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Supplemental Information

HOXB4 Promotes Hemogenic Endothelium Formation without Perturbing Endothelial Cell Development

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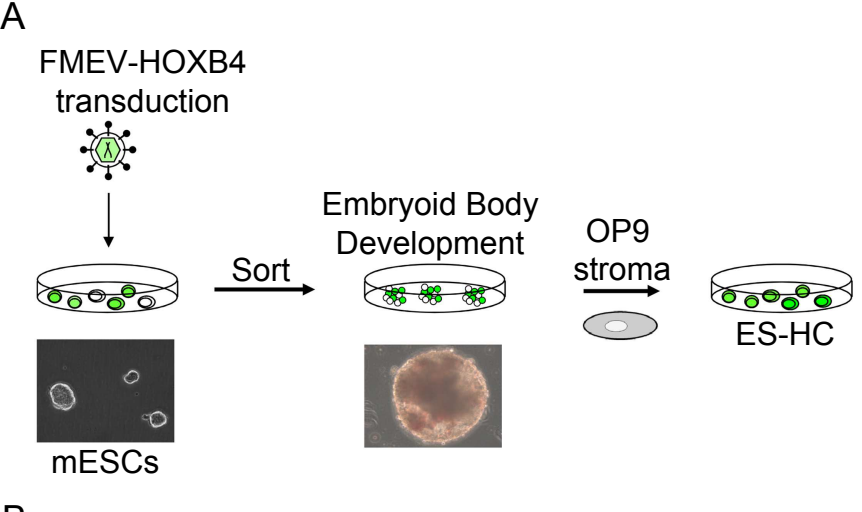
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OP9
stroma
ES-HC
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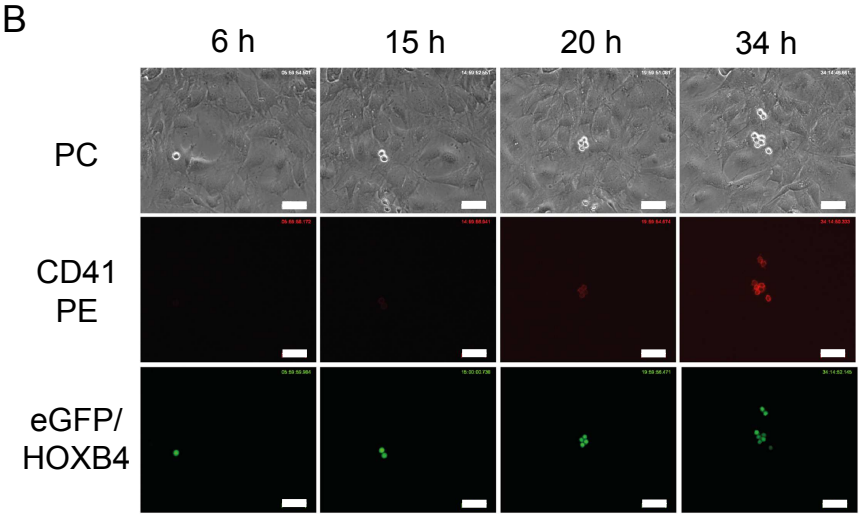
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20 h
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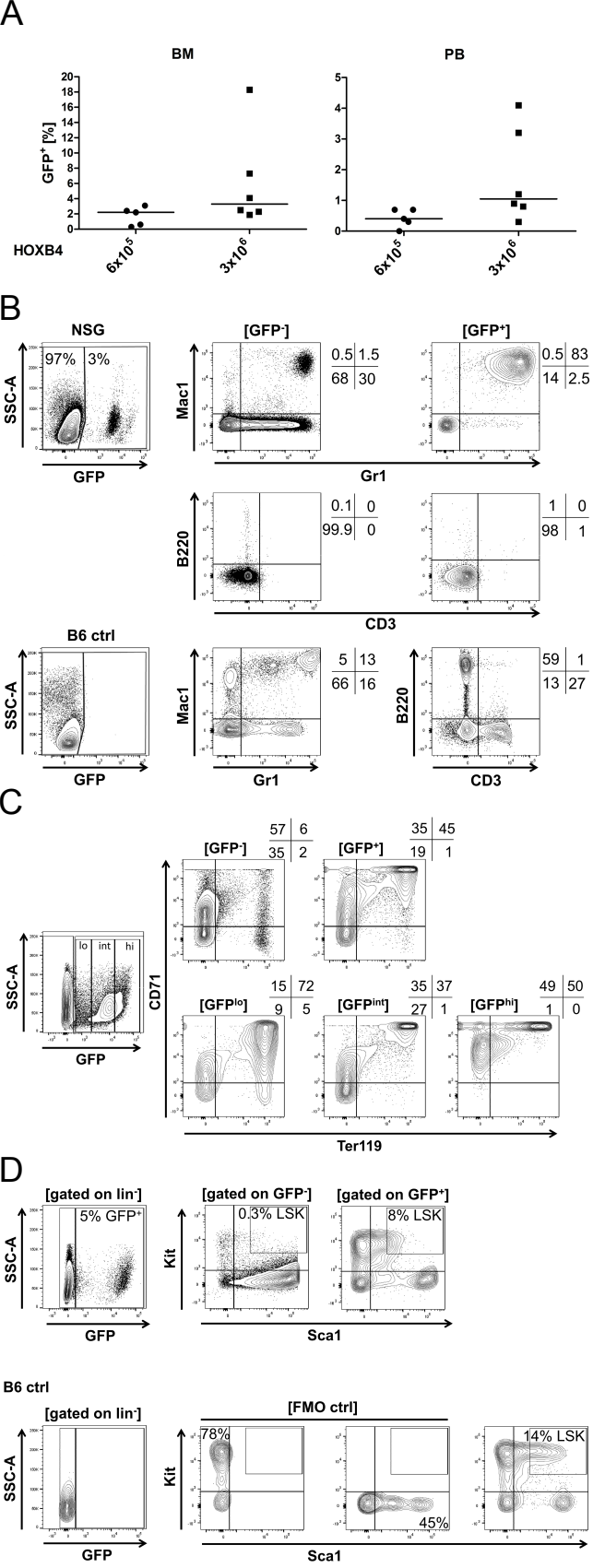
PC
CD41
PE
eGFP/
HOXB4


