Human definitive haemogenic endothelium and arterial vascular endothelium represent distinct lineages

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The generation of haematopoietic stem cells (HSCs) from human pluripotent stem cells (hPSCs) will depend on the accurate recapitulation of embryonic haematopoiesis. In the early embryo, HSCs develop from the haemogenic endothelium (HE) and are specified in a Notch-dependent manner through a process named endothelial-to-haematopoietic transition (EHT). As HE is associated with arteries, it is assumed that it represents a subpopulation of arterial vascular endothelium (VE). Here we demonstrate at a clonal level that hPSC-derived HE and VE represent separate lineages. HE is restricted to the CD34+CD73−CD184− fraction of day 8 embryoid bodies and it undergoes a NOTCH-dependent EHT to generate RUNX1C+ cells with multilineage potential. Arterial and venous VE progenitors, in contrast, segregate to the CD34+CD73medCD184+ and CD34+CD73+CD184+ fractions, respectively. Together, these findings identify HE as distinct from VE and provide a platform for defining the signalling pathways that regulate their specification to functional HSCs.

Human pluripotent stem cells (hPSCs) represent an unlimited source of therapeutically important haematopoietic cells, including transplantable haematopoietic stem cells for the treatment of haematological disorders. However, despite significant advances in our ability to differentiate hPSCs to diverse haematopoietic cell types1–4, the generation of HSCs in vitro has been challenging. This difficulty in deriving HSCs is due in part to the complex structure of the embryonic haematopoietic system that consists of separate programs that exhibit different potential and are specified at distinct times during development5. HSCs are generated from the definitive haematopoietic program that is initiated in different sites within the embryo following the onset of primitive haematopoiesis that develops at an earlier stage and generates a restricted subset of lineages6.

Studies from different model organisms have shown that HSCs develop from a progenitor population known as haemogenic endothelium (HE) that expresses endothelial markers and is thought to derive directly from the developing arterial vasculature6–9. Kinetic analyses of the haemogenic sites in the early embryo combined with time-lapse studies ex vivo have shown that during specification of the haemogenic endothelium, HE undergoes an endothelial-to-haematopoietic transition (EHT) to generate blood cell progenitors6–8 that subsequently mature to give rise to functional HSCs (ref. 9).

The identification of hPSC-derived HE has been challenging owing to the fact that the primitive program also transitions through a HE population that is indistinguishable from definitive HE on the basis of expression of cell surface markers10. Given these similarities, it is essential to be able to distinguish the two programs to monitor the development of definitive HE. We have recently shown that primitive and definitive haematopoiesis differ in their requirement for activin/nodal/TGFβ and Wnt/β-catenin signalling at the mesoderm specification stage and that through appropriate manipulation, it is possible to deplete the hPSC-derived populations of the primitive haematopoietic lineages11,10. Dependency on Notch signalling is also a distinguishing feature of these programs, as loss-of-function studies in vertebrate embryos have demonstrated that this pathway is essential for specification of HSCs and definitive progenitors, but dispensable for primitive haematopoiesis11–14.

Here, we have exploited these differences to isolate and characterize hPSC-derived definitive HE. We show that this HE can be distinguished from VE on the basis of cell surface marker...
Figure 1 Characterization of hPSC-derived definitive HE. (a) Experimental scheme. CD34+CD43− cells were isolated from EBs at day 8 of differentiation, reaggregated overnight in serum-free media supplemented with haematopoietic cytokines and then cultured for an additional 6 days on Matrigel-coated plates in the presence of haematopoietic cytokines to promote the EHT. This stage is referred to as the EHT culture, following which the cells were assayed as indicated. (b) Photomicrograph of day 8 CD34+CD43−-derived cells following 1 (upper) and 4 days (lower) of EHT culture. Non-adherent (haematopoietic) cells are visible in the day 4 culture. Scale bars, 100 μm. (c) Representative flow cytometric analysis of the frequency of CD34+ and CD45+ cells in the day 8 CD34+CD43−-derived populations at the indicated days of EHT culture. (d) Visualization of emerging round haematopoietic cells in EHT cultures by confocal imaging. Cells were stained for the endothelial marker CD144 (in green), the haematopoietic marker CD45 (in grey) and the EHT marker cKIT (in red). Scale bar, 5 μm. The dashed line demarcates a cell co-expressing CD144, CD45 and cKIT (white arrow). (e) Gating strategy used to define the different CD34/CD45 fractions in the CD34+CD43−-derived population following 7 days of EHT culture. (f) T-cell potential of the different CD34/CD45 fractions indicated in e measured by the development of CD4+CD8− cells within a CD45+CD56− gate following culture on OP9-DLL4 stromal cells for 24 days. (g) Haematopoietic colony-forming potential of the different CD34/CD45 fractions indicated in e following 7 days of EHT culture of day 8 CD34+CD43− cells. n=4, independent experiments (mean ± s.e.m.). ** ANOVA P<0.002. BFU-E: progenitors that generate large segmented erythroid colonies, CFU-E: progenitors that give rise to small erythroid colonies, Myeloid: includes macrophage and mast cell progenitors. (h) qRT-PCR analysis of MYB, RUNX1C, TAL1 and GATA2 expression in the different CD34/CD45 fractions isolated as in e. The day 8 CD34+CD43− population before culture is included as a control (ctrl). Cells were derived from H1 hESCs, n=4, independent experiments (mean ± s.e.m.). ** ANOVA P<0.0001. Images in b and plots in c are representative of 6 independent experiments, in f of 3 independent experiments.
expression and that it can progress through the EHT in a NOTCH-dependent fashion to generate myeloid, erythroid and lymphoid progeny. Together, these findings provide strong evidence that the hPSC-derived definitive HE represents the in vitro equivalent of the HE that in the early embryo gives rise to the HSCs.

Figure 2. RUNX1C–EGFP is expressed during the EHT of the definitive HE. (a) Representative flow cytometric analysis of the frequency of CD45+ cells (upper panel) and CD34+ RUNX1C–EGFP+ cells (lower panel) in the day 8-derived population following 7 days of EHT culture. (b) Representative flow cytometric analysis of the frequency of CD144+ and CD45+ cells (lower panels) in the CD34+ RUNX1C–EGFP+ fraction (upper panels) generated at the indicated times of EHT culture of day 8 CD34+CD43− cells. (c) Gating strategy used for the isolation of the CD34+ and RUNX1C–EGFP+ fractions in the CD34+ derived population following 5 days of EHT culture. (d) T-cell potential of the different CD34/CD45 fractions (indicated in c) measured by the development of CD4+CD8− cells within a CD45+CD56− gate following culture on OP9-DLL4 stromal cells for 24 days. (e) qRT-PCR analysis of MYB, RUNX1C, TAL1 and GATA2 expression in the different CD34/RUNX1C–EGFP fractions. Values relative to TBP for the CD34+GFP+ population are set to 1. n = 3, independent experiments (mean ± s.e.m.). ** ANOVA MYB, RUNX1C, GATA2 P < 0.0001, TAL1 P = 0.0003. (f) Representative flow cytometric analysis of the frequency of CD34+CD45+ and CD34+ RUNX1C–EGFP+ cells generated after 7 days in EHT culture from KDR+CD235a− (left panels) and KDR+CD235a+ (right panels) derived CD34+CD43− cells. Plots in a are representative of 5 independent experiments, in b,d and f of 3 independent experiments.

