Universitat de Barcelona

Facultat de Farmàcia

Departament de Bioquímica i Biologia Molecular

Role of Notch/RBPjκ signaling pathway in embryonic hematopoiesis

Alexandre Robert i Moreno

2007

Universitat de Barcelona
Facultat de Farmàcia
Departament de Bioquímica i Biologia Molecular
Doctorat en Biomedicina
Bienni 2001-03

"Role of Notch/RBPjκ signaling pathway in embryonic hematopoiesis"

Memòria presentada per Alexandre Robert i Moreno per optar al títol de doctor europeu per la Universitat de Barcelona.

Thesis presented by Alexandre Robert i Moreno to obtain the title of PhD (European Doctorate) by the Universitat de Barcelona.

This thesis has been supervised by doctor:

Anna Bigas Salvans, PhD

PhD student: Alexandre Robert Moreno

Tutor: Carme Caelles Franch, PhD

Alexandre Robert i Moreno

2007

Contents

Presentation 7

Acknowledgements 8

Abbreviations 9

Chapter 1. General introduction

Section 1. Embryonic and adult hematopoiesis

1.1 The hematopoietic system 15

1.1.1 Introduction to the hematopoietic system 15

* Definition of hematopoietic stem cell

1.1.2 Regulation of the hematopoietic system 18

1.1.2.1 The hematopoietic stem cell niche

1.1.2.2 Cytokines and cytokine receptor signaling

1.1.2.3 Hematopoietic transcription factors

1.1.2.4 MicroRNAs as hematopoietic regulators

1.1.2.5 Transdifferentiation / Reprogramming

1.1.3 Methodological approaches to study HSCs and hematopoietic progenitors 23

1.2 Ontogeny of the hematopoietic system in the mouse embryo 25

1.2.1 Brief introduction to mammalian embryonic development 25

1.2.2 Brief introduction to the hematopoietic system development in the mouse embryo 26

1.2.3 Primitive hematopoiesis 28

1.2.3.1 The yolk sac

1.2.3.2 Primitive hematopoiesis

1.2.4 Definitive hematopoiesis 29

1.2.4.1 Generation of the AGM region

1.2.4.2 Hematopoietic activity in the AGM region

1.2.4.3 Direct relationship between hematopoietic and endothelial lineages

1.2.4.4 Proposed models for AGM-derived HSCs emergence

1.2.5 Regulators of embryonic hematopoiesis 33

1.2.5.1 Developmental signaling pathways

1.2.5.2 Hematopoietic transcription factors

1.2.5.3 Cytokines

1.3 Hematologic disorders 38

1.3.1 Leukemias 38

1.3.2 Myelodysplastic syndromes 39

Section 2. Erythropoiesis

2.1 Erythroid differentiation 40

2.1.1 Primitive and definitive erythropoiesis 40

2.1.2 Erythroid markers 40

2.2 Regulation of embryonic and adult erythropoiesis 41
2.2.1 Cytokine-mediated regulation 41
2.2.2 Erythropoietic transcription factors 42
2.2.3 Apoptosis 43

2.3 Erythroid pathologies 45

Section 3. The Notch signaling pathway
3.1 Notch signaling and the control of cell fate 46
3.1.1 Key players and mechanism 46
3.1.2 Control of cell-fate decisions 48

3.2 Role of the Notch signaling pathway in hematopoiesis 50
3.2.1 Expression of Notch members in the hematopoietic system 50
3.2.2 Role of Notch in HSC self-renewal 50
3.2.3 Notch regulation of lymphoid cell-fate decisions 51
3.2.4 Notch in myeloid differentiation 51
3.2.5 Notch in apoptosis 51
3.2.6 Notch implication in the ontogeny of the hematopoietic system 52

3.3 Altered Notch signaling and disease 52

3.4 Animal models 53

Chapter 2. Aims 57

Chapter 3. Results

Section 1. RBPjκ-dependent Notch function regulates *Gata2* and is essential for the formation of intra-embryonic hematopoietic cells 61

Section 2. The Notch ligand Jagged1 is required for intra-embryonic hematopoiesis 77

Section 3. The notch pathway positively regulates programmed cell death during erythroid differentiation 95

Chapter 4. General discussion and future prospects 113

Chapter 5. Conclusions 123

Chapter 6. Resum de la tesi 127

Chapter 7. About the author 159

Chapter 8. References 163

Chapter 9. Publications 173

Presentation

The work presented in this thesis has been developed in the laboratory of Transcriptional Regulation of Stem Cells and Cancer sited in the Molecular Oncology Department in the *Institut de Investigació Biomèdica de Bellvitge (IDIBELL)* in Barcelona, Spain. This laboratory is co-directed by Dr. Anna Bigas Salvans and Dr. Lluís Espinosa Blai.

This study was the continuation of the work initiated in the lab about the role of the Notch signaling pathway in the hematopoietic system although it signified the initiation of the *in vivo* studies of Notch implication in the regulation of hematopoietic stem cells.

The present thesis exposes the implication of the Notch signaling pathway in the generation of hematopoietic cells during the first stages of embryonic development. This work gave rise to two publications: one about the role of Notch in intra-embryonic AGM hematopoiesis (published in Development) and the other about Notch involvement in extra-embryonic yolk sac hematopoiesis (published in Leukemia). Thus, this thesis has been written in the papers compilation format beginning with a General Introduction about embryonic and adult hematopoiesis and the Notch signaling pathway; the Results chapter that contains the two published papers (each one with their own Material and Methods description and Discussion) and the more recent results (that are not published yet) and finally, a General Discussion that integrates the whole work.

This thesis has been written in English plus a summary in catalan with the aim of obtaining the title of PhD with the European Doctorate mention by the *Universitat de Barcelona*.

Acknowledgements

Now that the work is done and looking back I would like to thank the support to those people who contributed to this thesis:

Dr. Anna Bigas and Dr. Lluís Espinosa for the supervision of the whole work during these years; specially to Dr. Anna Bigas, my supervisor. Also to Dr. Carme Caelles, my thesis tutor.

Dr. Júlia Inglés-Esteve for all the help in the diary work and all the questions about techniques, products…

To all my lab collegues, the ones that left (Dr. Lluís Riera, Dr. Cristina Aguilera…), the ones that remain (Vanessa Fernández-Majada, Cristina Ruiz, Verónica Rodilla) and the new ones (Tiago Guimaraes, Mª Eugènia López). It has been a pleasure to share the lab with all of you and good luck with your work! Also to Jessica González, Irene Mérida and to all the technicians that were “in practices”, for their technical support. Thanks as well to many of the people in the Molecular Oncology Department of IRO-IDIBELL.

Thanks to the people in the animal facility for their advisement, explanations and help. Specially to Mila González, Blanca Luena, Rosa Bonavia and Joana Visa. I would not like to forget the people in the Serveis Científico-Tècnics in Bellvitge: Ester Castaño for her teaching in the “hard world” of Flow Cytometry and Benjamín Castrejón for the confocal assistance.

Many thanks to the people that act as “godmothers” when I was alone in such a rainy country as The Netherlands during my 3 months stage in the Erasmus Medical College in Rotterdam. Specially to Dr. Claudia Orelio and Prof. Dr. Elaine Dzierzak, who supervised my work there. Thanks to the rest of Elaine’s lab for their dairly support.

Finally, thanks to the Department of Research and Universities (AGAUR) from the Catalan Government (Generalitat de Catalunya) and Institut de Recerca Oncològica (IRO-IDIBELL) for their financial support [CIRIT predoctoral fellowship (2002-SI00791) and other grants].

Last but not the least I would like to specially thank to those people who helped me outside the lab, specially to my girlfriend Mireia Mercé, my sister Mª del Mar Robert and my parents.

Abbreviations

(mi)RNA or miR: micro-interfering ribonucleic acid
(si)RNA: small-interfering ribonucleic acid
[E(spl)-C]: enhancer of split complex
7-AAD: 7-aminoactinomicin-D
AGM: aorta-gonad-mesonephros
AHSP: α-hemoglobin stabilizing protein
Alad: 5-aminolevulinic acid dehydratase
ALAS-E: δ-amino levulinic acid synthase-erythroid
ALL: acute lymphoblastic leukemia
AMKL: acute megakaryoblastic leukemia
AML: acute myeloid leukaemia
ANK: ankyrin repeats domain
APC: antigen presenting cells
B-ALL: B-cell acute lymphoblastic leukemia
bFGF: basic fibroblast growth factor
BFU-e: burst-forming unit-erythroid
b-HLH: basic helix-loop-helix
BM: bone marrow
BMP: bone morphogenetic protein
C/EBPα: CCAAT/Enhancer-Binding Protein-α
CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CFC: colony-forming cell
CFU-C: colony-forming unit-culture
CFU-e: colony-forming unit-erythroid
CFU-S: spleen colony-forming unit
ChIP: chromatin immunoprecipitation
CLL: chronic lymphocytic leukemia
CLP: common lymphoid progenitor
CML: chronic myeloid leukemia
CMP: common myeloid progenitor
CSL: CBF1, Supressor of hairless, Lag-1
CXCR4: C-X-C chemokine (SDF1) receptor 4
DAPI: 4’6-diamidino-2-phenylindole
DC: dendritic cells
DD: death domain
DISC: death-inducing signaling complexe
DISH: double in-situ hybridization
DMSO: dimethyl sulfoxide
DNA: deoxy-ribonucleic acid
DSL: Delta, Serrate and Lag-2
E*10.5*: embryonic day *10.5*
EB: embryoid bodies
EBF: early B-cell factor
EBP: early B-cell factor
EGF: epidermal growth factor
EKLF: erythroid Krüppel-like factor
EPO: erythropoietin
EpoR: erythropoietin receptor
EryP: primitive erythroid progenitors
ESC: embryonic stem cell
ETP: early T-lineage progenitor
FACS: fluorescence-activated cell sorting
FasL: Fas ligand
FasR: Fas receptor
FBS: fetal bovine serum

FGFR-1: fibroblast growth factor receptor-1
FOG-1: Friend of Gata-1
FTOC: fetal thymic organ culture
G-CFC: granulocyte colony-forming cell
G-CSF: granulocyte colony-stimulating factor
G-CSFR: granulocyte colony-stimulating factor receptor
GFP: green fluorescent protein
GM-CFC: granulocyte-macrophage colony-forming cell
GM-CSF: granulocyte-macrophage colony-stimulating factor
GMP: granulocyte-macrophage progenitor
GSK3β: glycogen-synthetase kinase 3β
HDAC: histone deacetylases
Herp: Hes-related protein
Hes: Hairy and Enhancer of Split
HMBA: hexametinelene-bisacetamide
HPC: hematopoietic progenitor cell
HPP-CFC: high proliferative potential colony-forming cell
Hrt: Hes-related
HSC: hematopoietic stem cell
IFN-γ: interferon-γ
IgM: immunoglobulin M
Ihh: indian hedgehog
IL: interleukin
Jag1: Jagged1
Jag2: Jagged2
JAK2: Janus kinase 2
KLF: Krüppel-like factor
KSL: c-Kit^{+} Sca-1^{+}Lin^{-} cells
LEF: lymphoid enhancer factor
LIF: leukemia inhibitory factor
LNR: LIN/Notch repeats
LTR-HSC: long-term repopulating hematopoietic stem cell
Mac-CFC: macrophage colony-forming cell
M-CSF: macrophage colony-stimulating factor
MDS: myelodysplastic syndrome
MEF: murine embryonic feeder
Meg-CFC: megakaryocyte colony-forming unit
MEL: murine erythroleukemia cell line
MEP: megakaryocyte-erythroid progenitor
Mix-CFC: erythroid and myeloid colony-forming cell
MPP: multipotent progenitor
mRNA: messenger ribonucleic acid
NK: natural killer cells
NOD-SCID: non-obese diabetic/severely compromised immunodeficient mice
NotchIC/NICD: intracel.lular domain of Notch
OSMR: oncostatin M receptor
PBS: phosphate-buffered saline
PECAM-1: platelet endothelial cell adhesion molecule-1
PI3K: phosphoinositide-3-kinase
P-Sp: para-aortic splanchnopleura
Ptch: patched
PV: polycythemia vera
qRT-PCR: quantitative reverse transcriptase-polymerase chain reaction
RBPjκ: recombinant binding protein-Jκ
RNAi: interference ribonucleic acid
SAPs: subaortic patches
Sca-1: stem cell antigen-1
SCF: stem cell factor
Scl: stem cell leukemia

SDF1: stromal cell derived factor 1
Shh: sonic hedgehog
Smo: smoothened
Sp: somite pairs
STR-HSC: short-term repopulating hematopoietic stem cell
Su[H]: Supressor of Hairless
TACE: TNF-α-converting enzyme
Tal-1: T-cell acute leukemia-1
T-ALL: T-cell acute lymphoblastic leukaemia
TCF: T-cell factor
TCR: T-cell receptor
TGFβ: transforming growth factor-β
TMD: transient myeloproliferative disorder
TNF-R: tumor necrosis factor receptor
TNF-α: tumor necrosis factor-α
TPO: thrombopoietin
UGR: urogenital ridges
UTR: untranslated region
VE-C: vascular endothelial cadherin
VEGF: vascular endothelial growth factor
WISH: whole mount in-situ hybridization
WT: wild-type
YS: yolk sac
Z-VAD-FMK:Z-Val-Ala-DL-Asp-fluoromethylketone

CHAPTER 1:

GENERAL INTRODUCTION

Section 1. Embryonic and adult hematopoiesis

1.1 The hematopoietic system

1.1.1 Introduction to the hematopoietic system

The hematopoietic system has developed through evolution to ensure nutrient supply and protection from external challenges in multicellular organisms. The blood is composed of a large variety of mature cell types with a limited life-span (i.e two days for neutrophils, thirty days for erythrocytes), thus blood cells need constantly to be replenished from a pool of hematopoietic stem cells (HSCs). This process is known as hematopoiesis [reviewed in (Godin and Cumano, 2002)].

The different hematopoietic cell types play different physiological functions throughout life. For example, red blood cells or erythrocytes are specialized in gas exchange within the different tissues (providing oxygen and eliminating carbon dioxide), whereas platelets are responsible for clotting processes in vessel fissures or wounds [reviewed in (Godin and Cumano, 2002)]. The other hematopoietic cell types are components of the immune system.

The innate immune system consists of a physical and chemical barrier formed by the epithelium with its secreted antimicrobial substances and a pool of specialized cells: macrophages, neutrophils, eosinophils, basophils and natural killer cells. Macrophages are phagocytic cells that clear exogenous particles and cellular debris; neutrophils are specialized in destroying bacteria, whereas eosinophils mainly act on parasites. Neutrophils and eosinophils are called granulocytes since they are filled with granules and lysosomes that contain cytotoxic agents. Mast cells and basophils are also granulocytes and help to the immune response against pathogens. Finally, the lymphoid-derived natural killer cells (NK) induce cytotoxicity in the antigen presenting target cells and the dendritic cells are also involved in the innate immune response.

The adaptive immune system is responsible for the specific defence against pathogens and the elimination of abnormal cells. This function is carried out by lymphocytes, a group that includes B and T lymphoid cells and cytotoxic T cells (Abas, Lichtman & Pober, "Immunología Celular y Molecular", McGraw-Hill, 1999).

During embryonic development, the major site of hematopoiesis shifts from one organ to another in a dynamic temporal and spatial manner. However, the bone marrow (BM) is the main hematopoietic organ after birth and is responsible for the generation of all the hematopoietic hierarchy in the adult [reviewed in (Orkin, 2000)]. Other important hematopoietic organs in the adult are the spleen and thymus (responsible for the final maturation of B and T cells, respectively), together with the lymph nodes.

For many years, hematopoiesis was conceived as a cascade of binary decisions, resumed in the Akashi-Kondo-Weissman model (see Figure 1A) (Akashi et al., 2000; Kondo et al., 1997). A limited number of HSCs are at the basis of the hematopoietic hierarchy and they have the ability to self-renew and differentiate into the common lymphoid or the common myeloid progenitors (CLP and CMP). These progenitors give rise to specific lineage committed progenitors that differentiate into mature cells. Once a cell is committed along a lineage the ability to self-renew and its plasticity decreases. However, recent experimental data suggest that

alternative developmental pathways generate myeloid and lymphoid cells from already committed hematopoietic progenitors that transdifferentiate to distant hematopoietic lineages, suggesting a more complicated hierarchical tree (see Figure 1B) (Adolfsson et al., 2005).

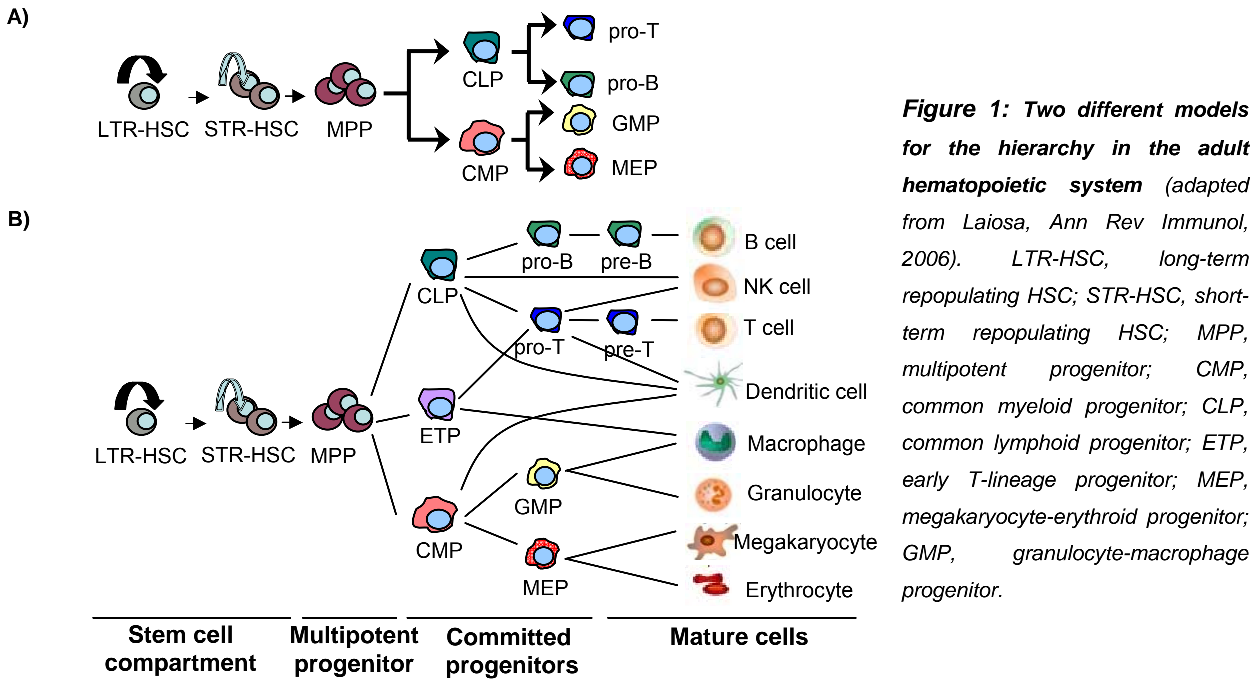


Figure 1: Two different models for the hierarchy in the adult hematopoietic system *(adapted from Laiosa, Ann Rev Immunol, 2006). LTR-HSC, long-term repopulating HSC; STR-HSC, short-term repopulating HSC; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; ETP, early T-lineage progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor.*

- **Definition of hematopoietic stem cell (HSC)**

Stem cells can be distinguished between embryonic and somatic stem cells (also referred as adult stem cells). Embryonic stem (ES) cells derive from early embryos and are totipotent since they are able to generate all the cell types and tissues in the adult animal. On the other hand, somatic stem cells are located in specific organs of the body and are responsible for replenishing specific tissue cells [reviewed in (Graf, 2002)].

Hematopoietic stem cells (HSC) are somatic stem cells that give rise to all the blood cell types in the organism [reviewed in (Orkin, 2000)]. They are first generated during embryonic development in the aorta surrounded by gonad and mesonephros (a region called AGM). Between 500 and 1000 HSCs are formed during E8.5 and E13 in the mouse embryo [reviewed in (Godin and Cumano, 2002).

HSCs represent a mostly quiescent pluripotent population that self-renews and are able to repopulate the whole hematopoietic system when transplanted into adult irradiated mice. In the adult mouse, HSCs are located in the bone marrow in a proportion of 1 to 10 HSC per 100,000 cells. There, they receive microenvironmental signals to either proliferate or differentiate to specific lineages (Cheshier et al., 1999). Recent studies of the *in vivo* repopulation capacity of clonally derived HSCs suggest that the HSC compartment consists of a limited number of HSCs each one with characteristic and limited repopulation capacity (Sieburg et al., 2006).

An important function of the stem cell niche is to regulate the balance between self-renewal and cell differentiation. This may be achieved by regulating asymmetric and symmetric HSC division (see Figure 2). Asymmetric division refers to the formation of two different daughter cells; one that remains in the niche as a

stem cell and the other one that leaves to differentiate. In contrast, symmetric division refers to cells that divide into two identical daughter cells, both remaining in the niche as stem cells.

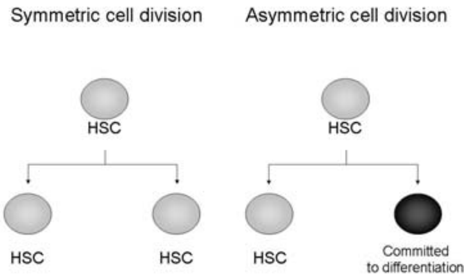


Figure 2: ***Symmetric and asymmetric cell division.*** *Symmetric division refers to HSCs that divide into two identical daughter HSCs, whereas asymmetric division refers to the formation of two different daughter cells, one that remains in the niche as a HSC and the other one that leaves to differentiate.*

Whether stem cells normally undergo symmetric, asymmetric or both types of divisions is yet to be determined. However, the stability in the number of HSCs in the adult bone marrow suggests that these cells are likely to perform asymmetric divisions under normal physiological conditions [reviewed in (Yin and Li, 2006)]. In contrast to the homeostatic behavior of adult HSCs, the number of HSCs dramatically increases during development (Ema and Nakauchi, 2000), which could be explained by a symetric division model.

The best-known and widely used technique for separating HSCs by Flow Cytometry is based on their expression of cell surface antigens. In the adult, mouse HSCs express Stem cell antigen-1 (Sca-1), high levels of c-Kit and are negative for lineage-specific markers (Lin^-) such as Mac1, B220, Gr1 and Ter119. This $c\text{-}Kit^+$ $Sca\text{-}1^+Lin^-$ population (KSL) contains long-term and short-term repopulating HSCs (LTR-HSCs/STR-HSCs) and multipotent progenitors without repopulation ability (Osawa et al., 1996). Selection for other cell-surface markers can divide HSCs into LTR-HSCs ($Thy1^{low}Flk2/Flt3^-$), STR-HSCs ($Thy1^{low}/Flt3^+$) and multipotent progenitors ($Thy1^-Flt3^+$). In humans, CD34 is expressed on HSCs but in the mouse, adult LTR-HSCs are mostly $CD34^-CD38^+$ whereas STR-HSCs are enriched in the $CD34^+CD38^-$ population (Zhao et al., 2000). Combination of cell surface markers and Hoechst 33342 exclusion defines a more undifferentiated subpopulation known as side population (see Figure 3).

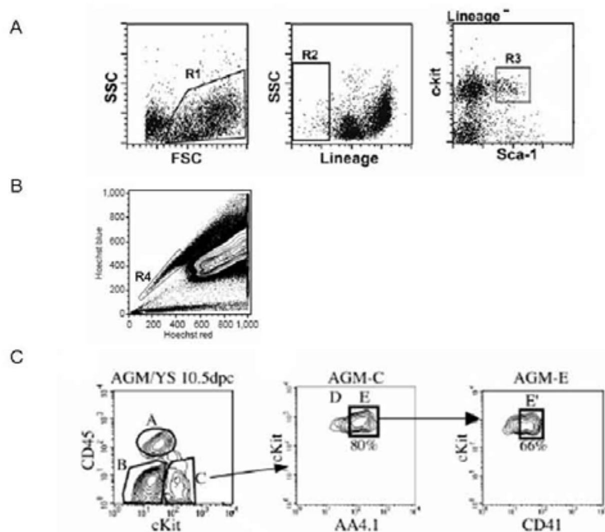


Figure 3: ***Characterization of embryonic and adult HSCs. A)*** *Forward and side scatter (R1) and lineage-negative (R2) sorting gates. Lineage-negative cells sorted based on c-Kit and Sca-1 expression (KSL cells: R3.) From Baumann et al. Blood, 2004.* ***B)*** *Bone marrow cells loaded with Hoechst 33342. Cells with the highest dye efflux define a stem cell-enriched population known as side population (R4). From Duncan et al. Nat Immunol 2005.* ***C)*** *Embryonic HSCs defined as cells that are $CD45^{low}c\text{-}Kit^+$ (region C) $AA4.1^+$ (region E) and $CD41^+$ (region E'). From Bertrand et al. PNAS 2005.*

In murine embryos, HSCs express c-Kit, CD41 and AA4.1 (Bertrand et al., 2005), CD31/PECAM (Baumann et al., 2004) and VE-cadherin (Kim et al., 2005) although they express low levels of Sca-1 and Mac1. In contrast to the quiescent nature of bone marrow HSCs, they are mainly in S-phase in the embryo [reviewed in (Godin and Cumano, 2002)]. These differences in the cell cycle kinetics may account for some

of the differences in the cell-surface markers whereas other proteins such as CD41 and Mac1 receptors are related with the ability of embryonic HSCs to migrate within the embryo (Sanchez et al., 1996).

1.1.2 Regulation of the hematopoietic system

Interactions between cells and their environment regulate development of hematopoietic cells either through cell-cell interactions or by secreted factors such as cytokines or growth factors. Altogether characterizes the hematopoietic stem cell niche.

1.1.2.1 The hematopoietic stem cell niche

The bone marrow from the major long bones is the most important hematopoietic organ during adult-life. Hematopoietic cells are retained within the bone cavity until they achieve the appropriate stage of maturation and then are released into the bloodstream. HSCs and progenitor cells are surrounded by different mesenchymal-derived stromal cells including chondrocytes, endothelial cells, fibroblasts and osteoblasts and by the extracellular matrix that these cells produce (which includes fibronectin, laminin, collagen and proteoglycans) [reviewed in (Dazzi et al., 2006)]. Interaction of both hematopoietic and stromal cells together with signals mediated by soluble and membrane-bound growth factors are important in the regulation of adult bone marrow hematopoiesis. All these elements form the stem cell niche, which offer the proper microenvironment for stem cells to either self-renew or differentiate into their progeny. In the last years it has been demonstrated that osteoblasts (cells responsible for bone growth) and the endothelial cells of sinusoidal vessels are required for proper HSCs function, leading to the notion that two different niches support HSCs development, the osteoblastic and the vascular niche. The former may maintain the HSCs in a quiescent state whereas the latter may promote proliferation and further differentiation into the different hematopoietic lineages [reviewed in (Yin and Li, 2006)].

Ligands with their corresponding receptors that mediate the interaction between the HSC and the niche include Notch [reviewed in (Li and Li, 2006)], Stem Cell Factor (SCF)/c-Kit [reviewed in (Linnekin, 1999)], Wnt (Reya et al., 2003), basic Fibroblast Growth Factor (bFGF) or hedgehog [reviewed in (Yin and Li, 2006)] signaling pathways.

For example, Notch receptor is expressed in HSCs whereas the Notch ligand Jagged1 is expressed in osteoblasts and bone marrow stromal cells, supporting the hypothesis that activated Notch induces self-renewal of HSCs and hematopoietic progenitors (Varnum-Finney et al., 2000) (see Notch section in page 46).

- **Chemokines and CXC receptors**

In addition to the ligand-receptor interactions, HSC-niche specific chemokines (cytokines with chemotactic activity) are important in regulating HSC behavior. Chemokines have conserved cysteine residues that allow them to be assigned to four groups which are C-C chemokines (RANTES, MCP-1, MIP-1α, and MIP-1β), C-X-C chemokines (SDF1), C chemokines (Lymphotactin), and CXXXC chemokines (Fractalkine). Stromal cell derived factor 1 (SDF1/CXCL12) participates in the mobilization of HSC from bone marrow to the blood stream. In one hand, SDF1 expressed in endothelial cells regulates the transendothelial migration of HSCs that express CXCR4 receptor, whereas SDF1 expression in osteoblast mediates HSC homing to the bone marrow. SDF1 is also involved in the HSCs mobilization to peripheral blood induced by granulocyte colony-

stimulating factor (G-CSF) treatment. G-CSF treatment is clinically used for HSC mobilization and stem cell transplantation for the treatment of leukemias [reviewed in (Juarez and Bendall, 2004)].

1.1.2.2 Cytokines and cytokine receptor signaling

Several cytokines and growth factors are responsible for the modulation of HSC survival, proliferation and differentiation by interacting with their specific receptors (summarized in table 1). This includes Interleukin 1 (IL-1), which activates T cells; IL-2, which stimulates proliferation of antigen-activated T and B cells; IL-4, IL-5, IL6 and IL-7, which stimulate proliferation and differentiation of B cells; Interferon-gamma (IFNγ), which activates macrophages; and IL-3, Granulocyte-Monocyte Colony-Stimulating Factor (GM-CSF), G-CSF or Flt3 ligand which stimulate myeloid lineages [reviewed in (Lotem and Sachs, 2002)]. Cytokines that regulate hematopoiesis in the embryo such as SCF, bFGF, Vascular Endothelial Growth Factor (VEGF) and IL-3 are specifically covered in section 1.2.5.3.

Table1: ***Important cytokines for hematopoiesis.*** *Adapted from Lotem and Sachs, 2002; and htpp://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=imm.table.2499*

Family	Cytokine	Receptor	Principal source	Primary activity
Hematopoietins	EPO (erythropoietin)	EpoR	Kidney cells and hepatocytes	Erythroid progenitor proliferation, survival and differentiation
Hematopoietins	TPO (thrombopoietin)	c-Mpl	Kidney	Proliferation of myeloid cells and HSCs
Unassigned	IL-1 (interleukin-1)	CD121	Macrophages and antigen presenting cells (APC)	T-cell activation, inflammation
Hematopoietins	IL-2 (interleukin-2)	CD122, CD132	Activated TH_1 cells and NK cells	Antigen activated T- and B-cell proliferation
Hematopoietins	IL-3 (interleukin-3)	CD123	Activated T cells and thymic cells	Proliferation of early hematopoietic progenitors
Hematopoietins	IL-4 (interleukin-4)	CD124, CD132	TH_2 and mast cells	B-cell proliferation
Hematopoietins	IL-5 (interleukin-5)	CD125	TH_2 and mast cells	Eosinophil differentiation
Hematopoietins	IL-6 (interleukin-6)	CD126	Activated TH_2 cells, APCs	B-cell proliferation
Hematopoietins	IL-7 (interleukin-7)	CD127, CD132	Thymic and marrow stromal cells	T and B lymphopoiesis
Interferons	IFNγ (interferon-gamma)	IFNGR2	Activated T cells and NK cells	Macrophages, neutrophils and NK cells activation
Hematopoietins	GM-CSF	CD116	Macrophages, T cells	Macrophage proliferation and differentiation
Hematopoietins	G-CSF	G-CSFR	Fibroblasts and monocytes	Granulocyte proliferation and differentiation
Unassigned	Flt3	Flt3 receptor	Marrow stroma	Myeloid lineage proliferation
Hematopoietins	Oncostatin M	OSMR	T cells, macrophages	Proliferation of early progenitors

1.1.2.3 Hematopoietic transcription factors

Specific transcription factors have been identified for their ability to control lineage specific gene programs during hematopoietic development. Some of them are summarized in table2 and in the following section.

Table2: ***Important transcription factors for the regulation of the hematopoietic system***

Transcription factor	Class	Time of Death(E)	Primitive hematopoiesis (yolk sac)	Definitive hematopoiesis (fetal liver)	Hematopoietic defect	References
Pu.1	ETS	E18.5	Normal	Reduced	-Defects in myeloid and lymphoid development	McKercher, 1996
Ikaros	Zinc finger	Viable	Normal	Reduced	-Decreased HSC/HPC generation or proliferation. -Lymphoid defects	Nichogiannopoulou, 1999
C/EBPα	Basic leucine zipper	Perinathally	Normal	Reduced	-Completely absence of GMP and mature neutrophils/macrophages -Increased number of HSCs and myeloblasts	Zhang, 1997 Zhang, 2004
E2A	bHLH	Viable	Normal	B-cell blockage	-Defective B-cell commitment	Bain, 1994
Pax5	paired	Alive but die within 3 weeks	Normal	B-cell blockage	-Defective B-cell differentiation	Urbánek, 1994 Nutt, 1997; Souabni, 2002
GATA3	Zinc finger	E11.5-E12.5	Normal	Defective	-T-cell blockage at the earliest stages	Ting, 1996
Runx1/AML1	bHLH	E11.5-E12.5	Normal	Blocked	-Impaired HSC/HPC generation and/or proliferation	Okuda, 1996; North, 1999 and 2002; Burns, 2005; Lacaud, 2003
GATA2	Zinc finger	E10.5-E11.5	Reduced	Markedly reduced	-Impaired HSC/HPC generation and/or proliferation. -Reduced expansion of the various lineages	Tsai, 1994; Shivdasani, 1996; Minegishi, 2003; Ling, 2004
Scl/Tal-1	bHLH	E9-E10.5	Markedly reduced	Absent	-Lack of precursor determination or maintenance.	Elefanty, 1999; Shivdasani, 1995; Robb, 1995 &1996
c-Myb	Leucine zipper	E15	Normal	Reduced	-Impaired proliferation of hematopoietic progenitors	Mucensky, 1991; Emambokus, 2003; Mukouyama, 1999
GATA1	Zinc finger	E10.5-E11.5	Markedly reduced	Absent	-Erythrocytes arrested at the proerythroblast stage	Fujiwara, 1996; Weiss and Orkin, 1995; Takahashi, 1997; Suwabe, 1998
FOG-1	Zinc finger	E10.5-E11.5	Markedly reduced	Absent	-Erythroid and megakaryocytic development arrest	Tsang, 1998

- **Common Myeloid and Lymphoid transcription factors**

Pu.1 belongs to the Ets family of transcription factors. Mice with a targeted mutation of Pu.1 display a complete absence of granulocytic, monocytic and B- and T-cell lineages whereas erythroid and megakaryocytic development is not affected. This indicates that this transcription factor plays a specific role in myeloid and lymphoid differentiation (McKercher et al., 1996). Consistent with this, Pu.1 is expressed in adult spleen, thymus and bone marrow and different myeloid and lymphoid cell lines [reviewed in (Ling and Dzierzak, 2002)]. In early erythroblasts Pu.1 is expressed together with GATA1 and GATA2. Since GATA factors promote erythroid proliferation and differentiation and Pu.1 mediates myeloid differentiation it is likely that the previously reported mutual inhibition regulate the commitment of common hematopoietic progenitor into erythroid or myeloid lineages (Zhang et al., 1999).

The Ikaros transcription factor is a member of the Krüppel family of zinc-finger proteins and was first described as a key factor in activating B- and T-cell specific gene expression. Moreover, Ikaros is expressed in HSCs and lymphoid restricted progenitors (Klug et al., 1998) and null mice for Ikaros display reduced number of HSCs (Nichogiannopoulou et al., 1999).

- **Granulocytic-macrophage transcription factors**

C/EBPα is required for granulocytic differentiation since null mice for this factor display a complete lack of neutrophils and eosinophils (Zhang et al., 1997). Moreover, studies with conditional knockouts suggest that C/EBPα is required for the transition from the Common Myeloid Progenitor to the Granulocyte-Macrophage Progenitor (Zhang et al., 2004). In addition, C/EBP$\alpha^{-/-}$ mice display increased numbers of HSCs and myeloblasts (resembling human acute myeloid leukemia) in the bone marrow suggesting a role for this protein in limiting self-renewal of HSCs and hematopoietic progenitors (Zhang et al., 2004).

- **B-cell lineage transcription factor**

The basic helix-loop-helix (bHLH) transcription factor E2A and the early B-cell factor (EBF) are required for the initiation of B lymphopoiesis since targeted mutations trigger B-cell blockage at the pro-B cell stage (Bain et al., 1994). Both proteins cooperate to activate the expression of several B-lineage specific genes such as those involved in the B-cell receptor rearrangements explaining the block of differentiation at the pro-B stage [reviewed in (Laiosa et al., 2006)].

Pax5 is another B-cell specific transcription factor. The null mice for Pax5 display a similar effect than E2A and EBF in arresting B-cell development in the bone marrow at the pro-B stage (Urbanek et al., 1994), without affecting expression of E2A and EBF (Nutt et al., 1997). This suggests that Pax5 is acting downstream of E2A and EBF in B-cell differentiation. Conversely, overexpression of Pax5 in bone marrow HSCs and progenitors strongly induces B-cell development at the expense of the T-cell fate by repressing Notch1 (Souabni et al., 2002).

- **T-cell lineage transcription factors**

Notch1 is the most important inductor of the T-cell fate at expenses of the B-cell one and it functions not only in T-cell specification but also in the whole hematopoietic system. Due to its relevance and because this is the main subject of this work the role of Notch in hematopoiesis will be discussed in Chapter 1, Section 3.

GATA3 is a very recently characterized Notch-target that is involved in T-cell development (Dontje et al., 2006). GATA3 null mice display blockage in T-cell generation at the earliest stages. Moreover, in chimeric mice, GATA3$^{-/-}$ embryonic stem cells failed to contribute to the T-cell lineage (Ting et al., 1996).

- **Natural killer transcription factors**

Natural killer cells represent a subtype of lymphoid cells that induces cytotoxicity in the target cells. The phenotype of Ikaros-, Pu.1- and Id-2-null mice revealed that this three transcription factors are required for NK cell development. Ikaros deficiency leads to the most severe phenotype with a complete absence of this lymphoid cell lineage, indicating that this transcription factor plays a key role in the development of NK cells [reviewed in (Laiosa et al., 2006)].

- **Dendritic cells**

Dendritic cells (DC) are responsible for the uptake, processing and presentation of antigens to T and B cells. Many studies revealed that dendritic cells originate from both lymphoid and myeloid progenitors and these two different DC populations require different transcription factors. In this sense, RelB and Pu.1 null mice lack myeloid-derived DC, Id-2 null mice lack lymphoid-derived DCs and Ikaros mutants lack both DC types [reviewed in (Laiosa et al., 2006)].

1.1.2.4 MicroRNAs as hematopoietic regulators

Recent studies indicate that small non-coding RNAs regulate many different cellular and developmental processes in animals and plants. This group of regulators called interference RNAs (RNAi) is composed by the small-interfering (si)RNAs and the micro (mi)RNAs. Both types are small RNAs (22 nucleotides approximately) and while the former bind to the target mRNA leading to its degradation, the latter leads to both translational inhibition and mRNA degradation [reviewed in (Chen and Lodish, 2005)].

The first miRNAs were found in *C. elegans* but nowadays it is known that multi-cellular organisms express hundreds of miRNAs in different cell types and their target sites are mainly found in the 3'-untranslated region (UTRs) of the mRNA.

Emerging studies reveal that miRNAs regulate different developmental processes such as muscle differentiation and limb formation. For example, miR-1 likely regulates Hand2 expression, a transcription factor that controls cardiomyocyte expansion whereas miR-196 represses HoxB8 mRNA [reviewed in (Shivdasani, 2006)]. In the last few years, many examples of miRNAs that regulate different hematopoietic processes have been described. Ectopic expression of specific c-Kit microRNAs miR-221 and miR-222 on $CD34^+$ human cord blood cells leads to arrested proliferation and accelerated differentiation (Felli et al., 2005). Moreover, the stem cell activity of these cells is abrogated as shown by transplantation experiments in NOD-SCID immunodeficient mice (Felli et al., 2005). On the other hand, miR-223 that is activated by C/EBPα is suggested to induce human granulocytic differentiation through inhibition of the transcription factor NF1-A (Fazi et al., 2005).

Although not much is known about the genes regulated by miRNAs and most of the data comes from bioinformatic predictions, it is tempting to speculate that the study of miRNAs will have an important contribution in the understanding of hematopoiesis.

1.1.2.5 Transdifferentiation / Reprogramming

Current models of hematopoietic differentiation suggest that stem cells become gradually restricted in their differentiation potential by activating gene expression programs and simultaneously inactivating alternative lineage choices. However, the notion of a strictly hierarchical branching model of hematopoiesis has been challenged by many experimental data which suggest that both HSCs and committed hematopoietic cells can transdifferentiate or switch into cells of another lineage [reviewed in (Graf, 2002)].

There are many examples of differentiated cells that can reprogramme their gene expression patterns and switch into cells of a different lineage. For example, $Pax5^{-/-}$ B-cell progenitors can be induced to differentiate into T cells when transplanted into immunodeficient Rag2-null mice. Alternatively, they can differentiate into NK cells, dendritic cells, macrophages or neutrophils in response to different cytokines *in vitro*. Moreover transdifferentiation within the myeloid and erythroid compartments can be achieved by

overexpressing or inhibiting transcription factors such as Pu.1, GATA1, FOG-1 and C/EBPα [reviewed in (Graf, 2002)]. Thus, it is likely that some hematopoietic progenitors maintain their plasticity and are able to transdifferentiate into other lineages even when committed to a given one. However, these experiments rely in alterations of physiological conditions and it is not known whether hematopoietic progenitors are able to transdifferentiated into different lineages *in vivo* [reviewed in (Graf, 2002)].

More exciting are the recent experiments that suggest that HSCs can transdifferentiate into nonhematopoietic cells such as myocytes, neurons, hepatocytes and endothelial cells following transplantation of adult irradiated mice with bone marrow HSCs. All these experiments are controversial since angiogenic precursors and mesenchymal stem cells from the bone marrow may be responsible for these results. Moreover it has been shown that HSC can fuse to different cell types thus giving an alternative explanation to transdifferentiation results [reviewed in (French et al., 2002)].

1.1.3 Methodological approaches to study HSCs and hematopoietic progenitors.

Classification of HSCs and hematopoietic progenitors is based on their ability to long-term repopulate the hematopoietic system of irradiated/chemically-ablated mice (LTR-HSCs), generate macroscopic colonies in the spleen of adult irradiated mice (STR-HSCs and/or CFU-S) or form colonies *in vitro* (committed hematopoietic progenitors or colony-forming cells CFC) [reviewed in (Dzierzak and Medvinsky, 1995)]. Considering the particularities of this methodology, a detailed explanation of the most common used techniques to identify HSC and progenitors is included below.

- **Short and Long-term multilineage repopulation assays**

This assay allows the detection of long-term repopulating hematopoietic stem cells (LTR-HSCs), the most undifferentiated hematopoietic progenitor capable of generating the entire hematopoietic system (Muller et al., 1994).

Two different approaches of this *in vivo* assay have been developed. The first one consists in the intravenously transplantation of HSCs or progenitor cells (accompanied of more mature progenitors to facilitate survival during the first weeks) from a donor mouse into an adult recipient whose hematopoietic system has been depleted by lethal irradiation. One month after transplantation, the contribution from the donor short-term repopulating HSCs (STR-HSCs) to the hematopoiesis of the recipient can be detected by the presence of the donor specific marker in the peripheral blood. At four months post-transplantation, detection of donor hematopoietic cells indicates the long-term repopulation ability of the transplanted cells (LTR-HSC) (Muller et al., 1994). Limiting dilution and competitive long-term repopulation experiments can be used to determine in a more quantitative and qualitative manner the repopulation ability of HSCs. In addition, secondary and tertiary transplantations can be performed to assay self-renewal capacity that is characteristic of the HSCs.

A modification of this methodology consists in the usage of a chemotherapeutic agent (busulfan) that depletes the endogenous hematopoiesis of the recipient. This protocol is used when the experimental approach requires the transplantation of donor cells into the liver of newborn pups. Such is the case for very immature HSCs that are unable to engraft adult recipients (i.e. yolk sac HSCs) [reviewed in (Palis and Yoder, 2001)].

- **CFU-S and HPP-CFC**

Committed hematopoietic progenitors arise from multipotential precursors that are detected by two different assays: ***1)*** the spleen colony-forming assay (CFU-S assay) that allows the detection of multipotent precursors, some of which are able to self-renew. In this case, progenitor cells are intravenously injected into lethally irradiated mouse recipients and after 8-16 days the presence of macroscopic colonies in the spleen determined (Medvinsky and Dzierzak, 1996) and ***2)*** the high proliferative potential colony-forming cell assay (HPP-CFC) permits the detection of multipotent precursors because they generate large macroscopic colonies of myeloid cells in soft-agar in the presence of the appropriate cytokines (Bertoncello, 1992).

- **Colony forming unit-culture assay**

The colony forming unit-culture (CFU-C) allows the detection of the most-differentiated progenitors (see Figure 4). Hematopoietic cells are seeded in methylcellulose supplemented with a cocktail of cytokines. After 5-14 days in culture, hematopoietic progenitors give rise to mature blood cells that form a colony which can be easily distinguished under the inverted microscope. Early erythroid progenitors are recognized by the generation of large colonies of red cells after 7-10 days and are referred as burst-forming unit erythroid (BFU-e). More mature erythroid progenitors generate smaller colonies within 3-4 days in culture and are called colony-forming unit erythroid (CFU-e). Progenitors committed to the megakaryocitic lineage (Meg-CFC) are also detected in the presence of thrombopoietin TPO). Myeloid progenitors can be distinguished into: Mac-CFC (macrophage-containing colonies), G-CFC (granulocyte-containing colonies), GM-CFC (both macrophage and granulocyte colonies), and Mix-CFC (both erythroid and myeloid mature cells in the same colony).

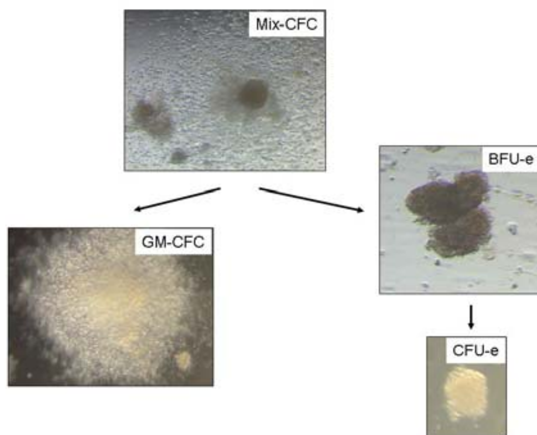


Figure 4: ***Hematopoietic progenitors detected in the CFC assay.*** *Photographs of the hematopoietic progenitors that can be detected by the colony-forming cell assay. Magnification 80x.*

- **Assays to detect lymphoid progenitors**

Lymphoid progenitors require very special conditions for their survival and differentiation. B-lymphoid progenitors can be cultured in the presence of the S17 stromal cell line. After 14 days of culture, cells expressing mature B-cell markers with the ability to produce IgM are detected (Fluckiger et al., 1998).

On the other hand T-lymphoid progenitors can be detected on fetal thymic organ culture [FTOC; reviewed in (Hare et al., 1999)]. Recently the use of the stromal cell line OP9 overexpressing the Notch-ligand Delta1 has shown to be sufficient to allow T-cell differentiation *in vitro* (Schmitt et al., 2004; Schmitt and Zuniga-Pflucker, 2002). Finally, both B and T-lymphoid progenitor activity can be analized by intravenous or intrathymic injection of cells into immunodeficient NOD/SCID or Rag mice (Godin et al., 1993).

- **Hematopoietic cell lines**

Many cell lines have been established from primary cells and they have become very important tools to study hematopoietic regulation. Some of the most common cell lines used in hematopoietic research are summarized.

Embrionic stem cells are derived from the inner cell mass of mouse blastocysts, which can self-renew or differentiate into all adult tissues. ES cells are key tools to generate gene targeted mutant mice by homologous recombination. In general, ES cells are maintained *in vitro* by co-culture with murine embryonic feeder cell layer with leukemia inhibitory factor (LIF) for murine cells or bFGF for human cells. ES cells can form aggregates of differentiated cells called embryoid bodies (EB) when LIF is removed from the media. EB development recapitulates the hematopoietic ontogeny *in vitro*, including generation of hemangioblasts from mesodermal precursors and the development of primitive and definitive hematopoiesis in the embryo. This system has proved to be extremely powerful to study the effect of genetic manipulation *in vitro* (Keller et al., 1993; Olsen et al., 2006).

Stromal cell lines are also useful tools for the study of hematopoiesis. Several cell lines have been established that support the survival, proliferation and maintenance of HSCs. The most commonly used are OP9, which was established from bone marrow of newborn macrophage colony-stimulating factor (M-CSF)-deficient mice. AGM-S3 is another stromal endothelial cell line derived from murine embryonic day 10.5 (E10.5) AGM (aorta-gonad-mesonephros) region that is able to support hematopoiesis [reviewed in (Olsen et al., 2006)]. Other stromal cell lines such as S17 and OP9-Delta1 are specifically used to support B-cell and T-cell differentiation respectively (Fluckiger et al., 1998; Schmitt et al., 2004; Schmitt and Zuniga-Pflucker, 2002).

Finally, many different hematopoietic cell lines have been developed from leukemic cells or by immortalizing hematopoietic progenitors and some of them can be induced to differentiate *in vitro*. For example, 32D can be maintained as undifferentiated progenitors in the presence of IL-3 or induced to granulocytic differentiation with G-CSF. Murine erythroleukemia (MEL) cells are spleen-derived cells transformed by the Friend leukemia virus that are arrested at the proerythroblast stage. Differentiation can be induced *in vitro* by hexamethylene bisacetamide (HMBA) [reviewed in (Marks and Rifkind, 1988)]. K562 is a human erythroid cell line obtained from a chronic myeloid leukemia patient that can be induced to differentiate by hemin or sodium butyrate treatment (Lozzio et al., 1981).

1.2 Ontogeny of the hematopoietic system in the mouse embryo

1.2.1 Brief introduction to mammalian embryonic development

Once fertilization occurs, the zygote starts several mitotic divisions to form a compact morula with 16 to 64 pluripotent cells or blastomeres. In the stage of blastula (E4.5) an outer monolayer of cells called trophoblasts generate the chorion and the amnion (fetal membranes) which line an inner cavity filled of fluid that contains the inner cell mass or embryoblast. Gastrulation is the next morphogenic process and results in the formation of the three germ layers: ectoderm, mesoderm and endoderm that give rise to the different organs of the embryo.

From the inner cell mass, cells proliferate and migrate to form a new cell layer inside the trophoblast. This new layer of cells is called the hypoblast and will form the yolk sac (YS). The remaining inner cell mass, the epiblast, will form the primitive streak which defines the longitudinal axis of the embryo and indicates the start of germ layer formation. These cells will later migrate through the streak depression and form the endoderm and mesoderm layers (E6-E6.5). The first migrating cells join the hypoblast layer forming the embryonic endoderm that will originate organs such as the gut, kidney and pancreas. The rest of the migrating cells enter the coelomic cavity to become the mesoderm (paraxial, intermediate and lateral) that will form among others, the heart, blood and muscles. Finally, the remaining epiblast becomes ectoderm that will form skin epidermis and the nervous system.

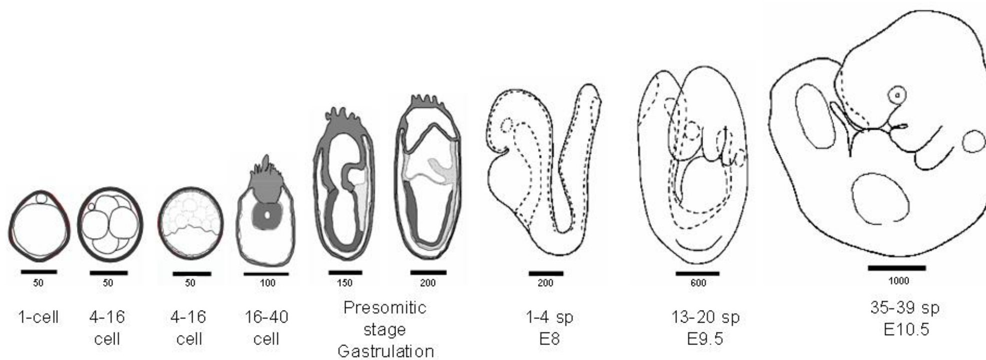


Figure 5:* *First stages of mouse embryonic development. *Schematic representation of developmental stages from zygote to a 10.5 day embryo adapted from e-map database (http://genex.hgu.mrc.ac.uk). From embryonic day 8 onwards the number of somites is used to accurately measure the developmental age. Thus, embryos with 1 to 10 somite pairs are at embryonic day 8-8.5 (E8-8.5), embryos with 11 to 20 somite pairs corresponds to E8.5-9.5, embryos with 21 to 30 somite pairs are the ones between E9.5-10 and 31 to 39 somite pairs, are embryos at E10-10.5 (The Atlas of Mouse Development, M. H. Kaufman).*

Early embryogenesis finishes with the process of organogenesis, with the formation of the notochord, appearance of the nervous system (neurulation), and the generation of the buds of the different organs. Somite formation occurs at this stage of embryogenesis and segregation from the paraxial mesoderm of somite pair progresses from rostral to caudal over time. Somites are blocks of mesoderm located lateral to the notochord and the number of somites in an embryo is the most accurate measure of the developmental age (see Figure 5). Later, somites will differentiate into vertebrae, ribs and basal bones of the skull, skin dermis and skeletal muscles.

1.2.2 Brief introduction to the hematopoietic system development in the mouse embryo

In the extra-embryonic yolk sac, at E7 the mesoderm layer develops into structures referred as blood islands, responsible for the first wave of hematopoiesis or primitive hematopoiesis (Silver and Palis, 1997). Primitive erythrocytes are characterized for the presence of nuclei and the expression of embryonic hemoglobins (ε and βH1). During the 6-8 somite pair stages (E8-8.5), the mouse embryo suffers the process of “turning” or axial rotation, in which achieves the characteristic “fetal” position (The Atlas of Mouse Development, M. H. Kaufman), and shortly after, starts the second wave of embryonic hematopoiesis or definitive hematopoiesis. At E8.5 circulation is established between the embryo and the yolk sac through the vitelline arteries, thus blood cells from the yolk sac are found in the embryo. Beginning E9, the intra-embryonic para-aortic splanchnopleura (P-Sp) mesoderm gives rise to the fused aorta surrounded by

gonads and mesonephros, a region referred as AGM. The first adult HSCs originate from this AGM region between E9 to E12 (Godin et al., 1995; Medvinsky and Dzierzak, 1996), although as early as E8.5, mesodermal-derived regions within the embryo body are able to generate hematopoietic cells (Cumano et al., 1996). The HSCs and other progenitors develop from the ventral part of the dorsal aorta (Garcia-Porrero et al., 1995) but also other major vessels such as the umbilical and vitelline arteries (de Bruijn et al., 2000) (see Figure 6).

From E11, fetal liver becomes active as hematopoietic site. Since HSCs appear in the embryo before fetal liver is formed, hematopoietic activity from other hematopoietic niches may colonize the liver, and next further differentiation and expansion of HSCs likely occur within this organ. Fetal liver erythrocytes expel the nuclei and express adult forms of hemoglobin (β-major and α). Colonization of both the fetal thymus (where T-cell differentiation occurs) and the spleen (responsible for B-cell generation) by HSCs starts around E12 (Godin et al., 1999). Near the end of gestation (E15-16), both fetal liver and spleen hematopoiesis regresses concomitant with the migration of HSCs to the bone marrow and this tissue will remain the main hematopoietic organ through adult life (Metcalf et al., 1971).

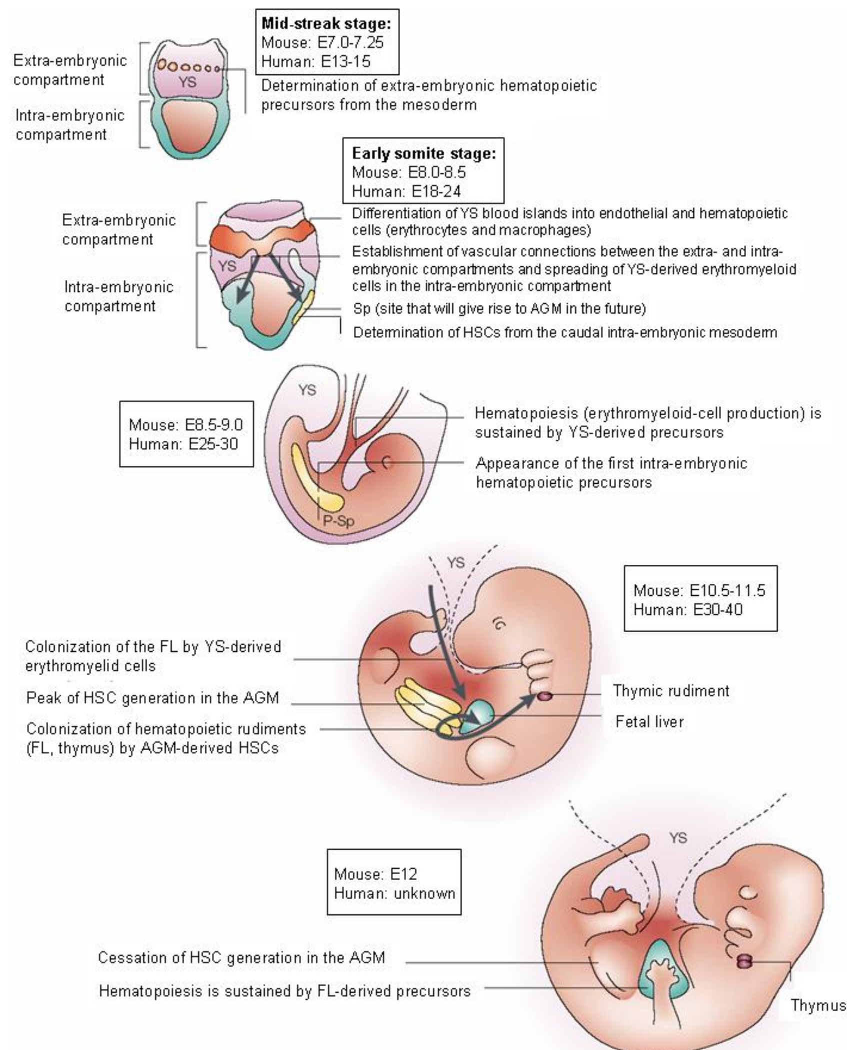


Figure 6: ***First stages of murine embryonic hematopoiesis*** *(adapted from Godin and Cumano, Nature Reviews Immunology 2002). For each murine developmental stage, its equivalent in human development is shown.*

The ontogeny of the human hematopoietic system is very similar to the mice. The first blood islands appear in the human embryo around day 18 of gestation, and YS primitive erythropoiesis takes place from weeks 3-6 of gestation. HSCs generation in the AGM occurs at weeks 5-7. From weeks 6-22, fetal liver acts as the major hematopoietic site and finally the bone marrow becomes the lifelong site of blood-cell production [reviewed in (Palis and Yoder, 2001)].

1.2.3 Primitive hematopoiesis

As mentioned before, generation of blood cell precursors in the embryo occurs in two different hemogenic tissues, quite different in space and time, the YS and the AGM. Primitive hematopoiesis occurs in the yolk sac and it is the first wave of hematopoietic cell production in the developing embryo.

1.2.3.1 The yolk sac

The murine yolk sac is composed by a layer of extra-embryonic mesoderm cells closely associated to a layer of visceral endoderm cells. The endoderm functions as a supporting layer for mesoderm and metabolizes maternally-derived molecules and synthesizes serum proteins. Furthermore, this layer seems to be the source of some inductive signals necessary for both blood cell generation (Belaoussoff et al., 1998) and endothelial network formation in the yolk sac (Palis et al., 1995). In agreement with this, embryoid bodies derived from GATA4 null embryonic stem cells which lack visceral endoderm display reduced blood island formation (Bielinska et al., 1996).

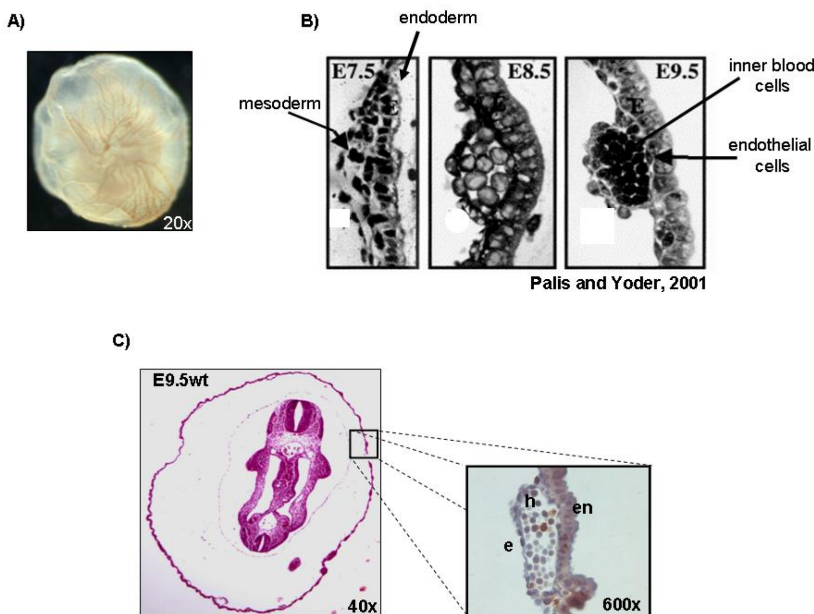


Figure 7: ***Yolk sac structure. A)*** *E9.5 mouse embryo inside the yolk sac.* ***B)*** *Photographs showing the generation of the yolk sac blood islands from E7.5 to E9.5 (adapted from Palis and Yoder, 2001). Note that E7.5 mesoderm gives rise to both hematopoietic cells and endothelial cells.* ***C)*** *Transversal section of an E9.5 embryo with the yolk sac. Hematoxiline-eosin staining. (h: hemato-poietic cells; e: endodermal cell layer).*

The first blood cells are derived from the mesoderm within the blood islands. In the mouse, the first blood islands appear between E7 and E7.5 as mesodermal cell masses lined by endodermal cells facing the yolk sac cavity. At this time the external cells acquire an endothelial-like morphology becoming true endothelial cells, whereas the rest of the inner cells progressively lose their intercellular attachments and differentiate into primitive erythroid cells. These cells will circulate after the formation of the vascular network between the YS and the embryo proper (see Figure 7).

1.2.3.2 Primitive hematopoiesis

Morphological studies revealed that blood cells generated in the YS resembled those found in lower vertebrates and were called primitive blood cells. Primitive mature erythrocytes (also called megaloblasts) are large nucleated cells that express embryonic globins (ε and βH1) and differ from definitive anucleated adult-type erythrocytes that express adult-type globins (β-globin). During development, yolk sac produces macrophages and megakariocytes that are different from their adult counterparts. More specifically, primitive macrophages lack lysozyme and peroxidase activity, whereas megakaryocytes display reduced ploidy [reviewed in (Godin and Cumano, 2002; Xu et al., 2001)]. In general, YS hematopoiesis produces red cells to ensure oxygen supply, megakaryocytes to clot the new vessels that are being formed and macrophages to clear the increasing amount of apoptotic cells present during early organogenesis in the developing embryo.

From E7 to E9, yolk sac erythroid progenitor cells are primitive erythroid progenitors (EryP) (Palis et al., 1999), which can be distinguished in CFC assays because they generate colonies of about a hundred large nucleated primitive erythroid cells expressing embryonic globins after 3 days of culture. From E9, definitive hematopoietic progenitors are detected in the yolk sac as indicates the presence of BFU-e, CFU-e, GM-CFC and Mix-CFC colonies. However, definitive erythrocytes at these stages are only detected in *in vitro* cultures whereas *in vivo*, they are first evident by E12, when the liver is the most important hematopoietic organ. This suggests that YS definitive erythroid progenitors do not physiologically differentiate in the YS (Palis et al., 1999) and is in agreement with the observation that explants of YS required the coculture with liver primordium to produced definitive erythrocytes (Cudennec et al., 1981).

Near 90% of YS-derived blood cells are erythrocytes. However, the yolk sac also contains Mac-CFC, GM-CFC (Palis et al., 1999) and microglial progenitors which migrate to the developing central nervous system (Kurz and Christ, 1998). Multipotent hematopoietic progenitors appear in the YS at E8.5 detected as HPP-CFCs (Palis and Yoder, 2001) and at day E9.5 detected as CFU-S (Medvinsky et al., 1993). Finally, whether the yolk sac has lymphoid potential is still controversial as to date no evidence of lymphopoiesis has been found in yolk sac stromal co-cultures [reviewed in (Palis and Segel, 1998; Yokota et al., 2006)].

1.2.4 Definitive hematopoiesis

Definitive hematopoiesis originates in an intra-embryonic region, formed by the aorta, gonads and mesonephros and referred as the AGM region (Medvinsky and Dzierzak, 1996). The HSCs and other progenitors generated in this region will move most likely to the fetal liver, where they proliferate and differentiate into adult-type hematopoietic cells. Finally, near birth, HSCs migrate to the bone marrow niches contributing to the adult-life hematopoietic system.

1.2.4.1 Generation of the AGM region

The AGM region extends from the forelimbs to the hindlimbs of the E9.5-E12.5 mouse embryo. It comes from the mesoderm germ layer and is composed of the dorsal aorta, the genital ridges (which will form the gonads) and the mesonephros (a mesodermally derived embryonic kidney) (see Figure 8). Around E8.5, there is a pair of dorsal aortas that will connect to the yolk sac vasculature through the vitelline vessels. These aortas fuse in a single dorsal aorta starting at E8.5 from caudal to rostral (Garcia-Porrero et al., 1995). Simultaneously, the umbilical artery forms the connection between the dorsal aorta and the placenta.

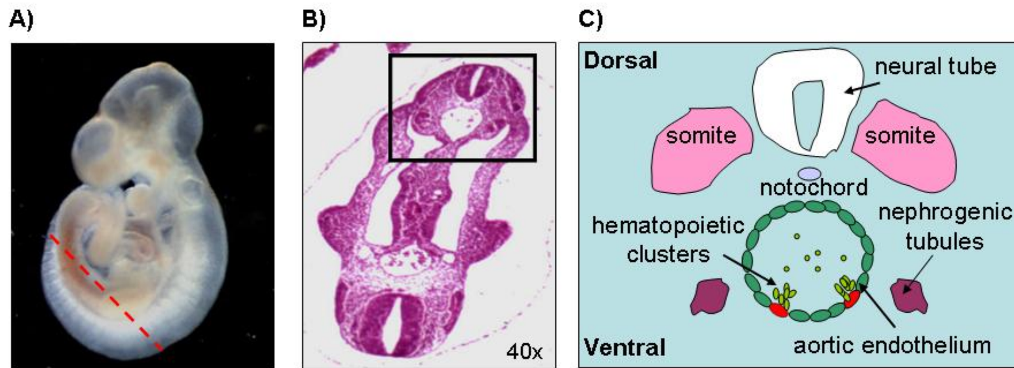


Figure 8: ***The AGM region.*** ***A)*** *E10.5 mouse embryo. The discontinuous red line marks the section in B in the region of the trunk.* ***B)*** *Transversal section through the region of the trunk of an E10.5 embryo. The black square marks the region represented in C; Hematoxiline-eosin staining.* ***C)*** *Representation of the aorta in the AGM region. From E10 to E12.5, clusters of hematopoietic cells appear as budding mainly from the ventral endothelium of the aorta in the AGM region.*

1.2.4.2 Hematopoietic activity in the AGM region

The para-aortic splanchnopleura mesoderm will give rise to the AGM region and contains B- and T-lymphoid progenitor activity at E8-9 (Godin et al., 1993) and CFU-spleen activity at E9 (Medvinsky et al., 1993). However, no adult HSC activity (in the sense that they are able to repopulate adult irradiated mice hematopoiesis) is found in the AGM region until day 10 (Muller et al., 1994). Historically, yolk sac was assumed as the origin site of HSCs. It was until 1975 when Dieterlen-Lièvre showed that in chicken yolk sac and quail embryo chimeras explanted before the establishment of the vascular network, only the quail cells contributed to the adult hematopoietic system (Dieterlen-Lievre, 1975). Similarly, in amphibian embryos, Turpen *et al* demonstrated that most of the HSCs come from the dorsal lateral plate, a homologous region to the AGM, and that contribution to definitive hematopoiesis of the ventral blood islands (homologous to the yolk sac) was less important (Turpen et al., 1997). Most recently, fate-mapping studies in *Xenopus* embryos revealed that ventral blood islands and dorsal lateral plate regions develop from different blastomeres in the blastula embryo and only the dorsal lateral plate contributes to the adult hematopoietic system (Ciau-Uitz et al., 2000).

In mammalian embryos, it has been demonstrated that HSC are autonomously generated from E10 in the AGM. From E11 HSCs most likely move to the fetal liver (Medvinsky and Dzierzak, 1996; Muller et al., 1994) as previously mentioned. Further subdissection of E10.5 AGMs into aorta and urogenital ridges (UGR) revealed that not only the aorta but also the umbilical and vitelline arteries generate definitive HSCs (de Bruijn et al., 2000).

Although yolk sac and para-aortic splanchnopleura at E9-10 failed to engraft hematopoiesis of irradiated adult recipients, these tissues engrafted in the liver of sublethally myeloablated newborn mice, being able to long-term reconstitute all blood cell lineages, and bone marrow (Yoder and Hiatt, 1997; Yoder et al., 1997; Yoder et al., 1997). These experiments indicate that immature pre-HSCs were present in the YS and early splanchnopleura and they require an appropriated microenvironment supplied by the new-born liver. A comparable result was obtained by Matsuoka *et al* by repopulating lethally irradiated adult mice with E8 yolk sac or P-sp co-cultured with AGM-S3 cells, a clonal endothelial cell line from E10.5 AGM region (Matsuoka et al., 2001).

More recently, the embryonic placenta has been proposed as the third source of HSCs (Gekas et al., 2005; Mikkola et al., 2005; Ottersbach and Dzierzak, 2005). HSC activity has been shown in this tissue at E10.5-11 thus paralleling the emergence in the aorta of the AGM region. However, it is possible that these HSCs come from other tissues and the placenta acts only as a maturation or expansion site. Alternatively, the fact that the umbilical artery, which derives from the allantoic mesoderm, has been shown to generate HSCs, it is plausible that HSC generation takes place in other allantoic mesodermal-derived regions such as the umbilical cord or the placenta. Further research is required to answer the origin of placental HSCs.

All these results reflect that the origin of HSC in the embryo is still an unresolved question and raises the possibility that HSCs that are formed in one of these sites can migrate to the others, or that they are simultaneously formed in several mesodermal tissues (see Figure 9). However, one general trait of all these sites is that they are all in close association with the endothelium.

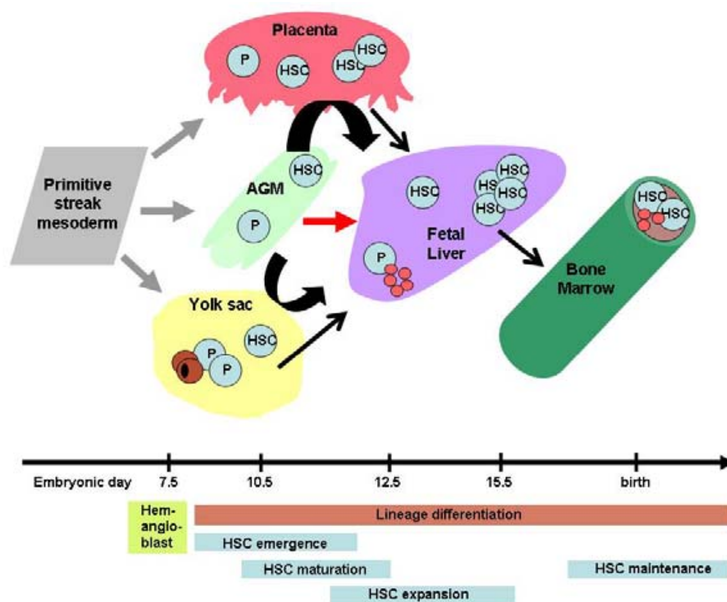


Figure 9: ***Schematic model of embryonic HSC generation*** *(adapted from Mikkola H, Exp. Hematology 2005). The primitive streak mesoderm is the origin of the hemangioblast, the common progenitor between endothelial and hematopoietic lineages. HSCs arise from the yolk sac, AGM region and placenta, colonize the fetal liver (the main organ for HSC expansion and lineage differentiation during fetal life) and finally seed the bone marrow for adult-life hematopoiesis. Arrows indicate possible routes of hematopoietic cell trafficking. Black arrows represent HSC precursor migration through bloodstream whereas gray arrows represent migration before the onset of circulation.*

1.2.4.3 Direct relationship between hematopoietic and endothelial lineages

In 1917, Sabin showed that hematopoietic cells in the blood islands of the yolk sac developed closely in time and space to endothelial cells [re-published in (Sabin, 2002). This observation raised the possibility that a common mesodermal progenitor for both lineages (the hemangioblast) exists. In fact, this could also be the case in the AGM region since clusters of hematopoietic cells bud from the ventral part of the dorsal aorta in close association with or within the endothelial cell layer (de Bruijn et al., 2002; de Bruijn et al., 2000; Garcia-Porrero et al., 1995). In the mouse, these clusters contain HSCs that express hematopoietic (stem) cell markers such as CD34, CD45 and c-Kit (North et al., 1999; North et al., 2002; Tavian et al., 1996) and resemble the ones described for birds, zebrafish, amphibian and humans (North et al., 1999; Tavian et al., 1996; Thompson et al., 1998). Cells within these clusters also express transcription factors required for definitive hematopoiesis such as Runx1, c-Myb, Gata2 and Scl [reviewed in (Godin and Cumano, 2002)].

