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## Supplemental Information

# CXCL12/CXCR4 Signaling Enhances Human PSC-Derived Hematopoietic Progenitor Function and Overcomes Early *In Vivo* Transplantation Failure

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## INVENTORY OF SUPPLEMENTAL ITEMS

## SUPPLEMENTAL FIGURES AND LEGENDS

Figure S1, related to Figure 1.

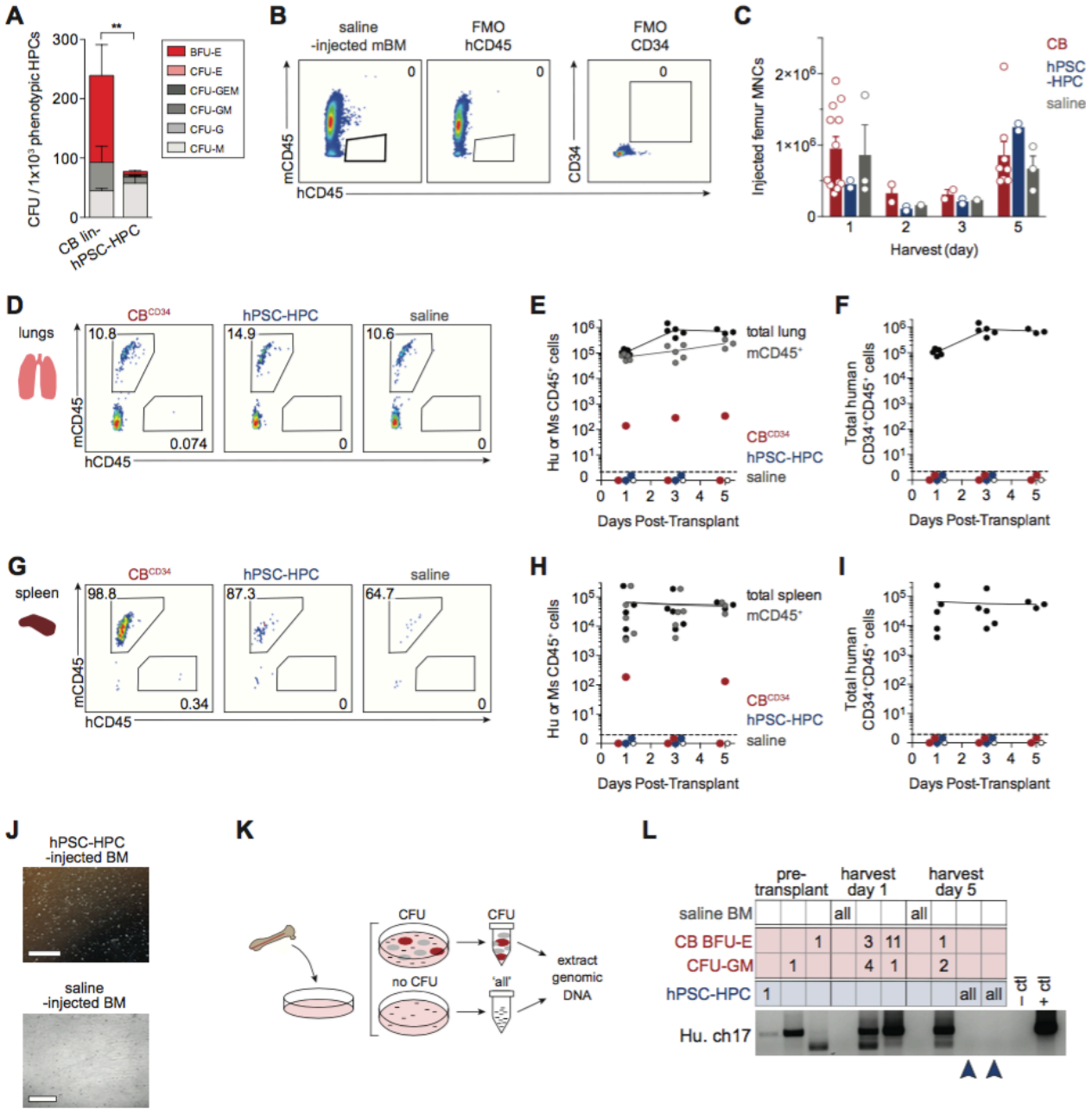

**Figure S1, related to Figure 1. Expanded transplantation kinetics**
(A) Total CFU per 1000 phenotypic ($CD34^+CD45^+$) progenitors. Data points represent *n=6* independently assayed wells, pooled from two independently performed experiments, tested on Day 0. Burst-forming unit-erythroid (BFU-E), colony forming unit (CFU)-erythroid (E), -granulocyte (G), -monocyte (M), -granulocyte/monocyte (GM), or -mixed lineage (GEM). Unpaired T Test, $p<0.01$**. Data are represented as mean ± SEM. (B) Flow cytometry of saline-injected BM harvested on day 5, and fluorescence minus one (FMO) controls for hCD45 and CD34 using pooled BM from CB-injected femurs harvested on day 5. Paired CB and hPSC-HPC flow plots shown in Figure 1E. (C) Total BM mononuclear cell (MNC) counts of *n* transplanted mice (precise *n* values indicated in Figure 1D), pooled from three independently performed experiments with six harvest analyses. Two-way ANOVA, no statistical differences. Data are represented as mean ± SEM. (D) Flow cytometry of lung tissue harvested on day 3. (E-F) Total human and mouse $CD45^+$ cells (E), and human HPCs ($CD34^+hCD45^+mCD45^-$cells; F), per lung sample. Data points represent *n* transplanted mice. Total lung MNCs in black. (G) Flow cytometry of mouse spleen tissue harvested on day 3. (H-I) Total human and mouse $CD45^+$ cells (H), and $CD34^+hCD45^+mCD45^-$ cells (I) per spleen. Data points represent *n* transplanted mice. Total spleen MNCs in black. (J) No CFU were observed from hPSC-HPC or saline-injected BM, at any harvest. (K) BM MNCs were seeded into MethoCult for CFU analysis. CFU were picked by micropipette. If no CFU were observed, the entire well was collected. Genomic DNA from each sample was extracted separately. (L) CFU were analyzed for human sequences by conventional PCR.

