SUPPLEMENTARY APPENDIX

Novel lineage depletion preserves autologous blood stem cells for gene therapy of Fanconi anemia complementation group A

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SUPPLEMENTAL INFORMATION for Adair, J.E., et al., Novel lineage depletion preserves autologous blood stem cells for gene therapy of Fanconi anemia complementation group A

List of Supplemental Items:

1. **Supplemental Table S1**
2. **Supplemental Figures 1-5**
3. **Supplemental Materials and Methods**

Table S1. FA-A Complementation Group Assignment for Each Enrolled Patient

Patient	Certified Test Performed	Results
1	Sequencing of the *FANCA* gene – Exon 22*	Homozygous splice variant defined as c.1827-1 G>A
2	Multiplex Ligation-dependent Probe Amplification (MLPA) of the *FANCA* gene†	Homozygous deletion of *FANCA* exons 6-31 (delExons 6-31)
3	Lentivirus transduction of *FANCA*, *FANCC* or *FANCG* cDNA with flow cytometric assessment of correction by reversal of G2/M arrest following DNA damage.ǂ	Correction observed with *FANCA* vector transduction, but not with *FANCC* or *FANCG*.

*****A previous test was performed by an un-named research laboratory to define this patient's splice variant mutation. Test results received to determine eligibility for entry into the current trial sequenced only the relevant exon to confirm presence of the splice variant.

†A previous test was performed by an un-named research laboratory to establish FA-A as the likely complementation group for this patient. Test results received to determine eligibility for entry into the current trial analyzed only the *FANCA* gene to confirm complementation.

ǂDescribed in Chandra, S. *et al. Molecular Therapy* (2005), Vol. 12(5):976-84.

