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ORIGINAL ARTICLE

The Notch ligand DLL4 specifically marks human hematoendothelial progenitors and regulates their hematopoietic fate

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Notch signaling is essential for definitive hematopoiesis, but its role in human embryonic hematopoiesis is largely unknown. We show that in hESCs the expression of the Notch ligand DLL4 is induced during hematopoietic differentiation. We found that DLL4 is only expressed in a sub-population of bipotent hematoendothelial progenitors (HEPs) and segregates their hematopoietic versus endothelial potential. We demonstrate at the clonal level and through transcriptome analyses that $DLL4^{high}$ HEPs are enriched in endothelial potential, whereas $DLL4^{low/-}$ HEPs are committed to the hematopoietic lineage, albeit both populations still contain bipotent cells. Moreover, DLL4 stimulation enhances hematopoietic differentiation of HEPs and increases the amount of clonogenic hematopoietic progenitors. Confocal microscopy analysis of whole differentiating embryoid bodies revealed that $DLL4^{high}$ HEPs are located close to $DLL4^{low/-}$ HEPs, and at the base of clusters of CD45+ cells, resembling intra-aortic hematopoietic clusters found in mouse embryos. We propose a model for human embryonic hematopoiesis in which $DLL4^{low/-}$ cells within hemogenic endothelium receive Notch-activating signals from $DLL4^{high}$ cells, resulting in an endothelial-to-hematopoietic transition and their differentiation into CD45+ hematopoietic cells.

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INTRODUCTION

Definitive hematopoietic stem cells (HSCs) are generated during embryonic development and represent the life-long source of functional hematopoietic cells.[1] Human embryonic stem cells (hESCs) and induced pluripotent stem cells represent an ideal source of HSCs for clinical use.[2] However, attempts at producing hESC-derived HSCs capable of robust immune reconstitution have been unsuccessful.[3] Increasing our knowledge on the signaling pathways involved in human embryonic hematopoiesis will help designing new protocols for *in vitro* generation of HSCs.

In the embryo, definitive hematopoiesis cannot occur in the absence of endothelial cell development and arterial specification, which are regulated by the conserved signaling pathways Sonic Hedgehog, Wnt and Notch, with a prominent role for the ligand DLL4.[4–7] Once the dorsal aorta (DA) is formed, definitive HSCs emerge from the hemogenic endothelium (HE) on the ventral wall of the DA, by a process known as endothelial-to-hematopoietic transition (EHT).[8] The limited studies available suggest that similar processes may be occurring during human embryonic hematopoiesis.[9,10] *In vitro,* hematopoietic differentiation of hESCs occurs through the generation of a KDR+VE-cadherin+CD31+CD34+CD45− bipotent hematoendothelial progenitor (HEP) that can originate both endothelial and hematopoietic cells and could be considered equivalent to the HE.[11,12] However, the molecular identity of hemogenic and non-hemogenic endothelial cells, and the extrinsic and intrinsic mechanisms leading to hematopoietic commitment of HE cells remain unclear.

The Notch pathway is essential for definitive HSC emergence in the embryo. Embryos from Notch1−/−, Jagged1−/− and CSL/RBP-Jκ−/− knock-out mice lack intraembryonic hematopoiesis,[13,14] and blocking Notch signaling in zebrafish also abrogates definitive hematopoiesis.[15] However, data on its role in humans are scarce. In hESCs, transient Notch activation increases the generation of CD45+ cells from human embryoid bodies (hEBs),[16] and Notch signaling via Hes1 is necessary for hematopoietic differentiation of hESCs.[17] However, there is no data on which ligands are responsible for Notch activation in human HE and their contribution to EHT and hematopoietic differentiation.

Here we analyzed the involvement of Notch signaling in human embryonic hematopoietic differentiation using hESCs. We found that the ligand DLL4 exclusively marks a subset of HEPs, and its surface expression segregates at the clonal level hematopoietic- versus endothelial-committed HEPs. Moreover, DLL4 activates Notch in HEPs and induces their hematopoietic differentiation. Using whole-mount hEB confocal microscopy, transcriptomics and *in vitro* assays, we provide evidence to sustain a model of human hematopoiesis in which DLL4-expressing cells within the HEP population signal to neighboring $DLL4^{low/-}$ cells to differentiate

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into CD45+ hematopoietic cells, resembling what occurs in mouse AGM hematopoietic clusters.

MATERIALS AND METHODS

hESC culture

The hESCs lines AND1 and H9 were cultured in Matrigel-coated T25 flasks (BD Biosciences) in mesenchymal stem cell conditioned media supplemented with 8 ng/ml basic fibroblast growth factor (Miltenyi) with daily media changes. Cells were split weekly by dissociation with 200 U/ml of collagenase IV (Invitrogen). hESC cultures were visualized daily by phase contrast microscopy. The production of mesenchymal stem cell conditioned media has been extensively reported elsewhere.[18,19] All the work with hESCs has the approval from the Spanish National Embryo Ethical Committee.

Hematopoietic differentiation from hESCs through embryoid body formation

Undifferentiated hESCs at confluence were treated with collagenase IV and scraped off of the Matrigel attachments. To allow hEB formation, hESC clumps were transferred to low-attachment plates (Corning) and incubated overnight in differentiation medium (DM; Knock-out-Dulbecco's modified Eagle's medium supplemented with 20% non-heat-inactivated fetal bovine serum, 1% nonessential amino acids, 1 mmol/l l-glutamine, and 0.1 mM β-mercaptoethanol). The medium was changed the next day (day 1) with the same DM supplemented with hematopoietic cytokines: 300 ng/ml stem cell factor (SCF), 300 ng/ml Flt3L, 10 ng/ml interleukin (IL)-3, 10 ng/ml IL-6, 50 ng/ml granulocyte-colony-stimulating factor and 25 ng/ml bone morphogenetic protein 4.[20,21] hEBs were dissociated using collagenase B (Roche Diagnostic) for 2 h at 37 °C followed by 10 min incubation at 37 °C with enzyme-free Cell Dissociation Buffer (Invitrogen) at days 7, 10, 15 and 22 of development. A single-cell suspension was obtained by gentle pipetting and passage through a 70-μm cell strainer. The dissociated cells were stained with anti-CD34-PE-Cy7(BD Biosciences), anti-CD31-PE, anti-CD45-APC or -FITC and DLL4-APC or -PE antibodies (all from Miltenyi) and 7-actinomycinD (BD Biosciences). Live cells identified by 7-actinomycinD exclusion were analyzed using a FACSCanto II flow cytometer equipped with FACS Diva software (Becton Dickinson).

Generation of OP9-DLL1 and OP9-DLL4 cell lines

The pLZRS-IRES-eGFP retroviral constructs encoding either the full-length human DLL1 or the green fluorescent protein (GFP) empty vector (EV) were kindly provided by Dr L Parreira (Instituto de Histologia e Embriologia, Lisboa, Portugal). Human DLL4 full-length complementary DNA (cDNA) expressed in the pcDNA3.1/myc-His expression vector was a generous gift of Dr G Tosato (National Institutes of Health, Bethesda, MD, USA). DLL4 cDNA was excised from the pcDNA3.1/myc.His expression vector and cloned into the *Bam*HI and *Eco*RI restriction sites of the pLZRS-IRES-eGFP retroviral vector. Restriction and sequence analyses were performed to confirm proper ligation of the human DLL4 open reading frame into the pLZRS-IRES-eGFP vector. DLL1, DLL4 and EV retroviral constructs were lipofected (Fugene) into the packaging 293T cell line. Transfected 293T cells were selected with 2.5 μg/ml of puromycin (Clontech). Two weeks after transfection, puromycin-selected cells were plated at confluency in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% HEPES (Gibco) without puromycin to obtain high titers of retrovirus. EV, DLL1 or DLL4 retroviral supernatants were used for transduction of the OP9 stromal cells (ATCC) that were seeded at semiconfluency onto p24-well plates. Transduction was performed by centrifugation in the presence or 8ng/ml of polybrene and OP9 cells were analyzed for GFP expression at 48 h post-transduction by flow cytometry and then sorted for enrichment (100%) in GFP-expressing cells. OP9-Jagged2 are a kind gift of Dr Tom Taghon (Ghent University, Belgium).

hESCs-OP9 co-cultures

hESC-OP9 co-cultures were performed as previously described[22] with minor modifications. Briefly, OP9 stroma was prepared by plating OP9 cells in gelatine-coated 10-cm dishes in alpha-minimum essential media basal medium supplemented with 20% non-heat-inactivated fetal bovine serum. OP9 cells were let to grow for 8 days to form an overgrown monolayer. The hESC lines AND1 and H9 grown in Matrigel-coated flasks were prepared as a suspension of small aggregates using collagenase IV treatment followed by gentle scraping in DM (alpha-minimum essential media basal medium, 10% non-heat-inactivated fetal bovine serum, 100 mM monothioglycerol and 50 mg/ml ascorbic acid). One-tenth of this suspension was plated on top of the 8-day overgrown OP9 stroma in 10 ml of DM. Next day, media was replaced by 20 ml of DM to remove unattached cells. Where indicated, we added the following inhibitors: 200 nM compound E (CpE; Calbiochem), 500 ng/ml bevacizumab (Grifols Laboratories, a kind gift from Dr Rodriguez Manzaneque) or 350 nM PKF-115-584 (Novartis). From day 3 of co-culture, a half-volume media change was performed every other day. Hematopoietic differentiation was assessed by flow cytometry (at days 6, 8 and 10 of co-culture). Cells were treated for 1 h with collagenase IV followed by 20 min with Tryple (Gibco), dissociated by pipetting and filtered through a 70 μm strainer. Single-cell suspensions were stained with anti-mouse CD29-FITC and anti-human CD31-PE, CD34-PE-Cy7 and CD45-APC. In the case of transgenic OP9 cell lines, we skipped the staining with anti-CD29-FITC because these cells express GFP. The proportion of hemogenic progenitors (CD31+CD45−), primitive blood cells (CD34+CD45+) and total blood cells (CD45+) was analyzed within the hESC-derived cell population identified as either CD29 or GFP-negative cells.

