Changes in cell fate and identity are essential for endothelial-to-haematopoietic transition (EHT), an embryonic process that generates the first adult populations of haematopoietic stem cells (HSCs) from hemogenic endothelial cells. Dissecting EHT regulation is a critical step towards the production of in vitro derived HSCs. Yet, we do not know how distinct endothelial and haematopoietic fates are parsed during the transition. Here we show that genes required for arterial identity function later to repress haematopoietic fate. Tissue-specific, temporally controlled, genetic loss of arterial genes (Sox17 and Notch1) during EHT results in increased production of haematopoietic cells due to loss of Sox17-mediated repression of haematopoietic transcription factors (Runx1 and Gata2). However, the increase in EHT can be abrogated by increased Notch signalling. These findings demonstrate that the endothelial haematopoietic fate switch is actively repressed in a population of endothelial cells, and that derepression of these programs augments haematopoietic output.
The first haematopoietic stem cells (HSCs) emerge in the embryo from a specialized subset of endothelial cells (ECs), collectively termed as hemogenic endothelium (HE). The concept of endothelial-derived HSCs has broad clinical implications as it may open new avenues for in vitro blood production. However, the hemogenic capacity of the endothelium is transient and its precise regulation remains unknown. During a narrow developmental time period (approximately embryonic day (E)10–12 in the mouse 1, 2, and 4–6 weeks in the human 3), a narrow developmental time period (approximately embryonic day 10–12 in the mouse 1,2, and 4–6 weeks in the human 3), a transient and its precise regulation remains unknown. During a narrow developmental time period (approximately embryonic day 10–12 in the mouse 1, 2, and 4–6 weeks in the human 3), a narrow developmental time period (approximately embryonic day 10–12 in the mouse 1, 2, and 4–6 weeks in the human 3), a transient and its precise regulation remains unknown. During a narrow developmental time period (approximately embryonic day 10–12 in the mouse 1, 2, and 4–6 weeks in the human 3), a transient and its precise regulation remains unknown.

In vivo endothelial genetic deletion of Sox17 during EHT (induction at E9.5, evaluation at E11; Fig. 2a) was evaluated using a endothelial-specific Cre recombinase (Cdhn5(PAC)-CreERT2 (ref. 47)) mouse line crossed to a Sox17 floxed line25 with a ROSA26Cre reporter48 (RTom, tdTomato, Td+). The induction strategy is similar to that used in fate-tracing studies49 and allows for timing of Sox17 endothelial recombination early in the hemogenic window and during EHT. Transcript analysis of sorted ECs after in vivo induction uncovered a significant increase in Runx1 and Gata2, two haematopoietic transcription factors known to be critical for HSC development during EHT27,28,30 (Fig. 2a). Notch1 transcripts are also notably decreased (Fig. 2a), in agreement with previous studies that show Sox17 positively regulates the Notch pathway22,26. In addition, other members of the SoxF family (Sox7 and Sox18) were increased, possibly due to a compensatory response (Fig. 2a). There were no observed differences in endothelial labelling or cell number across homozygous Sox17+/−, heterozygous Sox17+/−, or control animals (Supplementary Fig. 2a). Immunohistochemical analysis demonstrates the presence of HSPC clusters in the aorta with a marked decrease of endothelial SOX17 in Sox17+/− mutants (Fig. 2b). Also, we did not observe any obvious changes in endothelial morphology as evaluated by scanning electron microscopy (Supplementary Fig. 2b).

Currently, it is not possible to predict which specific EC within a hemogenic vascular bed will transition to a haematopoietic fate. Also, not known is whether ECs comprising the same hemogenic site are all capable of EHT. So whether the actual cell fate conversion is a stochastic event or a predetermined fate change remains to be seen. To circumvent the current obstacles of EHT prediction, we adopted a fate-tracing strategy49 that allows measurement of traced haematopoietic cell populations from...
labelled endothelial precursors within a specific hemogenic vascular site. By inducing endothelial recombination of Sox17 in AGM explants using the Cdh5(PAC)-CreERT2/RTom/Sox17flox-transgenic mouse line, the number of EHT-derived haematopoietic cells can be quantified by fate mapping (Fig. 2c; Supplementary Fig. 2c; Supplementary Table 2). Tamoxifen induction in vitro with the active metabolite 4-hydroxytamoxifen at E11.0 allows immediate ablation in AGM explants during EHT, and the calculation of a HE ratio, which we define as traced haematopoietic cells (HCS) compared with traced ECs. Using this assay to temporally and conditionally ablate Sox17, we demonstrate that timed loss of endothelial Sox17 promotes conversion to haematopoietic cell fate in situ (Fig. 2c–f). Sox17+/fmutants exhibit a significant threefold increase in HE ratios indicating increased haematopoietic output, in addition to significantly increased labelled hemogenic cluster populations.

