Inventory of Supplemental Materials:

Figure S1, related to Figure 2. Prom1-Sca1-CD34- cells are displayed for the expression of CD49f, CD31, CD105, VeCad, Tie2, CD41 and cKit as a control for the data displayed in Figure 2.

Figure S2, related to Figure 4. mRNA-Seq Profiling Supporting Data, Expression of Surface Markers, Transcription Factors and Gene Set Enrichment Analysis.

Figure S3, related to Figure 5. mRNA-Seq Integration Supporting Data, Clustering Methods, Pathways and Gene Ontologies.

Figure S4, related to Figure 6. Prom1-positive cells during mid-gestation contribute to adult myeloid and lymphoid lineages. Homozygous Prom1-CreERT2 mice were crossed with R26StopYFP mice and Cre recombinase was activated with 4-hydroxytamoxifen at E10.5. The progeny were analyzed by flow cytometry for the presence of YFP positive hematopoietic cells.

Figure S5, related to Figure 7. PS34 Cells Generate Mixed Colonies *in vitro* after co-cultures with OP-9 that Contain Megakaryocytes, Granulocytes, Functional Macrophages and Definitive Erythroblasts.

Figure S6, related to Figure 7. PS34 Cells Generate B and T cell precursors after Co-culture with OP-9DL1 cells.

Figure S7, related to Figure 7. PS34 Cells Do Not Directly Engraft Adult Mice. E10.5 to E12.5 placentas were dissociated and PS34 cells isolated and transplanted into congenic or immunodeficient mice.

Table S1, related to Figure 2D. Numbers of Placental-derived Cells Tested in Methylcellulose Colony Forming Assays.

Table S2, related to Figures 4 and 5. mRNA-seq data and analyses.

Table S3, related to Figure 5. Top 30 Functional Annotation Clusters in HPs and HSPCs.

Table S4, related to Figures 3 and 5. Frequency of cells positive for endothelial and trophoblastic markers.

Supplemental Experimental Procedures

Supplemental References

Supplemental Figures

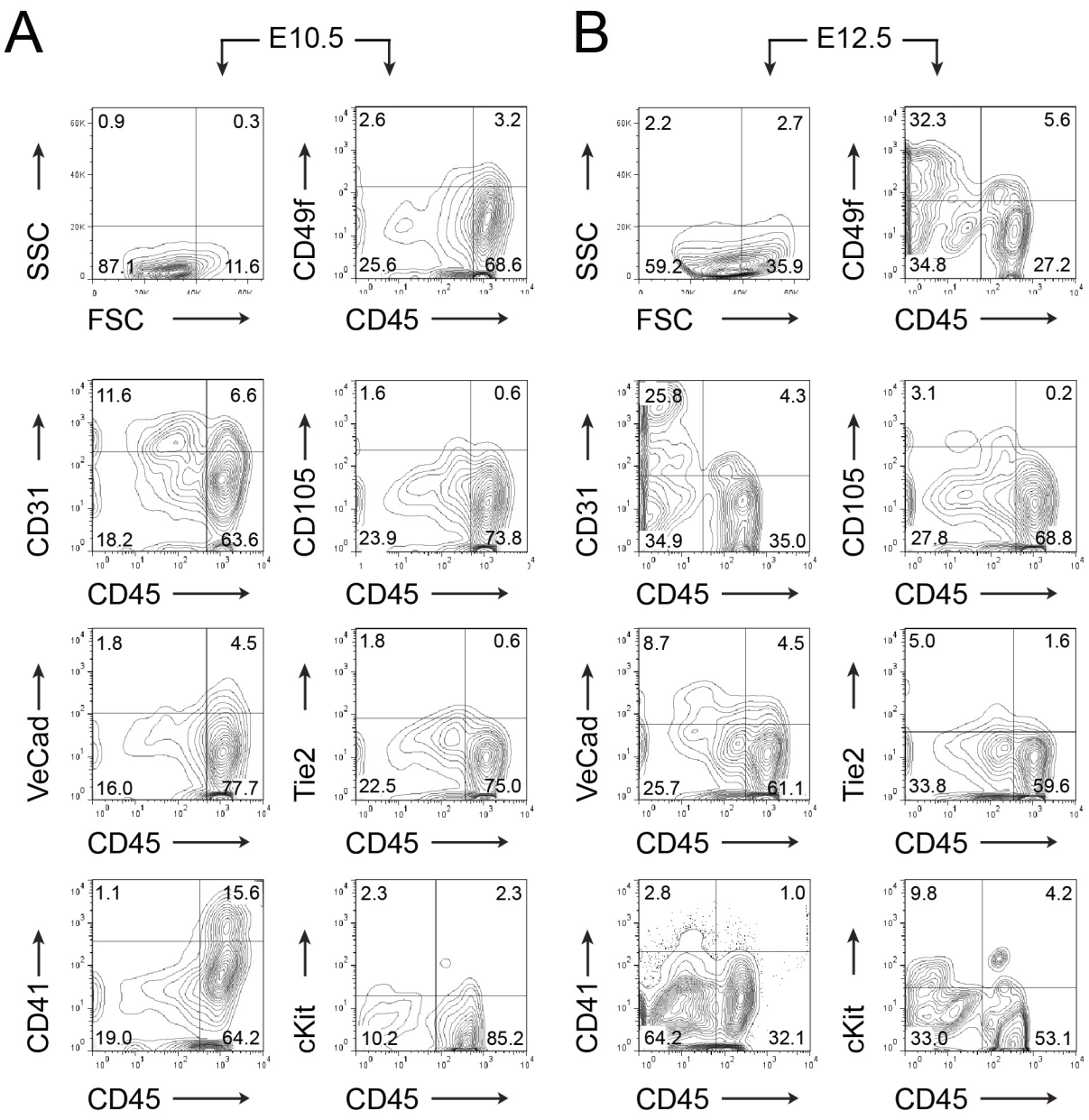


Figure S1. PS34 Cells Express Endothelial and Hematopoietic Markers, Related to Figure 2. (**A**) E10.5 and (**B**) E12.5 placentas were isolated and dissociated to a single cell suspension and analyzed. Prom1-Sca1-CD34- cells are displayed for the expression of CD49f, CD31, CD105, VeCad, Tie2, CD41 and cKit as a control for the data displayed in Figure 2.

