Notch signal strength controls cell fate in the haemogenic endothelium

Leonor Gama-Norton1,*, Eva Ferrando1,*, Cristina Ruiz-Herguido1,*, Zhenyi Liu2, Jordi Guiu1, Abul B.M.M.K. Islam3,4, Sung-Uk Lee5, Minhong Yan6, Cynthia J. Guidos7, Nuria López-Bigas3,8, Takahiro Maeda5, Lluis Espinosa1, Raphael Kopan2 & Anna Bigas1

Acquisition of the arterial and haemogenic endothelium fates concurrently occur in the aorta–gonad–mesonephros (AGM) region prior to haematopoietic stem cell (HSC) generation. The arterial programme depends on Dll4 and the haemogenic endothelium/HSC on Jag1-mediated Notch1 signalling. How Notch1 distinguishes and executes these different programmes in response to particular ligands is poorly understood. By using two Notch1 activation trap mouse models with different sensitivity, here we show that arterial endothelial cells and HSCs originate from distinct precursors, characterized by different Notch1 signal strengths. Microarray analysis on AGM subpopulations demonstrates that the Jag1 ligand stimulates low Notch strength, inhibits the endothelial programme and is permissive for HSC specification. In the absence of Jag1, endothelial cells experience high Dll4-induced Notch activity and select the endothelial programme, thus precluding HSC formation. Interference with the Dll4 signal by ligand-specific blocking antibodies is sufficient to inhibit the endothelial programme and favour specification of the haematopoietic lineage.
Haematopoietic stem cells (HSCs) are generated during embryonic life in the aorta–gonad–mesonephros (AGM) region. This process requires gain of haematopoietic competence from cells displaying endothelial traits located in the embryonic aorta (also known as endothelial-to-haematopoietic transition (EHT)). Recently, it has been demonstrated that the first molecular event in the EHT process requires the silencing of the endothelial programme; however, the molecular signals governing the sequence of events to obtain a functional HSC are mainly unknown.

Notch1 signalling is indispensable for the specification of the arterial programme and the generation of HSCs. Ligand specificity for each process has been suggested since deletion of Delta-like 4 (Dll4) results in strong arterial defects, while Jagged1 (Jag1) deletion impairs definitive haematopoiesis. The main structural difference between both types of ligands resides in the number of epidermal growth factor (EGF)-like repeats (6–8 for Delta and 16 for Jagged) and in the presence of C-rich domain in Jag1; however, ligand-mediated cleavage is thought to be a ‘no molecular’ process in relation to the identity of the ligand involved. Glycosylation of Notch by the fringe family of glycosyl-transferases was found to favour the association of Notch to Delta instead of Jagged ligands, likely affecting Notch signal strength.

We have recently developed two mouse lines that trace cells that activate the Notch pathway and their descendants. Importantly, N1IP::CreLO is a low-sensitivity line that only traps cells experiencing high levels of Notch1 activation, whereas N1IP::CreHI is high sensitive and traps cells experiencing both low and high levels of Notch activation. HI and LO designations reflect the differential sensitivity of these reporters defined here as the number of Notch intracellular domain (NICD) molecules released. We here demonstrate that, whereas N1IP::CreHI labels both haematopoietic and arterial cells, N1IP::CreLO specifically labels the arterial population, indicating that arterial and haematopoietic cells originate from different Notch-traceable populations. In addition, Jag1 restricts Notch activation in the haemogenic endothelium, which results in reduced expression of the endothelial gene programme and increased haematopoietic-specific transcription. Together, these results indicate that Jag1 is required to maintain the low Notch signal that is required for haematopoietic specification, whereas Dll4 secures the high Notch activity and the success of the arterial programme.

To further investigate this possibility, we pursue a strategy to trap cell lineages experiencing low levels of Notch activity. We found that removal of the tag from the Cre recombinase improved Cre activity, and consequently labelling efficiency (we refer to this transgene as N1IP::CreHI). In contrast to that observed in the haematopoietic lineages of the N1IP::CreLO mice, we found a consistent YFP+ staining in the different haematopoietic organs and cell lineages of the N1IP::CreHI,ROSA26YFP mice (Fig. 1b,c). Comparative analysis of E10.5 N1IP::CreHI and N1IP::CreLO embryos using whole-mount immunostaining demonstrated that both lines contained YFP+ cells in the aortic endothelium (Supplementary Fig. 1), but only the N1IP::CreHI haematopoietic cluster cells (Kit+) were YFP+ (Fig. 1d). In addition, YFP+ cells isolated from the fetal liver or bone marrow of the N1IP::CreHI,ROSA26YFP reconstituted the haematopoietic system of lethally irradiated hosts (Fig. 1e,f).

Jag1 and Dll4 distribution in the embryonic aortic cells. Both Dll4 and Jag1 ligands were strongly and homogeneously distributed along the rostral-caudal and dorsoventral axis of the aortic endothelium by E10.5 (Fig. 2a and Supplementary Fig. 2a). Initially, Kit+ cluster structures showed variable patterns of ligand distribution, most frequently being the positive signal for Dll4 (Supplementary Fig. 2b). However, the majority of sorted Kit+ endothelial cells (89%) co-expressed the Jag1 and Dll4 ligands, and only a few cells expressed either Jag1 (3.8%) or Dll4 (4.6%) or were negative for both (2.5%; Supplementary Fig. 2d).