Figure 1 | Haematopoietic cell clusters downregulate arterial gene expression. (a–e) Single channels in black and white, scale bars as shown. E10.5 wild-type dorsal aorta (DA). (a) Haematopoietic cell clusters of the AGM at E10.5. The endothelial layer and attached haematopoietic cell clusters are CD31+ (red). RUNX1 (grey) is notable in cells comprising the haematopoietic cluster (arrowhead). SOX17 (green) expression is localized to the endothelial layer (arrow). DAPI in blue. (b) GATA2 (green) is notable in the haematopoietic cell cluster (arrowhead). CD31 (red) and DAPI (blue). (c) SOX17 (green) immunofluorescence is noted in the cell nuclei of the endothelial layer (arrowheads), as compared with the associated cell cluster. CD31 in red, and DAPI in blue. (d) Notch pathway activation (green) as measured in the TP1 Venus mouse line is notable in the endothelial layer (arrow) but less so in the associated haematopoietic cell cluster, CD31 in red. DAPI in blue. (e) CD144 (red) labels the endothelium and haematopoietic cluster cells (arrowhead), Sox17 in grey, and Runx1 in green. (f) Embryos at E10.5 were sorted based on cell surface markers to isolate endothelial cells (CD31 + CD117 /CD45 /), haematopoietic cluster cells (CD31 + CD117 + CD45 -), maturing cluster cells and HSPCs (CD31 + CD117 + CD45 +) and mature haematopoietic cells (CD31 - CD45 +). Bar graphs depict transcript expression (RT–PCR) in each subgroup for Runx1, Gata2, Sox17, Notch1 and Cdh5 (CD144). Differing letters represent significance between groups where a versus b, or b versus c, or a versus c, is significant to a P value <0.01 or less, n = 3 litters, 24 embryos.
Figure 2 | Endothelial to haematopoietic conversion is increased after Sox17 loss. (a) Schema and bar graph of qRT–PCR analyses of sorted endothelial cells from E11 embryos after in vivo Sox17 ablation at E9.5. Error bars indicate standard error of the mean (n = 3 litters, embryos pooled by genotype). LOF, loss of function. (b) Immunofluorescence of Sox17 heterozygous and homozygous embryos at E10.5 after in vivo Cre induction (tamoxifen induction at E9.5). Haematopoietic clusters are labelled by CD117 (green), Cre traced endothelial and cluster cells in red (Tdc/+). Sox17 (grey) is absent in homozygous mutant endothelium. DAPI in blue. DA, dorsal aorta. Scale bar, 10 μm. Single channels in black and white. (c) Schematic of AGM explant analysis depicts in vitro Cre lineage tracing and calculation of hemogenic output (HE ratio); the ratio between per cent labelled (Tdc+) haematopoietic cell (CD45+CD31+) to per cent labelled (Tdc+CD41+) endothelial cells (CD31+CD45−). 4OHT, 4-hydroxytamoxifen. (d-f) Each data point represents a separate embryo/AGM explant, littersmates are depicted by the same data point colour and shape. Bars indicate group mean. P values calculated on Student’s t-test between groups, significance also validated by two-way analyses of variance (Supplementary Table 2). (d) The HE ratio of Sox17 homozygous (f/f) and heterozygous (f/+ ) mutant explants, f/+ n = 45, f/f n = 38, 15 litters. (e) Percentage of traced Tdc+ hemogenic endothelial cluster cells, designated as CD31+CD41+. f/+ n = 37, f/f n = 26, 9 litters. (f) Percentage of traced (Tdc+) maturing HSPCs (identified as CD31+CD41+CD45+), f/+ n = 14, f/f n = 27, 7 litters. (g) Schema depicts overexpression analyses in wild-type AGM explants at E11.0. (h) Immunofluorescence of E11 AGM explant after human adeno viral Sox17-GFP infection, GFP in green, Sox17 in magenta and DAPI in blue. Scale bar as indicated. (i) Cell sorting strategy for endothelial cells (CD31+) after exposure to AdhSOX17-GFP (GFP), where GFP+ and GFP− populations were gated. (j) Bar graph of qRT–PCR analyses of sorted E11 AGM CD31+ cells after AdhSOX17-GFP infection. Error bars indicate s.e.m. CD31+GFP− population served as a control, set to one for comparisons of fold change, n = 3 litters, embryos pooled, P values as indicated. (a-j) P values reflect Student’s t-test.

