Supplemental Experimental Procedures

Animals

C57BL/6 and B6.SJL mice were purchased from Jackson Laboratories and Taconic and bred in house. UBC-GFP mice were purchased from Jackson Laboratories (strain #004353) (Schaefer et al., 2001). Experiments were carried out with IACUC approval from CHB.

Bone Marrow Preparations

Femurs and tibias from neonatal and adult mice were isolated then crushed with mortar and pestle in PBS and strained through a 70 um filter. The sample was incubated with red blood cell lysis buffer on ice for 20-30 minutes then resuspended in PBS for transplantation or PBS with 2% serum for flow cytometry.

Embryonic Tissue Preparations

Embryonic day was determined by counting the day of vaginal plugging as 0.5 days post coitus. Staging of embryos was performed by somite counting. Embryos were collected from timed-mated females at E9.5 (16-26 somites), E10.5 (30-39 somites), E11.5 (40-48 somites), and E14.5 (53-55 somites). The AGM/PSp was dissected, dissociated with collagenase/dispase (1mg/mL in DMEM) for 30-60 minutes at room temperature, and strained through a 70 um filter. The fetal and neonatal liver was dissected and mashed over a 70 um filter. The sample was incubated with red blood cell lysis buffer on ice for 20-30 minutes. Cells were resuspended in PBS for transplantation or PBS with 2% serum for cell sorting.

Bone Marrow Transplantation

Donor cells were injected via the tail vein in adult mice and the facial vein in neonatal mice. Post-transplantation, mice were kept on antibiotic-treated drinking water. Engraftment was monitored in adult recipients starting 4 weeks post-transplant and in neonatal recipients starting 6 weeks post-transplant. Neonatal recipient analysis was delayed due to runted size as a result of irradiation. Continued engraftment was monitored every 4 weeks by flow cytometric analysis of peripheral blood.

Secondary Transplantations

Adult secondary recipients were lethally conditioned with a split dose of irradiation, 10 Gy total dose split by 3 hours. One million to 5 million whole BM cells from the primary recipient were injected via the tail vein into each of 3 to 4 secondary recipients.

FACS Analysis and Sorting

Multicolor FACS analysis was carried out on a 5-laser-LSRII or LSRFortessa flow cytometer (Beckton-Dickinson), and sorting was performed on a FACS-Aria+UV (Beckton-Dickinson). Leukocyte enriched peripheral blood cells were stained on ice for 15-30 minutes with the following antibody cocktail for lineage analysis: CD45.1 – FITC, CD45.2 – PE/Cy7, Ter119 – PE/Cy5, Gr1 – PE, Mac1 – Alexa700, B220 – Pacific Blue, CD19 – APC/Cy7, CD3 – APC, and

propidium iodide and for homing: CD45.1 – PE and CD45.2 – PE/Cy7. The cells were washed and resuspended in PBS with 2% serum.

To assay the proliferative state of HSCs, isolated HSCs were fixed and permeabilized using the BD Cytofix/Cytoperm kit according to the manufacturer's protocol. DAPI was used as a nuclear stain and Ki-67 – FITC marked proliferating cells. The cells were washed and resuspended in PBS with 2% serum.

To isolate HSCs from BM, samples were stained with biotinylated antibodies against Mac1, Gr-1, B220, CD3, and Ter-119, then lineage depleted with anti-biotin MACS beads. The remaining cells were stained with the following antibody cocktail on ice for 15-30 minutes: Lineage (Mac1, Gr1, B220, CD3, Ter119) – Alexa450, Sca1 – PerCP-Pc5.5, c-Kit – APC/Cy7, CD150 – PE/Cy7, CD48 – PE, and DAPI. Preparations for E14.5 FL were identical to BM except for the use of Mac1 in the lineage cocktail. To isolate prospective HSCs from E11.5 AGM, cells were stained with a purified VE-Cadherin antibody followed by a goat anti-rat Alexa680 secondary antibody then CD45 – PE and propidium iodide (Figure S2 A).

Peripheral Blood Isolation

Peripheral blood samples were collected by retro-orbital bleeds with heparinized capillary tubes. The red blood cells were allowed to settle out of suspension in PBS with 1% dextran and 0.25M EDTA at room temperature. The buffy layer was pelleted and resuspended in red blood cell lysis buffer on ice for 20-30 minutes to remove lingering red blood cells. The remaining leukocyte enriched samples were prepared for flow cytometry.

Homing

VE-Cadherin^{+} CD45^{+} cells were isolated from E11.5 AGM of UBC-GFP embryos. LT-HSCs (Lineage^{-} Sca-1^{+} c-Kit^{+} CD150^{+} CD48^{-}) were isolated from adult BM of UBC-GFP mice. Approximately 10^4 isolated cells were transplanted into conditioned B6.SJL neonatal and adult recipients. Transplanting purified LT-HSCs in addition to injecting a small number of cells reduces the likelihood of donor cells being trapped in the lung. Adult recipients received 3×10^5 helper cells from B6.SJL BM. At 15 hours post-injection, the lung, peripheral blood, BM from the long bones of the leg, spleen, liver, and thymus were collected. Peripheral blood and BM from adult recipients were subjected to red blood cell lysis. Solid tissues were mashed over a 70 um filter. Cells from the collected tissues were stained with antibodies for CD45.1 and CD45.2. Donor cells were detected using two markers: CD45.2 and GFP expression.

Microarray Analysis

The microarray data were analyzed per standard protocol using R/Bioconductor. Briefly, raw microarray signal intensities were RMA-summarized and quantile normalized (Bolstad et al., 2003, Irizarry et al., 2003) and corrected for batch effects using ComBat batch correction (Johnson et al., 2007). Data was obtained from GEO accession: GSE14361 (Essers et al., 2009), GSE1559 (Venezia et al., 2004), GSE37000 (McKinney-Freeman et al., 2012), and GSE55095 (Schuettpelz et al., 2014). Differential expression was determined using the cut-off fold change log2(1.5) and nominal p-value of 0.01. Hierarchical clustering was determined via

average linkage. Gene ontology (Ashburner et al., 2000) enrichment was determined on commonly up-regulated or down-regulated genes via hypergeometric test.

Limiting Dilution Analysis (LDA)

LDA was performed online using software provided by WEHI bioinformatics http://bioinf.wehi.edu.au/software/elda/index.html (Hu and Smyth, 2009). In all cases, the threshold for engraftment was 1% donor chimerism at 18 weeks post-transplantation.

Statistical Analysis

The Fisher exact test was used for statistical analysis of the difference in frequency of engraftment. In cases where a majority of the animals were engrafted, the student's t-test was used for statistical analysis of the engraftment levels. The LDA algorithm performed statistical analysis on the limiting dilution transplants.