## RESULTS

**hPSC-derived HE undergoes EHT to generate haematopoietic progeny**

We previously identified a definitive CD34+CD43− population that expressed HE markers (CD31+CD144+KDR+cKITlo) and exhibited
Figure 3 Haematopoietic specification of definitive HE is NOTCH-dependent. (a) Representative flow cytometric analysis of the frequency of CD34<sup>+</sup>CD43<sup>-</sup> cells in DMSO- (upper panel) or GSI- (lower panel) treated day 8 EBs generated from H1 ESCs. GSI or DMSO was added to the cultures every 60 h from day 3 to 8 of differentiation. (b) Representative flow cytometric analysis of the frequency of CD34<sup>+</sup> and CD45<sup>+</sup> cells in populations following 7 days of EHT culture of CD34<sup>+</sup>CD43<sup>-</sup> cells isolated from DMSO- (upper panel) or GSI- (lower panel) treated EBs measured by the development of CD4<sup>+</sup>CD8<sup>+</sup> cells within a CD45<sup>-</sup><sup>CD56</sup> gate following culture on OP9-DLL4 stromal cells for 24 days. (d) Photomicrograph of day 8 CD34<sup>+</sup>CD43<sup>-</sup>-derived populations following addition of either DMSO or GSI to the EHT culture. (e,f) Representative flow cytometry analysis of the frequency of CD34<sup>+</sup>CD45<sup>+</sup> cells (e) or CD34<sup>+</sup> RUNX1C–EGFP<sup>+</sup> cells (f) in populations following 7 days of EHT culture of H1- and R1C-GFP-derived CD34<sup>+</sup>CD43<sup>-</sup> cells respectively. DMSO or GSI was added throughout the 7-day culture period. (g) Haematopoietic colony-forming potential of the H1 hESC-derived day 7 EHT population treated with either DMSO and GSI. n=3 independent experiments (mean ± s.e.m.). Student’s t-test, **P < 0.001. (h) Gating strategy used for the isolation of the CD34/CD45 fractions from the population generated following 7 days of EHT culture of day 8 CD34<sup>+</sup>CD43<sup>-</sup> cells in the presence of GSI. (i) T-cell potential of the different CD34/CD45 fractions following 24 days of culture on OP9-DLL4 stromal cells. The lack of any CD45<sup>+</sup> cells indicates that GSI treatment during the EHT culture inhibited T-cell development. (j) Representative flow cytometric analysis of the frequency of CD34<sup>+</sup> and CD45<sup>+</sup> cells in day 8 CD34<sup>+</sup>CD43<sup>-</sup>-derived EHT populations treated for the indicated times with GSI. Cells were analysed at day 7 of culture. Images in a,b,e and f are representative of 6 independent experiments, in c and i of 3 independent experiments, in j of 4 independent experiments.

the capacity to generate T-lymphoid, erythroid and myeloid cells following culture on stromal cells<sup>2,10</sup>. To be able to monitor the EHT of this population, we isolated human embryonic stem cell (hESC)-derived CD34<sup>+</sup> cells and cultured them on Matrigel, in the presence of haematopoietic cytokines known to promote and sustain haematopoietic differentiation<sup>15–17</sup> (EHT culture, Fig. 1a). Under these
conditions, the cells rapidly formed an adhesive monolayer that underwent the EHT as demonstrated by the emergence of round cells within 3 to 4 days of culture and of a population of CD45⁺ cells by day 7 (Fig. 1b,c). Examination of the EHT cultures with time-lapse imaging revealed that the adherent cells gradually acquire CD45 expression and then give rise to non-adherent CD45⁺ haematopoietic cells (Supplementary Video 1). Immunostaining analyses showed that the emerging round cells co-express endothelial (CD144) and haematopoietic (CD45) surface markers as well as cKIT, a marker indicative of the EHT (refs 8,18; Fig. 1d and Supplementary Video 2).

To monitor definitive haematopoiesis in the day 8+7 population, we isolated the different CD34⁺/CD45⁺ fractions and assayed them for their haematopoietic potential and for expression of key genes associated with embryonic haematopoiesis including MYB, RUNX1C, TAL1, DLL4, and NR2F2 (Fig. 1e–h). T-lymphoid progenitors were detected only in the two CD34⁺/CD45⁺ CD45⁺ fractions,
whereas myelo/erythroid progenitors were found in all 3 CD45+ fractions (Fig. 1f,g). Quantitative real-time PCR (qRT-PCR) analyses showed that expression of MYB, RUNX1C, TAL1 and GATA2 was significantly higher in the 3 CD45+ haematopoietic fractions than in the CD34CD45− fraction and the input day 8 CD34+ population (Fig. 1h). The CD34+CD45−-derived fraction showed no haematopoietic potential, suggesting that it consists of only endothelial progenitors. The erythroid colonies generated from CD34+CD45−-derived progenitors at the end of EHT culture expressed levels of fetal globin HBG similar to that found in total mononuclear fetal liver cells and significantly higher than the levels in hESC-derived primitive erythroid colonies (EryR; Supplementary Fig. 1). As previously reported2-10, these HE-derived colonies retain expression of the embryonic globin HBE and show very low levels of the adult globin HBB, a pattern similar to that observed in human fetal liver-derived erythroid colonies19. Collectively, these findings suggest that the erythroid colonies generated from the HE represent the onset of definitive haematopoiesis.

**T-cell progenitors generated following the EHT express RUNX1C**

To provide better resolution of the emerging definitive HE and its EHT, we next differentiated a reporter hESCs treated with DMSO, GSI (L-685, 458 10 μM), 4-OHT (1 μM), MEKi (PD0325901, 1 μM) or PI(3)Ki (LY294002, 10 μM) between day 3 and day 8 of differentiation. (h,i) Graphs showing the total percentage of CD34+CD73−CD184− (h) and the estimated total percentage of T-cell progenitors (i) in day 8 HE-derived total EB populations following DMSO or GSI treatment from day 3 to day 8 of differentiation (Student’s t-test; P value is shown; h) n=6 independent experiments; (i) n=3, independent experiments). Images in a-f are representative of 4 mice from 4 independent experiments, in g of 3 independent experiments.

