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Contrasting Roles for C/EBP α and Notch in Irradiation-Induced Multipotent Hematopoietic Progenitor Cell Defects

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Abstract

Ionizing radiation (IR) is associated with reduced hematopoietic function and increased risk of hematopoietic malignancies, although the mechanisms behind these relationships remain poorly understood. Both effects of IR have been commonly attributed to the direct induction of DNA mutations, but evidence supporting these hypotheses is largely lacking. Here we demonstrate that IR causes long-term, somatically heritable, cell-intrinsic reductions in hematopoietic stem cell (HSC) and multipotent hematopoietic progenitor cell (mHPC) self-renewal that are mediated by C/EBP α and reversed by Notch. mHPC from previously irradiated (>9 weeks prior), homeostatically restored mice exhibit gene expression profiles consistent with their precocious differentiation phenotype, including decreased expression of HSC-specific genes and increased expression of myeloid program genes (including C/EBP α). These gene expression changes are reversed by ligand-mediated activation of Notch. Loss of C/EBP α expression is selected for within previously irradiated HSC and mHPC pools, and is associated with reversal of IR-dependent precocious differentiation and restoration of self-renewal. Remarkably, restoration of mHPC self-

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renewal by ligand-mediated activation of Notch prevents selection for C/EBP α loss of function in previously irradiated mHPC pools. We propose that environmental insults prompt HSC to initiate a program limiting their self-renewal, leading to loss of the damaged HSC from the pool while allowing this HSC to temporarily contribute to differentiated cell pools. This “programmed mediocrity” is advantageous for the sporadic genotoxic insults animals have evolved to deal with, but becomes tumor promoting when the entire HSC compartment is damaged, such as during total body irradiation, by increasing selective pressure for adaptive oncogenic mutations.

Keywords

Irradiation; Hematopoietic Stem Cells; Differentiation; Notch; Leukemia

Introduction

Ionizing radiation (IR), including X-rays and γ -rays, is a form of electromagnetic radiation able to penetrate human skin and damage underlying tissues. Humans are regularly exposed to low doses of IR by natural sources found in the air, soil, and water. The increasing use of high-dose IR in medicine, illustrated by the application of IR therapy to treat roughly 60% of solid tumors and the growing application of CT scans [1, 2], exemplify the critical need to understand the mechanisms underlying IR-associated human disease.

Studies following atomic bomb survivors revealed reduced hemoglobin levels, peripheral blood cytokine levels, and cellular immunity as long as 50 years after initial exposure [3, 4]. Occupational IR exposure has also been associated with decreased humoral immunity and peripheral blood CD4⁺ T cell counts [5], and survivors of pediatric leukemia treated with chemo- or radiotherapy are at increased risk for developmental defects and secondary malignancies [6]. Indeed, exposure to IR in multiple contexts is highly associated with the development of malignancies, primarily myeloid leukemias, which has become particularly relevant in the last few decades as the numbers of long-term survivors of cancer treatments increases [7, 8].

The persistence of panhematopoietic defects following IR exposure suggests that hematopoietic stem cells (HSC) may be the primary reservoir for IR-induced effects, as these pluripotent stem cells are responsible for continuously replenishing the adult hematopoietic system, including the HSC compartment itself. Hematopoiesis is a highly regulated and complex process, involving numerous microenvironmental, signaling, epigenetic and transcriptional cues. One key regulator of HSC maintenance is Notch, a transmembrane receptor that upon ligation undergoes proteolytic cleavages to release the intracellular domain, ICN, for nuclear translocation and transcriptional activation [9]. Notch signaling has been implicated in neural, skin, and muscle stem cell functions as well as in T lymphopoiesis [10]. Previous studies suggest a positive role for Notch in HSC self-renewal during stressed hematopoiesis but not at steady state [11–18].

In opposition to self-renewal, HSC undergo differentiation in order to replenish the hematopoietic compartment. One transcription factor known to direct differentiation, C/EBP α , has been found to be recurrently mutated or silenced in acute myeloid leukemia

(AML) patient samples [19–21]. Though a clear role in granulopoiesis has been shown [22], C/EBP α has been shown to play both positive and negative roles in HSC maintenance under different experimental contexts [23, 24].

Previous studies have identified IR-induced alterations in HSC function, including increased senescence, persistent oxidative stress, and decreased reconstitution potential *in vivo* [25–28]. Transplant studies have shown that irradiated host environments can influence donor cell function via radiation-induced bystander effects, such as through reactive oxygen species (ROS) and inflammation [29, 30]. Additionally, we have previously shown that ICN expression is selected for within previously irradiated HSC pools in the bone marrow, enhancing leukemogenesis [25]. In the current studies, we explored the mechanism underlying sustained reductions in multipotent hematopoietic progenitor cell (mHPC) fitness (ability to contribute epigenotype/genotype to subsequent cell generations) post-IR, and how this fitness reduction could influence leukemogenesis. We demonstrate that IR-mediated reductions in mHPC self-renewal persist long after the initial exposure, are mediated by C/EBP α -driven precocious myeloid differentiation, and are reversed by activation of Notch. Notably, restoration of self-renewal by ligand-mediated Notch activation prevents selection for reduced C/EBP α expression. These results suggest that the persistent IR-induced reductions in mHPC function are reversible, and thus amenable to therapeutic intervention.

