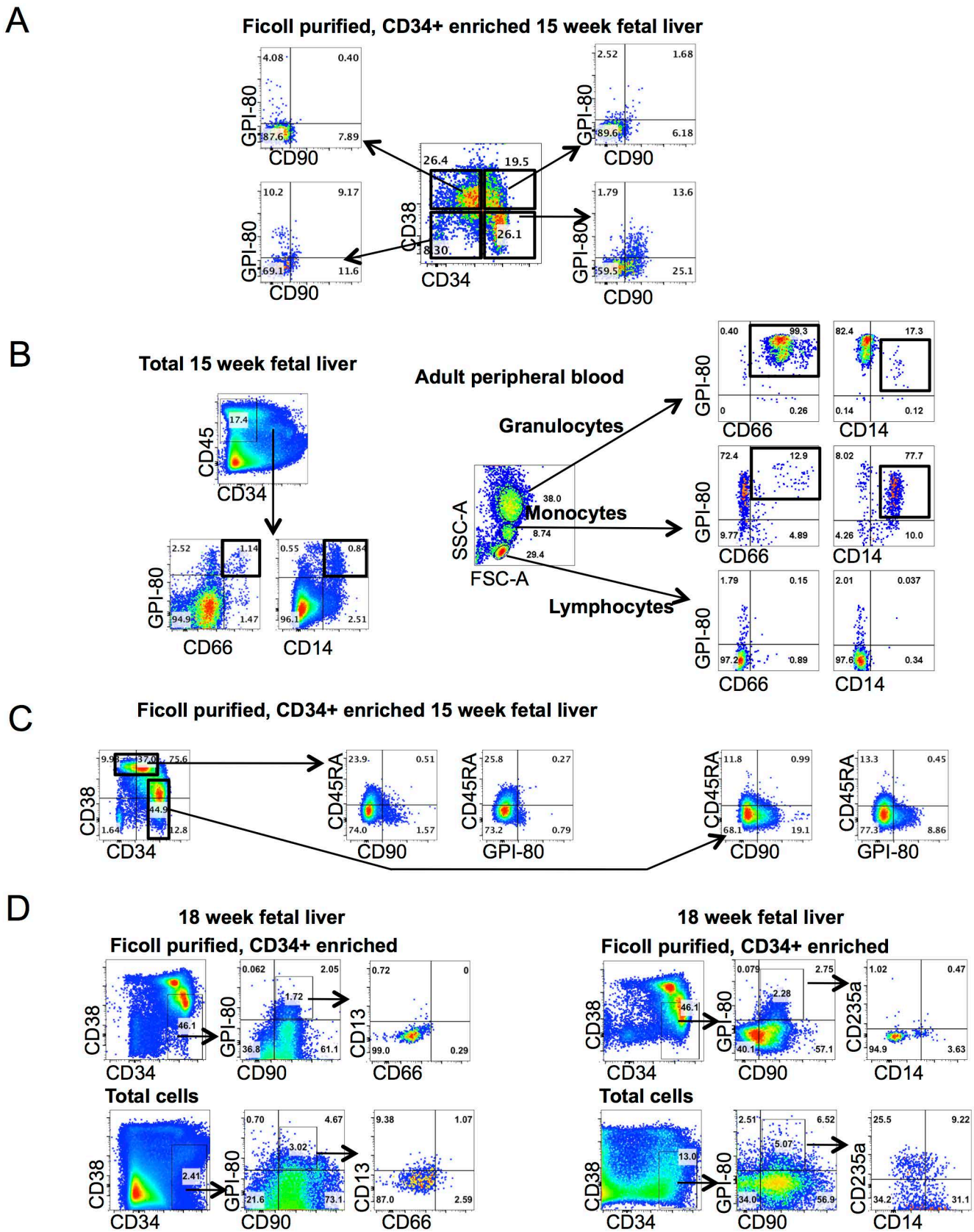

Figure S1, related to Figure 1. GPI-80 expression in HSPC and myeloid cells. A. Expression of GPI-80 in hematopoietic populations in Ficoll-purified, CD34+ enriched fetal liver cells. **B.** Flow cytometry of total fetal liver and adult peripheral blood with CD66, CD14 and GPI-80 verifies expression of GPI-80 in myeloid cells. Fetal liver cells are gated from CD45+CD34- population. **C**. CD45RA expression in fetal liver CD34^{+} cells is restricted CD90^{-} GPI-80^{-} cells. **D.** Flow cytometry analysis of 18 week fetal liver with lineage markers CD13, CD66, CD235, and CD14 with and without Ficoll purification shows depletion of Lin+ cells with Ficoll purification.

