Supplemental Information

Early Development of Definitive Erythroblasts from Human Pluripotent Stem Cells Defined by Expression of Glycophorin A/CD235a, CD34, and CD36

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Figure S1

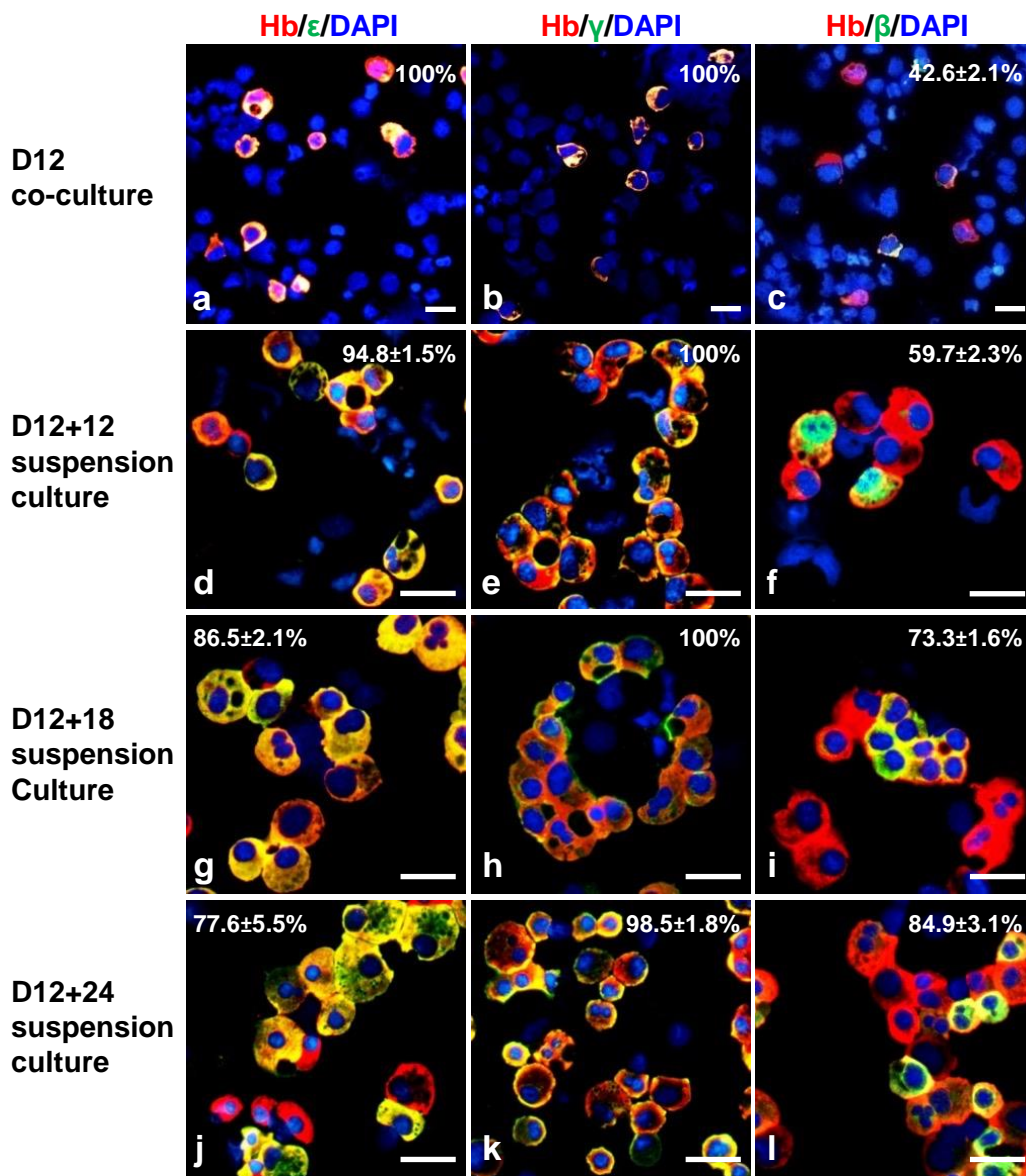|	Hb/ε/DAPI	Hb/γ/DAPI	Hb/β/DAPI
D12 co-culture	**a** 100%	**b** 100%	**c** 42.6±2.1%
D12+12 suspension culture	**d** 94.8±1.5%	**e** 100%	**f** 59.7±2.3%
D12+18 suspension Culture	**g** 86.5±2.1%	**h** 100%	**i** 73.3±1.6%
D12+24 suspension culture	**j** 77.6±5.5%	**k** 98.5±1.8%	**l** 84.9±3.1%

