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Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1

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Specific deletion of Notch1 and RBPjκ in the mouse results in abrogation of definitive haematopoiesis concomitant with the loss of arterial identity at embryonic stage. As prior arterial determination is likely to be required for the generation of embryonic haematopoiesis, it is difficult to establish the specific haematopoietic role of Notch in these mutants. By analysing different Notch-ligand-null embryos, we now show that Jagged1 is not required for the establishment of the arterial fate but it is required for the correct execution of the definitive haematopoietic programme, including expression of GATA2 in the dorsal aorta. Moreover, successful haematopoietic rescue of the Jagged1-null AGM cells was obtained by culturing them with Jagged1-expressing stromal cells or by lentiviral-mediated transduction of the GATA2 gene. Taken together, our results indicate that Jagged1-mediated activation of Notch1 is responsible for regulating GATA2 expression in the AGM, which in turn is essential for definitive haematopoiesis in the mouse.

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Introduction

Haematopoietic cells originate from different embryonic sites during development and are generated in close association with the vascular endothelium. Some of the best characterized haematopoietic sites in the embryo include the yolk sac (Yoder and Hiatt, 1997; Yoder *et al*, 1997a, b), the intra-embryonic para-aortic splanchnopleura (P-sp)/AGM (aorta-gonad-mesonephros) (Medvinsky and Dzierzak, 1996; Cumano *et al*, 2001), umbilical and vitelline arteries (de Bruijn *et al*, 2000), allantois (Zeigler *et al*, 2006; Dieterlen-Lievre, 2007) and placenta (Gekas *et al*, 2005; Ottersbach and Dzierzak, 2005). The AGM region generates the first adult repopulating haematopoietic stem cells (HSCs) but it is as yet unclear whether some or all these tissues can *de novo* generate such cells. It is thought that niche-dependent signals can influence the properties of the presumptive haematopoieitc cells as they emerge and function to meet the haematopoietic needs of the embryo. Thus, a variety of haematopoietic cells (as defined by functional assays) emerge, including myeloid progenitors, multipotent progenitors and *in vivo* repopulating HSC.

The Notch pathway is involved in the regulation of cell-fate decisions by cell–cell interactions in a variety of developmental systems, including haematopoiesis. In adult haematopoiesis, the best characterized function of Notch is in the development of T (Radtke *et al*, 1999) and B cells (Pui *et al*, 1999; Tanigaki *et al*, 2002) (reviewed in Radtke *et al*, 2004). In addition, Notch is likely to participate in other aspects of haematopoietic homoeostasis including stem cell maintenance as shown by the ability to immortalize pluripotent haematopoietic cells (Varnum-Finney *et al*, 2000). It has been shown that germline mutant embryos deficient for Notch1 and RBPjκ cannot generate intra-embryonic HSC in different organisms, whereas no major haematopoietic defects have been found in the yolk sac haematopoiesis of these mutants (Kumano *et al*, 2003; Burns *et al*, 2005; Robert-Moreno *et al*, 2005, 2007). Moreover, Notch1-deficient ES-cell derived chimaeras never contributed to adult haematopoiesis but did contribute to yolk sac-derived haematopoiesis (Hadland *et al*, 2004). Several studies in zebrafish also support a role for Notch signalling in definitive but not primitive haematopoiesis (Burns *et al*, 2005; Gering and Patient, 2005). Although these observations suggest that Notch is involved in the generation of the definitive HSC in the embryo, *in vivo* experiments in which Notch1 or Jagged1 was conditionally deleted in the adult by the interferon-inducible Mx-cre failed to support the idea that Notch has a similar function in adult HSCs (Radtke *et al*, 1999; Mancini *et al*, 2005). In addition, a conclusive proof for the haematopoietic Notch function in the embryo remains elusive, mainly because of the abnormal arterial development of all the analysed Notch mutants.

The onset of the haematopoietic programme in the aortic endothelial-like cells is characterized by the transcriptional activation of the Runx1 and GATA2 genes, which are required for the formation of definitive HSC (Tsai *et al*, 1994; Okuda *et al*, 1996; North *et al*, 2002; Ling *et al*, 2004). We previously described that GATA2 was a direct target of Notch in the AGM region (Robert-Moreno *et al*, 2005), and data on

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haematopoietic specification in zebrafish support Runx1 as a downstream factor of the genetic cascade initiated by Notch signalling (Burns *et al*, 2005). However, there is no evidence that Runx1 is a direct target of Notch.

To further identify and characterize the genetic cascade involving Notch and its role in haematopoietic specification, we examined the putative haematopoietic defects on embryos deficient for Jagged1, Jagged2 or Delta4, the three ligands that are present in the dorsal aorta of the mid-gestation embryo. Notch ligands Jagged1, Jagged2 and Delta4 are known to interact and activate all Notch receptors (Shimizu *et al*, 2000; Shutter *et al*, 2000). Targeted disruption of each of these genes results in embryonic (Jagged1 and Delta4) or perinatal (Jagged2) lethality, suggesting that all have essential, non-redundant functions (Jiang *et al*, 1998; Xue *et al*, 1999; Duarte *et al*, 2004; Krebs *et al*, 2004). *In vitro* cultures with Jagged1 (Varnum-Finney *et al*, 1998; Karanu *et al*, 2000), Delta1 (Varnum-Finney *et al*, 2003) and Delta4 (Dando *et al*, 2005) have been used for expansion of haematopoietic progenitors or stem cells with the purpose of improving cell therapy protocols. However, to date there is no proof for the involvement of Notch ligands in the embryonic haematopoiesis and maintenance of adult haematopoietic progenitors as well as the individual contribution of these ligands *in vivo*.

Here, we describe that the Notch ligand Jagged1 is directly involved in activating Notch in AGM haematopoietic cells, non-cell autonomously. This results in the transcriptional activation of GATA2 in these cells and the formation of the HSC and their progeny.

Results

Heterogeneous expression of Notch family members within the aortic haematopoietic clusters

Previously, we showed that *Notch1*, *Notch4*, *Jagged1*, *Jagged2* and *Delta4* are expressed in the mid-gestation AGM region in the mouse embryo (Robert-Moreno *et al*, 2005). We further studied the expression pattern of these molecules in the haematopoietic clusters emerging from the aortic endothelium at E9.5–10.5 (20–40sp). We found that Notch1 is expressed in most of the cells of the haematopoietic clusters similar to Jagged1 expression. In contrast, Notch4 is strongly decreased in the clustered cells compared with the surrounding endothelium (Figure 1A). Profound heterogeneity was found in the expression patterns of Jagged2 and Delta4 ligands in these structures. Jagged2 was heterogeneously expressed within the cells of a single cluster, whereas Delta4 was expressed in individual clusters and completely absent in others. Detection of activated Notch1 (Figure 1B) with specific antibody recognizing cleaved-Notch1 (Souilhol *et al*, 2006; Del Monte *et al*, 2007), concomitant with the presence of the Notch target genes hes1, hrt1 and GATA2 mRNA (Figure 1C) further indicates that Notch signalling is active and functional in the cells of the haematopoietic clusters in the aorta. These results together with the haema-

