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Supplemental Information

A Functional Bioluminescent Zebrafish Screen for Enhancing Hematopoietic Cell Homing

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Supplemental Figures S1 – S3

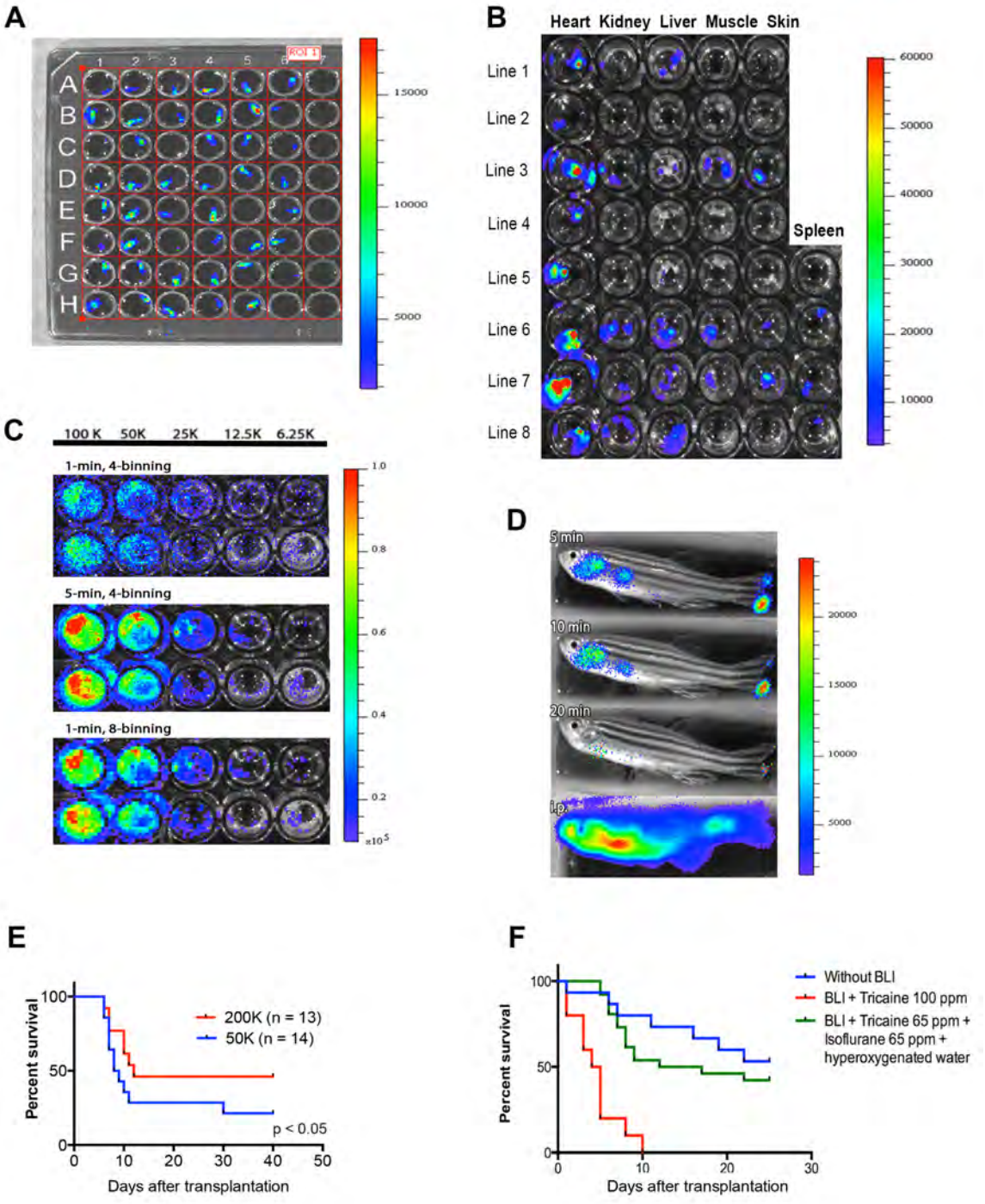

Figure S1. Luciferase expression in *ubi:luc* zebrafish and modifications of BLI to detect WKM cells.
(A) Single cell stage zebrafish were injected with 100 ng/μL of the Tol2 *ubi:luc* construct. Founder *ubi:luc* zebrafish embryos (96 hours post fertilization) were placed in a 96-well plate after the addition of D-luciferin to the water .