(CD31+CD41+Tdc+) and maturing HSPC populations (CD31+CD117−Sca1+CD45+Tdc+; Supplementary Fig. 2e). The observed increase in HE ratios and HSPC number is not due to proliferation effects (as measured by BrdU incorporation; Supplementary Fig. 2d) nor is the higher HE ratio due to changes in cell death (Annexin-V staining; Supplementary Fig. 2d). We also observe increases in other haematopoietic populations (CD31−CD117+Sca1+CD45+Tdc+; Supplementary Fig. 2e). In addition, when a similar strategy is applied to earlier explants (E9.5) before haematopoietic cell cluster emergence, we observe similar trends in the HE ratio (Supplementary Fig. 2f). So while Sox17 has been shown to be critical for HE specification before EHT, the loss of Sox17 actually promotes haematopoietic fate over endothelial fate during EHT. To further evaluate the role of...
Sox17 in this process, we undertook gain-of-function studies in wild-type AGM explants using adenoviral-mediated overexpression of human SOX17 (AdhSOX17-GFP; Fig. 2g). Green fluorescent protein (GFP) expression in explants overlapped with Sox17 co-staining (Fig. 2h), allowing for cell sorting of AGM ECs (CD31⁺) that were either successfully infected (GFP⁺) or not infected (GFP⁻) by AdhSOX17-GFP (Fig. 2i). Transcript analysis of ECs with SOX17 overexpression demonstrates significant increases in Sox17 and Notch1 transcripts with significant reduction in Runx1, Gata2, Sox7 and Sox18 transcripts (Fig. 2j). The data altogether suggest that Sox17 negatively regulates haematopoietic fate through repression of Runx1 and Gata2. We also show the known positive regulation of Notch1 by Sox17, and regulation of other SoxF family members, Sox7 and Sox18.

