

RBPj κ -dependent Notch function regulates *Gata2* and is essential for the formation of intra-embryonic hematopoietic cells

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Summary

Definitive hematopoiesis in the mouse embryo originates from the aortic floor in the P-Sp/AGM region in close association with endothelial cells. An important role for Notch1 in the control of hematopoietic ontogeny has been recently established, although its mechanism of action is poorly understood. Here, we show detailed analysis of Notch family gene expression in the aorta endothelium between embryonic day (E) 9.5 and E10.5. Since Notch requires binding to RBPj κ transcription factor to activate transcription, we analyzed the aorta of the para-aortic splanchnopleura/AGM in RBPj κ mutant embryos. We found specific patterns of expression of Notch receptors, ligands and Hes genes that were lost in RBPj κ mutants. Analysis of these mutants revealed the absence of hematopoietic progenitors, accompanied by the lack of expression of the hematopoietic transcription factors

Aml1/Runx1, *Gata2* and *Scf/Tal1*. We show that in wild-type embryos, a few cells lining the aorta endothelium at E9.5 simultaneously expressed *Notch1* and *Gata2*, and demonstrate by chromatin immunoprecipitation that Notch1 specifically associated with the *Gata2* promoter in E9.5 wild-type embryos and 32D myeloid cells, an interaction lost in RBPj κ mutants. Consistent with a role for Notch1 in regulating *Gata2*, we observe increased expression of this gene in 32D cells expressing activated Notch1. Taken together, these data strongly suggest that activation of *Gata2* expression by Notch1/RBPj κ is a crucial event for the onset of definitive hematopoiesis in the embryo.

Key words: Notch, Mouse, *Rbpsuh*

Introduction

Hematopoietic cells differentiate from mesoderm during embryogenesis, in close association with endothelial cells. Definitive hematopoietic progenitors and stem cells originate in distinct sites in the embryo, including the yolk sac (YS) (Yoder et al., 1997), the umbilical and vitelline arteries (de Bruijn et al., 2000), the para-aortic splanchnopleura (P-Sp) (Cumano et al., 2001) and the aorta/genital ridge/mesonephros (AGM) region (Medvinsky and Dzierzak, 1996). The first hematopoietic cells detected during mouse embryonic development are the primitive erythroid cells of the YS at embryonic day (E) 7. One day later, before circulation between the embryo and YS is established, multipotent hematopoietic stem cells (HSCs) have been isolated from the intra-embryonic P-Sp (Cumano et al., 2001) indicating that intra-embryonic hematopoietic cells can originate independently of the YS. In the mouse, the P-Sp forms from the splanchnic mesoderm (the endoderm-associated mesoderm) and the whole region develops into aorta, gonads and mesonephros and is subsequently called AGM. Around E10–11, the HSC activity is autonomously generated in this region (reviewed by Ling and Dzierzak, 2002).

The developmental origin and the genetic program of embryonic HSC emergence in the YS and the P-Sp/AGM in

some aspects are divergent. Yolk sac blood cells originate simultaneously with the surrounding endothelial cells, consistent with the idea of developing from a common progenitor or hemangioblast (Palis and Yoder, 2001). By contrast, P-Sp/AGM hematopoietic cells emerge in close association to the presumably differentiated aortic endothelium. The lineage relationships and molecular events leading to their differentiation are not completely understood. Immunohistochemical analyses of the AGM region reveal overlapping expression of hematopoietic and endothelial markers in the clusters of cells that emerge from the ventral wall of the aorta. However, *Aml1/Cbfa2* (*Runx1* – Mouse Genome Informatics) transcription factor has been shown specifically to be involved in the development of intra-embryonic hematopoiesis without affecting the main vasculature (North et al., 1999). The analysis of recently developed transgenic mice, which enable specific labeling of emerging HSCs, provides supportive evidence that true HSCs originate among the cells residing in the endothelial layer (Ma et al., 2002). Besides *Aml1* (North et al., 2002), *Gata2* (Tsai et al., 1994; Tsai and Orkin, 1997) and *Scf* (*Tal1* – Mouse Genome Informatics) (Porcher et al., 1996; Robb et al., 1996) are also expressed in hematopoietic clusters and endothelial-like cells lining the ventral wall of the dorsal aorta at E10–11 and there is now strong evidence that all these transcription

factors are important for the onset of definitive hematopoiesis in the embryo.

Signaling through the Notch receptors is a widely used mechanism for cell fate specification and pattern formation in embryonic development and adulthood (Artavanis-Tsakonas et al., 1999; Lai, 2004; Lewis, 1998). The interaction between Notch receptors and ligands results in the cleavage of the intracellular domain of Notch that translocates to the nucleus and together with RBPjk (Rbpsuh – Mouse Genome Informatics) activates gene transcription. The best-characterized Notch-target genes are the orthologs of the Hairy and enhancer of split (Hes) and Hes-related (Hrt) proteins (for a review, see Iso et al., 2003). Notch family members have been identified in several hematopoietic cell types from diverse origin and there is now strong evidence that they participate in the control of hematopoietic differentiation in many different lineages (Han et al., 2002; Radtke et al., 1999; Stier et al., 2002).

The first evidence showing the involvement of Notch in the onset of embryonic hematopoiesis has recently been published, confirming that development of hematopoietic cells from the hemogenic endothelium is a Notch1-regulated event and it is impaired in Notch1-deficient embryos (Hadland et al., 2004; Kumano et al., 2003). We show here that this is an *RBPjk*-dependent event, since *RBPjk* mutant embryos also lack intra-embryonic hematopoiesis. Endothelial cells are not affected, as previously seen in the *Notch1* mutant embryos. We identify several Notch family members showing distinct expression patterns in presumptive E9.5 and 10.5 hemogenic endothelium, suggesting that different Notch signals may operate in this system. We also present evidence that Notch1 directly regulates the expression of *Gata2*, thus suggesting that one of the first events in embryonic hematopoietic determination consists in the activation of *Gata2* expression by Notch1/RBPjk.

Materials and methods

Animals

RBPjk null mice have been previously described (Oka et al., 1995). Whole embryos were dissected from the decidual tissue of timed-pregnant females (E9.5–10.5 gestation embryos) under a dissecting microscope. Embryos were genotyped according to morphological criteria or by PCR (Oka et al., 1995).

Cell lines

32Dcl3 wild-type (32D-wt) and activated Notch1-expressing 32D cells (32D-N1^{IC}) have been extensively characterized (Bigas et al., 1998; Milner et al., 1996). Cells were maintained in Iscove's 10% fetal bovine serum (FBS) and 10% IL-3-conditioned media.