(B) Organs dissected from F2 zebrafish and BLI performed after the addition of D-luciferin to the water (150 mg/mL) indicating BLI variability in several lines.

(C) Limiting dilution with WKM from *ubi:luc* zebrafish in a 96-well plate followed by the addition 150 μg/mL D-luciferin to the water. Acquisition time set at 1 minute and CCD resolution set at 4 pixel binning gave good signal sensitivity but high background scatter. When acquisition time was increased to 5 minutes (middle panel) or resolution set to 8 pixel binning (lower panel), both BLI sensitivity and localization improved without interference from background signal.

(D) Bathing versus intraperitoneal injection of D-luciferin in transplant recipients. Lethally irradiated wild-type zebrafish received 100,000 *ubi*:*luc* WKM cells and allowed to engraft for 28 days. Imaging was performed using a Xenogen IVIS50 (Stage A). Exposure time was set at 1 minute. Animals were immobilized using 0.005% tricaine in a 6-well plate filled with 1% agarose gel with a portion removed to isolate the animal. Bathing was performed by placing zebrafish in D-luciferin in fish water at 150 μg/mL for the indicated lengths of time (top three panels). Intraperitoneal injection (lower panel) of 75 μg D-luciferin was performed immediately prior to BLI imaging (exposure time was set at 1 minute as before).

(E) Short-term survival is increased in zebrafish receiving a higher cell dose after HCT. Recipients received 30 Gy, followed by HCT with 50,000 or 200,000 *ubi*:*luc* WKM cells. Animals were followed for 40 days. P-value from a Student's t-test.

(F) Modification to anesthesia protocol. Wild-type recipients underwent HCT with 200,000 *ubi*:*luc* donor cells with BLI performed at 1 dpt and 5 dpt using the anesthesia as listed, n = 10/group.

All luminescence scale bars show radiance (p/sec/cm^2/sr).