HEPs-OP9 co-cultures

hEBs at day 10 or 11 of differentiation were dissociated as described above and the HEP population was purified by MACS separation using the human CD34 Microbead kit and the AutoMACS Pro separator (Miltenyi Biotech) as per the manufacturer's instructions. Purity of the CD34 fraction was assessed by flow cytometry and only CD34 fractions showing ⩾95% purity were used. Purified HEPs were then plated on either OP9-EV, OP9-DLL4, OP9-DLL1, OP9-Jagged1 or OP1-Jagged2 stroma ± 200 nM CpE on six-well plates using DM supplemented with hematopoietic cytokines (300 ng/ml SCF, 300 ng/ml Flt3L, 10 ng/ml IL-3, 10 ng/ml, IL-6 and 50 ng/ml granulocyte-colony-stimulating factor). At day 7 of co-culture, the cells were dissociated as described above, analyzed by flow cytometry in a similar way and plated for CFUs assays in methylcellulose. Alternatively, DLL4^{high} and DLL4$^{low/-}$ HEPs were FACS purified using anti-human CD31-PE, CD45-FITC, CD34-PE-Cy7 and DLL4-APC (all from Miltenyi) and plated on OP9 stroma. At day 7 of co-culture, the cells were dissociated as described above and analyzed by flow cytometry.

CFU assays

CFU assays were performed by plating 35 000 cells from day 7 HEPs-OP9 co-cultures into serum-free methylcellulose H4436 (Stem Cell Technologies) supplemented with SCF (50 ng/ml), erythropoietin (3 U/ml), granulocyte-macrophage colony-stimulating factor (10 ng/ml), and IL-3 (10 ng/ml). Cells were incubated at 37 °C in a 5% CO_2-humidified atmosphere, and colonies were counted after 14 days using standard morphological criteria.

Real-time reverse transcriptase–polymerase chain reaction

Total RNA was extracted from hEBs, hESCs-OP9 co-cultures or CFUs using either Total RNA Purification Kit (Norgen Biotek) or Trizol (Invitrogen). First-strand cDNA synthesis was performed using the First-Strand cDNA Synthesis Kit (Amersham). The resulting cDNA was analyzed for differential gene expression by using Brilliant II SYBR Green QPCR master mix on a Mx3005P Q-PCR System (Stratagene). Primer sequences are listed in Supplementary Table S6.

To confirm gene expression changes from the microarray data, we used a quantitative PCR (qPCR) array to analyze the expression levels of 84 genes involved in the Notch pathway (RT^2 Profiler PCR Array Human Notch Signaling Pathway, PAHS-059Z, SA Biosciences, QIAGEN), 40 of which were present in the microarray. A total of 10^5 purified CD31+CD34+CD45− DLL4^{high} and CD31+CD34+CD45− DLL4$^{low/-}$ HEPs were used for total RNA extraction using RNeasy Plus kit (QIAGEN). RNA quality (RIN 9.0) was assessed using a Bioanalyzer (Agilent Technologies) before cDNA synthesis using 150 ng total RNA per sample. The resulting cDNA was used as template to perform qPCR analysis. The raw data were analyzed using SA Biosciences web-based tool (www.sabiosciences.com/pcrarraydataanalysis.php), after which we discarded genes categorized as 'C' for their low-quality quantitative PCR data, as recommended by the manufacturer. Genes showing a change in expression >1.5-fold were considered differentially expressed between the two groups.

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Endothelial differentiation from purified HEPs and *in situ* immunocytochemistry

DLL4^{high} HEPs and DLL4$^{low/-}$ HEPs were FACS isolated from hEBs at day 11 of development. To promote endothelial differentiation, 2×10^4 isolated HEPs were seeded on 0.1% gelatine-coated 24-well plates in complete EGM-2 media with microvasculature supplements (Lonza, Walkersville, MD, USA) for 7 days. After fixation and permeabilization, cells were stained with rabbit anti-human VE-cadherin (Cayman, Miami, FL, USA), and mouse anti-human eNOS (BD Biosciences), mouse anti-human von Willebrand factor (DAKO) followed by Alexa488-conjugated (Invitrogen) anti-rabbit or Cy3-conjugated anti-mouse antibodies (Jakson Immunoresearch), respectively. The nuclei were counterstained with 4,6-diamidino-2-phenylindole. Pictures were obtained using an EVOS fluorescence inverted microscope.

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HEPs differentiation in immobilized DLL4 protein

In all, 2x10^4 FACS-isolated DLL4^{high} and DLL4$^{low/-}$ HEPs were cultured for 12 days in StemSpan supplemented with SCF, FLT3, granulocyte-colony-stimulating factor, IL-6 and IL-3 in 24-well plates coated with either 0.2% bovine serum albumin or 500 ng/µl purified DLL4 (R&D Systems) diluted in 0.2% bovine serum albumin. After this time, the cells were collected and hematopoietic differentiation assessed by flow cytometry.

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Gene expression profiling

In total, 10^5 DLL4^{high} and DLL4$^{low/-}$ HEPs purified by FACS from H9 and AND1 hEBs at day 15 of differentiation were used for gene expression analysis using Whole Human Genome Oligo Microarray chips (Agilent Technologies). Analysis of gene functions and canonical pathways was performed using the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems Inc., Redwood City, CA, USA), and the Gene Set Enrichment Analysis software (http://www.broadinstitute.org/gsea/index.jsp). The microarray data have been deposited in the public Gene Expression Omnibus database, accession number GSE56881.

Clonal experiments with FACS-sorted single DLL4^{high} and DLL4$^{low/-}$ HEPs

Single DLL4^{high} and DLL4$^{low/-}$ HEPs FACS isolated from day 11 hEBs were deposited into individual wells of 96-well plates coated with 0.1% gelatine in the presence of a 1:1 mixture of hematopoietic (100 µl of StemSpam supplemented with SCF, FLT3 and IL-3) and endothelial (100 µl of complete EGM-2 media) supportive media. Nine 96-well plates (~850 single cells) were used per condition. Single-cell sorting was carried out using a FACSAria sorter equipped with an Automatic Cell Deposition Unit (ACDU). Sorted single cells were allowed to expand and differentiate for 12 days and the resulting clonal outgrowth in each well was analyzed *in situ* by phase contrast morphology and immunocytochemical staining for CD45 (hematopoietic) and VE-cadherin (endothelial). The nuclei were counter-stained with 4,6-diamidino-2-phenylindole.

Q16

Confocal analysis of whole-mount hEBs

hEBs undergoing hematopoietic differentiation for 11 or 15 days were fixed using fresh methanol:dimethylsulphoxide (4:1) and stored at -20 °C until used. hEBs were then rehydrated in methanol, washed in PBT (phosphate-buffered saline containing 0.1% Tween 20), and incubated with blocking solution (TBST containing 2% nonfat milk carnation and 0.5% Tween) for 1 h. hEBs were stained at 4 °C overnight with primary antibodies (all at 1:50 dilution): rat monoclonal anti-CD45 (Abcam), mouse monoclonal anti-CD31 (Dako), rabbit polyclonal anti-DLL4 (Abcam), rabbit polyclonal anti-NICD1 (Sigma). After washing in TBST for 5 h, hEBs were incubated with their corresponding secondary antibodies (anti-rabbit-Alexa488, anti-rat-Alexa546, anti-mouse-Alexa647; 1:1000 dilution) and 4,6-diamidino-2-phenylindole (1:10 000 dilution) overnight at 4 ºC. After washing in TBST for 5 h, the stained hEBs where mounted in glycerol 80%. Images were acquired in a Zeiss LSM710 confocal microscope, using the ZEN 2009 software.

Q17 Q18

Mice xenotransplantation and analysis of engraftment

We used the mice strain NOD/LtSz-scidIL2Rγ$^{-/-}$ (NSG). Animals were housed under pathogen-free conditions and all the procedures were approved by the Animal Care Committee of Parc de Recerca Biomèdica de Barcelona and Generalitat de Catalunya. CB-derived CD34+ cells (2×10^5 cells/20 µl), as well as day 4 EV-OP9 or DLL4-OP9 co-cultured hematopoietic derivatives (5×10^5 cells/20 µl) were transplanted intra-bone marrow to sublethally irradiated (2.25 Gy) mice at 7–12 weeks of age. They were monitored throughout the entire experiment and killed 7 weeks after transplantation. Injected tibiae, contra-lateral tibiae, spleen, liver and peripheral blood were collected and analyzed for human hematopoietic engraftment by flow cytometry using anti-HLA-ABC-PE and anti-humanCD45-APC-Cy7 antibodies (BD Biosciences).

Statistical analysis

We applied *T*-test and ANOVA tests for statistical analysis of all data, except for the single-cell cloning experiment where we applied a Wald's test using R script (http://cran.r-project.org).

RESULTS

Notch receptors and ligands are differentially expressed during hESC hematopoietic differentiation

To address how the Notch pathway contributes to human embryonic hematopoiesis, we differentiated two hESCs cell lines (AND1 and H9) toward a hematopoietic fate through the formation of hEBs. This differentiation process consists of a stage I of HEPs specification followed by a stage II of hematopoietic commitment of these HEPs, marked by the appearance of hematopoietic progenitors in CFU assays[12,21,23] (Figures 1a and b). We analyzed by qPCR the expression of the four human Notch receptors (Notch1–4) and four human ligands (DLL1, DLL4, Jagged1 and Jagged2) in hEBs at different time points of hematopoietic differentiation. Notch1 and Notch2 are already expressed in undifferentiated hESCs (Supplementary Figure S1A) and become moderately upregulated as differentiation progresses (Figure 1c). Notch4 is barely expressed in hESCs (Supplementary Figure S1A) but it is strongly upregulated during hematopoietic differentiation (Figure 1c). As for the ligands, hESCs only express Jagged1 (Supplementary Figure S1A), but its expression barely changes throughout hEB differentiation. In contrast, from day 7 onward there is an upregulation of Jagged2 (~3-fold) and DLL4 (~50-fold) (Figure 1c).

In order to find associations between gene expression and generation of hematopoietic cells, we correlated the expression values of each gene with the percentages of the populations of interest (HEPs, CD45+ hematopoietic cells and CD34+CD45+ hematopoietic progenitors; Supplementary Figure S1B) at each differentiation time point. Several statistically significant correlations were observed for Notch1, Notch2, Notch3 and DLL4 (Supplementary Table S1). Despite its upregulation, we found no significant correlation of Notch4 with any hematopoietic population. We focused on the correlations involving DLL4, as DLL4 expression during stage II of hematopoietic commitment positively correlated with the output of CD34+CD45+ and CD45+ cells (Figure 1d). Thus, we generated a working hypothesis proposing that DLL4-mediated Notch activation favors hematopoietic commitment of HEPs.