Figure S2, related to Figure 3

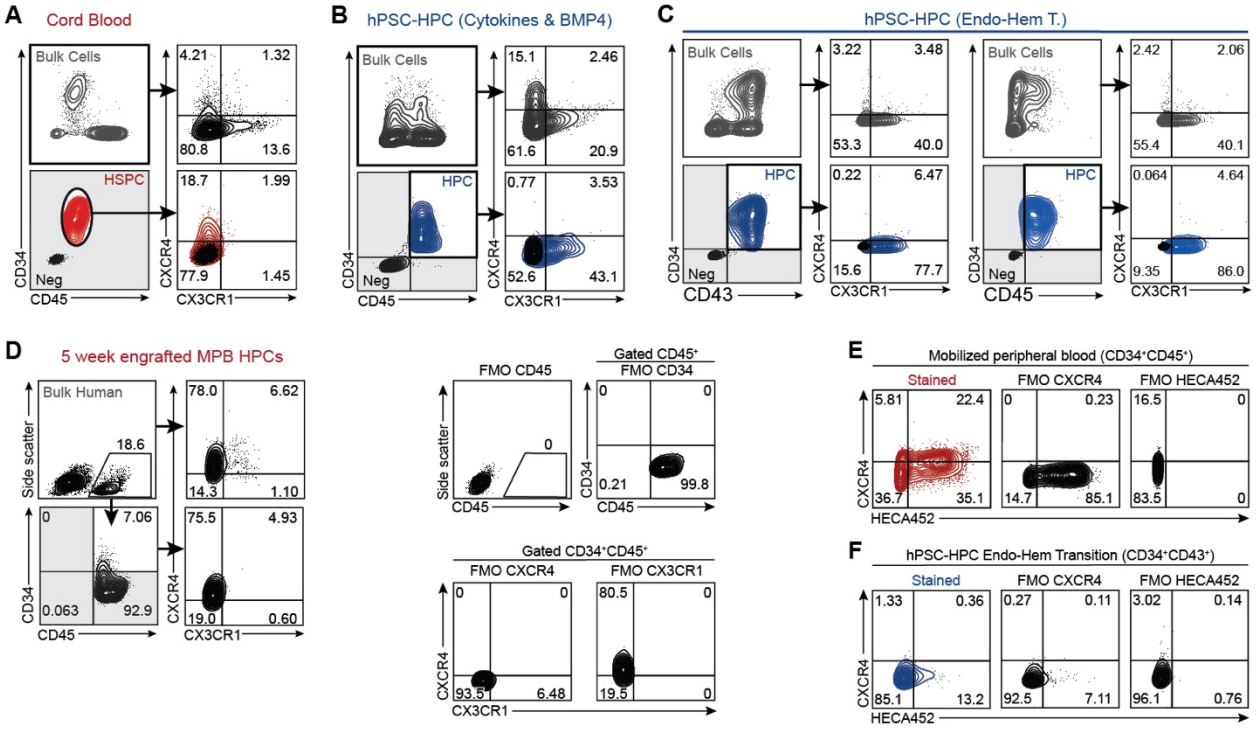


**Figure S2, related to Figure 3. Additional supporting immunophenotyping of somatic and hPSC-derived HPCs** (A-B) Flow cytometry of CXCR4 and CX3CR1 on bulk hematopoietic MNCs (grey) and of CB HPCs (red; A), Cytokines & BMP4-derived HPCs (blue; B), or EHT in hypoxia-derived HPCs (blue; C). Negative stain in black. (D) Immunophenotyping of BM aspirate taken on the fifth week post-transplant of an NSG mouse injected with MPB HPCs. Gates were set with FMO staining conducted on a pool of four MPB engrafted NSG mice. (E,F) CXCR4 and HECA452 staining of MPB (A; $CD34^+CD45^+$). and EHT-generated hPSC-HPCs (B; $CD34^+CD43^+$). Gates were set with FMO staining using pooled replicate samples.

