Jagged1 and Notch1 involvement in Haematopoietic Stem Cell Development

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Declaration

I declare that the work present in this thesis is my own except where otherwise stated.

The work has not been submitted for any other degree or professional qualification.

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Abstract

Previous studies have identified the Notch signalling pathway as an important regulator of haematopoietic development. However its role in definitive haematopoietic stem cell (dHSC) development is still unclear mainly due to the fact that Notch mutants die around mid-gestation before the emergence of the first dHSC. Here I investigated the role of the Notch signalling pathway in dHSC development focusing on the ligand Jagged1 and the receptor Notch1. I carried out a detailed characterisation of the expression pattern of Notch1 and Jagged1 in the aorta-gonad-mesonephros (AGM) region, where dHSCs first emerge in the embryo, by immunocytochemistry and immunohistochemistry. I then determined, by sorting cells from the AGM region based on their level of Notch1, that Notch1 was highly expressed in endothelial cells, precursors of dHSCs (called pre-HSC) and dHSCs, and its expression then decreases in haematopoietic progenitors.

I also generated a Jagged1 dtTomato knock-in reporter mouse using a combination of recombineering and traditional cloning to produce a targeting vector, followed by targeting a Bl6 ES cell line, and producing a mouse line from a correctly targeted ES cell clonal line. This mouse line allowed me to visualise Jagged1 expression during dHSC development. With the line I showed that pre-HSCs express both Jagged1 and Notch1 and that Jagged1$^+$Notch1$^+$ cell surface marker phenotype can enrich the pre-HSC population 1 in 8.

To further investigate the implication of Jagged1 in dHSC development the gene was conditionally deleted in the HSC lineage using a CD41-Cre. Our result indicated that Jagged1 is not required for HSC development in a cell autonomous manner. Furthermore, I carried out experiments with a conventional Jagged1 knock-out mouse line. It has previously been shown that Jagged1 null embryos die around E10.5 and contain fewer intra-haematopoietic progenitors. I used an explant culture system to culture E10.5 AGM regions from Jagged1$^{-/-}$ embryos past the point of embryo lethality and in culture HSCs were produced. This result indicates that Jagged1$^{-/-}$ embryos contain pre-HSCs that can mature efficiently into dHSC *in vitro*.

Lay Summary

In adult organisms all blood cells are derived from blood stem cells, called Haematopoietic Stem Cells (HSC). In the mouse the first HSC emerge in the main artery of the embryo around mid-gestation. The appearance of HSCs in the embryo results from the expansion and maturation of precursors that are called pre-HSCs. A cell signalling pathway, called Notch, has previously been shown to play an important role in the formation of the blood system in the mouse embryo. However its exact role in HSC formation is still unclear. The aim of my project is to determine the roles of two important molecules involved in the Notch pathway, Jagged1 and Notch1, during HSC development. First I showed that Notch1 is expressed by cells involved in the development of HSCs and that the levels of Notch1 expression decreases as the cells get closer to becoming mature blood cells. Then I created a reporter mouse line in which we can easily detect cells expressing Jagged1. Using this mouse I showed that pre-HSCs express both Notch1 and Jagged1. Finally to further investigate the role of Jagged1 in the development of HSCs, I used a mouse line that is deficient for Jagged1. The mutant embryos are defective and die before the first HSC develops. To overcome this problem, the embryos were harvested before their death and they were cultured for a few days. The results of this experiment showed that even in absence of Jagged1 pre-HSCs are present in the embryos and can mature efficiently into HSCs during the culture. Altogether I showed that even though Jagged1 is expressed by the HSC lineage in the mouse embryo, it is dispensable for their development.

Abbreviations

7AAD	7-aminoactinomycin D
AGM	Aorta Gonad Mesonephros
Amp	Ampicillin
BFU-E	Burst Forming Unit-Erythroid
Carb	Carbenicilin
Cat	Chloramphenicol
CcdB	Cell division B
CFU	Colony Forming Unit
CFU-C	Colony Forming Unit-Culture
CFU-GEMM	CFU-Granulocyte/Erythrocyte/Macrophage/Megakaryocyte
CFU-GM	CFU-Granulocyte/Macrophage
CFU-Mac	CFU-Macrophage
CFU-Mast	CFU-Mast cell
CFU-S	Colony Forming Unit-Spleen
DAPI	4-6 Diamidino-2-phenylindole
Dll	Delta-Like
DTA	Diphtheria Toxin Gene
E	Embryonic Day
EB	Embryonic Body
ee	Embryo equivalent
EHT	Endothelial to Haematopoietic Transition
ES	Embryonic Stem
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FL	Fetal Liver
Flk3l	Fms-like tyrosine kinase 3-ligand
GFP	Green Fluorescent Protein
hBact	Human β-actin Promoter
HIAC	Haematopoietic Intra-Aortic Cluster
HSC	Haematopoietic Stem Cell
IL	Interleukin

Jag1	Jagged1
KI	Knock-In
KO	Knock-out
LSK	Lin⁻Sca1⁺c-kit⁺
LTR	Long-Term Repopulating
Neo	Neomycin
ori	Origin of Replication
P/S	Penicillin/Streptomycin
pA	Polyadenylation Signal
PBS	Phosphate Buffered Saline
PGK	Phosphoglycerate Kinase
preHSC I	pre-HSC Type I
preHSC II	pre-HSC Type II
P-Sp	Para-aortic Spanchnopleura
RI	Random Insertion
RT	Room Temperature
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SAP	Sub-Aortic Patch
SCF	Stem Cell Factor
SCL	Stem Cell Leukaemia
SLAM	Signalling Lymphocyte Activation Molecule
sp	Somite Pair
Spec	Spectinomycin
Tet	Tetracycline
UGR	Urogenital Ridge
UTR	Untranslated Region
VECad	VE-Cadherin
VEGF	Vascular Endothelial Growth Factor
WT	Wild-type
YS	Yolk Sac
Zeo	Zeocin

Table of Contents

1 Introduction

1.1 Stem Cells

The first studies of stem cells started over 40 years ago with the works of Stevens, Pierce, and Kleinsmith on teratocarcinomas, and Till and McCulloch on haematopoietic stem cells (HSCs) (Kleinsmith & Pierce Jr 1964; Till & McCulloch 1961). Since then, many studies to characterise different populations of stem cells have been carried out and the concept of stem cells has evolved. A consensual definition of stem cells has arisen based on their functional properties: A stem cell (SC) is defined as an undifferentiated cell that possesses the ability to self-renew and differentiate to give rise to multiple mature and functional cell types. The process of differentiation occurs when cells mature into a cell type with a more distinct form and function. Cells undergo self-renewal by dividing and maintaining the undifferentiated state, stem cells can replicate for the life of the organism. Stem cells differentiate into functional cell types through intermediate cells type called progenitors. Progenitors have a more limited self-renewal capacity and more limited differentiated potential than their parent cell and are a step further towards becoming fully differentiated cells.

It is possible to classify stem cells based on their differentiation potential:

- Pluripotent stem cells can give rise to all cell lineages from all 3 germ layers (endoderm, mesoderm, and ectoderm). Some examples of pluripotent stem cells include: embryonic stem (ES) cells; epiblast stem cells (EpiSC); and induced pluripotent stem (iPS) cells. These cells can also be maintained in an undifferentiated state *in vitro* and used as a source for the production of differentiated cells. ES cells are derived from the inner cell mass of the pre-implantation developing blastocyst (Evans & Kaufman 1981; Martin 1981; Thomson et al. 1998). Induced pluripotent stem cells (iPS cells) are produced *in vitro* from differentiated adult cells by enforced gene expression (Takahashi & Yamanaka 2006; Yu et al. 2007). These cells are pluripotent like ES cells and present an exciting opportunity for the production of patient tailored cells for treatment and drug discovery. EpiSC are derived from the post-implantation embryo (Tesar et al. 2007; Brons et al. 2007).

- Multipotent stem cells have the capacity to differentiate into multiple cell types that constitute a tissue. For instance HSC are multipotent as they can give rise to all blood cells.

- Oligopotent/Unipotent stem cells generate a small number or only one cell type in a tissue for example satellite cells in the muscle (Sacco et al. 2008).

Multipotent/Oligopotent/Unipotent stem cells are also called tissue specific stem cells and differentiate to regenerate the specialised cells of that tissue during the lifespan of the individual.

However, stem cells do not always play a physiological role. In 1994 it was discovered that human acute myeloid leukaemia was transplantable (Lapidot et al. 1994) and the concept of a cancer stem cell (CSC) arose. It is thought that many cancers arise through a hierarchy of differentiation with a CSC at the top, similar to how normal tissue is maintained. A CSC is defined as "a cell within a tumour that possesses the capacity to self-renew and to cause heterogeneous lineages of cancer cells that comprise that tumour". It is thought that in that majority of cases CSCs arise from the accumulation of mutations in normal stem cells leading to their transformation. Like normal stem cells they are difficult to phenotypically define, and isolate, and understanding of the process is further complicated by a possible "dynamic stemness" of non-CSC in a tumour under certain conditions. It is however clear that understanding the process of tumour production and targeting of CSC in cancer therapy are very important factors in cancer therapeutics (reviewed in Sugihara & Saya 2013).

Understanding the processes involved in stem cell generation, self-renewal, and differentiation therefore has important applications in regenerative medicine, and cancer studies. The remainder of this introduction will focus on haematopoietic stem cells (HSC).

1.2 Adult HSC

1.2.1 HSC and Haematopoiesis

The adult HSCs maintain the adult blood system throughout the life of the organism, in both steady and stressed states, through a process called haematopoiesis. This is achieved by producing different haematopoietic progenitors which contribute to all blood cells lineages through a hierarchy of differentiation, as a multi-step process, of which one simplified version is shown in figure 1.1. This process is well defined in mammals due to the characterization of immunophenotypic markers, the existence of powerful *in vitro* and *in vivo* functional tests, and the use of genetic tools.

Downstream of HSCs in the adult haematopoietic hierarchy a heterogeneous population of multipotent progenitors (MMPs) still possess multilineage differentiation potential but lack unlimited self-renewal ability. Further downstream of MMPs, common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) possess limited self-renewal capacity and their differentiation potential is restricted to the lymphoid and myeloid lineages respectively. Through a range of intermediate progenitors which are progressively more lineage restricted and less able to self-renew CLPs give rise to mature lymphocytes (B cells, T cells and NK cells) and CMPs give rise to erythrocytes, platelets, granulocytes (neutrophils, basophils, eosinophils) and monocytes (when within tissues called macrophages). (Reviewed by (Seita & Weissman 2010)) This hierarchy has been shown to be flexible with other route of differentiation possible.

The HSC population is quite heterogeneous. Some HSCs remain quiescent in the bone marrow and rarely divide or differentiate, some HSCs give rise to more myeloid lineages than lymphoid and *vice versa* (Challen et al. 2010; Dykstra et al. 2007; Müller-Sieburg et al. 2002; Sieburg et al. 2006; Oguro et al. 2013). Thanks to functional assays described in the next paragraph, it has been shown that there is approximately 1 HSC in 10,000 nucleated murine bone marrow cells and that HSCs are highly enriched in the Lin$^-$Sca1$^+$c-kit$^+$CD150$^+$CD48$^-$CD34$^-$Flt3$^-$ population (Kiel et al. 2005; Kumaravelu et al. 2002; Osawa et al. 1996; Spangrude et al. 1989; Wilson et al. 2008; Boyer et al. 2011). Lineage negative (Lin-) refers to the exclusion of cells expressing known markers of lineage committed haematopoietic cells and

can include: CD3, CD4, CD5, CD8, B220, Gr1, Mac1 and Ter119 (Boyer et al. 2011). However, we still cannot identify with certainty an individual HSC based only on the expression of its immunophenotypic markers. This is an important experimental limitation when trying to isolate HSCs by fluorescence activated cell sorting (FACS) or visualize them in their niche.