**Figure 5** Engrafted CD73medCD184+ and CD73hiCD184− cells maintain their vascular identity. (a-f) Photomicrographs of immunostained histological sections of vascular grafts derived from day 8 CD73medCD184+ (a,c,e) and CD73hiCD184− (b,d,f) cells. Green depicts the presence of human CD31 cells and red the presence of α-smooth muscle actin (α-SMA)+ cells (a,b,e,f) and ephrin receptor B4 (EPHB4)+ cells (c,d). Nuclei are visualized by DAPI (blue) staining. The anti-CD31 antibody is specific for human CD31. Arrow indicates a murine vessel. * indicates autofluorescent red blood cells. Scale bars, 100 μm. (g) Representative flow cytometric analysis of the frequency of CD184+ and CD73+ cells in day 8 CD34+CD43− populations generated from Hes2-ICN1-ERtm hESCs treated with DMSO, GSI (L-685, 458 10 μM), 4-OHT (1 μM), MEKi (PD0325901, 1 μM) or PI(3)Ki (LY294002, 10 μM) between day 3 and day 8 of differentiation. (h,i) Graphs showing the total percentage of CD34+CD73−CD184− (h) and the estimated total percentage of T-cell progenitors (i) in day 8 HE-derived total EB populations following DMSO or GSI treatment from day 3 to day 8 of differentiation (Student’s t-test; P value is shown; h) n=6 independent experiments; (i) n=3, independent experiments). Images in a-f are representative of 4 mice from 4 independent experiments, in g of 3 independent experiments.
day 8 HE CD34<sup>+</sup> population nor in CD34<sup>+</sup> populations identified at earlier stages of development (Supplementary Fig. 3a). Following the EHT culture, the RIC–GFP-derived CD34<sup>+</sup> cells generated the same spectrum of CD34<sup>+</sup>CD45<sup>+</sup> fractions observed in the H1-derived population (Fig. 2a upper panel). A distinct RUNX1C–<br>EGFP<sup>+</sup>CD34<sup>med</sup> fraction was also detected at this time (Fig. 2a lower panel). Kinetic analyses showed that RUNX1C–EGFP<sup>+</sup>CD34<sup>med</sup> cells emerged as early as day 4 of EHT culture and by day 8+5, approximately half of the fraction also co-expressed CD144 and CD45 (Fig. 2b), a pattern of interest given that HSCs in the E11.5 mouse embryo restricted expression pattern in the hESC-derived cultures as it does in the mouse embryo<sup>20</sup>, we next analysed RIC–GFP-derived primitive haematopoietic populations. As a first approach we isolated the KDR<sup>+</sup>CD235a<sup>+</sup> and KDR<sup>+</sup>CD235a<sup>+</sup> fractions from day 3 activin A-induced embryoid bodies (EBs) as we have recently shown that they contain progenitors of the primitive and definitive haematopoietic programs, respectively<sup>10</sup>. The sorted cells were aggregated and cultured for 6 days to generate either primitive or definitive CD34<sup>+</sup>CD45<sup>+</sup> HE, as described in our previous study<sup>10</sup>. These respective HE populations were isolated and assayed in our EHT culture conditions. Although both HE populations generated CD45<sup>+</sup> cells, only the definitive KDR<sup>+</sup>CD235<sup>+</sup> progenitors gave rise to RUNX1C–EGFP<sup>+</sup> cells (Fig. 2f). With the second approach, we analysed different staged EBs generated in the presence of the WNT inhibitor IWP2 as we have shown that this manipulation promotes primitive haematopoiesis at the expense of the definitive haematopoietic program<sup>10</sup>. RUNX1C–EGFP expression was not detected in the EB cells at any stage, or in the EHT cultures generated from the day 8 CD34<sup>+</sup>CD43<sup>+</sup> progenitors (day 8+7; Supplementary Fig. 3b). Taken together, these findings provide strong evidence that RUNX1C–EGFP expression is restricted to the definitive haematopoietic program in the hPSC differentiation cultures.

**NOTCH signalling is essential for the EHT**

If the emergence of the CD34<sup>+</sup>CD45<sup>+</sup>RUNX1C<sup>+</sup> population is reflective of the onset of human definitive haematopoiesis its...
development should, at some stage, be dependent on NOTCH signalling. To investigate this, we treated CHIR-induced hPSC-derived cells at 2 different time intervals (day 3–8 EBs or day 8–14 EBs) with the chemical NOTCH inhibitor, γ-secretase inhibitor L-685, 458 (GSI). Inhibition of NOTCH between days 3 and 8 of differentiation during the haematopoietic and vascular lineage
specification stage (Fig. 1a) reduced NOTCH target gene expression (HES1, HEY1 and HES5; Supplementary Fig. 4a) and led to a twofold increase in the proportion of CD34+ cells detected at day 8 (Fig. 3a). Blocking the pathway at this stage did not markedly affect the emergence of the definitive haematopoietic program, as the treated CD34+ population underwent EHT and contained T-cell progenitors (Fig. 3b,c).

In contrast, treatment of the isolated CD34+ cells with GSI during the EHT culture prevented the formation of the non-adhesive round cells, reduced the size of the CD45 population that developed in the H1-derived cultures, and blocked the emergence of RUNX1C–EGFP+ cells in the R1C–GFP-derived cultures (Fig. 3d–f). Consistent with these observations, GSI treatment at this stage also inhibited the development of myelo/erythroid and T-cell progenitors (Fig. 3g–i). These findings indicate that the haematopoietic specification of HE during the EHT is NOTCH-dependent.

To examine more precisely the NOTCH-dependent stage, we next added GSI for defined intervals during the EHT culture. As shown in Fig. 3j and Supplementary Fig. 4b the largest reduction in the size of the CD45+ population was observed when GSI was included for the first 3 days of culture, the stage coinciding with onset of EHT (Fig. 1c). Collectively, these findings demonstrate that haematopoietic specification of the CD34+ HE population is dependent on NOTCH signalling, indicating that the emerging blood cell lineages represent the definitive program. Furthermore, they show that the NOTCH pathway is essential for the EHT stage of development, but not for the specification of the CD34+ HE.

Enrichment of HE in the CD34+ population
To enrich for HE within the CD34+ population, we next analysed it for expression of CD73 as recent studies showed that this marker distinguishes haemogenic and non-haemogenic progenitors at early stages in hPSC differentiation cultures.23,24,25 We also monitored CD184 expression, which demarcates arterial endothelium in the mouse embryo and in mESC-derived endothelial populations.23,24 The expression of these 2 markers resolved the following 3 fractions in the day 8 CD34+ population: CD73+CD184+, CD73medCD184+ and CD73medCD184– (Fig. 4a). When cultured under EHT conditions, all 3 populations generated an adhesive monolayer consisting of cells with endothelial morphology. However, only the CD73+CD184+– derived population underwent EHT and gave rise to CD45+ cells (Fig. 4b), CD34+RUNX1C+ cells (Fig. 4c) and myeloid/erythroid (Fig. 4d) and T-cell progenitors (Fig. 4e). Limiting-dilution analyses (LDA) revealed a frequency of 1/35 T-cell progenitors in the CD73+CD184+ population compared with 1/700 in the non-fractionated CD34+ population, indicating a significant enrichment in HE. As observed with the unfractonated CD34+ population, the transition of the CD73–CD184+ cells to a haematopoietic fate seems to be a NOTCH-dependent event, as addition of GSI during the EHT culture strongly reduced the development of the myeloid and erythroid progenitors (Fig. 4d).

Kinetic analyses revealed that a small CD34+CD184+ population was detectable as early as day 4 of differentiation (Supplementary Fig. 5a, left panels). All 3 fractions were present by day 6 (Supplementary Fig. 5a, right panels), the earliest stage at which we were previously able to measure T-cell progenitors. As observed with the day 8 population, the EHT potential and lymphoid and erythroid/myeloid progenitors were detected only in the CD73+CD184+– fraction at day 6 (Supplementary Fig. 5b–d). Generation of the erythroid/myeloid progenitors from the day 6 HE was also dependent on NOTCH signalling as their development was inhibited by the addition of GSI during the EHT culture (Supplementary Fig. 5d).

The day 8 CD34+ population isolated from EBs generated from the R1C–GFP cell line also contained the same 3 CD184 and CD73 factions. As with the H1 hESC-derived fractions, HE as measured by EHT, as well as lymphoid and myeloid/erythroid potential segregated to the R1C–GFP-derived CD73+CD184+– fraction (Supplementary Fig. 5e–g). Taken together, these findings demonstrate that day 8 CD34+ definitive HE can be distinguished from VE on the basis of expression of CD73 and CD184.

