Deletion of proapoptotic Puma selectively protects hematopoietic stem and progenitor cells against high-dose radiation

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Bone marrow injury is a major adverse side effect of radiation and chemotherapy. Attempts to limit such damage are warranted, but their success requires a better understanding of how radiation and anticancer drugs harm the bone marrow. Here, we report one pivotal role of the BH3-only protein Puma in the radiosensitivity of hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs). *Puma* deficiency in mice confers resistance to high-dose radiation in a hematopoietic cell–autonomous manner. Unexpectedly, loss of one *Puma* allele is sufficient to confer mice radioresistance. Interestingly, null mutation in *Puma* protects both primitive and differentiated hematopoietic cells from damage caused by low-dose radiation but selectively protects HSCs and HPCs against high-dose radiation, thereby accelerating hematopoietic regeneration. Consistent with these findings, Puma is required for radiation-induced apoptosis in HSCs and HPCs, and *Puma* is selectively induced by irradiation in primitive hematopoietic cells, and this induction is impaired in *Puma*-heterozygous cells. Together, our data indicate that selective targeting of p53 downstream apoptotic targets may represent a novel strategy to protecting HSCs and HPCs in patients undergoing intensive cancer radiotherapy and chemotherapy. (*Blood*. 2010;115(23):4707-4714)

Introduction

Maintaining a steady supply of mature blood cells is essential for defense against infection, tumor surveillance, and maintenance of homeostasis. Normal hematopoiesis can be perturbed by DNA-damaging agents such as γ-radiation and chemotherapy drugs. Morbidity rates associated with cancer chemotherapy and radiotherapy limit the effectiveness of these treatments. Radiation and anticancer drugs cause apoptosis in both hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs), leading to severe anemia, bleeding, and infections.[1,2]

Bone marrow (BM) injury and gastrointestinal toxicity are the 2 most common limiting factors for cancer therapies of anticancer DNA-damaging agents, such as ionizing radiation and chemotherapeutical drugs. It was established that γ-irradiation (7-14 Gy) primarily causes damage to HSCs and HPCs in the BM. As a result, mice have severe BM failure and die between 20 and 30 days after radiation.[3] In contrast, a radiation dose greater than 14 Gy in C57B6 wild-type mice caused lethality due to damage to the small intestine and gastrointestinal syndrome in addition to BM failure.[3] The damage is not limited to radiation; some chemotherapeutic drugs may also have similar detrimental effects. Therefore, approaches for improving the safety of anticancer DNA-damaging agents must include measures to either increase tumor sensitivity or reduce toxicity to critical stem cells or progenitors, or both. The latter requires a careful analysis of DNA damage of signaling pathways in HSCs.

Mammalian cells evolved a network of interacting pathways that comprise the DNA damage response, leading to (1) cell-cycle arrest, (2) DNA damage repair, (3) senescence, or (4) apoptosis, depending on the cell type and extent of DNA damage. The specific conserved genes that participate in the DNA damage response are still being defined. In addition, the detailed pathways governing DNA damage response and outcomes, especially those within HSCs, are not completely understood. Therefore, efforts to enhance the effectiveness of intensive γ-radiation and chemotherapeutic cancer therapies have been limited. In vivo studies showed that inactivation of *p53* increased radioresistance in HSCs and HPCs, thereby protecting mice against a lethal dose of radiation.[4,5] It is thought that p53-mediated apoptosis is executed by several of proapoptotic target genes[6]; however, molecular mechanisms of the DNA damage response in HSCs and HPCs remain elusive.

We previously showed that a highly conserved antiapoptotic transcription factor, Slug, promoted the survival of HPCs by down-modulating radiation-induced up-regulation of *Puma*, thereby conferring radioresistance against 6.5-Gy total body irradiation (TBI), which is a sublethal dose of radiation for wild-type mice. *Puma* encodes a p53-responsive BH3-only proapoptotic factor. Deletion of *Puma* converted the radiosensitivity of the *Slug*-deficient mice to radioresistance to 6.5-Gy TBI.[7] These results implicate a key role of Puma in radiation-induced apoptosis in these critical cells that are responsible for radioresistance. This finding prompted us to explore whether the deletion of *Puma* alone is sufficient to allow mice to withstand a higher or lethal dose of ionizing radiation by protecting HSCs and HPCs. Studies of the apoptotic pathways and molecules that selectively act in HSCs and

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HPCs will facilitate the possibility of finding small molecules that improve the therapeutic index of cancer radiotherapy or chemotherapy, or both.

In the present study, we demonstrate that the deletion of *Puma* allows mice to withstand lethal dose radiation in a hematopoietic cell–autonomous manner, and loss of one *Puma* allele renders mice radioresistance to 9-Gy TBI. Remarkably, deletion of *Puma* selectively protects primitive but not differentiated hematopoietic cells from lethal dose radiation, thereby accelerating hematopoietic regeneration. Consistently, *Puma* deficiency suppresses radiation-induced apoptosis in HSCs and HPCs. Interestingly, loss of one *Puma* allele impairs the full radiation induction of Puma in hematopoietic cells, and *Puma* is selectively induced in primitive hematopoietic cells. Thus, it suggests that radiosensitivity is determined by *Puma* gene dosage and radiation dosages. Collectively, our findings here provide the proof of principle that inhibition of Puma is probably a novel strategy for protecting HSCs and HPCs in patients undergoing intensive cancer radiotherapy and chemotherapy.

Methods

Animal studies

All animal studies were evaluated and approved by the Institutional Animal Care and Use Committee of Maine Medical Center. $PUMA^{-/-}$ mice were generated and used after 5 backcrosses to C57BL/6N mice. $p53^{-/-}$ mice were purchased from The Jackson Laboratory. C57BL/6N mice (Ly5.2) and B6.SJL-ptprcα Pep3b (B6.SJL) congenic mice (LY5.1) were purchased from Taconic. All mice were housed in the pathogen-free animal facility at the Maine Medical Center Research Institute. For TBI experiments, the mice were given acidified antibiotic water (1.1 g/L neomycin sulfate and 10^6 U/L polymyxin B sulfate) 3 days before γ-irradiation and then continuously for 30 days to reduce the chance of spontaneous infection. The mice were 4 to 14 weeks old, and γ-irradiation was performed in a ^{137}Cs γ-ray Model 143-45 irradiator (J.L. Shepherd and Associates). The irradiated mice were inspected daily for up to 40 days for radiation-induced death.

