SUPPLEMENTARY APPENDIX

# Secreted factors from mouse embryonic fibroblasts maintain repopulating function of single cultured hematopoietic stem cells

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**Supplementary files to:**

# Secreted factors from mouse embryonic fibroblasts maintain repopulating function of single cultured hematopoietic stem cells

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**Contents**

## Supplementary Methods

### Mice

C57BL/6.J (B6; CD45.2), B6.SJL-Ptprca.Pep3b/BoyJ (Ly5.1; CD45.1), 129S2/SvPasCrl (129; CD45.2) were obtained at an age of eight- to ten weeks from Charles River Laboratories (Lyon, France). In transplantation experiments, we used (129S2 x B6) F1 (129B6) or (129S2 x C57BL/6.SJL) F1 (129Ly5.1) mice unless otherwise specified. All experiments were approved by the Government of Upper Bavaria. All animals were housed in microisolators under specific pathogen-free conditions, according to Federation of Laboratory Animal Science Associations and institutional recommendations.

### Primary Cells and Mouse Embryonic Fibroblasts

Primary mouse embryonic fibroblasts (MEFs) were generated from pools of E11.5 or E13.5 129B6 embryos using a generic protocol[10] without trypsin digestion of the embryonic tissue. In brief, after dissection of the head and all internal organs, embryo trunks were chopped with scalpel to mince the tissue of 1-2 mm in Petri dish filled with MEF medium (DMEM high glucose (Gibco-Thermofisher), 10% FCS (Sigma-Aldrich) and antibiotics (Gibco)). To increase the surface area for cell growth, the bottom of the dish was lightly scratch. After 3-4 days of culture at 37°C, MEF colonies become visible. MEFs were passaged four times every 3-4 days with 1x Trypsin (Gibco-Thermofisher) for cell detachment. In every new passage, fresh culture medium was mixed with 20% of the cell-debris-free conditioned medium of the previous passage.

### Generation of Conditioned Medium (CM)

CM was prepared essentially as described previously[6]. Therefore, 70.000/cm$^2$ MEFs (passage 4) were cultured at 37°C. Twelve hours after culture, mitosis was inactivated with $\gamma$--irradiation (30 Gy X-Ray; Gulmay Type RS225), cells were washed twice with Dulbecco's PBS (DPBS; Gibco) and cultured with serum-free medium (SFM; StemSpan; Stemcell Technologies) containing antibiotics and $10^{-4}$ M ß-mercaptoethanol (Gibco) at 37°C. CM was harvested after 72 hours, centrifuged at 200g for 10 minutes, 0.22 µm filtered and either used immediately or stored at -20°C.

### Flow Cytometry Analysis and Cell Sorting

Surface antigens were stained with antibodies from either Invitrogen- or eBioscience-ThermoFisher (Supplementary Table) in HF2+ buffer (Hanks balanced salt solution (Gibco) with 2% FCS, 10 µM HEPES buffer, and antibiotics). Fluorescence-activated cell sorting (FACS) analyses for immature and mature lineages were performed on a CyAn ADP Lx P8 (Coulter-

Cytomation). Data were analyzed with FlowJo software (TreeStar). For single cell cultures, $CD34^-$ $CD48^-$ $CD150^+$ LSK cells ($CD34^-$ SLAM cells) were sorted on an Astrios high speed cell sorter (BeckmanCoulter).

**Single cell culture (SCC)**

$CD34^-$ SLAM cells were sorted into round-bottomed 96-well plates preloaded with 100 µl of filtered (0.2 µm filter) CM or SFM, and supplemented with two growth factors (2GFs): recombinant mouse SCF (100 ng/ml) and recombinant mouse IL-11 (20 ng/ml), both from R&D Systems (BioTechne), or additionally supplemented with collagen 1 (300 µg/ml; BioVendor) and recombinant human NGF (250 ng/ml; R&D Systems-BioTechne) where indicated as 4GFs. Immediately after sorting the plates were centrifuged for 5 minutes at 200g and microscopically inspected for the presence of single cells. Each well was inspected every 24 hours for clonal growth. After 5 days, the number of colonies was counted and cells that had divided at least once (≥2 cells/well) were harvested and stained with antibodies against lineage markers, KIT and SCA1 (Supplementary Table). Immunofluorescence staining was measured on a CyAn ADP Lx P8 (Coulter-Cytomation) and analyzed with FlowJo software (TreeStar). In some experiments, the harvested clones were studied for colony formation in growth factor-supplemented methylcellulose (M3434; Stemcell Technologies).