We also analyzed which signaling pathways are involved in the regulation of DLL4 expression during hESC hematopoietic differentiation. We used small molecules that inhibit the three main pathways reported to regulate DLL4 expression in endothelial cells:[5,24,25] the blocking antibody bevacizumab, which prevents activation of vascular endothelial growth factor (VEGF) receptors; the γ-secretase inhibitor CpE, which blocks the activation of Notch receptors; PKF-115-584, which inhibits the transcriptional activity of β-catenin.[26] Owing to the low permeability of the hEB system to small molecules, and to ensure the efficacy of these inhibitors, we performed hESC hematopoietic differentiation using OP9 co-cultures.[22] In these co-cultures, hESCs also generate HEPs and CD45+ hematopoietic cells (Supplementary Figure S1C), and DLL4 expression is also upregulated at the time of HEPs appearance (Supplementary Figure S1C). As shown in Figure 1e, inhibition of Notch activation by CpE reduced DLL4 expression at day 8 of co-culture to ~40% of the levels present in control co-cultures,

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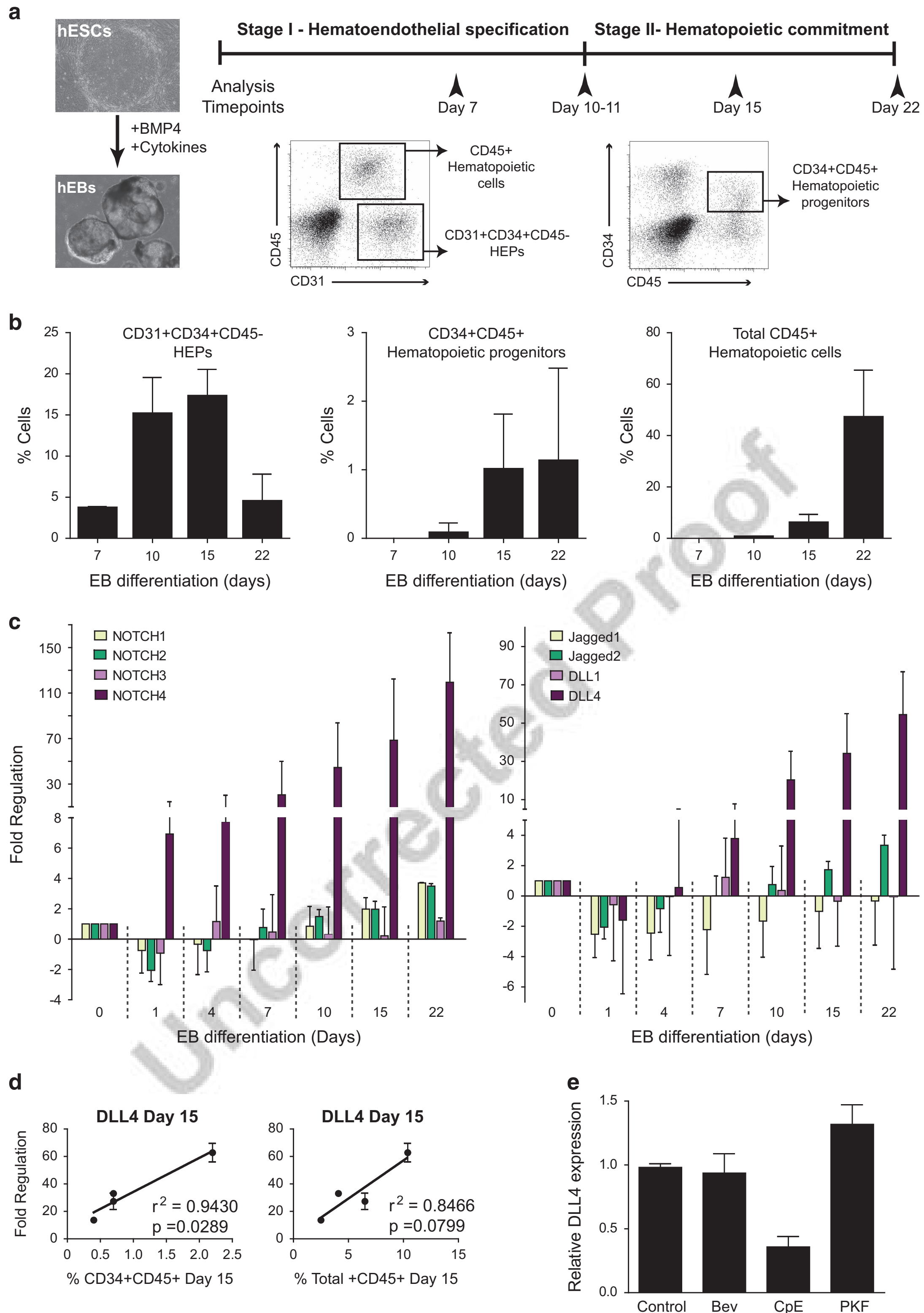

Figure 1. Expression kinetics of Notch ligands and receptors during hESC hematopoietic differentiation. **(a)** Schema of hESC hematopoietic differentiation system based on hEB formation and flow cytometry analysis. The differentiation process can be divided in stage I of HEPs specification, and stage II of hematopoietic commitment, based on the appearance of hematopoietic progenitors in CFU assays at day 10 of differentiation. **(b)** Kinetics of appearance of HEPs (CD31+CD34+CD45-), hematopoietic progenitors (CD34+CD45+) and total hematopoietic cells (CD45+ cells) in the differentiation experiments ($n = 4$). **(c)** Relative changes in the expression of Notch receptors (left graph) and ligands (right graph) during hEBs hematopoietic differentiation. qPCR data are presented as fold regulation considering expression in undifferentiated hESCs (Day 0) as 1. **(d)** Correlations between DLL4 expression and emergence of hematopoietic cells (CD45+) and hematopoietic progenitors (CD45+CD34+), respectively ($n = 4$). See also Supplementary Figure S1 and Supplementary Table S1. **(e)** Analysis of signaling pathways regulating DLL4 mRNA levels in hESC undergoing hematopoietic differentiation. Bev, bevacizumab; PKF, PFK-115-584.

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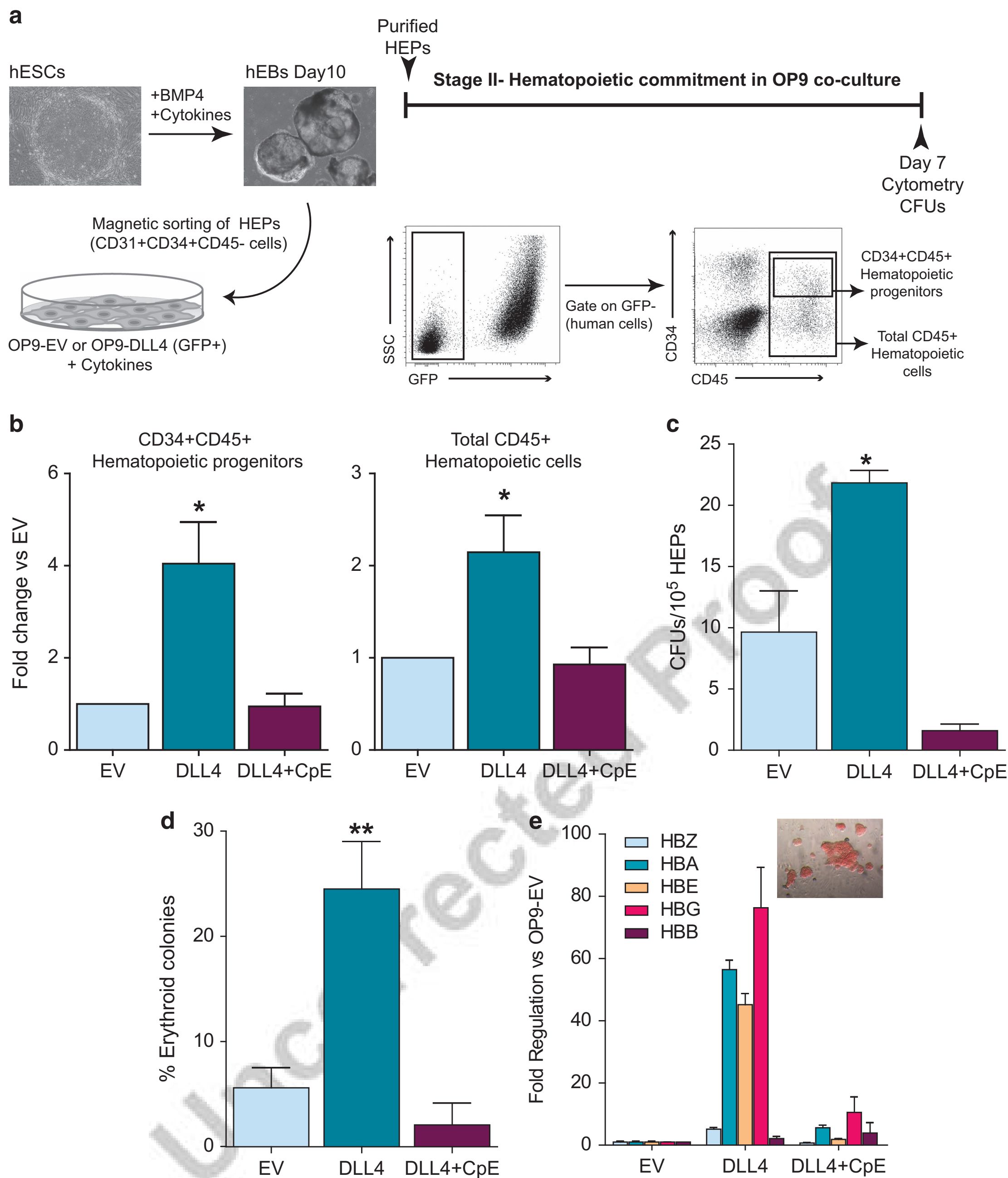

Figure 2. Activation of Notch by DLL4 enhances the differentiation of HEPs toward a hematopoietic fate. **(a)** HEPs were purified by magnetic sorting from hEBs at day 10 of differentiation and co-cultured with either OP9-EV or OP9-DLL4 stroma (both GFP+) in the presence of hematopoietic cytokines. Differentiating hematopoietic progenitors (CD34+CD45+) and hematopoietic cells (CD45+) were identified within the human GFP− population. **(b, c)** Effect of DLL4 on the appearance of hematopoietic progenitors (left panel) and total hematopoietic cells (right panel) in HEPs-OP9 co-cultures (**b**), and on the generation of hematopoietic colonies (CFUs) from HEPs-OP9 co-cultures (**c**). **(d)** Abundance of erythroid colonies in CFUs formed from HEPs co-cultured with the different OP9 stromas described in **c**. **(e)** Expression of the different hemoglobins in CFUs. qPCR data are presented as fold regulation considering expression in CFUs from OP9-EV co-cultures as 1. Inset shows a representative erythroid CFU. $n=5$; * $P<0.05$, ** $P<0.01$. See also Supplementary Figure S2.

whereas the other two inhibitors did not have a significant impact on DLL4 mRNA levels. Thus, in hESCs undergoing hematopoietic differentiation the Notch pathway is the main regulator of DLL4 expression.