BM histology

$Puma^{+/+}$, $Puma^{+/-}$, and $Puma^{-/-}$ mice (8 weeks old) were nonirradiated or γ-irradiated (9 Gy) and then killed at 10 days after irradiation. Femurs were collected and fixed in Bouin fixative (Labchem Inc) for an additional 8 days. Sections were made and stained with hematoxylin and eosin. The images were captured using Axiovision 4.8 image-acquisition software and an AxioCam MRM camera (Zeiss) on a Zeiss microscope (40×/0.75 NA objective).

BM transplantation

For BM transplantation experiments, mice (4-8 weeks old) were fed with acidified antibiotic water and then lethally irradiated with 9-Gy TBI or a total of 13 Gy, given in 2 fractions of 6.5 Gy 3 hours apart. BM cells were injected into the retroorbital venous sinus of irradiated recipients after depletion of red blood cells.

cDNA synthesis and real-time quantitative reverse transcription–polymerase chain reaction

RNA was extracted with Trizol (Invitrogen) according to the manufacturer's protocol. The cDNA was synthesized by a standard reverse transcription reaction with an oligo-dT primer. Real-time polymerase chain reaction (PCR) was performed on a Bio-Rad system with the use of SYBR Green-based assays with AmpliTaq Gold (Applied Biosystems). The *Puma* transcripts were analyzed by quantitative PCR (QPCR), as previously described.[7] The gene expression values were normalized to the geometric mean of the expression values of the housekeeping gene *Hprt* to obtain relative expression levels. All reactions were performed in triplicate.

Western blot analysis of protein expression level

BM cells were harvested from $Puma^{+/+}$ and $Puma^{+/-}$ mice at 0, 4, and 6 hours after irradiation (5 Gy) and lysed in sodium dodecylsulfate extraction buffer [6% sodium dodecylsulfate, 4% β-mercaptoethanol, 20% glycerol, Tris (tris(hydroxymethyl)aminomethane)–HCl, pH6.8]. The protein samples were boiled for 5 minutes before separation by polyacrylamide gel and blotted with anti-Puma polyclonal antibody (Abcam). β-actin was detected as a loading control.

Flow cytometric analysis of different lineages and apoptotic cells

The mice were killed at the indicated time points after TBI, and the BM cells were flushed from the femurs with phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS), filtered through a nylon mesh, and the red blood cells were depleted. BM cells (1×10^6) were blocked with the staining buffer (PBS plus 2% FBS) containing 5% rat serum and Fc-block for 10 minutes before antibody staining. For analysis of differentiated cells, the cells were stained with fluorescein isothiocyanate (FITC)–anti-CD11b (for myeloid cells), FITC–anti-CD3e (for T cells), or FITC–anti-B220 (for B cells) for 20 minutes on ice, followed by incubation with 7-amino-actinomycin D for 10 minutes on ice. The stained cells were washed with staining buffer and resuspended in 0.3 mL of staining buffer. For analysis of HPCs ($Lin^-Sca\text{-}1^+$), the cells were costained with FITC-labeled antibodies specific for lineage markers (CD3e, B220, TER119, CD11b, and Gr-1), phycoerythrin (PE)–anti-Scal-1, and 7-amino-actinomycin D. For analysis of HSCs, the cells were stained for 20 minutes on ice with FITC-labeled antibodies specific for lineage markers, washed with PBS containing 2% FBS, and then stained with a combinations of antibodies specific for c-Kit, Scal-1, Flk2, and CD150 to identify $CD150^+LSK$ ($Lin^-Sca1^+c\text{-}kit^+$), $Flk2^{low}LSK$, and $Flk2^{high}LSK$ cells.

For analysis of apoptotic cells in LSK and differentiated cell populations,[8] BM cells were harvested from nonirradiated and irradiated $PUMA^{-/-}$ and $PUMA^{+/+}$ mice (9 Gy) and cultured in serum-free expansion medium (StemSpan; 10 ng/mL interleukin-3) for HSCs and HPCs (Stemcell Technologies). Six hours after irradiation, BM cells were stained with biotinylated antibodies specific for lineage markers and PE–anti-Scal-1 and Alexa Fluor 647–anti–c-Kit antibodies, followed by Avid-PE/cyanine 7 staining. The stained cells were further stained with annexin V according to the manufacturer's instruction (Biotium). Annexin V^+ LSK cells were gated as apoptotic hematopoietic stem and progenitor cells. All of the stained cells were analyzed by the FACSCalibur instrument (Becton Dickinson). All antibodies were purchased from eBioscience and BioLegend.

Results

Radiosensitivity is determined by *Puma* gene dosage

Numerous studies implicate p53 as a pivotal factor that regulates apoptosis in hematopoietic cells exposed to DNA-damaging drugs or γ-radiation.[6] Targets for p53 include a growing list of proapoptotic genes, including *BAX, BAK, Puma,* and *NOXA*.[6] HSCs and HPCs are the most critical cells for survival of mammalian organisms after exposure to DNA-damaging agents[9]; however, it remains to be determined whether one or a combination of p53 targets mediate apoptosis in these key cells after a high dose of radiation. Our previous study indicated that Slug radioprotects HPCs through direct inhibition of *Puma* transcription in sublethally irradiated mice.[7] Inhibition of Puma by Slug is not sufficient to protect mice against a higher than 8.5-Gy TBI, a lethal dose for wild-type mice.[7]