A
BM
PB
GFP+ [%]
HOXB4
6x10^5
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B
NSG
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GFP
Mac1
Gr1
B220
CD3
97%
3%
0.5 1.5
68 30
0.5 83
14 2.5
0.1 0
99.9 0
1 0
98 1
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int
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27 1
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1 0
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Kit
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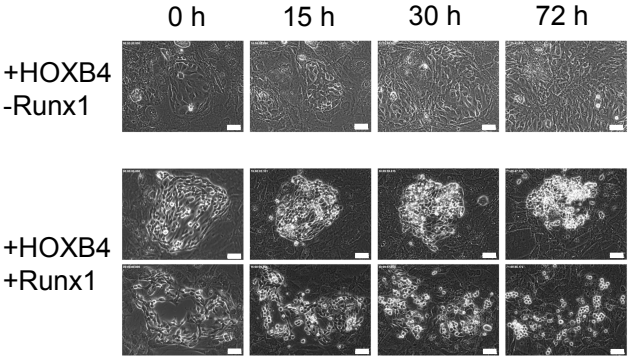
45%
14% LSK

A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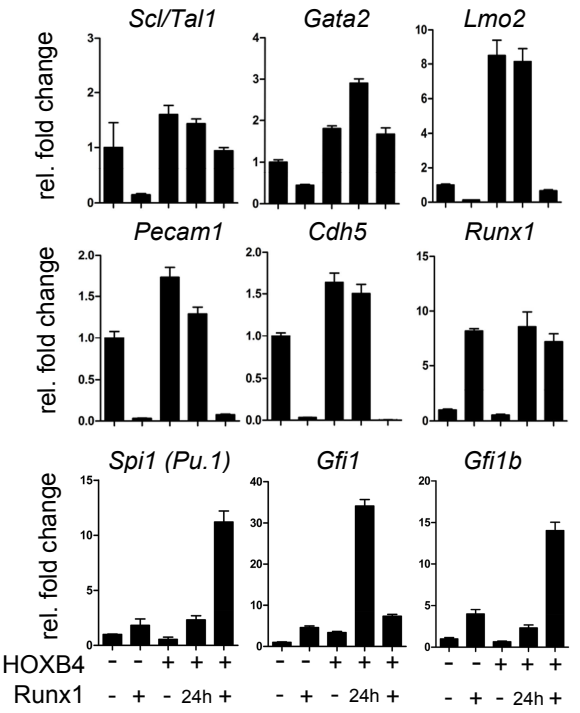
+Runx1

