MicroRNA126 contributes to granulocyte colony-stimulating factor-induced hematopoietic progenitor cell mobilization by reducing the expression of vascular cell adhesion molecule 1

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Supplementary Design and Methods

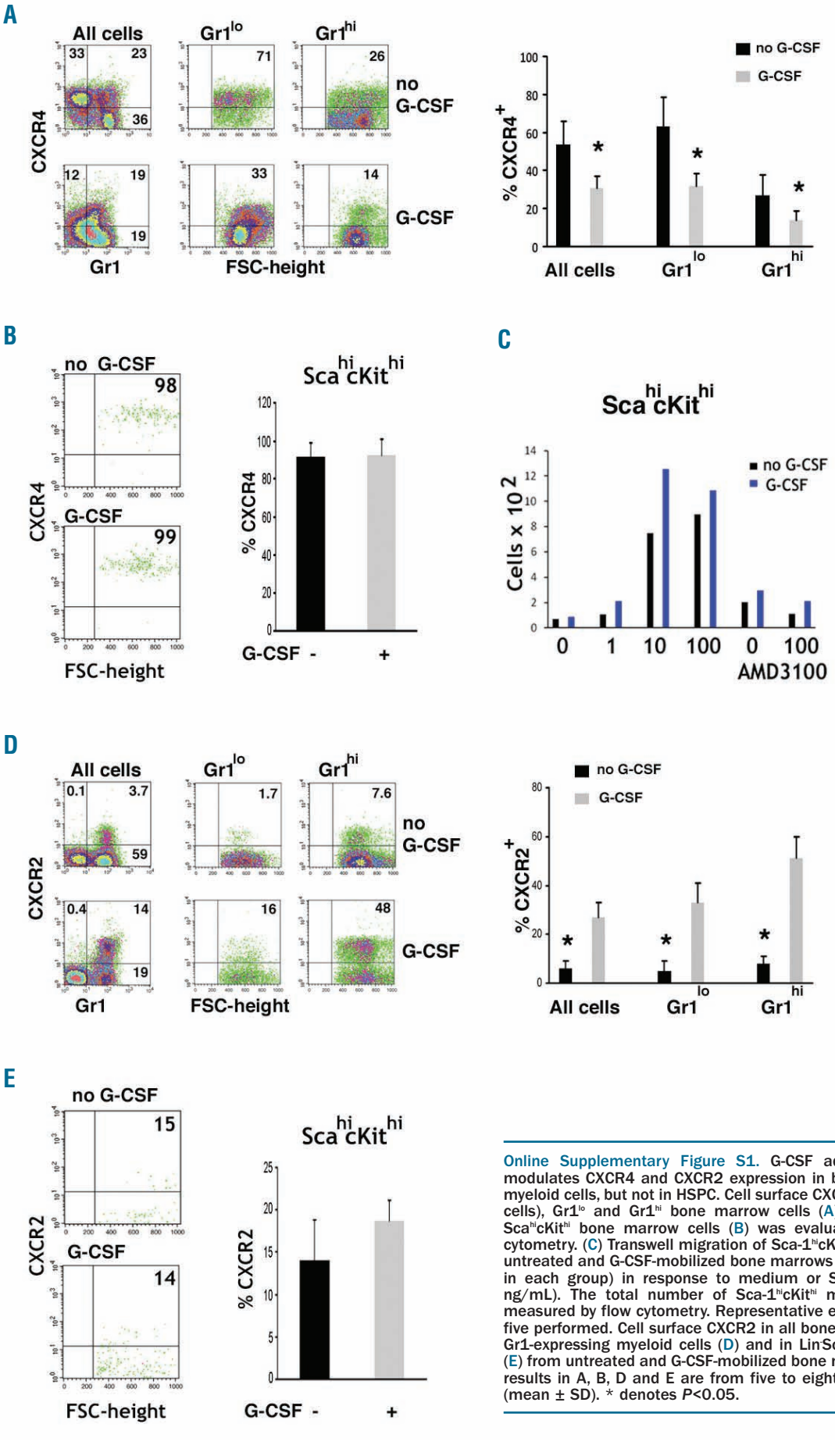
Microvesicle preparation and cell cultures