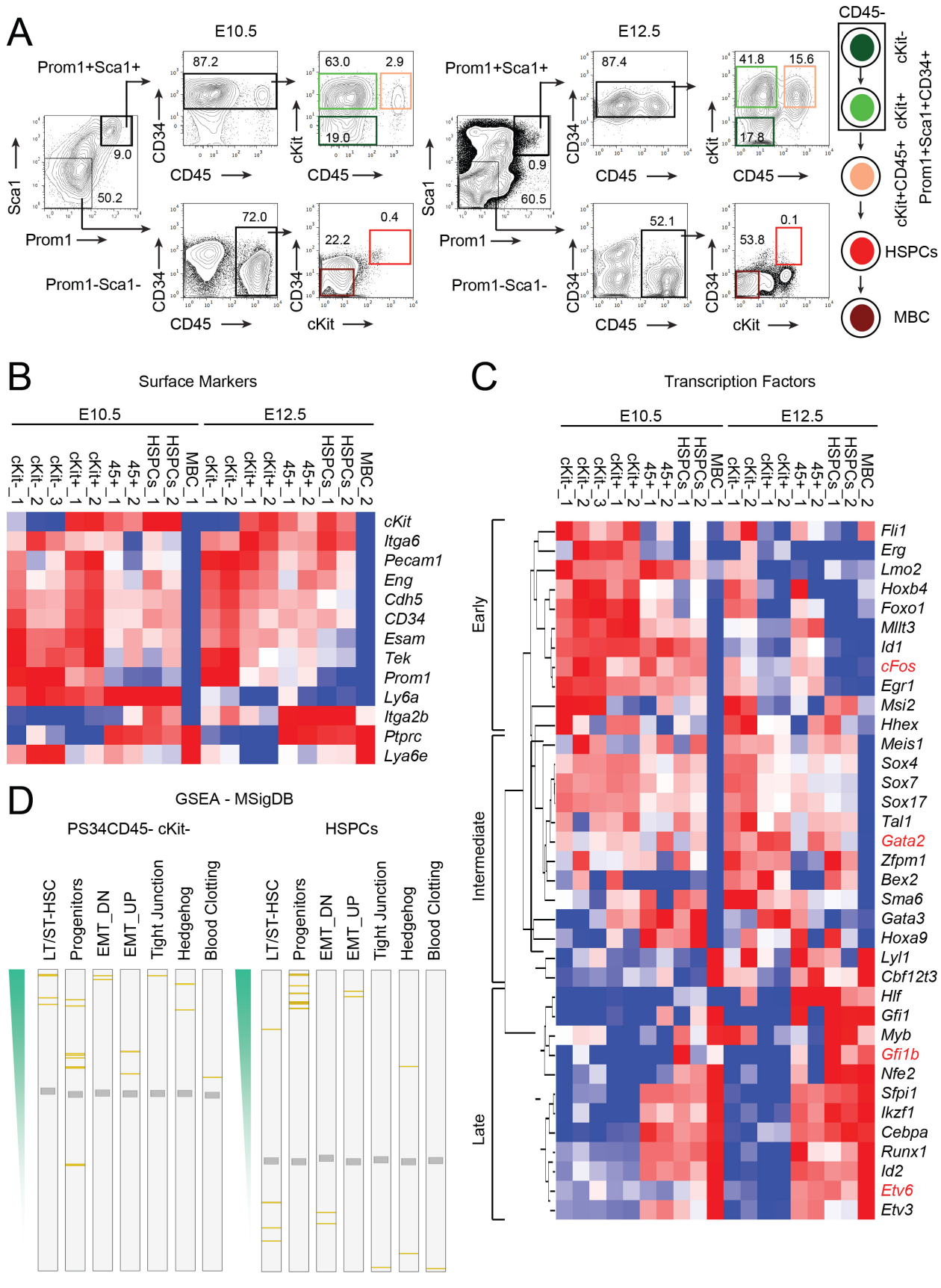
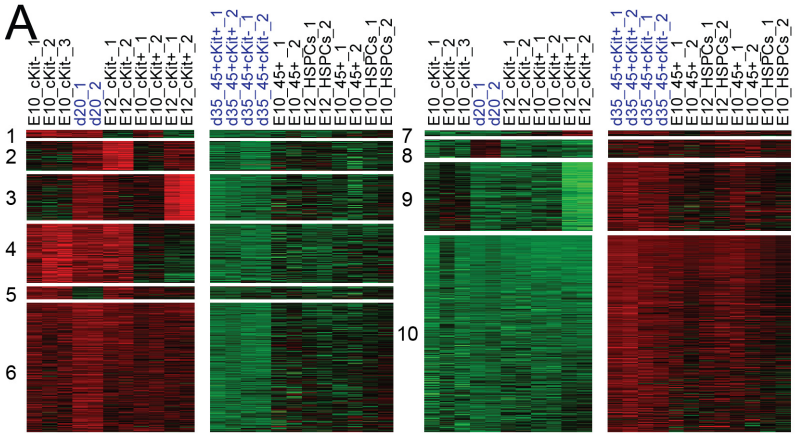

A
E10.5
E12.5
Prom1+Sca1+
Prom1-Sca1-
Sca1
Prom1
CD34
CD45
cKit
87.2
9.0
50.2
63.0
2.9
19.0
72.0
22.2
0.4
87.4
0.9
60.5
41.8
15.6
17.8
52.1
53.8
0.1
CD45-
cKit-
cKit+
cKit+CD45+
Prom1+Sca1+CD34+
HSPCs
MBC
B
Surface Markers
E10.5
E12.5
cKit_1
cKit_2
cKit_3
cKit+_1
cKit+_2
45+_1
45+_2
HSPCs_1
HSPCs_2
MBC_1
MBC_2
cKit
Itga6
Pecam1
Eng
Cdh5
CD34
Esam
Tek
Prom1
Ly6a
Itga2b
Ptprc
Lya6e
C
Transcription Factors
Early
Intermediate
Late
Fli1
Erg
Lmo2
Hoxb4
Foxo1
Mllt3
Id1
cFos
Egr1
Msi2
Hhex
Meis1
Sox4
Sox7
Sox17
Tal1
Gata2
Zfpm1
Bex2
Sma6
Gata3
Hoxa9
Lyl1
Cbf12t3
Hlf
Gfi1
Myb
Gfi1b
Nfe2
Sfpi1
Ikzf1
Cebpa
Runx1
Id2
Etv6
Etv3
D
GSEA - MSigDB
PS34CD45- cKit-
HSPCs
LT/ST-HSC
Progenitors
EMT_DN
EMT_UP
Tight Junction
Hedgehog
Blood Clotting

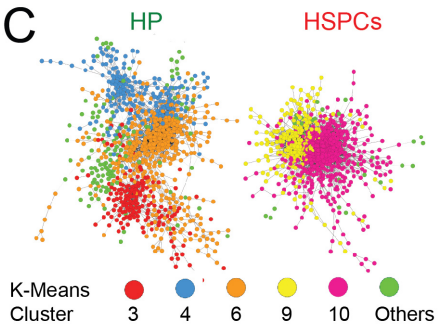
Figure S2. mRNA-Seq Profiling Supporting Data, Expression of Surface Markers, Transcription Factors and Gene Set Enrichment Analysis, Related to Figure 4. (**A**) Hemogenic PS34 cells from E10.5 and E12.5 placentas were fractioned into CD45-cKit-, CD45-cKit+ and CD45+cKit+ populations (upper panels). HSPCs (CD45+cKit+CD34+) and mature blood cells (CD45+cKit-CD34-) were also sorted as controls (lower panels). (**B**) Global gene-expression levels were profiled by mRNA-seq (biological replicates: 1, 2 and 3). Heatmap shows the expression of endothelial and hematopoietic cell surface markers. Red indicates increased expression, whereas blue indicates decreased expression over the mean. (**C**) Heatmap showing the expression of transcription factors associated with HSC biology. Unsupervised hierarchical clustering was applied. Transcriptions factors are grouped according to the stage of endothelial-to-hematopoietic transition. Early, intermediate and late groups are highlighted. Gene expression data were analyzed by Cluster 3.0 and displayed by Treeview. Red indicates increased expression, whereas blue indicates decreased expression over the mean. The hemogenic programming transcription factors Gata2, cFos, Gfi1b and Etv6 are highlighted in red. (**D**) Gene set enrichment analysis (GSEA) for hemogenic PS34CD45-cKit- cells and HSPCs. Gene-expression lists were analyzed for enrichment of gene sets present in the Molecular Signatures Database (MSigDB; 1,888 gene sets, gene size 0-5,000). Gene sets are ordered according to the normalized enrichment score (NES) and the grey horizontal bar shows the enrichment cut-off (FDR = 0.25). Orange lines highlight the labelled gene sets.

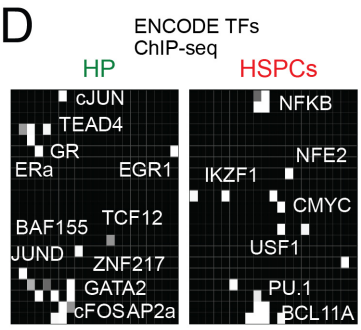


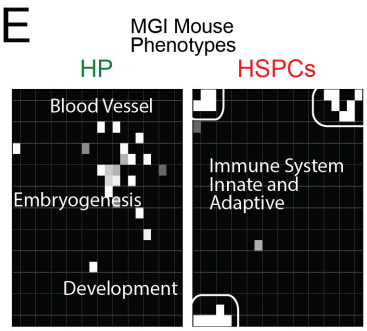
B

HSPCs

Pathways	P value
Infammation mediated cytokine	3.1E-19
Toll Recept. Signalling	1.2E-12
T-cell Activation	4.0E-09
Apoptosis Signalling	4.8E-08
B-cell Activation	9.4E-08
EGF Signalling	3.7E-06
Interleukin Signalling	2.8E-05
PDGF Signalling	3.3E-05
Heterotrimeric G-prot. Signalling	3.3E-05
Integrin Signalling	1.1E-04
p38 MAPK Pathway	1.1E-04
VEGF Signalling	1.2E-04
Endothelin Signalling	2.4E-04
Heterotrimeric G-prot. Signalling	2.7E-04
Cytoskeletal by Rho GTPase	2.7E-04
Gonadotropin Hormone Recept.	1.2E-03
N-acetylglucosamine Metabol.	1.2E-03
Angiotensin-II Signalling	1.8E-03
Ras Pathway	1.9E-03
FGF Signalling	2.6E-03