**Figure 3 | SOX17 directly binds Runx1 and Gata2 for repression of haematopoietic fate.** (a) SOX17 chromatin immunoprecipitation (ChIP) qRT–PCR of E11.0 sorted endothelial cells. Letters denote regions with SOX17-binding site consensus sequences upstream of Runx1 and Gata2 promoters, and Left as a positive control. Error bars indicate s.e.m. IgG control set to one for comparisons of fold change, n = 3 litters, embryos pooled, P values as indicated. (b) Electrophoretic mobility shift assay (EMSA) of putative SOX17-binding sites within ChIP sequences designated by letters in a. Each lane represents biotin-labelled duplexed oligonucleotides containing the Left promoter SOX17-binding site (Left_Biot). Addition of rSOX17-Flag produces a specific shift, indicating protein–DNA complex (lane 2), which is competed away by unlabelled Left (Left_s1), while mutant probe does not compete (Left_A_s1). Similar designations are used for putative binding sites (and mutants) in Runx1 and Gata2 sequences. Asterisks denote competitive binding. (c) Bar graph depicts luciferase activity of Gata2 and Runx1 promoters after Sox17 siRNA versus control (scramble). P values as indicated. Error bars represent s.e.m. (d) Immunofluorescence of haematopoietic cell clusters (arrowhead) in E10.5 dorsal aorta (DA) of Sox17f/f and Sox17f/+ mutants (after tamoxifen mediated Cre induction at E9.5). Traced cells labelled in red (Td⁺), Sox17 in grey and RUNX1 in green. DAPI in blue. Scale bar, 10 μm. Single channels in black and white. (e) Gata2 (green) and Sox17 (grey) immunofluorescence of haematopoietic cell clusters in E10.5 DA of Sox17f/+ and Sox17f/f (arrowhead) mutants (Cre induction at E9.5). Traced cells in red (Td⁺), Sox17 in grey and RUNX1 in green. DAPI in blue. Scale bar, 10 μm. Single channels in black and white. (f) SOX17 siRNA knockdown in HUAECs and qRT–PCR analysis. Control represents treatment with lipofectin alone, SOX17 siRNA compared with scrambled (n = 3 experiments, error bars indicate s.e.m.). P values as indicated. (g) Adenoviral-mediated overexpression of hSOX17 in HUAECs and qRT–PCR analyses, P values calculated with respect to Adeno-GFP-infected cells, control represents uninfected cells (n = 3 experiments, error bars indicate s.e.m.).
luciferase reporter assays, which demonstrate derepression of both Runx1 and Gata2 activity after Sox17 short interfering RNA (siRNA) knockdown (Fig. 3c). In vivo loss of Sox17 demonstrates intact haematopoietic clusters with normal localization of RUNX1 and GATA2 expression (Fig. 3d,e). To investigate how Sox17 may regulate Runx1 and Gata2 in mature endothelium in the human system, we conducted in vitro gain- and loss-of-function experiments. Sox17 siRNA inhibition of human umbilical arterial cell lines resulted in significantly elevated RUNX1 transcripts, at similar levels to the control LEF1 (ref. 51), a Sox17 repressive target (Fig. 3f). In addition, genes important in arterial and venous identity are altered with decreased arterial gene transcripts (DLL4) and elevated transcript levels of COUP-TFI, an important determinant of venous fate21 (Fig. 3f). In contrast, when Sox17 is overexpressed after adenoviral infection, RUNX1 and GATA2 transcript levels are significantly decreased (Fig. 3g). Sox17 overexpression also altered levels of DLL4 (increased) and COUP-TFI (decreased) (Fig. 3g). The data suggest a novel role of Sox17 as a repressor of haematopoietic fate, while confirming Sox17 as a pro-arterial fate regulator.

Intersecting roles of Sox17, Runx1 and the Notch pathway. As Sox17 was previously shown to promote arterial identity upstream of the Notch pathway22, we evaluated SOX17 regulation of Notch pathway members in our system. SOX17 ChIP demonstrates enriched occupancy upstream of the Notch1 5'-untranslated region, and of the Notch ligand Dll4 (Fig. 4a). In addition, we also observe occupancy upstream of Coup-TfII, which has not been previously described (Fig. 4a). Similar enrichment of these sites was observed in HUAECs (Supplementary Fig. 4a). We further validated direct binding of SOX17 within the enriched ChIP sites via EMSA, and demonstrated multiple SOX17-binding sites are capable of outcompeting LEF1 controls (Fig. 4b; Supplementary Fig. 4b,c). To understand whether Notch1, a putative downstream target of SOX17, also plays a repressive role in EHT, we evaluated Notch1 loss of function. Similar to Sox17, loss of Notch1 in AGM explants increased the HE ratio, as well as populations of HSPCs (Fig 4e–f; Supplementary Fig. 4d; Supplementary Table 5). We also observed increased HE ratios after AGM explants were exposed the γ-secretase inhibitor DAPT (Supplementary Fig. 4e). However, when BrdU incorporation was evaluated in Notch1 mutant explants, significantly higher levels of incorporation occurred in the haematopoietic compartment (Fig. 4g), suggesting the observed changes may be due to haematopoietic cell proliferation, and not due to an increase in EHT. Annexin-V levels were not notably changed (Supplementary Fig. 4g).