Murine HE cells lack expression of CD184 and CD73 in vivo
To determine whether HE in vivo exhibits a CD184–CD73– phenotype, we next analysed the Runx1+CD31+ population in the AGM and yolk sac (YS), of E8.5, E9.5 and E10.5 Runx1–GFP mouse embryos for expression of these markers. Co-expression of Runx1 and CD31 marks HE in both tissues at these stages.23 Neither the AGM-nor YS-derived HE cells expressed CD184 or CD73 (Supplementary Fig. 6a–d), indicating that HE in vivo can also be characterized as a CD184–CD73– progenitor.

CD184 and CD73 expression distinguishes HE from arterial and venous VE
qRT-PCR analyses showed that the hESC-derived CD34+CD43+CD184+CD73–CD184– fraction expressed the highest levels of early haematopoietic genes including RUNX1C, MYB and TAL1, whereas arterial endothelial genes, such as EPHRINB2 and DLL4 (ref. 26), were detected at the highest levels in the CD73medCD184+– fraction (Fig. 4f). Conversely, expression of NR2F2, the master regulator of venous endothelium development,27, was highest in the CD73medCD184– fraction (Fig. 4f). These patterns suggest that the expression of CD184 and CD73 can be used to separate the CD34+ VE population into distinct fractions with arterial and venous potential.

To confirm that the CD73medCD184+ and CD34+CD184– populations represent distinct endothelial cell types, we transplanted them subcutaneously in a Matrigel plug into immunocompromised NOD.Cg-Prkdcreid Il2rgtm1Wjt/Sj (NSG) mice. Four weeks following transplantation, hESC-derived vascular structures comprised of human CD31+ cells were detected in the grafts from both populations (Fig. 5a,b). The vessels generated from the CD73medCD184+ cells were considerably larger than those derived from the CD34+CD184– cells. They also had larger numbers of α-SMA+ cells associated with them, probably reflecting the recruitment of vascular smooth muscle cells (Fig. 5a,b). The CD31+ cells in these grafts did not express the venous endothelial marker ephrin receptor B4 (EPHB4; Fig. 5c). These characteristics suggest that the CD73medCD184+– derived structures represent arterial vessels. The smaller vessels derived from the CD34+CD184– cells expressed EPHB4 indicative of developing venous vasculature (Fig. 5d). Autofluorescent erythrocytes were detected in the lumen of both types of vessel, demonstrating integration into the vasculature network of the host (Fig. 5e,f).
Studies in different model organisms have shown that notch and mitogen-activated protein kinase (MAPK) signalling promote the arterial fate whereas signalling through the phosphoinositide 3-kinase (PI(3)K) pathway supports venous development\(^\text{27,28}\). To determine whether these pathways regulate development of the CD73\textsuperscript{med}CD184\textsuperscript{hi} and CD73\textsuperscript{hi}CD184\textsuperscript{−} populations, we used a HES2 hESC line containing a tamoxifen (4-OHT)-responsive NOTCH1 intracellular domain (Hes2-ICN1-ER\(^\text{tm}\)) to activate the NOTCH pathway and the small molecules LY294002 and PD0325901 to inhibit the PI(3)K and MEK/ERK pathways respectively. Induction of NOTCH or inhibition of PI(3)K between days 3 and 8 of differentiation led to a significant increase in the size of the CD73\textsuperscript{med}CD184\textsuperscript{−} arterial population (Fig. 5g). The reverse pattern was observed following the inhibition of NOTCH with GSI or MEK/ERK with PD0325901, as addition of these molecules led to an increase in the size of the CD73\textsuperscript{hi}CD184\textsuperscript{−} venous population. Collectively these observations indicate that hPSC-derived CD73\textsuperscript{med}CD184\textsuperscript{−} arterial and CD73\textsuperscript{hi}CD184\textsuperscript{−} venous fates are regulated by the same pathways that regulate these fates in the embryo.

In addition to decreasing the size of the arterial fraction, NOTCH inhibition also led to a reduction in the size of the CD73\textsuperscript{−}CD184\textsuperscript{−} fraction (Fig. 5g). Although our studies above showed that addition of GSI between days 3 and 8 did not completely block haematopoietic development (Fig. 3b,c), it is possible that this manipulation of NOTCH signalling during this stage led to some reduction of haematopoietic potential. LDA analysis of T-cell progenitors revealed that inhibition of NOTCH signalling between days 3 and 8 of differentiation did not significantly affect T-cell progenitor frequency within the CD34\textsuperscript{+}CD73\textsuperscript{−}CD184\textsuperscript{−} fraction. Although NOTCH inhibition reduced the size of the CD34\textsuperscript{+}CD73\textsuperscript{−}CD184\textsuperscript{−} fraction, it also increased the size of the CD34 population. As a consequence, the percentage of CD34\textsuperscript{+}CD73\textsuperscript{−}CD184\textsuperscript{−} cells and T-cell progenitors generated in the total EB population did not differ between GSI-treated and control cultures (Fig. 5h,i). Activation of NOTCH signalling in Hes2-ICN1-ER\(^\text{tm}\)-derived cells by 4-OHT treatment between days 3 and 8 of differentiation did not alter the size of the total CD34 population, the size of the CD34\textsuperscript{+}CD73\textsuperscript{−}CD184\textsuperscript{−} fraction or the frequency of T-cell progenitors within the CD34\textsuperscript{+}CD73\textsuperscript{−}CD184\textsuperscript{−} fraction compared to the control. These findings confirm that manipulation of the NOTCH pathway between days 3 and 8 of differentiation does not significantly impact the generation of the HE lineage.

HE cells lack expression of the endothelial progenitor marker DLL4

To further characterize the progenitor potential of the CD73\textsuperscript{−}CD184\textsuperscript{−} fraction, we carried out a clonal analysis under EHT culture conditions. Out of 384 cells analysed, 83 generated progeny (Fig. 6a) and, of these, 10 (3% of starting) exhibited HE potential and underwent the EHT to generate round CD45\textsuperscript{+} haematopoietic cells (Fig. 6b). The remaining 73 (20%) gave rise to adhesive CD144\textsuperscript{+} endothelial cells (Fig. 6c). Haematopoietic and endothelial cells were never detected in the same clone. Wells containing both cell types were, however, observed when larger numbers (10) of CD73\textsuperscript{−}CD184\textsuperscript{−} cells were plated (Fig. 6d), confirming that the conditions support the development of both lineages together. Single CD73\textsuperscript{med}CD184\textsuperscript{+} and CD73\textsuperscript{hi}CD184\textsuperscript{−} cells gave rise to only CD144\textsuperscript{+} endothelial progeny, at cloning frequencies of 20–25%. Together, these findings demonstrate that the CD73\textsuperscript{−}CD184\textsuperscript{−} fraction contains both HE and VE progenitors further indicating that they represent distinct lineages. Furthermore, they show that HE is haematopoietic restricted, as these progenitors did not give rise to endothelial progeny.

We next sought to determine the heterogeneity in the CD73\textsuperscript{med}CD184\textsuperscript{+}, CD73\textsuperscript{hi}CD184\textsuperscript{−} and CD73\textsuperscript{−}CD184\textsuperscript{−} populations by analysing the expression of MYB (haemogenic), EFNB2 (arterial) and NR2F2 (venous) at the single-cell level. These analyses revealed that 71% of the CD73\textsuperscript{med}CD184\textsuperscript{+} cells expressed only the arterial marker EFNB2 whereas 75% of the CD73\textsuperscript{hi}CD184\textsuperscript{−} cells expressed the venous marker NR2F2, indicating little contamination between the cell types (Fig. 6e). A subpopulation (21%) of the CD73\textsuperscript{hi}CD184\textsuperscript{−} cells (5 out 18 cells) co-expressed the arterial and venous marker, a pattern that has been observed during the formation of the main vessels in the mouse embryo\(^\text{29}\). As expected, the CD73\textsuperscript{−}CD184\textsuperscript{−} population contained MYB-expressing HE (15%, 6 out 40) as well as vascular progenitors as demonstrated by the presence of cells that express either NR2F2 or EFNB2 (in both cases 10%, 4 out of 40 cells).