Microvesicles were derived from flushing femora and tibiae with 10 mL ice-cold phosphate-buffered saline (PBS), cell removal through centrifugation (10 min at 300g), filtration through 0.45 mm filters followed by two sequential ultracentrifugations (SW40; Beckman Instruments) for 30 min at 10,000 g and 60 min at 70,000 g. Individual 70,000 g pellets were suspended in 0.1 mL PBS, fixed (2% gluteraldehyde in 0.1M cacodylate buffer) for electron microscopy (Electron Microscopy Laboratory, SAIC Frederick, MD, USA) or labeled with PKH26 (red) or PKH67 (green) fluorescent dyes (Sigma-Aldrich) following the manufacturer's instructions.

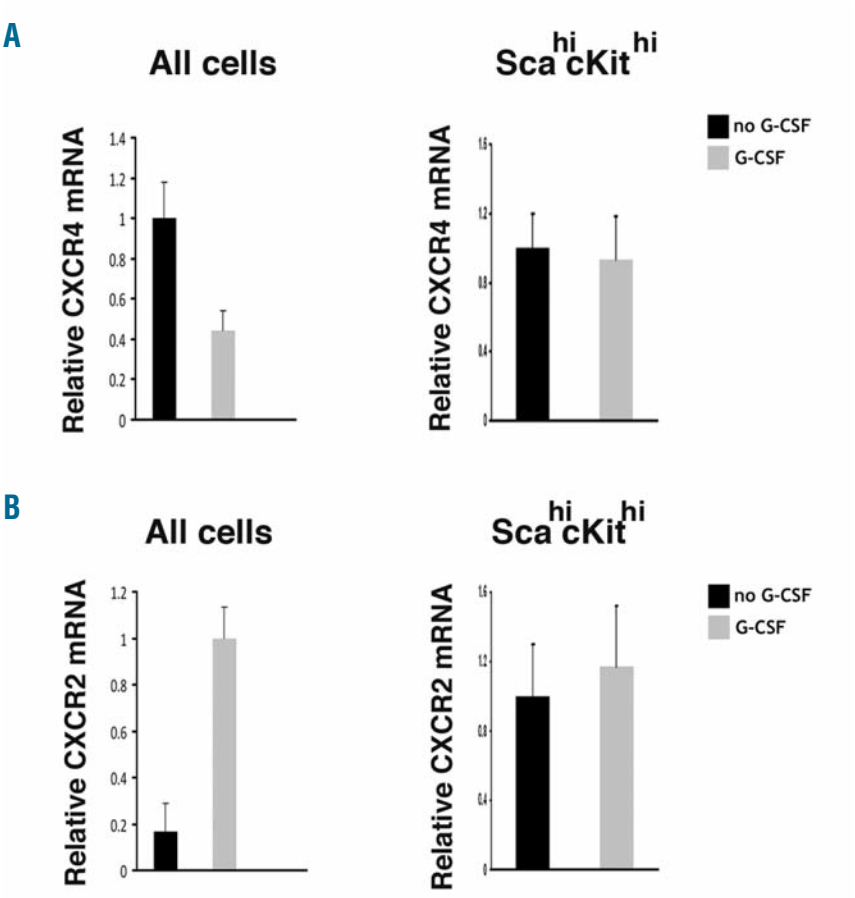
Labeled microvesicles were washed twice in PBS (10 mL) by ultracentrifugation (60 min at 70,000 g). Bone marrow cells (1×10^5/mL) were cultured (18-24 h) in Iscove's DMEM with 10% fetal bovine serum alone or with labeled microvesicle preparations. The ratio between cells and microvesicles was chosen to reflect the expected ratios in bone marrow (typically 50×10^6 bone marrow cells generated microvescicles that we suspended in 100 μL PBS; thus, 1×10^6 cells were cultured with 2 μL microvesicle preparations). Human umbilical vein endothelial cells (HUVEC) or MS-5 cells were incubated (6-10 h) alone or with labeled exosomes (at 1/10th the ratio used for bone marrow cells). Colony assays were performed using methylcellulose medium supplemented with a cocktail of recombinant growth factors (Methocult M3434, Stem Cell Technologies). Heparinized blood (10 and 20 μL) and bone marrow cells (2×10^4) were plated in duplicate on methylcellulose medium (2.5 mL) and incubated (5% CO_2, 37°C) for 7-12 days of culture. Colonies (at least 50 cells) were counted; for calculations we assumed a blood volume of 1.8 mL.