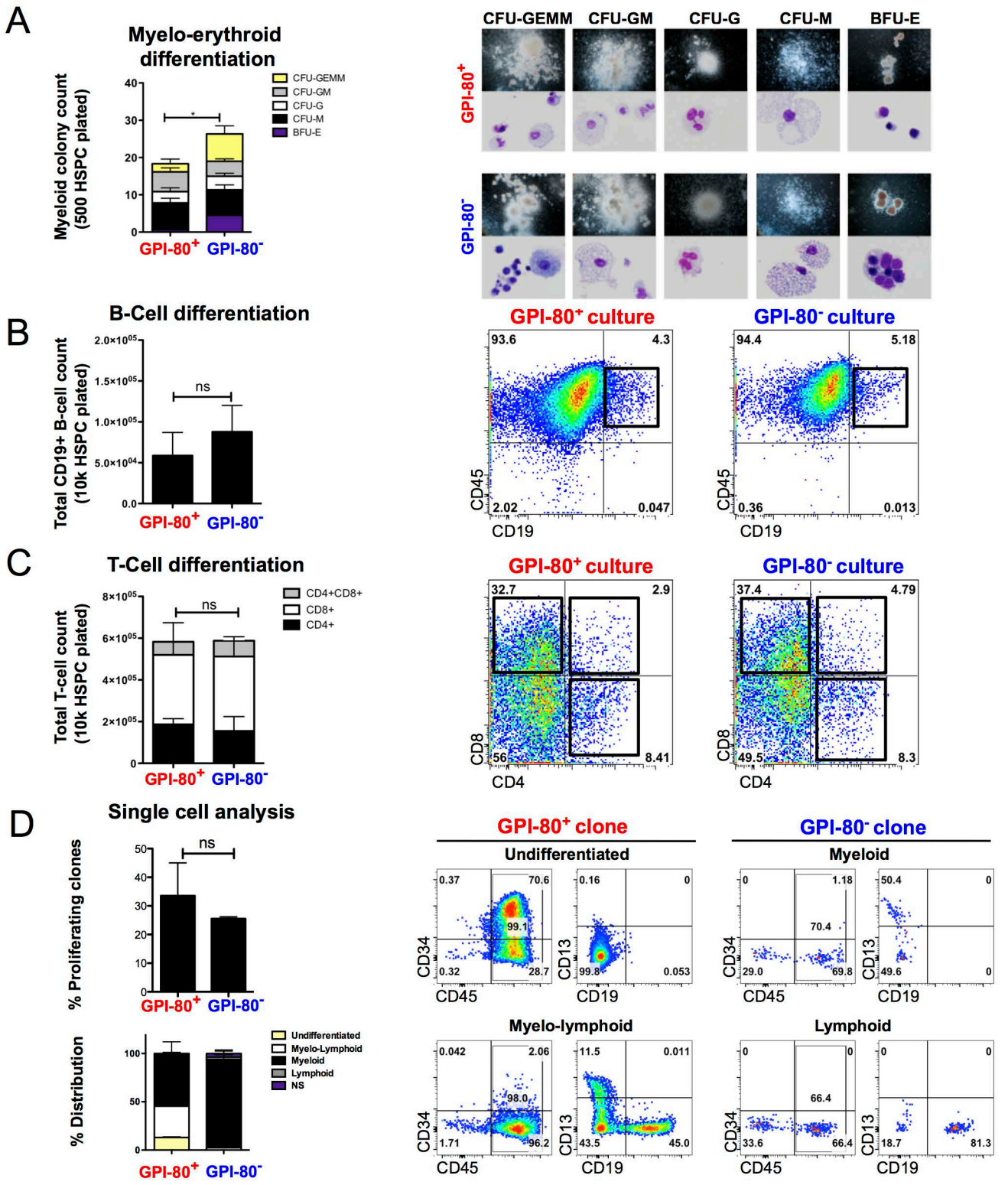

Figure S2, related to Figure 2. Multipotency of GPI-80⁺ HSPC. A. Myelo-erythroid differentiation potential of GPI-80⁺ and GPI-80⁻ HSPC on methylcellulose assay is shown (n=6 donors). Error bars represent mean ± SEM. **B.** Flow cytometry analysis for B-cell marker CD19 on GPI-80⁺ and GPI-80⁻ HSPC after 2 weeks culture on OP9M2 is shown (n=3 donors). Error bars represent mean ± SEM. **C.** Flow cytometry analysis of T cell markers CD4 and CD8 after 2 weeks of culture on OP9-Dll1 (n=3 donors). Error bars represent mean ± SEM. **D.** Myeloid and lymphoid potential of GPI-80⁺ and GPI-80⁻ HSPC at the single cell level is shown. Quantification of proliferating clones (defined as >200 cells, n=2 donors), distribution of clone types (40 clones analyzed), and representative clones from GPI-80⁺ and GPI-80⁻ HSPC after two weeks of culture on OP9M2 are shown. Though bulk cultures demonstrate multilineage potential of GPI-80⁻ HSPC, single cell analysis reveals enrichment of multipotent cells in GPI-80⁺ population. Error bars represent mean ± SEM.

