SUPPLEMENTARY APPENDIX

In vitro human embryonic stem cell hematopoiesis mimics MYB-independent yolk sac hematopoiesis

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Supplemental materials and methods

MATERIALS AND METHODS

Cell lines, culture of cell lines and isolation of primary cells

All experiments were approved by the Medical Ethical Committee of Ghent University Hospital (Belgium). The WA01 (National Institutes of Health code: WA01) human embryonic stem cell (hESC) line was used in all experiments. The hESC line was kept in an undifferentiated state on MEFs as described. Single cell adapted hESC were cultured as described (Costa et al. Nature Protocols. 2007, Vol 2, No 4, pp 792-796). Briefly, single cell suspensions were made from hESC cultures using TrypLE select (Gibco, Life Technologies, Carlsbad, CA, USA) and were subsequently cultured on MEFs at a density of 21.000c/cm^2. Cultures were split every 3 days. OP9 cells, MOLT4 and K562 were purchased from ATCC (LGC Standards SARL, Molsheim, France). Cell lines were cultured in medium supplemented with L-glutamine, streptomycin and penicillin with 10-20% FCS (all from Invitrogen, Life Technologies, Carlsbad, CA, USA). Peripheral blood monocytes were isolated after hydroxyethyl starch (HES) sedimentation for 30' at room temperature and subsequently sorted by FACS.

Hematopoietic differentiation of hESC in spin embryoid bodies (EB)

To differentiate hESC into hematopoietic cells, the protocol from Ng et al. was used with minor modifications (Ng et al. Nature Protocols. 2008, Vol 3, No 5, pp 768-776). In brief, 5x10^3 single cell-adapted hESC were spun at 480 g into each well of a 96-well low attachment plates and subsequently cultured in APEL medium containing 10 µM Rock inhibitor Y-27632 (Selleckchem, Houston, TX, USA), 40 ng/ml SCF (Peprotech, Rocky Hill, NJ, USA), 2 ng/ml BMP4 (R&D, Minneapolis, MN, USA) and 20 ng/ml VEGF165 (Peprotech), further referred to as "EB mix". After 4 days, spin EB were transferred on an OP9 cell layer and further cultured in EB mix for a total of 7-14 days. Half of the medium was changed on day 7, with APEL medium containing EB mix cytokines, unless a different combination of cytokines is specified. These cytokines were added at following concentrations: 50 ng/ml IL-3 (R&D), 50 ng/ml Flt3-L (R&D), 10 ng/ml TPO (Peprotech) and/or 50 ng/ml IL-6 (R&D).

For myeloid differentiation, spin EBs were dissociated at day 11 and transferred onto OP9 cells in MEM-a with 20% FCS, supplemented with 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech), 10 ng/ml IL3 (R&D) and 20 ng/ml IL-6 (R&D). For erythro-megakaryocytic differentiation, spin EBs were dissociated and transferred onto OP9 cells in MEM-a with 20% FCS, supplemented with 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech) and 50 ng/ml EPO (eBioscience, San Diego, CA, USA).

Methocult assay

Methocult assay was performed according the manufacturers protocol. In brief, 2x10e3c CD34+CD43+ cells were isolated using FACS at indicated time points. Cells were resuspended in Methocult GF H84434 medium (Stemcell technologies, Vancouver, British Columbia, Canada) and plated in 35mm tissue culture dishes. Colonies were scored after 14 days of culture.

Single cell progeny assay

For single cell progeny assay d11 or d14 embryoid body cultures were dissociated and stained for FACS sorting following standard procedures. Cells were deposited in Terasaki 60well format plates (Nunc, Roskilde, Denmark) using FACS ARIA III with ACDU. Cells were cultured on OP9 feeders in MEM-a with 20% FCS, supplemented with 50 ng/ml SCF (Peprotech), 50 ng/ml TPO (Peprotech), 10 ng/ml IL3 (R&D), 20 ng/ml IL-6 (R&D) and 50 ng/ml EPO (eBioscience). Cells were scored for growth by microscopy, and expanding clones were assayed by flow cytometry after 7 days of culture for indicated markers.