RT-PCR

Total RNA from dissected wild-type and *RBPjk* mutant embryonic P-Sp was isolated using TRIzol Reagent (Invitrogen). Poly-AT Tract System IV (Promega) and RT-First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech) was used to obtain mRNA and cDNA respectively. PCR product was analyzed at 35 and 40 cycles to avoid saturation. Quantity One software (Biorad) was used for densitometry. Oligonucleotide sequences will be given under request.

Hematopoietic colony assay

The P-Sp from E9.5 wild-type and *RBPjk* mutant embryos was digested in 0.1% collagenase (Sigma) in PBS, 10% FBS and 10% IL3-

and stem cell factor (SCF)-conditioned medium for 1 hour at 37°C. One hundred thousand cells were plated in 1% methylcellulose (Stem Cell Technologies) plus Iscove's with 10% FBS, 10% IL3- and SCF-conditioned medium, 2.5% L-glutamine, 0.1% monothioglycerol (Sigma), 1% Pen/Strep (Biological Industries), 2 IU/ml erythropoietin (Laboratorios Pensa), 20 ng/ml GM-CSF (PeproTech) and 100 ng/ml of G-CSF (Aventis Pharma). After 7 days, the presence of hematopoietic colonies was scored under a microscope. For liquid cultures, the P-Sp region was dissected from embryos and dissociated by gentle pipetting. One hundred thousand cells were plated in Iscove's with 10% FBS, 10% IL3- and SCF-conditioned medium, 0.1% monothioglycerol, 2.5% L-glutamine and 1% Pen/Strep. Non-adherent cells were recovered and analyzed after 6 days.

Flow cytometry analysis

For flow cytometry (FACS) assay, 75,000 non-adherent cells were stained with anti-CD45-FITC or IgG-FITC (Pharmingen). Cells were analyzed by FACScalibur (Becton&Dickinson) and WinMDI2.8 software. Dead cells were excluded by 7-aminoactinomycin-D staining.

Immunostaining

Wild-type and *RBPjk* null embryos (E9.5) were frozen in tissue-tek OCT (Sakura) and sectioned (10 μm). Slides were fixed with -20°C methanol for 15 minutes and blocked-permeabilized in 10% FBS, 0.3% Surfact-AmpsX100 (Pierce) and 5% non-fat milk in PBS for 90 minutes at 4°C. Samples were stained with rat anti-PECAM (Pharmingen) at 1:50 in 10% FBS, 5% non-fat milk in PBS overnight and HRP-conjugated rabbit anti-rat antibody (Dako) at 1:100 for 90 minutes and developed with Cy3-coupled tyramide (PerkinElmer). Sections were mounted in Vectashield medium with 4'6-diamidino-2-phenylindole (DAPI) (Vector).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (Aguilera, 2004). In brief, crosslinked chromatin from 32D cells or whole E9.5 embryos was sheared by sonication with a UP50H Ultrasonic Processor (2 minutes, four times), incubated overnight with anti-N1 antibody (sc-6014) or α-N1 (Huppert et al., 2000) and precipitated with protein G/A–Sepharose. Cross-linkage of the co-precipitated DNA-protein complexes was reversed, and DNA was used as a template for semiquantitative PCR to detect the mouse *Gata2*_{IG} (from -435 to -326), *Hes1* (from -175 to +13), β-globin (from +125 to +309) promoters. PCR primers will be given under request.

Whole-mount in-situ hybridization

Whole-mount in-situ hybridization (WISH) was performed according to standard protocols (de la Pompa et al., 1997). For histological analysis, 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.

Double in-situ hybridization

Wild-type embryos (E10.5) were frozen in OCT and sectioned (10 μm). Sections were fixed in 4% paraformaldehyde for 10 minutes, digested with 1 μg/ml proteinase K (Roche) in 50 mmol/l TrisHCl pH 7.5, 5 mmol/l EDTA buffer and permeabilized with 1% Surfact-Amps X100 (Pierce) in PBS. After incubation with 3% H₂O₂ (Sigma) in PBS, slides were prehybridized for 1 hour and hybridized overnight at 70°C with fluorescein-tagged or digoxigenin-tagged probes. Anti-fluorescein and anti-digoxigenin-POD antibodies (Roche) were used at 1:1000 in Blocking reagent (Roche). Slides were developed using the tyramide amplification system, TSA-Plus Cyanine3/Fluorescein System (PerkinElmer) and mounted in glycerol:water.

Image acquisition

Images were acquired with an Olympus BX-60 for embryonic sections and with a Leica MZ125 for whole embryos using a Spot camera and Spot3.2.4 software (Diagnostic Instruments). Images for liquid cultures were acquired with an Olympus IX-70 using a video camera and Image-Pro-Plus4.5.1 software. Adobe Photoshop 6.0 software was used for photograph editing.

Results

Notch1 and *Notch4* are expressed in the endothelium of the P-Sp/AGM region

In the embryo, hematopoietic cells originate from the aortic floor in the P-Sp/AGM region in close association with endothelial cells. Hemogenic activity in this region is concentrated between E8.5 and 12.5 (Cumano et al., 2001; Medvinsky and Dzierzak, 1996), and expression of genes that are critical for the generation of hematopoietic cells are first detected in the endothelium of the P-Sp/AGM as early as E9.5 (North et al., 1999; Minegishi et al., 1999). Thus, crucial

decisions that specify the hematopoietic phenotype and are likely to involve the Notch pathway are occurring at this embryonic stage. In order to identify the Notch family members that may be involved in the onset of definitive hematopoiesis, we studied their expression in the endothelium of the aorta on transverse sections through the trunkal region of E9.5 and 10.5 mouse embryos (Fig. 1A). WISH revealed that *Notch4* mRNA was widely distributed in the aorta endothelium, whereas *Notch1* was restricted to a few individual cells at the ventral wall of the dorsal aorta in E9.5 and 10.5 embryos (Fig. 1B). *Notch2* or *Notch3* expression was not detected in the aorta, although there was expression in other tissues, such as heart or neural tube. This is consistent with the lack of hematopoietic defects in the *Notch2* mutant embryos (Kumano et al., 2003). Interestingly, the *Notch1* patched pattern was specifically detected in the aorta of sections

