™ X-100 in PBS for 5–10 min. Primary antibody mixtures were prepared at manufacturer-suggested dilutions in 5% BSA. Cells were incubated with primary antibody mixture overnight at 4°C then washed with PBS three times for 5 min each. Secondary antibody solution was prepared at manufacturer-suggested dilutions in PBS and added to cells for 1 h at room temperature. After washing with PBS three times for 5 min each, 0.5 µg/ml DAPI in PBS was added for 10–20 min at room temperature to label nuclei. Cells were mounted and allowed to dry for 30 min. Pictures were taken using fluorescence microscopes at University of Pittsburgh Center for Biologic Imaging (Pittsburgh, PA). For each sample, three fields were subjected to analysis. Data are presented as mean $\pm$ standard error of the percentage of cells positive for target protein.

The primary antibodies used include p21 mouse monoclonal antibody (Santa Cruz Biotechnology® Inc., Dallas, TX). Secondary antibodies used include goat anti-mouse antibody Alexa Fluor® 488 (Thermo Fischer Scientific).

Immunohistochemistry

Organs were harvested and mounted in Tissue-Tek®, O.C.T., (Optimum Cutting Temperature; Sakura Finetek Inc., Torrence, CA) then sliced at 4 µm per section. Slides were

stained for chromogenic beta-galactosidase (Cell Signaling Technology) according to manufacturer's protocol. Briefly, slides were washed with PBS, then incubated for 4 min with 1× fixative solution. Slides were washed with PBS twice for 1 min each, then beta-galactosidase solution was added. Beta-galactosidase solution was prepared fresh for each assay and titrated to pH 6.0. Polypropylene plastic staining jars were used to hold slides and beta-galactosidase solution. Staining jars were wrapped with paraffin and incubated overnight at 37°C in 0% CO₂. Slides were washed with PBS twice, then mounted with aqueous mounting media. Slides were stored at -20°C. Separate slides were also stained for H&E. At 400× magnification, the number of blue beta-galactosidase-positive (β-gal-positive) cells and total cells were counted. For each mouse, 2 sections were counted with 4 fields per section. Data are presented as mean ± standard error of percentage of β-gal-positive cells.

Peripheral Blood Cell Counts

Blood samples from 129/Sv Fanca^{+/+} and 129/Sv Fanca^{-/-} mice were collected at baseline and after TBI prior to sacrifice at one week or one year post-TBI. Submandibular blood (100 μl) was collected in tubes with K₂EDTA (BD Microtainer®; Becton, Dickinson and Co., Franklin Lakes, NJ). Complete blood count and differential was performed on the same day using the Abaxis Vet Scan HM5 (Allied Analytic®, Tampa, FL) at the University of Pittsburgh Molecular Imaging Laboratory.

Statistics

GraphPad Prism (LaJolla, CA) was used for statistical analysis and plotting graphs. Graphs were plotted using mean ± standard error (SEM). The data were analyzed using two-tailed Student's *t* test. In this exploratory experiment, *P* value was not corrected for multiple comparisons. Differences were considered significant at *P* < 0.05 (27). At each week of the LTBMCS for each of the end points (i.e., weekly nonadherent cells per flask, percentage confluence, day 7 colonies and day 14 colonies), data were summarized with mean ± standard deviation. The following comparisons were made, using oneway ANOVA *F* test at each time point, followed by Tukey's multiple comparisons.

RESULTS

Radiation-Induced Senescence in Fanca^{-/-} Bone Marrow Stromal Cell Lines

We tested the hypothesis that the level of radiation-induced senescence in Fanca^{-/-} bone marrow stromal cell lines would be greater than that detected in Fanca^{+/+} cells. We first measured senescence-associated beta-galactosidase staining in nonirradiated compared to irradiated cell lines. Fanca^{-/-} and Fanca^{+/+} bone marrow stromal cell lines were irradiated to 5 Gy, then assayed for chromogenic beta-galactosidase at days 1, 3, 5 and 7 postirradiation. We did not see an increase in beta-galactosidase-positive cells (indication of senescence) without irradiation in our system at days 1, 3, 5 or 7. At baseline, there were no differences in β-gal levels between Fanca^{+/+} and Fanca^{-/-} (0.68% ± 0.21% vs. 1.13% ± 0.32%, respectively, *P* = 0.7481) (Fig. 1). At days 3, 5 and 7 after 5 Gy irradiation, Fanca^{+/+} bone marrow stromal cell lines showed significantly elevated levels of β-gal staining (*P* = 0.0162, 0.0365 and 0.0435, respectively). In contrast, at days 1, 3 5 and 7 postirradiation, Fanca^{-/-}

bone marrow stromal cell lines demonstrated a greater increase in β -gal-positive cells ($P=0.0006, 0.0004, 0.0002$ and 0.0335) (Fig. 1). Furthermore, $Fanca^{-/-}$ bone marrow stromal cell lines displayed greater levels of β -gal staining compared to $Fanca^{+/+}$ at day 3, 5 and 7 postirradiation ($P=0.0197, 0.0062$ and 0.0335 , respectively). When higher doses of 7.5 and 10 Gy radiation were administered, we did not detect significantly higher frequencies of radiation-induced senescence. These experiments were performed to optimize the dose and time of irradiation. At higher radiation doses, while increased cell death was observed, no increase in senescence was observed, as indicated by the percentage of β -gal-positive cells.