Supplemental Figures

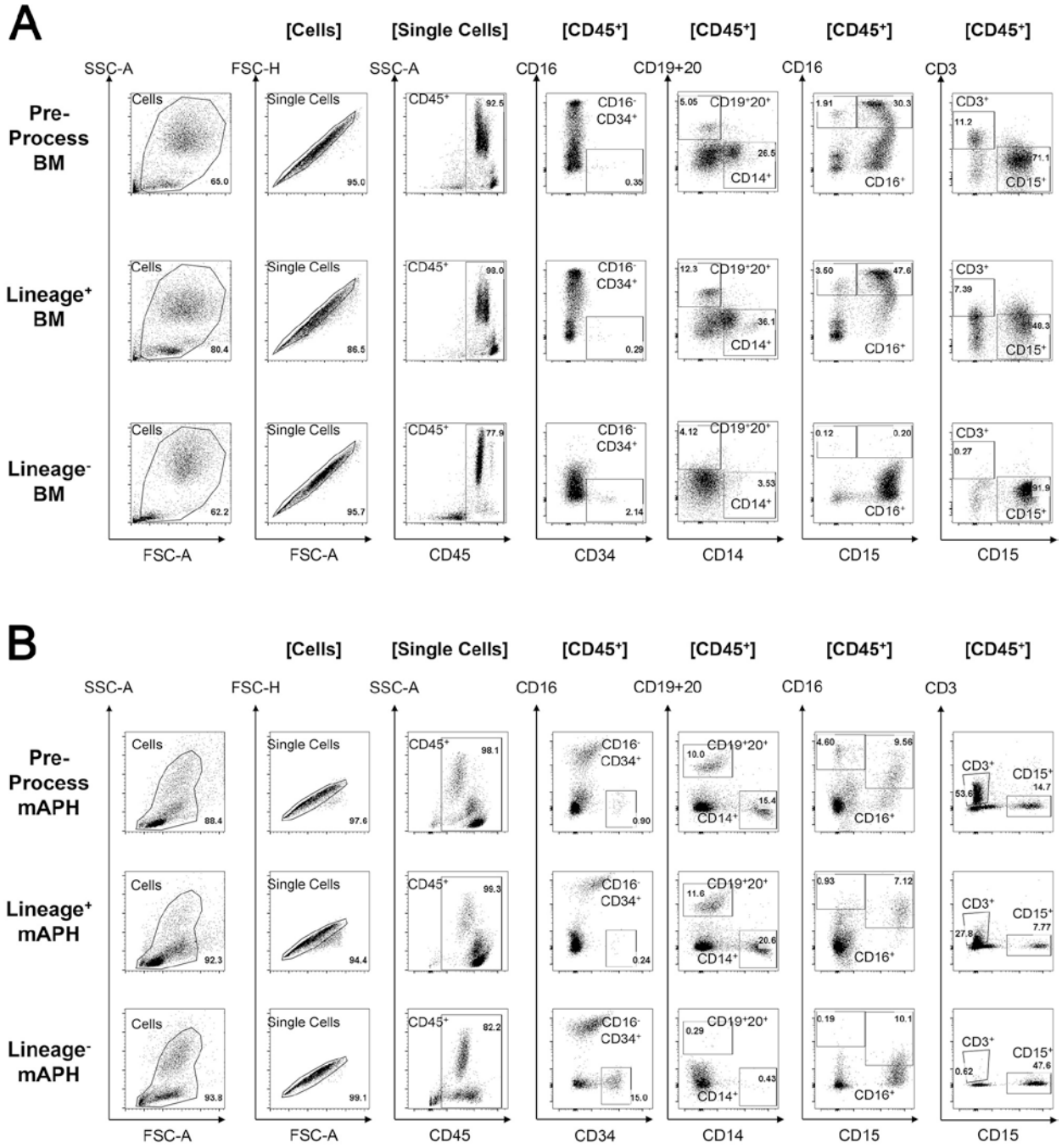

Figure S1. Flow cytometry analysis and gating strategy of BM and mAPH samples during lineage depletion. Figure depicts typical profiles of cell samples collected from initial, lineage depleted and lineage enriched products for both BM (**A**) and mAPH (**B**). Subsets represented within square brackets ([ ]) above each column represent the parent population for the respective gates. All lineage markers (CD3, CD14, CD15, CD16, CD19, CD20, and CD34) are gated within the $CD45^+$ cell population. The $CD45^+$ cell population in turn is gated within the single cell population identified using forward and side scatter parameters.

