SUPPLEMENTARY APPENDIX

Stress-induced hematopoietic failure in the absence of immediate early response gene X-1 (IEX-1, *IER3*)

Haley Ramsey,[1] Qi Zhang,[1] Diane E. Brown,[2,3] David P. Steensma,[4] Charles P. Lin,[1,5] and Mei X. Wu[1,5]

[1]Wellman Center for Photomedicine, Department of Dermatology, Massachusetts General Hospital, Harvard Medical School, Boston, MA; [2]Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, MA; [3]Center for Comparative Medicine, Massachusetts General Hospital, Boston, MA; [4]Dana-Farber Cancer Institute, Department of Medical Oncology, Harvard Medical School, Boston, MA; and [5]Harvard-MIT Division of Health Sciences and Technology, Cambridge, USA

 doi:10.3324/haematol.2013.092452
Manuscript received on May 30, 2013. Manuscript accepted on September 12, 2013.
Correspondence: mwu2@partners.org

Online supplement

1. Methods

Flow cytometry

For HSC analysis, BM cells were stained with a lineage cocktail of rat anti-mouse antibodies against mature blood cell markers including Mac-1, Gr-1, CD3, CD4, CD8, B220, and Ter-119. These lineage positive cells were excluded using PE or PE-Cy7-conjugated goat anti-rat antibody. LSK cells were identified as lineage-negative Sca-1^{+}c-Kit^{+} (Lin^{-} Sca-l^{+} c- Kit^{+}) cells by additional antibody combinations against Sca-1 (clone D7) from Biolegend and c-Kit (clone 2B8) from eBioscience. Long-term (LT) and short-term (ST) LSK cells were further distinguished with antibodies specific for CD135 or Flk2 (A2F10.1) and CD34 (MEC14.7) as Lin^{-} Sca-l^{+}c-Kit^{+} CD34^{-} Flk2^{-}cells or Lin^{-} Sca-l^{+}c-Kit^{+} CD34^{+} Flk2^{+}cells, respectively. Myeloid progenitors were recognized as IL7Rα^{-} Lin^{-}c-Kit^{+}Sca-l^{-} cells by antibody against CD127 from Biolegend, and more committed myeloid progenitors with antibody directed at FcGRII/III (clone 93) from eBioscience in which common myeloid progenitor (CMP) was marked as Lin^{-} IL7Rα^{-}c-Kit^{+}Sca-1^{-}CD34^{+} FcRII/III^{-} cells, granulocyte-monocyte progenitor (GMP) as Lin^{-} IL7Rα^{-}c-Kit^{+}Sca-l^{-} CD34^{+} FcRII/III^{+} cells, and megakaryocyte-erythrocyte progenitor (MEP) as Lin^{-} IL7Rα^{-} c-Kit^{+}Sca-l^{-} CD34^{-} FcRII/III^{-} cells, respectively[1]. For macrophage detection, cells prepared from BM and splenic tissues were stained with PE-conjugated F4/80 antibody directly.

To determine chimerism in transplantation assays, CD3 (17A2), CD45R/B220 (RA3-6B2) and CD11b (M1/70) antibodies from Biolegend were used to define T cells, B cells,

and myeloid cells, respectively. Antibodies specific for CD45.1 (A20) from eBioscience and CD45.2 (104-2) from Abcam were employed to differentiate donor from recipient cells. Erythropoietic lineages were stained with Ter-119 (Ter-119) antibody, and megakaryocytes and platelets with CD41 (MWReg30) antibody (eBiosciences). Flow cytometric studies were performed on a FACSAria (BD Bioscience) and data were analyzed by FlowJo software (Tree Star).

BM Transplantation

BM cells were harvested from femur and tibia of gender- and age-matched cognate donor mice, washed, resuspended, and counted in M199 (Sigma M 4530) medium containing DNase (Sigma D-4527), gentamicin, and HEPES. Recipient animals were conditioned one day earlier by lethal irradiation with 1100 rad, administered in 2 fractions with 550 rad (5.5Gy) each at 3 hours apart, using a ^{137}Cs gamma irradiator (Mark I, 30 J.L. Shepherd). Donor BM cells at $1x10^6$ cells/mouse were transferred to lethally irradiated recipient mice by tail vein injection. For serial transplantations, secondary recipients were conditioned similarly above and administered next day 1×10^6 BM cells collected from primary recipients employing a similar protocol.