Materials and Methods

Mice

C57Bl/6 and C57Bl/6-Ly5.1 mice were obtained from NCI at 6–8 weeks of age. Nrf2 KO mice were obtained from Jackson Labs. Irradiation was conducted using Cs¹³⁷ at a dose rate of 1.069 gray (Gy)/min. Previously irradiated, homeostatically restored (IR^P) mice were generated by dosing mice with 2.5Gy total body irradiation and allowing the mice to recover for a minimum of 9 weeks. For myeloablative bone marrow transplants, recipient mice were sublethally irradiated with 5.0Gy 48 hours prior to transplantation or lethally irradiated with 2 doses of 5.0Gy separated by 4 hours on the day of transplantation. Nonmyeloablative transplantation were performed by injecting 6.6×10^5 Ctrl or IR^P WT bone marrow cells into Ctrl or IR^P congenic recipients without any conditioning at the time of transplant. All transplantations were conducted by tail vein injection of cells in normal saline.

Flow Cytometry

Bone marrow was harvested from the tibias, femurs, hip bones, radius, and ulnas. Samples were hemolyzed and cells were counted on a Millipore Guava 8HT. Cells were stained in FACS buffer (5% FBS, 4mM EDTA) with 5% anti-FC (24G2 hybridoma lysate) and antibodies (Table S1). For analysis of peripheral blood, 20uL of peripheral blood was harvested from the tail into 50uL heparin, hemolyzed, and stained for the indicated surface markers (Table S1).

LSK cultures

For sorted cultures, LSK were flow sorted into each well of a 96-well U-bottom suspension plate using a Beckman Coulter MoFlo XDP70. For cKit⁺ cultures, whole bone marrow was

enriched for cKit⁺ cells using Miltenyi CD117 (cKit) microbeads, Magnetic-Activated Cell Sorting (MACS), and 3×10^4 – 1×10^5 cKit⁺ cells were cultured per well of 96 well plate. Cells were cultured as described previously [17]. In short, cells are cultured in IMDM (Invitrogen), 20% fetal bovine serum (HyClone), 50uM β -mercaptoethanol and 100ng/mL human interleukin (hIL)-6, hFlt3-L, murine stem cell factor (mSCF), and 10ng/mL hIL-11 (Peprotech and R&D). IgG-DL1 was coated at 2.5ug/mL in DPBS (Invitrogen) and incubated at 4°C overnight.

Statistics

Two-way ANOVA or unpaired T-Tests were used to analyze experiments unless otherwise specified. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

See Supplemental Materials and Methods for additional information.

Results

Radiation results in persistent defects within the HSC population *in vivo*

To examine long-term IR effects on HSC, two common phenotypic HSC populations were analyzed from previously irradiated (IR^P) and age- and gender-matched non-irradiated control (Ctrl) mice. Mice and bone marrow cells from mice that were irradiated 9–12 weeks prior, possessing hematopoietic systems that have returned to homeostasis, are referred to as IR^P. The Lineage^{neg}Sca1⁺cKit⁺ (LSK; Figure 1A left panel) population contains both HSC and multipotent progenitor populations, and the Lineage^{neg}CD34^{neg}CD135^{neg}Sca1⁺cKit⁺ fraction is further enriched for long-term HSC (LT-HSC; Figure 1A) [31]. IR^P mice possessed significantly reduced fractions and numbers of both LSK and LT-HSC with no significant reductions in total bone marrow cellularity (Figure 1B; S1A). Additionally, numbers of common myeloid progenitors (Lineage^{neg}Sca1^{neg}cKit⁺CD34⁺CD16/32^{neg}; CMP) were reduced in IR^P bone marrow, but numbers of both granulocyte/macrophage progenitors (Lineage^{neg}Sca1^{neg}cKit⁺CD34⁺CD16/32⁺; GMP) and megakaryocyte/erythroid progenitors (Lineage^{neg}Sca1^{neg}cKit⁺CD34^{neg}CD16/32^{neg}; MEP) were unaffected (Figure 1C, S1B).

Previous studies demonstrated increased lymphoid differentiation of HSC within 24 hours of IR exposure [26]. To determine whether IR results in long-term alterations in the frequency of either HSC population, we assessed the surface expression of CD150, a SLAM surface marker expressed at high levels on myeloid-biased and low levels on lymphoid-biased HSC [32, 33]. We found no significant change in the frequency of CD150^{low}LSK 9 weeks post IR, but the frequency of CD150^{hi}LSK was significantly increased in IR^P mice (Figure S1C). However, frequencies of B220⁺ and CD11b⁺ bone marrow cells were unaltered in IR^P mice (Figure S1D).

Previous reports show reduced LT-HSC activity in IR^P bone marrow [25, 26, 28]. In order to confirm LT-HSC functional defects, unfractionated bone marrow from Ctrl or IR^P congenic (CD45.1⁺) mice was mixed at a 9:1 ratio with competitor bone marrow from a Ctrl C57Bl/6 (CD45.2⁺) mouse and transplanted into lethally irradiated CD45.2⁺ recipients. IR^P donor bone marrow failed to compete with CD45.2⁺ Ctrl competitor cells as measured by

contributions to peripheral blood (Figure 1D), indicating reduced LT-HSC function within IR^P bone marrow. To account for the difference in HSC frequency, Ctrl and IR^P C57BL/6 (CD45.2⁺) bone marrow was normalized for the transplantation of 35 HSC (LSKCD34^{neg}) per sublethally irradiated congenic (CD45.1⁺) recipient. Ctrl donor cells contributed to long-term, multi-lineage hematopoiesis (Figure S1E), with contribution to the myeloid population as far out as 150 days (Figure S1F). In contrast, the contribution of IR^P donor cells to hematopoiesis was significantly reduced, with contribution to myelopoiesis near background by 150 days post transplant.