1.2.2 Assessment of HSC activity

HSC were initially characterized based on their capacity to generate colonies on the spleen of irradiated mice after transplantation (Till and McCulloch, 1961; Wu et al., 1968). However these cells, called colony-forming unit-spleen (CFU-S), are progenitors that are more mature than HSCs (Purton & Scadden 2007). The gold standard assay to identify a HSC is to show engraftment after transplantation into irradiated adult recipients. This shows the HSC is able to home to the bone marrow, engraft, regenerate, and contribute to all the blood cell lineages in the recipient (Harrison 1980). Subsequent transplantation of the bone marrow of recipient mice into secondary recipients and detection of cells derived from the primary donor show the capacity of the HSC to self-renew (they can be transplanted 5 or 6 times) (Lemischka et al. 1986). Large numbers of tested HSCs can be transplanted without carrier cells, such as secondary transplantation of bone marrow. However, transplantation is usually carried out with co-transplanted competitor/carrier cells HSCs which are distinguishable from the recipient and test donor cells. These carrier cells have two purposes: firstly, to provide short-term haematopoiesis to the recipient before HSC engraftment, and secondly, they can be used to measure the ability of the test HSCs to engraft and survive relative to the competitor HSCs (competitive repopulating units (CRU). Limiting dilution studies can be carried out to estimate the number of HSCs in a sample using Poisson statistics (Szilvassy et al. 1990). The CD45.1/CD45.2 system can be used to distinguish donors, recipients and carrier cells. (Reviewed (Purton & Scadden 2007; van Os et al. 2004))

Other less reliable methods for HSC assessment include *in vitro* clonogenic growth assays: the cobblestone area-forming cells (CAFC) and long-term culture-initiating cell (LTC-IC) co-culture systems. The frequency of day 28 CACFs correlates well with HSCs though interlaboratory variability of these assays is observed due to the

use of different feeder layers (Reviewed (Purton & Scadden 2007; van Os et al. 2004)).

The colony-forming cell culture (CFU-C) assay uses commercially available methylcellulose-based culture medium, to assess the presence of myeloid lineage restricted progenitors, by the formation of colonies of haematopoietic cells in culture. The colony types formed after 10 days culture include: BFU-E: burst forming unit erythroid; CFU-Mac: colony-forming cell macrophage; CFU-GM: granulocyte, macrophage; CFU-GEMM: granulocyte, erythroid, macrophage, megakaryocyte. B and T cell production can be assessed from haematopoietic progenitors by co-culture with cell lines (Whitlock & Witte 1982; Schmitt & Zúñiga-Pflücker 2002).

The mechanisms that regulate HSC functions are complex and the next sections will briefly discuss some examples of intrinsic and extrinsic processes that together permit the coordination of HSC activity with the physiologic needs of the individual.

1.2.2 The role of the niche

HSC reside in the bone marrow in close association with the endosteum and also are found adjacent to sinusoidal blood vessels. Endosteum residing osteoblasts and osteoclasts, sinusoidal endothelial cells, and perivascular cells have all been shown to be involved in HSC maintenance. Experiments which lead to an increase in osteoblast numbers resulting in an increase in HSCs (Calvi et al. 2003). Ablation of osteoblasts using Col2.3Δ-TK transgenic mice resulted in decreased haematopoietic cells and haematopoietic progenitors (Zhu et al. 2007; Visnjic et al. 2004), however, using a biglycan-deficient (reduced osteoblasts) transgenic line showed no defects in HSC frequency or haematopoiesis (Kiel et al. 2007). Therefore the role of osteoblast in HSC maintenance and haematopoiesis remains unclear. Ablation of perivascular nestin-expressing mesenchymal stem cells (Nes$^+$ MSC) also reduced the number of LTR-HSCs (Méndez-Ferrer et al. 2010) and a very recent study showed that deletion of Jagged1 in bone marrow endothelial cells resulted in HSC exhaustion (Poulos et al. 2013). It is not known exactly through which mechanisms these cells regulate HSCs, by direct cell contact, secreted factors, or actions on intermediate cells. For example the cytokine CXCL12 is known to be important in HSC homeostasis through its activation of CXCR4. It is expressed by many cells in the bone marrow

including osteoblasts, perivascular CXCL12-abundant reticular cells (CAR cells) (Sugiyama et al. 2006), and perivascular mesenchymal progenitors. This means it is unclear which cell type is the effector CXCL12 function on HSCs or if all the cells expressing CXCL12 play a role. Whether these cell types constitute separate microenvironments or cooperate as part of one HSC niche is unclear. It is likely that different factors play a role at different stages of HSC quiescence, self-renewal and differentiation. The HSC niche remains and unresolved open question. Other factors known to be important in HSC homeostasis found in the niche include: angiopoieitn (Arai et al. 2004) secreted by osteoblast and perivascular cells; Ca_2^+ ions released from bone by osteoclasts; sonic hedgehog (SHH) (Trowbridge et al. 2006); Osteopontin produced by osteoblasts (Nilsson et al. 2005); stem cell factor (SCF) (Bowie et al. 2007); Thrombopoieitin expressed by osteoblasts (Kaushansky 2003); oxygen tension mediated through HIF1α (Takubo et al. 2010); and sheer force. The various components of the bone marrow HSC niche are summarised in figure 1.2. (Reviewed in (Wang & Wagers 2011; Kiel & Morrison 2008)).

It is thought that long-term repopulating haematopoietic stem cells (LTR-HSC) only arise during development *de novo*. The next part of the introduction will describe the mechanisms involved in the generation of HSC, focusing on the mouse model. Determining the many factors involved in the ontogeny and maintenance of HSCs has important implications, such as use in developing an *in vitro* system for the production of HSC, progenitors and mature blood cells from alternative sources (such as ES cells and iPS cells) for clinical use, and to develop a better understanding of blood related diseases, such as leukaemia, allowing development of treatments.

Figure 1.1: Adult haematopoietic hierarchy.
All blood cells are derived from HSCs through a series of downstream progenitors.
LT-HSC: long-term haematopoietic stem cell; MPP: multipotent progenitor; CMP:
common myeloid progenitor; CLP: common lymphoid progenitor; MEP:
megakaryocyte/erythroid progenitor; GMP: granulocyte–macrophage progenitor;
CD135: also known as FLK2 and FLT3; IL-7R: interleukin-7 receptor; lin: lineage
markers (which are a combination of markers found on mature blood cells but not
HSCs or progenitors); NK: natural killer; SCA1: surface cell antigen 1. Adapted
from (Wang & Wagers 2011).
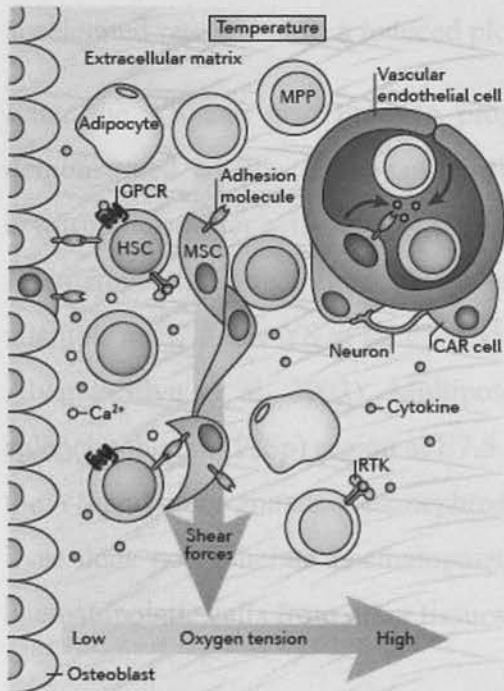
**Figure 1.2: Components of the HSC
niche in adult bone marrow.**
The niche consists of supportive factors
for HSC renewal and differentiation.
These factors include cells, cytokines,
cell adhesion molecules and physical
factors.
CAR, CXCL12-abundant reticular;
GPCR, G protein-coupled receptor; MPP,
multipotent progenitor; MSC,
mesenchymal stem cell; RTK, receptor
Tyr kinase.
From (Wang & Wagers 2011).

16

1.3 Haematopoietic development

Haematopoiesis occurs in the embryo in different tissues including the yolk sac (YS), aorta-gonad-mesonephros (AGM) region, placenta and fetal liver (FL). There are two distinguishable waves of haematopoiesis: embryonic haematopoiesis, which generates a transitory hematopoietic cell population and definitive haematopoiesis that occurs later during development from dHSCs. These are distinguished by genetic control and lineage output. Early embryonic haematopoiesis is summarized in figure 1.3.

1.3.1 Embryonic haematopoiesis

The first wave of haematopoiesis, the embryonic wave, begins in the yolk sac (YS) where differentiated primitive nucleated erythrocytes and progenitors of primitive macrophages and megakaryocytes arise from E7.5 in the YS blood islands (Moore & Metcalf 1970; Silver & Palis 1997). The role of these primitive blood cells is to support blood function during embryonic development before the emergence of HSCs. Hematopoietic cells produced by progenitors in the YS differ from their adult counterparts: The primitive erythrocyte is nucleated and synthesizes embryonic haemoglobins such as ζ globin (Leder et al. 1992), primitive macrophages undergo accelerated maturation and synthesize a different set of enzymes compared to adult macrophages (Naito et al. 1996), primitive megakaryocytes produce platelets at an accelerated rate and have a reduced ploidy (Xu M et al. 2001).

Later the YS can also produce progenitors with an adult blood cell output as demonstrated by the ability of eyrthroid progenitors to contribute to definitive erythropoiesis after activation of the EpoR (erythropoietin receptor) (Lee et al. 2001). Haematopoietic progenitors of the erythroid myeloid lineage are found in the allantois at E7.5, the YS at E8.25, and the placenta at E9.0 (Dzierzak & Speck 2008; Alvarez-Silva et al. 2003). Multipotent progenitors are found in the para-aortic splanchnopleura (P-Sp) region at E7.5 (Dzierzak & Speck 2008). CFU-S are found in the YS and aorta-gonads-mesonephros (AGM) at E9.0 (Medvinsky et al. 1993). The liver does not generate haematopoietic cells *de novo*, it is colonized at E9 with haematopoietic cells from other tissues (Johnson & Moore 1975).
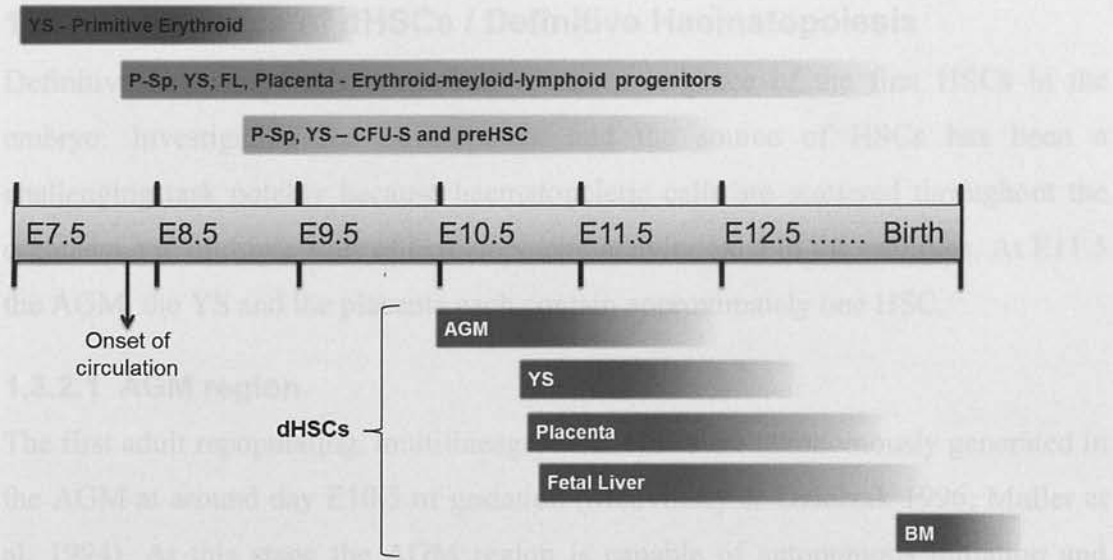
Figure 1.3 Summary of haematopoietic emergence in the embryo.
Haematopoiesis begins in the YS at E7.5 with the emergence of primitive erythrocytes and macrophages through primitive haematopoiesis. After the onset of circulation at E8 multipoent progenitors are detected in the P-Sp, YS, fetal liver and placenta. Pre-HSCs are detected in the E9 P-Sp and YS by transplantation of condition newborns at E9. Adult repopulating HSCs are first detected in the AGM region around E10.5-11.5 and subsequently in the YS, placenta and fetal liver. HSC eventually migrate to the bone marrow where they reside for the life of the organism. YS: yolk sac; P-Sp: para-aortic splanchnopleura; FL: fetal liver; CFU-S: colony forming unit spleen.