***In Vivo* Transplantation Assay**

*In vivo* repopulation assay using competitive transplantation into lethally irradiated (8.5Gy; Gulmay) recipient mice was performed as we described previously[11,12]. 24 hours after irradiation, clones grown from 129Ly5.1 (Ly5.1xLy5.2) $CD34^-$ SLAM cells which had divided at least once in cultures in SFM 4GFs or in either MEF-CM 2GFs or 4GFs for 5 days, were then harvested, pooled, and transplanted at 20 clones/mouse in 100 µl HF2+ buffer into the tail vein of 129B6 (CD45.2) recipients. After 16 weeks, mice were sacrificed, and hematopoietic tissues were analyzed by flow cytometry for lymphoid and myeloid engraftment. Mice were considered positive when ≥ 1 % myeloid and ≥ 1 % lymphoid donor cells were detected in the peripheral blood. In addition, we analyzed the BM for regeneration of immature hematopoiesis, as described previously[11,12].

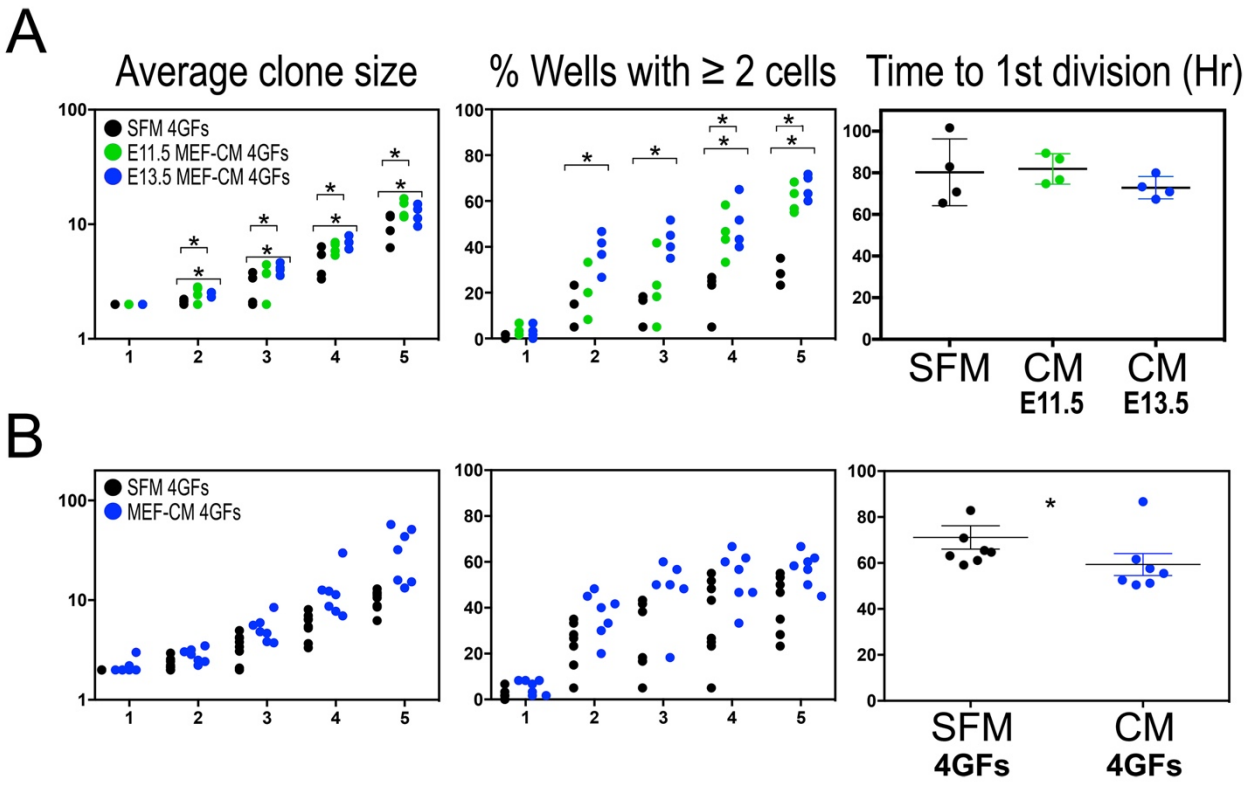
**Apoptosis Assay**

For analysis of apoptosis, 3000 LSK cells were sorted and cultured for 48 hours in a 12 well plate, prefilled with 2 ml SFM 4GFs or either MEF-CM 2GFs or 4GFs. After culture, LSK cells were harvested with HF2+ buffer. Each sample was stained with 500 µl Annexin Buffer (BD-Biosciences) containing 0.1 µg/ml PI and 2 µl AnnexinV-FITC for 15 minutes at 4°C in the dark.

