

EXPERIMENTAL PROCEDURES

Human Pluripotent stem cell culture

The BJ foreskin fibroblast-derived induced pluripotent stem cell line (1), the Pearson syndrome patient-derived induced pluripotent stem cell line CH2N2 (2), and the H1 and H9 human embryonic stem cell lines (Wicell Research Institute) were maintained on Matrigel matrix (Corning). Cells were cultured in mTeSR™1 (Stemcell Technologies) with 0.5% penicillin/streptomycin and 25ng/ml human bFGF (Peprotech). Cells were maintained at 37°C, 5% O₂, 5% CO₂ and 90% N₂ conditions, with daily media changes.

Hematopoietic differentiation of human pluripotent stem cells

The BJ hiPSC line (1) was used for all hematopoietic differentiation experiments except where specifically stated otherwise. Human pluripotent stem cells were harvested using TrypLE Select Enzyme without phenol red (Thermo Fisher Scientific) and dissociated into single cells by pipetting. 6,000 cells were re-suspended in differentiation media composed of 75% IMDM, 25% DMEM/F12, 0.5x N-2 supplement (Thermo Fisher Scientific), 0.5x B-27 supplement minus Vitamin A (Thermo Fisher Scientific), 28mM ascorbic acid (Sigma), 0.46mM monothioglycerol (Sigma), 90mM folic acid (Sigma), 0.05% AlbuMAX I (Thermo Fisher Scientific) and 0.5% penicillin/streptomycin. The suspension cells were pelleted into Poly 2-hydroxyethyl methacrylate (Sigma) -coated 96 well plates by centrifugation at 1,500rpm for 1 minute to generate embryoid bodies. For human pluripotent stem cells grown on Matrigel matrix, mitomycin C-treated mouse embryonic fibroblasts were supplemented to support embryoid body formation before centrifugation. 10ng/ml BMP-4, 50ng/ml VEGF, 25ng/ml bFGF, 20ng/ml IL-6, 50ng/ml TPO, 100ng/ml Flt3L, 100ng/ml SCF (all from Peprotech), 3 ng/ml Activin (R&D Systems), 500uM 8-bromo-cyclic AMP sodium salt, 10µM Y-27632, 10µM SB431542 (all

from Cayman Chemical), were added at indicated days (Figure. 1A). At day 4, embryoid bodies were dissociated into single cells using TrypLE Select Enzyme without phenol red and plated onto fibronectin (Thermo Fisher Scientific) coated 6 well plates. At day 10, the differentiation media was switched to StemSpan SFEM II (Stem cell Technologies). In one set of experiments, BJ hiPSC were differentiated to HSPC using alternative protocols described by Keller (3) and Kiem (4)

Hematopoietic colony forming assays

Colony forming assays were performed using MethoCult H4435 Enriched (Stem cell Technologies) as per the manufacturer's instructions. At the days indicated, differentiated HSPCs were collected using TrypLE (day4-8), or by gentle pipetting to minimize mixing of adherent cells (d10,12) and indicated numbers of cells were re-suspended in 0.3ml IMDM with 2% FBS. The solution was mixed with 3ml MethoCult H4435 Enriched (Stem cell Technologies) and 1.1ml was dispensed into 35mm dishes. The cells were incubated at 37°C, and 5% CO₂. Quantification and classification of colonies were assessed after 14 days

SDF-1- Transwell Migration Assay

SDF-1 transwell migration assays were performed based on protocols described in Vagima et al (5), with modifications. Human iPS cell-derived HSPCs (hiPS-HSPCs) or human PBMC (sampled by our group from healthy volunteers at University of Massachusetts Medical School or Brigham and Women's Hospital) were suspended at 1×10^6 /ml in RPMI media supplemented with 25mM HEPES, 10% FBS, and 1% penicillin/streptomycin (full RPMI) +/- 50µM CXCR4 antagonist AMD3100. 1.5×10^5 cells were seeded into uncoated 6.5mm transwell chambers with 5.0 µm or 8.0µm pore size (Costar), and transwells were placed into individual wells of a 24-well plate pre-loaded with 600µl full RPMI (control), full RPMI +125ng/ml SDF-1 (SDF-1), or full RPMI +125ng/ml SDF-1 +50µM CXCR4 antagonist