At the transcriptional level, lower levels of ligand mRNA were observed in the haematopoietic lineages of the AGM region. This process requires gain of haematopoietic competence from cells displaying endothelial traits located in the embryonic aorta (also known as endothelial-to-haematopoietic transition (EHT)). Recently, it has been demonstrated that the first molecular event in the EHT process requires the silencing of the endothelial programme; however, the molecular signals governing the sequence of events to obtain a functional HSC are mainly unknown.

Notch1 signalling is indispensable for the specification of the arterial programme and the generation of HSCs. Ligand specificity for each process has been suggested since deletion of Delta-like 4 (Dll4) results in strong arterial defects, while Jagged1 (Jag1) deletion impairs definitive haematopoiesis. The main structural difference between both types of ligands resides in the number of epidermal growth factor (EGF)-like repeats (6–8 for Delta and 16 for Jagged) and in the presence of C-rich domain in Jag1; however, ligand-mediated cleavage is thought to be a ‘no molecular’ process in relation to the identity of the ligand involved. Glycosylation of Notch by the fringe family of glycosyl-transferases was found to favour the association of Notch to Delta instead of Jagged ligands, likely affecting Notch signal strength.

We have recently developed two mouse lines that trace cells that activate the Notch pathway and their descendants. Importantly, N1IP::CreLO is a low-sensitivity line that only traps cells experiencing high levels of Notch1 activation, whereas N1IP::CreHI is high sensitive and traps cells experiencing both low and high levels of Notch activation. HI and LO designations reflect the differential sensitivity of these reporters defined here as the number of Notch intracellular domain (NICD) molecules released. We here demonstrate that, whereas N1IP::CreHI labels both haematopoietic and arterial cells, N1IP::CreLO specifically labels the arterial population, indicating that arterial and haematopoietic cells originate from different Notch-traceable populations. In addition, Jag1 restricts Notch activation in the haemogenic endothelium, which results in reduced expression of the endothelial gene programme and increased haematopoietic-specific transcription. Together, these results indicate that Jag1 is required to maintain the low Notch signal that is required for haematopoietic specification, whereas Dll4 secures the high Notch activity and the success of the arterial programme.

Jag1 inhibits the endothelial programme in AGM cells. The ligand distribution observed in the aortic endothelium of the AGM suggests the existence of a functional interplay between Jag1 and Dll4. Thus, we aimed to investigate the relative contribution of each ligand in the process of haematopoietic and endothelial production from AGM endothelial progenitor cells. With this purpose, we dissected E10.5 and E11.5 AGM tissues and sorted the cells based on CD31 positivity (excluding Ter119+) and CD45+ cells. We then incubated the CD31+ AGM cells for 7 days in the presence of OP9 stroma overexpressing either Jag1 (OP9-Jag1) or Dll4 (OP9-Dll4) ligands (Supplementary Fig. 3c) and analysed the cultures for the presence of cells expressing the haematopoietic markers Kit and CD45, as a measure of haematopoietic cell production. Generation of haematopoietic cells (CD45+) and haematopoietic progenitors (Kit+CD45+) from E10.5 and E11.5 AGM cells was increased both in percentage (eightfold increase; Supplementary Fig. 3d) and in total cell number (Fig. 2c) when comparing OP9-Jag1 with OP9-Dll4 cultures. To investigate whether Jag1 and Dll4 affect the haematopoietic cell production by regulating the EHT process, we performed a similar experiment; however, in
this case the sorted CD31⁺CD45⁻ E11.5 AGM cells were incubated on OP9-Jag1 or OP9-Dll4 for 2 h, resorted according to Kit expression and processed for transcriptome analysis using Affymetrix arrays. Of note that the final analysis included four populations generated in the experiment (Kit⁻ and Kit⁺ after incubation with Jag1 (Kit⁻ J and Kit⁺ J) or with Dll4 (Kit⁻ D and Kit⁺ D)) and two controls corresponding to the untreated populations (CD31⁺Kit⁺CD45⁻ (Kit⁻) and CD31⁺Kit⁺CD45⁻ (Kit⁺)) that were directly sorted from freshly dissected E11.5 AGMs (Fig. 2d). Independent triplicates of each sample were obtained and processed using the Affymetrix gene chips. Bioinformatic analysis detected global changes in gene expression on ligand incubation, relative to the basal transcriptome of the Kit⁺ and Kit⁻ cell populations. Principal component analysis

