SUPPLEMENTARY APPENDIX

Granulocyte colony-stimulating factor directly acts on mouse lymphoid-biased but not myeloid-biased hematopoietic stem cells

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Supplemental Methods

Single-cell sorting

BM cells were isolated from tibiae, femora and iliac crests of 8- to 10-week-old female CD45.1 or CD45.2-B6 mice, and c-Kit-positive cells were enriched using anti-c-Kit antibody-conjugated MACS beads (Miltenyi Biotechnology, catalog no.130091224) and LS columns (Miltenyi Biotechnology, catalog no.130042401).

For HSC1, HSC2 and HPC1 sorting, cells were incubated with the following antibodies for 60 minutes: allophycocyanin (APC)-eFlour780-conjugated anti-Gr-1 (RB6-8C5, 47593182, eBioscience), -B220 (RA3-6B2, 47045282, eBioscience), and -TER-119 (TER-119, 47592182, eBioscience) antibodies, fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (RAM34, 11034185, eBioscience), APC-conjugated anti-c-Kit (2B8, 17117182, eBioscience), phycoerythrin-cyanine 7 (PE-Cy7)-conjugated anti-Sca-1 (D7, 25598182, eBioscience), PE-conjugated anti-CD150 (TC15-12F12.2, 115904, Biolegend), Brilliant Violet (BV) 510-conjugated anti-CD41(MWReg30, 133923, Biolegend), BV421-conjugated anti-CD48 (HM48-1, 562745, eBioscience), and PerCp-eFlour710-conjugated anti-CD201 (eBio1560, 46201280, Biolegend) antibodies.

For HPC2, HPC3 and HPC4 sorting, cells were stained with a lineage-marker cocktail, FITC-conjugated anti-CD34 (RAM34, 11034185, eBioscience), APC-conjugated anti-c-Kit (2B8, 17117182, eBioscience), PE-Cy7-conjugated anti-Sca-1 (D7, 25598182, eBioscience), BV785-conjugated anti-CD150 (TC15-12F12.2, 115937, Biolegend), and PE-conjugated anti-Flt-3 (A2F10, 12135181, eBioscience) antibodies.

Cell surface markers for HSC1, HSC2, HPC1, HPC2, HPC3, and HPC4 are shown in the Supplemental Table S1. Antibodies are listed in Supplemental Table S2.

Serum-free medium

Ham's F-12 medium (Thermo Fisher Scientific, 21700026) was supplemented with 0.5 mg/ml recombinant human serum albumin (HAS, Albumin Bioscience, 1001), 2 mM L-glutamine (Thermo Fisher Scientific, 25030081), 1× ITS-X (Thermo Fisher Scientific, 51500-056), 10 mM HEPES (Sigma Aldrich, H0887), 0.1 mM MEM nonessential amino acids (Thermo Fisher Scientific, 11140050), 0.5 mg/ml penicillin-streptomycin-glutamine (Thermo Fisher Scientific, 10378016), and 5×10^{-5} M 2-mercaptoethanol (Thermo Fisher Scientific, 21985023).

Single-cell culture

Single cells were cultured in serum-free medium supplemented with 50 ng/ml recombinant mouse SCF (Peprotech, 250-03) plus 50 ng/ml recombinant mouse TPO (Peprotech, 315-14), 10 ng/ml recombinant human G-CSF (Peprotech, 300-23), or 10 ng/ml recombinant mouse GM-CSF (Peprotech, 315-03). Cells were cultured for 7 days at 37°C with 5% CO_2 in the air. Number of cells per well were daily counted under inverted microscope.

Serial competitive repopulation

Twenty HSC1 or HSC2 cells from CD45.1-B6 mice were cultured with cytokines for 7 days, and cells were transplanted into lethally irradiated CD45.2-B6 mice with 5×10^5 BM competitor cells from CD45.2-B6 mice. As a control, 20 freshly isolated HSC1 or HSC2 cells from CD45.1-B6 mice were similarly transplanted into CD45.2-B6 mice.

For secondary transplantation of HSC1 cells, 2×10^7 BM cells from primary recipients were transplanted into lethally irradiated CD45.2-B6 mice. PB cells were analyzed at the indicated time points after transplantation.

Single-cell transplantation

Single HSC1 cells from CD45.1-B6 mice were cultured with SCF + TPO for 1 day, and the surviving single HSC1 cells were selected and transplanted into lethally irradiated CD45.2-B6 mice with 5×10^5 BM competitor cells from CD45.2-B6 mice. For the cultured cells group, single HSC1 cells were cultured with cytokines for 7 days, and cells of each well were transplanted into lethally irradiated CD45.2-B6 mice with 5×10^5 BM competitor cells from CD45.2-B6 mice. PB cells were analyzed at the indicated time points after transplantation. Identification of My-bi, Bala, Ly-bi HSCs from each group is shown in Supplemental Table S5-8.

