# Maintenance and repair of the lung endothelium does not involve contributions from marrow-derived endothelial precursor cells

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ONLINE DATA SUPPLEMENT

Supplemental Methods:

*Hematopoietic stem cell (HSC) purification:* HSCs were purified from the bone marrow of mice by the Hoechst dye efflux method(1) with modifications published previously (2). This method identifies the bone marrow side population (SP; as shown in the supplemental figures) that is highly enriched in hematopoietic stem cell activity. Bone marrow single cell suspensions and purified HSCs were prepared and transplanted according to standardized methods exactly as previously published(3). This freely downloadable, detailed protocol for bone marrow Hoechst staining to sort SP cells is available on line at: http://www.bumc.bu.edu/stemcells/protocols/.

*Flow cytometry sorting*: Cell sorting was performed on a MoFlo triple laser instrument (DakoCytomation, Fort Collins, CO) using Summit 3.1 software. Analysis of raw data was completed with FlowJo software (Treestar, Ashland, OR). The laser emissions were 488, 350, and 647 nms. Fluorescence was detected with the following bandpass filters; 530/40 for FITC and GFP, 580/30 for PE, 670/30 for PI, 405/30 for Hoechst Blue, 570/20 for Hoechst Red, 670/20 for APC (Omega Optical Inc., Brattleboro, VT). Hoechst blue and red fluorescence was detected in linear scale acquisition. First a live cell gate was created excluding cell fragments (low forward scatter) or events that contained high PI fluorescence. Cells within this live cell gate were displayed on a Hoechst-Red- Hoechst-Blue histogram, and 200 side population (SP) cells per recipient were sorted for transplantation studies. A detailed protocol for isolating HSCs according to this method is available for free download from www.kottonlab.com.

*Competitive long-term blood repopulation and analysis of peripheral blood chimerism:* Recipient mice were lethally irradiated with either 11 Gy of radiation in a single dose or 14

Gy, delivered as 2 doses of 7 Gy given 3 hours apart on the day before transplantation. Where indicated in the text, 200 donor HSCs obtained from the indicated transgenic donor mice were mixed with $2x10^5$ unfractionated unlabeled competitor bone marrow cells and intravenously injected retro-orbitally into unlabeled recipient mice. Levels of blood chimerism were calculated as the proportion of CD45 labeled white blood cells that expressed the donor GFP marker gene in recipients of B-actin-GFP labeled bone marrow. For whole marrow transplantation 2-10 million unfractionated donor bone marrow cells were injected without competitor into recipients. Robust long-term hematopoietic reconstitution (>50% blood chimerism for >3 months post transplantation) was documented in all recipients prior to further analysis.

*Single Hematopoietic Stem Cell Transplantation:* Single bone marrow SP cells from a donor GFP+ transgenic mouse (B-actin-GFP, Jackson Labs) were sorted into separate wells of a 96 well plate that contained 100 mcl of HBSS/2%FBS and $3x10^5$ Sca-1 negative competitor marrow cells from a wild-type mouse. The entire contents of each well were then injected into the retro-orbital venous plexus of each recipient. A separate syringe was used for each well to avoid cross-contamination. An additional 96 well plate sorted with single cells by this method was examined by microscope to confirm that 100% of wells contained only 1 sorted cell. 18 mice that received single cell transplants were then followed to select recipients with >40% GFP+ blood chimerism. Self-renewal of the transplanted single stem cells was documented by serial transplantation of bone marrow from these recipients into secondary recipients.

*Immunostaining of lung and bone marrow samples for FACS:* Lung tissue was harvested for FACS and tissue sectioning as previously published(3). Briefly, the right lung was inflated in 4% paraformaldehyde for overnight fixation followed by embedding in optimal tissue