Figure 1 | Haematopoietic and arterial specification requires different levels of Notch1 activity. (a) Schematic representation of Notch activation history mouse reporters by replacing the intracellular domain of mouse Notch1 with low sensitivity (N1IP::CreLO) and high sensitivity (N1IP::CreHI) Cre-recombinase. Reporter activation of N1IP::CreLO requires a high threshold of Notch activity, while N1IP::CreHI is induced in response to low or high Notch activity. (b) Flow cytometry analysis of peripheral blood of adult mice. Cells were stained with Lineage (lin) markers (CD3, B220, Gr1, Mac1 and Ter119) gated on lin⁺ cells. Numbers indicate the percentage of YFP⁺ cells. (c) Graph represents the percentage of YFP⁺ cells within haematopoietic cell types in the bone marrow (BM), spleen and thymus of N1IP::CreLO (grey bars) and N1IP::CreHI (blue bars) as detected using flow cytometry. (d) Representative confocal images of three-dimensional whole-mount immunostaining in N1IP::CreHI and N1IP::CreLO embryos (E10.5) detecting YFP (green), c-Kit (cyan) and CD31 (red). General view of the dorsal aorta (left panel) and details of haematopoietic cluster (right panels). White arrows indicate cluster structures. D, dorsal; DA, dorsal aorta, HC, haematopoietic cluster; V, ventral. Scale bars, 100 μm for DA, 25 μm for HC in N1IP::CreHI and 50 μm in N1IP::CreLO. See also Supplementary Fig. 1. (e,f) Graphs show the percentage of reconstituted cells in animals transplanted with YFP⁺ and YFP⁻ fractions of E13-14 fetal liver and BM at 4-month post-transplantation (e). Representative dot plots from analysis (f). Donor CD45.2 N1IP::CreHI cell fractions together with 500,000 supporting CD45.1 spleen cells were transplanted into CD45.1/CD45.2 chimeras.
Figure 2 | Expression of Jag1 and Dll4 ligands in the embryonic dorsal aorta. (a) Representative confocal images of E10.5 embryo transverse section with CD31 (red) and Jag1 (green, left) and Dll4 (green, right). Details of ventral part (lower panels) corresponding to boxed areas. Scale bars, 25 μm. Nuclear staining with 4,6-diamidino-2-phenylindole is shown (D, dorsal; V, ventral). (b) Experimental design to test the effects of OP9-Jag1 and OP9-Dll4 on purified CD31+/CD45- Ter119- AGM cells after 7 days of culture. (c) Quantification of haematopoietic lineage generated from CD31+/CD45- Ter119- AGM cells on culture on OP9-Jag1 or OP9-Dll4. Bars represent the total number of cells on 7-day culture; n=4 or more samples of at least two independent experiments. s.e.m. is represented. P value for t-test is indicated. (d) Schematic representation for purification of E11.5 AGM CD31+/Kit- CD45- Ter119- and CD31+/Kit+ CD45- Ter119- (Kit- and Kit+). In parallel, CD31+/CD45+ Ter119- cells were incubated for 2 h in OP9-Jag1 or OP9-Dll4. Cells were resorted on the basis of Kit expression (Kit- J and Kit+ J or Kit- D and Kit+ D). (e) Principal component analysis (PCA) of global gene expression profiles of samples included in the study. Each dot of the same colour represents arrays from replicates of the same sample. Dotted lines arbitrarily reunite replicates of a specific condition. (f) Unsupervised hierarchy clustering of transcriptional profiles from selected cell populations. (g) Venn diagram displaying the number of genes differentially expressed in cluster-containing versus endothelial populations (Kit+ J, Kit– J, Kit+ D and Kit– D). Fold change expression levels from MicroArray analysis of 23 angiogenic-related genes are listed. Green-colour grade represents the range of fold change (FC) values on Kit+ J or Kit– J populations compared with Kit– D cells. All FC values represent statistically significant differences on gene expression (P value-adjusted<0.05). Grey cells represent genes in which FC is not statistically significant. (h) Biological process enrichment analysis of genes differentially expressed in Kit– J, Kit+ J and Kit– D comparisons. Only selected GO terms are presented, and all significant terms are given in Supplementary Tables 2 and 3. (e–h) Analysis performed on three independent experiments; one Kit– J sample was excluded for technical reasons.
Kit with downregulation of several endothelial-related genes on the instead, exposure of the Kit⁺ (Fig. 2h and Supplementary Tables 2 and 3).

specification and maintenance of endothelial cell identity analysis of the data indicated that genes downregulated in the (Fig. 2g and GSE59344). Accordingly, Gene Ontology (GO) clustered together with the Kit—cells exposed to Jag1 (Kit—; Fig. 2f). Together, these analyses strongly suggest that Jag1 imposes a haematopoietic-like signature to the Kit— population, before the acquisition of detectable amounts of the Kit haematopoietic marker.

Next, we compared the expression profiles of the endothelial population exposed to Jag1 (Kit—), the haematopoietic precursors (Kit+) and the endothelial population (Kit—). Unexpectedly, we did not identify a shared haematopoietic signature in Kit+ and Kit— cells exposed to Jag1 (Kit—). Instead, exposure of the Kit— population to Jag1 was associated with downregulation of several endothelial-related genes on the Kit— population such as Flk1, Nrp1 and Cdhl2 among others (Fig. 2g and GSE59344). Accordingly, Gene Ontology (GO) analysis of the data indicated that genes downregulated in the Kit— cells by exposure to Jag1 fall into categories related to specification and maintenance of endothelial cell identity (including angiogenesis, regulation of cell migration and cell adhesion), or are associated with the Notch and Wnt pathways (Fig. 2h and Supplementary Tables 2 and 3).

To confirm that Jag1 imposes the downregulation of a pre-existing endothelial signature in the Kit— cells, we performed reverse transcriptase–PCR (RT–PCR) analysis of genes identified in our GO analysis (n = 39) from independent pools of cells sorted and processed as detailed above. The 95% of all tested genes were found to be downregulated in the different pools of Kit+ cells when compared with Kit— AGM cells, as expected. Importantly, in two independent experiments 62% of all tested genes showed a reduction in their mRNA levels in the Kit— compared with the original endothelial population (Kit—; Fig. 3a). Comparable results were obtained from sorted endothelial CD31⁺ Kit— CD45— cells grown on the OP9-Jag1 for 2 or 5 h (Supplementary Fig. 4a,b, respectively). These results exclude the possibility that the observed transcriptional changes are originated from Kit+ cells that lost the Kit marker during Jag1 incubation. In addition, we detected a general decrease in flk1 levels in the entire endothelial population after 5-h incubation on Jag1 as determined using flow cytometry (Supplementary Fig. 4c), suggesting that the effects of ectopic Jag1 are not restricted to a minor haemogenic endothelial cell population.