Peripheral blood analysis

Red blood cells from PB were lysed with red blood cell lysis buffer, and the cells were stained with FITC-conjugated anti-CD45.1 (A20, 11045385, eBioscience), PE-conjugated anti-CD45.2 (104, 12045483, eBioscience), PE-CY7-conjugated anti-CD4, APC-conjugated anti-CD8a (53-6.7, 17008182, eBioscience), PerCP-CY5.5-conjugated anti-B220 (RA3-6B2, 45045282, eBioscience), and APC-eFluor780-conjugated anti-Mac-1/Gr-1 antibodies (M1/70, 47011282 and RB6-8C5, 47593182, eBioscience).

Single-cell RT-PCR

For single-cell RT-PCR for 6 populations, 48 single HSC1, HSC2, HPC1, HPC2, HPC3, and HPC4 cells were sorted into each well of a 96-well plate that contained 10 μl of RT-STA master mix. For single-cell RT-PCR for cultured cells, single HSC1 cells were cultured with SCF, SCF+G-CSF, and SCF+TPO for 7 days. Single cells were randomly picked up from 48 wells (one cell per well) by a micromanipulator and were placed into the RT-STA master mix. Freshly isolated 48 single HSC1 cells were used as a control.

Reverse transcription was performed at 50°C for 15 min. The samples were incubated at 95°C for 2 min, followed by specific target amplification in 22 cycles of 95°C for 15 s and 60°C for 4 min. A 5 μl cDNA sample was diluted with 20 μl Tris-EDTA buffer and used for real-time PCR. For the sample loading mix, 2.7 μl cDNA was mixed with 3 μl Taqman universal PCR master mix (Applied Biosystems) and 0.3 μl sample loading buffer. For the assay loading mix, 3 μl of each set of 20× primers was mixed with 3 μl assay loading buffer. A 5 μl sample loading mix and 5 μl assay loading mix were applied to a 48×48 chip. The chip was first placed in an integrated fluidic circuit controller to mix 48×48 reactions. Then the chip was set on the Fluidigm Biomark system and incubated at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Data were analyzed by Biomark real-time PCR analysis software (Fluidigm). All PCR primers were purchased from Thermo Fisher Scientific. Genes set used for six populations and cultured cells are listed in Supplemental Tables S3 and 4, respectively.

Supplemental Table S1. Cell surface markers used for the identification of six populations by flow cytometry.

Cell types	Surface markers
HSC1	$CD201^{+}CD150^{+}CD48^{-}CD41^{-}CD34^{-}KSL$
HSC2	$CD201^{+}CD150^{-}CD48^{-}CD41^{-}CD34^{-}KSL$
HPC1	$CD201^{+}CD150^{+}CD48^{-}CD41^{+}CD34^{-}KSL$
HPC2	$CD150^{+}Flt\text{-}3^{-}CD34^{+}KSL$
HPC3	$CD150^{-}Flt\text{-}3^{-}CD34^{+}KSL$
HPC4	$CD150^{-}Flt\text{-}3^{+}CD34^{+}KSL$

Supplemental Table S2. Antibodies used for flow cytometry.

Antibody conjugate	Clone No.	REF No.	Supplier
Gr-1 APC-eFlour780	RB6-8C5	47593182	eBioscience
B220 APC-eFlour780	RA3-6B2	47045282	eBioscience
TER119 APC-eFlour780	TER-119	47592182	eBioscience
CD34 FITC	RAM34	11034185	eBioscience
c-Kit APC	2B8	17117182	eBioscience
Sca-1 PE-Cy7	D7	25598182	eBioscience
CD150 PE	TC15-12F12.2	115904	Biolegend
CD150 BV785	TC15-12F12.2	115937	Biolegend
CD41 BV510	MWReg30	133923	Biolegend
CD48 BV421	HM48-1	562745	eBioscience
CD201 PerCp-eFlour710	eBio1560	46201280	Biolegend
Flt3 PE	A2F10	12135181	eBioscience

Supplemental Table S3. Gene set for single-cell RT-PCR for six populations.