B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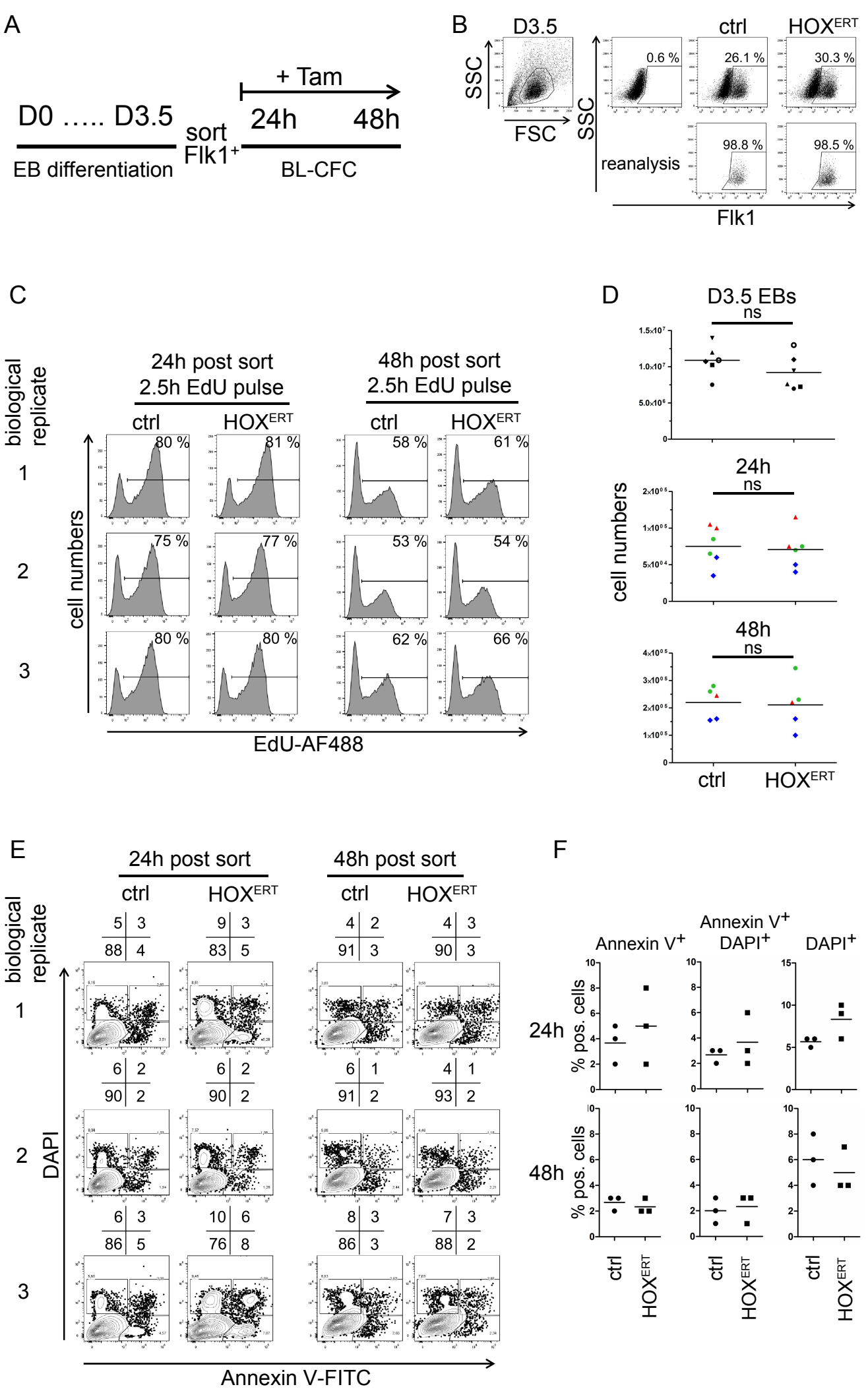
Lmo2
Pecam1
Cdh5
Runx1
Spi1 (Pu.1)
Gfi1
Gfi1b
rel. fold change
HOXB4
Runx1
24h


A
+ Tam
D0 ….. D3.5
24h
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Flk1+
EB differentiation
BL-CFC
B
D3.5
ctrl
HOXERT
SSC
FSC
0.6 %
26.1 %
30.3 %
reanalysis
98.8 %
98.5 %
Flk1
C
biological replicate
24h post sort
2.5h EdU pulse
48h post sort
2.5h EdU pulse
ctrl
HOXERT
80 %
81 %
58 %
61 %
75 %
77 %
53 %
54 %
80 %
80 %
62 %
66 %
cell numbers
EdU-AF488
D
D3.5 EBs
ns
24h
ns
48h
ns
cell numbers
ctrl
HOXERT
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48h post sort
ctrl
HOXERT
5 3 88 4
9 3 83 5
4 2 91 3
4 3 90 3
6 2 90 2
6 2 90 2
6 1 91 2
4 1 93 2
6 3 86 5
10 6 76 8
8 3 86 3
7 3 88 2
DAPI
Annexin V-FITC
F
Annexin V+
Annexin V+ DAPI+
DAPI+
24h
48h
% pos. cells
ctrl
HOXERT