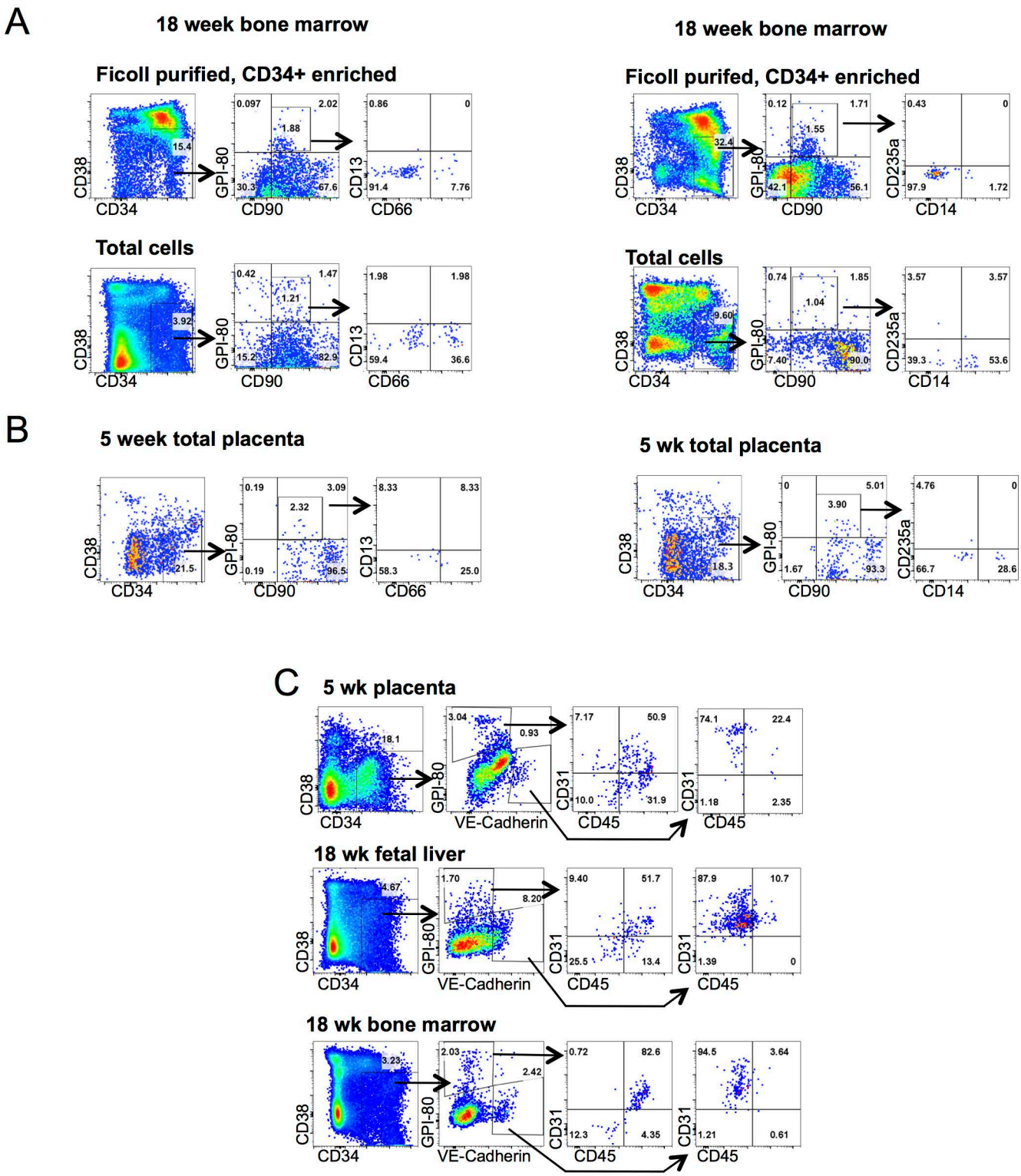

Figure S3, related to Figure 3. GPI-80 expression in multiple fetal hematopoietic sites. A. Lineage analysis of total vs ficoll purified, CD34+ enriched second trimester bone marrow with lineage markers CD13, CD66, CD235a, and CD14 shows depletion of Lin+ cells with Ficoll purification. **B.** Lineage analysis of 5 week total placenta with differentiation markers CD13, CD66, CD235a, and CD14 shows the presence of a subpopulation of GPI-80 HSPC that are devoid of lineage marker expression. **C.** Representative flow cytometry plots of endothelial cells show that GPI-80 expression in the placenta, fetal liver and fetal bone marrow is confined to hematopoietic cells.