Kit	*IL-2rg*	*Mki67*	*Cdkn1b*
Mpl	*IL-3ra*	*Ccnd1*	*Cdkn1c*
Epor	*IL-4ra*	*Ccnd2*	*Cdkn2c*
Flt3l	*IL-6ra*	*Ccnd3*	*Cdkn2d*
Csf1r	*IL-6st*	*Ccne1*	*Chk1*
Csf2ra	*IL7R*	*Ccne2*	*Chk2*
Csf2rb	*IL10ra*	*Cdk1*	*Gadd45a*
Csf3r	*IL10rb*	*Cdk2*	*Cd150*
IL-1r2	*IL-11ra1*	*Cdk4*	*Cd48*
IL1rap	*IL-12rb1*	*Cdk6*	*Procr*
IL-2ra	*IL-12rb2*	*Cdk7*	*Cxcr4*
IL-2rb	*Gapdh*	*Cdkn1a*	*Cxcl12*

The 48 genes included cytokine receptors, cell cycle regulators, and cell surface markers. *Gapdh* was used as a positive control.

Supplemental Table S4. Gene set for single-cell RT-PCR for cultured HSC1 cells.

Kit	*Mki67*	*Egr1*	*Tyk2*
Mpl	*Cdk2*	*Egr2*	*Stat1*
Csf3r	*Cdk4*	*Egr3*	*Stat3*
CXCR4	*Cdk6*	*Socs1*	*Stat5a*
Ccna1	*Cdkn1a*	*Socs2*	*Stat5b*
Ccnb1	*Cdkn1b*	*Socs3*	*Bax*
Ccnd1	*Cdkn1c*	*Socs4*	*Bid*
Ccnd2	*Cdkn2a*	*Socs5*	*Puma*
Ccnd3	*Cdkn2b*	*Socs6*	*Bcl2*
Ccne1	*Cdkn2c*	*Socs7*	*Bclxl*
Ccne2	*Cdkn2d*	*Jak1*	*Mcl*
Gadd45a	*Trp53*	*Jak2*	*Gapdh*

The 48 genes included cytokine receptors, cell cycle regulators, apoptosis-associated genes, Socs family, and signaling molecules. *Gapdh* was used as a positive control.

Supplemental Table S5. Identification of My-bi, Bala, and Ly-bi HSCs 6 months after single-cell transplantation (control).

Mouse ID	Cell No. on day 7	% CD45.1 cells			L/M ratio	HSC/HPC
		1 mon	3 mons	6 mons		
1	1	1.84	36.5	36.7	1.70	LT-My-bi HSC
2	1	14.4	23.1	30.3	1.54	LT-My-bi HSC
3	1	6.1	11.8	16.3	1.63	LT-My-bi HSC
4	1	1.9	7.57	7.27	0.50	LT-My-bi HSC
5	1	13.2	4.46	4.71	1.13	LT-My-bi HSC
6	1	0.3	1.1	1.6	1.85	LT-My-bi HSC
7	1	54.9	37.7	26.6	79.10	ST-Ly-bi HSC
8	1	76.6	54.0	23.3	13.03	ST-Ly-bi HSC
9	1	14.2	0.43	0.18	0	HPC
10	1	2.08	0.6	0.08	0	HPC
11	1	3.86	1.11	0	0	HPC

Data used in Figure 4. Myeloid-biased (My-bi), balanced (Bala), and lymphoid-biased (Ly-bi) HSCs were defined by the ratio of lymphocytes to myeloid cells (L/M ratio) in peripheral blood 6 months after transplantation. My-bi HSCs were defined by the L/M ratio < 3, Ly-bi HSCs were defined by the L/M ratio > 10, and Bala HSCs were defined by $3 < L/M < 10$.[1,2] Long-term (LT) HSCs were defined when the percentage of myeloid cells maintained or increased by 6 months after transplantation. Short-term (ST) HSCs were defined when the percentage of myeloid cells decreased by 6 months, with

myeloid, B-lymphoid, and T-lymphoid lineage reconstitution at a time after transplantation.[3] Hematopoietic progenitor cells (HPCs) were defined when one or two lineages lacked from the definition of ST-HSCs.

Supplemental Table S6. Identification of My-bi, Bala, and Ly-bi HSCs 6 months after transplantation with cells from SCF single-cell culture.

Mouse ID	Cell No. on day 7	% CD45.1 cells			L/M ratio	HSC/HPC
		1 mon	3 mons	6 mons		
1	3	57.9	74	88.4	2.18	LT-My-bi HSC
2	2	25.9	56.1	56.2	16.13	ST-Ly-bi HSC
3	4	57.0	20.8	7.3	3.82	ST-Bala HSC
4	3	49.4	25.4	8.1	14.79	ST-Ly-bi HSC
5	2	37.4	4.4	1.3	57.69	ST-Ly-bi HSC
6	2	3.7	1.4	0.6	10.24	ST-Ly-bi HSC
7	2	11.6	0	0.4	0	HPC
8	2	0.9	0.2	0	0	HPC
9	4	0	1.0	0	0	HPC
10	2	0	0.7	0	0	HPC