Figure S3, related to Figure 5

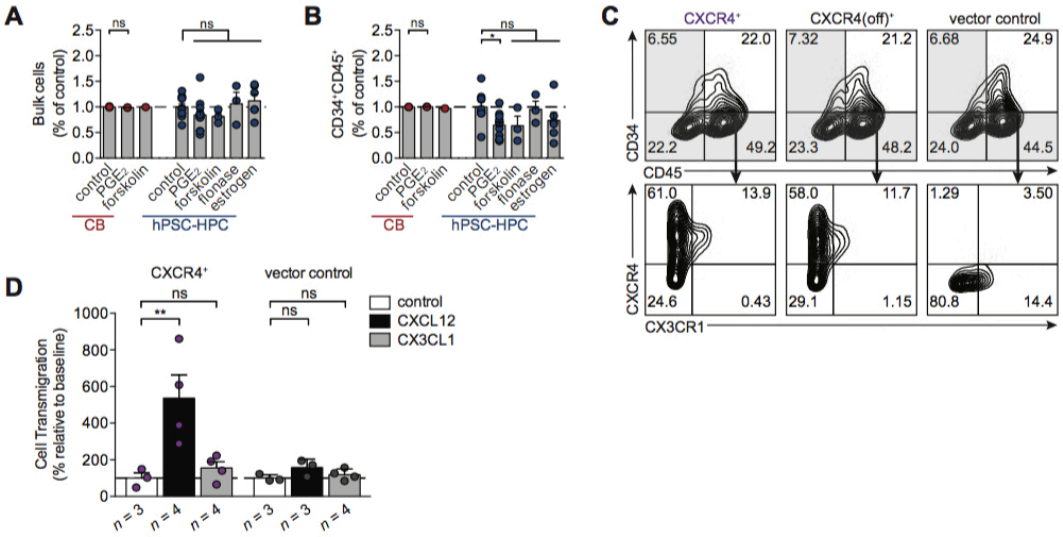


**Figure S3, related to Figure 5. Pharmacological effects on hematopoietic cells**
(A-B) CB were treated ±10 μM $PGE_2$ in 0.01% BSA, or ±10 μM forskolin in 0.1% DMSO in IMDM for 2 h at 37°C. Cells were analysed by flow cytometry for viability (A), and total number of $CD34^+CD45^+$ cells (B). Differentiating hPSC-derived hematopoietic cells were treated with compounds previously reported to induce CXCR4 expression (see also Supplemental Experimental Procedures), counted, and quantified by flow cytometry for total live cells (A), and HPCs (B; $CD34^+CD45^+$), relative to controls (0.1% DMSO or 0.01% BSA). Data points represent *n* independently assayed wells (precise *n* values indicated in the Supplemental Experimental Procedures), pooled from independently performed experiments. One-way ANOVA, $p<0.05$*. Data are represented as mean ± SEM. (C) Flow cytometry at 48 h post-transduction on EB day 16, showing comparable hPSC-HPC frequency ($CD34^+CD45^+$), robust $CXCR4^+$ expression, and continued CX3CR1 expression. (D) Transwell assay was conducted with 200 ng/mL CXCL12, 200 ng/mL CX3CL1, or control (0.001% BSA), and quantified by flow cytometry at 48 h post-transduction on EB day 16. Data points represent *n* independently assayed wells (precise *n* values indicated in the figure). Two-way ANOVA, $p<0.01$**. Data are represented as mean ± SEM. Control and CXCL12 data also shown in Figure 5F.