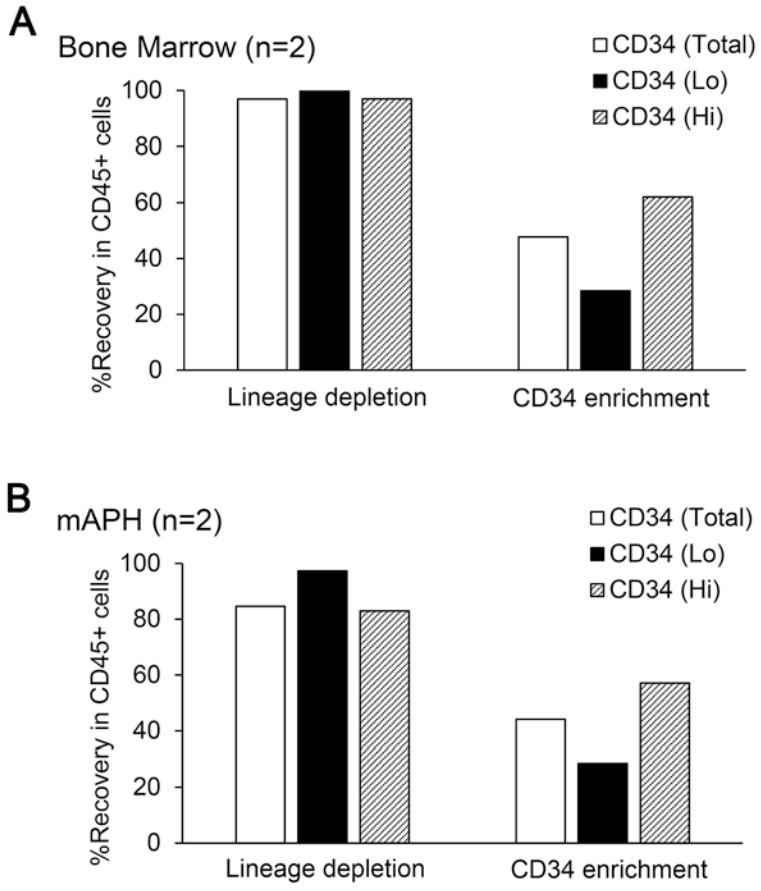

Figure S2. Lineage depletion preserves $CD34^{Hi}$ cells and increases the proportion of $CD34^{Lo}$ cells relative to direct CD34 enrichment in healthy donors. We compared our lineage depletion protocol with the clinical standard protocol for direct CD34 enrichment within both bone marrow (**A**) and mobilized apheresis products (mAPH) (**B**) from healthy donors. Initial cell products were split to directly compare recovery of $CD34^{Hi}$ and $CD34^{Lo}$ cells within the same donor cell source.

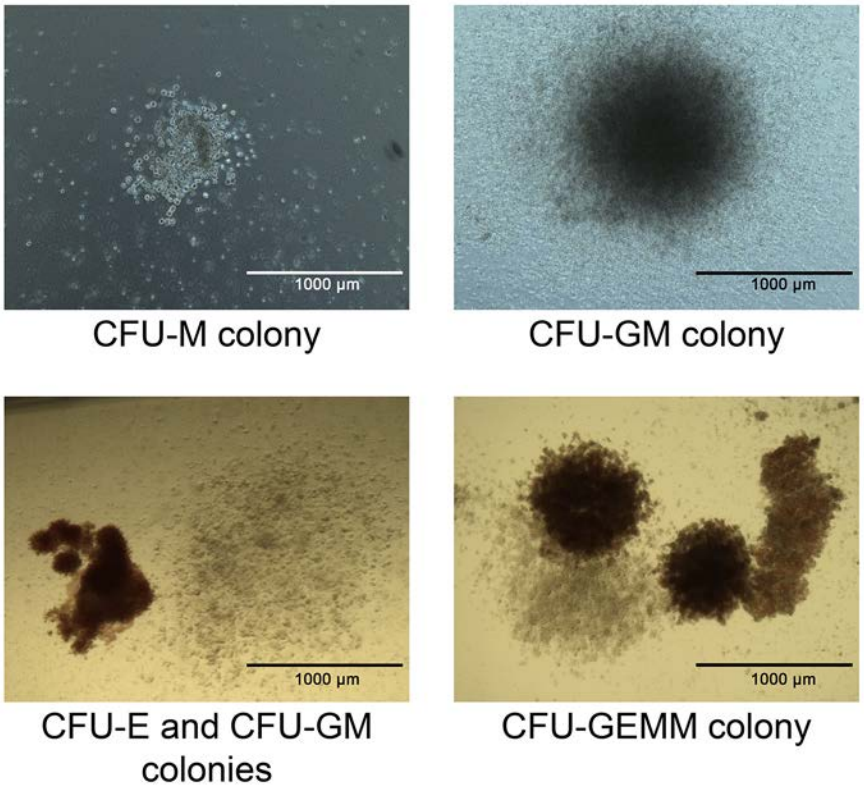

Figure S3. Representative images of colony morphologies. Lineage depleted and transduced cells from BM or mAPH products were seeded in standard CFC assays. Hematopoietic colonies that arose in the assay were viewed under 4X magnification, wide field and scored for morphology as CFU-macrophage (M) colonies with larger cell appearance and loose association, CFU-granulocyte macrophage (GM) with diffuse appearance, CFU-erythroid (E) with hemoglobinization and tight association and CFU-granulocyte erythrocyte macrophage megakaryocyte (GEMM) with mixed appearance and partial hemoglobinization.