Altogether, the results strongly suggest that Jag1 counteracts a transcriptional endothelial programme that is likely imposed by Dll4 in the Kit— cell population.

**Jag1 enhanced haematopoietic gene expression.** The EHT process involves the acquisition of a haematopoietic transcriptional programme that was not detected as differentially regulated in our microarray analysis. Thus, we specifically determined the expression levels of particular haematopoietic genes in the Kit— and Kit+ AGM cell populations, and in comparison with the Jag1-incubated Kit— cells (Fig. 3b). We detected a remarkable upregulation of Kit, CD41, Runx1 and Gata2 expression in E10.5 or E11.5 Kit— AGM cells exposed to Jag1 (Fig. 3b and Supplementary Fig. 4b), expected from cells undergoing haematopoietic commitment. Taken together, our results suggest that Jag1 promotes the EHT process through both the downregulation of the endothelial signature and the activation of the haematopoietic-specific transcription.

**Jag1 is required to inhibit the endothelial signature in EHT.** Jag1+/— embryos die around E11 with impaired definitive haematopoietic development'. A detailed analysis of the subpopulations present in the Jag1+/— AGM at E10.5 identified an increase in the number of endothelial-like cells (CD31⁺ Kit— CD45—), but also CD31⁺ Kit⁺ CD45— haematopoietic precursors, associated with a decline in the number of CD45+ cells (Fig. 4a). Immunofluorescence analysis of AGM sections confirmed the presence of Kit+ cells in the Jag1+/— embryos; however, both the total number and the morphology of these haematopoietic-like clusters were severely affected (Fig. 4b–d). Specifically, Jag1-deficient Kit+ cells were distributed as single cells along the aortic endothelium or aggregate in clusters that do not evaginate into the aortic lumen, thus invading the mesenchymal tissue underneath the endothelial layer instead (Fig. 4b). To characterize the haematopoietic defects in Jag1+/— embryos at the molecular level, we sorted E10.5 AGM CD31⁺ Kit— CD45— (Kit—) and CD31⁺ Kit⁺ CD45— (Kit+) cells from Jag1+/— and Jag1+/— embryos, obtained the total RNA from each population and compared their gene transcriptional patterns using qRT–PCR. Our results showed that Jag1+/— embryos have a prominent upregulation of the endothelial-related signature both in the Kit+ and Kit— populations compared with Jag1+/+ (Fig. 4e, Supplementary Fig. 5a). More importantly, comparison of Kit+ and Kit— populations demonstrated that Jag1+/— cells maintain a consistent endothelial signature during the Kit— to Kit+ transition (Fig. 4f). Further evidence for this endothelialization of the Kit+ cell population in the Jag1+/— embryos is provided by the observed decrease in the expression of C-Kit and Runx1 when compared with Jag1+/+ littermates (Supplementary Fig. 5b).

Because HSCs are known to reside inside the Kit+ CD45+ population, which is still present in the Jag1+/— embryos (not shown), we functionally measured the frequency of haematopoietic progenitors (CFUs-S[F]-1) and HSCs in these mutant embryos compared with Jag1+/+ littermates. We established E10 AGM explant cultures from Jag1+/+, Jag1+/— and Jag1+/— embryos and, after 72 h, cells were injected into lethally irradiated mice. On day 11 post transplantation, we found that Jag1+/— AGM-injected mice contained a very low number of colonies in the spleen (colony-forming units in spleen, CFU-S[F]-1) that was comparable to the non-injected/irradiated controls. The number of these colonies was increased up to eightfold in the animals injected with wild-type AGM explant cells (Fig. 4g). To determine the HSC activity, β-actin-GFP/Jag1+/+, β-actin-GFP/Jag1+/— or β-actin-GFP/Jag1+/— AGM explants (from E10 to 10.5 embryos) and transplanted (together with 5 x 10⁷ spleen supporting cells) into lethally irradiated recipients. Donor engraftment was analysed by the presence of GFP at 1 and 4 months after transplantation. We found that 14 out of 23 Jag1+/+ or +/— embryos contained long-term multilineage reconstitution activity (engraftment >1% GFP+ cells), whereas we did not detect any HSC activity in the animals transplanted with the Jag1+/— AGMs (Fig. 4h).
the embryonic haematopoietic precursors, we found that Hey2, EfnB2 and Cend1 (well-known targets of Notch signalling) were markedly downregulated in the Kit− cells exposed to the Jag1-expressing stroma (Fig. 5a,b), while nonsignificant changes in the transcription of these genes were detected in Kit+ cells exposed to Dll4 compared with the original Kit− population (P > 0.05). Because most Kit− cells expressed both Dll4 and Jag1, we speculated that Jag1 presented by OP9 cells produces a low Notch signal while outcompeting Dll4-mediated signalling originated from adjacent Kit− cells. In agreement with this possibility, the expression levels of several Notch-target genes were significantly upregulated in the aortic endothelium of Jag1−/− embryos (Fig. 5c) and we detected high Notch activity in multiple areas around the AGM aorta of the Jag1−/− embryos, as determined using immunofluorescence with the antibody recognizing the active form of Notch (ICN1; Fig. 5d). To further investigate whether Jag1 negatively regulates the endothelial programme by competing with Dll4-mediated Notch1 signalling, we evaluated the effect of anti-Dll4 and anti-Jag1-blocking antibodies on the in vitro generation of haematopoietic cells from E10.5 AGMs CD31+/CD45− Ter119− AGM cells incubated 2 h on OP9-Jag1. Quantification was performed in Kit−, Kit−/J and Kit−J. The bars represent the average expression level of four to six replicates from two independent experiments, normalized to Kit− expression. Average fold change (± s.d.) is represented. Student’s t-test for comparisons of Kit+/, Kit− and Kit− J populations with Kit− population was performed (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001). (b) Expression of haematopoietic genes on E10.5 and E11.5 Kit− and Kit− J populations (2 h on OP9-Jag1). The bars represent the average (± s.e.m.) expression level of six to nine replicates from three or four independent experiments, normalized to Kit− expression. Statistical significance was assessed by Student’s t-test (*P ≤ 0.05; **P ≤ 0.001 when significant). nd, not detected.
for generating HSC in the embryo. The N1HPCre mice data indicate that endothelial and haematopoietic specification associates with a differential Notch signal strength, and we propose that regulation of the correct Notch1 signal strength during embryonic development is achieved by the competition betweenDll4 and Jag1 ligands.