Flow cytometry

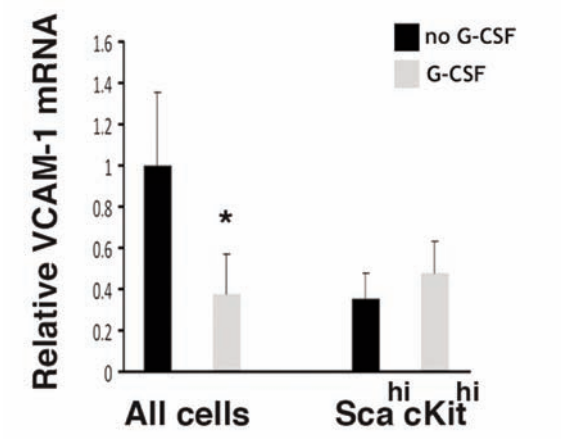
Cells were stained with the antibodies: FITC, PE or APC anti-mouse Gr1 (LY-6G and Ly-6C); biotin rat anti-mouse CXCR4/CD184; PE-labeled rat anti-mouse CXCR2 (R&D Systems); biotin anti-human/mouse CXCR7 (clone 8F11-M-16, Biolegend); PE or biotin rat anti-mouse CD106/VCAM-1, biotin rat anti-mouse CD49d (α_4 integrin); PE, biotin or PECy5 (rat anti-mouse Sca-1; Ly-6A-E, AbD Serotec); biotin anti-mouse Sca-1 (Ly-6A/E); FITC, PE or APC rat anti-mouse cKit/CD117; PE-Cy5 rat anti-mouse CD45 (LCA, Ly5); PE anti-mouse VE-cadherin/CD144 (BioLegend), Lineage markers (CD45, TER119, 7-4 clone/neutrophils, CD11b, CD19, Ly-6G/C and CD5, all biotin-labeled from StemCell Technologies), FITC rat anti-mouse F4/80 antigen (AbD Serotec); APC rat anti-mouse CD45R/B220, APC rat anti-mouse CD11b. Unless otherwise specified, all antibodies and appropriate isotype control antibodies were from BD Pharmingen. Streptavin-FITC (Invitrogen)-APC (Biolegend) and -PE (BD Biosciences) was used for detection of biotin-labeled antibodies. Live cells were identified using DAPI or a LIVE/DEAD Fixable Far Red Dead Cell Stain Kit (Invitrogen/Molecular Probes).