DLL4-mediated Notch activation enhances the differentiation of HEPs toward a hematopoietic fate

To analyze whether DLL4-mediated Notch activation participates in the hematopoietic commitment of HEPs, we isolated HEPs from hEBs at days 10–11 of differentiation (before CD45+ cells emerge) and co-cultured them on OP9-EV or OP9-DLL4 (Figure 2a). DLL4 stimulation enhanced the appearance of both CD34+CD45+ hematopoietic progenitors (~4-fold) and total CD45+ hematopoietic cells (~2-fold) from HEPs (Figure 2b). CFU assays confirmed that DLL4 also enhanced the production of clonogenic hematopoietic progenitors (Figure 2c) and promoted a skew toward an erythroid lineage (Figure 2d), as previously reported for fetal liver CD34+ cells and mouse ES cells.[27,28] qPCR showed that different hemoglobin genes (including adult HBA) were highly upregulated

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in CFUs from OP9-DLL4 co-cultures, confirming the promotion of erythroid lineage (Figure 2e). All these effects were reversed by the γ-secretase inhibitor CpE, demonstrating that DLL4 is acting in a Notch-dependent manner. It has been postulated that this skew toward an erythroid lineage may be mediated by DLL4-regulated expression of ephrinB2,[28] which will act through its receptor EphB4 that is expressed in erythroid progenitors.[29] We analyzed by qPCR the expression levels of ephrinB2 in OP9-DLL4 cells and found that the exogenous overexpression of DLL4 was not increasing endogenous ephrinB2 levels in these cells. Then, we quantified ephrinB2 and EphB4 levels in CFUs and found that none of them were upregulated in the CFUs originating from HEPs/OP9-DLL4 co-cultures (data not shown). Based on these results, we hypothesize that the ephrinB2/EphB4 pathway is not responsible for the erythroid skew caused by DLL4. Importantly, both the increase in clonogenic progenitors and the skew toward erythroid lineage is specific of DLL4, as DLL1 did not significantly change the amount of CFUs or the proportion of erythroid colonies (Supplementary Figure S2). We did not observe either a significant increase in hematopoietic differentiation of HEPs when co-cultured with OP9-Jagged1 or OP9-Jagged2 (data not shown).

DLL4 is exclusively expressed in a sub-population of HEPs

As exogenous DLL4 stimulation enhances hematopoietic differentiation of HEPs, we asked which population in the differentiating hEBs expresses DLL4. We analyzed the expression levels of Notch receptors and ligands by qPCR in FACS-purified HEPs, hematopoietic cells and non-hematopoietic/remaining cells from hEBs (Figure 3a). We found that HEPs differentially express Notch1, Notch4 and DLL4 at both days 10 and 15 of differentiation, a pattern also present in endothelial cells of the DA[14,30–32] (Figure 3b). Based on this expression pattern, we can speculate that DLL4-mediated hematopoietic differentiation of HEPs (Figure 2) may be occurring through activation of either Notch1 or Notch4. HEPs differentially express the endothelial marker VEGFR2/FLK1 and the HE marker SCL/TAL1, whereas CD45+ cells are enriched in the hematopoietic transcription factor PU.1 (Figure 3b), confirming the identity of the purified populations.

Q20

By flow cytometry, DLL4 could be detected exclusively in a sub-population of HEPs (Figure 3c), confirming our qPCR data. Confocal microscopy on whole-mount hEBs showed a consistent staining of CD31+ cells arranging themselves in vessel-like structures. Some of these CD31+ cells (5–20%) also showed strong, punctuated, intracellular DLL4 staining, possibly due to the recycling of DLL4 in the signaling cell,[14,33,34] although we were also able to see in some instances DLL4 present at the membrane co-localizing with CD31 (Figure 3d). CD31+DLL4+ cells were intermingled with CD31+DLL4– cells (Figure 3d), confirming the existence of two CD31+ populations with different levels of DLL4.

Gene expression profiling of DLL4^{high} and DLL4$^{low/-}$ HEPs

Next, we FACS-isolated DLL4^{high} and DLL4$^{low/-}$ HEPs at day 15 of differentiation and performed gene expression analysis using microarrays (Figure 4a). We found 5606 genes differentially expressed in DLL4$^{low/-}$ compared with DLL4^{high} HEPs (Supplementary Table S2), including DLL4 itself (~7.5-fold upregulation in DLL4^{high} HEPs). To get insight into the biological functions affected by these genes, we performed ingenuity pathway analysis comparing DLL4$^{low/-}$ with DLL4^{high} HEPs. The top biological functions included 'cellular movement', 'cardiovascular development and function' and 'hematological development and function' (Figure 4b), compatible with the bipotent nature of HEPs[12] and in agreement with gene expression data from AGM-derived HSCs.[35]

Using ingenuity pathway analysis, we further analyzed which specific biofunctions are either activated or inhibited in DLL4$^{low/-}$ HEPs, based on z-score value. All the activated 'cell movement' functions are related to the movement of hematopoietic cells, whereas the few inhibited functions correspond to endothelial cell movement. Moreover, all the 'cardiovascular development and function' biofunctions are inhibited, whereas all the 'hematological development and function' biofunctions are activated in DLL4$^{low/-}$ HEPs (Figure 4c). A gene set enrichment analysis showed that genes differentially expressed in DLL4$^{low/-}$ HEPs show very significant enrichment in gene sets obtained from HSCs, megakaryocytes/platelets and hematopoietic cell lineage pathways, whereas genes differentially expressed in DLL4^{high} HEPs are enriched in gene sets obtained from endothelial cells (Supplementary Tables S3 and S4 and Supplementary Figure S3). DLL4$^{low/-}$ HEPs express genes essential for EHT (RUNX1, GFI1, GFI1B and TAL1), hematopoietic transcription factors (GATA1, GATA2, SPI1, IKZF1 and MYB), as well as the early HSC markers CD41 and CD43[36,37] (Figure 4d). Of note, DLL4$^{low/-}$ HEPs express only a few components of the Notch pathway, as already reported for ESC-derived HSC compared with definitive HSCs[35] (Supplementary Figure S4A). On the other hand, DLL4^{high} HEPs differentially express important genes involved in endothelial cell function, such as VEGFs, VEGFRs and co-receptors (neuropilins), ephrins and VE-cadherin, as well as known transcriptional regulators of DLL4, such as FOXC1/2[ref.38] (Figure 4d and Supplementary Figure S4B).

To confirm our microarray data, we quantified the expression of 40 selected genes by qPCR and obtained a high (~0.75) concordance correlation coefficient between the microarray and the qPCR data (Figure 4e and Supplementary Table S5). By flow cytometry, we confirmed the enriched expression of the endothelial markers CD31, CD34 and KDR/VEGFR2 in DLL4^{high} HEPs, whereas the early hematopoietic markers CD41 and CD43 are expressed exclusively in a small fraction of DLL4$^{low/-}$ HEPs (Figure 4f). Taken together, these analyses indicated that DLL4$^{low/-}$ HEPs are undergoing an EHT leading to their differentiation into hematopoietic cells, whereas DLL4^{high} HEPs retain an endothelial identity.

DLL4 expression segregates HEPs that retain an endothelial fate from HEPs that are committed to a hematopoietic fate

We next wanted to verify whether DLL4^{high} and DLL4$^{low/-}$ HEPs were already committed progenitors or they still retained their bipotent nature (Figure 5a). In conditions promoting endothelial differentiation, DLL4^{high} HEPs grow into a monolayer of endothelial cells expressing VE-cadherin at cell–cell contacts, as well as the mature endothelial marker von Willebrand factor. However, DLL4$^{low/-}$ HEPs can barely grow in these conditions, express VE-cadherin in a disorganized manner and lack von Willebrand factor expression (Figure 5b). On the other hand, when cultured in conditions promoting hematopoietic differentiation, DLL4$^{low/-}$ HEPs produce 3–4 times more hematopoietic cells than DLL4^{high} HEPs (Figure 5c).

To confirm these results, we performed single-cell cloning assays under conditions promoting both endothelial and hematopoietic differentiation. DLL4^{high} HEPs generated a much higher proportion of wells with purely endothelial growth, whereas DLL4$^{low/-}$ HEPs gave rise to a significantly higher proportion of wells with hematopoietic cells (Figure 5d). Of note, both populations originated similar numbers of wells with a mix of hematopoietic and endothelial cells, indicating that both DLL4^{high} and DLL4$^{low/-}$ HEPs retain some degree of plasticity and are still bipotent cells characteristic of HE.[39,40] The hematopoietic cells generated in this assay expressed VE-cadherin, resembling the primitive HSC population described in mouse and human AGM.[10,41,42] Therefore, the differential expression of DLL4 discriminates between HEPs that retain endothelial characteristics and those that are undergoing EHT to acquire an hematopoietic