Tracking cell division in the BM by calvarium intravital microscopy

LSK cells were sorted from WT and KO mice, stained with DiI or DiD (Invitrogen), respectively, mixed at 1:1 ratio, and injected into naïve WT mice at 1×10^5 cells per mouse. Recipient mice were subcutaneously administered 15 μg anti-LYVE-1 antibody (R&D Systems) conjugated with Alexa Fluor-647 (Invitrogen) into a hind footpad 16 hr

before imaging to label mouse microvasculature. The labeled LSK cells were tracked by calvarium intravital microscopy as previously described[2]. LSK cells were tracked and calculated for each video segment using ImageJ software.

Cell cycle analysis

BM cells were flushed from femur and tibia of indicated mice and stained with antibodies against various lineages, Sca-1 and c-Kit as above. The stained cells were then fixed, permeabilized, and counter-stained overnight with antibody directed at Ki67 (BD Bioscience) followed by a 5 minute incubation with 2μg/ml DAPI (Millipore). The stained cells were then washed with Permwash (BD Bioscience) and analyzed on a FACSAria.

MDS induction and monitoring

Eight-weeks-old mice of indicated phenotypes were exposed to a single dose of 300 rad (3 Gy) total body irradiation in a ^{137}Cesium gamma irradiator. Monthly blood smears and complete blood cell counts (CBCs) were evaluated for cytopenias and dysplasia for 8 months. For CBCs, blood (50μl) was collected via tail vein into EDTA-coated microtainer tubes (BD Bioscience) and analyzed on a HemaTrue veterinary hematology analyzer (Heska Corporation). Peripheral blood smears were stained with Wright-Giemsa (Sigma-Aldrich) or new methylene blue (Poly Scientific). Formalin fixed tissues were stained with H&E and reticulin for histological analysis. Microscopic analysis was conducted with a Zeiss Axiophot and images were captured using Picture Frame 2.3 software.

Apoptosis detection

Apoptosis was assayed by flow cytometry using an Annexin V/propidium iodide (PI) Apoptosis Detection Kit (BD Bioscience) or an In Situ Cell Death Detection Kit (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling or TUNEL) from Roche Applied Science, in accordance to the manufacturer's instructions.

Colony-formation assays

Colony forming assays were performed using cytokine-enriched Methocult M3434 methylcellulose (StemCell Technologies). Colonies were scored under a microscope using protocols provided by Stem Cell Technologies.

Tail bleeding times

Bleeding time was assessed by transecting the mouse tail at 5mm from the tip while in a restrainer (Braintree Scientific). The bleeding tail was immersed in a 15-ml test tube containing phosphate-buffered saline (PBS), which was pre-warmed to 37°C, and time to cessation was measured.

Transmission electron microscopy

BM cells were fixed in Karnovsky's fixative at 4°C for overnight, followed by centrifugation and removal of the fixative. The cell pellets were washed with 0.1 M sodium cacodylate buffer, postfixed in 2% OsO4 in sodium cacodylate buffer, dehydrated, and embedded in Epon t812 (Tousimis). Ultrathin sections were cut on a

microtome (Reichert-Jung Ultracut E), collected on 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined on a Philips CM-10 transmission electron microscope (Eindhoven). Images were taken using an undermount XR41M 4 Mpixel cooled camera.

In vivo tracking of platelet formation from megakaryocytes

Megakaryocytes were sorted from the BM of WT and KO mice after labeling with CD41 antibody, resuspended in 2.5% FBS in PBS, and stained for 15 minutes with 7μM CFSE (Invitrogen). The cells were washed twice with PBS, resuspended, and administered into WT mice at $5x10^5$cells/mouse via tail vein. The resultant $CD41^+CFSE^+$ platelets were analyzed by flow cytometry in blood samples collected every other day following the injection.

RBC and Platelet life-span studies

RBC turnover was measured by *in vivo* biotinylation assays. In brief, 3mg EZ-link-*N*-Hydroxysulfosuccinimide-Biotin (NHS-Biotin) (Sigma) was intravenously injected into mice, which is a well-established method to label on average 95% of peripheral RBCs and platelets after one treatment. EZ-NHS-biotin irreversibly binds to the amine rich surface of RBCs and platelets with high affinity, and the labeling can last for their entire life-span. Blood samples were obtained at indicated days and incubated with flourescein-labeled avidin and phycoerythrin-conjugated TER119 or CD41, followed by flow cytometry.