18

1.3.2 Emergence of dHSCs / Definitive Haematopoiesis

Definitive haematopoiesis is marked by the emergence of the first HSCs in the embryo. Investigating the development and the source of HSCs has been a challenging task notably because haematopoietic cells are scattered throughout the organism and multiple sites of hematopoietic activity exist in the embryos. At E11.5 the AGM, the YS and the placenta each contain approximately one HSC.

1.3.2.1 AGM region

The first adult repopulating, multilineage, LTR-HSCs are autonomously generated in the AGM at around day E10.5 of gestation (Medvinsky & Dzierzak 1996; Muller et al. 1994). At this stage the AGM region is capable of autonomous initiation and expansion of HSCs indicating its crucial role in HSC development. By E11.5 the AGM region contains one HSC, and between E11.5 and E12.5 HSCs are generated at an extremely high rate via maturation of HSC precursors called pre-HSC type I (CD41^{lo}CD45$^-$VE-Cadherin$^+$) and pre-HSC type II (CD45$^+$VE-Cadherin$^+$) populations (Kumaravelu et al. 2002; Taoudi et al. 2008; Rybtsov et al. 2011). Subsequently HSCs are found in the foetal liver and eventually the bone marrow (Medvinsky & Dzierzak 1996; Muller et al. 1994; Gekas et al. 2005; Ottersbach & Dzierzak 2005).

More specifically, HSCs arise in the dorsal aorta of the AGM region, in the ventral domain and the developing aorta maintains a polarity that is important in HSC emergence (Taoudi & Medvinsky 2007; de Bruijn et al. 2000). Haematopoietic intra-aortic clusters (HIAC), which are clusters of haematopoietic precursors within the lumen of the major arteries, are first detected in the ventral part of the main arteries (aorta, vitelline and umbilical arteries) (de Bruijn et al. 2000) at E10.5 (Garcia-Porrero et al. 1995). HIAC are shown to express markers of the haematopoietic lineage such as CD41 and CD45, and express factors that are required for haematopoiesis in the embryo such as Runx1, c-myb and GATA2 (Godin & Cumano 2002). The size and number of HIACs peak when the number of haematopoietic progenitors in the AGM (Godin et al. 1999) peaks, and at this stage, precursors become detectable in the blood stream (Delassus & Cumano 1996).

1.3.2.2 Yolk Sac

As the yolk sac is the first site of haematopoiesis it was suggested to be the site of HSC development. However experiments performed producing chicken/quail chimeras by grafting chick YS onto quail embryos showed that in the avian model the adult haematopoietic hierarchy is derived from the intra-embryonic region (Dieterlen-Lievre 1975). This grafting strategy is not currently possible for mouse embryos but transplantation of cultured pre-circulatory (E8) YS and P-Sp region into adult immunodeficent mice showed long-term multilineage engraftment from P-Sp only (Cumano et al. 2001) indicating that the P-Sp has HSC potential before YS precursors can migrate there through blood flow. In addition, CFU-S from the E9.5 P-Sp region are significantly higher than in YS at the same stage (Medvinsky et al. 1993). These results indicate that the definitive haematopoietic system does not have a YS origin but it cannot yet be ruled out that early precursors of definitive haematopoiesis, which are not detectable by these assays, arise in the YS. Yoder and colleagues showed that injection of E9 YS into conditioned newborns gave rise to long-term mulitlineage engraftment suggesting the presence of pre-HSCs (Yoder et al. 1997). Culture of E11 YS shows only one HSC before and after culture indicating that it is unable to expand HSCs or mature pre-HSCs, if present at this stage (Kumaravelu et al. 2002). This haematopoietic activity is observed after the onset of circulation and it is therefore unclear whether YS can autonomously generate pre-HSCs and HSCs. It is however clear that the P-Sp/AGM region has far greater multilineage long term repopulation potential than the YS.

1.3.2.3 Placenta

The placenta is a major site of haematopoiesis in the embryo containing multilineage progenitors from around E9 and from E10 onwards, containing higher numbers of haematopoietic progenitors than found in the YS (Alvarez-Silva et al. 2003). High level repopulating HSC activity is detected in the placenta, around the time of HSC emergence in the AGM, approximately E11, and numbers increase dramatically between E11 and E12 (Gekas et al. 2005; Ottersbach & Dzierzak 2005). Ly-6A, CD34, and CD31 staining co-localise in the placenta and some of these cells are seen in the endothelium of the vascular of the placenta suggesting that HSCs emerge in the vasculature, as in the AGM (Ottersbach & Dzierzak 2005). Explant culture of

placenta was not able to produce HSCs suggesting that the placenta does not contain pre-HSCs and is colonised by HSC emerging elsewhere but it is possible that the explant organ culture systems used do not support placental pre-HSC maturation due to technical reasons (Robin et al. 2006; Ottersbach & Dzierzak 2005). In addition, experiments in embryos lacking a heartbeat (Nxc1) show multipotent haematopoietic cells in the placenta as early as E8.5, prior to the onset of circulation, though no transplantation experiments were done to assess if the placenta could autonomously produce HSCs (Rhodes et al. 2008). It therefore remains unclear whether the placenta can autonomously generate pre-HSCs and HSCs or if it is seeded by cells from other sites.

1.3.3 Possible origins of dHSCs

It has been shown that HSCs arise through the maturation of pre-HSCs but it is still unclear from where these cells originate and in what location the pre-HSC to HSC maturation takes place. Some possible theories, summarised in figure 1.4, are discussed further in this section.

1.3.3.1 Identification of precursors of HSCs

E9.5 P-Sp cells have been shown to give long term multilineage engraftment of conditioned newborns and Rag2γc$^{-/-}$, NK cell deficient, mice (Kumano et al. 2003; Kieusseian et al. 2012). These cells cannot however contribute to long term multilineage engraftment of adult irradiated recipients (Muller et al. 1994). After explant organ culture adult repopulating HSCs can be detected in E10 AGM region at higher numbers than found in uncultured AGM (<1) (Muller et al. 1994; Medvinsky & Dzierzak 1996; Robin et al. 2006). These results indicated the existence of an immature "pre-HSC" population of cells that can mature into HSCs: *in vivo*; in certain *in vitro* conditions; or in a newborn/immunodeficient environment (discussed (Medvinsky et al. 2011). To further investigate these immature cells a re-aggregate culture system was developed in our lab (Taoudi et al. 2008). This technique involves the dissociation of an organ into a single cell suspension thus allowing manipulation of cell populations of interest before the cells are re-aggregated and cultured. Using this technique it was determined that the generation of HSCs that occurs in the E11.5 AGM regions is through the maturation of pre-HSC

Type II cells (CD45$^+$VE-Cadherin$^+$) (Taoudi et al. 2008). Investigation of earlier HSC precursors through a similar technique which involved co-aggregation of the populations of interest with OP9 cells, rather than AGM cells, showed that pre-HSC Type I cells (CD41$^+$CD45-VE-Cadherin$^+$) mature to HSC through the pre-HSC Type II stage in hierarchical organised way (Rybtsov et al. 2011). Cells with the pre-HSC surface marker phenotype are found throughout the developing embryo, however, only those found in the AGM possess the ability to mature into HSCs. Identification of further surface markers of the pre-HSC is required.

1.3.3.2 Haemangioblast

The close association of the development of haematopoietic progenitors and angioblasts in the blood islands of the YS has led to the idea of the existence of a common progenitor termed the haemangioblast. The haemangioblast is a precursor that is restricted to differentiate into endothelial and haematopoietic cells (Sabin 1920). The cells which give rise to endothelial cells, haematopoietic cells and smooth muscle cell are detected in the mouse during the mid-streak stage of gastrulation, these cells are mesoderm precursors rather than bi-potent haemangioblast (Huber et al. 2004). Blast colony forming cells which can give rise to haematopoietic colonies and vascular endothelium in the presence of certain cytokines have been isolated from ESC-derived embryoid bodies (EB) (Choi et al. 1998). However it is not clear that if certain culture conditions were met that smooth muscle cells would also be present, suggesting the cell is merely a mesodermal progenitor. *In vivo* lineage tracing of presumptive haemangioblasts is required to further investigate this hypothesis.

1.3.3.3 Haematogenic Endothelium

Haematogenic endothelium is thought to give rise to haematopoietic progenitors from differentiated endothelium by a transdifferentiation process that is dependent on Runx1. Experimental evidence that supports this hypothesis includes the close association of haematopoietic progenitors, in the HIAC, with endothelial cells (Smith & Glomski 1982) and the incorporation of fluorescent acetyl low-density lipoprotein (Ac-LDL-DiI) into haematopoietic progenitors and HSCs (Sugiyama et al. 2005). In addition, presumptive endothelial cells (CD45$^-$Ter119$^-$VE-Cadherin$^+$) from E9.5

AGM and YS produced endothelial and haematopoietic cells *in vitro* and reconstituted newborn recipients (Nishikawa et al. 1998; Fraser et al. 2002). Presumptive haematogenic endothelium has been show to form *in vitro* from mouse ES cells (Eilken et al. 2009; Lancrin et al. 2009). Haematopoietic progenitors are also labelled by Cre recombinase expressed under the control of VE-Cadherin (Zovein et al. 2008) and removal of Runx1 from cells of the VE-Cadherin lineage results in loss of HSC activity (Chen et al. 2009). However, this evidence does not take into account that the incorporation of acetyl low-density lipoprotein into a cell has not been shown to be endothelial specific, and that VE-cadherin has been shown to be a marker of pre-HSCs (Taoudi et al. 2008; Rybtsov et al. 2011). Pre-HSC Type I cells are CD45⁻ which coincides with the endothelial populations studied in Nishikawa et al. 1998 and Fraser et al. 2002. As the pre-HSC Type I population also expresses CD41, a marker of the haematopoietic system, it cannot be considered purely endothelial (Medvinsky et al. 2011). In 2010 live imaging techniques of zebrafish and mouse explant cultures showed haematopoietic cells arising from the ventral part of the dorsal aorta and entering circulation (Kissa & Herbomel 2010; Boisset et al. 2010). These experiments relied on phenotypical identification of HSC and functional analysis of cells which emerge from the aorta in this manner is required to confirm HSCs arise from this process.