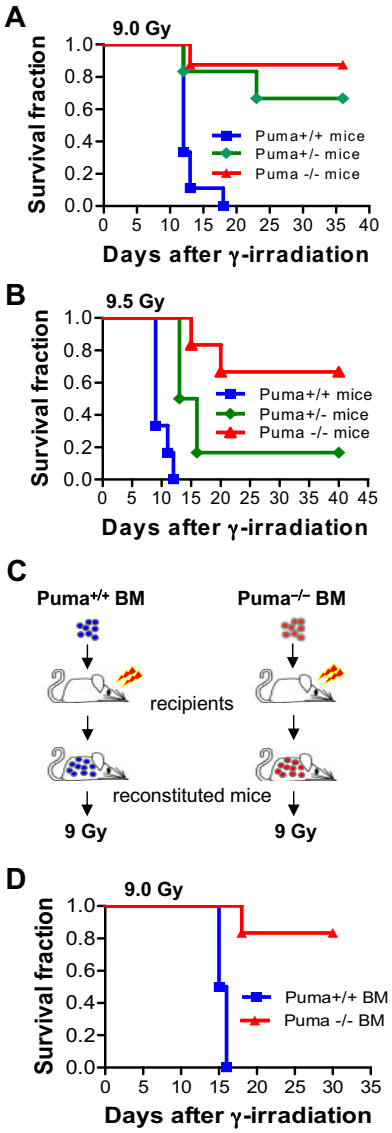

Figure 1. Deletion of *Puma* renders mice resistant to γ-irradiation. (A) Kaplan-Meier survival curves of mice exposed to 9 Gy TBI. *Puma*$^{+/+}$ (n = 9), *Puma*$^{+/-}$ (n = 6), and *Puma*$^{-/-}$ (n = 8) mice were given a single dose of TBI (9 Gy) and were monitored for survival. The enhanced survival rate of *Puma*$^{-/-}$ and *Puma*$^{+/-}$ mice was significantly greater than *Puma*$^{+/+}$ mice ($P < .001$ and $P < .01$, respectively), but the survival rate was not significantly different between *Puma*$^{-/-}$ mice versus *Puma*$^{+/-}$ mice ($P > .05$). (B) Kaplan-Meier survival curves of mice exposed to 9.5 Gy TBI. *Puma*$^{+/+}$ (n = 6), *Puma*$^{+/-}$ (n = 9), and *Puma*$^{-/-}$ (n = 7) mice were then administrated a lethal TBI dose (9.5 Gy) and monitored daily for survival. The survival rate of *Puma*$^{-/-}$ and *Puma*$^{+/-}$ mice was significantly greater than *Puma*$^{+/+}$ mice (*Puma*$^{-/-}$ mice vs *Puma*$^{+/+}$ mice, $P < .001$; *Puma*$^{+/-}$ mice vs *Puma*$^{+/+}$ mice, $P < .01$). (C) Diagram for generation of reconstituted mice. C57BL6/N recipients were lethally irradiated for a total of 13 Gy (6.5 Gy, 2 times, 3 hours apart) and received 1×10^7 total BM cells from *Puma*$^{+/+}$ mice or *Puma*$^{-/-}$ mice (n = 6 mice/group). (D) Kaplan-Meier survival curves of reconstituted mice after 9 Gy TBI. After 8 weeks, the mice in panel C were given a second course of TBI (9 Gy) and monitored for survival. The survival rate of mice reconstituted with *Puma*$^{-/-}$ BM cells was significantly higher than in mice reconstituted with *Puma*$^{+/+}$ BM cells ($P < .001$).

To test the effects of Puma on the BM failure and death of all wild-type mice (LD100) after exposure to a lethal dose of irradiation, we lethally irradiated *Puma*$^{+/+}$, *Puma*$^{+/-}$, and *Puma*$^{-/-}$ mice with 9-Gy TBI. As expected, all of the wild-type mice died within 20 days. Remarkably, deletion of *Puma* allowed 90% of the irradiated mice to survive more than 35 days after γ-irradiation (Figure 1A). Thus, mice lacking *Puma* survive an otherwise lethal dose of γ-irradiation, even though the mice are wild-type for *p53* and *Slug*.

We also found that 70% of *Puma*$^{+/-}$ mice (with one *Puma* copy) withstood 9-Gy TBI (Figure 1A). When the radiation dose was increased from 9 Gy to 9.5 Gy, 20% of *Puma*$^{+/-}$ mice survived for more than 40 days after γ-irradiation (Figure 1B). In comparison to *Puma*$^{+/-}$ mice, the deletion of *Puma* (*Puma*$^{-/-}$) rendered most (70%) of the lethally irradiated mice to survive beyond 40 days after irradiation (Figure 1B). These results show that *Puma*$^{-/-}$ mice phenocopy *p53*$^{-/-}$ mice in terms of their radioresistance.[10] Taken together, these data indicate that *Puma* gene dosage is a critical determinant of radiosensitivity, implying that *Puma* serves as a convergence point downstream of p53 for the DNA damage-induced signaling pathway in HSCs and HPCs.

Radioresistance conferred by *Puma* deficiency is intrinsic to the transplanted BM cells

To investigate whether *Puma* deficiency confers resistance to lethal doses of radiation in a hematopoietic cell–autonomous manner, we reconstituted 2 groups of lethally γ-irradiated wild-type mice with *Puma*$^{+/+}$ or *Puma*$^{-/-}$ BM cells as donor cells with the use of our previously published approach.[7] Thus, these reconstituted mice had the same microenvironment of BM, but they had a different hematopoietic compartment (Figure 1C). As expected, these mice had an equivalent level of white blood cell counts (data not shown). After a second course of 9-Gy TBI given at 2 months after reconstitution, all of the mice reconstituted with *Puma*$^{+/+}$ BM cells died within 20 days, as expected (Figure 1D). By contrast, 90% of the chimeric mice reconstituted with *Puma*$^{-/-}$ BM cells survived more than 30 days after γ-irradiation (Figure 1D). These results show that *Puma* deficiency confers radioresistance to lethal dose of radiation in a transplanted hematopoietic cell–autonomous manner, and the effect does not depend on the BM marrow microenvironment.

Puma deficiency selectively protects HSCs and HPCs against lethal dose radiation

HPCs and HSCs have predominant roles in radioprotection, and they are required for short-term and long-term radioprotection, respectively.[9] We and others showed that Puma has an important role in apoptosis in myeloid progenitor cells[7] and lymphocytes[11-13] after exposure to a sublethal dose of irradiation. In addition, Puma deficiency also afforded radioresistance to intestinal progenitor cells.[14] Therefore, we decided to determine whether the radioresistance of *Puma*$^{-/-}$ mice against lethal dose radiation (Figure 1) is because of increased survival of in vivo HSCs and HPCs. To address this possibility, we determined survival of hematopoietic primitive cells versus differentiated cells (T, B, and myeloid cells) in *Puma*$^{+/+}$, *Puma*$^{+/-}$, and *Puma*$^{-/-}$ mice before and after a sublethal dose of TBI (5 Gy). We found that the number of LSK cells (Figure 2A), HPCs (Figure 2B), T cells (Figure 2C), B cells (Figure 2D), and myeloid cells (Figure 2E) in nonirradiated mice was equivalent, regardless of *Puma* genotypes. Thus, under physiologic conditions, loss of *Puma* does not lead to abnormal hematopoiesis.

