

Supplemental Materials and Methods

Lentivirus-mediated transduction of human cord blood (hCB) CD34⁺ cells. Briefly, hCB CD34⁺ cells were pre-stimulated in QBSF-60 media (Quality Biological, Inc, Gaithersburg, MD) with a cytokine cocktail including 100 ng/mL recombinant human stem cells factor (rhSCF), 100 ng/mL recombinant human thrombopoietin (rhTPO), 100 ng/mL recombinant human interleukin-6 (rhIL6), and 20 ng/mL rhIL3 for 24 hours and then transduced in sterile 6-well plates (Falcon-Becton Dickinson) coated with 20 $\mu\text{g}/\text{cm}^2$ Retro Nectin (PanVera Corp., Madison, WI) with 2bF8 LV. After transduction, cells were harvested, washed, and resuspended in QBSF-60 media for transplantation.

Human cell chimerism analysis. Human platelets were identified by a mouse anti-human GPIb monoclonal antibody, AP1, which was produced by our laboratory and directly conjugated with AlexaFluor 647 (Invitrogen, Carlsbad, CA). Mouse platelets were identified by anti-mouse CD41 mAb directly conjugated with phycoerythrin (PE) (Santa Cruz Biotechnology Inc, CA). We also analyzed human engraftment in transplanted recipients by staining peripheral blood leukocytes (white blood cells) using mouse anti-human CD45-PE monoclonal antibody (BD Biosciences). Mouse leukocytes were identified by rat anti-mouse CD45-FITC monoclonal antibody (eBioscience, San Diego, CA). For platelet staining, 3 μL of whole blood was incubated in a total volume of 50 μL of PBS buffer with 2% bovine serum albumin (BSA), 8 $\mu\text{g}/\text{mL}$ of anti-mouse CD41-PE and 5 $\mu\text{g}/\text{mL}$ of AP1-Alexa 647 for 30 minutes at room temperature. For leukocyte staining, 50 μL of blood was collected and red cells were lysed using Red Blood Cell Lysing Buffer (Sigma, St. Louis, MO). Cells were stained with anti-human CD45-PE (1:10) and anti-mouse CD45-FITC (1:100) in a total volume of 50 μL of PBS buffer with 2% BSA for 30

minutes at room temperature. All samples were run on BD LSRII Flow Cytometry (BD Biosciences, Sparks, MD) and analyzed using FlowJo Version 9.3.2 software. Samples from untransplanted mice and humans were used as controls. Appropriate isotype IgG controls were used in parallel for each staining.

PCR and quantitative real-time PCR (qPCR) analysis. Briefly, genomic DNA was purified from peripheral leukocytes using QIAamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA) and a 650 bp fragment from the 2bF8 cassette was amplified using PCR GoTaq Master Mix (Promega, Madison, WI). Since peripheral blood cell-derived DNA from hCBT recipients contained both mouse and human genomic DNA, two internal controls were used for PCR amplification. One was a 233 bp fragment from wild type mouse FVIII using a set of primers as previously reported.¹ The other was a 100 bp fragment from the human GAPDH (hGAPDH) gene, which was amplified using forward primer 5'-CCC CAC ACA CAT GCA CTT ACC-3' and reverse primer 5'-CCT ACT CCC AGG GCT TTG ATT-3'. NSG mice and normal human individuals were used as controls. DNA from a known 2bF8 transgenic mouse² served as a 2bF8 positive control and water was used as a negative control.

The average copy number of 2bF8 proviral DNA per cell in recipients was determined by qPCR as described in our previous report.³ Briefly, peripheral blood cell-derived genomic DNA was analyzed for quantification of the 2bF8 expression cassette sequence, with normalization to the hGAPDH gene using iQ Supermix (BioRad, Hercules, CA, USA). The sequences of primers used for hGAPDH were the same as those used for PCR as described above. The sequence of the

probe used for hGAPDH was 5'-(VIC) AAA GAG CTA GGA AGG ACA GGC AAC TTG GC (TAMRA)-3'. DNA from NSG mice and humans were used as controls.

Immunogold electron microscopy (EM). Briefly, platelet pellets were fixed and embedded in Lowicryl K4M resin. Ultrathin sections (60 nm) were collected on formvar/carbon coated copper grids. Sections were then incubated with mouse anti-human FVIII monoclonal antibody 103.3, and rabbit anti-human VWF polyclonal antibody (Dako), which recognizes both human and mouse VWF, and then probed with goat anti-mouse (6 nm) and goat anti-rabbit (10 nm) colloidal gold probes. The sections were observed using a JEM2100 transmission electron microscope (Japanese Electron Optics Ltd) operating at 80kv. Platelets from NSG mice and humans were used as controls. Isotype IgG controls were used in parallel for each staining.

2bF8 LV integration site analysis. Similar to the LAM-PCR strategy used in our previous report,¹ single stranded linear amplification was performed using a single biotinylated primer positioned near the end of the 2bF8 LV 3' LTR to allow extension into the flanking genomic DNA. The linear DNA products were captured by streptavidin-coated magnetic beads. Greater than 150 bp ssLinear PCR products were purified using the NucleoSpin Extract II Kit (Clontech, Mountain View). A 5'-phosphorylated and 3'-modified (dideoxycytidine, ddC) single strand linker (ssLinker) was ligated to bead-bound ssLinear PCR products using T4 RNA Ligase 1 (New England BioLabs Inc, Ipswich, MA). Two rounds of nested PCR using LTR and linker-specific primers were performed to amplify the DNA that flanks the 5' end of the lentiviral insertion site. The products were then cloned into pCR2.1-TOPO (Invitrogen) and sequenced to determine chromosomal vector integration sites.

Reference List

1. Kuether EL, Schroeder JA, Fahs SA et al. Lentivirus-mediated platelet gene therapy of murine hemophilia A with pre-existing anti-factor VIII immunity. *J.Thromb.Haemost.* 2012;10:1570-1580.
2. Shi Q, Wilcox DA, Fahs SA et al. Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies. *J.Clin.Invest* 2006;116:1974-1982.
3. Shi Q, Fahs SA, Wilcox DA et al. Syngeneic transplantation of hematopoietic stem cells that are genetically modified to express factor VIII in platelets restores hemostasis to hemophilia A mice with preexisting FVIII immunity. *Blood* 2008;112:2713-2721.