(AMD3100). Equal numbers of cells were also added directly into similar wells without transwells (as counting controls). After incubation at 37°C for 4 hours, transwells were lifted and the bottom surface of the transwell was gently rinsed 3x with media from the bottom well. The number of cells in each well was quantified by adding a known quantity of absolute counting beads (CountBright, Molecular Probes) and acquiring by FACS. Baseline cell transmigration was determined by dividing the calculated number of cells in the bottom chamber of the control (no SDF-1) transwell by the number of cells in the corresponding counting control well. This baseline percentage of transmigrated cells was set as 1. The relative transmigration activity in the SDF-1 and SDF-1+AMD3100 groups were determined in a similar fashion, by dividing the calculated number of transmigrated cells in the bottom chamber by the number of cells in the corresponding containing counting control well, and the percentage transmigration was presented as a proportion of the baseline transmigration for each experiment.

Measurements of Integrin Activation

Freshly harvested day 10 hiPS-HSPCs were divided into 3 treatment groups: No treatment at 4°C (baseline), 4mM MnCl₂ for 30 minutes at 37°C, and 100ng/ml SDF-1 for 75 minutes at 37°C. After completion of treatment, cells were stained at 4°C for CD29 (β1-integrin) using the activation specific monoclonal antibodies HUTS4 (catalog FCMA389F, Millipore) and N29 (catalog MAB2252, Millipore) and the non-activation specific antibody 4B4 (catalog 6603113, Beckman Coulter). Secondary staining was performed using goat anti-mouse IgG-PE (Southern Biotech). Fluorescence intensities were calculated as logarithmic means.

Flow cytometry

Antibodies used for flow cytometry were as follows: HECA452-FITC (catalog 321308, Biolegend), hCD45-APC (catalog 304018, Biolegend), hCD34-PECy7 (catalog 343616, Biolegend), hCD43-PE (catalog 343204, Biolegend), hCD31-Pacific Blue (catalog 303114, Biolegend), hKDR-PerCPy5.5 (catalog 359908, Biolegend), mCD45-PECy7 (catalog 103114, Biolegend), hCD44-PE (catalog 130-095-180, BD Pharmingen), hCXCR4-PE (catalog FAB170P-100, R&D Systems), hCD49d-FITC (catalog IM1404U, Beckman Coulter), unlabeled hCD49e (catalog 555651, BD Pharmingen), followed by goat anti-mouse Ig-FITC (Southern Biotech). Acquisitions were performed using a Cytomics FC 500 MPL (Beckman Coulter) or a FACSCanto II (Becton Dickinson).

Modified-mRNA synthesis

Modified-mRNA was synthesized as described previously (6). Briefly, human fucosyltransferase 3, 6, and 7 (FUT3, FUT6, FUT7) cDNAs were ligated into a vector containing T7 promoter, 5' UTR and 3' UTR. Templates bearing FUT3, 6 or 7 ORF with 5' and 3' UTR were PCR-amplified using HiFi Hotstart (KAPA Biosystems) and were purified using a gel purification kit (Quiagen) for further *in vitro* transcription. 1.6 µg of purified PCR product was used as template for RNA synthesis using the MEGAscript T7 kit (Thermo Fisher Scientific), including 6mM 3'-O-Me-m⁷G(5')ppp(5')G ARCA cap analog (New England Biolabs), 7.5mM adenosine triphosphate, 1.5mM guanosine triphosphate (USB), 7.5mM 5-methylcytidine triphosphate and 7.5mM pseudouridine triphosphate (TriLink Biotechnologies). The mixtures were incubated 3-6 hours at 37°C followed by DNase treatment as the manufacturer's instructions. Modified-mRNA product was purified using MEGAclean spin columns (Thermo Fisher Scientific), and aliquots were kept frozen for future use at -20°C. Nuclear destabilized EGFP modified-mRNA was similarly prepared and used as a control modified-mRNA (1).

Modified-mRNA transfection

Modified-mRNA transfections were carried out with Stemfect (Stemgent) as per manufacturer's instructions. Two tubes were prepared separately with one tube containing 1 µg of modified-mRNA in 60µl of buffer and the other tube containing 2 µl of reagent in 60 µl of buffer, and then the two complexes were mixed together and incubated for 15 minutes at room temperature. The mixture was added to 1×10^6 of d10 human pluripotent stem cell derived-HSPCs in StemSpan SFEM II (Stem cell technologies). Following modified-mRNA transfection, the B18R interferon inhibitor (eBioscience) was added as a media supplement at the concentration of 200 ng/ml.