To enrich for HE, we next analysed the CD34\textsuperscript{−}CD73\textsuperscript{−}CD184\textsuperscript{−} fraction for expression of the NOTCH ligand DLL4 that is found on VE progenitors early in development\(^\text{30}\). As shown in Fig. 7a, both DLL4\textsuperscript{−} and DLL4\textsuperscript{+} cells were detected. The percentage of DLL4\textsuperscript{+} cells within the DLL4\textsuperscript{−} fraction varied between experiments and ranged from 28% to 79%, (51% ± 7, mean ± s.e.m., n = 6, independent). Gene expression analysis showed that RUNX1C, MYB and GATA2 were expressed at significantly higher levels in the DLL4\textsuperscript{+} than in DLL4\textsuperscript{−} cells (Fig. 7b). Clonal analysis revealed that the DLL4\textsuperscript{−} subfraction was enriched for HE as 70% of the cells that formed a clone in the EHT assay (176 out of 480) generated only haematopoietic cells (Fig. 7c). The remaining 30% gave rise to only endothelial progeny. LDA analyses revealed a T-cell progenitor frequency of 1/11 (ranging from 1/1 to 1/30, n = 4, independent) in the DLL4\textsuperscript{−} fraction. The DLL4\textsuperscript{−} fraction, in contrast, was enriched in vascular potential, as 89% of the cells that formed a clone (111 out of 480) were endothelial restricted, whereas only 11% showed haematopoietic potential. T-cell potential was detected only when 1,000, or more, cells were plated. Together, these findings show that the CD34\textsuperscript{−}CD73\textsuperscript{−}CD184\textsuperscript{−}DLL4\textsuperscript{−} fraction is highly enriched for HE and provide additional evidence that HE and VE progenitors represent distinct cell types that can be separated on the basis of the expression of DLL4.

CD34\textsuperscript{−}CD73\textsuperscript{−}CD184\textsuperscript{−}DLL4\textsuperscript{−} HE generates multipotent haematopoietic progenitors

To determine whether the T-lymphoid, myeloid and erythroid lineages that develop from the HE derive from the same progenitor, single CD34\textsuperscript{−}CD184\textsuperscript{−}DLL4\textsuperscript{−} cells were sorted directly onto OP9-DLL4 stroma and cultured for 7 days to promote EHT and haematopoietic expansion. The developing clones were collected and cells from each were plated both into methylcellulose to measure erythroid/myeloid progenitors and onto fresh OP9-DLL4 stromal cells to monitor T-lymphoid potential (Fig. 7d). Out of a total of 252 cells analysed, 68 exhibited haematopoietic potential and of these 5 gave rise to lymphoid, myeloid and erythroid progeny. Eight cells showed lymphoid/myeloid potential, whereas the remainder differentiated to...
either lymphoid or myeloid and/or erythroid progeny (Fig. 7c,f). These findings demonstrate that a subset of CD34+CD73−CD184−DLL4− HE cells are able to give rise to multiple blood cell lineages, probably through the generation of a multipotent haematopoietic progenitor during the EHT process.

**DISCUSSION**

Although the close association of HE with the arterial vasculature in the developing embryo supports the hypothesis that these progenitors derive from the arterial endothelium,5,31,32, direct evidence establishing a progenitor–progeny relationship between the two populations is lacking. The findings in this study, demonstrating that arterial VE and definitive HE can be separated on the basis of CD184 and CD73 expression and that clones derived from the CD184+CD73−DLL4− fraction contain either haematopoietic or endothelial cells, provide strong evidence that these populations represent distinct lineages. These observations are consistent with those from a recent study showing that prospective HE cells isolated from murine AGM give rise to only haematopoietic or endothelial lineage, but never to both.33 The finding in both studies that the haematopoietic clones do not contain endothelial cells strongly suggests that HE cells are haematopoietic-restricted progenitors.

Our demonstration that the EHT of the CD34+ HE is NOTCH-dependent provides strong evidence that the derivative haematopoietic lineages represent the definitive program, as studies in other models have shown that definitive, but not primitive haematopoiesis requires Notch signalling44. Although this requirement for Notch signalling for definitive haematopoiesis is well established, it has remained unclear whether the block in this program was due to an absence of HE or to its inability to undergo EHT. By manipulating the pathway at specific stages, we show for the first time that NOTCH signalling is not essential for generation of the CD34+ HE but rather seems to be required for the cells to progress through the EHT. A role for Notch signalling at the EHT stage is supported by recent findings in the zebrafish that showed a cell-autonomous requirement for this pathway for the generation of HSC progenitors just before their emergence from the aortic floor55.

With the high frequency of HE in the CD73−CD184−DLL4− fraction, we were able to demonstrate that a subpopulation of these progenitors are able to generate lymphoid, erythroid and myeloid progeny. Our interpretation of these findings is that following the EHT, these HE cells generate a multipotent haematopoietic progenitor that may represent the equivalent of the HSC precursor identified in the mouse embryo.56 The identification of this multipotent progenitor, in particular its erythroid potential, rules out the possibility that our T-cell assays are detecting only the equivalent of the immune-restricted progenitors found during mouse embryonic development before the emergence of the HSCs (refs 37,38).

In summary, the findings from our study support a model (Fig. 7g) in which HE is characterized as a CD184+CD73−DLL4− haematopoietic-restricted progenitor that is specified as a lineage distinct from the VE at the mesoderm stage of development. Following a NOTCH-dependent EHT, we propose that the HE gives rise to CD45+ multipotent progenitors that represent the HSC precursor stage of haematopoietic development. The hPSC-derived HE and themultipotent haematopoietic progenitors provide target populations for further investigating the pathways that regulate the generation of HSCs *in vitro*.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**REFERENCES**