Recent work with transgenic mice that express the green fluorescent protein (GFP) under the control of Ly-6A/Sca-1 promoter, a well characterized HSC marker, revealed the presence of a GFP^{+} cell population in

the AGM with long-term repopulating ability that resides in the endothelial layer lining the wall of the dorsal aorta, strongly suggesting that HSCs come from the endothelium (de Bruijn et al., 2002).

Hematopoietic and endothelial cells share a great number of cell surface markers: PECAM-1/CD31 (platelet endothelial cell adhesion molecule-1), angiopoietin receptor Tie-2, CD34 and the VEGF receptor-2, Flk-1 (Baumann et al., 2004; Hamaguchi et al., 1999; Hsu et al., 2000; North et al., 1999; Young et al., 1995). Moreover, several targeted mutations in endothelial genes strongly compromise the hematopoietic development in mice. For example, mice deficient for Flk-1 (Shalaby et al., 1995) or the transcription factor Scl (Robb et al., 1995) die at early stages of development due to severe hematopoietic and endothelial disorders. Moreover, Flk-1 deficient ES cells failed to contribute to the vascular endothelium and to primitive and definitive hematopoiesis as well (Shalaby et al., 1997). These results indicate that hematopoiesis and endothelial vascular formation share a similar genetic program. In fact, there is strong evidence that endothelial cells (characterized by the presence of exclusive endothelial markers) are able to generate hematopoietic cells (Eichmann et al., 1997). Moreover, murine embryoid bodies contain blast-colony forming cells that can differentiate into endothelial or hematopoietic cells upon secondary replating (Choi et al., 1998). More recently, a cell population that expresses the Brachyury mesodermal marker and Flk-1, first detected in the primitive streak of the mouse embryo, was shown to be the precursor of both endothelial and hematopoietic cells of the yolk sac (Huber et al., 2004). Finally, Wang's group identified an endothelial-like subpopulation (PECAM-1, VE-cadherin, Flk-1 positive) within human ES cells with hemangioblastic properties (Wang et al., 2004).

1.2.4.4 Proposed models for AGM-derived HSCs emergence

Endothelium of the dorsal aorta is formed before the emergence of adult repopulating HSCs, in contrast with the simultaneous appearance of hematopoietic and endothelial cells in the yolk sac. Three different models have been proposed to explain the generation of HSCs in the embryonic AGM [reviewed in (Godin and Cumano, 2002)]:

1) HSCs are generated from cells in the ventral part of the dorsal aorta with an endothelial phenotype that transdifferentiate into HSCs. This hypothesis would explain the great number of surface markers coexpressed in endothelial and HSCs, although its expression is also found in mesodermal cells.

2) HSCs develop from different cell populations within the aortic endothelium either intra-embryonic hemangioblasts or less differentiated mesodermal cells. Supporting this hypothesis Pardanaud *et al* demonstrated in the avian model that aorta formation rises from two different mesodermal populations, one of them with hemogenic potential and the other without (Pardanaud et al., 1996). Moreover, the use of Ly6/Sca-1-GFP mice revealed that HSC activity arises within the endothelium lining the dorsal aorta (de Bruijn et al., 2002).

3) HSCs develop from subaortic patches in the mesenchyme of the aortic floor and then migrate towards the ventral endothelium of the aorta, to form the hematopoietic clusters and release into the bloodstream. This hypothesis was based in the presence of $CD31^{+}CD41^{+}$ HSCs cells in these subaortic patches (which may express Gata2) (Bertrand et al., 2005). Nowadays, all three models are still valid and more research is needed to decipher how HSCs are generated.

1.2.5 Regulators of embryonic hematopoiesis

1.2.5.1 Developmental signaling pathways

Although not much is known, there is increasing evidence for a role of conserved developmental pathways in the regulation of HSC formation and maintenance. Some of the current data is summarized below.

- **Wnt signaling pathway**

Wnt proteins are secreted glycoproteins involved in cell fate determination, survival, proliferation and migration in a wide variety of tissues [reviewed in (Khan and Bendall, 2006)]. Activation of the canonical Wnt/β-catenin pathway by several Wnt molecules (including Wnt1, 3a, 8 or 8b) through Frizzled receptors results in the inhibition of glycogen synthetase kinase 3β (GSK3β)-mediated phosphorylation of β-catenin. This protects β-catenin from degradation by the proteosome and leads to an increase in protein levels and nuclear translocation. In the nucleus, β-catenin binds to LEF/TCF family of transcription factors to specifically activate gene expression. However, non-canonical Wnt pathways, independent of β-catenin have been described and are mainly involved in motility, apoptosis and planar cell polarity [reviewed in (Khan and Bendall, 2006)].

Several members of the Wnt family have been found to affect hematopoiesis in several species. Wnt5a and 10b are expressed in the yolk sac and the fetal liver where they are supposed to promote progenitor cell expansion (Austin et al., 1997). Wnt3a protein is able to induce self-renewal of HSC *in vitro* (Willert et al., 2003) and activates the expression of brachyury, a mesodermal gene required for commitment of the hematopoietic cell fate in ES cells (Arnold et al., 2000). Moreover, overexpression of an activated form of β-catenin in HSCs increased self-renewal *in vitro* and reconstitution of lethally irradiated adult mice by enhancing expression of both HoxB4 and Notch1 (Reya et al., 2003). It has been reported that Notch signaling is required for Wnt-mediated maintenance of undifferentiated HSCs but not for their survival and cell cycle entry (Duncan et al., 2005). All these reports indicate that Wnt signaling may be required for promoting expansion and self-renewal of HSCs, although recent work shows that Wnt activation leads to HSC differentiation but not self-renewal (Kirstetter et al., 2006).

Finally, increasing evidences that the Wnt signaling pathway is involved in T- and B-cell development have been provided by the analysis of different mutant mice for different members of the Wnt signaling pathway. In this sense, TCF1 knockout mice show a dramatic decrease in thymocyte number likely due to impaired proliferation and enhanced apoptosis (reviewed in (Staal and Clevers, 2005).

- **Smad-mediated signaling and hematopoiesis**

The transforming growth factor-β (TGFβ) superfamily is composed by a wide variety of peptide growth factors. Apart from TGFβ1, 2 and 3 other members are the activins and bone morphogenetic proteins (BMPs). These ligands transduce their signals through transmembrane serine/threonine kinase receptors which in turn, phosphorylate the intracellular mediators called Smad which heterodimerize to regulate gene expression [reviewed in (Larsson and Karlsson, 2005)].

In the last years, many studies revealed a key role of BMPs and other TGFβ members in the hematopoietic system. BMP4 mutant mice die at E7.5-9.5 displaying impaired mesoderm formation,

defective generation of blood islands with reduced numbers of red blood cells (Winnier et al., 1995) similar to the phenotype of TGFβ1 null mice (Dickson et al., 1995). In contrast, Smad5 null mice display increased numbers of hematopoietic progenitors, indicating its negative role in either hematopoietic commitment or progenitor expansion (Liu et al., 2003). Hence, TGFβ family members exert both positive and negative regulation in the hematopoietic system.

- **Hedgehog signaling**

Hedgehog is a family of secreted proteins composed by three members: Indian, Desert and Sonic. Binding of Hedgehog to the receptor Patched (Ptch) results in the activation of a second transmembrane protein, Smoothened (Smo) that transduces the signal through the zinc finger transcription factor Gli [reviewed in (Baron, 2001)]. Coculture experiments of mesodermal tissue from early gastrulating embryo and visceral endoderm tissue revealed that the latter is required for the mesoderm to generate primitive hematopoietic red blood cells. This effect is likely mediated by secreted Indian Hedgehog (Ihh) that upregulates BMP4 expression in the mesodermal tissue (Dyer et al., 2001). Consistent with this observation, Ihh and Smo null mice display hemato-vascular defects (Byrd et al., 2002; Dyer et al., 2001). In zebrafish embryos, the analysis of both Hedgehog mutants and cyclopamine-treated embryos (which is a Smo inhibitor) revealed that Hedgehog signaling is required for definitive but not for primitive hematopoiesis (Gering and Patient, 2005). Finally, Sonic hedgehog (Shh) induces proliferation of human $CD34^{+}CD38^{-}Lin^{-}$ cord blood hematopoietic progenitors *in vitro* via BMP4 (Bhardwaj et al., 2001).

- **The Notch signaling pathway**

Notch is an evolutionary conserved signaling pathway, widely used for the control of cell fate decisions during development. In mammals, Notch regulates vasculogenesis, neurogenesis, miogenesis, somitogenesis and many other processes. Since the Notch pathway is the main scope of this thesis, its role in the control of hematopoiesis will be discussed in detail separately.

1.2.5.2 Hematopoietic transcription factors

Transcription factors are key regulators of all biological processes including hematopoiesis since they modulate the expression of downstream genes induced by (extracellular) signals. Several transcription factors are responsible for the onset of the hematopoietic program in the embryo. The most important ones are described in this section.

- **Runx1**

Runx1 (also referred as AML-1, CBFα2 and PEBPα2B) is one of the members of the conserved runt homology domain transcription factor family. Runx1 is a helix-loop-helix protein that regulates the expression of several hematopoietic genes such as IL-3, Pu.1, c-myb, Flk-2 and the GM-CSF and M-CSF receptors (Okada et al., 1998).

Runx1 null mice die around E12.5 lacking definitive fetal liver hematopoiesis whereas primitive hematopoiesis is not affected (Okuda et al., 1996; Wang et al., 1996), although Runx1 is expressed in primitive erythrocytes and endothelial cells in the E8 yolk sac (North et al., 1999). In the embryo proper, Runx1 is expressed from E8.5 in the endothelial cells at the ventral part of the dorsal aorta and other major

arteries, and by E11.5 in the fetal liver. Interestingly, Runx1$^{-/-}$ embryos lack hematopoietic clusters in the aorta (North et al., 1999) but also Runx1$^{+/-}$ embryos display changes in the distribution of HSCs (Cai et al., 2000) indicating that Runx1 dosage is important for hematopoiesis. However, Runx1$^{-/-}$ ES cells-derived embryoid bodies contain some blast-colonies progenitors indicating that hematopoiesis can occur in a Runx1-independent manner (Lacaud et al., 2002). In addition, there is some evidence for Runx1 in repressing VEGF receptor-2 (Flk-1) expression suggesting a possible role in the differentiation of endothelial-like cells to hematopoietic cells (Hirai et al., 2005).

- **GATA2**

The GATA family of transcription factors is characterized by the presence of two homologous zinc-finger domains: the C-terminus zinc-finger binds to the DNA GATA-consensus sequence (T/AGATAA/G), whereas the N-terminus domain interacts with other transcription factors. GATA1 is exclusively found in the hematopoietic system; GATA2 and GATA3 are expressed in the hematopoietic system but also in other tissues.

GATA2 null mice display profound reduction in blast/mixed colonies in the yolk sac and die by E10.5 due to severe anemia (Tsai et al., 1994). Analysis of GATA2-null ES chimeras revealed that these cells could not contribute to neither primitive nor definitve hematopoiesis (Tsai et al., 1994). Interestingly, GATA2 haploinsufficiency results in a reduced number of HSCs in the AGM (Ling et al., 2004). *In vitro*, GATA2 null ES cells generate multipotential progenitors that proliferate poorly and undergo massive apoptosis (Tsai and Orkin, 1997). GATA2 is expressed in progenitor cells and progressively downregulated during erythroid maturation and myeloid cell differentiation, in contrast with GATA1 that is progressively upregulated during erythroid differentiation. Thus, a cross-regulatory mechanism by which GATA1 and GATA2 reciprocally control their expression has been described possibly through GATA binding sites [reviewed in (Ohneda and Yamamoto, 2002)].

- **Scl / Tal-1**

Scl (stem cell leukemia) or tal-1 (T-cell acute leukemia-1) gene encodes a basic-helix-loop-helix transcription factor, which has been associated with many leukemogenic processes. In the developing embryo it is expressed first in the visceral mesoderm that will form the yolk sac blood islands and next in both hematopoietic and endothelial cells (Elefanty et al., 1999). Scl null embryos die between E8.5 and E10.5 displaying absence of yolk sac hematopoiesis and vitelline vessel formation. Moreover, GATA1, c-myb and embryonic βH1-globin expression is lost in this mutant indicating that Scl may be required for their activation (Robb et al., 1995; Shivdasani et al., 1995). Chimeric mice with Scl null ES cells revealed that this gene is required for both primitive and definitive hematopoiesis (Robb et al., 1996). In contrast with its crucial role during development, disruption of scl in adult mice does not affect the repopulation ability of the bone marrow HSCs (Mikkola et al., 2003).

- **c-Myb**

The c-Myb transcription factor is expressed in immature blood progenitors and in embryonic hematopoietic sites. c-Myb null mice die at E15 due to failure of fetal liver hematopoiesis whereas primitive hematopoiesis is not affected (Mucenski et al., 1991). Moreover, primary cultures from P-Sp at E9.5 and

AGM at E11.5 of c-Myb$^{-/-}$ embryos displayed a complete lack of hematopoiesis (Mukouyama et al., 1999) suggesting that the absence of hematopoietic progenitors in the fetal liver of murine c-Myb$^{-/-}$ embryos is likely due to a previous defect in the generation of HSCs in the AGM.

In addition, c-myb doses are important in the regulation of hematopoietic progenitor cell proliferation and differentiation since a knockdown allele of c-myb, which expresses 5-10% of the wild-type c-Myb levels allows *in vitro* progenitor expansion while blocking differentiation (Emambokus et al., 2003).

Finally and similar to GATA2, c-Myb is highly expressed in immature erythroid progenitors and its downregulation is required to allow terminal erythroid maturation (Bartunek et al., 2003).

- **HoxB4**

Hox transcription factors were identified as main regulators of developmental patterning. However, more recent work shows that they also function in adult tissues including hematopoiesis. There are four hox clusters (A-D) located on different chromosomes, and a total number of 39 hox genes assigned to 13 paralog groups in each cluster, based on homeobox sequence similarities. Although several hox genes may be involved in hematopoiesis, hoxB4 is the best characterized. Retroviral overexpression of HoxB4 expands HSCs in murine bone marrow (Antonchuk et al., 2002) and human cord blood cultures without affecting their repopulating ability or terminal differentiation capacity (Buske et al., 2002). Moreover, hoxB4 infection of primitive murine yolk sac hematopoietic progenitors (E8.25, prior to the onset of circulation) results in the acquisition of definitive HSCs characteristics such as long-term repopulating capacity in irradiated adult mice (Kyba et al., 2002). This also supports the idea that yolk sac contains cells that are capable of generating definitive HSCs when the appropriate gene program is induced.

1.2.5.3 Cytokines

- **Vascular endothelial growth factor**

The vascular endothelial growth factor is an important cytokine for both hematopoietic and endothelial cells in the developing embryo. Studies with both mice and ES cells lacking the VEGF receptor-2 (Flk-1) suggested that signaling through VEGF is required for hemangioblast commitment from mesoderm (Shalaby et al., 1997; Shalaby et al., 1995). Conversly, injection of VEGF mRNA in *Xenopus* embryos results in excessive production of endothelial cells at expense of blood cells indicating that proper levels of VEGF may be required to maintain hematopoietic/angioblastic differentiation (Koibuchi et al., 2006).

In chimeras, Flk1$^{-/-}$ ES cells fail to contribute to primitive and definitive blood cells and they aberrantly accumulate on the surface of the amnion (Shalaby et al., 1997). Thus, VEGF signaling may be involved in migration of Flk1-positive precursors from the mesoderm to hematopoietic sites, a hypothesis supported by studies in *Drosophila* which indicate that VEGF may be involved in migration of hemocytes during embryonic life (Cho et al., 2002).

VEGF effects are dose dependent. Embryos with two VEGF hypomorphic alleles which reduce VEGF expression to a 50% compared with the wild-type (VEGF$^{lo/lo}$ embryos) die at E9.5 displaying the same hematopoietic and vascular defects than VEGF$^{+/-}$ and VEGF$^{-/-}$ embryos (Martin et al., 2004). VEGF$^{lo/lo}$ embryos display increased apoptosis in the Ter119^{+} population in the yolk sac indicating that proper VEGF dose is necessary for survival of primitive erythroid cells. Moreover, Scl overexpression in VEGF$^{lo/lo}$ embryos

decreases the percentage of apoptotic Ter119^{+} cells indicating that Scl acts downstream of VEGF to ensure survival of primitive erythroid cells (Martin et al., 2004).

- **Stem cell factor / Stem cell factor receptor**

The Stem Cell Factor (SCF, also Kit ligand) is a hematopoietic cytokine that exerts its functions by binding to its tyrosine kinase receptor c-Kit. Two natural occurring mutations in this locus (Steel and White spotting) lead to severe anemia and perinatal lethality. Several studies revealed that c-Kit activation is required for proliferation and maintenance of hematopoietic progenitors, mast cells and primordial germ cells [reviewed in (Broudy, 1997)].

In the developing embryo, SCF and c-Kit receptor expression is found in the yolk sac, fetal liver and bone marrow and other non-hematopoietic tissues such as the gut and the central nervous system. c-Kit expression is also found in HSCs, progenitor cells and mature cell types [(Sanchez et al., 1996); reviewed in (Broudy, 1997)]. Transplantation of c-Kit null fetal liver cells in Rag2$^{-/-}$ mice (which are deficient for B and T cells) revealed that SCF/c-Kit are required for T lymphopoiesis but not for B-cell development (Takeda et al., 1997).

SCF can be found in both soluble and membrane bound forms resulting from alternative splicing. The soluble form is likely to be required for proliferation and differentiation of progenitors/stem cells in combination with other cytokines such as IL-3, IL-1, IL-6 and erythropoietin (EPO). However, in the absence of specific cytokines SCF promotes viability rather than proliferation [reviewed in (Broudy, 1997)]. Alternatively, the membrane bound form of SCF is thought to be required for adhesion of HSCs and progenitor cells to bone marrow stromal elements through its binding to c-Kit [reviewed in (Linnekin, 1999)].

- **Fibroblast growth factor**

In the last years it has been shown that the fibroblast growth factor (FGF) is also a regulator of hematopoiesis. FGFR1 (fibroblast growth factor receptor-1) null ES cells do not generate hematopoietic cells whereas bFGF enhances blast-CFC (or hemangioblast) generation (Faloon et al., 2000). Conversely, different FGFs such as FGF-2 and FGF-4 or more recently FGF-1 have been shown to regulate lineage differentiation [reviewed in (Kashiwakura and Takahashi, 2005)] and *ex vivo* self-renewal of HSCs (de Haan et al., 2003), respectively.

- **Interleukin-3**

IL-3 has been widely used for both expansion of hematopoietic progenitors and colony growth in semisolid media *in vitro*. Mice carrying IL-3 or IL-3 receptor mutations display minor alterations of adult hematopoiesis (Lantz et al., 1998; Nishinakamura et al., 1996). However, recent studies indicate a possible embryonic role of IL-3 as a proliferation and/or survival factor for the earliest HSCs in the embryo. IL-3 is a putative target of Runx1 and it has been shown that IL-3 alone is able to rescue the Runx1$^{+/-}$ HSC defect in the AGM, indicating that this gene is important for proper generation of embryonic HSCs (Robin et al., 2006).

1.3 Hematologic disorders

Regulation of the hematopoietic system is extremely complex. Thus, mutations and alterations in genes regulating hematopoiesis often develop into life-threatening disorders, indicating that blood cell homeostasis is essential for survival of an organism.

In the following section, an overview of the most frequent pathologies of the hematopoietic system caused by known hematopoietic regulators is discussed. Erythroid pathologies will be analysed on Chapter 1, Section 2.

1.3.1 Leukemias

Neoplasms of hematopoietic cells are termed leukemias. Transformation to malignancy likely occurs in a single cell, which starts proliferating, clonal expanding and avoiding apoptosis. This fact usually occurs at the pluripotent stem cell level but sometimes it may involve a committed stem cell. Leukemic cells accumulate and/or replace bone marrow cells causing abnormal hematopoiesis, which leads to anemia, thrombocytopenia and granulocytopenia. In addition, they can infiltrate organs such as liver, spleen, lymph nodes and kidney leading to their dysfunction.

Leukemias are classified according to cell differentiation stages and the severity of the disease. Thus, acute leukemia is usually a rapidly progressing disease characterized by replacement of normal bone marrow and other organs by malignant blast cells. They are divided into acute lymphoblastic leukemias (ALL) and acute myeloid leukemias (AML). Chronic leukemia shows a slower progression and many patients can be asymptomatic for years. Chronic lymphocytic leukemia (CLL) is due to the clonal expansion of mature-appearing lymphocytes in lymph nodes and other lymphoid tissues with progressive infiltration of bone marrow and peripheral blood. On the other hand, chronic myeloid leukemia (CML) is caused by clonal myeloid cell proliferation likely from a transformed pluripotent cell resulting in overproduction of granulocytes in both bone marrow and extramedullary sites (spleen and/or liver).

- **Abnormalities leading to leukemia**

Leukemogenesis needs a critical first step, which usually is an acquired genetic aberration or initiating mutation which perturbs normal hematopoietic development. This “first hit” is necessary but not sufficient for development of a leukemic process and frequently occurs in genes encoding regulators of normal hematopoiesis. The progression to leukemia depends on “second hits” in additional pathways that control survival and proliferation of the developmentally arrested leukemic cells [reviewed in (Izraeli, 2004)].

ALL is the most common malignancy in children but can also appear in adults. The t(1;14)(p32;q11) chromosomal translocation causes T-cell acute lymphoblastic leukemia (T-ALL) due to the insertion of the scl locus in the regulatory elements of the T-cell receptor locus leading to aberrant expression of Scl. Moreover, a small deletion in the sil gene promoter, a gene upstream of scl, results in overexpression of Scl and deregulation of cell cycle genes in proliferating T cell precursors [reviewed in (Izraeli, 2004)].

Other chromosomic rearrangements such as the Tel-Runx1 translocation and those affecting the E2A locus cause B-cell acute lymphoblastic leukemia (B-ALL). The t(12;21) translocation generates a Tel-Runx1 fusion protein, involving two important regulators of the bone marrow HSCs population [reviewed in (Izraeli, 2004)]. Another frequent translocation associated with AML which involves Runx1 is the t(8;21) runx1-eto (also known aml1-eto). Besides preventing Runx1-dependent activities, the Runx1-Eto protein also inhibits

C/EBPα and Pu.1, thus blocking granulocytic differentiation (de Guzman et al., 2002). Gain-of-function mutations on the hoxA9 gene (both inducing its constitutive expression or activity) also lead to AML [reviewed in (Moore, 2005)].

Finally, in 95% of the patients with CML, a reciprocal translocation t(9;22) known as “Philadelphia chromosome” between the oncogene c-abl and the gene bcr generates the aberrant fusion protein Bcr-Abl. This chimeric protein inhibits C/EBPα translation, thus leading to a blockage in myeloid differentiation. Inactivation of C/EBPα also leads to different types of AML [reviewed in (Leroy et al., 2005)].

1.3.2 Myelodysplastic syndromes (MDS)

Myelodysplastic syndromes are hematopoietic stem cell disorders characterized by impaired hematopoiesis which result in anemia, neutropenia and/or thrombocytopenia of variable severity. The first stages of MDS are characterized by excessive apoptosis of progenitor cells leading to ineffective hematopoiesis. In response to this defect, the hematopoietic system increases proliferation of progenitor cells and as MDS progresses; cells become resistant to apoptosis and in most of the cases develop into acute myeloid leukemia (AML) [reviewed in (Catenacci and Schiller, 2005)]. Recently it was shown that GATA1 was mutated in Down Syndrome patients which develop a transient myeloproliferative disorder (TMD) and acute megakaryoblastic leukemia (AMKL) suggesting a high implication of this transcription factor in MDS and/or AMKL [reviewed in (Izraeli, 2004)].

On the other hand, a constitutively active form of human c-Kit (D816V) has been found with high frequency in patients with mastocytosis and associated hematological disorders. This mutation results in SCF-independent proliferation as well as transforming abilities leading to leukemia [reviewed in (Linnekin, 1999)].

Section 2. Erythropoiesis

2.1 Erythroid differentiation

2.1.1 Primitive and definitive erythropoiesis

Erythropoiesis is a multistep process that involves the differentiation from HSCs to mature erythrocytes. In the adult hematopoietic system, erythropoiesis takes place in the bone marrow and first implies the differentiation of HSC into the common myeloid progenitor, a multipotent progenitor that generates granulocytic-macrophage and megakaryocytic-erythroid progenitors. In the CFU-culture assay, the CMP generates mixed colonies of myeloid and erythroid cells (Mix-CFC). When commited to the erythroid lineage CMPs give rise to burst-forming unit-erythroid (BFU-e), which generates large colonies of erythroid cells *in vitro* and the late erythroid progenitor CFU-e [reviewed in (Testa, 2004)]. After several mitotic divisions, the CFU-e differentiates into morphologically recognizable erythroid cells. During this differentiation process, the cells gradually express erythropoietin receptor thus becoming sensitive to erythropoietin (EPO). This cytokine regulates both proliferation and survival of erythroid cells. The last stages of erythroid differentiation involve a proliferative-maturative compartment composed by proerythroblasts, basophilic erythroblasts and polychromatophilic erythroblasts and a maturative non-proliferative compartment involving maturation of orthochromatic erythroblast, reticulocytes and mature erythrocytes. During last stages of maturation, erythroid cells decrease their size, accumulate hemoglobin and increase their chromatin density to exclude the nucleus. Finally, they are released to the bloodstream and take part in the gas exchange for a half-period of thirty days [reviewed in (Testa, 2004)].

During embryonic development, there are two major waves of erythroid cell production, the first one starting at E7.5 in the yolk sac and the second one beginning at E11 in the fetal liver. Yolk sac erythropoiesis is characterized by the appearance of primitive nucleated large erythrocytes (6-fold larger than adult ones) expressing embryonic (ζ, βH1 and εy) and adult globins (α1, α2, β1 and β2) differentiated from primitive erythroid progenitors (EryP-CFC). However, from E8.5 adult-like progenitors (BFU-e and CFU-e) are found in the yolk sac, which in culture generate enucleated erythrocytes expressing adult globins (Palis et al., 1999).

Adult erythropoiesis is well characterized based on cell morphology, expression of cell-surface markers and hemoglobin content. Nevertheless, not much is known about primitive erythroid development. Primitive erythroid cells, generated in the blood islands of the yolk sac, enter the bloodstream once circulation between embryo and yolk sac is established, at the stage of basophilic erythroblasts. While circulating primitive erythroblasts undergo maturation characterized by a decrease in size, accumulation of hemoglobin resulting in decreased cytoplasmic basophilia and chromatin condensation, similar to that occurring in fetal liver or adult-life bone marrow erythropoiesis [reviewed in (McGrath and Palis, 2005)].

2.1.2 Erythroid markers

Several cell surface markers have been identified in different erythroid subpopulations and are related to specific maturation stages (see Figure 10).

Ter119 is expressed in mouse erythroid cells and is associated with the erythroid cell-surface glycophorin A protein (used to characterized human erythrocytes) (Kina et al., 2000). Ter119/Glycophorin A markers are not detected in early BFU-e or CFU-e progenitors and first appear at the proerythroblast stage.

CD71 is the transferrin receptor, which is essential for cellular growth in proliferating cells. In the erythroid lineage, it is required for iron uptake for hemoglobin synthesis and is highly expressed in erythroid progenitors while not detectable on mature erythrocytes (Shintani et al., 1994). On the other hand, c-Kit (CD117) is the SCF receptor and it is expressed in erythroid progenitors where is required for proliferation and differentiation.

CD41 is expressed in murine erythroid progenitors and is dowregulated during erytroid differentiation. It is also expressed in the yolk sac primitive erythroid cells (Ferkowicz et al., 2003) and in HSC in the embryo (Bertrand et al., 2005). Different erythroid population have been characterized based on CD41 expression: $CD41^{dim}Ter119^{-}$ (immature cells with larger cell size), $CD41^{dim}Ter119^{+}$ (basophilic erythroblasts) and $CD41^{-}Ter119^{+}$ (mature chromatophilic or late basophilic erythroblasts) (Otani et al., 2005).

Both primitive and definitive erythroid cells start expressing hemoglobin at the basophilic erythroblast stage being progressively accumulated and maximum at final steps of maturation (Otani et al., 2005).

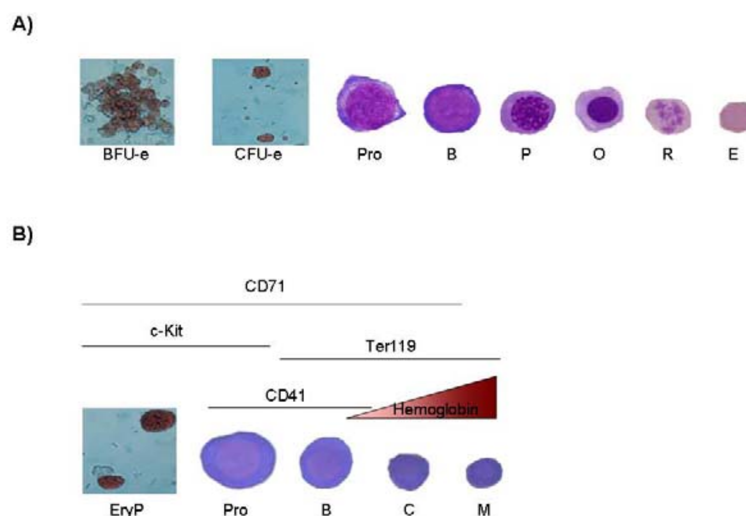


Figure 10:* The erythroid lineage. A)** *Bone marrow-derived definitive erythroid lineage: BFU-e and CFU-e erythroid progenitors (from Palis, 1999); Pro, proerythroblast; B, basophilic erythroblast; P, polychromatic erythroblast; O, orthochromatic erythroblast; R, reticulocyte; E, mature erythrocyte.* ***B) *E9.5 yolk sac-derived primitive erythroid lineage: EryP, primitive erythroid progenitor (from Palis, 1999); Pro, proerythroblast; B, basophilic erythroblast; C, chromatophilic erythroblast; M, mature nucleated erythrocyte. The different markers expressed by each population are represented.*

2.2 Regulation of embryonic and adult erythropoiesis

2.2.1 Cytokine-mediated regulation

From E10, passive difusion is not sufficient for oxygen and nutrient supply in the embryo. At this stage, hypoxia plays a critical role in erythropoiesis, by regulating EPO levels (Wang and Semenza, 1995). EPO signaling is the major regulatory mechanism for red blood cell production. Binding of EPO to its receptor (EpoR) activates the Janus kinase (Jak2), which phosphorylates its downstream effector, Stat5, leading to specific gene transcription. In addition, it induces other signaling pathways such as phosphoinositide-3-kinase (PI3kinase), Akt kinase and Ras that regulate apoptosis, proliferation and terminal erythroid differentiation [reviewed in (Testa, 2004)].

Targeted disruption of EPO signaling pathway components such as EPO, EpoR and Jak2 kinase (Lin et al., 1996; Neubauer et al., 1998; Wu et al., 1995) leads to embryonic death with severe anemia due to a huge reduction of primitive erythroblasts at E12.5. However, EPO signaling mutants develop normal numbers of erythroid progenitors indicating that this cytokine is not required at the first stages of erythroid commitment. The role of EPO in primitive erythropoiesis is still unclear although it has been shown that EPO increases primitive red blood cell production and prevents from death in E8.5 yolk sac cultures (Kimura et al., 2000).

The Stem cell factor (SCF) or Kit ligand is another important cytokine in erythropoiesis. Both SCF and c-Kit are highly expressed in uncommitted CD34 positive cells and erythroid committed progenitors (BFU-e and CFU-e) being downregulated with differentiation and absent in mature blood cells (Testa et al., 1996). Thus, SCF is likely involved in the expansion and survival of erythroid progenitors.

2.2.2 Erythropoietic transcription factors

GATA1 is the most important transcription factor in erythroid development although it is also required for proper maturation of mast and megakaryocytic precursors and for specification of eosinophils. It recognizes the WGATAR-binding motif present in the promoter and/or enhancers of all erythroid-specific genes [reviewed in (Patient and McGhee, 2002)]. This zinc finger transcription factor is expressed at basal levels in quiescent erythroid progenitors, but after EPO-induced differentiation, expression increases reaching its maximum between CFU-e and proerythroblasts stages [reviewed in (Cantor and Orkin, 2002)].

GATA1 null mutation is embryonic lethal at E10.5-11.5 due to severe anemia. The presence of erythroid progenitors in these embryos indicates that GATA1 is not essential for erythroid commitment, however they display a maturational arrest at the level of proerythroblasts (Fujiwara et al., 1996). In agreement with this, GATA1$^{-/-}$ erythroid precursors derived from embryoid bodies have a blockage at the proerythroblast stage and undergo apoptosis (Weiss and Orkin, 1995).

Surprisingly, in a mouse model in which GATA1 levels are less than 5% of the level present in wild-type, the mutant embryos die at E12.5 as well (Takahashi et al., 1997). However, erythroid cells are resistant to apoptosis and remain proliferative (Suwabe et al., 1998), leading to a defect that develops in leukemia (Shimizu et al., 2004). Thus, GATA1 levels seem to be crucial to regulate erythroid homeostasis.

Friend of GATA1 (FOG-1) is another zinc-finger transcription factor that interacts with GATA1. FOG-1 mutant mice display a similar phenotype than GATA1 null animals indicating that both proteins are required for erythroid differentiation. However, FOG-1 null phenotype is more severe compared to GATA1 as shown by the complete lack of megakaryocytes, suggesting that FOG-1 exerts some GATA1-independent functions (Tsang et al., 1998).

The basic helix-loop-helix transcription factor Scl /Tal-1 interacts with ubiquitously expressed E proteins to bind the promoters of erythroid and megakaryocytic specific genes. Enforced Scl expression favours erythroid commitment of hematopoietic progenitors (Valtieri et al., 1998), whereas Scl deficiency inhibits proliferation and self-renewal of erythroleukemia cells (Green et al., 1991).

Other transcription factors involved in erythroid development are the family of Krüppel-like zinc finger transcription factors or KLF. Among all the members of the family, KLF1/EKLF is required for the expression of the β-globin gene, heme group synthesis enzymes (such as ALAS-E or Alad), the α-hemoglobin stabilizing protein (AHSP) and other proteins involved in red cell membrane and cytoskeletal stability (Hodge et al.,

2006). Moreover, KLF6 null mice die at E12.5 due to primitive hematopoietic and vascular defects in the yolk sac, suggesting a role of this transcription factor in mesoderm specification into these two lineages. Interestingly, GATA1 and Scl expression was impaired in the knockout embryos suggesting that KLF6 works upstream of these two proteins (Matsumoto et al., 2006).

2.2.3 Apoptosis

Programmed cell death or apoptosis is a highly conserved mechanism for regulation of tissue remodelling and cell homeostasis. The balance between the rate of hematopoietic cell production and destruction is responsible for maintaining blood homeostasis. In addition, there should be high numbers of progenitors and continuous cell differentiation in order to ensure a rapid response in compromised conditions (i.e pathogen infections or anemia) [(reviewed in (Testa, 2004)]. Hence, red blood cell homeostasis is absolutely dependent on the appropriate balance between apoptotic versus antiapoptotic signals.

Consistent with an antiapoptotic role for GATA1, bcl-X_L promoter contains GATA binding sites and its expression is upregulated by GATA1 in ES cells (Gregory et al., 1999). Moreover, GATA1 null ES-derived red blood cells die from apoptosis (Weiss and Orkin, 1995). Bcl-X_L promoter also contains binding sites for Stat5, which is dowstream of EPO signaling (de Groot et al., 2000; Dolznig et al., 2006). Stat5 deficient embryos display severe anemia due to impaired survival of liver erythroid progenitors (Socolovsky et al., 1999). Bcl-X_L mutant mice die at E13 displaying massive neuronal cell death and erythroid apoptosis in the fetal liver (Motoyama et al., 1995).

EPO also inhibits apoptosis through the PI3K/Akt signaling pathway. PKB/Akt activity induces inactivation of proapoptotic Bad leading to the maintenance of mitochondrial integrity, together with downregulation of other death genes such as FasL and Bim [reviewed in (Vivanco and Sawyers, 2002)].

Figure 11:* *Intrinsic apoptotic pathway. *Apoptotic signals such as cytokine deprivation or DNA damage activates the death machinery induced or repressed by members of the Bcl-2 protein family. The multidomain proapoptotic subfamily (Bax, Box and Bok) and the BH3-only proapoptotic proteins (Bim, Bid, Bak) cooperate to disrupt mitochondrial membrane potential, allowing the release of cytocrom C and other components to the cytoplasm, leading to the activation of caspase-9 and the apoptotic executioner caspase-3, a cysteine protease responsible for the degradation of nucleus, organelles and DNA fragmentation, the classical features of apoptotic death (Hengartner, 2000). The multidomain antiapoptotic subfamily (Bcl-2, Bcl-X_L, Bcl-w) counteract pro-apoptotic molecules by protein-protein inhibition, but the latter can also inhibit the anti-apoptotic ones (adapted from U. Testa, Leukemia 2004).*

An extrinsic apoptotic pathway, which involves secreted factors or ligand-mediated signals, is also involved in regulating erythroid apoptosis (see Figure 12). Several studies revealed that activation of death receptors including Fas receptor (FasR) or tumor necrosis factor-α (TNF-α) receptor leads to apoptosis of erythroid progenitors (De Maria et al., 1999). FasR or Fas ligand (FasL) null mice display augmented extramedullary hematopoiesis as a result of increased progenitor cell death in the bone marrow (Schneider et al., 1999). Moreover, FasR-mediated caspase activation leads to GATA1 and Scl cleaveage in specific caspase recognition sites thus promoting apoptosis and inhibiting erythroid differentiation (De Maria et al., 1999; Zeuner et al., 2003). This mechanism is specific for regulating mature erythrocyte populations since both hematopoietic stem cells (c-Kit^{+}Sca-1^{+}Lin^{-}) and quiescent CD34^{+} progenitors are resistant to FasR-FasL apoptotic pathway (Bryder et al., 2001).

TNF receptor (TNF-R) cascade is also involved in erythroid apoptosis likely through regulation of FasR levels (Maciejewski et al., 1995). In agreement with this, TNF-α null mice display higher number of erythroid colonies in the bone marrow (Jacobs-Helber et al., 2003). Finally, the TRAIL/TRAIL-receptor system plays a similar role than FasR-FasL in the erythroid lineage (Zamai et al., 2000).

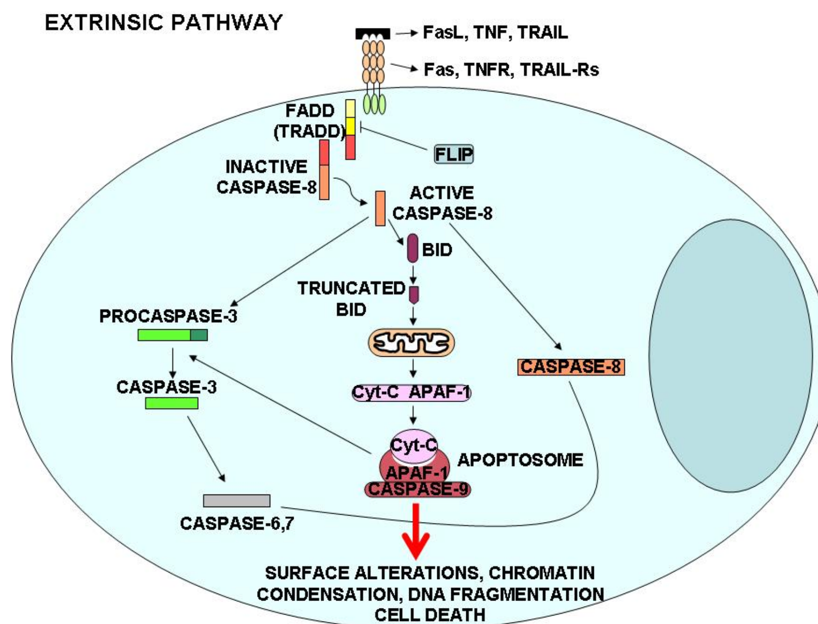


Figure 12: ***Extrinsic apoptotic pathway.*** *Activation of death cell receptors by their ligands triggers the extrinsic apoptotic pathway, by recruiting de death domain (DD) adaptors FADD or TRADD. This signal leads to the formation of a death-inducing signaling complex (or DISC) which in turn, activates the apoptosis initiating caspase-8 and caspase-10. The result, is the activation of the executioner caspase-3 and Bid, leading to release of apoptogenic factors from mitochondria, such as SMAC/ DIABLO, which prevents the activation of antiapoptotic proteins (adapted from U. Testa, Leukemia 2004).*

Finally, calcium has been described as apoptotic inducer as well, since increment in intracellular calcium concentration leads to destruction of red blood cells in an apoptotic-like manner, a process thought to be independent of caspases (Berg et al., 2001).

2.3 Erythroid pathologies

Many erythroid disorders are due to a deregulation of the apoptotic mechanisms that control red blood cell compartment.

- **Polycythemia vera (PV)**

Polycythemia vera (PV) patients display an acquired myelodysplastic syndrome (MDS) with erythroid, megakaryocytic and granulocytic cell overproduction, that usually develops into acute leukemia [reviewed in (Spivak, 2002)]. Some molecular alterations have been described in this syndrome that results in increased apostosis resistance in the erythroid lineage. However, most PV patients harbour a point mutation that activates Jak2 leading to a blockage of death receptor-mediated apoptosis concomitant with bcl-X_L overexpression. Moreover, PV erythroid cells also express high levels of FLICE-inhibitory protein (c-FLIP), a specific inhibitor or caspase-8, which confers resistance to the extrinsic apoptotic pathway (Zeuner et al., 2006).

- **Anemia**

Multiple myeloma associated anemia as well as Fanconi anemia are due to spontaneous apoptosis due to a higher sensitivity to FasL-mediated apoptosis (Silvestris et al., 2002), leading to GATA1 cleavage and cell death. Moreover, point mutations in the GATA1 gene cause dyserythropoietic anemia and thrombocytopenia due to a conformational change that impedes its binding to FOG-1 (Nichols et al., 2000). On the other hand, rheumatoid arthritis-linked anemia is caused by an excessive TNF-α release.

- **Hemoglobinopathies**

Abnormal globin expression leads to hemoglobinopathies commonly known as thalassemias. Thalassemias are characterized by unbalanced globin chain synthesis leading to free α-chains (β-thalassemias) or β-chains (α-thalassemias) that precipitate into the cytoplasm promoting hemolysis of the more mature erythroblasts (cells with higher hemoglobinization). GATA1 and EKLF control the expression of both β-globin and the α-haemoglobin stabilizing protein. Thus, the absence of EKLF in mice results in globin imbalance and embryonic death by E16 due to a phenotype resembling β-thalassemia.

On the other hand, sickle cell disease is also caused by β-chain precipitation leading to an aberrant erythroid morphology, which blocks small vessels [reviewed in (Testa, 2004)].

Section 3. The Notch signaling pathway

3.1 Notch signaling and the control of cell fate

During the evolution, multicellular organisms have developed regulatory mechanisms that ensure the orderly and reproducible development of the different organs and tissues. Cell-cell signaling is likely to govern most of these processes. Notch signaling is known to regulate many of the cell fate decisions in the organism through direct cell-cell interactions [reviewed in (Lai, 2004)]. Notch family members have been found in evolutionary diverged organisms from flies to mammals.

Notch was first identified almost a hundred years ago by genetic experiments in *Drosophila* and received its name from indentations (notches) displayed in the wing of mutant flies. Since then, Notch function has been the aim of extensive research and nowadays it is known that regulates a wide variety of developmental processes such as neurogenesis, miogenesis, hematopoiesis, wing formation and somite segregation [reviewed in (Lewis, 1998)].

3.1.1 Key players and mechanism

The Notch family members include the Notch receptors, the Delta and Serrate/Jagged ligands and the nuclear transcription factor CSL (that accounts for CBF1/recombinant binding protein-J kappa (RBPjκ), Suppressor of Hairless (Su[H]), Lag-1). All metazoan organisms contain one or more orthologues of these proteins as summarized in table 3.

Core component	*C. elegans*	*D. melanogaster*	*Mammals*
Receptor (Notch)	LIN-12 GLP-1	Notch	Notch1 Notch2 Notch3 Notch4
Ligand	LAG-2 APX-1 ARG-2 F16B12.2	Delta Serrate	Delta-like1 (Dll1) Delta-like3 (Dll3) Delta-like4 (Dll4) Jagged1 (JAG1) Jagged2 (JAG2)
Transcription factor (CSL)	LAG-1	Suppressor of Hairless [(Su(H)]	CBF1/RBPJk RBPL

Table 3: ***Basic components of the Notch signaling pathway*** *(adapted from Lai, 2004). Notch receptor, ligand and transcription factor in different species (Caenhorabditis elegans, Drosophila melanogaster and mammals).*

- **The Notch receptor**

Mammals contain four different Notch genes (Notch1-4). The Notch receptors are heterodimeric transmembrane proteins involved in transducing specific extracellular signals to the nucleus in response to ligand binding. The extracellular part of the receptor contains multiple epidermal growth factor (EGF) repeats and specifically, EGF repeats 11 and 12 are required for ligand-binding (Rebay et al., 1991). Another extracellular part of the receptor are the LIN/Notch repeats (LNR), which are involved in maintaining the heterodimeric structure of the functional receptor by disulphide bridges and likely prevent ligand-independent activation (Sanchez-Irizarry et al., 2004). The intracellular part of Notch (NotchIC) contains several functional domains required for signal transduction. These include the RAM domain and the ankyrin (ANK) repeats,

both required for the interaction with downstream effector proteins, nuclear localization signals, the transactivation domain and the C-terminal PEST domain that regulates protein stability. Notch molecule is translated from a single mRNA transcript, however during the maturation in the Golgi complex is first cleaved (S1 site) by a furin convertase and subsequently reassembled by disulphite bridges into a functional heterodimeric receptor at the cell surface [reviewed in (Maillard et al., 2003)] (see Figure 13).

- **Jagged and Delta ligands**

Signaling through the Notch receptor is triggered by interaction with one of the Notch ligands (see Figure 13). They are also transmembrane proteins that contain multiple EGF-like repeats and a characteristic DSL domain (DSL accounts for Delta, Serrate and Lag-2). Both EGF and DSL repeats are involved in Notch receptor interaction, although there is some evidence that DSL is the minimal unit that can activate Notch (Shimizu et al., 2000). The intracellular domain of Jagged and Delta is very short (only few aminoacids) and it is not known whether it displays any function in the presenting cell. Two different ligands have been identified in *Drosophila* (Delta and Serrate), whereas vertebrates have five different ligands (Jagged1 and Jagged2, Delta1, Delta3 and Delta4) although an additional ligand, Delta2, has been found in *Xenopus*. Serrate and its orthologues Jagged1 and Jagged2 differ from Delta ligands in that contain additional EGF-like repeats and a cysteine-rich domain [reviewed in (Ohishi et al., 2003)].

- **Activation of the Notch pathway**

Activation of the Notch receptor by one of its ligands expressed in the adjacent cell leads to two successive proteolytic cleavages in the Notch molecule (Kopan et al., 1996). The first cleavage (S2) occurs in the extracellular domain and it is mediated by an ADAM metalloprotease called TACE (TNF-α converting enzyme) in vertebrates or Kuzbanian in *Drosophila*. The truncated receptor is then a substrate for a multiprotein complex formed by presenilin, nicastrin, Aph1 and Pen-2 with γ-secretase activity that cleaves Notch within its transmembrane domain thus leading to the release of the intracellular domain (Notch^{intra} or NotchIC) [reviewed in (Lai, 2004)]. Blocking γ-secretase activity with pharmacological inhibitors such as DAPT or genetic inactivation of members of the γ-secretase complex prevents Notch signaling (Zhang et al., 2000).

After cleavage, intracellular Notch fragment (NotchIC) translocates to the nucleus where it binds its downstream effector, the transcription factor CSL. In the absence of Notch activation, CSL is bound to specific binding sites in the DNA of its target promoters (C/T)GTGGGAA) and repress the corresponding genes by recruiting corepressors and histone deacetylases (HDACs) (Kao et al., 1998). Once NotchIC enters the nucleus, it binds to CSL thus displacing transcriptional corepressors and recruiting coactivators leading to gene activation (see Figure 13). The best-characterized Notch target genes are the hes (hairy and Enhancer of Split) and hrt (hes-related) family of transcription factors. There are seven hes genes (hes1-7) in mammals, based on sequence homology, but only hes1 (Jarriault et al., 1995), hes5 (Ohtsuka et al., 1999) and hes7 (Bessho et al., 2001) are activated by Notch. Aditionally there are two hrt genes, herp1/hrt2 and herp2/hrt1 that are both Notch targets [reviewed in (Iso et al., 2003)]. Hes and Hrt are basic helix-loop-helix transcription factors that act as transcriptional repressors of other bHLH such as Mash1 and MyoD. In general, Hes targets are transcription factors involved in cell differentiation of several systems including neurogenesis, myogenesis, hematopoiesis or intestinal differentiation [reviewed in (Ohishi et al., 2003)].

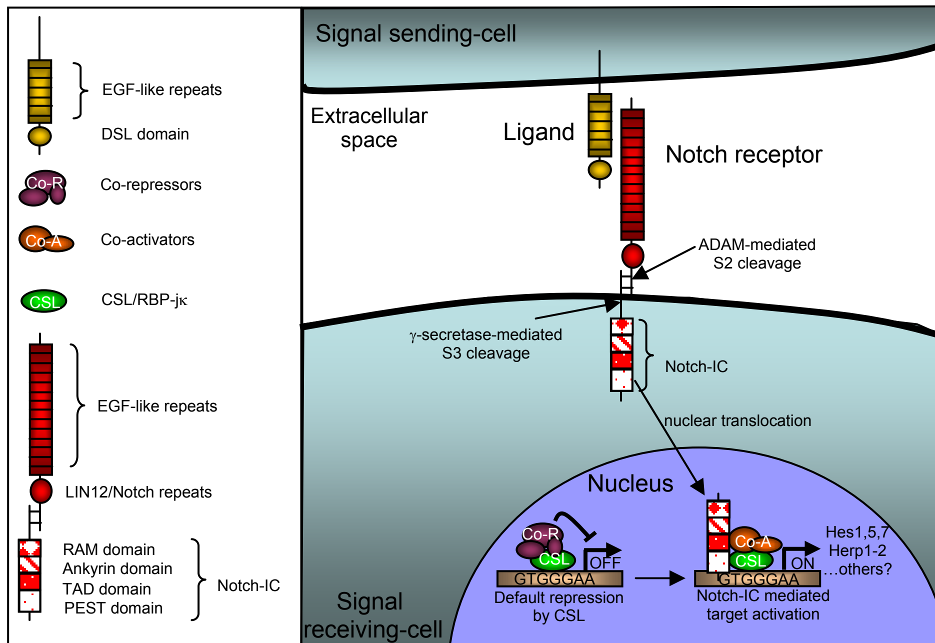


Figure 13: ***The canonical Notch signaling pathway.*** *Notch activation leads to a cascade of proteolytic events resulting in NotchIC translocation to the nucleus and NotchIC/RBPjκ dependent target gene expression (adapted from Lai, 2004).*

3.1.2 Control of cell-fate decisions

During the first stages of development of a given tissue, most of the cells have an equivalent developmental background and potentiality. In this homogeneous population, the interactions between Notch and Jagged/Delta (located in neighboring cells) are responsible for generating cell diversity through activating specific gene programs [reviewed in (Lewis, 1998)]. Two different mechanisms have been proposed to explain how Notch regulates cellular diversity: lateral inhibition and lateral induction processes.

- **Lateral inhibition model**

The lateral inhibition model implies that in a population of equivalent cells expressing low levels of both receptor and ligand, the cell that first produces more ligand, activates the Notch receptor in the neighboring cell that dowregulates ligand expression. This mechanism allows the maintenance and intensification of the differences in expression of receptor and ligand, since the ligand-expressing cell does not receive inhibitory signals from its surrounding cells. A salt-and-pepper mosaic of cells emerges and as a result, the ligand-expressing cell differentiates into a distinct lineage than the surrounding cells (see Figure 14) [reviewed in (Lewis, 1998)].

The classical example of lateral inhibition occurs during the neural-epidermal choice in *Drosophila*. Specification to the neural lineage needs the expression of the achaete-scute bHLH transcriptional activators. Notch activation results in the expression of the Enhancer of split Complex [E(spl)-C] which represses the expression of these proneural genes and results in the inhibition of the neural fate (Parks et al., 1997) leading to the epidermal fate. In mammals, Notch signaling represses neurogenesis and myogenesis via homologous Hairy/E(spl)-related bHLH repressors known as Hes, which inhibit the bHLH transcription factors Mash1 and MyoD [reviewed in (Artavanis-Tsakonas et al., 1999)].

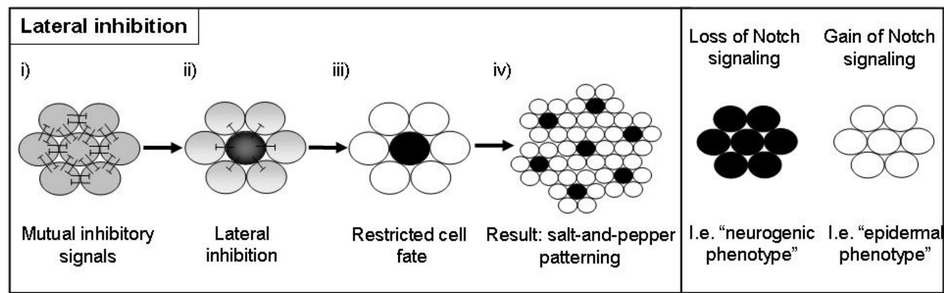


Figure 14:* *Lateral inhibition model. i) *Equivalent cells expressing receptor and ligand exert mutual inhibitory signals.* ***ii)*** *One of these cells expresses high levels of ligand, inhibiting the expression in the surrounding cells.* ***iii)*** *The ligand expressing cell switches on a different genetic program than the neighboring cells and adopts a different cellular fate.* ***iv)*** *As a result, a salt-and-pepper pattern is generated. Loss of Notch signaling drives all the cells to adopt the restricted cell fate. In Drosophila neuro-epidermis system, all the cells would differentiate into neural cells. Gain of Notch signaling inhibits differentiation and all the cells would appear as epidermis (adapted from Lai, 2004).*

- **Lateral induction model**

Notch can also function in a lateral induction model. In this case, activation of Notch in a given cell promotes the expression of the Notch ligands in the same cell. Thus, a ligand-expressing cell activates Notch signaling in the adjacent cell preventing the salt-and-pepper pattern, inducing their cell-fate choices cooperatively and forming defined boundaries of gene expression (see Figure 15) [reviewed in (Lewis, 1998)].

In *Drosophila*, the classical example of lateral induction occurs during the wing formation. Notch signaling between the dorsal and ventral compartments of the wing imaginal disc results in the formation of the wing margin. In this case, Notch activates the expression of vestigial, a transcriptional coactivator that is required for proper wing development (Couso et al., 1995). In vertebrates the classical example is the generation of boundaries during somitogenesis. Somites are regularly spaced blocks of mesoderm that split off from the presomitic mesoderm in a periodic oscillatory manner. This is mediated by Notch through oscillatory activation of hes genes (Jouve et al., 2000) and oscillatory inhibition of Notch activity by Lunatic fringe (Dale et al., 2003).

Thus, Notch principal role during development is the control of cell-fate decisions generating cell diversity in cells sending or receiving signals. Although these two models explain some developmental events, other developmental systems are likely to be more complex.

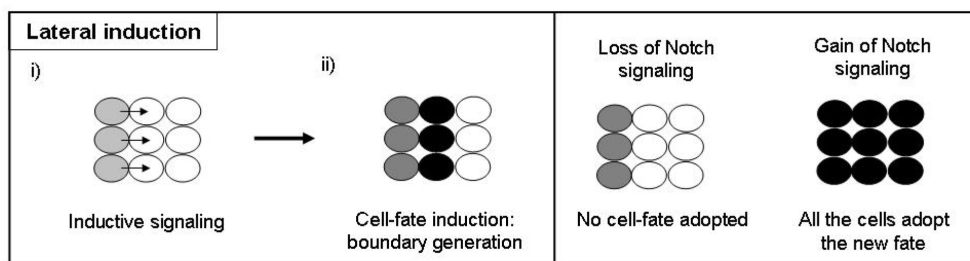


Figure 15:* *Lateral induction model. i) *A group of cells (in grey) signal to the adjacent white cells.* ***ii)*** *Subsequently, the Notch receptor expressing cells adopt a new fate, in this case, to form a boundary (black cells). Loss of Notch signaling results in the absence of the new fate, whereas excessive Notch signaling has the opposite effect (adapted from Lai, 2004).*

- **Other key players in Notch signaling**

Glycosilation of the Notch receptor by Fringe glycosyltransferases (Radical, Lunatic and Manic Fringe) is responsible for modulating ligand specificity. Fringe proteins are shown to regulate Notch activity and contribute to generation of cell diversity [reviewed in (Irvine, 1999)].

Ubiquitination has been shown to be required for endocytosis of Notch and their ligands. Neuralized and Mind bomb which belong to the RING-type E3 ubiquitin ligases ubiquitinate Delta ligands and mutations in these proteins result in Notch-loss of function phenotypes (Itoh et al., 2003; Lai et al., 2001). Recent studies suggest that Delta ubiquitination increases its affinity for Notch binding. Moreover, endocytosis of the Delta ligand bound to extracellular Notch facilitates the S2 cleavage of Notch receptor (Nichols et al., 2007).

3.2 Role of the Notch signaling pathway in hematopoiesis

Notch signaling pathway functions at various stages of hematopoietic development [reviewed in (Ohishi et al., 2003)]. Notch is activated by interactions between precursors and stromal supporting cells but also by interactions between hematopoietic cells.

3.2.1 Expression of Notch members in the hematopoietic system

Notch1 and Notch2 expression is found in $CD34^{+}Lin^{-}$ bone marrow hematopoietic precursors, suggesting a role for Notch very early in mammalian blood cell development (Milner et al., 1994; Ohishi et al., 2000), Conversely, Jagged1 expression is found in bone marrow stromal cells (Walker et al., 2001) as well as Delta1 and Delta4 (Karanu et al., 2001). All these ligands are also expressed in thymic epithelial cells, consistent with the important role of Notch in T-cell development (Felli et al., 1999; Mohtashami and Zuniga-Pflucker, 2006).

In the myeloid lineage, monocytes express high levels of Notch1 and 2 but expression has not been found in granulocytes (Ohishi et al., 2000). Finally, bone marrow erythroid progenitors express Notch1 but expression decreases in more mature erythroid cells such as acidophilic normoblasts (Ohishi et al., 2000; Walker et al., 2001).

3.2.2 Role of Notch in HSC self-renewal

There is important evidence supporting the idea that Notch plays a crucial role in HSC self-renewal. Recent work performed by Duncan *et al*, demonstrated that Notch signaling is active in the KSL subpopulation located in the bone marrow niche and downregulated as these cells differentiate. Moreover, Notch inhibition by a dominant negative CBF1 leads to accelerated differentiation of HSCs *in vitro* and depletion of HSC activity *in vivo* indicating that Notch is required for the maintenance of the undifferentiated state of HSCs (Duncan et al., 2005).

Retroviral transduction of the Notch intracellular domain in c-$Kit^{+}Sca1^{+}Lin^{-}$ murine hematopoietic progenitors leads to immortalization of these cells and repopulation of both myeloid and lymphoid lineages when transplanted into lethally irradiated mice (Varnum-Finney et al., 2000). Moreover, Notch1IC expands the number of bone marrow repopulating cells in secondary transplants and promotes their lymphoid

differentiation (Stier et al., 2002). A similar result was obtained by expressing Notch4IC in human Lin$^-$ cord blood cells (Vercauteren and Sutherland, 2004).

Ex vivo expansion of human hematopoietic cells is critical for clinical procedures that involve stem cell transplantation. Addition of soluble Jagged1 to *ex vivo* cultures of human CD34$^+$CD38$^-$Lin$^-$ cord blood cells expand human stem cells without loosing the ability to repopulate the hematopoiesis of NOD/SCID mice (Karanu et al., 2000). A similar effect was found by incubating bone marrow KSL progenitors with a Delta1 fusion protein (Varnum-Finney et al., 2003). Many of these effects are reproduced in CD34$^-$KSL cells retrovirally transduced with hes1, suggesting that this protein is responsible for the effects of activating Notch1 on HSCs self-renewal (Kunisato et al., 2003).

3.2.3 Notch regulation of lymphoid cell-fate decisions

Notch signaling regulates several cell-fate decisions in the lymphoid lineage. There is strong evidence from different studies that Notch activation promotes T- whereas inhibits B-cell fate. Deletion of RBPjκ in hematopoietic cells results in increased B-cell differentiation and blockage of T-cell development (Han et al., 2002). Conversely, expression of Notch1IC in progenitors blocks B-cell differentiation and leads to generation of immature CD4$^+$CD8$^+$ T cells (Pui et al., 1999). It is still unclear whether this B- versus T-cell decision is made in the CLP or in a recently described early T-cell progenitor (ETP) [reviewed in (Maillard et al., 2003)]. In addition, Notch2 is required to generate the marginal zone B cells in the spleen which are important for T-cell independent immune response [reviewed in (Maillard et al., 2003)].

Notch is also involved in the commitment of $\alpha\beta$ versus $\gamma\delta$ TCR (T-cell receptor) lineage. Notch1 activation favours the $\alpha\beta$ choice since decreased Notch1 levels results in an increase of $\gamma\delta$ cells (Washburn et al., 1997). Similarly, conditional inactivation of RBPjκ increases the number of $\gamma\delta$ T cells (Tanigaki et al., 2004).

At the last stage of T-cell differentiation, Notch is also involved in the decision between CD4$^+$ and CD8$^+$ from CD4$^+$CD8$^+$ double positive cells since expression of Notch1IC in thymocytes favours the generation of CD8$^+$ cells with a corresponding decrease in CD4$^+$ T cells (Robey et al., 1996).

3.2.4 Notch in myeloid differentiation

Many experimental models have been used to determine the role of Notch in myelopoiesis from cell lines to *in vivo* models. Early studies with myeloid cell lines showed that forced Notch1 or Notch2 activation could inhibit differentiation in a cytokine-dependent manner (Bigas et al., 1998; Milner et al., 1996), likely through expression of Gata2 (Kumano et al., 2001). Recently, a similar result has been reported with KSL progenitors co-cultured on an OP9 stromal cell line expressing Delta1 (de Pooter et al., 2006). However, several results suggest that Notch activity is mainly required for T and B lymphoid differentiation *in vivo* (Radtke et al., 1999).

3.2.5 Notch in apoptosis

There is increasing evidence that Notch1 activity is involved in regulating programmed cell death or apoptosis. In most cases, Notch activation inhibits apoptosis as described in T cells (Jehn et al., 1999), Kaposi's sarcoma (Curry et al., 2005) and Hodgkin lymphoma cells (Jundt et al., 2002), however and likely due to cell context specificity, there are several examples that show Notch as a positive regulator of

apoptosis. In B-ALL cell lines, activation of Notch induces apoptosis, most likely through Hes1 (Zweidler-McKay et al., 2005) comparable to human peripheral blood monocytes cultured in the presence of immobilized Delta1 ligand and M-CSF (Ohishi et al., 2000).

Whether Notch induces apoptosis in the erythroid lineage remains controversial. In K562 cell line, Notch inhibits erythroid differentiation and induces apoptosis, likely through Hes1, which inhibits GATA1 activity and bcl-X_L expression (Ishiko et al., 2005). However, Notch1 prevents apoptosis in the murine erythroleukemic cell line during HMBA-induced differentiation (Jang et al., 2004; Shelly et al., 1999).

3.2.6 Notch implication in the ontogeny of the hematopoietic system

During the recent years, the function of Notch in embryonic hematopoiesis has partially been elucidated. Some of the work is the aim of this thesis and will be discused in detail; however, while this work was in progress other important contributions came out and have been included in this section.

The first *in vivo* proof that Notch signaling plays a key role in the generation of HSCs during embryonic development came from Kumano *et al* by studying the Notch1 null mice. Using P-sp explant culture on OP9 stromal cell line, they found that definitive hematopoiesis is impaired in the Notch1$^{-/-}$ embryos due to the lack of HSC. In contrast, the number of CFC in the Notch1$^{-/-}$ yolk sacs was similar to wild-type, suggesting that primitive hematopoiesis is at least partially preserved. However, HSC from Notch1$^{-/-}$ YS failed to reconstitute myeloablated new-born mice. At E9.5 Notch1 null embryos display similar number of CD34^{+}c-Kit^{+} and VE-cadh^{+}CD45^{-} hemogenic endothelial cells in both YS and P-sp, suggesting that lack of definitive hematopoiesis is due to impaired commitment of HSC from endothelial cells (Kumano et al., 2003). Consistent with this finding, chimeric mice with Notch1-deficient ES cells do not contribute to long-term definitive hematopoiesis (Hadland et al., 2004). In contrast, analysis of Notch2$^{-/-}$ embryos indicates that Notch2 is dispensible for the generation of hematopoietic cells (Kumano et al., 2003).

Finally, important contributions for the role of Notch in the formation of HSC came from zebrafish studies *(Danio rerio).* Mind bomb mutants display normal primitive hematopoiesis but impaired HSC development. Consistent with this phenotype c-myb and runx1 expression is lost in the aorta of Mind bomb mutants. Conversely, transient notch1 expression revealed an expansion in the number of HSCs, dependent on Runx1 (Burns et al., 2005). Moreover, Runx1 but not GATA2 or Scl was able to reconstitute the generation of CFC progenitors but not HSCs in embryonic Notch1-null cells in mice (Nakagawa et al., 2006). These results indicate that Runx1 acts downstream of Notch signaling for HSC emergence in the AGM.

3.3 Altered Notch signaling and disease

Three different congenital diseases due to mutations in Notch ligands have been described. Mutations in the Jagged1 gene are responsible for Alagille sydrome, which results in impaired generation and function of different organs such as heart, eye, liver and skeleton (Li et al., 1997). Delta3 mutations are responsible for spondylocostal dysostosis, a developmental disease characterized by rib fusion and trunk dwarfism (Bulman et al., 2000). Finally, mutations in the extracellular EGF-like repeats of Notch3 results in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), characterized by migraines, strokes and dementia, further supporting that Notch is involved in neural development (Joutel et al., 1996).

Notch also participates in tumorigenic processes in different tissues including mammary gland, skin, cervix and prostate [reviewed in (Lai, 2004)], colon (Fernandez-Majada et al., 2007) or pancreas (Miyamoto et al., 2003).

- **Hematopoietic disorders**

The human Notch1 gene was first identified in a t(7;9)(q34;q34.3) chromosomal translocation in which Notch1 fused to the TCR-β resulted in constitutive activation of Notch1 in T cells leading to human T-cell leukemia (Ellisen et al., 1991). However, this rearrangment is a rare event in T-cell leukemias and point mutations occurring in the heterodimerization domain (aberrantly facilitating Notch activation) or in the PEST domain (resulting in a more stable Notch1IC protein) have been recently described in 50% of human T-ALL (Weng et al., 2004).

In the last few years, some reports have suggested a crosstalk between the Ikaros transcription factor (involved in T-cell development) and the Notch signaling pathway in T-cell tumorigenesis. In this sense, homozygous mice for a hypomorphic mutation in the Ikaros gene which develop thymic lymphomas have higher Notch activity and hes1 expression in thymocytes (Dumortier et al., 2006). Thus, in normal conditions Ikaros may be repressing the Notch target genes likely by competing with RBPjκ for a similar DNA-binding consensus.

In B cells, Epstein-Barr virus or Kaposi's sarcoma-associated herpes viruses are known to activate Notch-target genes by using viral proteins that bind to the RBPjκ consensus leading to cell immortalization and transformation [reviewed in (Milner and Bigas, 1999)].

3.4 Animal models

To study the role of the Notch in development and in adult organisms, transgenic and knockout mutants of several Notch signaling elements have been generated in species such as *Drosophila* (fly), *C. elegans* (nematodes), *Danio rerio* (zebrafish) and *Mus musculus* (mice).

The strongest Notch-loss-of-function mutation is obtained by deletion of the RBPjκ gene, the unique effector of the Notch signaling pathway (Oka et al., 1995). RBPjκ$^{-/-}$ embryos display a complex phenotype with impaired somitogenesis, defects in the neural tube formation and growth retardation (see Figure 16). Moreover, the fusion of the embryonic allantois to the maternal placenta does not occur in RBPjκ null embryos, thus umbilical vessels cannot form. All these features indicate that Notch signaling is required for vasculogenesis, somitogenesis and neurogenesis (Oka et al., 1995).

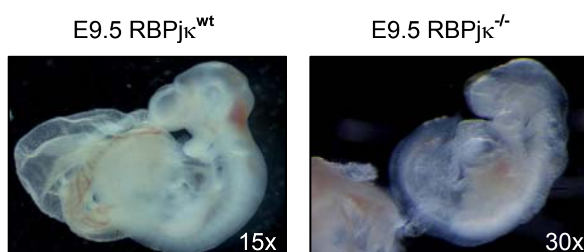


Figure 16: ***The RBPjκ null mice.*** *Photographs of E9.5 RBPjκ wild-type and knockout embryos. RBPjκ disruption is embryonic lethal by E10 and leads to growth retardation, defective somitogenesis, pericardium dilatation, neural tube contorsion and defective allantoid fusion.*

The phenotype of the Notch1 null embryos is quite similar to the RBPjκ null ones, although embryonic lethality occurs at E11.5. Notch1$^{-/-}$ mutants also display growth retardation, impaired neurogenesis and somite formation, but the chorioallantoic fusion defect is not found (Conlon et al., 1995).

Notch2 mutants die at E11.5 and display a massive apoptosis in the neuroephitelium and otic and optic vesicles (Hamada et al., 1999).

Notch3 null mutants are viable and fertile and only display some defects in vasculogenesis (Domenga et al., 2004; Krebs et al., 2003).

Finally, Notch4 mutant embryos are viable and fertile although it cooperates with Notch1 mutation in inducing vasculogenic defects (Krebs et al., 2000).

Jagged1 null mice die at E10. The mutant embryos can form the primitive blood vessels both in the yolk sac and the embryo proper but they fail to remodel the vascular plexus to form the large vitelline blood vessels, suggesting that angiogenesis but not vasculogenesis is impaired (Xue et al., 1999).

Jagged2 mutation is not lethal until birth and mutant fetuses display impaired craniophacial morphogenesis and palatal clefting, defects in limb formation and abnormal thymic development with a reduction of γδ T cells (Jiang et al., 1998).

Finally, Mind bomb mutant mice and double knockouts for presenilin1 and presenilin2 mice die at E10.5 and E9.5 respectively, and both display several Notch-associated defects, similar to RBPjκ$^{-/-}$ embryos (impaired neurogenesis, somitogenesis and vasculature remodelling) suggesting that all these proteins acts upstream of Notch and are required for Notch signaling (Donoviel et al., 1999; Koo et al., 2005).

