Materials and Methods

Complete blood counts and colony assays

Blood for cell counts were collected into Microtainer tubes with K_2EDTA (Becton Dickinson, Franklin Lakes, NJ). All samples were analyzed at Antech Diagnostics® (Lake Success, NY) by laser flow cytometry. Mouse methylcellulose complete media (Stem Cell Technologies, Vancouver, BC) and Megacult-C media (Stem Cell Technologies) were used to assay hematopoietic colony formation, which were enumerated according to the manufacturer's protocol. Megacult-C media was supplemented with recombinant mouse interleukin-3 (10ng/ml), recombinant human TPO (50ng/ml), recombinant mouse IL11 (50ng/ml) and recombinant human IL6 (20ng/ml) (Stem Cell Technologies).

Histology and marrow sinusoid vascular density

Femurs and spleens from Pf4/FF1 mice and age-matched littermate controls (which express the Pf4-Cre transgene but not the FF1 transgene) were fixed in 10% (vol/vol) formalin for 24 hr. Femurs were then decalcified and paraffin sections (5-µm thickness) were stained with hematoxylin and eosin (H&E). Images were taken using an Olympus BX51 microscope.

Marrow sinusoids were identified as red blood cell engorged vessels lined by a single layer of flat endothelial cells.[1] The percentage of the total area occupied by sinusoid vessels on each image was recorded and the mean marrow sinusoid vascular density was calculated by averaging the percentages from all images examined.

Flow cytometry

Hematopoietic cell lineage analysis was performed using CD71, Gr-1, Mac-1, B220 antibody or isotype control staining (all Biolegend) and analyzed on a FACSCalibur (Becton Dickinson). For EC surface marker analysis, platelet endothelial cell adhesion molecule (PECAM-1 or CD31, Clone 390), vascular endothelial-cadherin (VE-cadherin or CD144, Clone BV13), vascular endothelial growth factor 2 (VEGFR2 or CD309, Clone Avas 12) antibody or isotype control was used.

Bone Marrow Transplantation Assays

Recipient mice were lethally irradiated (950 cGy) and then received 5 $x10^6$ nucleated marrow cells from donor mice by standard intravenous tail vein injection using a 27G insulin syringe. Peripheral blood was obtained every 4 weeks after transplantation and CD45.2 percentage chimerism and complete blood counts were measured.

Polymerase Chain Reaction

Human JAK2-specific primers GAGCAAGCTTTCTCACAAGC and AATTCTGCCCACTTTGGTGC (the same primers used for FF1 genotyping) were used to detect the expression of human $JAK2^{V617F}$ in MK and E-SLAM cells from Pf4/FF1 mice using reverse transcription polymerase chain reaction (RT-PCR). The primers amplify a 530-bp fragment which would be detected on 2% agarose gel.

The TaqMan® Gene Expression Assay (Applied Biosystems) was used for real-time quantitative polymerase chain reaction (qPCR) to verify differential expression of Fibroblast growth factor 1 (*FGF1*), Platelet factor 4 (*Pf4*), Transforming growth factor beta 1 (*TGFb-1*), Chemokine (C-X-C motif) ligand 12 (*CXCL12*), Vascular endothelial growth factor A (*VEGFa*), Chemokine (C-X-C motif) receptor 4 (*CXCR4*), and *c-MPL* on an ABI ViiA™ 7 Real-Time PCR machine (Applied Biosystems). The gene expression levels were normalized to Transferrin receptor (Tfrc) and/or Actin beta (Actb) expression and relative fold changes was calculated by the $2^{\Delta\Delta CT}$ method. All assays were performed in triplicate.

Isolation of murine lung endothelial cells

14-18wk old C58BL/6 mice were euthanized using 100% CO_2 inhalation followed by cervical dislocation. The chest was immediately opened through a midline sternotomy. The left ventricle was identified and the ventricular cavity was entered through the apex with a 27-gauge needle. The right ventricle was identified and an incision was made in the free wall to exsanguinate the animal and to allow the excess perfusate to exit the vascular space. The animal was perfused with 30 ml of cold PBS. The lung tissue was collected and minced finely with scissors. The tissue fragments were digested in DMEM medium containing 1 mg/mL Collagenase D (Roche, Switzerland), 1 mg/mL Collagenase/Dispase (Roche) and 25 U/mL DNase (Sigma, St. Louis, MO) at 37°C for 2hr with shaking, after which the suspension was homogenized by

triturating. The homogenate was filtered through a 70μm nylon mesh (BD Biosciences, San Jose, CA) and pelleted by centrifugation (400g for 5 min). Cells were first depleted for $CD45^+$ cells (Miltenyi Biotec, SanDiego, CA) and then positively selected for $CD31^+$ cells (Miltenyi Biotec) using magnetically labeled microbeads according to the manufacturer's protocol. Isolated ECs ($CD45^-CD31^+$) were cultured in EC culture medium with no medium change for the first 72hrs to allow EC attachment followed by medium change every 2-3 days. Cells were re-selected for $CD31^+$ cells when they reach >70-80% confluence (usually after 3-4 days of culture).