The competitive transplantation assays utilized lethal irradiation of recipient mice, and therefore donor cells are transplanted into an inflammatory environment where they are rapidly stimulated to proliferate and differentiate in order to reconstitute the recipient hematopoietic compartment. To assess IR^P LT-HSC function under more homeostatic conditions, Ctrl or IR^P CD45.2⁺ competitor cells were transplanted into Ctrl or IR^P congenic (CD45.1⁺) recipients without concomitant myeloablation of recipients at the time of transplant. Contributions to hematopoiesis were measured by assessing frequency of donor cells as in the previous experiment. Ctrl donor cells transplanted into IR^P recipients expanded to a significantly greater extent than Ctrl donor cells transplanted into Ctrl recipients (Figure 1E). Additionally, IR^P donor cells were unable to expand when transplanted into either Ctrl or IR^P recipients. Collectively, these studies reveal that IR results in long-term reductions in mHPC frequency and function.

IR^P LSK exhibit cell-intrinsic self-renewal reductions *in vitro*

To further elucidate the impact of IR on LSK functional parameters, *in vitro* mHPC expansion was performed as previously described [17]. This culture model promotes the self-renewal of early mHPC for several weeks, although these mHPC lose LT-HSC activity with time *in vitro*. LSK from Ctrl or IR^P mice were sorted and cultured with various cytokines promoting proliferation and survival. The total cell expansion of IR^P cells at day 6 of culture was similar to that of Ctrl, but was significantly reduced by day 12 (~3-fold reduction; Figure 2A). No significant difference was identified between Ctrl and IR^P in culture-initiating cell frequency (Figure S2A,B), cell cycle profiles on day 6 (Figure S2C), or cell survival, as measured by DAPI exclusion, through day 12 (Figure 2B).

Self-renewal and differentiation were assessed using the mHPC markers Sca1 and cKit and the myeloid markers Gr1 and CD11b (Figure 2C). B220⁺ and CD3⁺ lymphoid and Ter119⁺ erythroid populations were not detected (data not shown), consistent with the inability of the culture conditions to support differentiation of these lineages [17]. As Sca1 expression has been shown to decrease upon differentiation of HSC *in vivo* [34–36], the culture LSK population was divided into LSK Sca-hi (LSK^{hi}) and LSK Sca-low (LSK^{low}) populations. Re-plating of sort-purified LSK, LS, LK, and L[−]S[−]K[−] cells purified from 4 day cultures indicated that the LSK population has the greatest potential to give rise to all culture populations, with LK or L[−]S[−]K[−] cells only giving rise to terminally differentiated myeloid progeny (Figure S2D).

This culture model supports a >100X expansion of Ctrl LSK cells over 6 days, indicating self-renewing divisions of these cells. The proportion of both LSK^{low} and LSK^{hi} cells were

significantly reduced in IR^P cultures by day 6, and coincided with a striking increase in the proportion of more differentiated myeloid cells, particularly Gr1⁺ cells, within IR^P cultures (Figure 2D,E). Additionally, cultures of the more primitive LT-HSC population revealed similar numbers of total cells and LS^{low}K cells, but significantly reduced LS^{hi}K numbers on day 6 for IR^P relative to Ctrl (Figure S2E,F). This resulted in a significant reduction in the total number of IR^P LSK on day 6. Thus, as the total number of cells on day 6 is unaffected by prior IR exposure, the ratio of undifferentiated LSK to differentiated myeloid cells is dramatically reduced. This LSK expansion defect was apparent in mice exposed to X-ray or γ -rays (Cs¹³⁷) (Figure S2G), and required a dose of greater than 0.8Gy (Figure 2F). Moreover, expansion of IR^P LSK cells was not affected by co-culture with Ctrl LSK and, conversely, IR^P LSK exerted no effect on the expansion of Ctrl LSK (Figure 2G), indicating that the IR-mediated LSK expansion defect is cell intrinsic.

Finally, the potential of cultured Ctrl and IR^P LSK to contribute to multilineage hematopoiesis was analyzed by transplantation of total progeny arising from 600 LSK on day 6 of culture into sublethally irradiated congenic recipients. Ctrl LSK cultures were able to contribute to multilineage hematopoiesis in recipient mice for at least 100 days (Figure 2H), although contributions to the CD11b⁺ (myeloid) population were not sustained at 4 months post-transplant for all recipients of Ctrl LSK cultures (Figure S2H). Thus, Ctrl cultures maintain mHPC activity, but not robust HSC activity, as previously shown [17]. In contrast, IR^P LSK cultures failed to contribute to hematopoiesis of recipient mice beyond 27 days, suggesting that IR^P LSK lose mHPC potential faster than Ctrl LSK *in vitro*. Collectively, these results indicate that high-dose IR exposure results in long-term, cell-intrinsic reductions in the self-renewal of mHPC.

Notch activation rescues IR^P LSK expansion *in vitro*

Previous studies have shown that the intracellular Notch1 mutant, ICN1, is selected for within IR^P HSC *in vivo* but not in Ctrl HSC pools [25]. To determine whether Notch activation is selected for within the IR^P LSK compartment due to a correction of the IR^P mHPC self-renewal defect, Ctrl and IR^P LSK were cultured in the presence or absence of the Notch ligand DL1. On day 6, total cell expansion in both Ctrl and IR^P cultures was reduced with activation of Notch (Figure 3A). Notch activation increased the proportion of LSK, specifically within the LS^{hi}K population, and decreased the proportion of differentiated progeny in both Ctrl and IR^P cultures (Figure 3B), resulting in a rescue of LS^{hi}K numbers in IR^P cultures on day 6 (Figure 3C). As the DL1-mediated increase in LSK was composed primarily of LS^{hi}K cells, further analyses focused on the LS^{hi}K population. The reduction in total cell number and increased proportion of LSK in DL1 cultures on day 6 is consistent with increased quiescence and self-renewal of mHPC. Analysis of Notch surface expression revealed only a slight reduction (~10%) in Notch2, but not Notch1, on freshly isolated IR^P LSK (Figure S3A).