Figure 4 | Jag1 mutants fail to generate functional HSCs and retain endothelial signature. (a) Representative analysis of population distribution in Jag1+/+, Jag1+/− and Jag1−/− animals. Endothelial population (CD31+Kit−CD45−) in red (frame and graph bars), cluster-containing population (CD31+Kit−CD45+) in orange (frame and graph bars) and mature haematopoietic population (CD31+Kit−CD45+) in blue (frame and graph bars). Bars represent the average population distribution in Jag1+/+ (n = 6), Jag1+/− (n = 10) and Jag1−/− (n = 6) embryos, normalized to Jag1+/+. * and ** denote statistically significant differences on gene expression (P < 0.05 and **P < 0.01 when significant). (b) Representative confocal images of transversal sections of E10.5 AGM from Jag1+/+ and Jag1−/− stained for CD31 (red) and Kit (green). Nuclear staining with DAPI. White arrows point to cluster-like structures. Scale bar, 25 µm. Asterisks indicate autofluorescent circulating cells. D, dorsal; V, ventral. (c,d) Quantification of Kit+ cells (c) and Kit+ clusters (group of at least four positive cells; d) per 100 µm of AGM from Jag1+/+, Jag1+/− and Jag1−/− embryos. Bars represent the average ± s.e.m. of cells per embryo (n = 4). Student’s t-test was performed (**P < 0.01). (e,f) FC in the expression levels of the indicated genes as detected using qRT–PCR from E10.5 Jag1−/− compared with Jag1+/+ and Jag1−/−. (e) or in Kit+ cells (f) compared with Kit− populations in the Jag1+/+ and Jag1−/−. FC in the expression levels of the indicated genes as detected using qRT–PCR from E10.5 Jag1−/− compared with Jag1+/+ and Jag1−/−. (f) Two independent pooled samples for each genotype were analysed. Colour grades reflect the FC values of each gene expression. All FC values represent statistically significant differences on gene expression (P value < 0.05). Genes that do not show a statistically significant alteration in pairwise comparison are listed in grey. See also Supplementary Fig. 5a. (g) CFU-S1 from E10.5 Jag1+/+, Jag1−/− or Irrad ctrl. Irrad ctrl: irradiated/non-injected control. Bars represent the mean ± s.e.m. of colonies per tissue. Significance is assessed by Student’s t-test (**P < 0.01; not assigned if not significant). (h) Percentage of reconstitution at 4 months post transplantation. Each dot represents a single animal. n represents the number of transplanted mice per genotype.
Discussion

Understanding the complex regulatory signals that govern the generation of HSC is clinically relevant for regenerative medicine applications.

In the present study we particularly focused on the regulation and contribution of the Notch pathway during HSC specification in the mouse embryo. We had previously demonstrated that Notch1 activation through the Jag1 ligand was required for the expression of the haematopoietic programme\(^\text{21}\). However, analysis of the Notch1 reporter N1IP::Cre\(^{\text{LO}}\) (ref. 17) indicated that the haematopoietic lineage was not labelled, and therefore we deduced that the aortic endothelial cells receiving high levels of...
Figure 6 | Blockage of Dll4 promotes haematopoietic commitment. (a) Schematic of experimental design. E10.5 AGM CD31+ CD45− Ter119− cells were sorted and incubated in the medium with anti-Dll4- or anti-Jag1-blocking antibodies or irrelevant Ig for 7 days. (b) Graphs represent the average FC of total cell number obtained after culturing with anti-Dll4 or anti-Jag1 compared with each Ig control ± s.e.m. from three independent experiments. Student’s t-test was used to assess the significance (**P<0.01; not assigned if not significant). (c) Relative number of CFC haematopoietic progenitors obtained in b. Average results from three independent experiments ± s.d. determination. Student’s t-test was used to assess the significance (**P<0.01; **P<0.001). (d) E11.5 CD31+ Kit+ CD45− Ter119− cells were sorted and incubated for 5h on the medium supplemented with anti-Dll4-blocking antibody or irrelevant Ig control. Cells were injected in 3-Gy-irradiated SCID-Beige mice or resorted for mRNA extraction and qRT–PCR analyses. (e) Quantification of spleen colonies (CFU-S11). Bars represent the average of six replicates from three independent experiments (coloured dots) ± s.e.m. Student’s t-test was used to assess significance. (f) FC in expression levels of a panel of angiogenic- and haematopoietic-related genes using qRT–PCR. Colour grades reflect the fold change values of each gene expression (normalization to expression levels in control conditions). All coloured FC values represent statistically significant differences on gene expression (P≤0.05, using Student’s t-test). Grey is shown for non-statistical significant differences.