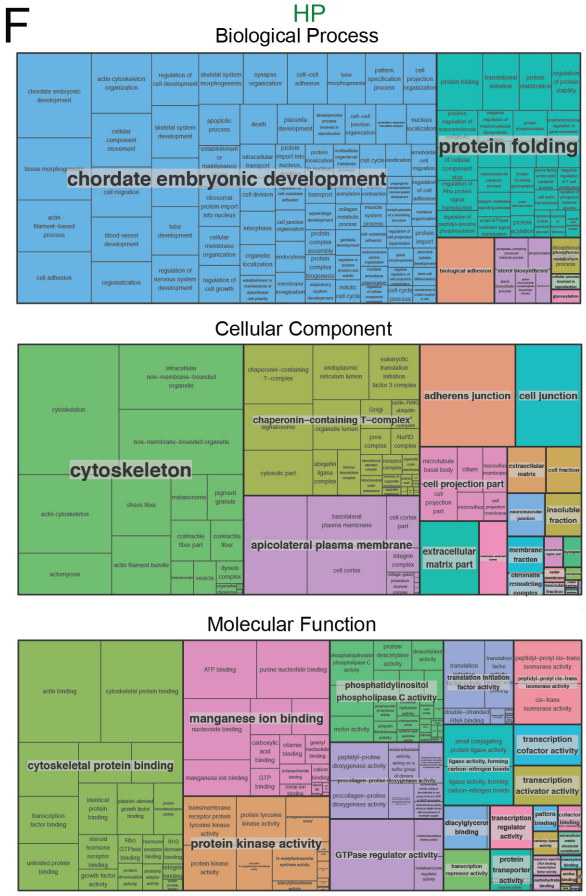
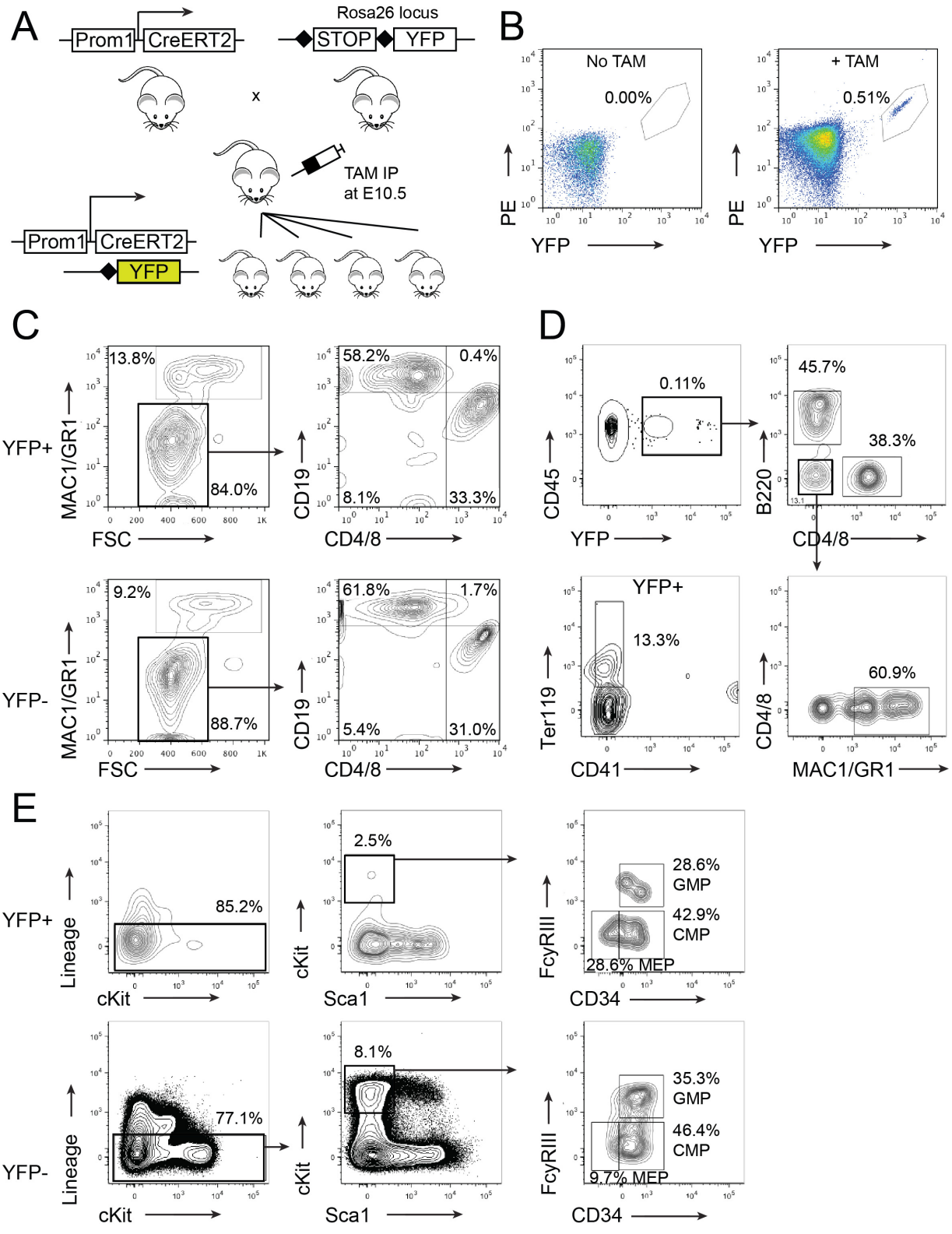


Figure S3. mRNA-Seq Integration Supporting Data, Clustering Methods, Pathways and Gene Ontologies, Related to Figure 5. (**A**) K-means clustering of integrated data reveals 10 clusters of genes enriched in hemogenic cells (cluster 1-6) and HSPCs (cluster 7-10). Red indicates increased expression and green decreased expression relative to the mean. (**B**) Pathway enrichment analysis was performed on the genes enriched in the HSPCs clusters using Panther software (www.pantherdb.org). The pathways enriched in the HSPC clusters and corresponding P values are shown. (**C**) Integration of the two independent clustering methods used: k-means clustering and co-expression clustering. The figure shows the co-expression network overlayed with the genes identified by k-means clustering. k-means main gene clusters for HP (cluster 3, 4 and 6) and HSPCs (cluster 9 and 10) map to the clusters identified by the co-expression network. (**D, E**) HP and HSPCs gene clusters were analyzed for gene-list enrichment with gene-set libraries created from ENCODE transcription factor Chip-Seq (**D**) and level 4 of the MGI mouse phenotype ontology (**E**). Enriched terms are highlighted and brightness represents increasingly significant P values. (**F**) Gene ontology (biological process, cellular component and molecular function) was performed on the HE and HSPCs gene clusters using DAVID clustered functional analysis and displayed by REVIGO (revigo.irb.hr). Treemaps show the main categories of enrichment and the box sizes represent the respective P values. Treemaps for HP (left panels) and HSPCs (right panels) can be directly compared.


A
Prom1
CreERT2
Rosa26 locus
STOP
YFP
x
TAM IP
at E10.5
Prom1
CreERT2
YFP
B
No TAM
0.00%
+ TAM
0.51%
PE
YFP
C
YFP+
MAC1/GR1
FSC
13.8%
84.0%
CD19
CD4/8
58.2%
0.4%
8.1%
33.3%
YFP-
9.2%
88.7%
61.8%
1.7%
5.4%
31.0%
D
CD45
YFP
0.11%
B220
CD4/8
45.7%
38.3%
YFP+
Ter119
CD41
13.3%
CD4/8
MAC1/GR1
60.9%
E
YFP+
Lineage
cKit
85.2%
cKit
Sca1
2.5%
FcγRIII
CD34
28.6%
GMP
42.9%
CMP
28.6% MEP
YFP-
77.1%
8.1%
35.3%
GMP
46.4%
CMP
9.7% MEP

Supplementary Figure S4, related to Figure 6. Prom1-positive cells during mid-gestation contribute to adult myeloid and lymphoid lineages. (**A**) Homozygous Prom1-CreERT2 mice (Zhu et al, 2009) were crossed with R26StopYFP mice (Cooley et al, 2014). Cre recombinase was activated with an intraperitoneal maternal injection (IP) of 4-hydroxytamoxifen (TAM) at E10.5. (**B**) The progeny were analyzed by flow cytometry for the presence of YFP positive cells in the peripheral blood of the progeny at 2 months of age. (**C**) YFP-positive and YFP-negative cells were analyzed for the expression of the myeloid (Gr1 and Mac1) and lymphoid (CD19, CD4 and CD8) markers. (**D**) YFP-positive from mice at 15 months of age were analyzed for the expression of the myeloid (Gr1 and Mac1), lymphoid (B220, CD4 and CD8) and erythroid (Ter119) markers. (**E**) Bone marrow analyses of 15-month old mice for the presence of granulocyte-macrophage progenitors (GMP), common myeloid progenitors (CMP) and megakaryocyte-erythroid progenitors (MEP).