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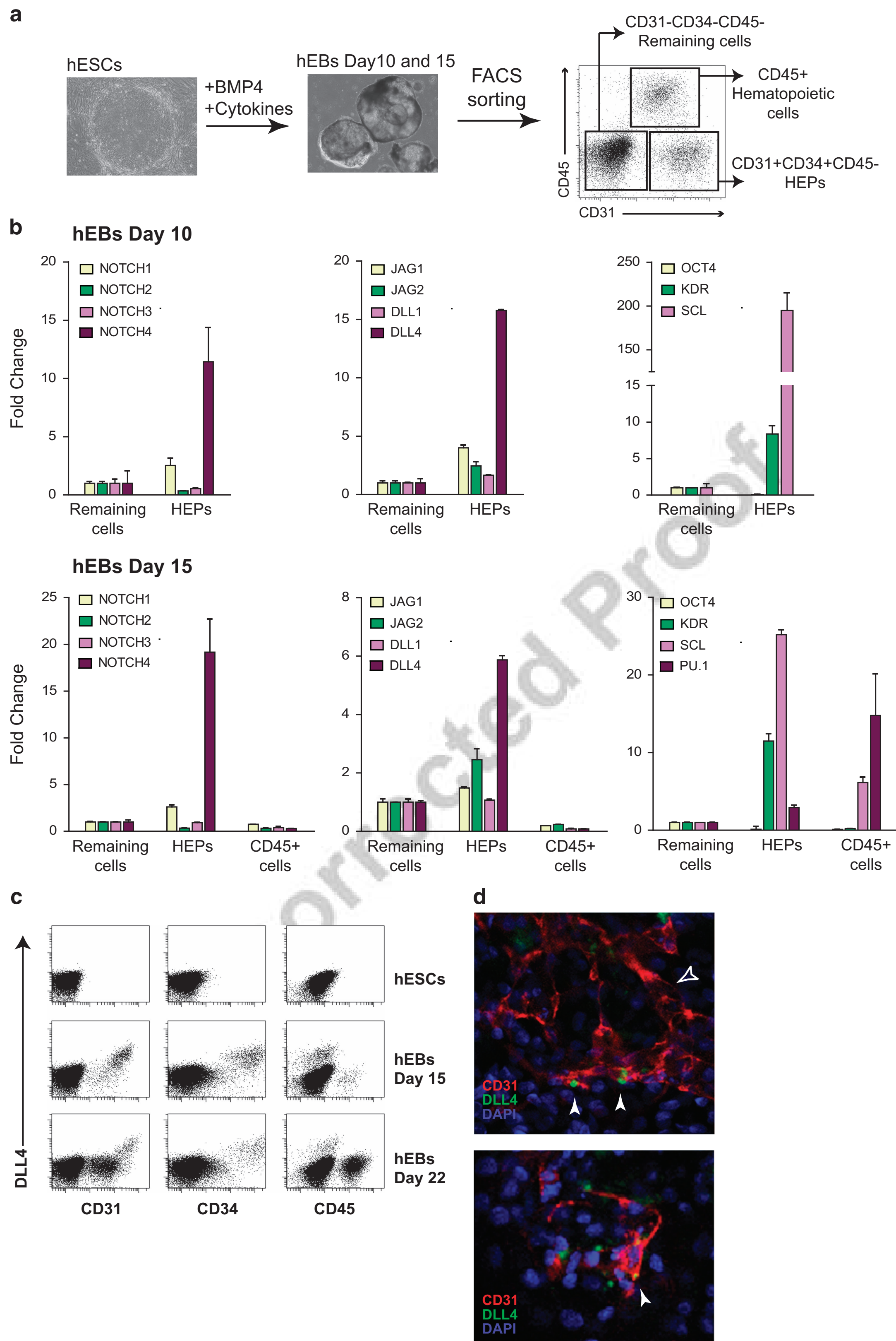

Figure 3. DLL4 is expressed in a sub-population of CD31+CD34+CD45– HEPs in the differentiating hEBs. **(a)** Sorting strategy used to purify our populations of interest from hEBs at day 15 of differentiation. **(b)** Relative levels of expression of Notch receptors (left graphs) and ligands (middle graphs) in the purified HEPs from day 10 hEBs and HEPs and CD45+ hematopoietic cells from day 15 hEBs. The pluripotency marker OCT4, the HEP markers KDR and SCL, and the hematopoietic transcription factor PU.1 were used to verify the identity of the purified populations (right graphs). qPCR data are presented as fold change considering expression in the remaining population (CD31–CD34–CD45– cells) as 1. **(c, d)** We confirmed the expression of DLL4 within a sub-population of HEPs by flow cytometry (**c**) and confocal microscopy of whole-mount hEBs (**d**).

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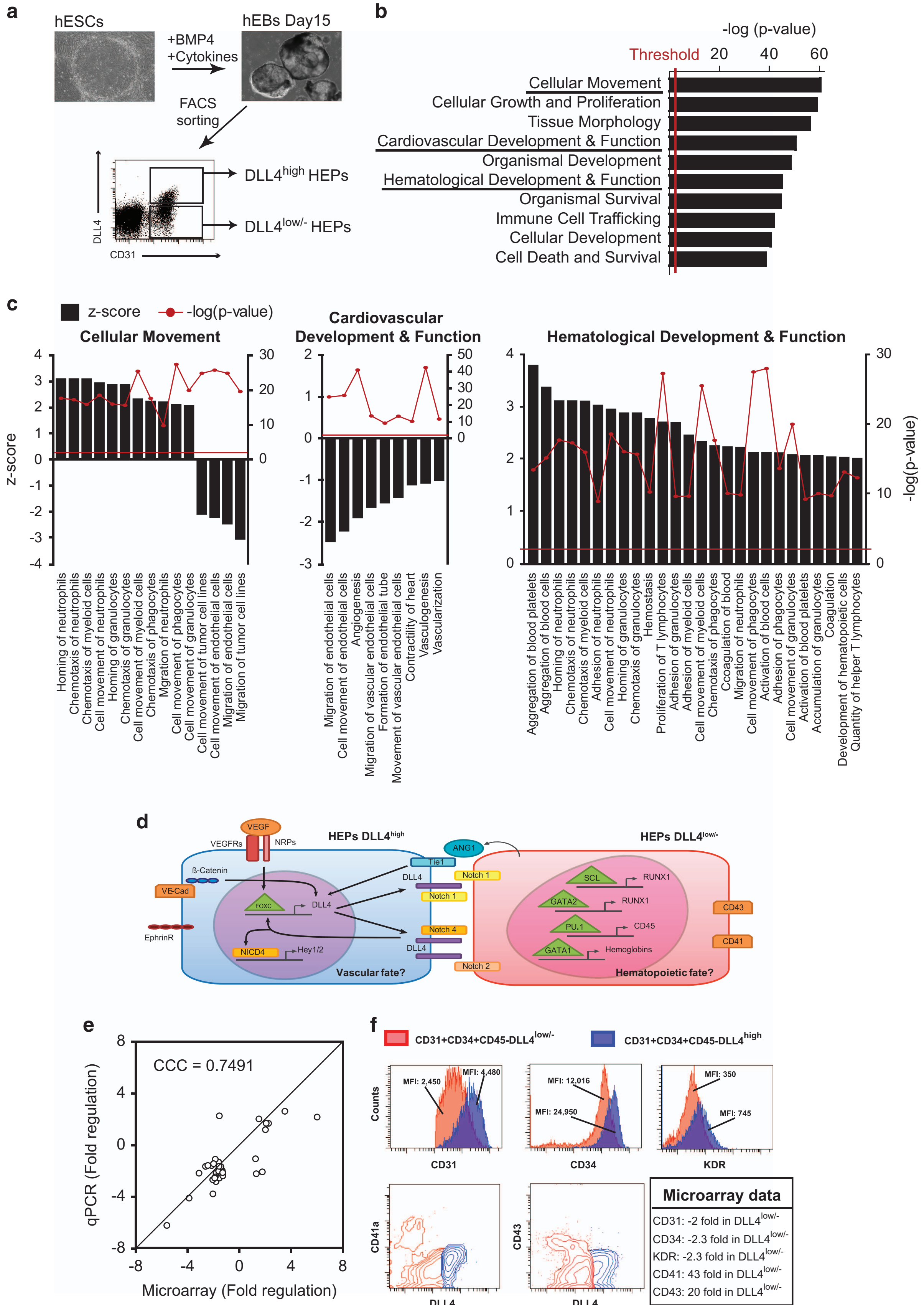

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fate, likely representing two different stages in the hematopoietic commitment of the HE.

DLL4 provides a positive signal for hematopoietic differentiation of $DLL4^{low/-}$ HEPs

We have shown that stimulation with exogenous DLL4 increased hematopoietic differentiation of HEPs (Figure 2). We hypothesized that $DLL4^{high}$ HEPs within the hEB are providing a DLL4-mediated signal to activate Notch in $DLL4^{low/-}$ HEPs, which will then differentiate into CD45+ hematopoietic cells. We FACS-purified $DLL4^{low/-}$ HEPs and cultured them with immobilized DLL4. Recombinant DLL4 was able to double the amount of hematopoietic cells generated from $DLL4^{low/-}$ HEPs, more specifically increasing the percentage of CD34+CD45+ hematopoietic progenitors (Figure 5e). As $DLL4^{high}$ HEP population contains bipotent cells, we next asked whether some $DLL4^{high}$ HEPs can themselves transition to $DLL4^{low/-}$ HEPs to undergo EHT and become CD45+ hematopoietic cells. We purified $DLL4^{high}$ HEPs and plated them on OP9-EV stroma. After 7 days in culture, a fraction of them (~12%) have differentiated into CD45+ hematopoietic cells (Figure 5f). Next, we tested if a strong DLL4 signal (DLL4 MFI ~ 7,500) coming from OP9-DLL4 cells could enhance the transition of $DLL4^{high}$ HEPs (DLL4 MFI ~ 2,500) to hematopoietic cells. Indeed, $DLL4^{high}$ HEPs co-cultured with OP9-DLL4 produce twice the amount of CD45+ cells (~24%), although they do not reach the levels of differentiation of $DLL4^{low/-}$ HEPs (~47%) (Figure 5f). These results confirm that DLL4 signaling is involved in the induction of hematopoietic differentiation of HEPs.

$DLL4^{high}$ HEPs are located at the base of hematopoietic clusters arising from $DLL4^{low/-}$ HEPs

Finally, we performed confocal microscopy on whole-mount hEBs and studied the three-dimensional localization of CD31+$DLL4^{high}$ and CD31+$DLL4^{low/-}$ HEPs together with CD45+ hematopoietic cells (see Supplementary Figure S5A for negative control stainings). In the majority (87%) of hEBs analyzed (35/40), we could observe CD31+ structures resembling cysts or sacs (Figure 6a), which contained interspersed CD31+DLL4+ cells (Figure 6b). CD45+ hematopoietic cells were located in a disorganized manner in the periphery of the hEBs (Figures 6a and b). However, 20% (8/40) of hEBs contained well-defined clusters of round CD31+CD45+ hematopoietic cells inside CD31+ cysts in close contact with the CD31+ lining cells (Figure 6c, Supplementary Videos 1 and 2), resembling the intra-aortic hematopoietic clusters arising from the DA in the mouse embryo.[14,43,44] Importantly, we could always (8/8) detect CD31+DLL4+CD45− cells located at the base of these clusters (Figure 6c, Supplementary Videos 1 and 2), suggesting that this association is specific, and making it physically possible for CD31+DLL4+ cells to be signaling to the differentiating CD31+DLL4− cells. We also analyzed Notch1 activation in the differentiating hEB and in ~ 31% (4/13) of analyzed hEBs, we could detect clusters of CD31+CD45+ cells showing nuclear staining of NICD1 (Figure 6d and Supplementary Figure S5B), suggesting a DLL4-mediated Notch1 activation in CD45+ hematopoietic clusters. Based on our data, we propose a model of human embryonic hematopoiesis in which the HE is formed by DLL4+ HEPs that retain an endothelial phenotype, which signal to neighboring $DLL4^{low/-}$ HEPs to undergo EHT and differentiate into CD45+ hematopoietic cells that emerge in clusters that resemble intra-aortic hematopoietic clusters formed in mouse DA (Figure 6e).