The ability of *in vitro* Notch activation to restore mHPC activity was assessed by transplantation of the total progeny arising from 200 sorted Ctrl or IR^P LSK cultured with or without DL1 for 6 days. Ctrl donors, but not IR^P donors, contributed to hematopoiesis up to 11 weeks post transplant (Figure S3B). Activation of Notch during culture did not affect

contribution to hematopoiesis by either Ctrl or IR^P donor cells. Therefore, although Notch activation restores IR^P mHPC expansion *in vitro*, the short 6-day stimulation is unable to permanently restore IR^P mHPC activity *in vivo*.

Time course studies were performed for Ctrl and IR^P LSK cultures to assess cell fate dynamics over time. Mathematical modeling was used to determine the probability of

differentiation per LSK division (D) as $D = 1 - \frac{1}{2} \left(\frac{S_t}{S_0} \right)^{\frac{1}{\log_2 \left(\frac{P_t}{S_0} \right)}}$ using the initial number of LSK (S_0), and experimentally determined numbers of total cells (P_t) and LSK (S_t) on days 3 and 5 (Figure 3D and S3C). For an explanation of the derivation for this equation, refer to Supplemental Materials and Methods. IR^P LSK had a significantly increased probability of differentiation per cell division (~0.38) as compared to Ctrl LSK (~0.28) for both days 3 and 5 (Figure 3E). Notch activation led to significantly decreased probabilities of differentiation for Ctrl and IR^P on both days. Equivalent D values for day 3 and 5 reveals that the reduced propensity for self-renewal of IR^P LSK remains a static property over many cell divisions. The ~40% increase in differentiation probability for IR^P LSK may appear modest, but is amplified over the course of many cell cycles, resulting in a substantial loss in LSK fitness (Figure 3F). Notch activation reverses the increased probability of IR^P LSK differentiation, resulting in a restoration of IR^P LSK fitness.

IR^P LSK display hallmarks of oxidative stress

Next Generation RNA-seq and Gene Set Enrichment Analysis (GSEA) were employed to identify potential mechanisms of the IR-mediated LSK expansion defect. LSK from Ctrl or IR^P mice were sorted and used immediately for RNA-seq to determine transcriptional changes in LSK at steady state ("Fresh LSK", Figure 4A). To assess the effect of Notch activation on gene expression, Ctrl and IR^P LSK were expanded in culture, with or without DL1, and after 6 days LSK were resorted and subsequently used for RNA-seq analysis ("Cultured LSK"). Hierarchical clustering showed high correlation between biological replicates within each condition for both freshly isolated and culture expanded LSK (Figure S4A). Note that the impact of DL1 treatment dominates over prior IR exposure, as IR^P+DL1 gene expression profiles are much more closely related to Ctrl+DL1 than IR^P profiles. The entire data set is available in GEO (GEO Accession #GSE61602; significant changes are in Table S2).

RNA-seq revealed a significant enrichment for Nrf2-regulated genes [37–39] in both freshly isolated and culture expanded IR^P LSK, which was reduced upon Notch activation (Figure 4B and S4B,C). Nrf2 is an oxidative stress sensor that when activated, localizes to the nucleus and drives transcriptional activation of downstream antioxidant genes [40]. Previous studies have shown increased levels of ROS within IR^P LSK [27]. We analyzed lipid peroxidation in multiple bone marrow populations. Lin^{neg} and myeloid progenitor (Lin^{neg} cKit⁺; LK) populations of Ctrl and IR^P mice contained comparable levels of lipid peroxidation (Figure 4C, S4D). Notably, IR^P LSK exhibited significantly elevated levels of lipid peroxidation. Interestingly, we were unable to detect any significant increase in 2',7'-dichlorodihydrofluorescein diacetate (DCF) staining for intracellular hydrogen peroxide within IR^P LSK (Figure S4E). These results indicate that IR causes persistent and active

generation of oxidative stress specifically within LSK that appears localized to membranes, as would be measured by lipid peroxidation and potentially missed when assessing cell-wide oxidative stress by DCF.

To determine whether persistent activation of Nrf2 impacts long-term self-renewal defects in IR^P mHPC, we irradiated (or not) sets of NRF2 knockout (KO) mice together with age-matched wild-type (WT) mice. IR^P WT and NRF2 KO mice exhibited similar reductions in LSK and LT-HSC numbers in the bone marrow 9 weeks after IR exposure (Figure 4D, S4F). In addition, Nrf2 KO IR^P LSK exhibited a similar reduction in expansion *in vitro* as compared to WT IR^P LSK (Figure 4E, S4G). Notch activation rescued the expansion of Nrf2 KO IR^P LSK similarly to WT IR^P LSK. Therefore, Nrf2 activation via persistent oxidative stress does not contribute to or mitigate IR-mediated long-term LSK self-renewal defects, and Notch-mediated rescue of IR^P LSK self-renewal does not require Nrf2.

Monocyte differentiation programs are enriched within IR^P LSK

As previous studies indicate that IR^P LSK exhibit reduced self-renewal and increased differentiation propensity, GSEA was used to analyze expression of a previously identified HSC specific gene expression signature [41]. Both fresh and cultured IR^P LSK expressed significantly reduced HSC gene expression signatures relative to Ctrl (Figure 5A, S5A,B), indicating decreased stemness long-term post IR. To further investigate HSC self-renewal, expression of known Notch activated and repressed genes were analyzed [42]. Ctrl LSK cultured with DL1 exhibited increased expression of genes activated by Notch and decreased expression of genes repressed by Notch (Figure S5C,D), indicating that these targets are directionally modulated by DL1 *in vitro*. Surprisingly, previous IR exposure did not significantly alter expression of genes that were activated upon Notch signaling (Figure S5E). However, genes repressed by Notch, including C/EBP α , were enriched within IR^P LSK (Figure S5F). These results suggests that although increased Notch activation rescues IR^P LSK self-renewal, it is not through the direct correction of a defect in Notch transcriptional activation, but may act in part by restoring Notch-dependent transcriptional repression.