1.3.3.4 Sub-aortic patches

The sub aortic patches (SAPs) are mesodermal structures located below the aorta in E10.5-E11.5 AGM. This model hypothesises that pre-HSCs, are generated below the aortic floor in SAPs. They then migrate up through the endothelium of aortic floor to integrate into the endothelium and form the HIAC before entering the circulation. The SAPs have been shown to reside below the ventral domain of the dorsal aorta (Manaia et al. 2000) and early haematopoietic progenitors have been shown to be present in the SAPs (Bertrand et al. 2005). Proteins involved in haematopoietic development such as BMP4 (bone morphogenic protein 4), GATA3 and AA4.1 are also present in the SAPs (Godin & Cumano 2002). In the absence of Runx1, which results in loss of HSC production, there are no SAPs present in the AGM (North et al. 1999). It has also been shown that at E11.5, some pre-HSC Type I reside in the

sub-endothelial layers, whereas pre-HSC Type II and dHSCs reside in the endothelial layer (Rybtsov et al. 2011).
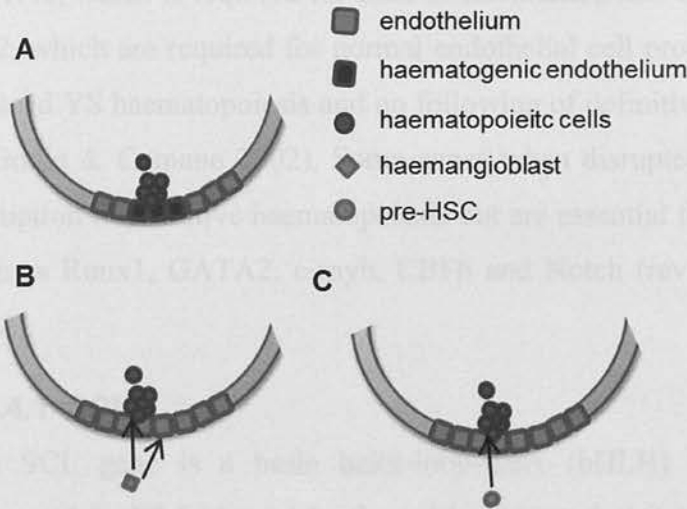
Figure 1.4: Mechanisms of intra-aortic cluster formation.
Representatives of ventral domain of aorta. **A:** Specialised endothelium in the ventral wall of the dorsal aorta called haematogenic endothelium produces haematopoietic cells. **B:** A bi-potent precursor called the haemangioblast produces endothelial cells and haematopoietic precursors which both integrate into the aorta endothelium. **C:** Haematopoietic precursors emerge in the sub-aortic mesenchyme and integrate or pass through the endothelium of the aorta.

1.3.4 Factors involved in the ontogeny of HSCs

Disruption of genes and other external factors has revealed important information about the function of certain genes in the ontogeny of the haematopoietic system. The removal of genes that are important for primitive and definitive haematopoiesis, results in embryonic death around E8.5-E10.5. Disruption of SCL and Lmo2, which have a role in the early stage of haematopoietic determination in the embryo, GATA1, which is required for final differentiation into an erythrocyte, and Flk1 and Tie2, which are required for normal endothelial cell production, results in markedly reduced YS haematopoiesis and no following of definitive haematopoiesis (reviewed in Godin & Cumano 2002). Some genes when disrupted do not cause a significant disruption to primitive haematopoiesis but are essential for definitive haematopoiesis such as Runx1, GATA2, c-myb, CBFβ and Notch (reviewed in Godin & Cumano 2002).

1.3.4.1 SCL

The SCL gene is a basic helix-loop-helix (bHLH) transcription factor and is expressed in E7.5 blood islands and haematopoietic and vascular cells (Begley & Green 1999). SCL null embryos, which die in utero between E8.5 and E10.5, fail to initiate both primitive and definitive haematopoiesis shown by the absence of primitive erythrocytes and CFU-Cs (Robb et al. 1995; Shivdasani et al. 1995). In addition, SCL$^{-/-}$ ES cells do not contribute to adult haematopoiesis in chimeric mice (Porcher et al. 1996) suggesting an important role for SCL in haematopoietic commitment.

Conditional inactivation of SCL in the adult revealed that SCL was dispensable for maintenance, engraftment, self-renewal and multilineage differentiation of LTR-HSCs (Mikkola et al. 2003). However, in a lyl1/SCL conditional double knockout HSCs failed to repopulate irradiated recipients and underwent rapid apoptosis (Souroullas et al. 2009) indicating that lyl1 is essential for HSC function in the absence of SCL. Lyl1 is a bHLH transcription factor that is highly related to SCL suggesting a functional redundancy between the two in HSC function.

1.3.4.2 Lmo2

Lmo2 is a LIM-domain nuclear protein which functions as a bridging protein in SCL and GATA1 protein complexes (Warren et al. 1994). Lmo2$^{-/-}$ cells fail to undergo erythroid differentiation but can still produce myeloid cells, similar to the phenotype seen in GATA1$^{-/-}$ cells, suggesting primitive haematopoiesis is not completely abolished (Warren et al. 1994; Weiss et al. 1994). Lmo2 is expressed in the E8-11 aorta endothelium and HIACs, and Lmo2$^{-/-}$ ES cells are unable to contribute to definitive haematopoiesis in chimeras, suggesting a possible role for Lmo2 in HSC development (Yamada et al. 2000; Manaia et al. 2000).

1.3.4.3 GATA2

GATA2 is a zinc finger protein that influences cell fate in adult and developing HSCs. GATA2 is expressed in immature haematopoietic cells, and in the endothelium of the E9.5-10.5 aorta, of the AGM region (Minegishi et al. 1999; Robert-Moreno et al. 2005; Robert-Moreno et al. 2008). Disruption of the GATA2 gene results in embryonic lethality at around E10.5 and reduced expansion of haematopoietic lineages (Tsai et al. 1994). Conditional deletion of GATA2 in VE-Cadherin^{+} cells resulted in loss of FL HSCs (Lim et al. 2012). GATA2$^{-/-}$ ES cells fail to contribute to definitive haematopoiesis in the embryo or adult (Tsai et al. 1994). In addition, GATA2$^{+/-}$ haploinsufficient embryos have impaired HSC expansion/maturation compared to wild-type (Ling et al. 2004). Therefore it is clear that GATA2 plays a role in HSC development but the mechanism of this action is unclear.

1.3.4.4 GATA3

GATA3 is from the GATA zinc finger protein family. GATA3 gene inactivation results in embryonic lethality around E11.75-12.5 due to extensive internal haemorrhages, growth retardation and central nervous system malformations (Pandolfi et al. 1995). GATA3 has been shown to be expressed in the SAPs, which may be involved in HSC development (Yoon et al. 2008). In GATA3$^{-/-}$ embryos at E11.5 YS progenitors are unaffected while progenitors in the FL are markedly decreased suggesting a role in definitive but not primitive haematopoiesis (Pandolfi et al. 1995). However, GATA3$^{-/-}$, RAG2$^{-/-}$ ES cells contribute to the myeloid and B-

cell lymphoid lineage in adult chimeras, but not the T-cell lineage, suggesting GATA3 is not an essential factor for HSC development but is required in T-cell differentiation (Ting et al. 1996). In GATA3$^{-/-}$ embryos no direct analysis of pre-HSCs or HSCs during HSC development has been carried out, therefore it is not clear if the process is dependent on GATA3 or what is it's role might be.

1.3.4.5 C-myb

C-myb is a DNA-binding, proto-oncogene. Disruption of the c-myb gene results in embryonic lethality at E15 due to a proliferative defect of immature progenitors which results in a failure of definitive haematopoiesis (Mucenski et al. 1991).

1.3.4.6 Runx1

Runx1 (AML1, CBFα2) contains the Runt DNA-binding domain that can bind to many genes expressed in haematopoietic cells, such as genes encoding cell surface markers and is a subunit of the core-binding factor (CBF). CBFβ is a non DNA binding subunit of the CBF required for Runx1 function and CBFβ$^{-/-}$ embryos have a similar phenotype to Runx1 mutants (Q Wang et al. 1996). Runx1 is also involved in many oncogene translocations (Lam & Zhang 2012).

Runx1 is expressed in the ventral part of the dorsal aorta and in the under lying mesenchyme (North et al. 1999). Targeted deletion of the Runx1 gene leads to haemorrhaging and embryonic lethality at around E12.5 (Qing Wang et al. 1996). YS haematopoiesis was normal but FL haematopoiesis is impaired (Qing Wang et al. 1996). Runx1 is necessary for the formation of intra-aortic clusters, LTR-HSCs express Runx1 and Runx1 is required for HSC generation (Cai et al. 2000; North et al. 1999). Runx1 deletion in the adult bone marrow leads to a deregulation of the HSC pool and defects of downstream lineages (megakaryocyte and lymphoid differentiation) (Ichikawa et al. 2004; Kumano & Kurokawa 2010).

The level of Runx1 expression also plays a role in haematopoietic development and spatial and temporal patterns of HSC development are disrupted in Runx1$^{+/-}$ embryos. In Runx1$^{+/-}$ embryos HSC are readily detectable in the E10.5 AGM region and YS (Cai et al. 2000). This is in contrast to wild-type embryos where HSCs are infrequent in the AGM region and not found in the YS at E10.5 (Muller et al. 1994; Medvinsky

& Dzierzak 1996). In Runx1$^{+/-}$ embryos HSCs are detected in the CD45$^-$ compartment at E10.5 and E11.5 (North et al. 2002). In wild-type they are only detected in the CD45$^+$ compartment (Taoudi et al. 2005; North et al. 2002). This data led to the hypothesis that Runx1 may be involved in the maintenance and differentiation of non-haematopoietic populations.

Conditional Runx1 knock-out with VE-Cadherin cre resulted in loss of HSCs and HIACs but use of Vav cre (expressed by early HSCs) resulted in normal HSC development (Chen et al. 2009). In addition to this data, experiments *in vitro* have shown that partial rescue of the haematopoietic programme is possible in cultured cells prepared from E9.5 but not E11.5 AGM by introduction of Runx1 cDNA (Mukouyama et al. 2000). This data suggests a role for Runx1 before HSC emergence in the VE-Cadherin$^+$ populations possibly in the control of the endothelial to haematopoietic transition.

1.3.4.7 β-catenin

β-catenin is a downstream effector of canonical Wnt signalling pathway. Canonical Wnt signalling leads to the inhibition of β-catenin destruction which then accumulates and enters the nucleus to activate gene specific transcription (Bigas et al. 2013). Experiments involving inhibition of β-catenin activation in E10.5 and E11.5 AGM explant cultures showed that blocking of β-catenin activation at E10.5 but not at E11.5 inhibited HSC production (Ruiz-Herguido et al. 2012). In addition, deletion of β-catenin in VE-Cadherin$^+$ cells but not Vav$^+$ cells led to a decrease in haematopoietic output from the E10.5 and E11.5 AGM region (Ruiz-Herguido et al. 2012). These results suggest that like Runx1, β-catenin may be involved in cell maintenance before HSC emergence and possibly in the haematopoietic to endothelial transition. In the adult, the role of β-catenin in the adult remains unknown with conflicting reports of β-catenin reduction causing HSC exhaustion or having no effect on HSC maintenance in the adult (reviewed in Bigas et al. 2013).

1.3.4.8 Extrinsic factors involved in the ontogeny of HSCs

Explant culture experiments indicated that ventral tissues (gut) induce AGM region HSC potential whereas dorsal tissues (neural tube and notochord) had a negative effect (Peeters et al. 2009). This highlights the importance of surrounding tissues in

the establishment of the dorso-ventral polarity in the dorsal aorta and their influence on HSC development (Jaffredo et al. 2013). BMP4 has been shown to be expressed in the ventral mesenchyme of the dorsal aorta (Durand et al. 2007; Pimanda et al. 2007). The use of a BMP antagonist results in E10 AGM region explant culture results in loss of HSC potential (Durand et al. 2007). In addition, the BMP pathway has also been shown to be connected to SCL and Runx1 (Pimanda et al. 2007). This data suggests BMP4 as a critical extrinsic factor in HSC development and its ventral expression may be involved in establishing dorso-ventral polarity in the dorsal aorta. In zebrafish it has been established that Hedgehog is required for tbx20 expression in the dorsal side of the dorsal aorta conferring a non-haematopoietic fate to these cells (Gering & Patient 2005; Wilkinson et al. 2009).