CHAPTER 2:

AIMS

AIMS

Although Notch signaling pathway was previously reported to regulate many cell-fate decisions in the hematopoietic system, its role in the ontogeny of embryonic hematopoiesis was completely unknown at the beginning of this thesis. Thus, by using the RBPjκ$^{-/-}$ mice as a model, we aimed to study whether the Notch signaling pathway was involved in the generation of both primitive and/or definitive hematopoiesis in the mice embryo.

The following specific aims were proposed for this project:

- To study whether the Notch/RBPjκ signaling plays a specific role in the ontogeny of the primitive hematopoietic system in the murine yolk sac from E7.5 to E9.5.

- To determine whether Notch/RBPjκ signaling is required for the generation of definitive HSCs and other progenitors from the murine P-sp/AGM region at E9.5-11.5.

CHAPTER 3:
RESULTS

E9.5 yolk sac vasculature

E11.5 Sca-1-GFP AGM

SECTION 1: RBPjκ-dependent Notch function regulates *Gata2* and is essential for the formation of intra-embryonic hematopoietic cells

(This chapter was published in Development 132, 1117-1126, 2005).

The intra-embryonic region encompassing the aorta surrounded by gonad and mesonephros tissues has been described as one of the first embryonic hematopoietic organs involved in the generation of hematopoietic progenitors (HP) and hematopoietic stem cells (HSC) that will later seed the bone marrow and sustain hematopoiesis in the adulthood (Medvinsky, 1996; de Bruijn, 2000; de Bruijn, 2002). Previous to our work there were many evidences that the Notch signaling pathway regulates different biological processes in many hematopoietic cell lines (Bigas, 1998; Karanu, 2000; Varnum-Finney, 2000; Kumano, 2001) although little work was done *in vivo*. Thus, taking advantage of the RBPjκ null mice (Oka, 1995), our aim was to determine whether Notch signaling was required for the generation of HP/HSC from the AGM.

Here we show that in wild-type embryos, members of the Notch pathway including the Notch1 and Notch4 receptor, and the Delta4, Jagged1 and Jagged2 ligands are expressed in endothelial cells from the AGM aorta at E9.5-10.5. Moreover, Notch target genes such as hes1, hrt1 and hrt2 are also expressed indicating that Notch pathway is active in this region. This expression pattern of Notch receptors and ligands is lost in the RBPjκ null embryos (which lack the common nuclear effector thus impeding downstream signaling from all four Notch receptors) demonstrating that positive and negative feed-back loops regulate their expression.

We also demonstrate that RBPj$\kappa^{-/-}$ embryos display impaired hematopoietic potential from the AGM region with a complete lack of hematopoietic progenitors (determined by CFC assay) and CD45^{+} hematopoietic cells after 6 days in culture. Absence of hematopoiesis correlates with a complete lack of the critical hematopoietic transcription factor Gata2, Runx1 and Scl expression and with an increase of endothelial cells suggesting the presence of a common progenitor (or hemangioblast) for hematopoietic and endothelial lineages.

By double in-situ hybridization (DISH) and chromatin immunoprecipitation we describe that Notch directly binds through RBPjκ to the gata2 promoter thus regulating its expression not only in the 32D cell line but also in the embryo. Finally, by DISH we describe which ligands are expressed in the adjacent cell next to the Notch1-expressing cell thus suggesting that Notch1-induced activation of gata2 expression in endothelial cells from the ventral part of the AGM aorta and activation of the hematopoietic program in that cell may be induced by Jagged2 or Delta4.

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

AUTHORS: Àlex Robert-Moreno[1], Lluís Espinosa[1], José Luis de la Pompa[2] and Anna Bigas[1]*

INSTITUTION: [1]Centre Oncologia Molecular.
IDIBELL-Institut de Recerca Oncologica.
Hospitalet, Barcelona 08907, Spain.
[2]Dept. of Immunology and Oncology, Centro Nacional de Biotecnología,
CSIC. Darwin, 3. Campus de Cantoblanco, Madrid 28049, Spain

RUNNING TITLE: Notch regulates *Gata2* in early hematopoiesis

*Corresponding author.
Mailing address: Centre Oncologia Molecular.
Institut de Recerca Oncològica
Gran Via Km 2.7.
08907 - Hospitalet, Barcelona. Spain
Phone: 932 607 404
Fax: 932 607 426
e-mail: abigas@iro.es

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 *RBPjκ* transcription factor to activate transcription, we analyzed the aorta of the Para-aortic Splanchnopleura/AGM in *RBPjκ* mutant embryos. We found specific patterns of expression of Notch receptors, ligands and Hes genes that were lost in *RBPjκ* 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 *Scl/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 *RBPjκ* 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/*RBPjκ* is a crucial event for the onset of definite hematopoiesis in the embryo.

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 (HSC) 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 to specifically 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 HSC, 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 *Scl* (*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 *RBPjκ* (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 *RBPjκ*-dependent event, since *RBPjκ* 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 E10.5 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/*RBPjκ*.

MATERIALS AND METHODS

Animals

RBPjκ 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 *RBPjκ* 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 *RBPjκ* 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 37ºC. 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), 20ng/ml GM-CSF (PeproTech) and 100ng/ml of G-CSF (Aventis Pharma). After 7 days, the presence of hematopoietic colonies was scored under a microscope. For liquid cultures, 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-aminoactinomicin-D staining.

Immunostaining

Wild-type and *RBPjκ* null embryos (E9.5) were frozen in tissue-tek OCT (Sakura) and sectioned (10µm). Slides were fixed with –20ºC methanol for 15 minutes and blocked-permeabilized in 10% FBS, 0.3% Surfact-AmpsX100 (Pierce), 5% non-fat milk in PBS for 90 minutes at 4ºC. 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 anti-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 4ºC in 4% paraformaldehyde, dehydrated and embedded in Paraplast (Sigma). Embryos were sectioned in a Leica-RM2135 at 7µm.

Double in-situ hybridization

Wild-type embryos (E10.5) were frozen in OCT and sectioned (10µm). Sections were fixed in 4% paraformaldehyde for 10 minutes, digested with 1 µg/ml proteinase K (Roche) in 50mmol/l TrisHcl ph 7.5, 5mmol/l EDTA buffer and permeabilized with 1%Surfact-Amps X100 (Pierce) in PBS. After incubation with 3% H_2O_2 (Sigma) in PBS, slides were prehybridized for 1 hour and hybridized overnight at 70ºC 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 sofware (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-E12.5 (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 is 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 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*).

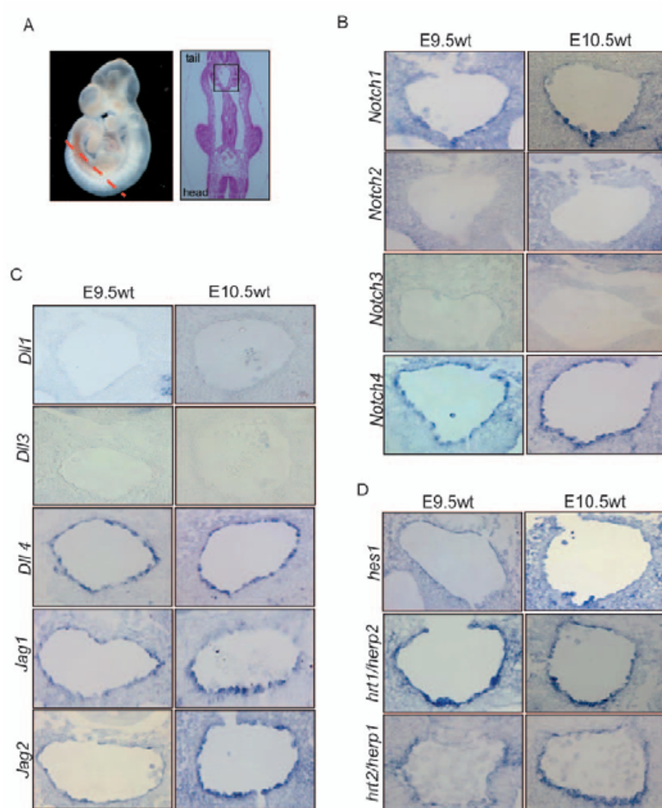


Fig.1: *Expression of Notch family members in the endothelium of the P-Sp/AGM aorta. (A) E9.5 embryo, indicating the site for P-Sp/AGM aorta and hematoxilin-eosin staining of a transverse section at the indicated level (100x). (B,C,D) Whole-mount of (B) Notch receptors, (C) Notch-ligands and (D) Notch-target genes in 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).*

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.

RBPjκ 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 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 *RBPjκ* 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 the *RBPjκ* 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 *RBPjκ* 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 *RBPjκ* 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 *RBPjκ* 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 impared in the *RBPjκ* mutant embryos

To investigate whether Notch/*RBPjκ* signaling plays a role in hematopoietic determination in the aorta, we next assayed the hematopoietic activity contained in the P-Sp/AGM region of *RBPjκ* mutant embryos compared with wild-type. Despite the presence of several developmental abnormalities and disorganized vasculature, the majority of the *RBPjκ* mutant embryos (more than 80%) display a regular fused aorta in the trunkal region at E9.5 (Oka et al., 1995). As *RBPjκ* 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 *RBPjκ* mutant littermates in the same conditions (Fig. 2C). We speculated that *RBPjκ* 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 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 *RBPjκ* mutant cultures (Fig. 2F). These results indicate that Notch signaling through *RBPjκ* is required for the generation of the hematopoietic progenitors in the P-Sp/AGM.

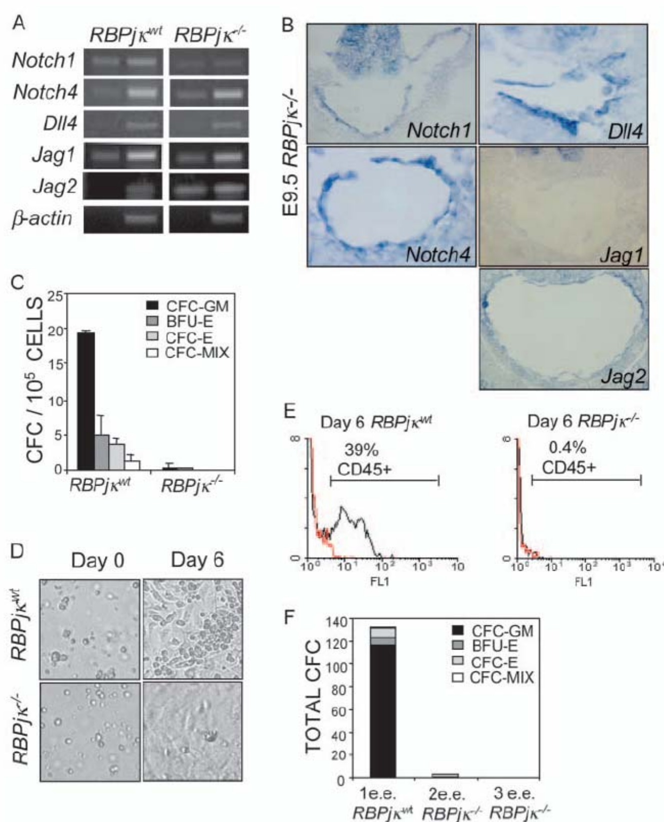


Fig.2: *Intra-embryonic hematopoiesis is impaired in the RBPjκ mutant embryos and they display an aberrant expression of Notch family members in the aorta. (A) Semiquan-titative RT-PCR analysis in dissected P-Sp of E9.5 wild-type and RBPjκ mutants. Representative PCR products after 35 and 40 cycles of two independent experiments are shown. (B) WISH, with the indicated probes and transverse sections of E9.5 aortas of RBPjκ mutants. Orientation of the aortas is dorsal (up) to ventral (down). (C) Hematopoietic CFC from dissected P-Sp of E9.5 wild-type and RBPjκ 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 RBPjκ mutants at day 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.*

Absence of hematopoietic cells and increase of endothelial cells in the P-Sp/AGM of *RBPjκ* 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 hemapotoietic 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 *RBPjκ* 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 *RBPjκ* mutants, compared with wild-type embryos (Fig. 3A).

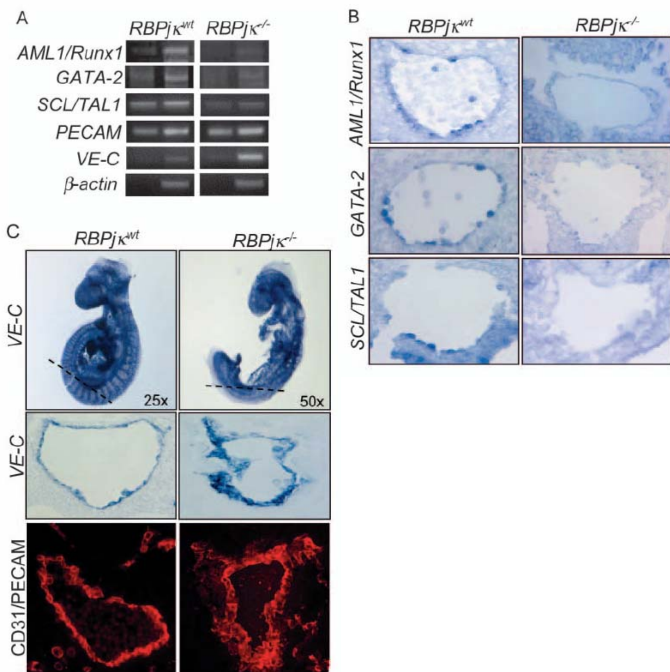


Fig.3: *Absence of hematopoietic cells and increase of endothelial cells in the P-Sp/AGM of RBPjκ mutant embryos. (A) Semiquantitative RT-PCR analysis in dissected P-Sp of E9.5 wild-type and RBPjκ mutants. Representative PCR products after 35 and 40 cycles of two independent experiments are shown. (B) WISH of hematopoietic transcription factors and transverse sections of E9.5 aortas of wild-type and RBPjκ 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 RBPjκ mutants. Orientation of the aortas is dorsal (up) to ventral (down).*

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 *RBPjκ* mutant embryos (Fig. 3B). These results are consistent with the lack of hematopoietic precursors in these mutants (Fig. 2). In addition, we detected expression of *VE-C* gene in a multiple-layered endothelium in some regions of the aorta in the *RBPjκ* 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 *RBPjκ*

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 *RBPjκ* 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 three-fold increase in *Gata2* mRNA levels, and a two-fold increase in *Scl* levels in 32D-N1^{IC} cells compared to 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^{IC} 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/*RBPj*κ 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 *RBPj*κ binding sites in the *Gata2* promoter (Minegishi et al., 1997), we tested whether the association of Notch1 to *Gata2* was dependent on *RBPj*κ. By immunoprecipitating chromatin-associated Notch1, we specifically detected the *Gata2* promoter in the precipitates from wild-type embryos but not in those from *RBPj*κ 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 *RBPj*κ (Fig. 4E). Altogether, these results indicate that Notch1, together with *RBPj*κ, regulates the expression of *Gata2* not only in hematopoietic cell lines but also in the mouse embryo.

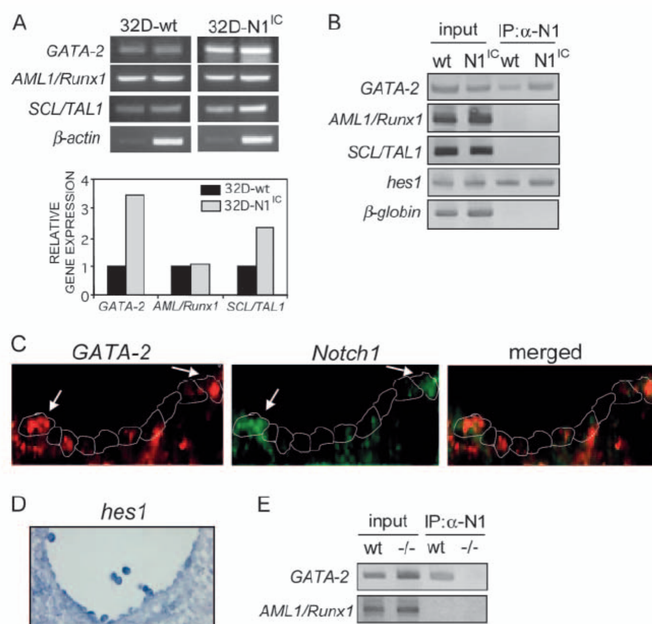
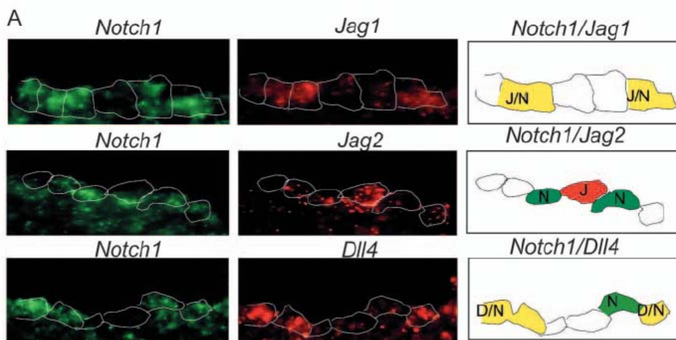


Fig.4: *Notch1/RBPjκ regulates Gata2 transcriptional activity. (A) Semiquantitative RT-PCR analysis of Gata2, Aml1 and Scl expression in 32D wild-type of N1IC-expressing cells. Representative PCR products after 35 and 40 cycles of two independent experiments are shown. Quantitated relative mRNA levels 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, 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 RBPjκ mutant whole E9.5 embryos. PCR detection of the Gata2 and Aml1 promoter is shown.*

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 *Jag*1 (Fig. 5A, upper panels), whereas *Jag*2 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.



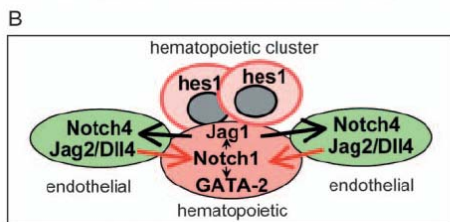


Fig.5: *Notch1*$^{+}$*/Gata2*$^{+}$ *cells in the P-Sp/AGM endothelium are Jag1*$^{+}$*/Jag2*$^{-}$*. Double in-situ hybridization on transverse sections of wild-type E10.5 aortas. (A) Hybridization of Notch1 with Jag1 (upper), Jag2 (middle) and Dll4 (lower panels). Representative photographs of at least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

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 *RBPjκ* and several members of the Notch family are likely to be involved. Consistent with the phenotype described for the *Notch1* mutant embryos, *RBPjκ* 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).

RBPjκ-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 *RBPjκ* 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 *RBPjκ*. Although the expression of other hematopoietic genes such as *Scl* and *Aml1* is severely affected in the *RBPjκ* mutants, we showed that only *Gata2* is a direct target of Notch1/*RBPjκ* 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 *RBPjκ* 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, *Jag*1 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 *RBPjκ* 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 the 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 *RBPjκ*-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.

ACKNOWLEDGEMENTS

We thank R. Kageyama, N. A. Speck, D. Martin and D.Srivastava for cDNA probes. We sincerely thank Elaine Dzierzak for helpful discussions in various aspects of this work. We thank all the members of the lab for many constructive discussions, Irene Merida for technical support and Serveis Cientifico-Tècnics, UB-Bellvitge, for confocal microscopy technical support. L.E. is an investigator from the Carlos III program (ISCIII/02/3027). A.R.M. is a recipient of a CIRIT predoctoral fellowship (2002-SI00791). This work was supported by a grant from the Comisión Interministerial de Ciencia y Tecnología, Plan Nacional de Salud (SAF2001-1191).

REFERENCES

- **Aguilera, C., Hoya-Arias R., Haegeman, G., Espinosa, L., Bigas, A.** (2004). Recruitment of IkBalpha to the hes1 promoter is associated with transcriptional repression. *Proc Natl Acad Sci U S A*, **101**, 16537-16542.
- **Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J.** (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-6.
- **Artavanis-Tsakonas, S. and Simpson, P.** (1991). Choosing a cell fate: a view from the notch locus. *Trends Genet* **7**, 403-408.
- **Barrantes, I. B., Elia, A. J., Wunsch, K., De Angelis, M. H., Mak, T. W., Rossant, J., Conlon, R. A., Gossler, A. and de la Pompa, J. L.** (1999). Interaction between Notch signaling and Lunatic fringe during somite boundary formation in the mouse. *Current Biology* **9**, 470-80.
- **Bigas, A., Martin, D. I. and Milner, L. A.** (1998). Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. *Mol.Cell Biol* **18**, 2324-2333.
- **Cai, Z., de Bruijn, M., Ma, X., Dortland, B., Luteijn, T., Downing, R. J. and Dzierzak, E.** (2000). Haploinsufficiency of AML1 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo. *Immunity* **13**, 423-31.
- **Chitnis, A. B.** (1995). The role of Notch in lateral inhibition and cell fate specification. *Mol.Cell Neurosci.*, 311-321.
- **Cumano, A., Ferraz, J. C., Klaine, M., Di Santo, J. P. and Godin, I.** (2001). Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity* **15**, 477-85.
- **de Bruijn, M. F., Ma, X., Robin, C., Ottersbach, K., Sanchez, M. J. and Dzierzak, E.** (2002). Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* **16**, 673-83.
- **de Bruijn, M. F., Peeters, M. C., Luteijn, T., Visser, P., Speck, N. A. and Dzierzak, E.** (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *Blood* **96**, 2902-4.
- **de Celis, J. F., Mari-Beffa, M. and Garcia-Bellido, A.** (1991). Cell-autonomous role of Notch, an epidermal growth factor homologue, in sensory organ differentiation in Drosophila. *Proc Natl Acad Sci U S A* **88**, 632-6.
- **de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. et al.** (1997). Conservation of the Notch signaling pathway in mammalian neurogenesis. *Development* **124**, 1139-1148.
- **Fortini, M. E., Rebay, I., Caron, L. A. and Artavanis-Tsakonas, S.** (1993). An activated notch receptor blocks cell-fate commitment in the developing drosophila eye. *Nature* **365**, 555-557.
- **Hadland, B. K., Huppert, S. S., Kanungo, J., Xue, Y., Jiang, R., Gridley, T., Conlon, R. A., Cheng, A. M., Kopan, R. and Longmore, G. D.** (2004). A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. *Blood* **104**, 3097-105.
- **Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K. and Honjo, T.** (2002). Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol* **14**, 637-45.
- **Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P.** (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signaling in Drosophila. *Development*, 161-171.
- **Huppert, S. S., Le, A., Schroeter, E. H., Mumm, J. S., Saxena, M. T., Milner, L. A. and Kopan, R.** (2000). Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1. *Nature* **405**, 966-70.
- **Iso, T., Kedes, L. and Hamamori, Y.** (2003). HES and HERP families: multiple effectors of the Notch signaling pathway. *J Cell Physiol* **194**, 237-55.
- **Kawamata, S., Du, C., Li, K. and Lavau, C.** (2002). Overexpression of the Notch target genes Hes in vivo induces lymphoid and myeloid alterations. *Oncogene* **21**, 3855-63.
- **Kumano, K., Chiba, S., Kunisato, A., Sata, M., Saito, T., Nakagami-Yamaguchi, E., Yamaguchi, T., Masuda, S., Shimizu, K., Takahashi, T. et al.** (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. and Hirai, H.** (2001). Notch1 inhibits differentiation of hematopoietic cells by sustaining GATA-2 expression. *Blood* **98**, 3283-9.
- **Lai, E. C.** (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-73.
- **Lewis, J.** (1998). Notch signaling and the control of cell fate choices in vertebrates. *Semin Cell Dev Biol* **9**, 583-9.
- **Ling, K. W. and Dzierzak, E.** (2002). Ontogeny and genetics of the hemato/lymphopoietic system. *Curr Opin Immunol* **14**, 186-91.
- **Ma, X., Robin, C., Ottersbach, K. and Dzierzak, E.** (2002). The Ly-6A (Sca-1) GFP transgene is expressed in all adult mouse hematopoietic stem cells. *Stem Cells* **20**, 514-21.
- **Medvinsky, A. and Dzierzak, E.** (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906.
- **Milner, L. A., Bigas, A., Kopan, R., Brashem-Stein, C., Bernstein, I. D. and Martin, D. I.** (1996). Inhibition of granulocytic differentiation by mNotch1. *Proc.Natl.Acad.Sci.U.S.A.* **93**, 13014-13019.
- **Minegishi, N., Morita, S., Minegishi, M., Tsuchiya, S., Konno, T., Hayashi, N. and Yamamoto, M.** (1997). Expression of GATA transcription factors in myelogenous and lymphoblastic leukemia cells. *Int J Hematol* **65**, 239-49.

- **Minegishi, N., Ohta, J., Yamagiwa, H., Suzuki, N., Kawauchi, S., Zhou, Y., Takahashi, S., Hayashi, N., Engel, J. D. and Yamamoto, M.** (1999). The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region. *Blood* **93**, 4196-207.
- **Nakagawa, O., McFadden, D. G., Nakagawa, M., Yanagisawa, H., Hu, T., Srivastava, D. and Olson, E. N.** (2000). Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of notch signaling. *Proc Natl Acad Sci U S A* **97**, 13655-60.
- **North, T., Gu, T. L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M. and Speck, N. A.** (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* **126**, 2563-75.
- **North, T. E., de Bruijn, M. F., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M., Dzierzak, E. and Speck, N. A.** (2002). Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity* **16**, 661-72.
- **Oka, C., Nakano, T., Wakeham, A., de la Pompa, J. L., Mori, C., Sakai, T., Okazaki, S., Kawaichi, M., Shiota, K., Mak, T. W. et al.** (1995). Disruption of the mouse RBP-J kappa gene results in early embryonic death. *Development* **121**, 3291-301.
- **Palis, J. and Yoder, M. C.** (2001). Yolk-sac hematopoiesis: the first blood cells of mouse and man. *Exp Hematol* **29**, 927-36.
- **Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D.** (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912.
- **Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F. W. and Orkin, S. H.** (1996). The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell*, 47-57.
- **Radtke, F. and Raj, K.** (2003). The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat Rev Cancer* **3**, 756-67.
- **Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R. and Aguet, M.** (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547-58.
- **Robb, L., Elwood, N. J., Elefanty, A. G., Kontgen, F., Li, R., Barnett, L. D. and Begley, C. G.** (1996). The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse. *EMBO Journa* **15**, 4123-4129.
- **Stier, S., Cheng, T., Dombkowski, D., Carlesso, N. and Scadden, D. T.** (2002). Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood* **99**, 2369-78.
- **Timmerman, L. A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J. M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J. C. et al.** (2004). Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev* **18**, 99-115.
- **Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. and Orkin, S. H.** (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-6.
- **Tsai, F. Y. and Orkin, S. H.** (1997). Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. *Blood* **89**, 3636-43.
- **Yoder, M. C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D. M. and Orlic, D.** (1997). Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* **7**, 335-44.

SECTION 2: The Notch ligand Jagged1 is required for intra-embryonic AGM hematopoiesis

(This chapter is unpublished).

We have described that Notch1/RBPjκ-mediated Gata2 expression is required for AGM hematopoiesis (Robert-Moreno, 2005). Our results are in agreement with Kumano's work with the Notch1 null mice (Kumano, 2003). However, nothing is known about the specific ligand that activates Notch to induce AGM hematopoiesis. Moreover, due to the strong vascular defects displayed by the Notch1 and RBPjκ mutants (Oka, 1995; Krebs, 2000) and to preclude the possibility that lack of intra-embryonic hematopoiesis is due to the lack of a previous specified artery, assessment of the Notch function in hematopoiesis of wild-type aortas was required.

In this chapter, we describe that hematopoietic potential of AGM aortas is severely compromised (albeit not totally impaired) in embryos lacking the Notch ligand Jagged1 but not Jagged2. Jagged1 null embryos completely lack Gata2 expression, display reduced number of hematopoietic progenitors and reduced number of Sca-1^{+} cells (a population of cells which includes HSCs). In addition and similar to the RBPjκ null embryos, loss of intra-embryonic hematopoiesis in the Jagged1 mutant embryos correlates with an increase of endothelial cells.

Moreover, γ-secretase inhibition of Notch signaling in wild-type E11 aortas cultured as explants, which promotes HSC/HPC generation and/or expansion (Medvinsky, 1996) significantly reduces the number of hematopoietic progenitors (determined by CFC assay) and a population of high repopulating HSCs (determined by repopulating studies).

Since we also describe that Notch1, Jagged1 and Hes1 are highly expressed in hematopoietic clusters budding from the ventral part of the AGM aorta we propose that Jagged1-induced activation leads to Gata2 and Hes1 expression and to an expansion of both HP and HSCs in the aortic endothelium and to a maintenance of the stemness state in these cells.

The Notch ligand Jagged1 is required for intra-embryonic AGM hematopoiesis

Àlex Robert-Moreno[1], Claudia Orelio[2], Ruiz-Herguido C, Lluis Riera[1], Thomas Gridley[3], Elaine Dzierzak[2], Lluis Espinosa[1] and Anna Bigas[1]*

[1]Centre Oncologia Molecular, IDIBELL-Institut de Recerca Oncològica. Gran Via km 2.7 Hospitalet, Barcelona, Spain.
[2]Department of Cell Biology and Genetics. Erasmus University Medical Center. Rotterdam, The Netherlands.
[3]The Jackson Laboratory, Bar Harbor, Maine, USA.

Scientific heading: Hematopoiesis

* Corresponding author
Mailing address: Oncologia Molecular. IDIBELL
Gran Via Km 2.7.
08907 - Hospitalet, Barcelona. Spain
Phone: 932 607 404
Fax: 932 607 426
e-mail: abigas@iro.es

Running title: Jagged1/Notch in intra-embryonic hematopoiesis

SUMMARY

The mechanisms that regulate the generation of both hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) during the ontogeny of the hematopoietic system in the embryo have been extensively studied in the last years. Among many others, the implication of the Notch signaling pathway in regulating the emergence of HPC/HSC from the P-sp/AGM region has been recently pinpointed (Burns et al., 2005; Hadland et al., 2004; Kumano et al., 2003; Robert-Moreno et al., 2005). In the present study we describe by in-situ hybridization that certain Notch signaling members including the *Notch1* receptor and the *Jagged1* and *Jagged2* ligands, are expressed in hematopoietic clusters emerging from the AGM aortic endothelium at E10.5. Moreover, Jagged1- (but not Jagged2-) mediated Notch activation is required to induce the expression of the hematopoietic transcription factor *Gata2* but not *Runx1*. In agreement with this, AGM hematopoiesis is severely reduced in Jagged1 but not Jagged2 null embryos. Finally, HSC/HPC emergence in aorta explants cultured in the presence of the γ-secretase inhibitor DAPT is also reduced, indicating that Notch signaling is required for activating the hematopoietic program of cells budding from the hemogenic aortic endothelium of the AGM region.

INTRODUCTION

Different embyronic hematopoietic sites such as the yolk sac (Yoder and Hiatt, 1997; Yoder et al., 1997; Yoder et al., 1997), the intra-embryonic para-aortic splanchnopleura (P-sp)/AGM (aorta-gonad-mesonephros) region (Cumano et al., 2001; Medvinsky and Dzierzak, 1996), other major vessels such as the umbilical and vitelline arteries (de Bruijn et al., 2000) and more recently the placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005) are responsible for generating or amplifying the pool of Hematopoietic Stem Cells (HSC) and more committed progenitors. HSC, defined as cells with the ability to reconstitute hematopoiesis in immunodepleted mice, are present in all this sites. However there is compelling evidence that niche-dependent- or cell-autonomous-induced signals confer different qualities to these generally called HSC. For example, yolk sac cells are able to reconstitute busulfan-treated new-born mice, but not adult irradiated mice (Yoder and Hiatt, 1997; Yoder et al., 1997; Yoder et al., 1997). In contrast, AGM-derived HSC show identical qualities as adult/definitive HSC in terms of hematopoietic reconstitution of adult animals (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996). Moreover, recent studies from several gene-targeted mice suggest that hematopoiesis from different embryonic sites have specific gene expression requirements (Kumano et al., 2003; Ling et al., 2004; Okuda et al., 1996; Robert-Moreno et al., 2005; Wang et al., 1996).

The Notch pathway is generally involved in the regulation of cell fate decisions and it is activated through cell-cell interaction in a variety of developmental systems including hematopoiesis. It has previously been characterized that several Notch pathway mutant embryos cannot generate intra-embryonic HSC both in the mouse and zebrafish (Burns et al., 2005; Kumano et al., 2003; Robert-Moreno et al., 2005). However, it is remarkably interesting that no major hematopoietic defects have been found in the yolk sac hematopoiesis of these mutants (Burns et al., 2005; Kumano et al., 2003; Robert-Moreno et al., 2005). This observation suggests that Notch may be involved in the acquisition of specific traits of AGM-definitive HSC.

We further investigate the mechanism underlying Notch activation in the AGM and whether the hematopoietic effects of Notch on this region depends on specific Notch/Notch-ligand interactions. Here we show that Jagged1- but not Jagged2-deficient embryos fail to activate *Gata2* in the AGM region but not

Runx1 which results in a reduced number of HSC and hematopoietic progenitors. Moreover, blocking Notch activation in E11 AGM wild-type cells by γ-secretase inhibitors highly compromised the capacity of AGM-derived HSC to efficiently repopulate hematopoiesis in an irradiated recipient. The overall data reinforce the idea that Notch signaling is required for the generation of HSC and HPC in the AGM aorta and for the first time highlights the importance of the Jagged1 ligand to activate the Notch-induced establishment of the hematopoietic program in this region.

MATERIALS AND METHODS

Animals

Ly-6A-GFP (Sca-1-GFP) mice (CD1 or B_{10}CBA background)(kindly given by Dr. E. Dzierzak) and Jagged1^{dDSL} and Jagged2^{dDSL} null mutant mice (C57BL/6J background; kindly given by Dr. Thomas Gridley) have been extensively characterized (de Bruijn et al., 2002; Jiang et al., 1998; Xue et al., 1999). Transgenic embryos were typed based on the presence of GFP positive cells using an Olympus IX70 fluorescent microscope and by PCR for the transgene as well. Jag1^{dDSL} and Jag2^{dDSL} were typed by PCR against the mutant allele. Sca-1-GFP $^{tg/+}$ mice were crossed with Jag1$^{dDSL/+}$ or Jag2$^{dDSL/+}$ mice and embryos were not used for experimental procedures before the fifth generation. Wild-type CD1 embryos were used for whole mount in-situ hybridization (WISH). Animals were kept under pathogen-free conditions and experiments approved by the Animal Care Committee. Embryos were obtained from timed pregnant females and somite pairs were counted for precisely timing.

Dorsal aorta explant culture

The P-sp/AGM region from E9.5 to E11.5 embryos was dissected and subsequently subdissected into the aorta with the surrounding mesenchyme using 27G needles. Aortas were cultured as explants for 3 days as previously described (Medvinsky and Dzierzak, 1996). Briefly, aortas were deposited on nylon filters (Millipore) placed on metallic grids and cultured in myeloid long-term culture medium (Stem Cell Technologies) supplemented with 10 μM hydrocortisone succinate (Sigma) in an air-liquid interphase culture for 3 days in the presence of DMSO (as a control) or the γ-secretase inhibitor DAPT (50μM). Explanted aortas were either used for hematopoietic colony assay or repopulation studies.

Hematopoietic colony assay

The 3-day explanted aortas were digested in 0.12% collagenase (Sigma) in PBS supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Biological Industries) for 1 hour at 37ºC and $3.5x10^4$ cells were plated in M-5323 semisolid medium (Stem Cell Technologies). After 7 days the presence of hematopoietic colonies was scored under the microscope, a part from CFU-e progenitors that were scored at day 4.

Hematopoietic liquid culture

The P-sp/AGM region from E9.5 wild-type embryos was dissociated by gentle pippeting and cells were split and treated with DMSO or 50μM DAPT in Iscove's with 10% FBS, 10% IL3- and SCF-conditioned medium, 0.1% monothioglycerol (Sigma), 2.5% L-glutamine and 1% Pen/Strep for 6 days.

Flow cytometry analysis

Following the 6-day liquid culture, cells from E9.5 P-sp were stained with anti-cd45-FITC or IgG-FITC (Pharmingen), for flow activated cell sorting (FACS) assay. Cells were analyzed by FACScalibur (Becton & Dickinson) and WinMDI2.8 software. Dead cells were excluded by 7-aminoactinomicin-D staining (Molecular Probes).

CD31 immunostaining

E10.5 embryos were fixed overnight in 4% paraformaldehyde (Sigma) at 4ºC, frozen in Tissue-tek (Sakura) and sectioned (10μm). Slides were fixed with -20ºC methanol for 15 minutes and block-permeabilized in 10% FBS, 0.3% Surfact-AmpsX100 (Pierce) and 5% non-fat milk in PBS for 90 minutes at 4ºC. Samples were stained with rat anti-CD31 (PECAM1; 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).

Whole mount in-situ hybridization (WISH)

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

Short-term and long-term multilineage repopulating activity

DMSO or DAPT-treated explanted aortas (genetically marked by the Ln72 transgene) were dissociated and cell suspensions were assayed for the presence of definitive HSCs by intravenous injection into irradiated adult recipients (de Bruijn et al., 2002; Medvinsky and Dzierzak, 1996). Briefly, C57BL/10 x CBA male recipients were irradiated with a 9.5Gy split dose of γ-irradiation. Adult spleen cells ($2x10^5$/mouse) were coinjected with the aorta cells to promote survival. Transplanted mice were bled at 1 and 4 months after injection to assay short- and long-term repopulation respectively by analyzing for donor contribution by donor marker-specific PCR (hβ-globin) on peripheral blood. Percentage of donor-cell contribution (engraftment/chimerism) was analyzed by normalizing for the myogenin gene.

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). Adobe Photoshop 6.0 software was used for photograph editing.

RESULTS

Expression of Notch family members within the E10.5 AGM hematopoietic clusters.

We previously showed that *Notch1*, *Notch4*, *Jagged1*, *Jagged2* and *Delta4* are expressed in the mid-gestation AGM region. We have now focused on the expression of these molecules in the hematopoietic clusters emerging from the aortic endothelium. We found *Notch1* expression in these clustered cells whereas *Notch4* is strongly downregulated compared to the surrounding endothelial cells (Fig. 1). We also detected high levels of the Notch ligand *Jagged1* in most of the cells in the cluster whereas *Jagged2* and *Delta4* expression was only found at low levels and in sporadic cells. Moreover, *Hes1* expression confirmed that the Notch signaling pathway is active in the cells within the hematopoietic clusters (Fig. 1), thus suggesting that Notch may participate in subsequent events other than the hematopoietic commitment from the hemogenic endothelium (Kumano et al., 2003; Robert-Moreno et al., 2005).

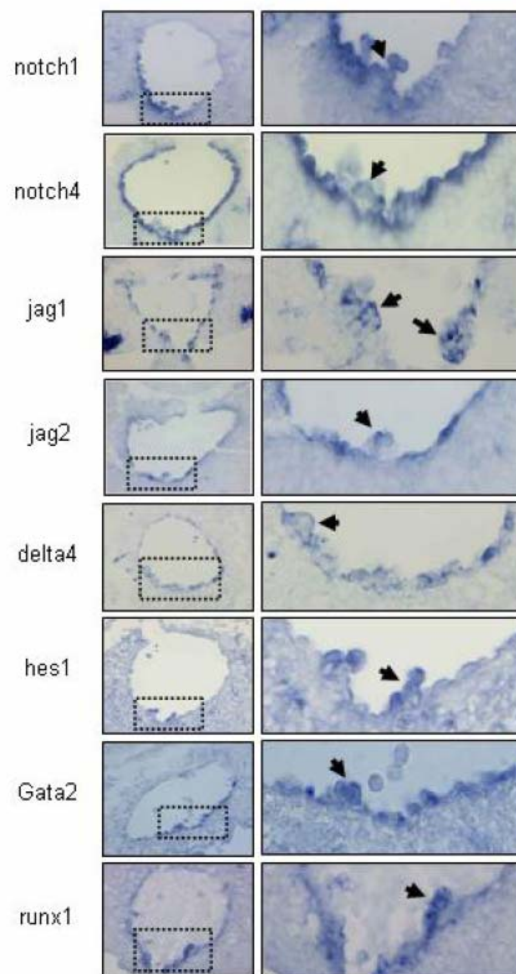


Figure 1: Notch family members are expressed in E10.5 aortic hematopoietic clusters. *WISH showing expression of the different Notch genes was performed in E10.5 wild-type embryos. Left panels show a representative aorta at 400x of magnification. Arrowheads in the right panels highlight hematopoietic clusters at 1000x of magnification. The orientation is dorsal (up) to ventral (down).*

Altered hematopoiesis in the AGM of Jag1^{dDSL} but not Jag2^{dDSL} embryos

To determine whether specific Notch ligands are required for Notch-dependent HSC determination we analyzed the expression pattern of early hematopoietic markers *Gata2* and *Runx1* in the AGM of previously reported Jagged1 and Jagged2 null embryos (Jiang et al., 1998; Xue et al., 1999). By WISH, we detected expression of both markers in Jagged2 mutant embryos at E10.5-E11 similar to the wild-type littermates, albeit 3 out of 4 Jag2^{dDSL} embryos showed lower number of *Gata2* expressing cells (Fig. 2A, 2B and Table 2). In contrast, Jagged1 embryos showed a complete lack of *Gata2* expression in the aorta whereas we detected *Runx1* expression in the AGM of 3 out of 6 mutant embryos (Fig. 2A, 2B and Table1). Lack of expression of *Gata2* and *Runx1* was also found in E9.5 Delta4$^{-/-}$ embryos, however strong malformations in the vasculature of these embryos precluded the study in this model (data not shown).

To investigate the functional implication of *Gata2* deficiency in the AGM of mutant embryos, we performed hematopoietic colony (CFC) assay from subdissected E10.5-E11 Jag1^{dDSL} and Jag2^{dDSL} aortas. Similar number and types of CFCs (myeloid, erythroid and Mix progenitors) were obtained from Jagged2 mutant embryos compared with the wild-type littermates (Fig. 2C). In contrast, the total number of CFCs from Jag1^{dDSL} aortas was significantly reduced with all colony types equally represented (Fig. 2C).

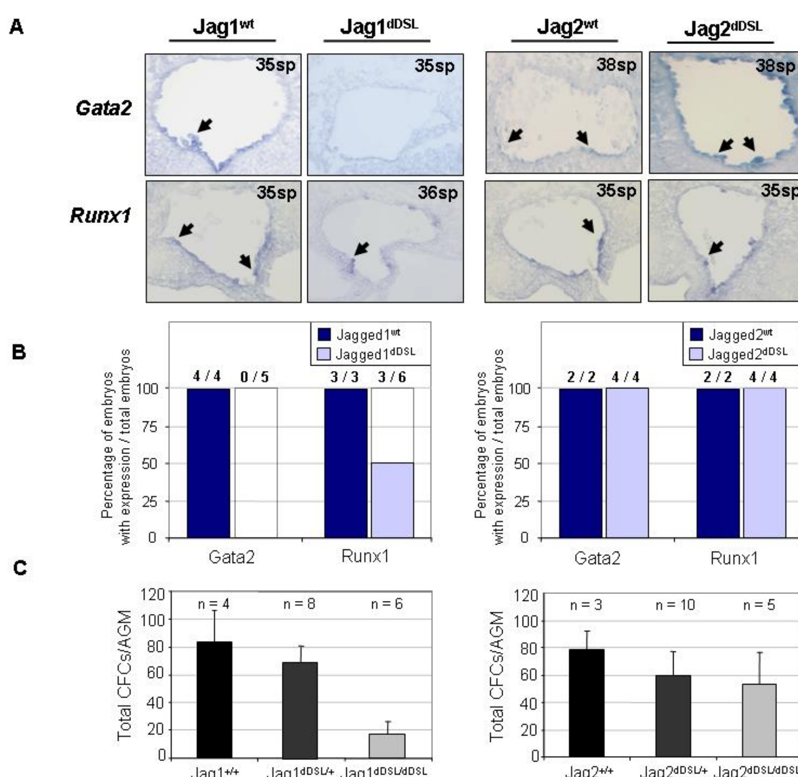


Figure 2: ***Impaired Gata2 expression and reduced intra-embryonic AGM hematopoiesis in Jagged1 but not Jagged2 null embryos. A)*** *WISH for the hematopoietic specific transcription factors Gata2 and Runx1 in the aortic endothelium of E10.5-E11 wild-type and Jag1*^{dDSL} *or Jag2*^{dDSL} *embryos. For comparison only embryos of the same number of somite pairs were used. The orientation is dorsal (up) to ventral (down). Magnification 400x.* ***B)*** *Graphs represent the percentage of embryos showing expression from the total embryos analyzed. The absolute number of embryos is shown above each bar.* ***C)*** *Hematopoietic progenitor potential of Jagged1*^{dDSL} *and Jagged2*^{dDSL} *embryos determined by CFC assay. Bars represent the average number of CFCs and standard deviation. Number of embryos assayed is shown above each bar.*

WISH in Jagged1 embryos

Gata2 expression	Jagged1 wt		Jagged1 dDSL	
	no. of somites	$\bar{x}$ cells/100 µm	no. of somites	$\bar{x}$ cells/100 µm
	33 sp +/-	33,61	33sp -/-	0
	35 sp +/+	30,61	35 sp -/-	0
	35 sp +/+	47,7	35 sp -/-	0
	38 sp +/-	76,43	38 sp -/-	0
			38 sp -/-	0

Runx1 expression	Jagged1 wt		Jagged1 dDSL	
	no. of somites	$\bar{x}$ cells/100 µm	no. of somites	$\bar{x}$ cells/100 µm
	34 sp +/+	57,13	34sp -/-	0
	35 sp +/-	38,39	35 sp -/-	0,61
			35 sp -/-	0
			35 sp -/-	16,47
			35 sp -/-	33,9
	38 sp +/+	36,94	38 sp -/-	25,14

Table1: ***WISH for Gata2 and Runx1 in Jagged1^{dDSL} embryos compared with their wild-type littermates.*** *For each embryo, the genotype, the number of somite pairs and the average number of positive cells in 100 µm of aorta is shown.*

WISH in Jagged2 embryos

Gata2 expression	Jagged2 wt		Jagged2 dDSL	
	no. of somites	$\bar{x}$ cells/100 µm	no. of somites	$\bar{x}$ cells/100 µm
			37 sp -/-	28,57
	38 sp +/-	54,9	38 sp -/-	25,42
			38 sp -/-	51,02
	40 sp +/+	34,64	40 sp -/-	17,85

Runx1 expression	Jagged2 wt		Jagged2 dDSL	
	no. of somites	$\bar{x}$ cells/100 µm	no. of somites	$\bar{x}$ cells/100 µm
	35 sp +/+	19,44	35 sp -/-	26,41
			35 sp -/-	28,21
	38 sp +/+	42,13	38 sp -/-	32,38
			38 sp -/-	42,86

Table2: ***WISH for Gata2 and Runx1 in Jagged2^{dDSL} embryos compared with their wild-type littermates.*** *For each embryo, the genotype, the number of somite pairs and the average number of positive cells in 100 µm of aorta is shown.*

To further characterize the hematopoietic role of Jagged1 and Jagged2 *in vivo*, we used the previously described Ly-6E.1-GFP (Sca-1-GFP) transgenic mice which labels hematopoietic cells emerging from the aorta including HSC (de Bruijn et al., 2002). We crossed Jag1$^{dDSL/+}$ or Jag2$^{dDSL/+}$ mice with Ly-6A-GFP mice and counted the number of GFP^{+} cells in the aorta of different precisely timed embryos. We found similar numbers of GFP^{+} cells in the AGM of E10.5-E11 Jagged2 null embryos compared with their wild-type littermates. However, the number of GFP^{+} cells found in the endothelium of the aorta of Jagged1-deficient embryos was extremely reduced indicating that lack of Notch activation by Jagged1 results in impaired intra-embryonic hematopoiesis lilkely due to defects on *Gata2* expression (Fig. 3).

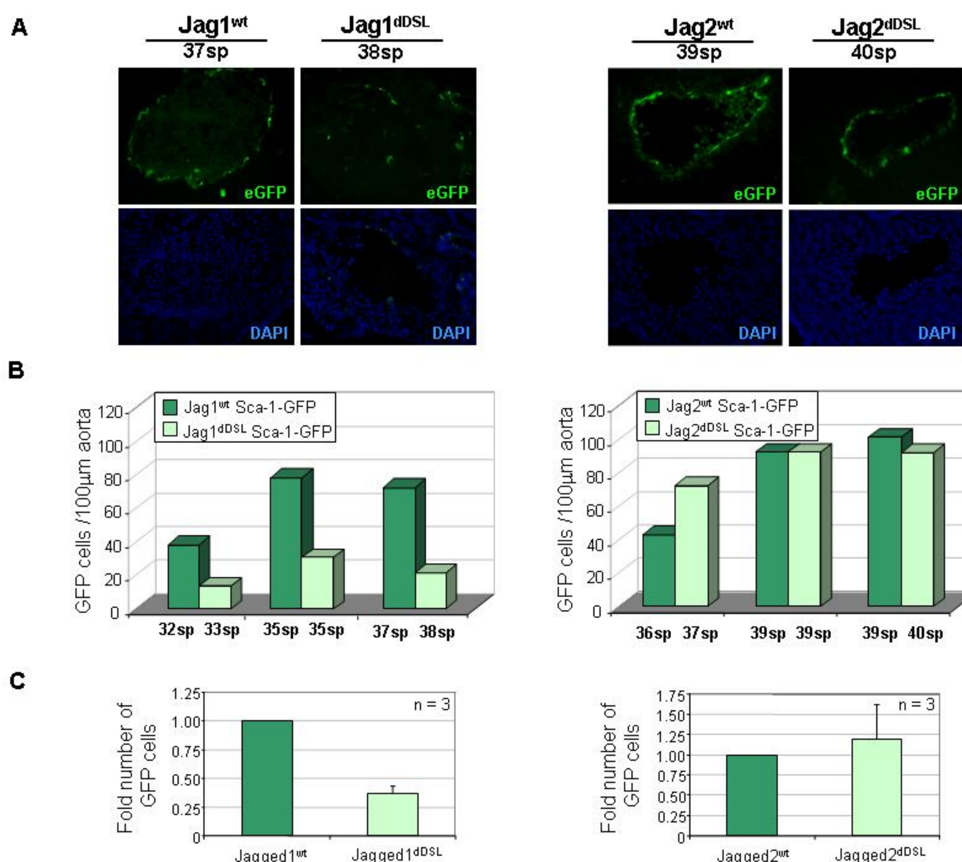


Figure 3:* Depletion of Sca-1-GFP cells in Jagged1 but not Jagged2 null embryos. A)** *Precisely timed E10.5-11 wild-type, Jag1^{dDSL} or Jag2^{dDSL} / Sca-1-GFP embryos were sectioned and the number of GFP^{+} cells lining the dorsal aorta was counted. Representative photographs from these embryos are shown. The orientation is dorsal (up) to ventral (down). Magnification: 400x.* ***B) *Bars represent the number of GFP^{+} cells found in 100µm of AGM aorta from three different Jag1^{dDSL} or Jag2^{dDSL} / Sca-1-GFP embryos compared with their Jag2^{wt} Sca-1-GFP littermates.* ***C)*** *Bars represent the fold reduction of GFP^{+} cells in three Jag1^{dDSL} or Jag2^{dDSL} embryos compared with their wild-type littermates.*

γ-secretase inhibitor DAPT affects hematopoietic reconstitution from AGM

Our results indicate that Notch plays important functions in the generation of hematopoietic cells in the AGM and most likely in regulating other events within the hematopoietic clusters. Since most of the previous work was performed with mutant animals displaying vascular abnormalities, we investigated whether

pharmacological inhibition of Notch signaling was sufficient to reproduce the hematopoietic defects of Notch-family mutants in wild-type cells. First, we tested the effect of DAPT on liquid cultures from disrupted E9.5 AGM cells. After 6 days, we found a 3- to 4-fold decrease in the number of $CD45^+$ hematopoietic cells in DAPT-treated cultures compared to the controls, similar to that observed in liquid cultures from $RBPj\kappa^{-/-}$ cells (Robert-Moreno et al., 2005) (Fig. 4A and 4B). This result further confirms that Notch signaling pathway plays a role in the generation of HSC/HPC from early aortic endothelium.

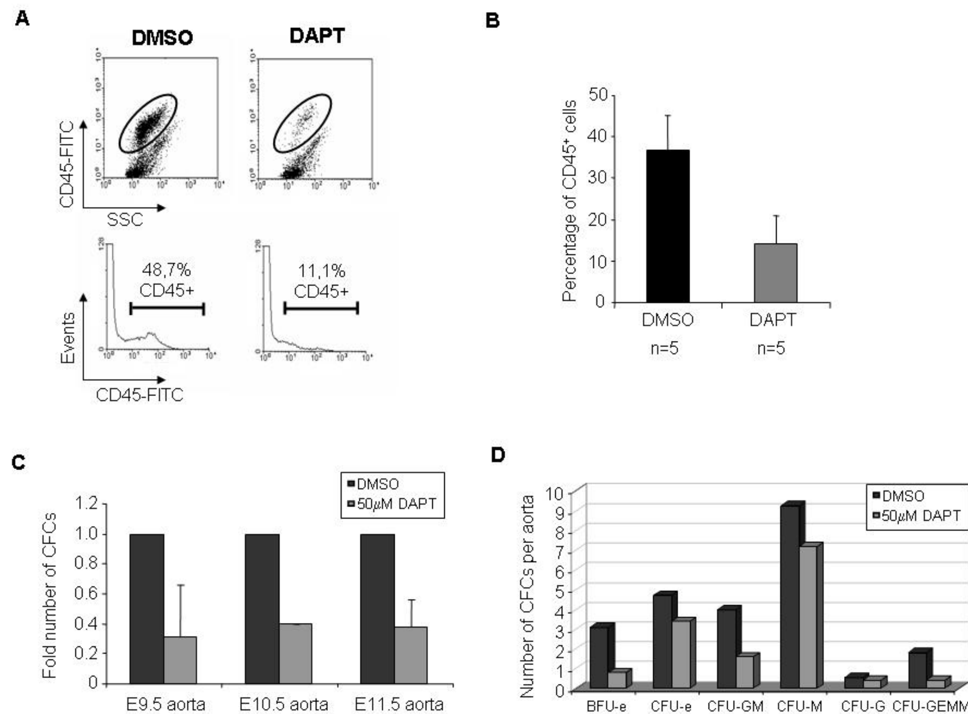


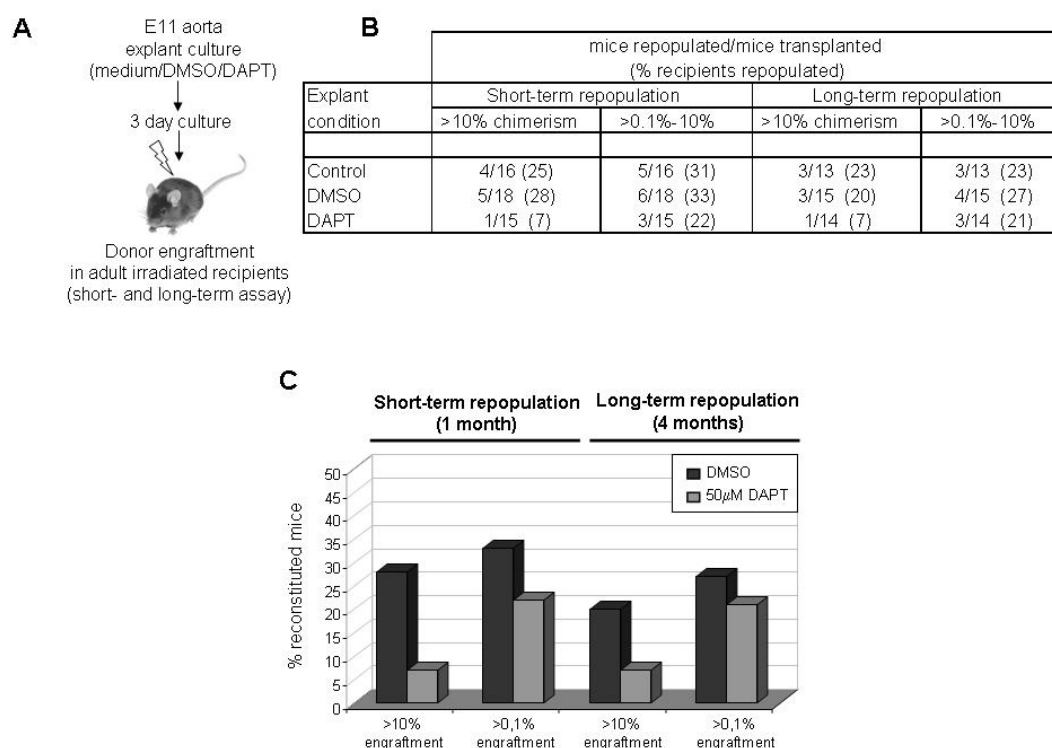
Figure 4: ***DAPT treatment on E9.5 to E11.5 wild-type aortas decreases hematopoiesis. A)*** *Dot plots of a representative experiment showing the percentage of CD45 positive cells in E9.5 P-sp wild-type cells cultured for 6 days in the presence of DMSO or 50μM DAPT.* ***B)*** *Graphs represent the average percentage and standard deviation of 5 different experiments.* ***C)*** *Relative number of hematopoietic colonies obtained from E9.5-E11.5 aorta explants cultured for three days in the presence of DMSO or 50μM DAPT. Bars represent the average and standard deviation of 2 (E9.5), 1 (E10.5) or 3 experiments (E11.5).* ***D)*** *Representative E11.5 experiment showing that all kind of progenitors are detected and no great differences in the percentage of hematopoietic progenitors from each lineage are found in the DAPT-treated aortas compared with the control ones.*

Since Notch signaling is highly dependent on cell-cell interactions, we next tested the effect of Notch inhibitors in intact AGM explant cultures that allows HSC expansion (Medvinsky and Dzierzak, 1996). Dissected aortas from wild-type E9.5, E10.5 and E11.5 AGM were cultured as explants in the presence of DMSO or DAPT for 3 days and plated in methylcellulose to determine the number of CFC. We found a reduction of 2 to 3-fold in the total number of hematopoietic progenitors from DAPT-treated aortas compared with their controls in all three developmental stages (Fig. 4C). Moreover, we did not detect a significant difference in the percentage of different CFC types indicating that Notch inhibition was not affecting any specific hematopoietic lineage (Fig. 4D).

Effects of pharmacological Notch inhibition on HSC repopulation activity from E11 aorta explants.

We have previously shown that RBPjκ deficiency results in the absolute abrogation of hematopoiesis in the AGM. However, Jagged1 deficient embryos contain few Sca-1^{+} cells in the AGM with reduced capacity to generate CFCs *in vitro*, suggesting that Jagged1-dependent Notch activity is not required for the generation of HSC but in the amplification of this compartment. To further investigate this possibility, we treated fully-developed E11 aortas from Ln72 transgenic mice with DMSO or DAPT in explant cultures, and after 3 days we transplanted the cells into sublethally irradiated mouse. We quantified the contribution of donor cells to hematopoietic reconstitution by detecting the Ln72 transgene by PCR at 1 month (short-term repopulation) and 4 months (long-term repopulation) after transplantation (Fig. 5A). We found a 3 to 4-fold reduction in the percentage of mice displaying more than a 10% engraftment in mice reconstituted with DAPT-treated aortas compared to the control (Fig. 5B and 5C). Interestingly, we did not find any significant difference when comparing mice with low levels of donor engraftment (0.1 to 10%; Fig. 5B and 5C). These results indicate that inhibition of Notch at E11 may specifically affect a subpopulation of both short-term and long-term repopulating HSC with high reconstitution potential (>10% of engraftment).

Altogether, our work suggests that Jagged1-induced Notch activation in the AGM hematopoietic cells is responsible for inducing *Gata2* expression thus permitting the expansion of highly efficient repopulating stem cells.



Explant condition	mice repopulated/mice transplanted (% recipients repopulated) Short-term repopulation >10% chimerism	Short-term repopulation >0.1%-10%	Long-term repopulation >10% chimerism	Long-term repopulation >0.1%-10%
Control	4/16 (25)	5/16 (31)	3/13 (23)	3/13 (23)
DMSO	5/18 (28)	6/18 (33)	3/15 (20)	4/15 (27)
DAPT	1/15 (7)	3/15 (22)	1/14 (7)	3/14 (21)

Figure 5: ***Impaired generation of a subpopulation of high reconstitutive HSCs in DAPT-inhibited aorta explants. A)*** *Experimental protocol. E11 aortas were dissected and cultured as explants for 3 days. Irradiated adult mice were injected with 1 or 2e.e. of aortic cells together with spleen cells for short-term survival.* ***B)*** *Table showing mice reconstituted with explanted aortas cultured in control medium, DMSO or DAPT.* ***C)*** *Short- and long-term repopulation of recipient mice reconstituted with aortas cultured either in DMSO or DAPT. Bars represent the percentage of reconstituted recipients. Mice displaying greater than 10% or 0.1%-10% donor reconstitution are represented separately. Results are from five independent experiments.*

DISCUSSION

There is evidence for a role of Notch signaling in the generation of HSC in the aortic endothelium (Kumano et al., 2003; Robert-Moreno et al., 2005). In this work, we show that Jagged1 is responsible for Notch activation in hematopoietic cells of the aorta in the AGM region. This is the first evidence about the specific Notch ligand which is involved in the AGM hematopoiesis. The interaction between Notch and Jagged1 induces the transcriptional activation of *Gata2* which likely regulates the proliferation of the stem cell pool in the AGM region. In addition, DAPT-treatment of E11 AGM explants confirms the involvement of Notch in the AGM hematopoiesis and reveals a new role for Notch in the acquisition of high repopulation capacity in HSC.

Our previous work suggested that *Delta4*, *Jagged1* and *Jagged2* were good candidates for Notch-activating ligands, since they are all expressed in the mid-gestation aortic endothelium (Robert-Moreno et al., 2005). Delta4 heterozigous embryos die at E10-10.5 displaying severe vasculogenic defects and loss of the endothelial arterial cell fate (Duarte et al., 2004), which strongly compromised the study of hematopoiesis in these animals. Thus, we focused in the study of the other two candidates, Jagged1 and Jagged2: Jag1$^{dDSL/dDSL}$ embryos display defects in the vasculature of the yolk sac and in the branching of blood vessels in the embryo head but no defects in aorta formation have been reported (Xue et al., 1999) whereas Jag2^{dDSL} homozygous embryos die perinatally due to craniophacial, thymus and limb bud formation defects (Jiang et al., 1998). We have now identified that Jagged1 ligand is responsible for specifically activating the Notch receptor, switching on the hematopoietic program in endothelial cells of the AGM. This is supported by the lower number of hematopoietic progenitors, Sca-1^{+} cells and the absence of *Gata2* expression. By contrast, these hematopoietic defects are not observed in the Jagged2-deficient embryos, although a lower number of *Gata2*-expressing cells were found in these embryos.

We previously described that Notch1/RBPjκ signaling leads to the expression of the hematopoietic transcription factor *Gata2* and presumably to the commitment of HPC/HSCs from the AGM region (Robert-Moreno et al., 2005). However, the Notch-Runx pathway establishes the commitment of the hematopoietic stem cell fate in the zebrafish embryo (Burns et al., 2005) and *Drosophila* hemocytogenesis needs the Notch-induced activation of the Runt family gene, *Lozenge* (Lebestky et al., 2003). Loss of *Gata2* expression in the Jag1^{dDSL} null embryos reinforces the idea that *Gata2* (but not *Runx1*) is a target gene of the Jagged1/Notch1/RBPjκ signaling pathway. In contrast, *Runx1* is expressed in the aorta in the 50% of the Jagged1 embryos indicating that Jagged1 is not required at this stage for *Runx1* expression. However, the possibility that the expression of *Runx1* may depend on another Notch ligand cannot be excluded.

Gene targeting studies revealed the importance of *Gata2* for hematopoiesis since Gata2$^{-/-}$ embryos have reduced numbers of hematopoietic cells (Tsai et al., 1994); there is no contribution of Gata2$^{-/-}$ ES-derived cells to any hematopoietic tissue (Tsai et al., 1994) and *Gata2* haploinsufficiency results in different HSC abnormalities (Ling et al., 2004; Rodrigues et al., 2005). Interestingly, several similarities are found between hematopoietic cells from Jagged1-deficient, DAPT-treated and Gata2-deficient hematopoietic cells since they all show defects in HSC proliferation and/or expansion. Thus, a possible explanation is that Notch acts on the first hematopoietic decision activating *Runx1* and on a posterior committed cell to induce *Gata2* resulting in the expansion of these cells. This interpretation is in agreement with our present work, since Jagged1 mutants contain some hematopoiesis in the AGM and express *Runx1* but not *Gata2*. Thus Jagged1

would be responsible for a secondary Notch decision in these cells whereas another ligand may be responsible for the first event that activates *Runx1* expression.

To exclude the possibility that vascular abnormalities in Notch-mutants was responsible for the hematopoietic defect (Kumano et al., 2003; Robert-Moreno et al., 2005), we have tested the effect of γ-secretase inhibitors in the amplification of HSC in AGM explants. Our results suggest that Notch signaling is required for the generation of HPC (as demonstrated by CFC assay of DAPT-treated explanted aortas) and HSC (determined by the lower adult repopulation of γ-secretase inhibited explants). Hematopoietic colonies found in the DAPT-treated aortas may come from cells generated previous to the γ-secretase treatment, thus suggesting that Notch would not be required for cell-fate decisions within the hematopoietic hierarchical tree, but may be required for *de novo* generation of a common undifferentiated hematopoietic progenitor from the aortic endothelium. Thus, Notch signaling is required in endothelial cells from a previously specified artery in order to generate HPC/HSCs. A very interesting observation is that Notch seems to be necessary for the generation of a subpopulation of short- and long-term repopulating HSCs with high reconstitution ability, suggesting that, different subsets of HSCs with distinct repopulation and self-renewal behavior are generated from the AGM, as previously described in the bone marrow (Sieburg et al., 2006). This specific Jagged1 function may have an enormous relevance in cell therapy when trying to use Notch ligands for HSC amplification.

Hematopoietic clusters emerging from the endothelium of the dorsal aorta are likely to be qualitatively different since they express different levels of hematopoietic and endothelial markers (reviewed in (Dieterlen-Lievre et al., 2006)]. We have now characterized the expression of Notch family members in these clusters and have shown that their expression also differ among clustered cells. The Notch target gene *hes1* is also expressed in the hematopoietic clusters, which is a measure of Notch activity. It has been proposed that Hes1 maintains the stemness of HSCs by inhibiting their differentiation (Kunisato et al., 2003). Consistent with this, we propose a model in which Notch1 activates both *hes1* and *gata2* expression thus maintaining the undifferentiated state and promoting proliferation of a subset of HSCs with high repopulating ability.

SUPPLEMENTAL INFORMATION

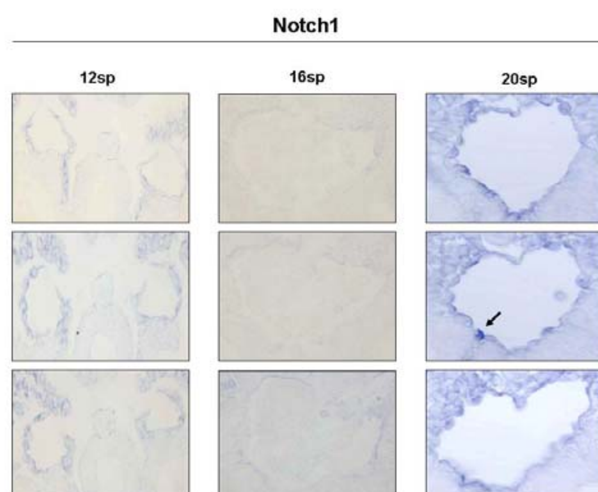
Temporal and spatial pattern of expression of Notch1 receptor and its ligands during the first stages of aorta formation

We previously reported by single and double in situ hybridization that several Notch receptors (*Notch1* and *Notch4*) and ligands (*Delta4*, *Jagged1* and *Jagged2*) are coexpressed in the aortic endothelium of the E9.5-E10.5 AGM region and functional studies indicated that Notch signaling (through RBPjκ) is required for the generation of the hematopoietic progenitor pool from the AGM region (Kumano et al., 2003; Robert-Moreno et al., 2005).

We previously showed that both Notch receptors and ligands are already expressed in some cells of the endothelium at E9.5 (20 somite pair). Our aim now was to characterize the first appearance of Notch receptor and ligand expressing cells in the endothelium in order to understand the mechanism followed by this pathway to determine HSC (lateral inhibition or lateral induction). For this purpose we used precisely timed embryos at 12 somite pairs (E8.5, two pair of dorsal aortas), 16 somite pairs (E9, fusion of the dorsal aorta) and 20 somite pairs (E9.5, definitive hematopoiesis starts).

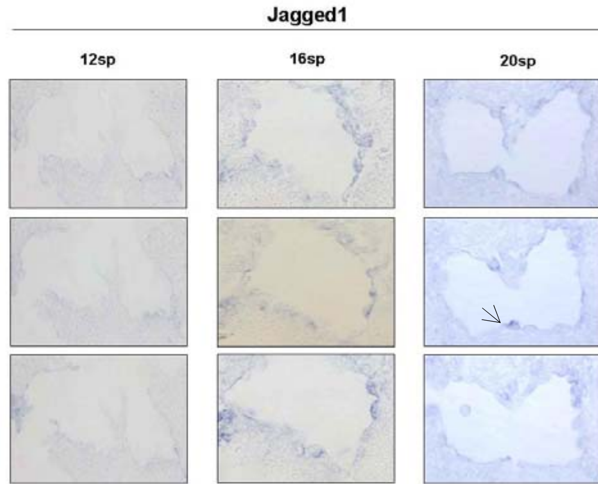
As shown in Supplementary Figure1, low *Notch1* expression is detected at 12 and 16 somite pair (sp) stages and increases in discrete cells of the ventral part or the aorta at the 20sp stage. *Jagged1* and *Jagged2* expression is also low at the 12sp stage (Supplementary Fig.2 and 3) suggesting that all these genes may not be involved in the fusion of the dorsal aorta. However, *Jagged2* expression becomes strong at the 16sp stage and maintained at E9.5 (Supplementary Fig.3), whereas *Jagged1* expression is not evident until the 20sp stage and displays a pattern of expression that resembles the one for *Notch1* (Supplementary Fig.2).

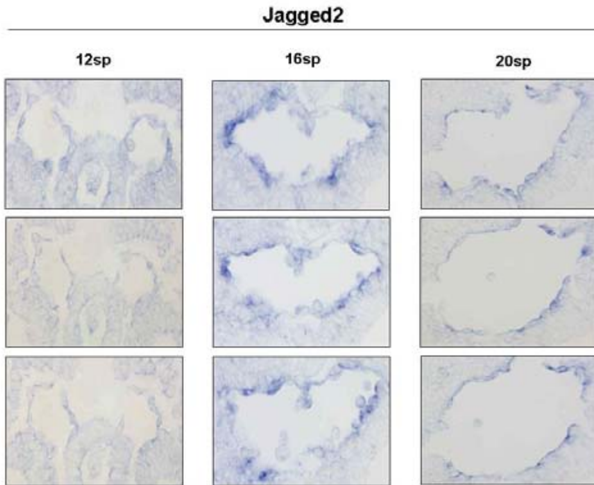
Finally, *Delta4* is expressed in most of the endothelial cells at the 12sp stage before dorsal aortas fuses and its expression is maintained at E9 and E9.5 (Supplementary Fig.4). Taken together, these results show that the different Notch members are differentially expressed during the first stages of definitive hematopoiesis in the P-sp/AGM region and the precise temporal and spatial pattern of expression of each one suggests that they may play different roles during aorta fusion and/or HSC generation. Moreover the temporal pattern of expression of *Notch1* resembles that suggested for a lateral inhibition process in which one cell that activates ligand expression would activate Notch receptor signaling in the adjacent cell thus preventing ligand expression in that cell and leading to a salt-and-pepper pattern of the receptor only expressed in few scattered cells.