To confirm the results with β -gal staining, as a biomarker for senescence, we measured levels of p21, which is another commonly used molecular marker of senescence. $Fanca^{-/-}$ and $Fanca^{+/+}$ bone marrow stromal cell lines were plated in multi-chamber slides and allowed to attach overnight, then exposed to 5 Gy. At days 1, 3, 5 and 7 postirradiation, cells were fixed and stained using immunofluorescence for detection of p21, as described in Materials and Methods. Levels of immunofluorescence were quantified as percentage of cells positive for p21 (Fig. 2A).

There were no significant differences in baseline percentage of p21-positive cells when $Fanca^{+/+}$ and $Fanca^{-/-}$ were compared in nonirradiated control cells ($11.7\% \pm 3.48\%$ vs. $14.57\% \pm 7.61\%$, $P=0.8602$). $Fanca^{+/+}$ bone marrow stromal cells showed clearly increased levels of p21 at days 1, 3, 5 and 7 after 5 Gy irradiation ($P=0.0258, 0.0064, 0.0063$ and 0.0489 , respectively). $Fanca^{-/-}$ bone marrow stromal cells showed a similar baseline level of p21 and comparable levels with $Fanca^{+/+}$ cells with increases in the percentage of positive cells at days 1, 3 and 7 postirradiation ($P=0.0139, 0.0033$ and 0.0398 , respectively) (Fig. 2A).

We confirmed the immunostaining results with levels of p21 (Fig. 2B) by measuring mRNA levels of p21 in bone marrow stromal cell lines after irradiation using RT-PCR, as described in Materials and Methods.

The baseline expression of p21 mRNA was similar between the $Fanca^{+/+}$ and $Fanca^{-/-}$ bone marrow stromal cell lines ($1.16 \times \pm 0.09 \times$ versus $1.39 \times \pm 0.17 \times$, respectively, $P=0.8298$) (Fig. 2C). In the $Fanca^{+/+}$ bone marrow stromal cell line, an increase in p21 mRNA was observed at day 1, 3 and 5 postirradiation ($P=0.0079, 0.0031$ and 0.0071 , respectively), but not at day 7 ($P=0.1129$). The same pattern was observed with the $Fanca^{-/-}$ cells (Fig. 2C). There were significant increases in p21 mRNA in $Fanca^{-/-}$ bone marrow stromal cell lines at day 1, 3 and 5 postirradiation ($P=0.0035, 0.0102$ and 0.0309 , respectively), but not at day 7 ($P=0.3791$). The levels of p21 measured by RT-PCR correlated with levels observed in p21 by immunofluorescence for both $Fanca^{-/-}$ and $Fanca^{+/+}$ bone marrow stromal cell lines. Although the results with p21 showed radiation-induced increases, the data with induction of β -gal staining were more sensitive and revealed more quantitative differences between cell lines derived from the two genotypes.

MMS350 Decreases Radiation-Induced Senescence of Bone Marrow Stromal Cell Lines

$Fanca^{+/+}$ and $Fanca^{-/-}$ bone marrow stromal cell lines were irradiated to 0, 5, 7.5 or 10 Gy. In both cell lines, the greatest induction of beta-galactosidase was detected at day 10 after 10

Gy irradiation (Fig. 3A). At day 10 postirradiation, *Fanca*^{-/-} bone marrow stromal cells demonstrated greater increase in percentage of β -gal-positive cells ($0.67\% \pm 0.59\%$ in control nonirradiated cells versus $16.15\% \pm 2.63\%$ in 10 Gy irradiated cells, $P < 0.0001$). *Fanca*^{+/+} bone marrow stromal cells also showed increased numbers ($5.817\% \pm 0.81\%$) of senescent cells at day 10 after 10 Gy irradiation compared to control nonirradiated cells ($0.48\% \pm 0.31\%$) ($P = 0.0925$).

We next compared the effects on radiation-induced senescence of each of three drugs reported to mitigate radiation effects: MMS350 (18), JP4-039 (26) and metformin (11) at day 10 after 10 Gy irradiation. MMS350 and metformin were dissolved in tissue culture media, JP4-039 in its diluent DMSO. DMSO was tested alone, as a control. In both *Fanca*^{-/-} and *Fanca*^{+/+} cell lines, there was no significant effect of the tested drugs or DMSO on increasing levels of senescence in control nonirradiated cells. *Fanca*^{+/+}, but not *Fanca*^{-/-}, bone marrow stromal cells showed a significant decrease in radiation-induced (10 Gy) β -gal staining after JP4-039 treatment ($P = 0.0189$) (Fig. 3B). In contrast, *Fanca*^{-/-} bone marrow stromal cells showed a significant decrease in radiation-induced β -gal staining after treatment with either DMSO or MMS350 ($P = 0.0084$ and $P = 0.0011$, respectively) (Fig. 3B). The similar results with DMSO and MMS350 may reflect the structure of MMS350 as a bifunctional, water-soluble analog of DMSO (16, 32, 33). The opposite effect of JP4-039 in *Fanca*^{+/+} compared to *Fanca*^{-/-} stromal cell lines may be attributable to the higher baseline level of senescence observed in the latter (control irradiated) and higher propensity of this cell line to respond to JP4-039. Because JP4-039 is dissolved in DMSO, DMSO alone was used as a control to determine whether the detectable effects were attributable to JP4-039 or the DMSO diluent. JP4-039 is a known radiation protector and mitigator for both *Fanca*^{+/+} and *Fanca*^{-/-} (25) cell lines. It is unknown why the effect of JP4-039 on senescence was opposite for the two cell lines in Fig. 3B. Based on the known effect of MMS350 as a radiation mitigator *in vivo* (18), and its safety during prolonged administration, we next tested the effect of this drug on TBI-induced senescence *in vivo* with *Fanca*^{-/-} mice.