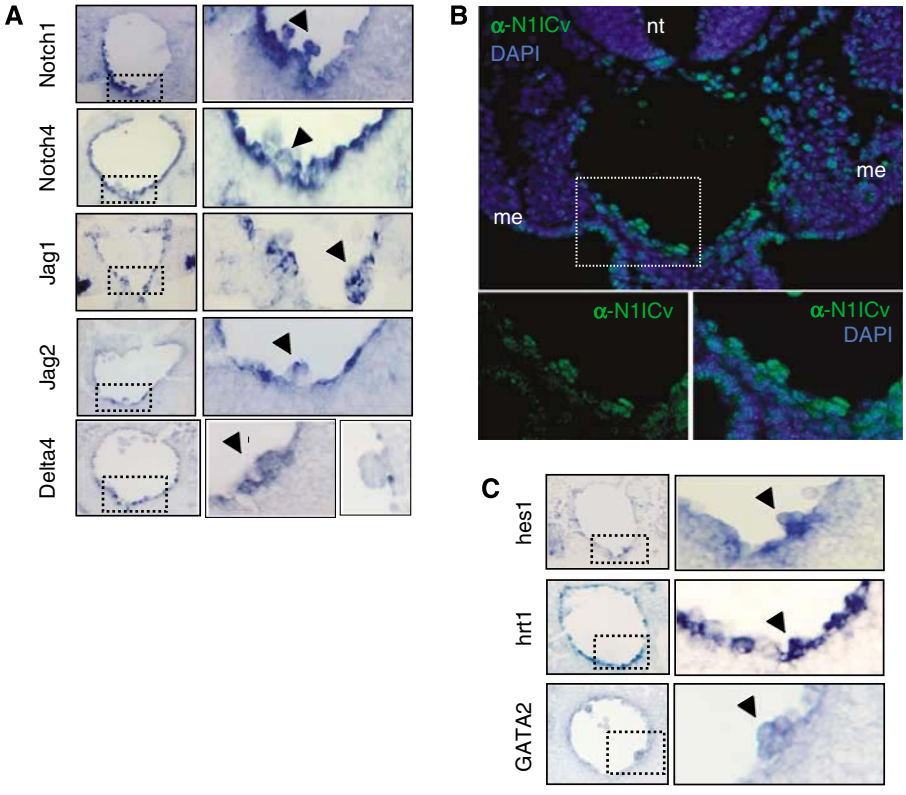

Figure 1 Heterogeneous expression of Notch family members within the aortic haematopoietic clusters. (**A**) Expression of the indicated genes in the AGM of E10.5 embryos by WISH. Transversal sections of dorsal aorta in a dorsal-to-ventral orientation (× 400). Details of clustered cells are in the right panels. (**B**) Immunofluorescence staining of anti-N1ICv (green) overlayed with DAPI (blue) in the AGM region. Details of clusters are in the lower panels. (**C**) WISH of Notch target genes in clustered cells budding from the dorsal aorta.

topoietic defects observed in the Notch1 and RBPjκ-null embryos (Kumano *et al*, 2003; Robert-Moreno *et al*, 2005) support the notion that Notch signalling has a pivotal function in the embryonic haematopoietic development.

Altered haematopoiesis in the AGM of Jagged1 but not Jagged2 mutant embryos

To examine the contribution of the different Notch ligands on embryonic haematopoiesis *in vivo*, we took advantage of the Ly-6A–GFP (Sca-1-GFP) transgenic mice (de Bruijn *et al*, 2002). These mice express GFP in the first HSCs, in cells of the haematopoietic clusters and haemogenic endothelium of the E10 aorta and have been used to quantitate haematopoietic clusters in normal and GATA2 mutant embryos (Ling *et al*, 2004). We crossed Jag1$^{+/\Delta}$ or Jag2$^{+/\Delta}$ (Jiang *et al*, 1998; Xue *et al*, 1999) with Ly-6A–GFP mice and determined the number of GFP^{+} cells in the haematopoietic clusters and endothelium of the aorta of precisely timed embryos. We found similar numbers of GFP^{+} cells in the AGM of E10–11 Jag2$^{\Delta/\Delta}$/Ly-6A–GFP embryos compared with their wild-type littermates. Conversely, the number of GFP^{+} cells found in the aortic endothelium of Jag1$^{\Delta/\Delta}$/Ly-6A–GFP embryos was reduced at least 50% at three different developmental stages (Figure 2A and B), suggesting an important role for Jagged1 in embryonic haematopoiesis. Although the Notch ligand Delta4 was also expressed in the aorta, the strong vascular defects of the Delta4$^{+/-}$ mutants precluded this study (Duarte *et al*, 2004; Krebs *et al*, 2004).

To further examine the influence of Notch ligands on haematopoietic development, haematopoietic progenitors in the different mutants were quantitated by colony-forming cell (CFC) assays. AGM tissues from E10–11 Jag1$^{\Delta/\Delta}$ embryos