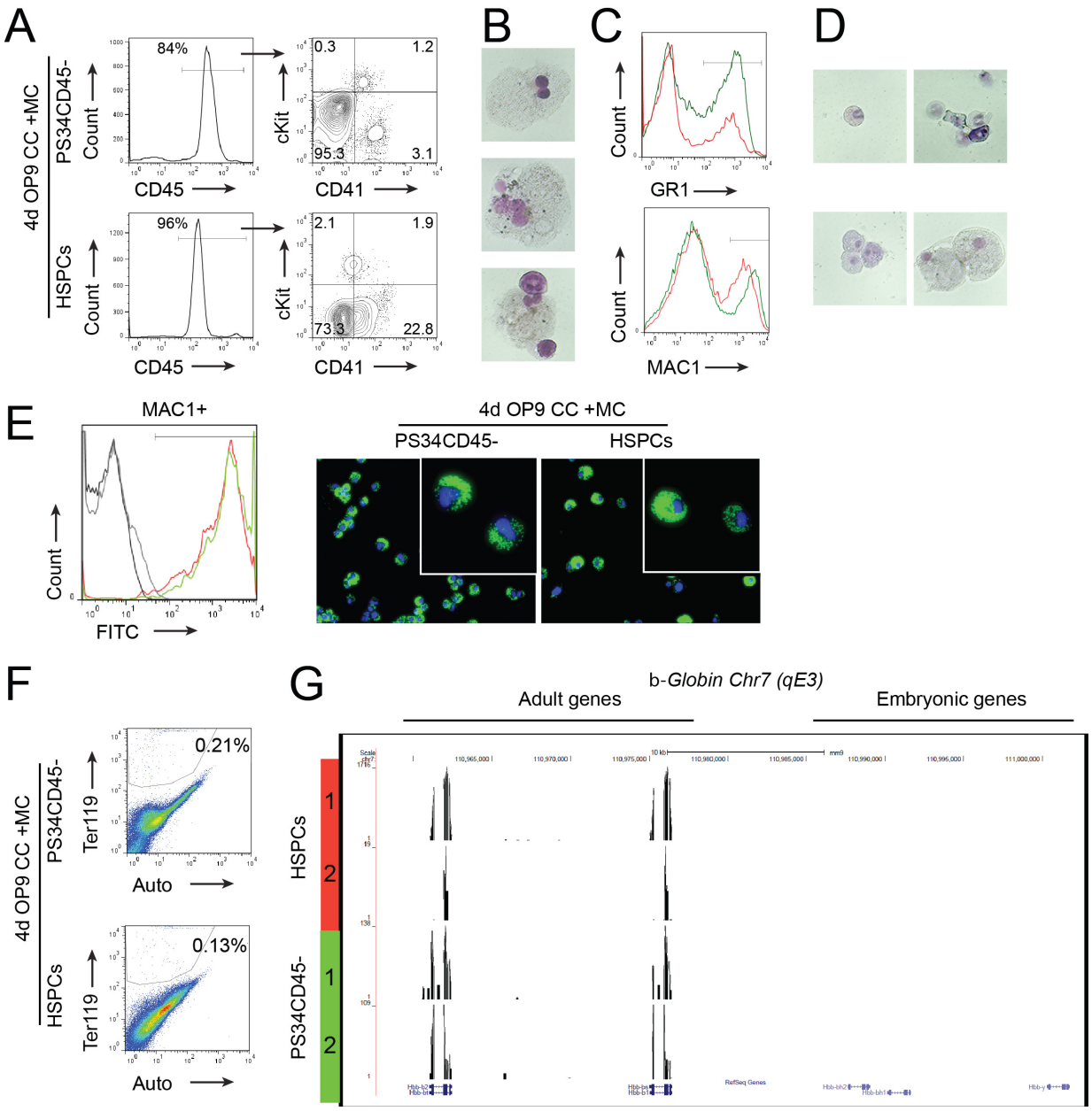


Figure S5. PS34 Cells Generate Mixed Colonies that Contain Megakaryocytes, Granulocytes, Functional Macrophages and Definitive Erythroblasts, Related to Figure 7. E12.5 placental PS34CD45- cells and HSPCs as controls were co-cultured (CC) with OP-9 in the presence of cytokines for 4 days and then transferred to myeloid CFU medium (MC) for 7 days. (**A**) Flow analyses show that generated CD45+ cells contain CD41+ cells that do not express the progenitor marker cKit. (**B**) Morphological analyses show multinucleated megakaryocytes generated from PS34 CD45- cells contained in CFU mixed colonies. (**C**) Flow analyses of colonies for the granulocyte marker (Gr-1) and the macrophage marker (Mac1) in HSPC-derived (red line) and PS34CD45- derived (green line) colonies. (**D**) Morphological analyses of mixed colonies show granulocytes (upper panels) and macrophages (lower panels) derived from PS34CD45- cells. (**E**) Gated Mac1+ cells derived from PS34CD45- cells (green line) or HSPCs (red line) are able to phagocytose FITC-coupled latex beads. The black lines represent no beads control. The right panel shows MAC1 sorted cells with engulfed beads in the cytoplasm (green). (**F**) Mixed colonies contain Ter119+ erythroblasts. (**G**) Ter119+ cells derived from HSPCs and PS34CD45- cells were sorted and analyzed by mRNA-Seq. The panel shows the β-globin gene cluster (Chromosome 7, qE3) with aligned reads (black bars). Ter119+ cells express the adult mouse globin genes *Hbb-b1* and *Hbb-b2* but not the embryonic *Hbb-bh1*, *Hbb-bh2* and *Hbb-γ*.

A

	Sorted cells	Sorted cell number	Total number of cells in co-cultures at day 15
B-cell (OP-9)	PS34CD45-	22,496	8.52 x 10e7
	HSPCs	11,416	7.56 x 10e7
T-cell (OP-9-DL1)	PS34CD45-	20,385	1.46 x 10e8
	HSPCs	8,247	2.56 x 10e8