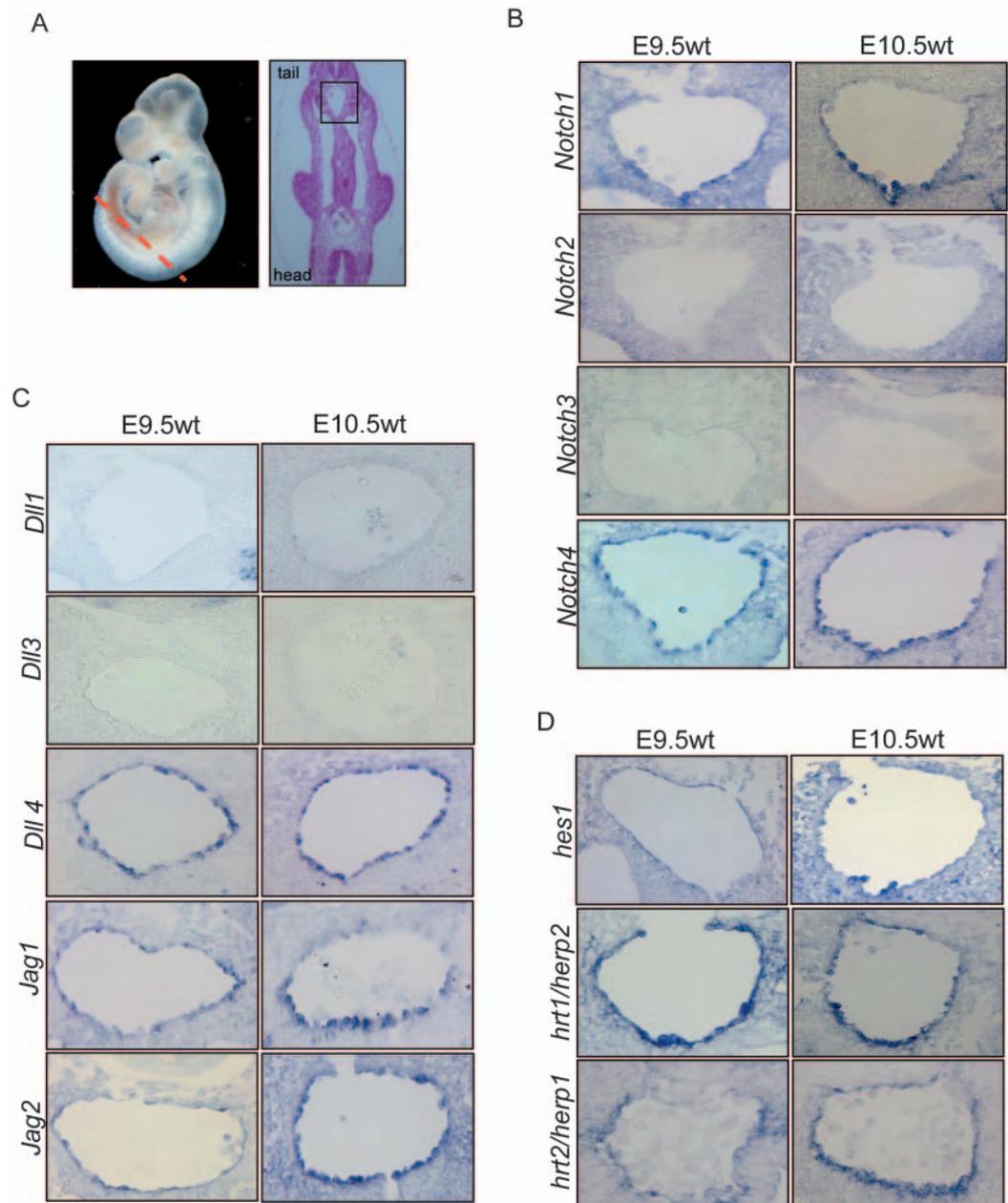


Fig. 1. Expression of Notch family members in the endothelium of the P-Sp/AGM aorta. (A) E9.5 embryo, indicating the site for P-Sp/AGM aorta and hematoxylin-eosin staining of a transverse section at the indicated level (100 \times) (B,C,D) Whole-mount of (B) Notch receptors, (C) Notch-ligands and (D) Notch-target genes and transverse sections of E9.5 and 10.5 aortas. (B) *Notch1* is expressed in few scattered cells at E9.5 and these cells increase at E10.5. *Notch2* and *Notch3* are not expressed in the aorta. *Notch4* shows a homogenous staining pattern in most of the cells of the endothelium at E9.5 and 10.5. (C) *Dll1* and *Dll3* are not expressed in the aorta. *Jag1*, *Jag2* and *Dll4* are expressed in few scattered cells at E9.5 and these cells increase at E10.5 (D) *Hes1* is not expressed at E9.5 but shows expression at E10.5 in cells budding from the endothelium. *Hrt1/herp2* is expressed in the ventral endothelium and hematopoietic clusters at E9.5 and 10.5. *Hrt2/Herp1* shows more diffused expression at E9.5 and ventral endothelium at E10.5. Orientation of the aortas is dorsal (up) to ventral (down).

containing mesonephric tissue, where hematopoietic precursors are generated, whereas in other regions of the aorta its distribution was more general and the patched pattern was lost (data not shown). Interestingly, this *Notch1* patched expression pattern was similar to that described for the transcription factors involved in the generation of the definitive hematopoietic cells in the embryo (Minegishi et al., 1999; North et al., 1999) (Fig. 3), in agreement with previous observations indicating a role for Notch1 in the determination of definitive hematopoietic cells (Kumano et al., 2003).

The Notch ligands *Jag1*, *Jag2* and *Dll4* are expressed in the ventral endothelium of the P-Sp/AGM region

Notch receptors exist in an inactive form on the cell surface until they interact with the appropriate ligand expressed in the neighboring cells (Fortini et al., 1993). To determine which Notch ligands may play a role in the activation of the Notch pathway in the P-Sp/AGM region at E9.5-10.5, we analyzed the expression pattern of the Jagged and Delta homologs by WISH. We detected that *Dll4*, *Jag1* and *Jag2* were specifically expressed in this region (Fig. 1C). *Dll4* was expressed in most of the aortic endothelial cells of the P-Sp/AGM region at E9.5

and 10.5. By contrast, *Jag1* and *Jag2* were expressed in scattered cells at E9.5 and were strongly increased throughout the ventral portion of the dorsal aorta at E10.5 (Fig. 1C). This characteristic expression pattern, restricted to individual cells on the floor of the aorta in the P-Sp/AGM region, was similar to that observed for *Notch1* (Fig. 1B). Altogether, these expression patterns suggest that Notch1 activation is involved in the onset of definitive hematopoiesis in this region of the aorta and presumably mediated by *Jag1*, *Jag2* and/or *Dll4* ligands.