Figure S1, Related to Figure 1. Changes in the expression of human hemoglobin in H1/mAGM-S3 co-culture-derived cells over time.

Immunostaining of embryonic ε-globin, fetal γ-globin and adult β-globin in erythroblasts derived from day 12 H1/AGM-S3 co-culture and subsequent suspension culture. (Red, Cy3; Green, FITC; Blue, DAPI; independent experiments, n=3; mean±SD; bar=20um)
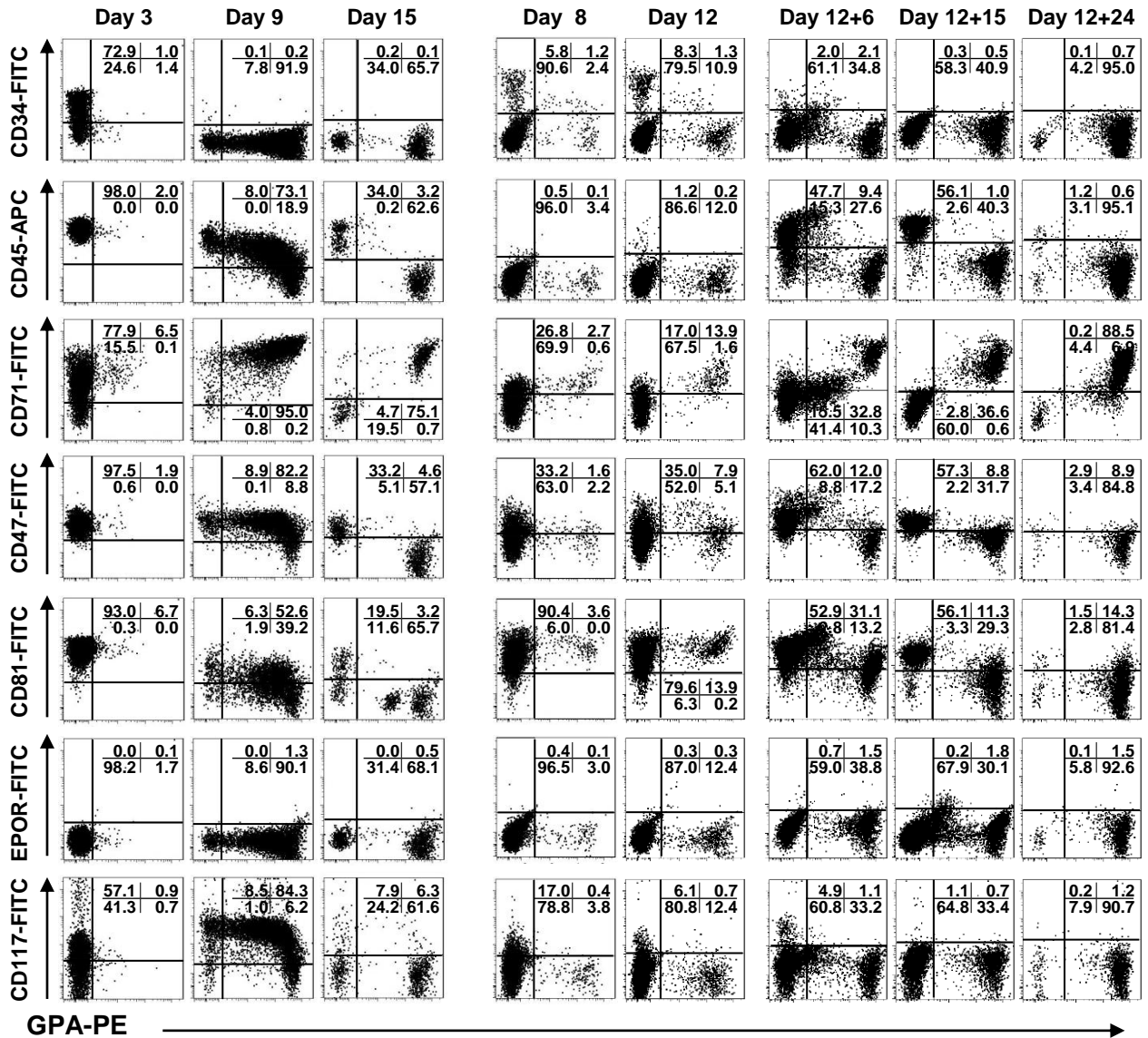
Figure S2