1. Kaufman, D. S. Toward clinical therapies using hematopoietic cells derived from human pluripotent stem cells. *Blood* **114**, 5315–5323 (2009).
2. Kennedy, M. et al. T lymphocyte potential marks the emergence of definitive haematopoietic progenitors in human pluripotent stem cell differentiation cultures. *Cell Rep.*, **2**, 1722–1735 (2012).
3. Klimchenko, O. et al. Monocytic cells derived from human embryonic stem cells and fetal liver share common differentiation pathways and homeostatic functions. *Blood* **117**, 3065–3075 (2011).
4. Takayama, N. et al. Generation of functional platelets from human embryonic stem cells *in vitro* via ES-sacs, VEFG-promoted structures that concentrate hematopoietic progenitors. *Blood* **111**, 5298–5306 (2008).
5. Clements, W. K. & Traver, D. Signalling pathways that control vertebrate haematopoietic stem cell specification. *Nat. Rev. Immunol.* **13**, 336–348 (2013).
6. Kissa, K. & Herbolomel, P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* **464**, 112–115 (2010).
7. Bertrand, J. Y. et al. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* **464**, 108–111 (2010).
8. Boisse, J. C. et al. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* **464**, 116–120 (2010).
9. Rybtsov, S. et al. Tracing the origin of the HSC hierarchy reveals an SCF-dependent, IL-3-independent CD43+ embryonic precursor. *Stem Cell Rep.* **3**, 489–501 (2014).
10. Sturgeon, C. M., Ditadi, A., Awong, G., Kennedy, M. & Keller, G. Wnt signalling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. *Nat. Biotechnol.* **32**, 554–561 (2014).
11. Bertrand, J. Y., Cisson, J. L., Stachura, D. L. & Traver, D. Notch signalling distinguishes 2 waves of definitive hematopoiesis in the zebrafish embryo. *Blood* **115**, 2777–2783 (2010).
12. Hadland, B. K. et al. A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. *Blood* **104**, 3097–3105 (2004).
13. Kumano, K. et al. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* **18**, 699–711 (2003).
14. Robert-Moreno, A., Espinosa, L., de la Pomp, J. L. & Bigas, A. RBPγ-dependent Notch function regulates Galα2 and is essential for the formation of intra-embryonic haematopoietic cells. *Development* **132**, 1117–1126 (2005).
15. Peeters, M. et al. Ventral embryonic tissues and Hedgehog proteins induce early AGM haematopoietic stem cell development. *Development* **136**, 2613–2621 (2009).
16. Robin, C. & Durand, C. The roles of BMP and IL-3 signalling pathways in the control of haematopoietic stem cells in the mouse embryo. *Int. J. Dev. Biol.* **54**, 1189–1200 (2010).
17. Zambidis, E. T. et al. Expression of angiotensin-converting enzyme (CD143) identifies and regulates primitive hemangioblasts derived from human pluripotent stem cells. *Blood* **112**, 3601–3614 (2008).

18. Yokomizo, T. & Dzierzak, E. Three-dimensional cartography of hematopoietic clusters in the vasculature of whole mouse embryos. *Development* **137**, 3651–3661 (2010).

19. Uenishi, G. et al. Tenasin C promotes hematoendothelial development and T lymphoid commitment from human pluripotent stem cells in chemically defined conditions. *Stem Cell Rep.* **3**, 1073–1084 (2014).

20. Bee, T. et al. Alternative Runx1 promoter usage in mouse developmental hematopoiesis. *Blood Cells Mol. Dis.* **43**, 35–42 (2009).

21. Choi, K. D. et al. Identification of the hemogenic endothelial progenitor and its direct precursor in human pluripotent stem cell differentiation cultures. *Cell Rep.* **2**, 553–567 (2012).

22. Rafii, S. et al. Human ESC-derived hemogenic endothelial cells undergo distinct waves of endothelial to hematopoietic transition. *Blood* **121**, 770–780 (2013).

23. Yamamizu, K. et al. Convergence of Notch and β-catenin signaling induces arterial fate in vascular progenitors. *J. Cell Biol.* **189**, 325–338 (2010).

24. Yurugi-Kobayashi, T. et al. Adrenomedullin/cyclic AMP pathway induces Notch activation and differentiation of arterial endothelial cells from vascular progenitors. *Arterioscler. Thromb. Vasc. Biol.* **26**, 1977–1984 (2006).

25. Tober, J., Yzaguirre, A. D., Piwarzyk, E. & Speck, N. A. Distinct temporal requirements for Runx1 in hematopoietic progenitors and stem cells. *Development* **140**, 3765–3776 (2013).

26. Marcelo, K. L., Goldie, L. C. & Hirschi, K. K. Regulation of endothelial cell differentiation and specification. *Circ. Res.* **112**, 1272–1287 (2013).

27. You, L. R. et al. Suppression of Notch signalling by the COUP-TFIII transcription factor regulates vein identity. *Nature* **435**, 98–104 (2005).

28. Hong, C. C., Peterson, Q. P., Hong, J. Y. & Peterson, R. T. Artery/vein specification is governed by opposing phosphatidylinositol-3 kinase and MAP kinase/ERK signaling. *Curr. Biol.* **16**, 1366–1372 (2006).

29. Lindskog, H. et al. Molecular identification of venous progenitors in the dorsal aorta reveals an aortic origin for the cardinal vein in mammals. *Development* **141**, 1120–1128 (2014).

30. Benedito, R. & Duarte, A. Expression of Dil4 during mouse embryogenesis suggests multiple developmental roles. *Gene Expr. Patterns* **5**, 750–755 (2005).

31. Richard, C. et al. Endothelio-mesenchymal interaction controls runx1 expression and modulates the notch pathway to initiate aortic hematopoiesis. *Dev. Cell* **24**, 600–611 (2013).

32. Ciau-Uitz, A., Wang, L., Patient, R. & Liu, F. ETS transcription factors in hematopoietic stem cell development. *Blood Cells Mol. Dis.* **51**, 248–255 (2013).

33. Swiers, G. et al. Early dynamic fate changes in haemogenic endothelium characterized at the single-cell level. *Nat. Commun.* **4**, 2924 (2013).

34. Bigas, A., Robert-Moreno, A. & Espinosa, L. The Notch pathway in the developing hematopoietic system. *Int. J. Dev. Biol.* **54**, 1175–1188 (2010).

35. Kim, A. D. et al. Discrete Notch signaling requirements in the specification of hematopoietic stem cells. *EMBO J.* **33**, 2363–2373 (2014).

36. Godin, I., Garcia-Porrero, J. A., Dieterlen-Lievre, F. & Cumano, A. Stem cell emergence and hematopoietic activity are incompatible in mouse intraembryonic sites. *J. Exp. Med.* **190**, 43–52 (1999).

37. Boiers, C. et al. Lymphomyeloid contribution of an immune-restricted progenitor emerging prior to definitive hematopoietic stem cells. *Cell Stem Cell* **13**, 535–548 (2013).

38. Luc, S. et al. The earliest thymic T cell progenitors sustain B cell and myeloid lineage potential. *Nat. Immunol.* **13**, 412–419 (2012).
METHODS