Supplemental Information

Supplemental Figures and Videos

Figure S1. Development of hematopoietic progenitors from differentiating ESCs, Related to Figure 1.
(A) Retrovirally transduced ESCs were purified by flow cytometry based on fluorescent protein expression, differentiated as embryoid bodies, which were dissociated and cocultered on OP9 stroma cells. ES-HC: ES-cell derived hematopoietic cells. **(B**, **related to Movie S1)** Time lapse microscopy was used to follow upregulation of CD41 expression during hematopoietic differentiation of HOXB4-ESCs. 48 hour time lapse recording of single suspension cells expressing the FMEV-eGFP-2A-HOXB4 vector was initiated 5 days after placing dissociated d6EBs on OP9 stroma cells. A PE-labeled anti-CD41 antibody was added to the culture medium to detect initiation of CD41 expression (an example from n=5 independent experiments is shown). Scale bars: 50 µm.

Figure S2. HOXB4$^+$ ESC-derived hematopoietic progenitors mediate myeloid-biased repopulation in NSG-mice, *in vivo*, Related to Figure 2A.
Analysis of mice 6 weeks after transplantation with 6x10^5 or 3x10^6 GFP$^+$/HOXB4$^+$ CCE-cells, which were harvested after 6 days of embryoid body differentiation and subsequent co-culture on OP9 cells for further 14 days. **(A)** shows the overall percentages and median values of GFP$^+$HOXB4$^+$ cells in the bone marrow (BM) and peripheral blood (PB) of single transplanted mice. **(B-D)** Flow cytometric analysis of bone marrow from a representative mouse is shown, together with that from a C57/Bl6 control mouse (B6 ctrl). The proportions of lineage positive cells within GFP$^+$ or GFP$^-$ cell populations are indicated in the quadrant analysis next to the contour plots. **(B)** GFP$^+$/HOXB4$^+$ strongly skews repopulation towards MAC1(CD11b)$^+$GR1$^+$ myeloid cells and inhibits development of B220$^+$ B- or CD3$^+$ T-lymphoid cells. **(C)** Erythroid expression of TER119$^+$/CD71$^+$ is shown, gated on the expression levels of GFP$^+$/HOXB4$^+$. Because of the 2A^{esterase} based coexpression strategy, GFP-fluorescence directly correlates with HOXB4 expression levels (Klump et al., 2001; Will et al., 2006). HOXB4 inhibits erythroid differentiation in a dosage dependent manner, reflected by the lack of more mature TER119$^+$CD71^{lo} cells when HOXB4 is highly expressed (Chen et al., 2009; Koulnis et al., 2011). **D)** Bone marrow cells were gated on the lineage$^-$ (Lin$^-$) population and expression of Kit and Sca1 determined. The proportion of LIN$^-$SCA1$^+$KIT$^+$ (LSK) hematopoietic progenitor cells is indicated in the insets.

Figure S3. Endothelial-to-Hematopoietic Transition (EHT) of HOXB4$^+$ hemogenic endothelium, Related to Figure 2 and Movie S2.
(A) Dissociated iRunx EBd6 were placed on OP9 cells and cultured for 2 days prior to the induction of *Runx1* with 0.1 µg/ml doxycycline. Endothelial-to-hematopoietic transition (EHT) was documented from day 4 of *Runx1* induction on, with images taken every 10 minutes. Morphological changes of two individual colonies are shown from four time points of a 72 hour period (lower two rows). Scale bars: 50 µm. **(B)** qRT-PCR analysis showing the relative amounts of the indicated transcripts in the EBd6/OP9d4 cultures, +/- induction of HOXB4 throughout differentiation and +/- induction of *Runx1* either from day 3 of EB-development on or only for 24 hours between day 3 and day 4 of OP9 co-culture ("24h"). Values were normalized against *Actb* and *Gapdh* housekeepers. Expression was related to the controls without *Runx1* and HOXB4 induction. Columns represent arithmetic mean values of n=3 independent experiments, error bars represent standard deviations.