1.4 Notch Signalling and hematopoietic development

1.4.1 The Notch Signalling Pathway

Notch signalling is an evolutionarily conserved system which controls cell fate and has been shown in all metazoans studied so far, in particular in Drosophila melanogaster (Portin 2002), Caenorhabditis elegans (Priess 2005), and vertebrates, to be mandatory in various developmental processes, including stem cell homeostasis, cell growth, cell differentiation, and survival (Koch et al. 2013).

Notch signalling takes place through cell-to-cell contact. In mammals the Notch signalling pathway consists of four receptors Notch 1-4 with ligands Jagged 1 and 2, and Delta-like 1, 3 and 4. The Notch receptor is a heterodimer consisting of 36 EGF-like repeats and Lin12 repeats linked non-covalently via a heterodimerization domain to a single pass transmembrane protein whose intra cellular domain consists of RAM (RBPjκ Associate Molecule), Ankyrin repeats and a PEST domain. The Notch ligands are single pass transmembrane proteins that contain an extracellular Delta/Serrate/LAG-2 (DSL) domain, several EGF-like repeats and in the case of Jagged1 and 2 a cystein-rich region. Notch receptors undergo S1 cleavage in the golgi before transport to the cell membrane. In the canonical Notch signalling pathway the Notch receptor binds to a Notch ligand, on a neighbouring cell, the ectodomain is shed and this exposes the S2 cleavage site to the transmembrane ADAM-family metalloproteases. After S2 cleavage the S3 cleavage site is exposed to the multi pass membrane protein, γ-secretase complex. These successive cleavages result in the release of the Notch intracellular domain (NICD) which translocates to the nucleus and interacts with the DNA-binding protein RBPjκ and co-activator Mastermind to promote transcription of Notch target genes which encode transcriptional repressors of the Hairy and Hey-related families. In the absence of NICD, RBPjκ recruits repressor complexes. Notch signalling is summarised in figure 1.45 (From Reviews (Bray 2006; Fortini 2009; Fiuza & Arias 2007; Lai 2004)) The best characterised target genes of the Notch signalling pathway are the bHLH genes of the HES class (Bray 2006; Bigas & Espinosa 2012). In general they function as DNA binding transcription repressors and are involved in Notch actions that involve

the suppression of one cell fate to allow determination of an alternate fate (Bigas & Espinosa 2012).

Differential gene expression of Notch receptors and ligands is not sufficient to explain the patterns of Notch activation in tissues and cells. Regulation of Notch activity involves precise post-translational modification of ligands and receptors. Notch ligand endocytosis is essential for ligand activation and is regulated by the E3 ubiquitin ligases, Mind bomb (Mib) and Neuralized (Neur). Other factors controlling ligand activation include ligand localization within the cell and proteolytic cleavage that may down regulate the Notch ligands. Production of a functional Notch receptor relies on the enzyme O-fucosyl transferase (O-fut) to glycosylate an EGF repeat and chaperone protein folding during Notch receptor processing in the endoplasmic reticulum. Further glycosyl-modifications by members of the glycosyl-tranferase family Fringe affect binding affinity of different ligands to the Notch receptor. Therefore regulation of Notch signalling can occur through the control of the enzymes involved in the post-translational modification of Notch receptors and ligands as well as the enzymes involved in the proteolytic cleavage of the Notch receptor during activation. (From Reviews (Bray 2006; Fortini 2009; Fiuza & Arias 2007; Lai 2004))

Notch has various modes of action. These include lateral inhibition, lineage decisions and boundary induction (figure 1.6). Lateral inhibition occurs when cells with the same potential are induced to different fates due to slight changes in the equilibrium of Notch signalling, one cell commits to a specialized fate and inhibits surrounding cells from adopting this fate. Lineage decisions between two daughter cells can be dependent on inheritance of Notch signalling pathway components. Asymmetrical division may lead to higher Notch activation in one daughter cell than in another and therefore direct the daughter cells to different fates. Another role of Notch is to promote the development of a cell type or body region, often by inducing the expression of positively acting regulatory molecules. In many of these cases, Notch signalling creates a new cell type as a result of cell-cell interactions at the boundary between distinct cell populations. (From Reviews (Bray 2006; Fortini 2009; Fiuza & Arias 2007; Lai 2004))

| 1: Ligand binds to Notch receptor | 2: Endocytosis of ligand exposes S2 cleavage site to ADAM | 3: Removal of the extracellular domain exposes S3 cleavage site to γ-secretase and the NICD is released | 4: The NICD translocates to the nucleus, it interacts with RBPjκ and Mastermind to promote transcription of target genes |
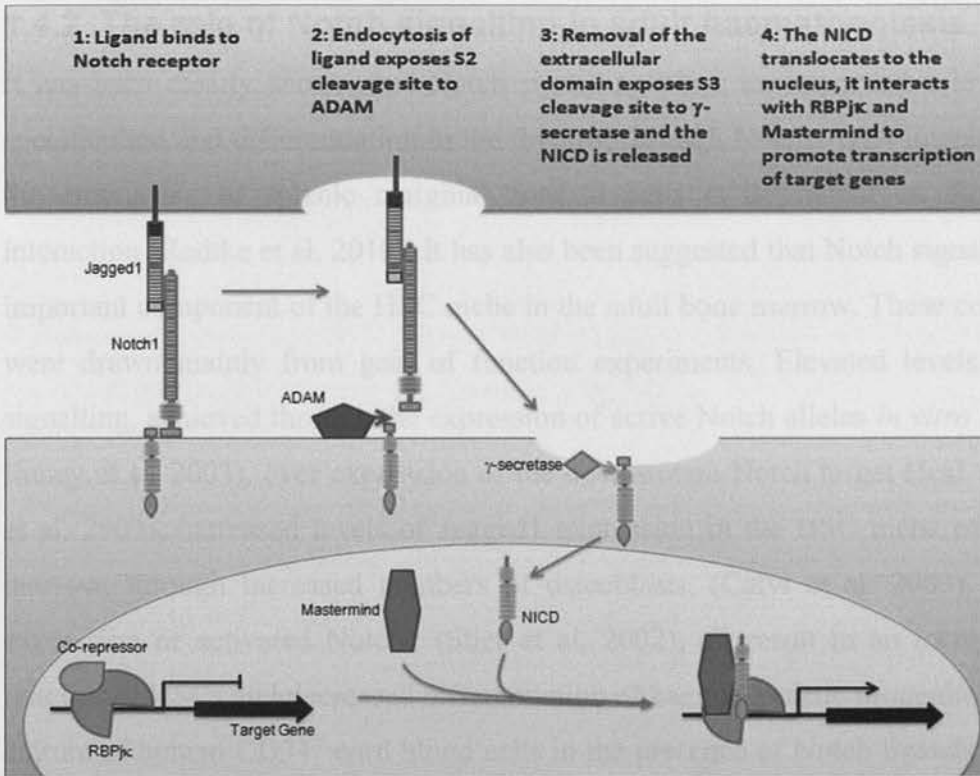
Figure 1.5: Schematic representation of the Notch Signalling pathway.
ADAM: a desintegrin and metallopeptidase; NICD: Notch intra-cellular domain.
Adapted from (Bray 2006).

Notch receiving cell
Notch sending cell
→ Notch signalling
▬ Notch receptors
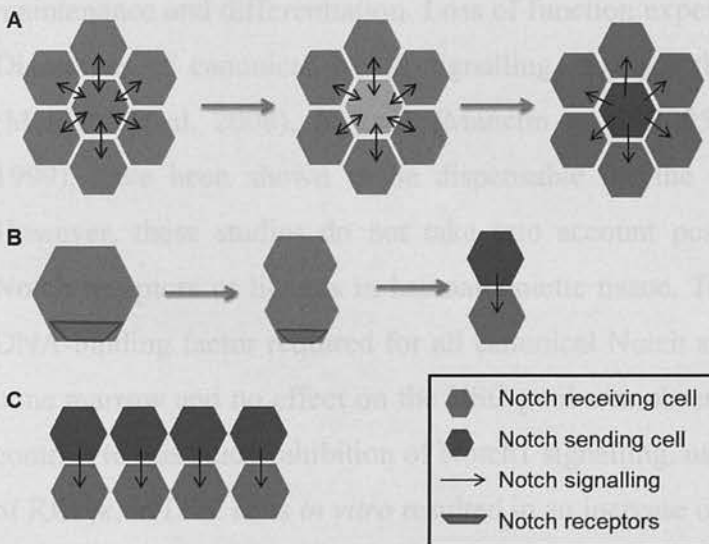
Figure 1.6: Different modes of Notch action.
A: Lateral inhibition. Notch signalling amplifies small difference between roughly equivalent cells that resolves cells into distinct cell fates. B: Lineage decisions. Asymmetrical inheritance of Notch signalling components can determining lineage output of daughter cells. Adapted from (Bray 2006).

1.4.2 The role of Notch signalling in adult haematopoiesis

It has been clearly shown that Notch signalling plays an important role in T cell specification and differentiation in the thymus, through Notch1/Dll4 interaction, and the emergence of splenic marginal zone B cells is dependent on Notch2/Dll1 interaction (Radtke et al. 2010). It has also been suggested that Notch signalling is an important component of the HSC niche in the adult bone marrow. These conclusions were drawn mainly from gain of function experiments. Elevated levels of notch signalling, achieved through the expression of active Notch alleles *in vitro* (Varnum-finney et al. 2003), over expression of the downstream Notch target Hes1 (Kunisato et al. 2003), increased levels of Jagged1 expression in the HSC niche of the bone marrow, through increased numbers of osteoblasts, (Calvi et al. 2003), and over expression of activated Notch1 (Stier et al. 2002), all result in an increased self-renewal of HSCs and decreased differentiation of haematopoietic progenitors. Indeed culture of human CD34$^+$ cord blood cells in the presence of Notch ligands showed a 100 fold increase in CD34$^+$ cells which repopulated NSG mice providing a powerful method for therapeutic expansion of HSC (Delaney et al. 2010). These gain of function experiments suggest that Notch signalling can expand the HSC pool but do not address the physiological role of Notch signalling in HSC expansion, maintenance and differentiation. Loss of function experiments give less clear results. Disruption of canonical Notch signalling, through the disruption of Mastermind (Maillard et al. 2008), Jagged1 (Mancini et al. 2005) and Notch1 (Radtke et al. 1999), have been shown to be dispensable for the maintenance of adult HSCs. However, these studies do not take into account possible redundancy with other Notch receptors or ligands in haematopoietic tissue. To address this issue RBPjκ, a DNA-binding factor required for all canonical Notch signalling was deleted in adult bone marrow and no effect on the HSC pool was observed (Maillard et al. 2008). In contrast to this study inhibition of Notch1 signalling, using a dominant negative form of RBPjκ, in LSK cells *in vitro* resulted in an increase of lineage restricted cells and a reduction HSCs suggesting a role for Notch signalling in controlling HSC maintenance and differentiation (Duncan et al. 2005). In addition, a recent study showed deletion of Jagged1 in bone marrow endothelium resulted in premature exhaustion of the HSC pool (Poulos et al. 2013). Assessment of Jagged1 deletion in

the VE-Cadherin cre mice compared to the classically used Mx1-cre mice showed that Jagged1 is not efficiently deleted in the bone marrow endothelial cells when the inducible Mx1-cre line was used (Poulos et al. 2013). So the question of Notch signalling's role in physiological HSC maintenance, differentiation and expansion remains open.

1.4.3 The role of Notch signalling in blood vessel development and haematopoietic development

The Notch signalling pathway plays a very important role in both blood vessel development and haematopoiesis. This is evident from the phenotypes that result from the disruption of genes encoding components of the Notch signalling pathway, as summarised in Table 1.

1.4.3.1 Notch in blood vessel development and specification

Notch signalling has been shown to have an important role in blood vessel development during embryogenesis and in angiogenesis (figure 1.7).