Maintenance and differentiation of human ESCs. The hESC lines H1, Hes2-ICN1-ER" and HES3-RUNXIC477-GFP were maintained on irradiated mouse embryonic fibroblasts in hESC media as described previously. For differentiation, hESCs were dissociated using trypsin-EDTA (BD Biosciences) for 2-3 h, followed by EB generation, as described previously. Briefly, the undifferentiated hESCs were dissociated with Tryp-LE ( Gibco) treatment, followed by scraping. Aggregates were resuspended in StemPro-34 (Invitrogen), supplemented with penicillin/streptomycin (10 ng ml⁻¹), 1,l-glutamine (2 mM), ascorbic acid (1 mM), monothioglycerol (MTG, 4 x 10⁻⁴ M; Sigma), transferrin (150 ng ml⁻¹) (ref. to ‘as supplemented StemPro-34) and BMP-4 (10 ng ml⁻¹), and cultured in 6-well low-attachment plates (Corning) in a volume of 2 ml per well. Following 24 h, BMP-4 was added to a final concentration of 5 ng ml⁻¹. At day 2, the developing EBs were collected, washed and resuspended in supplemented StemPro-34 with BMP-4, BFGF, and CHIR99021 (3 μM, Stemgent), IWP2 (3 μM, Tocris Biosciences) or activin A (1 ng ml⁻¹) as indicated. After 24 h, the EBs were again collected and resuspended in supplemented StemPro-34 containing VEGF (15 ng ml⁻¹), bFGF (5 ng ml⁻¹), IL-6 (10 ng ml⁻¹) and IL-11 (5 ng ml⁻¹) and cultured for 48 h. At this stage, the EBs were fed with 2 ml of the media containing also SCF (50 ng ml⁻¹ final), IGF-1 (25 ng ml⁻¹ final) and EPO (2 U ml⁻¹ final) and cultured until day 8. Cultures were maintained in a 5% CO₂/5% O₂/95% N₂ atmosphere. All recombinant factors are human and most were purchased from R&D Systems. Where indicated 4-OHT (1 μM) was added to a final concentration of 5 ng ml⁻¹. For the multilayer culture, hESCs were dissociated with Tryp-LE (GIBCO) treatment, followed by scraping. Aggregates were resuspended in StemPro-34 containing VEGF (15 ng ml⁻¹) and bFGF (5 ng ml⁻¹). EBs were collected, washed and resuspended in Supplement StemPro-34 containing VEGF (15 ng ml⁻¹) and bFGF (5 ng ml⁻¹) and cultured for 48 h. The EBs were fed with 2 ml of the media containing also SCF (50 ng ml⁻¹ final), IGF-1 (25 ng ml⁻¹ final) and EPO (2 U ml⁻¹ final) and cultured until day 8. Cultures were maintained in a 5% CO₂/5% O₂/95% N₂ atmosphere. All recombinant factors are human and most were purchased from R&D Systems. The exceptions are CD34-PE-CY7 and CD144-PE, which were purchased from eBioscience. CD184-Bright Brilliant Violet 421 (clone 12G5, 2:100), CD73-APC (clone AD2, 1:400), KDR (clone 4D5A1, 1:7) and the human ROSA26 locus, was electroporated with a Cre expression vector along with the ICN1ER-IRES-Puro donor vector. Successfully targeted Hes2-NICD-ER clones were expanded by transfection with a Cre-recombinase plasmid, pEFBOS-Cre-IRES-Puro, as described previously.

Endothelial-to-haematopoietic transition assay. Candidate cells (total CD34⁺CD75⁻CD14⁻CD13⁻CD16⁻CD38⁻CD45⁻CD123⁻APC-CY7⁺ or CD34⁺CD73⁺CD14⁻CD38⁻CD45⁻CD123⁻APC-CY7⁺) were aggregated overnight at a density of 2 x 10⁵ ml⁻¹ in supplemented StemPro-34 media, containing TPO (30 ng ml⁻¹), IL-3 (30 ng ml⁻¹), SCF (100 ng ml⁻¹), IL-6 (10 ng ml⁻¹), IL-11 (5 ng ml⁻¹), IGF-1 (25 ng ml⁻¹), EPO (2 U ml⁻¹), VEGF (5 ng ml⁻¹), bFGF (5 ng ml⁻¹), BMP4 (10 ng ml⁻¹), Flk-3L (10 ng ml⁻¹), SHH (20 ng ml⁻¹), and the chemical AGTR1 (angiotensin II receptor type 1) blocker losartan potassium (100 μM). All recombinant factors are human and most were purchased from R&D Systems. The exceptions are angiotensin II, which was purchased from Sigma, and EPO, which was purchased from Janssen-Cilag. Aggregates were then transferred onto thin-layer Matrigel-coated plasticware where they were cultured for an additional 4 to 6 days in the same media. Cultures were maintained in a 5% CO₂/5% O₂/95% N₂ atmosphere. Cells were visualized using a Leica Imaging System.

Haematopoietic colony assay. Analysis of haematopoietic colony potential was performed by plating 0.1 x 10⁶ - 1 x 10⁷ cells in 1% methylcellulose containing specific cytokines as described in detail previously. Colonies consisting of erythroid, erythroid/myeloid and myeloid (either macrophage or mast cell) cells were quantified after 14 days.

Multilineage clonal assay. Single CD34⁺CD73⁻CD184⁻DLL4⁺ cells were sorted directly into individual wells of a 96-well plate containing OP9-DLL4 cells, and cultured in α-MEM supplemented with penicillin/streptomycin and 20% FBS, rhFlt-3L (30 ng ml⁻¹), rhIL-11 (5 ng ml⁻¹), rhSCF (30 ng ml⁻¹) was added to the cultures for the first 5 days. Every five days co-cultures were transferred onto fresh OP9-DLL4 cells by vigorous pipetting and passing through a 40 μm cell strainer. Cells were analysed by flow cytometry on the days indicated. Populations scored as positive yielded greater than 100 gated CD45⁺ events.

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Flow cytometry and cell sorting. The following antibodies were used for these studies: CD34-APC (clone 8G12, 1:100), CD34-PE-CY7 (clone 4H11, 1:100), CD34-PE or FITC (clone 1G10, 1:30 and 1:10 respectively), CD4-PE-Cy7 (clone RPA-T4, 1:1000), CD8-PE (clone RPA-T8, 1:300), CD45-APC-Cy7 or eFluor450 (clone 2D1, 1:50), CD56-APC (clone Bi139, 1:300), CD144-PE (clone 123413, 1:50), CD184-Brilliant Violet 421 (clone 12G5, 2:100), CD73-APC (clone AD2, 1:400), KDR (clone 4D5A1, 1:7) and the human ROSA26 locus, was electroporated with a Cre expression vector along with the ICN1ER-IRES-Puro donor vector. Successfully targeted Hes2-NICD-ER clones were expanded by transfection with a Cre-recombinase plasmid, pEFBOS-Cre-IRES-Puro, as described previously. Single cell sorted subclones were screened by PCR for the removal of the PKGNeo or PKGHyg selection cassette. Targeted lines expressed surface markers of undifferentiated hESCs (Supplementary Fig. 2b), retained a nana karyotype (Supplementary Fig. 2c) and formed teratomas (Supplementary Fig. 2e-i) following injection of undifferentiated RUNXIC477-GFP hESCs under the kidney capsule of NOD/SCID/IL2Rγ⁻ mice as described below. The fidelity of the reporter was confirmed by demonstrating that expression of the RUNXIC isoform co-segregated with GFP expression.

Teratoma assay. Eight- to ten-week-old male or female NSG mice were anaesthetized and injected under the kidney capsule with 1 x 10⁶ undifferentiated RUNXIC477-GFP hESCs. Mice received postoperative analgesia to alleviate discomfort. Mice were euthanized humanely after 6 weeks or earlier if palpable tumours causing distress were present. Three mice were injected, all of which developed teratomas. Tumours were fixed in paraformaldehyde-containing solution, embedded in paraffin and sections stained with haematoxylin and eosin. This work was performed under the auspices of the Monash University animal ethics committee (approval number SOBSA-MIS-2009-007). No statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Quantitative real-time PCR. Total RNA was purified with the RNeasy RNA Isolation Kit (Ambion) and treated with RNase-free DNase (Qiagen). RNA (100 ng to 1 μg) was transcribed into cDNA using random hexamers and Oligo (dT) with Superscript III Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed on a MasterCycler EP RealPlex (Eppendorf). All experiments were carried out in triplicate Quantifast SYBR Green PCR Kit (Qiagen). The
To synthesize cDNA, the thawed plate of lysed cells was incubated for 90 s at 65°C. Following this step, 1 μl of a mix containing 10× SuperMIX VILO (Invitrogen) and 0.5 μl of 40× oligonucleotide sequences are available on request. Gene expression was evaluated as DeltaCt relative to control (TBP).