A hCB-CD34+ HSPC-derived cells

B H1/AGM-S3-derived cells

Co-culture cells   *Suspension culture cells*

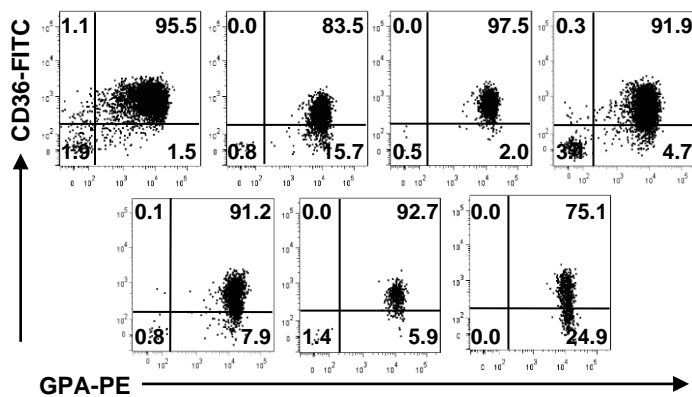**C** H1/AGM-S3-derived BFU-E colonies

Figure S2, Related to Figure 2. Changes of erythroid-lineage surface markers on cells derived from different origins.

(A) Representative FC profiles showing changes of GPA, CD34, CD45, CD71, CD47, CD81, EPO-R and CD117(c-Kit) on hCB-CD34^{+} HSPCs-derived cells over time.

(B) Representative FC profiles showing expression changes of GPA, CD34, CD45, CD71, CD47, CD81, EPO-R and CD117(c-kit) on H1/AGM-S3-derived co-culture cells and suspension culture cells from day 12 co-cultures over time.
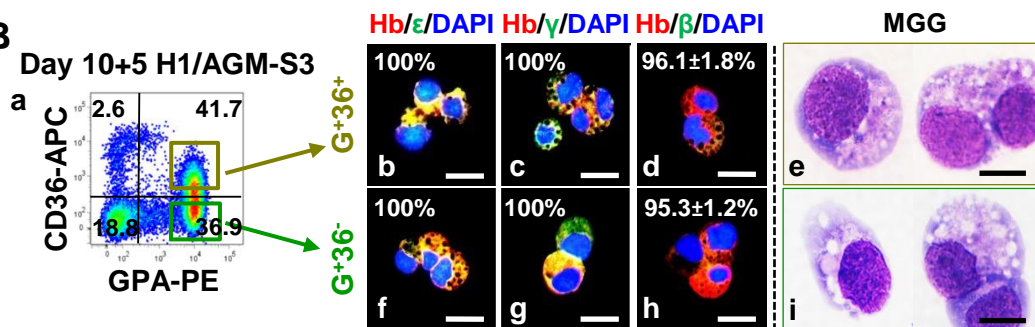
(C) Co-expression of GPA and CD36 on cells in BFU-E colonies. BFU-E colonies were derived from day 12 H1/AGM-S3 co-culture and re-cultured in semi-solid medium for 14 days. Individual BFU-E colonies were randomly picked up for FC analysis (colony numbers, n=7).
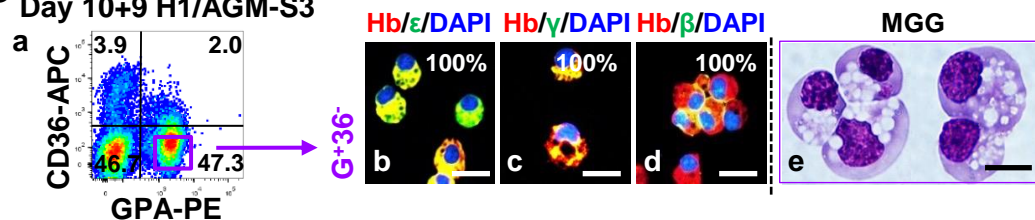
Figure S3