compound (OCT) for frozen tissue sectioning, and the left lung was excised for enzyme digestion and FACS analysis as follows: first the left lung tissue was finely minced with razor blades and enzymatically digested for 1 hour at 37°C in a solution of 0.1% Collagenase A in 2.4U/ml Dispase II (Roche, Indianapolis, IN) with 2.5mM CaCl2. The resulting digest was then filtered through a 70 um Falcon cell strainer (BD Biosciences, San Jose, CA) to remove debris and washed with HBSS+. Cell single suspensions were stained with Fc Block (BD Pharmingen) followed by the monoclonal anti-mouse antibody combination indicated in the figures: anti-CD45-PE, anti-CD31-APC, anti-CD31-PE, biotinylated anti-VE-Cadherin, or biotinylated anti-CD 45 (all from BD Pharmingen). Where biotinylated antibodies were used, the primary staining was followed by streptavidin conjugated-Cy7APC (BD Pharmingen) followed by PI staining of half the cells. Parallel aliquots stained with isotype control antibodies were included in all analyses (BD Pharmingen). The other half of the stained cells were fixed overnight and stained with anti-BrdU antibody label using the BrdU APC flow kit (BD Pharmingen) according to the manufacturer's instructions. Analysis of cells was performed by flow cytometry (LSR II, BD Biosciences or MoFlo, Dakocytomation). Raw data were then processed with FlowJo software (Treestar, Ashland, OR). Forward/side scatter properties were used to exclude cell fragments and red blood cells. PI staining was used to exclude dead cells (in unfixed samples; supplemental figure 2). The live lung cell subgate was then analyzed for GFP fluorescence or expression of each indicated fluorescent marker.

*Lectin staining and confocal microscopy:* Frozen lung tissue sections were blocked in CAS Block (Invitrogen, Carlsbad, CA) for 30 minutes at room temperature, and then incubated with biotinylated GSL 1-isolectin B4 (Vector Laboratories, Burlingame, CA) diluted 1:100 in CAS Block for 1 hour at room temperature. After washing with PBS, secondary staining was performed with streptavidin-conjugated Cy3 (Invitrogen, Carlsbad, California; diluted 1:5000

in CAS Block) for 1 hour at room temperature prior to washing and counterstaining nuclei with Prolong Gold antifade reagent with DAPI (Invitrogen). Each analysis included negative control sections treated with CAS Block alone in place of lectin, followed by SA-CY3. All sections were examined using a Zeiss LSM 510-Axiovert 200 Laser Scanning Confocal Microscope (Carl Zeiss, Oberkochen, germany) .

*Bleomycin-induced lung injury*: After long-term hematopoietic reconstitution (>3 months post transplantation) recipient mice were anesthetized with isoflurane, and a blunt end canula was used to intratracheally instill 0.05 units of bleomycin sulfate in 100 mcl of sterile PBS. 1 month following bleomycin exposure, recipients' lungs were harvested for analysis.

References

E1. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *The Journal of Experimental Medicine* 1996;183 1797-1805.
E2. Kotton DN, Fabian AJ, Mulligan RC. A novel stem cell population in adult liver with potent hematopoietic reconstitution activity. *Blood* 2005;106:1574-1580.
E3. Kotton DN, Fabian AJ, Mulligan RC. Failure of bone marrow to reconstitute lung epithelium. *AmJRespirCell MolBiol* 2005;33(4):328-334.

Supplemental Figures:

**Supplemental Figure 1: Long term blood reconstitution from a single transplanted bone marrow hematopoietic stem cell (HSC).** Peripheral blood chimerism of CD45+ cells is indicated by reconstitution of circulating peripheral blood deriving from a single GFP+ transplanted HSC. Long term self-renewal of the transplanted HSC is indicated by serial transplantation of bulk bone marrow from a primary single cell recipient into 2 secondary recipient mice 1 year after the first transplant. Peripheral blood reconstitution is indicated by GFP chimerism in the peripheral blood of each secondary recipient 3 months after the second serial transplant.