***Supplementary Figure 1:* Restricted pattern of Notch1 expression.** *WISH performed in 12, 16 and 20 somite pair embryos. For each embryo three serial sections of 5µm are shown. The orientation is dorsal (up) to ventral (down). The arrow indicates a discrete high Notch1-expressing cell lining the ventral part of the dorsal aorta. Magnification 400x.*

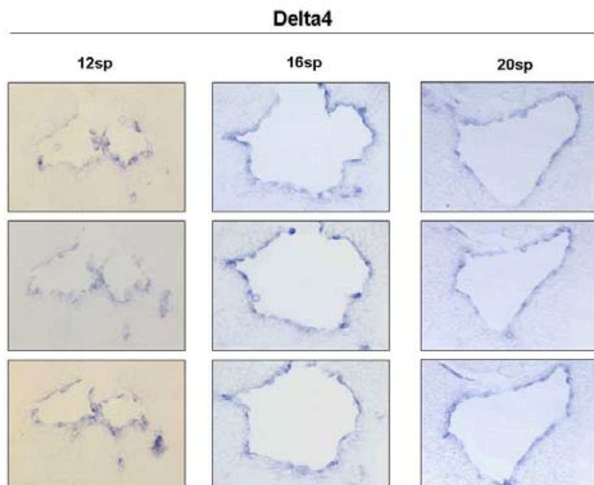
Supplementary Figure 2: Jagged1 displays a similar temporal and spatial pattern than Notch1. *WISH performed in 12, 16 and 20 somite pair embryos. For each embryo three serial sections of 5μm are shown. The orientation is dorsal (up) to ventral (down). The arrow indicates a discrete cell lining the ventral part of the dorsal aorta with high Jagged1 expression. Magnification 400x.*





Supplementary Figure 3: Jagged2 expression begins at the 16 somite pair stage. *WISH performed in 12, 16 and 20 somite pair embryos. For each embryo three serial sections of 5μm are shown. The orientation is dorsal (up) to ventral (down). Magnification 400x.*

Supplementary Figure 4: Delta4 is expressed before the pair of aortas fusion. *WISH performed in 12, 16 and 20 somite pair embryos. For each embryo three serial sections of 5μm are shown. The orientation is dorsal (up) to ventral (down). Magnification 400x.*

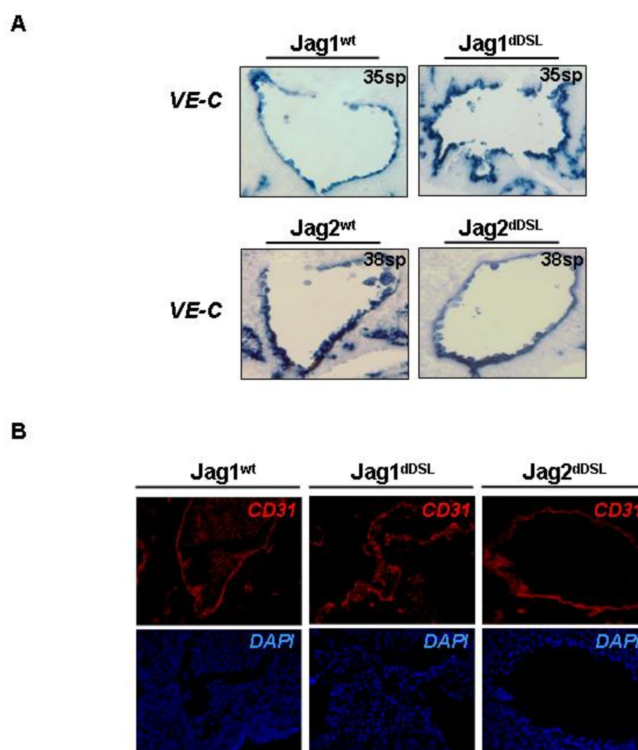


Study of the endothelial lineage in Jag1^{dDSL} or Jag2^{dDSL} mutant embryos

For many years, the hypothesis that hematopoietic and endothelial lineages come from a common progenitor or hemangioblast has been considered. There are many evidences that support the existance of a common progenitor in the yolk sac and the intra-embryonic aorta in the AGM region, as an early progenitor for both lineages (Choi et al., 1998; Eichmann et al., 1997; Huber et al., 2004; Sabin, 2002). For this reason, we investigated whether decreased hematopoiesis found in the Jag1^{dDSL} embryos affected the number of endothelial cells in the AGM aorta, similar to the RBPjκ (Robert-Moreno et al., 2005) or Notch1 null embryos.

WISH for the classical endothelial marker *VE-cadherin (VE-C)* revealed that wild-type embryos display a mixed pattern of cells with high and low levels of *VE-C* whereas, Jagged1 null embryos contained a hyperthrofic aorta with higher expression of *VE-C* compared with the wild-type littermates (Supplementary Fig. 5A). Moreover, a multiple-layered endothelium could be found in some regions of the aorta (data not shown), a pattern that resembles the one found in the RBPjκ null embryo. This result was confirmed by immunofluorescence with the PECAM/CD31 staining (Supplementary Fig. 5B). On the other hand, *VE-C* and PECAM/CD31 expression was normal in the Jag2^{dDSL} embryos, indicating that they do not display hematopoietic-endothelial defects in the aorta from the AGM region (Supplementary Fig. 5A and 5B).

Altogether these results indicate that abnormal hematopoiesis displayed in the Jag1^{dDSL} embryos (but not in the Jag2^{dDSL}) correlates with an enrichment of the number of endothelial cells and an abnormal aortic architecture and these results suggest that a balance between the hematopoietic and endothelial lineages must exist, supporting the idea of a putative common progenitor.



Supplementary Figure 5:* Increased expression of the endothelial markers VE-cadherin and CD31/PECAM1 in Jag1 null embryos. A)** *WISH for VE-cadherin in precisely timed E10.5-11 wild-type, Jag1^{dDSL} or Jag2^{dDSL} embryos. The orientation is dorsal (up) to ventral (down). Magnification 400x.* ***B) *PECAM/CD31 expression in wild-type, Jag1 or Jag2 null embryos by immunofluorescence on transverse sections of AGM aortas. The orientation is dorsal (up) to ventral (down). Magnification 400x.*

REFERENCES

- **Burns, C. E., Traver, D., Mayhall, E., Shepard, J. L. and Zon, L. I.** (2005). Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes Dev* **19**, 2331-42.
- **Cumano, A., Ferraz, J. C., Klaine, M., Di Santo, J. P. and Godin, I.** (2001). Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity* **15**, 477-85.
- **Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. and Keller, G.** (1998). A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-32.
- **de Bruijn, M. F., Ma, X., Robin, C., Ottersbach, K., Sanchez, M. J. and Dzierzak, E.** (2002). Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* **16**, 673-83.
- **de Bruijn, M. F., Speck, N. A., Peeters, M. C. and Dzierzak, E.** (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *Embo J* **19**, 2465-74.
- **de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. et al.** (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139-48.
- **Dieterlen-Lievre, F., Pouget, C., Bollerot, K. and Jaffredo, T.** (2006). Are intra-aortic hemopoietic cells derived from endothelial cells during ontogeny? *Trends Cardiovasc Med* **16**, 128-39.
- **Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., Costa, L., Henrique, D. and Rossant, J.** (2004). Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev* **18**, 2474-8.
- **Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Breant, C. and Le Douarin, N. M.** (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 U S A* **94**, 5141-6.
- **Gekas, C., Dieterlen-Lievre, F., Orkin, S. H. and Mikkola, H. K.** (2005). The placenta is a niche for hematopoietic stem cells. *Dev Cell* **8**, 365-75.
- **Hadland, B. K., Huppert, S. S., Kanungo, J., Xue, Y., Jiang, R., Gridley, T., Conlon, R. A., Cheng, A. M., Kopan, R. and Longmore, G. D.** (2004). A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. *Blood* **104**, 3097-105.
- **Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J. and Keller, G.** (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* **432**, 625-30.
- **Jiang, R., Lan, Y., Chapman, H. D., Shawber, C., Norton, C. R., Serreze, D. V., Weinmaster, G. and Gridley, T.** (1998). Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev* **12**, 1046-57.
- **Kumano, K., Chiba, S., Kunisato, A., Sata, M., Saito, T., Nakagami-Yamaguchi, E., Yamaguchi, T., Masuda, S., Shimizu, K., Takahashi, T. et al.** (2003). Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* **18**, 699-711.
- **Kunisato, A., Chiba, S., Nakagami-Yamaguchi, E., Kumano, K., Saito, T., Masuda, S., Yamaguchi, T., Osawa, M., Kageyama, R., Nakauchi, H. et al.** (2003). HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo. *Blood* **101**, 1777-83.
- **Lebestky, T., Jung, S. H. and Banerjee, U.** (2003). A Serrate-expressing signaling center controls Drosophila hematopoiesis. *Genes Dev* **17**, 348-53.
- **Ling, K. W., Ottersbach, K., van Hamburg, J. P., Oziemlak, A., Tsai, F. Y., Orkin, S. H., Ploemacher, R., Hendriks, R. W. and Dzierzak, E.** (2004). GATA-2 plays two functionally distinct roles during the ontogeny of hematopoietic stem cells. *J Exp Med* **200**, 871-82.
- **Medvinsky, A. and Dzierzak, E.** (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906.
- **Ottersbach, K. and Dzierzak, E.** (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell* **8**, 377-87.
- **Robert-Moreno, A., Espinosa, L., de la Pompa, J. L. and Bigas, A.** (2005). RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. *Development* **132**, 1117-26.
- **Rodrigues, N. P., Janzen, V., Forkert, R., Dombkowski, D. M., Boyd, A. S., Orkin, S. H., Enver, T., Vyas, P. and Scadden, D. T.** (2005). Haploinsufficiency of GATA-2 perturbs adult hematopoietic stem-cell homeostasis. *Blood* **106**, 477-84.
- **Sabin, F. R.** (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. 1917. *J Hematother Stem Cell Res* **11**, 5-7.
- **Sieburg, H. B., Cho, R. H., Dykstra, B., Uchida, N., Eaves, C. J. and Muller-Sieburg, C. E.** (2006). The hematopoietic stem compartment consists of a limited number of discrete stem cell subsets. *Blood* **107**, 2311-6.
- **Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. and Orkin, S. H.** (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-6.
- **Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster, G. and Gridley, T.** (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet* **8**, 723-30.
- **Yoder, M. C. and Hiatt, K.** (1997). Engraftment of embryonic hematopoietic cells in conditioned newborn recipients. *Blood* **89**, 2176-83.
- **Yoder, M. C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D. M. and Orlic, D.** (1997). Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* **7**, 335-44.
- **Yoder, M. C., Hiatt, K. and Mukherjee, P.** (1997). In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. *Proc Natl Acad Sci U S A* **94**, 6776-80.

SECTION 3: The notch pathway positively regulates programmed cell death during erythroid differentiation

(This chapter was published in Leukemia, May 2007).

Several gene target mutations revealed that hematopoiesis in the yolk sac and in the intra-embryonic AGM region are governed by two different genetic programs since some mutations affect both primitive and definitive hematopoiesis whereas others affect only the definitive one (reviewed in Cumano and Godin, 2002).

In this paper, we show that in contrast to the absolute Notch/RBPjκ signaling requirement for definitive AGM hematopoiesis, primitive erythropoiesis normally occurs in the yolk sac of RBPjκ mutants. All kind of hematopoietic progenitors are generated in these mutant embryos and development and maturation of the erythroid lineage does not display any defect. However, the percentage of $Ter119^{+}$ erythroid cells is higher in the $RBPj\kappa^{-/-}$ yolk sacs compared with their wild-type littermates and neither proliferation nor differentiation is responsible for this increase.

In contrast, apoptosis is reduced specifically in erythroid cells from the mutant yolk sacs, indicating that Notch signaling promotes programmed cell death in this lineage. By quantitative RT-PCR we demonstrate that loss of RBPjκ correlates with an increase of Epo and its receptor (Epo-R) expression and in the levels of the anti-apoptotic genes bcl-2 and bcl-xL in agreement with the higher survival of the $Ter119^{+}$ population.

Finally we show that Notch induces apoptosis in the erythroid lineage not only during embryonic development but in the adult as well, since bone marrow cells treated with the γ-secretase inhibitors DAPT and L685,458 display low apoptotic cell death and Notch-induced apoptosis by incubating bone marrow with Jagged1-expressing cells is abrogated by γ-secretase inhibitors treatment. In the same sense, murine erythroleukemic cells overexpressing the activated form of Notch1 display higher programmed cell death in hexametilene-bisacetamide-induced differentiation.

From all the data we suggest that Notch signaling is not essential for yolk sac hematopoiesis but specifically induces programmed cell death in the erythroid lineage not only during embryonic life but in adult tissues as well thus regulating homeostasis of this hematopoietic compartment.

The notch pathway positively regulates programmed cell death during erythroid differentiation

Running title: Notch induces apoptosis in erythroid cells

Àlex Robert-Moreno[1], Lluis Espinosa[1], M.José Sanchez[2], José L. de la Pompa[3] and Anna Bigas[1]*

[1]Centre Oncologia Molecular, IDIBELL-Institut de Recerca Oncològica. Gran Via km 2.7 Hospitalet, Barcelona, Spain.

[2] Centro Andaluz Biología Desarrollo, CSIC, Universidad Pablo Olavide, Sevilla

[3]Dept. Immunology and Oncology, Centro Nacional de Biotecnología, CSIC. Darwin, 3. Campus de Cantoblanco, Madrid 28049, Spain

* Corresponding author

Mailing address: Oncologia Molecular.
IDIBELL-Institut de Recerca Oncològica
Gran Via Km 2.7.
08907 - Hospitalet, Barcelona. Spain
Phone: 932 607 404
Fax: 932 607 426
e-mail: abigas@iro.es

SUMMARY

Programmed cell death plays an important role in erythropoiesis under physiological and pathological conditions. In this study, we show that the Notch/RBPjκ signaling pathway induces erythroid apoptosis in different hematopoietic tissues, including yolk sac and bone marrow as well as in murine erythroleukemia cells. In RBPjκ$^{-/-}$ yolk sacs, erythroid cells have a decreased rate of cell death that results in increased number of Ter119+ cells. A similar effect is observed when Notch activity is abrogated by incubation with the γ-secretase inhibitors, DAPT or L685,458. We demonstrate that incubation with Jagged1-expressing cells has a proapoptotic effect in erythroid cells from adult bone marrow that is prevented by blocking Notch activity. Finally, we show that the sole expression of the activated Notch1 protein is sufficient to induce apoptosis in hexametilene-bisacetamide-differentiating murine erythroleukemia cells. Together these results demonstrate that Notch regulates erythroid homeostasis by inducing apoptosis.

INTRODUCTION

Notch is a highly conserved signaling pathway that regulates cell fate specification during development and adult tissue homeostasis. Physiological activation of the Notch pathway requires the interaction between the Notch receptor and one of its ligands. This interaction leads to the cleavage of Notch receptor, releasing the intracellular domain that translocates to the nucleus to bind RBPjκ and activate specific gene transcription (reviewed by Bray[1] and Lai[2]).

Notch function is required for the generation of definitive hematopoiesis as shown by the lack of hematopoietic precursors in the aorta of Notch1$^{-/-}$ and RBPjκ$^{-/-}$ mouse embryos or in Mind bomb mutants in zebrafish[3-6]. In contrast, primitive hematopoiesis occurs in different Notch pathway mutants in both mouse and zebrafish[3-5]. In the mouse, primitive hematopoiesis originates in the blood islands of the yolk sac, starting at embryonic day 7.5 (E7.5). The main component of this primitive hematopoiesis is erythroid progenitor cells (EryP)[7] that generate large nucleated primitive erythrocytes that contain embryonic globins (βH1, ε-globin and ζ-globin) (reviewed by Palis and Segel[8]).

Erythropoiesis involves the progressive differentiation of uncommitted progenitors to mature erythrocytes. However, not only differentiation but also apoptosis participates in the regulation of cell survival and mature red cell turnover. The amount of erythropoietin (Epo), mainly dependent on hypoxia, is one of the key factors in controlling the survival of erythroid cells (reviewed in Mulcahy[9]). Expression of the antiapoptotic members of the Bcl-2 family, Bcl-2 and Bcl-x,[10] are some of the downstream effects of EpoR activation in this system. Consistent with this, bcl-x$^{-/-}$ embryos die of massive apoptosis in the nervous system and in fetal liver erythroid cells[11].

Other transcription factors including GATA1 and, more recently, p53 have been implicated in regulating apoptosis at different stages of erythroid maturation[12,13]. In this sense, GATA1 plays a key role in development and survival of erythroid cells since GATA1-deficient cells failed to develop beyond the proerythroblast stage and undergo rapid apoptosis[14].

Notch pathway has previously been shown to induce apoptosis in cell lines from different hematopoietic lineages most likely through the activation of its target gene hes1[15-17]. However the overall data linking Notch and erythroid apoptosis is controversial[18,19].

In this work, we demonstrate that the Notch signaling pathway is a positive regulator of apoptosis in primitive erythropoiesis in the yolk sac but also in erythroid cells from adult bone marrow (BM). Complementary studies using the murine erythroleukemia (MEL) cell line indicate that Notch induces erythroid-specific apoptosis.

MATERIALS and METHODS

Animals

RBPjκ$^{-/-}$ mice have been previously described[20]. Animals were kept under pathogen-free conditions and experiments approved by the Animal Care Committee. Yolk sacs were obtained from timed pregnant females at days 7.5 to 9.5 of gestation and dissected out from embryo and vitelline arteries. Embryos were genotyped by polymerase chain reaction (PCR) and morphology. BM was obtained from 8-12 week wild-type (WT) CD1 mice.

Cell lines and transfections

MEL cells[21] were maintained in RPMI 10% fetal bovine serum (FBS), 1% L-glutamine and 1% Pen/Strep. Stable clones of MEL cells expressing N1ΔE[22] or pcDNA.3 were obtained by electroporation and expression was confirmed by western blot (9E10 antibody). NIH-3T3 cells were transfected by calcium phosphate with Jagged1 construct[23] and clones overexpressing Jagged1 were selected in G418. Differentiation of MEL cells was performed with 5mM hexametilene-bisacetamide (HMBA; Sigma, St Louis, MI, USA) for 6 days.

Dianisidine staining

O-dianisidine (Sigma) was used to stain hemoglobin of both E9.5 WT and RBPjκ-mutant yolk sacs, and MEL Friend cells to assay erythroid differentiation as previously described[21].

Hematopoietic colony assay

Yolk sac from WT and RBPjκ$^{-/-}$ E7.5-9.5 embryos was digested in 0.1% collagenase (Sigma) in phosphate-buffered saline (PBS), 10% FBS for 30 min at 37ºC. Cells (30,000) were plated in duplicates in 1% methylcellulose (Stem Cell Technologies, Vancouver, Canada) plus Iscove's with 10% FBS, 10% IL3- and stem cell factor (SCF)-conditioned medium, 2.5% L-glutamine, 0.1% monothioglycerol (Sigma), 1% Pen/Strep (Biological Industries, Beit Haemek Kibbutz, Israel), 2 IU/ml erythropoietin (Laboratorios Pensa-Esteve, Barcelona, Spain), 20 ng/ml granulocyte-macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA) and 100 ng/ml of granulocyte colony-stimulating factor (Avantis Pharma, Paris, France). After 7 days, the presence of hematopoietic colonies was scored under a microscope. EryP colonies were scored at day 3.

Flow cytometry analysis

Collagenase-disrupted yolk sac cells were stained with fluorescein isothiocyanate FITC-conjugated CD71, CD41, ckit, CD45 and mac1 and PE-conjugated Ter119 and CD31 antibodies (Pharmingen, BD Biosciences, San Jose, CA, USA) or isotopic immunoglobulin G as a control. Cells were analyzed in a FACScalibur (Becton & Dickinson, BD Biosciences, San Jose, CA, USA) and WinMDI 2.8 software. Dead cells were excluded by 7-aminoactinomicin-D (7-AAD; Invitrogen, Carlsbad, CA, USA) staining. For the Annexin V binding analysis, cells were stained with rh AnnexinV-FITC kit (Bender Medsystems, Burlingame, CA, USA) and 7-AAD for 15 min according to the manufacturer's instructions.

For cell cycle analysis of total yolk sac, cells were fixed in 70% EtOH at -20^{0}C overnight, treated with 50 μg DNAse-free RNAse and stained with 25 μg of propidium iodide (Sigma). Ter119+ cell-cycle analysis was performed on fresh cells with 20 μM Draq5 (Biostatus Ltd, Leicestershire, UK). FlowJo 6.4.1 software was used for cell-cycle analysis.

Yolk sac and BM cultures

Collagenase-disrupted yolk sacs were cultured for 6 days in Iscove's with 10% FBS, 10% IL3- and SCF-conditioned medium, 0.1% monothioglycerol in the presence of 50 μM DAPT (Calbiochem), 2 μM L685,458 (Sigma) or dimethyl sulfoxide (DMSO) as control. For BM culture, 1.5×10^{5} whole BM cells were incubated with γ-secretase inhibitors in RPMI 10% FBS, 2IU/ml EPO for 2-3 days. Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK; Bachem, Budendorf, Switzerland) was used at 200μM.

Coculture on 3T3 or 3T3-Jag1 stromal cells was perfomed with 4×10^{5} whole BM cells in RPMI, 10% FBS for 16h. Cells were assayed for AnnexinV binding and analyzed by flow cytometry.

Immunohistochemisty

Yolk sacs were fixed with 4% paraformaldehyde (Sigma), embedded in Paraplast (Sigma) and sectioned (10 μm). Slides were dewaxed in xylene, antigen retrieval was performed by boiling for 2 min in sodium acetate, rehydrated and blocked-permeabilized in 10% FBS, 0.3% Surfact-Amps X100 (Pierce, Aalst, Belgium) and 5% non-fat milk in PBS for 90 min at 4ºC. Anti P-Ser10 H3 (Upstate, Charlottesville, VA, USA) was used at 1:500 dilution and developed with Dakocytomation kit (Dako, Glostrup, DK, Denmark) following manufacturer's instructions. Hematoxilin (Merck, Whitehouse Station, NJ, USA) was used for counterstaining.

For histological analysis, tissue samples were fixed overnight at 4ºC in 4% paraformaldehyde, dehydrated and embedded in Paraplast (Sigma). Samples were sectioned in a Leica-RM2135 at 4μm and stained with hematoxilin and eosin.

Images were acquired with an Olympus BX-60 using a Spot camera and Spot 3.2.4 software (Diagnostic Instruments, Sterling Heights, MI, USA). Adobe Photoshop 6.0 software was used for photograph editing.

Semiquantitative reverse transcriptase-polymerase chain reaction

Total RNA from subdissected E9.5 WT and $RBPj\kappa^{-/-}$ yolk sacs was isolated using TRIzol Reagent (Invitrogen). Poly-AT Tract System IV (Promega, Madison, WI, USA) and RT-First Strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK) were used to obtain mRNA and cDNA respectively. PCR product

was analyzed at different cycles to avoid saturation. Quantity One software (Biorad, Hempstead, UK) was used for densitometry. Primer pairs used in the experiments are listed in Supplementary Table S1.

Quantitative RT-PCR

Ter119+ cells were sorted from E9.5 collagenase-treated embryos in MoFlo Cell Sorter (Dakocytomation, BD Diagnostic, San Jose, CA, USA). mRNA was isolated with Rneasy minikit (Qiagen, Valencia, CA, USA) following manufacture's instructions. qRT-PCRs were performed with SYBR Green I Master (Roche, Basel, Switzerland) in LightCycler480 system.

Statistical analysis

Normal distribution of the samples was confirmed with one-sample Kolmogorow-Smirnoff test and Student's t-test was performed.

RESULTS

Absence of Notch signaling results in increased number of erythroid cells in the yolk sac

Notch signaling has previously been shown to influence differentiation and apoptosis of erythroid cells *in vitro* although controversial observations have been reported[15,18,19,24]. For this reason, we aimed to characterize the physiological role of the Notch pathway in erythropoiesis by comparing WT and RBPjκ$^{-/-}$ embryos. Despite the absence of intra-embryonic hematopoiesis in the RBPjκ$^{-/-}$ embryos and the presence of different angiogenic abnormalities in the yolk sac, we found that primitive hematopoiesis does occur in the yolk sac of the RBPjκ$^{-/-}$ embryos (Figure 1a), similar to the Notch1$^{-/-}$ mutants[25-27].

To determine whether Notch pathway plays a role in regulating hematopoiesis in the yolk sac, we first analyzed the expression of different Notch receptors, ligands and Notch-target genes in the yolk sac of WT and RBPjκ$^{-/-}$ embryos by semiquantitative RT-PCR and we observed that all Notch family genes are expressed in the yolk sac at E9.5 (Figure 1b). In RBPjκ$^{-/-}$, we found reduced expression of all ligands and receptors, whereas Notch3 and Jagged2 were upregulated. We also tested the expression of Notch-target genes and detected a consistent reduction in hes1 levels in RBPjκ$^{-/-}$ yolk sacs compared with WT (Figure 1b).

Since hematopoiesis in the yolk sac is mainly restricted to erythropoiesis, we determined the percentage of cells expressing the erythroid marker Ter119 in collagenase-treated yolk sacs at E7.5, E8.5 and E9.5. We detected a few positive cells in the yolk sac of E7.5 (Figure 1c) and E8.5 (data not shown). At day 9.5 the percentage of Ter119+ in the yolk sac ranged from 20 to 40% in the WT and 40-60% in the RBPjκ$^{-/-}$ (Figure 1c and d) being similar the total number of cells per yolk sac (Figure 1a).

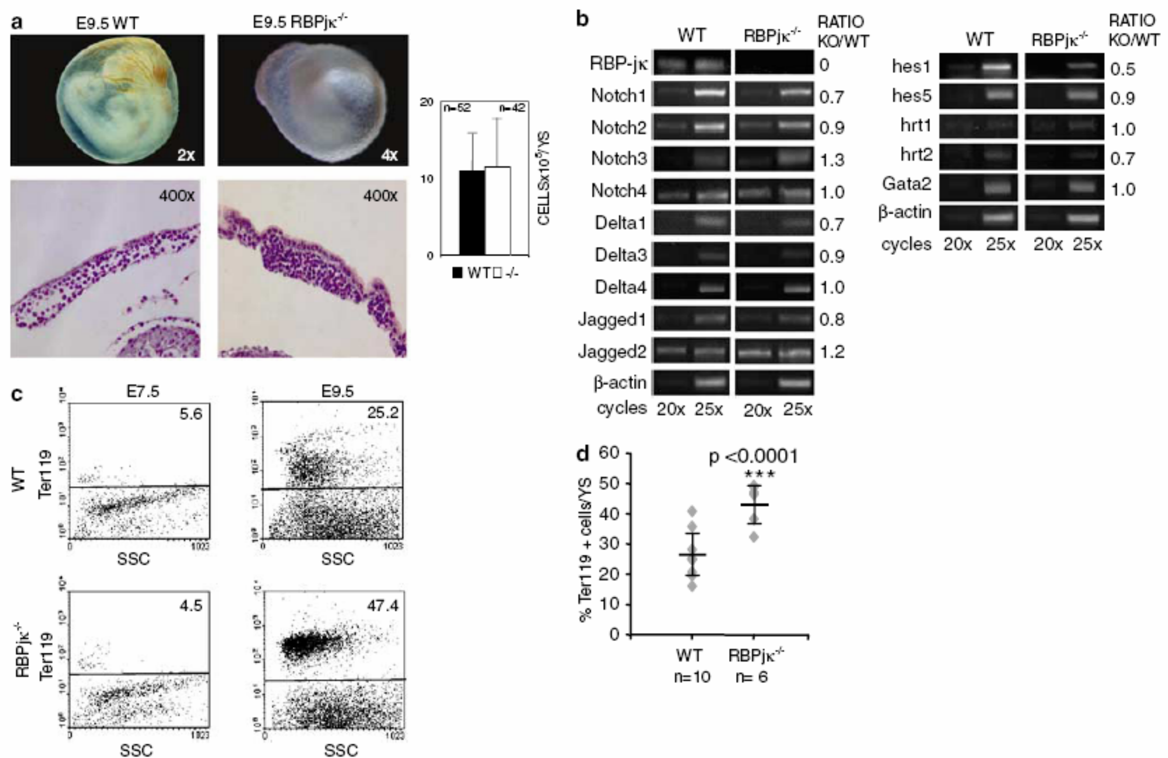


Figure1: *Increased number of erythroid cells in RBPjκ-/- yolk sacs. (a) Images of E9.5 WT and RBPjκ-/- embryos with the yolk sac (upper panel) and hematoxilin/eosin staining of yolk sac sections. Total number of cells obtained from disrupted yolk sacs (right panel). (b) Semiquantitative RT-PCR of Notch receptors, ligands and target genes from E9.5 WT and RBPjκ-/- yolk sacs. (c) Representative analysis of Ter119+ cells from E7.5 and E9.5 WT and RBPjκ-/- yolk sacs. (d) Percentage of Ter119+ cells in the analyzed E9.5 WT and RBPjκ-/- yolk sacs. Average and s.d. are represented.*

Proliferation is not responsible for increased erythropoiesis in RBPjκ-/- yolk sacs

To investigate whether the higher number of Ter119+ cells in the RBPjκ-/- yolk sac was owing to an increase in the number of progenitors, we performed colony-forming cell (CFC) assays with collagenase-treated yolk sac cells at E7.5, E8.5 and E9.5 from RBPjκ+/+, +/- and -/- embryos. We detected a similar percentage of myeloid, erythroid and mixed colonies in these cultures (Supplementary Figure S1), however; at E9.5, there was a twofold increase in the total number of CFC in mutant embryos (Figure 2a). To test whether this effect was due to increased proliferation, we analyzed the cell-cycle profile of WT and RBPjκ-/- yolk sac cells by flow cytometry. Surprisingly, the percentage of cells in S/G_2-M phase was slightly reduced in RBPjκ-/- compared with WT cells (from 53 to 45%), and this reduction in S/G_2-M phase was higher when cell cycle was analyzed in the Ter119+ cells (Figure 2b). To confirm this observation, we performed the P-H3 staining on yolk sac sections to assess the number of cells undergoing mitosis inside the blood islands. P-H3 staining showed that hematopoietic cells from the WT yolk sacs have a similar mitotic rate (19.8 %) than cells in RBPjκ-/- yolk sacs (12.9 %); (Figure 2c). Altogether these results indicate that proliferation is not increased in the RBPjκ-/- yolk sac erythroid cells.

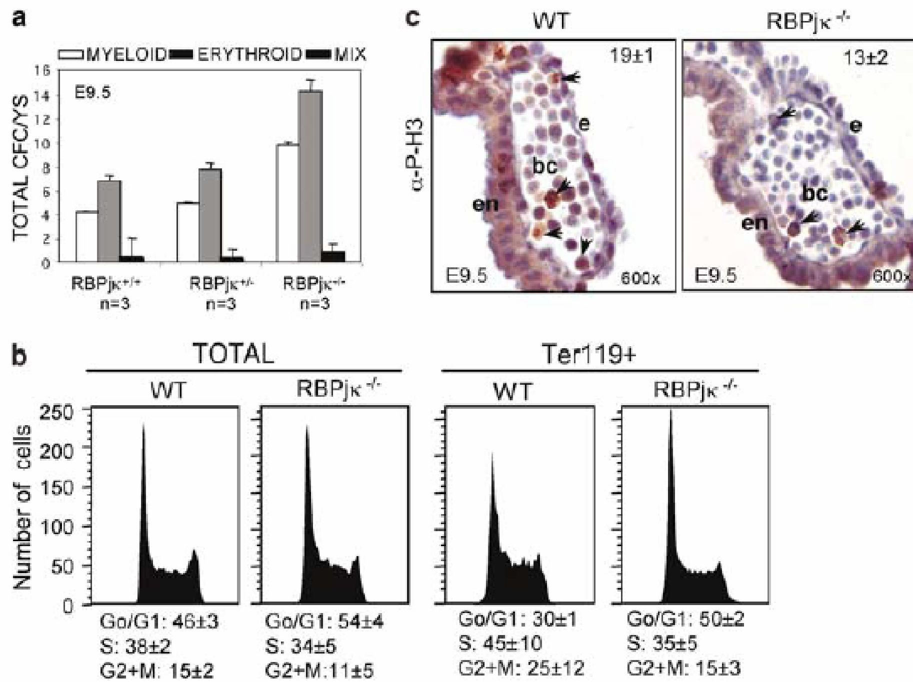


Figure2: *Proliferation in E9.5 WT and RBPjκ$^{-/-}$ yolk sacs. (a) Graphs represent the total number of CFC types obtained from WT or RBPjκ$^{-/-}$ yolk sacs. (b) Representative cell-cycle analysis from total and Ter119+ cells from E9.5 WT or RBPjκ$^{-/-}$ yolk sacs. Average values and s.d. from two yolk sacs are shown. (c) IHC of P-Histone3 in yolk sac sections. Numbers represent the average percentage of positive cells inside the blood islands found in four independent stainings. Arrowheads indicate cells with positive staining; e, endothelium; en, endoderm; bc, blood cells.*

Normal differentiation occurs in RBPjκ$^{-/-}$ yolk sac

Although hematopoiesis is mainly restricted to erythropoiesis in the yolk sac, different progenitor types and macrophages are also generated. As we detected increased number of different hematopoietic progenitors in the RBPjκ$^{-/-}$ (Figure 2a), we speculated that the decision between hematopoietic and endothelial lineages may be affected. To test this possibility we analyzed the expression of CD45 (hematopoietic excluding erythroid cells) and CD31 (endothelial) cell markers. We detected a similar number of cells expressing these markers, indicating that the non-erythroid hematopoiesis is normally occurring in the RBPjκ$^{-/-}$ yolk sac (Figure 3a). Consistent with this observation, we did not detect any difference in the percentage of endothelial cells (CD31+ and CD45-) (Figure 3a) or in the expression of PECAM or VE-cadh genes in these yolk sacs (Supplementary Figure S4), in contrast to that previously observed in the intra-embryonic endothelial/hematopoietic differentiation[3,6]. In addition, no major differences were found in the percentage of mac1+ cells between WT and RBPjκ$^{-/-}$ (Figure 3a).

We next investigated whether the higher number of Ter119+ cells in the RBPjκ$^{-/-}$ was due to a blockage in erythroid differentiation. Thus, we characterized the different erythroid subpopulations by analyzing the expression of specific differentiation markers CD71, CD41 and c-kit in the Ter119+ population by flow cytometry.

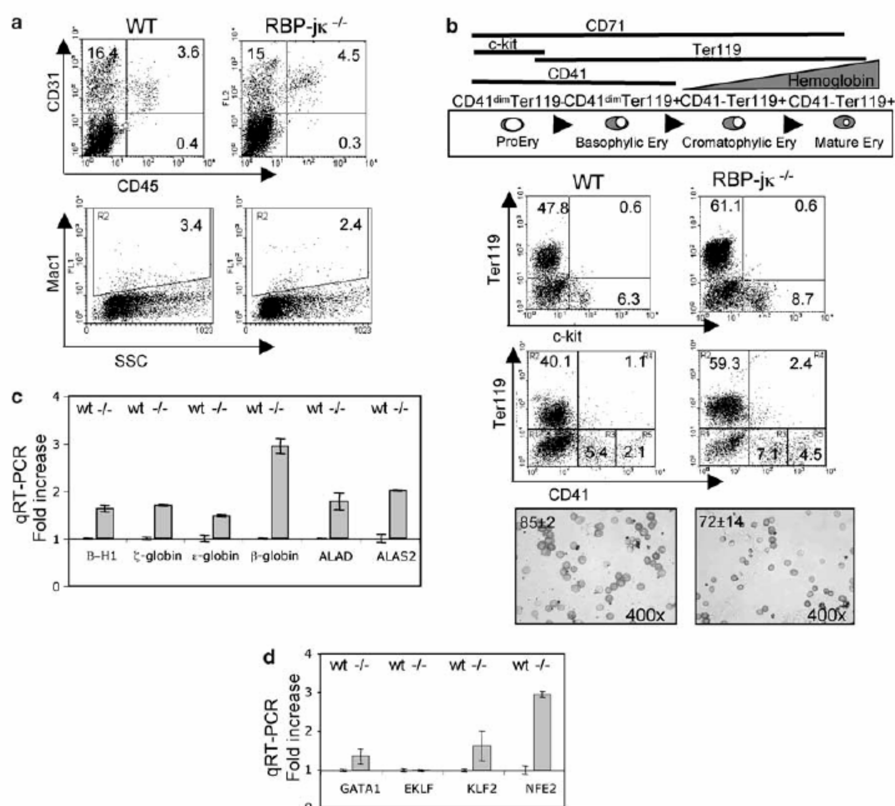


Figure3: *Erythroid differentiation is not impaired in RBPjκ$^{-/-}$ yolk sacs at E9.5. (a) Representative dot plots showing expression of CD45 (hematopoietic marker) versus CD31 (endothelial marker) and mac1 (macrophage marker). (b) Representation of erythroid differentiation markers (upper). Dot plots of representative erythroid subpopulations from three WT and RBPjκ$^{-/-}$ yolk sacs. Dianisidine staining of circulating cells from the yolk sac (lower panels). Numbers represent the average and s.d. of positive cells counted in three different samples. (c and d) qRT-PCR of (c) globin genes and hemo maturation enzime genes and (d) erythroid-specific transcription factors from WT and RBPjκ$^{-/-}$ yolk sacs.*

As shown in Supplementary Figure S2, CD71 was expressed in all Ter119+ cells in both WT and RBPjκ$^{-/-}$ yolk sacs, this result is surprising since this marker is downregulated during erythroid differentiation in BM[28]. Analysis of other differentiation markers showed that the different Ter119+ subpopulations were similarly represented in WT and mutant yolk sacs (Figure 3b). We also analyzed the expression of erythroid transcription factors and globin genes in purified Ter119+ cells from WT and RBPjκ$^{-/-}$ embryos by qRT-PCR. We did not detect major differences in the expression of the embryonic globins and hemo group maturation enzymes (ALAD and ALAS2; Figure 3c) or in the percentage of circulating yolk sac cells showing dianisidine staining (from 85% to 72%; Figure 3b); however, a three-fold increase in the expression of adult β-globin was observed in the RBPjκ$^{-/-}$ cells compared with the WT (Figure 3c). We also detected overexpression of the erythroid transcription factor NFE2 in RBPjκ$^{-/-}$ erythroid cells, whereas no significant differences were detected in GATA1, KLF2 and EKLF levels (Figure 3d). Surprisingly, we did not observed downregulation of the hes1 gene in the Ter119+ cells of the RBPjκ$^{-/-}$ embryos (data not shown).

Reduction of apoptosis in Notch-defective yolk sac erythroid cells

Apoptosis is a crucial mechanism for maintaining the homeostasis of the erythroid lineage. Since minor differences in proliferation or differentiation were found in the RBPjκ$^{-/-}$ mutants, we tested whether the increased number of erythroid cells in these embryos was due to differences in apoptosis. We detected a

significant reduction (P<0.05) in the apoptotic rate as measured by AnnexinV binding in the RBPjκ$^{-/-}$ yolk sacs that was specific for the erythroid Ter119+ population (Figure 4a and b).

Next, we analyzed the expression levels of different genes that are associated with apoptosis in purified Ter119+ cells. By qRT-PCR, we detected 3-4 fold increase in the expression levels of the erythroid survival factor EPO, its receptor and its downstream effectors, the antiapoptotic *bcl-2* and *bcl-x* genes in the RBPjκ$^{-/-}$ cells compared with the WT. This suggests that EpoR-mediated signaling may be participating in the increased survival of RBPjκ$^{-/-}$ Ter119+ cells. In contrast, no major differences were found in the expression levels of *p53* family genes although one of its proapoptotic targets *puma* was increased in the mutant cells compared with the WT (Figure 4c, right panel).

To further demonstrate that reduced apoptosis in the RBPjκ$^{-/-}$ erythroid cells was dependent on Notch function, we obtained WT cells from disrupted yolk sacs and incubated in liquid culture in the presence or absence of the γ-secretase inhibitors DAPT and L685,458. After 6 days of culture, both the total number and the percentage of TER119+ cells was significantly increased in the DAPT-treated cells compared with the control (Figure 4d) resembling the RBPjκ$^{-/-}$ phenotype. The increase in the Ter119+ population correlated with a three-fold reduction in the percentage of AnnexinV+ cells in the DAPT- and L685,458-treated cells (Figure 4e and f). In agreement with the differences in gene expression found in the RBPjκ$^{-/-}$ yolks sacs, upregulation of EPO, its receptor, *bcl-2* and *bcl-x* was detected in the γ-secretase-treated Ter119+ cells (Supplementary Figure S5). Together these results indicate that Notch activity regulates apoptosis in the erythroid lineage in the yolk sac.

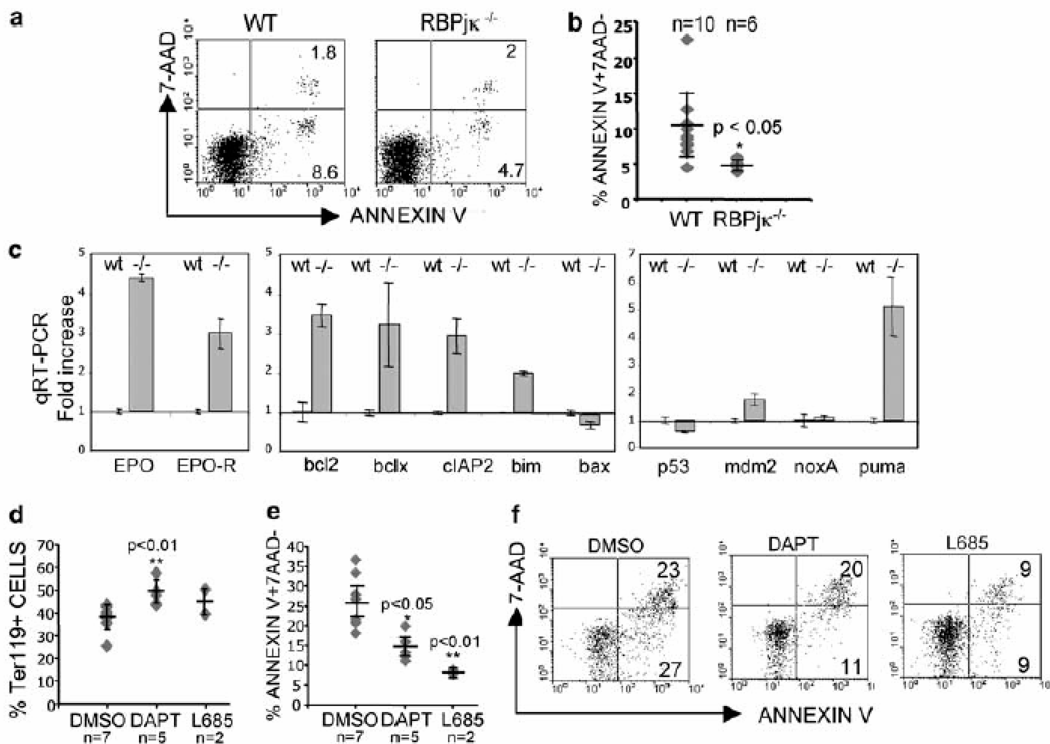


Figure4: *RBPjκ$^{-/-}$ and γ-secretase inhibitor-treated erythroid cells show reduced apoptosis. (a) Dot plot of AnnexinV and 7-AAD staining in a representative E9.5 WT and RBPjκ$^{-/-}$ yolk sac. (b) Percentage of AnnexinV+7AAD- cells in the Ter119+ cells in E9.5 WT and RBPjκ$^{-/-}$ yolk sacs. (c) Relative expression of pro- and antiapoptotic genes in E9.5 RBPjκ$^{-/-}$ compared with WT yolk sacs by qRT-PCR. (d and e) Graphs represent the percentage of Ter119+ cells (d) and AnnexinV+7AAD- cells (e) from E9.5 WT yolk sacs after 5 days of culture in DMSO, 50μM DAPT or 2μM L685,458. (f) Dot plots from a representative experiment.*

Notch activation induces apoptosis in adult BM erythroid and MEL cells

We next investigated whether Notch activation was regulating apoptosis not only in the yolk sac but also in adult erythropoiesis. Since RBPjκ$^{-/-}$ mutants do not generate definitive hematopoiesis and die at E10.5, we isolated BM from WT adult mice and performed cell cultures in the presence of DMSO, DAPT or L685,458. We observed a 30% decrease in the percentage of AnnexinV+ cells in the Ter119+ population after 2 days of culture in γ-secretase inhibitors compared with the control (Figure 5a). Interestingly, Ter119-negative cells were not protected from apoptosis by DAPT in these cultures (data not shown), indicating the specific proapoptotic effect of Notch activity on the erythroid lineage. Conversely, incubation of total BM cells from adult mouse on NIH-3T3-Jag1 cells resulted in a 2-3 fold increase in the percentage of AnnexinV+Ter119+ population compared with that incubated on NIH-3T3 control cells (P=0.04) (Figure 5b and c). Moreover, this effect was prevented in the presence of γ-secretase inhibitors DAPT (P=0.01) or L685,458 (P=0.002; Figure 5c) and the caspase inhibitor Z-VAD-FMK (data not shown).

To further demonstrate that Notch was sufficient to induce apoptosis in erythroid cells, we generated different clones of MEL cells expressing the active Notch1 fragment, N1ΔE. Cells expressing N1ΔE differentiated and proliferated similar to the controls (Supplementary Figure S3); however, these cells showed a higher percentage of Annexin V binding after 6 days in culture under differentiating conditions (HMBA) as expected (Figure 5d).

Altogether our results indicate that Notch positively regulates apoptosis in embryonic and adult erythropoiesis and in differentiating erythroleukemia cells.

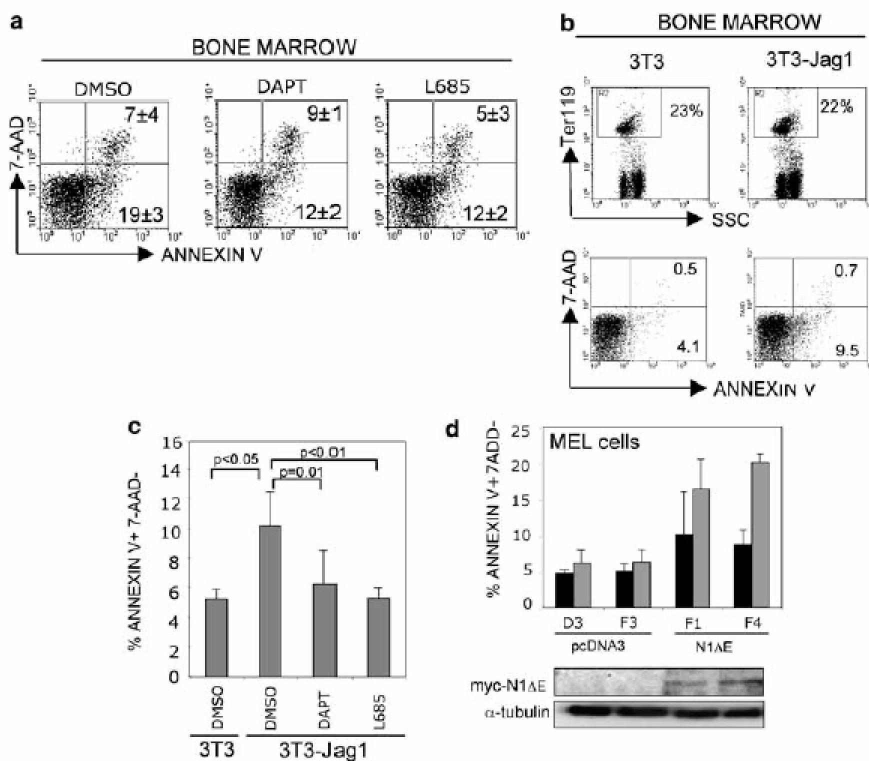


Figure5: Notch activity induces apoptosis in the Ter119+ cells from adult bone marrow and murine erythroleukemia cells. (a) Graphs represent the percentage of AnnexinV+7AAD- in the Ter119+ population of BM cultured in the presence of DMSO, DAPT or L685,458. Average percentage and s.d. of three different experiments are shown. (b and c) Representative flow cytometry analysis of AnnexinV binding in the Ter119+ cells from BM incubated on 3T3 or 3T3-Jag1 for 16h (b). Graphs represent the average and s.d. of 3T3-Jag1-induced apoptosis observed in three different experiments incubated with DAPT and L685,458 as measured by AnnexinV+7AAD- in Ter119+ cells (c). (d) Graphs represent the average percentage of AnnexinV+7AAD- cells in Notch1ΔE expressing MEL clones (F1, F4) compared with control clones (D3, F3) in proliferation media (black bars) or after 6 days of differentiation in 5mM HMBA (gray bars). Western blot shows the expression of Notch1ΔE.

DISCUSSION

Notch signaling had been linked previously to regulate apoptosis in erythroid cells, although it was unclear whether Notch inhibits or promotes apoptosis. We have now used primary cells from yolk sac and BM to elucidate the physiological effect of Notch activation on the erythroid lineage. We demonstrate that the Notch pathway is a positive inducer of apoptosis in erythroid cells from different tissues.

Although different cytokines regulate the integrity of the erythroid compartment, production of definitive erythroid cells is mainly dependent on Epo[29]. Signaling from EpoR through Jak2/Stat5[30,31] prevents apoptosis on this cell lineage from late erythroid progenitors (CFU-E) until the onset of hemoglobinization[32,33], and one important target gene of this cascade is bcl-x[34]. Studies using bcl-x-null mice demonstrated a critical role for this gene at the end of erythroid maturation when maximal hemoglobin synthesis occurs[35]. RBPjκ$^{-/-}$ erythroid cells show a clear upregulation of genes involved in this antiapoptotic pathway, such as EPO, EPO-R, bcl-x and bcl-2, suggesting that Notch may induce apoptosis by impinging on this pathway. Previous reports have attempted to link Notch induction of apoptosis through the p53 pathway in other systems[36,37]. We have also explored that possibility in erythroid cells, since there is increasing evidence of p53-mediated apoptosis at different stages of erythroid differentiation[13,38]; however, our results show that crucial p53 pathway genes are not affected in the RBPjκ$^{-/-}$ erythroid cells suggesting that this pathway is not responsible for the protection of apoptosis in the RBPjκ mutants. In fact, we found an erythroid-specific upregulation of the proapoptotic p53-target gene, *puma*, in the RBPjκ$^{-/-}$ Ter119+ cells, which is surprising since this population has a lower apoptotic rate.

Although previous reports have attempted to decipher which is the Notch function in apoptosis of erythroid cells[15,19], this is the first time that this question is addressed in primary cells. The use of cell lines in the previous published reports may explain the controversial data. Our data is in agreement with the results from Ishiko et al[15] using K562 cells in which Notch activity induces apoptosis. In this system, Notch activation led to downregulation of bcl-x, likely through repression of GATA1 by hes1 under differentiation conditions[15]. Similarly we found increased levels of bcl-x and bcl-2 in the Ter119+ cell population of RBPjκ$^{-/-}$; however we did not detect any change in hes1 and GATA1 levels in this model. The observation that hes1 is not affected by the lack of RBPjκ in particular cell types has already been described and explained by the repressor function of RBPjκ in the absence of Notch signaling (reviewed by Lai[2]).

We now have used different approaches including the Notch loss-of-function RBPjκ$^{-/-}$ embryos, γ-secretase inhibitors and Jag1-expressing cell co-culture to investigate the role of Notch signaling in regulating apoptosis on erythroid cells. Together, our results confirm that Notch activation favors apoptosis in this particular lineage and this finding may be particularly relevant in some erythroid disorders. In fact, erythropoiesis in the RBPjκ$^{-/-}$ embryos resembles the one observed in myelodysplastic syndrome (MDS)-derived leukemias or polycytemia vera. These human myeloproliferative syndromes cause the overproduction of erythroid cells, mainly because of activating mutations in the Jak2 kinase and this leads not only to increased cell proliferation but also upregulation of *bcl-x* (reviewed by Campbell and Green[39]) and protection from apoptosis. In this scenario and based on our results, it is particularly interesting to investigate whether the Notch pathway is deregulated in these tumors. In this sense, higher expression of the Notch-like ligand, dlk, in MDS compared with myeloid leukemia cells has been detected by microarray analysis[40].

Notch activation has previously been associated to inhibition of apoptosis in normal endothelial[41] and T cells[42], likewise in neoplastic cells including Kaposi's sarcoma[43], glioma[44] and Hodgkin's lymphoma[45] cells. Conversely, Notch activation can induce apoptosis during the development of the retina[46] and the serotonin lineage[47] in *Drosophila*. Notch also induces apoptosis in B-cell lymphomas[16], normal monocytes[48] or hepatocellular carcinoma cells[49]. Based on this and our results, it is likely that apoptosis is another mechanism used by Notch to control specific tissue homeostasis.

Altogether, our results indicate that the activation of the Notch pathway is able to regulate the erythroid lineage in different hematopoietic tissues by inducing apoptosis.

ACKNOWLEDGEMENTS

We thank Liheng Li for Jagged1 plasmid, Raphael Kopan for N1ΔE plasmid and M. Caudy for the hes1 plasmid. Thanks to I Fabregat and J Gil for extremely helpful suggestions and reagents. Many thanks to L Riera and J Inglés-Esteve for critical comments of the manuscript. We thank E Castaño from Serveis Cientifico-Tècnics, UB-Bellvitge, and J Petriz for flow cytometry technical support. LE is an investigator from the Carlos III program (ISCIII/02/3027). ARM is a recipient of a CIRIT predoctoral fellowship (2002-SI00791). This work was supported by the grants from Ministerio de Educación y Ciencia (SAF2004-03/98) and (SAF205-23978-E) and (SAF2003-07214).

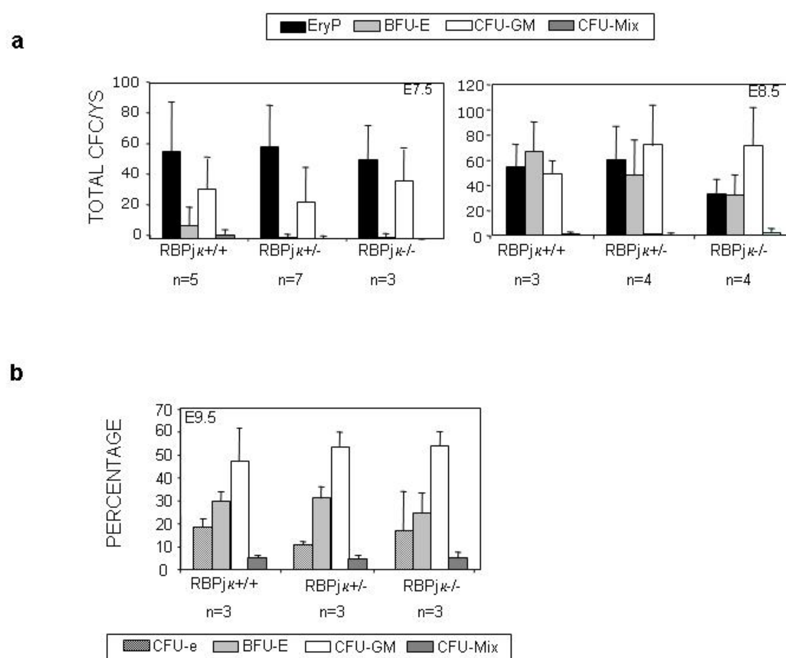
REFERENCES

1. Bray, SJ, (2006) Notch signaling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 7: 678-89.
2. Lai, EC, (2004) Notch signaling: control of cell communication and cell fate. Development 131: 965-73.
3. Kumano, K, Chiba, S, Kunisato, A, Sata, M, Saito, T, Nakagami-Yamaguchi, E et al., (2003) Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. Immunity 18: 699-711.
4. Gering, M and Patient, R, (2005) Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. Dev Cell 8: 389-400.
5. Burns, CE, Traver, D, Mayhall, E, Shepard, JL and Zon, LI, (2005) Hematopoietic stem cell fate is established by the Notch-Runx pathway. Genes Dev 19: 2331-42.
6. Robert-Moreno, A, Espinosa, L, de la Pompa, JL and Bigas, A, (2005) RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. Development 132: 1117-26.
7. Palis, J, Robertson, S, Kennedy, M, Wall, C and Keller, G, (1999) Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. Development 126: 5073-84.
8. Palis, J and Segel, GB, (1998) Developmental biology of erythropoiesis. Blood Rev 12: 106-14.
9. Mulcahy, L, (2001) The erythropoietin receptor. Semin Oncol 28: 19-23.
10. Silva, M, Grillot, D, Benito, A, Richard, C, Nunez, G and Fernandez-Luna, JL, (1996) Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. Blood 88: 1576-82.
11. Motoyama, N, Wang, F, Roth, KA, Sawa, H, Nakayama, K, Nakayama, K et al., (1995) Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science 267: 1506-10.
12. Ganguli, G, Back, J, Sengupta, S and Wasylyk, B, (2002) The p53 tumour suppressor inhibits glucocorticoid-induced proliferation of erythroid progenitors. EMBO Rep 3: 569-74.
13. Peller, S, Frenkel, J, Lapidot, T, Kahn, J, Rahimi-Levene, N, Yona, R et al., (2003) The onset of p53-dependent apoptosis plays a role in terminal differentiation of human normoblasts. Oncogene 22: 4648-55.
14. Weiss, MJ and Orkin, SH, (1995) Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. Proc Natl Acad Sci U S A 92: 9623-7.
15. Ishiko, E, Matsumura, I, Ezoe, S, Gale, K, Ishiko, J, Satoh, Y et al., (2005) Notch signals inhibit the development of erythroid/megakaryocytic cells by suppressing GATA-1 activity through the induction of HES1. J Biol Chem 280: 4929-39.
16. Zweidler-McKay, PA, He, Y, Xu, L, Rodriguez, CG, Karnell, FG, Carpenter, AC et al., (2005) Notch signaling is a potent inducer of growth arrest and apoptosis in a wide range of B-cell malignancies. Blood 106: 3898-906.
17. Romer, S, Saunders, U, Jack, HM and Jehn, BM, (2003) Notch1 enhances B-cell receptor-induced apoptosis in mature activated B cells without affecting cell cycle progression and surface IgM expression. Cell Death Differ 10: 833-44.
18. Jang, MS, Miao, H, Carlesso, N, Shelly, L, Zlobin, A, Darack, N et al., (2004) Notch-1 regulates cell death independently of differentiation in murine erythroleukemia cells through multiple apoptosis and cell cycle pathways. J Cell Physiol 199: 418-33.

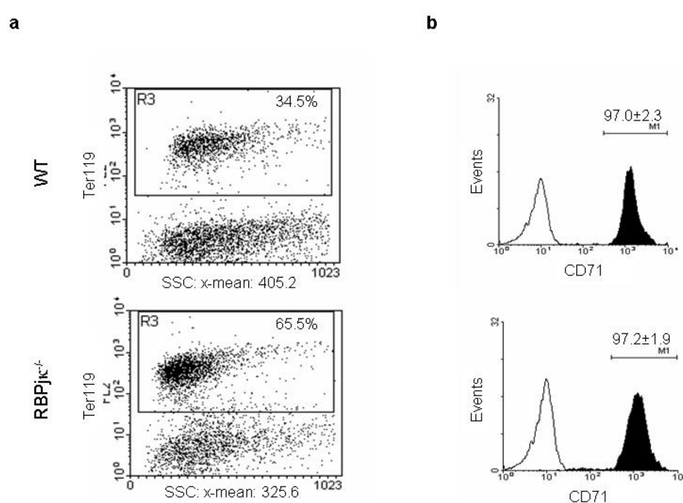
19. Shelly, LL, Fuchs, C and Miele, L, (1999) Notch-1 inhibits apoptosis in murine erythroleukemia cells and is necessary for differentiation induced by hybrid polar compounds. J Cell Biochem 73: 164-75.
20. Oka, C, Nakano, T, Wakeham, A, de la Pompa, JL, Mori, C, Sakai, T et al., (1995) Disruption of the mouse RBP-J kappa gene results in early embryonic death. Development 121: 3291-301.
21. Friend, C, Preisler, HD and Scher, W, (1974) Studies on the control of differentiation of murine virus-induced erythroleukemic cells. Curr Top Dev Biol 8: 81-101.
22. Schroeter, EH, Kisslinger, JA and Kopan, R, (1998) Notch-1 signaling requires ligand-induced proteolytic release of intracellular domain. Nature 393: 382-386.
23. Li, L, Milner, LA, Deng, Y, Iwata, M, Banta, A, Graf, L et al., (1998) The human homolog of rat jagged1 expressed by marrow stroma inhibits differentiation of 32d cells through interaction with notch1. Immunity 8: 43-55.
24. Lam, LT, Ronchini, C, Norton, J, Capobianco, AJ and Bresnick, EH, (2000) Suppression of erythroid but not megakaryocytic differentiation of human K562 erythroleukemic cells by notch-1. J Biol Chem 275: 19676-84.
25. Xue, Y, Gao, X, Lindsell, CE, Norton, CR, Chang, B, Hicks, C et al., (1999) Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. Hum Mol Genet 8: 723-30.
26. Krebs, LT, Xue, Y, Norton, CR, Shutter, JR, Maguire, M, Sundberg, JP et al., (2000) Notch signaling is essential for vascular morphogenesis in mice. Genes Dev 14: 1343-52.
27. Krebs, LT, Shutter, JR, Tanigaki, K, Honjo, T, Stark, KL and Gridley, T, (2004) Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev 18: 2469-73.
28. Loken, MR, Shah, VO, Dattilio, KL and Civin, CI, (1987) Flow cytometric analysis of human bone marrow: I. Normal erythroid development. Blood 69: 255-63.
29. Ihle, JN, Quelle, FW and Miura, O, (1993) Signal transduction through the receptor for erythropoietin. Semin Immunol 5: 375-89.
30. Neubauer, H, Cumano, A, Muller, M, Wu, H, Huffstadt, U and Pfeffer, K, (1998) Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. Cell 93: 397-409.
31. Socolovsky, M, Nam, H, Fleming, MD, Haase, VH, Brugnara, C and Lodish, HF, (2001) Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. Blood 98: 3261-73.
32. Koury, MJ and Bondurant, MC, (1990) Control of red cell production: the roles of programmed cell death (apoptosis) and erythropoietin. Transfusion 30: 673-4.
33. Yu, H, Bauer, B, Lipke, GK, Phillips, RL and Van Zant, G, (1993) Apoptosis and hematopoiesis in murine fetal liver. Blood 81: 373-84.
34. Dolznig, H, Habermann, B, Stangl, K, Deiner, EM, Moriggl, R, Beug, H et al., (2002) Apoptosis protection by the Epo target Bcl-X(L) allows factor-independent differentiation of primary erythroblasts. Curr Biol 12: 1076-85.
35. Motoyama, N, Kimura, T, Takahashi, T, Watanabe, T and Nakano, T, (1999) bcl-x prevents apoptotic cell death of both primitive and definitive erythrocytes at the end of maturation. J Exp Med 189: 1691-8.
36. Yang, X, Klein, R, Tian, X, Cheng, HT, Kopan, R and Shen, J, (2004) Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. Dev Biol 269: 81-94.
37. Huang, Q, Raya, A, DeJesus, P, Chao, SH, Quon, KC, Caldwell, JS et al., (2004) Identification of p53 regulators by genome-wide functional analysis. Proc Natl Acad Sci U S A 101: 3456-61.
38. Maetens, M, Doumont, G, De Clercq, S, Francoz, S, Froment, P, Bellefroid, E et al., (Epub 2006) Distinct Roles of Mdm2 and Mdm4 in Red Cell Production. Blood.
39. Campbell, PJ and Green, AR, (2006) The myeloproliferative disorders. N Engl J Med 355: 2452-66.
40. Miyazato, A, Ueno, S, Ohmine, K, Ueda, M, Yoshida, K, Yamashita, Y et al., (2001) Identification of myelodysplastic syndrome-specific genes by DNA microarray analysis with purified hematopoietic stem cell fraction. Blood 98: 422-7.
41. MacKenzie, F, Duriez, P, Wong, F, Noseda, M and Karsan, A, (2004) Notch4 inhibits endothelial apoptosis via RBP-Jkappa-dependent and -independent pathways. J Biol Chem 279: 11657-63.
42. Jehn, BM, Bielke, W, Pear, WS and Osborne, BA, (1999) Cutting edge: protective effects of notch-1 on TCR-induced apoptosis. J Immunol 162: 635-8.
43. Curry, CL, Reed, LL, Golde, TE, Miele, L, Nickoloff, BJ and Foreman, KE, (2005) Gamma secretase inhibitor blocks Notch activation and induces apoptosis in Kaposi's sarcoma tumor cells. Oncogene 24: 6333-44.
44. Purow, BW, Haque, RM, Noel, MW, Su, Q, Burdick, MJ, Lee, J et al., (2005) Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. Cancer Res 65: 2353-63.
45. Jundt, F, Anagnostopoulos, I, Forster, R, Mathas, S, Stein, H and Dorken, B, (2002) Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. Blood 99: 3398-403.
46. Miller, DT and Cagan, RL, (1998) Local induction of patterning and programmed cell death in the developing Drosophila retina. Development 125: 2327-35.
47. Lundell, MJ, Lee, HK, Perez, E and Chadwell, L, (2003) The regulation of apoptosis by Numb/Notch signaling in the serotonin lineage of Drosophila. Development 130: 4109-21.
48. Ohishi, K, Varnum-Finney, B, Flowers, D, Anasetti, C, Myerson, D and Bernstein, ID, (2000) Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. Blood 95: 2847-54.
49. Qi, R, An, H, Yu, Y, Zhang, M, Liu, S, Xu, H et al., (2003) Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. Cancer Res 63: 8323-9.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

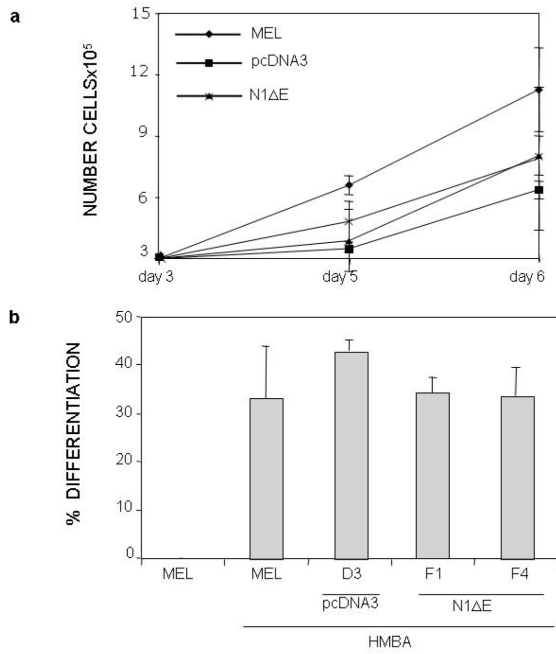
SUPPLEMENTARY INFORMATION



Supplementary Figure S1: *Analysis of myeloid and erythroid progenitors from collagenase-treated yolk sacs. (a) Total CFC/YS at E7.5 and E8.5. (b) Percentage of CFC at E9.5*

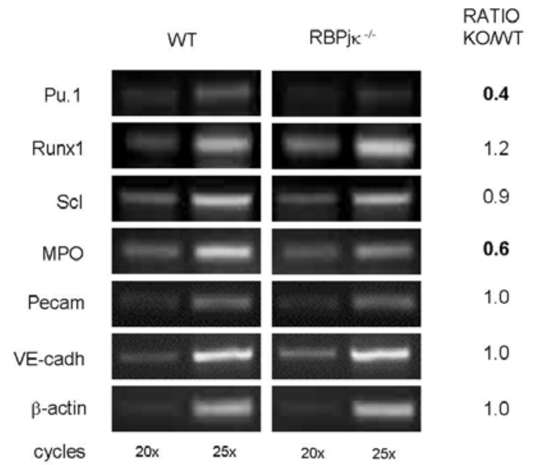


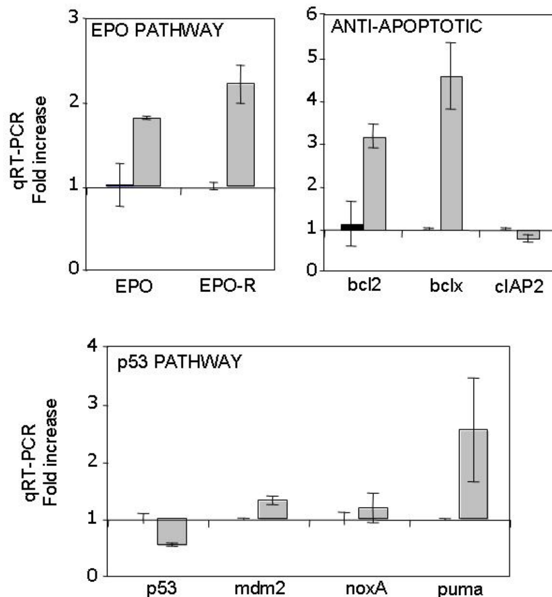
Supplementary Figure S2: *Ter119+ cells from the yolk sac express CD71. (a) Ter119+ cells from WT and mutant yolk sacs. X-mean of SSC is shown for both samples. (b) Percentage of gated Ter119+ cells expressing CD71. Numbers represent the average percentage and standard deviation of 3 different yolk sacs.*



Supplementary Figure S3: *Differentiation and proliferation of pcDNA3 and N1ΔE MEL cells. (a) Cell numbers for MEL, pcDNA3 and N1ΔE clones at day 3, 5 and 6. (b) Percentage of dianisidine positive cells after 6 days in HMBA-differentiating media.*

Supplementary Figure S4: *Semiquanti-tative RT-PCR of hematopoietic and endothelial genes from E9.5 WT and RBPjκ $^{-/-}$ yolk sacs.*





Supplementary Figure S5: *qRT-PCR from sorter Ter119+ cells from YS cultured for 6 days in DMSO and γ–secretase inhibitor L685,458.*

CHAPTER 4:

GENERAL DISCUSSION AND FUTURE PROSPECTS

Hematopoietic stem cells are very important for clinical transplantation and regenerative medicine. This fact has encouraged scientists to study the mechanisms that control self-renewal of HSC and how microenvironmental regulates their behavior. Our work has contributed to clarify the role of Notch in the embryonic hematopoiesis of the yolk sac as well as in the formation of HSC in the AGM, one of the best-characterized niches for generation and amplification of HSC in the embryo. However the precise site where first HSCs are originated in the embryo is still controversial.

The never-ending story: what is the origin of HSCs?

The current knowledge is that two main waves of hematopoiesis take place during the first stages of embryonic development, the primitive and transient hematopoiesis in the yolk sac and the definitive hematopoiesis that originates in the intra-embryonic region of the aorta surrounded by gonad and mesonephros (AGM region) and other major arteries.