In vitro cultures

E-SLAM HSPCs were sorted and cultured in StemSpan serum-free expansion medium (SFEM) (Stem Cell Technologies) containing recombinant mouse SCF (300 ng/mL) and recombinant mouse IL-11 (20 ng/mL) (Stem Cell Technologies) for a total of 12 days. Fresh medium with cytokines were added on Day 4 and 8 of the culture period. CD41+ MK cells were cultured in SFEM with 25ng/ml recombinant mouse SCF and 25ng/ml recombinant human TPO (Stem Cell Technologies). Prospectively collected MKCM was added to E-SLAM medium at 1:1 ratio together with the specific cytokines in designated E-SLAM culture wells.

ECs were cultured on 1% gelatin coated plates in complete EC medium which is consisted of advanced DMEM/F12 (ThermoFisher, Waltham, MA) medium containing 20% FBS, 50μg/ml endothelial cell growth supplement (Alfa Aesar, Ward Hill, MA), 1% Antibiotic-antimyotic solution (Cat. 15240-062, ThermoFisher), 10mM HEPES buffer (ThermoFisher), 5μM SB431542 small molecule (R&D, Minneapolis, MN), 50μg/ml Heparin (Sigma), 1% Glutamax 100x solution (ThermoFisher), 1% non-essential amino acid (ThermoFisher), recombinant mouse VEGF 10ng/ml (PeproTech, Rocky Hill, NJ; add fresh when changing medium) and recombinant human FGF2 20ng/ml (PeproTech; add fresh when changing medium). Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere with medium change every 2-3 days until they reach 70-80% confluence. 0.05 % trypsin-EDTA solution was used for cell passaging.

In vitro DiI-acetylated– low density lipoprotein labeling of ECs

The uptake of acetylated-low density lipoprotein conjugated to a fluorescent dye (DiI-Ac-LDL) is relatively specific to ECs.[2,3] ECs were incubated with 10ug/ml of DiI-Ac-LDL (Alfa Aesar) in complete EC

medium at 37°C for 4 hours. The cells were then washed twice with PBS and observed using an Olympus IX70 microscope using the standard rhodamine excitation/emission filter combinations.

Assays to examine endothelial cell in vitro angiogenesis and migration

EC tube formation assay was performed as a measure of angiogenesis *in vitro*. Matrigel® matrix (10mg/ml, Corning Inc., Corning, NY) were thaw overnight at 4°C and kept on ice until use. 150ul Matrigel per well was added to pre-chilled 48-well culture plate. After gelation at 37°C for 30minutes, gels were overlaid with 6 x 10^4 primary murine lung ECs (passage 3-4) in 300ul of complete EC medium with SFEM, or Pf4/FF1 MKCM, or control MKCM (all at 1:1 volume ratio). Tube formation was inspected after a period of 2, 4, 6, and 8hrs and images were captured with a phase-contrast microscope (AMEX-1200, AMG, Bothell, WA). The quantification of the capillary tube formation was performed using the ImageJ® software (National Institute of Health, Bethesda, MD) by counting the number of tubes and nodes (branch points) in 4 non-overlapping areas at x40 magnification in two duplicate wells.

Scratch assay was used to assess EC migration *in vitro*. Primary murine lung ECs (passage 3-4) were seeded in 1% gelatin coated 24-well plates and cultured at 37°C to near confluence. Three lines were draw with a marker on the bottom of EC wells. A lesion was produced by scraping through the adherent EC monolayer using a sterile 1ml pipet tip moving perpendicular to the marker lines (time 0hr). The cells were rinsed gently with PBS to remove dislodged cells and cellular debris. Cells were then cultured in complete EC medium with SFEM, or Pf4/FF1 MKCM, or control MKCM (all at 1:1 volume ratio) at 37°C in a humidified 5% CO_2 atmosphere. Images were captured with a phase-contrast microscope (AMEX-1200, AMG) at different time points after wound creation. The distance between the sides of the scratch wound was measured using ImageJ® software at 6-12 different locations in two duplicate wells for each culture condition.

Person who analyzes the images was blinded to the specific culture condition in both the tube formation assay and the scratch assay.

Supplementary Figure Legends

Supplementary Figure 1. Isolation of murine lung endothelial cells. (**A**) Lung ECs were isolated after intravascular perfusion, enzymatic digestion, CD45^{+} cell depletion and CD31^{+} cell selection. (**B**) Isolated ECs were labeled positively by acetylated-low density lipoprotein conjugated with the fluorescent dye (DiI-Ac-LDL). (**C**) Flow cytometry analysis revealed positive EC markers CD144, CD31, and CD309 on isolated ECs.

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2. Voyta JC, Via DP, Butterfield CE, Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. The Journal of cell biology 1984;99:2034-40.

3. Li XM, Hu Z, Jorgenson ML, Slayton WB. High levels of acetylated low-density lipoprotein uptake and low tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (Tie2) promoter activity distinguish sinusoids from other vessel types in murine bone marrow. Circulation 2009;120:1910-8.