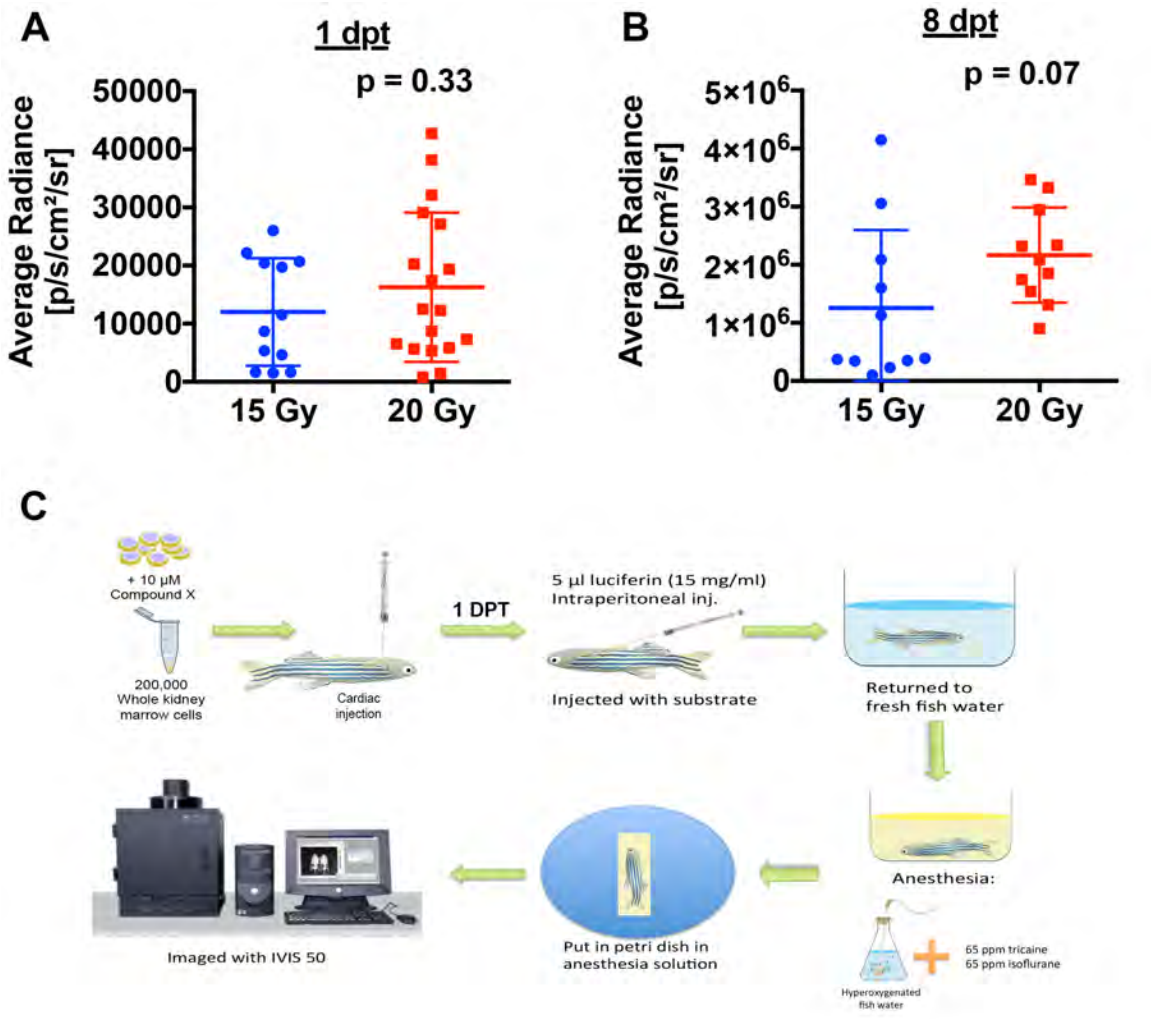

Figure S2. Radiation dose testing and compound screening schema.
(A and B) Recipient fish either received 15 Gy or 20 Gy x-ray irradiation followed by HCT of 500,000 donor *ubi:luc* WKM. BLI was measured as described previously at 1 dpt and 8 dpt. Shown are means ± SD, p-values are from a Student's t-test.

(C) Schema for medium-throughput small molecule screening to determine compounds that enhance HSPC homing.