Primitive hematopoiesis is responsible for generating primitive nucleated erythrocytes that provide oxygen supply to the growing embryo [reviewed in (McGrath and Palis, 2005)]. Definitive hematopoiesis is responsible for generating the first adult-type HSCs capable of engrafting in the bone marrow of lethally irradiated adults. These HSCs are first found at E10 (de Bruijn et al., 2002; de Bruijn et al., 2000) and by E11 they are detected in other tissues such as fetal liver, yolk sac, placenta and bloodstream (Gekas et al., 2005; Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996; Muller et al., 1994; Ottersbach and Dzierzak, 2005). Nevertheless, the idea that yolk sac contributes to the hematopoiesis in the adult has never been discarded. Indeed, E9 yolk sac and para-aortic splanchnopleura (mesoderm that will form the AGM) fail to engraft in irradiated adult recipients but are able to reconstitute hematopoiesis of myeloablated newborn mice (Yoder and Hiatt, 1997; Yoder et al., 1997; Yoder et al., 1997). These evidences rise the possibility that E9 YS and P-Sp contain immature HSCs that need to undergo further maturation to acquire the ability to home the adult bone marrow. In this sense, experiments done with YS or P-sp before the onset of circulation (E8-E8.5) revealed that these organs contain immature HSCs that become adult-HSCs when co-cultured with the E10.5 AGM-derived cell line AGM-S3 (Matsuoka et al., 2001). Thus, the current model is that the AGM serves as the main site for the generation of adult-type HSCs, but other organs such as the YS and more recently the placenta also contribute to adult hematopoiesis. However, a controversial report recently suggested that yolk sac is the origin of a significant percentage of definitive HSCs (Samokhvalov et al., 2007). Samokhvalov *et al* used a tamoxifen-inducible LacZ labelling technique to follow the cells from E7.5 that express runx1/aml1. The authors report the existence of runx1-marked cells at E7.5, coming from the yolk sac that contribute to the adult HSCs and persist in bone marrow for at least 15 months after birth (Samokhvalov et al., 2007). However, tamoxifen induction is not only achieved in the YS but also in the embryo proper where runx1 was previously described to be expressed as early as E8 (North et al., 1999). Moreover, Samokhvalov studies were done with Runx1$^{+/-}$ embryos where HSCs could be ectopically generated due to runx1 heterozigousity, as previously reported (Cai et al., 2000). Early cell tracking experiments performed in the avian model reinforce the idea that the AGM, but not the extra-embryonic yolk sac, serves as the HSC source. In 1970's, chicken YS and quail embryo proper chimeras were explanted before the vascular establishment and showed for the first time that the intra-embryonic region rather than the YS contributes to the adult hematopoietic compartment (Dieterlen-Lievre, 1975). The ventral endothelium of the aorta is the main source of HSCs in the AGM since cells expressing hematopoietic markers are

generally detected within the endothelial layer (Bertrand et al., 2005; de Bruijn et al., 2002; North et al., 1999; Robert-Moreno et al., 2005). An alternative model, proposes that embryonic hematopoiesis takes place in a structure referred as subaortic patches (SAPs) that are located in the mesenchyme under the floor of the dorsal aorta and express gata2, gata3 and lmo2. HSC in the SAP are $CD45^{low}/ckit^{+}/AA4.1^{+}/flk\text{-}1^{-}$ and are capable of reconstituting hematopoiesis in $Rag2\gamma c^{-/-}$ immunodeficient mice (Bertrand et al., 2005; Manaia et al., 2000). It is hypothesized that intra-embryonic HSCs are originated in the SAP and then migrate to the ventral part of the aorta, cross the endothelium and contribute to the formation of the hematopoietic clusters before they are released to the bloodstream. This possibility is not supported by our results from in situ hybridization since we did not detect any hematopoietic cells in the mesenchyme underlying the ventral wall of the aorta.

On the other hand, heterotopic grafts of quail splanchnopleural mesoderm into chicken embryos revealed that HSCs in the AGM come from mesodermal cells that integrate into the floor of the aorta and only once in this site they generate hematopoietic clusters (Pardanaud et al., 1996). Moreover, hematopoiesis in the AGM aorta ceases when splanchnopleura-derived cells become exhausted and are replaced by somite-derived non-hemogenic angioblasts, suggesting the idea that hemogenic endothelium in the floor of the aorta is a temporary structure of non-renewable hemogenic endothelial cells (Pouget et al., 2006).

Altogether, these results may reflect that the endothelial or mesenchymal origin of HSCs is not irreconciliable and that the integration of all the present data may result in a better understanding of HSC generation.

The aim of our work was to study the role of the Notch signaling pathway in both the primitive and definitive waves of hematopoiesis in the mouse embryo. Our data from the RBPjκ null embryos (Robert-Moreno et al., 2005) and the aorta explant cultures (Chapter 3, Section 2) suggest that Notch signaling is required for the commitment of HSC/HPC from the hemogenic endothelium of the AGM aorta. We propose that Notch activity in the ventral aorta generates a “salt and pepper” pattern that restricts the hemogenic potential to a discrete number of HSCs/HPCs among a majority of aortic endothelial cells, thus reminiscent of the lateral inhibition process described in *Drosophila* neurogenesis (Parks et al., 1997). In this sense, WISH for the different Notch ligands and Notch receptors in early embryos (Supplemental information, Chapter 3 Section 2) agree with this lateral inhibition model since Notch ligand-expressing cells appear earlier in development (at the 14 somite pair stage) than Notch-expressing ones (by the 20 somite pair stage) and only in discrete cells from the ventral part of the aortic endothelium.

Is there only one site of HSCs emergence in the embryo or conversely it is a redundant event that takes place in different tissues throughout fetal development? Our results are in agreement with the idea that primitive and definitive hematopoiesis are the result of two well differentiated genetic programs. RBPjκ null embryos completely lack intra-embryonic hematopoiesis (Robert-Moreno et al., 2005) whereas primitive yolk sac hematopoiesis does occur (Robert-Moreno et al., 2007). The same phenotype was described in null embryos for other Notch pathway molecules such as Notch1 in mice (Kumano et al., 2003) and Mind bomb in zebrafish (Burns et al., 2005). As previosly described in this thesis, the effect of RBPjκ deficiency in the yolk sac is restricted to the homeostasis of the erythroid lineage where prevents apoptosis (Robert-Moreno et al., 2007). However, loss of Notch signaling has a more severe effect in definitive P-sp/AGM hematopoiesis resulting in a complete abrogation of both HSCs and HPCs.

Although we did not assay whether the RBPjκ mutant yolk sacs are able to generate embryonic HSCs, Notch1$^{-/-}$ embryos completely lack E9.5 yolk sac and P-sp HSCs (Kumano et al., 2003) indicating that Notch is required for the generation/commitment of all definitive HSCs.

Notch signaling regulates generation and/or expansion of HSCs in the AGM region

Notch signaling has been reported to regulate developmental cell fate decisions in many systems [reviewed in (Lai, 2004)]. One of them is the arterial/vein decision. In this sense, Notch induces gridlock (the zebrafish orthologue of hrt1) expression in blood vessels to promote arterial specification (Zhong et al., 2001) whereas Notch-inhibition by the transcription factor COUP-TFII results in vein specification (You et al., 2005). Since AGM hematopoiesis occurs only in the main arteries (de Bruijn et al., 2002; de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996) and not in veins, the lack of intra-embryonic hematopoiesis in the Notch1 (Kumano et al., 2003) and RBPjκ mutants (Robert-Moreno et al., 2005) may be due to a defect in aorta specification. For this reason, we used the aorta/AGM explant culture system (Medvinsky and Dzierzak, 1996) to investigate the role of Notch signaling in HSC generation. Indeed repopulation studies performed with E11 wild-type aortas incubated with the γ-secretase inhibitor DAPT revealed a direct role for Notch signaling in the generation and/or expansion of HSCs in the embryo that is independent from aorta specification (Chapter 3; Section 2). In these experiments, we found a decrease in the number of irradiated mice repopulated by DAPT-treated aortas. Three different possibilities may explain these results: ***1)*** Notch inhibition may affect the new HSC generation or ***2)*** it may decrease proliferation of the pre-existing HSC, since this culture system allows HSC expansion (Medvinsky and Dzierzak, 1996) and since Notch activity usually correlates with the maintenance of an undifferentiated state in many systems [reviewed in (Lai, 2004)], ***3)*** it could be that Notch inhibition may be inducing differentiation of HSCs. However, this possibility is not consistent with the lower number of committed hematopoietic progenitors found in CFC assays from γ-secretase treated aortas (Chapter 3; Section 2).

Recently it has been reported that adult bone marrow contains a discrete number of different HSCs each one with its own repopulation kinetics and self-renewal behavior (Sieburg et al., 2006). Our results indicate that DAPT-treated AGM showed similar low repopulation efficiency (HSC with less of 10% engraftment capacity) than untreated AGM (Chapter 3; Section 2). However, the number of animals repopulated with HSCs with more than 10% engraftment capacity was reduced by 4-fold in the case of the γ–secretase treated ones. This result suggests that Notch is required to amplify a hypothetical population of HSC with high repopulating activity. It should be of great importance to study the different signals that regulate the generation and expansion of HSCs in the different hemogenic tissues.

Upstream of the Notch receptor

We have reported that notch1 and 4, together with the jagged1 and 2 and delta4 ligands are expressed in the E9.5-E10.5 aortic endothelium (Robert-Moreno et al., 2005). Notch4 null embryos are viable whereas Notch1/Notch4 double null embryos display more severe vasculogenic defects than the Notch1 single mutants (Conlon et al., 1995; Krebs et al., 2000) indicating a common vascular function for Notch1 and Notch4 receptors. However, expression of Notch family members and their target gene hes1 in the hematopoietic clusters of the AGM is suggestive of a hematopoietic function for Notch signaling (Chapter 3; Section 2). Thus, two different Notch signals may be required in the mid-gestation aortic endothelium, the

first one to establish artery/vein identity and the second one for generating/amplifying the HSC compartment.

Notch1 but not Notch4 is essential for angiogenic vascular remodelling albeit some functional redundancy is found between both receptors (Conlon et al., 1995; Krebs et al., 2000). On the other hand, Notch1 is required for P-sp/AGM hematopoiesis (Kumano et al., 2003) and notch4 (but not notch1) expression is downregulated in E10.5 aortic hematopoietic clusters (Chapter 3, Section 2). Moreover we found that Notch1 (through RBPjκ) associates to the promoter of the hematopoietic transcription factor Gata2 and double in situ hybridization revealed that both genes are coexpressed in the same cells from E10.5 aortic endothelium (Robert-Moreno et al., 2005). All these evidences reinforce the specific role of Notch1 in activating the hematopoietic program in the endothelium of the aorta. Thus, it would be worthwhile to understand how specificity is achieved and which set of genes are activated in each case. One possibility could be that the Notch1 receptor may be activated by a different ligand to specify the vasculogenic or the hematopoietic program. Since Delta4 null embryos die at E9.5 displaying severe vasculogenic defects and loss of endothelial arterial cell fate (Duarte et al., 2004) and lethality occurs even in heterozygosity due to severe vasculogenic malformations (Krebs et al., 2004), presumably activation of Notch1 receptor by Delta4 is the right combination to specify vascular development. However, Notch4 may partially compensate Notch1 defficiency in vasculogenesis.

Double in situ hybridization studies of E10.5 AGM sections revealed that jagged2-expressing cells were mostly located adjacent to the notch1/jagged1 positive cells (Robert-Moreno et al., 2005). Since cell-cell interactions between adjacent cells govern the activation of Notch receptors, the Jagged2 ligand was the best candidate to activate Notch1 and induce the establishment of the hematopoietic fate in the hemogenic endothelium of the AGM region. In order to fully address this issue, we studied whether Jagged1 or Jagged2 null embryos displayed abnormal intra-embryonic hematopoiesis *in vivo*. In fact, when we assayed the hematopoietic potential of both null mutants (by CFC or by counting Sca-1-GFP$^+$ cells), we found that Jagged1 but not Jagged2 mutant embryos had decreased numbers of all types of hematopoietic cells, indicating an important role for Jagged1 in this system (Chapter 3; Section 2). Thus, although Jagged2 expression pattern seem to better explain how Notch could be activated, we have not found any hematopoietic deficiency in the Jagged2 null embryos. One explanation could be that Jagged2 may have a minor hematopoietic role and this deficiency may be compensated by Jagged1. To test this, we are currently studying whether Jag1/Jag2 double deficiency has a more severe hematopoietic phenotype than the Jagged1 knockout embryos.

The decrease in the number of hematopoietic cells found in the Jagged1 mutants may result from a self-renewal or amplification defect of the HSC or more committed progenitors. Repopulation experiments of adult irradiated mice with the Jagged1 mutant aortas are required to answer this question.

We have used the Ly-6A/Sca-GFP mice (de Bruijn et al., 2002) to identify the HSC emerging from the aorta in the different Notch-mutant embryos. Although this is an excellent system for these studies, some issues should be considered when analyzing these mice. Sca-1 (Stem cell antigen-1) is a cell-surface glycoprotein used as a marker for embryonic and adult HSCs (de Bruijn et al., 2002; Miles et al., 1997) although it is also found in HPCs, vascular endothelium and kidney. Thus, although it is well characterized that the GFP$^+$ fraction in the aorta contains the HSC activity (de Bruijn et al., 2002), there is also a few non-hematopoietic endothelial cells expressing GFP. By crossing the Jagged1 and Jagged2 mutant mice with Ly-6A-GFP transgenic mice, we detected a profound decrease in the number of GFP$^+$ cells in the aorta of

Jagged1 but not Jagged2 mutant mice. This is consistent with a hematopoietic defect in these embryos since they also lack expression of the hematopoietic transcription factor Gata2 and show decreased number of CFC. Another possible explanation to this phenotype would be that Notch through Jagged1 controls Sca/Ly-6A gene expression. However Ly-6A transgene expression is found in other tissues (such as the nephrogenic cord tissue) in the Jagged1 null embryos, indicative that Sca/Ly-6A expression is not dependent of the Jagged1/Notch signal, at least in non-hematopoietic tissues.

We have observed that impaired aortic hematopoiesis in the RBPjκ and the Jagged1 null embryos correlates with an increase in the number of endothelial cells and expression of endothelial markers [(Robert-Moreno et al., 2005); Supplemental information, Chapter 3, Section 2)]. These results suggest that in the absence of Notch signaling cells follow an endothelial fate at expenses of a hematopoietic fate. This is in agreement with the idea that the AGM region serves as a site of HSC specification from the endothelium, suggesting that hematopoietic and endothelial lineages share a common progenitor as hypothesized for a long time [reviewed in (Dieterlen-Lievre et al., 2006)].

Although some defects in the yolk sac vasculature and in the branching of the head blood vessels were reported in the $Jag1^{dDSL}$ homozygous embryos no defects in the aorta development have been described neither in the $Jag1^{dDSL}$ nor in the $Jag2^{dDSL}$ null embryos (Jiang et al., 1998; Xue et al., 1999). However, since hematopoietic cells develop in close contact to endothelial cells from the aorta, the arterial fate specification may directly affect the capability of blood formation. We are currently investigating whether Jagged1 null embryos express arterial markers such as EphrinB2 that will be indicative of proper aorta specification.

Characterization of GATA2 as a new Notch target gene in the AGM region.

We have investigated whether different hematopoietic master genes may be Notch targets in the AGM. Although Notch plays many different functions in cell differentiation, not many target genes have been identified. However, some genes have been recently described, including ephrinB2 in heart development (Grego-Bessa et al., 2007), c-myc in T-cell lymphoma cells (Weng et al., 2006) or gata3 and il-4 in T cells (Amsen et al., 2004; Dontje et al., 2006); however only hes1 and hes-related genes are well-characterized Notch-target genes [reviewed in (Iso et al., 2003; Jarriault et al., 1995). Interestingly, most of the recently identified Notch-targets are only dependent on Notch in specific tissues and it is tempting to speculate that this will be a general trait for Notch-target genes. In the aorta, RBPjκ mutant embryos lacked expression of at least three hematopoietic transcription factors (Runx1, Gata2 and Scl) (Robert-Moreno et al., 2005). Although this may be a secondary effect of the hematopoiec defficiency, we investigated whether their expression directly depended on Notch. Chromatin immunoprecipitation of both myeloid progenitors (the 32D cell line) and E9.5 embryos suggested that gata2 was directly regulated by binding of Notch1/RBPjκ to its promoter (Robert-Moreno et al., 2005). Moreover, double in situ hybridization for notch1 and gata2 as well as the specific loss of gata2 expression in the aortic endothelium of E10.5-11 $Jag1^{dDSL}$ null embryos (Chapter 3; Section 2) reinforce the idea that gata2 is a target gene of the Jagged1/Notch1/RBPjκ signaling pathway in mammals (Robert-Moreno et al., 2005). In this sense, Notch also induces serpent (the *Drosophila* orthologue of Gata2) expression leading to the emergence of hemocyte progenitors in the fly lymph gland in *Drosophila* larvae (Mandal et al., 2004). Analysis of Gata2 null embryos and ES cell chimeric mice revealed that this is a clue hematopoietic transcription factor for primitive and definitive hematopoiesis (Tsai et al., 1994). More recently, it has been proposed that Gata2 is required for HSC/HPC generation, proliferation and survival.

Gata2 haploinsufficient embryos display reduced generation of HSCs from the AGM region (Ling et al., 2004), an observation that agrees with our results from the Jagged1 null embryos. Moreover, the bone marrow stem cell compartment of Gata2$^{+/-}$ mice shows a higher apoptotic ratio due to downregulation of the antiapoptotic gene bcl-X_L (Ling et al., 2004; Rodrigues et al., 2005) and Gata2$^{-/-}$ multipotential progenitors proliferate poorly and display massive cell death further indicating that Gata2 is required for proliferation and survival of early progenitors (Tsai and Orkin, 1997). Thus we propose that Jagged1/Notch1/RBPjκ-induced activation of gata2 is required for generation, proliferation and/or survival of HSC/HPC from the hemogenic endothelium of the AGM region. However, Jagged1 mutant embryos show reduced but not complete lack of hematopoiesis suggesting that HSC specification may occur in the absence of Jagged1 and Gata2.

The hematopoietic transcription factor Runx1/Aml1 is also required for the generation of HSCs from the aortic endothelium since Runx1$^{-/-}$ embryos completely lack definitive hematopoiesis (North et al., 1999; Okuda et al., 1996). Runx1 has been recently described to act downstream of Notch in the establishment of the hematopoietic stem cell fate in the zebrafish embryo (Burns et al., 2005). Consistently, in *Drosophila*, hemocytogenesis occurs through Notch-induced activation of lozenge, the fly orthologue of Runx1 (Lebestky et al., 2003). In addition, ectopic expression of runx1 (but not gata2 or scl) in the AGM-derived cells from Notch1 null embryos partially rescues the hematopoietic defect *in vitro* (Nakagawa et al., 2006). Although there is compiling evidence that Notch-Runx1 is also required for the initiation of the hematopoietic program in the AGM region, we did not detect the runx1 gene in the chromatin precipitated with anti-Notch1 antibody from E9.5 embryos (Robert-Moreno et al., 2005). Moreover, we have observed runx1 expression in 50% of the Jagged1 null embryos. One possible explanation is that activation of Notch through different ligands specifically activates runx1 or gata2 in specific populations of HSC and at different time points. However, since runx1 infected Notch1 null P-sp cells are unable to repopulate the hematopoiesis of busulfan compromised newborn mice (Nakagawa et al., 2006) it seems that both Runx1 and Gata2 transcription factors act synergistically to determine or expand the pool of HSCs from the AGM region.

We have also investigated whether scl/tal-1 was a Notch-target. Although we were not able to detect Notch1 binding to the scl promoter in our ChIP assays, scl was upregulated in the 32D cell line overexpressing the intracellular domain of Notch (Robert-Moreno et al., 2005). Gata2 upregulates scl in the BL-CFC population (Lugus et al., 2007) and scl is also transcriptionally increased after a brief pulse of Notch1IC in the zebrafish embryo (Burns et al., 2005). These evidences together with our results suggest that Scl could be acting downstream of Gata2.

We have also described expression of the classical Notch target genes hes1, hrt1 and hrt2 in the mid-gestation AGM aorta [(Robert-Moreno et al., 2005) and Chapter 3, Section 2)]. Both Hes and Hrt are basic helix-loop-helix proteins (bHLH) and they are reported to act as transcriptional repressors, usually leading to an inhibition of differentiation [reviewed in (Iso et al., 2003)]. Some hrt1 and hrt2 expression was found in E10.5 hematopoietic aortic clusters (data not shown) but the fact that Hrt1/Hrt2 double mutants show severe vascular remodelling defects and dorsal aorta lacks EphrinB2 expression and display the vein marker EphB4 (Fischer et al., 2004) suggests that Hrt proteins may be related to arterial specification rather than hematopoiesis.

On the other hand, we show hes1 expression not only in the endothelium lining the dorsal aorta but also in the E10.5 aortic hematopoietic clusters [(Robert-Moreno et al., 2005) and Chapter 3, Section 2] which suggest a role for this Notch target gene in this system. There is some evidence for a role of Hes1 inhibiting

the differentiation and maintaining the stemness of HSCs (Kunisato et al., 2003), thus one possibility is that Jagged1/Notch1/RBPjκ would activate gata2 and hes1 expression in a subset of HSCs with high repopulating ability in the AGM region (Figure1). Gata2 may regulate proliferation of HSCs, in agreement with the fact that Jagged1 mutant embryos contain a reduced number of hematopoietic progenitors, whereas Hes1 may be involved in the maintenance of the stem cell state.

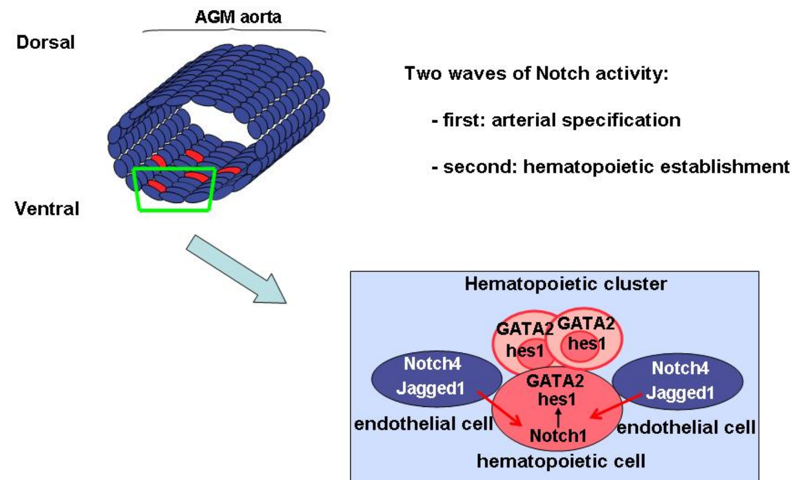


Figure 1: Model for Notch signaling implication in definitive hematopoiesis

Pleiotropy and context specificity of Notch in hematopoiesis

It is widely demonstrated that Notch signaling regulates many processes during embryonic and adult hematopoiesis. However, the high complexity of the Notch pathway itself, with a wide variety of ligands, receptors and intra and extracellular modulators leads to a huge diversity of responses depending on the tissue or cell context. For example, Notch activation inhibits differentiation in the majority of tissues [reviewed in (Artavanis-Tsakonas et al., 1999)] however, in keratinocytes Notch activation promotes terminal differentiation (Rangarajan et al., 2001). The mechanisms leading to Notch specificity may include 1) different Notch receptors (Cheng et al., 2007), 2) accesibility of Notch-presenting cell to different Notch ligands due to specific expression patterns (Robert-Moreno et al., 2005) or 3) ligand modifications through fringe glycosyltransferases (Hicks et al., 2000; Moloney et al., 2000), that would induce different gene expression programs.

The role of Notch in the hematopoietic system cannot be explained without considering the idea of context specificity. Notch activation effects are different in each hematopoietic lineage. During embryonic development, the generation of primitive and definitive hematopoiesis seems to have completely different Notch requirements. From the study of Notch1 and RBPjκ null mutants, others and we have concluded that Notch signaling is required for definitive hematopoiesis but dispensable for the primitive hematopoiesis in the yolk sac (Burns et al., 2005; Kumano et al., 2003; Robert-Moreno et al., 2005).

However, in the adult it is not so clear whether Notch is required for HSC self-renewal/maintenance. Whereas notch1IC retrovirally infected in bone marrow cells, expands the number of repopulating HSCs (Stier et al., 2002) and a dominant negative form of RBPjκ promotes their differentiation (Duncan et al., 2005), adult mice carrying an inducible inactivation of Notch1 only display lymphoid defects (Radtke et al.,

1999). Similarly, Jagged1 (Mancini et al., 2005) and RBPjκ (Han et al., 2002) are dispensable for HSC self-renewal and repopulation ability in the adult bone marrow in constrast with our results in AGM hematopoiesis. These results indicate that Notch may be specifically required for the generation of HSCs during embryonic life.

On the other hand, the effect of Notch in the regulation of different cell-fate decisions within the lymphoid lineage has extensively been demonstrated. Conditional inactivation of Notch1 (Radtke et al., 1999) or RBPjκ (Han et al., 2002) in bone marrow progenitors lead to a depletion of T cells because the common lymphoid progenitors commit to the B-cell fate. Moreover, a requirement of Notch signaling for the generation of $\alpha\beta$ T cells at expenses of $\gamma\delta$ T cells (Tanigaki et al., 2004) and $CD8^+$ cells at expenses of $CD4^+$ cells has been also described (Robey et al., 1996).

We have recently described that Notch/RBPjκ signaling induces apoptosis in the erythroid lineage of the yolk sac most likely by downregulating the expression of molecules from the Epo/EpoR survival erythroid pathway (Robert-Moreno et al., 2007). Binding of EPO to its receptor induces activation of Jak2 tyrosine kinase and the downstream effectors PI3-kinase/AKT, STAT5-bcl-X_L and ERK/MAPK leading to erythroid progenitor expansion, terminal erythroid maturation and apoptosis protection [reviewed in (Testa, 2004)]. Another important effector of EPO signaling is GATA1 that leads to bcl-X_L overexpression (Gregory et al., 1999). However we did not detect GATA1 overexpression in the RBPjκ null yolk sacs although we cannot exclude that this regulation could be at the post-translational level since we found upregulation of bcl-X_L. Moreover, it was previously published that Notch induces apoptosis in the human erythroleukemic cell line K562 through Hes1 repression of GATA1 leading to downregulation of bcl-X_L (Ishiko et al., 2005).

Another possibility is that the p53 tumor suppressor may be inducing apoptosis in primitive erythroid cells. The Notch target genes hes1 and hey1 were found to transcriptionally repress the p53 inhibitor hdm2 in a genome-wide screening (Huang et al., 2004). In agreement with this, Notch1-induced apoptosis in early neural progenitor cells was suppressed in a $p53^{-/-}$ background (Yang et al., 2004). Moreover, conditional deletion of mdm2 and mdm4 in $EpoR^+$ erythroid cells revealed that Mdm2 is crucial for inhibiting p53-induced apoptosis of yolk sac erythrocytes (Maetens et al., 2007). Although we did not find significant differences in the p53 or mdm2 levels and of p53-dependent gene transcription (bax and noxa (albeit puma) in RBPjκ null yolk sacs compared to wild-types, p53 phosphorylation should be assayed to further discard that Notch1 exerts its proapoptotic function through p53 [reviewed in (Chipuk and Green, 2006)].

Notch induction of apoptosis has previously been described in monocytes (Ohishi et al., 2000) and B-cell lymphomas (Zweidler-McKay et al., 2005), and opposite effects has been found in T cells (Jehn et al., 1999) indicating, once again, that Notch function is lineage and context-dependent. However, some of the reported observations come from cell lines and cultured systems and should be cautiously considered. However, our results have been generated from RBPjκ-deficient erythrocytes directly obtained from the yolk sac. The fact that this is an *in vivo* system, strongly suggest that Notch may induce apoptosis in this lineage and contribute to the homeostasis of erythroid cells.

In summary, Notch signaling is crucial not only in the generation/expansion of HSCs in the embryo, but also in differentiation and programmed cell death in many of the committed hematopoietic lineages derived from these HSCs. As a consequence, the fine tuned homeostasis of the hematopoietic system is reached.

CHAPTER 5:

CONCLUSIONS

CONCLUSIONS

In this thesis we have achieved the following conclusions:

1. Loss of the nuclear Notch pathway effector RBPjκ leads to a complete abrogation of intra-embryonic hematopoiesis, concomitantly with a lack of the hematopoietic transcription factors Gata2, Runx1 and Scl expression in the aortic endothelium.

2. Notch1 through RBPjκ directly binds to the Gata2 promoter and regulates its expression in the 32D cell line and in the mid-gestation embryo.

3. Loss of Jagged1 results in impaired expression of Gata2, reduced hematopoietic progenitor generation and Sca-1^{+} cells in the AGM region.

4. Inhibition of Notch activation in wild-type aortas by γ-secretase inhibitors diminishes the number of HSCs from the AGM, thus resembling the phenotype described for the Notch, RBPjκ and Jagged1^{dDSL} mutants.

5. Notch signaling is required to generate and/or expand a pool of high repopulating HSCs from the AGM region.

6. Lack of intra-embryonic AGM hematopoiesis correlates with an increase of the endothelial layer in the aorta of the RBPj$\kappa^{-/-}$ and Jagged1^{dDSL} embryos, suggesting that a common progenitor for the hematopoietic and endothelial lineages (or hemangioblast) does exist in the AGM aorta.

7. Yolk sac hematopoietic cells develop normally in RBPjκ deficient embryos with and increased number of erythropoietic cells.

8. Notch signaling regulates erythroid homeostasis in the yolk sac by inducing apoptosis.

9. The apoptotic protection in the erythroid lineage of RBPjκ null yolk sacs is due to the upregulation of Epo/Epo receptor pathway members leading to the overexpression of the antiapoptotic genes bcl-2 and bcl-X_L.

CHAPTER 6:
RESUM DE LA TESI

“Paper de Notch i RBPjκ en l’hemopoesi embrionària”

6.1 INTRODUCCIÓ GENERAL

6.1.1 EL SISTEMA HEMOPOÈTIC

6.1.1.1 Introducció al sistema hemopoètic

El sistema hemopoètic va aparèixer al llarg de l'evolució amb la finalitat d'assegurar l'arribada de nutrients i la protecció en contra de perills externs en organismes multicel.lulars. La sang està composada d'una gran varietat de tipus cel.lulars madurs amb una vida mitja limitada (dos dies pels neutròfils, trenta dies pels eritròcits) i per tant les cèl.lules sanguínies necessiten constantment ser formades a partir d'un grup de cèl.lules mare hemopoètiques (hematopoietic stem cells o HSCs en anglès). Aquest procès és conegut amb el nom d'hemopoesi (revisat a Godin i Cumano, 2002). Els diferents tipus cel.lulars sanguinis compleixen diferents funcions. Així, els eritròcits estan especialitzats en l'intercanvi gasós entre els diferents teixits (proveïnt d'oxigen i eliminant el diòxid de carboni), mentre que les plaquetes són responsables de processos de coagulació sanguínia en ferides. Finalment, altres tipus cel.lulars com macròfags, neutròfils, eosinòfils, basòfils i cèl.lules natural killer pertanyen al sistema immunitari innat, mentre que limfòcits B i T i limfòcits T citotòxics són responsables de la resposta immune adaptativa en front de patògens (revisat a Godin i Cumano, 2002).

En l'adult l'organ hemopoètic principal és el moll de l'òs (Orkin, 2000) tot i que l'hemopoesi també té lloc en la melsa i timus (responsables de la maduració final de cèl.lules B i T, respectivament) i en els nòduls limfàtics. Tot i això, durant el desenvolupament embrionari, l'òrgan principal hemopoètic va canviant d'un a altre òrgan d'una forma dinàmica en temps i en espai, com a continuació veurem.

Durant molt de temps es va pensar que la jerarquia en el sistema hemopoètic consistia en una cascada de decisions binàries resumides en el model Akashi-Kondo-Weissman (Chapter1, Figura 1A) en el qual un nombre limitat de HSC a la base d'aquesta jerarquia hemopoètica tindrien l'habilitat de proliferar i diferenciar-se cap al progenitor comú limfoide (CLP en anglès) o el progenitor comú mieloide (CMP) (Akashi, 2000; Kondo, 1997). Aquests progenitors donarien lloc a progenitors més determinats a cada llinatge en concret i finalment a cèl.lules sanguínies madures. No obstant, actualment es creu que això no seria tan senzill i que progenitors hemopoètics ja determinats a un llinatge en concret podrien transdiferenciar-se cap a altres llinatges hemopoètics, suggerint un arbre jeràrquic hemopoètic més complicat (Chapter1, Figura 1B) (Adolfsson, 2005).

Definició de cèl.lula mare hemopoètica (HSC)

Les cèl.lules mare poden distingir-se entre cèl.lules mare embrionàries o cèl.lules mare adultes o somàtiques. Les cèl.lules mare embrionàries (ESC en anglès) deriven d'embrions i són totipotents, és a dir, tenen la capacitat de generar tots els tipus cel.lulars i teixits presents en un animal adult. En canvi, les cèl.lules mare adultes, estan localitzades en determinats òrgans del cos i tenen la funció d'anar generant tipus cel.lulars específics de cada teixit (revisat en Graf, 2002).

Per tant, les cèl.lules mare hemopoètiques són cèl.lules mare adultes que donen lloc a tots els tipus cel.lulars sanguinis en l'organisme (Orkin, 2002). Tot i que en l'adult es localitzen en el moll de l'òs, es creu que entre dia 8.5 i dia 13 del desenvolupament embrionari en ratolí es generen entre 500 i 1000 HSCs

principalment en l'artèria aorta rodejada per la gònada i el mesonefros (una regió anomenada AGM). Les HSCs representen una població cel.lular pluripotent i quiescent amb la capacitat tant d'autoregenerar-se (proliferar) com de repoblar tot el sistema hemopoètic quan es transplanten en ratolins adults irradiats. Seria en el moll de l'òs i concretament en els nínxols de cèl.lules mare, on les HSCs rebrien senyals microambientals que farien que proliferessin (per divisió simètrica o assimètrica: Chapter1, Figura 2) o es diferenciessin cap a determinats llinatges sanguinis (Cheshier, 1999).

Actualment, la tècnica més utilitzada per a separar HSCs és per citometria de fluxe, basat en que les HSCs expressen determinats antígens de superfície cel.lular (Chapter1, Figura3). Així, en ratolí, les HSCs adultes expressen Sca-1, nivells elevats de c-Kit i són negatives pels marcadors específics de llinatge (Lin^-). Aquesta població $c\text{-}Kit^+Sca\text{-}1^+Lin^-$ o KSL està enriquida en cèl.lules mare repobladores a llarg termini (LTR-HSCs) i a curt termini (STR-HSCs) tot i que també conté progenitors multipotents sense habilitat repobladora (Osawa, 1996). Altres marcadors de superfície que s'utilitzen per aïllar HSCs són Thy, Flt3, CD34 i CD38 (Zhao, 2000). En canvi, les HSCs murines embrionàries expressen c-Kit, CD41 i AA4.1 (Bertrand, 2005), nivells baixos de Sca-1 (de Bruijn, 2002) a més de marcadors de tipus endotelial com CD31/PECAM (Baumann, 2004) i VE-cadherina (Kim, 2005).

Regulació del sistema hemopoètic

Tant l'interacció d'una cèl.lula amb d'altres cèl.lules, com l'interacció també amb el seu entorn regulen el desenvolupament de les cèl.lules hemopoètiques, ja sigui per interaccions directes cèl.lula-cèl.lula com per factors secretats com són les citoquines o els factors de creixement. En conjunt, tots aquests senyals que rep una cèl.lula determinada caracteritzen el nínxol de les cèl.lules mare hemopoètiques.

Com s'ha dit, el moll dels principals ossos llargs és l'òrgan hemopoètic més important durant la vida adulta. Les cèl.lules sanguínies són retingudes en aquest òrgan fins que adquireixen l'estat de maduració adequat i aleshores són alliberades al torrent sanguini. Tant les HSCs com els progenitors hemopoètics estan rodejats per l'estroma del moll de l'òs format per condròcits, cèl.lules endotelials, fibroblastes i osteoblastes a més de la matriu extracel.lular que produeixen (revisat a Dazzi, 2006). Tots aquests elements formen **el nínxol** de les cèl.lules mare hemopoètiques i ofereixen un microambient adequat per a que les HSCs proliferin o bé es diferencïin en la seva progènie. Entre els lligands i els seus corresponents receptors que medien la interacció entre les HSCs i el seu nínxol s'inclou les vies de senyalització a través de Notch (revisat en Li i Li, 2006), Stem Cell Factor (SCF)/c-Kit (revisat a Linnenkin, 1999), Wnt (Reya, 2003), basic Fibroblast Growth Factor o Hedgehog (revisat a Yin i Li, 2006). Per exemple, el receptor Notch es trobaria expressat en HSCs mentre que el seu lligand Jagged1 s'expressaria en osteoblastes i cèl.lules estromals del moll de l'òs, suggerint que Notch activat induïria la proliferació de HSCs i progenitors hemopoètics (Varnum-Finney, 2000).

Moltes són les **citoquines i factors de creixement** responsables de regular la supervivència cel.lular, la proliferació i la diferenciació de les HSCs, mitjançant la interacció amb els seus receptors específics (resumits a la Taula1, Chapter 1). Entre ells, s'inclouria l'interleukina 1 (IL-1) que activa cèl.lules T; IL-2, que promouria la proliferació de cèl.lules B i T activades per antigen; IL-4, IL-5, IL-6 i IL-7 estimularien la proliferació i diferenciació de limfòcits B; l'Interferó-gamma (IFNγ) que activaria macròfags; i IL-3, GM-CSF, G-CSF o Flt3 que estimularien el llinatge mieloide. Pel que fa a les citoquines que regulen l'hemopoesi en

l'embrió, tals com SCF, bFGF, Vascular Endothelial Growth Factor (VEGF) o IL-3 s'explicaran més endavant.

Molts són els **factors de transcripció** que s'han identificat per la seva habilitat de controlar programes genètics específics per cada llinatge hemopoètic i alguns d'ells es troben resumits a la Taula 2, Chapter 1. Així per exemple Pu.1 s'ha vist que està implicat específicament en la diferenciació mieloide i limfoide (McKercher, 1996), mentre que Ikaros es troba expressat tant en HSCs com en progenitors limfoides (Klug, 1998). Pel que fa a C/EBPα, se sap que està implicat en la diferenciació de granulòcits ja que ratolins mutants per aquesta proteïna careixen totalment de neutròfils i eosinòfils (Zhang, 1997) i es creu que també regularia negativament la proliferació de les HSCs (Zhang, 2004). Els factors de transcripció E2A i EBF, en canvi, són necessaris per la limfopoesi de cèl.lules B (Bain, 1994), mentre que Pax5 induïria la diferenciació cap a llinatge limfoide de cèl.lula B a expenses de cèl.lula T, a través de la inhibició de Notch (Souabni, 2002). Finalment, Notch seria el principal inductor de la diferenciació cap a limfòcits T a través de l'activació del gen diana GATA3 (Dontje, 2006), mentre que Ikaros, Pu.1 i Id-2 actuarien en la generació tant de cèl.lules natural killer com de cèl.lules dendrítiques (revisat a Laiosa, 2006).

Recentment s'ha suggerit que RNAs petits i no-codificants podrien estar regulant molts processos cel.lulars tant en animals com en plantes. Aquest grup de reguladors, anomenat RNAs d'interferència (RNAi) estaria format pels RNA d'interferència petits (siRNAs) i pels micro (mi)RNAs. En els darrers anys s'ha descrit alguns exemples de **microRNAs** que regularien diferents processos hemopoètics. Així s'ha vist que l'expressió ectòpica de miR-221 i miR-222 (que inhibirien específicament c-Kit) evitaria la proliferació i induïria la diferenciació de progenitors humans de cordó umbilical $CD34^+$ (Felli, 2005). En canvi, miR-223, que seria activat per C/EBPα, induïria la diferenciació granulocítica a través de la repressió del factor de transcripció NF1-A (Fazzi, 2005).

Finalment, es creu que la **transdiferenciació** (la diferenciació de HSCs en altres tipus cel.lulars no hemopoètics tals com miòcits, neurones, hepatòcits o cèl.lules endotelials), com la **reprogramació** (la diferenciació d'un progenitor hemopoètic d'un llinatge en cèl.lules madures d'un altre llinatge sanguini), podrien regular l'hemopoesi, encara que aquesta hipòtesi genera encara força controvèrsia.

Metodologia per a l'estudi de HSCs i progenitors hemopoètics

La classificació de les HSCs i els diferents progenitors hemopoètics es basa en l'habilitat de repoblar a llarg termini el sistema hemopoètic de ratolins irradiats o químicament immunosuprimits (LTR-HSCs), generar colònies macroscòpiques en la melsa de ratolins adults irradiats (STR-HSCs i/o CFU-S) o formar colònies hemopoètiques *in vitro* (progenitors hemopoètics determinats a llinatge o cèl.lules formadores de colònies-CFCs) (revisat a Dzierzak i Medvinsky, 1995).

Els **assajos de repoblació multi-llinatge a curt i llarg termini** permeten la detecció de LTR-HSCs, el progenitor més indiferenciat i capaç de donar a lloc tot el sistema hemopoètic sencer (Müller, 1994). Consisteix en injectar intravenosament les HSCs en ratolins en els que s'ha deplecionat el seu sistema hemopoètic mitjançant irradiació. Un i quatre mesos després del transplantament, la contribució de les STR-HSCs i LTR-HSCs respectivament del donant es detecta per la presència del marcador específic del donant en la sang perifèrica del receptor (Müller, 1994). Una varietat consisteix en deplecionar l'hemopoesi del receptor amb un agent quimioterapèutic anomenat busulfan i que s'usa en el cas que es vulgui transplantar

en ratolins nou-nats per a detectar HSCs molt immadures i que no es poden detectar per transplantament en adults irradiats.

Per a detectar progenitors multipotents es fa servir **l'assaig de colònies en melsa (CFU-S assay)** que consisteix en injectar els progenitors en ratolins adults irradiats i al cap de 8-16 dies apareixen colònies macroscòpiques a la melsa (Medvinsky, 1996) o **l'assaig de cèl.lules formadores de colònies amb gran potential proliferatiu (HPP-CFC)** que formen macrocolònies en agar en presència de les citoquines adequades (Bertoncello, 1992).

El **cultiu d'unitats formadores de colònies (CFU-C)** permet la detecció de progenitors diferenciats quan es cultiven en metilcel.lulosa i les citoquines adequades. Després de 5-14 dies es formen colònies de cèl.lules madures que es poden distingir fàcilment al microscopi invertit. Entre els progenitors que es poden reconèixer es troba el progenitor eritroide més indiferenciat (BFU-e), el progenitor eritroide més madur (CFU-e), el progenitor de megacariòcits (CFU-Meg), el de macròfags (Mac-CFC), el de granulòcits (G-CFC), el de macròfags i granulòcits (GM-CFC) i el progenitor de cèl.lula eritroide i mieloide (Mix-CFC). Alguns d'aquests progenitors es troben a la Figura4 Chapter1. En canvi, per a detectar progenitors limfoides es requereixen condicions més especials per la seva supervivència i diferenciació. Així per cultivar progenitors de limfòcits B es requereix la línia cel.lular d'estroma S17 (Fluckiger, 1998) mentre que per a detectar progenitors de limfòcits T es requereix el cultiu de timus fetal (revisat a Hare, 1999) o la línia cel.lular d'estroma OP9 que sobreexpressa el lligand de Notch, Delta1 (Schmitt, 2002; Schmitt, 2004).

També són àmpliament utilitzades les **cèl.lules mare embrionàries (ES)** per la seva capacitat d'autoregenerar-se i diferenciar-se en teixits adults. Les cèl.lules ES, en absència de la citoquina LIF generen agregats de cèl.lules diferenciades anomenats embryoid bodies, que recapitulen l'hemopoesi embrionària *in vitro* (Keller, 1993).

Finalment, s'ha establert moltes **línies cel.lulars** que s'utilitzen com a eina per a estudiar la regulació del sistema hemopoètic. Entre elles, destaquen la lína cel.lular OP9 (provinent del moll d'òs de ratolins nou-nats deficients per M-CSF) o la línia AGM-S3 provinent de la regió AGM d'embrions murins a dia 10.5 de gestació, a més de les esmentades S17 i OP9-Delta1 utilitzades per a diferenciar progenitors de limfòcits B i T respectivament. La línia cel.lular 32D cultivada en presència de IL3 es manté com a progenitors indiferenciats mentre que amb G-CSF s'indueix la diferenciació granulocítica. Finalment la línia cel.lular d'eritroleucèmia murina (MEL) són proeritroblastes provinents de cèl.lules de melsa transformades i que es poden diferenciar *in vitro* en presència d'hexametilè bisacetamida (HMBA) (revisat a Marks, 1978).

6.1.1.2 Ontogènia del sistema hemopoètic en l'embrió de ratolí

Breu introducció al desenvolupament del sistema hemopoètic

El sac vitel.lí (en anglès yolk sac; YS) extraembrionari és una estructura que embolcalla l'embrió i que a dia 7 de desenvolupament (E7) forma unes estructures anomenades illes sanguínies (en anglès blood islands; BI), responsables de la primera onada d'hemopoesi o hemopoesi primitiva (Silver, 1997). Durant l'estadi de 6-8 parells de somites (E8-8.5), l'embrió murí sofreix el procès de rotació axial en el qual adquireix la posició fetal característica i inmediatament després comença la segona onada d'hemopoesi embrionària o hemopoesi definitiva. A E8.5 s'estableix la circulació entre embrió i sac vitel.lí a través de les

artèries vitelines i per tant cèl.lules del sac es troben en l'embrió. A dia 9, el mesoderm intraembrionari de l'esplancnopleura para-aòrtica (P-sp) dóna lloc a una única aorta fusionada rodejada de teixit gonadal i mesonefros (AGM). És en aquesta regió on es generen les primeres HSCs entre dia E9 i E12 (Godin, 1995; Medvinsky and Dzierzak, 1996), tot i que ja a dia E8.5, regions del mesoderm intraembrionari ja generen cèl.lules hemopoètiques (Cumano, 1996). Aquestes HSCs i altres progenitors es generen de la part ventral de l'aorta dorsal (Garcia-Porrero, 1995) tot i que també d'altres vasos majors com les artèries vitel.lines i umbilicals (de Bruijn, 2000).

A dia 11, el fetge fetal comença a ser l'òrgan hemopoètic principal, sent col.lonitzat per les HSCs que s'han generat prèviament en altres òrgans de l'embrió i iniciant tant la diferenciació com l'expansió de les HSCs. La col.lonització del timus fetal (òrgan de diferenciació de cèl.lules T) i la melsa (de cèl.lules B) per part de les HSCs ocorre a dia E12 (Godin, 1999). Cap a finals de la gestació (E15-16) tant el fetge com la melsa deixen de ser els òrgans principals i és el moll de l'òs qui és col.lonitzat per les HSCs i acabarà sent el principal òrgan hemopoètic en l'adult (Metcalf i Moore, 1971). A la Figura 6, Chapter 1 es pot veure una il.lustració de tot aquest procés embrionari.

Hemopoesi primitiva

El sac vitel.lí està format per una capa de cèl.lules de mesoderm extraembrionari íntimament associat a una altra capa de cèl.lules d'endoderm visceral. Es creu que és aquest endoderm visceral la font de senyals inductives per la generació de cèl.lules sanguínies (Belaousoff, 1999) i la red endotelial del sac (Palis, 1995). Les primeres cèl.lules sanguínies de l'embrió deriven del mesoderm dins de les illes sanguínies. Aquestes estructures apareixen en l'embrió de ratolí a E7 com a masses de cèl.lules mesodermals que acaben diferenciant-se en una capa de cèl.lules endotelials que rodeja la resta de cèl.lules, les quals perden progressivament les seves unions intercel.lulars i es diferencien bàsicament en cèl.lules eritroides primitives (Chapter1, Figura 7).

A partir de dia E7.5 el sac vitel.lí genera eritròcits primitius madurs caracteritzats per la presència de nucli i l'expressió de globines embrionàries (ε i βH1) i que difereixen dels eritròcits definitius anucleats i que expressen globines adultes (β-globin) (revisat a Palis i Segel, 1998). A més també genera macròfags i megacariòcits lleugerament diferents que els presents en l'adult. Entre E7 i E9, el sac vitel.lí dóna lloc a progenitors eritroides primitius (EryP) que es distingeixen en assajos de CFU-C perquè generen colònies d'un centenar de cèl.lules eritroides nucleades. A partir de dia 9, es poden trobar progenitors hemopoètics definitius (BFU-e, CFU-e, CFU-GM i CFU-Mix). No obstant, els eritròcits madurs definitius no es troben fins a dia 12 que és quan el fetge fetal és l'òrgan hemopoètic principal.

Els progenitors multipotents apareixen en el YS a E8.5 detectats com a HPP-CFCs (Palis, 2001) i a dia 9.5 com a CFU-S (Medvinsky, 1993). Finalment, encara hi ha controvèrsia sobre si el sac vitel.lí té potencial limfoide, ja que no s'ha trobat encara cap evidència de que en tingui (Yokota, 2006).

Hemopoesi definitiva

L'hemopoesi definitiva s'origina en la regió intraembrionària de l'AGM (Medvinsky i Dzierzak, 1996). Aquesta regió s'extén des dels primordis de pota anteriors als posteriors i prové de la capa de mesoderm germinal que acaba originant l'aorta dorsal, les crestes genitals (que formaran les gònades) i el mesonefros (teixit embrionari que formarà el ronyó). L'aorta dorsal conecta a dia 8.5 amb els vasos vitel.lins del sac

embrionari i simultàneament amb l'artèria umbilical, que conecta l'aorta amb la placenta (Chapter 1, Figura 8).

El mesoderm de l'esplancnopleura para-aòrtica (teixit que dóna lloc a l'AGM) conté activitat progenitora de limfòcits B i T a dia 8-9 (Godin, 1993) i activitat CFU-S a dia 9 (Medvinsky, 1996). No obstant, les HSCs adultes (capaces de repoblar l'hemopoesi d'adults irradiats) no es troben fins a dia 10 (Müller, 1994). Tot i que el sac vitel.lí i la P-sp a dia 9-10 no són capaços de repoblar l'hemopoesi d'adults irradiats, aquests teixits sí que poden repoblar a llarg termini l'hemopoesi quan s'injecten al fetge de nou-nats deplecionats químicament (Yoder, 1997). Aquests experiments suggereixen que el YS i la P-sp contenen pre-HSCs immadures, que requereixen el microambient apropiat que els hi dóna el fetge dels nou-nats per a esdevenir HSCs madures.

Recentment, s'ha proposat que una tercera font de HSCs en l'embrió podria ser la placenta (Ottersbach and Dzierzak, 2005; Gekas, 2005). S'ha descrit activitat generadora de HSCs en la placenta a dia E10.5-11, per tant al mateix temps que en l'AGM, però seria possible que aquestes HSCs vinguessin d'altres teixits i que la placenta actués només com un lloc de maduració o d'expansió d'aquestes HSCs. No obstant, tots aquests resultats reflecteixen que l'origen de les HSCs en l'embrió encara és una qüestió no resolta i suggereix que les HSCs podrien ésser formades en algún d'aquests llocs i podrien migrar cap a altres teixits, o que en canvi, es generen simultàniament en diferents òrgans que deriven del mesoderm. El que sí és comú per a tots aquests llocs és que sembla que les HSCs es generarien en íntima associació amb l'endoteli d'aquests òrgans (Chapter 1, Figura 9).

Relació directa entre els llinatges hemopoètic i endotelial

A principis del segle passat, Sabin va observar que les cèl.lules hemopoètiques dins de les illes sanguínies del sac vitel.lí es generaven properes en temps i espai amb les cèl.lules endotelials. Això va dur a la idea que existiria un precursor mesodermal comú entre ambdós llinatges: l'hemangioblast. En la regió de l'AGM s'ha descrit que grups de cèl.lules hemopoètiques sobresurten de la part ventral de l'aorta en íntima associació amb o entre la capa endotelial (Garcia-Porrero, 1995; de Bruijn, 2002). Aquestes cèl.lules expressarien marcadors hemopoètics com c-Kit, CD45 i factors de transcripció hemopoètics claus com Runx1, c-myb, Gata2 i Scl (revisat a Godin i Cumano, 2002) però compartirien altres marcadors també expressats en cèl.lules endotelials com PECAM1/CD31, el receptor d'angiopoietina Tie-2, CD34 i el receptor-2 de VEGF (citoquina endotelial) o Flk-1 (Young, 1995; North, 1999; Hamaguchi, 1999; Hsu, 2000; Lacaud, 2001; Baumann, 2004).

Recentment, l'estudi de ratolins transgènics que expressen la proteïna verda fluorescent (GFP) sota el control del promotor Ly-6A/Sca-1, que és un marcador de HSC ben caracteritzat va revelar que hi havia una població de cèl.lules GFP^+ amb habilitat repobladora a llarg-termini i que residien en la capa de cèl.lules endotelials de l'aorta, suggerint que les HSCs provenen de l'endoteli (de Bruijn, 2002). A més, se sap que cèl.lules que expressen marcadors exclusivament endotelials poden originar cèl.lules hemopoètiques (Eichmann, 1997). En aquest sentit, s'ha identificat una subpoblació de cèl.lules endotelials que expressen PECAM1, VE-cadherina i Flk-1 entre les cèl.lules ES humanes amb propietats hemangioblàstiques (Wang, 2004) i també s'ha descrit una població mesodermal que expressa Brachyury i Flk-1, com el progenitor comú de cèl.lula hemopoètica i endotelial en el YS de ratolí (Huber, 2004).

Models proposats per la generació de HSCs de l'AGM

S'ha proposat tres models diferents que explicarien la generació de HSCs de la regió de l'AGM:

1) Les HSCs es generarien de cèl.lules de la part ventral de l'aorta amb un fenotip endotelial i que es transdiferenciarien a HSCs. Aquesta hipòtesi s'explicaria per la gran quantitat de marcadors de superfície compartits entre ambdós tipus cel.lulars.

2) Les HSCs provindrien de diferents poblacions cel.lulars dins de l'endoteli aòrtic, ja sigui hemangioblastes intraembrionaris o cèl.lules mesodermals menys diferenciades. A favor d'aquesta hipòtesi hi hauria els estudis en models d'aus que suggereixen que l'aorta dorsal es formaria a partir de dues poblacions mesodermals, una d'elles amb potencial hemògenic (Pardanaud, 1996).

3) Les HSCs es generarien en els parxes sub-aòrtics del mesènquima al voltant de la part ventral de l'aorta i migrarien cap a l'endoteli de l'aorta, formant els grups de cèl.lules hemopoètics i extravasant cap al torrent sanguini. En aquest sentit, s'ha trobat HSCs que expressen CD31 i CD41 en aquesta regió (Bertrand, 2005).

Avui en dia, tots tres models segueixen sent vàlids ja que es requereix més investigació per desxifrar com es generen les HSCs en l'embrió.

Reguladors de l'hemopoesi embrionària

Tot i que fins fa poc no se'n sabia gaire cosa, actualment hi ha cada cop més evidències que determinades **vies de senyalització** intervindrien en la regulació de la generació i manteniment de les HSCs en l'embrió. En aquest sentit s'ha descrit que el lligand de la via de Wnt/β–catenin, Wnt3a indueix l'autogeneració de HSCs *in vitro* (Willert, 2003). A més, la sobreexpressió d'una forma activada de β-catenina en les HSCs incrementa la proliferació *in vitro* i la repoblació d'adults irradiats *in vivo*, incrementant l'expressió de HoxB4 i Notch1 (Reya, 2003). A més, la via de Notch seria necessària per al manteniment de la indiferenciació de les HSCs (Duncan, 2005), tot i que s'ha descrit recentment que l'activació de Wnt conduïria a la diferenciació de les HSCs i no a la seva proliferació (Kirstetter, 2006).

D'altra banda, s'ha descrit que diferents membres de la família del TGFβ regularien tant positivament com negativament el sistema hemopoètic, ja que els ratolins mutants per BMP4 i els mutants per TGFβ1, careixen totalment de illes sanguínies i eritròcits en el sac vitel.lí (Winnier, 1995; Dickson, 1995). En canvi, els ratolins mutants per Smad5 mostren un increment en el nombre de progenitors hemopoètics, suggerint que Smad5 té un paper inhibidor en la determinació hemopoètica o en l'expansió dels progenitors (Liu, 2003).

Pel que fa a la via de senyalització de Hedgehog, s'ha descrit que els peixos zebra mutants per Hedgehog o tractats amb un inhibidor de la via, anomenat ciclopamina careixen d'hemopoesi definitiva però no primitiva (Gering and Patient, 2005). A més, Sonic hedgehog indueix la proliferació *in vitro* de progenitors hemopoètics de cordó umbilical $CD34^{+}CD38^{+}Lin^{-}$ via BMP4 (Bhardwaj, 2001).

Finalment, la via de senyalització a través del receptor Notch regula molts processos del desenvolupament embrionari i com que la funció de Notch en l'hemopoesi embrionària és l'objectiu d'aquesta tesi, la seva funció es discutirà amb més detall, separadament.

Hi ha també una gran quantitat de **factors de transcripció** involucrats en la regulació del programa hemopoètic en l'embrió. Els ratolins deficients per Runx1 moren a dia 12.5 i careixen totalment d'hemopoesi

definitiva en l'AGM i fetge fetal, tot i que l'hemopoesi primitiva del sac vitel.lí no es veu afectada (Okuda, 1996). Aquests mutants no presenten grups de cèl.lules hemopoètiques que sobresurten de l'endoteli aòrtic (North, 1999) i els embrions heterozigots, presenten problemes en la generació en temps i espai de HSCs (Cai, 2000) indicant que Runx1 és clau per la generació apropiada de HSCs.

En canvi, els ratolins mutants per Gata2 moren a dia 10.5 degut a una severa anèmia causada per una gran reducció del nombre de progenitors hemopoètics en el sac vitel.lí (Tsai, 1994). L'anàlisi de ratolins quimera indicà que les cèl.lules ES mutants per Gata2 no contribuïen ni a l'hemopoesi primitiva ni a la definitiva (Tsai, 1994). A més, els ratolins heterozigots presenten un nombre reduït de HSCs de l'AGM (Ling, 2004) i *in vitro*, les cèl.lules ES mutants per Gata2 dónen lloc a progenitors hemopoètics que proliferen poc i moren per apoptosi (Shivdasani, 1996).

Els mutants per c-Myb moren a dia 15 degut a la manca d'hemopoesi definitiva del fetge fetal tot i que no presenten anomalies en l'hemopoesi primitiva (Mucenski, 1991). A més, cultius primaris de P-sp a dia 9.5 i d'AGM a dia 11.5 dels embrions mutants per c-Myb careixen totalment d'hemopoesi (Mukouyama, 1999) suggerint que l'absència de progenitors hemopoètics en el fetge fetal podria ser causat per un defecte previ en la generació de HSCs de l'AGM.

Finalment, la sobreexpressió de HoxB4 expandeix el nombre de HSCs en el moll de l'òs murí (Antonchuk, 2002) i cultius humans de cordó umbilical (Buske, 2002) sense afectar l'habilitat repobladora o diferenciadora d'aquestes cèl.lules. A més, la infecció de progenitors hemopoètics de sac vitel.lí de dia 8.25 de gestació amb HoxB4 resulta en l'adquisició de les característiques de HSCs definitives (Kyba, 2002).

Entre les **citoquines i factors de creixement** que juguen un paper més important en l'hemopoesi embrionària trobem el VEGF (factor de creixement de cèl.lula endotelial vascular). L'estudi de tant els mutants pel receptor-2 de VEGF (Flk-1) com de ratolins quimera amb cèl.lules ES mutants, revelaren que la senyalització a través de VEGF és necessària per la determinació de l'hemangioblast a partir del mesoderm (Shalaby, 1995; Shalaby, 1997). A més, s'ha descrit que la dosi adquada de VEGF és necessària per la supervivència dels eritròcits primitius del YS (Martin, 2003).

Els mutants de Stem Cell Factor i el seu receptor c-Kit moren perinatalment mostrant anèmia severa. A més, s'ha vist que la senyalització a través de SCF/c-Kit és necessària per a la generació de limfòcits T però no B (Takeda, 1997) i que podria estar implicada en l'adhesió de HSCs i progenitors a l'estroma del moll de l'òs (revisat a Linnekin, 1998).

Les cèl.lules ES mutants pel receptor del factor de creixement de fibroblastes (FGF) no són capaces de generar cèl.lules hemopoètiques (Faloon, 2000) i s'ha descrit que FGF-2 i FGF-4 serien necessàries per la diferenciació de determinats llinatges (revisat a Kashiwakura, 2005) mentre que FGF-1 promouria la proliferació de HSCs (de Haan, 2003).

Finalment, els ratolins mutants per la IL-3 o el seu receptor no mostren alteracions greus de l'hemopoesi adulta (Lantz, 1998; Nishinakamura, 1995), però en canvi, s'ha descrit darrerament que IL-3 podria funcionar com un factor de proliferació i supervivència de les HSCs generades en l'AGM (Robin, 2006).

6.1.1.3 Desordres hemopoètics

Com s'ha pogut veure, la regulació del sistema hemopoètic és extremadament complexa. Per això mutacions i alteracions en gens reguladors de l'hemopoesi acaben provocant desordres hemopoètics, indicant que l'homeostasi de cèl.lules sanguínies és essencial per la supervivència de l'organisme.

Els neoplasmes en cèl.lules hemopoètiques s'anomenen **leucèmies**. Sovint la transformació ocorre en una única cèl.lula mare pluripotent, que comença a proliferar, expandir-se clonalment i evitar la mort per apoptosi, tot i que pot ocórrer també en un progenitor hemopoètic no tan indiferenciat. Les leucèmies es classifiquen segons l'estadi de diferenciació cel.lular i la severitat de la malaltia. Així podem trobar leucèmies agudes o cròniques i segons el tipus cel.lular, limfoblàstiques o mieloides. Diferents translocacions cromosòmiques que donen lloc a proteïnes de fusió han estat associades a la formació de leucèmies. Per exemple, la translocació cromosòmica t(1;14)(p32;q11) que provoca la inserció del locus de Scl en els elements reguladors del locus del receptor de cèl.lula T produeix leucèmia limfoblàstica aguda de cèl.lula T (T-ALL) que afecta principalment a nens. Altres translocacions involucrades en leucèmies són la Tel-Runx1, la Runx1-Eto i la Bcr-Abl (revisat a Izraeli, 2004; revisat a Leroy, 2005).

En canvi, els **síndromes mielodisplàstics (MDS)** són desordres de HSCs caracteritzats per alteracions que resulten en anèmia, neutropènia i/o trombocitopènia. En molts casos, desemboquen en leucèmies mieloides agudes (AML). S'ha descrit que tant mutacions en el factor de transcripció GATA1 com en el receptor de SCF, c-Kit poden desenvolupar aquest tipus de desordres (revisat a Izraeli, 2004; revisat a Linnekin, 1999).

6.1.2 <u>ERITROPOESI</u>

6.1.2.1 <u>Diferenciació eritroide</u>

L'eritropoesi és un procès amb múltiples etapes que involucra la diferenciació des de les HSCs fins a eritròcits madurs. En l'adult, l'eritropoesi té lloc en el moll de l'òs i implica la diferenciació des de les HSCs en el progenitor comú mieloide (CMP), progenitor multipotent que genera el progenitor de granulòcits-macròfags (GMP) i el progenitor de megacariòcits-eritròcits (MEP). En l'assaig de CFU-Culture, el CMP genera colònies mixtes de cèl.lules eritroides i mieloides (Mix-CFC). Quan es determina cap al llinatge eritroide, el progenitor CMP dóna lloc al progenitor BFU-e, que genera grans colònies de cèl.lules eritroides i al progenitor eritroide més diferenciat CFU-e (revisat a Testa, 2004), el qual després de determinades divisions mitòtiques, es diferencia en cèl.lules eritroides morfològicament diferenciables. Durant el procés de diferenciació, les cèl.lules expressen gradualment el receptor d'eritropoietina esdevenint sensibles a aquesta citoquina, que regula tant la proliferació com la supervivència de cèl.lules vermelles. Els últims estadis de diferenciació eritroide estan composats per un compartiment proliferatiu-maduratiu format per proeritroblastes, eritroblastes basofílics i eritroblastes policromatofílics i un altre compartiment maduratiu però no proliferatiu format per eritroblastes ortocromàtics, reticulòcits i eritròcits madurs. Durant els últims estadis de maduració, les cèl.lules eritroides es fan més petites, acumulen hemoglobina i incrementen la densitat de la cromatina per excloure el nucli. Finalment, s'alliberen al torrent sanguini prenent part en l'intercanvi gasós (revisat a Testa, 2004).

L'eritropoesi adulta està ben caracteritzada basada en la morfologia cel.lular, l'expressió de marcadors de superfície cel.lular i el contingut d'hemoglobina (Chapter 1, Figura 10). No obstant, se sap molt poc del desenvolupament eritroide primitiu. Els eritròcits primitius generats en les illes sanguínies del sac vitel.lí entren en el torrent sanguini un cop s'estableix la circulació entre sac i embrió, en l'estadi d'eritroblastes basofílics. Mentre circulen, els eritròcits acaben la seva maduració, caracteritzada per una acumulació

d'hemoglobina resultant en una reducció de la basofília citoplasmàtica i una condensació de la cromatina (revisat a McGrath i Palis, 2005).

Marcadors eritroides

Hi ha una sèrie de marcadors de superfície cel.lular que s'ha identificat en diferents subpoblacions eritroides i que s'ha relacionat amb diferents estadis de maduració. Així, el marcador Ter119 s'expressa en cèl.lules eritroides murines i es troba associat a la proteïna de superfície cel.lular glicoforina A (Kina, 2000). El marcador Ter119 no s'expressa en els progenitors eritroides BFU-e i CFU-e i apareix a partir de l'estadi de proeritroblaste. En canvi, CD71 és el receptor de transferrina, que és essencial per a cèl.lules en proliferació. En el llinatge eritroide és necessari per la captació de ferro per la síntesi d'hemoglobina i s'expressa en progenitors eritroides però deixa de ser detectable en eritròcits madurs (revisat a Gubin, 1999). Pel que fa a c-Kit (CD117) és el receptor de SCF i s'expressa en progenitors eritroides degut a que és requerit tant per la proliferació com per a la diferenciació.

En canvi, CD41 s'expressa en eritròcits primitius del sac vitel.lí (Ferkowicz, 2003) i en les HSCs de l'embrió (Bertrand, 2005). S'ha caracteritzat diferents subpoblacions dins el llinatge eritroide en funció de l'expressió d'aquest marcador. Aixi, les cèl.lules $CD41^{dim}Ter119^{-}$ són cèl.lules inmadures, les $CD41^{dim}Ter119^{+}$ són eritroblastes basofílics i les $CD41^{-}Ter119^{+}$ són eritroblastes cromatofílics o eritroblastes basofílics tardans (Otani, 2005). Finalment, tant els eritròcits primitius com els definitius comencen a expressar hemoglobina en l'estadi d'eritroblaste basofílic, acumulant-se fins arribar al màxim en les etapes finals de maduració (Otani, 2005).

6.1.2.2 Regulació de l'eritropoesi embrionària i adulta

La hipòxia que experimenta un embrió que creix per difusió passiva té un paper actiu en l'eritropoesi, regulant els nivells de la **citoquina** més important per la producció de cèl.lules sanguínies vermelles: l'eritropoietina. La unió d'EPO al seu receptor (EpoR) activa la kinasa Janus (Jak2), la qual fosforila l'efector nuclear Stat5, conduïnt a una transcripció gènica específica. A més, indueix altres vies de senyalització com la via de la PI3 kinasa, la de la kinasa Akt i la via de Ras, que regulen processos d'apoptosi, proliferació i diferenciació eritroide terminal (revisat a Testa, 2004). Els embrions mutants per a components de la via de senyalització tals com EPO, EpoR i Jak2 moren a dia 12.5 degut a anèmia severa i a una gran reducció del nombre de proeritroblastes primitius, tot i que tenen nombres normals de progenitors eritroides indicant que aquesta citoquina no és indispensable per a les primeres etapes de determinació eritroide (Wu, 1995; Lin, 1996; Neubauer, 1998).

D'altra banda, SCF és l'altra citoquina important per a l'eritropoesi. Tant SCF com el seu receptor c-Kit estan expressats en cèl.lules indiferenciades $CD34^{+}$ i progenitors eritroides BFU-e i CFU-e (Testa, 1996) suggerint que aquesta citoquina estaria involucrada en l'expansió i/o supervivència de progenitors eritroides.

Pel que fa als **factors de transcripció** eritroides, GATA1 és el més important tot i que també és necessari per la maduració de mastòcits i megacariòcits. GATA1 s'expressa a nivells basals en progenitors eritroides però quan s'indueix la diferenciació per EPO, la seva expressió augmenta arribant al màxim entre els estadis de CFU-e i proeritroblast (Cantor, 2002). La manca d'expressió de GATA1 provoca letalitat embrionària a dia 10.5-11.5 degut a una anèmia severa. Aquests mutants presenten progenitors eritroides

indicant que no és requerit per a la determinació a llinatge eritroide, però presenten un segrest de la maduració en l'estadi de proeritroblast (Fujiwara, 1996) i moren per apoptosi (Weiss and Orkin, 1995). En canvi, FOG-1 és un factor de transcripció que interacciona amb GATA1. Els embrions mutants per aquesta proteïna presenten un fenotip similar als mutants de GATA1 però amb una total manca de megacariòcits suggerint que FOG-1 també té funcions independents de GATA1 (Tsang, 1998).

Scl també té un paper en el llinatge eritroide. La sobreexpressió de Scl afavoreix la determinació a aquest llinatge dels progenitors hemopoètics (Valtieri, 1998) mentre que la seva manca inhibeix la proliferació de cèl.lules d'eritroleucèmia (Green, 1991).

Finalment, altres factors de transcripció involucrats en aquest llinatge són els de la família Krüppel-like o KLF. KLF1/EKLF regula l'expressió d'un gran nombre de gens específics de la diferenciació eritroide, com el de la β-globina (Hodge, 2005) mentre que els mutants de KLF6 tenen una manca d'expressió de GATA1 i Scl, suggerint que aquest factor podria estar actuant per sobre dels altres dos (Matsumoto, 2006).

L'**apoptosi** també és un regulador molt important de l'homeostasi del llinatge eritroide. El promotor del gen anti-apoptòtic bcl-x_L conté llocs d'unió a GATA1 i la seva expressió és controlada per GATA1 en cèl.lules mare embrionàries (Gregory, 1999). A més, també té llocs d'unió a Stat5, que es troba per sota de la senyalització per EPO (Dolznig, 2002). En aquest sentit, els embrions deficients en Stat5 mostren anèmia severa degut a una menor supervivència dels progenitors eritroides de fetge (Socolovsky, 1999), un fenotip compartit pels mutants de Bcl-x_L (Motoyama, 1995). A més, l'apoptosi en el llinatge eritroide també està regulada per factors secretats o senyals mediades per lligand, actuant a través de la via d'apoptosi extrínseca. En aquest sentit, l'activació de receptors de mort cel.lular com el receptor Fas o el receptor de TNF-α provoquen l'apoptosi de progenitors eritroides (De Maria, 1999; Schneider, 1999; Jacobs-Helber, 2003).

6.1.2.3 Desordres eritropoètics

Molts desordres del llinatge eritroide es deuen a la desregulació dels mecanismes apoptòtics que controlen aquest compartiment. Per exemple, els pacients amb **policitèmia vera** (PV) presenten un quadre clínic de síndrome mielodisplàstic amb una sobreproducció d'eritròcits, megacariòcits i granulòcits que acaben desembocant en leucèmies agudes (Spivak, 2002), degudes a alteracions moleculars que resulten en una resistència a l'apoptosi del llinatge eritroide (Zeuner, 2006).

En el cas dels pacients amb **anèmia** de Fanconi o anèmia associada a mieloma múltiple, la causa principal és una major apoptosi espontània degut a una major sensitivitat a l'apoptosis mediada per FasL (Silvestris, 2002).

Tot i això hi ha d'altres malalties del llinatge eritroide que no es deuen principalment a una desregulació de la mort cel.lular per apoptosi. Les **hemoglobinopaties** (conegudes comuntment com a talassèmies) es deuen a una expressió anormal de les globines. Els factors de transcripció GATA1 i EKLF controlen l'expressió tant de la β-globina com de la proteïna estabilitzadora de la α-hemoglobina (AHSP). Per tant, els ratolins EKLF$^{-/-}$ moren a dia 16 de gestació degut a un fenotip que s'assembla a la β-talassèmia (revisat a Testa, 2004).

6.1.3 LA VIA DE SENYALITZACIÓ DE NOTCH

La senyalització a través del receptor de membrana Notch s'ha descrit que regula moltes de les decisions de destí cel.lular, tant en el desenvolupament embrionari com en l'adult, mitjançant la senyalització directa cèl.lula-cèl.lula (revisat a Lai, 2004). Els membres d'aquesta família s'ha trobat conservats en organismes tan evolutivament divergents com les mosques i els humans i regulen processos del desenvolupament tant variats com la neurogènesi, la miogènesi, l'hemopoesi o la segregació de somites (revisat a Lewis, 1998).

6.1.3.1 Membres de la via de Notch i mecanisme

Els membres de la família Notch inclouen bàsicament els receptors de membrana Notch, ells lligands Delta i Serrate/Jagged i el factor de transcripció nuclear CSL (que significa CBF1/recombinant binding protein J kappa (RBPjκ), Suppressor of Hairless Su[H], Lag-1). Tots els organismes metazous contenen un o més ortòlegs d'aquestes proteïnes com es pot veure a la Taula 3, Chapter 1.