Flow cytometry and cell sorting

Before adding mAb, FcR blocking was performed using human IgG (Miltenyi Biotec). Flow cytometric analysis was performed on a LSR II system (BD biosciences, San Jose, California, United States of America). Cell sorting was performed with a FACS ARIA IIIU system (BD Biosciences). The following (conjugated) anti-human mAbs were used: CD10-PE (eBioscience), CD13-PE (eBioscience), CD11b-PE (Becton Dickinson); CD11c-PE (Becton Dickinson); CD14-PE (Miltenyi Biotec); CD15-PE (Miltenyi Biotec); CD16-PE (Coulter); CD33-PE (Miltenyi Biotec); CD34-APC (Miltenyi Biotec); CD41a-PE (Becton Dickinson); CD43-PE (Becton Dickinson), -V450 (Becton Dickinson), CD45-APCVio770 (Miltenyi Biotec); CD66-PE (eBioscience), CD86-PE (eBioscience), CD117-PECy7 (c-kit, eBioscience), CD123 (IL-3R)- PE (eBioscience); CD235A (GlycophorinA)-PE (Beckman-Coulter, Brea, CA, USA). Data were analyzed using FacsDIVA software (Beckton Dickinson) and Flowjo software (Treestar, Ashland, OR, USA).

Real time RT-PCR

Cells were lysed and cDNA was synthesized using the SYBR power cells-to-ct system (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Primers were designed and *in silico* specificity was determined using NCBI primer BLAST and manufactured by Biolegio (Biolegio, Nijmegen, The Netherlands). All PCRs were validated for efficiency and linearity on four point dilution series using a relevant control cDNA. All primers had efficiencies between 90 and 110%. Primer sequences can be found in supplemental TableS1. PCR reagents and SYBR GreenI master were obtained from Roche and used according to the manufacturer's instructions. The

reactions were run on a lightcycler480 384well system (Roche, Penzberg, Germany). The following cycling conditions were used: 5 min at 95°C for initial denaturation, 40 cycles at 95°C for 15 s, and 60°C for 30 s. After amplification, a melting curve was generated for every PCR product and compared with melting curve of control cells. The relative expression was calculated for each gene by the dCt method, relative to the mean of reference genes used as indicated.

Generation of reporter Bacterial Artificial Chromosome

BAC plasmid clone CH17-400L19 containing the MYB gene flanked by approximately 100kbp of genomic sequence on either side of the start codon was obtained from CHORI BACPAC (Children 's Hospital Oakland Research Institute, Oakland, CA, USA). To generate reporter BAC plasmids, an eGFPpA-LoxP-PGK-Gb2-Neo-pA-LoxP vector was targeted to the first start codon of the *MYB* gene using recombination-mediated genetic engineering. In brief, CH17-400L19 BAC plasmid containing DH10b were grown under Chlor-Amphenicol antibiotic selection (Sigma-Aldrich). pSC101BADgbaA[tet] (Genebridges, Heidelberg, Germany) was transfected into electro competent BAC containing cells and grown at 32°C under tetracyclin antibiotic resistance (Sigma-Aldrich). Bacterial cells containing both plasmids were then transfected with PCR amplified targeting construct, with primer integrated 50bp homology arms, and grown overnight at 37°C under Chlor-Amphenicol and Kanamycin resistance (Sigma-Aldrich).

BAC plasmids were purified using an adapted miniprep protocol, briefly, overnight bacterial cultures were pelleted and resuspended in Qiagen (Qiagen, Venlo, The Netherlands) minelute buffer P1, lysed using Qiagen minelute buffer P2 and the reaction was neutralized using Qiagen minelute buffer N3. Cell lysate was then spun down for 10' at 13200rpm in a chilled centrifuge (4°C), supernatant was transferred to a fresh tube and precipitated using isopropanol (Sigma-Aldrich). Isolated plasmid was then resuspended in nuclease free water (Ambion). For analysis of BAC plasmids, 50µg of plasmid DNA was digested for up to 16h with BamHI restriction enzyme (NEB, Ipswich, Massachusetts, USA). Restriction fragments were separated on a 0.8% agarose gel and visualized using UV light after incubation with Ethidium Bromide.

The MYBeGFP reporter construct was functionally validated by transfection in MOLT4 and K562, which express levels of *MYB* within a biologically relevant range. After transfection and neomycin selection, both cell lines expressed eGFP as expected. Subsequent transfection of the cell line with Cre recombinase induced an eGFP bright population in both cell lines suggesting that removal of the selection cassette caused increased activity of the *MYB* promoter (Figure S1).