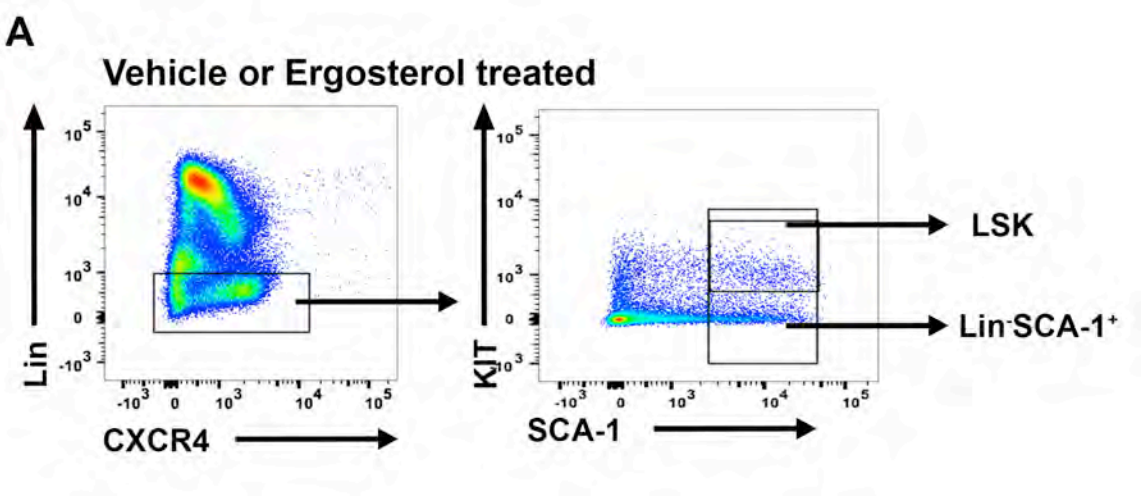

Figure S3. Flow cytometry gating of CXCR4 expression on WBM cells and progenitors.

(A) Murine WBM treated for 12 hours with 10 μM ergosterol or equal v/v vehicle followed by flow cytometry for CXCR4 and lineage cocktail as in Figure 4D. Initial gates were used to identify mononuclear cells that were PI negative. Flow cytometry gating for the identification of CXCR4 expression on murine lineage negative cells as shown in Figure 4D.The above gates indicate the flow cytometry gating for $Lin^{-}SCA\text{-}1^{+}$ and LSK.

Supplemental Methods

Cell migration and *Cxcr4* Expression

For transwell migration assays, the bottom chamber of a 24-well, 3 μm pore sized transwell plate (Corning, Corning, NY) was prepared by adding 600 μL of StemSpan SFEM II (StemCell Technologies, Vancouver, Canada) containing 100ng/mL mouse recombinant Sdf1a (R&D systems, Minneapolis, MN). Mouse whole bone marrow (WBM) cells were harvested as described above and suspended in StemSpan SFEM II treated with ergosterol (Sigma, St. Louis, MO) or ethanol equivalent, at a concentration of 5 x 10^6 cells/mL. 100μL of this cell suspension was then plated in the top chamber of the transwell and allowed to migrate overnight (approximately 12 hours). The following day, the transwell inserts were removed and the migrated cells were enumerated on a BD FACSCanto flow cytometer. CountBright Absolute Counting Beads were used to quantify absolute numbers of cells and Propidium Iodide was used to determine viability.

CXCR4 expression was analyzed via flow cytometry and qRT-PCR. Mouse WBM cells were prepared as described above. 5 x 10^6 mouse WBM cells were plated in a 1mL volume in wells of a 12-well plate in StemSpan SFEM II media containing ergosterol or DMSO equivalent and allowed to incubate overnight (12 hours). The following day, 500μL of each well was designated for flow cytometry and the other 500 μL of cells were pelleted for RNA. Mouse cells were stained for flow cytometry with anti-mouse Lineage Cocktail-PacBlue (#133306, Biolegend, San Diego, CA), Anti-Mouse Ly-6A/E (SCA-1) PE (#12-5981-82, eBioScience), anti-mouse CD117 (KIT) APC (#47-1172-82, eBioscience), and anti-mouse CXCR4 Alexa Fluor® 488 (#53-9991-80, eBioScience). CountBright Absolute Counting were used to quantify absolute numbers of cells and Propidium Iodide was used to determine viability. From the remaining cell pellets, RNA was prepared using RNeasy Mini Kit (Qiagen, Venlo, Limburg). cDNA was prepared using Superscript VILO cDNA synthesis kit (Life Technologies, Carlsbad, CA). qRT-PCR was performed using Taqman MasterMix (Life Technologies, Carlsbad, CA) on a StepOnePlus Real Time PCR System (Life Technologies, Carlsbad, CA). Mouse probes used were Taqman *beta-2-microglobulin* Mm00437762_m1 and *Cxcr4* Mm99999055_m1.