Els mamífers contenen quatre gens diferents que codifiquen per a quatre **receptors de membrana** (Notch1-4). Es tracta d'heterodímers de transmembrana involucrats en transduir senyals extracel.lulars específiques, cap al nucli en resposta a la unió del seu lligand. El domini extracel.lular conté una regió de múltiples repeticions en tàndem del factor de creixement epidèrmic (EGF) involucrats en la unió al seu lligand (Rebay, 1991) així com un domini anomenat LNR que manté l'estructura d'aquest heterodímer i evita l'activació independent de lligand (Sánchez-Irizarry, 2004) En canvi, el domini intracel.lular de Notch (NotchIC) conté el domini RAM i les repeticions de tipus ankirina (ANK) que es requereixen per la interacció amb d'altres proteïnes efectores, senyals de localització nuclear, el domini de transactivació i el domini PEST que regula l'estabilitat de la proteïna (revisat a Maillard, 2003) (Figura 13, Chapter 1).

L'activació de la senyal a través de Notch s'origina per la interacció del receptor amb un dels seus **lligands**. També són proteïnes de transmembrana que contenen múltiples repeticions de tipus EGF i un domini característic DSL, ambdós involucrats en la interacció amb el receptor. En *Drosophila* s'ha descrit dos lligands diferents (Delta i Serrate), mentre que en vertebrats n'hi ha cinc (Jagged1 i 2, Delta1, Delta3 i Delta4), tot i que un lligand addicional, Delta2 s'ha descrit en *Xenopus.*

Pel que fa al **mecanisme d'activació**, aquest s'inicia quan un dels lligands expressats en la cèl.lula adjacent interacciona amb el receptor Notch (Kopan, 1996). Això provoca un primer processament proteolític (anomenat S2, perquè el primer trencament, l'S1 es produeix en el complexe de Golgi per a crear un receptor heterodimèric funcional a la superfície cel.lular) mediat per una metal.loproteasa de la família ADAM, anomenada TACE (TNF-α converting enzyme). Aquest receptor truncat és aleshores substrat d'un complexe multiproteic amb activitat γ-secretasa que trenca el receptor Notch pel domini transmembrana, alliberant el domini intracel.lular Notch (NotchIC; revisat a Lai, 2004). El bloqueig de l'activitat γ-secretasa amb inhibidors farmacològics com el DAPT o la inactivació genètica de membres del complexe γ-secretasa inhibeix la senyalització a través de Notch (Zhang, 2000). Després del seu alliberament, el frament intracel.lular de Notch transloca al nucli on s'uneix al seu efector, el factor de transcripció RBPjκ. En absència d'activació de Notch, RBPjκ es troba unit a la seqüència d'unió dels seus promotors diana, reprimint l'expressió a través del reclutament de corepressors i histona deacetilases (HDACs) (Kao, 1998).

Un cop al nucli NotchIC s'uneix a RBPjκ desplaçant els corepressors i reclutant coactivadors activant-se l'expressió gènica (Figura 13, Chapter 1).

Entre els gens diana de Notch es troben les famílies de factors de transcripció hes (hairy and Enhancer of Split) i hrt (hes-related). Fins al moment, s'ha descrit 7 gens hes (hes1-7) en mamífers, tot i que només hes1 (Jarriault, 1995), hes5 (Ohtsuka, 1999) i hes7 (Bessho, 2001) són activats per Notch. A més, hi ha dos gens hrt (herp1/hrt2 i herp2/hrt1) que són gens diana de Notch (revisat a Iso, 2003). Hes i Hrt són factors de transcripció del tipus bHLH que actúen com a repressors transcripcionals d'altres bHLH involucrats en la diferenciació cel.lular de molts sistemes tals com neurogènesi, miogènesi, hemopoesi o diferenciació intestinal (revisat a Ohishi, 2003).

6.1.3.2 Control de decisions de destí cel.lular

Durant les primeres etapes del desenvolupament d'un determinat teixit, la majoria de cèl.lules tenen una potencialitat equivalent. En aquesta població homogènia, les interaccions entre Notch i Jagged/Delta són responsables de la generació de la diversitat cel.lular a través de l'activació de programes gènics específics (revisat a Lewis, 1998). S'ha proposat dos mecanismes diferents per a explicar com Notch regula la diversitat cel.lular:

El **model d'inhibició lateral** implica que en una població de cèl.lules equivalents que expressen nivells baixos de receptor i lligand, la cèl.lula que primer produeix més lligand, activa el receptor Notch en la cèl.lula veïna, la qual acaba disminuïnt l'expressió de lligand. Aquest mecanisme permet el manteniment i la intensificació de les diferències d'expressió d'ambdós proteïnes, ja que la cèl.lula que expressa lligand no rep senyals inhibitòries de les cèl.lules veïnes. Al final s'acaba aconseguint un mosaic cel.lular que es coneix amb el nom de sal-i-pebre, que condueix a que la cèl.lula que expressa lligand es diferencïi cap a un llinatge diferent al de les cèl.lules veïnes (Figura 14, Chapter 1). El clàssic exemple d'inhibició lateral ocorre durant la decisió entre cèl.lula neural i epidèrmica a *Drosophila* (Parks, 1997). En mamífers, la via de Notch reprimeix la neurògenesi i la miogènesi a través de repressors bHLH de la família Hes, els quals a la vegada inhibeixen altres factors de transcripció del tipus bHLH com Mash1 o MyoD (revisat a Artavanis-Tsakonas, 1999).

El segon mecanisme d'actuació de Notch és el **model d'inducció lateral**. En aquest cas, l'activació de Notch en una cèl.lula determinada promou l'expressió del lligand en la mateixa cèl.lula. Per tant, la cèl.lula que expressa lligand activa Notch en la cèl.lula adjacent prevenint el patró de sal-i-pebre i induïnt les seves decisions de destí cooperativament i formant marges definits d'expressió gènica (Figura 15, Chapter 1) (revisat a Lewis, 1998). En *Drosophila*, l'exemple clàssic d'inducció lateral ocorre durant la formació de l'ala (Couso, 1995). En vertebrats, el clàssic exemple és la generació de límits durant la somitogènesi, mediat a través de l'activació oscil.latòria de hes1 i hes7 per part de Notch (Jouve, 2000).

Altres membres claus de la via de Notch

La **glicosilació** del receptor de Notch a través de les glicosiltransferases Fringe (Radical, Lunatic i Manic Fringe) té la funció de modular l'especificitat de lligand. Les proteïnes Fringe regulen doncs, l'activitat Notch i contribueixen a la generació de diversitat cel.lular (revisat en Irvine, 1999).

En canvi, l'**ubiqüitinació** és requerida per a l'endocitosi del receptor Notch i els seus lligands. Neuralized i Mind bomb pertanyen a les RING-type E3 ubiqüitin lligases, ubiqüitinen el lligand Delta i mutacions en

aquestes proteïnes resulta en fenotips de pèrdua de funció de Notch (Itoh, 2003; Lai, 2001). Estudis recents suggereixen que la ubiqüitinació de Delta incrementa l'afinititat d'unió a Notch. A més, l'endocitosi del lligand Delta unit al domini extracel.lular de Notch facilita el processament proteolític S2 del receptor Notch (Nichols, 2007).

6.1.3.3 Funció de la via de senyalització de Notch en l'hemopoesi

La via de Notch funciona en diferents estadis del desenvolupament hemopoètic i la seva activació pot ser a través d'interaccions entre progenitors i cèl.lules de l'estroma però també entre cèl.lules hemopoètiques.

Expressió de receptors i lligands en el sistema hemopoètic

Els precursors hemopoètics de moll de l'òs $CD34^{+}Lin^{-}$ expressen el receptor Notch1 i Notch2, suggerint que Notch estaria implicat en el desenvolupament temprà del sistema hemopoètic (Milner, 1994; Ohishi, 2000). A més, les cèl.lules de l'estroma del moll de l'òs expressen Jagged1 (Walker, 2001), així com Delta1 i Delta4 (Karanu, 2001). Aquests mateixos lligands es troben expressats en cèl.lules d'epitel.li tímic, indicant que Notch jugaria un paper important en el desenvolupament de cèl.lula T (Felli, 1999; Mohtashami, 2006).

En el llinatge mieloide, els monòcits expressen elevats nivells de Notch1 i Notch2 però els granulòcits no (Ohishi, 2000). Finalment, els progenitors eritroides de moll de l'òs expressen Notch1 però l'expressió disminueix en cèl.lules més madures com els normoblastes acidòfils (Ohishi, 2000; Walker, 2001).

Paper de Notch en l'autoregeneració de HSCs

Actualment hi ha força evidències que indiquen que Notch té un paper crucial en l'autoregeneració de HSCs. Duncan *et al.* demostraren que la via de Notch està activa en la subpoblació KSL del moll de l'òs i s'inhibeix a mida que les cèl.lules diferencien. A més, la inhibició de Notch amb un dominant negatiu de CBF1 condueix a una diferenciació accelerada de les HSCs *in vitro* i una depleció de l'activitat repobladora *in vivo*, indicant que Notch és necessari pel manteniment de l'estat indiferenciat de les HSCs (Duncan, 2005).

La transducció del domini intracel.lular de Notch en progenitors hemopoètics murins $c\text{-}Kit^{+}Sca\text{-}1^{+}Lin^{-}$ duu a la immortalització d'aquestes cèl.lules i a la repoblació quan es transplanten en ratolins adults irradiats (Varnum-Finney, 2000). A més, Notch1IC expandeix el nombre de cèl.lules repobladores de moll de l'òs (Stier, 2002), un resultat similar al que s'obté expressant Notch4IC en cèl.lules humanes Lin^{-} de cordó umbilical (Vercauteren, 2004). A més, l'adició de Jagged1 soluble a cultius *ex vivo* de cèl.lules $CD34^{+}CD38^{-}Lin^{-}$ de cordó umbilical incrementa el nombre de cèl.lules mare hemopoètiques sense perdre l'habilitat repobladora (Karanu, 2000). L'incubació amb una proteïna de fusió Delta1 tindria el mateix efecte (Varnum-Finney, 2003). Molts d'aquests efectes són reproduïbles en cèl.lules $CD34^{-}KSL$ transduïdes amb Hes1, suggerint que aquesta proteïna seria responsable dels efectes de l'activació de Notch1 en la proliferació de les HSCs (Kunisato, 2003).

Regulació de decisions de destí cel.lular limfoide

Notch regula vàries decisions de destí cel.lular en el llinatge limfoide. L'activació de Notch promou la diferenciació cap al llinatge T a expenses del llinatge B. En aquest sentit, la deleció de RBPjκ en cèl.lules

hemopoètiques resulta en un increment de la diferenciació de cèl.lula B i un bloqueig del desenvolupament de cèl.lula T (Han, 2002). Contràriament, l'expressió de Notch1IC en progenitors bloqueja la limfopoiesi B i condueix a la generació de limfòcits T $CD4^{+}CD8^{+}$ (Pui, 1999). A més, Notch regula la determinació de cèl.lula $\alpha\beta$ *versus* $\gamma\delta$ dintre del llinatge TCR. L'activació de Notch afavoreix el destí $\alpha\beta$ ja que la disminució dels nivells de Notch1 resulta en un increment de cèl.lules $\gamma\delta$ (Washburn, 1997), un resultat que també s'obtingué amb la inactivació condicional de RBPjκ (Tanigaki, 2004). Finalment i en l'últim estadi de diferenciació de limfòcits T, Notch també estaria involucrat en la presa de decisió entre cèl.lula $CD4^{+}$ o $CD8^{+}$, ja que l'expressió de Notch1IC en timòcits afavoreix la generació de cèl.lules T $CD8^{+}$ en detriment de les cèl.lules T $CD4^{+}$ (Robey, 1996).

Notch en la diferenciació mieloide

La implicació de Notch en mielopoiesi s'ha estudiat tant en models *in vivo* com en línies cel.lulars *in vitro*. En aquest sentit, la sobreactivació de Notch1 i Notch2 inhibeix la diferenciació de línies cel.lulars mieloides de forma citoquina depenent (Milner, 1995; Bigas, 1998), segurament a través de l'expressió de Gata2 (Kumano, 2001). Més recentment, s'obtingué un resultat similar quan es va co-cultivar progenitors KSL amb la línia cel.lular d'estroma OP9 que expressa Delta1 (de Pooter, 2006). Tot i això, la funció de Notch en el llinatge mieloide no està tant clara, ja que hi ha resultats que suggereixen que l'activitat de Notch és requerida només per la diferenciació de limfòcits T i B *in vivo* (Radtke, 1999).

Notch i apoptosi

Molts estudis suggereixen que Notch també regula la mort cel.lular programada o apoptosi. En molts, casos, l'activació de Notch inhibeix l'apoptosi, com s'ha descrit en cèl.lules T (Jehn, 1999), cèl.lules de sarcoma de Kaposi (Curry, 2005) i cèl.lules de limfoma de Hodgkin (Jundt, 2002), tot i que degut a l'especificat de Notch segons el context, hi ha molts exemples que Notch actúa també al contrari. Per exemple, en línies cel.lulars de leucèmia limfoblàstica aguda de cèl.lules B, l'activació de Notch indueix apoptosi, segurament a través de Hes1 (Zweidler-McKay, 2005), així com en monòcits de sang perifèrica cultivats en presència de lligand Delta1 inmobilitzat i M-CSF (Ohishi, 2000).

Pel que fa a la funció de Notch en la regulació de l'apoptosi en el llinatge eritroide, encara ara hi ha força controvèrsia. En la línia cel.lular d'eritroleucèmia humana K562, Notch inhibeix la diferenciació i indueix apoptosi, a través de Hes1, el qual inhibeix l'activitat GATA1 i en últim lloc, l'expressió de bcl-X_L (Ishiko, 2005). Contràriament, Notch1 preve l'apoptosi en la línia cel.lular d'eritroleucèmia murina (MEL) durant la diferenciació induïda per HMBA (Shelly, 1999; Jang, 2004).

Implicació de Notch en l'ontogènia del sistema hemopoètic

En els darrers anys, la funció de Notch en l'hemopoesi embrionària ha estat parcialment descrita. Part del que se sap ha estat l'objectiu d'aquesta tesi i per tant es discutirà amb detall. Tot i això mentre estàvem treballant-hi, van aparèixer d'altres treballs que inclourem en aquesta secció.

La primera evidència que la via de Notch actúa en la generació de HSCs durant el desenvolupament embrionari aparegué dels treballs de Kumano *et al.* amb l'estudi dels ratolins mutants per Notch1. Cultivant cèl.lules de P-sp sobre la línia cel.lular d'estroma OP9, varen trobar que l'hemopoesi definitiva està totalment afectada en els embrions mutants de Notch1 degut a una manca de HSCs. Contràriament, el

nombre de CFC en els sacs vitel.lins Notch1$^{-/-}$ era similar als embrions salvatges, suggerint que l'hemopoesi primitiva estava almenys parcialment intacta. Tot i això, les HSCs tant del sac vitel.lí com de la P-sp no eren capaces de reconstituir l'hemopoesi de ratolins nou-nats hematològicament compromesos, suggerint que la manca d'hemopoesi definitiva era deguda a un problema de determinació de les HSCs des de l'endoteli aòrtic (Kumano, 2003). En aquest sentit es va demostrar que en ratolins quimera amb cèl.lules mare embrionàries mutants per Notch1, aquestes no contribueixen a l'hemopoesi definitiva (Hadland, 2004). Contràriament, l'anàlisi dels embrions mutants per Notch2 indica que Notch2 no és requerit per la generació de cèl.lules hemopoètiques (Kumano, 2003).

Finalment, estudis amb peix zebra també han ajudat a esclarir la funció de Notch en la formació de les HSCs. Els mutants per la proteïna Mind bomb tenen una hemopoesi primitiva normal però careixen de desenvolupament de HSCs, així com expressió de c-myb i runx1 en l'aorta. Contràriament, l'expressió transient de Notch1 revelà una expansió del nombre de HSCs, depenent de l'expressió de Runx1 (Burns, 2005). Per tant Runx1 actuaria per sota de Notch en la generació de HSCs de l'AGM.

6.1.3.4 Malalties degudes a alteracions de la via de Notch

S'ha descrit tres malalties congènites degudes a mutacions en membres de la família Notch. Així, mutacions en el gen jagged1 són responsables de la síndrome Alagille, que resulta en una generació i funció aberrant de diferent òrgans com són el cor, l'ull i el fetge (Li, 1997). En canvi, mutacions de Delta3 són responsables de la disostosi espondilocostal, una malaltia del desenvolupament caracteritzada per alteracions de l'esquelet entre les quals la fusió de les costelles (Bulman, 2000). Finalment, mutacions en les repeticions de tipus EGF de Notch3 resulta en la arteriopatia cerebral autosòmica dominant amb infarts subcorticals i leucoencefalopatia (CADASIL), caracteritzada per migranyes i demència (Joutel, 1996). A més, Notch també participa en processos tumorigènics en diferents teixits entre els que s'inclouen la glàndula mamària, pell, cèrvix i pròstata (revisat a Lai, 2004), còlon (Fernández-Majada, 2007) o pàncreas (Miyamoto, 2003).

Desordres hemopoètics

El gen Notch1 es va identificar en humans com a responsable de leucèmies de limfòcits T degut a que es veia involucrat en la translocació cromosòmica t(7;9)(q34;q34.3) que provocava una proteïna de fusió entre Notch1 i el receptor de cèl.lula T (TCR-β) resultant en la activació constitutiva de Notch1 en aquestes cèl.lules (Ellisen, 1991). No obstant, la majoria de leucèmies produïdes per Notch es deuen a mutacions puntuals que afecten el domini d'heterodimerització (provocant l'activació aberrant de Notch) o en el domini PEST (resultant en una major estabilitat de Notch1IC) (Weng, 2004). A més en els darrers anys s'ha descrit que darrera d'algunes leucèmies de tipus T podria haver-hi una cooperació entre Notch i Ikaros. En aquest sentit, ratolins homozigòtics per un al.lel hipomòrfic d'Ikaros, desenvolupen limfomes tímics per una major activitat Notch i expressió de hes1 en timòcits (Dumortier, 2006).

6.1.3.5 Models animals

Per tal d'estudiar la funció de Notch tant en el desenvolupament com en organismes adults, s'ha generat organismes transgènics i mutants per a diferents membres de la família Notch, tant en *Drosophila* (mosca), *C. elegans* (nemàtodes), *Danio rerio* (peix zebra) com *Mus musculus* (ratolí).

La mutació de pèrdua de funció de Notch amb un fenotip més server s'obté de la deleció del gen rbpjκ, ja que és l'únic efector de la via de senyalització de Notch (Oka, 1995). Els embrions RBPjκ$^{-/-}$ moren a dia 10.5 i presenten un fenotip complexe que inclou alteracions en la somitogènesi, defectes en la formació del tub neural i creixement retardat (Figura 16, Chapter1). A més, la fusió de l'alantoides embrionari amb la placenta materna no ocorre i per tant no es poden formar els vasos umbilicals. Tots aquestes característiques indiquen que la senyalització a través de Notch és requerida per a la vasculogènesi, somitogènesi i neurogènesi (Oka, 1995).

El fenotip dels embrions Notch1$^{-/-}$ és similar al dels mutants de RBPjκ tot i que la mort ocorre una mica després, a dia 11.5. Els mutants de Notch1 presenten també retard del creixement, alteració de la neurogènesi i la formació dels somites tot i que la fusió corioalantoidea sí que es produeix (Conlon, 1995). Els mutants de Notch2 moren a 11.5 i presenten una apoptosi massiva en el neuroepiteli de les vesícules òtiques i òptiques (Hamada, 1999). Pel que fa als mutants de Notch3 són viables i només mostren alguns defectes en la vasculogènesi (Krebs, 2003). Finalment, els mutants de Notch4 són viables i fèrtils tot i que aquest receptor coopera amb la mutació de Notch1 en la inducció de defectes vasculogènics (Krebs, 2000).

Els mutants de Jagged1 moren a dia 10. Aquests embrions poden formar els vasos primitius del sac vitel.lí i de l'embrió però tenen defectes en la remodelació del plexe vascular per a formar vasos vitel.lins grans, suggerint que l'angiogènesi però no la vasculogènesi està afectada (Xue, 1999). En canvi, la mutació Jagged2 no és letal fins etapes properes al naixement, i aquests mutants presenten defectes en la morfogènesi craniofacial i en la fusió palatal, defectes en la formació de les extremitats així com desenvolupament tímic anormal (Jiang, 1998).

Finalment, el mutant de Mind bomb i els doble mutants per Presenilin1 i Presenilin2 (proteïnes del complexe γ-secretasa) moren a dia 10.5 i 9.5 respectivament, amb un fenotip molt similar al dels mutants de RBPjκ suggerint que aquestes proteïnes actúen per damunt de Notch i són necessàries per a la senyalització (Koo, 2005; Donoviel, 1999).

6.2 OBJECTIUS D'AQUESTA TESI

Tot i que ja s'havia descrit prèviament que la via de Notch regula diferents decisions de destí cel.lular en el sistema hemopoètic, la seva funció durant l'ontogènia de l'hemopoesi embrionària no es coneixia en l'inici d'aquesta tesi. Per tant, mitjançant l'ús del model de ratolí mutant per RBPjκ, el nostre objectiu era estudiar si la senyalització a través de Notch estava involucrada en la generació de l'hemopoesi primitiva i/o definitiva en l'embrió murí.

Per tant, els objectius específics que ens vam proposar per aquest projecte foren:

* Estudiar si la senyalització a través de Notch/RBPjκ té una funció específica en l'ontogènia del sistema hemopoètic primitiu en el sac vitel.lí murí des d'E7.5 a E9.5.

* Determinar si la senyalització a través de Notch/RBPjκ és necessària per a la generació de cèl.lules mare hemopoètiques i altres progenitors definitius en la regió de la P-sp/AGM murina des d'E9.5 a E11.5.

6.3 RESULTATS

CAPÍTOL 6.3.1: La funció de Notch depenent de RBPjκ regula *Gata2* i és essencial per la formació de cèl.lules hemopoètiques intraembrionàries

(Aquest capítol va ser publicat a Development 132, 1117-1126, 2005).

La regió intraembrionària que inclou l'aorta rodejada per gònada i mesonefros ha estat descrita com el primer òrgan involucrat en la generació de progenitors hemopoètics (HP) i de cèl.lules mare hemopoètiques (HSC) que més tard col.lonitzaran el moll de l'òs i mantindran l'hemopoesi en l'adult (Medvinsky, 1996; de Bruijn, 2000; de Bruijn, 2002). Prèviament al nostre treball hi havia força evidències que la via de senyalització de Notch regula diferents processos biològics en moltes línies cel.lulars hemopoètiques (Bigas, 1998; Karanu, 2000; Varnum-Finney, 2000; Kumano, 2001) tot i que hi havia poca informació de què passava *in vivo*. Per tant, fent ús dels ratolins mutants per RBPjκ (Oka, 1995), el nostre objectiu era determinar si la senyalització per Notch és necessària per la generació de HP/HSC en l'AGM.

Aquí mostrem que en embrions salvatges, alguns membres de la via de Notch entre els que s'inclouen els receptors notch1 i notch4 i els lligands delta4, jagged1 i jagged2 es troben expressats en cèl.lules endotelials de l'aorta de l'AGM a dia 9.5 i 10.5 de desenvolupament. A més, gens diana de Notch tals com hes1, hrt1 i hrt2 també es troben expressats indicant que la via de Notch està activada en aquesta regió. Aquest patró d'expressió de receptors Notch i lligands es troba alterat en els embrions mutants per RBPjκ (als quals els hi falta l'efector nuclear comú, per tant manca la senyalització per sota dels quatre receptors Notch) demostrant que la regulació de la seva expressió depèn de feed-back loops positius i negatius.

També demostrem que els embrions RBPj$\kappa^{-/-}$ mostren una manca de potencial hemopoètic en la regió de l'AGM amb una completa falta de progenitors hemopoètics (determinat per l'assaig de CFC) i de cèl.lules hemopoètiques $CD45^+$ després de 6 dies en cultiu. L'absència d'hemopoesi correlaciona amb una completa manca d'expressió dels factors de transcripció hemopoètics claus com són Gata2, Runx1 i Scl i amb un increment dels nombre de cèl.lules endotelials, suggerint la presència d'un progenitor comú (o hemangioblast) pels llinatges hemopoètic i endotelial.

Finalment, per doble hibridació in situ i immunoprecipitació de cromatina, describim que Notch s'uneix directament a través de RBPjκ al promotor de gata2, regulant la seva expressió no només a la línia cel.lular 32D però també en l'embrió. Finalment, describim quins lligands s'expressen en la cèl.lula adjacent a la que expressa notch1 en l'endoteli aòrtic i suggerim que l'activació de l'expressió de gata2 induïda per Notch1 en algunes de les cèl.lules de l'endoteli ventral de l'aorta de l'AGM i l'activació del programa hemopoètic en aquesta cèl.lula podria ser induït pels lligands Jagged2 o Delta4.

CAPÍTOL 6.3.2: El lligand de Notch, Jagged1 és requerit per l'hemopoesi intraembrionària de l'AGM

(Aquest capítol conté resultats encara no publicats)

Nosaltres hem descrit que l'expressió de gata2 mediada per Notch1/RBPjκ és necessària per l'hemopoesi de l'AGM (Robert-Moreno, 2005). Aquests resultats concordaven amb el treball de Kumano *et al.* analitzant el ratolí mutant per Notch1 (Kumano, 2003). Tot i això, no se'n sap gaire sobre quin lligand específic és el que activa Notch i indueix l'hemopoesi de l'AGM. A més, degut als severs defectes vasculars que mostren els mutants de Notch1 i RBPjκ (Oka, 1995; Krebs, 2000) i per tal d'evitar la possibilitat que la manca d'hemopoesi intraembrionària és deguda a la manca d'una artèria prèviament especificada, l'estudi de la funció de Notch en l'hemopoesi d'aortes salvatges era necessària.

En aquest capítol, describim que el potencial hemopoètic de l'aorta de l'AGM dels embrions mutants pel lligand Jagged1 però no dels mutants de Jagged2 es troba severament compromès (tot i que no totalment afectat). Els embrions mutants per Jagged1 careixen totalment d'expressió de gata2, mostren un nombre menor de progenitors hemopoètics i una reducció en el nombre de cèl.lules Sca-1$^+$ (una població enriquida per HSCs). A més i de forma similar als embrions mutants per RBPjκ, la manca d'hemopoesi intraembrionària en els mutants de Jagged1 correlaciona amb un increment del nombre de cèl.lules endotelials.

També mostrem que la inhibició de la senyalització per Notch amb l'inhibidor de l'activitat γ-secretasa en aortes salvatges d'embrions de dia 11 de gestació, cultivades com a explants, redueix significativament el nombre de progenitors hemopoètics (determinat amb l'assaig de CFCs) i de HSCs amb alta capacitat repobladora (determinat per estudis de repoblació d'adults irradiats).

Degut a que també describim que notch1, jagged1 i hes1 estan expressats en els grups de cèl.lules hemopoètiques que sobresurten de la part ventral de l'aorta de l'AGM, proposem que l'activació induïda per Jagged1 condueix a l'expressió de gata2 i hes1 conduïnt a l'expansió del nombre de progenitors hemopoètics i HSCs en l'endoteli aòrtic i a la vegada, a un manteniment de l'estat indiferenciat d'aquestes cèl.lules.

CAPÍTOL 6.3.3: La via de Notch regula positivament la mort cel.lular programada durant la diferenciació eritroide

(Aquest capítol va ser publicat a Leukemia, Maig 2007)

Una sèrie d'estudis de mutagènesi gènica dirigida revelaren que l'hemopoesi en el sac embrionari i en la regió intraembrionària de l'AGM estan governades per dos programes genètics diferents, ja que algunes mutacions afecten tant a l'hemopoesi primitiva com la definitiva, mentre que d'altres només afecten a la definitiva (revisat a Cumano i Godin, 2002).

En aquest article, mostrem que en contrast amb l'absolut requeriment de la senyalització a través de Notch/RBPjκ per l'hemopoesi definitiva de l'AGM, l'eritropoesi primitiva ocorre normalment en els sacs vitel.lins dels mutants per RBPjκ. Aquests embrions mutants generen tots els tipus diferents de progenitors hemopoètics i el desenvolupament i maduració del llinatge hemopoètic no mostra cap defecte. No obstant, el percentatge de cèl.lules eritroides $Ter119^+$ és major en els sacs vitel.lins mutants per RBPjκ comparat amb els embrions salvatges. Ni una major proliferació, ni diferències en la diferenciació eritroide poden explicar aquest increment cel.lular.

En aquest treball describim que l'apoptosi es troba específicament reduïda en les cèl.lules eritroides dels sacs vitel.lins mutants, indicant que la via de Notch promou la mort cel.lular programada en aquest llinatge. Mitjançant RT-PCR quantitativa, demostrem que la pèrdua de RBPjκ correlaciona amb un increment de l'expressió dels gens EPO i del receptor d'Epo (EpoR) així com dels gens anti-apoptòtics bcl-2 i bcl-X_L, concordant amb la major supervivència de la població $Ter119^+$ en els mutants.

Finalment, mostrem que Notch indueix apoptosi en el llinatge eritroide no només durant el desenvolupament embrionari però també en d'adult, ja que cèl.lules de moll de l'òs tractades amb els inhibidors de l'activitat γ-secretasa DAPT i L685,458 mostren menor mort cel.lular per apoptosi. A més, la inducció de l'apoptosi a través de Notch, mitjançant la incubació de moll d'òs amb cèl.lules que expressen Jagged1, és revertit amb el tractament amb inhibidors de l'activitat γ-secretasa. En aquest sentit, cèl.lules d'eritroleucèmia murina que sobreexpressen la forma activada de Notch1 presenten una major mort cel.lular programada en condicions de diferenciació induïda per hexametilè-bisacetamida (HMBA).

Per tant, amb tota aquesta informació proposem que la senyalització a través de Notch no és essencial per l'hemopoesi del sac embrionari però pot induïr específicament la mort cel.lular programada en el llinatge eritroide no només durant la vida embrionària però també en teixits adults, regulant l'homeostasi d'aquest compartiment hemopoètic.

6.4 DISCUSIÓ GENERAL i PERSPECTIVES FUTURES

Les cèl.lules mare hemopoètiques tenen una gran importància tant pels transplantaments clínics com per la medicina regenerativa. Aquest fet ha motivat enèrgicament l'estudi dels mecanismes que controlen l'autoregeneració de les HSCs i com el microambient regula el seu comportament. El nostre treball ha contribuït a esclarir la funció de Notch en l'hemopoesi embrionària del sac vitel.lí així com la formació de HSCs en l'AGM, un dels nínxols de generació i amplificació de HSCs de l'embrió millor caracteritzats. Tot i això encara ara hi ha controvèrsia sobre el lloc precís on es formen les HSCs en l'embrió.

La història interminable: quin és l'origen de les HSCs?

L'actual model diu que les primeres HSCs adultes capaces de nïar en el moll de l'òs d'adults irradiats es troben en l'embrió murí a dia 10 (de Bruijn et al., 2002; de Bruijn et al., 2000) mentre que a dia 11 es detecten en altres teixits tals com el fetge fetal, el sac vitel.lí, la placenta i el torrent sanguini (Gekas et al., 2005; Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996; Muller et al., 1994; Ottersbach and Dzierzak, 2005). No obstant, la idea que el sac vitel.lí contribueix a l'hemopoesi de l'adult no s'ha descartat mai, tot i que clàssicament s'ha cregut que la seva funció bàsica era la de generar eritròcits pel creixement de l'embrió. De fet, el sac vitel.lí i el mesoderm de la P-Sp de dia 9 de gestació, no són capaços de repoblar adults irradiats però sí que reconstitueixen l'hemopoesi de nou-nats (Yoder and Hiatt, 1997; Yoder et al., 1997; Yoder et al., 1997). Aquestes evidències suggeririen la idea que a dia 9, tant el sac vitel.lí com la P-sp contenen HSCs immadures que necessiten madurar per adquirir l'habilitat de nïar en el moll de l'òs adult. Per tant, el model actual suggereix que l'AGM serveix com a l'òrgan principal de generació de HSCs adultes, però que d'altres teixits com el sac vitel.lí i la placenta també contribueixen a l'hemopoesi adulta.

No obstant, un treball recent el qual proposa que el sac embrionari és l'origen d'un percentatge elevat de les HSCs de l'adult ha afegit encara més controvèrsia (Samokhvalov et al., 2007). No obstant, als anys 70, experiments de quimeres entre embrions de pollastre i guatlla demostraven que era l'embrió qui contribuïa al compartiment hemopoètic definitiu (Dieterlen-Lievre, 1975), mentre que més recentment s'ha demostrat que l'aorta està formada per dues poblacions de cèl.lules mesodermals i només una d'elles amb potencial hemopoètic (Pardanaud et al., 1996). A més, l'expressió de marcadors hemopòetics entre les cèl.lules de l'endoteli aòrtic reforçarien encara més la idea que l'AGM és l'òrgan generador de HSCs (Bertrand et al., 2005; de Bruijn et al., 2002; North et al., 1999; Robert-Moreno et al., 2005). Finalment un model alternatiu suggereix que les HSCs s'originen en el mesènquima sota la part ventral de l'aorta dorsal, unes estructures anomenades parxes subaòrtics (SAPs) i que expressen marcadors hemopoètics com gata2, gata3 i lmo2 i que contenen HSCs $CD45^{low}/ckit^{+}/AA4.1^{+}/flk\text{-}1^{-}$ capaces de reconstituir l'hemopoesi de ratolins immunodeficients $Rag2\gamma c^{-/-}$ (Bertrand et al., 2005; Manaia et al., 2000). Els nostres resultats no donarien suport a aquesta possibilitat ja que no hem detectat per hibridació in situ cap tipus de cèl.lula hemopoètica en el mesènquima sota la part ventral de l'aorta. Tots aquest models reflecteixen que les idees sobre l'origen endotelial o mesenquimàtic de les HSCs són variades, però que la integració de tota la informació que hi ha en l'actualitat resultaria en un millor coneixement de la generació de les HSCs.

L'objectiu principal del nostre treball era estudiar la funció que podia estar realitzant la via de Notch tant en l'hemopoesi primitiva com en la definitva de l'embrió murí. Els nostres resultats de l'estudi dels embrions mutants per RBPjκ (Robert-Moreno et al., 2005) i dels cultius d'explant d'aorta (Chapter 3, Section 2) suggereixen que la via de Notch és necessària per la determinació de les HSC i altres progenitors hemopo-

ètics de l'endoteli hemogènic de l'aorta de l'AGM. Per tant, proposem que l'activitat de Notch a l'aorta ventral genera un patró d'expressió de sal-i-pebre que restringeix el potencial hemogènic a un nombre determinat de HSCs/HP entre una majoria de cèl.lules aòrtiques endotelials, recordant el procès de la inhibició lateral descrit en la neurogènesi de *Drosophila* (Parks et al., 1997). En aquest sentit, la hibridació in situ (WISH) pels diferents lligands i receptors Notch en embrions temprans (Supplemental information; Chapter 3, Section 2) recolsarien aquest model d'inhibició lateral ja que les cèl.lules que expressen lligand apareixen abans en el desenvolupament (en l'estadi de 14 parells de somites) que les cèl.lules que expressen notch1 (cap a l'estadi de 20 parells de somites) i només en cèl.lules aïllades de la paret ventral de l'endoteli aòrtic.

Per tant, hi ha només un lloc on es generen HSCs en l'embrió o per contra és un procès redundant que té lloc en diferents teixits al llarg del desenvolupament fetal? Els nostres resultats concordarien amb la idea que l'hemopoesi primitiva i la definitiva són resultat de dos programes genètics ben diferenciats. Els embrions mutants per RBPjκ careixen totalment d'hemopoesi intraembrionària (Robert-Moreno et al., 2005) mentre que l'hemopoesi primitiva del sac vitel.lí té lloc de forma normal (Robert-Moreno et al., 2007). Aquest mateix fenotip va ser descrit en el cas d'embrions mutants per altres molècules de la via de Notch, com per exemple els ratolins mutants per Notch1 (Kumano et al., 2003) o els mutants de Mindbomb en el peix zebra (Burns et al., 2005). Tot i que no hem estudiat si els sacs vitel.lins serien capaços de generar HSCs, els embrions mutants per Notch1 no en generen (ni al sac ni a la P-sp) (Kumano et al., 2003) indicant que Notch és requerit per la generació/determinació de totes les HSCs definitives.

La senyalització a través de Notch regula la generació i/o expansió de HSCs en la regió de l'AGM

Molts estudis conclouen que la via de Notch regula decisions de destí final durant el desenvolupament de molts sistemes (revisat a Lai, 2004). Un d'ells és la decisió entre artèria i vena. En aquest sentit Notch indueix l'expressió de gridlock (l'ortòleg en el peix zebra de hrt1) en els vasos sanguinis per a promoure l'especificació a artèria (Zhong et al., 2001) mentre que la inhibició de Notch pel factor de transcripció COUP-TFII resulta en l'especificació de vena (You et al., 2005). Degut a que l'hemopoesi de l'AGM ocorre només en les principals artèries (de Bruijn et al., 2002; de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996) i no en venes, la manca d'hemopoesi intraembrionària en els mutants de Notch1 (Kumano et al., 2003) i RBPjκ (Robert-Moreno et al., 2005) podria ser deguda al defecte d'especificació d'una aorta. Per aquesta raó, vam fer ús dels cultius d'explant d'aorta/AGM (Medvinsky and Dzierzak, 1996) per investigar la funció de Notch en la generació de HSCs. De fet, els estudis de repoblació que es van fer amb aortes salvatges de dia 11 de gestació incubades amb l'inhibidor de l'activitat γ-secretasa DAPT revelaren un paper directe de Notch en la generació i/o expansió de HSCs a l'embrió i que és independent de l'especificació d'aorta (Chapter 3, Section 2). En aquests experiments, vam trobar una disminució del nombre de ratolins irradiats que varen ser repoblats per les aortes que havien estat tractades amb DAPT. Aquests resultats es podrien explicar de tres maneres diferents: ***1)*** La inhibició de Notch podria estar afectant la generació de noves HSCs o ***2)*** podria estar disminuïnt la proliferació de les HSCs pre-existents, ja que aquest cultiu permet l'expansió d'aquestes cèl.lules mare (Medvinsky and Dzierzak, 1996), o ja que l'activitat Notch normalment correlaciona amb el manteniment d'un estat indiferenciat en molts sistemes (revisat a Lai, 2004) ***3)*** podria ser que la inhibició de Notch estigués induïnt la diferenciació de les HSCs. Tot i això, aquesta última

possibilitat no seria consistent amb els nostres resultats, ja que vam trobar un nombre menor de progenitors hemopoètics en el cas de les aortes tractades amb l'inhibidor de γ-secretasa (Chapter 3, Section 2).

Recentment, s'ha proposat que el moll de l'òs adult conté un nombre determinat de HSCs diferents, cada una amb la seva pròpia cinètica de repoblació i comportament proliferatiu (Sieburg et al., 2006). Els nostres resultats però indicaren que les aortes tractades amb DAPT mostraven la mateixa eficiència repobladora (en el cas de les HSCs amb una capacitat repobladora menor al 10%) que les aortes sense tractar, però en canvi, el percentatge de ratolins repoblats amb HSCs amb una capacitat repobladora major al 10% era 4 vegades menor en el cas de les aortes tractades amb el DAPT. Per tant suggeririen que Notch és necessari per amplificar una hipotètica subpoblació de HSCs amb gran capacitat repobladora (Chapter 3, Section 2).

Qui activa el receptor Notch?

Vàrem descriure que notch1 i notch4, juntament amb jagged1, jagged2 i delta4 estaven expressats en l'endoteli aòrtic a dia 9.5-10.5 (Robert-Moreno et al., 2005). Els embrions mutants per Notch4 són viables, mentre que els doble mutants per Notch1/Notch4 presenten un defecte vasculogènic més sever que els mutants per Notch1 (Conlon et al., 1995; Krebs et al., 2000) indicant una funció vascular comú per Notch1 i Notch4. No obstant, l'expressió de gens de la família de Notch i el gen diana hes1 en els grups de cèl.lules hemopoètiques de l'endoteli aòrtic (Chapter 3, Section 2) suggereix que la via de Notch també té una funció en l'hemopoesi. Per tant, podria ser que dues senyals de Notch diferents coexistissin en l'endoteli aòrtic en aquest estadi, el primer per establir la identitat artèria/vena i el segon per generar o amplificar el compartiment de HSCs.

Notch1 però no Notch4 és essencial per la remodelació angiogènica dels vasos sanguinis, tot i que hi ha certa redundància entre ambdós receptors (Conlon et al., 1995; Krebs et al., 2000). D'altra banda, Notch1 és necessari per l'hemopoesi de la P-sp/AGM (Kumano et al., 2003) i a més l'expressió de notch4 (però no la de notch1) es troba disminuïda en els grups de cèl.lules hemopoètiques aòrtiques a dia 10.5 (Chapter 3, Section 2). A més vàrem trobar que Notch1 (a través de RBPjκ) s'associa al promotor del factor de transcripció Gata2 i per doble hibridació in situ vàrem descriure que tots dos gens són coexpressats en les mateixes cèl.lules de l'endoteli a dia 10.5 (Robert-Moreno et al., 2005). Totes aquestes evidències reforcen la idea de que seria el receptor Notch1 el que estaria activant el programa hemopoètic en l'endoteli de l'aorta. Per tant seria interessant conèixer com s'aconsegueix l'especificitat i quin grup de genes s'activa en cada cas. Una possibilitat podria ser que el receptor Notch1 fóra activat per un lligand diferent per a especificar el programa vasculogènic o l'hemopoètic. Ja que els embrions mutants per Delta4 moren a dia 9.5 amb uns defectes vasculogènics severs i una manca d'especificació del destí arterial (Duarte et al., 2004) podríem suggerir que l'activació de Notch pel receptor Delta4 seria la combinació que especificaria el desenvolupament vascular. Tot i això Notch4 podria compensar parcialment la deficiència de Notch1 en la vasculogènesi.

Els estudis que vam realitzat per doble hibridació in situ en aortes a dia 10.5 van revelar que les cèl.lules que expressaven jagged2 es trobaven majoritàriament adjacents a les que eren positives per notch1/jagged1 (Robert-Moreno et al., 2005). Encara que aquests resultats suggerien que el lligand Jagged2 era el millor candidat a activar Notch1, per demostrar-ho, vam estudiar si els embrions mutants per Jagged1 com per Jagged2 presentaven defectes en l'hemopoesi intraembrionària *in vivo*. De fet, quan vàrem estudiar el potencial hemopoètic d'ambdós mutants (ja sigui per CFC o comptant el nombre de

cèl.lules Sca-1-GFP$^+$ cells), vàrem trobar que els mutants per Jagged1 però no per Jagged2 presentaven un menor nombre de cèl.lules hemopoètiques d'ambdós tipus, indicant que Jagged1 tenia una funció important en aquest sistema (Chapter 3, Section 2). Per tant, tot i que els primers resultats apuntaven a que Jagged2 podria ser el responsable d'activar Notch, no hem trobat cap defecte hemopoètic en els embrions mutants per Jagged2. Una possible explicació seria que Jagged2 podria tenir una funció hemopoètica menor i que la seva deficiència fos compensada per Jagged1. Per a comprovar aquesta possiblitat actualment estem estudiant si els dobles mutants per Jagged1 i Jagged2 tenen un fenotip hemopoètic més sever comparat amb els mutants de Jagged1. A més, la disminució en el nombre de cèl.lules hemopoètiques que vàrem observar en els mutants de Jagged1 podria ser el resultat d'un defecte en l'amplificació o autoregeneració de les HSCs o de progenitors menys indiferenciats. Per tal de poder abordar aquesta qüestió tenim pensat fer experiments de repoblació de ratolins adults irradiats amb aortes d'embrions mutants per Jagged1.

En aquest sentit, hem fet ús dels ratolins Ly-6A/Sca-1 GFP (de Bruijn et al., 2002) per tal d'identificar les HSCs que emergeixen de l'aorta en els diferents embrions mutants pels lligands de Notch. Tot i que aquest és un excel.lent sistema per a aquest tipus d'estudis s'hauria de tenir en compte que Sca-1 (Stem cell antigen-1) s'utilitza com a marcador de HSCs (de Bruijn et al., 2002; Miles et al., 1997) tot i que també s'expressa en progenitors hemopoètics, endoteli vascular i ronyó. Per tant, tot i que està ben caracteritzat que la fracció GFP$^+$ conté l'activitat HSC (de Bruijn et al., 2002) cal saber que també hi ha cèl.lules endotelials no hemopoètiques que expressen GFP. Mitjançant el creuament dels mutants de Jagged1 i Jagged2 amb els ratolins transgènics per Ly-6A-GFP, vàrem detectar una gran disminució del nombre de cèl.lules GFP$^+$ a l'aorta dels mutants de Jagged1 però no de Jagged2. Això seria consistent amb un defecte hemopoètic en aquests embrions ja que recordem, no tenen expressió del factor de transcripció gata2 i presenten menor nombre de progenitors (CFCs). Una altra possible explicació a aquest fenotip seria que Notch a través de Jagged1 podria estar regulant l'expressió gènica de ly-6A/sca. Tot i això el fet que s'observi expressió del transgen en d'altres teixits no-hemopoètics com en teixit nefrogènic, fa pensar que l'expressió de Ly-6A/Sca no depèn de Jagged1/Notch.

També hem observat que el defecte en l'hemopoesi aòrtica en els mutants de RBPjκ i de Jagged1 correlaciona amb un increment del nombre de cèl.lules endotelials i de l'expressió de marcadors endotelials [(Robert-Moreno et al., 2005); Supplemental information; Chapter 3, Section 2)]. Aquests resultats suggereixen que en absència de senyalització per Notch les cèl.lules segueixen el destí endotelial a expenses del destí hemopoètic. Això doncs, estaria d'acord amb la idea que la regió de l'AGM serveix com un lloc d'especificació de HSCs des de l'endoteli, suggerint que els llinatges hemopoètics i endotelials comparteixen un progenitor comú com s'havia ja hipotetizat fa molt de temps (revisat a Dieterlen-Lievre, 2006).

Tot i que s'ha descrit alguns defectes en la vasculatura del sac vitel.lí i dels vasos del cap en els embrions mutants per Jagged1, no s'ha descrit cap defecte en el desenvolupament de l'aorta, ni en els mutants de Jagged1 ni en els de Jagged2 (Jiang et al., 1998; Xue et al., 1999). Tot i això, ja que les cèl.lules hemopoètiques es desenvolupen en contacte amb les cèl.lules endotelials de l'aorta, l'especificació del destí arterial podria estar afectant la capacitat de generació de cèl.lules sanguínies. Per tant, actualment estem investigant si els mutants de Jagged1 expressen marcadors arterials tals com ephrinB2, que seria indicatiu de que s'ha especificat correctament l'aorta.

Caracterització de gata2 com a gen diana de Notch en la regió de l'AGM

Hem investigat també si diferent gens claus per l'hemopoesi podien ser gens diana de Notch en l'AGM. Tot i que Notch regula diverses funcions de la diferenciació cel.lular, fins al moment no s'ha descrit molts gens diana. Tot i això alguns gens s'han descrit, com per exemple ephrinB2 en el desenvolupament del cor (Grego-Bessa et al., 2007), c-myc en cèl.lules de limfoma (Weng et al., 2006) o gata3 i il-4 en cèl.lules T (Amsen et al., 2004; Dontje et al., 2006), tot i que només hes1 i els hrts s'han caracteritzat extensivament [revisat a (Iso et al., 2003; Jarriault et al., 1995). És interessant que la majoria dels gens diana de Notch que s'ha descrit recentment ho són només en determinats teixits. A l'aorta, els embrions mutants per RBPjκ careixen totalment l'expressió d'almenys tres dels factors de transcripció claus: Runx1, Gata2 i Scl (Robert-Moreno et al., 2005). Tot i que això podria ser degut a un efecte secundari de la manca d'hemopoesi, vàrem investigar si la seva expressió depenia directament de Notch. Mitjançant la inmunoprecipitació de cromatina tant en progenitors mieloides (línia cel.lular 32D) com en embrions de dia 9.5, vàrem trobar que el gen gata2 estava directament regulat per la unió de Notch1/RBPjκ al seu promotor (Robert-Moreno et al., 2005). A més, mitjançant la doble hibridació in situ per notch1 i gata2, així com la manca d'expressió de gata2 en l'endoteli aòrtic dels embrions mutants de Jagged1 a dia 10.5-11 (Chapter 3, Section 2) reforcen la idea que gata2 és un gen diana de la via de senyalització formada per Jagged1/Notch1/RBPjκ en mamífers (Robert-Moreno et al., 2005). En aquest sentit, Notch també indueix l'expressió de serpent (l'ortòleg a *Drosophila* de gata2) conduïnt a l'aparició dels progenitors hemopoètics en la glàndula limfàtica de la larva de mosca (Mandal et al., 2004). De l'anàlisi dels embrions mutants per Gata2 i dels embrions quimera amb cèl.lules mare Gata2$^{-/-}$ se n'obtingué que aquest és un factor de transcripció clau per l'hemopoesi primitiva i definitiva (Tsai et al., 1994). Recentment, s'ha proposat que Gata2 és necessari per la generació, proliferació i supervivència de les HSCs i altres progenitors hemopoètics. Els embrions heterozigots per gata2 presenten un menor nombre de HSCs generades a l'AGM (Ling et al., 2004), una observació que aniria en la mateixa direcció que els nostres resultats amb els mutants de Jagged1. A més, el compartiment de cèl.lules mare del moll de l'ós dels ratolins Gata2$^{+/-}$ presenta major apoptosi degut a la downregulació de l'expressió del gen anti-apoptòtic bcl-X_L (Ling et al., 2004; Rodrigues et al., 2005) i els progenitors multipotencials mutants per Gata2 proliferen menys i mostren una mort massiva per apoptosi indicant que Gata2 és necessari tant per la proliferació com per la supervivència de progenitors indiferenciats (Tsai and Orkin, 1997). Per tant, proposem que l'activació de l'expressió de gata2 induïda per Jagged1/Notch1/RBPjκ és necessària per la generació, proliferació i/o supervivència de les HSCs i altres progenitors de l'endoteli hemogènic de l'AGM. El fet que els mutants de Jagged1 presentin un menor nombre però no una total manca d'hemopoesi suggeriria però que l'especificació de les HSCs podria estar ocorreguent en absència de Jagged1 i de Gata2.

El factor de transcripció hemopoètic Runx1/Aml1 també és necessari per la generació de HSCs de l'endoteli aòrtic ja que els embrions Runx1$^{-/-}$ careixen completament d'hemopoesi definitiva (North et al., 1999; Okuda et al., 1996). S'ha descrit recentment que Runx1 actuaria per sota de Notch en l'establiment del destí hemopoètic en l'embrió de peix zebra (Burns et al., 2005). De la mateixa forma, l'hemocitogènesi de *Drosophila* ocorre a través de l'activació induïda per Notch de lozenge, l'ortòleg de runx1 a la mosca (Lebestky et al., 2003). A més, l'expressió ectòpica de runx1 (però no de gata2 o scl) en cèl.lules derivades de l'AGM d'embrions mutants per Notch1 rescataria parcialment el defecte hemopoètic *in vitro* (Nakagawa et al., 2006). Per tant, tot i que hi ha evidències que Notch-Runx1 també és indispensable per la iniciació del

programa hemopoètic en l'AGM, no vàrem detectar el gen runx1 en la cromatina precipitada amb l'anticòs anti-Notch1 dels embrions a dia 9.5 (Robert-Moreno et al., 2005). A més, vàrem trobar expressió de runx1 en el 50% dels embrions mutants per Jagged1. Una possible explicació a aquests resultats seria que l'activació de Notch a través de diferents lligands podria estar activant específicament runx1 o gata2 en diferents poblacions de HSC i/o a diferents estadis. No obstant, ja que les cèl.lules de la P-sp dels embrions deficients en Notch1 infectades amb runx1 no són capaces de repoblar l'hemopoesi de nou-nats (Nakagawa et al., 2006) semblaria que tant Runx1 com Gata2 estarien actuant sinergísticament per a determinar i/o expandir les HSCs de l'AGM.

També vàrem investigar si scl/tal-1 podria ser un gen diana de Notch. Tot i que no vam detectar Notch1 unit al promotor de scl en els nostres ChIPs, l'expressió de scl estava incrementada en les cèl.lules 32D que sobreexpressen el domini intracel.lular de Notch (Robert-Moreno et al., 2005). Està descrit que Gata2 augmenta els nivells de scl en la població BL-CFC (Lugus et al., 2007) i que scl també està transcripcionalment incrementat després d'un breu pols de NotchIC en l'embrió de peix zebra (Burns et al., 2005). Aquestes evidències juntament amb els nostres resultats suggeririen que Scl podria estar actuant per sota de Gata2.

També hem descrit l'expressió dels clàssics gens diana de Notch hes1, hrt1 i hrt2 a l'aorta de l'AGM (Robert-Moreno et al., 2005) i Chapter 3, Section 2). Tant Hes com els Hrts són proteïnes bHLH descrites com a repressors transcripcionals, normalment conduïnt a la inhibició de la diferenciació [revisat a (Iso et al., 2003)]. Tot i que vàrem detectar certa expressió de hrt1 i hrt2 en els grups de cèl.lules hemopoètiques de l'aorta a dia 10.5 (dada no mostrada), el fet que els dobles mutants per Hrt1 i Hrt2 mostrin defectes severs en la remodelació vascular i que l'aorta dorsal careixi de l'expressió de ephrinB2 però expressi el marcador de vena ephB4 (Fischer et al., 2004) fa pensar que les proteïnes Hrt estarien més relacionades amb l'especificació arterial i no en l'hemopoesi.

D'altra banda, hem ensenyat expressió de hes1 no només en l'endoteli aòrtic però també en els grups de cèl.lules hemopoètiques de l'endoteli a dia 10.5 de gestació [(Robert-Moreno et al., 2005) i Chapter 3, Section 2] suggerint que aquest gen diana de Notch podria estar actuant en aquest sistema. Hi ha certes evidències d'un possible paper de Hes1 en la inhibició de la diferenciació i el manteniment de l'estat indiferenciat de les HSCs (Kunisato et al., 2003) i per tant seria possible que Jagged1/Notch1/RBPjκ estigués activant l'expressió tant de gata2 com de hes1 en una subpoblació de HSCs amb gran capacitat repobladora en l'AGM (Figura1, Chapter 5). Gata2 podria estar regulant la proliferació de les HSCs (en concordància amb el fet que els mutants de Jagged1 contenen un menor nombre de progenitors) mentre que Hes1 podria estar involucrat en el manteniment de l'estat indiferenciat.

Especificitat de Notch depenent de contexte en l'hemopoesi

La via de Notch regula molts processos del desenvolupament tant en l'etapa embrionària com en l'adult. Tot i això la gran complexitat de la via de Notch, amb una gran varietat de lligands, receptors i moduladors intra i extracel.lulars comporta una gran varietat de respostes depenent del teixit o del contexte cel.lular. Per exemple, l'activació de Notch inhibiria la diferenciació en la majoria de teixits [revisat a (Artavanis-Tsakonas et al., 1999)] mentre que en queratinòcits, l'activació de Notch promouria la diferenciació terminal (Rangarajan et al., 2001). Els mecanismes que condueixen a l'especificitat de Notch podrien ser ***1)*** diferents receptors de Notch (Cheng et al., 2007), ***2)*** l'accessibilitat de la cèl.lula que expressa Notch a diferents

lligands degut a patrons d'expressió específics (Robert-Moreno et al., 2005) o ***3)*** modificacions dels lligands a través de les glicosiltransferases Fringe (Hicks et al., 2000; Moloney et al., 2000), que conduïrien a l'expressió de diferents programes gènics.

Per tant, la funció de Notch en el sistema hemopoètic no es podria entendre sense considerar la idea de l'especificitat segons el contexte. L'activació de Notch duu a efectes diferents segons el llinatge hemopoètic. Durant el desenvolupament embrionari, la generació de l'hemopoesi primitiva i definitiva sembla que té diferents requeriments de Notch. De l'estudi dels mutants Notch1 i RBPjκ, d'altra gent i nosaltres mateixos hem proposat que la senyalització a través de Notch és requerida per l'hemopoesi definitiva però dispensable per l'hemopoesi primitiva del sac vitel.lí (Burns et al., 2005; Kumano et al., 2003; Robert-Moreno et al., 2005).

Tot i això, en l'adult no està tan clar que Notch sigui indispensable pel manteniment i/o autoregeneració de les HSCs. Mentre que la infecció retroviral de notch1IC en les cèl.lules de moll de l'òs expandeix el nombre de HSCs repobladores (Stier et al., 2002) i en canvi, la infecció d'un dominant negatiu de RBPjκ promou la seva diferenciació (Duncan et al., 2005), la inactivació induïble de notch1 en ratolins adults només provoca defectes en el llinatge limfoide (Radtke et al., 1999). De la mateixa forma, en l'adult, sembla ser que Jagged1 (Mancini et al., 2005) i RBPjκ (Han et al., 2002) són dispensables per l'autoregeneració de HSCs i l'habilitat repobladora d'aquestes cèl.lules del moll de l'òs en contrast amb els nostres resultats de l'hemopoesi de l'AGM. Aquests resultats serien indicatius que Notch seria específicament requerit per la generació de HSCs només durant la vida embrionària.

D'altra banda, l'efecte de Notch en la regulació de diferents decisions de destí cel.lular dins del llinatge limfoide ha estat extensament demostrat. Així, la inactivació condicional de Notch1 (Radtke et al., 1999) o RBPjκ (Han et al., 2002) en progenitors de moll d'òs provoca una depleció de limfòcits T perquè el progenitor limfoide comú es diferencia cap al destí de cèl.lula B. A més, també s'ha descrit la necessitat de Notch per a la generació de cèl.lules T $\alpha\beta$ a expenses de les cèl.lules T $\gamma\delta$ (Tanigaki et al., 2004) i de les cèl.lules T $CD8^+$ a expenses de les $CD4^+$ (Robey et al., 1996).

Nosaltres hem descrit recentment que la senyalització a través de Notch/RBPjκ indueix l'apoptosi específicament en el llinatge eritroide del sac vitel.lí degut a la disminució de l'expressió de membres de la via de supervivència eritroide de Epo/EpoR (Robert-Moreno et al., 2007). La unió de l'EPO al seu receptor indueix l'activació de la tyrosin-kinasa Jak2 i dels efectors PI3-kinasa/Akt, STAT5-bcl-X_L i ERK/MAPK que provoca la proliferació dels progenitors eritroides, la maduració eritroide i una protecció de l'apoptosi [revisat a (Testa, 2004)]. Un altre important efector de la via de EPO és GATA1, que controla l'expressió de bcl-X_L i per tant indueix supervivència (Gregory et al., 1999). En el nostre cas, no vam observar una sobreexpressió de GATA1 en els sacs mutants per RBPjκ tot i que no podem excloure que aquesta regulació pogués ser a nivell post-transcripcional ja que sí que vam observar una sobreexpressió de bcl-X_L. A més, prèviament s'havia descrit que Notch indueix l'apoptosi en la línia d'eritroleucèmia humana K562, mitjançant la repressió de GATA1 per Hes1, conduïnt a una downregulació de l'expressió de bcl-X_L (Ishiko et al., 2005).

Una altra possibilitat seria que el supressor tumoral p53 estigués induïnt apoptosi en les cèl.lules eritroides primitives. Es va descriure que els gens diana de Notch hes1 i hey1 eren els candidats a reprimir transcripcionalment l'expressió de l'inhibidor de p53, hdm2 (Huang et al., 2004). En concordància amb això, l'apoptosi induïda per Notch1 en progenitors neurals és suprimida en un background mutant per p53 (Yang et al., 2004). A més, la deleció condicional de mdm2 i mdm4 en cèl.lules eritroides $EpoR^+$ revelà que Mdm2

és crucial per inhibir l'apoptosi induïda per p53 en eritròcits de sac vitel.lí (Maetens et al., 2007). Per tant, tot i que no vàrem trobar diferències significatives en els nivells transcripcionals de p53 o mdm2, ni en els nivells dels gens depenents de p53 (bax i noxa, apart de puma) en els sacs vitel.lins mutants per RBPjκ comparat amb els salvatges, s'hauria d'estudiar la fosforilació de p53 per a descartar completament que Notch1 exerceix la seva funció pro-apoptòtica a través de p53 [revisat a (Chipuk and Green, 2006)].

La funció de Notch induïnt apoptosi s'havia descrit prèviament en monòcits (Ohishi et al., 2000) i en limfoma de cèl.lules B (Zweidler-McKay et al., 2005) i els efectes oposats s'havien descrit en cèl.lules T (Jehn et al., 1999) indicant altra vegada que la funció de Notch és llinatge i contexte depenent. Tot i això algunes de les evidències que s'han descrit provenen de l'estudi de línies cel.lulars i de sistemes *in vitro* i per tant s'haurien de considerar amb cautela. En canvi, els nostres resultats han estat generats a partir d'eritròcits directament obtinguts de sacs vitel.lins mutants per RBPjκ. El fet que haguem obtingut els resultats en un sistema *in vivo*, indica que Notch estaria induïnt l'apoptosi en aquest llinatge i per tant estaria contribuïnt a l'homeostasi de les cèl.lules eritroides.

En resum, la senyalització a través de Notch és crucial no només en la generació/expansió de HSCs en l'embrió, però també en la diferenciació i en la mort cel.lular programada d'alguns llinatges hemopoètics derivats d'aquestes HSCs. Com a conseqüència de tota aquesta regulació, s'aconseguiria l'homeostasi adequada del sistema hemopoètic.

6.5 CONCLUSIONS

En aquesta tesi hem arribat a les següents conclusions:

1. La manca de l'efector nuclear de la via de Notch, RBPjκ, comporta una completa inhibició de l'hemopoesi intraembrionària conjuntament amb una pèrdua total d'expressió dels factors de transcripció hemopoètics Gata2, Runx1 i Scl en l'endoteli aòrtic.

2. Notch1 a través de RBPjκ s'uneix directament al promotor de gata2 i regula la seva expressió tant en la línia cel.lular 32D com en l'embrió de ratolí.

3. La manca de Jagged1 resulta en una pèrdua d'expressió de gata2, una reducció de la generació de progenitors hemopoètics i un menor nombre de cèl.lules Sca-1^{+} en la regió de l'AGM.

4. La inhibició de l'activació de Notch mitjançant inhibidors de l'activitat γ-secretasa en aortes salvatges, disminueix el nombre de HSC en l'AGM, fenotip semblant al descrit pels mutants de Notch, RBPjκ i Jagged1^{dDSL}.

5. La senyalització a través de Notch és necessària per a la generació i/o expansió d'una subpoblació de HSCs amb gran potencial repoblador de la regió de l'AGM.

6. La manca d'hemopoesi intraembrionària de l'AGM correlaciona amb un increment de la capa endotelial de l'aorta en els embrions mutants per RBPjκ i Jagged1, suggerint que en l'aorta de l'AGM hi ha un progenitor comú (hemangioblast) dels llinatges hemopoètic i endotelial.

7. L'hemopoesi del sac vitel.lí es desenvolupa de forma normal en els embrions mutants per RBPjκ tot i que aquests mostren un increment en el nombre de cèl.lules eritroides.

8. La senyalització a través de Notch regula l'homeostasi eritroide en el sac vitel.lí mitjançant la inducció de l'apoptosi.

9. La protecció davant de l'apoptosi en el llinatge eritroide dels sacs embrionaris mutants per RBPjκ és deguda a la sobreexpressió de gens de la via de Epo/receptor d'Epo, que condueix a una sobreexpressió dels gens anti-apoptòtics bcl-2 i bcl-X_L.

CHAPTER 7:
ABOUT THE AUTHOR

PERSONAL DATA

Name: Alexandre Robert i Moreno

Date/Place of birth: 1st February 1978 / Lleida, Spain

EDUCATION

1996-2001: Faculty of Biology, Universitat Autònoma de Barcelona, Spain. Specialised in "Genethics and Cellular Biology".

2000-2001: Undergraduate student in the Human Genethics Department. Universitat Autònoma de Barcelona, Spain. Supervised by Dr. Francesc Xavier Barquinero. Research project "Chromosomal aberrations in irradiation-exposed peripheral blood".

July 2001 - 2002: Trainee student in the Molecular Oncology Department from the Institut D'Investigació Biomèdica de Bellvitge (IDIBELL, Barcelona), under the supervision of Dr. Anna Bigas Salvans. Research project "Cross-talk between Notch an NF-κB pathways"

2002-to date: PhD student in the Molecular Oncology Department from the Institut D'Investigació Biomèdica de Bellvitge (IDIBELL, Barcelona), under the supervision of Dr. Anna Bigas Salvans. Research project "Role of Notch/RBPjκ signaling pathway in embryonic hematopoiesis"

FELLOWSHIPS

2002-2005: CIRIT predoctoral fellowship (2002-SI00791) from the *Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR)* of the catalan government.

April 2005: Fellowship for "young researchers" for attendance to EMBO Workshop in Rome, Italy.

July 2005: CIRIT fellowship (BE 00215) for a 3 month stay in Prof. Dr. Elaine Dzierzak's lab in Erasmus MC, Rotterdam, The Netherlands.

PUBLICATIONS

* **Robert-Moreno A**, Espinosa Ll, Sanchez MJ, de la Pompa J, Bigas A.
  "The Notch Pathway Positively Regulates Programmed Cell Death during Erythroid Differentiation".
  Leukemia, May 2007

* Fernandez-Majada V, Aguilera C, Villanueva A, Vilardell F, **Robert-Moreno A**, Aytes A, Real FX, Capella G, Mayo MV, Espinosa L, Bigas A.
  "Nuclear IKK activity leads to dysregulated notch-dependent gene expression in colorectal cancer".
  Proc Natl Acad Sci USA Jan 2; 104 (1): 276-81. Epub 2006 Dec 26

* **Robert-Moreno A,** Espinosa Ll, De la Pompa JL, Bigas A.
  "RBPjk-dependent Notch function regulates GATA-2 and is essential for the formation of intraembryonic hematopoietic cells".
  Development 132(5): 1117-26, 2005

* Espinosa L, Ingles-Esteve J, **Robert-Moreno A**, Bigas A.
  "IkappaBalpha and p65 regulate the cytoplasmic shuttling of nuclear corepressors: cross-talk between Notch an NFkappaB pathways".
  Mol. Biol. Cell, 2003

PARTICIPATION IN INTERNATIONAL MEETINGS

* **Robert-Moreno A** and Bigas A. “Altered Hematopoiesis in RBPjκ null embryos”.
  European Journal of Biochemistry, Vol 270, supl1, Brussels 2003

* EMBO Workshop: “The Notch signaling pathway in development and cancer”.
  Rome, Italy (April, 2005). Participation: oral communication.

* **Robert-Moreno A** and Bigas A. “Notch signaling in embryonic hematopoiesis”.
  12th congress du Club Hématopoïèse et Oncogenèse, France. October 2005.

* 1st Developmental Meeting (Bioregion). November, 2005. Barcelona, Spain.
  Participation: oral communication.

* **Robert-Moreno A**, Ruiz C, Espinosa L, Bigas A. “Notch functions in the embryonic
  Development of hematopoietic stem cells by regulating GATA2 expression”.
  EMBO workshop: “The role and control of GATA factors in tissue development and disease”
  Capri, Italy, April 2007.

* Molecular Hematopoiesis workshop. 12th congress of the European Hematology Association.
  Viena, June 2007.

CHAPTER 8:

REFERENCES

References

- **Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C. T., Bryder, D., Yang, L., Borge, O. J., Thoren, L. A. et al.** (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* **121**, 295-306.
- **Aguilera, C., Hoya-Arias R., Haegeman, G., Espinosa, L., Bigas, A.** (2004). Recruitment of IkBalpha to the hes1 promoter is associated with transcriptional repression. *Proc Natl Acad Sci U S A* **in press**.
- **Akashi, K., Traver, D., Miyamoto, T. and Weissman, I. L.** (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**, 193-7.
- **Amsen, D., Blander, J. M., Lee, G. R., Tanigaki, K., Honjo, T. and Flavell, R. A.** (2004). Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* **117**, 515-26.
- **Antonchuk, J., Sauvageau, G. and Humphries, R. K.** (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell* **109**, 39-45.
- **Arnold, S. J., Stappert, J., Bauer, A., Kispert, A., Herrmann, B. G. and Kemler, R.** (2000). Brachyury is a target gene of the Wnt/beta-catenin signaling pathway. *Mech Dev* **91**, 249-58.
- **Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J.** (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-6.
- **Artavanis-Tsakonas, S. and Simpson, P.** (1991). Choosing a cell fate: a view from the notch locus. *Trends Genet* **7**, 403-408.
- **Austin, T. W., Solar, G. P., Ziegler, F. C., Liem, L. and Matthews, W.** (1997). A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. *Blood* **89**, 3624-35.
- **Bain, G., Maandag, E. C., Izon, D. J., Amsen, D., Kruisbeek, A. M., Weintraub, B. C., Krop, I., Schlissel, M. S., Feeney, A. J., van Roon, M. et al.** (1994). E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell* **79**, 885-92.
- **Baron, M.** (2001). Induction of embryonic hematopoietic and endothelial stem/progenitor cells by hedgehog-mediated signals. *Differentiation* **68**, 175-85.
- **Barrantes, I. B., Elia, A. J., Wunsch, K., De Angelis, M. H., Mak, T. W., Rossant, J., Conlon, R. A., Gossler, A. and de la Pompa, J. L.** (1999). Interaction between Notch signalling and Lunatic fringe during somite boundary formation in the mouse. *Curr Biol* **9**, 470-80.
- **Bartunek, P., Kralova, J., Blendinger, G., Dvorak, M. and Zenke, M.** (2003). GATA-1 and c-myb crosstalk during red blood cell differentiation through GATA-1 binding sites in the c-myb promoter. *Oncogene* **22**, 1927-35.
- **Baumann, C. I., Bailey, A. S., Li, W., Ferkowicz, M. J., Yoder, M. C. and Fleming, W. H.** (2004). PECAM-1 is expressed on hematopoietic stem cells throughout ontogeny and identifies a population of erythroid progenitors. *Blood* **104**, 1010-6.
- **Belaoussoff, M., Farrington, S. M. and Baron, M. H.** (1998). Hematopoietic induction and respecification of A-P identity by visceral endoderm signaling in the mouse embryo. *Development* **125**, 5009-18.
- **Berg, C. P., Engels, I. H., Rothbart, A., Lauber, K., Renz, A., Schlosser, S. F., Schulze-Osthoff, K. and Wesselborg, S.** (2001). Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell Death Differ* **8**, 1197-206.
- **Bertoncello, I.** (1992). Status of high proliferative potential colony-forming cells in the hematopoietic stem cell hierarchy. *Curr Top Microbiol Immunol* **177**, 83-94.
- **Bertrand, J. Y., Giroux, S., Golub, R., Klaine, M., Jalil, A., Boucontet, L., Godin, I. and Cumano, A.** (2005). Characterization of purified intraembryonic hematopoietic stem cells as a tool to define their site of origin. *Proc Natl Acad Sci U S A* **102**, 134-9.
- **Bessho, Y., Miyoshi, G., Sakata, R. and Kageyama, R.** (2001). Hes7: a bHLH-type repressor gene regulated by Notch and expressed in the presomitic mesoderm. *Genes Cells* **6**, 175-85.
- **Bhardwaj, G., Murdoch, B., Wu, D., Baker, D. P., Williams, K. P., Chadwick, K., Ling, L. E., Karanu, F. N. and Bhatia, M.** (2001). Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat Immunol* **2**, 172-80.
- **Bielinska, M., Narita, N., Heikinheimo, M., Porter, S. B. and Wilson, D. B.** (1996). Erythropoiesis and vasculogenesis in embryoid bodies lacking visceral yolk sac endoderm. *Blood* **88**, 3720-30.
- **Bigas, A., Martin, D. I. and Milner, L. A.** (1998). Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. *Mol.Cell Biol* **18**, 2324-2333.
- **Bray, S. J.** (2006). Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* **7**, 678-89.
- **Broudy, V. C.** (1997). Stem cell factor and hematopoiesis. *Blood* **90**, 1345-64.
- **Bryder, D., Ramsfjell, V., Dybedal, I., Theilgaard-Monch, K., Hogerkorp, C. M., Adolfsson, J., Borge, O. J. and Jacobsen, S. E.** (2001). Self-renewal of multipotent long-term repopulating hematopoietic stem cells is negatively regulated by Fas and tumor necrosis factor receptor activation. *J Exp Med* **194**, 941-52.
- **Bulman, M. P., Kusumi, K., Frayling, T. M., McKeown, C., Garrett, C., Lander, E. S., Krumlauf, R., Hattersley, A. T., Ellard, S. and Turnpenny, P. D.** (2000). Mutations in the human delta homologue, DLL3, cause axial skeletal defects in spondylocostal dysostosis. *Nat Genet* **24**, 438-41.
- **Burns, C. E., Traver, D., Mayhall, E., Shepard, J. L. and Zon, L. I.** (2005). Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes Dev* **19**, 2331-42.
- **Buske, C., Feuring-Buske, M., Abramovich, C., Spiekermann, K., Eaves, C. J., Coulombel, L., Sauvageau, G., Hogge, D. E. and Humphries, R. K.** (2002). Deregulated expression of HOXB4 enhances the primitive growth activity of human hematopoietic cells. *Blood* **100**, 862-8.
- **Byrd, N., Becker, S., Maye, P., Narasimhaiah, R., St-Jacques, B., Zhang, X., McMahon, J., McMahon, A. and Grabel, L.** (2002). Hedgehog is required for murine yolk sac angiogenesis. *Development* **129**, 361-72.
- **Cai, Z., de Bruijn, M., Ma, X., Dortland, B., Luteijn, T., Downing, R. J. and Dzierzak, E.** (2000). Haploinsufficiency of AML1 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo. *Immunity* **13**, 423-31.
- **Cantor, A. B. and Orkin, S. H.** (2002). Transcriptional regulation of erythropoiesis: an affair involving multiple partners. *Oncogene* **21**, 3368-76.
- **Catenacci, D. V. and Schiller, G. J.** (2005). Myelodysplasic syndromes: a comprehensive review. *Blood Rev* **19**, 301-19.
- **Ciau-Uitz, A., Walmsley, M. and Patient, R.** (2000). Distinct origins of adult and embryonic blood in Xenopus. *Cell* **102**, 787-96.
- **Conlon, R. A., Reaume, A. G. and Rossant, J.** (1995). Notch1 is required for the coordinate segmentation of somites. *Development* **121**, 1533-45.
- **Couso, J. P., Knust, E. and Martinez Arias, A.** (1995). Serrate and wingless cooperate to induce vestigial gene expression and wing formation in Drosophila. *Curr Biol* **5**, 1437-48.
- **Cudennec, C. A., Thiery, J. P. and Le Douarin, N. M.** (1981). In vitro induction of adult erythropoiesis in early mouse yolk sac. *Proc Natl Acad Sci U S A* **78**, 2412-6.
- **Cumano, A., Dieterlen-Lievre, F. and Godin, I.** (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell* **86**, 907-16.
- **Cumano, A., Ferraz, J. C., Klaine, M., Di Santo, J. P. and Godin, I.** (2001). Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity* **15**, 477-85.
- **Curry, C. L., Reed, L. L., Golde, T. E., Miele, L., Nickoloff, B. J. and Foreman, K. E.** (2005). Gamma secretase inhibitor blocks Notch activation and induces apoptosis in Kaposi's sarcoma tumor cells. *Oncogene* **24**, 6333-44.
- **Chen, C. Z. and Lodish, H. F.** (2005). MicroRNAs as regulators of mammalian hematopoiesis. *Semin Immunol* **17**, 155-65.
- **Cheng, H. T., Kim, M., Valerius, M. T., Surendran, K., Schuster-Gossler, K., Gossler, A., McMahon, A. P. and Kopan, R.** (2007). Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron. *Development* **134**, 801-11.
- **Cheshier, S. H., Morrison, S. J., Liao, X. and Weissman, I. L.** (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci U S A* **96**, 3120-5.
- **Chipuk, J. E. and Green, D. R.** (2006). Dissecting p53-dependent apoptosis. *Cell Death Differ* **13**, 994-1002.
- **Chitnis, A. B.** (1995). The role of Notch in lateral inhibition and cell fate specification. *Mol.Cell Neurosci.*, 311-321.