The Notch pathway is activated in the P-Sp/AGM aorta

To confirm that the Notch pathway is activated in the P-Sp/AGM aorta, we next determined the expression of different Notch-target genes such as *Hes1* and Hes-related protein 1 and 2 (*Hrt1* and *Hrt2*). Consistent with previous reports, *Hrt1* and *Hrt2* are expressed in endothelial cells of the aorta (Nakagawa et al., 2000), although their expression patterns are not completely homogenous, showing a preferential ventral staining in the AGM region at E9.5 and 10.5 (Fig. 1D). We could not detect *Hes1* expression in E9.5 aorta, whereas a strong upregulation was observed in few ventral cells and in hematopoietic clusters arising from the endothelium at E10.5 (Fig. 1D). Thus, different Notch-target genes display specific temporal and spatial expression patterns in the aorta, suggesting that they could be playing different roles in early hematopoietic/endothelial decisions.

RBPj κ mutant embryos display an aberrant expression of Notch receptors and ligands in the P-Sp/AGM region

There is strong evidence from a variety of systems that Notch signaling participates in

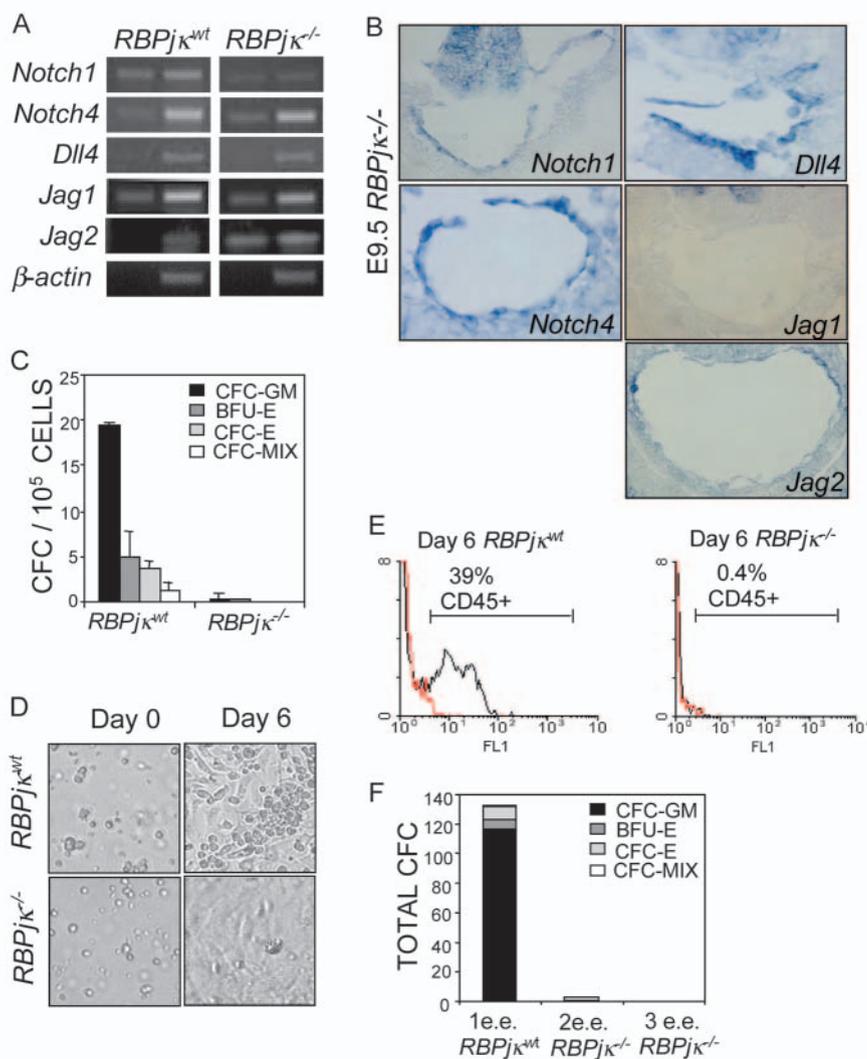


Fig. 2. Intra-embryonic hematopoiesis is impaired in the *RBPj κ* mutant embryos and they display an aberrant expression of Notch family members in the aorta. (A) Semiquantitative RT-PCR analysis in dissected P-Sp of E9.5 wild-type and *RBPj κ* mutants. Representative PCR products after 35 and 40 cycles of two independent experiments are shown. (B) WISH, with the indicated probes and transverse sections of E9.5 aortas of *RBPj κ* mutants. Orientation of the aortas is dorsal (up) to ventral (down). (C) Hematopoietic CFC from dissected P-Sp of E9.5 wild-type and *RBPj κ* mutants. Bars represent the average number of CFCs and standard deviation from three different embryos. (D) Liquid cultures with IL3 and SCF-conditioned media from P-Sp of E9.5 wild-type and *RBPj κ* mutants at days 0 and 6. (E) After 6 days in culture, cells were assayed for the expression of CD45 by flow cytometry and (F) the number of CFCs generated. Bars represent the average number of CFCs obtained from one wild-type embryo and pools of two or three mutant embryos equivalent (e.e.) in three independent different experiments.

the transcriptional regulation of several Notch receptors and ligands by positive (Barrantes et al., 1999; Timmerman et al., 2004) or negative (Chitnis, 1995; de la Pompa et al., 1997; Heitzler et al., 1996) feedback mechanisms. Since most of these regulatory networks depend on the *RBPjk* transcription factor (Heitzler et al., 1996; Timmerman et al., 2004), we investigated whether the expression of the different Notch family members is affected in the aorta of *RBPjk* mutant embryos (Oka et al., 1995). We first compared the expression by semi-quantitative RT-PCR of Notch receptors and ligands in the dissected P-Sp/AGM region from wild-type and mutant embryos at E9.5. We consistently observed a decrease in the expression of *Notch1* in the *RBPjk* mutant embryos compared with the wild type, while we did not detect important changes in the level of expression of *Notch4* or the different Notch ligands (Fig. 2A).

When we specifically studied the expression of these genes in the aorta endothelium using WISH, we observed decreased *Notch1* mRNA levels in the *RBPjk* mutant embryos (Fig. 2B) compared with the restricted but strong expression observed in the wild-type aortas (see Fig. 1B), as detected by RT-PCR. By contrast, expression of *Jag1* and *Jag2* was specifically impaired in the aorta endothelial cells (Fig. 2B), whereas their

expression was not affected in adjacent tissues in this region (data not shown). These results further confirm that expression and distribution of different Notch ligands and receptors depend on *RBPjk* as previously published (Heitzler et al., 1996) and points out the possibility that specific interactions between these proteins may regulate the proper cellular specification in the P-Sp/AGM aorta.