Figure S4, related to Figure 6

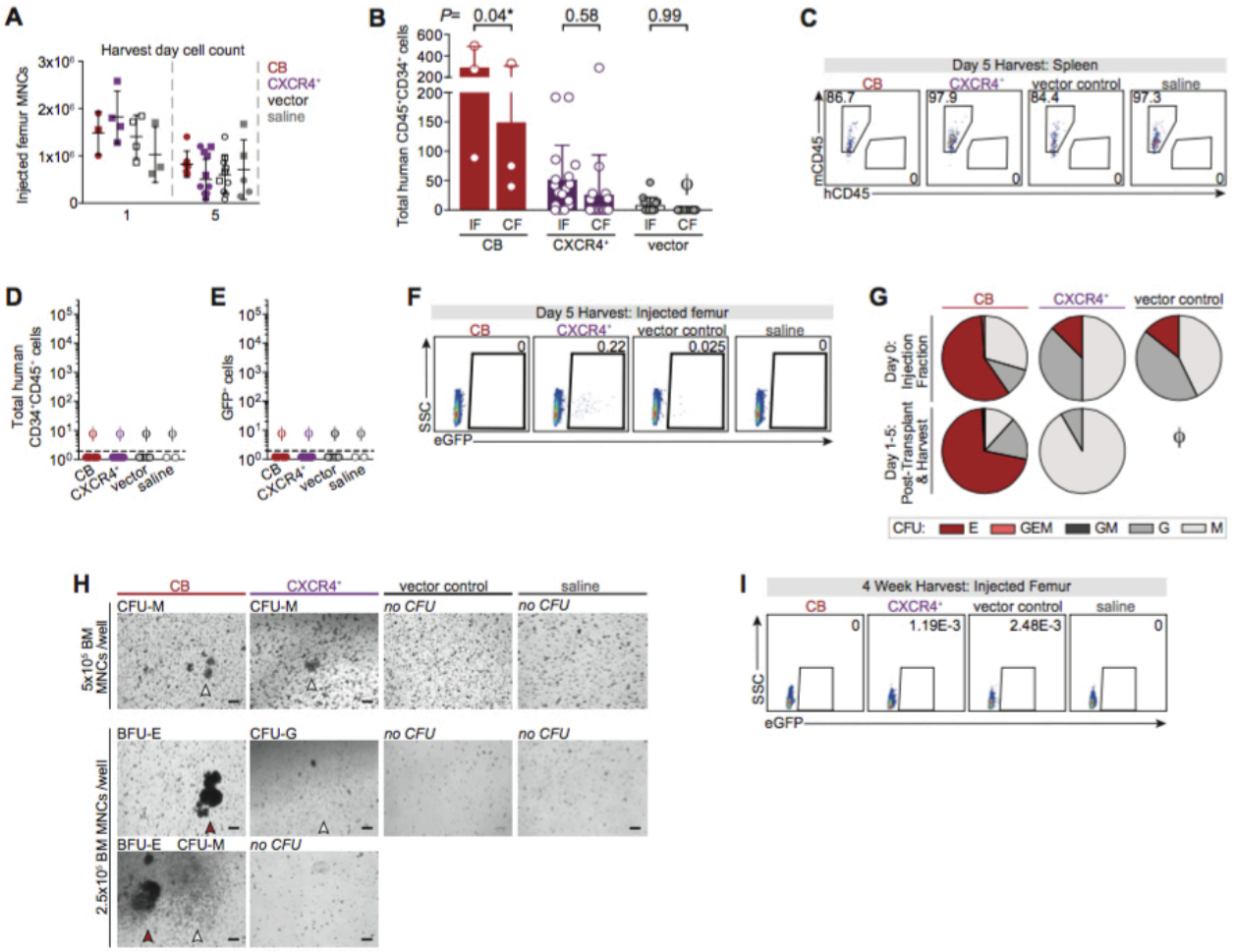


**Figure S4, related to Figure 6. Extended CXCR4⁺ transplant characterization**
(A) Total BM mononuclear cell (MNC) counts of *n* transplanted mice (precise *n* values indicated in Figure 6A), pooled from two independently performed experiments with three harvest analyses. Two-way ANOVA, no statistical differences. Two-way ANOVA, no statistical differences. Data are represented as mean ± SEM. (B) Total mCD45⁻ hCD45⁺CD34⁺ cells retained in the BM of injected (IF) and contralateral (CF) femurs. To assess BM retention separately from cellular proliferation and expansion, only 24 h retention data for CB shown; day 5 data omitted. Data points represent *n* transplanted mice, Ø is zero for group. Two-way ANOVA. Data are represented as mean ± SEM. (C) Flow cytometry of mouse spleen MNCs harvested on day 5. (D-E) Total CD34⁺hCD45⁺mCD45⁻ cells (D), and GFP⁺ cells (E) per spleen sample. Data points represent *n* transplanted mice. Ø is zero for group. (F) GFP analysis of mouse BM MNCs harvested on day 5. (G) CFU lineage at indicated time points. Ø is zero CFU in all vector control mice. (H) Morphology of CFU and background BM debris. CFU-G/M and BFU-E indicated by white and red arrowheads, respectively. Initial cell dose per 6-well is indicated on the left. (I) GFP analysis of mouse BM MNCs harvested at 4 wk.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### hPSC culture and differentiation