Western blot analysis

Day 10 hiPS-HSPCs were transfected with *FUT3*, *FUT6*, or *FUT7* modified-mRNAs, or mock treated with Stemfect reagents alone. 24 hours later, cells were harvested, washed, counted, and pelleted. Lysates were prepared in 2%NP40, 150mM NaCl, 50mM Tris-HCl (pH7.4), 20µg/mL PMSF, and 1x protease inhibitor cocktail (Roche), by sonication and repeated vortexing. 1.5x Reducing SDS-sample buffer (Boston BioProducts) was added and the samples were boiled for 10 minutes. The samples were separated on 7.5% Criterion Tris-HSC SDS-PAGE gels and transferred to PVDF membrane. Membranes were blocked with 5% milk and then stained consecutively with mouse E-selectin human-Ig chimera (E-Ig, R&D Systems), rat anti-mouse E-selectin (clone 10E9.6, BD Biosciences), and goat anti-rat IgG conjugated to horseradish peroxidase (HRP, Southern Biotech). All staining and washes were performed in Tris-buffered saline plus 0.1% Tween[®]20 plus 2 mM CaCl₂. Blots were visualized with chemiluminescence using Lumi-Light Western Blotting Substrate (Roche) as per the manufacturer's instructions. To confirm equal loading, membranes were subsequently

stained with rabbit anti-human beta-actin (ProSci) followed by goat anti-rabbit IgG-HRP (SouthernBiotech), and visualized with chemiluminescence as described.

Time course of enforced sLe^x expression following FUT6 modified-mRNA transfection

hiPS-HSPCs were FUT6 modified-mRNA transfected at d10 or left untransfected, and an aliquot was taken for flow cytometry experiments for expression of sLe^x using mAb HECA452. Every 24 hours, cells were harvested and sLe^x expression was examined using HECA452. A time course of cell surface sLe^x expression was obtained by comparing the % HECA452 positivity of each sample.

Parallel plate flow chamber studies

Parallel plate flow experiments were performed using a Bioflux-200 system and 48-well low-shear microfluidic plates (Fluxion Biosciences). Microfluidic chambers were coated with 250 µg/ml fibronectin (BD Biosciences) and seeded with human umbilical vein endothelial cells (HUVECs, Lonza), then cultured in endothelial growth media prepared from the EGM-2 BulletKit (EGM-2 media, Lonza) until confluent monolayers were formed. 4-6 hours prior to assay, HUVECs were activated with 40 ng/ml rhTNF- α (R&D Systems) to induce E-selectin expression. FUT6 modified-mRNA or nuclear destabilized EGFP (control) modified-mRNA transfected hiPS-HSPCs were suspended at 1.0-1.5x10⁶/ml in EGM-2 media and infused initially at a flow rate representing shear stress of 0.5 dynes/cm², increasing at 1-minute intervals to 1, 2, 4, 8, and 16 dynes/cm². The number of rolling cells captured per field was counted for two separate 10-second intervals at each flow rate, and averaged. Cell counts were corrected for starting cell number by visually determining the total number of cells visible per field in the initial infusate at 0.5 dynes/cm², and expressing the captured cell numbers as a proportion of

the starting cell number normalized to the number of cells at 1.0×10^6 cells/ml. Data is thus presented as the number of rolling cells captured per mm^2 , normalized to 1×10^6 cells/ml infusate. To determine the specificity of binding of the fucosylated cells, controls were performed using HUVECs not activated with TNF- α , and also with activated HUVECs blocked with anti-CD62E (E-selectin) antibody (clone 68-5H11, BD Pharmingen). The blocking antibody was suspended at 20 $\mu\text{g/ml}$ in EGM-2 media, infused onto the HUVECs and incubated for 20 minutes prior to washing and infusing the FUT6 modified-mRNA transfected hiPS-HSPCs. Rolling velocities were calculated by measuring the distance travelled in each 10 second interval for all rolling cells, converting to velocities measured in $\mu\text{m/second}$, and reporting the average rolling velocity for all rolling cells at each shear stress.