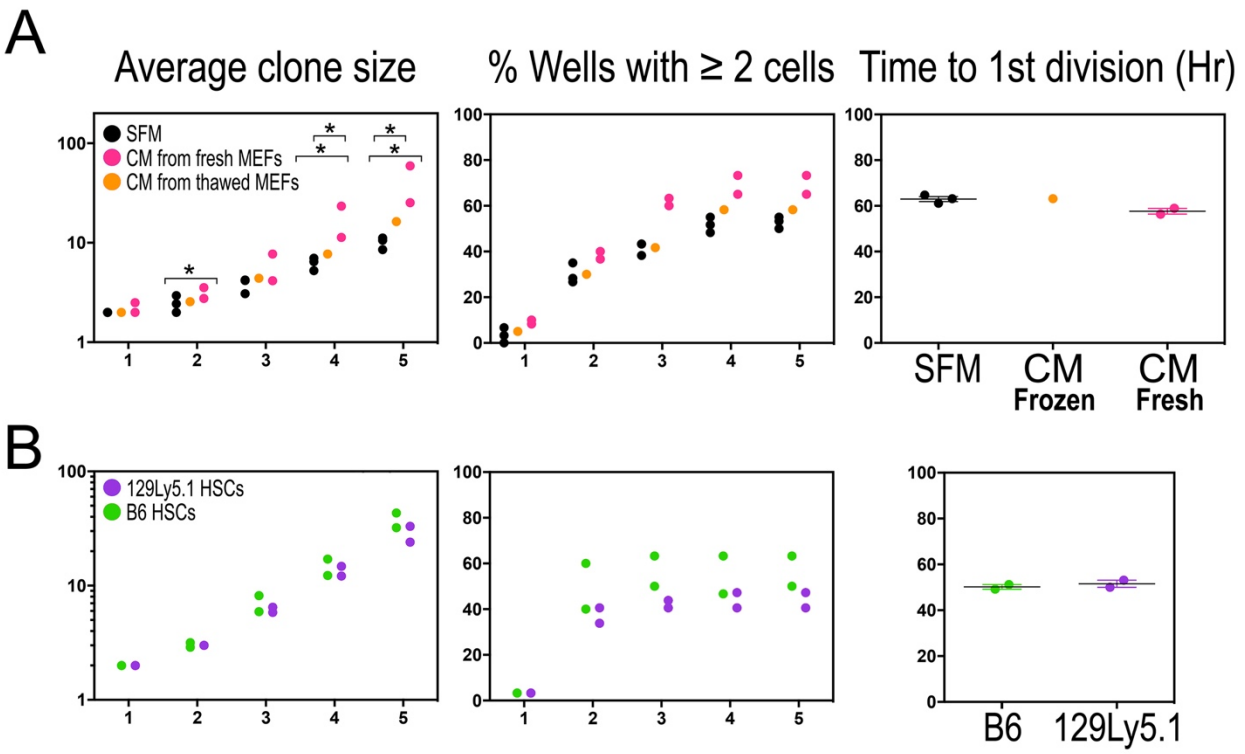
Fluorescence staining of cells was measured using flow cytometry and analyzed using FlowJo analysis software.

**Statistical evaluations**

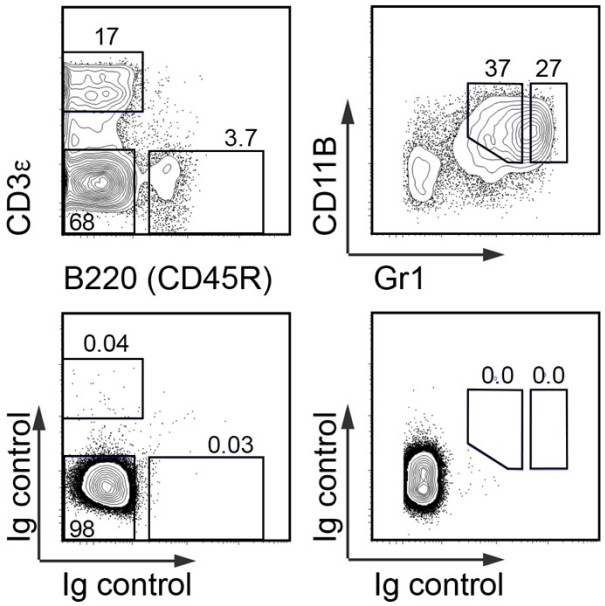
For statistical analysis, due to the sometimes large range of variation, we used the non-parametric Mann-Whitney U test (Prism, GraphPad Software). A p value<0.05 was considered statistically significant. Data are presented as dot plots or columns with means ± standard deviation.