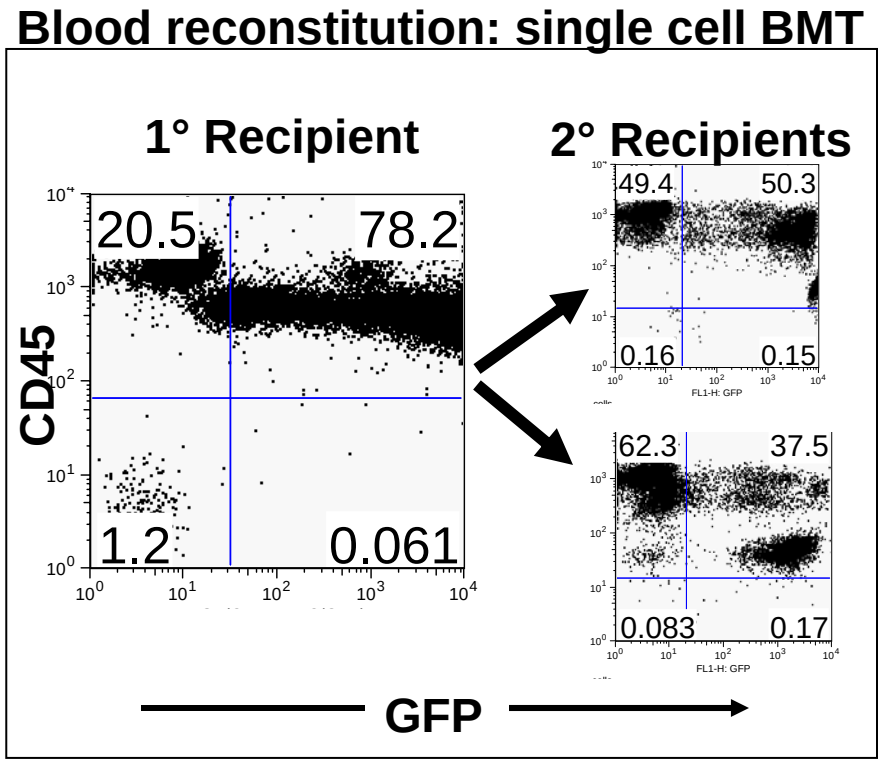
**Supplemental Figure 2: Gating algorithm used to analyze live lung single cell suspensions from donor (Tie2-GFP transgenic) mice, wildtype (WT) control mice, or wildtype recipient mice of bone marrow transplants from donor Tie2-GFP mice**. (A) Live cells are identified by exclusion of propidium iodide (PI), and autofluorescent live lung cells are excluded by gating out cells that fluoresce equally in the GFP and PE (autofluor-PE) channels. (B) Beta galactosidase staining for lacZ expression (blue) in lung sections from a recipient of bone marrow carrying a thrombomodulin-lacZ (TM-lacZ) endothelial selective reporter vs a control Beta galactosidase-stained lung section taken from a recipient of wildtype marrow (lower panel).

**Supplemental Figure 3**: Z stack of confocal microscopy images from lung tissue sections used in Figure 3 of the main text. The lung was taken from a recipient mouse that received a bone marrow transplant from a donor actin-GFP mouse. GFP fluorescence indicates bone marrow-derived cells and GSL-IB4 lectin staining (red) indicates either endothelial cells or alveolar macrophages. Note: GFP+ bone marrow derived cell is separated from the vascular lumen by an endogenous (red) endothelial cell. No bona fide GFP+ bone marrow-derived endothelial cells were found on any section.

**Supplemental Figure 4: Purification of hematopoietic stem cells from mouse bone marrow by Hoechst staining.** Bone marrow SP cells are identified after Hoechst staining of Ficolled bone marrow. The SP cells represent 0.1% of Ficolled marrow and 0.01% of whole bone marrow (before Ficoll). SP cells are highly enriched for Sca1+,

ckit+, Lin- cells compared to the non-SP gated cells. In addition, the SP gated cells are 100% CD45+ and possess robust competitive blood repopulating capacity, the defining characteristic of hematopoietic stem cells. The SP sort gate shown was used to purify HSCs for transplantations indicated in the text.