Vital dye staining, intravenous infusion, and intravital imaging of hiPS-HSPCs in NSG mice

All intravital imaging experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital. NOD-*scid IL2rgtm1Wjl/Sz* (NSG) mice (Jackson Laboratory) were exposed to 2.25 Gy of gamma irradiation. Approximately three hours later, the mice were anesthetized with vaporized isoflurane (3-4% for induction, 1.5% for maintenance). Day 10 hiPS-HSPCs were transfected with FUT6 modified-mRNA or nuclear destabilized EGFP modified mRNA (control), and cultured with B18R. 2 days later, modified-mRNA-transfected cells were harvested. Dead cells were excluded using Annexin V- Biotin antibody (eBioscience) with Streptavidin beads (Miltenyi Biotec). Aliquots of all samples were taken for flow cytometry analysis with HECA452 antibody before dye staining to confirm the increase in sLe^x by FUT6 modified-mRNA. hiPS-HSPCs from each of the two treatments were split in two, suspended at 1×10^6 cells/ml in PBS + 0.1% BSA and

stained with 10 μ M Vybrant[®] DiD or Vybrant[®] Dil dyes (Thermo Fisher Scientific) for 20 minutes at 37°C. Cells were gently washed twice with PBS + 0.1% BSA, and 1:1 reciprocal mixtures (FUT6 modified-mRNA transfected hiPS-HSPCs mixed 1:1 with nuclear destabilized EGFP modified mRNA (control) transfected hiPS-HSPCs) were prepared. Aliquots of the cell mixtures were imaged on a glass slide to confirm a precise starting ratio of DiD and Dil labeled hiPS-HSPCs for each mouse. The homing ratios obtained from the *in vivo* images were adjusted accordingly based on the starting ratio. 0.5 x 10⁶ to 1.5 x 10⁶ cells of each color per mouse were injected into each mouse via a retro-orbital injection two hours before imaging. To control for variability in dye staining and sensitivity, we labeled a second set of cells with the opposite dye combination and injected them into another mouse for comparison. 1.5 hours later, mice were anesthetized again with vaporized isoflurane and a small u-shaped incision was made in the scalp revealing the dorsal skull surface. Mice were positioned in a heated skull stabilization mouse holder for imaging. To label vasculature, AngioSense 750EX (3 nmol/mouse, NEV10011EX, PerkinElmer) was injected into the mouse retro-orbitally immediately before imaging. The mouse holder was mounted to the stage of a home-built video-rate confocal/two-photon laser-scanning microscope (34). A water-immersion 60 \times (NA 1.00, Olympus) or 63 \times (NA 1.15, Zeiss) objective was used to image approximately 2x2 mm of the mouse calvarial bone marrow in equivalent locations across all mice 2 hours after injection of the cells and then again at 24 hours in the same exact locations. Between the imaging sessions, the mice were removed from the microscope, the exposed skull was extensively irrigated with sterile 0.9% saline, the scalp was closed with size 6-0 surgical sutures (1956G, Ethicon), and the mice were returned to their cages. After the second imaging time point at 24 hours, the mice were euthanized.

In vivo image analysis

For imaging, Dil was excited with a 561nm continuous wave laser, and detected through a 593 ± 20 nm (FF01-593-40-25, Semrock) bandpass filter. DiD-labeled cells were excited with a 635 nm continuous wave laser, and detected through a 695 ± 27.5 nm bandpass filter (XF3076 695AF55, Omega Optical). Angiosense 750EX was excited linearly with a Titanium Sapphire laser (Mai Tai HP, Spectra-Physics) tuned to ~ 750 nm, and was detected through a 770 nm longpass filter (HQ770LP, Chroma Technologies). All colors were detected using separate confocal pinholes and photomultiplier tubes (R3896, Hamamatsu Photonics). To distinguish Dil and DiD from autofluorescence in the bone marrow, an autofluorescence channel was also collected by a fourth confocal pinhole and photomultiplier tube combination using a 491 nm continuous wave laser and a 528 ± 19 nm bandpass filter (FF01-528/38-25, Semrock). For quantification, pictures from ~ 30 representative imaging locations across the bone marrow of the calvarium were captured, and the numbers of DiD and Dil stained cells in that regions were counted. All counting was performed manually in a blinded fashion. A minimum diameter of ~ 10 μm cells were counted as events. The numbers of DiD to Dil stained cells counted in each mouse were counted and compared within each mouse pair with the membrane dye combination swapped, with equivalent homing assigned a baseline ratio of 1. Fold change in homing of the FUT6 modified-mRNA-transfected hiPS-HSPCs compared to control modified-mRNA-transfected hiPS-HSPCs was measured for each pair of mice to provide a relative measurement of homing efficacy. 6 mice (3 mouse pairs) representing 3 independent experiments were imaged. Extravasated cells were defined as those that were completely discrete from the Angiosense labeled vessels, and % extravasation was determined as the number of extravasated cells relative to all the dye-stained cells of that color within the same image stacks. All images were

analyzed using raw images and the brightness and contrast were adjusted for display purpose only.