Intra-embryonic hematopoiesis is impaired in the *RBPjk* mutant embryos

To investigate whether Notch/*RBPjk* signaling plays a role in hematopoietic determination in the aorta, we next assayed the hematopoietic activity contained in the P-Sp/AGM region of *RBPjk* mutant embryos compared with wild type. Despite the presence of several developmental abnormalities and disorganized vasculature, the majority of the *RBPjk* mutant embryos (more than 80%) display a regular fused aorta in the trunkal region at E9.5 (Oka et al., 1995). As *RBPjk* mutants die at E10, we performed direct hematopoietic colony assays with cells obtained from P-Sp/AGM at E9.5. Hematopoietic colony forming cells (CFCs) of the different lineages were generated in cell cultures from wild-type embryos whereas few rare colonies were obtained from the cultures from *RBPjk*

mutant littermates in the same conditions (Fig. 2C).

We speculated that *RBPjk* mutant embryos contained lower numbers of HSC that may be undetectable in the direct CFC cultures. To test this possibility, we expanded the number of progenitors by incubating cells from single wild-type P-Sp/AGM compared with pools of two or three mutant P-Sp/AGM in liquid cultures with cytokines for 6 days. As shown in Fig. 2D, liquid cultures from both wild-type and mutant embryos formed equivalent stromal cell layers after 6 days, although only wild-type cultures contained non-adherent, round-shaped, hematopoietic-like cells (Fig. 2D). By flow cytometry, we demonstrated that liquid cell cultures from wild-type embryos contained 30-50% of CD45+ cells (Fig. 2E) that corresponded to the non-adherent population (data not shown). In agreement with the absence of hematopoietic-like cells, this CD45+ population was not detected in the mutant cultures (Fig. 2E). Cells

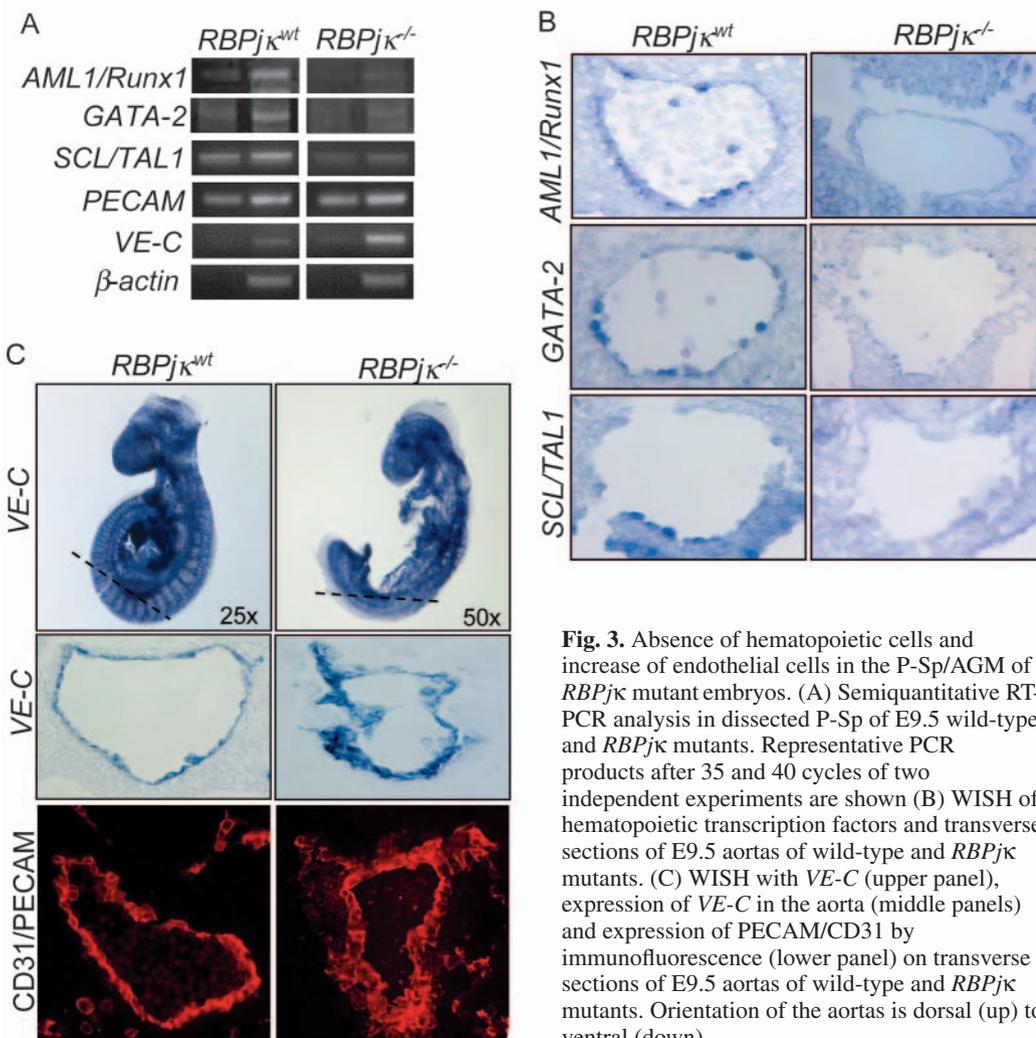


Fig. 3. Absence of hematopoietic cells and increase of endothelial cells in the P-Sp/AGM of *RBPjk* mutant embryos. (A) Semiquantitative RT-PCR analysis in dissected P-Sp of E9.5 wild-type and *RBPjk* mutants. Representative PCR products after 35 and 40 cycles of two independent experiments are shown (B) WISH of hematopoietic transcription factors and transverse sections of E9.5 aortas of wild-type and *RBPjk* mutants. (C) WISH with *VE-C* (upper panel), expression of *VE-C* in the aorta (middle panels) and expression of PECAM/CD31 by immunofluorescence (lower panel) on transverse sections of E9.5 aortas of wild-type and *RBPjk* mutants. Orientation of the aortas is dorsal (up) to ventral (down).