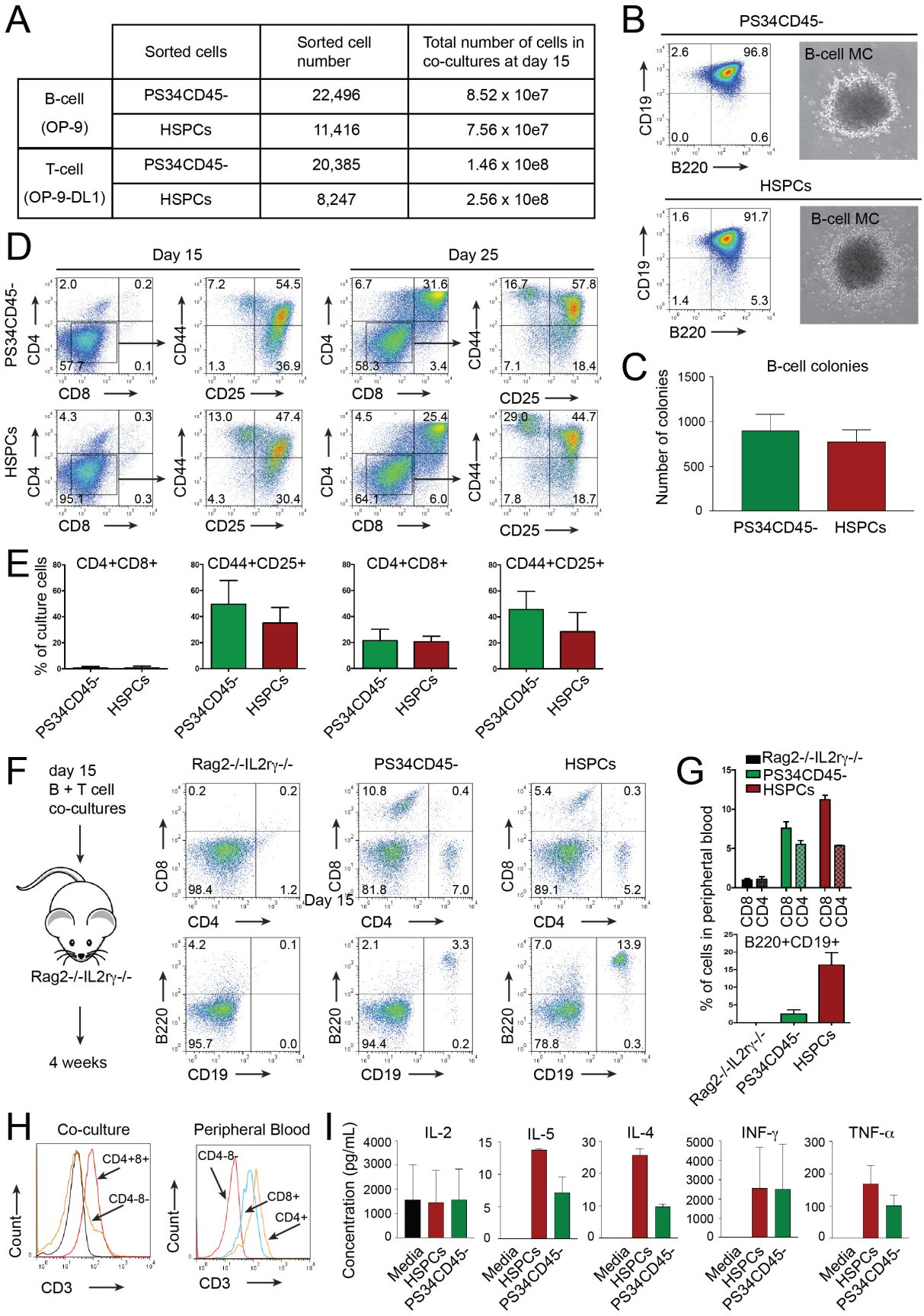


Figure S6. PS34 Cells Generate B and T cell precursors *In Vitro* After Co-culture, Related to Figure 7. (**A**) E12.5 PS34CD45- cells were co-cultured with OP-9 or OP-9-DL1 in the presence of SCF, Flt3l and IL-7 for 15 days. Table shows cell numbers at day 0 and day 15 of co-culture. (**B**) At day 15 OP-9 co-cultures were collected and cultured in B-cell methylcellulose. FACS analysis shows the generation of CD19+B220+ B cells (CD45+ gated) derived from PS34CD45- (upper panel) or HSPCs (lower panel). (**C**) B cell colonies were counted after 7 days (mean ± SD, n=3). (**D**) The potential to generate T-lymphocytes was tested in co-cultures with OP9-DL1 cells in the presence of SCF, Flt3l and IL-7 for 15-25 days. FACS analyses at day 15 show the emergence of CD44+CD25+ T-cell precursors (left panels) and CD4+CD8+ T cells at day 25 (right panels). (**E**) Shows the percentage of CD4+CD8+ and CD44+CD25+ cells (CD4-CD8- gated) at day 15 (left panel) and day 25 (right panel) (mean ± SD, n=3). (**F**) B and T cell precursors at day 15 were injected into RAG2/IL2rγ immunodeficient mice. After 4 weeks peripheral blood of recipient mice was analyzed by flow cytometry for mature, single positive B and T cells. (**G**) The percentages of single positive CD4+, CD8+ and B220+CD19+ cells in peripheral blood are displayed (mean ± SD, n=2). (**H**) FACS analysis for the expression of CD3 in T cells derived from PS34CD45- cells after 25 days of co-culture (left panel) or from peripheral blood of transplanted RAG2/IL2rγ immunodeficient mice (right panel). (**I**) CD4+ and CD8+ T-cells were sorted from spleen, bone marrow and peripheral blood of recipient RAG2/IL2rγ immunodeficient mice, activated in the presence of IL-2 and assayed for the secretion of IL-5, IL-4, INF-γ and TNF-α using a cytometric bead array (mean ± SD, n=2).

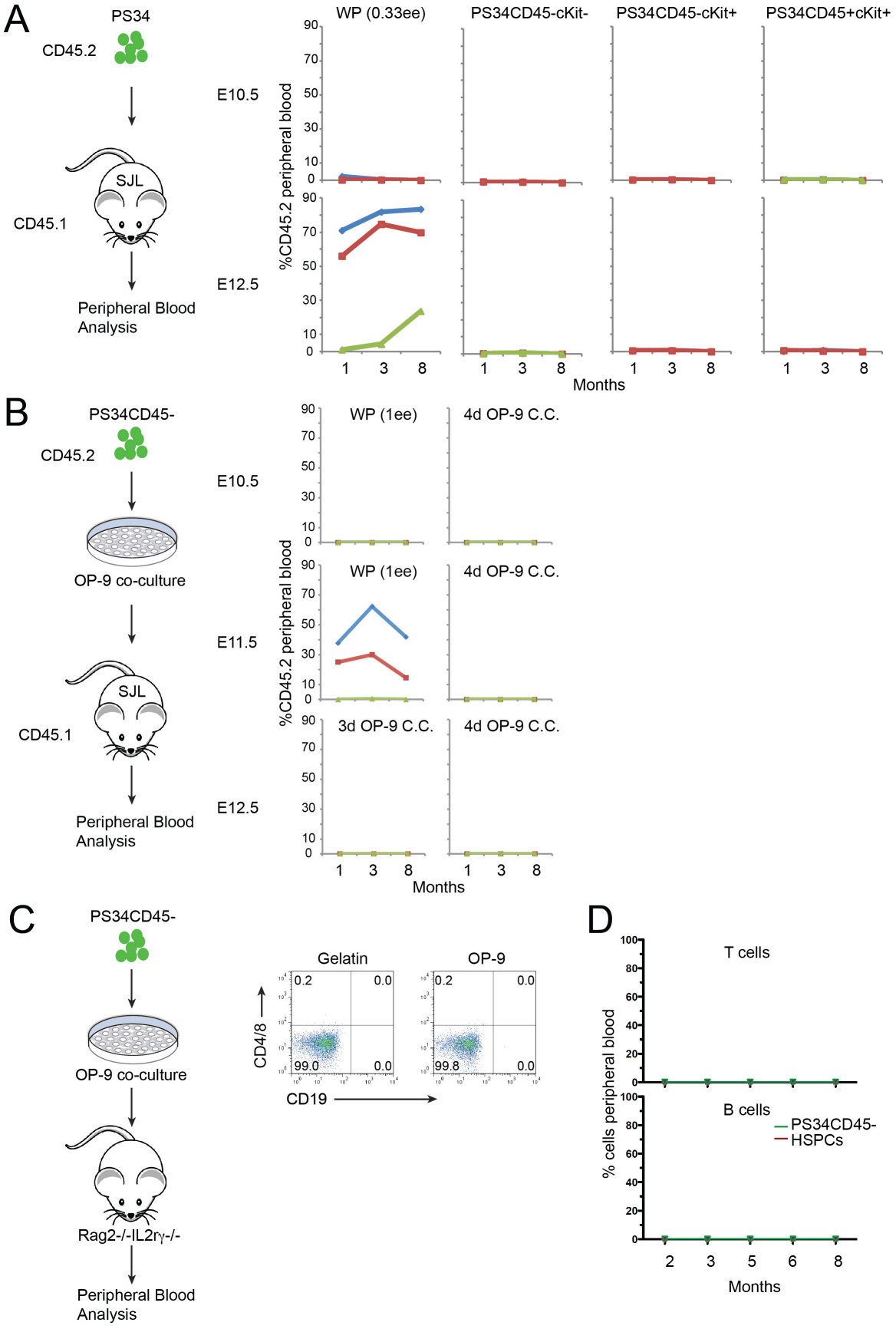

A
PS34
CD45.2
SJL
CD45.1
Peripheral Blood Analysis
E10.5
E12.5
WP (0.33ee)
PS34CD45-cKit-
PS34CD45-cKit+
PS34CD45+cKit+
%CD45.2 peripheral blood
Months
B
PS34CD45-
CD45.2
OP-9 co-culture
SJL
CD45.1
Peripheral Blood Analysis
E10.5
E11.5
E12.5
WP (1ee)
4d OP-9 C.C.
3d OP-9 C.C.
%CD45.2 peripheral blood
Months
C
PS34CD45-
OP-9 co-culture
Rag2-/-IL2rγ-/-
Peripheral Blood Analysis
Gelatin
OP-9
CD4/8
CD19
0.2
0.0
99.0
99.8
D
T cells
B cells
PS34CD45-
HSPCs
% cells peripheral blood
Months

Figure S7. PS34 Cells Do Not Directly Engraft Adult Mice, Related to Figure 7. (**A**) E10.5 and E12.5 placentas were dissociated and PS34 cells isolated and fractioned in CD45-cKit-, CD45-cKit+ and CD45+cKit+. Freshly isolated cells were injected into SJL (congenic CD45.1 mice) intravenously. Whole placentas (0.33 embryo equivalents) were used as a control. After 1, 3 and 8 months CD45.2 and CD45.1 cells in peripheral blood were analyzed by flow cytometry. Graphs show the percentage of CD45.2 single positive cells at any given time point. The lines represent engraftment in individual mice. (**B**) E10.5, E11.5 and E12.5 placentas were dissociated and PS34CD45- cells were isolated and co-cultured with OP-9 cells in the presence of cytokines for 4 days. Co-cultures were dissociated and transplanted into SJL mice intravenously. After 1, 3 and 8 months CD45.2 and CD45.1 cells in peripheral blood were analyzed by flow cytometry. (**C**) E12.5 placentas were dissociated and PS34CD45- cells isolated and cultured in gelatin-coated dishes or co-cultured with OP-9 cells in the presence of cytokines for 4 days. Co-cultures were dissociated and transplanted intravenously into RAG2/IL2rγ immunodeficient mice. After 12 weeks the presence of B (CD19+) and T (CD4+/CD8+) cells in peripheral blood was analyzed by flow cytometry. (**D**) The number of B and T cells in peripheral blood was followed periodically until 8 months post-transplant. WP, whole placentas; ee, embryo equivalent; C.C., co-culture.