Data used in Figure 4. Myeloid-biased (My-bi), balanced (Bala), and lymphoid-biased (Ly-bi) HSCs were defined by the ratio of lymphocytes to myeloid cells (L/M ratio) in peripheral blood 6 months after transplantation. My-bi HSCs were defined by the L/M ratio < 3, Ly-bi HSCs were defined by the L/M ratio > 10, and Bala HSCs were defined by 3 < L/M < 10.[1,2] Long-term (LT) HSCs were defined when the percentage of myeloid cells maintained or increased by 6 months after transplantation. Short-term (ST) HSCs were defined when the percentage of myeloid cells decreased by 6 months, with myeloid, B-lymphoid, and T-lymphoid lineage reconstitution at a time after

transplantation.[3] Hematopoietic progenitor cells (HPCs) were defined when one or two lineages lacked from the definition of ST-HSCs.

Supplemental Table S7. Identification of My-bi, Bala, and Ly-bi HSCs 6 months after transplantation with cells from SCF + G-CSF single-cell culture.

Mouse ID	Cell No. on day 7	% CD45.1 cells			L/M ratio	HSC/HPC
		1 mon	3 mons	6 mons		
1	3	30.4	20.20	26.30	0.27	LT-My-bi HSC
2	3	0.8	4.1	2.2	0.26	LT-My-bi HSC
3	4	34.0	38.5	28	66.29	ST-Ly-bi HSC
4	6	39.4	2.3	1.5	154.0	ST-Ly-bi HSC
5	3	25.5	8.7	2.6	1.04	ST-My-bi HSC
6	3	14.2	2.6	1.0	158.38	ST-Ly-bi HSC
7	2	6.4	1.0	1.0	136.38	ST-Ly-bi HSC
8	8	7.0	1.4	0.6	39.40	ST-Ly-bi HSC
9	4	53.8	0	0	0	HPC
10	3	26.7	0	0	0	HPC
11	2	1.9	0.2	0	0	HPC
12	11	2.8	0	0	0	HPC

Data used in Figure 4. Myeloid-biased (My-bi), balanced (Bala), and lymphoid-biased (Ly-bi) HSCs were defined by the ratio of lymphocytes to myeloid cells (L/M ratio) in peripheral blood 6 months after transplantation. My-bi HSCs were defined by the L/M ratio < 3, Ly-bi HSCs were defined by the L/M ratio > 10, and Bala HSCs were defined by 3 < L/M < 10.[1,2] Long-term (LT) HSCs were defined when the percentage of myeloid cells maintained or increased by 6 months after transplantation. Short-term (ST) HSCs

were defined when the percentage of myeloid cells decreased by 6 months, with myeloid, B-lymphoid, and T-lymphoid lineage reconstitution at a time after transplantation.[3] Hematopoietic progenitor cells (HPCs) were defined when one or two lineages lacked from the definition of ST-HSCs.

Supplemental Table S8. Identification of My-bi, Bala, and Ly-bi HSCs 6 months after transplantation with cells from SCF + TPO single-cell culture.

Mouse ID	Cell No. on day 7	% CD45.1 cells			L/M ratio	HSC/HPC
		1 mon	3 mons	6 mons		
1	>50	12.7	1.5	0.9	82.07	ST-Ly-bi HSC
2	>100	9.8	3.4	1.2	112.47	ST-Ly-bi HSC
3	>100	7.4	2.1	1.2	254.17	ST-Ly-bi HSC
4	>100	0.2	0	0	0	HPC

Data used in Figure 4. Myeloid-biased (My-bi), balanced (Bala), and lymphoid-biased (Ly-bi) HSCs were defined by the ratio of lymphocytes to myeloid cells (L/M ratio) in peripheral blood 6 months after transplantation. My-bi HSCs were defined by the L/M ratio < 3, Ly-bi HSCs were defined by the L/M ratio > 10, and Bala HSCs were defined by 3 < L/M < 10.[1,2] Long-term (LT) HSCs were defined when the percentage of myeloid cells maintained or increased by 6 months after transplantation. Short-term (ST) HSCs were defined when the percentage of myeloid cells decreased by 6 months, with myeloid, B-lymphoid, and T-lymphoid lineage reconstitution at a time after transplantation.[3] Hematopoietic progenitor cells (HPCs) were defined when one or two lineages lacked from the definition of ST-HSCs.

Supplemental Figure Legends

Supplemental Figure S1. The relationship between different classifications of HSCs and HPCs.

(A) The relationship of HSC1 with HSCs from the classification by Wilson *et al*..[4] HSC2 and HPC1 did not have corresponding populations in their classification.

(B) The relationship of HPC2 and HPC3 with MPP2 and MPP3 from the classification by Wilson *et al*.,[4] and HPC4 with MPP4 from the classification by Adolfsson *et al*..[5] HPC2 contained MPP1 and MPP2. HPC3 overlapped MPP3, and HPC4 overlapped MPP4.