Thermo-Fast 96 PCR Plate Non-Skirted 96-well PCR plates (Thermo Scientific) were used for the collection of single cells. Single cells were collected directly into 5 μl lysis solution consisting of 5 × VILO Reaction Mix (Invitrogen); 0.5% NP40 (Thermo Scientific); 0.4 units μl⁻¹ RNaseOut (Invitrogen). Lysed cells were frozen on dry ice.

To wash the sections twice with PBS and then blocked with 10% donkey serum in PBS/BSA 2%. Following these steps, the sections were processed for immunostaining. For immunostaining, the Matrigel plug dissected out and processed for paran sectioning. For immunostaining, the 12-week-old female NSG mice. Four weeks later, mice were euthanized, the Matrigel were used for the collection of single cells. Single cells were collected directly into 5 μl lysis solution consisting of 5 × VILO Reaction Mix (Invitrogen); 0.5% NP40 (Thermo Scientific); 0.4 units μl⁻¹ RNaseOut (Invitrogen). Lysed cells were frozen on dry ice.

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Supplementary Figure 1 Haemoglobin genes expression analysis of the BFU-derived colonies generated from CD34⁺CD43⁻ cells after EHT culture. qRT-PCR analysis of globin gene expression in the BFU-derived erythroid colonies generated from the CD34⁺CD45⁺ cells and in Ery⁺ CFC-derived colonies generated from day 8 CD43⁺ cells isolated from Activin A-induced EBs. FL: RNA from total fetal liver mononuclear cells, BM: adult bone marrow mononuclear cells. Ery⁺CFC n=13 individual colonies from 4 independent experiments, BFU-E n=7 individual colonies from 3 independent experiments, (Mean ± SEM). Student’s t-test. ** p = 0.0086.
Supplementary Figure 2 Generation and characterisation of RUNX1C<sup>GFP/w</sup> targeted hESCs. 

**a**, Schematic depicting the organization of the RUNX1 genomic locus with exons shown as boxes. Non-coding exons are shown in white and coding exons in black. The distal (D) and proximal promoters (P) and the transcripts emanating from each are indicated. The targeting vector is shown with black triangles marking loxP sites. The targeted allele is shown before and after CRE recombinase mediated excision of the antibiotic resistance cassette (NEO).

**b**, Flow cytometric analysis of undifferentiated RUNX1C<sup>GFP/w</sup> cells showing expression of the following surface markers associated with pluripotent cells, ECAD (CDH1), CD9, TRA 1 81 and GCTM2.

**c**, RUNX1C<sup>GFP/w</sup> cells demonstrate a normal female karyotype.

**d**, Pluritest (www.pluritest.org) analysis of transcriptional profiles of undifferentiated RUNX1C<sup>GFP/w</sup> cells, parental HES3 cells, H9 and MEL1 hESCs, scores all lines as pluripotent, with high pluripotency and low novelty scores.

**e**, Stained haematoxylin and eosin stained paraffin sections of a teratoma derived from undifferentiated RUNX1C<sup>GFP/w</sup> cells injected under the kidney capsule of an immunocompromised mouse demonstrate diverse cell types derived from three germ layers within the same field, including pigmented epithelium (**f**), primitive muscle and mesenchyme (**g**), glandular epithelium (**h**) and neural rosettes (**i**). Scale bar (**e**) 200 µM, (**f**) – (**i**) 50 µM. All images are representatives of three independent experiments.
Supplementary Figure 3  Kinetics of RUNX1C-EGFP expression. a, Representative flow cytometric analysis of CD34 and RUNX1C-EGFP expression in day 4, 6 and 8 EBs. b, Representative flow cytometric analysis of CD34, CD43 or CD45 (upper panels) and RUNX1C-EGFP expression (lower panels) in day 4, day 6 and day 8 EBs and after 7 days of EHT culture of day 8 CD34+CD43− cells in IWP2-induced cultures. All images are representatives of three independent experiments.
**Supplementary Figure 4** Inhibition of NOTCH signalling by GSI during EHT inhibits T cell potential. 

**a,** qRT-PCR analyses of expression of the Notch target genes HES1, HEY1 and HES5 in day 8 CD34+CD43- populations isolated from EBs treated with DMSO or GSI between days 3 and 8 of differentiation. Cells were derived from H1 hESCs. n=3, independent experiments. (Mean ± SEM). Student’s t-test, **p < 0.01.

**b,** Quantification of the effect of GSI treatment during the indicated times on the generation of CD45+ cells at day 7 of EHT culture. n=4, independent experiements. (Mean ± SEM). ANOVA, **p < 0.01, *p < 0.05.
Supplementary Figure 5 Expression of CD73 and CD184 distinguishes HE and VE CD34+CD43- cells derived from different hPSC lines. a, Kinetic analysis of the expression of CD184 and CD73 in day 4 and day 6 H1-derived CD34+CD43- cells and gating strategy used for the isolation of the CD184+ and CD73+ fractions from the day 6 CD34+CD43- population. b, Flow cytometric analyses of the frequency of CD34+ and CD45+ cells in populations generated after 7 days of EHT culture from the 3 H1-derived CD184/CD73 fractions isolated at day 6. c, T cell potential of the different H1-derived CD184/CD73 fractions measured by the development of CD4+ and CD8+ cells following culture on OP9-DLL4 stromal cells for 24 days. d, Haematopoietic colony-forming potential of CD184/CD73-derived populations following 7 days of EHT culture. The CD73-CD184- derived population was treated with GSI during the EHT culture to evaluate NOTCH-dependency. n = 3, independent. (Mean ± SEM). ** ANOVA p < 0.01. e, Flow cytometric analyses of the frequency of CD34+ and CD45+ cells in populations generated from the 3 day 8 R1C-GFP-derived CD184/CD73 fractions following 7 days of EHT culture. f, T cell potential of the different R1C-GFP-derived CD184/CD73 fractions measured by the development of CD4+ and CD8+ cells following culture on OP9-DLL4 stromal cells for 24 days. g, Haematopoietic colony-forming potential of CD184/CD73-derived populations following 7 days of EHT culture. The CD73-CD184- derived population was treated with GSI during the EHT culture to evaluate NOTCH-dependency. n = 3, independent. (Mean ± SEM). ** ANOVA p < 0.01. All images are representatives of three independent experiments.
Supplementary Figure 6 CD184 and CD73 expression on HE cells in vivo. a-d, Representative flow cytometric analysis of the frequency of CD184+ and CD73+ cells in the E10.5 aorta-gonad-mesonephros (AGM) (a), E10.5 yolk sac (YS) (b), E8.5 (c) and E9.5 (d) para-aortic splanchnopleura (p-Sp) populations isolated from Runx1-GFP mouse embryos. The proportion of CD184+ and CD73+ cells was measured in the indicated CD31/Runx1-GFP fractions gated to exclude Ter119+ CD41+ CD45+ cells (central panels). E10.5 embryos, n=2; E8.5 and E9.5 embryos, n=1.
Supplementary Video Legends

Supplementary Video 1
Time-lapse movie showing an adherent cell rounding up and gradually acquiring CD45 expression (in red) during EHT culture. This cell undergoes EHT and cell division giving rise to a round and an adherent cell, both positive for CD45. Scale bars: 50 μm

Supplementary Video 2
This movie shows the 3D reconstruction of confocal images of a cluster of emerging round haematopoietic cells in the EHT cultures. Cells were stained for the endothelial marker CD144 (in green), the haematopoietic marker CD45 (in gray) and for the EHT marker cKIT (in red). Scale bar: 5 μm.