Supplemental figure 1



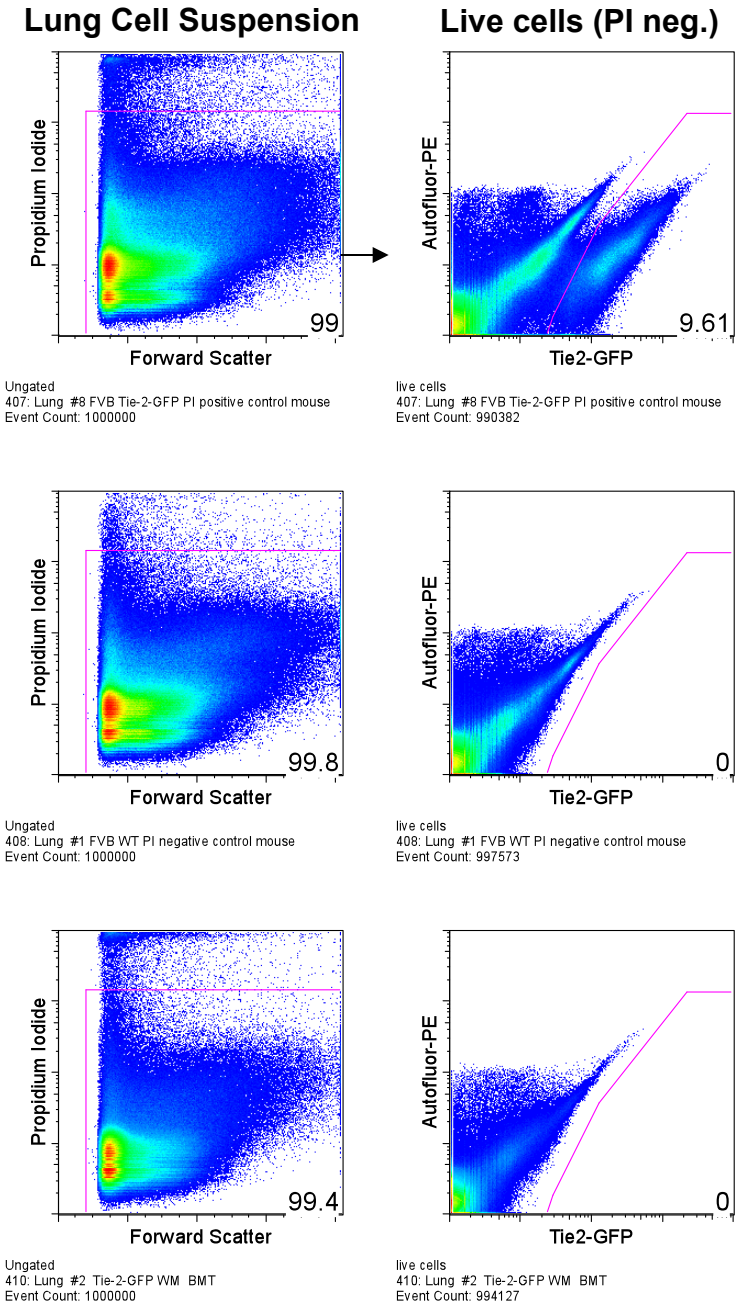
Supplemental figure 2

A

Pos. Control (Tie2-GFP Mouse lung)

Neg. Control (WT Mouse lung)