A Sorted from day 10+5 H1/AGM-S3 and re-cultured
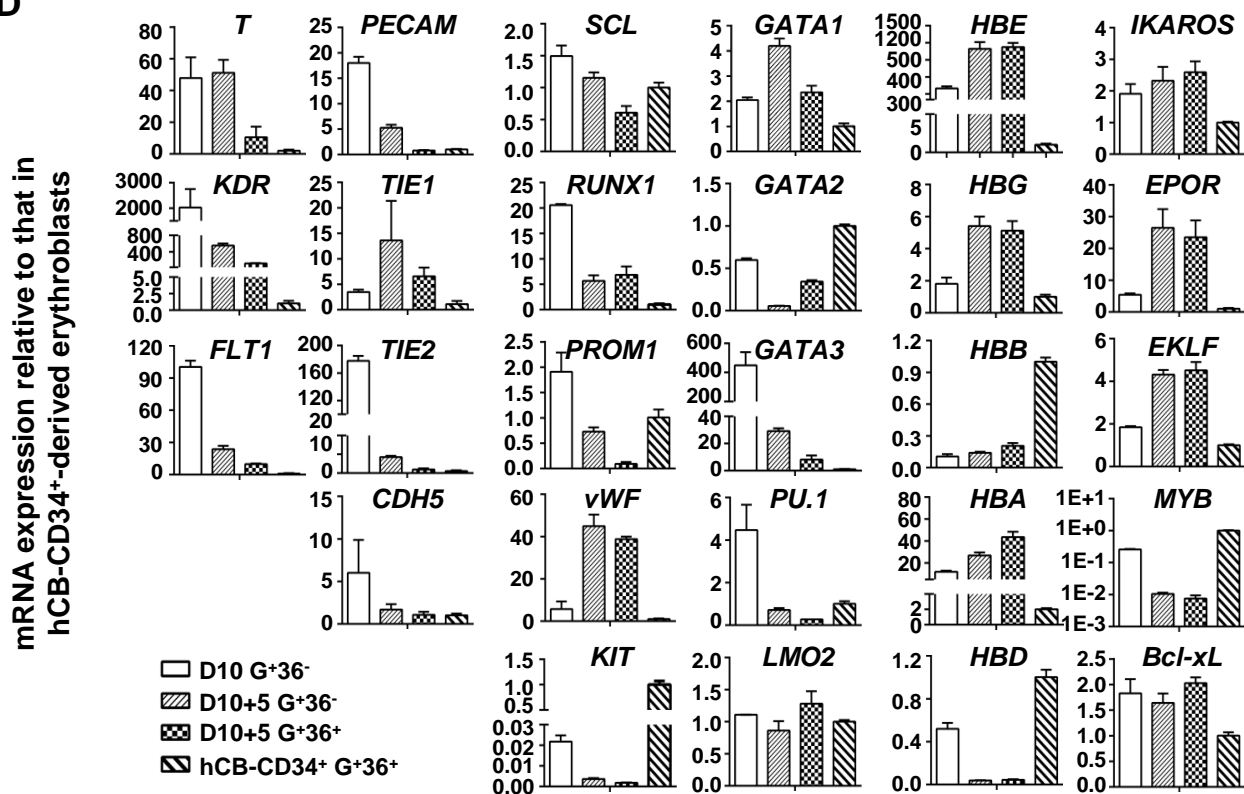