Supplemental Tables

Table S1. Numbers of Placental-derived Cells Tested in Methylcellulose Colony Forming Assays, Related to Figure 7A.

	E10.5		E12.5			
	PS34CD45-	**PS34CD45+**	**PS34CD45-**	**PS34CD45+**	**HSPCs**	**MBCs**
Exp. 1	1622 (2ee)	525 (2ee)	1124 (2ee)	751 (2ee)	1613 (0.3ee)	10133 (0.3ee)
Exp. 2	5222 (2ee)	2250 (2ee)	2057 (2ee)	895 (2ee)	1820 (0.3ee)	13146 (0.3ee)
Exp. 3			7545 (3ee)	798 (3ee)		

ee, embryo equivalent; HSPCs, hematopoietic stem/progenitor cells; MBCs, mature blood cells.

Table S3. Top 30 Functional Annotation Clusters in HPs and HSPCs, Related to Figure 5.

Cluster	ES	HPs (326 clusters)	ES	HSPCs (334 clusters)
1	10.2	Cytoskeleton	26.9	Lysosome
2	8.19	Embryonic Development	13	Inflammatory response
3	7.42	Actin-Binding	12.6	Innate Immunity
4	6.52	PDZ Domains	10.9	PH domain
5	6.37	Adherens Junction	10.5	Lymphocyte activation
6	5.92	Tight Junction	8.72	Membrane lipid metabolic process
7	5.64	Cell Adhesion	8.48	Endocytosis
8	5.42	Cytoskeleton Organization	7.9	Vesicle
9	4.76	Cell Motion	6.73	Activation of immune system
10	4.68	Embryonic Morphogenesis	6.62	GTPase regulator activity
11	4.28	Microtubule	6.06	Regulation of chemokine production
12	4.15	Endoplasmic Reticulum	5.72	Cell chemotaxis
13	4.14	Tube Morphogenesis	5.23	T cell activation
14	3.94	SH3 Domain	5.15	Adaptive immune response
15	3.78	Angiogenesis/Cell Motion	5.08	Regulation of apoptosis
16	3.73	PH Domain	4.73	Apoptosis
17	3.7	Actomyosin	4.35	NF-kappaB transcriptional activity
18	3.61	Negative Regulation of Cell Differentiation	4.28	Cell membrane
19	3.48	LIM Domain	4.25	Interleukin-6 production
20	3.48	Chaperone	4.19	GTPase activation
21	3.31	GTPase regulator activity	3.94	ATP metabolic process
22	3.27	ATP binding	3.93	SH2 domain
23	3.26	Skeletal System Morphogenesis	3.88	ATP binding
24	3.05	CRIB Domain	3.86	Late endosome
25	2.78	Signalosome	3.74	Lymphocyte homeostasis
26	2.75	Small GTPase regulator activity	3.67	Regulation of adaptive immune response
27	2.73	Apoptosis	3.57	Lipid binding
28	2.66	Protein Stabilization	3.55	SH3 domain
29	2.64	Regulation of cell morphogenesis	3.55	Blood Cell Homeostasis
30	2.53	Protein folding	3.54	SH3 domain binding

Gene Ontology Functional Annotation Clustering performed in DAVID. Complete lists can be found in Table S2. ES, Enrichment Score.

Table S4. Frequency of cells positive for endothelial and trophoblastic markers, Related to Figure 3 and 5.

Gene	E10.5 PS34 (48)		E12.5 PS34 (48)		E12.5 HSPCs (48)	
CD34	47	(98%)	48	(100%)	43	(90%)
CD31/Pecam1	47	(98%)	47	(98%)	24	(50%)
Krt7	4	(8%)	2	(4%)	2	(4%)
Krt19	7	(15%)	2	(4%)	1	(2%)
Krt17	0	(0%)	0	(0%)	0	(0%)
Hand1	1	(2%)	2	(4%)	0	(0%)
COUPTF/Nr2f1	1	(2%)	1	(2%)	0	(0%)
Efnb2	46	(96%)	39	(81%)	2	(4%)

Positive FPKM >1; Negative FPKM <1

Supplemental Experimental Procedures

Primary Antibodies Used in This Study

Antibody/Antigen	Clone	Conjugate	Source
CD133 (Prom1)	13A4	APC, Biotin, PE	eBioscience
Ly-6A/E (Sca1)	D7	Pacific Blue, APC-CY7	Biolegend
CD34	RAM34	Alexa Fluor 700	eBioscience
CD45	30-F11	PE, APC-CY7, APC	BD Pharmingen
CD49f	GoH3	PE	BD Pharmingen
CD31 (PECAM-1)	390	PE	eBioscience
CD105	MJ7/18	PE	eBioscience
CD144 (VE-CAD)	eBioBV13	APC	eBioscience
CD202b (Tie2)	TEK4	PE	eBioscience
CD41	eBioMWReg30	APC	eBioscience
CD117 (c-Kit)	2B8	PE, APC	eBioscience, Biolegend
CD45.1	A20	APC	BD Pharmingen
CD45.2	104	PE	Biolegend
Ter119 (Ly-76)	TER-119	FITC, Biotin	BD Pharmingen, eBioscience
CD4	GK1.5	PE-CY5, PE-CY7	eBioscience
CD8a	53-6.7	PE-CY5, APC	eBioscience
CD44	IM7	PE	BD Pharmingen
CD25	eBio3C7	eFluor 450	eBioscience
CD19	eBio1D3	PE-CY7	eBioscience
CD45R (B220)	RA3-6B2	FITC, Biotin	eBioscience
CD3e (CD3 ε chain)	145-2C11	FITC, Biotin	BD Pharmingen, eBioscience
CD11b (Mac1)	M1/70	APC-CY7, Biotin	Biolegend, eBioscience
Ly-6G/Ly-6C (Gr-1)	RB6-8C5	APC-CY7, APC, Biotin	Biolegend, eBioscience
CD150	TC15-12F12.2	PE-CY7	Biolegend
CD48	HM48-1	Biotin	Biolegend
CD16/CD32 (FcyRIII)	93	PE-CY7	eBioscience

34/H2BGFP Mouse Embryo Genotyping

To identify double and single transgenic embryos (and corresponding placentas) the embryonic head was removed and used for genotyping with the following primers: GFP 5'-AGCTGACCCTGAAGTTCATCTG, GFP 3'-GTCGGCCATGATATAGACGTTG and hCD34 5'-AGAAGAGATGAGGTGTGAGGAT, hCD34 3'-GGATCCACAAGAATGAGCATGTA.

AFT024, OP-9 and OP-9-DL1 Stromal Cell culture
The AFT024 cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS; Benchmark), 1mM L-Glutamine and penicillin/streptomycin (10 μgml^{-1}; Invitrogen) at 32°C and mitotically inactivated by irradiation as previously described (Moore et al., 1997). The OP-9 and OP-9-DL1 cell lines were cultured in Minimum Essential Medium (MEM) Alpha (Gibco) containing 20% fetal bovine serum (FBS; Hyclone), 1mM L-Glutamine and penicillin/streptomycin (10 μgml^{-1}; Invitrogen) in 5% CO_2 at 37ºC. OP-9 and OP-9-DL1 were never allowed to reach 100% confluency.

Methylcelulose clonogenic assays
Clonogenic progenitors were assayed in 1% methylcellulose media (Methocult M3434, Stem Cell Technologies) supplemented with TPO (10 $ngml^{-1}$). Hematopoietic colonies were scored and counted after 7-10 days of culture in 5% CO_2 at 37ºC. CFU-Mix, CFU-GM, CFU-G, CFU-M and CFU-E were identified.

B and T-cell *in vitro* differentiation assays
For B and T-cell differentiation assays the OP-9 and OP9-DL1 stromal cell lines were respectively used. PS34CD45- cells were sorted and cultured in Minimum Essential Medium (MEM) Alpha (Gibco) containing 20% fetal bovine serum (FBS; Hyclone), 1mM L-Glutamine and penicillin/streptomycin (10 μgml^{-1}; Invitrogen) in the presence of 10 $ngml^{-1}$ SCF, 10 $ngml^{-1}$ Flt3L and 10 $ngml^{-1}$ IL-7 (R&D) in 5% CO_2 at 37ºC. Cells were passaged 1:3-1:6 onto a fresh layer (70% confluent) of live OP-9 or OP-9DL1 cells every 4 days using TrypLE Express (Gibco). At day 15 one well of a 6-well plate from OP-9 co-cultures were collected and cultured in B-cell methylcellulose M3630 medium (Stem Cell Technologies). Colony number was determined 7 days later. To increase the maturation of differentiated lymphocytes *in vitro* the cytokine concentration was reduced at day 15 of differentiation to 1 $ngml^{-1}$ SCF, 1 $ngml^{-1}$ Flt3L and 1 $ngml^{-1}$ IL-7 (R&D). Cells were analyzed by flow cytometry until day 25.