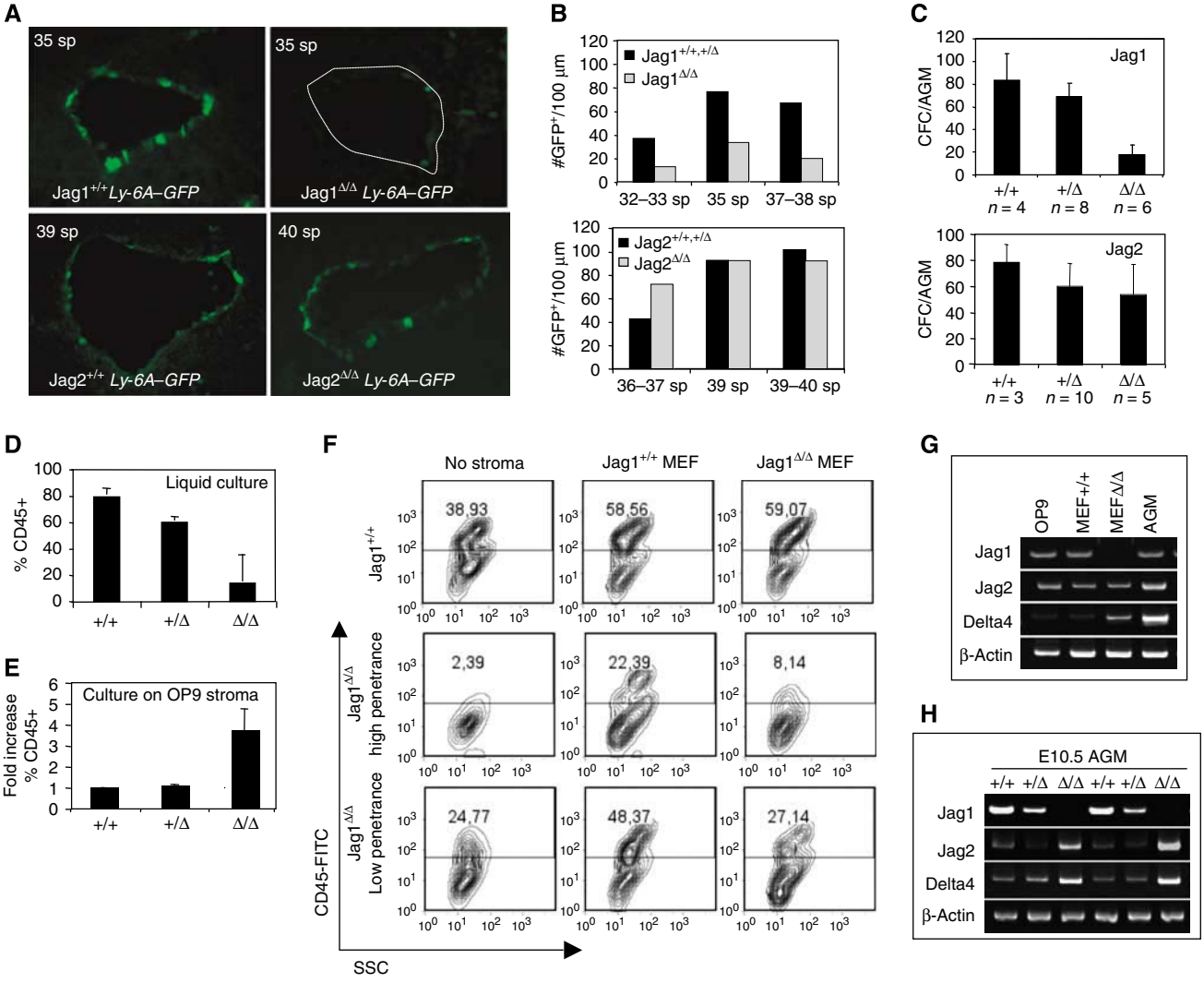

Figure 2 Altered haematopoiesis in the AGM of Jagged1 but not Jagged2 mutant embryos. (**A**) Precisely timed E10.5–11 Ly-6A–GFP, Jag1$^{\Delta/\Delta}$/Ly-6A–GFP or Jag2$^{\Delta/\Delta}$/Ly-6A–GFP embryos were sectioned and the number of GFP^{+} cells lining the dorsal aorta was counted. Representative photographs from these embryos are shown. The orientation is dorsal-to-ventral (×400). (**B**) Bars represent the number of GFP^{+} cells found in 100 µm of AGM aorta from three different Jag1$^{\Delta/\Delta}$/Ly-6A–GFP or Jag2$^{\Delta/\Delta}$/Ly-6A–GFP embryos compared with their wild-type littermates. (**C**) Bars represent the number of CFC from Jag1$^{\Delta/\Delta}$ or Jag2$^{\Delta/\Delta}$ E10.5 embryos compared with their wild-type littermates. (**D**) Percent of CD45+ cells obtained in liquid culture of AGM cells from Jag1$^{+/+}$ and Jag1$^{\Delta/\Delta}$. (**E**) Fold increase in the percent of CD45+ cells when cells are cultured on the OP9 cells. (**F**) Percent of CD45+ cells obtained from Jag1$^{+/+}$ and Jag1$^{\Delta/\Delta}$ AGM cells cultured without stroma or on Jag1$^{+/+}$ or Jag1$^{\Delta/\Delta}$ MEF. (**G**) Expression levels of different Notch ligands in the OP9 cells compared with Jag1$^{+/+}$ and Jag1$^{\Delta/\Delta}$ fibroblast and (**H**) in dissected E10.5 AGM from littermates of different Jag1 genotypes.

contained a severely reduced number of total haematopoietic progenitors (Figure 2C) and all progenitor types (myeloid, erythroid and mix colonies) were reduced (data not shown) compared with both Jag1$^{+/+}$ and Jag1$^{+/\Delta}$. In contrast, we did not detect any variation in the number and type of CFC in Jagged2 mutant AGMs. These results are in agreement with the absence of Ly-6A–GFP^{+} cells in the aorta of Jagged1 but not Jagged2 mutant embryos.

The capacity of Jag1$^{\Delta/\Delta}$ AGM tissues to generate haematopoietic cells was tested in liquid cultures. In the absence of stromal cells, a strong reduction in the percentage of CD45+ cells generated from Jag1$^{\Delta/\Delta}$ AGMs was observed as compared with the wild-type or heterozygous mutants (from 80% in Jag1$^{+/+}$ to 20% in Jag1$^{\Delta/\Delta}$) as determined by flow cytometry (Figure 2D). However, we identified a profound heterogeneity between different littermate null embryos with a range from 0 to 35% of CD45+ cells generated after liquid culture. This indicates variation in the penetrance of the Jagged1 deficiency (0–10% high penetrance and 10–35% low penetrance). We next co-cultured these cells with the OP9 stromal cell line that endogenously express Jagged1 (Figure 2G), to test whether the haematopoietic defect observed in the Jagged1 embryos was cell autonomous. When Jag1$^{\Delta/\Delta}$ AGM cells were co-cultured with OP9 stroma, the percentage of CD45+ cells generated increased up to fourfold (in both high and low penetrance null embryos) as compared with cells cultured without OP9, whereas we did not find any change in Jag1$^{+/+}$ and Jag1$^{+/\Delta}$ AGM cell cocultures (Figure 2E). Recovery of the CD45+ population is concomitant with an increase in GATA2 expression in these cells (Supplementary Figure 1). Overexpression of Jagged1 in OP9 cells did not further increase the recovery of CD45+ cells obtained from Jag1$^{\Delta/\Delta}$ AGMs (data not shown). To further confirm that Jagged1 was responsible for the haematopoietic recovery observed in the co-culture experiments, we isolated Jag1$^{+/+}$ or Jag1$^{\Delta/\Delta}$ murine embryonic fibroblast (MEF) (from E10.5 embryos) and used these cells as stromal layer. We observed a partial recovery in the number of CD45+ haematopoietic cells when Jag1$^{\Delta/\Delta}$ AGM cells were plated on Jag1$^{+/+}$ MEF but not on Jag1$^{\Delta/\Delta}$ MEF cells (Figure 2F). Taken together, these results indicate that Jagged1 expression in the aorta is required in a non-haematopoietic cell autonomous manner for haematopoietic cell development. Interestingly, we found comparable levels of Jagged2 and Delta4 in both MEFs (Figure 2G) and AGM from Jag1$^{\Delta/\Delta}$ (Figure 2H) embryos indicating that Jagged1 deficiency cannot be rescued by other Notch ligands.

Arterial fate is not affected in the Jagged1 mutant embryos

Notch function is required for aorta specification in different organisms (Lawson *et al*, 2001; Duarte *et al*, 2004; Krebs *et al*, 2004). As arterial determination precedes the haematopoietic cluster emergence in the mouse AGM, we tested whether the haematopoietic defects in the Jagged1 mutant embryos were due to a failure in the arterial programme. The expression of ephrinB2, which distinguishes arteries from veins before any structural, physiologic or functional distinctions (Wang *et al*, 1998; Adams *et al*, 1999) was examined by immunostaining of E10.5 AGM sections. We found that ephrinB2 is expressed in the arteries, including the aorta, of the Jag1$^{\Delta/\Delta}$ embryos similar to the wild type and it is absent from the venous