Generation of reporter hESC

A total of $2x10^6$ single cell adapted hESC were nucleofected with 5µg purified BAC reporter using Lonza Amaxa human stem cell nucleofector kit 2 (Lonza) with program F16. Cells were replated at high density ($1x10e5c/cm^2$) in single cell conditions on drug resistant DR4 MEFs (a kind gift of Dr. Hochepied, VIB, Ghent, Belgium). A total of 10µM of the Rock inhibitor Y-27632 (Selleckchem) was added. G418 (50µg/ml) (Gibco) was added from day three onwards and retained for three weeks. Single colonies were picked, expanded and screened for transgene integration. Colonies showing transgene integration were transfected using pCAGGS-NLS-Cre-PGK-Puro (plasmid 7779, BCCM/LBMP, Ghent, Belgium) and kept under puromycin (Sigma-Aldrich) selective pressure (300ng/ml) for three days to remove the neomycin selection cassette. Single colonies were expanded and screened by PCR. Genomic DNA was isolated using Genelute mammalian genomic DNA miniprep kit (Sigma-Aldrich) according to the manufacturer's instructions. Colonies containing successfully floxed cells were cloned using single cell deposition on the FACS Aria.

FISH analysis

Metaphase slides were pretreated with RNase and pepsin. Slides were denatured with 70% formamide/2× sodium saline citrate phosphatase (SSCP) at 80°C for 5 min. Probe mix was denatured at 75°C for 5 min, incubated at 37°C for 30 min and subsequently applied to the slides under a 18 × 18 mm coverslip. After 2–4 days of hybridization, slides were washed with 50% formamide/2× sodium saline citrate (SSC) (pH 7.3–7.5) at 42°C (3 × 5 min), followed by 3 washes in 2 × SSC (42°C). Metaphases were stained using the Vysis MYB SpectrumAqua FISH Probe Kit (Abbott Molecular, North Chicago, Illinois, United States of America) Slides were mounted in Vectashield (Vector, Burlingame, California, United States of America) containing DAPI counterstain.

High-resolution arrayCGH

DNA was hybridized to 1M human Genome CGH Microarray slides from Agilent Technologies (Santa Clara, CA, USA) according to the manufacturer's instructions with minor modifications. 1µg of genomic DNA was labeled with Cy3 and 1µg of reference DNA (Kreatech, Amsterdam, The Netherlands) was labeled with Cy5 using the BioPrime Array CGH Genomic Labeling System (Invitrogen) and subsequently purified by precipitation. After 24 h of hybridization at 65 °C, microarrays were washed and scanned at 5 µm resolution using a DNA microarray scanner (Agilent). The scan images were processed with Feature Extraction Software 10.1 (Agilent) and further analyzed and visualized with our in-house developed and freely available software tool ViVar (http://www.cmgg.be/ViVar/) (Sante et al. Submitted). Copy number variants (CNVs) were identified

by circular binary segmentation requiring a minimum of 5 consecutive oligonucleotides exceeding an absolute log_2-ratio threshold of 0.35 (≈2.5× standard deviation).