All experiments were performed using human ESC lines, H9 and CA2, maintained on Matrigel (BD) in mouse embryonic fibroblast-conditioned media (MEF-CM) with 8 ng/mL basic fibroblast growth factor (bFGF), as previously described (Chadwick et al., 2003). In a subset of experiments, CB-induced PSCs (Lee et al., 2014) were additionally tested. Media was changed daily, and cells were passaged as clumps weekly using collagenase IV. Daily morphological evaluation of cells was performed with light microscopy with routine monitoring of pluripotency marker expression (TRA-1-60 and Oct4) by flow cytometry. hPSC-HPCs were produced using the Cytokines and BMP4 protocol, unless stated otherwise, where EB were generated in suspension as previously described (Chadwick et al., 2003). Other hPSC-HPC differentiation methods include OP9 co-culture: MEF-CM-cultured hPSCs were passaged as clumps on to over-confluent OP9 and cultured as previously described (Choi et al., 2011); and EHT: H9 were maintained in mTeSR1 on Matrigel and differentiated as previously described (Lee et al., 2017), in normoxia (5% $CO_2$ incubator) or hypoxia (5% $O_2$/5% $CO_2$/90% $N_2$). Upon personal communication with Dr. Lee; ascorbic acid (0.28 mM) and folic acid (0.09 mM) were used at concentrations lower than reported (Lee et al., 2017). All reagents were purchased from the suppliers listed in each study. Additional compounds were supplemented into Cytokines and BMP4 EB media during hPSC hematopoietic differentiation, as follows:

*Small molecules tested for inducing CXCR4*

| Compound | Common Name | Dose | Treatment Duration | Biological Replicates | Supplier | Reference |
|---|---|---|---|---|---|---|
| ß-estradiol | estrogen | 10 nM | 48 h, or 72 h | 3 per time point | Sigma | (Rodriguez-Lara et al., 2017) |
| 16,16-dimethyl-prostaglandin E2 | $PGE_2$ | 10 $\mu$M | 2 h, 24 h, 48 h, or 5 d | 3 per time point | Cayman Chemical | (Cutler et al., 2013) |
| fluticasone propionate | flonase | 100 nM | 24 h | 3 | Selleck Chemicals | (Guo et al., 2017) |
| forskolin | forskolin | 10 $\mu$M | 72 h | 3 | Abcam | (Saxena et al., 2016) |

### Immunophenotyping and cell sorting

For CFU analysis, cells were FACS purified based on CXCR4 and CX3CR1 expression using a MoFlo XDP Cell Sorter (Beckman Coulter). For new microarray gene expression samples, Cytokines & BMP4-generated hPSC-HPC were FACS purified as $CD34^+CD45^+GFP^{\pm}$ (see Figure 7A), EHT cells as $CD34^+CD43^+$, and CB and BM cells as $CD34^+CD45^+$, using a FACSAria II (BD). For all live staining experiments, $<1x10^6$ cells/200 $\mu$L were incubated with antibodies for 30 min at 4°C, and then washed before flow cytometry. 7AAD (Beckman Coulter) or Live/Dead Fixable Violet Dead Cell Stain (Thermo Fisher) was used to exclude nonviable cells. An LSRII Flow Cytometer (BD Biosciences) was used for phenotyping.

*Antibody details*

| Antigen | Reactivity | Conjugated | Dilution | Clone | Supplier |
|---|---|---|---|---|---|
| CD34 | Human | APC, APC-Cy7, PE | 1:100 | 581 | BD Biosciences |
| CD38 | Human | PE | 1:100 | HB7 | BD Biosciences |
| CD43 | Human | FITC, PE | 1:100 | 1G10 | BD Biosciences |
| CD45 | Human | FITC, v450 | 1:100 | 2D1 | BD Biosciences |
| Cutaneous lymphocyte antigen (CLA) | Human | FITC | 1:100 | HECA452 | BioLegend |
| CXCR4 | Human | APC, PE | 1:50 | 12G5 | BD Biosciences |
| CX3CR1 | Human | PE-Vio770 | 1:100 | 2A9-1 | Miltenyi |
| CD45 | Mouse | PE-Cy7 | 1:3000 | 30-F11 | BD Pharmingen |

### Gene expression profiling

Total RNA was extracted from $0.5\text{-}3.0x10^4$ FACS-purified cells using the RNeasy Micro Kit (Qiagen) following the manufacturer's protocol. RNA was processed using the GeneChip WT Pico Kit and analyzed with Affymetrix Human Gene 2.0 ST microarray (London Regional Genomics Centre, Ontario, Canada). Gene expression analysis was conducted using Partek Gene Suite (v6.6, Partek Inc). Expression levels of RNA-seq data were obtained from a series matrix sheet in the GEO repository (NCBI). Log2 transformation of RNA-seq data was completed as previously described (Nakamura et al., 2016). For comparison of microarray data to RNA-seq data, the mean probe intensity was used for genes with multiple probes. Datasets were merged by common gene symbols and batch effect was removed

using Partek Gene Suite. Genes were considered differentially regulated with fold-change > | 2 | and false detection rate (FDR) p value < 0.05.