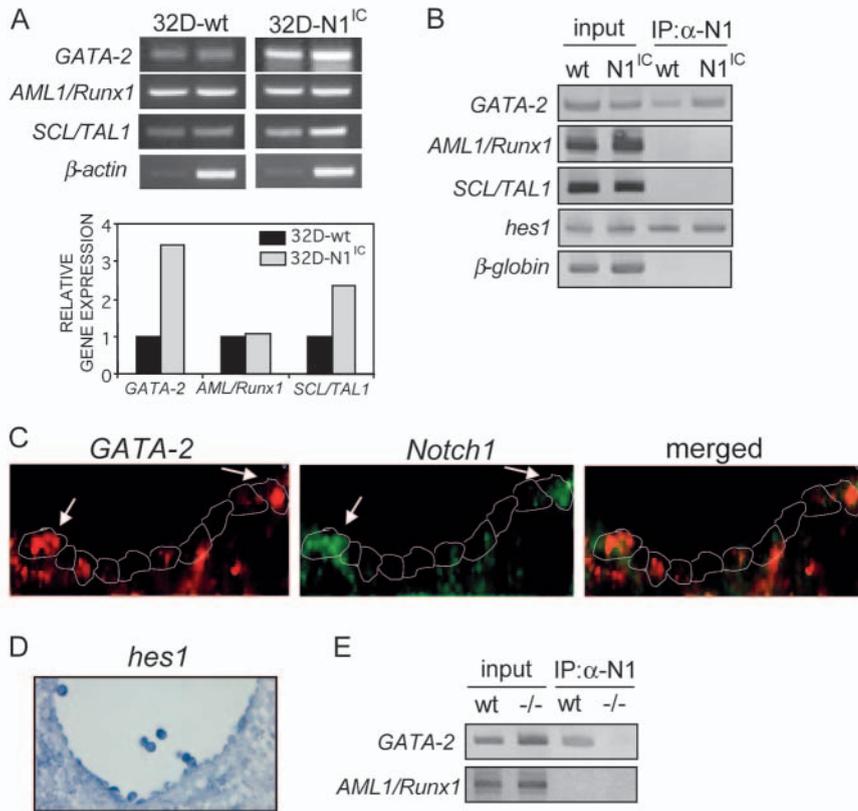


Fig. 4. Notch1/RBPjk regulates *Gata2* transcriptional activity. (A) Semiquantitative RT-PCR analysis of *Gata2*, *Aml1* and *Scl* expression in 32D wild-type or N1IC-expressing cells. Representative PCR products after 35 and 40 cycles of two independent experiments are shown. Quantitated relative mRNA levels of *Gata2*, *Aml1* and *Scl* are shown in the lower graph. (B) Chromatin immunoprecipitation with anti-N1 from 32D wild-type cells and 32D-N1^{IC} cells. PCR detection of the *Gata2*, *Aml1* and *Scl*, *Hes1* and β -globin promoters from the precipitates is shown. (C) Double in-situ hybridization with *Gata2* and *Notch1* on transverse section of wild-type E10.5 aortas. (D) Section of WISH that shows *Hes1* expression in hematopoietic clusters budding from the aorta from E10.5. (E) Chromatin immunoprecipitation with anti-N1 (α -N1) from wild-type and *RBPjk* mutant whole E9.5 embryos. PCR detection of the *Gata2* and *Aml1* promoter is shown.

from wild-type cultures generated CFCs with a predominant granulo-monocytic morphology, although colonies from other lineages were also observed (Fig. 2F). By contrast, we did not observe any hematopoietic colonies from the *RBPjk* mutant cultures (Fig. 2F). These results indicate that Notch signaling through *RBPjk* is required for the generation of the hematopoietic progenitors in the P-Sp/AGM.

Absence of hematopoietic cells and increase of endothelial cells in the P-Sp/AGM of *RBPjk* mutant embryos

Difficulties in characterizing HSCs in the P-Sp/AGM endothelium reside in the lack of specific HSC markers. In fact, endothelial markers were expressed in all the cells in the P-Sp/AGM endothelium, including the cells that would generate the HSCs. Thus, specific hematopoietic transcription factors such as *Aml1*, *Gata2* and *Scl* are widely used to identify these endothelial-like cells that will generate the hematopoietic clusters (Minegishi et al., 1999; North et al., 1999). These hematopoietic markers are expressed in individual rare cells in the floor of the dorsal aorta of the AGM region (North et al., 2002; Porcher et al., 1996; Tsai and Orkin, 1997) (Fig. 3B). To better understand the mechanisms by which definitive hematopoiesis is abrogated in *RBPjk* mutant embryos, we studied the expression of these genes together with endothelial genes in the P-Sp/AGM region in wild-type and mutant E9.5 embryos. RT-PCR showed reduced expression of the hematopoietic transcription factors *Aml1*, *Gata2* and *Scl* but higher expression of the classical endothelial marker *VE-cadherin* (*VE-C*) in dissected P-Sp/AGM regions of *RBPjk* mutants, compared with wild-type embryos (Fig. 3A). Next, we

investigated the expression of these transcription factors specifically in the endothelium of the aorta using WISH. We observed few cells expressing *Aml1*, *Gata2* and *Scl*, mainly localized in the ventral wall of the dorsal aorta in wild-type embryos as expected, whereas no expression was detected in the aorta endothelium of *RBPjk* mutant embryos (Fig. 3B). These results are consistent with the lack of hematopoietic precursors in these mutants (Fig. 4). In addition, we detected expression of *VE-C* gene in a multiple-layered endothelium in some regions of the aorta in the *RBPjk* mutant embryos (Fig. 3C). The endothelial nature of these cells was confirmed by PECAM/CD31 immunofluorescence staining. By contrast, in wild-type embryos *VE-C*/PECAM-expressing cells were restricted to a one-cell layer in the aorta (Fig. 3C). In addition, we detected a moderate increased percentage of PECAM/CD31-positive cells by flow cytometry in the mutant embryos (data not shown). These observations may reflect that the impairment of hematopoietic determination in the aorta results in an increase in the endothelial lineage.

Notch1 regulates *Gata2* transcriptional activity through *RBPjk*

Results from both RT-PCR and WISH indicate that *Gata2*, *Aml1* and *Scl* expression was greatly reduced not only in the aorta (Fig. 5) but also in other tissues in *RBPjk* mutants (data not shown). In previous work we have extensively characterized 32D cell lines stably expressing activated Notch1 (32D-N1^{IC}) (Bigas et al., 1998; Milner et al., 1996). Consistent with a role for Notch1 regulating hematopoietic transcription factors, we detected a threefold increase in *Gata2* mRNA levels, and a twofold increase in *Scl* levels in 32D-N1^{IC} cells compared with 32D wild-type (32Dwt) by RT-PCR (Fig. 4A), whereas there were no changes in *Aml1* expression. To test whether Notch1 was controlling the expression of these genes by a direct association with their promoters, we performed chromatin immunoprecipitation assays with anti-Notch1 antibody from both cell types. We consistently detected the

Gata2 promoter in the precipitates from both 32Dwt and 32D-N1^C cells (Fig. 4B). The amount of *Gata2* promoter was higher in the precipitates from cells expressing activated Notch1 as expected. By contrast, we could not detect *Scl* or *Aml1* promoters in the Notch1 precipitates. As a control, we detected binding of Notch1 to the Notch-target gene *Hes1*, while no interaction was detected with the β -globin promoter (Fig. 4B). Together, these results suggest that, unlike *Gata2*, *Aml1* and *Scl* are not direct targets of Notch1. As *Gata2* is crucial for the development of HSCs in the P-Sp/AGM region (Tsai et al., 1994), we hypothesized that the role of Notch1/RBPjk in the formation of embryonic HSCs may involve the transcriptional activation of *Gata2*. We next investigated whether cells in the endothelium of the aorta were co-expressing *Notch1* and *Gata2* by double in-situ hybridization. We observed that presumptive hematopoietic cells in the ventral wall of the aorta that expressed *Gata2* corresponded to the high *Notch1*-expressing cells (Fig. 4C). Moreover, the expression of *Hes1* in the emerging hematopoietic clusters (Fig. 4D) demonstrates that the Notch pathway is active in these cells.