Consistent with their precocious differentiation, genes upregulated in monocytes [41] were significantly enriched in freshly isolated and culture-expanded IR^P LSK (Figure 5B, S6A,B). Genes expressed in granulocytes [41] were also enriched in culture-expanded IR^P LSK (Figure S6C,D). The expression signature for the myeloid differentiation factor C/EBP α was altered in freshly isolated and culture expanded IR^P LSK (Figure 5C), with a subset of genes upregulated and a subset downregulated. Increased expression of C/EBP α in cultured IR^P LSK was validated by qPCR (Figure S6E), although the more dramatic impact on the C/EBP α transcriptional program in IR^P LSK suggests an effect beyond C/EBP α mRNA levels. IR^P LSK were also significantly enriched for genes regulated by PU.1, a transcription factor involved in both lymphoid and myeloid differentiation [43–45] (Figure S6F–H). Transgenically-increased expression of C/EBP α or PU.1 has previously been shown to result in decreased HSC maintenance [46]. Gene signatures of E2F and Myc, which are suppressed by C/EBP α [47], were down regulated in IR^P LSK (Figures S7A–D). Importantly, Notch activation with DL1 largely counteracted all of these IR-dependent

changes, partially restoring self-renewal signatures and E2F/Myc target gene expression, and inhibiting differentiation-associated target gene expression. In all, these results indicate that IR^P LSK express decreased LT-HSC, increased myeloid, and altered C/EBPα signatures *in vivo* and *in vitro*, all of which are in good measure reversible upon Notch activation.

Inhibition of C/EBPα expression rescues the IR^P self-renewal defect *in vitro*

Knockdown of C/EBPα was used to determine whether C/EBPα contributes to the IR^P LSK self-renewal defect. cKit⁺ bone marrow from Ctrl and IR^P mice was transduced with lentivirus co-expressing GFP and an shRNA targeting C/EBPα (shCEBPA#1) or a control shRNA (sh0). The degree of C/EBPα knockdown for shCEBPA#1 was measured in the murine myeloblast-like 32D cell line (Figure S8A). By analyzing the fraction of cells expressing GFP within different culture populations over time, selection for or against each shRNA can be determined. Expression of sh0 was maintained at low levels in both Ctrl and IR^P LSK on days 4, 6 and 9 of culture, indicating neutral selection for sh0 (Figures 6A). In contrast, the proportion of shCEBPA#1⁺ cells within LS^{hi}K populations increased over time, particularly within the IR^P LS^{hi}K, demonstrating positive selection for inhibition of C/EBPα within the LSK population.

To determine whether inhibition of C/EBPα rescued the IR^P LSK expansion defect, cell population frequencies within the total GFP^{neg} and GFP⁺ populations were analyzed on day 6 (Figures 6B). The population distribution within GFP⁺ cells in sh0 cultures resembled that of GFP^{neg} cells, with a decrease in IR^P LSK expansion relative to Ctrl. In contrast, the frequency of LSK and differentiated progeny increased and decreased, respectively, within the GFP⁺ populations of shCEBPA#1 cultures, indicating that inhibition of C/EBPα augments LSK expansion and inhibits precocious differentiation *in vitro*. Similar to Notch activation, inhibition of C/EBPα significantly decreased the probability of differentiation per LSK cell cycle (Figure 6C, S8B) and increased the relative fitness of IR^P LSK as assessed by the rate of LSK generation *in vitro* (Figure 6D). Together, these results indicate that inhibition of C/EBPα is selected for within and is able to rescue precocious differentiation of IR^P LSK *in vitro*.

C/EBPα inhibition is selected for within IR^P LSK *in vivo*

To determine whether selection for shCEBPA occurs *in vivo*, cKit⁺ bone marrow from Ctrl and IR^P WT mice was transduced with sh0 or shCEBPA#1 and transplanted into sublethally irradiated congenic recipients. Expression of GFP within peripheral blood over time was used to assess selection for or against each shRNA (Figure 6E). Additionally, as frequencies of short-lived mature myeloid cells can be used as a surrogate for HSC activity [48], expression of GFP within the CD11b⁺ myeloid compartment was used as a proxy for selection within the HSC population (Figure 6F). The frequency of sh0⁺ cells was maintained at low levels in both the total peripheral blood and the myeloid compartment for both Ctrl and IR^P donors. However, the percentage of shCEBPA#1⁺ donor cells within the peripheral blood population, both total and myeloid, was increased to a significantly greater extent in IR^P donor cells compared to Ctrl donor cells. These results demonstrate that prior irradiation promotes selection for inhibition of C/EBPα within IR^P HSC *in vivo*.