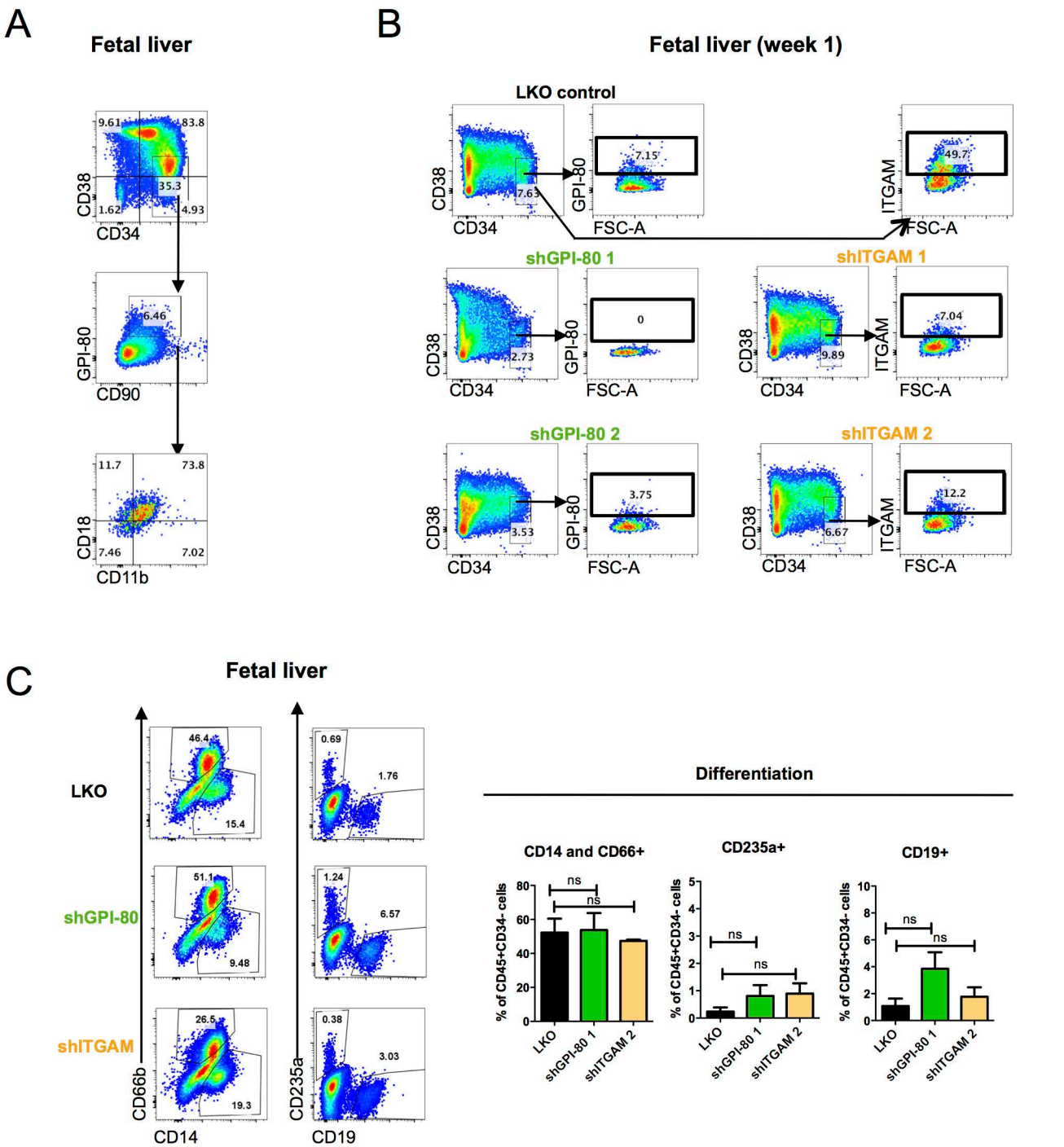

Figure S4, related to Figure 4. Lentiviral shRNA knockdown of GPI-80 and ITGAM. A. Representative flow cytometry plot of fetal liver showing expression of CD11b(ITGAM) and CD18(ITGB2) on GPI-80^{+} HSPC. **B.** Representative flow cytometry plots of GPI-80 and ITGAM expression one week after lentiviral transduction, documenting reduction of GPI-80 and ITGAM protein on HSPC with two different shRNA vectors. C. Differentiation ability of HSPC after knockdown of GPI-80 or ITGAM (n=4 donors). Error bars represent mean ± SEM.

Table S2, related to Figure 2. Human engraftment in bone marrow of NSG mice transplanted with GPI-80$^+$ and GPI-80$^-$ HSPC

Mouse ID	Population Transplanted (human fetal liver cells)	Percent CD45 Engraftment in Bone Marrow	Percent CD13$^+$,CD66$^+$ (of total cells)	Percent CD19$^+$ (of total cells)	Percent CD3$^+$ (of total cells)
Group A-1	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^+$	55.6	27.1	19.0	0.7
Group A-2	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^+$	44.6	17.1	19.1	1.0
Group B-1	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^+$	73.2	13.8	51.3	3.2
Group B-2	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^+$	38.6	3.6	18.2	0.6
Group C-1	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^+$	20.6	8.3	3.7	0.2
Group C-2	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^+$	19.6	2.1	10.1	0.1
Group C-3	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^+$	72.7	0.9	2.8	33.7
Group A-3	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^-$	0.5	0.1	0.3	0.1
Group B-3	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^-$	0	0.1	0.1	0
Group B-4	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^-$	0.1	0.1	0.1	0
Group C-4	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^-$	0.3	0.2	0.2	0.1
Group C-5	CD34$^+$CD38$^-$ CD90$^+$GPI-80$^-$	0.1	0.1	0	0.1

Table S4, related to Figure 4. Human engraftment in bone marrow of NSG mice transplanted with LKO, shGPI-80, or shITGAM transduced cells