Live confocal analysis

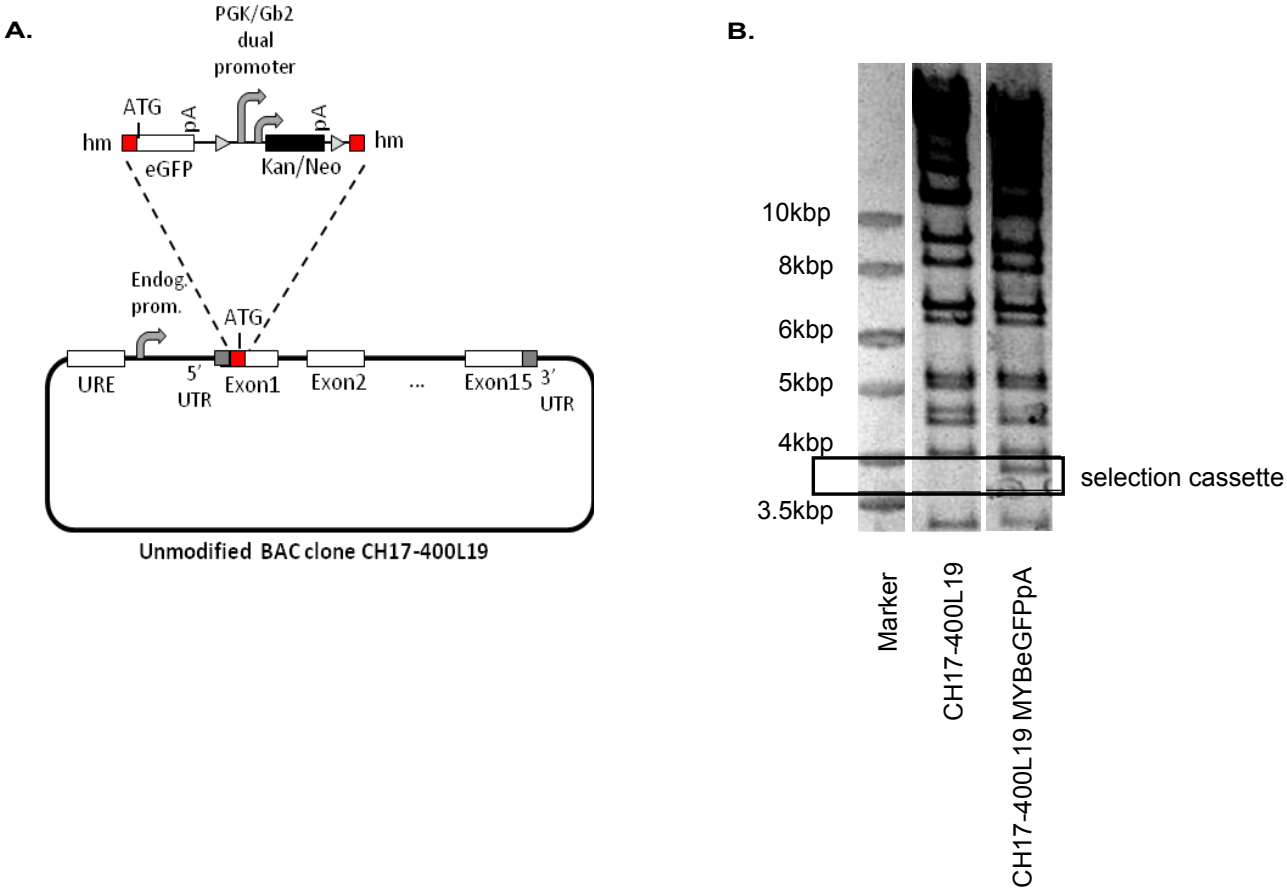
Live confocal analysis were performed on a Leica SPE confocal microscope with CO_2 and temperature control. All analysis were done at 37°C in MEMα containing 20% FCS and indicated cytokines. Images were made using an ACS APO 10.0x0.30 DRY lense. Fluorochrome-labeled mAbs were added directly to the cultured medium. Folowing mAbs were used: CD34-APC (Miltenyi biotec), CD43-PE(Beckton Dickinson), CD14-PE (Miltenyi biotec). Azide was removed from these antibodies using D-Tube™ Dialyzer Mini, MWCO 12-14 kDa (Novagen, EMD Millipore, Billerica, MA, USA). Images were acquired using LAS AF software (Leica microsystems, Wetzlar, Germany). Images were processed using FiJi (FiJi is Just imageJ) software. Adjustment of brightness and contrast and cropping of the images were performed on the complete image and all channels.