During the first stage of blood vessel development blood vessels are formed *de novo* from the lateral plate mesoderm, this process is called vasculogenesis. The angioblast, the precursor of blood vessels, differentiates to form endothelial cells and tubes that connect to form the primary capillary plexus. Vasculogenesis is directed by 3 main factors, basic fibroblast growth factor (FGF2), vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1) (Gilbert 2000; Risau & Flamme 1995; Flamme et al. 1997). In the next stage of blood vessel development, called angiogenesis, the primary capillary plexus is remodelled and vessel sprouting occurs. During angiogenesis new blood vessels are formed by sprouting from existing blood vessels, the main factor in this process is VEGF-A (Gilbert 2000). Endothelial cells that are stimulated by VEGF-A to become tip cells which contain numerous filopodia, are specialized for guided migration, rarely proliferate and do not contain a lumen (Gerhardt et al. 2003). These cells are followed by stalk cells which proliferate when stimulated by VEGF-A, form a lumen, tight adherence junctions and deposit a basement membrane (Gerhardt et al. 2003). The control of vessel branching and migration, i.e. tip cell stimulation, is controlled by the interaction of the Notch pathway with VEGF-A (figure 1.8). It has been shown that endothelial cells are

stimulated by VEGF-A through the VegfR2/3 receptor. This up regulates the expression of Dll4 on the endothelial cells and stimulates Notch signalling through the Notch1 receptor in neighbouring cells. Notch signalling in the neighbouring cells prevents these cells from becoming tip cells by inhibiting their response to VEGF-A, this is achieved through down regulation of VegfR2/3 and up regulation of VegfR1, which sequesters VEGF-A, another example of lateral inhibition (Roca & Adams 2007). The Notch ligand Jagged1 has also been shown to be involved in tip and stalk cell specification. In this case Jagged1 antagonizes the Dll4-Notch signalling, this further down regulates Notch signalling in the tip cells and down regulates Dll4-Notch signalling in stalk cells which allows expression of VegfR2/3 receptors and VEGF-A to stimulate proliferation (Roca & Adams 2007). Dll4-Notch signalling in the rest of the artery is in balance, stabilising the artery endothelial cells by reducing VEGF signalling which decreases cell proliferation and allows the cells to mature (Benedito et al. 2008).

Table 1.1: Summary of the effect of Notch Signalling on blood vessel and haematopoietic development. Homozygous knock-outs. CFU-C: Colony forming unit - culture; p-Sp: para-aortic splanchnopleura; YS: Yolk Sac; AGM: aorta-gonads-mesonephros.

Gene	Lethality	Endothelial Phenotype	Haematopoietic Phenotype	References
Notch1	E10.5	No major defects in vasculogenesis but dorsal aortae not properly formed and have a collapsed appearance; Impaired angiogenesis	Reduced CFU-C in the p-Sp, normal in YS; No reconstitution after transplantation of E9.5 p-Sp and YS into busulfan-conditioned newborns	(Kumano et al. 2003; Swiatek et al 1994; Krebs et al. 2000)
Notch2	E11.5		CFU-C and newborn reconstitution of YS and p-SP comparable to wild type	(Hamada et al 1999; Kumano et al. 2003)
Notch3	None	No obvious phenotype	No obvious phenotype	(Krebs et al. 2003)
Notch4	None	No obvious phenotype	No obvious phenotype	(Krebs et al. 2000)
Notch1 Notch4	E9.5	Similar phenotpye but more severe than Notch1 knock out		(Krebs et al. 2000)
Jagged1	E10.5	Defective vascular remodelling; Expression of arterial markers; Reduced angiogenesis	CFU-C in E10 AGM severely reduced	(Xue et al. 1999; Robert-Moreno et al. 2008; Benedito et al. 2009)
Jagged2	Shortly after birth		CFU-C in E10 AGM comparable with wild type	(Jiang et al. 1998; Robert-Moreno et al. 2008)
Delta-like1	E12	Haemorrhage; Required for post natal arteriogenesis		(Hrabě de Angelis et al. 1997; Limbourg et al. 2007)
Delta-like3	Adults have severe skeletal defects			(Kusumi et al. 1998)
Delta-like4	E10.5	Severe vascular remodelling defects; Loss of arterial markers; Reduced artery diameter		(Krebs et al. 2004; Gale et al. 2004; Duarte et al. 2004)
RBPjk	E8.5	No vascular remodelling; Loss of arterial markers	Severely reduced CFU-C in the pSp/AGM	(Krebs et al. 2004; Oka et al. 1995; Robert-Moreno et al 2005)
Hey1 Hey2	E12	Defective vascular remodelling; Defective angiogenesis; Loss of arterial markers		(Fischer et al. 2004)
Hes1 Hes5	E12	Normal areterial specification; Expression of arterial markers	Severe reduction in CFU-C and no HSCs after culture of E10.5 AGM	(Guiu et a. 2013)
Mind-Bomb1	E10.5	Defective vasculogenesis; Loss of arterial identity	CFU-C in E8.5 YS comparable to wild type; No CFU-C obtained from E9.5 p-Sp	(Koo et al 2005; Yoon et al. 2008)
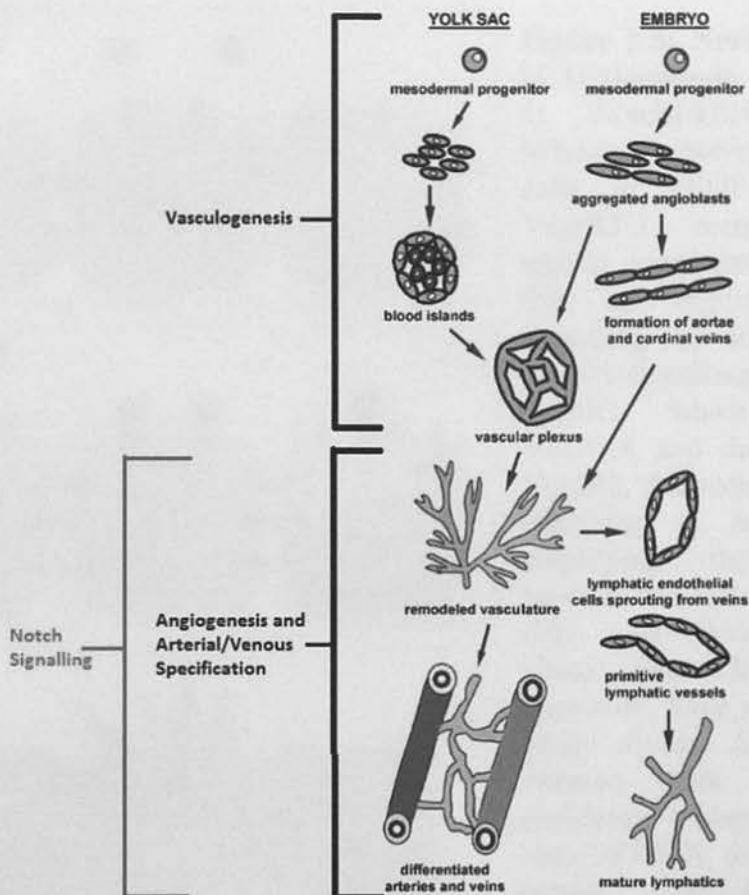
Figure 1.7: Representation of the development of endothelium from mesodermal progenitors, vasculogensis and angiogenesis. From (De Val & Black 2009).
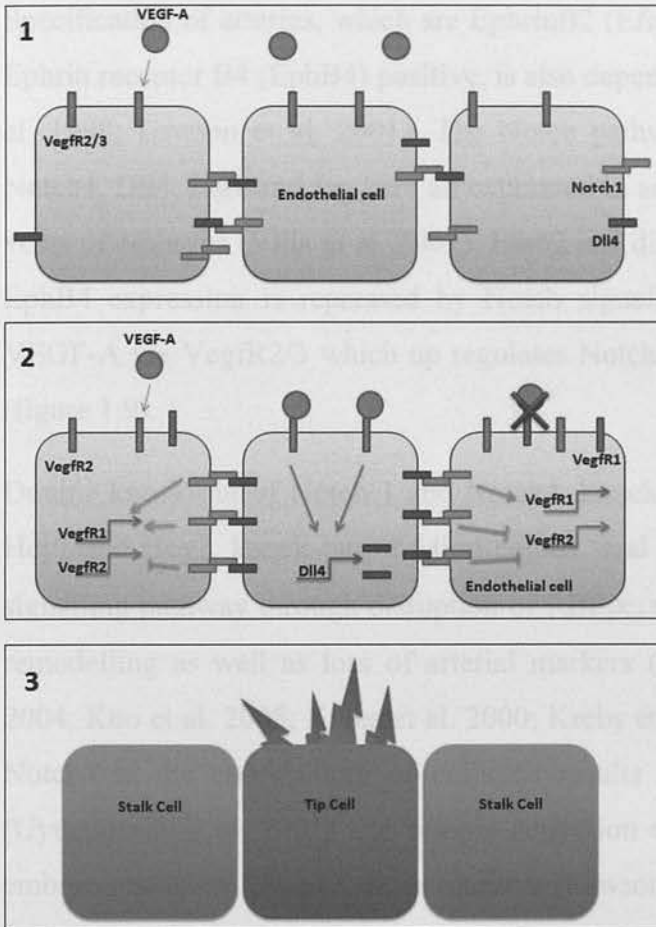
Figure 1.8: Notch Signalling in Angiogenesis.
1: Notch1/Dll4 signalling balance between endothelial cells. **2:** VEGF-A binds to VegfR2/3 receptor which signals upregulation of Dll4, this up regulates Notch1 signalling in adjacent cells. Notch1 signalling up regulates VegfR1 which sequesters VEGF-A and down regulates VegfR2, this reduces VEGF-A signalling in adjacent cells amplifying the difference between the cells. **3:** The high Dll4 cells become tip cells which have filopodia, are migratory, have no lumen and rarely divide. Adjacent cells become stalk cells, they proliferate when stimulated with VEGF-A, form a vascular lumen, establish adherence junctions and deposit a basement membrane (adapted from Phng & Gerhardt 2009).
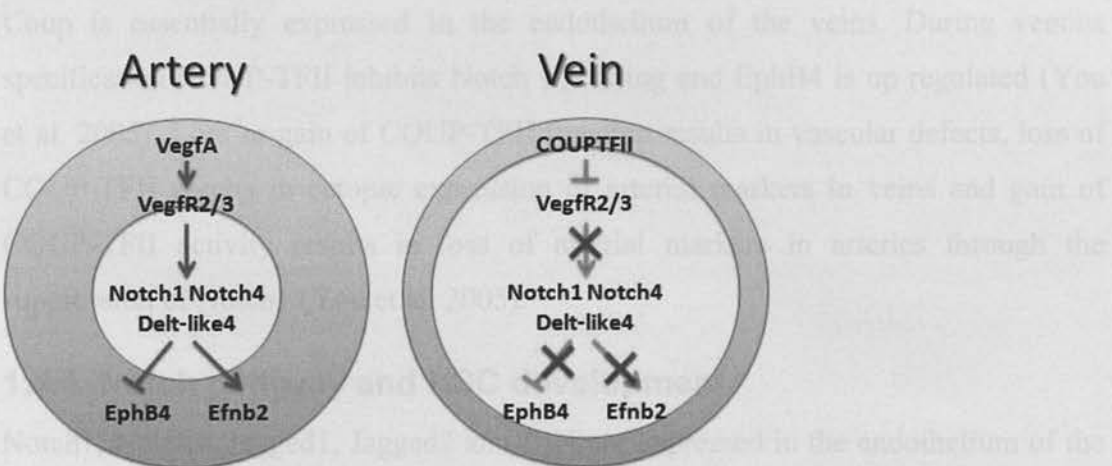
Figure 1.9: Notch Signalling in Arterial versus Venous specification.
VEGF-A up regulates Notch components in arterial specification, Notch signalling inhibits EphB4 a venous marker and up regulates Efnb2 an arterial marker. During venous specification COUPTFII inhibits Notch signalling therefore Efnb2 is not up regulated and EphB4 is not repressed.