Mouse ID	Population Transplanted (human fetal liver cells)	Percent CD45 Engraftment in Bone Marrow	Percent $CD13^+$,$CD66^+$ (of total cells)	Percent $CD19^+$ (of total cells)	Percent $CD3^+$ (of total cells)
Group A-1	LKO	18.4	3.9	7.22	0.4
Group A-2	LKO	34.8	12.4	16.0	0.6
Group A-3	LKO	16.2	5.0	8.4	0.2
Group B-1	LKO	22.0	7.4	13.0	0.2
Group B-2	LKO	4.9	1.5	2.5	0.6
Group C-1	LKO	13.8	2.2	6.0	0.3
Group C-2	LKO	17.3	2.1	10.7	0.4
Group C-3	LKO	24.2	2.3	19.0	0.5
Group A-4	shGPI-80	0.3	0.2	0.5	0.1
Group A-5	shGPI-80	0.5	0.1	0	0.2
Group A-6	shGPI-80	0.5	0.1	0.4	0
**Group B-3*	*shGPI-80*	*20.2*	*1.8*	*19.3*	*0.2*
Group B-4	shGPI-80	0.9	0.2	0.3	0.1
Group B-5	shGPI-80	0.8	0.5	0	0.2
Group C-4	shGPI-80	0.1	0	0	0
Group C-5	shGPI-80	0.1	0	0	0
Group C-6	shGPI-80	0.1	0	0	0
Group A-7	shITGAM	4.9	1.1	0.2	0.1
Group A-8	shITGAM	1.7	0.3	0	0.1
Group A-9	shITGAM	1.3	0.7	0	0.1
Group B-6	shITGAM	0.7	0.5	0	0.2
Group B-7	shITGAM	7.7	3.5	0.6	0.3
Group B-8	shITGAM	0.5	0.3	0.2	0.1
Group C-7	shITGAM	0.3	0	0	0
Group C-8	shITGAM	0.9	0.3	0.1	0.1
Group C-9	shITGAM	0.4	0	0.1	0

*engrafted bone marrow cells failed to show shRNA vector-associated puromycin resistance upon in vitro culture

Supplementary Experimental Procedures

Isolation of CD34$^+$ cells from human fetal hematopoietic tissues. Fetal livers were mechanically dissociated into single cell suspensions using scalpels and syringes (16, 18 and 20 gauge). Mononuclear cells were isolated on a Ficoll gradient (Lymphoprep, Stem Cell Technologies). CD34$^+$ cells were isolated using magnetic beads (Miltenyi Biotec, CD34 MicroBead Kit). Fetal bone marrow was ground in sterile mortars. Placentas were mechanically dissociated and incubated with collagenase (Worthington), dispase and DNase (Van Handel et al, 2010). The same enzymatic dissociation protocol performed on placentas was used also for fetal liver and bone marrow when co-expression of GPI-80 on endothelium was assessed. All cell suspensions were strained through a 70μm nylon mesh. The stage of the hematopoietic tissues is indicated as developmental age, which is two weeks less than the gestational age (from last menstrual period).

Flow cytometry and sorting. 7-amino-actinomycin D was used to identify and remove dead cells. Mouse anti-human antibodies were used to detect CD34, CD90, CD45, CD66b, CD13, CD3, CD4 and CD8, CD14, CD19, CD235a, CD31, CD45RA (BD Biosciences), CD38, CD19, ITGAM, VE-Cadherin (eBioscience) , CD34, CD45 (Biolegend), and GPI-80 (MBL International). Cells were analyzed on BD LSRII flow cytometer, and cell sorting was conducted on BD Aria II.

Microarray. RNA was purified from sorted HSPC using RNeasy Mini Kit (QIAGEN). Extracted RNA was hybridized on Affymetrix arrays (u133plus2.0 array). Microarrays were completed by the Clinical Microarray Core, Department of Pathology & Laboratory Medicine at UCLA.

Bioinformatic analysis. Analysis of microarray results was performed as previously described (Van Handel et al, 2012). Briefly, R package *Limma* provided through Bioconductor (Gentleman et al, 2004). was used for assessing differential expression (> 2-fold and p-value <0.05). Robust Multiarray Averaging was used to obtain absolute expression mRNA levels (Bolstad et al, 2003). For genes with multiple probe

sets, probe sets with the lowest p-value were chosen. Diagnostic plots using the Bioconductor package array QualityMetrics were generated to assess quality of all arrays. The normalized expression were standardized and analyzed in Cluster 3.0 and Java Treeview for heatmap visualization.

Myeloerythroid colony forming assay. Sorted HSPC were plated on MethoCult GF+H443 (Stem Cell Technologies) containing SCF, GM-CSF, IL-3 and EPO, and supplemented with TPO (10ng/ml, Peprotech), 1% penicillin-streptomycin and 1% amphotericin B (GIBCO/Invitrogen). Myeloerythroid colonies were scored 14 days after plating.