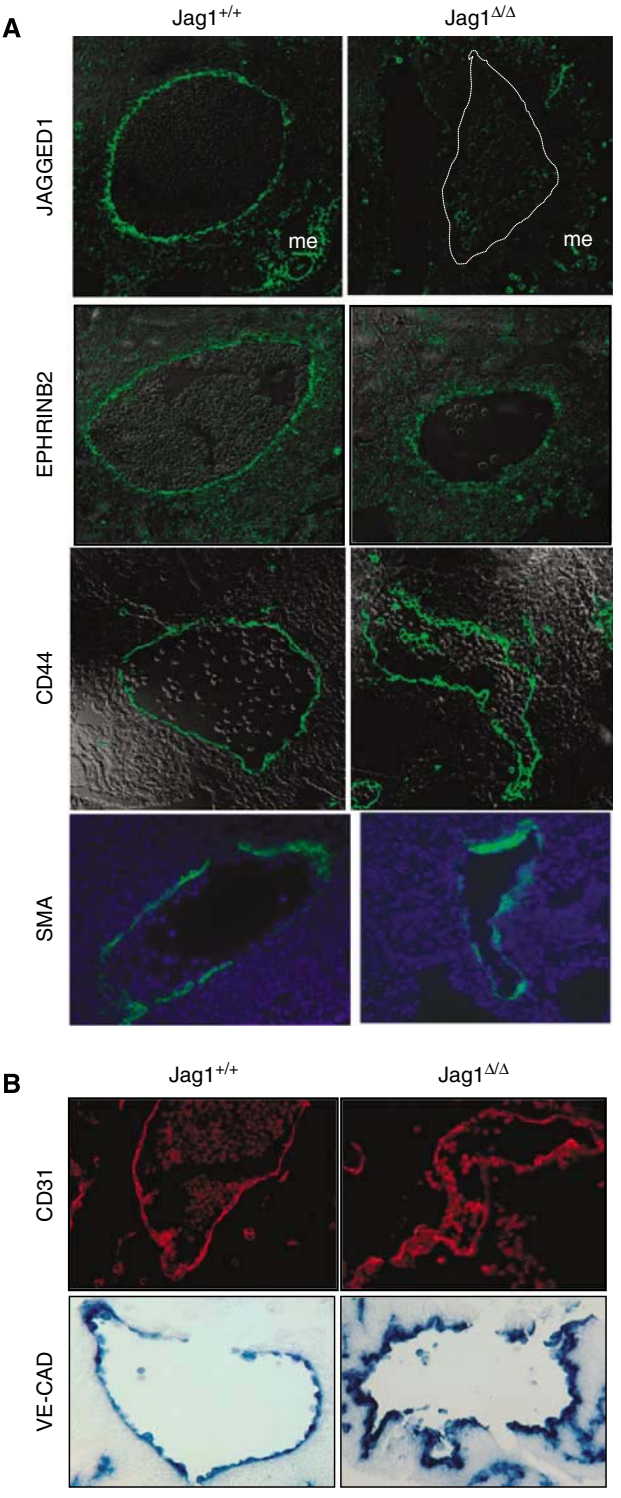

Figure 3 Arterial fate is not affected in the Jagged1 mutant embryos. (**A**, **B**) Sections from Jag1$^{+/+}$ and Jag1$^{\Delta/\Delta}$ E10.5–11 embryos showing expression of Jag1, EfnB2, CD44, SMA and CD31 by immunofluorescence or VE-cad by WISH. Confocal images for Jag1, EfnB2 and CD44 merged with the Nomarsky image are shown (×630). Detection of VE-cadherin (WISH) and immunofluorescence of SMA (merged with DAPI) and CD31 were obtained in Olympus BX60 at ×400.

network (Figure 3A). To further confirm that arterial determination is not affected in these mutants, we tested the expression pattern of other arterial markers such as CD44

and α-smooth muscle actin (SMA). We found comparable expression of CD44 and SMA in $Jag1^{+/+}$ and $Jag1^{\Delta/\Delta}$ (Figure 3A). Taken together, these results indicate that Jagged1-mediated activation of Notch is not required for arterial specification.

Previous studies of aortas from Notch1 and RBPjk mutant embryos showed an increase in the number of endothelial cells concomitant with a reduction in haematopoietic cells, suggesting a switch in the balance between endothelial and haematopoietic cell fates from a common precursor. To analyse whether the endothelial lineage was favoured in the Jagged1 mutants, AGM sections from wild-type and mutant embryos were examined for the expression of endothelial markers CD31 and VE-cadherin. Increased expression levels of both endothelial markers were observed in the $Jag1^{\Delta/\Delta}$ embryos compared with wild-type littermates (Figure 3B). Moreover, the aortic endothelial monolayer expressing CD31 and VE-cadherin lining the dorsal aorta displays, in these mutants, an abnormal architecture that resembles a multi-stratified endothelium. This phenotype is reminiscent of the one observed in the RBPjκ mutant embryos (Robert-Moreno *et al*, 2005).

Altogether these results indicate that the haematopoietic phenotype of the Jagged1 mutant embryos is not due to defective arterial specification, but instead suggest a defect in haematopoietic determination from the haemogenic endothelium of the AGM region.

GATA2 expression is compromised in Jagged1 mutants

Although we have previously shown that GATA2 is a direct target of Notch1 signalling, we next examined whether the lack of Jagged1 or Jagged2 influences GATA2 expression in the aortic endothelium and haematopoietic clusters. The expression of Runx1, another pivotal haematopoietic transcription factor in the emergence of the aortic haematopoietic clusters, was also examined by *in situ* hybridization. E10.5–11 $Jag1^{\Delta/\Delta}$ showed a complete lack of GATA2 expression in the aorta (Figure 4A). Interestingly, only a few cells expressing Runx1 were found in three out of six mutant embryos. Expression of Runx1 and GATA2 was also absent from E9.5 Delta4−/− embryos (data not shown). In contrast, both transcription factors were expressed in E10.5–11 $Jag2^{\Delta/\Delta}$ embryos and their expression patterns were similar to those found in wild-type littermates (Figure 4). However, some reduction in the total number of GATA2-expressing cells was found in the AGM region of these embryos (Table I).

As a direct target for Notch1 in the AGM, we hypothesized that recruitment of Notch1 to the GATA2 promoter may be affected and this may influence GATA2 expression in the Jagged1 mutant embryos. By chromatin IP assay followed by qPCR analysis, we detected Notch1 recruitment on $Jag1^{+/\Delta}$ E10.5 AGM cells, whereas no Notch1 recruitment was detected in the GATA2 promoter of $Jag1^{\Delta/\Delta}$ AGM at the same developmental stage (35–40 sp) (Figure 4C).

Ectopic GATA2 expression rescues Jagged1 mutant haematopoiesis

As shown in Figure 2, haematopoietic cells cannot be properly generated from $Jag1^{\Delta/\Delta}$ AGM in liquid cultures unless exogenous Jagged1 signalling is provided. As GATA2 expression is downstream of Notch activation in the AGM and it is absent in the haematopoietic clusters from Jagged1-null embryos, we next tested whether enforced expression of GATA2 on E10.5 AGM cells was sufficient to rescue $Jag1^{\Delta/\Delta}$ haematopoietic defects *in vitro*.

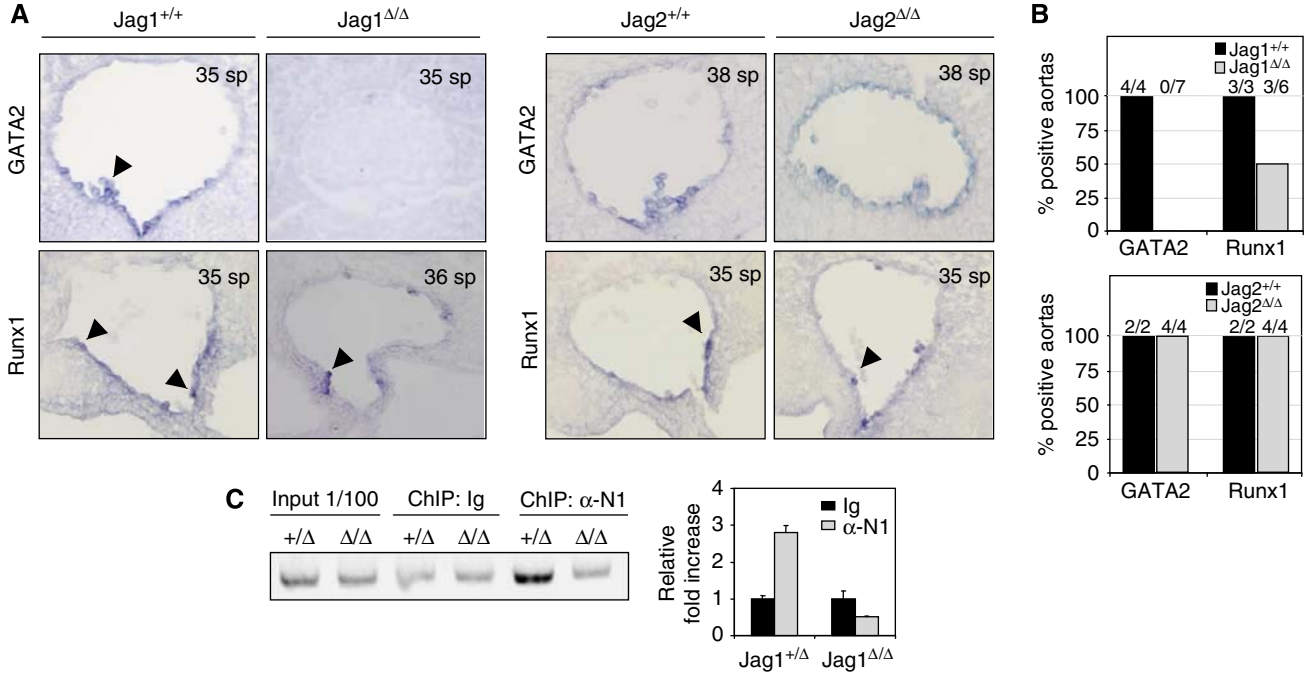

Figure 4 GATA2 expression is compromised in Jagged1 mutants. (**A**) WISH for the haematopoietic transcription factors GATA2 and Runx1 in the aortic endothelium of E10.5–11 wild types, $Jag1^{\Delta/\Delta}$ or $Jag2^{\Delta/\Delta}$ embryos. Somite pair precisely timed embryos were used to compare. The orientation is dorsal-to-ventral (× 400). (**B**) Graphs represent the percentage of embryos showing expression of the indicated genes from total analysed embryos. (**C**) Chromatin IP with the indicated antibodies from six pooled E10.5 dissected AGMs. PCR amplification of the precipitates (left) and relative fold enrichment of the GATA2 promoter as detected by qPCR (right) are shown.