Specification of arteries, which are EphrinB2 (Efnb2) positive, and veins, which are Ephrin receptor B4 (EphB4) positive, is also dependent on Notch signalling (Wang et al. 1998; Lawson et al. 2001). The Notch pathway components Notch1, Notch3, Notch4, Dll4, Jag1 and Jag2 are all expressed in arteries, but are not expressed in the veins of embryos (Villa et al. 2001). Efnb2 is a direct target of Notch signalling and EphB4 expression is repressed by Notch signalling. This is again stimulated by VEGF-A via VegfR2/3 which up regulates Notch 1/4 and Dll4 (Duarte et al. 2004) (figure 1.9).

Double knock-out of Notch 1 and Notch4, knock-out of Dll4, double knock-out of Hey1 and Hey2, knock-out of Mind-bomb1, and disruption of the canonical Notch signalling pathway through disruption of RBPjκ, result in severe defects of vascular remodelling as well as loss of arterial markers (Duarte et al. 2004; Fischer et al. 2004; Koo et al. 2005; Krebs et al. 2000; Krebs et al. 2004). Expression of activated Notch4 in the endothelium of embryos results in defects of vascular patterning (Uyttendaele et al. 2001) and ectopic activation of Notch1 signalling the zebrafish embryo results in loss of venous markers (Lawson et al. 2001). Deletion of Notch1 in Tie2$^+$ cells produces embryos with a vascular phenotype similar to Notch1 full knock-outs indicating the cell autonomous role of Notch1 in vascular development. Coup is essentially expressed in the endothelium of the veins. During venous specification COUP-TFII inhibits Notch signalling and EphB4 is up regulated (You et al. 2005). Loss or gain of COUP-TFII function results in vascular defects, loss of COUP-TFII results in ectopic expression of arterial markers in veins and gain of COUP-TFII activity results in loss of arterial markers in arteries through the suppression of Notch1 (You et al. 2005).

1.4.4 Notch pathway and HSC development

Notch1, Notch4, Jagged1, Jagged2 and Dll4 are expressed in the endothelium of the E9.5 and E10.5 aorta (Robert-Moreno et al. 2008). Notch1 and Jagged1 are expressed in HIAC clusters, Notch4 is not, and Jagged2 and Dll4 have a heterogeneous expression (Robert-Moreno et al. 2008). In addition, Notch1, Mind-bomb1 and Dll1 are also expressed in the sub-aortic patches (Yoon et al. 2008).

Triple positive Notch1, Jagged1 and GATA2 expressing cells were also observed in the E10.5 aorta endothelium (Robert-Moreno et al. 2005).

A requirement for Notch signalling in the development of intra-embryonic haematopoietic progenitors has been shown in various studies (Table1.1). P-Sp from E9.5 Notch1 knock-outs produce little to no CFU-Cs after culture, have reduced GATA2 and Runx1 expression, and contain increased proportions of VE-Cadherin$^+$CD45$^-$ cells (Kumano et al. 2003). YS from the same embryos produce similar numbers of CFU-Cs as wild type YS after culture suggesting an effect on intra-embryonic but not primitive haematopoiesis (Kumano et al. 2003). In RBPjκ mutant's intra-embryonic haematopoiesis is impaired in a similar way as in Notch1 mutants. No CFU-Cs are produced from E9.5 RBPjκ$^{-/-}$ p-Sp, GATA2 and Runx1 expression are decreased and a multi-layered endothelium is present in the dorsal aorta (fig 1.10b) (Robert-Moreno et al. 2005). Again this phenotype is observed in Mind-bomb1 mutants (Yoon et al. 2008). Mind-bomb1 is an E3 ubiquitin ligase essential for generation of functional Notch ligands. In these mutants again no CFU-Cs are generated in the E9.5 p-Sp and the aorta contains a disorganized morphology (Yoon et al. 2008).

Embryos lacking genes of the Notch signalling pathway such as RBPjκ (Krebs et al. 2004), Notch1 (Krebs et al. 2000), Jagged1 (Xue et al. 1999), Dll1 (Hrabě de Angelis et al. 1997), Dll4 (Duarte et al. 2004) or Hey1/Hey2 (Fischer et al. 2004) and genes involved in the regulation of the Notch pathway such as Mind-bomb1 (Koo et al. 2005), die before or around E10.5, the time of HSC emergence meaning direct assessment of HSC activity cannot be carried out. Transplantation of E9.5 Notch1$^{-/-}$ p-Sp into conditioned newborns, as a direct assessment of pre-HSCs, showed no repopulation by Notch1$^{-/-}$ cells indicating that HSC development is impaired in these mutants (Kumano et al. 2003). However these mutants along with embryos of other Notch mutant, RBPjκ and Mind-Bomb1, present with systemic defects, retarded development and disrupted arterial specification. This has made the study of the effects of Notch signalling disruption on HSC development difficult. As the Notch1, and RBPjκ mutant embryos are severely retarded any effects observed in the haematopoietic lineage may be a result of general systematic defects (fig 1.10 a and

b). In addition, Notch signalling plays a vital role in determination of arterial identity as discussed in the previous section. Arterial identity may be an important prerequisite for HSC emergence as HSC emerge in close association with the endothelium of the dorsal aorta. In COUP-TFII mutants notch signalling is no longer suppressed in veins, they up regulate arterial markers and cell clusters are found in veins similar to the HIACs seen in arteries (You et al. 2005). HIACs are also found in veins of other mutants with arterial malformations (Sorensen et al. 2003). Blood flow has also been shown to be essential for HSC development (Adamo et al. 2009) therefore development of the cardiovascular system may play an indirect role in haematopoietic development.

To circumvent this issue Notch1 null ES cells were shown to contribute to primitive haematopoiesis but not definitive haematopoiesis when injected into mouse embryos at the blastocyst stage (Hadland et al. 2004) suggesting a cell autonomous requirement for Notch1 in definitive haematopoiesis. In addition, it has very recently been shown that Hes1$^{-/-}$Hes5$^{-/-}$ double knock-outs maintain normal arterial specification but contain increased numbers of HIACs (Guiu et al. 2013) and after culture of E10.5 AGM little to no CFU-Cs and no HSCs are produced in these mice (Guiu et al. 2013). These results indicate a direct effect of Notch signalling on HSC development although further work into the process is required.

In the Jagged1 mutant it was described that intra-embryonic haematopoiesis was decreased and that, as seen in other Notch mutants, YS haematopoiesis was comparable to wild type (Robert-Moreno et al. 2008). These embryos also contained a multi-layered dorsal aorta endothelium (fig 1.10c) but contrary to Notch1, RBPjκ and Mind-Bomb1 mutants maintained arterial specification (Robert-Moreno et al. 2008). In addition these mutants do not present with a severe developmental retardation as do some other Notch mutants. At E9.5 they are indistinguishable from littermate controls, and at E10 approximately half present with gross defects (Robert-Moreno et al. 2008; Xue et al. 1999 and own observations). These results lead to the idea that Jagged1 was the ligand involved with Notch1 in the AGM region during HSC development. However, though intra-embryonic CFU-Cs are reduced in these

mutants no transplantation assays were carried out to determine if HSC development was affected.

Loss of Notch signalling by disruption of RBPjκ, Notch1, or Jagged1 results in reduced haematopoietic activity in the AGM but also a corresponding increase in endothelial cells in the AGM (fig 1.10 b and c) (Robert-Moreno et al. 2008; Robert-Moreno et al. 2005). This suggests a possible role of Notch signalling in the cell fate determination of the haemangioblast or control of differentiation of the haematogenic endothelium. The mechanism of Notch action in definitive haematopoiesis is still unclear; a summary of what is so far thought to occur is shown in figure 1.11. Levels of Runx1 and GATA2 are reduced in RBPjκ and Jagged1 mutant AGM regions and ectopic expression of Runx1 and GATA2 partially rescue the haematopoietic phenotype in these mutants (Robert-Moreno et al. 2005; Robert-Moreno et al. 2008; Nakagawa et al. 2006). Control of GATA2 by Notch signalling has been shown to be by a feed-forward loop. The GATA2 promoter has two RBPjκ binding sites allowing Notch signalling to activate GATA2 expression after which GATA2 is repressed by Notch target Hes genes (Guiu et al. 2013). Hes1/Hes5 double knock outs show up regulation of GATA2 and loss of HSC activity (Guiu et al. 2013). Further studies in zebrafish have contributed to the picture of the mechanism of Notch actions in HSC development. It has been shown that Runx1 expression is required for successful EHT. In the absence of Runx1 cells begin EHT but then disintegrate (Kissa & Herbomel 2010). In addition c-myb$^+$ HSC have been rescued by ectopic expression of Runx1 in mind bomb mutant zebrafish (Burns et al. 2005). These observations suggested Runx1 is a downstream effect of Notch signalling in HSC development and through Runx1 Notch may play a role in EHT. It has been shown that loss of function of all Notch ligands, through the deletion of Mind bomb-1, results in loss of intra-embryonic haematopoiesis (Koo et al. 2005; Yoon et al. 2008). However, targeted deletion of Mind bomb-1 in the aortic endothelium only results only in a reduction of intra-embryonic haematopoiesis (Yoon et al. 2008) suggesting a site for intra-embryonic haematopoiesis outside the aortic endothelium, the sub-aortic patch. It was shown that GATA3, Notch1 and Dll1 were expressed in the sub-aortic patch suggesting they are the effectors of Notch signalling's role in haematopoiesis at this site (Yoon et al. 2008).
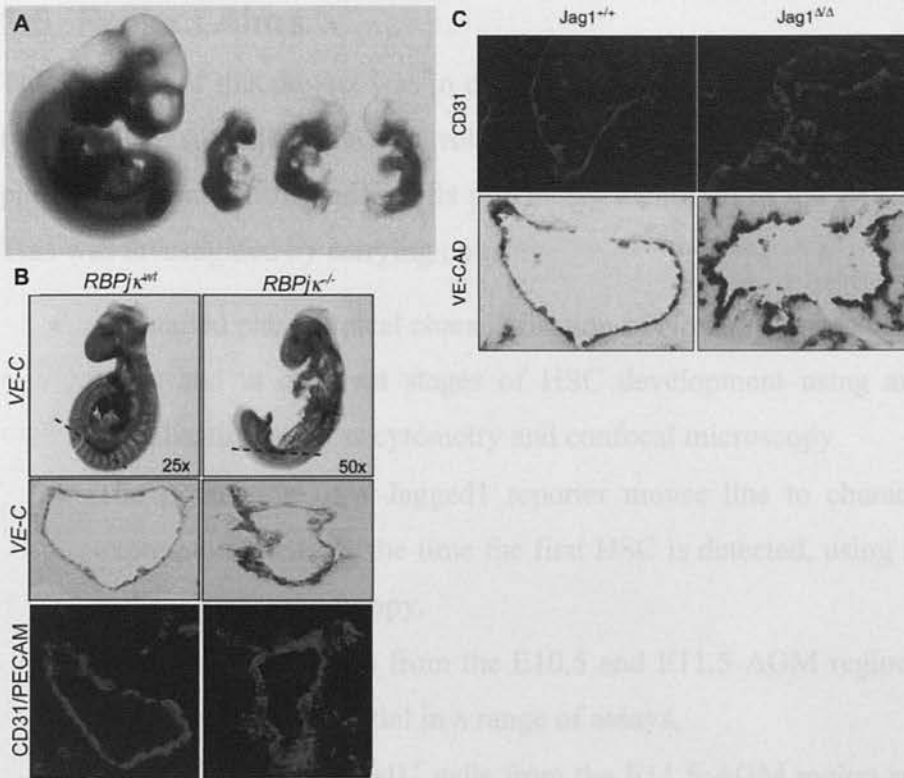
Figure 1.10: Notch mutants.
A: E9.5 wild-type embryo on left and 3 Notch knock-outs on right (Kumano et al. 2003). **B:** E9.5 RBPjκ mutants and transverse sections of dorsal aorta (Robert-Moreno et al. 2005). **C:** E10.5 transverse sections of dorsal aorta from Jagged1 mutants (Robert-Moreno et al. 2008).
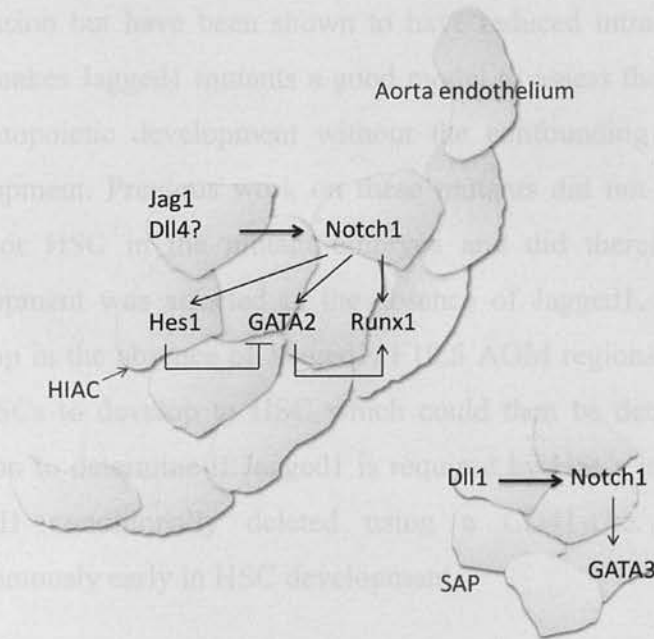
Figure 1.11: Schematic representation of the mechanism of Notch action in haematopoietic development.
HIAC: Haematopoietic intra-aortic cluster, SAP: Sub-aortic patch.