Finally, we wanted to test if the stimulation with DLL4 was sufficient to generate fully functional HSCs/hematopoietic progenitor cells capable of engraftment in immunodeficient mice. We transplanted NSG mice with HEPs co-cultured with either OP9-EV or OP9-DLL4, using cord blood CD34+ cells as positive control. As can be seen on Supplementary Figure S6, we were not able to detect any human chimerism in the mice transplanted with any of the co-cultures, whereas we detected up to 90% of human hematopoietic cells in mice transplanted with cord blood CD34+ cells. So, DLL4 signaling participates in the hematopoitic specification of hESCs, but it is not sufficient to generate fully functional hESC-derived HSCs.

DISCUSSION

We have used hESCs to ascertain the role of Notch signaling during human embryonic hematopoiesis, as nearly all data come from animal models.[45] We found that DLL4 is consistently upregulated during hESC hematopoietic differentiation, and that there is a positive correlation between DLL4 expression and the specification of CD34+CD45+ and CD45+ hematopoietic cells from HEPs. DLL4 expression is mainly regulated by the Notch pathway itself and it is restricted to a sub-population of CD31+ HEPs, allowing for discrimination between $DLL4^{high}$ and $DLL4^{low/-}$ HEPs. In the whole hEB, CD31+DLL4+ cells are positioned in contact with CD31+ cells devoid of DLL4 expression mimicking the generation of DLL4+ aortic endothelial cells in the embryo.[14,31]

The ventral side of the DA contains the HE that undergoes an EHT to generate hematopoietic cells,[8,46] but the identity of the cells within the HE that finally undergo EHT and the signaling cascades involved are not fully described. $DLL4^{high}$ HEPs display an endothelial transcriptome, expressing higher levels of endothelial markers and are enriched in endothelial precursors. On the other hand, $DLL4^{low/-}$ HEPs still retain some endothelial markers but they already express key hematopoietic proteins and the early hematopoietic precursor markers CD41 and CD43,[36,37] but do not express CD45 at the cell surface. When cultured in hematopoietic conditions $DLL4^{low/-}$ HEPs give rise to VE-cadherin+CD45+ cells, similar to pre-HSCs type I, which express the VE-cadherin and CD41, but still lack expression of CD45.[41] Nevertheless, both $DLL4^{high}$ and $DLL4^{low/-}$ populations are still bipotent, suggesting that they may represent two different stages in the hematopoietic commitment of the HE.[47]

DLL4 expression not only marks the fate of the HEPs within the hEB but it also has a functional role in the hematopoietic differentiation process. DLL4 enhanced HEPs hematopoietic

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Figure 4. Gene profiling of $DLL4^{high}$ and $DLL4^{low/-}$ HEPs indicates that DLL4 expression discriminates between HEPs with an endothelial fate and HEPs that have acquired a hematopoietic fate. **(a)** Sorting strategy followed to purify $DLL4^{high}$ and $DLL4^{low/-}$ HEPs used for gene expression analysis. **(b)** Top 10 biological functions of genes differentially expressed in $DLL4^{low/-}$ compared with $DLL4^{high}$ HEPs, ranked by *P*-value. **(c)** Predicted activation/inhibition ($\pm$ z-score, respectively) of different biofunctions included within the categories 'cell movement', 'cardiovascular development and function' and 'hematological development and function' in $DLL4^{low/-}$ compared with $DLL4^{high}$ HEPs. Z-score: black bars, left Y axis; -log *P*-value: filled red circle with red line, right Y axis. **(d)** Key hematopoietic and endothelial genes upregulated in $DLL4^{low/-}$ and $DLL4^{high}$ HEPs, respectively, suggesting that HEPs with low DLL4 expression have already acquired a hematopoietic fate, whereas HEPs with high DLL4 expression retain an endothelial fate. **(e)** Correlation of gene expression values obtained by microarray and qPCR data of a subset of 40 genes differentially regulated in $DLL4^{low/-}$ HEPs determined by the concordance correlation coefficient (CCC). **(f)** Confirmation of differential protein expression at the cell surface by flow cytometry of the endothelial markers CD31, CD34, KDR, and the hematopoietic markers CD41 and CD43, in $DLL4^{low/-}$ and $DLL4^{high}$ HEPs. See also Supplementary Figures S3 and S4, and Supplementary Tables S2–S4.

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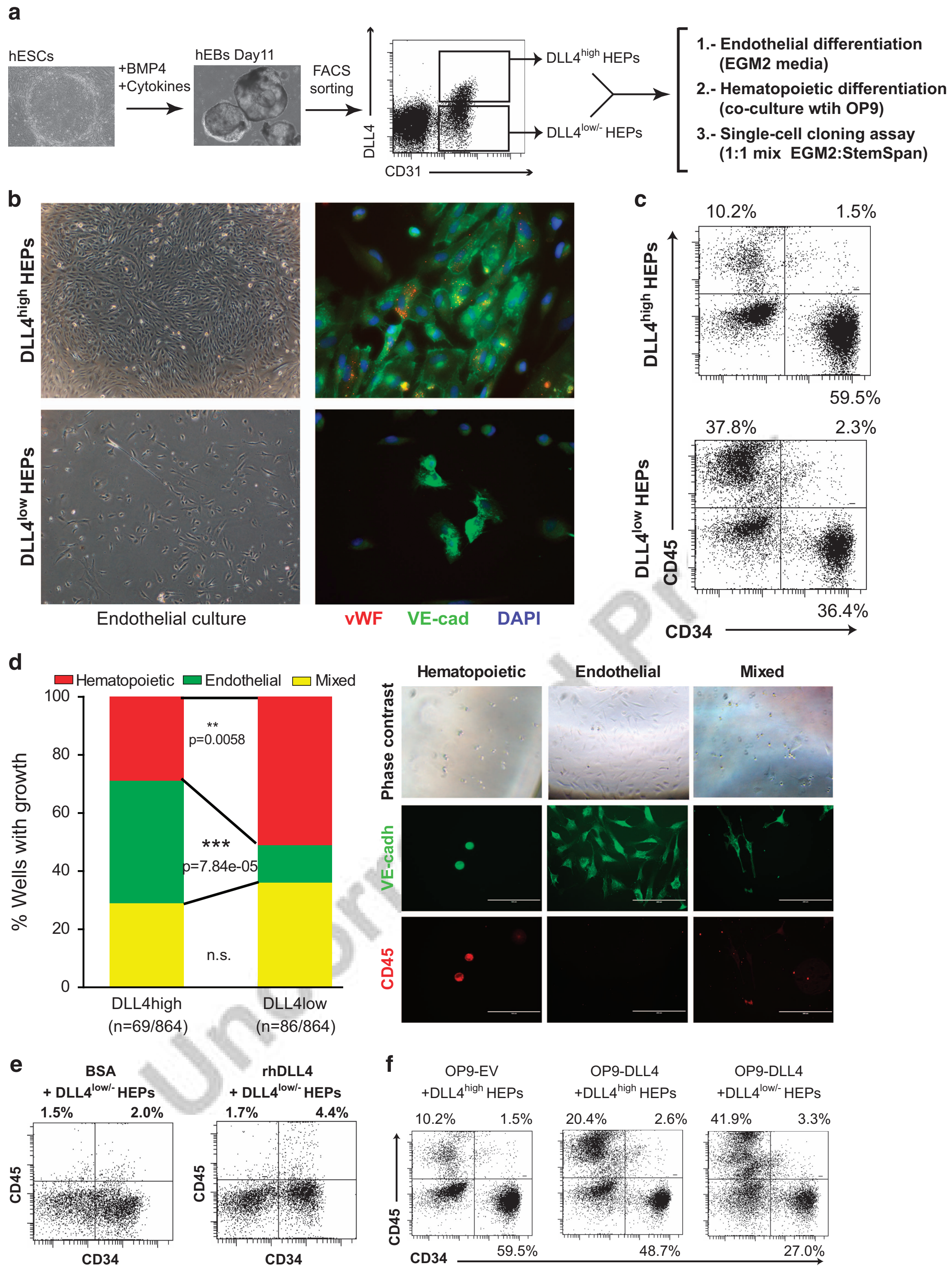

Figure 5. DLL4$^{low/-}$ HEPs are committed to a hematopoietic lineage, whereas DLL4^{high} HEPs retain an endothelial fate. **(a)** Sorting strategy used to purify DLL4^{high} and DLL4$^{low/-}$ HEPs from hEBs at day 11 used in the different functional assays performed to determine the developmental fate of these HEPs. **(b)** Purified DLL4^{high} and DLL4$^{low/-}$ HEPs were plated on gelatin-coated wells and cultured for 7 days in EGM-2 medium. Pictures show cell cultures growth and expression of the endothelial markers VE-cadherin and von Willebrand factor (vWF) by immunofluorescence. **(c)** Purified DLL4^{high} and DLL4$^{low/-}$ HEPs were co-cultured with OP9 stroma for 7 days to assess their ability to differentiate toward CD34+CD45+ hematopoietic progenitors and total CD45+ hematopoietic cells. Figure shows dot plots from a representative experiment ($n=5$). **(d)** Single purified DLL4^{high} and DLL4$^{low/-}$ HEPs were deposited in 10 wells of 96-well plates in the presence of a 1:1 mixture of EGM-2 and StemSpan media, supplemented with hematopoietic cytokines. Cells were left to grow for 10 days and their phenotype was confirmed by phase contrast microscopy and immunostaining with VE-cadherin and CD45 antibodies. **(e)** Purified DLL4$^{low/-}$ HEPs by cell sorting were cultured for 12 days in StemSpan with cytokines in either control wells (bovine serum albumin) or wells coated with immobilized rhDLL4 protein, and their hematopoietic differentiation was assessed by flow cytometry. Figure shows dot plots from a representative experiment ($n=2$). **(f)** Hematopoietic differentiation and expression of DLL4 in purified DLL4^{high} and DLL4$^{low/-}$ HEPs co-cultured with either OP9-EV or OP9-DLL4 for 7 days.