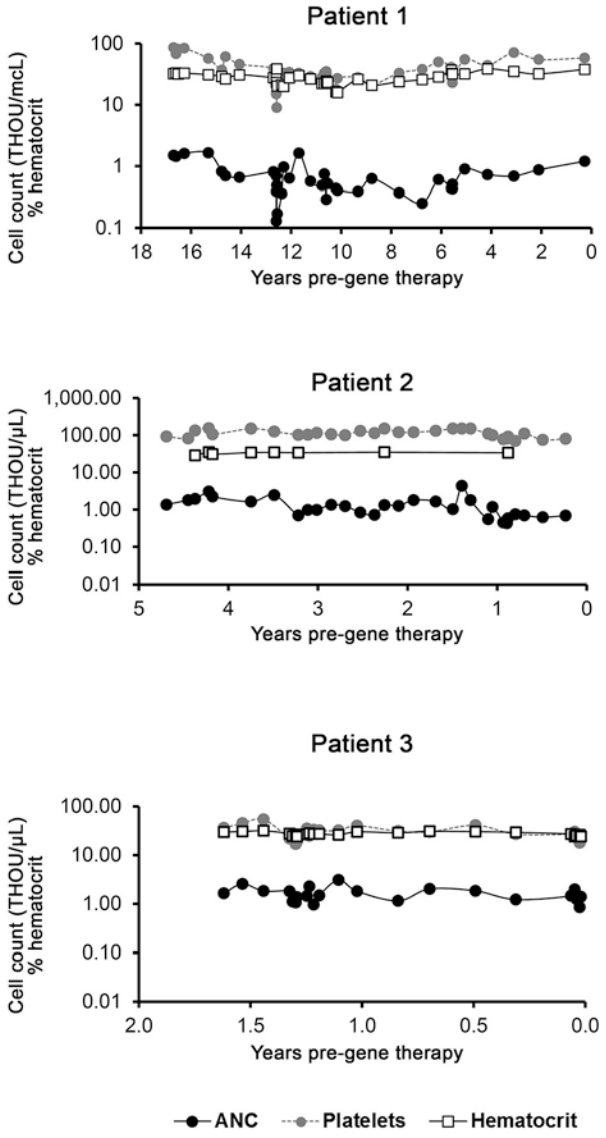

Figure S4. Blood cell counts for three FA-A patients prior to HSC gene therapy. Graphs depict peripheral blood cell counts in neutrophils (solid black circle), and platelets (solid gray circles) and percent hematocrit (open squares) over time prior to HSC gene therapy intervention.

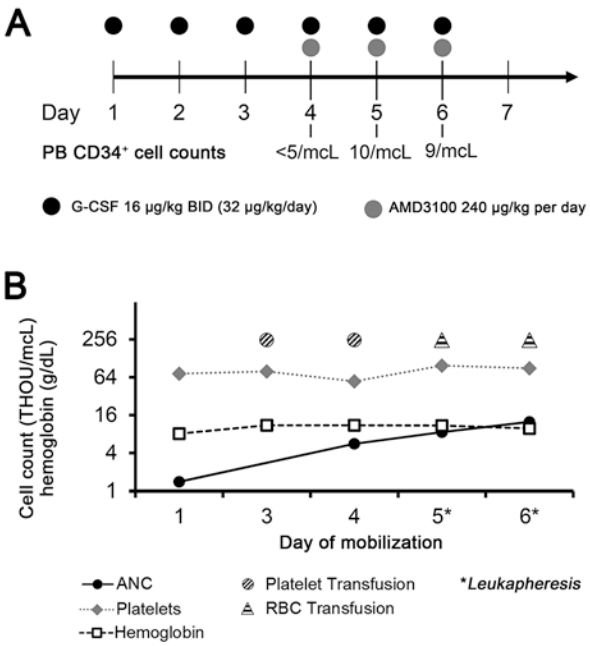

Figure S5. Mobilization and Leukapheresis protocol for FA-A Patient 3. Successful mobilization of CD34$^+$ cells was achieved with combination G-CSF (16µg/kg BID) and plerixafor (240 µg/kg). **Panel A** outlines the mobilization drug regimen and the peripheral blood CD34$^+$ cell counts evaluated daily by flow cytometry from day 4 of mobilization. Peripheral blood counts for absolute neutrophils (ANC), platelets and hemoglobin recorded daily through mobilization is depicted in **Panel B**.