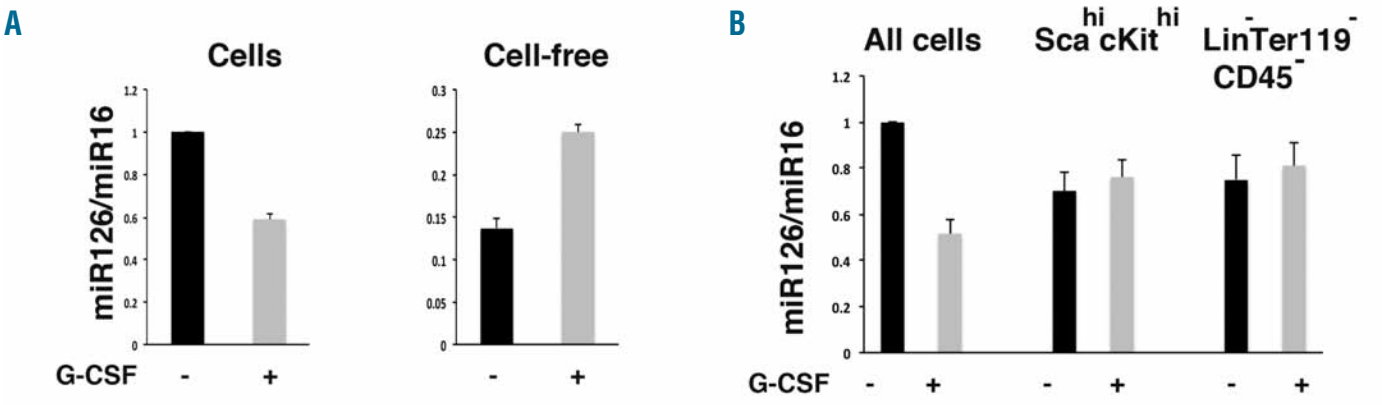
Online Supplementary Figure S1. G-CSF administration modulates CXCR4 and CXCR2 expression in bone marrow myeloid cells, but not in HSPC. Cell surface CXCR4 in all (All cells), Gr1^{lo} and Gr1^{hi} bone marrow cells (A) and in Lin^{-} Sca^{hi}cKit^{hi} bone marrow cells (B) was evaluated by flow cytometry. (C) Transwell migration of Sca-1^{hi}cKit^{hi} cells from untreated and G-CSF-mobilized bone marrows (3 combined in each group) in response to medium or SDF-1 (1-100 ng/mL). The total number of Sca-1^{hi}cKit^{hi} migrated was measured by flow cytometry. Representative experiment of five performed. Cell surface CXCR2 in all bone marrow and Gr1-expressing myeloid cells (D) and in Lin^{-}Sca^{hi}cKit^{hi} cells (E) from untreated and G-CSF-mobilized bone marrows. The results in A, B, D and E are from five to eight mice/group (mean ± SD). * denotes $P<0.05$.



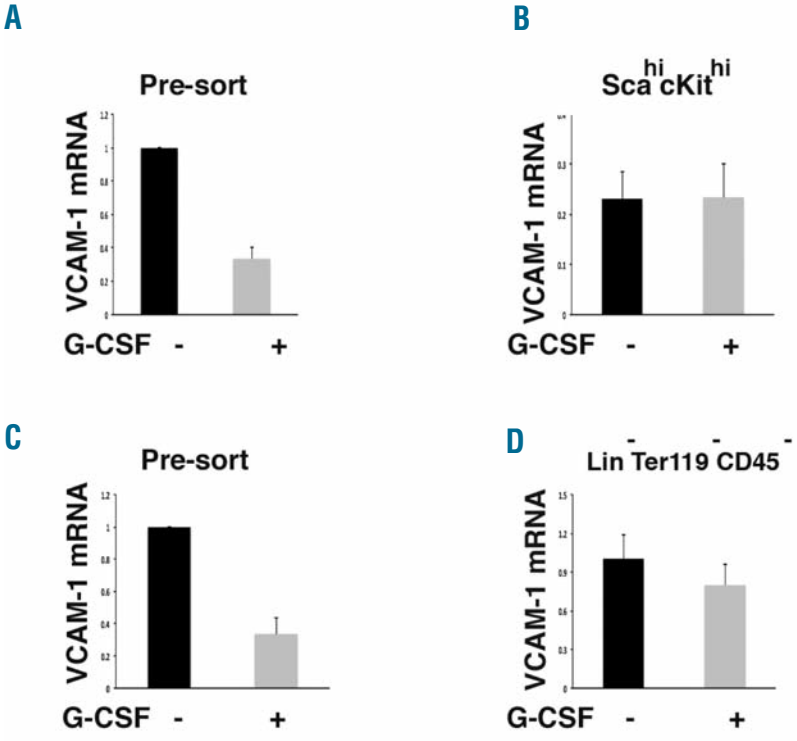
Online Supplementary Figure S2. Analysis of relative levels of CXCR4 (A) and CXCR2 (B) mRNA in non-fractionated bone marrow cells ("All cells") and in sorted Lin$^-$Sca^{hi}cKit^{hi} HSPC from mice mobilized with G-CSF or left untreated. The results are from quantitative PCR and are expressed as arbitrary units. The results are expressed as group (n=5-6) means ± SD.