Effects of Total-Body Irradiation on Senescence in *Fanca*^{+/+} and *Fanca*^{-/-} Mouse Tissues In Vivo

The paradigm of *in vivo* studies is shown in Fig. 4. All animals, which were followed for one year, survived. The 7.5 Gy TBI dose was chosen for its sublethal effect relative to the LD_{50/30} dose of 9.0 Gy in this 129/Sv background mouse strain. Thus, there was no difference in survival of the *Fanca*^{+/+} compared to the *Fanca*^{-/-} mice. To determine the effects of sublethal TBI and MMS350 on cell senescence *in vivo*, organs from mice were removed at one week or one year post-TBI, and stained for quantitation of β -gal-positive cells immediately after sacrifice. At one week after 7.5 Gy TBI, both *Fanca*^{+/+} and *Fanca*^{-/-} mouse spleens had increased levels of senescence compared to preirradiation levels (Fig. 5A). Both the *Fanca*^{+/+} and *Fanca*^{-/-} spleens showed significant radiation-induced senescence ($P = 0.0025$ and $P = 0.0064$, respectively). At this acute time point, MMS350 did not prevent radiation-induced senescence in *Fanca*^{+/+} and *Fanca*^{-/-} spleens ($P = 0.8885$ and $P = 0.2222$, respectively).

We compared results with senescence-associated beta-galactosidase in spleen with a representative slowly proliferating tissue, the brain. In the brain, at one week postirradiation, Fanca^{+/+} and Fanca^{-/-} tissue had similar levels of senescence compared to preirradiation levels (Fig. 5D). At one week after 7.5 Gy TBI, the brain of Fanca^{-/-} mice showed $0.50 \pm 0.28\%$ and Fanca^{+/+} mice showed $0.98 \pm 0.45\%$ (Fig. 5D).

At one year post-TBI, the percentage of β -gal-positive cells in the spleens of sham-irradiated Fanca^{+/+} mice were significantly higher than that in Fanca^{-/-} mice (Fig. 5B and C). Fanca^{+/+} compared to Fanca^{-/-} spleen results for percentage of β -gal-positive cells were $22.21 \pm 3.27\%$ vs. $1.72 \pm 0.34\%$, $P = 0.0002$. MMS350 decreased β -gal expression at one year in sham-irradiated Fanca^{+/+} mouse spleens ($P = 0.048$). In spleens from Fanca^{+/+} mice on regular water, TBI was also associated with a significant decrease in percentage of β -gal-positive cells at one year ($P = 0.0068$). Spleens from Fanca^{+/+} mice given MMS350 after TBI continued to show significant decrease in β -gal compared to spleens from control mice on regular water at one year ($P = 0.0364$).

In contrast, spleens from Fanca^{-/-} mice on regular water demonstrated radiation-induced β -gal at one year post-TBI ($P = 0.0498$). MMS350 administered to sham-irradiated Fanca^{-/-} mice resulted in increased β -gal-positive cells ($P = 0.0145$). MMS350 administered to irradiated Fanca^{-/-} mice showed increased β -gal as well (Fig. 5B and C). The data demonstrate that MMS350 reduced the baseline spleen expression of senescence-associated β -gal in Fanca^{+/+} mice but induced β -gal in both control and 7.5 Gy TBI Fanca^{-/-} mouse spleen.

At one year post-TBI, both Fanca^{+/+} and Fanca^{-/-} mouse brain explants showed no increase in radiation-induced β -gal ($P = 0.067$ and $P = 0.3423$, respectively) (Fig. 5E). There was no significant effect of MMS350 on brain cell senescence after TBI in Fanca^{+/+} or Fanca^{-/-} mouse brain explants compared to each genotype baseline level ($P = 0.602$ and $P = 0.8051$, respectively) (Fig. 5F).

In control mice that were given regular drinking water, Fanca^{-/-} mice had significantly lower levels of β -gal levels in the brain at one year compared to Fanca^{+/+} ($P = 0.014$) (Fig. 5E). Increased baseline levels of apoptosis in Fanca^{-/-} compared to Fanca^{+/+} neural progenitor cells may explain the decreases in β -gal-positive senescent cells.

MMS350 Modulation of Radiation-Induced Decreased Bone Marrow Growth in LTBMcs in Fanca^{-/-} Mice

To quantitate the effects of TBI and MMS350 on hematopoiesis, peripheral blood cell counts were obtained from mice prior to sacrifice at one week or one year post-TBI. Peripheral blood cell counts showed a radiation-induced effect with little effect of MMS350 on white blood cells or platelet blood counts with either genotype at one week (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR15199.1.S1>). At one year post-TBI, peripheral blood cell counts were improved with both genotypes, but there were persistent low white blood cell counts and platelet counts in Fanca^{-/-} mice. These data indicate that aging and radiation had more profound effects on Fanca^{-/-} bone marrow function, as reflected in peripheral blood

counts. These data are consistent with that of many FA patients with Fanca mutations who have peripheral blood evidence of marrow failure (19–21, 34–35).