Table I Number of GATA2- and Runx1-positive cells per AGM as detected by WISH in the endothelium of the dorsal aorta in different Jagged1 and Jagged2 mutants

	Jagged1^{wt}			Jagged1$^{\Delta/\Delta}$	
	Somite pairs	Genotype	Cells/ AGM	Somite pairs	Cells/ AGM
GATA2 expression					
	33	+/Δ	80	33	0
	35	+/+	120	35	3
	35	+/+	197	35	0
	38	+/Δ	428	38	3
				38	0
Runx1 expression					
	34	+/+	259	34	0
	35	+/Δ	151	35	2
	38	+/+	165	35	0
				35	56
				35	113
				38	62
	Jagged2^{wt}			Jagged2$^{\Delta/\Delta}$	
GATA2 expression					
	38	+/Δ	246	37	94
	40	+/+	177	38	89
				38	175
				40	85
Runx1 expression					
	35	+/+	49	35	61
	38	+/+	233	35	79
				38	158

Single collagenase-treated AGMs from Jag1$^{+/+}$ ($n=2$), Jag1$^{+/\Delta}$ ($n=2$) and Jag1$^{\Delta/\Delta}$ ($n=3$) embryos were infected with either pHRGFP or pHRhGATA2 lentivirus and maintained in liquid culture. After 7 days, cultures were assayed for the percentage of CD45+ cells by flow cytometry. We detected variable infection efficiency as measured by GFP$^+$ (20–50%) in both wild-type and null AGM cells. We did not observe any difference in the percentage of haematopoietic cells (CD45+) in Jag1$^{+/+}$, Jag1$^{+/\Delta}$ AGM cells transduced with GATA2 compared with the control within the GFP$^+$ population. However, a 2- to 3-fold enrichment in the percentage of CD45+ cells was consistently observed in the Jag1$^{\Delta/\Delta}$ cultures (either from high penetrance or low penetrance embryos). Thus, enforced expression of GATA2 was sufficient to partially rescue the generation of haematopoietic cells from Jag1$^{\Delta/\Delta}$ dissected AGMs (Figure 5A and B).

To further demonstrate that GATA2 is able to rescue the haematopoietic Notch signalling defect avoiding the putative previous defects in Jag1$^{\Delta/\Delta}$ during AGM development, we used the γ-secretase inhibitor DAPT to block Notch activation in the AGM cultures and transduced with pHRGFP or pHRhGATA2 lentivirus. After 7 days, we found a decrease in the percentage of CD45+ cells in the control-transduced cells treated with DAPT compared to DMSO-treated cultures ($P=0.05$). Interestingly, this effect was abrogated by GATA2 expression (Figure 5C). Moreover, a strong inhibitory effect of DAPT (100-fold) was observed on the number of progenitors (CFC) that was significantly recovered in GATA2-transduced cells ($P=0.002$) (Figure 5D). Consistent with the higher effect of Notch inhibition on CFC assay, we found that DAPT preferentially affects the number of ckit+CD45+ progenitors compared with total CD45+ cells in AGM cultures (data not shown).

Altogether our results indicate that Jagged1-mediated activation of Notch is required for the proper expression of GATA2 in the haematopoietic cells of the embryonic aorta, and that GATA2 is a required haematopoietic effector of Notch signalling in these cells.

Discussion

Our present work demonstrates that the Notch ligand Jagged1 is responsible for the Notch activation that permits the proper execution of the haematopoietic programme in the dorsal aorta of the AGM region. This occurs, at least partially, through GATA2, a direct transcriptional target of Notch1.

We have previously shown that *Delta4*, *Jagged1* and *Jagged2* are expressed in the mid-gestation aortic endothelium; therefore, they were all candidates to activate Notch in the AGM (Robert-Moreno *et al*, 2005). We have now focused on studying the loss-of-function mutant embryos for Jagged1 (Xue *et al*, 1999), Jagged2 (Jiang *et al*, 1998) and Delta4 (Duarte *et al*, 2004; Krebs *et al*, 2004) to identify the putative haematopoietic Notch ligand. We found that Jagged1, but not Jagged2, is responsible for Notch activation in haematopoietic cells in the dorsal aorta. Different Notch ligands, including Jagged1, had been previously shown to influence HSCs *in vitro* and Jagged1 may be an important component of the osteoblastic niche for maintaining adult HSC in the bone marrow (Calvi *et al*, 2003). Our results demonstrate for the first time that the Notch ligand Jagged1 is required for the embryonic haematopoietic development *in vivo*.

Although preliminary studies with the Delta4 heterozygous embryos also showed lack of haematopoiesis, these mutants display severe vasculogenic defects together with the loss of the arterial cell fate, which strongly compromised the study of haematopoiesis in these mutants. Further studies of the Delta4 deficiency in AGM, by using cell-specific cre-recombinase expression in the appropriate cells, without disturbing the arterial identity should determine the importance of Delta4 in embryonic haematopoiesis.

The role of Notch in actively promoting arterial identity is well documented in several systems (Lawson *et al*, 2001; Duarte *et al*, 2004; Krebs *et al*, 2004). Conversely, COUP-TFII is required in veins to repress Notch signalling and maintain vein identity (You *et al*, 2005). The link between arterial determination and haematopoiesis development is supported by the abnormal generation of haematopoeitic progenitors in the veins of mutant embryos showing vein-to-artery conversion, such is the case for activin receptor such as kinase1 (ACVRL1) (Urness *et al*, 2000) or COUP-TFII (You *et al*, 2005)-deficient embryos. However, it is not well established whether haematopoiesis can occur in the absence of arterial specification, although in zebrafish, haematopoietic cells can originate from vessels that do not express EphrinB2 after N1IC induction (Burns *et al*, 2005). We now demonstrate that Jagged1-null embryos fail to generate haematopoietic cells but display normal expression of arterial markers such as ephrinB2 or CD44. This observation is extremely relevant as it is the first time that Notch signalling is directly associated with the generation of haematopoietic cells independently of its role in arterial development. This is in contrast with the previously analysed Notch mutants in which arterial deter-