Figure 4 | The role of the Notch pathway in endothelial to haematopoietic fate decisions. (a) SOX17 (Chip) qRT-PCR of E11.0 sorted endothelial cells. Letters denote regions upstream of Notch1, Dll4, and Coup-TFI promoters, and LEF1 as a positive control. Error bars indicate s.e.m.. IgG control set to one for comparisons of fold change, n = 3 litters, embryos pooled. P values as indicated. (b) EMSA of putative SOX17-binding sites within ChIP sequences (designated by letters in a). Each lane represents biotin-labelled duplexed oligonucleotides spanning the LEF1 promoter SOX17-binding site (LEF1_Biot). Addition of rSOX17-Flag produces a specific shift, indicating protein-DNA complex (lane 2), which is competed away by unlabelled LEF1 (LEF1_s1), while mutant probe does not compete (LEF1_s2). Similar designations are used for putative binding sites (and mutants) in Notch1, Dll4 and Coup-TFI sequences. Asterisks denote competition. (c) Schematic of AGM explant analysis depicts in vitro Cre lineage tracing and calculation of hemogenic output (HE ratio); the ratio between per cent labelled (Td+) haematopoietic cells (CD45+ CD31−) to per cent labelled (Td+) endothelial cells (CD31+ CD45−). (d–g) Each data point represents a separate embryo/AGM explant, littersmates are depicted by the same data point colour and shape. Bar indicates group mean. P values calculated on Student’s t-test between groups, significance also validated by two-way analysis of variance (Supplementary Table 5) (d) The HE ratio of Notch1 homozygous (+/+) and heterozygous (+/−) mutant explants. /+ /+ n = 18, f/f n = 21, 6 litters. (e) Percentage of traced Td+ hemogenic endothelial cluster cells, designated as CD31+ CD41−. /+ /+ n = 12, f/f n = 13, 4 litters. (f) Percentage of traced Td+ maturing HSPCs (identified as CD31+ CD117+ Sca1− CD45−) /+ /+ n = 10, f/f n = 6, 3 litters. (g) BrdU+ cells measured after 2-h incubation with BrdU in traced ECs (left) and traced HCs (right) demonstrates a significant increase in HC proliferation, /+ /+ n = 6, f/f n = 10, 3 litters. (h) Schema and bar graph of qRT-PCR analyses of sorted endothelial cells from E11 embryos after in vivo Notch1 ablation at E9.5. Error bars indicate s.e.m. (n = 3 litters, embryos pooled by genotype). LOF, loss of function.
loss of Notch1 (induction at E9.5) demonstrates expected changes in arterial and venous identity genes (EfnB2 and EphB4)23 within sorted ECs (Fig. 4h). No changes in Runx1 transcripts were noted, while expected Hes1 transcripts were decreased (Fig. 4h). There were no observed differences in endothelial labelling or cell number across homozygous Notch1+/−, heterozygous Notch1+/− or control animals (Supplementary Fig. 4h). Interestingly, we also noted expected changes in endothelial morphology24 (Supplementary Fig. 4i).

To understand the role of Notch1 signalling in the context of Sox17 loss, we bred R26RNotch1IC-NEGFp lines25 (+ mNITCD-GFP) that overexpress the Notch1 intracellular domain (NICD) upon Cre activation into our temporal endothelial-specific Sox17 loss-of-function models (Fig. 5a). Increased Notch activation in E11.0 AGM explants was capable of abrogating the observed EHT increase in Sox17 mutants (Fig. 5b) with normal appearing HSPC clusters in vivo after induction of Sox17 loss and NICD overexpression at E9.5 (Fig. 5c). Thus, the conversion to haematopoietic fate in HE requires loss of arterial identity programs in addition to derepression of haematopoietic genes by SOX17. While our data have shown the regulation of Runx1 by SOX17, previous reports suggest that RUNX1 may directly bind and repress Sox17 (ref. 56). To evaluate whether there may be bidirectional requirements, elucidated here, explains previous data where continued or overexpression of Sox17 was noted to prevent haematopoietic conversion in culture26,52. In addition, the reciprocal repression of Sox17 by RUNX1 introduces another unique aspect of fate determination where once endothelial Sox17 levels decrease, Runx1 levels can rapidly rise during the fate switch, and together they function as a classical bistable system; similar to those described in mesodermal progenitors53. Last, the data also demonstrate that the EHT program can be manipulated for increased haematopoietic output, suggesting that hemogenic EC number may not be a fixed entity. If EHT is not restricted to a fixed number of ECs within a hemogenic vascular compartment, but instead occurs as a more global transient stochastic process of developing endothelium, it allows for the possibility of endothelial expansion for HSC production.