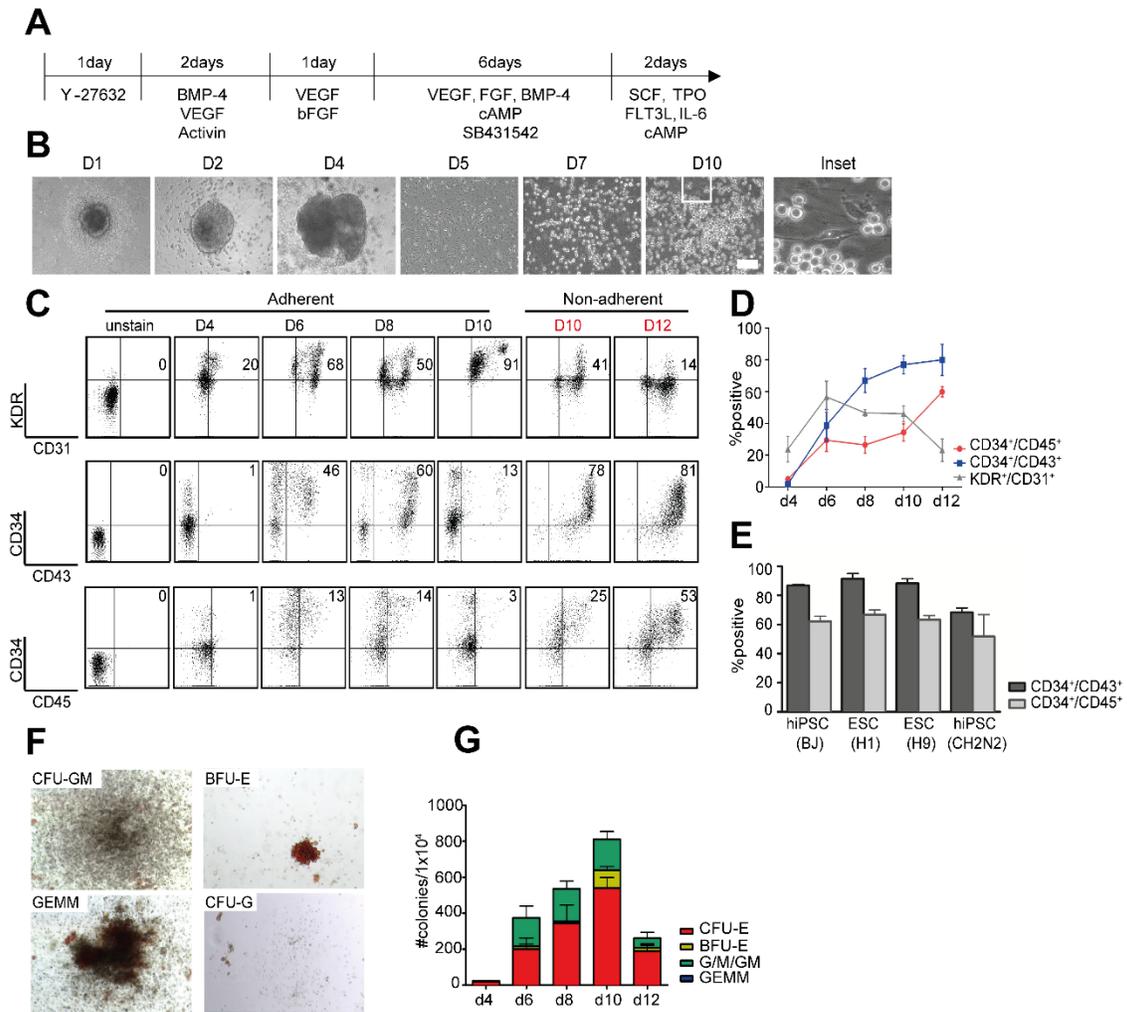
In vivo xenotransplantation assay to measure homing and engraftment of human hematopoietic cells

For measurement of short-term homing, 7.5×10^4 hiPS-HSPCs or 5×10^4 CD34⁺ mobilized peripheral blood cells were stained with CellTraceTM CFSE (Thermo Fisher Scientific) or SNARF-1 (Thermo Fisher Scientific) as described (7), and injected into the tail vein of sub-lethally irradiated (220cGy) immune-compromised NOD-*scid* *IL2rgtm1Wjl/Sz* (NSG) mice (Jackson Laboratory). 16 hours post transplantation, bone marrow was harvested from both femurs and analyzed by FACS for the presence of homed CFSE or SNARF positive cells. Counting beads (Thermo Fisher Scientific) were added to bone marrow samples to enable calculation of absolute homing efficiencies, with the cells collected from 2 femurs presumed to represent 13.4% of the total bone marrow of the mouse (8).