Supplemental Figure S2. Heatmaps of single-cell RT-PCR of six populations.

Heatmaps of single HSC1 cells (A), HSC2 cells (B), HPC1 cells (C), HPC2 cells (D), HPC3 cells (E), and HPC4 cells (F).

Each column represents a specific gene, and each row represents an individual cell. Color scale reflects the threshold cycle (Ct) values. Gene set is shown in Supplemental Table S3.

Supplemental Figure S3. Heatmaps of single-cell RT-PCR of cultured cells.

(A) Heatmap of freshly isolated single HSC1 cells.

(B) Heatmap of single cells from the SCF culture.

(C) Heatmap of single cells from the SCF+G-CSF culture.

(D) Heatmap of single cells from the SCF+TPO culture.

Each column represents a specific gene, and each row represents an individual cell. Color scale reflects the threshold cycle (Ct) values. Gene set is shown in Supplemental

Table S4.

Supplemental Figure S4. Single-cell RT-PCR of cultured HSC1 cells.

(A) Gene expression in a single cell. A column represents one gene, and a row represents a single cell. Gene-expressing cells are shown as dots, which are defined by the threshold cycle (Ct) value < 27.65.

(B) Violin density plots show the relative gene expression levels of gene-expressing cells. The relative expression level is defined as the (27.65-Ct) values.

Statistical significance was analyzed by ANOVA with Tukey's multiple comparisons test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$. ns, no significance.

Supplemental References

1. Muller-Sieburg CE, Cho RH, Karlsson L, Huang JF, Sieburg HB. Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. Blood. 2004;103(11):4111-8.

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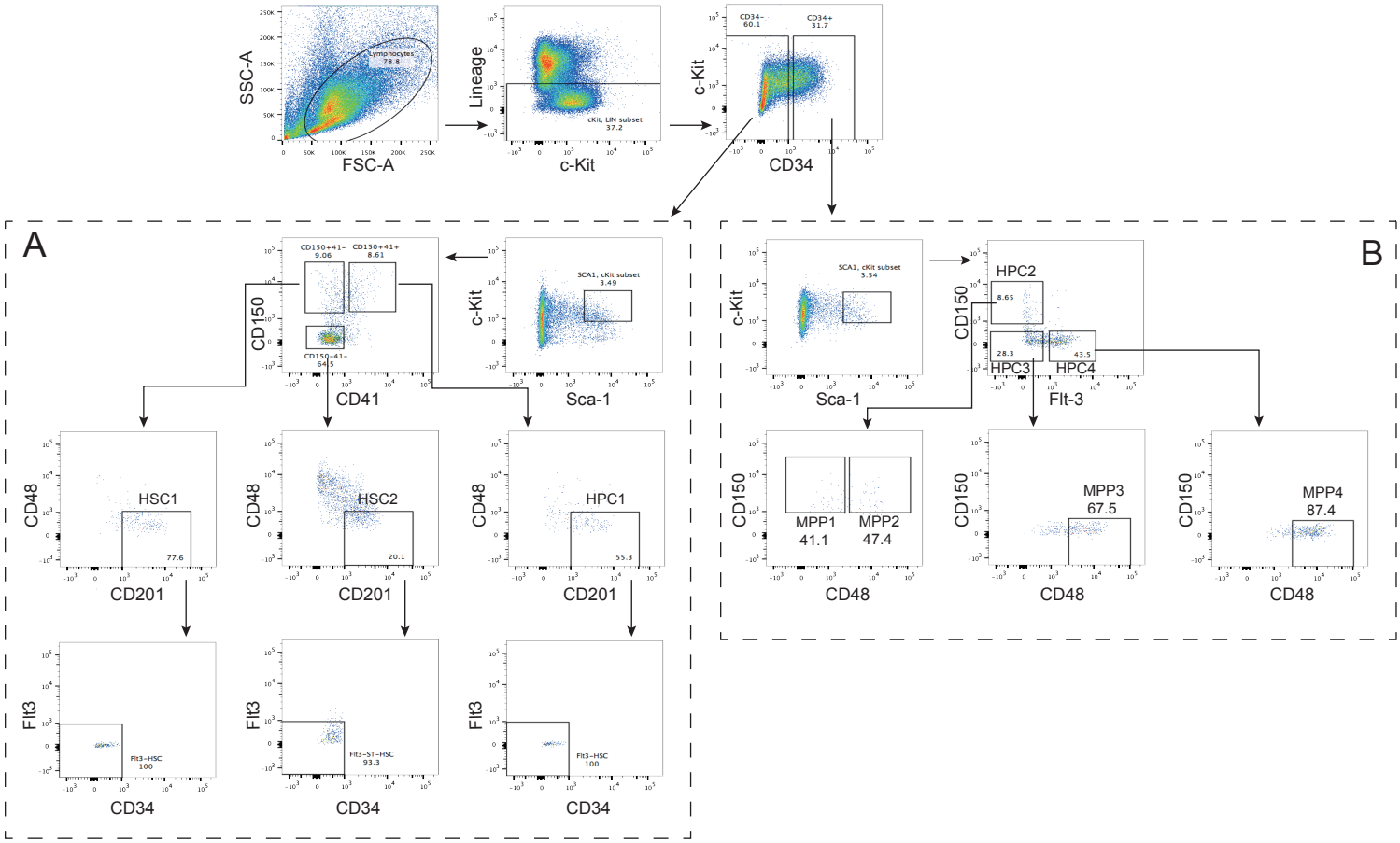
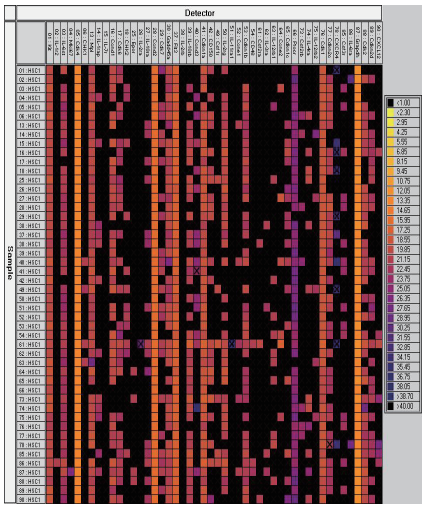
4. Wilson A, Laurenti E, Oser G, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell. 2008;135(6):1118-29.