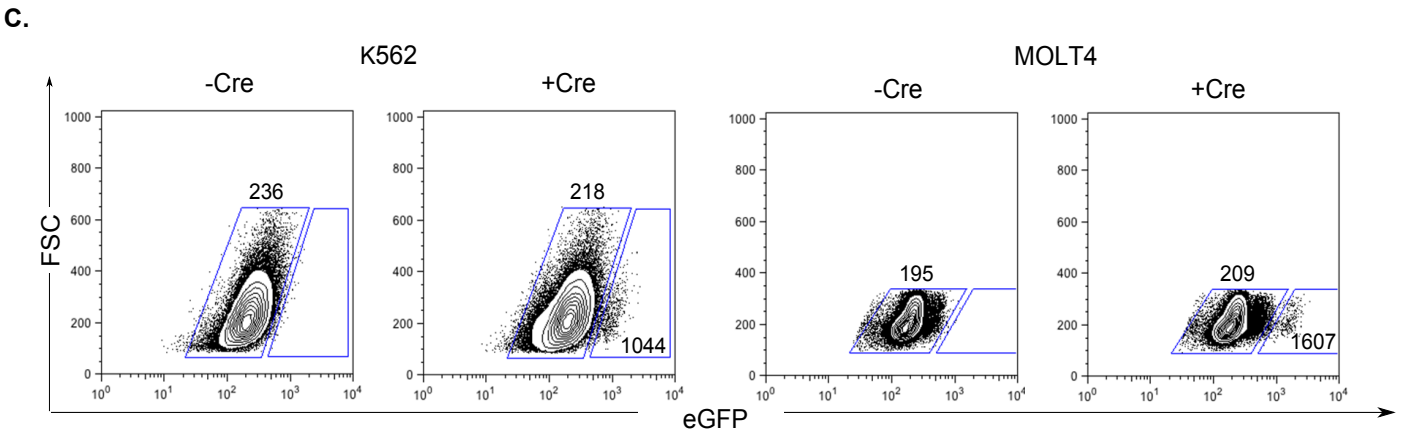
Statistical analysis

All statistical analyses were performed using SPSS V22.0 (IBM, New York, USA). Significance was assessed using Mann-Whitney U statistical analysis with significance level set at $p \leq 0.05$.

Supplemental table I: primer sequences used in this study

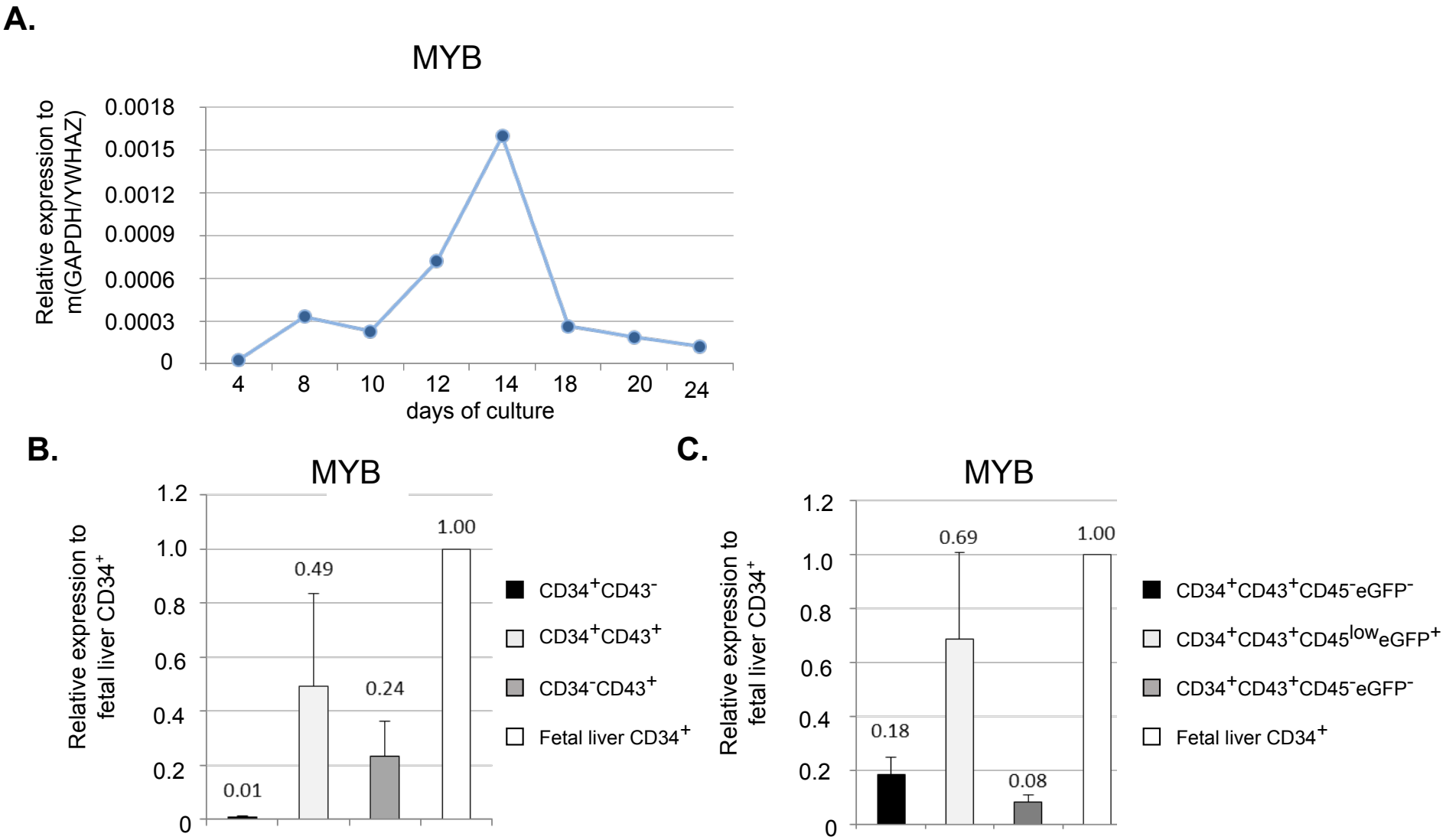
Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
MPL	GAGATGCAGTGGCACTTGGA	CCAGATGGGGTACCTGTCTCTG
MYB	GTCTCCAGTCATGTTCCATACC	TGAATGGCTGCGGCAGCT
GATA1	CAAGAAGCGCCTGATTGTCAG	AGTGTCGTGGTGGTCGTCTG
LTF	TGCTGGAGACGTTGCATTTG	CTCZGTCACAGGCTTCCGTT
MBP	TCGGCTCACAAGGGATTCAA	AAGCTGAGGACAGGATTCCG
MPO	TGCATCATCGGTACCCAGTTC	AGATGTTGTTCTTAGACACGGTGG
PU1	GGATCTATACCAACGCCAAACG	GGGTGGAAGTCCCAGTAATGG