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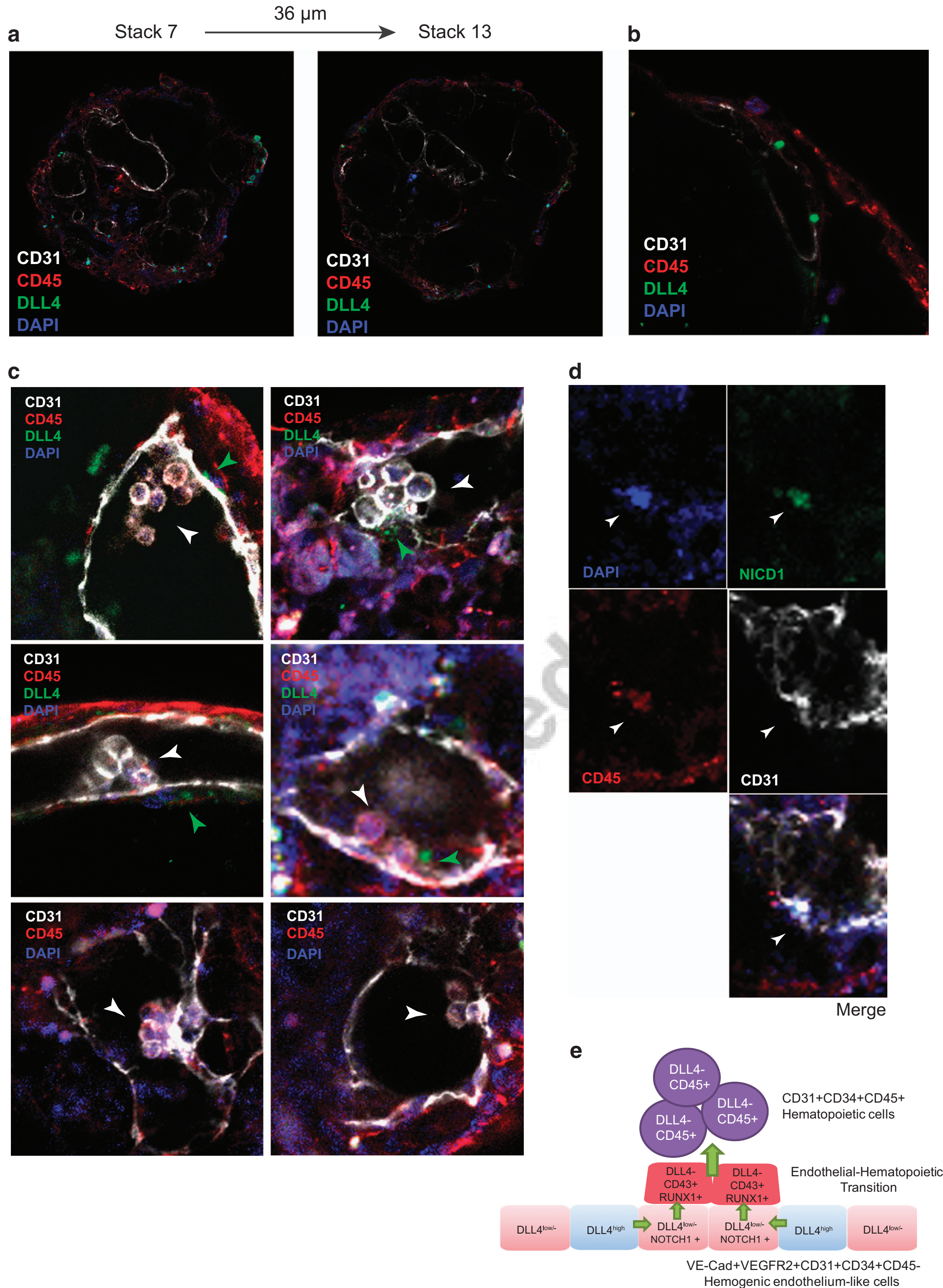

Figure 6. hEBs contain CD31+CD45+ hematopoietic cell clusters within CD31+ cyst-like structures and close to DLL4+CD31+ cells, resembling hematopoietic clusters found in AGM. (**a**) Series of two stacks at different depths within the differentiating hEB stained with CD31, CD45, DLL4 and 4,6-diamidino-2-phenylindole (DAPI) depicting a typical hEB structure with several cyst-like sacs lined by endothelial CD31+CD45– cells. (**b**) Detailed view of a CD31+ sac with two DLL4+ cells, but with no hematopoietic cluster formation. (**c**) Representative images of cyst-like sac structures of CD31+ cells containing clusters of CD31+CD45+ hematopoietic cells in its interior, with CD31+DLL4+ cells at or very close to the base of the cluster. White arrowheads indicate the localization of the clusters; green arrowheads indicate the localization of DLL4-positive cells close to the clusters. See also Supplementary Movies S1 and S2. (**d**) Representative image of a CD31+CD45+ cluster of cells that show nuclear staining for activated Notch1 (NICD1). (**e**) Proposed model for human embryonic hematopoietic differentiation in which cells with high expression of DLL4 signal to cells with low DLL4 expression within the HE, probably activating Notch1 in the latter. DLL4 stimulation of these DLL4^{low} HEPs induces them to undergo hematopoietic differentiation, probably through an EHT mediated by SCL and RUNX1, resulting in the generation of CD45+ blood cells.

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differentiation in a Notch-dependent manner, increasing the amount of clonogenic hematopoietic progenitors, as well as skewing their fate toward erythroid lineage. Despite the well-known role for DLL4 in arterial development[48,49] and T-cell differentiation,[50] this is the first time that DLL4 is directly involved in the specification of hematopoietic cells from HEPs during human embryonic development. A recent report showed that DLL4 expressed in endothelial cells biased the differentiation of hESC-derived CD45+ multipotent hematopoietic progenitor cells toward a myeloid fate.[51] We did not observe any significant changes in the amount of myeloid colonies produced (data not shown), possibly because HEPs represent an earlier developmental stage than the hematopoietic progenitor cells.

In the developing hEB, endothelial CD31+DLL4+ cells are intermingled with CD31+DLL4– cells within well-organized cyst-like structures, which in some instances contained clusters of CD31+DLL4–CD45+ cells in close proximity to CD31+DLL4+ cells, resembling intra-aortic hematopoietic clusters found in AGM.[36,44,52] This three-dimensional localization in the hEB indicates that CD31+DLL4+ cells can signal and activate Notch in CD31+DLL4– cells to undergo EHT and generate CD45+ hematopoietic cells, similar to the EHT described using mouse embryonic and ES cells.[39,53] We propose a model for human embryonic hematopoiesis (Figure 6e), in which clusters of DLL4+ cells within HE signal to DLL4– cells to undergo hematopoietic differentiation and become CD45+ cells. This role of DLL4 in blood specification could be conserved over evolution. During *Drosophila* development, clusters of delta-expressing cardioblasts activate Notch in neighboring delta-negative pre-lymph gland cells, leading to the expression of the *lethal of scute* (SCL) and the formation of blood progenitors expressing *serpent* (GATA homolog),[54] resembling our findings in hEBs.

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Many efforts are being made to produce functional HSCs from hESCs and hiPSCs, but with little success.[3] The CD31+ endothelial structures containing clusters of CD31+CD45+ cells that we report clearly resemble AGM intra-aortic hematopoietic clusters, and they may potentially be the niche for *bona fide* HSC-like cells that we can produce *in vitro*. However, the frequency of hEBs containing these well-defined structures was relatively low (23%). This could be due to an inefficient generation of DLL4+ aortic endothelium, together with a deficient activation of the Notch pathway.[35] In the mouse embryo, DLL4 cells are present in most of the cells of the endothelial layer of the DA, but they also co-expressed Jagged1 and Jagged2.[44] In our system, neither Jagged1 nor Jagged2 correlate with the generation of HEPs or hematopoietic precursors. This can be interpreted as the human HSCs ontogeny depends on different Notch ligands, but also that other ligands important for generating functional HSCs[44] are not expressed in these conditions. Efforts to improve hESCs/hiPSCs hematopoietic differentiation may be directed at improving the organization of the developing structures, which will probably be linked to a correct activation of Notch signaling in these *in vitro* systems. If we increase the capacity to produce arterial specification within the developing endothelium inside the hEB, we will most probably increase the amount of DLL4-expressing HEPs able to signal to DLL4$^{low/-}$ HEPs and induce EHT more efficiently, leading to an enhancement of HSC-like cells production.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

VA designed and did the experiments, interpreted the data and wrote the manuscript; PM conceived the study, designed the experiments, interpreted the data and wrote the manuscript; CB, VR-M, TR, CP, ONM, PJR and MJG-L did experiments; MLT and AB contributed key reagents and interpreted the data. All authors have read the current version of the MS and agreed with its submission.