While peripheral blood counts were improved at one year post-TBI (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR15199.1.S1>) in both genotypes, the late effects of TBI at one year on hematopoiesis were not restored to normal levels by continuous MMS350 administration in either Fanca^{+/+} or Fanca^{-/-} mice when measured in peripheral blood counts.

Long-term marrow cultures were established from marrow of mice in all treatment groups at one week or one year postirradiation and showed a significant effect after 7.5 Gy TBI (Fig. 6). There was a significant effect at one week postirradiation on the marrow in LTBMCS of Fanca^{+/+} and Fanca^{-/-} mice (Fig. 6; Supplementary Fig. S2, Supplementary Tables S1–4; <http://dx.doi.org/10.1667/RR15199.1.S1>), but no significant and/or consistent effect of MMS350 (Supplementary Fig. S2, Supplementary Tables S1–4). Fanca^{-/-} mouse LTBMCS showed a modest stimulatory effect of MMS350 at one week post-TBI with respect to stromal cell growth (Fig. 6).

LTBMCS from Fanca^{-/-}, as well as, Fanca^{+/+} mice displayed a significant reduction in confluence of cells in the adherent cell layer at one week post-TBI (Fig. 6). There was no restorative effect of one week MMS350 administration in drinking water on the explanted marrow adherent layers in LTBMCS from Fanca^{+/+} mice. We quantitated hematopoiesis in LTBMCS by measuring weekly nonadherent cell production. These nonadherent cells were then plated in secondary cultures to determine the number of day 7 scored colony-forming cells and more primitive day 14 scored colony-forming cells [colony-forming unit-granulocyte/macrophage and colony-forming unit-granulocyte/erythrocyte/megakaryocyte/macrophage (CFU-GM and CFU-GEMM)], respectively. LTBMCS from marrow ex- planted at one week post-TBI from both Fanca^{+/+} and Fanca^{-/-} mice showed a significant reduction in nonadherent cell production (Supplementary Fig. S2, Supplementary Tables S1–4; <http://dx.doi.org/10.1667/RR15199.1.S1>), and there was little improvement if mice had received continuous MMS350 administration with marrow from either Fanca^{+/+} or Fanca^{-/-} mice (Supplementary Fig. S2, Supplementary Tables S1–4).

At one year post-TBI, Fanca^{+/+} mouse LTBMCS established showed complete recovery of stromal cell growth (Fig. 6). Fanca^{-/-} marrow stromal cells did not recover proliferative capacity, but there was a clear restorative effect of marrow growth if mice had received continuous MMS350 (Fig. 6). At one year after 7.5 Gy TBI, the parameters of hematopoiesis in marrow explanted to long-term marrow cultures from both Fanca^{-/-} and Fanca^{+/+} mice were not restored by MMS350 (Fig. 6, Supplementary Fig. S2, Supplementary Tables S5–8; <http://dx.doi.org/10.1667/RR15199.1.S1>). While marrow cultures from Fanca^{-/-} mice at one year post-TBI showed a persistent decrease in stromal cell proliferation (Fig. 6), there was significant restoration by continuous administration of MMS350 in the drinking water (Fig. 6). In Fanca^{-/-} LTBMCS, there was no comparable recovery of hematopoiesis (Supplementary Fig. S2, Supplementary Tables S5–8). In control nonirradiated Fanca^{+/+} mice, continuous MMS350 administration for one year increased nonadherent cell growth and colony-forming progenitor cell production in explanted marrow

in LTBMcs (Supplementary Fig. S2, Supplementary Tables S5–8). Thus, in LTBMcs from Fanca^{-/-} mice, MMS350 did not mitigate the effects of radiation as effectively, and there was depressed hematopoietic cell production at one year post-TBI (Supplementary Fig. S2, Supplementary Tables S5–8; <http://dx.doi.org/10.1667/RR15199.1.S1>). The current results establish that partial restoration of marrow stromal cell proliferation in explanted LTBMcs from Fanca^{-/-} mice by MMS350 administration for one year post-TBI was not accompanied by recovery of hematopoiesis. The effect of MMS350 on bone marrow stromal cells in our marrow culture assay system was relatively small; however, a previously published study demonstrated that MMS350 may be more important as a mitigator of the late effects in another organ measuring radiation pulmonary fibrosis (18).

MMS350 Restores Cloning Capacity of Bone Marrow Stromal Cells Removed at One Year after 7.5 Gy TBI

We quantitated the capacity of single bone marrow stromal cells to generate permanent cell lines (cloning capacity) *in vitro*. This assay is a reliable measurement of the proliferative capacity of explanted marrow stromal cells in mice at various times after receiving TBI (12). Marrow was extracted from femur bone marrow of irradiated mice at either one week or one year post-TBI in sublethal groups of mice treated with or without continuous oral administration of MMS350 in the drinking water. We compared freshly explanted bone marrow stromal cells, with bone marrow stromal cell lines that were established *in vitro* from explanted marrow and then passaged for five weeks before quantitation of single cell cloning capacity.