Statistical analysis

Statistical significance was determined by 2-tailed student's t-test for two group comparison or one way ANOVA for multiple group comparison using Graphpad Prism 5 (Graphpad Software).

Reference List

1. Challen GA, Boles N, Lin KK, Goodell MA. Mouse hematopoietic stem cell identification and analysis. Cytometry A. 2009;75 (1) :14-24.

2. Lo CC, Lin CP, Scadden DT. In vivo imaging of transplanted hematopoietic stem and progenitor cells in mouse calvarium bone marrow. Nat Protoc. 2011;6 (1) :1-14.

2. Results

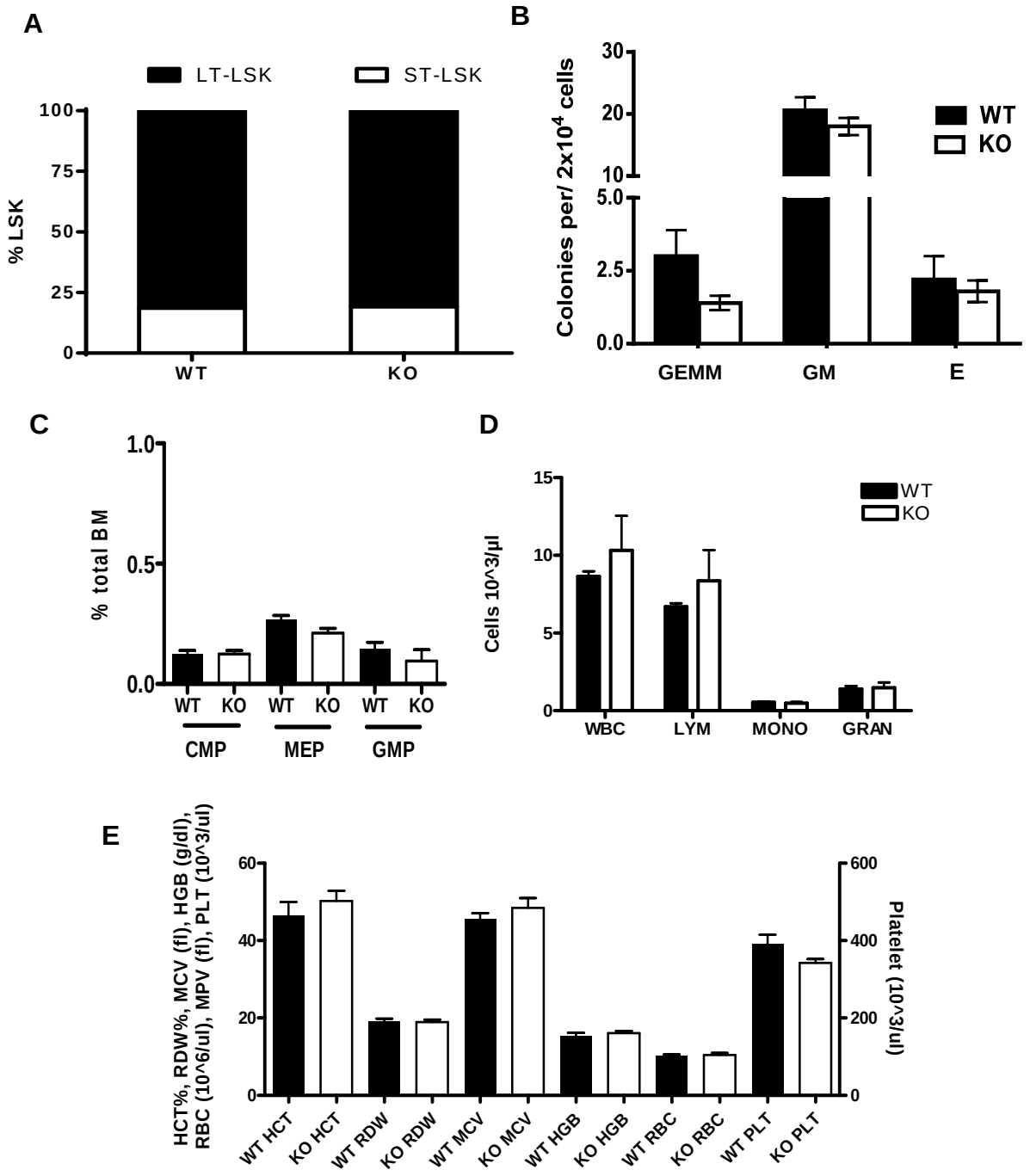

Figure S1. Hematopoietic analysis in naïve WT and KO mice.