Subsequently, we irradiated *Puma*$^{+/+}$, *Puma*$^{+/-}$, and *Puma*$^{-/-}$ sex-matched littermates with a sublethal dose of TBI (5 Gy) and used flow cytometry to quantify each surviving hematopoietic lineages in the BM of these animals at 3 days after irradiation. According to the report from Wang et al,[15] LSK cells or BM cells decreased to their lowest numbers at 3 days after irradiation. As

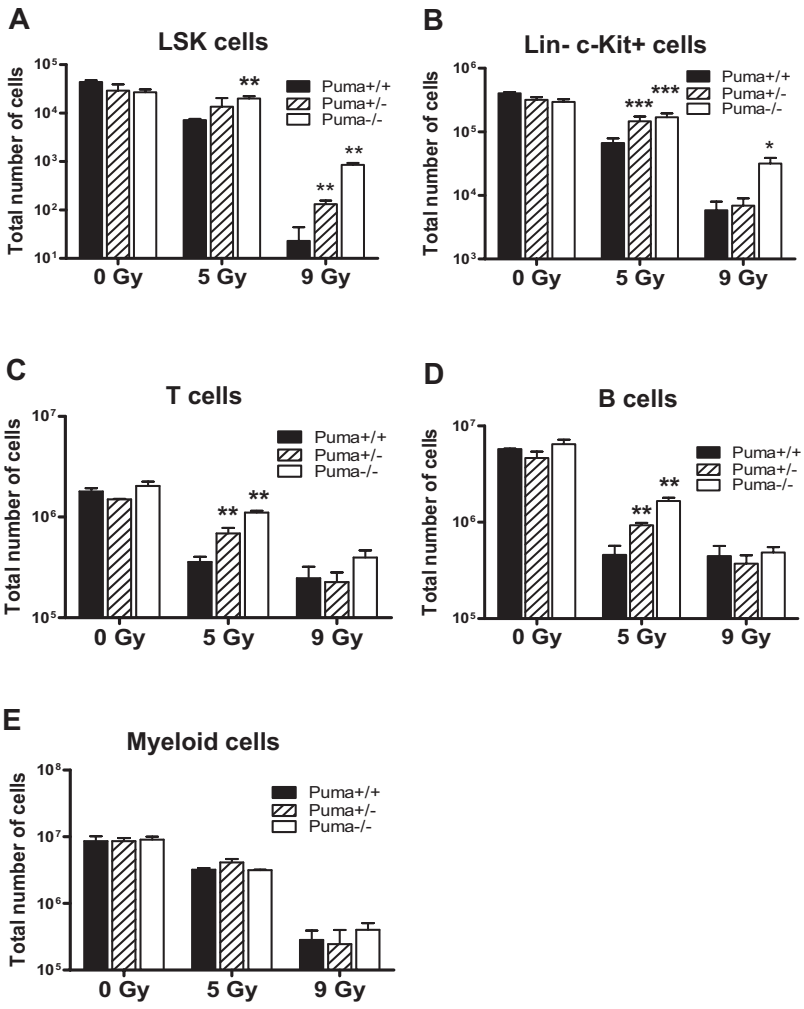

Figure 2. Radiosensitivity of hematopoietic cells is determined by *Puma* gene dosages and radiation dosages. (A) Total number of LSK cells in mice (n = 3 per group). The number of surviving LSK cells in 1 pair of femurs of *Puma*$^{-/-}$ mice was comparable with that from *Puma*$^{+/+}$ mice before irradiation, but this number was significantly higher in *Puma*$^{-/-}$ than in *Puma*$^{+/+}$ mice at 3 days after 5 Gy TBI and was significantly higher in *Puma*$^{-/-}$ and *Puma*$^{+/-}$ mice than in *Puma*$^{+/+}$ mice at 3 days after 9 Gy TBI. $^{**}P < .01$. (B) Total number of HPCs in 1 pair of mouse femurs before and after radiation. The number of HPCs (Lin$^-$c-Kit$^+$) in *Puma*$^{-/-}$, *Puma*$^{+/-}$, *Puma*$^{+/+}$ mice was similar before irradiation but is significantly higher in *Puma*$^{-/-}$ and *Puma*$^{+/-}$ mice than in *Puma*$^{+/+}$ mice at 3 days after 5 Gy TBI. There was a significantly higher number of Lin$^-$c-Kit$^+$ cells in *Puma*$^{-/-}$ mice than in *Puma*$^{+/-}$ and *Puma*$^{+/+}$ mice at 3 days after 9 Gy TBI (n = 3 mice per group). $^{*}P < .05$, $^{***}P < .001$. (C-D) Total number of T and B lymphocytes in 1 pair of femurs of mice. There was no significant difference in cell numbers of differentiated cells in nonirradiated mice, regardless of Puma genotype. The number of surviving differentiated T cells (CD3e$^+$) and B cells (B220$^+$) in *Puma*$^{-/-}$ and *Puma*$^{+/-}$ mice was significantly greater than those in *Puma*$^{+/+}$ mice at 3 days after 5 Gy TBI. Three days after 9-Gy TBI, there was no difference in cell numbers of T and B cells in these mice (n = 3 per group). $^{**}P < .01$. (E) Total number of myeloid cells in 1 pair of femurs of mice before and after exposure to radiation. There was no significant difference in myeloid cell numbers between *Puma*$^{-/-}$ or *Puma*$^{+/-}$ mice or *Puma*$^{+/+}$ mice (n = 3) before and after radiation (5 or 9 Gy TBI). All data are shown as mean ± SD.

expected, the number of surviving LSK cells (Figure 2A), progenitors (Figure 2B), T and B lymphocytes (Figure 2C-D), and myeloid cells (Figure 2E) in sublethally irradiated (5 Gy) mice were fewer than those in nonirradiated animals. Specially, we observed that *Puma*$^{-/-}$ mice retained 3 times more LSK cells, 2.5 times more HPCs (Lin$^-$c-Kit$^+$), at least 4 times more T cells (CD3e$^+$), and 3 times more B cells (B220$^+$) cells in their femurs, respectively, than did their *Puma*$^{+/+}$ littermates after 5-Gy TBI (Figure 2A-D). Interestingly, the total number of myeloid cells is nearly equal in irradiated mice, regardless of *Puma* genotype (Figure 2E). The behavior of T and B cells after 5-Gy TBI is in agreement with the previous report.[12]

Next, we irradiated mice with a high dose of TBI (9 Gy), a lethal dose of irradiation for wild-type mice. The number of surviving LSK cells (Figure 2A) and HPCs (Figure 2B) in the femurs of *Puma*$^{-/-}$ mice was at least 36-fold and 6-fold higher, respectively, than in *Puma*$^{+/+}$ counterparts who received 9-Gy TBI. Surprisingly, we found that the number of surviving T and B lymphocytes and myeloid cells in *Puma*$^{+/+}$, *Puma*$^{+/-}$, and *Puma*$^{-/-}$ mice was essentially the same after 9-Gy TBI, regardless of their genotypes (Figure 2C-E). These results suggest that the absence of *Puma* cannot protect these differentiated hematopoietic cells (T, B, and myeloid cells) against 9-Gy TBI.