The single cell cloning of freshly explanted marrow from Fanca^{+/+} or Fanca^{-/-} mice, measured at one week or one year post-TBI, revealed no detectable cell doublets in over (600–2,592) single cell containing wells followed for 10 weeks in culture (Table 1). MMS350 in the drinking water did not increase single cell cloning capacity at either time with freshly removed marrow. These results reflect the expected lack of cloning capacity of single hematopoietic cells in the absence of growth factors or semisolid media (27) and the low abundance of stromal cell progenitors in fresh marrow.

The cloning capacity of stromal cells after four sequential passages of bone marrow stromal cell cultures from 4-week-old LTBMcs established at one week post-TBI from irradiated Fanca^{+/+} or Fanca^{-/-} mice was undetectable, and reflected the inability of acutely irradiated explanted marrow stromal cells to sustain passage *in vitro*. One week after 7.5 Gy TBI, adherent cells from 4-week-old long-term bone marrow cultures from each genotype failed to sustain four passages *in vitro*, and there were no passaged cell lines available for the single cell cloning experiment. Thus, there was persistence of acute radiation damage detected by this assay using an attempted passage of confluent adherent cell monolayers from LTBMcs. These results establish the failure of bone marrow stromal cells to recover from the acute radiation response of TBI when tested for cloning capacity at one week post-TBI. Those LTBMcs from MMS350-treated mice at one week post-TBI also did not show capacity for stromal cell line passage or cloning. Thus, there was failure to passage bone marrow-derived stromal cell lines even after cells initially grew to a confluent monolayer in

LTBMC and even though they could sustain a low level of hematopoiesis by supported hematopoietic cells *in vitro*.

The results with cloning of single cells from the adherent cell layers of long-term bone marrow cultures established at the one year time point after 7.5 Gy TBI, and the effect of continuous administration of MMS350 were very different. All LTBMCs derived from bone marrow of both genotypes at one year after 7.5 Gy TBI (with or without prior continuous administration of MMS350 in drinking water), demonstrated successful adherent cell line passage, and there were detectable single cell-derived clonal lines from marrow of all groups (Table 2). As shown in Table 2, both *Fanca*^{+/+} and *Fanca*^{-/-} bone marrow stromal cell lines, derived from nonirradiated explanted marrow at one year post-TBI, showed $33.8 \pm 1.0\%$ and $61.0 \pm 2.4\%$, respectively, of single cells forming clonal cell lines. Furthermore, the cloning capacity of stromal cells was significantly increased with marrow of both genotypes from mice that had been irradiated one year previously if those mice had been treated with continuous MMS350 after TBI compared to no treatment: with *Fanca*^{+/+} irradiated mouse marrow ($22.6 \pm 1.0\%$ compared to $8.2 \pm 1.0\%$) and with *Fanca*^{-/-} irradiated mouse marrow ($37.7 \pm 2.7\%$ compared to $19.7 \pm 5.7\%$) (Table 2). Thus, bone marrow stromal cells from both *Fanca*^{+/+} and *Fanca*^{-/-} mice removed at one year postirradiation showed a radiation mitigation effect of continuous MMS350 treatment *in vivo*.

Figure 7 shows a summary of the hypothesized effect of continuous administration over one year post-TBI of MMS350 on bone marrow stromal cell recovery of proliferative capacity.

DISCUSSION

Irradiated cells respond by entry into one of several pathways, including cell death, quiescence, carcinogenesis, transdifferentiation or senescence (1, 5, 36–40). In particular, the mechanism by which cells enter senescence and whether this condition is reversible is a subject of recent interest (41–45). Senescent cells show lost proliferative capacity and accumulate in tissues in an age-dependent manner (5, 45). Senescent cells release both cell contact mediated, and humoral factors, the latter of which are described as the senescence-associated secretory phenotype (5, 40). Unlike quiescence, which is associated with longterm survival of bone marrow stem cells and stable capacity for meeting proliferative demands of hematopoiesis, the accumulation of senescent cells in the bone marrow has not been associated with a beneficial effect on organ integrity or tissue longevity (42, 44). We analyzed several methods to quantitate senescence in mouse bone marrow-derived cell lines and organs after irradiation. We concluded that histochemical staining for β -gal was the most specific of the assays tested. Quantitative PCR was utilized to measure expression of p21 but was not more specific than the histochemical β -gal stain. Senescence was not uniform throughout the tissue, thus, the site of sampling for extraction of RNA may have produced a result that did not agree with the β -gal staining in another section of the same organ. This explanation may also have contributed to the lack of specificity in the quantitative PCR.

The results of the current study provide evidence that both *Fanca*^{-/-} and control mouse bone marrow stromal cells, of the supportive hematopoietic microenvironment, express late