(A) Long-term- and short-term LSK cells; (B) colony forming units; (C) progenitor and (D-E) peripheral cell analysis in naïve WT and KO mice. No significant differences were noted in all populations measured. n=8 per group

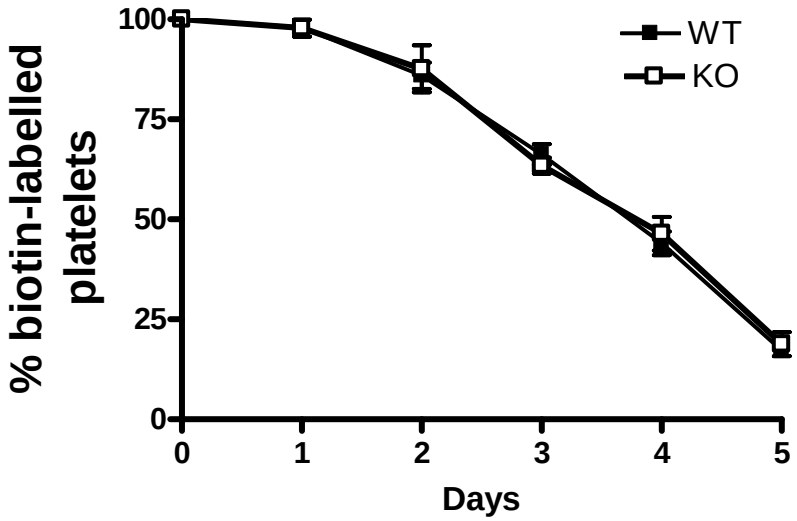

Figure S2. No differences in lifespan between WT and KO platelets

Platelets were isolated from blood of indicated mice and biotinylated, followed by tracking the number of biotin-labeled platelets in the circulation daily in cognate recipient mice. The data are expressed as mean percentages ± SD relative to the number of labeled platelets on day 0 in the same mice. n=5 for each group.

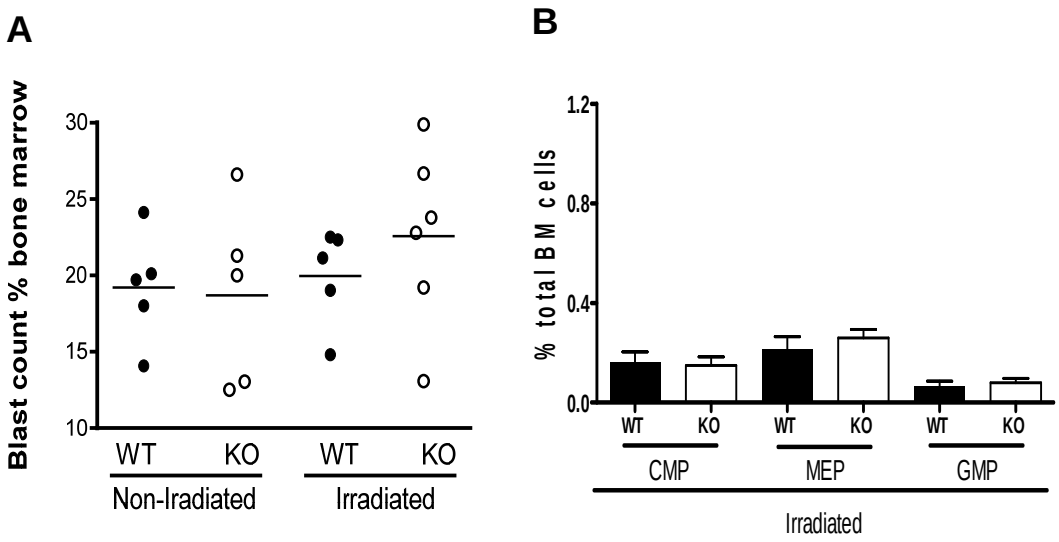

Figure S3. No significant differences in blast counts (A) and myeloid progenitors (B)

Blast counts were performed on Wright-Giemsa stained BM brushings prepared from WT and IEX-1 KO mice 8 months post-irradiation or before irradiation. Each symbol in (A) represents a mean percentage of blast counts from 10 different sections prepared from individual mice. pulations in 8 months post-irradiation. The mean percentages ± SD relative to total BM cells in (B) were determined in common myeloid (CMP), myeloid erythroid (MEP) or granulocyte macrophage (GMP) progenitors 8 months post-irradiative treatment. n=6 for each group.