Figure S4: HOXB4 does not alter proliferative activity or apoptosis of FLK-1$^+$ progenitor cells, Related to Figure 5.
(A) Scheme of the experimental strategy. **(B)** 500,000 ESCs from each sample (ctrl and HOXB4^{ERT} without 4-Hydroxytamoxifen (Tam)) were used for EB differentiation. At day 3.5 of EB differentiation, FLK-1$^+$ cells were isolated by FACS and 50,000 cells placed into blast culture medium. A representative plot is shown. **(C)** 24 hours or 48 hours later, replicating DNA was labeled with EdU for 2.5 hours. After addition of Alexa Fluor 488 picolyl azide, the proportion of EdU-AF488 positive cells was determined by flow cytometry. The results of three independent, biological replicates are shown. **(D)** Total cell numbers of day 3.5 EBs and purified FLK-1$^+$ cells after 24 and 48 hours of growth in blast culture medium were determined. Biological replicates are depicted by different symbols, technical replicates by the same colors. Bars indicate arithmetic means of cell numbers. P values were calculated based on the two-sided, unpaired Student´s t-test. A P-value < 0.05 was considered statistically significant. **(E)** Proportions of Annexin V and/or DAPI positive cells were determined by flow cytometry after 24 and 48 hours growth of isolated FLK-1$^+$ cells. Quadrant analyses showing the percentages are given above each plot. Figure **(F)** summarizes the proportions of early apoptotic (Annexin V$^+$/DAPI$^-$), late apoptotic (Annexin V$^+$/DAPI$^+$) and dead (Annexin V$^-$/DAPI$^+$) cells after 24 and 48 hours. Bars represent arithmetic means of the percentages.

Table S1: Gene expression changes associated with HOXB4 expression, Related to Figure 6A,B.

Table S2: Gene sets used for GSEA, Related to Figure 6C.

Movie S1. Genesis of CD41$^+$ hematopoietic cells, *in vitro*, promoted by HOXB4, Related to Figure 1C.
Movie S1 shows an example of a suspension cell generated from differentiating HOXB4$^+$ CCE ESCs whose daughter cells upregulate CD41 expression indicating the genesis of very first hematopoietic cells, *in vitro*. 48 hour time lapse recording of single cells in suspension retrovirally coexpressing eGFP and HOXB4 was initiated 5 days after placing dissociated d6EBs on OP9 stroma cells. Images were taken in 15 min. intervals. A PE-labeled anti-CD41 antibody was added to the culture medium (final concentration: 20 ng/ml) to detect initiation of CD41 expression. **(A)** Fluorescence **(B)** Phase contrast. Scale bars: 50 µm.

Movie S2. Endothelial-to-Hematopoietic Transition of HOXB4$^+$ hemogenic endothelium, Related to Figure 2E.
Movie S2 shows the process of endothelial-to-hematopoietic transition (EHT) of HOXB4 expressing mouse iRunx ESC-derived endothelial circular sheets formed from day 4 of OP9 coculture on, +/- induction of *Runx1* expression. Phase contrast images were taken every 10 minutes. **(A)** without Runx1, **(B)** after *Runx1* induction. Scale bars: 50 µm.

Supplemental Experimental Procedures

Production of gammaretroviral particles
293T packaging cells were transfected in 175 cm^2 flasks with 15 µg of each FMEV-derivative together with 15 µg of a plasmid expressing MLV gag-pol and 6 µg of an expression plasmid encoding the VSV-G gene driven by the CMV promoter. Transfection was performed via the $Ca_3(PO_4)_2$ precipitation method. Cell culture supernatants containing the retroviral vector particles were collected after 24 and 48 hours. Titration of vector-containing supernatants was performed using 10^5 HT1080 fibroblasts.

ES-cell culture, retroviral transduction and embryoid body differentiation
Mouse embryonic stem cells were either grown in gelatin-coated flasks (CCE ES cells)(Pilat et al., 2013) or growth-arrested murine CF1-embryonic fibroblasts (iRunx ES cells) in knockout DMEM medium containing 15% ($^v/_v$) pretested fetal calf serum (FCS), 2 mM L-glutamine (L-Gln), $1.5x10^{-4}$ M Monothioglycerol (MTG) (Sigma-Aldrich, Taufkirchen, Germany), 10 ng/ml Leukemia Inhibitory Factor (LIF, R&D) and 1% ($^v/_v$) Penicillin/Streptomycin. For isolation and subsequent culture of retrovirally transduced, fluorescent ESCs, flow cytometrical sorting was performed using a BD FACSAria III (Beckton-Dickinson, USA). DMEM was exchanged for IMDM 2 days prior to initiation of *in vitro* differentiation. Embryoid body (EB) formation was performed in suspension, as previously described with slight modifications (Lesinski et al., 2012), after removal of MEFs by magnetic activated cell sorting (MACS; Feeder Removal MicroBeads mouse; Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, 5000 ES-cells/ml were plated in IMDM medium (Lonza, Switzerland), 15% ($^v/_v$) fetal calf serum, 5% ($^v/_v$) Protein-Free Hybridoma Medium II (PFHM-II; Gibco, ThermoFisher Scientific), 2 mM L-Glutamine (L-Gln), 0.5% ($^v/_v$) Penicillin/Streptomycin (Sigma-Aldrich), 50 µg/ml ascorbic acid (Sigma Aldrich), 300 µg/ml iron saturated transferrin (Sigma-Aldrich), and $4x10^{-4}$ MTG. EBs were dissociated by 0.05% Trypsin-EDTA treatment at 37°C for 2-5 minutes. As soon as the solution started to become turbid, enzymatic activity was inhibited by addition of FCS and the EBs immediately mechanically dissociated by vigorous pipetting.