Together, these results showed that the deletion of *Puma* selectively protected HSCs (LSK cells) and HPCs in mice exposed to a lethal dose of TBI (9 Gy) but not to a sublethal dose of TBI (5 Gy). Notably, loss of one *Puma* allele (*Puma*$^{+/-}$) was sufficient to partially protect LSK cells against 9-Gy TBI (Figure 2A) and HPCs from 5-Gy TBI (Figure 2B), which may explain why *Puma*$^{+/-}$ mice were partially resistant to radiation (Figure 1A-B). In addition, heterozygosity of *Puma* radioprotected T and B cells against a sublethal dose but not a lethal dose of TBI (Figure 2C-D).

Although LSK cells enrich HSCs, LSK markers do not mark HSCs with high specificity. To further confirm that deletion of *Puma* indeed radioprotects HSCs, we used different combinations of surface markers to analyze long-term HSCs in *Puma*$^{+/+}$ and *Puma*$^{-/-}$ mice before and after exposure to 9-Gy TBI. Our analysis indicated that *Puma*$^{-/-}$ mice have a similar number of long-term (LT) HSCs marked by CD150$^+$LSK cells[16] or Flk2^{low}LSK cells, compared with *Puma*$^{+/+}$ mice, but had 3 times more CD150$^+$LSK cells and 4 time more Flk2^{low}LSK cells at 3 days after 9-Gy TBI (Figure 3A-B). Similarly, the total number of short-term (ST) HSCs (Flk^{high} LSK cells) was nearly the same in nonirradiated mice but was 2.5 times higher in *Puma*$^{-/-}$ mice than in *Puma*$^{+/+}$ mice (Figure 3C). In contrast, these animals had similar numbers of multipotent progenitors marked as CD150$^-$ CD48$^-$CD244$^+$,[16] regardless of *Puma* genotype (Figure 3D). Thus, these data indicated that Puma is a critical mediator of radiation-induced apoptosis in LT-HSCs and ST-HSCs, but not in multipotent progenitors. Taken together, our data indicate that the deletion of *Puma* shielded both differentiated cells (lymphocytes and myeloid cells) and undifferentiated cells (HSCs and HPCs) from sublethal doses of radiation but selectively protected HSCs and HPCs in mice exposed to lethal doses of radiation.

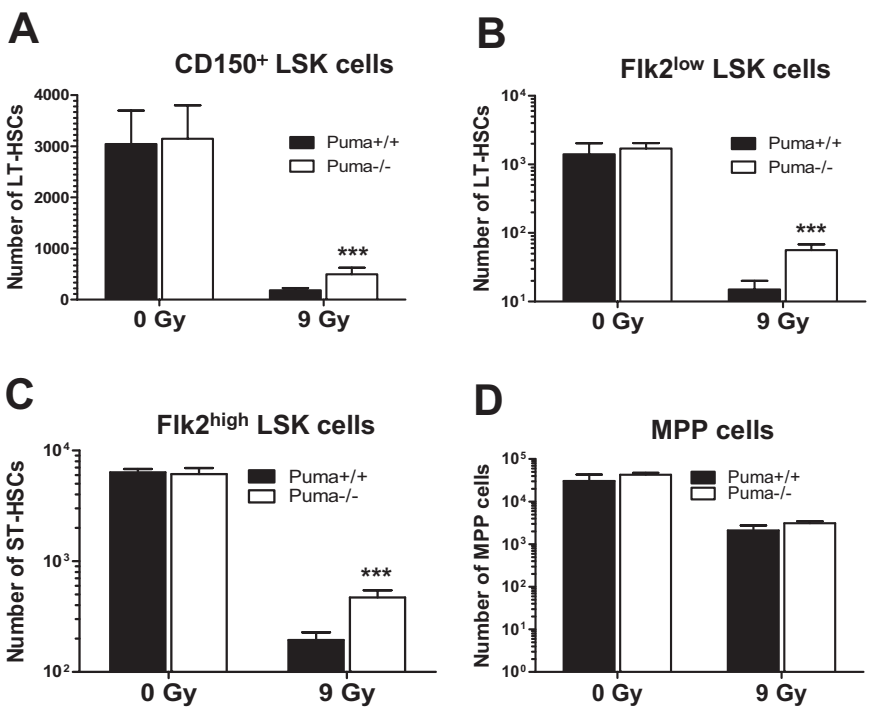

Figure 3. Ablation of *Puma* confers protection of primitive hematopoietic cells against a single dose of γ-irradiation. (A-D) Total number of surviving $CD150^+$ LSK cells (A), $Flk2^{low}$ LSK cells (B), $Flk2^{high}$ LSK cells (C), and multipotent progenitor (MPP; $CD150^-CD48^-CD244^+$) cells (D) in mice before and after 9 Gy TBI. The total number of $CD150^+$ LSK cells, $Flk2^{low}$ LSK cells, and $Flk2^{high}$LSK cells in 1 pair of femurs of $Puma^{-/-}$ mice (n = 6) was comparable with those in $Puma^{+/+}$ mice (n = 5) under normal conditions but was significantly higher in $Puma^{-/-}$ mice than in $Puma^{+/+}$ mice at 3 days after 9 Gy TBI. In contrast, the number of MPP cells ($CD150^-CD48^-CD244^+$) was similar in both $Puma^{-/-}$ mice and $Puma^{+/+}$ mice before and 3 days after 9 Gy TBI, respectively; ***$P < .001$. All data are shown as mean ± SD.

