Fanca$^{-/-}$ hematopoietic stem cells demonstrate a mobilization defect which can be overcome by administration of the Rac inhibitor NSC23766

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SUPPLEMENTARY APPENDIX

MATERIALS AND METHODS

Mice

All animals were maintained in a specific pathogen-free environment and all experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Cincinnati Children's Research Foundation. *Fanca*$^{+/-}$ mice (1, 2) were obtained from Dr Markus Grompe (Oregon Health Science University, Portland, OR) (3) and were backcrossed with the C57Bl6/J strain for 10 generations. *Fanca* $^{-/-}$ mice were generated by the mating of heterozygous knockout mice, with the resulting wild type (WT) littermates reserved as a source of control animals. The identity of knockout and WT mice was established by PCR of genomic DNA. C57Bl6/J and B6.SJL$^{Ptprca\ Pep3b/boyJ}$ mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA).

Bone Marrow Competitive Repopulation Assay

Bone marrow (BM) was isolated from femora and tibiae by flushing with phosphate buffered saline using a 23G needle (Beckton Dickinson, San Jose, CA, USA), followed by passing through a 40 µm nylon cell strainer (Beckton Dickinson). The cell content of 1/10 of a femur from either *Fanca*$^{-/-}$ or WT mice (CD45.2^{+}) was mixed with 3 x 10^6 congenic CD45.1^{+} (B6.SJL$^{Ptprca\ Pep3b/boyJ}$) competitor BM cells and then injected intra-venous (i.v.) into lethally irradiated (11.75 Gy, 56 cGy/min, split dose from a ^{135}Cs source) B6.SJL$^{Ptprca\ Pep3b/boyJ}$ recipients. The relative contribution of each donor cell population to the peripheral blood of recipient mice was determined by flow analysis following staining with monoclonal antibodies directed against either CD45.1 or CD45.2 (BD Biosciences, San Diego, CA, USA). The frequency of competitive repopulating units (CRU) was calculated as described (4) using the actual number of donor test cells transplanted in the calculation and assuming that the frequency of CRU in competitor BM cells is 1/10^5.

Immunophenotyping

Cells were stained with fluorescent labeled monoclonal antibodies (FITC-conjugated anti-mouse CD3 (KT3), CD4 (RM4-5), CD8 (53-6.7), CD45R/B220 (RA3-6B2), Mac-1 (M1/70), Gr-1 (RB6-8C5) and Ter119 (Ly-76); PE-conjugated anti-mouse Sca-1 (D7); APC-conjugated anti-mouse c-Kit (2B8); biotin-conjugated anti-mouse CD49d (9C10), CD49e (5H10-27), CD62L (MEL-14) or CXCR4 (2B11/CXCR4), followed by Streptavidin-APC-Cy7, according to the manufacturer's instructions (all BD Biosciences). Cells were subsequently analysed by flow cytometry (FACS Canto, Beckton Dickinson).

Homing Assay

Ten million BM cells were injected i.v. into lethally irradiated C57Bl6/J recipients. Sixteen hours after injection, mice were sacrificed following which BM and splenocytes were harvested. Harvested cells were plated out in methylcellulose (1 x

MethoCult, Stem Cell Technologies, Vancouver, Canada) supplemented with 30% fetal calf serum (FCS, Omega Scientific, Tarzana, CA), 2 mM L-Glutamine (Hyclone), 200 U Penicillin/Streptomycin (Hyclone), 100 μM β-mercaptoethanol (Thermo Fisher), 1 % BSA (Roche, Indianapolis, IN), 100 ng/ml rrSCF, 100 ng/ml mIL3, and 4 U/ml Epogen (Amgen). Plates were then incubated in a humidified atmosphere at 37ºC, 5% CO_2. Colonies consisting of ≥50 cells were scored at day 7. The input frequency of colony forming units (CFU) was also determined by plating out an aliquot of BM in methylcellulose concurrent with the remaining BM being injected into recipient mice. The recovery of CFU in the BM was determined under the assumption that 2 femora and 2 tibiae comprise 18% of the total BM (5)

HSC Mobilization and Engraftment

Mice were administered with 100 μg/Kg/day G-CSF (Neupogen, Amgen, Thousand Oaks, CA) for 6 days, intraperitoneally. On the sixth day, they were either co-administered PBS (G-CSF alone group) or NSC23766 (2.5 mg/Kg), intraperitoneally. Peripheral blood was harvested from mice 6 hours after treatment with G-CSF/G-CSF + NSC23766 and subject to red cell lysis (Pharm Lyse, Becton Dickinson). For CFU analysis, the cell number equivalent of 100 μl whole blood was plated in methylcellulose as described above. For CRU analysis, the cell number equivalent to 400 μl of whole blood was co-injected i.v. into lethally irradiated B6.SJL$^{Ptprca\ Pep3b/boyJ}$ recipients along with 5 x 10^5 B6.SJL$^{Ptprca\ Pep3b/boyJ}$ competitor BM cells. As before, antibody staining for CD45.1 or CD45.2 was used to evaluate relative donor chimerism. At 5 months post-transplant, BM was harvested from primary recipients and 1 x 10^7 cells were injected i.v. into lethally irradiated secondary recipients.

Statistical Analysis

Unless otherwise stated, mean values are quoted ± standard deviation. Comparison of statistical significance was performed using two tailed, non-paired Student's t-test.

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