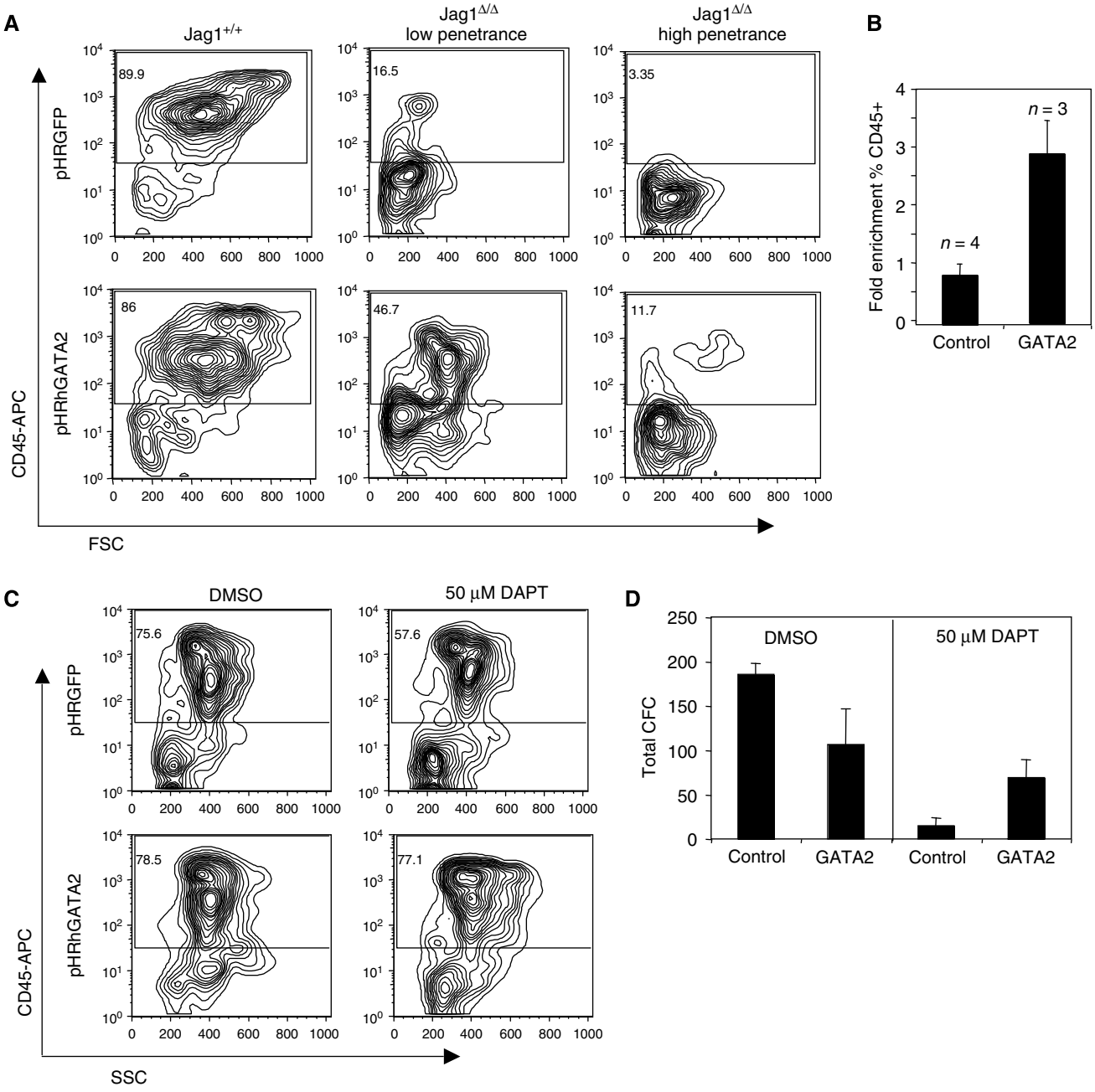

Figure 5 Ectopic GATA2 expression rescues Jagged1 mutant haematopoiesis. (**A**) Analysis of CD45+ cells by flow cytometry in the GFP+ population obtained from E10.5 AGM cultures transduced with control pHRGFP and pHRhGATA2 lentivirus. Representative $Jag1^{+/+}$ and two $Jag1^{\Delta/\Delta}$ with different penetrance are shown. (**B**) Graph represents the relative fold increase in the percent of CD45+ cells obtained in the GATA2- compared with GFP-transduced $Jag1^{\Delta/\Delta}$ cultures. (**C**) Flow cytometry analysis of one representative of three cultures of AGM cells transduced with the indicated lentivirus and incubated with DMSO or DAPT. The percentage of CD45+ cells within the GFP^{+} population is indicated. (**D**) Bars represent the average and standard deviation of CFCs obtained from three different cultures.

mination, most likely a requirement for haematopoiesis to progress, was also affected (Duarte *et al*, 2004; Krebs *et al*, 2004; Gridley, 2007).

We previously described that Notch1/RBPjκ signalling participates in the transcriptional regulation of the haematopoietic transcription factor GATA2 (Robert-Moreno *et al*, 2005). We have now found that recruitment of Notch1 to the GATA2 promoter depends on Jagged1. It has been proposed that loss of Runx1 is responsible for the haematopoietic defects in mouse Notch1 (Nakagawa *et al*, 2006) and zebrafish mind bomb mutants (Burns *et al*, 2005). However, we have found that Runx1 is expressed at low levels in the aorta of 50% of Jagged1 null mutants, which absolutely fail to express GATA2. The fact that GATA2 is involved in regulating Runx1 expression (Nottingham *et al*, 2007) may explain the reduced levels of this transcription factor found in the Jagged1 null mutants. Indeed, we have been able to detect increased expression of Runx1 in the CD45+ population in one out of two $Jag1^{\Delta/\Delta}$ AGM cultures transduced with GATA2 lentivirus (Supplementary Figure 2). Gene targeting studies revealed the importance of GATA2 for haematopoiesis as $GATA2^{-/-}$ embryos have reduced numbers of haematopoietic

cells (Tsai *et al*, 1994); there is no contribution of GATA2$^{-/-}$ ES-derived cells to any haematopoietic tissue (Tsai *et al*, 1994) and GATA2 haploinsufficiency results in different HSC abnormalities (Ling *et al*, 2004; Rodrigues *et al*, 2005). Our experiments demonstrate that re-expression of GATA2 partially reverts the haematopoietic defects of Jagged1 null embryos, suggesting that GATA2 is not the only Notch target required for embryonic haematopoiesis.

There is genetic evidence showing that haematopoietic and endothelial cells share a common genetic programme and come from a common progenitor (the haemangioblast). For example, the targeted disruption of the vascular endothelial growth factor and the tyrosine kinase Flk1 (Shalaby *et al*, 1995) results in complete deficiencies of both vascular and haematopoietic systems (Ferrara *et al*, 1996; Carmeliet *et al*, 1999). Moreover, *in vivo* labelling studies in the chick showed the endothelial origin of the haematopoietic clusters emerging in the aorta (Jaffredo *et al*, 1998). There are many evidences that support the existence of a common progenitor in the yolk sac and the AGM region, as an early progenitor for both lineages (Eichmann *et al*, 1997; Choi *et al*, 1998; Sabin, 2002; Huber *et al*, 2004). We have consistently found that different Notch pathway mutants contain an excess of endothelial cells that is concomitant with the absence of haematopoietic clusters in the aorta (Kumano *et al*, 2001; Robert-Moreno *et al*, 2005). This observation strongly suggests that haematopoietic progenitors assume the endothelial fate in the absence of Notch signalling.

Taken together, our results indicate that Jagged1-mediated activation of Notch1 is responsible for regulating GATA2 expression in the AGM, which in turn is essential for maintaining intraembryonic haematopoiesis in the mouse.

Materials and methods

Animals

Ly-6A/sca–GFP mice and Jagged1$^{\Delta/\Delta}$- and Jagged2$^{+/\Delta}$-null mutant mice have been extensively characterized (Jiang *et al*, 1998; Xue *et al*, 1999; de Bruijn *et al*, 2002). Mice and embryos were genotyped by PCR, or under the UV microscope for GFP expression. Jag1$^{\Delta/+}$ and Jag2$^{\Delta/+}$ were crossed with Ly6A/sca–GFP and bred into C57BL6/J. CD1 and C57BL6/J embryos were used indistinctly for whole mount *in situ* hybridization (WISH). Animals were kept under pathogen-free conditions and all procedures were approved by the Animal Care Committee. Embryos were obtained from timed pregnant females and somite pairs were counted for precise timing.