To measure hematopoietic reconstitution, 2×10^7 HSPCs or 1×10^5 CD34 positive cord blood cells were injected into the tail vein of sub-lethally irradiated NSG mice. At various time points, peripheral blood was assessed by flow cytometry for human chimerism by staining with antibodies specific for human CD45 (clone H130, Biolegend) and mouse CD45 (clone 30-F11, Biolegend). Data was analyzed using FlowJo software (Tree Star).

All animal use was in accordance with the guidelines of the Animal Care and Use Committee of the University of Massachusetts Medical School and The Jackson Laboratory and conformed to the recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Supplemental Figure 1

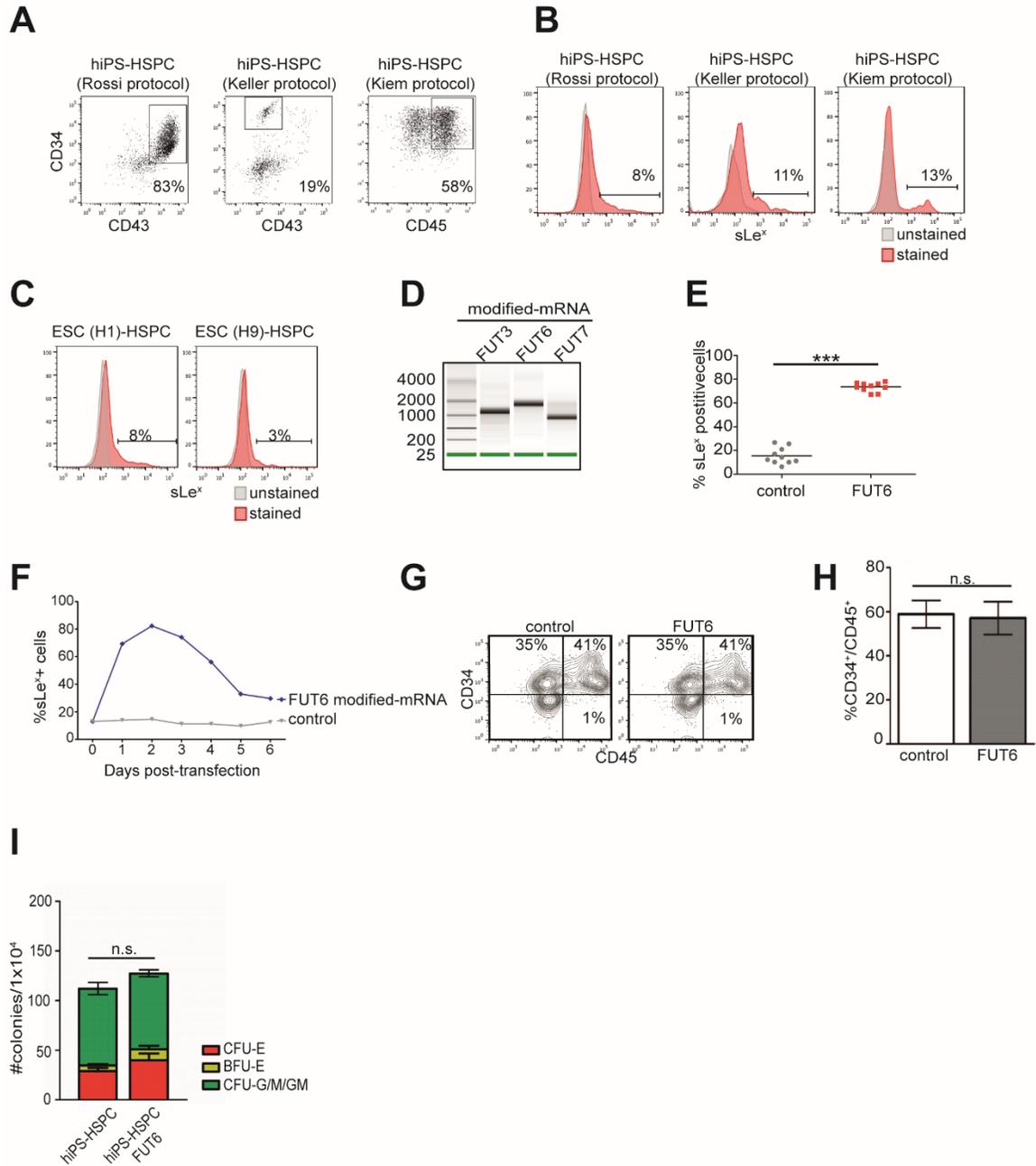


Supplemental Figure 1. Optimized hematopoietic differentiation protocol generates HSPCs from human PSCs with high efficiency

(A) Schematic of hematopoietic differentiation protocol. (B) Microscopic images showing morphologies of differentiating cells from BJ hiPS cells. At day 10, two cell populations exist- adherent flat cells and non-adherent round cells. Scale bar= 200 μ m. (C) Expression of hematopoietic markers on HSPCs, measured by flow cytometry. Red denotes non-adherent round cells. (D) Expression of hematopoietic (CD34, CD43, CD45) or endothelial (KDR, CD31) markers from day 4 to day 12 of differentiation of BJ hiPSC cells. N=3. (E) Hematopoietic differentiation of multiple human pluripotent stem cell lines. H1 and H9: human embryonic stem cell lines, CH2N2: Pearson syndrome iPSC