Antibodies used for Flow Cytometry Analysis and Cell Sorting
Differentiating ESCs were analysed by flow cytometry using the following fluorochrome-conjugated rat anti-mouse monoclonal antibodies, purchased from eBioscience/ThermoFisher (eBio) or BioLegend (BL):

specificity	provider	clone	conjugate	cat.no.
FLK-1 (CD309)	eBio	Avas12a1	APC	17-5821-80
FLK-1 (CD309)	eBio	Avas12a1	PE	12-5821-81
FLK-1 (CD309)	eBio	Avas12a1	Biotin	13-5821-81
TIE-2 (CD202b)	eBio	TEK4	PE	12-5987-81
TIE-2 (CD202b)	eBio	TEK4	Biotin	13-5987-82
TIE-2 (CD202b)	BL	TEK4	APC	124009
VE-Cadherin (CD144)	eBio	BV13	PE	12-1441-80
VE-Cadherin (CD144)	eBio	BV13	Biotin	13-1441-80

VE-Cadherin (CD144)	eBio	BV13	APC	17-1441-80
VE-Cadherin (CD144)	BL	BV13	BV 421	138013
CD41a	eBio	MWReg30	PE	12-0411-81
CD41a	eBio	MWReg30	Biotin	13-0411-82
CD41a	eBio	MWReg30	APC	17-0411-80
CD41a	eBio	MWReg30	APC-eFluor 780	47-0411-82
CD45	eBio	30-F11	PE-Cy7	25-0451-81
CD45	eBio	30-F11	Biotin	13-0451-81
CD45	eBio	30-F11	PE	12-0451-82
CD45	eBio	30-F11	APC	17-0451-82
CD45	BL	30-F11	BV 711	103147
KIT (CD117)	eBio	2B8	APC	17-1171-81
KIT (CD117)	eBio	2B8	PE-Cy7	25-1171-82
KIT (CD117)	BL	2B8	BV 421	105827
PDGFRB (CD140b)	eBio	APB5	PE	12-1402-81
PDGFRB (CD140b)	eBio	APB5	Biotin	13-1402-82
PDGFRA (CD140a)	eBio	APA5	Biotin	13-1401-80
PDGFRA (CD140a)	eBio	APA5	PE-Cy7	25-1401-82

For analysis of bone marrow and peripheral blood of transplanted mice, the following additional antibodies against surface markers were used:

specificity	**provider**	**clone**	**conjugate**	**cat.no.**
CD11b (MAC-1)	eBio	M1/70	PerCP-Cy5.5	45-0112-82
CD11b (MAC-1)	eBio	M1/70	APC	17-0112-82
CD150	BL	TC15-12F12.2	BV 421	115926
CD48	BL	HM48-1	PerCP-Cy5.5	103422
SCA1 (LY-6A/E)	eBio	D7	PE-Cy7	25-5981-82
B220 (CD45R)	eBio	RA3-6B2	PE-Cy7	25-0452-82
CD3ε	eBio	145-2C11	PerCP-Cy5.5	45-0031-82
CD3ε	BL	BM10-37	BV 421	100341
KIT (CD117)	eBio	2B8	APC	17-1171-82
CD71	BL	RI7217	PE-Cy7	113812
TER-119	eBio	TER-119	APC	17-5921-82
GR-1 (LY-6G)	eBio	RB6-8C5	eFluor 450	48-5931-82

For lineage cell detection in mouse bone marrow, the biotin-conjugated antibody cocktail was used from Miltenyi Biotec (Bergisch-Gladbach, Germany)(cat.no. 130-092-613).
Biotin-labeled antibodies were detected with Streptavidin conjugated to either APC-eFluor 780 (TF, cat.no. 47-4317-82) or PE-Cy7 (TF, cat.no. 25-4317-82).