*Gene expression sample details*

| Lab | GEO ID | Symbol in study | Samples Used | Sample IDs | Platform | Total Annotated Genes |
|---|---|---|---|---|---|---|
| Bhatia | GSE106721 | hexagon | 15 | All; GSM2849362 to GSM2849376 | HG 2.0 ST Array, and HG U133A | 34661 |
| Bhatia | GSE92778 | circle | 6 | GSM2437567 to GSM2437572 | HG 1.0 ST Array | 20796 |
| Bhatia | GSE3823 | circle | 9 | U133A; GSM87705 to GSM87716, GSM87729 to GSM87734 | HG U133A | 13462 |
| Daley | GSE49938 | diamond | 17 | GSM1210379 to GSM1210384, GSM1210388 to GSM1210392, GSM1210401 to GSM121406 | HG U133A Plus2 | 23520 |
| Daley | GSE83719 | triangle | 5 | All; GSM2214010 to GSM2299187 | Illumina NextSeq 500 | 25855 |

**Gene set enrichment analysis**

Global GSEA was performed with default parameters (Subramanian et al., 2005), with gene sets from the Molecular Signatures Database. False discovery rate (FDR) < 0.25 with $P < 0.05$ was considered significant (Sugimura et al., 2017).

*Percent (%) overlap of gene sets used in Pearson correlation and GSEA, with global transcriptome dataset*

| Figure 7 Group | | Molecular Signatures Database Standard Name | Overlap |
|---|---|---|---|
| GPCR | CXCR4 | BIOCARTA_CXCR4_PATHWAY | 96 |
| | | BIOCARTA_AGPCR_PATHWAY | 100 |
| | Migration | REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES | 74 |
| | | GO_G_PROTEIN_COUPLED_CHEMOATTRACTANT_RECEPTOR_ACTIVITY | 84 |
| | | GO_LEUKOCYTE_CHEMOTAXIS | 81 |
| | | GO_LEUKOCYTE_MIGRATION | 86 |
| Upstream of CXCR4 | Hypoxia | BIOCARTA_HIF_PATHWAY | 100 |
| | | BIOCARTA_VEGF_PATHWAY | 93 |
| | Notch | REACTOME_SIGNALING_BY_NOTCH2 | 83 |
| | | REACTOME_SIGNALING_BY_NOTCH | 78 |
| | Steroid | GO_GLUCOCORTICOID_RECEPTOR_BINDING | 93 |
| | PGE | GO_CELLULAR_RESPONSE_TO_PROSTAGLANDIN_E_STIMULUS | 100 |
| | | GO_RESPONSE_TO_PROSTAGLANDIN_E | 96 |
| Down-stream of CXCR4 | Calcium | GO_CALCIUM_MEDIATED_SIGNALING | 73 |
| | | GO_REGULATION_OF_CALCIUM_ION_TRANSPORT | 80 |
| | | GO_REGULATION_OF_CYTOSOLIC_CALCIUM_ION_CONCENTRATION | 88 |
| | Kinases | GO_ACTIVATION_OF_PROTEIN_KINASE_ACTIVITY | 86 |
| | | GO_ACTIVATION_OF_MAPK_ACTIVITY | 85 |
| | JAK STAT | KEGG_JAK_STAT_SIGNALING_PATHWAY | 85 |
| | mTOR | KEGG_MTOR_SIGNALING_PATHWAY | 90 |
| HSC Identity | hESC | BENPORATH_ES_1 | 83 |
| | HSC | EPPERT_HSC_R | 76 |
| | | JAATINEN_HEMATOPOIETIC_STEM_CELL_UP | 70 |
| | | JAATINEN_HEMATOPOIETIC_STEM_CELL_DN | 84 |
| | | GEORGANTAS_HSC_MARKERS | 85 |
| | | GO_HEMATOPOIETIC_STEM_CELL_PROLIFERATION | 77 |

**STRING Analysis**

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis was performed with default settings (Szklarczyk et al., 2015), using 120 significantly differentially regulated genes which were upregulated by somatic HPCs compared to hPSC-HPCs, listed in Table S2. Disconnected nodes were removed.