Methods

Animal care and use. Animal protocols were conducted in accordance with University of California at San Francisco and Baylor College of Medicine Laboratory Animal Research Committee guidelines. Cdh5(PAC)-CreERT2 (Tg(Cdhs-Cre;cre/ERT2)Rha) mice54, Notch1tm3Rbk and Sox17tm2jimjien flexed lines25,37, and R26RNotch1IC-NEGFp (Gt(Rosa)26Sortm1(Notch1Cre)) lines25 were crossed with Tp1-Venus (Tg(Rbp5*Venus)) mice53,31 were generously provided by RIKEN BioResource Centre. Myosin light chain 2 alpha (Mlc2α)−/− mutant lines were provided by Mary Dickinson (Baylor College of Medicine)60,64. Pregnancies were dated by the presence of a vaginal plug (day 0.5 of gestation). Genomic DNA from isolated tail tips or conceptus yolk sac was genotyped using a MyTaQ Extract PCR Kit (Bioline, BIO21127). Genotype PCR was performed using the primers listed in Supplementary Table 6.

Immunofluorescence and confocal microscopy. E10.5 to E11.5 embryos (in vivo induction with maternal tamoxifen injection at E9.5) were fixed in 2% paraformaldehyde solution overnight and frozen in Tissue-Tek OCT Compound (Sakura Finetek, 4383). Cryosections (20–30 μm) were obtained (Thermo Scientific Micron, HM500). Slides were dried for 1 h at room temperature, washed with PBS (0.5%, Tween or Triton-X-100) and incubated in blocking buffer (PBST, 1% bovine serum albumin and 5% donkey serum) for 1 h. Primary antibodies (for full list of antibodies please see Supplementary Table 7) were incubated at 4°C overnight or room temperature for 6 h in blocking buffer. Slides were washed with PBST and incubated with the secondary antibody for 2 h, washed, stained with 2 μg ml−1 4,6-diamidino-2-phenylindole (DAPI) and mounted in Vectorshield (H-1400) or Vectamount (Vector Laboratories, H-5000). Images were captured on a Leica SPE Confocal Microscope and compiled using the ImageJ and Imaris 7.6 (Bitplane, Belfast, UK) software.

Flow cytometric analyses and cell sorting. Whole embryos or AGMs underwent mechanical dissociation by pipetting to single cell suspension in Hank’s Balanced Salt Solution (HBSS) containing 10−3 M hydrocortisone (Stem Cell Technologies), at E10.5 (and E9.5 for mouse). Placental vascular beds that are not overtly arterial15–18. In vivo induction of the induced endothelium, lineage traced CD31-APC−CD41-FITC−, CD45-FITC−CD11b-FITC−, CD11c-FITC− and R26RtdTdT (Tg(Rbp4*-Venus)#Okn) mice30,31 were generously provided by TP1-Venus (Tg(Rbp5*Venus)), Sox17tm2jimjien mice54 were provided by John Gutierrez, and M2α-cre/ERT2 (Tg(Cdhs-Cre;cre/ERT2)Rha) mice54. Immunofluorescence and confocal microscopy were performed on a FACSDiva 8.0 software (BD Biosciences) and data analysed using the FlowJo v10.0.7 (Tree Star). Gating strategy in Supplementary Figs 1h, 2e, see Supplementary Table 7 for a list of antibodies.

Real-time RT-PCR expression analysis. For in vivo transcriptional characterization of the induced endothelium, lineage traced CD31-APC−CD41-FITC−, CD45-FITC−, CD11b-FITC−, CD11c-FITC−, CD45-FITC−, CD11b-FITC− and R26RtdTdT (Tg(Rbp4*-Venus)#Okn) mice30,31 were generously provided by TP1-Venus (Tg(Rbp5*Venus)), Sox17tm2jimjien mice54 were provided by John Gutierrez, and M2α-cre/ERT2 (Tg(Cdhs-Cre;cre/ERT2)Rha) mice54. Immunofluorescence and confocal microscopy were performed on a FACSDiva 8.0 software (BD Biosciences) and data analysed using the FlowJo v10.0.7 (Tree Star). Gating strategy in Supplementary Figs 1h, 2e, see Supplementary Table 7 for a list of antibodies.