Immunofluorescence
Detection of CD45 after hemogenic cell culture was done directly on 6-well plates (gelatin-coated or with AFT024 feeder layer) under an inverted microscope. For live staining, phycoerythrin (PE)-conjugated sterile rat monoclonal antibodies against CD45 were used at a 1:100 dilution. Emergent colonies were washed twice with PBS5% FBS to remove unbound antibody. For detection of Prom1/CD133 in tissues, whole E10.5, E11.5 and E12.5 placentas and E13.5 fetal livers were fixed in 4% paraformaldehyde/PBS for 3 hours at 4ºC and equilibrated in 30% sucrose/PBS overnight at 4ºC. To facilitate O.C.T. (Optimal Cutting Temperature Solution, Tissue-Tek) penetration half of the sucrose solution was removed and replaced with O.C.T for 2 hours at 4ºC and transfer to 100% O.C.T. for 2 hours at room temperature. Placentas were cut in half, frozen in dry ice and 10 µm sections were prepared using a cryotome and deposited on slides. After cryosectioning, tissues were treated in 100% cold acetone for 10 min, washed with wash buffer (PBS, 0.2% BSA, 0.05% Tween20), blocked with blocking buffer (PBS, 2.5% BSA, 0.05% Tween20 and 10% normal goat serum) for 30 min, washed three times, blocked with Biotin blocking solution (Vector Laboratories) for 15 min, and washed three times. Subsequently, sections were incubated with biotinylated antibodies for Prom1/CD133 (1:100 dilution) at room temperature for 2 hr, washed three times, incubated with Streptavidin-Alexa488 (Molecular Probes) for 45 min, washed three times, dehydrated in ethanol (from 70% to 100%), and mounted with Vectashield (Vector Laboratories) containing 4,6-diamidino-2-phenylindole (DAPI, 1 µg/mL, Sigma). Antibodies were diluted in blocking buffer. Cells and tissues were visualized on a Leica DMI4000 microscope and processed with Leica software and Adobe Photoshop.

Cell Isolation, FACS Sorting and Analysis
For isolating PS34 CD45- cell populations, placentas were dissociated to single cells, resuspended in PBS5% FBS and stained with PB-Sca1, APC-Prominin1/CD133, Alexa700-CD34, APCCY7-CD45 and PE-cKit. All antibodies were used at 1:100 dilution in PBS 5% FBS (Benchmark) with Normal Rat Serum (Thermo Scientific). 7-Amino-Actinomycin D (7-AAD, BD) was added before analysis to exclude dead cells. Cell populations were isolated on an InFlux cell sorter or FACSAriaII (BD) and immediately lysed in Trizol (Ambion) for RNA extraction or used for *in vitro* culture. Flow cytometric analysis was performed on a 5-laser LSRII with Diva software (BD) and analyzed using FlowJo software (Tree Star).

Flow cytometry analysis of AGM cell populations
Embryos were collected, dissected and staged as described (Swiers et al., 2013). Aorta-Gonad-Mesonephros (AGM) regions were dissected together with vitelline and umbilical vessels. Flow cytometry analysis was performed as

described (Swiers et al., 2013). Briefly, tissues were pooled and dissociated to a single cell suspension by collagenase digestion and pipetting. Cells were washed and stained at 4 ºC in the dark. Dead cells were excluded by Hoechst 33258 uptake. Data was acquired using a Cyan ADP analyser (Beckman Coulter) and analysed using Summit (BC) software.

Confocal Whole Mount Imaging
Embryos were collected, staged and dissected as described (Swiers et al., 2013). Yolk sac, head, limbs and body walls from both sides were removed. Embryos were then fixed in a PFA 4% solution in PBS for 1 hour at 4 ºC and stored in methanol at -20ºC. Whole mount immuno-labeling was performed as described (Yokomizo et al., 2012). The following primary antibodies were used: Rabbit anti-GFP (Life Technologies), Goat anti-CD31 (R&D Systems), purified Rat anti-CD133/Prom1. Secondary antibodies used were Donkey anti-Rabbit conjugated with Alexa 488, Donkey anti-Rat conjugated with Alexa 594 and Donkey anti-Goat conjugated with Alexa 647 (all from Life Technologies). Samples were imaged on a Zeiss Examiner.Z1 microscope connected to a Zeiss LSM 780 confocal module, using a 25x/0.8 DIC Imm Korr (UV) VIS-IR objective. Images were acquired using Zeiss Zen software. Data was analysed and 3D reconstruction were made using Imaris software (Bitplane).

qRT-PCR of AGM cell populations
Cells were collected and processed as described (Swiers et al., 2013). Microfluidic Quantitative RT-PCR was performed using the Fluidigm Biomark platform and data was analysed as described (Swiers et al., 2013). Data shown are the mean of 6 pools of 25-sorted cells from 2 independent biological experiments.

T-Cell Cytokine Assay
T-cells were isolated from Rag2/IL2rγ peripheral blood, spleen and bone marrow 4 weeks after transplantation with OP-DL1 co-cultured PS34CD45- or HSPCs. 2,000 $CD3^{+}CD4+/CD8+$ T cells were FACS sorted and cultured in MEM Alpha (Gibco) in the presence of IL-2 (10 $ngmL^{-1}$, R&D) and anti-CD3 and anti-CD28-coated beads (BD Dynabeads T-Activator). After 3 days, the supernatant was harvested and processed with the mouse Th1/Th2 cytokine kit (BD Cytometric Bead Array) according to the manufacturer's instructions. LSRII flow cytometer (BD) was used for acquisition and data were analyzed using the FCAP array software (BD). Data show average of independent T-cell pools derived from 3 transplanted Rag2/IL2rγ deficient mice.

Phagocytosis Assay
Hematopoietic cells derived from PS34CD45- or HSPCs were differentiated on OP-9 feeder layers with cytokines and re-plated with 10 µL of 2.5% yellow-green fluorescent coupled solid latex beads, (carboxylate-modified polystyrene, Sigma). Sixteen hours later, cells were collected washed twice in PBS 5% FBS and stained with anti-$CD11b^{+}$ (Mac1+) antibody and analyzed by flow cytometry along with untreated cells. Mac1+ cells were sorted and deposited on slides by cytospin to confirm bead incorporation by immunofluorescence. DAPI (1 µg/mL, Sigma) was used to stain nuclei.

Cytospin and Benzidine Staining
3,000-100,000 cells were washed twice in cold 5% FBS in PBS and diluted in 200 µl of cold 1% FBS in PBS. The samples were loaded into the appropriate wells of the Cytospin centrifuge equipment. Samples were spun at 500 r.p.m. for 3 min to allow cellular adherence to the slides. Slides were fixed and stained using DIFF Stain Kit (IMEB inc), followed by 10 min in PBS and a quick wash in distilled water. Slides were viewed on a Nikon Eclipse TE2000-U microscope and images acquired using Nikon ACT-1 software. For Benzidine staining of hemoglobin, 1 tablet of 3,3',5,5'-Tetramethylbenzidine (Sigma) was dissolved in 10 ml of PBS, filtered and supplemented with 10 µl of 30% H_2O_2 before use. To directly stain mixed colonies in the methylcellulose media, 1 ml of staining solution was added and incubated at 37°C for 30 minutes.

***In vivo* Maturation of T-Cells and Long-Term Repopulation Assays**
Rag2/IL2rγ (B10;B6-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl}, Taconic) and congenic SJL (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) mice were used as recipients for directly isolated placental cells or cells co-cultured with stromal cells. After 3-4 days of co-culture, cells were dissociated with Accutase Cell detachment solution (Innovative Cell Technologies). Animals were transplanted with cells intravenously by retro-orbital injection. Up to 6 hours before transplantation with placental-derived cells Rag2/IL2rγ or SJL mice received a sublethal total body irradiation dose of 600 cGy. The number of cells transplanted per animal were in the range of 2-4 embryo equivalents cells per animal. Starting 4 weeks after transplantation, mice were bled on the orbital venous plexus and donor contribution was assessed by FACS with

anti-CD45.1/CD45.2 (for SJL) and anti- CD19/CD4/CD8 (for Rag2/IL2rγ) antibodies. Red blood cells were lysed with Pharm Lyse Buffer (BD) for 5 min on ice. Dead cells were excluded by staining with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/mL, Sigma).