**Colony forming unit (CFU) assay**
Primary somatic HPCs, hPSC-derived HPCs, and BM transplant samples were plated at $5.0x10^2$-$1.0x10^4$ cells/0.5 mL, $1.0x10^3$-$5.0x10^4$ cells/0.5 mL, and $1.0x10^5$/0.5 mL respectively, in Methocult H4434 (StemCell Technologies). Cells were incubated at 37°C for 14 d and manually scored. Each CFU well represents an independent biological assay, as input cells and MethoCult formulations were individually prepared for testing in single wells. CFU were stained with calcein green (Invitrogen) in Hank's Buffered Salt Solution (HBSS) for 30 min, and imaged with the Operetta High Content Imaging System (PerkinElmer). CFU from transplants were picked by micropipette, genomic DNA was extracted by DNA Micro Kit (Qiagen) following manufacturer's protocol, and analyzed by conventional PCR.

*Conventional PCR primer sequences*

| **Human DNA / Gene** | **Forward (5' – 3')** | **Reverse (5' – 3')** |
|---|---|---|
| alpha-satellite, chromosome 17 | GGGATAATTTCAGCTGACTAAACAG | TTCCGTTTAGTTAGGTGCAGTTATC |
| genomic CXCR4 (gen. CXCR4) | GGTGGTCTATGTTGGCGTCT | TCGATGCTGATCCCAATGTA |
| viral CXCR4 (vir. CXCR4) | TGGAATTTGCCCTTTTTGAG | TTGTCCGTCATGCTTCTCAG |
| viral eGFP (vir. GFP) | CACATGAAGCAGCACGACTT | TGCTCAGGTAGTGGTTGTCG |

**Cytospin**
CFU were centrifuged onto glass microscope slides using the Shandon Cytospin 3 (Block Scientific, Inc). Differential staining was performed with the Shandon Kwik-Diff Stain Kit (Thermo Scientific).

**Lentivirus transgene expression**
*CXCR4* was subcloned into the pHIV(IRES)EGFP vector (#21373, Addgene). Site directed mutagenesis targeted N123K; termed CXCR4(off). Lentivirus was produced from HEK 293FT cells with 2nd generation pMD2.G and psPAX2 packaging plasmids, and collected after 72 h. The multiplicity of infection (MOI) was calculated by a dilution series on HEK cells, and used at MOI of 100. Experimental cells were incubated with concentrated lentivirus for 48 h in the presence of 8 μg/mL polybrene (Sigma) in Cytokines and BMP4 media (Chadwick et al., 2003).

**Transwell assay**
$1.0\text{-}1.5x10^5$ cells were seeded in the upper Transwell (EMD Millipore) compartment in 0.4 mL, with 200 ng/mL CXCL12 or CX3CL1 (PeproTech), or 0.01% BSA in 0.6 mL Iscove's Modified Dulbecco's Medium (IMDM, Gibco) in the bottom. Cells in the bottom well were collected after 4 h and counted by flow cytometry. Each Transwell represents an independent biological assay, as input cells and media formulations were individually prepared for testing in single wells.

**Calcium flux assay**
$1.0\text{-}1.5x10^5$ cells were adhered to 24-well plates pre-coated with Cell-Tak (Corning) for 3 h. Cells were loaded with 1 μM Fura Red AM (Thermo Fisher) for 60 min at RT, washed twice, and incubated at 60 min RT; all in 25 mM HEPES, 20 mM glucose, in HBSS. Dynamic fluorescent imaging of intracellular calcium concentration at a single focal plane was acquired with an inverted confocal microscope, with an image pair (415 and 485 nm excitation) collected every 2 s for a total of 8 min 40 sec (250 frames). At 50th frame, AMD3100 (10 $\mu$M, Mozobil, Genzyme) or DMSO (0.01%) was added, at 100th frame CXCL12 (200 ng/mL) was added, and at 200th frame Ionomycin (10 $\mu$M, Sigma-Aldrich) was added as a positive control. Ratiometric analysis of Fura Red intensity over time was quantified using ImageJ.

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