AGM explant culture and in vivo induction. AGMcs from Cdh5(PAC)-CreERT2/ R26RtdTdT/Sox17 and Notch1 floxed embryos were dissected and cultured for 24 h on 40 μm filters at an air liquid interface in 10 μl 1 4-hydroxytamoxifen (Sigma H7904) in myelocyt medium (Stem Cell Technologies) supplemented with 10% FBS (Stem Cell Technologies) and 100 U ml−1 penicillin/streptomycin (Sigma) in vitro induction was achieved by intraperitoneal injection of 0.8 mg of tamoxifen of pregnant dams at E9.5. Tamoxifen powder (MP Biomedical, 156738)

Discussion

An important obstacle in recapitulating HE in culture for in vitro blood production is identification of possible activators and silencers of the hemogenic program. Here we demonstrate important altering requirements for Sox17 and Notch1, which highlights the refinements needed for translational models recapitulating EHT. Previous studies have identified Runx1 (ref. 27), Gata2 (ref. 28), Notch1 (refs 24,58) and Sox17 (refs 25,26) as critical for EHT. However, dissecting the contributions of these pathways to vascular development versus the process of haematopoietic emergence from the endothelium has not been previously reported. Notch1 and Sox17 both have important roles in arterial specification22,23,43. As the major vessels that harbour HE are arterial sites9,13, it may be that arterial identity is a prerequisite to hemogenic endothelial activity. However, hemogenic activity also occurs in yolk sac and placental vascular beds that are not overtly arterial15–18. In addition, recent evidence in human ESC cultures suggest that while hemogenic ECs incorporate into arterial vascular walls, they have differential surface marker expression profiles than arterial cells29. There is also evidence that arterial identity can be uncoupled from hemogenic capacity28,30,61. So it may be that hemogenic endothelial specification requires the same pathways mobilized in the acquisition of arterial identity, but not arterial identity per se. However, for the direct transition to haematopoietic fate, the expression levels of arterial/hemogenic specifiers need to be reduced. The complex temporal

requirements, elucidated here, explains previous data where continued or overexpression of Sox17 was noted to prevent haematopoietic conversion in culture26,52. In addition, the reciprocal repression of Sox17 by RUNX1 introduces another unique aspect of fate determination where once endothelial Sox17 levels decrease, Runx1 levels can rapidly rise during the fate switch, and together they function as a classical bistable system; similar to those described in mesodermal progenitors53. Last, the data also demonstrate that the EHT program can be manipulated for increased haematopoietic output, suggesting that hemogenic EC number may not be a fixed entity. If EHT is not restricted to a fixed number of ECs within a hemogenic vascular compartment, but instead occurs as a more global transient stochastic process of developing endothelium, it allows for the possibility of endothelial expansion for HSC production.
was dissolved in a sunflower seed oil/ethanol (10:1) mixture at 10 mg ml\(^{-1}\) (ref. 49). DAPT \(-\)secretase inhibitor (Sigma, D5942) was prepared in dimethyl-sulphoxide and added directly to explant culture medium at final concentrations of 25, 50, 100 or 200 \(\mu\)M. For overexpression studies, AGMs were incubated with 8\(\times\)10\(^7\) adenoviral particles per millilitre at 37\(^\circ\)C with agitation for 1 h before explant culture 49. Adeno-CMV-hSox17-GFP (AdhSox17-GFP) was produced by Vector Biolabs (ADV-224019, RefSeq: BC140307).

BrdU. AGM explants were incubated for 2 h with BrdU (10 \(\mu\)M), disaggregated and stained for extracellular markers CD45-percp and CD31-APC for 30 min. Cells were then fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, 554714) according to the manufacturer instructions. Cell pellet was washed and incubated in DNase I (300 \(\mu\)g ml\(^{-1}\)) for 1 h at 37\(^\circ\)C, stained with DAPI and anti-BrdU conjugated with FITC for 30 min, and analysed by flow cytometry.