Notch signal did not produce HSC. Our newly engineered N1IP::CreHI reporter labelled cells in both the arterial and haematopoietic systems, suggesting that in the dorsal aorta endothelial cells with low Notch1 activity (labelled in the N1IP::CreHI but not in the N1IP::CreLO) sort out from the ones with high Notch activity (labelled in both lines) to become haematopoietic precursors. Of note that the N1IP::CreHI activity (as reported by YFP positivity) is detected earlier in development and more intense in the dorsal aorta, and subsequently only the N1IP::CreHI displays positive cells in the haematopoietic clusters. Our explanation is that the haemogenic endothelium differs from endothelial cells in the magnitude of Notch activity, which will never reach the levels required to induce the N1IP::CreLO reporter. Alternatively, there is a common precursor that is specified to the arterial or haemogenic lineage during the period in between N1IP::CreHI and N1IP::CreLO induction (as detected by YFP).

Analysis of N1IP::CreHI HSC indicated that YFP+ cells contain bona fide HSCs in fetal liver and bone marrow; however, some long-term repopulating activity is also found in the YFP− population that may have escaped recombination. However, YFP− cells do not show secondary engraftment, strongly suggesting that it correspond to a qualitatively different type of HSCs.

It has been shown that Notch ligands can deliver different Notch signal strengths,20,22. Jag1 and Dll4 ligands are expressed in the embryonic aorta and both can activate the Notch1 receptor. Deletion of either ligand results in distinct endothelial/haematopoietic phenotypes, highlighting a functional difference between the ligands: Dll4 deletion results in an arterialization defect10,12,13, whereas Jag1 deletion specifically affects the establishment of definitive haematopoiesis (ref. 7 and this work).

We combine all available data with our analysis to propose how the balance between Notch1-Dll4 and Notch1-Jag1 signalling guarantees the correct establishment of the endothelial and haematopoietic cell fates in the AGM. Our data indicate that the lack of Jag1 ligand results in higher Notch activity in the aortic endothelium of the AGM, which enhanced endothelial fates at the expense of HSC formation. This observation supports existing models in which Dll4-Notch1 signals maintain the endothelial/
arterial programme. We hypothesize that precursor haemogenic cells responding to Jag1 attenuate/inhibit the strong DLL4-Notch1 signal, replacing it with a productive low Notch1 signal necessary and sufficient to activate haematopoietic genes, such as Gata2 (ref. 21), but not to activate the endothelial programme.

In addition, we found that Jag1 induced the transcriptional activation of several microRNAs (GSE59934) that may contribute to the active repression of the endothelial programme, a possibility that we are currently investigating. The requirement of a Jag1-dependent productive Notch signal for haematopoietic specification is further supported by the fact that treatment of AGM cells with anti-Jag1-blocking antibody prevents production of haematopoietic progeny similar to γ-secretase inhibition10. Finally, although our results obtained in the OP9-Jag1 co-culture experiments are compatible with Jag1 delivering a proliferation signal on the kit+ population, the fact that culture conditions are optimized for haematopoietic cell growth precluded to obtain any conclusion about target-cell specificity. Moreover, these effects of Jag1 on cell proliferation are not supported by gene expression analysis on the kit+ population.

Antagonistic interactions between Jagged and Delta ligands to attenuate Notch signalling have been previously proposed for angiogenic sprouting23 or prosenory specification in the inner ear22. In the vascular network of the retina, Jag1 antagonizes strong DLL4-Notch1 signalling in a stalk cell to promote the tip fate that requires a weak/no Notch1 signal. Fringe enhances the ability of Notch1 to respond to DLL4 while lowering activation by Jag1 in the tip and the developing inner ear22,24. Fringe is likely to be involved in EHT; however, the specific role of fringe in HSC development has not been addressed yet.

Lines of evidence in different organisms support the model known as cis-inhibition, in which activation of Notch by a ligand expressed in a neighbouring cell (sending cell) is prevented by the ligands that are co-expressed with Notch in the receiving cell25–29. Our observation that endothelial genes in Kit— cells are repressed by incubation with OP9-Jag1 (signal in trans) points against a pure cis-inhibitory model, thus suggesting the existence of a trans-inhibition mechanism.

The importance of Jag1 signal in the maintenance of the haematopoietic identity was previously demonstrated in the Jag1–/– mouse2; however, a precise analysis of the haemogenic and HSC subpopulations in these animals was lacking. We now show that Kit+ cells are produced in Jag1-deficient aortas in a similar frequency as the wild type; however, mutant cells fail to downregulate the endothelial programme, are not properly localized in the emerging clusters and show a subaortic mesenchymal localization.

In summary, our study identifies a novel two-step process in the specification of definitive HSC by Jag1 during mammalian development has not been addressed yet.

Methods

Animals. CD1, C57BL/6j wild-type, B6SJL-Ptprc-Pep3b/BoyJ, SCID-Beige mice (Charles River Laboratories), Jag1−/− (ref. 30), β-actin-GFP (ref. 31) and ROSA26S화요 (ref. 32) NIP-Cre (ref. 17) and NIP-CreERT (ref. 19) strains were used. Animals were kept under pathogen-free conditions, and all procedures were approved by the Animal Care Committee of the Parc de Recerca Biomèdica de Barcelona (registration of Generalitat de Catalunya). Embryos were obtained from timed pregnant females and staged by somite counting: E10.5 (31–40 sp) E11.5 (43–48 sp). The detection of the vaginal plug was designated as day 0.5. Mice and embryos were genotyped using PCR when justified.