In vitro Hematopoietic Cell Expansion

Freshly prepared bone marrow was lineage depleted using an EasySep™ Mouse Hematopoietic Progenitor Isolation Kit (STEMCELL Technologies). Lineage negative cells were then stained with Mouse Ly-6A/E (SCA-1) PE (eBioScience, San Diego, CA), Anti-Mouse CD117 APC e-Fluor 780 (KIT) (BioLegend, San Diego, CA) antibodies. Lineage$^-$, SCA-1$^+$, KIT$^+$ (LSK) cells were isolated on a FACSAria II cell sorter. 2,500 LSK cells in a volume of 400 μL were plated per well of a 24-well plate. Cells were cultured in StemSpan media (STEMCELL Technologies) containing 100 ng/mL murine stem-cell factor, 100 ng/mL human FLT-3 ligand, 100 ng/mL human IL-6, and 10 ng/mL human IL-11 (all Peprotech, Rocky Hill, NJ). Ergosterol stock was added to the media to obtain a final concentration of 10 μM. 250μL of additional media with ergosterol was added at day 7 of culture. Readout was performed at day 14 of culture on a BDFACSCanto. Cells numbers and phenotype were determined as in the homing experiments. Counting beads and Propidium Iodide were also used as described above.

Reporter Assays

For analysis of transcription of the vitamin D response element (VDRE), the Cignal Lenti VDRE Reporter (luc) Kit (#CLS-9029L) was used according to the manufacturer's instructions (Qiagen, Valencia, CA). The Lenti VDRE reporter is a preparation of replication incompetent, VSV-g pseudotyped lentivirus particles expressing the firefly luciferase gene under the control of a minimal cytomegalovirus promoter and tandem repeats of the VDR transcriptional response element (TRE). Briefly, HEK293 cells were transduced with the Lenti VDRE reporter and selected in puromycin for 7 days. Cells were then plated in a white-opaque 96-well plate at 25,000 cells per well. Ergosterol (10 μM) or equal v/v ethanol was added for 12 hours. Luciferase quantification was performed using Molecular Devices SpectraMaxL plate reader. Calcitriol at 100 nM was used as a positive control molecule. For experiments in which the VDR was expressed via a transgene, the mouse vitamin D receptor encoding plasmid [catalog # MC207441 (NM_0095494), Origene Technologies, Rockville, MD] was transduced into Lenti VDRE transduced HEK293 cells using NanoJuice® (MilliporeSigma, Darnstadt, Germany) according to manufacturer's instructions (5 μg DNA / 3 μL NanoJuice® / well of a 6-well plate). Twenty-four hours after *Vdr* transduction, HEK293 cells were plated at 25,000 cells per well in a 96-well plate. Ergosterol exposure followed by luciferase readout was performed as before.

For SP1 activity assay, the Cignal SP1 Reporter (luc) Kit: (#CCS-6027L) was used according to the manufacturer's instructions (Qiagen). The SP1 reporter is a mixture of a SP1-responsive firefly luciferase construct and a constitutively expressing *Renilla* luciferase construct (40:1). The SP1-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal (m)CMV promoter and tandem repeats of the GC box transcriptional response element. Additionally, the *Renilla* luciferase reporter gene is under the control of a constitutive CMV immediately early enhancer/promoter as an internal control for normalizing transfection efficiencies. The reporter plasmid was transfected into HEK293 cells using NanoJuice® according to manufacturer's instructions (5 μg DNA / 3 μL NanoJuice® / well of a 6-well plate). Cells were then plated at 25,000 cells per well in a 96-well plate. Ergosterol exposure followed by luciferase readout was performed as before. Firefly luciferase was normalized to *Renilla* luciferase activity. For experiments involving transgenic VDR expression, the *Vdr*-encoding plasmid was transfected into cells 24 hours prior to SP1 transfection following the same protocol as before.