cells. N=3. (F) Microscopic images showing different colony types from BJ hiPS-HSPCs in methylcellulose assay. (G) Colony-forming activity of BJ hiPS-HSPCs. N=3.

Supplemental Figure 2



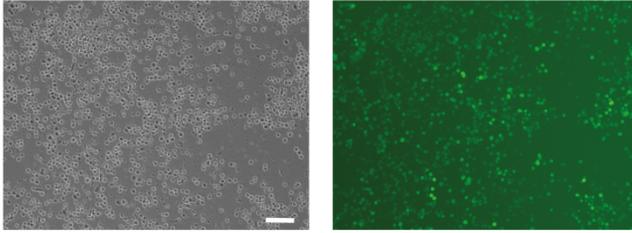
Supplemental Figure 2. E-selectin ligand deficiency of hiPS-HSPCs can be corrected via FUT6 modified-mRNA mediated glycoengineering.

(A) Gating on putative HSPCs to detect sLe^x expression. hiPS-HSPCs were generated from three different protocols and analyzed by flow cytometry. For our protocol, we pre-

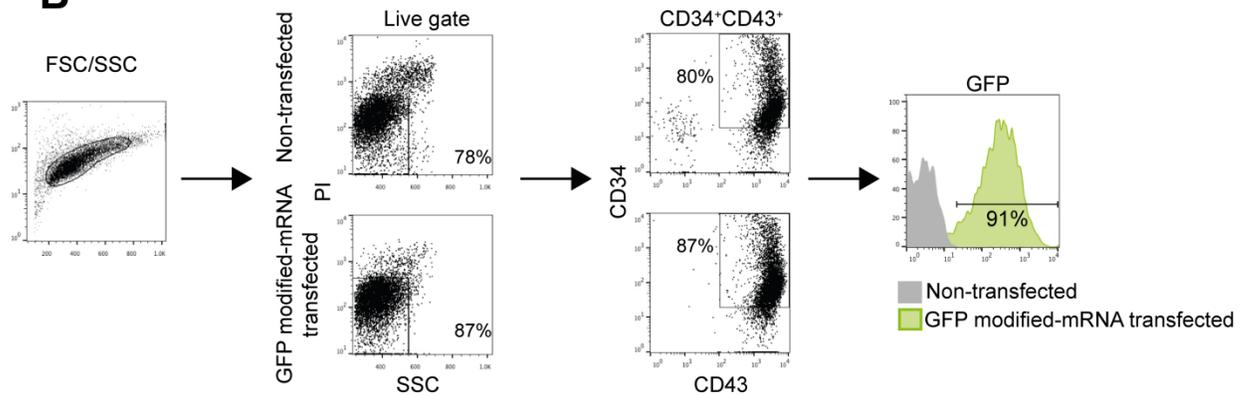
gated on CD34 and CD43 double positive cells, on CD34 positive cells for Keller protocol, and on CD34 and CD45 positive cells for Kiem protocol. (B) sLe^x expression level in hiPS-HSPCs generated from three different differentiation protocols. (C) sLe^x expression level in HSPCs generated from two different human ESC lines. (D) Bioanalyzer analysis of synthesized modified-mRNA encoding $\alpha(1,3)$ fucosyltransferases 3, 6, and 7 (FUT3, 6, 7). (E) Robust and consistent increase in sLe^x expression at 48 hours post-transfection by FUT6 modified-mRNA on hiPS-HSPCs. N=10 independent experiments. *** = P < 0.001. (F) Time course of sLe^x expression on hiPS-HSPCs transfected with FUT6 modified-mRNA. (G) Representative FACS plot and (H) graph showing CD34 and CD45 hematopoietic marker expression of hiPS-HSPCs transfected with control modified-mRNA or FUT6 modified-mRNA. N=3. (I) Colony-forming activity of control or FUT6-modified-mRNA transfected hiPS-HSPC. N=3. n.s.=not significant. Error bars in panels H and I represent SEM. P-values calculated by student's t-test.