B

C
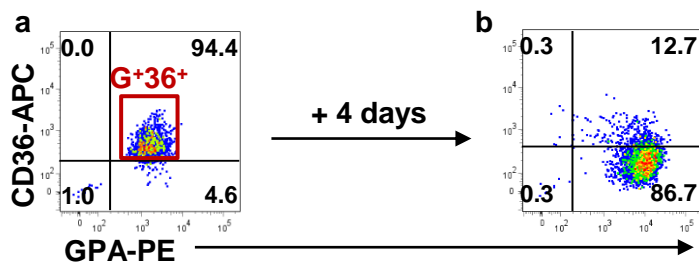
D

Figure S3, Related to Figure 5. Erythroid cell fractions sorted from day 10+5 and day 10+9 cultures defined by expression of GPA and CD36.

(A) (a) FC profile showing the purity of sorted GPA$^+$CD36$^+$ (G$^+$36$^+$) cell fraction from day 10+5 H1/AGM-S3 co-culture. (b) FC profile showing the expression of CD36 on the progeny of sorted G$^+$CD36$^+$ cells re-cultured in SFEM supplemented with 100 ng/mL SCF, 5 ng/mL IL-3, 4 IU/mL EPO and 10^{-6} M dexamethasone for additional 4 days.

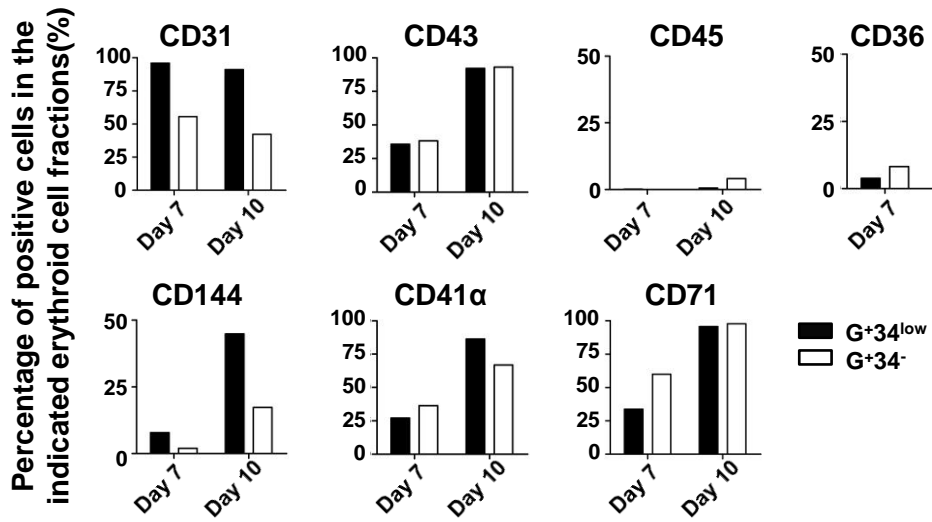(B) (a) GPA$^+$CD36$^+$ (G$^+$36$^+$) and GPA$^+$CD36$^-$ (G$^+$36$^-$) cell fractions were sorted by FACS from day 10+5 H1/AGM-S3 co-culture. IF analysis showing co-expression of human Hb and ε-, γ- and β-globins in G$^+$36$^+$ (b–d) and G$^+$36$^-$ (f–h) erythroid cell fractions (bar=20μm; independent experiments, n=3; mean±SD). (e, i) MGG staining showing the morphology of G$^+$36$^+$ and G$^+$36$^-$ cell fractions (bar=10μm).