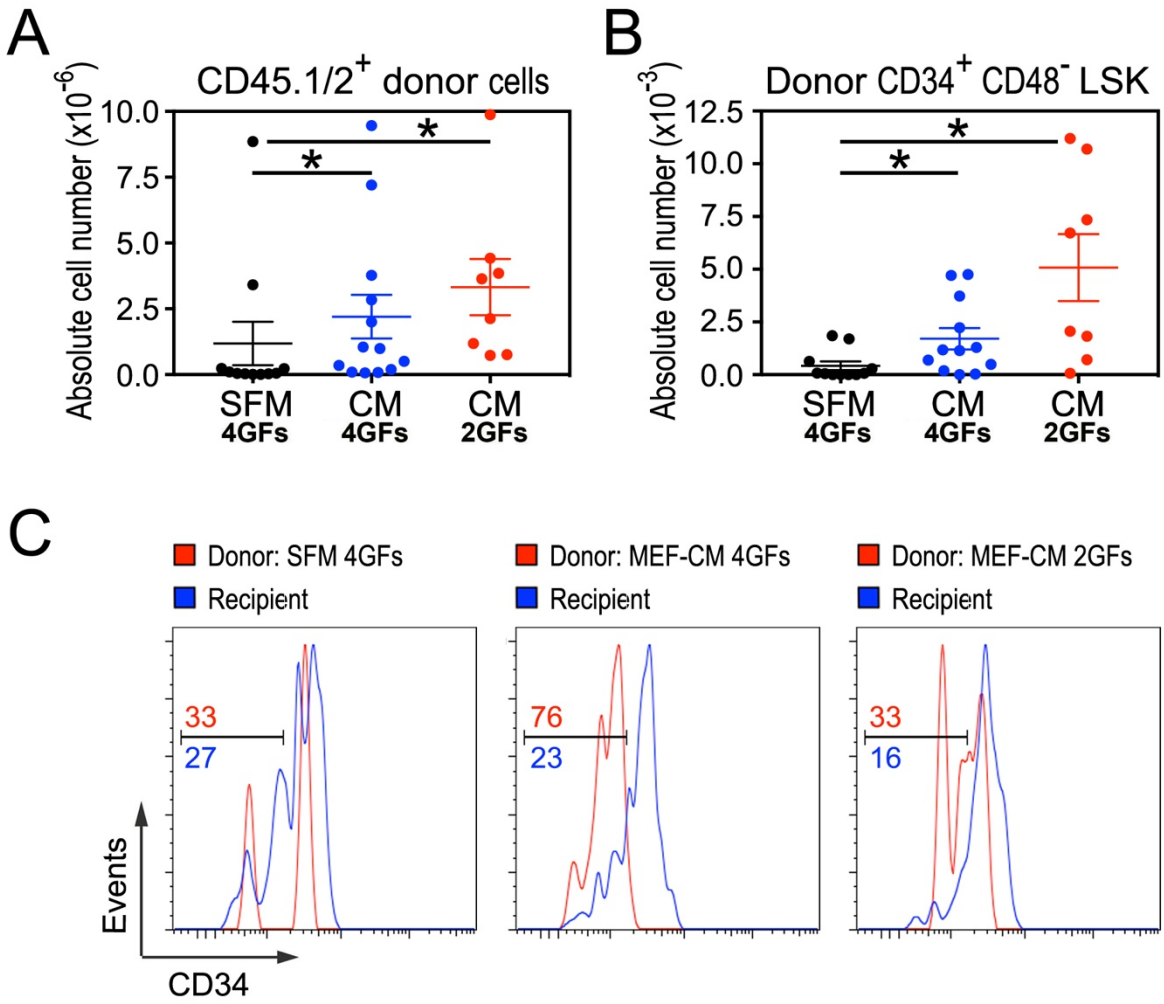

**Supplementary Figure 1. MEF-CM increases the number of dividing CD34$^-$ SLAM cells and reduces the time to first division.** (A) CM was prepared from MEFs generated from either E11.5 or E13.5 embryos as described in the Materials and Methods. Single CD34$^-$ SLAM cells of 129xLy5.1 were cultured under 4GFs conditions. Each dot represents the mean result of all clones in one 96-well plate in four independent comparisons. Left plot: average clone size of wells cultured in either SFM or MEF-CM (E11.5 or E13.5), all with 4GFs; Middle plot: Percentage of wells with ≥ 2 cells cultured in either SFM or MEF-CM (E11.5 or E13.5) with 4GFs conditions; Right plot: time to 1$^{st}$ division in hours of cells cultured in either SFM or MEF-CM (E11.5 or E13.5) with 4GFs conditions. (B) Variations of E13.5 MEF-CM from seven individual embryos in cultures of single CD34$^-$ SLAM cells. Single CD34$^-$ SLAM cells were cultured in either SFM or MEF-CM with 4GFs conditions. Together, the results represent three independent experiments. Left plot: average clone size of wells cultured in either SFM or E13.5 MEF-CM with 4GFs conditions; Middle plot: Percentage of wells with ≥ 2 cells cultured in either SFM or E13.5 MEF-CM with 4GFs conditions; Right plot: time to 1$^{st}$ division in hours of cells cultured in either SFM or E13.5 MEF-CM with 4GFs conditions. Black dots represent SFM 4GFs, green dots represent E11.5 MEF-CM 4GFs and blue dots represent E13.5 MEF-CM 4GFs. *: $p<0.05$ using the Mann Whitney U-test.




**Supplementary Figure 2. Optimization of single cell cultures of CD34$^-$ SLAM cells in MEF-CM.