BMT Recipient Lung

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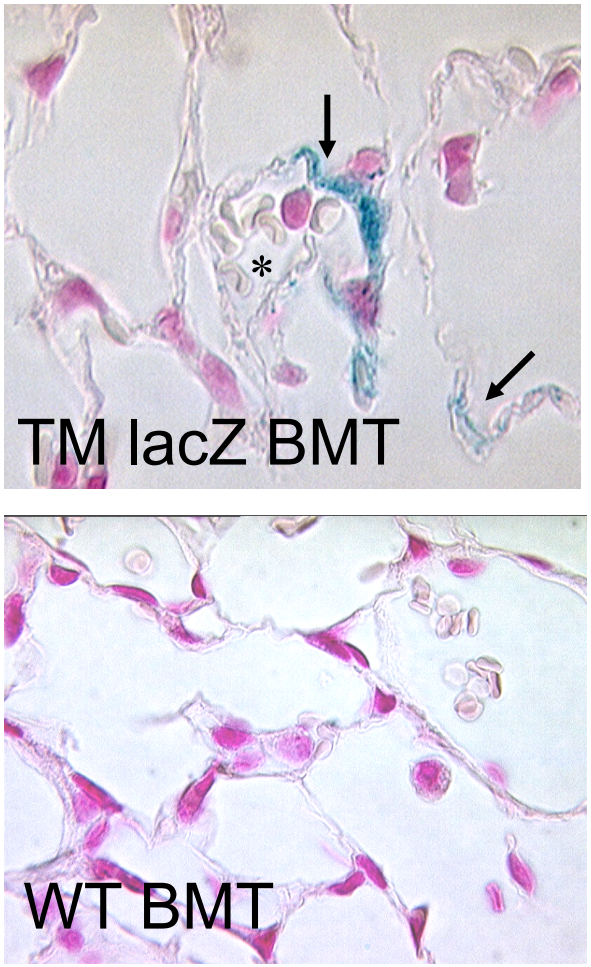
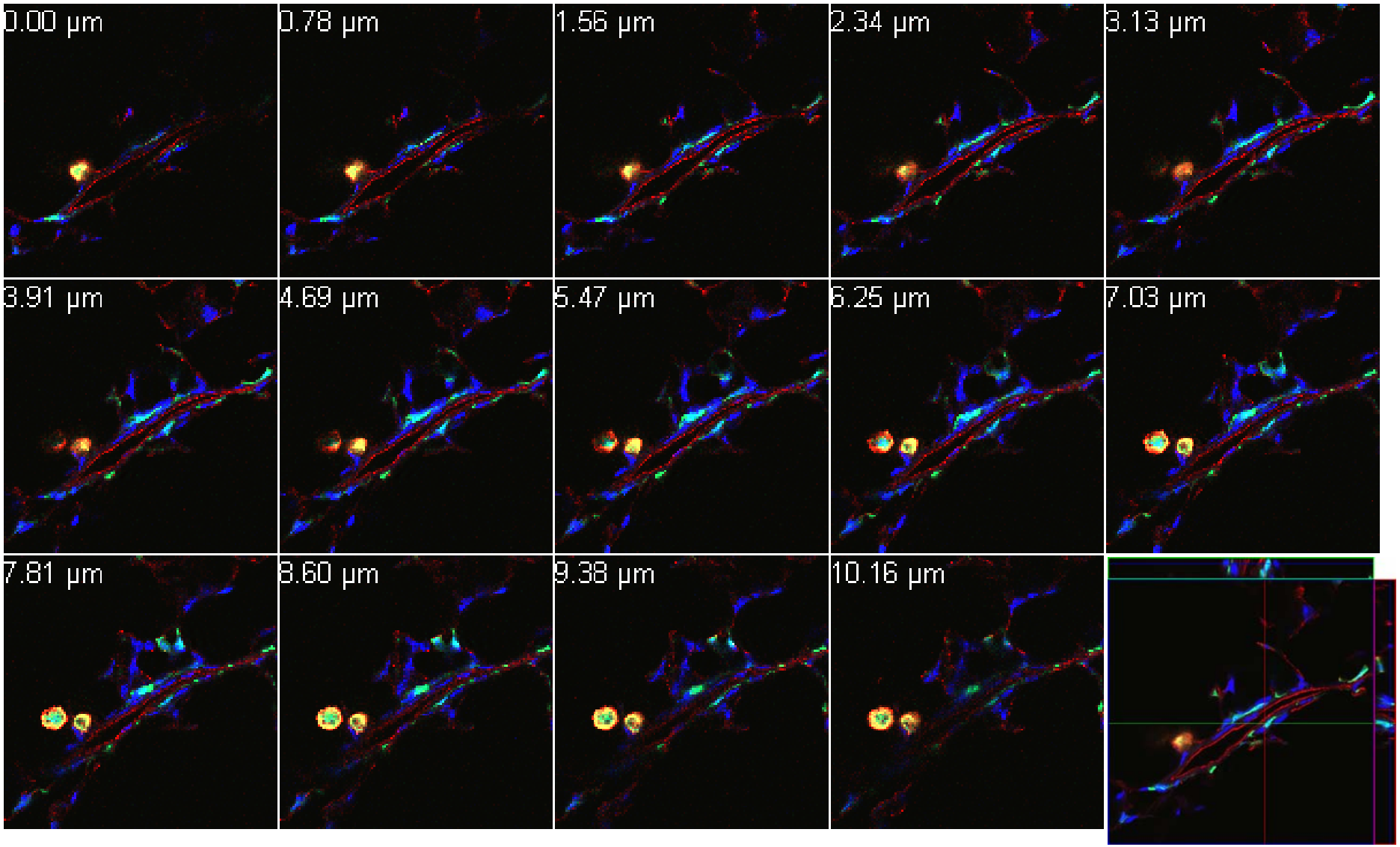


Supplemental figure 3



Supplemental Figure 4: Purification of hematopoietic stem cells