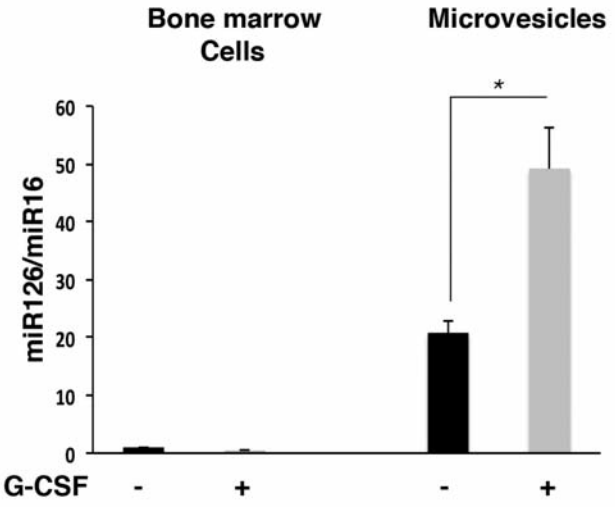
Online Supplementary Figure S3. Relative levels of VCAM-1 mRNA measured by quantitative PCR in unfractionated bone marrow cells and in the Lin$^-$Sca$^+$cKit$^+$ cells sorted from the marrow from mice treated with G-CSF or untreated. The results are expressed as group (n=6) means ± SD.