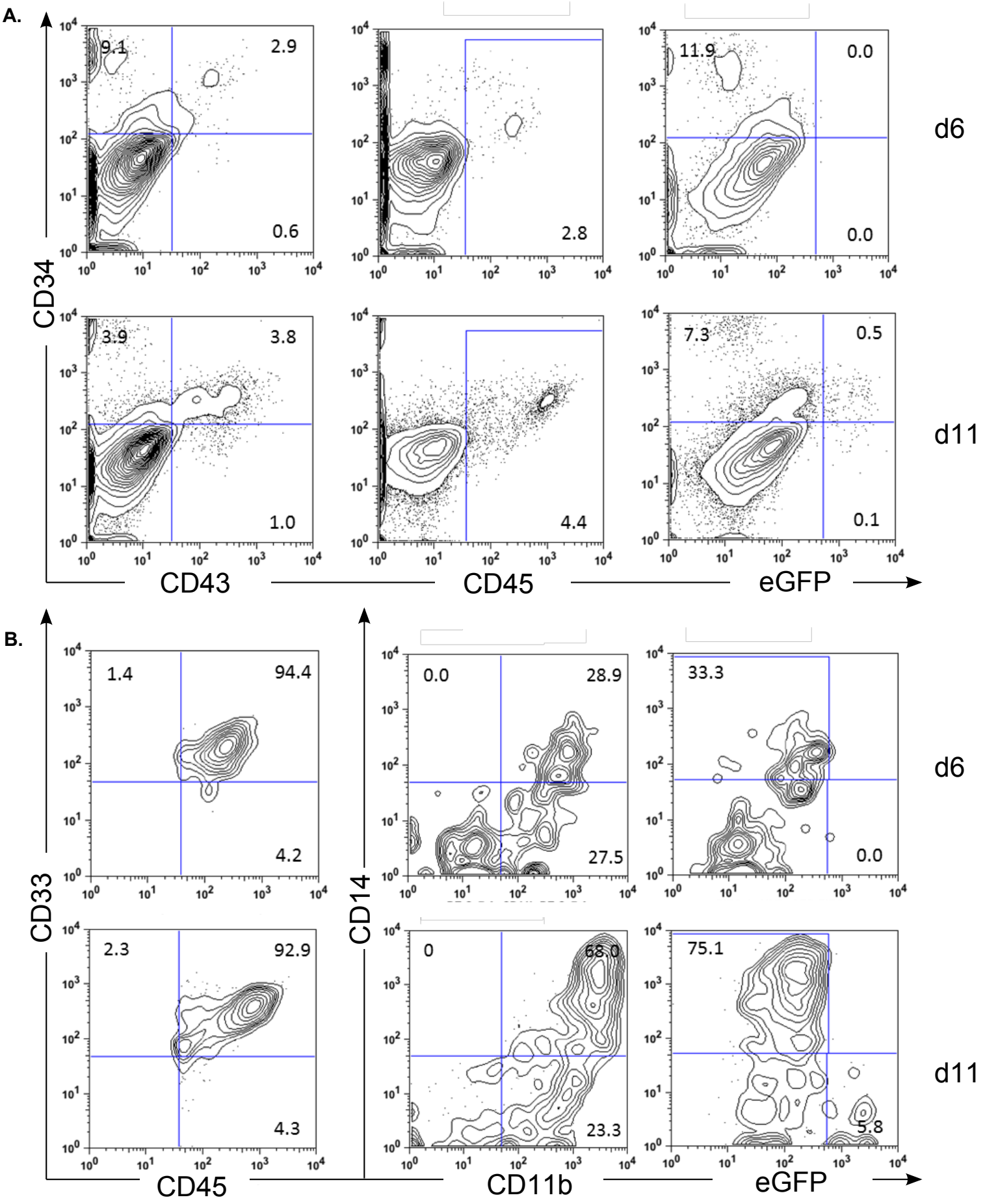
FigureS1: Generation of MYB-eGFP reporter hESC.