OP9 cell culture and stromal-free culture of AGM-derived sorted cells. OP9 stromal cell lines overexpressing Jag1 or DLL4 ligands33 were maintained in a minimum essential medium ( Gibco, Life Technologies) supplemented with 20% fetal serum (FBS) and 100 U ml−1 penicillin/streptomycin and were cultured at 37 °C in a humidified atmosphere with 5% CO2. Cells were plated at 1 × 104 cells cm−2 24 h before experiment.

Sorted cells were plated in Iscoves medium (Gibco, Life Technologies) supplemented with 10% inactivated FBS, 10 ng ml−1 interleukin (IL)-3, 10 ng ml−1 stem cell factor (SCF), 20 ng ml−1 IL-6, 10 ng ml−1 insulin-like growth factor-1 (IGF-1), 10 ng ml−1 fibroblast growth factor-basic (FGF-B), 10 ng ml−1 vascular endothelial growth factor (VEGF), 2 μl ml−1 erythropoietin, 4.5 × 10−4 M monothioglycerol, 10 μg ml−1 Heparin and 50 ng ml−1 bovine pituitary extract.

Cells were incubated for 2.5 h or 7 days depending on experiment. Incubation with 1 μg ml−1 of blocking anti-Jag1 N-17 (sc-34473, Santa Cruz Biotechnology), 5 μg ml−1 of blocking anti-DLL4 (Genentech) or with the IgG mock controls at the correspondent concentration (irrelevant Goat IgG, Sigma 19140 or Goat Anti-Human Ig, Southern Biotech 2010-01, respectively).

Haematopoietic progenitor assay. On culture, AGM-derived cells were harvested and seeded in duplicates in Methocult M-3434 semi-solid medium (Stem Cell Technologies). Cells were incubated at 37 °C with 5% CO2 and colony-forming units were counted after 5 days.

CFU-S. AGM-derived cells on culture were harvested and washed with PBS. Cells were resuspended in 330 μl per sample and injected intravenously into adult sublethally irradiated (3 Gy) C57BL/6j wild-type or SCID-Beige recipients (Figs 4 and 6, respectively). After 11 days, the animals were killed and the presence of macroscopic haematopoietic colonies in the spleen was scored under a stereo-scope (KL200 LED, Leica).

Single-cell suspensions and antibody staining. AGMs were dissected from embryos at E10.5 or E11.5, incubated for 20 min at 37 °C in 0.2% collagenase (Sigma-Aldrich) in PBS + 10% FCS and dissociated by pipetting to single-cell suspensions. Cultured cells or primary cells were washed with PBS + 10% FBS before antibody staining. Antibody staining was performed in PBS supplemented with 10% FCS in the dark, at room temperature for 15 min, or carried out on ice for 30 min. The antibodies CD45-PeCy7, CD45-FTTC, Ter119-PeCy7, CD31-PE and Kit-APC were purchased from BD Biosciences. Dead cells were excluded using Hoechst 33342 (Invitrogen) or 4-6-diamidino-2-phenylindole (DAPI, Invitrogen).

Flow cytometry and cell sorting. Flow cytometry analysis was performed on FACSCalibur (BD Biosciences) or LSRII (BD Biosciences). Cell sorting was performed on FACSVantage (70-μm nozzle), FACSAria (85-μm nozzle) or Influx (100-μm nozzle) and Influx (100-μm nozzle) or Influx (100-μm nozzle) and Influx (100-μm nozzle) and Influx (100-μm nozzle) and Influx (100-μm nozzle) and Influx (100-μm nozzle). The data were analysed with the FlowJo software (Tree Star) or FACSDiva software (BD Biosciences). Sorted cells were collected either in medium or Qiangen RLRT buffer (for culture or mRNA extraction, respectively). When possible, cells were analysed for sorting purity; neither Kit+ nor CD45− cell contamination was detected in Kit— population.

cDNA amplification and quantitative RT–PCR. Total RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was obtained with RT First Strand cDNA Synthesis (GE Healthcare) according to the manufacturer’s instructions. cDNA was pre-amplified before qRT–PCR reaction using TaqMan PreAmp Master Mix Kit (Applied Biosystems) according to the manufacturer’s instructions. The primers used for pre-amplification and qRT–PCR are listed in Supplementary Table 1.

Microarray analysis. For microarray study, AGM regions were obtained from E11.5 mouse embryos, digested with 0.1% collagenase and single-cell suspension stained with anti-Ter19, anti-CD45 and anti-CD31. The CD31+ CD45+ Ter19+ population was sorted in Iscoves-based medium and seeded on OP9-Jag1 or OP9-DLL4 stromal cells for 2h, as described above. After 2h, cells from each of the culture conditions were re-stained with anti-CD45, anti-CD31 and anti-Kit, and CD31+ Kit− CD45+ and CD31+ Kit+ CD45− directly recovered in RLRT buffer (Qiagen). Total RNA from three independent sorting experiments was extracted using RNeasy mini or mini Kit (Qiagen) and was assessed using Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Only samples with high integrity (RNA integrity number > 7) were subsequently used in microarray experiments. Microarray expression profiles were obtained using the Affymetrix GeneChip Mouse Gene 1.0 ST array (Affymetrix, Santa Clara, CA) and the GCSS3000 Affymetrix small plate format. From each sample was generated using the Affymetrix array WTA System (NuGEN Technologies, San Carlos, CA) and sense transcript cDNA (ST-cDNA) was generated using the WT-Ovation Exon Module.
Haematopoietic stem cells derive directly from aortic endothelium by a Notch pathway.