effects one year after sublethal TBI. Fanconi anemia marrow is known to accumulate greater numbers of senescent cells with age (38, 39). Since there is no known difference between age-related senescence and radiation-induced premature senescence, it is logical to conclude that in the current experimental systems, cellular senescence (replicative or/and stress-induced premature senescence) may have also contributed to other aging effects. Our results demonstrate that the continuous administration of water-soluble radiation mitigator, MMS350, significantly ameliorates the effects of TBI on stromal cells of the hematopoietic microenvironment. We used the non-water-soluble radiation mitigator, JP4-039 (25, 31) as a control for *in vitro* experiments, but restricted our *in vivo* studies to MMS350. The data show detectable bone marrow stromal cell retention of radiation damage expressed *in vitro* at one year post-TBI and damage-mitigation by MMS350 administration *in vivo*. The observed radiation-induced damage *in vitro* was not associated with detectable capacity of bone marrow stromal cells to function *in situ* to support hematopoietic stem cell differentiation, and the proliferative for mature blood cell production (30, 31). Irradiated Fanca^{-/-} and control mice demonstrated partially restored peripheral blood counts, including white cell counts, hematocrit, hemoglobin and platelet counts at one year post-TBI. However, when explanted to culture, bone marrow demonstrated significant persistent defects in support capacity for hematopoiesis. These data confirm a previously published study showing that 3.0 Gy TBI CBA/Ca mouse bone marrow stromal cells maintained *in situ* fail to proliferate *in vitro* three months postirradiation (12). We now extend these observations to marrow from another mouse strain tested at one year after the higher dose of 7.5 Gy TBI.

We used two other mitigators (JP4-039 and metformin) as controls for MMS350. A previously reported study demonstrated that water-soluble MMS350, when delivered in drinking water, is easy and safe to administer (18). Because Fanca^{-/-} mice are radiosensitive and show rapid depletion of bone marrow stem cells (25), we chose this mouse strain to determine whether a positive effect of MMS350 could be detected. Because we were studying late effects, we needed to administer a mitigator continually over the one year observation period. One other mitigator tested, JP4-039, could not be given continuously, since it is not water-soluble and not easily administered (26). Studies with metformin given in drinking water are in progress. MMS350 is both a ROS scavenger and an antioxidant, with stability and less depletion of cellular glutathione and other antioxidant levels (16, 18). MMS350 clearly showed a therapeutic effect in Fanca^{-/-} mice, perhaps because the animals are more fragile, and differences were more easily detected compared to the results with Fanca^{+/+} mice (25).

The current results demonstrate that senescent cells accumulate rapidly when Fanca^{-/-} bone marrow stromal cells are irradiated *in vitro* and to a degree greater than that detected with control mouse bone marrow stromal cells. The data confirm other reports of greater senescence in FA marrow (39, 40). Quantitation of senescent cells at one week and at one year after sublethal TBI, measuring cell numbers in a proliferative tissue (spleen) and slowly proliferating tissue (brain) demonstrated a difference between Fanca^{-/-} and control mice. At one week post-TBI, senescent cell accumulation was greater in Fanca^{-/-} mouse tissues but was decreased at one year. The data suggest that the postirradiation pathway to senescence may be reduced in Fanca^{-/-} mice in favor of other radiation-response pathways including

cell death (34, 37, 45). MMS350-treated Fanca^{-/-} mice showed less senescent cells *in vivo* at one year post-TBI. It is unknown whether this decrease was attributable to greater cell death, increase in senescent cell clearance, more cells moving into quiescence or a combination of these factors. Additionally, it is unknown whether cells of the Fanca^{-/-} mouse blocked the induction of senescence. We have performed clonogenic radiation survival curves with Fanca^{+/+} compared to radiosensitive Fanca^{-/-} marrow stromal cell lines and the latter are radiosensitive (25). Previously published studies also showed that MMS350 is both a radiation protector and a mitigator in the clonogenic survival curve assay (18). Further studies will be required to determine the mechanism of the different response of Fanca^{-/-} organs to radiation-induced senescence in the acute, compared to late, time points.

The published biomarkers of senescence in irradiated cell lines and in tissues do not always correlate, and remain a subject of controversy (1, 2, 5, 28). Nonconcordance of senescence biomarkers was detected in both our *in vitro* and *in vivo* studies. Beta-galactosidase (senescence-associated galactosidase levels) did not always correlate with levels of p21 (41). Furthermore, we could not determine whether those cells positive for each of the three senescence associated markers were the same cells, since co-staining *in situ* was not performed. A published technique by which to sort and separate senescent cells from *in vitro* cultures or organs *in vivo* (36) was attempted with our cell lines and tissues but was not reproducible in our studies. New cellsorting methods and further studies with other organs in TBI Fanca^{-/-} mice, including analysis of lung, liver and intestine, will be required to determine the spectrum of accumulation of senescent cells in rapidly proliferating tissues, compared to slowly proliferating tissues (35). The current studies did not include analysis of radiation-induced changes in the senescence-associated secretory phenotype, nor did we study the physiologic function of organs expressing high levels of senescent cells (spleen) with respect to elaboration of cytokines known to be associated with senescence (28, 40). The current studies support the importance for further evaluation of the continuous administration of MMS350 in ameliorating the late effects of ionizing radiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by the NIAID/NIH (grant no. U19-A168021) and a grant from the Fanconi Anemia Research Fund. This project used the UPMC Hillman Cancer Center Animal, Flow Cytometry and In Vivo Imaging Facilities, which are supported in part by NCI Cancer Center Support Grant no. P30CA047904.

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FIG. 1. Radiation (5 Gy) induced beta-galactosidase (β -gal) in Fanca^{-/-} and Fanca^{+/+} bone marrow stromal cell lines *in vitro*. Panel A: Graph shows mean $\pm$ SEM from three separate experiments for percentage of β -gal-positive cells. Panel B: Representative pictures of β -gal-positive bone marrow stromal cells at 20 $\times$ magnification.