A) Schematic overview of BAC recombineering. Red squares indicate homology arms, grey triangles indicate LoxP sequences; B) Restriction digest of the targeted BAC vector shows successful integration of the selection cassette in between BamHI restriction sites; C) K562 and MOLT4 stably transfected with MYB-eGFP BAC plasmid were analyzed for eGFP expression, before and after transient Cre recombinase transfection. Mean Fluorescence Intensities (MFI) for gated populations are shown.



FigureS2: Expression profile of MYB in EB derived hematopoietic progenitors.

A) Kinetics of MYB expression in bulk hESC feeder differentiation culture. Expression is shown relative to the mean of GAPDH and YWHAZ expression; B) MYB expression was analyzed in indicated populations from d14 EB. dCt was calculated relative to the mean of GAPDH and YWHAZ expression. Expression is indicated relative to expression in fetal liver $CD34^+$. Error bars indicate standard deviation of the mean (n=3); C) MYB expression was analyzed in indicated populations within the $CD34^+CD43^+$ population. dCt was calculated relative to the mean of GAPDH and YWHAZ expression. Expression is indicated relative to expression in fetal liver $CD34^+$. Error bars indicate standard deviation of the mean (n=3).



FigureS3: CD34^{+}CD43^{-}eGFP^{-} endothelial cells give rise to eGFP^{-} hematopoietic precursors, before emergence of eGFP^{+}CD34^{+}CD43^{+} cells.

A) Analysis of progeny of CD34^{+}CD43^{-} hemogenic endothelial cells derived from d11 EB. Cells are analyzed after 6 and 11 days of culture respectively; B) Phenotypic analysis of CD45^{+} cells from panel A at indicated time points.

MovieS1: Time-lapse analysis of HPC generation from endothelial cells.

Time-lapse confocal analysis of MYBeGFP hESC d11 EB differentiation sorted $CD34^+$ cells cultured on OP9 with hematopoietic cytokines. Cells were live stained with CD34-APC and CD43-PE. Images were acquired every 30minutes, scale bare measures 100µM, movie is played at a frame rate of 4fps (or 2h per second). Arrow indicates a single $CD34^+$ sorted endothelial cell transforming into a hematopoietic progenitor cells.

MovieS2: Time-lapse analysis of macrophage differentiation.

Time-lapse confocal analysis of MYB-eGFP hESC d11 EB differentiation of sorted $CD34^+CD43^+CD45^+eGFP^-CD14^-$ cells cultured on OP9 with hematopoietic cytokines. Cells were live stained with CD14-PE. Images were acquired every 15 minutes, scale bare measures 100µM, movie is played at a frame rate of 4fps (or 1h per second). Arrow indicates a single progenitor developing into a $CD14^+$ macrophage.