Supplemental Figure 3

A



B

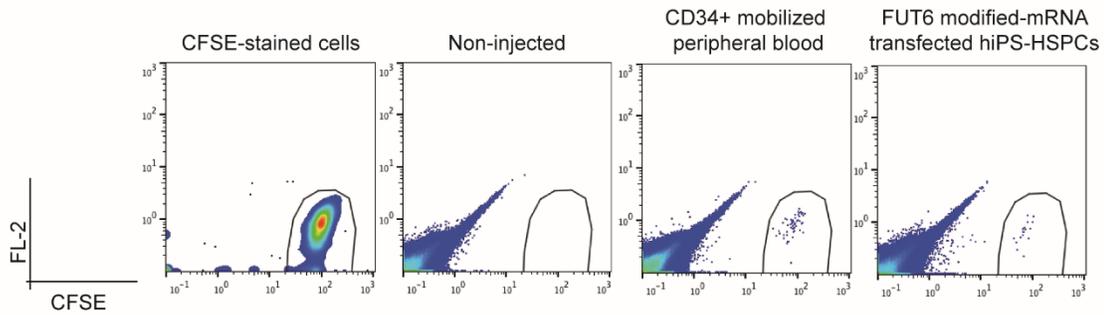


Supplemental Figure 3. High transfection efficiency of modified-mRNA in hiPS-HSPCs

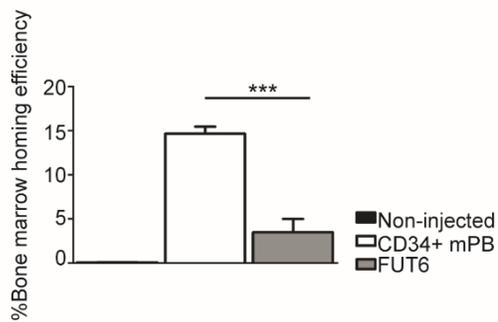
(A) Microscopic images showing transfection efficiency. Day 10 hiPS-HSPCs were transfected with GFP modified-mRNA and the cells were imaged using phase contrast microscope (left panel) and fluorescence microscope (right panel) at two days later. Scale bar= 200 μ m. (B) Cell viability and modified-mRNA transfectability in hiPS-HSPCs measured by flow cytometry. Day 10 hiPS-HSPCs were transfected with GFP modified-mRNA, stained with Propidium Iodide to detect live cells, and subsequent CD34⁺CD43⁺ cells were analyzed for GFP expression at two days later.

Supplemental Figure 4

A



B



Supplemental Figure 4. Short-term homing of hiPS-HSPCs. (A) Representative FACS plots for short-term homing. D12 FUT6 modified-mRNA transfected-hiPS-HSPCs or CD34⁺ mobilized peripheral blood cells were stained with CFSE before injection, and the staining intensity was confirmed. 16 hours after injection into NSG mice, bone marrow was harvested and analyzed by FACS to quantify homing efficiency. (B) CD34⁺ mobilized peripheral blood cells (CD34⁺ mPB) show higher 16-hour BM homing efficiency compared to FUT6 modified-mRNA transfected hiPS-HSPCs (FUT6). N=4. *** = P < 0.001. Error bars represent SEM. P-values calculated by student's t-test.

References for Experimental Procedures

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