Puma deficiency accelerates regeneration of hematopoiesis in BM after irradiation

Deletion of *Puma* renders HSCs and HPCs radioresistant, based on their phenotype analysis. To ensure that phenotype of these spared HSCs correlate with their function in vivo, we designed an assay in which the repopulation ability of $Puma^{-/-}$ HSCs and $Puma^{+/+}$ HSCs after radiation can be compared in each mouse. To this end, we generated reconstituted mice by transplanting a mixture of $Puma^{-/-}$ ($Ly5.2^+$) and wild-type ($Ly5.1^+/5.2^+$) BM cells into lethally irradiated recipient mice ($Ly5.1^+$) (Figure 4A). At 2 months after BM transplantation, the ratio of $Puma^{-/-}$ donor-derived cells was between 40% and 55% in peripheral blood of these reconstituted mice but increased to 85% at 2 months after a second course of 9-Gy TBI. This data further support the notion that *Puma* deficiency protects HSCs against γ-irradiation.

Furthermore, we directly examined the regeneration of hematopoiesis in the BM sections from lethally irradiated $Puma^{+/+}$, $Puma^{+/-}$, and $Puma^{-/-}$ mice. As expected, there was no noticeable difference in normal hematopoiesis, regardless of genotypes (Figure 4C upper panel). However, at day 10 after 9-Gy TBI, there were few organized hematopoietic cell clusters in $Puma^{+/+}$ mice but significantly more hematopoietic cell clusters evident in the $Puma^{-/-}$ mice (Figure 4C lower panel). Interestingly, the number of repopulated hematopoietic cells in $Puma^{+/-}$ mice varied between $Puma^{+/+}$ and $Puma^{-/-}$ mice, suggesting a *Puma* gene dosage effect. Collectively, our data indicated that loss of *Puma* accelerates the normal regeneration of hematopoietic compartments after γ-irradiation, suggesting that limiting apoptosis in HSCs is a primary mechanism for restoration of hematopoiesis in $Puma^{-/-}$ mice after irradiation.

Ablation of Puma limits apoptosis in primitive hematopoietic cells

In mice, BM is the primary tissue damaged by γ-irradiation at doses up to 14 Gy,[3] and HSCs and HPCs are critical for LT and ST radioprotection, respectively.[9] Being a BH3-only protein, Puma's proapoptotic function has been extensively documented.[11,17-19] Thus, we considered that Puma deficiency might render HSCs and HPCs more resistant to apoptosis after lethal dose radiation, which should accelerate recovery and regeneration of hematopoietic cells in the BM. To test this hypothesis, we irradiated $Puma^{+/+}$ and $Puma^{-/-}$ mice with 9 Gy, the same dose for the prior survival experiments (Figures 1-4). We harvested BM cells from irradiated and nonirradiated mice and cultured them in the StemSpan serum-free expansion medium (10 ng/mL interleukin-3 for suppressing spontaneous apoptosis). We analyzed LSK cells enriched in HSCs and HPCs to quantify apoptosis cells with the use of annexin V staining and found that the percentage of apoptotic LSK cells is 2-fold lower in $Puma^{-/-}$ versus $Puma^{+/+}$ mice before radiation and 3 times lower in $Puma^{-/-}$ versus $Puma^{+/+}$ mice at 6 hours after 9-Gy TBI (Figure 5). Notably, the percentage of apoptotic LSK cells (10.9%) is only slightly higher in irradiated $Puma^{-/-}$ mice than in nonirradiated $Puma^{+/+}$ mice. Thus, deletion of *Puma* renders LSK cells resistant to a higher dose of irradiation (9 Gy).

Puma is selectively up-regulated in primitive hematopoietic cells and its induction by radiation is in impaired in *Puma*-heterozygous hematopoietic cells

Our results indicated that deletion of one *Puma* allele is sufficient to protect animals and their LSK cells and HPCs from lethality caused by both sublethal and lethal-dose TBI (Figures 1-2). In addition, *Puma* expression is known to be up-regulated by γ-irradiation in a p53-dependent manner.[7,11] Thus, we investigated whether loss of one *Puma* allele resulted in impairment of *Puma* induction by p53 after γ-irradiation. In agreement with the previous reports,[7,11] *Puma* was indeed induced by γ-irradiation in $Puma^{+/+}$ hematopoietic cells, and its expression increased up to 2-fold at 1.5 hours and 5-fold at 3 hours after γ-irradiation (Figure 6A). In contrast, *Puma* expression in $Puma^{+/-}$ hematopoietic cells only increased up to 1.5-fold at 1.5 hours and 3.5-fold at 3 hours after