Notch activation prevents selection for C/EBP α knockdown within LSK *in vitro*

As Notch activation reversed the enrichment for myeloid differentiation gene signatures in IR^P LSK, *in vitro* LSK cultures were utilized to determine whether Notch activation prevents selection for CEBPA inhibition. For these experiments, a second independent shRNA (shCEBPA#2) with a more modest inhibition of CEPBA expression (~44%) was used (Figure S8A). cKit⁺ bone marrow from Ctrl or IR^P mice was transduced with sh0 or shCEBPA#2. As above, GFP expression served as readout of selection for or against the shRNAs. In both Ctrl and IR^P cultures, sh0⁺ cells were maintained at a low frequency over time (Figure 7A). In Ctrl cultures, the frequency of shCEBPA#2⁺ cells was significantly elevated within the LS^{hi}K population on day 6, but decreased by day 9. In contrast, the frequency of shCEBPA#2⁺ cells within IR^P LS^{hi}K was significantly elevated on day 6 and continued to increase by day 9. Moreover, DL1-mediated activation of Notch significantly reduced selection for shCEBPA#2 within IR^P LS^{hi}K. Thus, by restoring IR^P mHPC self-renewal, Notch activation prevents selection for C/EBP α inhibition. Furthermore, inhibition of CEBPA resulted in an increased proportion of LS^{hi}K cells within the GFP⁺ populations, but did not further enrich the fraction of LS^{hi}K cells within DL1 cultures (Figure S9), suggesting that CEBPA inhibition and Notch activation are acting through a similar pathway to restore LSK expansion in IR^P cultures.

Discussion

The long-term persistence of panhematopoietic defects after IR exposure is suggestive of a persistent, multipotent cellular reservoir for IR-induced effects. Here we show that IR results in persistent, somatically heritable, reversible reductions in the function of mHPC and HSC that propagate these IR-associated effects. IR^P mHPC possess decreased self-renewal and precocious differentiation *in vitro*. Additionally, both fresh and cultured IR^P LSK have decreased expression of genes associated with LT-HSC. Activation of Notch, a known promoter of HSC self-renewal, restores expansion of IR^P mHPC *in vitro* and reverses many of these gene expression changes. IR exposure is known to damage the bone marrow niche, including supporting cells that express Notch ligands [49, 50], which may disrupt Notch signaling. Gene expression analysis of revealed no alterations in expression of genes activated by Notch signaling (although genes downregulated by Notch signaling were upregulated in IR^P mHPC). Thus, at present, it is not clear how alterations in Notch signaling via IR-induced changes in the bone marrow microenvironment contribute to the initiation and persistence of IR-induced long-term reductions in mHPC self-renewal. Nonetheless, our data demonstrate that the ability of Notch activation to overcome IR^P mHPC self-renewal defects is linked to activation of self-renewal pathways and antagonism of differentiation.

Fresh and Cultured IR^P LSK did not exhibit increased expression of lymphoid-associated genes, previously shown to be elevated in a subset of both myeloid-biased and lymphoid-biased HSC at 12 hours post IR [26]. These differences are likely due to different microenvironmental contexts and HSC states, reconstitution versus homeostatic at early and late time points, respectively, following IR exposure. Both fresh and cultured IR^P mHPC did exhibit enrichment for gene signatures of differentiation, specifically for myelopoiesis, that

are reversed upon Notch activation coincident with the Notch-mediated reversal of precocious differentiation *in vitro*. One such Notch-reversible gene signature altered in IR^P mHPC is C/EBP α , a known regulator of granulopoiesis. Inhibition of C/EBP α was preferentially selected for within IR^P mHPC, and restored the expansion of IR^P mHPC, *in vitro* and *in vivo*. Selection for C/EBP α inhibition detected within Ctrl populations *in vitro* and *in vivo* is likely due to effects of culture conditions, such as elevated oxidative stress and the particular cytokine milieu, or to irradiator-induced bystander effects exerted by the irradiated environment of the recipient mice. Moreover, activation of Notch prevented selection for C/EBP α loss of function in LSK *in vitro*. Collectively, these results indicate that the IR-mediated mHPC self-renewal defect is mediated by increased differentiation, such as through persistently activated C/EBP α , and that inhibition of such precocious differentiation, such as via Notch activation, can restore IR^P mHPC self-renewal.

Though enhanced differentiation was identified as a mediator of the IR^P mHPC defect, the mechanism that stably maintains this phenotype within IR^P mHPC remains unknown. Elevated oxidative stress is likely a key component. Lipid peroxidation assays and enrichment for an Nrf2 transcriptional program indicate the persistence of oxidative stress specifically within the IR^P LSK population, suggesting a continuous active generation of ROS within LSK. It has been suggested that NADPH Oxidase 4 (NOX4) may be responsible for the persistent generation of ROS within LSK [27]. Though we did not detect a significant increase in NOX4 mRNA transcripts, IR^P LSK possessed elevated mRNA levels of the NOX4 binding partner, p22phox (GEO Accession # GSE61602, Table S2), which is required for NOX4 membrane localization and ROS generation [51]. Additionally, increased lipid peroxidation but not DCF staining within IR^P LSK suggests membrane localization and not a cell-wide distribution of ROS. Previous studies have shown that inhibition of oxidative stress *in vivo* can partially alleviate IR^P HSC competitive and reconstitution defects [25, 27]. Additionally, Notch-mediated rescue of IR^P LSK self-renewal *in vitro* correlates with reversal of the oxidative stress-associated gene expression. Therefore, although resolution of these issues will require additional experimentation, persistent generation of oxidative stress is a potential regulator of long-term IR^P HSC defects.

As IR is a known DNA damaging agent, IR-associated hematopoietic defects and oncogenesis have conventionally been ascribed to a direct induction of function-impairing and oncogenic mutations by IR, respectively [3, 52–54]. However, such IR-induced DNA damage should be random, and therefore effects should not be reproducible or easily reversible. We instead argue that IR-induced DNA damage induces a program that reduces self-renewal of the affected HSC as a means to maintain overall HSC pool fitness (Figure 7B). In this model, HSC in young, healthy individuals are highly fit, and constantly in competition with one another for niche space and nutrients, promoting maximal pool fitness. In the natural context of sporadic DNA damage, the rare damaged HSC institutes a program of limited self-renewal and augmented differentiation thereby effectively reducing its fitness and promoting removal of the rare damaged HSC while preserving the HSC's ability to temporarily contribute to the differentiated pools (which may limit immediate physiological stress to the organism). This “programmed mediocrity” restores HSC pool fitness and promotes tissue homeostasis, as the damaged HSC are gradually replaced by local healthy

neighboring HSC. However, in the more modern context of system-wide injury, such as with total body IR, the same program is initiated in all or almost all HSC, resulting in decreased function of the entire HSC compartment.