FIG. 2.

Increased radiation-induced senescence marker p21 in Fanca^{-/-} bone marrow stromal cell lines. Permanent bone marrow stromal cell lines were plated, then received sham irradiation or 5 Gy irradiation. At days 1, 3, 5 and 7 postirradiation, the cells were stained using chromogenic beta-galactosidase assay as described in Materials and Methods. Cells staining positive for senescence-associated b-gal were viewed by brightfield microscopy as blue-stained cells. Panel A: Graph shows mean $\pm$ SEM from three separate experiments for percentage of p21-positive cells. Panel B: Representative images at 40 $\times$ magnification of cells stained for p21 (green) and nuclei DAPI (blue). Panel C: RNA was extracted from cells to determine fold change in mRNA of p21 relative to 129/Sv Fanca^{+/+}. Student's *t* tests were used to determine differences within a genotype ($*P < 0.05$ compared to same genotype, day 1, sham irradiation) and between genotypes comparing cells at the same day and radiation dose. Results are representative of one of five separate experiments.

FIG. 3.

MMS350 ameliorates radiation-induced senescence in Fanca^{-/-} bone marrow stromal cells. Permanent bone marrow stromal cell lines were plated in logarithmic phase, then exposed to various doses ranging from 0–10 Gy. At day 3, 10 or 17 postirradiation, cells were stained using the chromogenic beta-galactosidase assay. Panel A: Percentage of β -gal-positive cells is shown as mean $\pm$ SEM of fields from a single exploratory experiment. Student's *t* test was used to determine radiation-induced significant differences ($P < 0.05$), by comparing, within each genotype, the irradiated to sham-irradiated (*) groups. Differences between genotypes were tested for groups of same radiation dose and time point (#). Panel B: Fanca^{+/+} and Fanca^{-/-} bone marrow stromal cell lines were then either sham irradiated or 10 Gy irradiated. Drugs added were DMSO, 100 μ M MMS350 dissolved in media, 10 μ M JP4-039 dissolved in DMSO or 100 μ M metformin dissolved in media. Drugs were supplemented in the media of cells 1 h before irradiation and then continuously afterwards. At day 10 after 10

Gy irradiation, the percentage of β -gal-positive cells was scored and graphed as mean $\pm$ SEM of two separate experiments. Student's t test was used to determine significant differences ($P < 0.05$) due to each drug by comparing to controls that were given no drug (*).

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FIG. 4.

Schematic of measuring MMS350 effects in TBI Fanca^{+/+} and Fanca^{-/-} mice. Subgroups of mice were either sham irradiated or 7.5 Gy irradiated. All mice survived one year, since this was a sublethal TBI dose. One week prior to irradiation, mice were started on drinking water supplemented with no drug (control, regular water) or 400 μ M MMS350. Mice remained on regular water or MMS350-supplemented water continuously after irradiation until time of sacrifice. Mice were sacrificed at one week or one year post-TBI to study markers of aging *in situ* and in explanted marrow in LTBM. C.

FIG. 5.

Effect of TBI on β -gal in Fanca^{+/+} and Fanca^{-/-} mouse spleen and brain at acute (one week) and long-term (one year) time points with or without MMS350. Subgroups of Fanca^{+/+} and Fanca^{-/-} mice were either sham irradiated or 7.5 Gy irradiated and given either regular water or 400 μ M MMS350-supplemented water, as shown in Fig. 4. Spleen and brain tissue were harvested from mice at one week and one year postirradiation, embedded in OCT media, frozen in hexanes, sectioned into 0.4- μ m slices, then freshly stained for β -gal using the chromogenic beta-galactosidase assay. Percentage of β -gal-positive spleen cells are shown at one week (panel A) and one year (panel B) postirradiation. Panel C: Photos of β -gal-positive cells in spleen at one year. Percentage of β -gal-positive brain cells are shown at one week (panel D) and one year (panel E) postirradiation. To highlight the decreased expression of senescence in the brain compared to spleen, we used the same y axis in panels A-E. Panel F: Photos of β -gal-positive cells in brain at one year postirradiation. Data in bar graphs are

presented as mean $\pm$ SEM. Student's t tests were used to determine differences within genotypes (* $P < 0.05$ compared to sham irradiation, regular water) and between genotypes [$\#P < 0.05$ compared to 129/Sv Fanca^{+/+}, same dose (Gy) and drug]. Photos were taken with brightfield microscopy at 40 $\times$ magnification. Blue indicates positive staining for β -gal. Student's t tests were used to determine differences within genotypes from controls, no-drug, sham-irradiated spleens (*) and between genotypes (#).

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FIG. 6.

Effect of treatment with MMS350 *in vivo* on hematopoiesis in long-term marrow cultures established from Fanca^{-/-} and Fanca^{+/+} mice one week or one year after 7.5 Gy TBI.

Percentage of the flasks covered by adherent bone marrow stromal cells is shown. Other LTBM data are shown in Supplementary Fig. S1 and Supplementary Tables S1–8 (<http://dx.doi.org/10.1667/RR15199.LS1>).

FIG. 7.