- **Cho, N. K., Keyes, L., Johnson, E., Heller, J., Ryner, L., Karim, F. and Krasnow, M. A.** (2002). Developmental control of blood cell migration by the Drosophila VEGF pathway. *Cell* **108**, 865-76.
- **Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. and Keller, G.** (1998). A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-32.
- **Dale, J. K., Maroto, M., Dequeant, M. L., Malapert, P., McGrew, M. and Pourquie, O.** (2003). Periodic notch inhibition by lunatic fringe underlies the chick segmentation clock. *Nature* **421**, 275-8.
- **Dazzi, F., Ramasamy, R., Glennie, S., Jones, S. P. and Roberts, I.** (2006). The role of mesenchymal stem cells in haemopoiesis. *Blood Rev* **20**, 161-71.
- **de Bruijn, M. F., Ma, X., Robin, C., Ottersbach, K., Sanchez, M. J. and Dzierzak, E.** (2002). Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* **16**, 673-83.
- **de Bruijn, M. F., Speck, N. A., Peeters, M. C. and Dzierzak, E.** (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *Embo J* **19**, 2465-74.
- **de Celis, J. F., Mari-Beffa, M. and Garcia-Bellido, A.** (1991). Cell-autonomous role of Notch, an epidermal growth factor homologue, in sensory organ differentiation in Drosophila. *Proc Natl Acad Sci U S A* **88**, 632-6.
- **de Groot, R. P., Raaijmakers, J. A., Lammers, J. W. and Koenderman, L.** (2000). STAT5-Dependent CyclinD1 and Bcl-xL expression in Bcr-Abl-transformed cells. *Mol Cell Biol Res Commun* **3**, 299-305.
- **de Guzman, C. G., Warren, A. J., Zhang, Z., Gartland, L., Erickson, P., Drabkin, H., Hiebert, S. W. and Klug, C. A.** (2002). Hematopoietic stem cell expansion and distinct myeloid developmental abnormalities in a murine model of the AML1-ETO translocation. *Mol Cell Biol* **22**, 5506-17.
- **de Haan, G., Weersing, E., Dontje, B., van Os, R., Bystrykh, L. V., Vellenga, E. and Miller, G.** (2003). In vitro generation of long-term repopulating hematopoietic stem cells by fibroblast growth factor-1. *Dev Cell* **4**, 241-51.
- **de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. et al.** (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139-1148.
- **De Maria, R., Testa, U., Luchetti, L., Zeuner, A., Stassi, G., Pelosi, E., Riccioni, R., Felli, N., Samoggia, P. and Peschle, C.** (1999). Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. *Blood* **93**, 796-803.
- **De Maria, R., Zeuner, A., Eramo, A., Domenichelli, C., Bonci, D., Grignani, F., Srinivasula, S. M., Alnemri, E. S., Testa, U. and Peschle, C.** (1999). Negative regulation of erythropoiesis by caspase-mediated cleavage of GATA-1. *Nature* **401**, 489-93.
- **de Pooter, R. F., Schmitt, T. M., de la Pompa, J. L., Fujiwara, Y., Orkin, S. H. and Zuniga-Pflucker, J. C.** (2006). Notch signaling requires GATA-2 to inhibit myelopoiesis from embryonic stem cells and primary hemopoietic progenitors. *J Immunol* **176**, 5267-75.
- **Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S. and Akhurst, R. J.** (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121**, 1845-54.
- **Dieterlen-Lievre, F.** (1975). On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *J Embryol Exp Morphol* **33**, 607-19.
- **Dieterlen-Lievre, F., Pouget, C., Bollerot, K. and Jaffredo, T.** (2006). Are intra-aortic hemopoietic cells derived from endothelial cells during ontogeny? *Trends Cardiovasc Med* **16**, 128-39.
- **Dolznig, H., Grebien, F., Deiner, E. M., Stangl, K., Kolbus, A., Habermann, B., Kerenyi, M. A., Kieslinger, M., Moriggl, R., Beug, H. et al.** (2006). Erythroid progenitor renewal versus differentiation: genetic evidence for cell autonomous, essential functions of EpoR, Stat5 and the GR. *Oncogene* **25**, 2890-900.
- **Domenga, V., Fardoux, P., Lacombe, P., Monet, M., Maciazek, J., Krebs, L. T., Klonjkowski, B., Berrou, E., Mericskay, M., Li, Z. et al.** (2004). Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. *Genes Dev* **18**, 2730-5.
- **Donoviel, D. B., Hadjantonakis, A. K., Ikeda, M., Zheng, H., Hyslop, P. S. and Bernstein, A.** (1999). Mice lacking both presenilin genes exhibit early embryonic patterning defects. *Genes Dev* **13**, 2801-10.
- **Dontje, W., Schotte, R., Cupedo, T., Nagasawa, M., Scheeren, F., Gimeno, R., Spits, H. and Blom, B.** (2006). Delta-like1-induced Notch1 signaling regulates the human plasmacytoid dendritic cell versus T-cell lineage decision through control of GATA-3 and Spi-B. *Blood* **107**, 2446-52.
- **Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., Costa, L., Henrique, D. and Rossant, J.** (2004). Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev* **18**, 2474-8.
- **Dumortier, A., Jeannet, R., Kirstetter, P., Kleinmann, E., Sellars, M., dos Santos, N. R., Thibault, C., Barths, J., Ghysdael, J., Punt, J. A. et al.** (2006). Notch activation is an early and critical event during T-Cell leukemogenesis in Ikaros-deficient mice. *Mol Cell Biol* **26**, 209-20.
- **Duncan, A. W., Rattis, F. M., DiMascio, L. N., Congdon, K. L., Pazianos, G., Zhao, C., Yoon, K., Cook, J. M., Willert, K., Gaiano, N. et al.** (2005). Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat Immunol* **6**, 314-22.
- **Dyer, M. A., Farrington, S. M., Mohn, D., Munday, J. R. and Baron, M. H.** (2001). Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo. *Development* **128**, 1717-30.
- **Dzierzak, E. and Medvinsky, A.** (1995). Mouse embryonic hematopoiesis. *Trends Genet* **11**, 359-66.
- **Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Breant, C. and Le Douarin, N. M.** (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**, 5141-6.
- **Elefanty, A. G., Begley, C. G., Hartley, L., Papaevangeliou, B. and Robb, L.** (1999). SCL expression in the mouse embryo detected with a targeted lacZ reporter gene demonstrates its localization to hematopoietic, vascular, and neural tissues. *Blood* **94**, 3754-63.
- **Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D. and Sklar, J.** (1991). TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**, 649-61.
- **Ema, H. and Nakauchi, H.** (2000). Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood* **95**, 2284-8.
- **Emambokus, N., Vegiopoulos, A., Harman, B., Jenkinson, E., Anderson, G. and Frampton, J.** (2003). Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. *Embo J* **22**, 4478-88.
- **Faloon, P., Arentson, E., Kazarov, A., Deng, C. X., Porcher, C., Orkin, S. and Choi, K.** (2000). Basic fibroblast growth factor positively regulates hematopoietic development. *Development* **127**, 1931-41.
- **Fazi, F., Rosa, A., Fatica, A., Gelmetti, V., De Marchis, M. L., Nervi, C. and Bozzoni, I.** (2005). A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell* **123**, 819-31.
- **Felli, M. P., Maroder, M., Mitsiadis, T. A., Campese, A. F., Bellavia, D., Vacca, A., Mann, R. S., Frati, L., Lendahl, U., Gulino, A. et al.** (1999). Expression pattern of notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand-receptor interactions in intrathymic T cell development. *Int Immunol* **11**, 1017-25.
- **Felli, N., Fontana, L., Pelosi, E., Botta, R., Bonci, D., Facchiano, F., Liuzzi, F., Lulli, V., Morsilli, O., Santoro, S. et al.** (2005). MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc Natl Acad Sci U S A* **102**, 18081-6.
- **Ferkowicz, M. J., Starr, M., Xie, X., Li, W., Johnson, S. A., Shelley, W. C., Morrison, P. R. and Yoder, M. C.** (2003). CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development* **130**, 4393-403.
- **Fernandez-Majada, V., Aguilera, C., Villanueva, A., Vilardell, F., Robert-Moreno, A., Aytes, A., Real, F. X., Capella, G., Mayo, M. W., Espinosa, L. et al.** (2007). Nuclear IKK activity leads to dysregulated notch-dependent gene expression in colorectal cancer. *Proc Natl Acad Sci U S A* **104**, 276-81.
- **Fischer, A., Schumacher, N., Maier, M., Sendtner, M. and Gessler, M.** (2004). The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev* **18**, 901-11.
- **Fluckiger, A. C., Sanz, E., Garcia-Lloret, M., Su, T., Hao, Q. L., Kato, R., Quan, S., de la Hera, A., Crooks, G. M., Witte, O. N. et al.** (1998). In vitro reconstitution of human B-cell ontogeny: from CD34(+) multipotent progenitors to Ig-secreting cells. *Blood* **92**, 4509-20.
- **Fortini, M. E., Rebay, I., Caron, L. A. and Artavanis-Tsakonas, S.** (1993). An activated notch receptor blocks cell-fate commitment in the developing drosophila eye. *Nature* **365**, 555-557.
- **French, S. W., Hoyer, K. K., Shen, R. R. and Teitell, M. A.** (2002). Transdifferentiation and nuclear reprogramming in hematopoietic development and neoplasia. *Immunol Rev* **187**, 22-39.
- **Fujiwara, Y., Browne, C. P., Cunniff, K., Goff, S. C. and Orkin, S. H.** (1996). Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc Natl Acad Sci U S A* **93**, 12355-8.

- **Ganguli, G., Back, J., Sengupta, S. and Wasylyk, B.** (2002). The p53 tumour suppressor inhibits glucocorticoid-induced proliferation of erythroid progenitors. *EMBO Rep* **3**, 569-74.
- **Garcia-Porrero, J. A., Godin, I. E. and Dieterlen-Lievre, F.** (1995). Potential intraembryonic hemogenic sites at pre-liver stages in the mouse. *Anat Embryol (Berl)* **192**, 425-35.
- **Gekas, C., Dieterlen-Lievre, F., Orkin, S. H. and Mikkola, H. K.** (2005). The placenta is a niche for hematopoietic stem cells. *Dev Cell* **8**, 365-75.
- **Gering, M. and Patient, R.** (2005). Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. *Dev Cell* **8**, 389-400.
- **Godin, I. and Cumano, A.** (2002). The hare and the tortoise: an embryonic haematopoietic race. *Nat Rev Immunol* **2**, 593-604.
- **Godin, I., Dieterlen-Lievre, F. and Cumano, A.** (1995). Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. *Proc Natl Acad Sci U S A* **92**, 773-7.
- **Godin, I., Garcia-Porrero, J. A., Dieterlen-Lievre, F. and Cumano, A.** (1999). Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites. *J Exp Med* **190**, 43-52.
- **Godin, I. E., Garcia-Porrero, J. A., Coutinho, A., Dieterlen-Lievre, F. and Marcos, M. A.** (1993). Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. *Nature* **364**, 67-70.
- **Graf, T.** (2002). Differentiation plasticity of hematopoietic cells. *Blood* **99**, 3089-101.
- **Green, A. R., DeLuca, E. and Begley, C. G.** (1991). Antisense SCL suppresses self-renewal and enhances spontaneous erythroid differentiation of the human leukaemic cell line K562. *Embo J* **10**, 4153-8.
- **Grego-Bessa, J., Luna-Zurita, L., del Monte, G., Bolos, V., Melgar, P., Arandilla, A., Garratt, A. N., Zang, H., Mukouyama, Y. S., Chen, H. et al.** (2007). Notch signaling is essential for ventricular chamber development. *Dev Cell* **12**, 415-29.
- **Gregory, T., Yu, C., Ma, A., Orkin, S. H., Blobel, G. A. and Weiss, M. J.** (1999). GATA-1 and erythropoietin cooperate to promote erythroid cell survival by regulating bcl-xL expression. *Blood* **94**, 87-96.
- **Hadland, B. K., Huppert, S. S., Kanungo, J., Xue, Y., Jiang, R., Gridley, T., Conlon, R. A., Cheng, A. M., Kopan, R. and Longmore, G. D.** (2004). A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. *Blood* **104**, 3097-105.
- **Hamada, Y., Kadokawa, Y., Okabe, M., Ikawa, M., Coleman, J. R. and Tsujimoto, Y.** (1999). Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. *Development* **126**, 3415-24.
- **Hamaguchi, I., Huang, X. L., Takakura, N., Tada, J., Yamaguchi, Y., Kodama, H. and Suda, T.** (1999). In vitro hematopoietic and endothelial cell development from cells expressing TEK receptor in murine aorta-gonad-mesonephros region. *Blood* **93**, 1549-56.
- **Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K. and Honjo, T.** (2002). Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol* **14**, 637-45.
- **Hare, K. J., Jenkinson, E. J. and Anderson, G.** (1999). In vitro models of T cell development. *Semin Immunol* **11**, 3-12.
- **Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P.** (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. *Development*, 161-171.
- **Hengartner, M. O.** (2000). The biochemistry of apoptosis. *Nature* **407**, 770-6.
- **Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F. and Weinmaster, G.** (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat Cell Biol* **2**, 515-20.
- **Hirai, H., Samokhvalov, I. M., Fujimoto, T., Nishikawa, S., Imanishi, J. and Nishikawa, S.** (2005). Involvement of Runx1 in the downregulation of fetal liver kinase-1 expression during transition of endothelial cells to hematopoietic cells. *Blood* **106**, 1948-55.
- **Hodge, D., Coghill, E., Keys, J., Maguire, T., Hartmann, B., McDowall, A., Weiss, M., Grimmond, S. and Perkins, A.** (2006). A global role for EKLF in definitive and primitive erythropoiesis. *Blood* **107**, 3359-70.
- **Hsu, H. C., Ema, H., Osawa, M., Nakamura, Y., Suda, T. and Nakauchi, H.** (2000). Hematopoietic stem cells express Tie-2 receptor in the murine fetal liver. *Blood* **96**, 3757-62.
- **Huang, Q., Raya, A., DeJesus, P., Chao, S. H., Quon, K. C., Caldwell, J. S., Chanda, S. K., Izpisua-Belmonte, J. C. and Schultz, P. G.** (2004). Identification of p53 regulators by genome-wide functional analysis. *Proc Natl Acad Sci U S A* **101**, 3456-61.
- **Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J. and Keller, G.** (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* **432**, 625-30.
- **Huppert, S. S., Le, A., Schroeter, E. H., Mumm, J. S., Saxena, M. T., Milner, L. A. and Kopan, R.** (2000). Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1. *Nature* **405**, 966-70.
- **Irvine, K. D.** (1999). Fringe, Notch, and making developmental boundaries. *Curr Opin Genet Dev* **9**, 434-41.
- **Ishiko, E., Matsumura, I., Ezoe, S., Gale, K., Ishiko, J., Satoh, Y., Tanaka, H., Shibayama, H., Mizuki, M., Era, T. et al.** (2005). Notch signals inhibit the development of erythroid/megakaryocytic cells by suppressing GATA-1 activity through the induction of HES1. *J Biol Chem* **280**, 4929-39.
- **Iso, T., Kedes, L. and Hamamori, Y.** (2003). HES and HERP families: multiple effectors of the Notch signaling pathway. *J Cell Physiol* **194**, 237-55.
- **Itoh, M., Kim, C. H., Palardy, G., Oda, T., Jiang, Y. J., Maust, D., Yeo, S. Y., Lorick, K., Wright, G. J., Ariza-McNaughton, L. et al.** (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Dev Cell* **4**, 67-82.
- **Izraeli, S.** (2004). Leukaemia -- a developmental perspective. *Br J Haematol* **126**, 3-10.
- **Jacobs-Helber, S. M., Roh, K. H., Bailey, D., Dessypris, E. N., Ryan, J. J., Chen, J., Wickrema, A., Barber, D. L., Dent, P. and Sawyer, S. T.** (2003). Tumor necrosis factor-alpha expressed constitutively in erythroid cells or induced by erythropoietin has negative and stimulatory roles in normal erythropoiesis and erythroleukemia. *Blood* **101**, 524-31.
- **Jang, M. S., Miao, H., Carlesso, N., Shelly, L., Zlobin, A., Darack, N., Qin, J. Z., Nickoloff, B. J. and Miele, L.** (2004). Notch-1 regulates cell death independently of differentiation in murine erythroleukemia cells through multiple apoptosis and cell cycle pathways. *J Cell Physiol* **199**, 418-33.
- **Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A.** (1995). Signalling downstream of activated mammalian Notch. *Nature* **377**, 355-8.
- **Jehn, B. M., Bielke, W., Pear, W. S. and Osborne, B. A.** (1999). Cutting edge: protective effects of notch-1 on TCR-induced apoptosis. *J Immunol* **162**, 635-8.
- **Jiang, R., Lan, Y., Chapman, H. D., Shawber, C., Norton, C. R., Serreze, D. V., Weinmaster, G. and Gridley, T.** (1998). Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev* **12**, 1046-57.
- **Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabriat, H., Mouton, P., Alamowitch, S., Domenga, V., Cecillion, M., Marechal, E. et al.** (1996). Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* **383**, 707-10.
- **Jouve, C., Palmeirim, I., Henrique, D., Beckers, J., Gossler, A., Ish-Horowicz, D. and Pourquie, O.** (2000). Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm. *Development* **127**, 1421-9.
- **Juarez, J. and Bendall, L.** (2004). SDF-1 and CXCR4 in normal and malignant hematopoiesis. *Histol Histopathol* **19**, 299-309.
- **Jundt, F., Anagnostopoulos, I., Forster, R., Mathas, S., Stein, H. and Dorken, B.** (2002). Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. *Blood* **99**, 3398-403.
- **Kao, H. Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C. R., Evans, R. M. and Kadesch, T.** (1998). A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes Dev* **12**, 2269-77.
- **Karanu, F. N., Murdoch, B., Gallacher, L., Wu, D. M., Koremoto, M., Sakano, S. and Bhatia, M.** (2000). The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. *J Exp Med* **192**, 1365-72.
- **Karanu, F. N., Murdoch, B., Miyabayashi, T., Ohno, M., Koremoto, M., Gallacher, L., Wu, D., Itoh, A., Sakano, S. and Bhatia, M.** (2001). Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. *Blood* **97**, 1960-7.
- **Kashiwakura, I. and Takahashi, T. A.** (2005). Fibroblast growth factor and ex vivo expansion of hematopoietic progenitor cells. *Leuk Lymphoma* **46**, 329-33.
- **Kawamata, S., Du, C., Li, K. and Lavau, C.** (2002). Overexpression of the Notch target genes Hes in vivo induces lymphoid and myeloid alterations. *Oncogene* **21**, 3855-63.
- **Keller, G., Kennedy, M., Papayannopoulou, T. and Wiles, M. V.** (1993). Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol Cell Biol* **13**, 473-86.
- **Khan, N. I. and Bendall, L. J.** (2006). Role of WNT signaling in normal and malignant hematopoiesis. *Histol Histopathol* **21**, 761-74.
- **Kim, I., Yilmaz, O. H. and Morrison, S. J.** (2005). CD144 (VE-cadherin) is transiently expressed by fetal liver hematopoietic stem cells. *Blood* **106**, 903-5.

- **Kimura, T., Sonoda, Y., Iwai, N., Satoh, M., Yamaguchi-Tsukio, M., Izui, T., Suda, M., Sasaki, K. and Nakano, T.** (2000). Proliferation and cell death of embryonic primitive erythrocytes. *Exp Hematol* **28**, 635-41.
- **Kina, T., Ikuta, K., Takayama, E., Wada, K., Majumdar, A. S., Weissman, I. L. and Katsura, Y.** (2000). The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. *Br J Haematol* **109**, 280-7.
- **Kirstetter, P., Anderson, K., Porse, B. T., Jacobsen, S. E. and Nerlov, C.** (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat Immunol* **7**, 1048-56.
- **Klug, C. A., Morrison, S. J., Masek, M., Hahm, K., Smale, S. T. and Weissman, I. L.** (1998). Hematopoietic stem cells and lymphoid progenitors express different Ikaros isoforms, and Ikaros is localized to heterochromatin in immature lymphocytes. *Proc Natl Acad Sci U S A* **95**, 657-62.
- **Koibuchi, N., Taniyama, Y., Nagao, K., Ogihara, T., Kaneda, Y. and Morishita, R.** (2006). The effect of VEGF on blood vessels and blood cells during Xenopus development. *Biochem Biophys Res Commun* **344**, 339-45.
- **Kondo, M., Weissman, I. L. and Akashi, K.** (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661-72.
- **Koo, B. K., Lim, H. S., Song, R., Yoon, M. J., Yoon, K. J., Moon, J. S., Kim, Y. W., Kwon, M. C., Yoo, K. W., Kong, M. P. et al.** (2005). Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. *Development* **132**, 3459-70.
- **Kopan, R., Schroeter, E. H., Weintraub, H. and Nye, J. S.** (1996). Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. *Proc Natl Acad Sci U S A* **93**, 1683-8.
- **Krebs, L. T., Shutter, J. R., Tanigaki, K., Honjo, T., Stark, K. L. and Gridley, T.** (2004). Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev* **18**, 2469-73.
- **Krebs, L. T., Xue, Y., Norton, C. R., Shutter, J. R., Maguire, M., Sundberg, J. P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R. et al.** (2000). Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev* **14**, 1343-52.
- **Krebs, L. T., Xue, Y., Norton, C. R., Sundberg, J. P., Beatus, P., Lendahl, U., Joutel, A. and Gridley, T.** (2003). Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. *Genesis* **37**, 139-43.
- **Kumano, K., Chiba, S., Kunisato, A., Sata, M., Saito, T., Nakagami-Yamaguchi, E., Yamaguchi, T., Masuda, S., Shimizu, K., Takahashi, T. et al.** (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. and Hirai, H.** (2001). Notch1 inhibits differentiation of hematopoietic cells by sustaining GATA-2 expression. *Blood* **98**, 3283-9.
- **Kumaravelu, P., Hook, L., Morrison, A. M., Ure, J., Zhao, S., Zuyev, S., Ansell, J. and Medvinsky, A.** (2002). Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development* **129**, 4891-9.
- **Kunisato, A., Chiba, S., Nakagami-Yamaguchi, E., Kumano, K., Saito, T., Masuda, S., Yamaguchi, T., Osawa, M., Kageyama, R., Nakauchi, H. et al.** (2003). HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo. *Blood* **101**, 1777-83.
- **Kurz, H. and Christ, B.** (1998). Embryonic CNS macrophages and microglia do not stem from circulating, but from extravascular precursors. *Glia* **22**, 98-102.
- **Kyba, M., Perlingeiro, R. C. and Daley, G. Q.** (2002). HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* **109**, 29-37.
- **Lacaud, G., Gore, L., Kennedy, M., Kouskoff, V., Kingsley, P., Hogan, C., Carlsson, L., Speck, N., Palis, J. and Keller, G.** (2002). Runx1 is essential for hematopoietic commitment at the hemangioblast stage of development in vitro. *Blood* **100**, 458-66.
- **Lai, E. C.** (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-73.
- **Lai, E. C., Deblandre, G. A., Kintner, C. and Rubin, G. M.** (2001). Drosophila neuralized is a ubiquitin ligase that promotes the internalization and degradation of delta. *Dev Cell* **1**, 783-94.
- **Laiosa, C. V., Stadtfeld, M. and Graf, T.** (2006). Determinants of lymphoid-myeloid lineage diversification. *Annu Rev Immunol* **24**, 705-38.
- **Lantz, C. S., Boesiger, J., Song, C. H., Mach, N., Kobayashi, T., Mulligan, R. C., Nawa, Y., Dranoff, G. and Galli, S. J.** (1998). Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites. *Nature* **392**, 90-3.
- **Larsson, J. and Karlsson, S.** (2005). The role of Smad signaling in hematopoiesis. *Oncogene* **24**, 5676-92.
- **Lebestky, T., Jung, S. H. and Banerjee, U.** (2003). A Serrate-expressing signaling center controls Drosophila hematopoiesis. *Genes Dev* **17**, 348-53.
- **Leroy, H., Roumier, C., Huyghe, P., Biggio, V., Fenaux, P. and Preudhomme, C.** (2005). CEBPA point mutations in hematological malignancies. *Leukemia* **19**, 329-34.
- **Lewis, J.** (1998). Notch signalling and the control of cell fate choices in vertebrates. *Semin Cell Dev Biol* **9**, 583-9.
- **Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J. et al.** (1997). Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet* **16**, 243-51.
- **Li, Z. and Li, L.** (2006). Understanding hematopoietic stem-cell microenvironments. *Trends Biochem Sci* **31**, 589-95.
- **Lin, C. S., Lim, S. K., D'Agati, V. and Costantini, F.** (1996). Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. *Genes Dev* **10**, 154-64.
- **Ling, K. W. and Dzierzak, E.** (2002). Ontogeny and genetics of the hemato/lymphopoietic system. *Curr Opin Immunol* **14**, 186-91.
- **Ling, K. W., Ottersbach, K., van Hamburg, J. P., Oziemlak, A., Tsai, F. Y., Orkin, S. H., Ploemacher, R., Hendriks, R. W. and Dzierzak, E.** (2004). GATA-2 plays two functionally distinct roles during the ontogeny of hematopoietic stem cells. *J Exp Med* **200**, 871-82.
- **Linnekin, D.** (1999). Early signaling pathways activated by c-Kit in hematopoietic cells. *Int J Biochem Cell Biol* **31**, 1053-74.
- **Liu, B., Sun, Y., Jiang, F., Zhang, S., Wu, Y., Lan, Y., Yang, X. and Mao, N.** (2003). Disruption of Smad5 gene leads to enhanced proliferation of high-proliferative potential precursors during embryonic hematopoiesis. *Blood* **101**, 124-33.
- **Lotem, J. and Sachs, L.** (2002). Cytokine control of developmental programs in normal hematopoiesis and leukemia. *Oncogene* **21**, 3284-94.
- **Lozzio, B. B., Lozzio, C. B., Bamberger, E. G. and Feliu, A. S.** (1981). A multipotential leukemia cell line (K-562) of human origin. *Proc Soc Exp Biol Med* **166**, 546-50.
- **Lugus, J. J., Chung, Y. S., Mills, J. C., Kim, S. I., Grass, J., Kyba, M., Doherty, J. M., Bresnick, E. H. and Choi, K.** (2007). GATA2 functions at multiple steps in hemangioblast development and differentiation. *Development* **134**, 393-405.
- **Ma, X., Robin, C., Ottersbach, K. and Dzierzak, E.** (2002). The Ly-6A (Sca-1) GFP transgene is expressed in all adult mouse hematopoietic stem cells. *Stem Cells* **20**, 514-21.
- **Maciejewski, J., Selleri, C., Anderson, S. and Young, N. S.** (1995). Fas antigen expression on CD34+ human marrow cells is induced by interferon gamma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoietic suppression in vitro. *Blood* **85**, 3183-90.
- **Maetens, M., Doumont, G., Clercq, S. D., Francoz, S., Froment, P., Bellefroid, E., Klingmuller, U., Lozano, G. and Marine, J. C.** (2007). Distinct roles of Mdm2 and Mdm4 in red cell production. *Blood* **109**, 2630-3.
- **Maillard, I., Adler, S. H. and Pear, W. S.** (2003). Notch and the immune system. *Immunity* **19**, 781-91.
- **Manaia, A., Lemarchandel, V., Klaine, M., Max-Audit, I., Romeo, P., Dieterlen-Lievre, F. and Godin, I.** (2000). Lmo2 and GATA-3 associated expression in intraembryonic hemogenic sites. *Development* **127**, 643-53.
- **Mancini, S. J., Mantei, N., Dumortier, A., Suter, U., MacDonald, H. R. and Radtke, F.** (2005). Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood* **105**, 2340-2.
- **Mandal, L., Banerjee, U. and Hartenstein, V.** (2004). Evidence for a fruit fly hemangioblast and similarities between lymph-gland hematopoiesis in fruit fly and mammal aorta-gonadal-mesonephros mesoderm. *Nat Genet* **36**, 1019-23.
- **Marks, P. A. and Rifkind, R. A.** (1988). Hexamethylene bisacetamide-induced differentiation of transformed cells: molecular and cellular effects and therapeutic application. *Int J Cell Cloning* **6**, 230-40.
- **Martin, R., Lahlil, R., Damert, A., Miquerol, L., Nagy, A., Keller, G. and Hoang, T.** (2004). SCL interacts with VEGF to suppress apoptosis at the onset of hematopoiesis. *Development* **131**, 693-702.
- **Matsumoto, N., Kubo, A., Liu, H., Akita, K., Laub, F., Ramirez, F., Keller, G. and Friedman, S. L.** (2006). Developmental regulation of yolk sac hematopoiesis by Kruppel-like factor 6. *Blood* **107**, 1357-65.

- **Matsuoka, S., Tsuji, K., Hisakawa, H., Xu, M., Ebihara, Y., Ishii, T., Sugiyama, D., Manabe, A., Tanaka, R., Ikeda, Y. et al.** (2001). Generation of definitive hematopoietic stem cells from murine early yolk sac and paraaortic splanchnopleures by aorta-gonad-mesonephros region-derived stromal cells. *Blood* **98**, 6-12.
- **McGrath, K. E. and Palis, J.** (2005). Hematopoiesis in the yolk sac: more than meets the eye. *Exp Hematol* **33**, 1021-8.
- **McKercher, S. R., Torbett, B. E., Anderson, K. L., Henkel, G. W., Vestal, D. J., Baribault, H., Klemsz, M., Feeney, A. J., Wu, G. E., Paige, C. J. et al.** (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *Embo J* **15**, 5647-58.
- **Medvinsky, A. and Dzierzak, E.** (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906.
- **Medvinsky, A. L., Samoylina, N. L., Muller, A. M. and Dzierzak, E. A.** (1993). An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature* **364**, 64-7.
- **Metcalf, D., Moore, A. S. and Shortman, K.** (1971). Adherence column and buoyant density separation of bone marrow stem cells and more differentiated cells. *J Cell Physiol* **78**, 441-50.
- **Mikkola, H. K., Gekas, C., Orkin, S. H. and Dieterlen-Lievre, F.** (2005). Placenta as a site for hematopoietic stem cell development. *Exp Hematol* **33**, 1048-54.
- **Mikkola, H. K., Klintman, J., Yang, H., Hock, H., Schlaeger, T. M., Fujiwara, Y. and Orkin, S. H.** (2003). Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature* **421**, 547-51.
- **Miles, C., Sanchez, M. J., Sinclair, A. and Dzierzak, E.** (1997). Expression of the Ly-6E.1 (Sca-1) transgene in adult hematopoietic stem cells and the developing mouse embryo. *Development* **124**, 537-47.
- **Milner, L. A. and Bigas, A.** (1999). Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. *Blood* **93**, 2431-48.
- **Milner, L. A., Bigas, A., Kopan, R., Brashem-Stein, C., Bernstein, I. D. and Martin, D. I.** (1996). Inhibition of granulocytic differentiation by mNotch1. *Proc.Natl.Acad.Sci.U.S.A.* **93**, 13014-13019.
- **Milner, L. A., Kopan, R., Martin, D. I. and Bernstein, I. D.** (1994). A human homologue of the Drosophila developmental gene, Notch, is expressed in CD34+ hematopoietic precursors. *Blood* **83**, 2057-62.
- **Minegishi, N., Morita, S., Minegishi, M., Tsuchiya, S., Konno, T., Hayashi, N. and Yamamoto, M.** (1997). Expression of GATA transcription factors in myelogenous and lymphoblastic leukemia cells. *Int J Hematol* **65**, 239-49.
- **Minegishi, N., Ohta, J., Yamagiwa, H., Suzuki, N., Kawauchi, S., Zhou, Y., Takahashi, S., Hayashi, N., Engel, J. D. and Yamamoto, M.** (1999). The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region. *Blood* **93**, 4196-207.
- **Miyamoto, Y., Maitra, A., Ghosh, B., Zechner, U., Argani, P., Iacobuzio-Donahue, C. A., Sriuranpong, V., Iso, T., Meszoely, I. M., Wolfe, M. S. et al.** (2003). Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell* **3**, 565-76.
- **Mohtashami, M. and Zuniga-Pflucker, J. C.** (2006). Three-dimensional architecture of the thymus is required to maintain delta-like expression necessary for inducing T cell development. *J Immunol* **176**, 730-4.
- **Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S. et al.** (2000). Fringe is a glycosyltransferase that modifies Notch. *Nature* **406**, 369-75.
- **Moore, M. A.** (2005). Converging pathways in leukemogenesis and stem cell self-renewal. *Exp Hematol* **33**, 719-37.
- **Motoyama, N., Wang, F., Roth, K. A., Sawa, H., Nakayama, K., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., Fujii, S. et al.** (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science* **267**, 1506-10.
- **Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J., Jr. and Potter, S. S.** (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell* **65**, 677-89.
- **Mukouyama, Y., Chiba, N., Mucenski, M. L., Satake, M., Miyajima, A., Hara, T. and Watanabe, T.** (1999). Hematopoietic cells in cultures of the murine embryonic aorta-gonad-mesonephros region are induced by c-Myb. *Curr Biol* **9**, 833-6.
- **Mulcahy, L.** (2001). The erythropoietin receptor. *Semin Oncol* **28**, 19-23.
- **Muller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F. and Dzierzak, E.** (1994). Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1**, 291-301.
- **Nakagawa, M., Ichikawa, M., Kumano, K., Goyama, S., Kawazu, M., Asai, T., Ogawa, S., Kurokawa, M. and Chiba, S.** (2006). AML1/Runx1 rescues Notch1-null mutation-induced deficiency of para-aortic splanchnopleural hematopoiesis. *Blood* **108**, 3329-34.
- **Nakagawa, O., McFadden, D. G., Nakagawa, M., Yanagisawa, H., Hu, T., Srivastava, D. and Olson, E. N.** (2000). Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of notch signaling. *Proc Natl Acad Sci U S A* **97**, 13655-60.
- **Neubauer, H., Cumano, A., Muller, M., Wu, H., Huffstadt, U. and Pfeffer, K.** (1998). Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell* **93**, 397-409.
- **Nichogiannopoulou, A., Trevisan, M., Neben, S., Friedrich, C. and Georgopoulos, K.** (1999). Defects in hemopoietic stem cell activity in Ikaros mutant mice. *J Exp Med* **190**, 1201-14.
- **Nichols, J. T., Miyamoto, A., Olsen, S. L., D'Souza, B., Yao, C. and Weinmaster, G.** (2007). DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. *J Cell Biol* **176**, 445-58.
- **Nichols, K. E., Crispino, J. D., Poncz, M., White, J. G., Orkin, S. H., Maris, J. M. and Weiss, M. J.** (2000). Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1. *Nat Genet* **24**, 266-70.
- **Nishinakamura, R., Miyajima, A., Mee, P. J., Tybulewicz, V. L. and Murray, R.** (1996). Hematopoiesis in mice lacking the entire granulocyte-macrophage colony-stimulating factor/interleukin-3/interleukin-5 functions. *Blood* **88**, 2458-64.
- **North, T., Gu, T. L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M. and Speck, N. A.** (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* **126**, 2563-75.
- **North, T. E., de Bruijn, M. F., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M., Dzierzak, E. and Speck, N. A.** (2002). Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity* **16**, 661-72.
- **Nutt, S. L., Urbanek, P., Rolink, A. and Busslinger, M.** (1997). Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev* **11**, 476-91.
- **Ohishi, K., Katayama, N., Shiku, H., Varnum-Finney, B. and Bernstein, I. D.** (2003). Notch signalling in hematopoiesis. *Semin Cell Dev Biol* **14**, 143-50.
- **Ohishi, K., Varnum-Finney, B., Flowers, D., Anasetti, C., Myerson, D. and Bernstein, I. D.** (2000). Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. *Blood* **95**, 2847-54.
- **Ohneda, K. and Yamamoto, M.** (2002). Roles of hematopoietic transcription factors GATA-1 and GATA-2 in the development of red blood cell lineage. *Acta Haematol* **108**, 237-45.
- **Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F. and Kageyama, R.** (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *Embo J* **18**, 2196-207.
- **Oka, C., Nakano, T., Wakeham, A., de la Pompa, J. L., Mori, C., Sakai, T., Okazaki, S., Kawaichi, M., Shiota, K., Mak, T. W. et al.** (1995). Disruption of the mouse RBP-J kappa gene results in early embryonic death. *Development* **121**, 3291-301.
- **Okada, H., Watanabe, T., Niki, M., Takano, H., Chiba, N., Yanai, N., Tani, K., Hibino, H., Asano, S., Mucenski, M. L. et al.** (1998). AML1(-/-) embryos do not express certain hematopoiesis-related gene transcripts including those of the PU.1 gene. *Oncogene* **17**, 2287-93.
- **Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G. and Downing, J. R.** (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84**, 321-30.
- **Olsen, A. L., Stachura, D. L. and Weiss, M. J.** (2006). Designer blood: creating hematopoietic lineages from embryonic stem cells. *Blood* **107**, 1265-75.
- **Orkin, S. H.** (2000). Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* **1**, 57-64.
- **Osawa, M., Hanada, K., Hamada, H. and Nakauchi, H.** (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**, 242-5.
- **Otani, T., Inoue, T., Tsuji-Takayama, K., Ijiri, Y., Nakamura, S., Motoda, R. and Orita, K.** (2005). Progenitor analysis of primitive erythropoiesis generated from in vitro culture of embryonic stem cells. *Exp Hematol* **33**, 632-40.
- **Ottersbach, K. and Dzierzak, E.** (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell* **8**, 377-87.
- **Palis, J., McGrath, K. E. and Kingsley, P. D.** (1995). Initiation of hematopoiesis and vasculogenesis in murine yolk sac explants. *Blood* **86**, 156-63.

- **Palis, J., Robertson, S., Kennedy, M., Wall, C. and Keller, G.** (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126**, 5073-84.
- **Palis, J. and Segel, G. B.** (1998). Developmental biology of erythropoiesis. *Blood Rev* **12**, 106-14.
- **Palis, J. and Yoder, M. C.** (2001). Yolk-sac hematopoiesis: the first blood cells of mouse and man. *Exp Hematol* **29**, 927-36.
- **Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D.** (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912.
- **Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L. M., Catala, M. and Dieterlen-Lievre, F.** (1996). Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development* **122**, 1363-71.
- **Parks, A. L., Huppert, S. S. and Muskavitch, M. A.** (1997). The dynamics of neurogenic signalling underlying bristle development in Drosophila melanogaster. *Mech Dev* **63**, 61-74.
- **Patient, R. K. and McGhee, J. D.** (2002). The GATA family (vertebrates and invertebrates). *Curr Opin Genet Dev* **12**, 416-22.
- **Peller, S., Frenkel, J., Lapidot, T., Kahn, J., Rahimi-Levene, N., Yona, R., Nissim, L., Goldfinger, N., Sherman, D. J. and Rotter, V.** (2003). The onset of p53-dependent apoptosis plays a role in terminal differentiation of human normoblasts. *Oncogene* **22**, 4648-55.
- **Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F. W. and Orkin, S. H.** (1996). The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell*, 47-57.
- **Pouget, C., Gautier, R., Teillet, M. A. and Jaffredo, T.** (2006). Somite-derived cells replace ventral aortic hemangioblasts and provide aortic smooth muscle cells of the trunk. *Development* **133**, 1013-22.
- **Pui, J. C., Allman, D., Xu, L., DeRocco, S., Karnell, F. G., Bakkour, S., Lee, J. Y., Kadesch, T., Hardy, R. R., Aster, J. C. et al.** (1999). Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* **11**, 299-308.
- **Radtke, F. and Raj, K.** (2003). The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat Rev Cancer* **3**, 756-67.
- **Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R. and Aguet, M.** (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547-58.
- **Rangarajan, A., Talora, C., Okuyama, R., Nicolas, M., Mammucari, C., Oh, H., Aster, J. C., Krishna, S., Metzger, D., Chambon, P. et al.** (2001). Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *Embo J* **20**, 3427-36.
- **Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S.** (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67**, 687-99.
- **Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R. and Weissman, I. L.** (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* **423**, 409-14.
- **Robb, L., Elwood, N. J., Elefanty, A. G., Kontgen, F., Li, R., Barnett, L. D. and Begley, C. G.** (1996). The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse. *Embo J* **15**, 4123-9.
- **Robb, L., Lyons, I., Li, R., Hartley, L., Kontgen, F., Harvey, R. P., Metcalf, D. and Begley, C. G.** (1995). Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. *Proc Natl Acad Sci U S A* **92**, 7075-9.
- **Robert-Moreno, A., Espinosa, L., de la Pompa, J. L. and Bigas, A.** (2005). RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. *Development* **132**, 1117-26.
- **Robert-Moreno, A., Espinosa, L., Sanchez, M. J., de la Pompa, J. L. and Bigas, A.** (2007). The notch pathway positively regulates programmed cell death during erythroid differentiation. *Leukemia*.
- **Robey, E., Chang, D., Itano, A., Cado, D., Alexander, H., Lans, D., Weinmaster, G. and Salmon, P.** (1996). An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell* **87**, 483-92.
- **Robin, C., Ottersbach, K., Durand, C., Peeters, M., Vanes, L., Tybulewicz, V. and Dzierzak, E.** (2006). An unexpected role for IL-3 in the embryonic development of hematopoietic stem cells. *Dev Cell* **11**, 171-80.
- **Rodrigues, N. P., Janzen, V., Forkert, R., Dombkowski, D. M., Boyd, A. S., Orkin, S. H., Enver, T., Vyas, P. and Scadden, D. T.** (2005). Haploinsufficiency of GATA-2 perturbs adult hematopoietic stem-cell homeostasis. *Blood* **106**, 477-84.
- **Romer, S., Saunders, U., Jack, H. M. and Jehn, B. M.** (2003). Notch1 enhances B-cell receptor-induced apoptosis in mature activated B cells without affecting cell cycle progression and surface IgM expression. *Cell Death Differ* **10**, 833-44.
- **Sabin, F. R.** (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. 1917. *J Hematother Stem Cell Res* **11**, 5-7.
- **Samokhvalov, I. M., Samokhvalova, N. I. and Nishikawa, S.** (2007). Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. *Nature* **446**, 1056-61.
- **Sanchez-Irizarry, C., Carpenter, A. C., Weng, A. P., Pear, W. S., Aster, J. C. and Blacklow, S. C.** (2004). Notch subunit heterodimerization and prevention of ligand-independent proteolytic activation depend, respectively, on a novel domain and the LNR repeats. *Mol Cell Biol* **24**, 9265-73.
- **Sanchez, M. J., Holmes, A., Miles, C. and Dzierzak, E.** (1996). Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. *Immunity* **5**, 513-25.
- **Schmitt, T. M., de Pooter, R. F., Gronski, M. A., Cho, S. K., Ohashi, P. S. and Zuniga-Pflucker, J. C.** (2004). Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nat Immunol* **5**, 410-7.
- **Schmitt, T. M. and Zuniga-Pflucker, J. C.** (2002). Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* **17**, 749-56.
- **Schneider, E., Moreau, G., Arnould, A., Vasseur, F., Khodabaccus, N., Dy, M. and Ezine, S.** (1999). Increased fetal and extramedullary hematopoiesis in Fas-deficient C57BL/6-lpr/lpr mice. *Blood* **94**, 2613-21.
- **Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., Bernstein, A. and Rossant, J.** (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89**, 981-90.
- **Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L. and Schuh, A. C.** (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376**, 62-6.
- **Shelly, L. L., Fuchs, C. and Miele, L.** (1999). Notch-1 inhibits apoptosis in murine erythroleukemia cells and is necessary for differentiation induced by hybrid polar compounds. *J Cell Biochem* **73**, 164-75.
- **Shimizu, K., Chiba, S., Saito, T., Kumano, K. and Hirai, H.** (2000). Physical interaction of Delta1, Jagged1, and Jagged2 with Notch1 and Notch3 receptors. *Biochem Biophys Res Commun* **276**, 385-9.
- **Shimizu, R., Kuroha, T., Ohneda, O., Pan, X., Ohneda, K., Takahashi, S., Philipsen, S. and Yamamoto, M.** (2004). Leukemogenesis caused by incapacitated GATA-1 function. *Mol Cell Biol* **24**, 10814-25.
- **Shintani, N., Kohgo, Y., Kato, J., Kondo, H., Fujikawa, K., Miyazaki, E. and Niitsu, Y.** (1994). Expression and extracellular release of transferrin receptors during peripheral erythroid progenitor cell differentiation in liquid culture. *Blood* **83**, 1209-15.
- **Shivdasani, R. A.** (2006). MicroRNAs: regulators of gene expression and cell differentiation. *Blood* **108**, 3646-53.
- **Shivdasani, R. A., Mayer, E. L. and Orkin, S. H.** (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* **373**, 432-4.
- **Sieburg, H. B., Cho, R. H., Dykstra, B., Uchida, N., Eaves, C. J. and Muller-Sieburg, C. E.** (2006). The hematopoietic stem compartment consists of a limited number of discrete stem cell subsets. *Blood* **107**, 2311-6.
- **Silva, M., Grillot, D., Benito, A., Richard, C., Nunez, G. and Fernandez-Luna, J. L.** (1996). Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. *Blood* **88**, 1576-82.
- **Silver, L. and Palis, J.** (1997). Initiation of murine embryonic erythropoiesis: a spatial analysis. *Blood* **89**, 1154-64.
- **Silvestris, F., Cafforio, P., Tucci, M. and Dammacco, F.** (2002). Negative regulation of erythroblast maturation by Fas-L(+)/TRAIL(+) highly malignant plasma cells: a major pathogenetic mechanism of anemia in multiple myeloma. *Blood* **99**, 1305-13.
- **Socolovsky, M., Fallon, A. E., Wang, S., Brugnara, C. and Lodish, H. F.** (1999). Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* **98**, 181-91.
- **Souabni, A., Cobaleda, C., Schebesta, M. and Busslinger, M.** (2002). Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1. *Immunity* **17**, 781-93.
- **Spivak, J. L.** (2002). Polycythemia vera: myths, mechanisms, and management. *Blood* **100**, 4272-90.
- **Staal, F. J. and Clevers, H. C.** (2005). WNT signalling and haematopoiesis: a WNT-WNT situation. *Nat Rev Immunol* **5**, 21-30.

- **Stier, S., Cheng, T., Dombkowski, D., Carlesso, N. and Scadden, D. T.** (2002). Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood* **99**, 2369-78.
- **Suwabe, N., Takahashi, S., Nakano, T. and Yamamoto, M.** (1998). GATA-1 regulates growth and differentiation of definitive erythroid lineage cells during in vitro ES cell differentiation. *Blood* **92**, 4108-18.
- **Takahashi, S., Onodera, K., Motohashi, H., Suwabe, N., Hayashi, N., Yanai, N., Nabesima, Y. and Yamamoto, M.** (1997). Arrest in primitive erythroid cell development caused by promoter-specific disruption of the GATA-1 gene. *J Biol Chem* **272**, 12611-5.
- **Takeda, S., Shimizu, T. and Rodewald, H. R.** (1997). Interactions between c-kit and stem cell factor are not required for B-cell development in vivo. *Blood* **89**, 518-25.
- **Tanigaki, K., Tsuji, M., Yamamoto, N., Han, H., Tsukada, J., Inoue, H., Kubo, M. and Honjo, T.** (2004). Regulation of alphabeta/gammadelta T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. *Immunity* **20**, 611-22.
- **Tavian, M., Coulombel, L., Luton, D., Clemente, H. S., Dieterlen-Lievre, F. and Peault, B.** (1996). Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood* **87**, 67-72.
- **Testa, U.** (2004). Apoptotic mechanisms in the control of erythropoiesis. *Leukemia* **18**, 1176-99.
- **Testa, U., Fossati, C., Samoggia, P., Masciulli, R., Mariani, G., Hassan, H. J., Sposi, N. M., Guerriero, R., Rosato, V., Gabbianelli, M. et al.** (1996). Expression of growth factor receptors in unilineage differentiation culture of purified hematopoietic progenitors. *Blood* **88**, 3391-406.
- **Thompson, M. A., Ransom, D. G., Pratt, S. J., MacLennan, H., Kieran, M. W., Detrich, H. W., 3rd, Vail, B., Huber, T. L., Paw, B., Brownlie, A. J. et al.** (1998). The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Dev Biol* **197**, 248-69.
- **Timmerman, L. A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J. M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J. C. et al.** (2004). Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev* **18**, 99-115.
- **Ting, C. N., Olson, M. C., Barton, K. P. and Leiden, J. M.** (1996). Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature* **384**, 474-8.
- **Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. and Orkin, S. H.** (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-6.
- **Tsai, F. Y. and Orkin, S. H.** (1997). Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. *Blood* **89**, 3636-43.
- **Tsang, A. P., Fujiwara, Y., Hom, D. B. and Orkin, S. H.** (1998). Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. *Genes Dev* **12**, 1176-88.
- **Turpen, J. B., Kelley, C. M., Mead, P. E. and Zon, L. I.** (1997). Bipotential primitive-definitive hematopoietic progenitors in the vertebrate embryo. *Immunity* **7**, 325-34.
- **Urbanek, P., Wang, Z. Q., Fetka, I., Wagner, E. F. and Busslinger, M.** (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell* **79**, 901-12.
- **Valtieri, M., Tocci, A., Gabbianelli, M., Luchetti, L., Masella, B., Vitelli, L., Botta, R., Testa, U., Condorelli, G. L. and Peschle, C.** (1998). Enforced TAL-1 expression stimulates primitive, erythroid and megakaryocytic progenitors but blocks the granulopoietic differentiation program. *Cancer Res* **58**, 562-9.
- **Varnum-Finney, B., Brashem-Stein, C. and Bernstein, I. D.** (2003). Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood* **101**, 1784-9.
- **Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W. S. and Bernstein, I. D.** (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat Med* **6**, 1278-81.
- **Vercauteren, S. M. and Sutherland, H. J.** (2004). Constitutively active Notch4 promotes early human hematopoietic progenitor cell maintenance while inhibiting differentiation and causes lymphoid abnormalities in vivo. *Blood* **104**, 2315-22.
- **Vivanco, I. and Sawyers, C. L.** (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* **2**, 489-501.
- **Walker, L., Carlson, A., Tan-Pertel, H. T., Weinmaster, G. and Gasson, J.** (2001). The notch receptor and its ligands are selectively expressed during hematopoietic development in the mouse. *Stem Cells* **19**, 543-52.
- **Wang, G. L. and Semenza, G. L.** (1995). Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* **270**, 1230-7.
- **Wang, L., Li, L., Shojaei, F., Levac, K., Cerdan, C., Menendez, P., Martin, T., Rouleau, A. and Bhatia, M.** (2004). Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. *Immunity* **21**, 31-41.
- **Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A. H. and Speck, N. A.** (1996). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci U S A* **93**, 3444-9.
- **Washburn, T., Schweighoffer, E., Gridley, T., Chang, D., Fowlkes, B. J., Cado, D. and Robey, E.** (1997). Notch activity influences the alphabeta versus gammadelta T cell lineage decision. *Cell* **88**, 833-43.
- **Weiss, M. J. and Orkin, S. H.** (1995). Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. *Proc Natl Acad Sci U S A* **92**, 9623-7.
- **Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P. t., Silverman, L. B., Sanchez-Irizarry, C., Blacklow, S. C., Look, A. T. and Aster, J. C.** (2004). Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* **306**, 269-71.
- **Weng, A. P., Millholland, J. M., Yashiro-Ohtani, Y., Arcangeli, M. L., Lau, A., Wai, C., Del Bianco, C., Rodriguez, C. G., Sai, H., Tobias, J. et al.** (2006). c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev* **20**, 2096-109.
- **Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., 3rd and Nusse, R.** (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448-52.
- **Winnier, G., Blessing, M., Labosky, P. A. and Hogan, B. L.** (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev* **9**, 2105-16.
- **Wu, H., Liu, X., Jaenisch, R. and Lodish, H. F.** (1995). Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* **83**, 59-67.
- **Xu, M. J., Matsuoka, S., Yang, F. C., Ebihara, Y., Manabe, A., Tanaka, R., Eguchi, M., Asano, S., Nakahata, T. and Tsuji, K.** (2001). Evidence for the presence of murine primitive megakaryocytopoiesis in the early yolk sac. *Blood* **97**, 2016-22.
- **Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster, G. and Gridley, T.** (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet* **8**, 723-30.
- **Yang, X., Klein, R., Tian, X., Cheng, H. T., Kopan, R. and Shen, J.** (2004). Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. *Dev Biol* **269**, 81-94.
- **Yin, T. and Li, L.** (2006). The stem cell niches in bone. *J Clin Invest* **116**, 1195-201.
- **Yoder, M. C. and Hiatt, K.** (1997). Engraftment of embryonic hematopoietic cells in conditioned newborn recipients. *Blood* **89**, 2176-83.
- **Yoder, M. C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D. M. and Orlic, D.** (1997). Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* **7**, 335-44.
- **Yoder, M. C., Hiatt, K. and Mukherjee, P.** (1997). In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. *Proc Natl Acad Sci U S A* **94**, 6776-80.
- **Yokota, T., Huang, J., Tavian, M., Nagai, Y., Hirose, J., Zuniga-Pflucker, J. C., Peault, B. and Kincade, P. W.** (2006). Tracing the first waves of lymphopoiesis in mice. *Development* **133**, 2041-51.
- **You, L. R., Lin, F. J., Lee, C. T., DeMayo, F. J., Tsai, M. J. and Tsai, S. Y.** (2005). Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature* **435**, 98-104.
- **Young, P. E., Baumhueter, S. and Lasky, L. A.** (1995). The sialomucin CD34 is expressed on hematopoietic cells and blood vessels during murine development. *Blood* **85**, 96-105.
- **Zamai, L., Secchiero, P., Pierpaoli, S., Bassini, A., Papa, S., Alnemri, E. S., Guidotti, L., Vitale, M. and Zauli, G.** (2000). TNF-related apoptosis-inducing ligand (TRAIL) as a negative regulator of normal human erythropoiesis. *Blood* **95**, 3716-24.
- **Zeuner, A., Eramo, A., Testa, U., Felli, N., Pelosi, E., Mariani, G., Srinivasula, S. M., Alnemri, E. S., Condorelli, G., Peschle, C. et al.** (2003). Control of erythroid cell production via caspase-mediated cleavage of transcription factor SCL/Tal-1. *Cell Death Differ* **10**, 905-13.

- **Zeuner, A., Pedini, F., Signore, M., Ruscio, G., Messina, C., Tafuri, A., Girelli, G., Peschle, C. and De Maria, R.** (2006). Increased death receptor resistance and FLIPshort expression in polycythemia vera erythroid precursor cells. *Blood* **107**, 3495-502.
- **Zhang, D. E., Zhang, P., Wang, N. D., Hetherington, C. J., Darlington, G. J. and Tenen, D. G.** (1997). Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci U S A* **94**, 569-74.
- **Zhang, P., Behre, G., Pan, J., Iwama, A., Wara-Aswapati, N., Radomska, H. S., Auron, P. E., Tenen, D. G. and Sun, Z.** (1999). Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. *Proc Natl Acad Sci U S A* **96**, 8705-10.
- **Zhang, P., Iwasaki-Arai, J., Iwasaki, H., Fenyus, M. L., Dayaram, T., Owens, B. M., Shigematsu, H., Levantini, E., Huettner, C. S., Lekstrom-Himes, J. A. et al.** (2004). Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity* **21**, 853-63.
- **Zhang, Z., Nadeau, P., Song, W., Donoviel, D., Yuan, M., Bernstein, A. and Yankner, B. A.** (2000). Presenilins are required for gamma-secretase cleavage of beta-APP and transmembrane cleavage of Notch-1. *Nat Cell Biol* **2**, 463-5.
- **Zhao, Y., Lin, Y., Zhan, Y., Yang, G., Louie, J., Harrison, D. E. and Anderson, W. F.** (2000). Murine hematopoietic stem cell characterization and its regulation in BM transplantation. *Blood* **96**, 3016-22.
- **Zhong, T. P., Childs, S., Leu, J. P. and Fishman, M. C.** (2001). Gridlock signalling pathway fashions the first embryonic artery. *Nature* **414**, 216-20.
- **Zweidler-McKay, P. A., He, Y., Xu, L., Rodriguez, C. G., Karnell, F. G., Carpenter, A. C., Aster, J. C., Allman, D. and Pear, W. S.** (2005). Notch signaling is a potent inducer of growth arrest and apoptosis in a wide range of B-cell malignancies. *Blood* **106**, 3898-906.

CHAPTER 9:
PUBLICATIONS

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

Àlex Robert-Moreno[1], Lluís Espinosa[1], José Luis de la Pompa[2] and Anna Bigas[1,*]

[1]Centre Oncologia Molecular, IDIBELL-Institut de Recerca Oncologica, Hospitalet, Barcelona 08907, Spain
[2]Department of Immunology and Oncology, Centro Nacional de Biotecnología, CSIC. Darwin, 3. Campus de Cantoblanco, Madrid 28049, Spain
*Author for correspondence (e-mail: abigas@iro.es)

Accepted 22 December 2004

Development 132, 1117-1126
Published by The Company of Biologists 2005
doi:10.1242/dev.01660

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 *RBPjκ* transcription factor to activate transcription, we analyzed the aorta of the para-aortic splanchnopleura/AGM in *RBPjκ* mutant embryos. We found specific patterns of expression of Notch receptors, ligands and Hes genes that were lost in *RBPjκ* 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 *Scl/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 *RBPjκ* 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/RBPjκ is a crucial event for the onset of definitive hematopoiesis in the embryo.

Key words: Notch, Mouse, *Rbpsuh*

Development

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 *Scl* (*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 RBPjκ (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 *RBPjκ*-dependent event, since *RBPjκ* 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/RBPjκ.

Materials and methods

Animals

RBPjκ 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 *RBPjκ* 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 *RBPjκ* 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-aminoactinomicin-D staining.

Immunostaining

Wild-type and *RBPjκ* 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% H_2O_2 (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.

Development

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

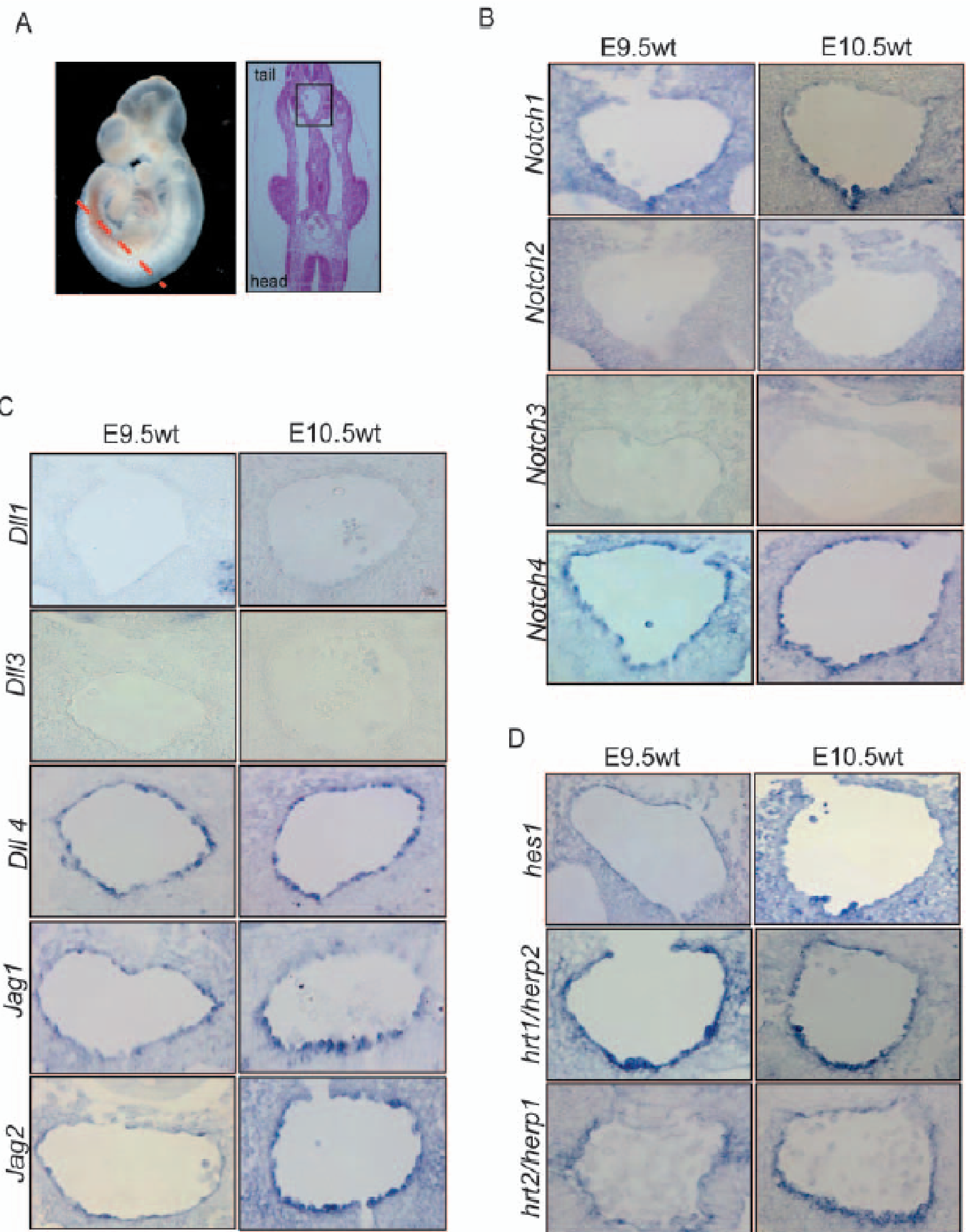


Fig. 1. Expression of Notch family members in the endothelium of the P-Sp/AGM aorta. (A) E9.5 embryo, indicating the site for P-Sp/AGM aorta and hematoxilin-eosin staining of a transverse section at the indicated level (100×) (B,C,D) Whole-mount of (B) Notch receptors, (C) Notch-ligands and (D) Notch-target 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).

Development

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.

RBPjκ 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

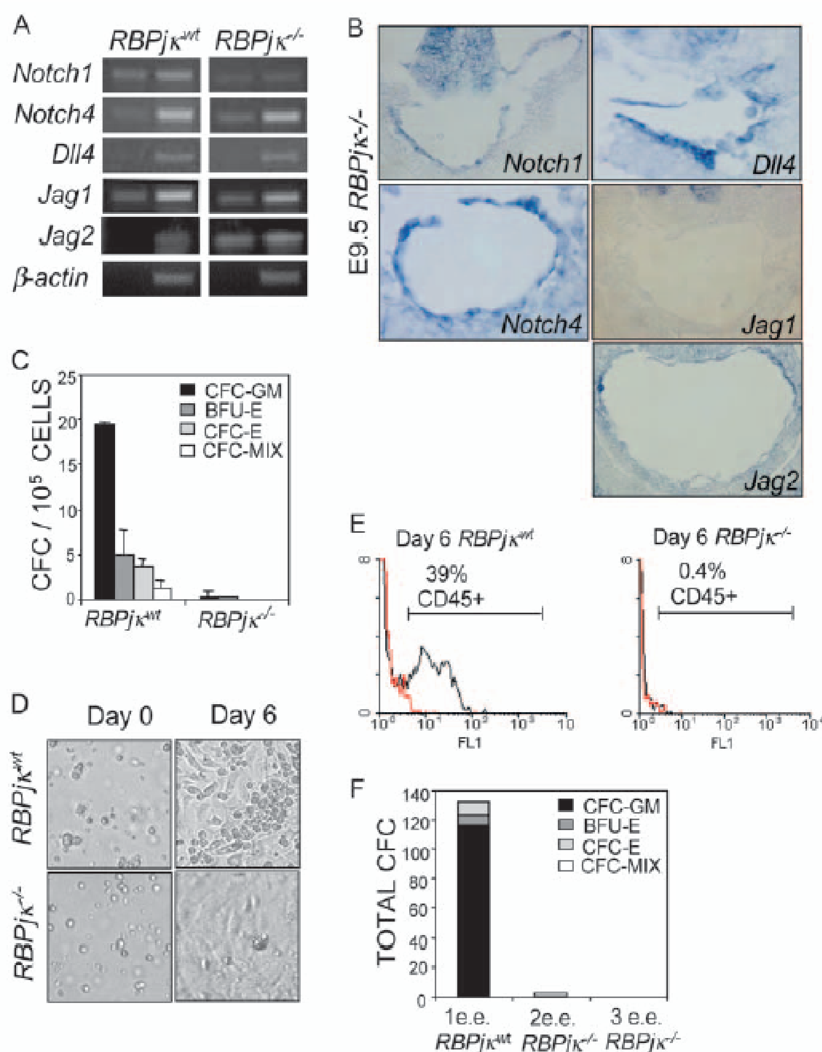


Fig. 2. Intra-embryonic hematopoiesis is impaired in the *RBPjκ* mutant embryos and they display an aberrant expression of Notch family members in the aorta (A) Semiquantitative RT-PCR analysis in dissected P-Sp of E9.5 wild-type and *RBPjκ* mutants. Representative PCR products after 35 and 40 cycles of two independent experiments are shown. (B) WISH, with the indicated probes and transverse sections of E9.5 aortas of *RBPjκ* mutants. Orientation of the aortas is dorsal (up) to ventral (down). (C) Hematopoietic CFC from dissected P-Sp of E9.5 wild-type and *RBPjκ* 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 *RBPjκ* 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 *RBPjκ* 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 *RBPjκ* 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 *RBPjκ* 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 *RBPjκ* 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 *RBPjκ* 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 *RBPjκ* mutant embryos

To investigate whether Notch/*RBPjκ* signaling plays a role in hematopoietic determination in the aorta, we next assayed the hematopoietic activity contained in the P-Sp/AGM region of *RBPjκ* mutant embryos compared with wild type. Despite the presence of several developmental abnormalities and disorganized vasculature, the majority of the RBPjκ mutant embryos (more than 80%) display a regular fused aorta in the trunkal region at E9.5 (Oka et al., 1995). As *RBPjκ* 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 *RBPjκ* mutant littermates in the same conditions (Fig. 2C). We speculated that *RBPjκ* 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

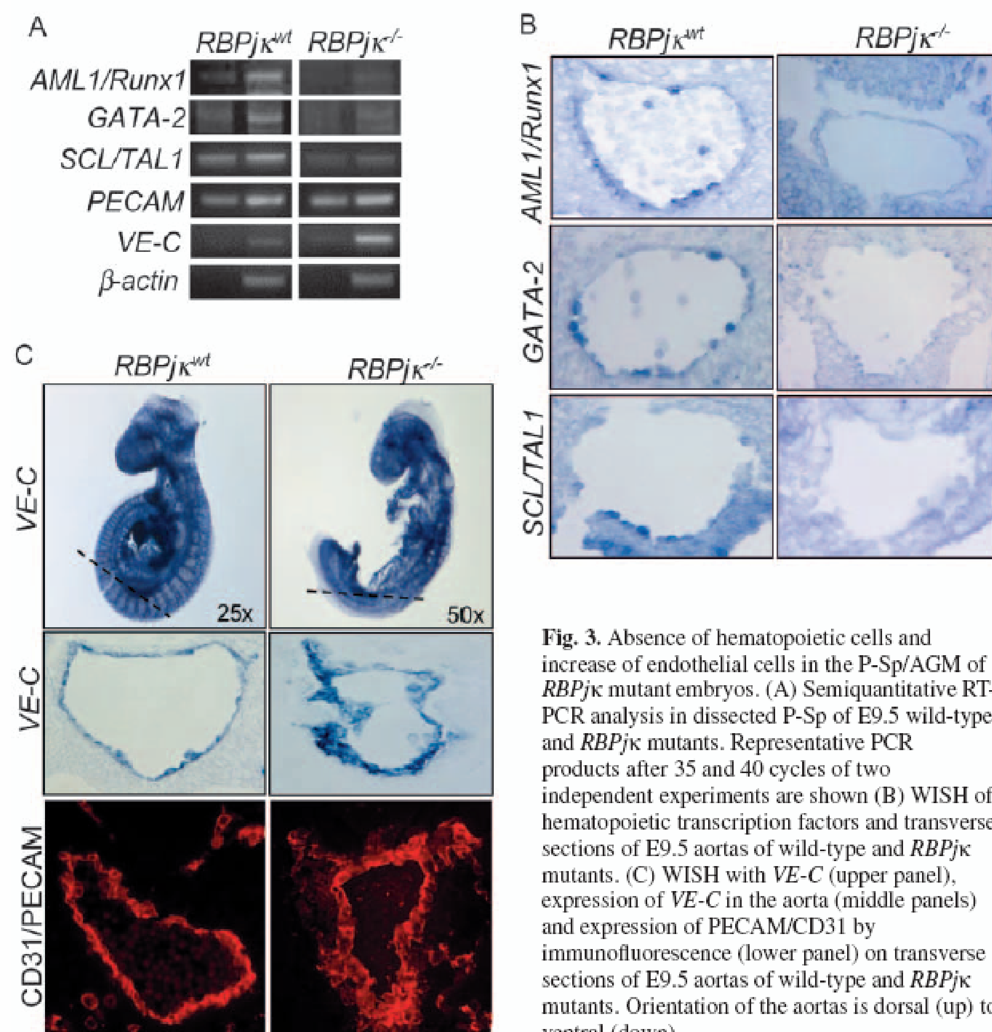


Fig. 3. Absence of hematopoietic cells and increase of endothelial cells in the P-Sp/AGM of *RBPjκ* mutant embryos. (A) Semiquantitative RT-PCR analysis in dissected P-Sp of E9.5 wild-type and *RBPjκ* mutants. Representative PCR products after 35 and 40 cycles of two independent experiments are shown (B) WISH of hematopoietic transcription factors and transverse sections of E9.5 aortas of wild-type and *RBPjκ* 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 *RBPjκ* mutants. Orientation of the aortas is dorsal (up) to ventral (down).