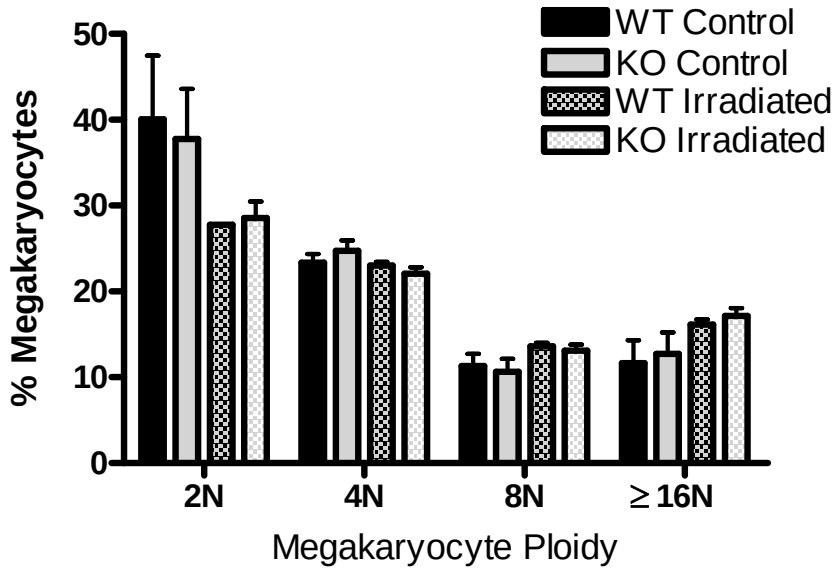

Figure S4. Megakayocyte ploidy analysis.

Intercellular propidium iodide staining was used to determine DNA ploidy of megakaryocytes in the control or irradiated WT and KO mice. The data are expressed as mean percentages ± SD of each subsets relative to total megakaryocytes. n=5.

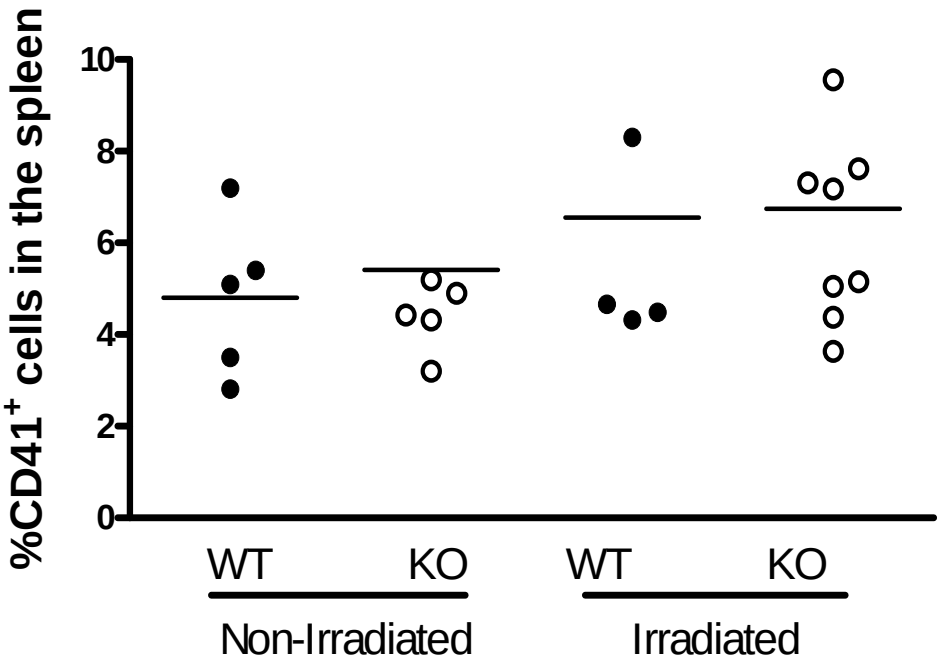

Figure S5. Splenic megakaryocyte analysis.

Splenic megakaryocytes were analyzed by flow cytometry after labeling with anti-CD41 antibody in WT and KO mice in 8 months post-TBI or before TBI. Each symbol represents data from individual mice and horizontal bars indicate the mean.