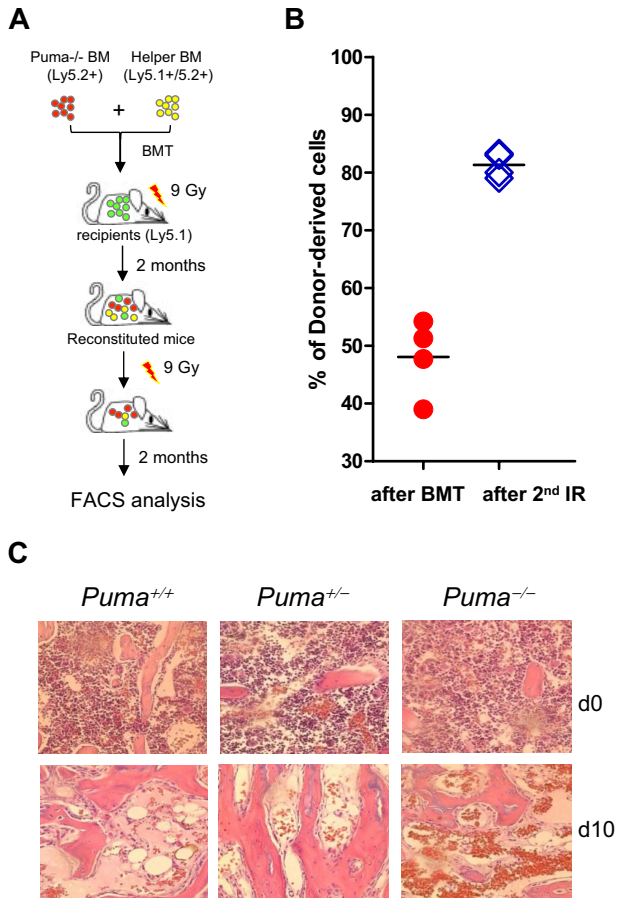

Figure 4. Deletion of *Puma* enhances repopulation of hematopoiesis after a single lethal dose of radiation. (A) Diagram for in vivo HSC competitive repopulating assay. *PUMA*$^{-/-}$ BM cells (Ly5.2$^+$) were mixed with an equal number (1×10^6) of helper BM cells (Ly5.1$^+$/Ly5.2$^+$), and then transplanted into lethally irradiated recipients (Ly5.1$^+$). The percentage of donor-derived cells (Ly5.2$^+$) among hematopoietic cells derived from transplanted BM cells (Ly5.1$^+$/Ly5.2$^+$ and Ly5.2$^+$) was determined by flow cytometric analysis in each recipient at 2 months after radiation. Reconstituted mice were then given a second course of γ-irradiation (9 Gy), and the percentage of donor-derived cells (Ly5.2$^+$) in each recipient was determined by flow cytometric analysis at 2 months after the second radiation. (B) The percentage of donor-derived cells (Ly5.2$^+$) in reconstituted mice in panel A. The percentage of donor-derived cells (Ly5.2$^+$) in each of the reconstituted mice was significantly increased after a second course of γ-irradiation (9 Gy). The data shown are the means ± SD (n = 4 mice/group). $P < .001$. (C) Hematoxylin and eosin staining of the BM cavities of femurs from mice after TBI (9 Gy) or without treatment. Ten days after irradiation, hematopoietic cell clusters were evident in the BM of *Puma*$^{-/-}$ mice but not those of *Puma*$^{+/+}$ mice.

γ-irradiation (Figure 6A). *Puma* mRNA level in *Puma*$^{+/-}$ hematopoietic cells was 1.5-fold lower compared with *Puma*$^{+/+}$ BM cells before γ-irradiation, 2-fold lower at 1.5 hours, and 2.5-fold lower at 3 hours after irradiation. Consistent with these results, Puma protein levels in *Puma*$^{+/-}$ BM cells were lower at each designated time points than in *Puma*$^{+/+}$ BM cells (Figure 6B). As expected, expression of *NOXA*, another p53-responsive gene, in these samples was not altered by *Puma* gene dosage (data not shown). These results, together with the other data (Figures 1-3), indicated that deletion of one *Puma* allele impaired full induction of Puma expression, which resulted in increased survival of LSK cells and HPCs of *Puma*$^{+/-}$ mice, rendering these animals resistant to lethal doses of radiation.

These studies could not answer the critical question: why does deletion of *PUMA* result in selective radioprotection of primitive hematopoietic cells but not differentiated cells? To address this question, we examined the expression level of *Puma* before and after irradiation in LSK cell versus Lin$^+$ cell populations. The QPCR analysis indicated that expression level of endogenous *Puma* is 3-times higher in LSK cells than in Lin$^+$ cells, and that a 2.5-fold increase of *Puma* transcripts was shown in LSK cells versus Lin$^+$ cells after irradiation (Figure 6C). Thus, *Puma* is endogenously expressed at a higher level and prominently induced by irradiation in primitive hematopoietic cells versus differentiated cells. Our data indicate that *Puma* is selectively expressed in primitive hematopoietic cells versus differentiated cells.

Discussion

Maintaining the integrity of hematopoiesis is essential for survival of mammalian organisms during lethal intrinsic and extrinsic insults. To accomplish this function, the hematopoietic system replenishes mature blood cells daily and maintains sufficient numbers of stem and progenitor cells to regenerate the hematopoietic system quickly. Therefore, apoptotic pathway(s) should operate differently in mature cells and in primitive hematopoietic cells when exposed uniformly to the same genotoxic agent. Although p53-deficient mice survive a lethal radiation dose due to increased resistance of HSCs and HPCs in the BM,[4,5] it remains to be determined whether a common key factor downstream of p53 mediates radiation-induced apoptosis in these key cells.[6]

Our results indicate that deletion of *Puma* protects mice from lethal doses of irradiation in a cell-autonomous manner. Interestingly, deletion of one *Puma* allele (*Puma*$^{+/-}$) rendered the mice resistant to 9-Gy TBI, but radioresistance declined when the γ-irradiation dose increased to 9.5 Gy, which is similar to the results noted with the *p53*$^{+/-}$ mice.[10] In addition, our data showed that both *Puma* mRNA and protein levels are significantly lower in *Puma*$^{+/-}$ BM cells than in wild-type (*Puma*$^{+/+}$) BM cells at specific time points after γ-irradiation, implying that the *Puma* mRNA level in *Puma*$^{+/-}$ mice is affected by its gene dosage. Consistent with these new findings, our previous studies indicate that Slug, a transcriptional repressor, protects HPCs in vivo by

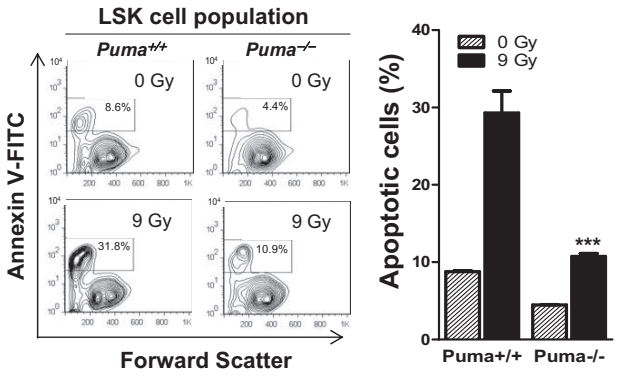

Figure 5. Puma deficiency diminishes radiation-induced apoptosis in primitive hematopoietic cells. Analysis of apoptotic rate in LSK cell population in BM with annexin V–based apoptosis assay. BM cells were harvested from nonirradiated and irradiated (9 Gy) *Puma*$^{-/-}$ and *Puma*$^{+/+}$ mice and cultured in the StemSpan serum-free expansion medium supplemented with 10 ng/mL interleukin-3 (for suppressing spontaneous apoptosis). Six hours later, BM cells were stained for LSK markers, and the apoptotic cells were identified by a fluorescence-labeled annexin V. Apoptotic LSK cells (annexin V$^+$ LSK) were analyzed by flow cytometry. The percentage of annexin V$^+$ LSK cells (the numbers in boxes in left panel) is significantly increased in BM cells from *Puma*$^{+/+}$ mice than those from *Puma*$^{-/-}$ mice before and after radiation. The data shown are representative of flow cytometric analysis (left) and the mean of the triplicate (right). ***$P < .001$ (*Puma*$^{-/-}$ vs *Puma*$^{+/+}$ after radiation). FACS indicates fluorescence-activated cell sorting. Bar graph data are mean ± SD.