Supplemental Materials and Methods

Processing of BM and mAPH products

All processing was conducted in a Class II, Type A2 biological safety cabinet or on a CliniMACS Prodigy™ device (Miltenyi Biotec GmbH, Auburn, CA). Prior to device loading, 1 L of PlasmaLyte A (Baxter Healthcare, Deerfield, IL) was supplemented with N-acetylcysteine (NAC) to a final concentration of 1 mM and mixed well. A 3 L bag of CliniMACS (PBS/ EDTA) buffer (Miltenyi Biotec) was supplemented with 60 mL of 25% human serum albumin (HSA; Baxter Healthcare) and NAC to a final concentration of 1 mM and mixed well. For BM products, a 600 mL bag of 6% hetastarch (HES) in 0.9% NaCl (Hospira Inc., Lake Forest, IL), was supplemented with NAC to a final concentration of 1 mM. Immunomagnetic bead reagents were concentrated on the device using a custom program and a TS 510 tubing set (Miltenyi Biotec GmbH), to a total volume of 14 mL or less and then transferred into a 60 mL syringe containing 3 mL of 10% IVIg (GAMMAGARD; Baxter Healthcare) and ~20mL of air, and stored at 2-8°C until needed. For device loading, a TS 510 tubing set was installed.

BM products were collected by standard clinical procedures. Upon receipt, complete blood counts were obtained and BM was diluted in PlasmaLyte A + NAC to obtain a hematocrit value of ≤25% prior to loading into the tubing set Application Bag. To facilitate HES sedimentation, 400 mL funneled cryobags (OriGen Biomedical, Austin, TX) were sterile-welded onto the tubing set prior to installation. When more than a single bag was used, bags were joined by sterile-welding onto a color-coded, trifurcated, standard bore extension set (Smiths Medical, Saint Paul, MN) prior to attachment to the tubing set.

Large-volume mAPH products were collected by standard clinical procedures on a COBE Spectra or Optia apheresis system (Terumo BCT, Lakewood, CO). For mAPH products, first day collections were sampled for complete blood cell counts and diluted in autologous plasma to a concentration of $\leq 200 \times 10^6$ cells/mL for overnight storage at 2–8°C as needed. Upon receipt of second day collection, products were pooled and complete blood cell counts were obtained. During TS 510 tubing set preparation, the provided target and non-target cell bags were removed and replaced with 3 L transfer packs (Fenwal, Lake Zurich, IL) to accommodate larger volumes of cell product. mAPH products were loaded onto the tubing set via the Application Bag.

Device Set-up and operation:

For all processes, valve configurations were as follows:

Valve 1: CliniMACS (PBS/EDTA) Buffer + NAC + HSA

Valve 2: Empty

Valve 3: Concentrated bead reagent + IVIg

Valve 4: Funnel cyrobag(s) (BM products ONLY)

Valve 5: HES + NAC (BM products ONLY)

Valve 6: Empty

Valve 7: Empty

Valve 8: Application Bag (standard with tubing set)

Valve 9: Pre-separation column

Valve 10: Priming bag

Valves 11-16 and 21: Directional valves

Valves 17-18: Centricult unit

Valve 19: Waste bag

Valve 20: Non-target cell bag

Valve 22: Target cell bag

Valve 23: Intermediate storage bag

Valve 24: Empty

Once the tubing set is loaded and the program is launched, the user chooses whether the initial product is BM or mAPH. If BM is selected, there are five user inputs required for processing to begin: (1) the volume of diluted BM product loaded (+10 mL for complete loading), (2) the measured post-dilution hematocrit value, (3) the number of stages required for loading (total diluted BM volume divided by 300 mL per stage and rounded up to the nearest whole number), (4) the estimated frequency of cells expressing a lineage marker targeted for depletion ("bead-bound") cells, and (5) the number of cells in millions loaded into the application bag. Following entry, the device prompts the user to perform several checks of the clamps and connections to ensure device loading was accurate and then the automated process begins. Once started, there is no user interface required until HES sedimentation has completed. The user can select to continue the process or permit longer sedimentation if required. Once sedimentation is complete, the user is prompted to begin RBC removal and enters volumes of RBC which are transferred from the bottom of the funnel cryobags (one at a time if more than one bag is used), until the user is satisfied with the volume of RBCs debulked. Once the user indicates RBS removal is sufficient, the program continues automatically to begin lineage depletion. Once lineage depletion is completed, the target cell product bag contains the desired lineage$^-$ cell fraction for transduction and is removed from the device by heat sealing the tubing set.