Schematic representation of hypothesized mechanism of late radiation effects at one year on the marrow microenvironment and amelioration by continuous MMS350 administration. Panel A: Some marrow stromal cell damage was repaired by one week post-TBI to allow formation *in situ* to support hematopoiesis *in vivo*. Panel B: Subtle marrow stromal cell damage was not repaired by one year postirradiation and is revealed by the stress of induction of proliferation *in vitro* in LTBMcs. Panel A images show the *in vivo* interaction of bone marrow stromal cells (mesenchymal stem cells) representing the hematopoietic microenvironment, with hematopoietic stem cells and their progeny, representing the

hematopoietic stem cell compartment. After 7.5 Gy TBI, the majority of differentiated hematopoietic cells *in vivo* and some hematopoietic stem cells are absent (middle image) compared to control sham-irradiated marrow (left-side image), and associated with the acute reduction in peripheral blood counts (leukocytes, red blood cells and platelets). While bone marrow stromal cell proliferation after explant at one week post-TBI is reduced, representing the acute effects of radiation (Fig. 6), this is not associated with impaired recovery *in vivo* (right-side image) for support of hematopoietic cells that were in quiescence and resisted radiation. The images in panel B demonstrate the effects of administration of MMS350 at one year after 7.5 Gy TBI when bone marrow is then explanted to culture. While there is recovery of hematopoiesis *in vivo* (upper middle and right-side images), there was impaired capacity of marrow stromal cells after explant to form a functioning hematopoietic microenvironment and support hematopoietic cells (lower middle and right-side images) (Fig. 6, Supplementary Fig S2A–F; <http://dx.doi.org/10.1667/RR15199.1.S1>). While MMS350 administration for one year post-TBI (lower right-side image) increased survival and proliferation of stromal cells, there was still incomplete recovery of support of functioning hematopoiesis *in vitro*.

TABLE 1:

Cloning Capacity of Fresh Marrow Removed from Controls or 7.5 Gy TBI Fanca^{+/+} and Fanca^{-/-} Mice at One Week or One Year with or without Continuous MMS350 Administration prior to Explant

Time post-TBI	Genotype	Gy	Water	No. of wells plated	Single cell-derived clonal lines/no. plated (%)
1 week	Fanca ^{+/+}	0	Regular	1,824	0
1 week	Fanca ^{+/+}	7.5	Regular	1,344	0
1 week	Fanca ^{+/+}	0	MMS350	1,728	0
1 week	Fanca ^{+/+}	7.5	MMS350	1,248	0
1 week	Fanca ^{-/-}	0	Regular	1,344	0
1 week	Fanca ^{-/-}	7.5	Regular	1,248	0
1 week	Fanca ^{-/-}	0	MMS350	1,344	0
1 week	Fanca ^{-/-}	7.5	MMS350	1,248	0
1 year	Fanca ^{+/+}	0	Regular	1,632	0
1 year	Fanca ^{+/+}	7.5	Regular	1,920	0
1 year	Fanca ^{+/+}	0	MMS350	1,056	0
1 year	Fanca ^{+/+}	7.5	MMS350	1,440	0
1 year	Fanca ^{-/-}	0	Regular	672	0
1 year	Fanca ^{-/-}	7.5	Regular	768	0
1 year	Fanca ^{-/-}	0	MMS350	2,592	0
1 year	Fanca ^{-/-}	7.5	MMS350	1,728	0

Notes. Fresh marrow was flushed from the tibia and femur of Fanca^{+/+} and Fanca^{-/-} mice as described in the Materials and Methods. Flow cytometry of unstained cells was used to sort single cells into individual wells of 96-well plates. Cultures were maintained for 10 weeks after initial plating and scored weekly for clonal lines.

TABLE 2:

Cloning Capacity of Tissue Culture Expanded Stromal Cells Removed from Controls or 7.5 Gy TBI Fanca^{+/+} and Fanca^{-/-} Mice at One Year with or without Continuous MMS350 Administration prior to Explant

Time post-TBI	Genotype	Dose (Gy)	Water	No. of wells plated	No. of wells containing clonal lines	Single cell-derived clonal lines/no. plated (%)
1 year	Fanca ^{+/+}	0	Regular	800	270	33.8 ± 1.0%
1 year	Fanca ^{+/+}	7.5	Regular	800	66	8.2 ± 1.0% ^a
1 year	Fanca ^{+/+}	0	MMS350	800	262	32.7 ± 1.1%
1 year	Fanca ^{+/+}	7.5	MMS350	800	180	22.6 ± 1.0% ^{a,b}
1 year	Fanca ^{-/-}	0	Regular	800	486	61.0 ± 2.4%
1 year	Fanca ^{-/-}	7.5	Regular	800	158	19.7 ± 5.7% ^a
1 year	Fanca ^{-/-}	0	MMS350	800	372	51.8 ± 3.2% ^a
1 year	Fanca ^{-/-}	7.5	MMS350	800	311	37.7 ± 2.7% ^{a,b}

Notes. Fresh marrow was flushed from the tibia and femur of Fanca^{+/+} and Fanca^{-/-} mice and LTBMCs were established as described in the Materials and Methods. After the establishment of bone marrow stromal cell lines from each 4-week LTBM, flow cytometry of removed adherent unstained cells was used to sort single cells into individual wells of 96-well plates. Cultures were maintained for 10 weeks after initial plating, then scored for number and percentage of single cells that grew to a clonal cell line.

^a $P < 0.05$ compared to 0 Gy (sham irradiated).

^b $P < 0.05$ compared to 7.5 Gy.