Haematopoietic colony assay

Dissected AGM was dissociated in 0.12% collagenase (Sigma) in PBS supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Biological Industries) for 1 h at 37°C and cells were plated in M-5323 semisolid medium (Stem Cell Technologies). After 7 days the presence of haematopoietic colonies was scored under the microscope.

Haematopoietic liquid cultures

The AGM region from E10.5 embryos was dissociated in 0.12% collagenase (Sigma) in PBS supplemented with 10% FBS and penicillin/streptomycin (Biological Industries) for 1 h at 37°C. Cells were incubated in Iscove's, 10% FBS, 10% IL3- and 10% SCF-conditioned medium plus 0.2 μg/ml IL6, 0.1 μg/ml flt3. Cells were harvested after 7–10 days for FACS analysis or CFC assay.

For OP9 cultures, OP9 cells were maintained in MEMα, 10% FBS and plated in 24-well plates 2 days before the AGM liquid cultures.

For MEF stromal cultures, embryos of different genotypes were mechanically dissociated with a scalpel and pipetting and plated on DMEM, 10% FBS until confluence. Twenty-four-well plates with MEF stromal cells were used for AGM liquid cultures.

RT–PCR

Total RNA was extracted with Qiagen kit, and RT-First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, GE Healthcare, Buckinghamshire, UK) was used. qRT–PCR was performed in LightCycler480 system using SYBR Green I Master kit (Roche, Basel, Switzerland).

Lentiviral constructs and infection

Flag-tagged GATA2 was subcloned into the pHR-SIN-CSGW-EGFP lentiviral expression construct under control of the SFFV promoter (Demaison *et al*, 2002). Vector components were a kind gift from Adrian Thrasher. Recombinant lentiviruses were produced by transient transfection of 293T cells according to Tronolab protocols (http://tronolab.epfl.ch/page58122.html). Briefly, subconfluent 293T cells were co-transfected with 20 μg of transfer vector (pHRGFP or pHRhGATA2), 15 μg of packaging plasmid (psPAX2) and 6 μg of envelope plasmid (pMD2.G) by calcium phosphate precipitation. After 3 days, supernatant was ultracentrifuged in Beckman L-70 at 26 000 r.p.m. for 2 h at 4°C in SW41 rotor, and viral pellet resuspended in 100 μl of PBS. Fresh viral suspension (20 μl) was used per infection.

Flow cytometry analysis

Following liquid culture, cells were analysed by flow cytometry with a FACScalibur (Becton & Dickinson) and FlowJo software. Dead cells were excluded by 7-aminoactinomicin-D staining (Molecular Probes). Cell sorting experiments in Supplemental data were performed in a FACSaria system (Becton & Dickinson).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (Aguilera *et al*, 2004). In brief, crosslinked chromatin from E10.5 dissected AGMs was sonicated with a UP50H Ultrasonic Processor, and precipitated with anti-N1 antibody (sc-6014). After crosslinkage reversal, DNA was used as a template for PCR to detect the mouse *GATA2* promoter (5′GCACGGTGTGAG AACCTGATTTC3′ and 5′TCCCAAAGTCTGTGTCTTG AGAGC3′. qPCR was performed with SYBR Green I Master (Roche) in LightCycler480 system.

Immunostaining

E10.5 embryos were fixed overnight in 4% paraformaldehyde (Sigma) at 4°C and frozen in Tissue-tek (Sakura). Sections (10 μm) were fixed with −20°C methanol for 15 min and block-permeabilized in 10% FBS, 0.3% Surfact-AmpsX100 (Pierce) and 5% non-fat milk in PBS for 90 min at 4°C. Samples were stained with α-CD31 (PECAM1; Pharmingen at 1:50) or α-CD44 (Pharmingen, 1:50) in 10% FBS, 5% non-fat milk in PBS overnight and HRP-conjugated secondary antibody (Dako) at 1:100 for 90 min and developed with Cy3- or FITC-coupled tyramide (PerkinElmer). α-SMA (Lab Vision) was used at 1:100 and Alexa Fluor 488-conjugated donkey–anti-rabbit (Invitrogen, 1:500) as a secondary antibody. For α-N1Icv (Cell Signaling at 1:100) or α-EphrinB2 (R&D systems at 1:200) or α-Jagged1 (sc-6011, 1:400) immunofluorescence was performed in paraffin-embedded sections, antigen retrieval was performed in 10 mM Na citrate pH = 6, 20 min in autoclave. Primary antibody was incubated in 3% BSA, 20 mM $MgCl_2$, 0.3% Tween20, 5% FBS in PBS overnight and HRP-conjugated secondary antibody (Dako) at 1:200 for 90 min and developed using the tyramide amplification system, TSA-Plus Cyanine3/Fluorescein System (PerkinElmer). Sections were mounted in Vectashield medium with 4′6-diamidino-2-phenylindole (DAPI) (Vector).

WISH

WISH was performed according to standard protocols (de la Pompa *et al*, 1997). For histological analysis, precisely timed embryos were fixed overnight at 4°C in 4% paraformaldehyde, dehydrated and embedded in Paraplast (Sigma). Embryos were sectioned in a Leica-RM2135 at 7 μm and mounted with DPX (Roche).

Image acquisition

Images were acquired with an Olympus BX-60 using a Spot camera and Spot3.2.4 software (Diagnostic Instruments) or a Leica TCS-NT laser scanning confocal microscope equipped with the ×63 Leitz Plan-Apo objective (NA 1.4). Representative images were edited on Adobe Photoshop 6.0 software.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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References

Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R (1999) Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev* **13:** 295–306

Aguilera C, Hoya-Arias R, Haegeman G, Espinosa L, Bigas A (2004) Recruitment of IkappaBalpha to the hes1 promoter is associated with transcriptional repression. *Proc Natl Acad Sci USA* **101:** 16537–16542

Burns CE, Traver D, Mayhall E, Shepard JL, Zon LI (2005) Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes Dev* **19:** 2331–2342

Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425:** 841–846

Carmeliet P, Lampugnani MG, Moons L, Breviario F, Compernolle V, Bono F, Balconi G, Spagnuolo R, Oostuyse B, Dewerchin M, Zanetti A, Angellilo A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter MC, Gittenberger-de Groot A, Poelmann R, Lupu F *et al* (1999) Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* **98:** 147–157

Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G (1998) A common precursor for hematopoietic and endothelial cells. *Development* **125:** 725–732

Cumano A, Ferraz JC, Klaine M, Di Santo JP, Godin I (2001) Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity* **15:** 477–485

Dando JS, Tavian M, Catelain C, Poirault S, Bennaceur-Griscelli A, Sainteny F, Vainchenker W, Peault B, Lauret E (2005) Notch/Delta4 interaction in human embryonic liver CD34+ CD38− cells: positive influence on BFU-E production and LTC-IC potential maintenance. *Stem Cells* **23:** 550–560

de Bruijn MF, Ma X, Robin C, Ottersbach K, Sanchez MJ, Dzierzak E (2002) Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* **16:** 673–683

de Bruijn MF, Speck NA, Peeters MC, Dzierzak E (2000) Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J* **19:** 2465–2474

de la Pompa JL, Wakeham A, Correia KM, Samper E, Brown S, Aguilera RJ, Nakano T, Honjo T, Mak TW, Rossant J, Conlon RA (1997) Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124:** 1139–1148

Del Monte G, Grego-Bessa J, Gonzalez-Rajal A, Bolos V, De La Pompa JL (2007) Monitoring Notch1 activity in development: evidence for a feedback regulatory loop. *Dev Dyn* **236:** 2594–2614

Demaison C, Parsley K, Brouns G, Scherr M, Battmer K, Kinnon C, Grez M, Thrasher AJ (2002) High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. *Hum Gene Ther* **13:** 803–813