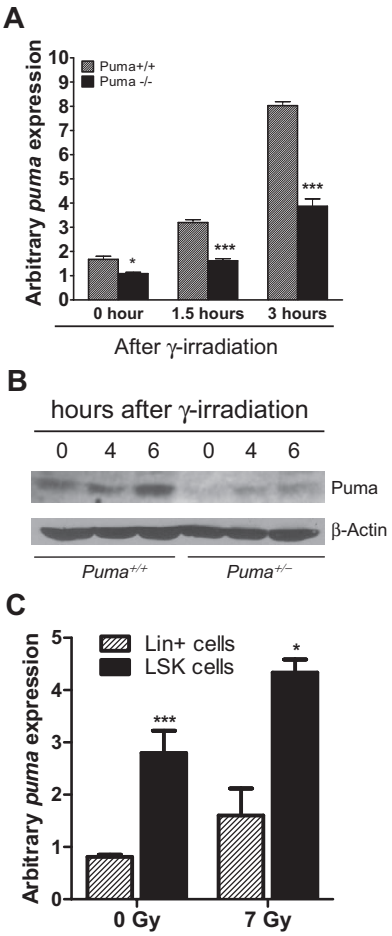

Figure 6. Puma is up-regulated by γ-irradiation in a gene dose-dependent manner and is selectively expressed in LSK cells. (A) QPCR analysis of *Puma* mRNA expression levels in primary hematopoietic cells from *Puma*$^{+/+}$ and *Puma*$^{+/-}$ mice at 1.5 and 3 hours after irradiation (5 Gy) or no treatment. *Puma* gene expression levels were normalized, based on the levels of the housekeeping gene *HPRT*. The cross comparison between *Puma*$^{+/+}$ and *Puma*$^{+/-}$ mice at each time point was highly significant (2-tailed Student *t* test). $^{*}P < .05$, $^{***}P < .001$ (*Puma*$^{+/+}$ vs *Puma*$^{+/-}$ at each time point). (B) Western blot analysis of Puma expression levels. Lysates extracted from BM of *Puma*$^{+/+}$ and *Puma*$^{+/-}$ mice at the indicated time points after irradiation (5 Gy) were probed with an anti-Puma and an anti–β-actin antibody (loading control). (C) QPCR analysis of *PUMA* induction by irradiation in LSK cells. LSK and Lin^{+} cells were sorted, respectively, from wild-type mice and cultured in the StemSpan serum-free expansion medium (10 ng/mL interleukin-3). The cells were untreated or treated with γ-irradiation (7 Gy) and then subjected to total RNA extraction at 3 hours after irradiation. QPCR analysis was used to qualify *Puma* mRNA level in nonirradiated and irradiated cells and normalized to *HPRT* expression level. $^{***}P < .001$ and $^{*}P < .05$ (LSK vs Lin^{+} cells). Data in panels A and C are mean ± SD.

down-modulating transcriptional up-regulation of *Puma* in response to ionizing radiation.[7] Taken together, these data clearly indicate that *Puma* is an essential target of p53 in HSCs and HPCs, and it mediates p53-induced apoptosis in these key cells.

In vivo proapoptotic functions of Puma in radiation-induced signaling were previously shown in lymphocytes and other nonhematopoietic cells.[11-14] To our surprise, in contrast to a sublethal dose (5 Gy) effect, deletion of *Puma* selectively protected HSCs and HPCs from a lethal radiation dose (9 Gy). In addition, we demonstrated that surviving HSCs identified by their phenotypes in irradiated *Puma*$^{-/-}$ mice retained repopulating potential.

How does deletion of *Puma* protect HSCs and HPCs but not mature hematopoietic cells from lethal dose radiation? p53, as a principle transcriptional factor, responds to γ-irradiation and up-regulates several target genes, including *Puma*, *NOXA*, *BAX*, *DR5*, and others.[17,18,20-23] The timing for p53 to induce its target genes is determined by both radiation dose and cell type.[24-26] It is conceivable that *Puma* is primarily induced in primitive cells and thus is a rate-limiting factor for lethal-dose radiation–induced apoptosis in these cells. Indeed, our QPCR data indicated that *Puma* is selectively expressed in primitive versus differentiated hematopoietic cells before and after irradiation. The molecular mechanisms underlying lineage-specific *Puma* transcription regulation should be extensively studied in the future.

The role of Puma in determining the fate of HSCs and HPCs is relevant to cancer therapy because therapeutic doses of radiation and chemotherapy are primarily limited by the sensitivity of these key cells to genotoxic agents. Interestingly, a small molecule inhibitor of p53, which was recently identified by high-throughput screening, protects mice from γ-irradiation. There are some safety concerns with this approach due to the critical roles of p53 in tumor suppression and DNA damage repair.[27]

It has been widely assumed that the inability of p53 to induce apoptosis would enable irradiated cells to propagate any chromosomal aberrations sustained from γ-irradiation exposure, thereby increasing the likelihood of generating new malignant cells. However, recent work from Christophorou et al[28] and Efeyan et al[29] surprisingly showed that, in mouse models, the inability of p53 to induce apoptosis did not increase the onset of tumor formation and also that oncogenes, rather than p53-mediated apoptosis, provided the key signal to p53 to suppress tumorigenesis.

Consistent with previous reports from others,[11,30-32] mice deficient in *Puma* are not inherently prone to form tumors. Interestingly, *Puma* deficiency accelerates Myc-induced lymphomagenesis,[32,33] implying that Puma may function differently in tumors induced by different oncogenic signals. Thus, it is necessary to further test whether Puma inhibition will promote tumor progression or drug resistance in preexisting tumors. Nevertheless, according to our findings, inhibition of Puma rather than p53 represents a safer approach to protecting HSCs and HPCs from the detrimental effects of radiation and possibly chemotherapy.

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Authorship

Contribution: L.S., Y.S., and Z.Z. designed and performed research and analyzed data; W.F. and Y.G. performed the research; Z.C. and Z.Z.W. contributed to research design; A.T.L. provided materials; W.-S.W. designed the research and wrote the paper.

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