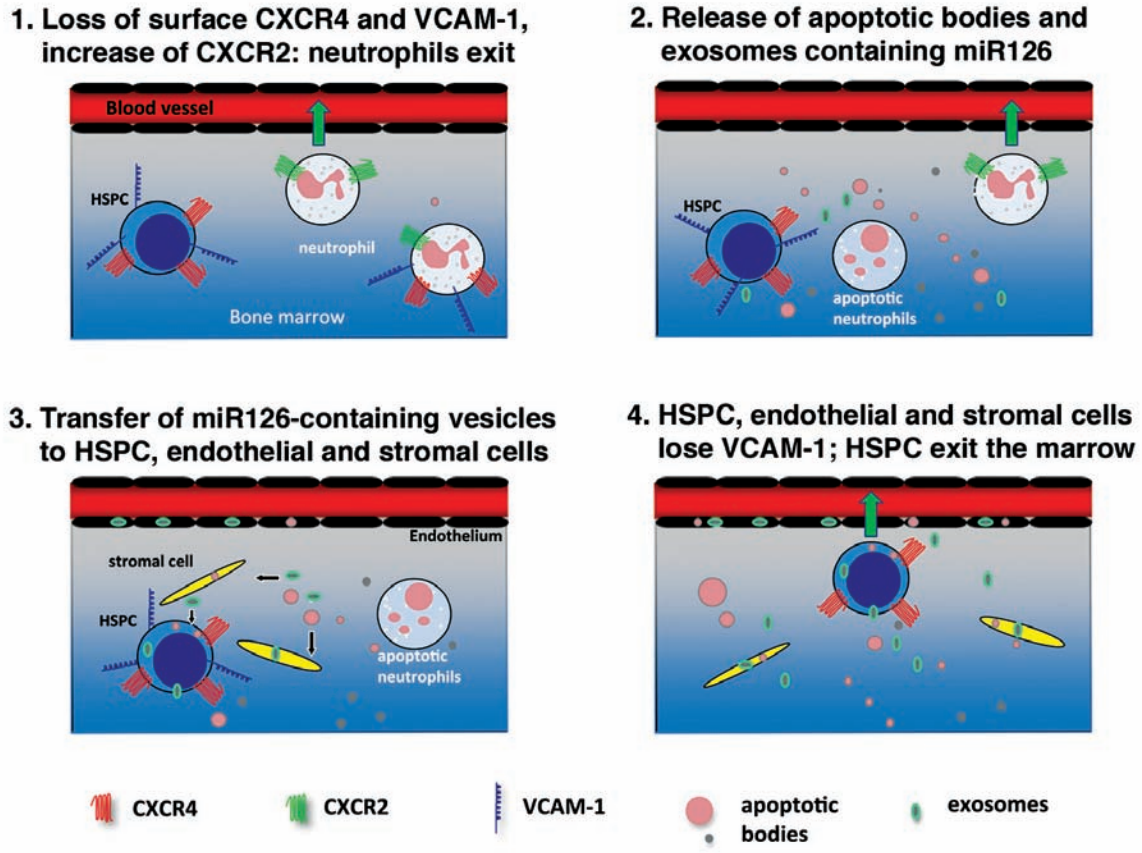
Online Supplementary Figure S4. G-CSF modulates miR126 expression in bone marrow cell cultures. (A) Unfractionated bone marrow cells were cultured (1x10^6 cells/mL) for 18 h with or without G-CSF (100 ng/mL) in complete culture medium. RNA was extracted from unfractionated cells and cell-free supernatant. The results from quantitative PCR reflect the mean ± SD (3 experiments) relative levels of miR126/miR16. (B) Unfractionated bone marrow cells were cultured (1x10^6 cells/mL) for 18 h with or without G-CSF (100 ng/mL) in complete culture medium. At the end of culture, Sca-1^{hi} and Lin$^-$Ter119$^-$CD45$^-$ cell populations were sorted. RNA was extracted from the unsorted bone marrow cells and the sorted populations. The results from quantitative PCR reflect the mean ± SD (3 experiments) relative levels of miR126/miR16.



Online Supplementary Figure S5. Administration of G-CSF regulates expression of VCAM-1 in the bone marrow. Combined bone marrows (3 mice) from untreated or G-CSF-treated mice were tested for relative levels of VCAM-1 mRNA in the unfractionated bone marrow cells (Pre-sort, A) and in the Sca-1^{hi}cKit^{hi} cells (after sorting, B). Combined bone marrows (3 mice) from untreated or G-CSF-treated mice were tested for relative levels of VCAM-1 mRNA in the unfractionated bone marrow cells (Pre-sort, C) and in the Lin$^-$Ter119$^-$CD45$^-$ cells (after sorting, D). The results reflect the mean ± SD of three experiments each (9 mice) in A, B, C and D.



Online Supplementary Figure S6. Detection of miR126 in micovesicles from bone marrow. Relative levels of miR126 in unfractionated bone marrow cells and microvesicle preparations from these bone marrow. Mice (5/group) were either untreated or treated with G-CSF. The results reflect the mean (± SD) levels of miR126/miR16 relative to levels detected in unfractionated bone marrow cells from control mice. * Denotes $P<0.05$.



Online Supplementary Figure S7. Schematic representation of HSPC and myeloid-lineage cell trafficking from the bone marrow to the blood; contribution of CXCR4, CXCR2, VCAM-1 and microvesicles containing miR126. See the *Discussion* for details.