Dieterlen-Lievre F (2007) Emergence of haematopoietic stem cells during development. *C R Biol* **330:** 504–509

Duarte A, Hirashima M, Benedito R, Trindade A, Diniz P, Bekman E, Costa L, Henrique D, Rossant J (2004) Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev* **18:** 2474–2478

Eichmann A, Corbel C, Nataf V, Vaigot P, Breant C, Le Douarin NM (1997) Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc Natl Acad Sci USA* **94:** 5141–5146

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380:** 439–442

Gekas C, Dieterlen-Lievre F, Orkin SH, Mikkola HK (2005) The placenta is a niche for hematopoietic stem cells. *Dev Cell* **8:** 365–375

Gering M, Patient R (2005) Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. *Dev Cell* **8:** 389–400

Gridley T (2007) Notch signaling in vascular development and physiology. *Development* **134:** 2709–2718

Hadland BK, Huppert SS, Kanungo J, Xue Y, Jiang R, Gridley T, Conlon RA, Cheng AM, Kopan R, Longmore GD (2004) A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. *Blood* **104:** 3097–3105

Huber TL, Kouskoff V, Fehling HJ, Palis J, Keller G (2004) Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* **432:** 625–630

Jaffredo T, Gautier R, Eichmann A, Dieterlen-Lievre F (1998) Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* **125:** 4575–4583

Jiang R, Lan Y, Chapman HD, Shawber C, Norton CR, Serreze DV, Weinmaster G, Gridley T (1998) Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev* **12:** 1046–1057

Karanu FN, Murdoch B, Gallacher L, Wu DM, Koremoto M, Sakano S, Bhatia M (2000) The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells [in process citation]. *J Exp Med* **192:** 1365–1372

Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T (2004) Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev* **18:** 2469–2473

Kumano K, Chiba S, Kunisato A, Sata M, Saito T, Nakagami-Yamaguchi E, Yamaguchi T, Masuda S, Shimizu K, Takahashi T, Ogawa S, Hamada Y, Hirai H (2003) Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* **18:** 699–711

Kumano K, Chiba S, Shimizu K, Yamagata T, Hosoya N, Saito T, Takahashi T, Hamada Y, Hirai H (2001) Notch1 inhibits differentiation of hematopoietic cells by sustaining GATA-2 expression. *Blood* **98:** 3283–3289

Lawson ND, Scheer N, Pham VN, Kim CH, Chitnis AB, Campos-Ortega JA, Weinstein BM (2001) Notch signaling is required for arterial–venous differentiation during embryonic vascular development. *Development* **128:** 3675–3683

Ling KW, Ottersbach K, van Hamburg JP, Oziemlak A, Tsai FY, Orkin SH, Ploemacher R, Hendriks RW, Dzierzak E (2004) GATA-2 plays two functionally distinct roles during the ontogeny of hematopoietic stem cells. *J Exp Med* **200:** 871–882

Mancini SJ, Mantei N, Dumortier A, Suter U, MacDonald HR, Radtke F (2005) Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood* **105:** 2340–2342

Medvinsky A, Dzierzak E (1996) Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86:** 897–906

Nakagawa M, Ichikawa M, Kumano K, Goyama S, Kawazu M, Asai T, Ogawa S, Kurokawa M, Chiba S (2006) AML1/Runx1 rescues Notch1-null mutation-induced deficiency of para-aortic splanchnopleural hematopoiesis. *Blood* **108:** 3329–3334

North TE, de Bruijn MF, Stacy T, Talebian L, Lind E, Robin C, Binder M, Dzierzak E, Speck NA (2002) Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity* **16:** 661–672

Nottingham WT, Jarratt A, Burgess M, Speck CL, Cheng JF, Prabhakar S, Rubin EM, Li PS, Sloane-Stanley J, Kong ASJ, de Bruijn MF (2007) Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer. *Blood* **110:** 4188–4197

Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84:** 321–330

Ottersbach K, Dzierzak E (2005) The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell* **8:** 377–387

Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S, Lee JY, Kadesch T, Hardy RR, Aster JC, Pear WS (1999) Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* **11:** 299–308

Radtke F, Wilson A, Mancini SJ, MacDonald HR (2004) Notch regulation of lymphocyte development and function. *Nat Immunol* **5:** 247–253

Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, Aguet M (1999) Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10:** 547–558

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

Robert-Moreno A, Espinosa L, Sanchez MJ, de la Pompa JL, Bigas A (2007) The notch pathway positively regulates programmed cell death during erythroid differentiation. *Leukemia* **21:** 1496–1503

Rodrigues NP, Janzen V, Forkert R, Dombkowski DM, Boyd AS, Orkin SH, Enver T, Vyas P, Scadden DT (2005) Haploinsufficiency of GATA-2 perturbs adult hematopoietic stem-cell homeostasis. *Blood* **106:** 477–484

Sabin FR (2002) Preliminary note on the differentiation of angioblasts and the method by which they produce blood-vessels, blood-plasma and red blood-cells as seen in the living chick. *J Hematother Stem Cell Res* **11:** 5–7

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376:** 62–66

Shimizu K, Chiba S, Saito T, Kumano K, Hirai H (2000) Physical interaction of delta1, jagged1, and jagged2 with notch1 and notch3 receptors. *Biochem Biophys Res Commun* **276:** 385–389

Shutter JR, Scully S, Fan W, Richards WG, Kitajewski J, Deblandre GA, Kintner CR, Stark KL (2000) Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev* **14:** 1313–1318

Souilhol C, Cormier S, Monet M, Vandormael-Pournin S, Joutel A, Babinet C, Cohen-Tannoudji M (2006) Nas transgenic mouse line allows visualization of Notch pathway activity *in vivo*. *Genesis* **44:** 277–286

Tanigaki K, Han H, Yamamoto N, Tashiro K, Ikegawa M, Kuroda K, Suzuki A, Nakano T, Honjo T (2002) Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nat Immunol* **3:** 443–450

Tsai FY, Keller G, Kuo FC, Weiss M, Chen J, Rosenblatt M, Alt FW, Orkin SH (1994) An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371:** 221–226

Urness LD, Sorensen LK, Li DY (2000) Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet* **26:** 328–331

Varnum-Finney B, Brashem-Stein C, Bernstein ID (2003) Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood* **101:** 1784–1789

Varnum-Finney B, Purton LE, Yu M, Brashem-Stein C, Flowers D, Staats S, Moore KA, Le Roux I, Mann R, Gray G, Artavanis-Tsakonas S, Bernstein ID (1998) The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood* **91:** 4084–4091

Varnum-Finney B, Xu L, Brashem-Stein C, Nourigat C, Flowers D, Bakkour S, Pear WS, Bernstein ID (2000) Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive notch1 signaling. *Nat Med* **6:** 1278–1281

Wang HU, Chen ZF, Anderson DJ (1998) Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93:** 741–753

Xue Y, Gao X, Lindsell CE, Norton CR, Chang B, Hicks C, Gendron-Maguire M, Rand EB, Weinmaster G, Gridley T (1999) Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet* **8:** 723–730

Yoder MC, Hiatt K (1997) Engraftment of embryonic hematopoietic cells in conditioned newborn recipients. *Blood* **89:** 2176–2183

Yoder MC, Hiatt K, Dutt P, Mukherjee P, Bodine DM, Orlic D (1997a) Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* **7:** 335–344

Yoder MC, Hiatt K, Mukherjee P (1997b) *In vivo* repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. *Proc Natl Acad Sci USA* **94:** 6776–6780

You LR, Lin FJ, Lee CT, DeMayo FJ, Tsai MJ, Tsai SY (2005) Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature* **435:** 98–104

Zeigler BM, Sugiyama D, Chen M, Guo Y, Downs KM, Speck NA (2006) The allantois and chorion, when isolated before circulation or chorio-allantoic fusion, have hematopoietic potential. *Development* **133:** 4183–4192