REFERENCES

1 Costa G, Kouskoff V, Lacaud G. Origin of blood cells and HSC production in the embryo. *Trends Immunol* 2012; **33**: 215–223.
2 Clapes T, Robin C. Embryonic development of hematopoietic stem cells: implications for clinical use. *Regen Med* 2012; **7**: 349–368.
3 Islam MS, Ni Z, Kaufman DS. Use of human embryonic stem cells to understand hematopoiesis and hematopoietic stem cell niche. *Curr Stem Cell Res Ther* 2010; **5**: 245–250.
4 Benedito R, Trindade A, Hirashima M, Henrique D, da Costa LL, Rossant J *et al.* Loss of Notch signalling induced by Dll4 causes arterial calibre reduction by increasing endothelial cell response to angiogenic stimuli. *BMC Dev Biol* 2008; **8**: 117.
5 Corada M, Nyqvist D, Orsenigo F, Caprini A, Giampietro C, Taketo MM *et al.* The Wnt/beta-catenin pathway modulates vascular remodeling and specification by upregulating Dll4/Notch signaling. *Dev Cell* 2010; **18**: 938–949.
6 Kim PG, Albacker CE, Lu YF, Jang IH, Lim Y, Heffner GC *et al.* Signaling axis involving Hedgehog, Notch, and Scl promotes the embryonic endothelial-to-hematopoietic transition. *Proc Natl Acad Sci USA* 2012; **110**: E141–E150.
7 Yamamizu K, Matsunaga T, Uosaki H, Fukushima H, Katayama S, Hiraoka-Kanie M *et al.* Convergence of Notch and beta-catenin signaling induces arterial fate in vascular progenitors. *J Cell Biol* 2010; **189**: 325–338.
8 Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E, Robin C. *In vivo* imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* 2010; **464**: 116–120.
9 Tavian M, Peault B. Embryonic development of the human hematopoietic system. *Int J Dev Biol* 2005; **49**: 243–250.
10 Ivanovs A, Rybtsov S, Anderson RA, Turner ML, Medvinsky A. Identification of the niche and phenotype of the first human hematopoietic stem cells. *Stem Cell Rep* 2014; **2**: 449–456.
11 Kennedy M, D'Souza SL, Lynch-Kattman M, Schwantz S, Keller G. Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. *Blood* 2007; **109**: 2679–2687.
12 Wang L, Li L, Shojaei F, Levac K, Cerdan C, Menendez P *et al.* Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. *Immunity* 2004; **21**: 31–41.
13 Kumano K, Chiba S, Kunisato A, Sata M, Saito T, Nakagami-Yamaguchi E *et al.* Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* 2003; **18**: 699–711.
14 Robert-Moreno A, Espinosa L, de la Pompa JL, Bigas A. RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. *Development* 2005; **132**: 1117–1126.
15 Bertrand JY, Cisson JL, Stachura DL, Traver D. Notch signaling distinguishes 2 waves of definitive hematopoiesis in the zebrafish embryo. *Blood* 2010; **115**: 2777–2783.
16 Yu X, Zou J, Ye Z, Hammond H, Chen G, Tokunaga A *et al.* Notch signaling activation in human embryonic stem cells is required for embryonic, but not trophoblastic, lineage commitment. *Cell Stem Cell* 2008; **2**: 461–471.
17 Lee JB, Werbowetski-Ogilvie TE, Lee JH, McIntyre BA, Schnerch A, Hong SH *et al.* Notch-HES1 signaling axis controls hemato-endothelial fate decisions of human embryonic and induced pluripotent cells. *Blood* 2013; **122**: 1162–1173.
18 Montes R, Ligero G, Sanchez L, Catalina P, de la Cueva T, Nieto A *et al.* Feeder-free maintenance of hESCs in mesenchymal stem cell-conditioned media: distinct requirements for TGF-beta and IGF-II. *Cell Res* 2009; **19**: 698–709.

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19 Ramos-Mejia V, Fernandez AF, Ayllon V, Real PJ, Bueno C, Anderson P *et al.* Maintenance of human embryonic stem cells in mesenchymal stem cell-conditioned media augments hematopoietic specification. *Stem Cells Dev* 2012; **21**: 1549–1558.
20 Chadwick K, Wang L, Li L, Menendez P, Murdoch B, Rouleau A *et al.* Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood* 2003; **102**: 906–915.
21 Ramos-Mejia V, Melen GJ, Sanchez L, Gutierrez-Aranda I, Ligero G, Cortes JL *et al.* Nodal/Activin signaling predicts human pluripotent stem cell lines prone to differentiate toward the hematopoietic lineage. *Mol Ther* 2010; **18**: 2173–2181.
22 Vodyanik MA, Slukvin II. Hematoendothelial differentiation of human embryonic stem cells. *Curr Protoc Cell Biol* 2007; **23**: Unit 23–Unit 26.
23 Real PJ, Ligero G, Ayllon V, Ramos-Mejia V, Bueno C, Gutierrez-Aranda I *et al.* SCL/TAL1 regulates hematopoietic specification from human embryonic stem cells. *Mol Ther* 2012; **20**: 1443–1453.
24 Caolo V, van den Akker NM, Verbruggen S, Donners MM, Swennen G, Schulten H *et al.* Feed-forward signaling by membrane-bound ligand receptor circuit: the case of NOTCH DELTA-like 4 ligand in endothelial cells. *J Biol Chem* 2010; **285**: 40681–40689.
25 Hayashi H, Kume T. Foxc transcription factors directly regulate Dll4 and Hey2 expression by interacting with the VEGF-Notch signaling pathways in endothelial cells. *PLoS One* 2008; **3**: e2401.
26 Ruiz-Herguido C, Guiu J, D'Altri T, Ingles-Esteve J, Dzierzak E, Espinosa L *et al.* Hematopoietic stem cell development requires transient Wnt/beta-catenin activity. *J Exp Med* 2012; **209**: 1457–1468.
27 Dando JS, Tavian M, Catelain C, Poirault S, Bennaceur-Griscelli A, Sainteny F *et al.* Notch/Delta4 interaction in human embryonic liver CD34+ CD38- cells: positive influence on BFU-E production and LTC-IC potential maintenance. *Stem Cells* 2005; **23**: 550–560.
28 Laranjeiro R, Alcobia I, Neves H, Gomes AC, Saavedra P, Carvalho CC *et al.* The Notch ligand delta-like 4 regulates multiple stages of early hemato-vascular development. *PLoS One* 2012; **7**: e34553.
29 Suenobu S, Takakura N, Inada T, Yamada Y, Yuasa H, Zhang XQ *et al.* A role of EphB4 receptor and its ligand, ephrin-B2, in erythropoiesis. *Biochem Biophys Res Commun* 2002; **293**: 1124–1131.
30 Chong DC, Koo Y, Xu K, Fu S, Cleaver O. Stepwise arteriovenous fate acquisition during mammalian vasculogenesis. *Dev Dyn* 2011; **240**: 2153–2165.
31 Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP *et al.* Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev* 2000; **14**: 1343–1352.
32 Shutter JR, Scully S, Fan W, Richards WG, Kitajewski J, Deblandre GA *et al.* Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev* 2000; **14**: 1313–1318.
33 Alcobia I, Gomes A, Saavedra P, Laranjeiro R, Oliveira S, Parreira L *et al.* Portrayal of the Notch system in embryonic stem cell-derived embryoid bodies. *Cells Tissues Organs* 2010; **193**: 239–252.
34 Martinez JC, Muller MM, Turley H, Steers G, Choteau L, Li JL *et al.* Nuclear and membrane expression of the angiogenesis regulator delta-like ligand 4 (DLL4) in normal and malignant human tissues. *Histopathology* 2009; **54**: 598–606.
35 McKinney-Freeman S, Cahan P, Li H, Lacadie SA, Huang HT, Curran M *et al.* The transcriptional landscape of hematopoietic stem cell ontogeny. *Cell Stem Cell* 2012; **11**: 701–714.
36 Boisset JC, Clapes T, Van Der Linden R, Dzierzak E, Robin C. Integrin alphaIIb (CD41) plays a role in the maintenance of hematopoietic stem cell activity in the mouse embryonic aorta. *Biol Open* 2013; **2**: 525–532.
37 Vodyanik MA, Thomson JA, Slukvin II. Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. *Blood* 2006; **108**: 2095–2105.
38 Seo S, Fujita H, Nakano A, Kang M, Duarte A, Kume T. The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. *Dev Biol* 2006; **294**: 458–470.
39 Lancrin C, Sroczynska P, Stephenson C, Allen T, Kouskoff V, Lacaud G. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature* 2009; **457**: 892–895.
40 Zovein AC, Hofmann JJ, Lynch M, French WJ, Turlo KA, Yang Y *et al.* Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell* 2008; **3**: 625–636.
41 Rybtsov S, Sobiesiak M, Taoudi S, Souilhol C, Senserrich J, Liakhovitskaia A *et al.* Hierarchical organization and early hematopoietic specification of the developing HSC lineage in the AGM region. *J Exp Med* 2011; **208**: 1305–1315.
42 Taoudi S, Gonneau C, Moore K, Sheridan JM, Blackburn CC, Taylor E *et al.* Extensive hematopoietic stem cell generation in the AGM region via maturation of VE-cadherin+CD45+ pre-definitive HSCs. *Cell Stem Cell* 2008; **3**: 99–108.
43 Guiu J, Shimizu R, D'Altri T, Fraser ST, Hatakeyama J, Bresnick EH *et al.* Hes repressors are essential regulators of hematopoietic stem cell development downstream of Notch signaling. *J Exp Med* 2013; **210**: 71–84.
44 Robert-Moreno A, Guiu J, Ruiz-Herguido C, Lopez ME, Ingles-Esteve J, Riera L *et al.* Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1. *EMBO J* 2008; **27**: 1886–1895.
45 Bigas A, D'Altri T, Espinosa L. The Notch pathway in hematopoietic stem cells. *Curr Top Microbiol Immunol* 2012; **360**: 1–18.
46 Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* 2010; **464**: 108–111.
47 Swiers G, Rode C, Azzoni E, de Bruijn MF. A short history of hemogenic endothelium. *Blood Cells Mol Dis* 2013; **51**: 206–212.
48 Duarte A, Hirashima M, Benedito R, Trindade A, Diniz P, Bekman E *et al.* Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev* 2004; **18**: 2474–2478.
49 Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev* 2004; **18**: 2469–2473.
50 Hozumi K, Mailhos C, Negishi N, Hirano K, Yahata T, Ando K *et al.* Delta-like 4 is indispensable in thymic environment specific for T cell development. *J Exp Med* 2008; **205**: 2507–2513.
51 Rafii S, Kloss CC, Butler JM, Ginsberg M, Gars E, Lis R *et al.* Human ESC-derived hemogenic endothelial cells undergo distinct waves of endothelial to hematopoietic transition. *Blood* 2013; **121**: 770–780.
52 Oberlin E, Fleury M, Clay D, Petit-Cocault L, Candelier JJ, Mennesson B *et al.* VE-cadherin expression allows identification of a new class of hematopoietic stem cells within human embryonic liver. *Blood* 2010; **116**: 4444–4455.
53 Eilken HM, Nishikawa S, Schroeder T. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* 2009; **457**: 896–900.
54 Grigorian M, Mandal L, Hakimi M, Ortiz I, Hartenstein V. The convergence of Notch and MAPK signaling specifies the blood progenitor fate in the *Drosophila* mesoderm. *Dev Biol* 2011; **353**: 105–118.

Uncorrected Proof

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)

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