Lymphoid differentiation assays. Sorted HSPC were differentiated into B cells on OP9M2 stroma and to T cells on OP9-DL1 stroma. Lymphoid co-cultures were plated in media containing αMEM (GIBCO/Invitrogen), 20% Fetal Bovine Serum (Hyclone) and 1% penicillin/streptomycin supplemented with SCF (25 ng/ml), FLT3 (10 ng/ml), (Peprotech) and IL-7 (20 ng/ml) (Invitrogen).

Single cell differentiation assay. Fetal liver HSPC were sorted into 96 well plates pre-seeded with 20,000 irradiated OP9-M2, in the presence of SCF (25 ng/ml), TPO (25 ng/ml), Flt3 (25 ng/ml) (Peprotech) and IL-7 (20 ng/ml) (Invitrogen). Wells that visually demonstrated cell expansion were collected and analyzed by FACS for myeloid, lymphoid and HSPC markers after 14 days.

***In vitro* self-renewal assay.** OP9M2 stromal cells were plated in media containing MEM-α (GIBCO/Invitrogen), 20% Fetal Bovine Serum of specific batches tested for HSC expansion (Hyclone or OMEGA), penicillin (100 U/ml), streptomycin (100 µg /ml) and glutamine (292µg/ml). For co-culture assays, OP9M2 stromal cells were irradiated at 2000 rads and plated in tissue culture-treated 24-well plates at a concentration of 50,000 cells/cm2. HSPC were plated in media (α-MEM, 20% FBS and 1% penicillin/strep) supplemented with TPO (25 ng/ml), FLT-3 (25 ng/ml), and SCF (25 ng/ml) (Peprotech). Half of the medium in each well was changed every other day. FACS analysis was used to evaluate the ability to maintain undifferentiated HSPC populations in culture.

Cell cycle analysis. Freshly isolated $CD34^+$ cells from 15-17 weeks fetal livers were cultured overnight on OP9M2 stroma and pulse labeled with 10μM BrdU for 35 min in culture. Cells were sorted for the indicated surface phenotypes and processed according to the FITC-BrdU flow kit (BD) instructions to detect the distribution between cell cycle stages.

Transplantation Assays. Human fetal liver hematopoietic populations were injected into sub-lethally irradiated (325 rad) NOD-scid IL2Rγ-null mice (Jackson Laboratories). Sorted subpopulations originating from 50,000 CD34+ cells were injected intravenously in each mouse. Mice were bled retro-orbitally for analysis of peripheral blood at 6 and 12 weeks. 16 weeks after transplantation, mice were sacrificed and hematopoietic organs (bone marrow and spleen) were harvested and analyzed for human engraftment based on detection of human CD45, CD66b, CD13, CD3 (BD Biosciences) and CD19 (eBioscience). Experimental protocols involving mice were reviewed and approved by the UCLA Animal Research Committee (Protocol number 2005-109).

CFSE labeling and homing assay. GPI-80^+ and GPI-80^- HSPC were sorted from 15 week human fetal liver and labeled with CFSE (Invitrogen) as conducted by Quah and Parish, 2010. After labeling, the GPI-80^+ and GPI-80^- HSPC equivalent of one liver was divided to three recipients and transplanted by retro-orbital injection into sub-lethally irradiated NOD-scid IL2Rγ-null mice (Jackson Laboratories). 16 hours post-transplantation, mice were sacrificed and bone marrow homing of assessed based on the presence of CSFE labeled cells.

Amnis Image Stream flow cytometry. Samples were analyzed on an Image Stream 100 flow cytometer, and data was analyzed using IDEAS software (Amnis). CD34+ cells magnetically isolated from human fetal liver were stained with CD90 (BD Biosciences), ITGAM (eBioscience) and GPI-80 (MBL International).

Production of shRNA lentiviral vectors. 293T cells were transfected with Lipofectamine 2000 (Invitrogen)-complexed plasmids (pLKO vectors: VSV-G: ΔR8.2 in a 2.5:1:1 ratio) in OPTI-MEM (Invitrogen). Viral supernatant was collected after 48 hours and ultracentrifuged at 20,200 rpm for 1.5 hrs. Viral pellet was resuspended in Serum-Free Expansion Medium (SFEM, STEMCELL Technologies) and stored at -80°C.