If mAPH is selected, there are two user inputs required for processing to begin: (1) the number of cells in millions loaded into the application bag, and (2) the estimated frequency of cells expressing a lineage marker targeted for depletion ("bead-bound") cells. Following entry, the device prompts the user to perform several checks of the clamps and connections to ensure device loading was accurate, and then the automated process begins. The user is prompted to

mix the Application Bag during two rinse steps at the start of the automated program. Following this interface, there is no other user interface required until the lineage depletion process is completed. Once lineage depletion is completed, the target cell product bag contains the desired lineage$^-$ cell fraction for transduction and is removed from the device by heat sealing the tubing set.

For Patient 1 alone, the RBC-debulked BM product was removed from the CliniMACS Prodigy device and further processed by labeling with CliniMACS anti-CD34 microbeads (Miltenyi Biotech GmbH) and then purified for CD34$^+$ cells using immunomagnetic positive selection on the CliniMACS Select Plus instrument (Miltenyi Biotech GmbH) per manufacturer's recommendations. No further enrichment strategy following RBC debulking was applied for the BM product from Patient 2. mAPH product collected on the first day from Patient 3 was held overnight at 4°C on an orbital shaker in the presence of 1mM concentration of NAC and pooled with the second collection on the following day for RBC debulking and lineage depletion on the CliniMACS Prodigy device.

Colony-forming assay methods

Colony-forming assays were plated into methylcellulose containing 2% heat inactivated fetal bovine serum (ThermoFisher, Waltham, MA) and 100 ng/mL each of the following growth factors: rh-interleukin3 (IL3), rhIL-6, rh-TPO, rh-erythropoietin (EPO), rhSCF, rh-granulocyte colony stimulation factor (G-CSF) and rh-granulocyte monocyte colony stimulating factor (GM-CSF) [IL-3, IL-6 and TPO were purchased from PeproTech; EPO, SCF, and G-CSF were purchased from Amgen; and GM-CSF was purchased from Miltenyi Biotech GmbH]. Cells were cultured for ~12–14 days until robust colony development was achieved and evaluated by colony count and phenotype as granulocyte-monocyte (GM) colonies distinguished as dense white colonies with a halo appearance, erythroid (E) colonies distinguished by a distinct red coloration due to

hemoglobinization, or mixed (GEMM) colonies distinguished by a mixture of hemoglobinized and white colonies. Individual colonies were also picked into QuickExtract DNA Extraction Solution 1.0 (Epicenter, Lucigen Corporation, Middleton, WI). Genomic DNA was isolated by heating at 65°C for 20 minutes and then at 99°C for 10 minutes to quench the enzymatic reaction. DNA was analyzed by PCR using primers specific to LV backbone (forward, 5'- AGAGATGGGTGCGAGAGCGTCA; reverse, 5'- TGCCTTGGTGGGTGCTACTCCTAA). The reaction mix was subjected to PCR conditions of 94°C for 2 minutes for initial denaturation followed by 38 cycles of 94°C for 1 minute (denaturation), 65°C for 0.5 minutes (annealing) and 72°C (extension), and finally 72°C for 10 minutes (final extension). DNA samples were also run in a separate reaction for β-actin as an internal DNA control (forward, 5'- TCCTGTGGCATCGACGAAACT; reverse, 5'- GAAGCATTTGCGGTGGACGAT). This reaction mix was subjected to PCR conditions of 95°C for 2 minutes for initial denaturation, followed by 37 cycles of 95°C for 1 minute (denaturation), 62°C for 1 minute (annealing) and 72°C (extension), and finally 72°C for 10 minutes (final extension). Colonies containing expected bands for both LV and β-actin were scored positive for transduction. Reactions which did not yield β-actin products were considered non-evaluable.

Flow-based cell sorting of high and low expressing CD34^{+} cells

Leukocytes from mAPH products were stained with an anti-human CD34 antibody (clone 563; BD Biosciences) per manufacturer's recommendations and sorted for high (CD34^{Hi}, MFI: 15,886) and low (CD34^{Lo}, MFI: 928.5) expression on the FACS Aria II. Dead cells and debris were excluded based on forward scatter/ side scatter gating.