(C) (a) The G$^+$36$^-$ cell fraction was sorted by FACS from day 10+9 cultures. (b–d) IF analysis showing co-expression of human Hb and ε-, γ- and β-globins in the G$^+$36$^-$ erythroid cell fraction (bar=20μm; independent experiments, n=3; mean±SD). (e) MGG staining showing the morphology of the G$^+$36$^-$ cell fraction from day 10+9 cultures (bar=10μm).
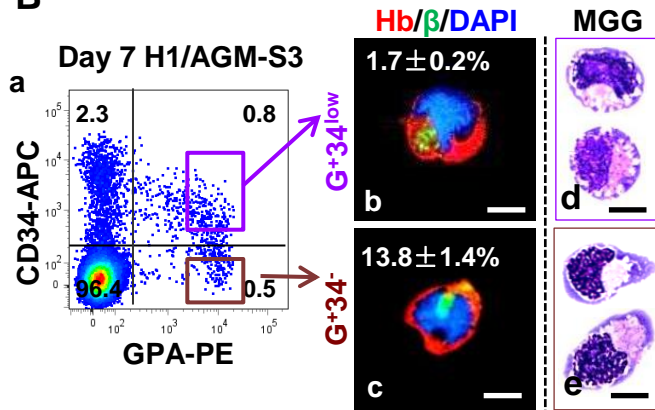
(D) Quantitative RT-PCR (qRT-PCR) analysis of hESC-derived induced erythroid cell fractions defined by expression of GPA and CD36. hCB-CD34$^+$ HSPC-derived erythroblasts (hCB-CD34$^+$ G$^+$36$^+$) were used as the control, which were representative of adult definitive erythroblasts. hCB-CD34$^+$ HSPCs differentiated into erythroid cells after 11 days and the purity of erythroid cells was almost 90%. Other erythroid cell fractions including GPA$^+$CD36$^-$ cells on day 10 (D10 G$^+$36$^-$) , G$^+$36$^-$ and G$^+$36$^+$ cells on day 10+5(D10+5 G$^+$36$^+$; D10+5 G$^+$36$^-$) and G$^+$36$^-$ cells on day 10+9 (D10+9 G$^+$36$^-$) were sorted by FACS. The purity of each sorted fraction was more than 90%. qRT-PCR analysis of transcripts in indicated cell fractions (independent experiments, n=3, mean±SD). The relative mRNA expression was normalized to *GAPDH* mRNA of each reaction. Each sample was compared to hCB-CD34$^+$ HSPC-derived erythroblasts.

Figure S4

A

Percentage of positive cells in the indicated erythroid cell fractions(%)

CD31

CD43

CD45

CD36

CD144

CD41α

CD71

■ G+34^low
□ G+34-

B

a Day 7 H1/AGM-S3

CD34-APC vs GPA-PE

2.3   0.8

96.4   0.5

G+34^low

G+34-

Hb/β/DAPI   MGG

b 1.7±0.2%   **d**

c 13.8±1.4%   **e**

C Daughter cells of G-34+ cells from day 10 co-culture

MGG

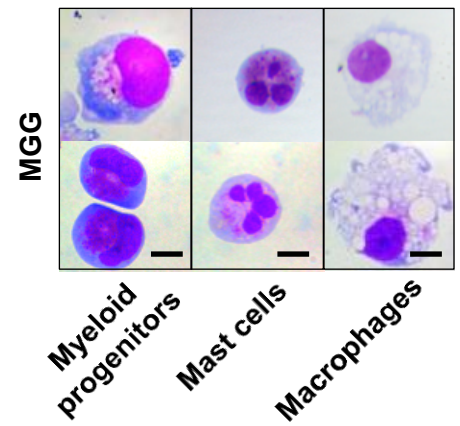Myeloid progenitors   Mast cells   Macrophages

Figure S4, Related to Figure 6. Characteristics of cells defined by GPA and CD34 from day 7 and day 10 co-culture.

(A) FC analysis showing representative phenotypic expression on cell fractions GPA$^+$CD34^{low} (G$^+$34^{low}) and GPA$^+$CD34$^-$ (G$^+$34$^-$) cells in day 7 and day10 co-culture, including endothelial, hematopoietic and erythroid lineage related surface makers.