As we identified two putative RBPjk binding sites in the *Gata2* promoter (Minegishi et al., 1997), we tested whether the association of Notch1 to *Gata2* was dependent on RBPjk. By immunoprecipitating chromatin-associated Notch1, we specifically detected the *Gata2* promoter in the precipitates from wild-type embryos but not in those from RBPjk mutants. This strongly suggests that the interaction between Notch1 and the *Gata2* promoter was occurring in the embryo and that this interaction is dependent on RBPjk (Fig. 4E). Altogether, these results indicate that Notch1, together with RBPjk, regulates the expression of *Gata2* not only in hematopoietic cell lines but also in the mouse embryo.

Notch1⁺Gata2⁺ cells in the P-Sp/AGM endothelium are Jag1⁺Jag2⁻

Different expression levels of Notch receptors and ligands dictate the specification of different cell lineages (for a review, see Lai, 2004). To investigate the specific ligands that activate Notch1 in the presumptive hematopoietic cells in the aorta, we performed double in-situ hybridizations. It is well established that the ligands responsible for activating Notch1 are expressed in cells adjacent to the Notch expressing one. We analyzed transverse sections of E10.5 embryos simultaneously hybridized with specific probes for *Notch1* and the different ligands that are expressed in the aorta endothelium at this developmental stage. We consistently observed that cells expressing *Notch1* (*Notch1*⁺) also expressed *Jag1* (Fig. 5A, upper panels),

whereas *Jag2* was specifically detected in cells adjacent to *Notch1*⁺ but not in the *Notch1*⁺ themselves (Fig. 5A, middle panels). *Dll4* showed a mixed pattern of co-expression with *Notch1*, in which some cells simultaneously expressed both *Notch1* and *Dll4* and other cells only expressed one of these genes (Fig. 5A, lower panels). Altogether, these results are consistent with a model in which *Jag2* or *Dll4* activate Notch1 in the ventral wall of the aorta. This event would initiate the hematopoietic program in the *Notch1*⁺ cells by activating the expression of *Gata2* (Fig. 5B). However, and considering that multiple ligands are simultaneously expressed in the endothelium of the aorta, it is tempting to speculate that back and forward signals between different members may occur.

Discussion

There is now evidence that Notch1 is required for the generation of intra-embryonic hematopoiesis (Hadland et al., 2004; Kumano et al., 2003). Here we show that this function is dependent on the transcription factor RBPjk and several members of the Notch family are likely to be involved. Consistent with the phenotype described for the *Notch1* mutant embryos, RBPjk mutants are deficient for intra-embryonic/definitive hematopoiesis. We propose that Notch1 activation in individual cells of the hemogenic endothelium regulates transcription of *Gata2*, which is essential for the generation and proliferation of HSCs (Tsai et al., 1994).

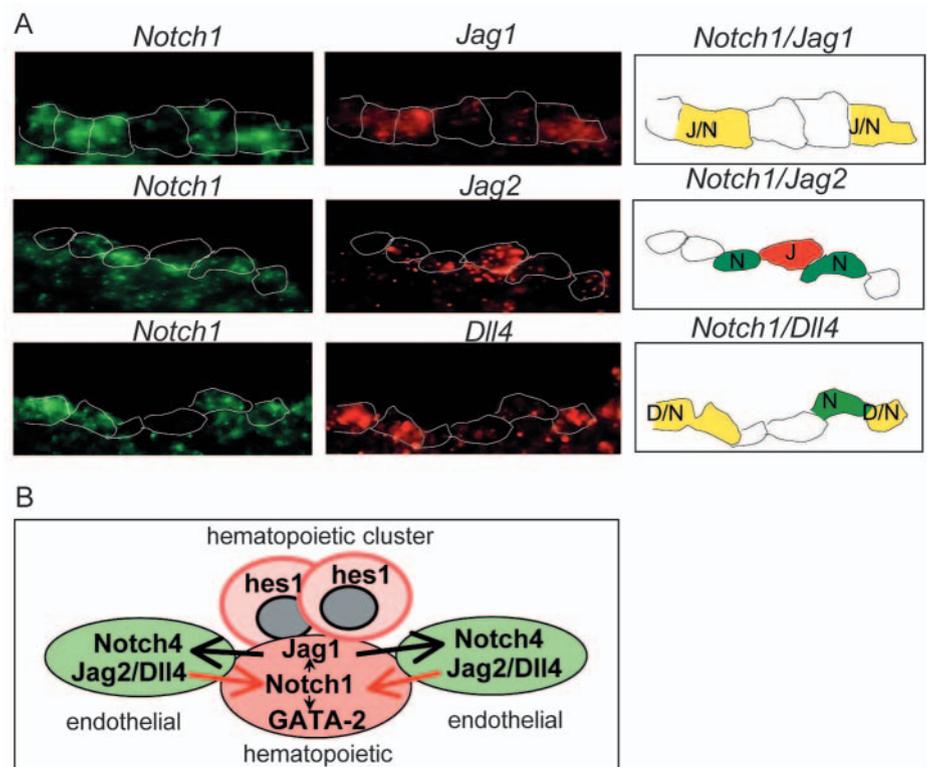


Fig. 5. *Notch1*⁺/*Gata2*⁺ cells in the P-Sp/AGM endothelium are *Jag1*⁺/*Jag2*⁻. Double in-situ hybridization on transverse section of wild-type E10.5 aortas. (A) Hybridization of *Notch1* with *Jag1* (upper), *Jag2* (middle) and *Dll4* (lower panels). Representative photographs of at least three hybridizations are shown (B) Model for Notch function in the formation of hematopoietic clusters from the aorta endothelium during development.