Lentiviral transduction protocol. $CD34^+$ cells were pre-stimulated for 6 hours in SFEM supplemented with SCF (100 ng/ml), TPO (100 ng/ml, and Flt3 (100 ng/ml) (Peprotech). Transduction was performed on 40 ug/ml RetroNectin (Takara)-coated plates according to manufacturer's protocol. For efficient transduction, $CD34^+$ cells were incubated twice with 5 µl of virus at 12 hour intervals. After infection, cells were washed in PBS supplemented with 5% FBS and seeded onto OP9M2 as described above. Cells were selected with puromycin 1µg/ml starting 48h after first transduction for the duration of the experiment. shRNA knockdown of GPI-80 and ITGAM was verified by FACS and q-RT-PCR. Two independent shRNA sequences that yielded robust knock-down and similar phenotype in HSPC culture were identified for both genes.

shRNA differentiation assay. After lentiviral transduction performed as described above, transduced cells were cultured on OP9M2 in the presence of SCF (25 ng/ml), TPO (25 ng/ml, Flt3 (25 ng/ml) (Peprotech), IL^-7 (20 ng/ml) (Invitrogen) and puromycin at 1µg/ml. After 2 weeks of culture, cells were lifted and assessed for the expression of differentiation markers CD14, CD66, CD19 and CD235a.

shRNA transplantation assay. After lentiviral transduction performed as described above, transduced cells were selected with puromycin at 1µg/ml for 8 days. Cells were transplanted by retro-orbital injection into sub-lethally irradiated NOD-scid IL2Rγ-null mice (Jackson Laboratories). Mice were bled retro-orbitally for analysis of peripheral blood. 10 weeks post-transplantation, mice were sacrificed and bone marrow was harvested and analyzed for human engraftment.

Supplemental References

Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185-193

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome biology 5: R80

Quah BJ and Parish CR (2010) The use of carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor Lymphocyte Proliferation. J Vis Exp 44.

Van Handel B, Montel-Hagen A, Sasidharan R, Nakano H, Ferrari R, Boogerd CJ, Schredelseker J, Wang Y, Hunter S, Org T, Zhou J, Li X, Pellegrini M, Chen JN, Orkin SH, Kurdistani SK, Evans SM, Nakano A, Mikkola HK (2012) Scl represses cardiomyogenesis in prospective hemogenic endothelium and endocardium. Cell 150: 590-605

Van Handel B, Prashad SL, Hassanzadeh-Kiabi N, Huang A, Magnusson M, Atanassova B, Chen A, Hamalainen EI, Mikkola HK (2010) The first trimester human placenta is a site for terminal maturation of primitive erythroid cells. Blood 116: 3321-333

Supplementary Table Legends

Table S1. Gene expression analysis of fetal liver hematopoietic subsets, Related to Figure 1. Gene expression analysis shows the comparison between $CD34^{+}CD38^{lo/-}CD90^{+}$ HSPC and $CD34^{+}CD38^{lo/-}CD90^{-}$ HPC in human fetal liver. Significantly upregulated and downregulated genes (≥2 fold, $p<0.05$) are shown.

Table S2. Human engraftment in the bone marrow of NSG mice transplanted with $GPI\text{-}80^{+}$ and $GPI\text{-}80^{-}$ HSPC, Related to Figure 2. Human engraftment at 16 weeks post-transplantation is shown.

Table S3. Gene expression analysis of fetal liver $GPI\text{-}80^{+}$ and $GPI\text{-}80^{-}$ HSPC, Related to Figure 4. Gene expression analysis shows comparison between $CD34^{+}CD38^{lo/-}CD90^{+}GPI\text{-}80^{+}$ and $CD34^{+}CD38^{lo/-}CD90^{+}GPI\text{-}80^{-}$ HSPC. Significantly upregulated and downregulated genes (≥2 fold, $p<0.05$) are shown.

Table S4. Human engraftment in the bone marrow of NSG mice transplanted with human fetal liver hematopoietic cells transduced with LKO, shGPI-80, or shITGAM lentiviral vectors, Related to Figure 4. Human engraftment at 10 weeks post-transplantation is shown.