Isolation of Tissues from Recipient Mouse and Isolation of HSCs from Bone Marrow.
At the end of each experiment mice were sacrificed and peripheral blood, bone marrow (BM), and spleen of engrafted mice were further analyzed by FACS. The BM from primary recipients was used in secondary transplantation experiments. Total BM cells were harvested from long bones (tibias and femurs) by crushing with pestle and mortar in PBS supplemented with 5% FBS (Benchmark). Bone debris was filtered away with 70 μm cell strainers (BD). Red blood cells were lysed with Pharm Lyse Buffer (BD) for 5 min on ice and further filtered through 45 μm cell strainers to obtain a single-cell suspension. Dead cells were excluded by staining with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/mL, Sigma). For HSC isolation, BM was harvested from the long bones and hips of primary transplant recipients as described. BM was depleted of lineage marker- (B220, CD3ε, Ter119, CD11b, Gr-1) and CD48-expressing cells via magnetic separation using a cocktail of biotinylated antibodies and streptavidin-coated magnetic beads (Dynabeads, Life Technologies). Lineage and CD48 depleted BM was then further stained for HSC markers prior to FACS sorting. Lineage-negative, CD48-negative, Sca-1-positive, cKit-positive and CD150-positive (LSK48-150+) HSCs were sorted from engrafted primary mice. 1,300-2,000 LSK48-150+ cells were transplanted per mouse for secondary transplantation assays.

Lineage Tracing
We crossed homozygous Prom1-CreERT2 mice (Zhu et al., 2009) with R26StopYFP mice (Cooley et al., 2014) and activated Cre with a 4-Hydroxytamoxifen (Sigma) injection (IP, 300 ng in 150μl per mouse) at E10.5. We analyzed the presence of YFP-positive cells in the peripheral blood of the progeny of injected and non-injected mice at 2 and 15 months of age. Analysis of the bone marrow and spleen was performed at 15 months of age. As an additional control we injected 4-Hydroxytamoxifen (IP) in adult heterozygous mice (Prom1-CreERT2 x R26StopYFP) and verify the lack of YFP positive cells in the peripheral blood 2 months after injection.

mRNA-seq Analysis
Sequence read files were pre-processed with the FASTX-toolkit suite (http://hannonlab.cshl.edu/fastx_toolkit/) for adapter trimming (ACACTCTTTCCCTACACGACGCTCTTCCGATCT) and quality filtering. Only reads >20nt were retained and aligned to the mouse genome (*Mus musculus* mm9 assembly) using TopHat v2.0.9 and v2.0.12 mapper (Langmead et al., 2009; Trapnell et al., 2009; Trapnell et al., 2010). Transcript assembly and expression estimation was conducted with the Cufflinks package v2.1.1 and v2.2.1 (Roberts et al., 2011; Trapnell et al., 2012; Trapnell et al., 2010) for known transcripts using geometric library normalization to obtain gene FPKM report and differential expression tables (Table S2). Inter-sample FPKM expression correlation was conducted by Spearman correlation and hierarchical clustering and plotted via the R v3.1.0 statistical package. Principal component analysis (PCA) was performed on the sample FPKM values scaled to unit variance and zero centered, PC1 and PC2 were displayed in Rv3.1.0. Gene set enrichment analysis (GSEA) (Subramanian et al., 2005) was performed using the gene FPKM output from Cufflinks for hemogenic precursors (E10.5_PS34cKit- and E12.5_PS34cKit-) and HSPC (E10.5_HSPCs and E12.5_HSPCs) sample classes and analysed against the Molecular Signatures Database version 2.0 (Subramanian et al., 2005) curated gene sets and pathway gene sets assembled from the Netpath-annotated immune signaling pathways (Kandasamy et al., 2010) for gene set sizes 0-5000 and ranked by Ratio_of_Classes. Bedgraph files from TopHat aligned reads were generated with Bedtools v2.20.1 and visualized on the UCSC Genome Browser (Kent et al., 2002) for examination of the mm9 assembly chr7(7qE3) region for β-globin gene expression in Ter119+ sorted samples.

Integration of Independently Obtained Gene Expression Data Sets
Microarray data from precursors and HSPCs from E8.5 concepti (PAS+YS) (Swiers et al., 2013) were downloaded from NCBI GEO database (GSE2075), RNA-seq data from programmed cells with Gata2, cFos, Gfi1b and Etv6 [(Pereira et al., 2013), GSE47497] and the RNA-seq data collected in the present study from placental HPs and HSPCs were subjected to processing and integration.

Processing RNA-seq and microarray data: The FPKM values were floored at one unit and converted to log-space. Genes differentially expressed among the samples were identified based on the consistency of their expression in biological replicates (ANOVA test, false-detection rate $p < 0.05$). Microarray data values were transformed to log-space and differentially expressed genes were found using ANOVA test ($p < 0.05$) as well.

Data integration: All samples from different datasets were subjected to quantile normalization in order to obtain comparable value ranges. Consequently, the expression values for each gene from each individual dataset were normalized using Z-score normalization method. Then the data from different datasets were integrated and the data for genes uniformly expressed in any given dataset removed based on the previously obtained ANOVA p-values (the corresponding expression values were set to 0 in the corresponding datasets).

Data clustering*:* An intersection of the integrated data representing only genes differentially expressed in the datasets analyzed was taken further to the clustering step. An intersection of 5,896 genes significantly changing in both placenta and programmed datasets and 1,677 genes in both placenta, programmed and AGM datasets has been found and the corresponding data from the initial datasets were merged. Clustering of samples was performed using Cluster 3.0 program, hierarchical clustering, complete linkage method with default parameters. Clustering data with respect to cell expression profiles was performed using two different methods: k-means clustering with default parameters using cluster 3.0 program (Pereira et al., 2013), and coexpression clustering using custom software and Cytoscape program (Smoot et al., 2011; Stuart et al., 2003). In the case of coexpression clustering, failed measurements (zeroes) were ignored and the corresponding probabilistic similarity matrix was constructed by calculating correlation P values, given the number of non-failed readings in all pairwise comparisons. Visualization of the resulting coexpression clusters (coexpression network) has been performed with the help of force-directed layout using Cytoscape program (Smoot et al., 2011).

Gene list enrichment analysis**:** Pathway enrichment analysis was performed on the genes enriched in the HPs and HSPCs clusters using Panther software (www.pantherdb.org). Gene ontology (biological process, cellular component and molecular function) was performed using DAVID clustered functional analysis (david.abcc.ncifcrf.gov/). Gene ontologies and genes identified were listed in Table S2 and Table S3 and were summarized using treemaps in REVIGO (Supek et al., 2011). For grid enrichment analysis the program Network to Canvas (Tan et al., 2013) was used with gene-set libraries created from ENCODE transcription factor Chip-Seq and the level 4 of the MGI mammalian phenotype ontology. Differential gene ontology (biological process, cellular component and molecular function), KEGG and REACTOME term enrichment analysis between HPs and HSPCs k-means-clustered genes was performed and visualized via ClueGOv2.0.8 (Bindea et al., 2009) plug-in in Cytoscape v3.1.0 using a two-sided hypogeometric test with a Benjamini-Hochberg correction and connection restriction Kappa score set at 0.7 and a p-value threshold ≤ 0.05.

Single Cell mRNA-seq Analysis

PS34CD45- cells at E10.5 and E12.5, and HSPCs (CD45+CD34+cKit+) at E12.5 were sorted and collected. The cDNA synthesis was performed following the manufacturers instruction using the C1 Single-Cell Auto Prep System (Fluidigm). The cDNA reaction products were quantified using the Quant-iT PicoGreen® dsDNA (double-stranded DNA) Assay Kit (Life Technologies) and then diluted to a final concentration of 0.15–0.30 ng/µl using C1 Harvest Reagent. Sequencing libraries were prepared using the Nextera XT DNA Library Prep Kit (Illumina). 48 single-cell libraries were mixed together. The concentration of the mixed libraries was assayed using Agilent Bioanalyzer. The libraries were sequenced yielding ~0.18 -5.7 Million 100nt reads on a HiSeq 2000 platform at Mount Sinai Genomics Core. For single cell mRNA-Seq analysis the raw fastq files were mapped to the mouse mm9 genome using Tophat with Bowtie2. Transcript assembly and expression estimation were conducted using the Cuffquant and Cuffnorm features in the Cufflinks package. Differential expression, gene clustering and time-series analysis for single-cell RNA-seq was performed with Monocle v1.0.0 package (Trapnell et al., 2014) in R v3.1.0.

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