(B) G$^+$34^{low} and G$^+$34$^-$ cell fractions in day 7 H1/AGM-S3 co-culture. (a) G$^+$34^{low} and G$^+$34$^-$ cell fractions were sorted by FACS from day 7 H1/AGM-S3 co-culture. (b, c) IF analysis showing co-expression of human Hb and β-globin in G$^+$34^{low} and G$^+$34$^-$ cell fractions (bar=10 μm; independent experiments, n=3; mean±SD). (d, e) MGG staining showing the morphology of G$^+$34^{low} and G$^+$34$^-$ cell fractions (bar=10 μm).

(C) G$^-$34$^+$ cells were sorted from day 10 co-culture and recultured in myeloid supporting medium for 6 days. MGG staining showing the morphology of their daughter cells (bar=10 μm).

Table S1. Antibodies used for flow cytometric analysis

Antigen	Fluor chrome Conjugated	Source	Clone	Isotype	Cat. No.
CD117	APC	eBioscience	YB5-B8	Ms IgG$_1$, κ	17-1179-42
CD144	FITC	BD	55-7H1	Ms IgG$_1$, κ	560411
CD31	FITC	BD	WM59	Ms IgG$_1$, κ	555445
CD34	FITC	BD	581	Ms IgG$_1$, κ	555821
CD36	FITC	eBioscience	eBioNL07	Ms, IgM	11-0369-42
CD36	FITC	BD	CB38	Ms IgG$_1$, κ	555454
CD36	FITC	BioLegend	5-271	Ms IgG2a, κ	336204
CD36	FITC	BECKMAN	FA6.152	Ms IgG1	PNIM0766U
CD36	APC	BioLegend	5-271	Ms IgG2a, κ	336208
CD41a	FITC	BD	HIP8	Ms IgG1, κ	555466
CD43	FITC	BD	1G10	Ms IgG1, κ	555475
CD45	APC	BioLegend	HI30	Ms IgG1, κ	304012
CD47	FITC	BD	B6H12	Ms IgG1, κ	556045
CD71	FITC	BD	M-A712	Ms IgG2a, κ	555536
CD81	FITC	BD	JS-81	Ms IgG1, κ	551108
EPO-R	FITC	R&D	38409	Ms IgG2b	FAB307F
GPA	PE	Dako	JC159	Ms, MoAb	R7078
GPA	PE	BD	GA-R2	Ms, IgG2b	555570
7-AAD		BD			559925

Table S2. Antibodies used for Hb immunostaining

Antigen	Source	Specificity	Cat. No.
Hemoglobin	Bethyl	Goat to Human	A80-134A
ε-globin	FITCCORTEX Biochem	Mouse to Human	CR8008M
γ-globin	Santa Cruz Biotech	Mouse to Human	sc-21756
β-globin	Santa Cruz Biotech	Mouse to Human	sc-21757
FITC-conjugated Secondary Ab	Jackson Immuno Research	Donkey to Goat	705-095-003
FITC-conjugated Secondary Ab	Jackson Immuno Research	Donkey to Mouse	715-095-150
Cy3-conjugated Secondary Ab	Jackson Immuno Research	Donkey to Goat	705-165-003