5. Adolfsson J, Månsson R, Buza-Vidas N, et al. Identification of Flt3+ Lympho-Myeloid Stem Cells Lacking Erythro-Megakaryocytic Potential. Cell. 2005;121(2):295-306.



Xie_Supplemental Fig.S1

A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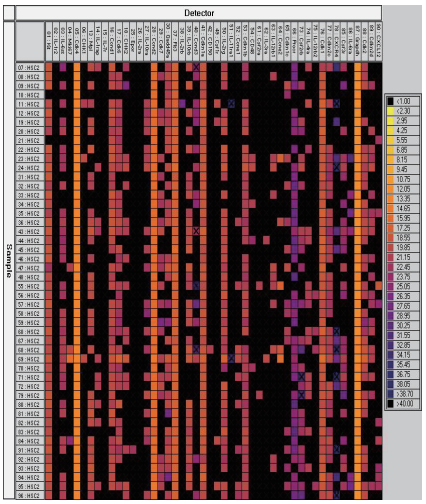
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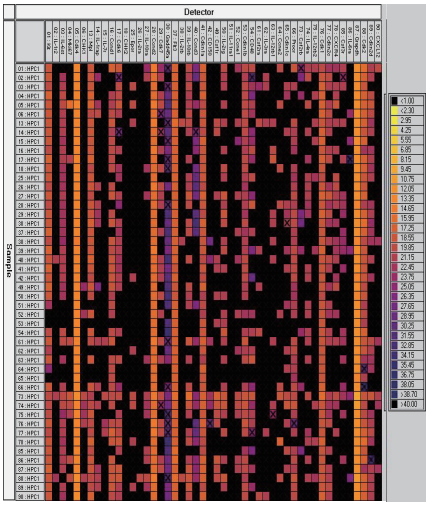
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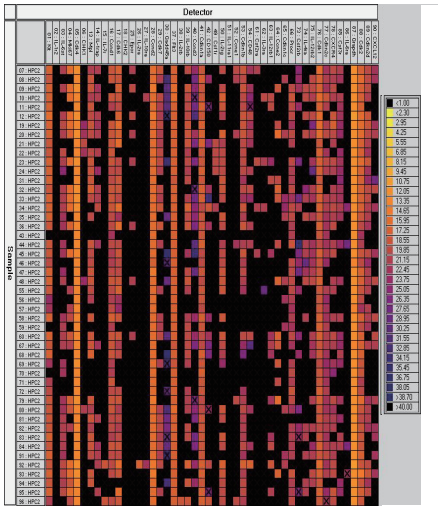
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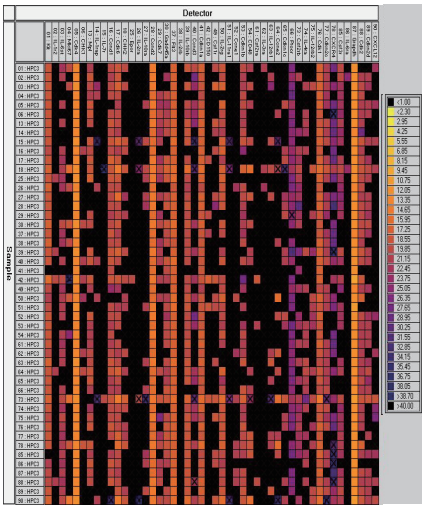
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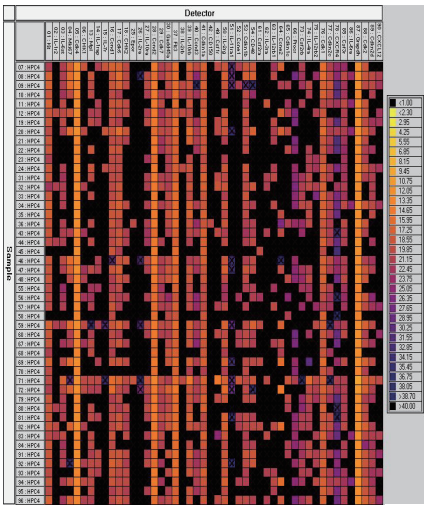
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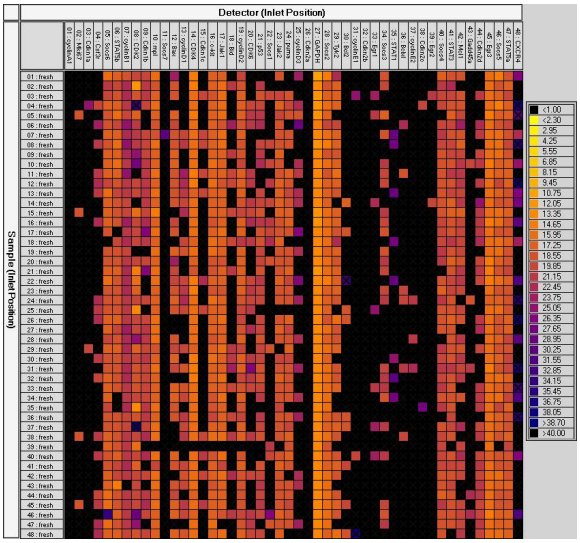
A