** (A) CM was prepared from MEFs generated from E13.5 embryos as described in the Materials and Methods. MEFs were used fresh or thawed before the experiment. Single CD34$^-$ SLAM cells of 129xLy5.1 were cultured under 4GFs conditions. Each dot represents the mean result of one to three independent comparisons. Left plot: average clone size of wells cultured in either SFM or E13.5 MEF-CM (fresh or thawed) with 4GFs conditions; Middle plot: Percentage of wells with ≥ 2 cells cultured in either SFM or E13.5 MEF-CM (fresh or thawed) with 4GFs conditions; Right plot: time to 1$^{st}$ division in hours of cells cultured in either SFM or E13.5 MEF-CM (fresh or thawed) with 4GFs conditions. Black dots represent SFM 4GFs, pink dots represent CM from fresh MEFs and yellow dots represent CM from thawed MEFs. (B) Variations in cultures of single CD34$^-$ SLAM cells from 129Ly5.1 and B6. Single CD34$^-$ SLAM cells were cultured in either SFM or E13.5 MEF-CM with 4GFs conditions. Together, the results represent two independent experiments. Left plot: average clone size from 129Ly5.1 and B6 of wells cultured in either SFM or E13.5 MEF-CM with 4GFs conditions; Middle plot: Percentage of wells with ≥ 2 cells from 129Ly5.1 and B6 cultured in either SFM or E13.5 MEF-CM with 4GFs conditions; Right plot: time to 1$^{st}$ division in hours of cells from 129Ly5.1 and B6 cultured in either SFM or E13.5 MEF-CM with 4GFs conditions. Black dots represent SFM 4GFs, green dots represent B6- and purple dots 129Ly5.1 HSCs cultured in MEF-CM 4GFs. *: $p<0.05$ using the Mann Whitney U-test.




**Supplementary Figure 3. Isotype controls for gating of differentiation studies in culture and engrafted animals.** Each dot plot shows a representative sample from BM cells stained for differentiation markers (top left: lymphoid; top right: myeloid in the non-lymphoid gate) and corresponding isotype controls (bottom row).