Hematopoietic differentiation on OP9 cells
Dissociated EBs were cocultured with OP9 cells in IMDM, 10% ($^{v}/_{v}$) FCS (PAA/GE-Healthcare), 2 mM L-Gln containing the following cytokines: 100 ng/ml rmSCF, 40 ng/ml rmTPO, 100 ng/ml rhFlt3, 40 ng/ml rhVEGF (STFV)(McKinney-Freeman et al., 2009). All employed cytokines were obtained from PeproTech (Hamburg, Germany). OP9 stroma cells were plated at a density of 250,000 cells per 6-well 24 hours prior to cocultivation, in α-MEM (GE-Healthcare) supplemented with 20% ($^{v}/_{v}$) FCS (Biochrom), 2 mM L-Gln and 1% ($^{v}/_{v}$) Penicillin/Streptomycin. Cell numbers and volumes were determined using an electronic cell counter (CASY, Schärfe Systems, Reutlingen, Germany).

Hematopoietic Colony Assays
For testing the ability to form hematopoietic colonies, ESC-derived hematopoietic cells were plated in semisolid methylcellulose supplemented with rmSCF, rmIL-3, rhIL-6 and rhEPO (M3434 MethoCult, Stem Cell Technologies) in 24 wells by seeding 500 ESC-HCs. All assays were done in triplicates and colonies evaluated after 12 days.

Proliferation measurements
For measuring cell proliferation, incorporation of EdU (5-ethynyl-2'-deoxyuridine) into DNA during replication was determined. In brief, day 3.5 embryoid bodies from iRunx-ESCs (ctrl and HOXB4^{ERT} without Tamoxifen) were dissociated, 50,000 FLK-1^{+}cells isolated by FACS and seeded on gelatinized plates in IMDM containing 10% ($^{v}/_{v}$) FCS (GE-Healthcare), 2 mM L-glutamine, 20% ($^{v}/_{v}$) D4T conditioned medium, 1% ($^{v}/_{v}$) Penicillin/Streptomycin, 4.5x10^{-4} M MTG, 25 ng/ml ascorbic acid, 300 µg/ml holotransferrin (Sigma-Aldrich), 10 ng/ml rhIL-6, 5 ng/ml rhVEGF and Tam (100 nM). Cells were incubated under hypoxic conditions (3% O_2) at 37^{0}C in a H_2O saturated atmosphere. 24 and 48 hours post sort, cells were pulse-labeled for 2.5 hours after addition of 10 µM EdU to the medium. As a negative control, medium containing 0,1% (v/v) DMSO (the solvent used for the 10 mM EdU stock) was used. Incorporated EdU was detected by staining with Alexa Fluor 488 picolyl azide and subsequent analysis by flow cytometry, according to the according to manufacturer's recommendations (Click-it^{TM} Plus EdU Flow Cytometry Assay Kit, Thermo Fisher Scientific, Germany).

Apoptosis assay
The Annexin V-FITC kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) in combination with DAPI staining was employed to determine the proportion of apoptotic and dead cells. EBd3.5 FLK-1^{+} iRunx cells were isolated and grown as described for the proliferation assay. On two consecutive days (24 and 48 hours post FLK-1^{+} sort), cells were stained with Annexin V-FITC and DAPI (final concentration 0.1 µg/ml). As a positive control for apoptosis, FLK-1^{+} sorted cells irradiated with 10 Gy were used.

Transplantation experiments and analysis
All animal experiments were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV)(reference number 84-02.04.2013.A350) and were performed according to official guidelines and regulations. 8-12 weeks old NOD.Cg-Prkdc^{scid}IL2rgtm1^{Wjl}/SzJ (NSG) mice were sublethally irradiated with 2 Gy and transplanted via retro-orbital injection. Prior to transplantation, OP9 stromal cells were removed using a monoclonal antibody directed against CD140b and subsequent MACS, according to the manufacturer´s guidelines (Miltenyi, Bergisch-Gladbach, Germany). 6x10^{5}, 3x10^{6} of unsorted ESC-derived hematopoietic cells were co-transplanted with 1x10^{5} freshly isolated, bone-marrow cells (NOD/SCID) per recipient mouse. For analysis, mice were sacrificed 6 weeks post transplantation. Total bone marrow cells were harvested from tibae and femora after crushing with pestle and mortar in MACS-buffer (1xDPBS, 0.5% BSA, 2 mM EDTA). After filtering through a 40 µm mesh (cell strainer, BD Bioscience), red blood cells were lysed with ACK lysing buffer (Lonza), for 8 min. on ice. Before flow cytometry analysis, whole bone marrow and peripheral blood samples were counted on an animal blood counter system (Scil VetABC hematology analyzer, animal care company, Viernheim, Germany). Lineage positive cells from BM were collectively labeled with biotinylated lineage marker antibodies (GR-1, CD11b, B220, CD3ε and TER119)(Miltenyi Biotec, Bergisch-Gladbach, Germany, cat.no. 130-092-613) and stained with Streptavidin-APC eFluor conjugate. Peripheral blood was obtained from mouse tail veins or from the retro-orbital sinus using heparin-coated capillary tubes. After erythrocyte lysis, cells were washed with 1xPBS, antibody staining performed in MACS-buffer.