Such “programmed mediocrity” can be either tumor suppressive or promoting depending on the context of the DNA damage. In undamaged, healthy HSC pools, the highly fit cells are well adapted to their environment, and acquisition of random oncogenic mutations is unlikely to provide an individual cell with a fitness benefit relative to neighboring cells. In fact, many oncogenic mutations experimentally induced in young, healthy HSC result in decreased fitness of the mutation-bearing cell by decreasing self-renewal or increasing differentiation (apparently the “cost” of increased cell cycling) [55]. Thus, in the more natural context of sporadic DNA damage, programmed mediocrity promotes tissue maintenance and is tumor suppressive by allowing for the removal of the rare damaged HSC, restoring HSC pool fitness, and thereby limiting selection for adaptive oncogenic mutations.

In the more modern context of system-wide injury, such as by total body IR, programmed mediocrity is implemented in the entire HSC pool, resulting in decreased HSC function throughout. This decrease in mean population fitness leaves “room for improvement”, and thus increases selective pressure for adaptive oncogenic mutations that are able to repair or circumvent the fitness defect. Our studies indicate that the IR^P-associated decrease in self-renewal and increase in differentiation of the HSC compartment increases selective pressure for mutations that restore self-renewal or inhibit differentiation, such as mutations activating Notch or impairing C/EBP α . Consistent with this hypothesis, many early driver mutations in leukemias inhibit cell differentiation [56–58]. Cells harboring an adaptive oncogenic mutation would preferentially expand, increasing the odds for subsequent oncogenic mutations to occur in a cell bearing the initiating mutation, thus promoting leukemogenesis. In addition, IR-induced DNA damage will increase the frequency of mutations and genomic rearrangements, which could provide fuel (in the form of fitness-changing and therefore selectable oncogenic events) upon which IR-promoted selection can act.

Programmed mediocrity is certainly not the only mechanism influencing tissue maintenance and oncogenesis following cellular insults, such as IR, but likely acts in concert with other cellular programs, including apoptosis and senescence. Previous studies have shown that loss of function for p53 is selected for in HSC during or shortly after IR exposure, when cells undergo apoptosis during the acute IR response, but is not selected for at time points long after the initial exposure when the acute IR response has subsided [59, 60]. Similarly, BATF induction immediately following irradiation has been shown to contribute to impaired HSC maintenance [26]. Other studies have shown increased senescence within murine HSC months after the initial IR exposure [28]. It is likely that these processes act in series, perhaps dependent on the level of IR-induced damage, to reduce HSC maintenance post-IR. Again, these programs would have negative effects when the insult is population wide, resulting in reduced fitness of the residual HSC pool and persistence of panhematopoietic defects. However, our studies indicate that “programmed mediocrity” is reversible, and therefore has the potential to be therapeutically alleviated. A further understanding of the mechanisms underlying IR-induced reductions in HSC self-renewal will serve as a

foundation for the development of future therapeutics to restore HSC function and thereby prevent irradiation-induced oncogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. IR reduces mHPC frequency and function

A) Representative flow cytometric profiles of bone marrow cells from Ctrl and IR^P mice. Bone marrow cells were assessed for surface expression of hematopoietic lineage markers (Lin), cKit and Sca1. Lin^{neg}Sca1⁺cKit⁺ (LSK) cells were further analyzed for expression of CD135 and CD34 to identify long-term HSC (LSKCD135^{neg}CD34^{neg}; LT), short-term HSC (LSKCD135^{neg}CD34⁺; ST) and multipotent progenitor cells (LSKCD135⁺CD34⁺; MPP). Numbers indicate percent of gated population. **B)** Total number of LT-HSC and LSK in the bone marrow of Ctrl and IR^P mice (n=3 mice each). **C)** Total numbers of common myeloid progenitors (Lin^{neg}Sca1^{neg}cKit⁺CD16/32^{neg}CD34⁺; CMP), granulocyte-monocyte progenitors (Lin^{neg}Sca1^{neg}cKit⁺CD16/32⁺CD34⁺; GMP), and megakaryocyte-erythrocyte progenitors (Lin^{neg}Sca1^{neg}cKit⁺CD16/32^{neg}CD34^{neg}; MEP) (n=4 mice each). **D)** Ctrl or IR^P CD45.2⁺ bone marrow was mixed 9:1 with congenic CD45.1⁺ bone marrow and transplanted into lethally irradiated recipient mice. Peripheral blood was analyzed over time for contribution of Ctrl or IR^P CD45.2⁺ donor cells to assess competitiveness. Statistics generated using Two-way ANOVA with multiple comparisons. (n=5 mice each) **E)** Ctrl or

IR^P CD45.2⁺ bone marrow was transplanted into Ctrl or IR^P CD45.1⁺ recipient mice without myeloablation at the time of transplantation. The contribution of CD45.2⁺ donor cells to hematopoiesis was assessed over time by flow cytometric analysis of the peripheral blood. (n=2 mice each, representative of 4 experiments). Data are represented as mean $\pm$ SEM.