Figure 5 | Parsing endothelial and haematopoietic fates during EHT. (a) Schematic depicting Sox17 or Notch1 loss of function (LOF) and strategy for evaluating Notch overexpression (mNICD-GFP) in Sox17 mutants. NICD, Notch1 intracellular domain. (b) HE ratios of E11 AGM explants in Sox17 mutants with and without Notch overexpression (+ N1). Centre lines represent median values, box represents 25th–75th percentiles and bars represent minimum and maximum values. f/ (+ N1) \(n = 7\), f/ (+ N1) \(n = 3\), f/ (– N1) \(n = 5\), f/ (+ N1) \(n = 8\), 3 litters. \(P\) values calculated on Student’s t-test between groups, significance also validated by two-way analysis of variance (Supplementary Table 2) (c) Immunofluorescence of a representative haematopoietic cluster in a E10.5 Sox17\((+/−)\) AGM after in vivo induction of Cre and NICD at E9.5. SOX17 in grey, traced ECs (Tд\(^+\)) in red and RUNX1\(^+\) in green. DAPI in blue. Scale bar, 10 \(\mu\)m. (d) RUNX1 chromatin immunoprecipitation (ChIP) PCR of E11.0 sorted endothelial cells. Letters denote evaluated regions containing RUNX1-binding site consensus sequences upstream of the Sox17 promoter. Error bars indicate s.e.m. IgG control set to one for comparisons of fold change, \(n = 3\) litters, embryos pooled, \(P\) values as indicated. (e) Adenoviral-mediated overexpression of hRUNX1 in HUAECs and qRT–PCR analyses, \(P\) values calculated with respect to Adeno-GFP-infected cells, control represents uninfected cells (\(n = 3\) experiments, error bars indicate s.e.m.). (f) Schematic depicting the cell fate switch from endothelial to haematopoietic fate, and the governing regulatory pathways of EHT. Sox17 inhibition of Runx1 and Gata2 maintains endothelial fate. Loss of Sox17 inhibition in the context of decreased Notch activity promotes haematopoietic fate conversion.
**Annexin-V.** AGM explants were dissociated, washed in PBS and resuspended in buffer (10 mM HEPES, 0.9% NaCl, 2.5 mM CaCl₂ and 0.1% bovine serum albumin) containing Annexin-V-Biocytin (BD Biosciences). Cells were incubated at room temperature in the dark for 15 min followed by the addition of buffer containing DAPI, and analysed by flow cytometry.

**siRNA.** Primary human umbilical arterial ECs (HUAEAC) (VEC Technologies) were cultured in MCDR-131 Complete medium (VEC Technologies). Sox17 Silencer Select siRNA (Ambion, s4626-8), scramble negative control siRNA (non-targeted control) and water (control) were administered using Lipofectamine 3000 (Life Technologies) according to manufacturer’s recommendations. After 48 h of culture, cells were lysed and luciferase activity assessed using the Dual-Luciferase Reporter Assay System reagents (Promega) in a GloMax 96 Microplate Luminometer with dual injectors. In technical triplicate, relative luciferase activity was calculated by dividing Firefly readings by Renilla readings for each well and then normalized according to baseline values for each treatment condition after transfection of pGL4.30-CGL4A without a fragment added.

**Statistical analyses.** Student’s t-test, one- and two-way analysis of variance were calculated as performed in all experiments where n ≥ 3 unless otherwise noted. Mean and standard error were calculated and graphed using GraphPad Prism 6 software. All statistical measurements are listed in Supplementary Tables 2 and 5.

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Author contributions
C.O.L. conducted the majority of the experiments, with support from J.S.H. as well as the data analysis, all under the guidance of A.C.Z. J.S.H. with supervision by C.O.L. and A.C.Z. optimized most of the protocols and technical approaches for colony assays, cell culture studies, EMSA and luciferase assays; with cloning strategy and troubleshooting support from C.E.S., reagents and EMSA troubleshooting help from H. B.-P., M.E.D. and J.D.W. F.L.B. provided technical expertise with immunofluorescence and scanning electron microscopy. J.P.Z. initiated pilot experiments with Notch loss of function and also provided immunofluorescence expertise, while K.M.C. generated critical reagents for pilot Sox17 viral experiments. A.M.R. and J.D.W. obtained and evaluated circulation deﬁcient mutant lines. H.Y. provided technical mouse support and the preliminary analysis of Sox17 and Notch1 cell culture expression. J.D.W. and M.E.D. helped with reagents and advice. C.O.L. and A.C.Z. designed the project. C.O.L., J.S.H. and A.C.Z. wrote the manuscript. All authors critically read and contributed to the manuscript.

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