Analysis of transcription by qRT-PCR
For quantitative, real-time PCR (qRT-PCR) measurements of gene specific transcripts, total RNA was extracted from cells using TRIzol reagent (Thermo Fischer Scientific Inc., Waltham, MA, USA) and subsequent purification of RNA using the RNeasy kit (Qiagen, Hilden, Germany). 1 µg of RNA were reverse transcribed into cDNA using a mixture of Oligo dT and random hexamer primers (QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany). The RNA template was then removed with RnaseH and 1/100 volume of the reaction used for a PCR reaction using the following desoxyoligonucleotide-primers:

Runx1:	5´- ATGACCAGCCTCTCTGCAGAACT- 3´and
	5´-AGATGGACGGCAGAGTAGGGAA -3´
Gfi1:	5´- CTGTGCTAGGTATGGGAGAGCCAG-3´ and
	5´- GCCTTCTTCAGAACTCCCTTGGG -3´
Gfi1b:	5´- CAGGATGGGGAATCACCACTC -3´and
	5´- GGGGTCTGTGTGTAGCTGT -3´
Lmo2:	5´- GACGGAAATTGTGCAGGAG -3´ and
	5´- GATGCACAGAGACCATCCT -3´
Sox17:	5´- ACGCTAGCTCAGCGGTCTACTAT -3´and
	5´- AAGGATTTCCTTAGCGCTTCCAG -3´
Pu.1 (Sfpi1):	5´- AGCAGAGCTCAGATGAGGAGGAG -3´and
	5´- GGAACTGGTACAGGCGAATCTTT -3´
Scl/Tal1:	5´-CCAACAACAACCGGGTGAAG -3´ and

	5´-GCCGCACTACTTTGGTGTGAG- 3´
Gata2:	5´-GCTGCAGAGCACCCTAACCG -3´ and
	5´-CTTCTCTTATGCGGGTACTAGCAC -3´
Pecam1:	5´-AGCAATGGCAACTGGAGCGAGC -3´ and
	5´-TCTTCTTTGGGCCTTCGGCAT -3´
Cdh5:	5´-CCCAGGGCACACCTTGCAGAA -3´ and
	5´-CAAGGGAGAGATGCAGAGAGGC -3´

The amplification efficiency of each primer pair was determined as described (Pfaffl, 2001). Amplification was carried out in 96-well PCR plates (Peqlab/VWR, Erlangen, Germany) in a Biorad CFX real-time-PCR machine, using the QuantiFast SYBRGreen reagents (Qiagen, Hilden, Germany). Relative differences in gene expression were calculated using the $2^{-\Delta CT}$ method by normalizing the CT-values for each gene to the CT-values of two housekeeping genes, *β-Actin* and *Gapdh* (Vandesompele et al., 2002). Cycling conditions were 95^0C for 5 min followed by 40 amplification cycles being 95^0C denaturation for 10 sec and a combined annealing and elongation step at 60^0C for 30 sec.

Time Lapse Microscopy

For time lapse analysis, cultures were kept at 37^0C, 5% CO_2, 3% O_2 in a humidified chamber (Incubator XL multiS1, Zeiss, Jena, Germany). Photos were taken using a Zeiss Axio Observer X.1 microscope. Axio Vision Software 4.8 was used for image processing, editing and animation (Carl Zeiss, Jena, Germany). For detection of CD41 expression on the cell surface of emerging hematopoietic cells, phase contrast and fluorescence images were taken in 15 minute intervals from day 5 of OP9 coculture on, over a 48 hour period. The PE-conjugated anti-CD41 mAb was directly added to the culture medium every 8 hours at a final concentration of 20 ng/ml (Movie S1A). For documentation of EHT, phase contrast photos of iRunx/OP9 cocultures were taken in 10 minute intervals, from day 4 of coculture on over a period of 72 hours. For induction of *Runx1* and documentation of ongoing EHT, doxycycline was added to the cultures on day 2 and day 4 of OP9 coculture in a final concentration of 0.1 µg/ml (Movie S1B).

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