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Figure 2. IR^P LSK exhibit cell intrinsic reductions in self-renewal *in vitro*

200 Sorted LSK from Ctrl (n=5) or IR^P (n=4) C57Bl/6 mice were cultured. **A)** Total cell numbers on days 6 and 12. **B)** Percent of viable cells in LSK cultures on days 6 and 12 as assessed by DAPI exclusion. **C)** Representative flow plots for cell population analysis of LSK cultures on day 6. Myeloid differentiation was assessed by surface expression of Gr1 and CD11b (left panels). Lin^{neg} cells were further assessed for expression of cKit and Sca1 (right panels). **D)** Population distribution in Ctrl and IR^P cultures. **E)** Number of LS^{low}K and LS^{hi}K on day 6 of LSK cultures. **F)** 200 LSK were sorted from mice irradiated with varying doses of Cs¹³⁷ IR (0–1.6Gy) and cultured for 6 days, after which the number of LSK were assessed by flow cytometry (n=10). **G)** 75 sorted CD45.1⁺ congenic (Cg) Ctrl or IR^P LSK were cultured 1:1 with sorted LSK from CD45.2⁺ (WT) Ctrl or IR^P mice. The number of

Gr1^{neg} Sca1⁺ cells on day 6 was assessed by flow cytometry (n=5). **H**) 600 LSK from Ctrl or IR^P mice were cultured for 6 days. The total progeny were transplanted into sublethally irradiated congenic recipients, and contribution to hematopoiesis was assessed by CD45.2⁺ donor frequencies in the peripheral blood over time post-bone marrow transplant (n=3, representative of 2 experiments). Statistics generated using Two-way ANOVA with multiple comparisons. Data are represented as mean $\pm$ SEM.

Figure 3. Activation of Notch restores IRP LSK self-renewal and reduces differentiation *in vitro*.

A) 200 LSK from Ctrl or IRP mice were sorted and cultured with or without Notch activation via IgG-DL1 (DL1) and total cell number was measured on day 6 (n=5). **B)** Cell population composition on day 6 of LSK culture with or without DL1. **C)** Number of LSK^{hi}K on day 6 of culture. **D)** Cell population composition on days 3 and 5 of LSK cultures (n=3). **E)** Probability of differentiation for a single LSK per cell cycle was calculated using the number of total cells (Figure S3) and percentage of LSK on days 3 and 5 (D). **F)** Relative Fitness, measured as the generation of new LSK per LSK per hour, was measured using experimental data derived from D. Data are represented as mean $\pm$ SEM.

Figure 4. Oxidative stress signature in IR^P LSK

A) Experimental design. LSK were sorted from Ctrl or IR^P mice, and used immediately for RNA-seq (Fresh; 3 mice pooled per sample; n=3), or cultured for 6 days after which LSK were resorted and used for RNA-seq (Cultured; 1 mouse per sample; n=3). **B)** Nrf2 signature in Fresh and Cultured Ctrl or IR^P LSK with or without DL1. **C)** Lipid peroxidation was measured in Lin^{neg}, LK, and LSK populations using Bodipy dye, which changes from red fluorescence to green upon oxidation (n=2, representative of 4 experiments). **D)** WT or Nrf2 KO mice were irradiated at 2.5Gy (IR^P) or left unirradiated (Ctrl) (n=3 mice). Numbers of LT-HSC and LSK in the bone marrow were analyzed 9 weeks later. Indicated fold decrease are for IR^P relative to Ctrl for each genotype. **E)** 200 LSK were sorted from WT or Nrf2 KO mice and cultured for 6 days with or without DL1. The number of LSK^{hi}K on day 6 was assessed by flow cytometry (n=3 mice; 4 wells/mouse). Data are represented as mean ± SEM.

Figure 5. Myeloid differentiation signatures are enriched in IR^P LSK and reversed by Notch activation

A–C) RNA-seq data from fresh and cultured LSK were analyzed for enrichment for HSC signature (genes uniquely expressed in HSC) (A), monocyte signature (genes expressed specifically in monocytes) (B), or C/EBPα (C) regulated genes.

Figure 6. C/EBP α inhibition rescues IRP LSK self-renewal and reverses precocious differentiation

A) cKit⁺ bone marrow was transduced with shCEBPA #1 or control sh0 (co-expressing GFP) and cultured for 9 days (n=5 wells, representative of 6 experiments). Flow cytometric analysis for GFP expression in the $LS^{hi}K$ population was assessed on days 4, 6, and 9. **B)** Cell population distribution was assessed in the total GFP^{neg} and GFP⁺ populations in cultures transduced with sh0 or shCEBPA #1. Cultures with >250 total GFP⁺ cells were assayed for population distribution. Numbers above X axis indicate assayed wells/5 total wells. **C)** The probability of differentiation was measured for GFP⁺ LSK from day 4 to day 6. **D)** The relative fitness, measured as the rate of new LSK generated per LSK per hour, was determined for GFP⁺ LSK from day 4 to day 6. **E–F)** Ctrl or IRP cKit⁺ cells transduced with sh0 or shCEBPA #1 were transplanted into sublethally irradiated recipient mice. Selection for each shRNA was assessed by measuring the percent of GFP⁺ cells in total $CD45.2^{+}$ donor peripheral blood (E) and in $CD45.2^{+}$ donor $CD11b^{+}$ peripheral blood (F)

over time. Two-way ANOVA with Multiple Comparisons: sh0 v shCEBPA (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$), IR^P v Ctrl shCEBPA (& $p < 0.0001$).

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Figure 7. Selection for C/EBP α inhibition is reversed by exogenous Notch activation

A) cKit⁺ bone marrow was transduced with sh0 or shCEBP α #2 and cultured with or without exogenous activation of Notch via DL1. Selection for the shRNAs was assessed by flow cytometric analysis for GFP expression in the Ls^{hiK} population on days 4, 6, and 9 (n=5, representative of 3 experiments). **B)** Programmed Mediocrity Model. See text for details.