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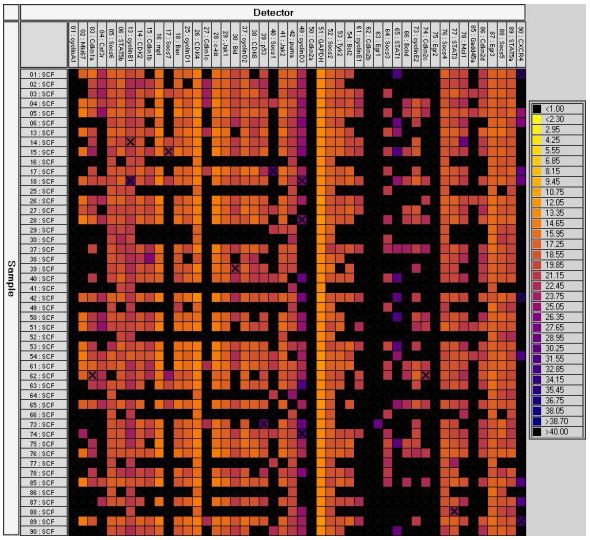

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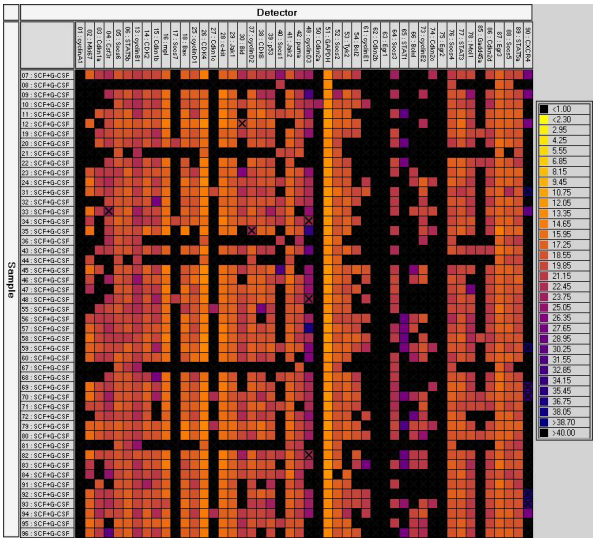