**Supplementary Figure 4. Histograms of donor LSK cells against CD34 (to Figure 3).** (A) shows the total number of donor cells in the BM of recipients from SFM 4GFs, MEF-CM 4GF, or MEF-CM 2GFs cultures. (B) Total number of donor-derived short term-repopulating cells in the BM of recipients from SFM 4GFs, MEF-CM 4GF, or MEF-CM 2GFs cultures. (C) Representative histograms from the LSK cells of recipients showing detectable BM engraftment. In the left-panel, $CD34^-$ donor cell engraftment after 16 weeks of transplantation from HSCs cultured in SFM 4GFs, shown is the histogram of one of the two engrafted recipients (total n=11). In the middle panel, the histogram is shown from one of seven engrafted recipients transplanted with cells from MEF-CM 4GFs cultures (total n=13). On the right panel, histogram from one of eight engrafted recipients transplanted with cells from MEF-CM 2GFs cultures (total n=8) is shown. in the three histograms, donor CD48- LSK cells are shown in red and recipient cells are shown as controls in blue lines. Percentages of HSC-enriched cells ($CD34^-$) are shown on the left side of each histogram.

| Antigen | Isotype | Clone | Conjugate | Dilution | Manufacturer |
|---|---|---|---|---|---|
| CD3ε | IgG (Hamster) | 145-2C11 | PECy5.5 | 1/10 | invitrogen |
| CD3ε | IgG (Hamster) | 145-2C11 | biotin | 1/10 | eBiosciences |
| CD11b | IgG2b, κ (rat) | M1/70 | APCCy7 | 1/10 | invitrogen |
| CD11b | IgG2b, κ (rat) | M1/70 | biotin | 1/10 | eBiosciences |
| CD34 | IgG2a, κ (rat) | RAM34 | FITC | 1/10 | eBiosciences |
| CD45 | IgG2b, κ (rat) | 30-F11 | FITC | 1/1 | invitrogen |
| CD45 | IgG2b, κ (rat) | 30-F11 | PE | 1/1 | invitrogen |
| CD45 | IgG2b, κ (rat) | 30-F11 | PECy5.5 | 1/1 | invitrogen |
| CD45 | IgG2b, κ (rat) | 30-F11 | PECy7 | 1/1 | invitrogen |
| CD45 | IgG2b, κ (rat) | 30-F11 | PB | 1/1 | invitrogen |
| CD45 | IgG2b, κ (rat) | 30-F11 | APC | 1/1 | invitrogen |
| CD45 | IgG2b, κ (rat) | 30-F11 | APCCy7 | 1/1 | invitrogen |
| CD45.1 (Ly5.1) | IgG2a, κ (mouse) | A20 | FITC | 1/10 | invitrogen |
| CD45.2 (Ly5.2) | IgG2a, κ (mouse) | 104 | PE | 1/10 | invitrogen |
| CD45R (B220) | IgG2a, κ (rat) | RA3-6B2 | PECy7 | 1/10 | invitrogen |
| CD45R (B220) | IgG2a, κ (rat) | RA3-6B2 | biotin | 1/10 | eBiosciences |
| CD48 | IgG (Hamster) | HM48-1 | biotin | 1/10 | invitrogen |
| CD117 | IgG2b, κ (rat) | 2B8 | APC | 1/10 | invitrogen |
| CD150 | IgG1, κ (mouse) | 9D1 | PE | 1/10 | invitrogen |
| Gr1 | IgG2b, κ (rat) | RB6-8C5 | PB | 1/10 | invitrogen |
| Gr1 | IgG2b, κ (rat) | RB6-8C5 | biotin | 1/10 | eBiosciences |
|  | IgG (Hamster) | eBio299Arm | PECy5.5 | 1/10 | eBiosciences |
|  | IgG2a, κ (mouse) | eBM2a | PE | 1/10 | eBiosciences |
|  | IgG2a, κ (mouse) | eBM2a | FITC | 1/10 | eBiosciences |
|  | IgG2a, κ (rat) | eBR2a | PECy7 | 1/10 | eBiosciences |
|  | IgG2b, κ (rat) | eB149/10H5 | APCCy7 | 1/10 | eBiosciences |
|  | IgG2b, κ (rat) | eB149/10H5 | PB | 1/10 | eBiosciences |
| TER-119 | IgG2b, κ (rat) | TER-119 | PE | 1/10 | invitrogen |
| TER-119 | IgG2b, κ (rat) | TER-119 | APC | 1/10 | invitrogen |
| TER-119 | IgG2b, κ (rat) | TER-119 | biotin | 1/10 | eBiosciences |
| Sca-1 | IgG2a, κ (rat) | D7 | PECy7 | 1/10 | invitrogen |
| Streptavidin |  |  | PECy5.5 | 1/10 | Invitrogen |

**Suppplementary Table**. Antibodies used for sorting of $CD34^-$ SLAM cells, flow cytometric analyses of cultures, isotype controls and analysis of transplanted mice.