Table S3. Primers used for qRT-PCR

Gene	Direction	Sequences
T	Forward	5' CCT TCA GCA AAG TCA AGC TCA CC 3'
	Reverse	5' TGA ACT GGG TCT CAG GGA AGC A 3'
LMO2	Forward	5' GCG CCT CTA CTA CAA ACT GGG C 3'
	Reverse	5' CTC ATA GGC ACG AAT CCG CTT 3'
KDR	Forward	5' GGA ACC TCA CTA TCC GCA GAG 3'
	Reverse	5' CCA AGT TCG TCT TTT CCT GGG C 3'
FLT1	Forward	5' CCTGCAAGATTCAGGCACCTATG 3'
	Reverse	5' GTT TCG CAG GAG GTA TGG TGC T 3'
Tie-1	Forward	5' GAC GCA CCT TCA CCT ACC A 3'
	Reverse	5' GAG GCA TAC TCT TTC AGC ATT T 3'
Tie-2	Forward	5' GGT CAA GCA ACC CAG CCT TTT C 3'
	Reverse	5' CAG GTC ATT CCA GCA GAG CCA A 3'
CDH5	Forward	5' GAC CTC TCT GTG AAG CAA CTG C 3'
	Reverse	5' CAC ATT GTC ACG GTA GTT GGT GG 3'
vWF	Forward	5' CCT TGA ATC CCA GTG ACC CTG A 3'
	Reverse	5' GGT TCC GAG ATG TCC TCC ACA T 3'
c-Kit	Forward	5' CAC CGA AGG AGG CAC TTA CAC A 3'
	Reverse	5' CAC CGA AGG AGG CAC TTA CAC A 3'
PECAM1	Forward	5' AAG TGG AGT CCA GCC GCA TAT C 3'
	Reverse	5' ATG GAG CAG GAC AGG TTC AGT C 3'
GATA1	Forward	5' CAC GAC ACT GTG GCG GAG AAA T 3'
	Reverse	5' TTC CAG ATG CCT TGC GGT TTC G 3'
GATA2	Forward	5' CAG CAA GGC TCG TTC CTG TTC A 3'
	Reverse	5' ATG AGT GGT CGG TTC TGC CCA T 3'
GATA3	Forward	5' ACC ACA ACC ACA CTC TGG AGG A 3'
	Reverse	5' TCG GTT TCT GGT CTG GAT GCC T 3'
PU.1	Forward	5' GAC ACG GAT CTA TAC CAA CGC C 3'
	Reverse	5' CCG TGA AGT TGT TCT CGG CGA A 3'
IKAROS	Forward	5' CGG CTT TGT CGG GAG TTG 3'
	Reverse	5' GCC CTT CTG GGT GAA TGA G 3'
RUNX1	Forward	5' CCA CCT ACC ACA GAG CCA TCA A 3'
	Reverse	5' TTC ACT GAG CCG CTC GGA AAA G 3'
SCL	Forward	5' GAC ACA GTG CAA GCT GGA AGA C 3'
	Reverse	5' AGT CAG GCT CTT GAT CCT CAC C 3'
PROM1	Forward	5' TTG TGG CAA ATC ACC AGG TA 3'
	Reverse	5' TCA GAT CTG TGA ACG CCT TG 3'
EKLF	Forward	5' TTG CGG CAA GAG CTA CAC CAA G 3'
	Reverse	5' GTA GTG GCG GGT CAG CTC GTC 3'
EPOR	Forward	5' GCC TCT TCA CCA CC CAC AA 3'
	Reverse	5' TCC ACT GCC TGC ATC GTC 3'
MYB	Forward	5' GGG AAC AGA TGG GCA GAA ATC G 3'
	Reverse	5' GCTGGCTTTTGAAGACTCCTGC 3'
HBE	Forward	5' AAC CTC AAG CCC GCC TTT GCT A 3'
	Reverse	5' GGT GAA CTC CTT GCC AAA GTG AG 3'
HBG	Forward	5' GGA AGA TGC TGG AGG AGA AAC C 3'
	Reverse	5' GTC AGC ACC TTC TTG CCA TGT G 3'
HBB	Forward	5' CAC CTT TGC CAC ACT GAG TGA G 3'
	Reverse	5' CCA CTT TCT GAT AGG CAG CCT G 3'
HBA	Forward	5' GAC CTG CAC GCG CAC AAG CTT 3'
	Reverse	5' GCT CAC AGA AGC CAG GAA CTT G 3'
HBD	Forward	5' GCT CAT GGC AAG AAG GTG CTA G 3'
	Reverse	5' ACA CCA GCA CAT TGC CCA AGA G 3'
BCL-xL	Forward	5' TGA CCA CCT AGA GCC TTG GA 3'
	Reverse	5' CTG AAG AGT GAG CCC AGC AG 3'