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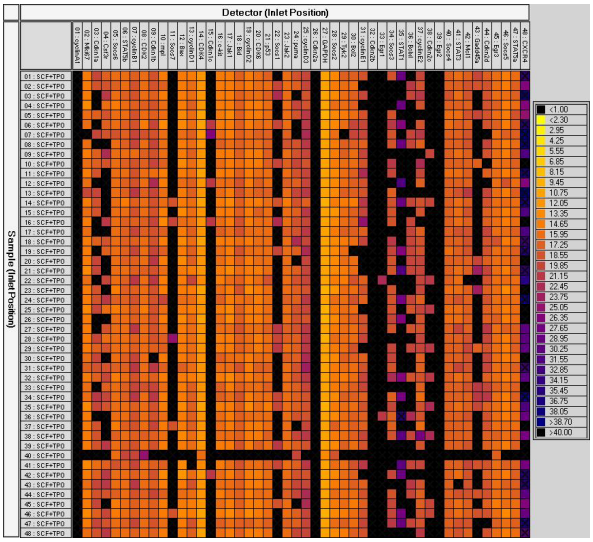

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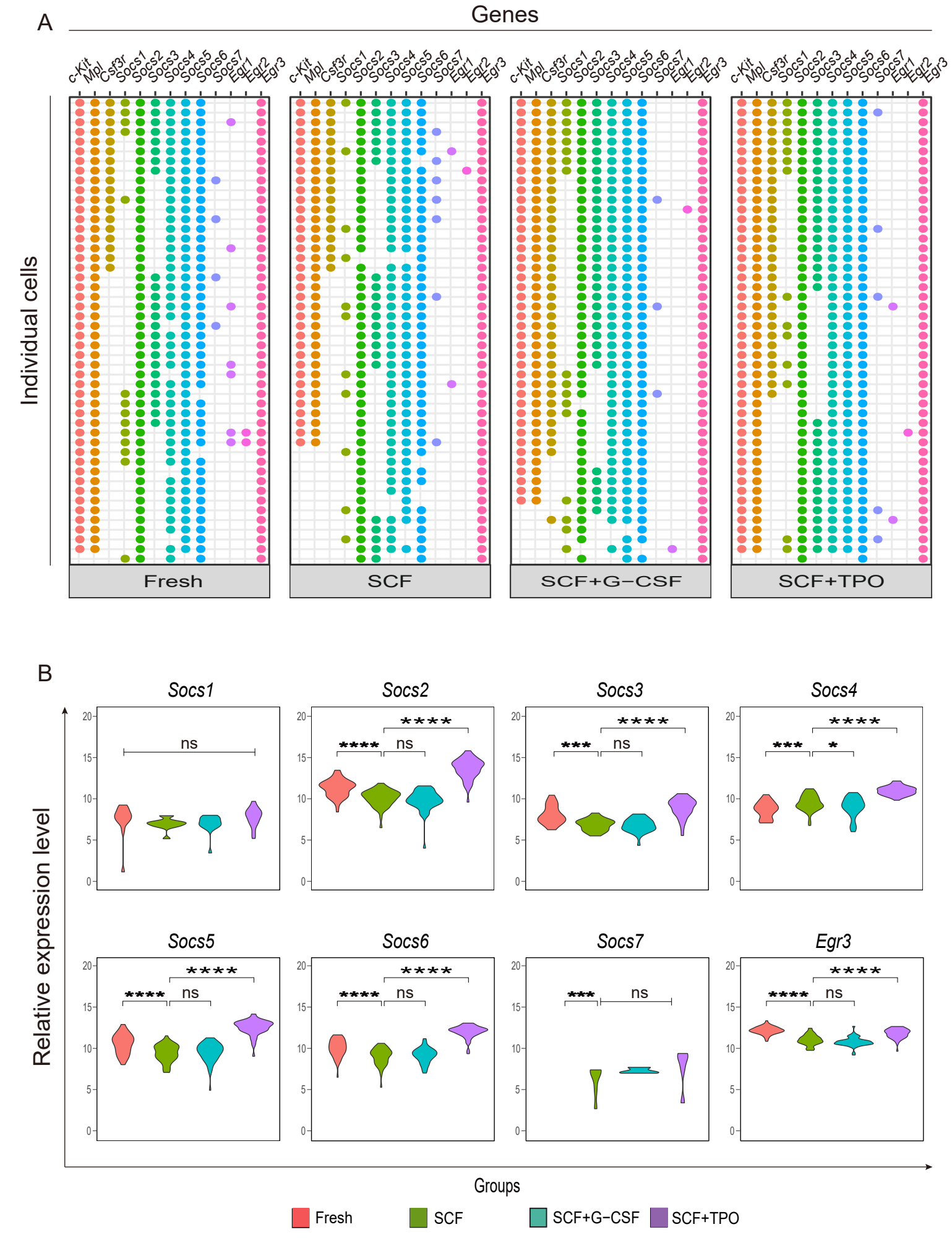

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