***RBPjk*-dependent Notch function in the generation of intra-embryonic hematopoiesis**

The origin of definitive HSCs from an endothelial/hematopoietic common progenitor known as hemangioblast is still controversial. While the yolk sac is a primary site of hematopoietic development, several lines of evidence support the idea that, under physiological conditions, HSCs are generated de novo within the endothelium lining the ventral wall of the aorta of the P-Sp/AGM region (Cai et al., 2000; de Bruijn et al., 2002). Our work demonstrates that intra-embryonic hematopoiesis is abolished in the *RBPjk* mutant embryos, presumably due to impaired hematopoietic progenitor determination from endothelial-like precursors in the aorta. This correlates with the absence of expression of hematopoietic transcription factors in this region in the mutant embryos compared with wild type. Furthermore, expression of classical endothelial markers, such as VE-cadherin and PECAM, is increased in the embryonic aortas of these mutants, suggesting that in the absence of Notch signaling, the endothelial lineage is favored at the expense of the hematopoietic one. While this work was in progress, it was reported that *Notch1*-deficient embryos have impaired intra-embryonic hematopoiesis due to a defect in hematopoietic determination from endothelial cells (Kumano et al., 2003), and that *Notch1*-deficient embryonic stem cells cannot contribute to definitive hematopoiesis in chimeric embryos (Hadland et al., 2004). Our results are in agreement with a role of Notch1 in the onset of definitive hematopoiesis through a transcriptional activation mechanism dependent on *RBPjk*. Although the expression of other hematopoietic genes such as *Scl* and *Aml1* is severely affected in the *RBPjk* mutants, we showed that only *Gata2* is a direct target of Notch1/*RBPjk* signaling. As *Gata2* is required to maintain the pool of undifferentiated hematopoietic progenitors (Tsai and Orkin, 1997), we speculate and present evidence that the absence of *Gata2* in the *RBPjk* mutants could be responsible for the lack of hematopoietic progenitors in these mutants and is likely in the *Notch1* mutants (Kumano et al., 2003). In agreement with this, the maintenance of undifferentiated 32D myeloid progenitors by Notch1 has been associated with *Gata2* expression (Kumano et al., 2001). Our work demonstrates that most of the cells in the aorta that express *Notch1* simultaneously express *Gata2*. This result, together with the demonstration by chromatin precipitation assays that intracellular Notch1 associates with the *Gata2* promoter, strongly suggests that Notch1 may regulate the generation and maintenance of hematopoietic progenitors by directly activating the expression of *Gata2*.

Using in-situ hybridization, we detected high levels of expression of the *Hes1* gene in a few endothelial cells as well as in the hematopoietic clusters of the aorta, thus suggesting that Notch activation is concomitant with the formation of these clusters. The function of *Hes1* in the maintenance of HSC has not been studied in vivo; however, several pieces of evidence confirm that *Hes1* is regulating cell differentiation in different hematopoietic cell types (Kawamata et al., 2002; Kumano et al., 2001). These studies together with our results suggest that *Hes1* could be involved in maintaining the immature phenotype of the hematopoietic precursors budding from the aorta and/or in repressing the expression of specific

endothelial markers in these cells. The detection of other Notch-target genes, such as *Hrt1* and *Hrt2* (E9.5), preceding *Hes1* expression confirms that Notch is active at this embryonic stage. However, the role of these *Hes*-related proteins in the cellular specification of the aorta remains to be determined.

Lateral inhibition or lateral induction in P-Sp/AGM hematopoietic determination

During the development of complex multicellular organisms, numerous cell-cell signaling events are required for proper cell-fate determination. Two different Notch signaling mechanisms have been proposed: lateral inhibition and lateral induction (reviewed by Lewis, 1998). Singling out an individual cell or group of cells from initially equivalent cells is known as lateral inhibition, whereas lateral induction implies the adoption of cellular fates cooperatively. In lateral inhibition Notch activation leads to Delta downregulation, while in lateral induction activation of Notch leads to Delta upregulation. A typical example of lateral inhibition mediated by Notch is the process of neurogenesis in *Drosophila* (Artavanis-Tsakonas et al., 1999) and vertebrates (Chitnis, 1995), while lateral induction occurs during wing margin development in *Drosophila* (Panin et al., 1997), somite formation (reviewed by Lewis, 1998) and endocardial development (Timmerman et al., 2004). To define whether the determination of hematopoietic cells in the mid-gestation aorta is compatible with one of these mechanisms, it is crucial to know the expression pattern of Notch receptors and ligands at this stage, as well as the characterization of the aorta hematopoietic potential of the different mutant embryos. Although Notch family members have been detected in many adult and embryonic hematopoietic tissues, this is the first time that E9.5-10.5 P-Sp/AGM aorta endothelium has been studied by single and double in-situ hybridization and the expression of these genes has been analyzed on transverse sections through the trunkal region. Our analysis reveals co-expression of multiple Notch-family members in these cells at this developmental stage, strongly suggesting that several Notch signals are likely to be involved in hematopoietic determination. For example, *Jag1* is co-expressed with *Notch1* in most of the endothelial cells, while the *Jag2* transcript is absent from these cells and specifically expressed in the cells neighboring the *Notch1*⁺ ones. Moreover, *Jag1* is absent from the endothelium of *RBPjk* mutant embryos, strongly suggesting that its expression depends on Notch1 activation in this tissue.

An important question to be determined is how specific expression patterns of Notch family members are acquired. For example, the endothelium covering the aorta outside the AGM region has a very homogenous pattern of *Notch1* or *Dll4* expression in the majority of cells (data not shown), while the scattered expression pattern is restricted to the AGM aorta. Considering this, it is tempting to speculate that the aorta endothelium originates as a pool of equivalent Notch- and ligand-expressing cells and lateral inhibition events will generate a 'salt and pepper' expression pattern that is reminiscent of that described for *Drosophila* neurogenesis (Artavanis-Tsakonas and Simpson, 1991). Once *Notch1* expression pattern in the P-Sp/AGM aorta is established, hemogenic endothelial cells have to undergo determination, proliferation and migration events that may require multiple local interactions with neighboring cells. Our results are

consistent with a model in which expression of *Notch1* in individual cells in the ventral wall of the aorta leads to the activation of *Gata2* that is crucial for the generation of a pool of definitive HSCs. Loss of *Gata2* expression in the *RBPjk*-deficient embryos results in the loss of the HSC pool and in the absence of definitive hematopoiesis (Fig. 5B). This model implies that, similarly to the situation in other developmental systems (de Celis et al., 1991), Notch1 acts cell-autonomously in promoting an HSC fate in the P-Sp/AGM aorta as previously proposed (Kumano et al., 2003). Our results support a role for Notch in the maintenance of a population of stem cells (HSCs) that are critical for the definitive hematopoiesis in the embryos and are consistent with the finding that alterations in the Notch function are responsible for leukemias (reviewed by Radtke and Raj, 2003). Gaining insight into the mechanism of Notch action will help to design therapeutical approaches for the treatment of such complex diseases.

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