Nature Communications | 6:8510 | DOI: 10.1038/ncomms9510 | www.nature.com/naturecommunications

Medvinsky, A. & Dzierzak, E. Definitive hematopoiesis is autonomously initiated by the AGM region. Cell 86, 897–909 (1996).

Kissa, K. & Herbomel, P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. Nature 464, 112–115 (2010).

Boisset, J. C. et al. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature 464, 116–120 (2010).

Bertrand, J. Y. et al. Haematopoietic stem cells derive directly from aortic endothelium during development. Nature 464, 108–111 (2010).

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

Lawson, N. D. et al. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development 126, 3623–3630 (2000).

Robert-Moreno, A. et al. Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1. EMBO J. 27, 1886–1895 (2008).

Bruns, C. E., Traver, D., Mayhall, E., Shepard, J. L. & Zon, L. I. Haematopoietic stem cell fate is established by the Notch-Runx pathway. Genes Dev. 19, 2331–2342 (2005).

Robert-Moreno, A., Espinoza, L., de la Pompa, J. L. & Bigas, A. RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. Development 132, 1117–1126 (2005).

Krebs, L. T. et al. Notch signaling is essential for vascular morphogenesis in mice. Genes Dev. 14, 1343–1352 (2000).

Krebs, L. T. et al. Notch1 and Notch2 is essential for generating hematopoietic stem cells from endothelial cells. Immunity 18, 699–711 (2003).

Duarte, A. et al. Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev. 18, 2474–2478 (2004).

Krebs, L. T. et al. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev. 18, 2469–2473 (2004).

Ong, C. T., Sedy, I. R., Murphy, K. M. & Kopan, R. Notch and presenilin provide a more comprehensive map of cells experiencing Notch1 activity. Development 142, 1193–1202 (2005).

Van de Walle, I. et al. Specific Notch receptor-ligand interactions control human TCR-α/β-gammadelta development by inducing differential Notch signal strength. J. Exp. Med. 210, 683–697 (2013).

Guinea, I. et al. Hes repression identifies essential regulators of hematopoietic stem cell development downstream of Notch signaling. J. Exp. Med. 210, 71–84 (2013).

Petrovic, J., Galvez, H., Neves, I., Abello, G. & Giraldez, F. Differential regulation of Hes/Hey genes during inner ear development. Dev. Neurobiol. 75, 703–720 (2014).

Benedict, R. et al. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. Cell 137, 1124–1135 (2009).

Beaou, L. et al. Notch-1/Notch-2 and Jagged1/2 induce the formation of Notch1+ cells that show increased expression of angiogenic markers. EMBO J. 25, 4697–4706 (2006).

Miller, A. C., Lyons, E. L. & Herman, T. G. cis-Inhibition of Notch by endogenous Delta biases the outcome of lateral inhibition. Curr. Biol. 19, 1379–1383 (2009).

Sprinzak, D. et al. cis-Interactions between Notch and Delta generate mutually exclusive signaling states. Nature 465, 86–90 (2010).

Sprinzak, D. et al. Notch and Delta transduce cis-inhibitory signals that shape neuroepithelial organisation. PLoS Comput. Biol. 7, e1001920 (2011).

Xue, Y. et al. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. Hum. Mol. Genet. 8, 723–730 (1999).

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. & Nishimune, Y. 'Green mice' as a source of ubiquitous green cells. FEBS Lett. 407, 313–319 (1997).

Srinivas, S. et al. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. 1, 4 (2001).

Van de Walle, I. et al. Jagged2 acts as a Delta-like Notch ligand during early hematopoietic cell fate decisions. Blood 117, 4449–4459 (2011).

Ruiz-Herguido, C. et al. Hematopoietic stem cell development requires transient Wnt/beta-catenin activity. J. Exp. Med. 209, 1457–1468 (2012).

Sturm, A., Quackenbush, J. & Trajanoski, Z. Genesis: cluster analysis of microarray data. Bioinformatics 18, 207–208 (2002).

Köster, T. et al. Bioconductor package for quality assessment of microarray data. Bioinformatics 21, 1378–1382 (2005).

Van der Walle, I. et al. Whole-mount three-dimensional imaging of internally localized immunostained cells within mouse embryos. Nat. Protoc. 7, 421–431 (2012).
L.G.-N. was a recipient of Marie Curie Intra-European Fellowship (PIEF-GA-2011-302226). E.F. and J.G. are recipients of FPI (BES-2011-048360 and BES-2008-005708, respectively). This research was funded by the Ministerio de Economía y Competitividad (PLE2009-0111, SAF2010-15450, SAF2013-40922-R), Red Temática de Investigación Cooperativa en Cáncer (RTICC) (RD12/0036/0054), Agència de Gestió d’Ajuds Universitaris i de Recerca (AGAUR; 2014SGR-124) to A.B.

Author contributions
L.G.-N., E.F., J.G., C.R.-H., Z.L., S.-U.L., T.M., L.E. and A.B. designed and performed experiments and analysed data; A.B.M.K.I. and N.L.-B. analysed data; M.Y. and C.J.G. provided reagents; L.G.-N., L.E., R.K. and A.B. wrote the manuscript.

Additional information
Accession codes: Microarray data have been deposited in the GEO database under accession codes GSE59344 and GSE35395.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: Minhong Yan is an employee and shareholder of Roche Genentech. The remaining authors declare no competing financial interest.

How to cite this article: Gama-Norton, L. et al. Notch signal strength controls cell fate in the haemogenic endothelium. Nat. Commun. 6:8510 doi: 10.1038/ncomms9510 (2015).