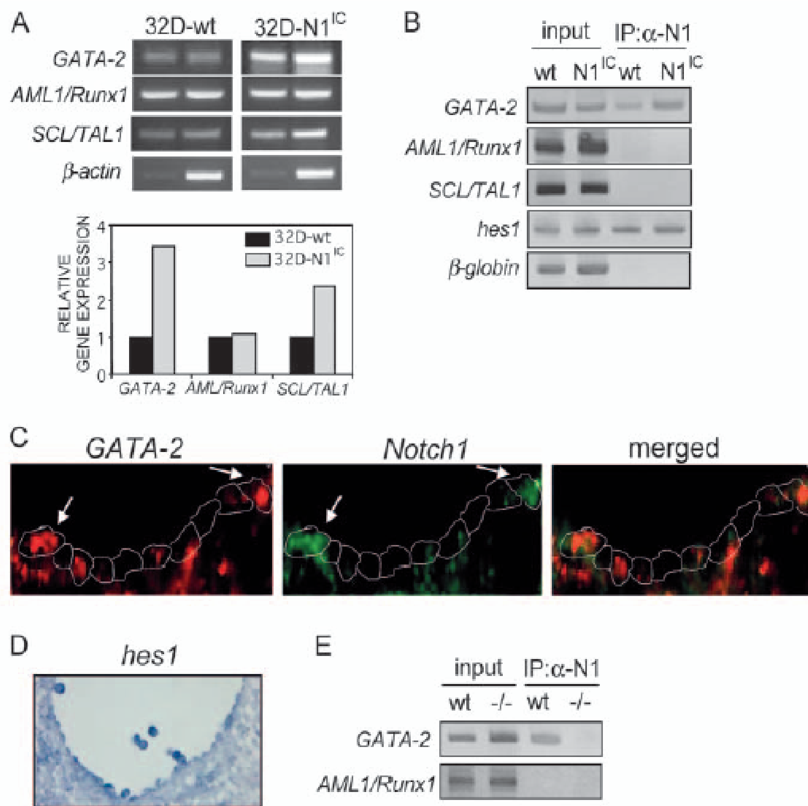


Fig. 4. Notch1/RBPjκ regulates *Gata2* transcriptional activity. (A) Semiquantitative RT-PCR analysis of *Gata2*, *Aml1 and Scl* expression in 32D wild-type or N1IC-expressing cells. Representative PCR products after 35 and 40 cycles of two independent experiments are shown. Quantitated relative mRNA 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 *RBPj*κ 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 *RBPj*κ mutant cultures (Fig. 2F). These results indicate that Notch signaling through *RBPj*κ 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 *RBPj*κ 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 *RBPJ*κ 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 *RBPj*κ 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 *RBPj*κ 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 *RBPj*κ 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 RBPjκ

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 *RBPj*κ 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-$N1^{IC}$ 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/RBPjκ 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 *RBPjκ* binding sites in the *Gata2* promoter (Minegishi et al., 1997), we tested whether the association of Notch1 to *Gata2* was dependent on RBPjκ. By immunoprecipitating chromatin-associated Notch1, we specifically detected the *Gata2* promoter in the precipitates from wild-type embryos but not in those from *RBPjκ* 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 RBPjκ (Fig. 4E). Altogether, these results indicate that Notch1, together with RBPjκ, 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 *RBPjκ* and several members of the Notch family are likely to be involved. Consistent with the phenotype described for the *Notch1* mutant embryos, *RBPjκ* 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).

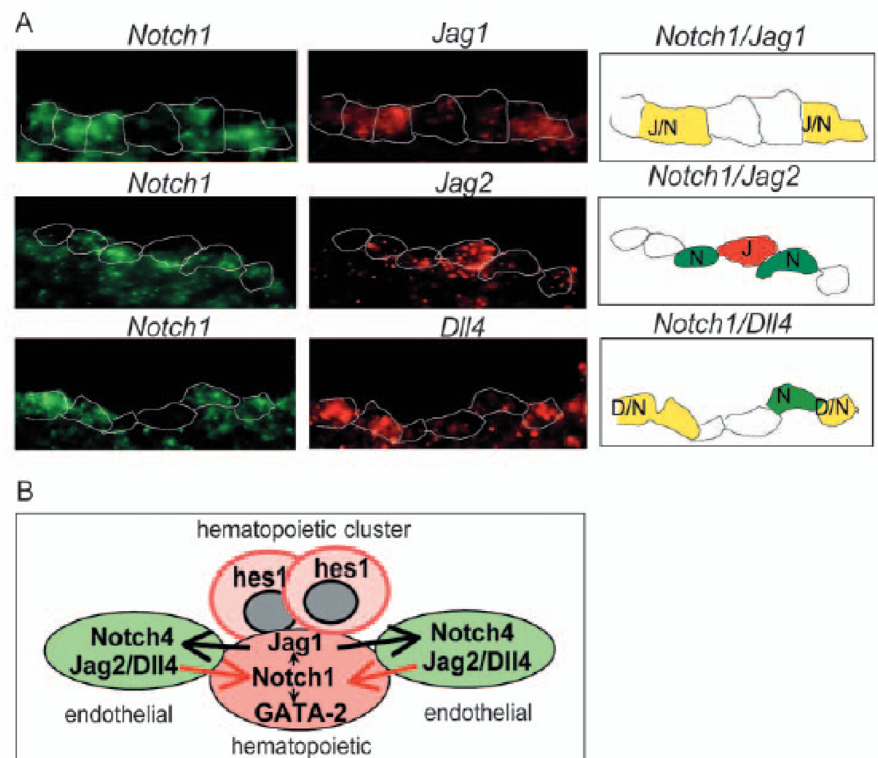


Fig. 5. *Notch1*$^+$/*Gata2*$^+$ cells in the P-Sp/AGM endothelium are *Jag1*$^+$/*Jag2*$^-$. Double in-situ hybridization on transverse section of wild-type E10.5 aortas. (A) Hybridization of *Notch1* with *Jag1* (upper), *Jag2* (middle) and *Dll4* (lower panels). Representative photographs of at least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

Development

*RBPj*κ-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 *RBPj*κ 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 RBPjκ. Although the expression of other hematopoietic genes such as *Scl* and *Aml1* is severely affected in the *RBPj*κ mutants, we showed that only *Gata2* is a direct target of Notch1/RBPjκ 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 *RBPj*κ 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 *RBPj*κ 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 *RBPjκ*-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.

We thank R. Kageyama, N. A. Speck, D. Martin and D. Srivastava for cDNA probes. We sincerely thank Elaine Dzierzak for helpful discussions in various aspects of this work. We thank all the members of the lab for many constructive discussions, Irene Merida for technical support and Serveis Cientifico-Tècnics, UB-Bellvitge, for confocal microscopy technical support. L.E. is an investigator from the Carlos III program (ISCIII/02/3027). A.R.M. is a recipient of a CIRIT predoctoral fellowship (2002-SI00791). This work was supported by a grant from the Comisión Interministerial de Ciencia y Tecnología, Plan Nacional de Salud (SAF2001-1191).

References

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

Artavanis-Tsakonas, S. and Simpson, P. (1991). Choosing a cell fate: a view from the notch locus. *Trends Genet* **7**, 403-408.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Barrantes, I. B., Elia, A. J., Wunsch, K., De Angelis, M. H., Mak, T. W., Rossant, J., Conlon, R. A., Gossler, A. and de la Pompa, J. L. (1999). Interaction between Notch signalling and Lunatic fringe during somite boundary formation in the mouse. *Curr. Biol.* **9**, 470-480.

Bigas, A., Martin, D. I. and Milner, L. A. (1998). Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. *Mol. Cell. Biol.* **18**, 2324-2333.

Cai, Z., de Bruijn, M., Ma, X., Dortland, B., Luteijn, T., Downing, R. J. and Dzierzak, E. (2000). Haploinsufficiency of AML1 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo. *Immunity* **13**, 423-431.

Chitnis, A. B. (1995). The role of Notch in lateral inhibition and cell fate specification. *Mol. Cell. Neurosci.* **6**, 311-321.

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

de Bruijn, M. F., Peeters, M. C., Luteijn, T., Visser, P., Speck, N. A. and Dzierzak, E. (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *Blood* **96**, 2902-2904.

de Bruijn, M. F., Ma, X., Robin, C., Ottersbach, K., Sanchez, M. J. and Dzierzak, E. (2002). Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* **16**, 673-683.

de Celis, J. F., Mari-Beffa, M. and Garcia-Bellido, A. (1991). Cell-autonomous role of Notch, an epidermal growth factor homologue, in sensory organ differentiation in Drosophila. *Proc. Natl. Acad. Sci. USA* **88**, 632-636.

de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. et al. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139-1148.

Fortini, M. E., Rebay, I., Caron, L. A. and Artavanis-Tsakonas, S. (1993). An activated notch receptor blocks cell-fate commitment in the developing drosophila eye. *Nature* **365**, 555-557.

Hadland, B. K., Huppert, S. S., Kanungo, J., Xue, Y., Jiang, R., Gridley, T., Conlon, R. A., Cheng, A. M., Kopan, R. and Longmore, G. D. (2004). A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. *Blood* **104**, 3097-3105.

Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K. and Honjo, T. (2002). Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int. Immunol.* **14**, 637-645.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P. (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. *Development* **122**, 161-171.

Huppert, S. S., Le, A., Schroeter, E. H., Mumm, J. S., Saxena, M. T., Milner, L. A. and Kopan, R. (2000). Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1. *Nature* **405**, 966-970.

Iso, T., Kedes, L. and Hamamori, Y. (2003). HES and HERP families: multiple effectors of the Notch signaling pathway. *J. Cell Physiol.* **194**, 237-255.

Kawamata, S., Du, C., Li, K. and Lavau, C. (2002). Overexpression of the Notch target genes Hes in vivo induces lymphoid and myeloid alterations. *Oncogene* **21**, 3855-3863.

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

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

Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-973.

Lewis, J. (1998). Notch signalling and the control of cell fate choices in vertebrates. *Semin. Cell Dev. Biol.* **9**, 583-589.

Ling, K. W. and Dzierzak, E. (2002). Ontogeny and genetics of the hemato/lymphopoietic system. *Curr. Opin. Immunol.* **14**, 186-191.

Ma, X., Robin, C., Ottersbach, K. and Dzierzak, E. (2002). The Ly-6A (Sca-1) GFP transgene is expressed in all adult mouse hematopoietic stem cells. *Stem Cells* **20**, 514-521.

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

Milner, L. A., Bigas, A., Kopan, R., Brashem-Stein, C., Bernstein, I. D. and Martin, D. I. (1996). Inhibition of granulocytic differentiation by mNotch1. *Proc. Natl. Acad. Sci. USA* **93**, 13014-13019.

Minegishi, N., Morita, S., Minegishi, M., Tsuchiya, S., Konno, T., Hayashi, N. and Yamamoto, M. (1997). Expression of GATA transcription factors in myelogenous and lymphoblastic leukemia cells. *Int. J. Hematol.* **65**, 239-249.

Minegishi, N., Ohta, J., Yamagiwa, H., Suzuki, N., Kawauchi, S., Zhou, Y., Takahashi, S., Hayashi, N., Engel, J. D. and Yamamoto, M. (1999). The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region. *Blood* **93**, 4196-4207.

Nakagawa, O., McFadden, D. G., Nakagawa, M., Yanagisawa, H., Hu, T., Srivastava, D. and Olson, E. N. (2000). Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of notch signaling. *Proc. Natl. Acad. Sci. USA* **97**, 13655-13660.

North, T., Gu, T. L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M. and Speck, N. A. (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* **126**, 2563-2575.

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

Oka, C., Nakano, T., Wakeham, A., de la Pompa, J. L., Mori, C., Sakai, T., Okazaki, S., Kawaichi, M., Shiota, K., Mak, T. W. et al. (1995). Disruption of the mouse RBP-J kappa gene results in early embryonic death. *Development* **121**, 3291-3301.

Palis, J. and Yoder, M. C. (2001). Yolk-sac hematopoiesis: the first blood cells of mouse and man. *Exp. Hematol.* **29**, 927-936.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912.
Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F. W. and Orkin, S. H. (1996). The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell* **86**, 47-57.
Radtke, F. and Raj, K. (2003). The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat. Rev. Cancer* **3**, 756-767.
Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R. and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1 *Immunity* **10**, 547-558.
Robb, L., Elwood, N. J., Elefanty, A. G., Kontgen, F., Li, R., Barnett, L. D. and Begley, C. G. (1996). The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse. *EMBO J.* **15**, 4123-4129.
Stier, S., Cheng, T., Dombkowski, D., Carlesso, N. and Scadden, D. T. (2002). Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood* **99**, 2369-2378.
Timmerman, L. A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J. M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J. C. et al. (2004). Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev.* **18**, 99-115.
Tsai, F. Y. and Orkin, S. H. (1997). Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. *Blood* **89**, 3636-3643.
Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. and Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-226.
Yoder, M. C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D. M. and Orlic, D. (1997). Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* **7**, 335-344.

Leukemia (2007), 1–8
© 2007 Nature Publishing Group All rights reserved 0887-6924/07 $30.00
www.nature.com/leu

ORIGINAL ARTICLE

The notch pathway positively regulates programmed cell death during erythroid differentiation

À Robert-Moreno[1], L Espinosa[1], MJ Sanchez[2], JL de la Pompa[3] and A Bigas[1]

[1]*Centre Oncologia Molecular, IDIBELL-Institut de Recerca Oncològica, Hospitalet, Barcelona, Spain;* [2]*Centro Andaluz Biología Desarrollo, CSIC, Universidad Pablo Olavide, Sevilla, Spain and* [3]*Department Immunology and Oncology, Centro Nacional de Biotecnología, CSIC, Campus de Cantoblanco, Madrid, Spain*

Programmed cell death plays an important role in erythropoiesis under physiological and pathological conditions. In this study, we show that the Notch/RBPjκ signaling pathway induces erythroid apoptosis in different hematopoietic tissues, including yolk sac and bone marrow as well as in murine erythroleukemia cells. In RBPjκ$^{-/-}$ yolk sacs, erythroid cells have a decreased rate of cell death that results in increased number of Ter119+ cells. A similar effect is observed when Notch activity is abrogated by incubation with the γ-secretase inhibitors, DAPT or L685,458. We demonstrate that incubation with Jagged1-expressing cells has a proapoptotic effect in erythroid cells from adult bone marrow that is prevented by blocking Notch activity. Finally, we show that the sole expression of the activated Notch1 protein is sufficient to induce apoptosis in hexametilene-bisacetamide-differentiating murine erythroleukemia cells. Together these results demonstrate that Notch regulates erythroid homeostasis by inducing apoptosis.
Leukemia advance online publication, 3 May 2007; doi:10.1038/sj.leu.2404705
Keywords: notch; erythropoiesis; apoptosis

Introduction

Notch is a highly conserved signaling pathway that regulates cell fate specification during development and adult tissue homeostasis. Physiological activation of the Notch pathway requires the interaction between the Notch receptor and one of its ligands. This interaction leads to the cleavage of Notch receptor, releasing the intracellular domain that translocates to the nucleus to bind RBPjκ and activate specific gene transcription (reviewed by Bray[1] and Lai[2]).

Notch function is required for the generation of definitive hematopoiesis as shown by the lack of hematopoietic precursors in the aorta of Notch1$^{-/-}$ and RBPjκ$^{-/-}$ mouse embryos or in mind bomb mutants in zebrafish.[3–6] In contrast, primitive hematopoiesis occurs in different Notch pathway mutants in both mouse and zebrafish.[3–5] In the mouse, primitive hematopoiesis originates in the blood islands of the yolk sac, starting at embryonic day 7.5 (E7.5). The main component of this primitive hematopoiesis is erythroid progenitor cells (EryP)[7] that generate large nucleated primitive erythrocytes that contain embryonic globins (βH1, ε-globin and ζ-globin) (reviewed by Palis and Segel[8]).

Erythropoiesis involves the progressive differentiation of uncommitted progenitors to mature erythrocytes. However, not only differentiation but also apoptosis participates in the regulation of cell survival and mature red cell turnover. The amount of erythropoietin (Epo), mainly dependent on hypoxia, is one of the key factors in controlling the survival of erythroid cells (reviewed in Mulcahy[9]). Expression of the antiapoptotic members of the Bcl-2 family, Bcl-2 and Bcl-x,[10] are some of the downstream effects of EpoR activation in this system. Consistent with this, bcl-x$^{-/-}$ embryos die of massive apoptosis in the nervous system and in fetal liver erythroid cells.[11]

Other transcription factors including GATA1 and, more recently, p53 have been implicated in regulating apoptosis at different stages of erythroid maturation.[12,13] In this sense, GATA1 plays a key role in development and survival of erythroid cells since GATA1-deficient cells failed to develop beyond the proerythroblast stage and undergo rapid apoptosis.[14]

Notch pathway has previously been shown to induce apoptosis in cell lines from different hematopoietic lineages most likely through the activation of its target gene hes1.[15–17] However, the overall data linking Notch and erythroid apoptosis is controversial.[18,19]

In this work, we demonstrate that the Notch signaling pathway is a positive regulator of apoptosis in primitive erythropoiesis in the yolk sac but also in erythroid cells from adult bone marrow (BM). Complementary studies using the murine erythroleukemia (MEL) cell line indicate that Notch induces erythroid-specific apoptosis.

Materials and methods

Animals

RBPjκ$^{-/-}$ mice have been described previously.[20] Animals were kept under pathogen-free conditions and experiments approved by the Animal Care Committee. Yolk sacs were obtained from timed pregnant females at days 7.5–9.5 of gestation and dissected out from embryo and vitelline arteries. Embryos were genotyped by polymerase chain reaction (PCR) and morphology. BM was obtained from 8 to 12-week wild-type (WT) CD1 mice.

Cell lines and transfections

MEL cells[21] were maintained in RPMI 10% fetal bovine serum (FBS), 1% L-glutamine and 1% Pen/Strep. Stable clones of MEL cells expressing N1ΔE[22] or pcDNA.3 were obtained by electroporation and expression was confirmed by western blot (9E10 antibody). NIH-3T3 cells were transfected by calcium

Correspondence: Dr A Bigas, Oncologia Molecular, IDIBELL-Institut de Recerca Oncològica, Gran Via Km 2.7, Hospitalet, Barcelona 08907, Spain.
E-mail: abigas@iro.es
Received 29 January 2007; revised 2 March 07; accepted 14 March 2007

phosphate with Jagged1 construct[23] and clones overexpressing Jagged1 were selected in G418. Differentiation of MEL cells was performed with 5 mM hexametilene-bisacetamide (HMBA; Sigma, St Louis, MI, USA) for 6 days.

Dianisidine staining

O-dianisidine (Sigma) was used to stain hemoglobin of both E9.5 WT and RBPjκ-mutant yolk sacs, and MEL friend cells to assay erythroid differentiation as described previously.[21]

Hematopoietic colony assay

Yolk sac from WT and RBPj$\kappa^{-/-}$ E7.5–9.5 embryos was digested in 0.1% collagenase (Sigma) in phosphate-buffered saline (PBS), 10% FBS for 30 min at 37°C. Cells (30 000) were plated in duplicates in 1% methylcellulose (Stem Cell Technologies, Vancouver, Canada) plus Iscove's with 10% FBS, 10% IL3- and stem cell factor (SCF)-conditioned medium, 2.5% L-glutamine, 0.1% monothioglycerol (Sigma), 1% Pen/Strep (Biological Industries, Beit Haemek Kibbutz, Israel), 2 IU/ml erythropoietin (Laboratorios Pensa-Esteve, Barcelona, Spain), 20 ng/ml granulocyte-macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA) and 100 ng/ml of granulocyte colony-stimulating factor (Avantis Pharma, Paris, France). After 7 days, the presence of hematopoietic colonies was scored under a microscope. EryP colonies were scored at day 3.

Flow cytometry analysis

Collagenase-disrupted yolk sac cells were stained with fluorescein isothiocyanate FITC-conjugated CD71, CD41, ckit, CD45, and mac1 and PE-conjugated Ter119 and CD31 antibodies (Pharmingen, BD Biosciences, San Jose, CA, USA) or isotopic immunoglobulin G as a control. Cells were analyzed in a FACScalibur (Becton & Dickinson, BD Biosciences, San Jose, CA, USA) and WinMDI 2.8 software. Dead cells were excluded by 7-aminoactinomicin-D (7-AAD; Invitrogen, Carlsbad, CA, USA) staining. For the AnnexinV binding analysis, cells were stained with rh AnnexinV-FITC kit (Bender Medsystems, Burlingame, CA, USA) and 7-AAD for 15 min according to the manufacturer's instructions.

For cell cycle analysis of total yolk sac, cells were fixed in 70% EtOH at −20°C overnight, treated with 50 μg DNAse-free RNAse and stained with 25 μg of propidium iodide (Sigma). Ter119+ cell-cycle analysis was performed on fresh cells with 20 μM Draq5 (Biostatus Ltd, Leicestershire, UK). FlowJo 6.4.1 software was used for cell-cycle analysis.

Yolk sac and BM cultures

Collagenase-disrupted yolk sacs were cultured for 6 days in Iscove's with 10% FBS, 10% IL3- and SCF-conditioned medium, 0.1% monothioglycerol in the presence of 50 μM N-S-phenylglycine-t-butylester (DAPT) (Invitrogen), 2 μM L685,458 (Sigma) or dimethyl sulfoxide (DMSO) as control. For BM culture, 1.5×10^5 whole BM cells were incubated with γ-secretase inhibitors in RPMI 10% FBS, 2 IU/ml EPO for 2–3 days. Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK; Bachem, Budendorf, Switzerland) was used at 200μM.

Coculture on 3T3 or 3T3-Jag1 stromal cells was perfomed with 4×10^5 whole BM cells in RPMI, 10% FBS for 16 h. Cells were assayed for AnnexinV binding and analyzed by flow cytometry.

Immunohistochemisty

Yolk sacs were fixed with 4% paraformaldehyde (Sigma), embedded in Paraplast (Sigma) and sectioned (10 μm). Slides were dewaxed in xylene, antigen retrieval was performed by boiling for 2 min in sodium acetate, rehydrated and blocked-permeabilized in 10% FBS, 0.3% Surfact-Amps × 100 (Pierce, Aalst, Belgium) and 5% non-fat milk in PBS for 90 min at 4°C. Anti P-Ser10 H3 (Upstate, Charlottesville, VA, USA) was used at 1:500 dilution and developed with Dakocytomation kit (Dako, Glostrup, DK, Denmark) following manufacturer's instructions. Hematoxilin (Merck, Whitehouse Station, NJ, USA) was used for counterstaining.

For histological analysis, tissue samples were fixed overnight at 4°C in 4% paraformaldehyde, dehydrated and embedded in Paraplast (Sigma). Samples were sectioned in a Leica-RM2135 at 4 μm and stained with hematoxilin and eosin.

Images were acquired with an Olympus BX-60 using a Spot camera and Spot 3.2.4 software (Diagnostic Instruments, Sterling Heights, MI, USA). Adobe Photoshop 6.0 software was used for photograph editing.

Semiquantitative reverse transcriptase-polymerase chain reaction

Total RNA from subdissected E9.5 WT and RBPj$\kappa^{-/-}$ yolk sacs was isolated using TRIzol Reagent (Invitrogen). Poly-AT Tract System IV (Promega, Madison, WI, USA) and RT-First Strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK) were used to obtain mRNA and cDNA respectively. PCR product was analyzed at different cycles to avoid saturation. Quantity One software (Biorad, Hempstead, UK) was used for densitometry. Primer pairs used in the experiments are listed in Supplementary Table S1.

Quantitative RT-PCR

Ter119+ cells were sorted from E9.5 collagenase-treated embryos in MoFlo cell sorter (Dakocytomation, BD Diagnostic, San Jose, CA, USA). mRNA was isolated with Rneasy minikit (Qiagen, Valencia, CA, USA) following manufacture's instructions. qRT-PCRs were performed with SYBR Green I Master (Roche, Basel, Switzerland) in LightCycler480 system.

Statistical analysis

Normal distribution of the samples was confirmed with one-sample Kolmogorow–Smirnoff test and Student's *t*-test was performed.

Results

Absence of Notch signaling results in increased number of erythroid cells in the yolk sac

Notch signaling has previously been shown to influence differentiation and apoptosis of erythroid cells *in vitro* although controversial observations have been reported.[15,18,19,24] For this reason, we aimed to characterize the physiological role of the Notch pathway in erythropoiesis by comparing WT and RBPj$\kappa^{-/-}$ embryos. Despite the absence of intraembryonic hematopoiesis in the RBPj$\kappa^{-/-}$ embryos and the presence of different angiogenic abnormalities in the yolk sac, we found that primitive hematopoiesis does occur in the yolk sac of the RBPj$\kappa^{-/-}$ embryos (Figure 1a), similar to the Notch1$^{-/-}$ mutants.[25–27]

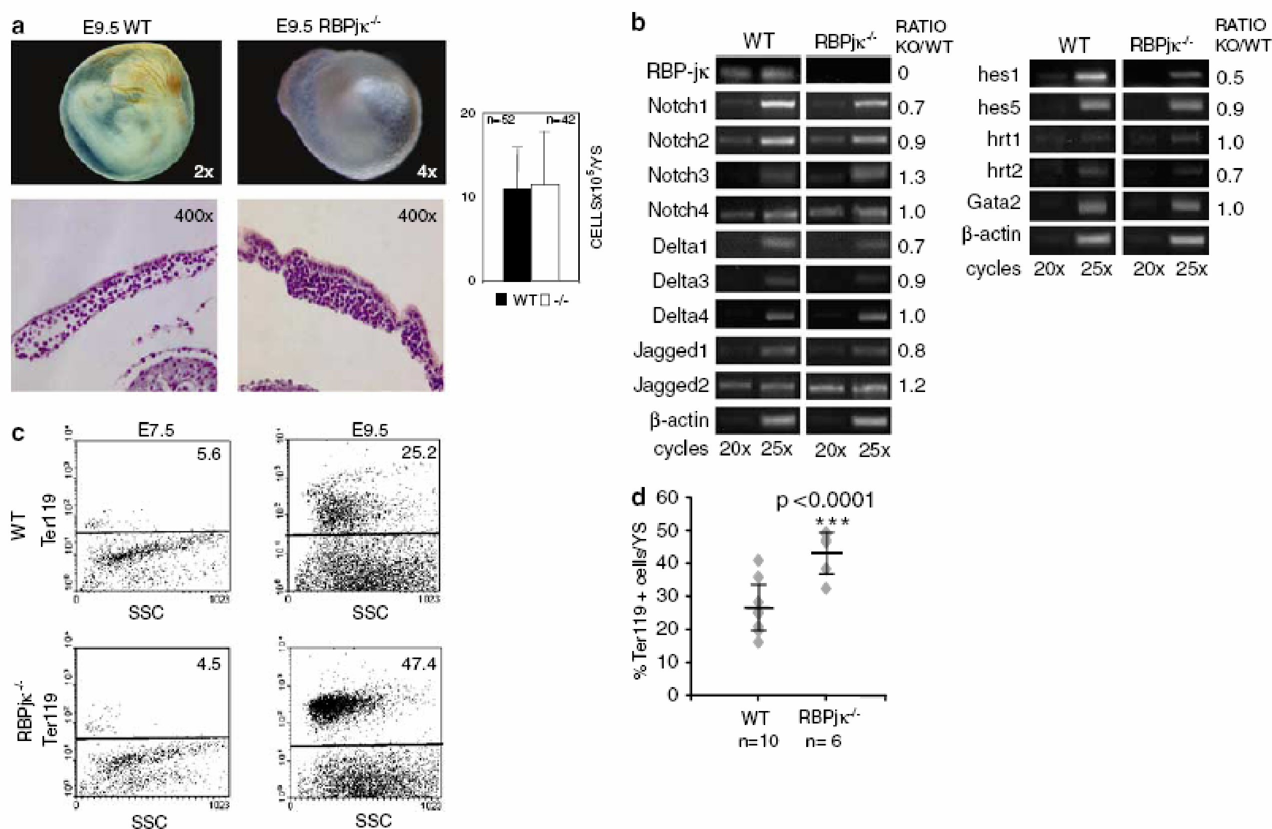


Figure 1 Increased number of erythroid cells in RBPjκ$^{-/-}$ yolk sacs. (**a**) Images of E9.5 WT and RBPjκ$^{-/-}$ embryos with the yolk sac (upper panel) and hematoxilin/eosin staining of yolk sac sections. Total number of cells obtained from disrupted yolk sacs (right panel). (**b**) Semiquantitative RT-PCR of Notch receptors, ligands and target genes from E9.5 WT and RBPjκ$^{-/-}$ yolk sacs. (**c**) Representative analysis of Ter119+ cells from E7.5 and E9.5 WT and RBPjκ$^{-/-}$ yolk sacs. (**d**) Percentage of Ter119+ cells in the analyzed E9.5 WT and RBPjκ$^{-/-}$ yolk sacs. Average and s.d. are represented.

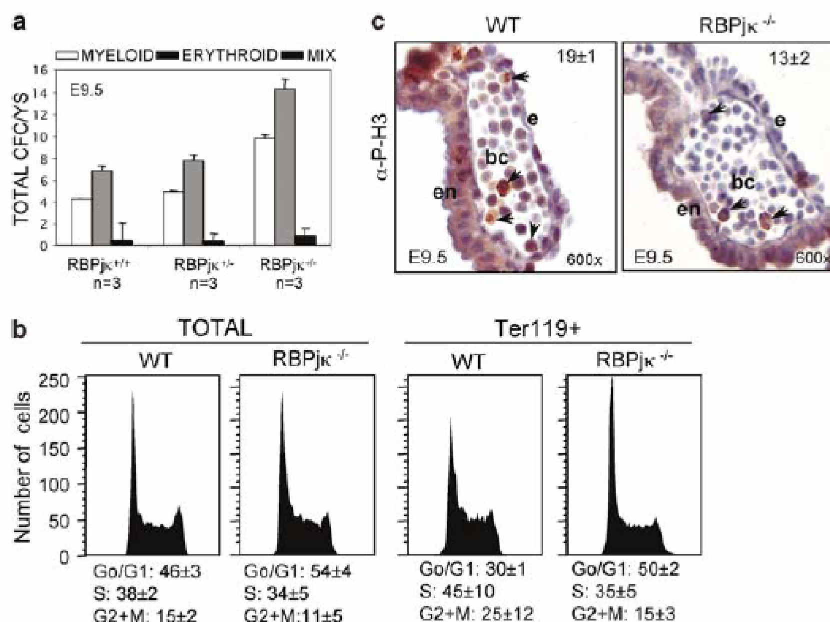


Figure 2 Proliferation in E9.5 WT and RBPjκ$^{-/-}$ yolk sac cells. (**a**) Graphs represent the total number of CFC types obtained from WT or RBPjκ$^{-/-}$ yolk sacs. (**b**) Representative cell-cycle analysis from total and Ter119+ cells from E9.5WT or RBPjκ$^{-/-}$ yolk sacs. Average values and s.d. from two yolk sacs are shown. (**c**) IHC of P-Histone3 in yolk sac sections. Numbers represent the average percentage of positive cells inside the blood islands found in four independent stainings. Arrowheads indicate cells with positive staining; e, endothelium; en, endoderm; bc, blood cells.

To determine whether Notch pathway plays a role in regulating hematopoiesis in the yolk sac, we first analyzed the expression of different Notch receptors, ligands and Notch-target genes in the yolk sac of WT and RBPj$\kappa^{-/-}$ embryos by semiquantitative RT-PCR and we observed that all Notch family genes are expressed in the yolk sac at E9.5 (Figure 1b). In RBPj$\kappa^{-/-}$, we found reduced expression of all ligands and receptors, whereas Notch3 and Jagged2 were upregulated. We also tested the expression of Notch-target genes and detected a consistent reduction in hes1 levels in RBPj$\kappa^{-/-}$ yolk sacs compared with WT (Figure 1b).

Since hematopoiesis in the yolk sac is mainly restricted to erythropoiesis, we determined the percentage of cells expressing the erythroid marker Ter119 in collagenase-treated yolk sacs at E7.5, E8.5 and E9.5. We detected a few positive cells in the yolk sac of E7.5 (Figure 1c) and E8.5 (data not shown). At day 9.5, the percentage of Ter119+ in the yolk sac ranged from 20 to 40% in the WT and 40–60% in the RBPj$\kappa^{-/-}$ (Figure 1c and d) being similar the total number of cells per yolk sac (Figure 1a).

Proliferation is not responsible for increased erythropoiesis in RBPj$\kappa^{-/-}$ yolk sacs

To investigate whether the higher number of Ter119+ cells in the RBPj$\kappa^{-/-}$ yolk sac was owing to an increase in the number of progenitors, we performed colony-forming cell (CFC) assays with collagenase-treated yolk sac cells at E7.5, E8.5 and E9.5 from RBPj$\kappa^{+/+,+/-}$ and $^{-/-}$ embryos. We detected a similar percentage of myeloid, erythroid and mixed colonies in these cultures (Supplementary Figure S1), however; at E9.5, there was a twofold increase in the total number of CFC in mutant embryos (Figure 2a). To test whether this effect was due to increased proliferation, we analyzed the cell-cycle profile of WT and RBPj$\kappa^{-/-}$ yolk sac cells by flow cytometry. Surprisingly, the percentage of cells in S/G_2–M phase was slightly reduced in RBPj$\kappa^{-/-}$ compared with WT cells (from 53 to 45%), and this reduction in S/G_2–M phase was higher when cell cycle was analyzed in the Ter119+ cells (Figure 2b). To confirm this observation, we performed the P-H3 staining on yolk sac sections to assess the number of cells undergoing mitosis inside the blood islands. P-H3 staining showed that hematopoietic

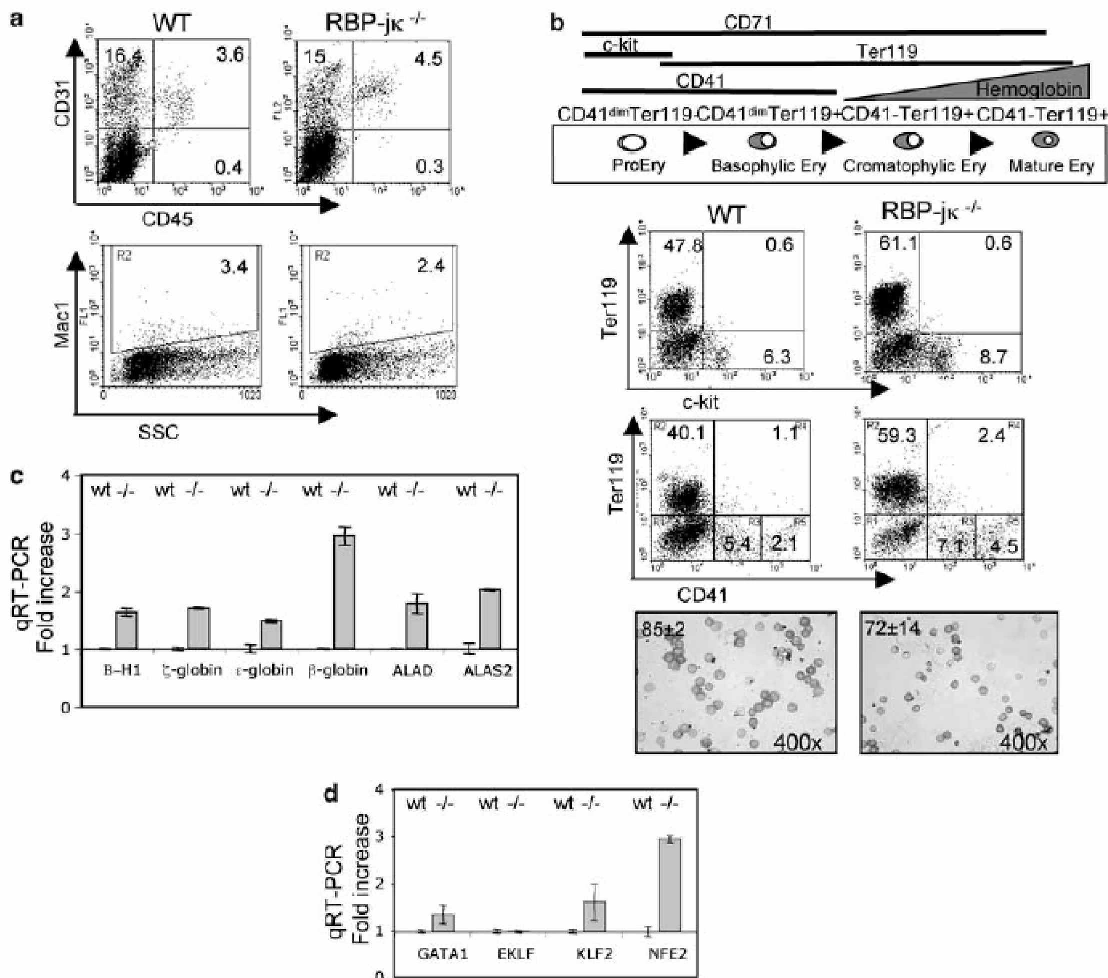


Figure 3 Erythroid differentiation is not impaired in RBPj$\kappa^{-/-}$ yolk sacs at E9.5. (**a**) Representative dot plots showing expression of CD45 (hematopoietic marker) versus CD31 (endothelial marker) and mac1 (macrophage marker). (**b**) Representation of erythroid differentiation markers (upper). Dot plots of representative erythroid subpopulations from three WT and RBPj$\kappa^{-/-}$ yolk sacs. Dianisidine staining of circulating cells from the yolk sac (lower panels). Numbers represent the average and s.d. of positive cells counted in three different samples. (**c** and **d**) qRT-PCR of (**c**) globin genes and hemo maturation enzime genes and (**d**) erythroid-specific transcription factors from WT and RBPj$\kappa^{-/-}$ yolk sacs.

cells from the WT yolk sacs have a similar mitotic rate (19.8 %) than cells in RBPj$\kappa^{-/-}$ yolk sacs (12.9 %); (Figure 2c). Altogether these results indicate that proliferation is not increased in the RBPj$\kappa^{-/-}$ yolk sac erythroid cells.

Normal differentiation occurs in RBPj$\kappa^{-/-}$ yolk sac

Although hematopoiesis is mainly restricted to erythropoiesis in the yolk sac, different progenitor types and macrophages are also generated. As we detected increased number of different hematopoietic progenitors in the RBPj$\kappa^{-/-}$ (Figure 2a), we speculated that the decision between hematopoietic and endothelial lineages may be affected. To test this possibility, we analyzed the expression of CD45 (hematopoietic excluding erythroid cells) and CD31 (endothelial) cell markers. We detected a similar number of cells expressing these markers, indicating that the non-erythroid hematopoiesis is normally occurring in the RBPj$\kappa^{-/-}$ yolk sac (Figure 3a). Consistent with this observation, we did not detect any difference in the percentage of endothelial cells (CD31 + and CD45−; Figure 3a) or in the expression of PECAM or VE-cadh genes in these yolk sacs (Supplementary Figure S4), in contrast to that previously observed in the intraembryonic endothelial/hematopoietic differentiation[3,6] In addition, no major differences were found in the percentage of mac1 + cells between WT and RBPj$\kappa^{-/-}$ (Figure 3a).

We next investigated whether the higher number of Ter119 + cells in the RBPj$\kappa^{-/-}$ was due to a blockage in erythroid differentiation. Thus, we characterized the different erythroid subpopulations by analyzing the expression of specific differentiation markers CD71, CD41 and c-kit in the Ter119 + population by flow cytometry. As shown in Supplementary Figure S2, CD71 was expressed in all Ter119 + cells in both WT and RBPj$\kappa^{-/-}$ yolk sacs, this result is surprising since this marker is downregulated during erythroid differentiation in BM.[28] Analysis of other differentiation markers showed that the different Ter119 + subpopulations were similarly represented in WT and mutant yolk sacs (Figure 3b). We also analyzed the expression of erythroid transcription factors and globin genes in purified Ter119 + cells from WT and RBPj$\kappa^{-/-}$ embryos by qRT-PCR. We did not detect major differences in the expression of the embryonic globins and hemo group maturation enzymes (ALAD and ALAS2; Figure 3c) or in the percentage of circulating yolk sac cells showing dianisidine staining (from 85% to 72%; Figure 3b); however, a threefold increase in the expression of adult β-globin was observed in the RBPj$\kappa^{-/-}$ cells compared with the WT (Figure 3c). We also detected overexpression of the erythroid transcription factor NFE2 in RBPj$\kappa^{-/-}$ erythroid cells, whereas no significant differences were detected in GATA1, KLF2 and EKLF levels (Figure 3d). Surprisingly, we did not observed downregulation of the hes1 gene in the Ter119 + cells of the RBPj$\kappa^{-/-}$ embryos (data not shown).

Reduction of apoptosis in Notch-defective yolk sac erythroid cells

Apoptosis is a crucial mechanism for maintaining the homeostasis of the erythroid lineage. Since minor differences in

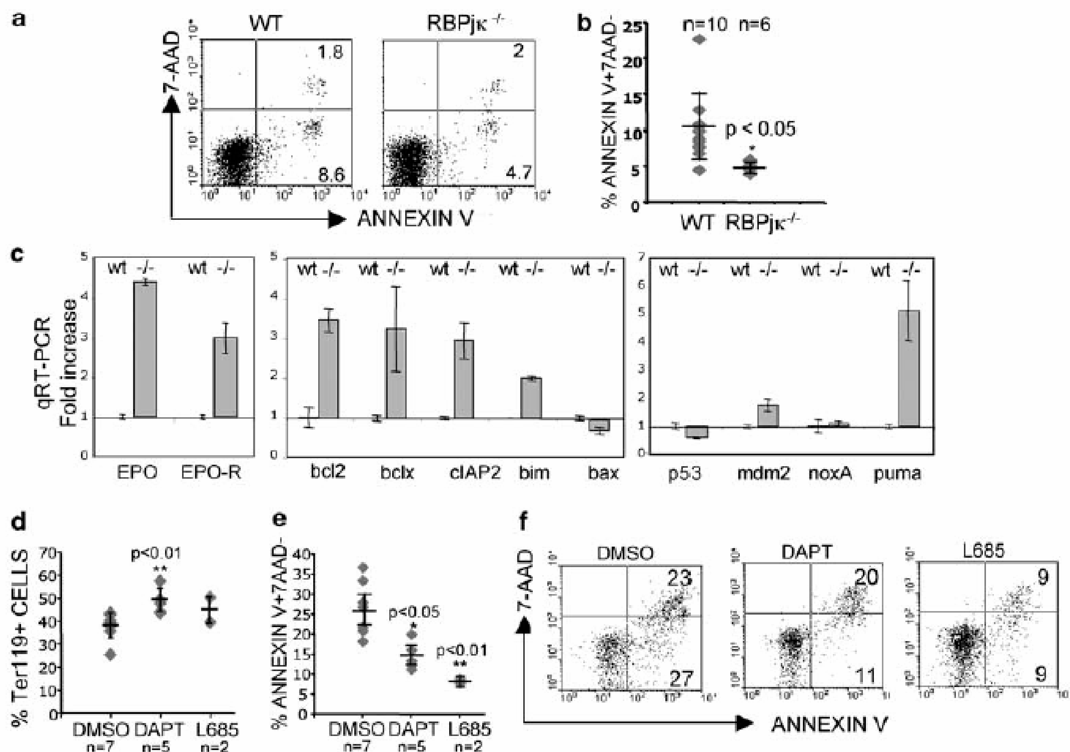


Figure 4 RBPj$\kappa^{-/-}$ and γ-secretase inhibitor-treated erythroid cells show reduced apoptosis. (**a**) Dot plot of AnnexinV and 7-AAD staining in a representative E9.5 WT and RBPj$\kappa^{-/-}$ yolk sac. (**b**) Percentage of AnnexinV + 7AAD– cells in the Ter119 + cells in E9.5 WT and RBPj$\kappa^{-/-}$ yolk sacs. (**c**) Relative expression of pro- and antiapoptotic genes in E9.5 RBPj$\kappa^{-/-}$ compared with WT yolk sacs by qRT-PCR. (**d** and **e**) Graphs represent the percentage of Ter119 + cells (**d**) and AnnexinV + 7AAD-cells (**e**) from E9.5 WT yolk sacs after 5 days of culture in DMSO, 50 μM DAPT or 2 μM L685,458. (**f**) Dot plots from a representative experiment.

proliferation or differentiation were found in the RBPj$\kappa^{-/-}$ mutants, we tested whether the increased number of erythroid cells in these embryos was due to differences in apoptosis. We detected a significant reduction ($P<0.05$) in the apoptotic rate as measured by AnnexinV binding in the RBPj$\kappa^{-/-}$ yolk sacs that was specific for the erythroid Ter119+ population (Figure 4a and b).

Next, we analyzed the expression levels of different genes that are associated with apoptosis in purified Ter119+ cells. By qRT-PCR, we detected 3–4-fold increase in the expression levels of the erythroid survival factor EPO, its receptor and its downstream effectors, the antiapoptotic *bcl-2* and *bcl-x* genes in the RBPj$\kappa^{-/-}$ cells compared with the WT. This suggests that EpoR-mediated signaling may be participating in the increased survival of RBPj$\kappa^{-/-}$ Ter119+ cells. In contrast, no major differences were found in the expression levels of *p53* family genes although one of its proapoptotic targets *puma* was increased in the mutant cells compared with the WT (Figure 4c, right panel).

To further demonstrate that reduced apoptosis in the RBPj$\kappa^{-/-}$ erythroid cells was dependent on Notch function, we obtained WT cells from disrupted yolk sacs and incubated in liquid culture in the presence or absence of the γ-secretase inhibitors DAPT and L685,458. After 6 days of culture, both the total number and the percentage of TER119+ cells was significantly increased in the DAPT-treated cells compared with the control (Figure 4d) resembling the RBPj$\kappa^{-/-}$ phenotype. The increase in the Ter119+ population correlated with a threefold reduction in the percentage of AnnexinV+ cells in the DAPT- and L685,458-treated cells (Figure 4e and f). In agreement with the differences in gene expression found in the RBPj$\kappa^{-/-}$ yolks sacs, upregulation of EPO, its receptor, *bcl-2* and *bcl-x* was detected in the γ-secretase-treated Ter119+ cells (Supplementary Figure S5). Together these results indicate that Notch activity regulates apoptosis in the erythroid lineage in the yolk sac.

Notch activation induces apoptosis in adult BM erythroid and MEL cells

We next investigated whether Notch activation was regulating apoptosis not only in the yolk sac but also in adult erythropoiesis. Since RBPj$\kappa^{-/-}$ mutants do not generate definitive hematopoiesis and die at E10.5, we isolated BM from WT adult mice and performed cell cultures in the presence of DMSO, DAPT or L685,458. We observed a 30% decrease in the percentage of AnnexinV+ cells in the Ter119+ population after 2 days of culture in γ-secretase inhibitors compared with the control (Figure 5a). Interestingly, Ter119-negative cells were not protected from apoptosis by DAPT in these cultures (data not shown), indicating the specific proapoptotic effect of Notch activity on the erythroid lineage. Conversely, incubation of total BM cells from adult mouse on NIH-3T3-Jag1 cells resulted in a

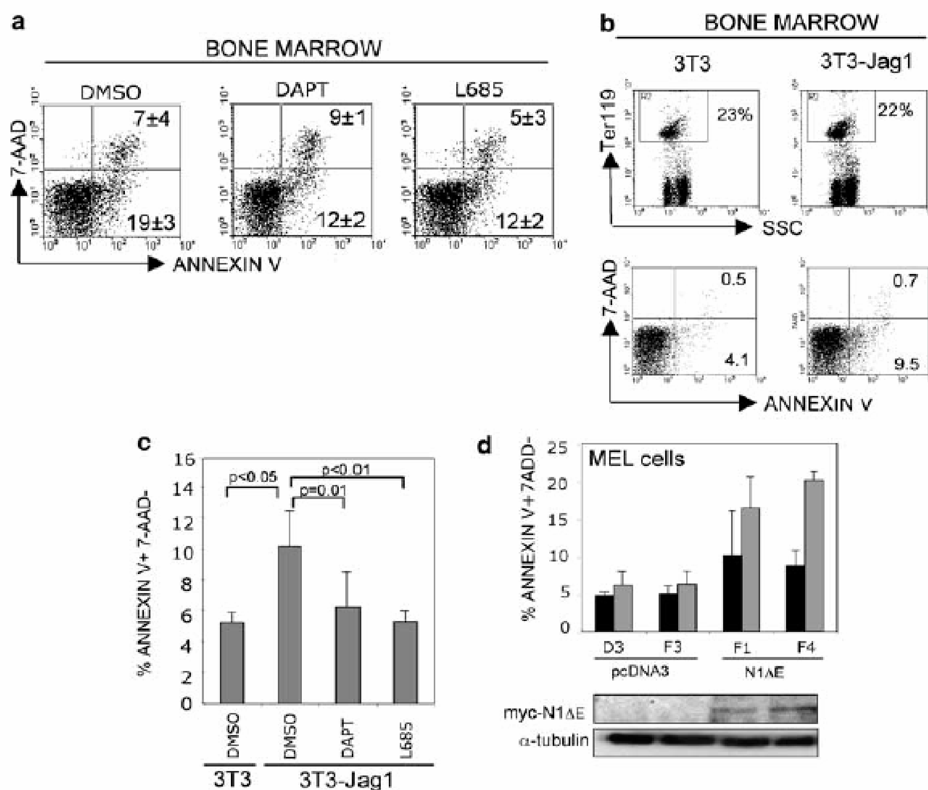


Figure 5 Notch activity induces apoptosis in the Ter119+ cells from adult bone marrow and murine erythroleukemia cells. (**a**) Graphs represent the percentage of AnnexinV+7AAD- in the Ter119+ population of BM cultured in the presence of DMSO, DAPT or L685,458. Average percentage and s.d. of three different experiments are shown. (**b** and **c**) Representative flow cytometry analysis of AnnexinV binding in the Ter119+ cells from BM incubated on 3T3 or 3T3-Jag1 for 16 h (**b**). Graphs represent the average and s.d. of 3T3-Jag1-induced apoptosis observed in three different experiments incubated with DAPT and L685,458 as measured by AnnexinV+7AAD- in Ter119+ cells (**c**). (**d**) Graphs represent the average percentage of AnnexinV+7AAD- cells in Notch1ΔE expressing MEL clones (F1, F4) compared with control clones (D3, F3) in proliferantion media (black bars) or after 6 days of differentiation in 5 mM HMBA (gray bars). Western blot shows the expression of Notch1ΔE.

2–3-fold increase in the percentage of AnnexinV + Ter119 + population compared with that incubated on NIH-3T3 control cells ($P = 0.04$) (Figure 5b and c). Moreover, this effect was prevented in the presence of γ-secretase inhibitors DAPT ($P = 0.01$) or L685,458 ($P = 0.002$; Figure 5c) and the caspase inhibitor Z-VAD-FMK (data not shown).

To further demonstrate that Notch was sufficient to induce apoptosis in erythroid cells, we generated different clones of MEL cells expressing the active Notch1 fragment, N1ΔE. Cells expressing N1ΔE differentiated and proliferated similar to the controls (Supplementary Figure S3); however, these cells showed a higher percentage of AnnexinV binding after 6 days in culture under differentiating conditions (HMBA) as expected (Figure 5d).

Altogether our results indicate that Notch positively regulates apoptosis in embryonic and adult erythropoiesis and in differentiating erythroleukemia cells.

Discussion

Notch signaling had been linked previously to regulate apoptosis in erythroid cells, although it was unclear whether Notch inhibits or promotes apoptosis. We have now used primary cells from yolk sac and BM to elucidate the physiological effect of Notch activation on the erythroid lineage. We demonstrate that the Notch pathway is a positive inducer of apoptosis in erythroid cells from different tissues.

Although different cytokines regulate the integrity of the erythroid compartment, production of definitive erythroid cells is mainly dependent on Epo.[29] Signaling from EpoR through Jak2/Stat5[30,31] prevents apoptosis on this cell lineage from late erythroid progenitors (CFU-E) until the onset of hemoglobinization,[32,33] and one important target gene of this cascade is bcl-x.[34] Studies using bcl-x-null mice demonstrated a critical role for this gene at the end of erythroid maturation when maximal hemoglobin synthesis occurs.[35] RBPj$\kappa^{-/-}$ erythroid cells show a clear upregulation of genes involved in this antiapoptotic pathway, such as EPO, EPO-R, bcl-x and bcl-2, suggesting that Notch may induce apoptosis by impinging on this pathway. Previous reports have attempted to link Notch induction of apoptosis through the p53 pathway in other systems.[36,37] We have also explored that possibility in erythroid cells, since there is increasing evidence of p53-mediated apoptosis at different stages of erythroid differentiation;[13,38] however, our results show that crucial p53 pathway genes are not affected in the RBPj$\kappa^{-/-}$ erythroid cells suggesting that this pathway is not responsible for the protection of apoptosis in the RBPjκ mutants. In fact, we found an erythroid-specific upregulation of the proapoptotic p53-target gene, *puma*, in the RBPj$\kappa^{-/-}$ Ter119 + cells, which is surprising since this population has a lower apoptotic rate.

Although previous reports have attempted to decipher which is the Notch function in apoptosis of erythroid cells,[15,19] this is the first time that this question is addressed in primary cells. The use of cell lines in the previous published reports may explain the controversial data. Our data is in agreement with the results from Ishiko *et al.*[15] using K562 cells in which Notch activity induces apoptosis. In this system, Notch activation led to downregulation of bcl-x, likely through repression of GATA1 by hes1 under differentiation conditions.[15] Similarly, we found increased levels of bcl-x and bcl-2 in the Ter119 + cell population of RBPj$\kappa^{-/-}$; however, we did not detect any change in hes1 and GATA1 levels in this model. The observation that hes1 is not affected by the lack of RBPjκ in particular cell types has already been described and explained by the repressor function of RBPjκ in the absence of Notch signaling (reviewed by Lai[2]).

We now have used different approaches including the Notch loss-of-function RBPj$\kappa^{-/-}$ embryos, γ-secretase inhibitors and Jag1-expressing cell co-culture to investigate the role of Notch signaling in regulating apoptosis on erythroid cells. Together, our results confirm that Notch activation favors apoptosis in this particular lineage and this finding may be particularly relevant in some erythroid disorders. In fact, erythropoiesis in the RBPj$\kappa^{-/-}$ embryos resembles the one observed in myelodysplastic syndrome (MDS)-derived leukemias or polycytemia vera. These human myeloproliferative syndromes cause the overproduction of erythroid cells, mainly because of activating mutations in the Jak2 kinase and this leads not only to increased cell proliferation but also upregulation of *bcl-x* (reviewed by Campbell and Green[39]) and protection from apoptosis. In this scenario and based on our results, it is particularly interesting to investigate whether the Notch pathway is deregulated in these tumors. In this sense, higher expression of the Notch-like ligand, dlk, in MDS compared with myeloid leukemia cells has been detected by microarray analysis.[40]

Notch activation has previously been associated to inhibition of apoptosis in normal endothelial[41] and T cells,[42] likewise in neoplastic cells including Kaposi's sarcoma,[43] glioma[44] and Hodgkin's lymphoma[45] cells. Conversely, Notch activation can induce apoptosis during the development of the retina[46] and the serotonin lineage[47] in *Drosophila*. Notch also induces apoptosis in B-cell lymphomas,[16] normal monocytes[48] or hepatocellular carcinoma cells.[49] Based on this and our results, it is likely that apoptosis is another mechanism used by Notch to control specific tissue homeostasis.

Altogether, our results indicate that the activation of the Notch pathway is able to regulate the erythroid lineage in different hematopoietic tissues by inducing apoptosis.

Acknowledgements

We thank Liheng Li for Jagged1 plasmid, Raphael Kopan for N1ΔE plasmid and M Caudy for the hes1 plasmid. Thanks to I Fabregat and J Gil for extremely helpful suggestions and reagents. Many thanks to L Riera and J Inglés-Esteve for critical comments of the manuscript. We thank E Castaño from Serveis Cientifico-Tècnics, UB-Bellvitge, and J Petriz for flow cytometry technical support. LE is an investigator from the Carlos III program (ISCIII/02/3027). ARM is a recipient of a CIRIT predoctoral fellowship (2002-SI00791). This work was supported by the grants from Ministerio de Educación y Ciencia (SAF2004-03/98) and (SAF205-23978-E) and (SAF2003-07214).

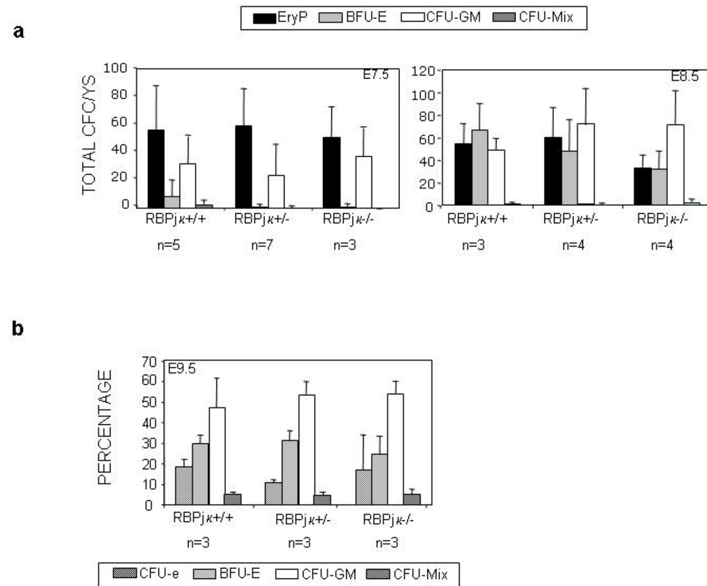
References

1 Bray SJ. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 2006; 7: 678–689.
2 Lai EC. Notch signaling: control of cell communication and cell fate. *Development* 2004; **131**: 965–973.
3 Kumano K, Chiba S, Kunisato A, Sata M, Saito T, Nakagami-Yamaguchi E *et al*. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* 2003; **18**: 699–711.
4 Gering M, Patient R. Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. *Dev Cell* 2005; **8**: 389–400.
5 Burns CE, Traver D, Mayhall E, Shepard JL, Zon LI. Hematopoietic stem cell fate is established by the Notch–Runx pathway. *Genes Dev* 2005; **19**: 2331–2342.

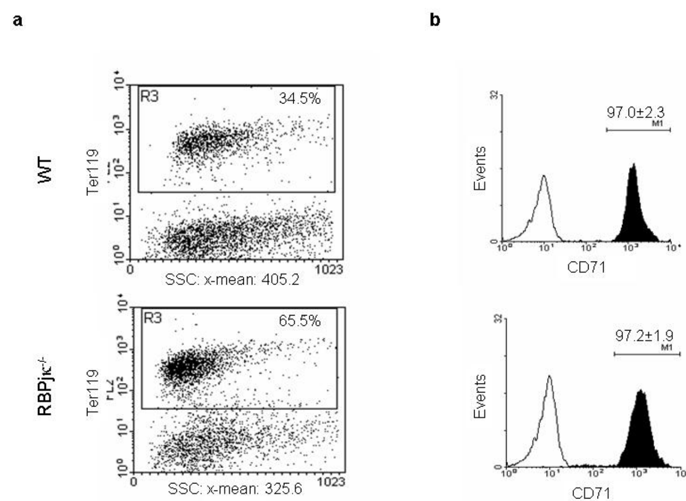
6 Robert-Moreno A, Espinosa L, de la Pompa JL, Bigas A. RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. *Development* 2005; **132**: 1117–1126.
7 Palis J, Robertson S, Kennedy M, Wall C, Keller G. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* 1999; **126**: 5073–5084.
8 Palis J, Segel GB. Developmental biology of erythropoiesis. *Blood Rev* 1998; **12**: 106–114.
9 Mulcahy L. The erythropoietin receptor. *Semin Oncol* 2001; **28**: 19–23.
10 Silva M, Grillot D, Benito A, Richard C, Nunez G, Fernandez-Luna JL. Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. *Blood* 1996; **88**: 1576–1582.
11 Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Nakayama K *et al*. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science* 1995; **267**: 1506–1510.
12 Ganguli G, Back J, Sengupta S, Wasylyk B. The p53 tumour suppressor inhibits glucocorticoid-induced proliferation of erythroid progenitors. *EMBO Rep* 2002; **3**: 569–574.
13 Peller S, Frenkel J, Lapidot T, Kahn J, Rahimi-Levene N, Yona R *et al*. The onset of p53-dependent apoptosis plays a role in terminal differentiation of human normoblasts. *Oncogene* 2003; **22**: 4648–4655.
14 Weiss MJ, Orkin SH. Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. *Proc Natl Acad Sci USA* 1995; **92**: 9623–9627.
15 Ishiko E, Matsumura I, Ezoe S, Gale K, Ishiko J, Satoh Y *et al*. Notch signals inhibit the development of erythroid/megakaryocytic cells by suppressing GATA-1 activity through the induction of HES1. *J Biol Chem* 2005; **280**: 4929–4939.
16 Zweidler-McKay PA, He Y, Xu L, Rodriguez CG, Karnell FG, Carpenter AC *et al*. Notch signaling is a potent inducer of growth arrest and apoptosis in a wide range of B-cell malignancies. *Blood* 2005; **106**: 3898–3906.
17 Romer S, Saunders U, Jack HM, Jehn BM. Notch1 enhances B-cell receptor-induced apoptosis in mature activated B cells without affecting cell cycle progression and surface IgM expression. *Cell Death Differ* 2003; **10**: 833–844.
18 Jang MS, Miao H, Carlesso N, Shelly L, Zlobin A, Darack N *et al*. Notch-1 regulates cell death independently of differentiation in murine erythroleukemia cells through multiple apoptosis and cell cycle pathways. *J Cell Physiol* 2004; **199**: 418–433.
19 Shelly LL, Fuchs C, Miele L. Notch-1 inhibits apoptosis in murine erythroleukemia cells and is necessary for differentiation induced by hybrid polar compounds. *J Cell Biochem* 1999; **73**: 164–175.
20 Oka C, Nakano T, Wakeham A, de la Pompa JL, Mori C, Sakai T *et al*. Disruption of the mouse RBP-J kappa gene results in early embryonic death. *Development* 1995; **121**: 3291–3301.
21 Friend C, Preisler HD, Scher W. Studies on the control of differentiation of murine virus-induced erythroleukemic cells. *Curr Top Dev Biol* 1974; **8**: 81–101.
22 Schroeter EH, Kisslinger JA, Kopan R. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* 1998; **393**: 382–386.
23 Li L, Milner LA, Deng Y, Iwata M, Banta A, Graf L *et al*. The human homolog of rat jagged1 expressed by marrow stroma inhibits differentiation of 32d cells through interaction with notch1. *Immunity* 1998; **8**: 43–55.
24 Lam LT, Ronchini C, Norton J, Capobianco AJ, Bresnick EH. Suppression of erythroid but not megakaryocytic differentiation of human K562 erythroleukemic cells by notch-1. *J Biol Chem* 2000; **275**: 19676–19684.
25 Xue Y, Gao X, Lindsell CE, Norton CR, Chang B, Hicks C *et al*. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet* 1999; **8**: 723–730.
26 Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP *et al*. Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev* 2000; **14**: 1343–1352.
27 Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev* 2004; **18**: 2469–2473.
28 Loken MR, Shah VO, Dattilio KL, Civin CI. Flow cytometric analysis of human bone marrow: I. Normal erythroid development. *Blood* 1987; **69**: 255–263.
29 Ihle JN, Quelle FW, Miura O. Signal transduction through the receptor for erythropoietin. *Semin Immunol* 1993; **5**: 375–389.
30 Neubauer H, Cumano A, Muller M, Wu H, Huffstadt U, Pfeffer K. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell* 1998; **93**: 397–409.
31 Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF. Ineffective erythropoiesis in Stat5a(−/−)5b(−/−) mice due to decreased survival of early erythroblasts. *Blood* 2001; **98**: 3261–3273.
32 Koury MJ, Bondurant MC. Control of red cell production: the roles of programmed cell death (apoptosis) and erythropoietin. *Transfusion* 1990; **30**: 673–674.
33 Yu H, Bauer B, Lipke GK, Phillips RL, Van Zant G. Apoptosis and hematopoiesis in murine fetal liver. *Blood* 1993; **81**: 373–384.
34 Dolznig H, Habermann B, Stangl K, Deiner EM, Moriggl R, Beug H *et al*. Apoptosis protection by the Epo target Bcl-X(L) allows factor-independent differentiation of primary erythroblasts. *Curr Biol* 2002; **12**: 1076–1085.
35 Motoyama N, Kimura T, Takahashi T, Watanabe T, Nakano T. bcl-x Prevents apoptotic cell death of both primitive and definitive erythrocytes at the end of maturation. *J Exp Med* 1999; **189**: 1691–1698.
36 Yang X, Klein R, Tian X, Cheng HT, Kopan R, Shen J. Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. *Dev Biol* 2004; **269**: 81–94.
37 Huang Q, Raya A, DeJesus P, Chao SH, Quon KC, Caldwell JS *et al*. Identification of p53 regulators by genome-wide functional analysis. *Proc Natl Acad Sci USA* 2004; **101**: 3456–3461.
38 Maetens M, Doumont G, De Clercq S, Francoz S, Froment P, Bellefroid E *et al*. Distinct Roles of Mdm2 and Mdm4 in Red Cell Production. *Blood* 2007; **109**: 2630–2633.
39 Campbell PJ, Green AR. The myeloproliferative disorders. *N Engl J Med* 2006; **355**: 2452–2466.
40 Miyazato A, Ueno S, Ohmine K, Ueda M, Yoshida K, Yamashita Y *et al*. Identification of myelodysplastic syndrome-specific genes by DNA microarray analysis with purified hematopoietic stem cell fraction. *Blood* 2001; **98**: 422–427.
41 MacKenzie F, Duriez P, Wong F, Noseda M, Karsan A. Notch4 inhibits endothelial apoptosis via RBP-Jkappa-dependent and -independent pathways. *J Biol Chem* 2004; **279**: 11657–11663.
42 Jehn BM, Bielke W, Pear WS, Osborne BA. Cutting edge: protective effects of notch-1 on TCR-induced apoptosis. *J Immunol* 1999; **162**: 635–638.
43 Curry CL, Reed LL, Golde TE, Miele L, Nickoloff BJ, Foreman KE. Gamma secretase inhibitor blocks Notch activation and induces apoptosis in Kaposi's sarcoma tumor cells. *Oncogene* 2005; **24**: 6333–6344.
44 Purow BW, Haque RM, Noel MW, Su Q, Burdick MJ, Lee J *et al*. Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. *Cancer Res* 2005; **65**: 2353–2363.
45 Jundt F, Anagnostopoulos I, Forster R, Mathas S, Stein H, Dorken B. Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. *Blood* 2002; **99**: 3398–3403.
46 Miller DT, Cagan RL. Local induction of patterning and programmed cell death in the developing *Drosophila* retina. *Development* 1998; **125**: 2327–2335.
47 Lundell MJ, Lee HK, Perez E, Chadwell L. The regulation of apoptosis by Numb/Notch signaling in the serotonin lineage of *Drosophila*. *Development* 2003; **130**: 4109–4121.
48 Ohishi K, Varnum-Finney B, Flowers D, Anasetti C, Myerson D, Bernstein ID. Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. *Blood* 2000; **95**: 2847–2854.
49 Qi R, An H, Yu Y, Zhang M, Liu S, Xu H *et al*. Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. *Cancer Res* 2003; **63**: 8323–8329.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

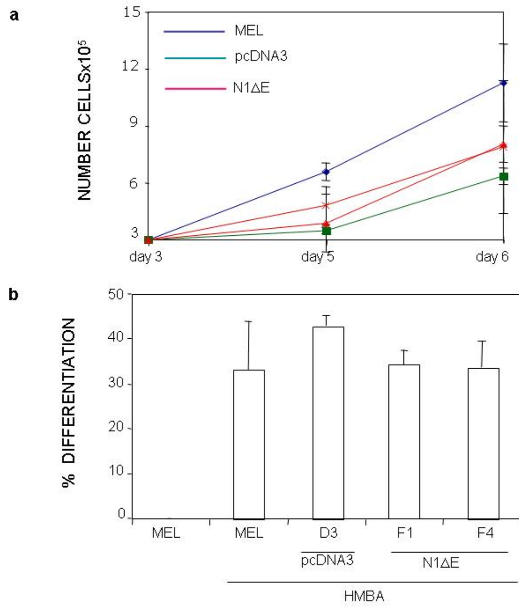
SUPPLEMENTARY INFORMATION



Supplementary Figure S1: *Analysis of myeloid and erythroid progenitors from collagenase-treated yolk sacs. (a) Total CFC/YS at E7.5 and E8.5. (b) Percentage of CFC at E9.5*

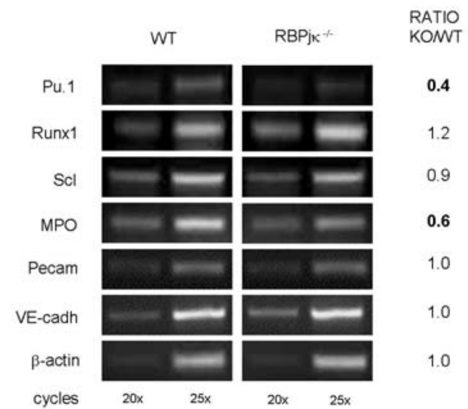


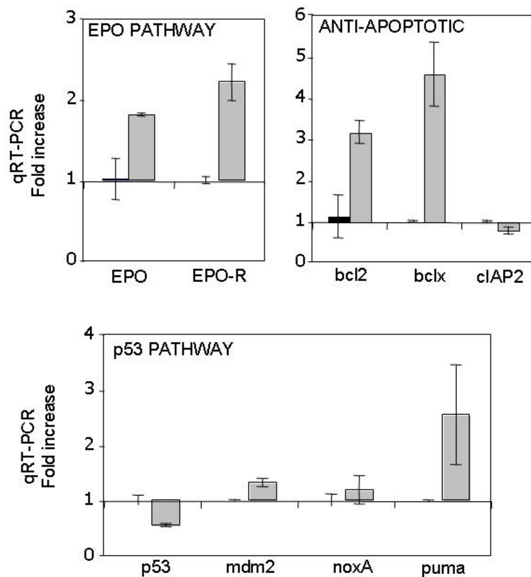
Supplementary Figure S2: *Ter119+ cells from the yolk sac express CD71. (a) Ter119+ cells from WT and mutant yolk sacs. X-mean of SSC is shown for both samples. (b) Percentage of gated Ter119+ cells expressing CD71. Numbers represent the average percentage and standard deviation of 3 different yolk sacs.*



Supplementary Figure S3: *Differentiation and proliferation of pcDNA3 and N1ΔE MEL cells. (a) Cell numbers for MEL, pcDNA3 and N1ΔE clones at day 3, 5 and 6. (b) Percentage of dianisidine positive cells after 6 days in HMBA-differentiating media.*

Supplementary Figure S4: *Semiquanti-tative RT-PCR of hematopoietic and endothelial genes from E9.5 WT and RBPjκ $^{-/-}$ yolk sacs.*





Supplementary Figure S5: *qRT-PCR from sorter Ter119+ cells from YS cultured for 6 days in DMSO and γ–secretase inhibitor L685,458.*