1.5 Project Aims

The first aim of this project was to determine at which stages and though which cell types Notch signalling plays a role in HSC development and to determine the potential of Notch1$^+$Jagged1$^+$ cells previously identified in the E11.5 AGM region. This was investigated by carrying out:

- A detailed phenotypical characterisation of Notch1 expression in various cells types and at different stages of HSC development using anti-Notch1 and visualisation by flow cytometry and confocal microscopy.

- The production of a Jagged1 reporter mouse line to characterise Jagged1 expression at E11.5, the time the first HSC is detected, using flow cytometry and confocal microscopy.

- Sorting Notch1$^+$ cells from the E10.5 and E11.5 AGM region and assessing their functional potential in a range of assays.

- Sorting Notch1$^+$Jagged1$^+$ cells from the E11.5 AGM region and determining by functional assay whether they are pre-HSCs.

The second main goal of the project was to more closely assess the role of Jagged1 in HSC development. As discussed, Jagged1 mutants have normal arterial marker expression but have been shown to have reduced intra-embryonic haematopoiesis. This makes Jagged1 mutants a good model to assess the role of Notch signalling in haematopoietic development without the confounding factor of impaired arterial development. Previous work on these mutants did not assess the presence of pre-HSC or HSC in the mutant embryos and did therefore not show if the HSC development was affected in the absence of Jagged1. To determine if HSCs can develop in the absence of Jagged1, E10.5 AGM regions were cultured to allow any pre-HSCs to develop to HSC which could then be detected by transplantation. In addition to determine if Jagged1 is required by HSCs in a cell autonomous manner Jagged1 conditionally deleted using a CD41-Cre. CD41 is expressed cell autonomously early in HSC development.

2 Material and Methods

2.1 General Solutions

Dissecting solution: Dulbecco's phosphate buffered saline (PBS) solution with Mg_2^+ and Ca_2^+ ions (Sigma or Gibco) containing 7% fetal calf serum (FCS) (Invitrogen) and 50units/ml penicillin and streptomycin (P/S) (Gibco).

Cell suspension/Staining solution/Washing solution: Dulbecco's PBS solution without $Mg2^+$ and $Ca2^+$, containing 7% FCS and 50units/ml P/S.

2.2 Animal Procedures

2.2.1 Animal Husbandry

C57BL6 wild-type and transgenic lines were housed and bred within the University of Edinburgh animal houses and according to the regulations of the Animals Scientific Procedures Act, UK, 1986. Animals were provided with a constant supply of water and chow food, and were housed in a constant environment with a 14 hours light/10 hours dark cycle. Litters obtained were left with parents for three weeks postnatally before weaning by separating parents and offspring. Only mice older than 6 weeks were used for matings. Mice used in this project are listed in table 2.1.

Table 2.1: Mouse Lines

Line	Reference
C57BL/6J	Jackson Laboratories
CD41-Cre	(Emambokus & Frampton 2003)
sGFP	(Gilchrist et al. 2003)
Jagged1 floxed	(Nyfeler et al. 2005)
Jagged1 knock-out	Produced from Jagged1 floxed mice
Jagged1 Tomato	Produced in house

2.2.2 Timed Matings

Time matings were organised to obtain embryonic tissue of a specific gestational stage. Matings were setup overnight and females examined the next morning for the

presence of a vaginal plug. The day of vaginal plug discovery was determined as E0.5.

2.2.3 Genotyping

After weaning ear biopsies were taken from pups for genotyping by PCR. Ear biopsies were incubated in 100μl lysis buffer (containing 10% Tween 20, NP40, and 10 mg/ml proteinase K in PCR buffer (Qiagen)) at 56°C overnight. The following day they were heat inactivated at 95°C for 10min. 5μl of this solution was used for PCR. Samples were stored at 4°C or -20°C.

2.3 ES cell and Stromal Cell Tissue Culture

Tissue culture procedures were carried out in class 2 laminar flow hoods (Nuaire and Bassaire) using aseptic technique. Cells were cultured in 5% CO2 at 37°C in humidified incubators (Sanyo). All medium were freshly prepared and kept for no longer than 2 weeks. Supplementary reagents were prepared by the tissue culture core facility were stored appropriately. Solutions were stored at 4°C and allowed to warm up to RT prior to use.

2.3.1 Solutions and Medium

PBS (Sigma) (no Ca$^+$, Mg$^+$)

Bl6 ES cell media
500 ml Knockout DMEM (Gibco)
50 ml FBS (Invitrogen)
5 ml L-Glutamine (Gibco)
500μl β-mercaptoethanol
5.5 ml 100X P/S Glutamine (Gibco)
Murine LIF 10x10^{4}IU/ml (in house)

Bl6 ES cell Trypsin
500ml PBS (Gibco)
0.1g EDTA (Sigma)
0.5g D-glucose (Sigma)
35ml Chicken Serum (Gibco)
20ml 2.5% Trypsin (Gibco)
(Aliquot and store at -20°C)

OP9 medium/Stromal Cell medium
150ml IMDM (Gibco)
40ml FCS (Gibco)
2ml L-Glutamine (Gibco)
200μl β-mercaptoethanol (Sigma)
2ml P/S (optional)

0.1% Gelatin Solution
25ml of 2% Gelatin solution (Sigma)
500ml PBS (Sigma)

Freezing Medium
Dimethylsulphoxide (DMSO) (Sigma)
10% v/v
Cell medium
Filter sterilise 22μm

46

Table 2.2: Appropriate volumes to use in protocols depending on flask size.

Dish/Flask/Plate	Gelatin/PBS	Trypsin	Medium	Freezing
96 well	100µl	50µl	800µl	
24 well	750µl	125µl	1.5ml	1ml
6 well	3ml	0.5ml	5ml	2ml
25cm^2 flask	5ml	1ml	10ml	4ml
75cm^2 flask	10ml	3ml	30ml	8ml
125cm^2 flask	10ml	5ml	40ml	
10cm dish	5ml	1.5ml	10ml	

2.3.2 Thaw Cells

10 ml medium was added to a universal tube. One well of a plate or a flask was coated with 0.1% gelatin (>2min), and excess solution aspirated off. A frozen vial of cells was quickly thawed in a water bath (or in hand) with agitation. The outside of tube was wiped with 70% ethanol and air dried. The cell suspension was added to the universal tube containing medium. The cells were gently resuspend by swirling the tube and spun down in a centrifuge for 3min (300 x g). The media was aspirated, taking care not to disturb the cell pellet. Cells were resuspended in the appropriate amount of media and gently pipette until cells dispersed. They were transfer to the gelantinised plate or flask. Cells were incubated overnight and medium changed the next morning.

2.3.3 Passaging Cells

When cells were healthy and near confluent, medium was aspirated and cells were washed with an appropriate amount of warm PBS which was then aspirated. The appropriate amount of trypsin was the added and cells incubated for 5min (or until cells have rounded up and lifted off the flask). Cell medium was added to inactivate trypsin and cells were disperse by gently pipetting. For Bl6 ES cells the appropriate number of cells were added to a freshly gelatinised plate/flask and topped up with the appropriate amount of medium. Cells were incubated overnight and the following day the medium was changed. For other cell types, after trypsin treatment and inactivation of trypsin, cells were transferred to a universal and spun down in a centrifuge for 3min (300 x g). Cells were resuspended in fresh medium and the

appropriate amount transferred into a freshly gelatinised flask, topped up with the appropriate volume of medium and incubated.

2.3.4 Freezing Cells

Cell passaging was started as in 2.3.3. Once cells were spun down they were resuspended in the appropriate volume of freezing medium, 1ml quickly transferred to each labelled cryovial (Nunc), and place directly onto dry ice. Cells were stored at 80°C overnight and transferred to liquid nitrogen cell bank for long term storage.

2.3.5 Electroporation of Cells

The cell medium was changed 4 hours prior to electroporation. Cell passaging was started as in 2.3.3. Once cells were spun down they were gently resuspend in 10mls of RT PBS. 10μl was taken and cells counted using a haemocytometer. The PBS cell suspension was spun down in a centrifuge for 3min (300 x g). PBS was aspirated and cells resuspend in a final volume of PBS that would mean 1×10^7 cells per 25μl. 3-5×10^7 cells were taken and added to PBS to make a final volume of 700μl. Cells were added to 100μl of 5-15mg linearized DNA, gently mixed, quickly added to the cuvette, and electroporated at 800V 3μF. Cells were left to recover at RT for 20min. 10cm dishes were gelatinised with 2% gelatin. 10ml medium was added to each plate. The electroporated cells were transferred to 10ml medium and 5×10^6, 2×10^6 and 1×10^6 cells added onto 10cm dishes. Plates were incubated overnight and next day growth was checked. The medium was changed to medium + 150ug/ml G418. G418 media was changed every 2 days. Colonies were picked after 8-10 days.

2.3.5.1 Production of Stromal Cell Lines

Cells from E10.5 AGM region were transfected with pB-PGK-TAG-CER-M20-2 (produced by Yiding Zhao in house) and helper plasmid pcylo43helper using the Neon Transfection System (Invitrogen). After culture single cells were plated in a 96 well plate.

2.3.6 Picking Colonies

A 96 well U bottomed (Sterlilin) plate was prepared by adding 25μl trypsin to each well. A gelatinised flat bottomed 96 well plate (Corning) was prepared by adding 100μl of 2% gelatin to each well, incubating for 10min and then aspirating the

gelatin. The medium was aspirated from the plate containing the colonies to be picked. 10ml RT PBS was gently added. Colonies were gently dislodged with a 20µl pipette and tip set to 15µl and taken up in 10-15µl PBS. The colony was then dispensed into a fresh well of the 96 well trypsin plate. Colonies were pick for no more than 30min or until the 96 well plate was full. The trypsin plate with colonies plate was then incubated at 37°C for 10min. 165µl of medium was added to each well using a multichannel pipette and cells gently dispersed 4-5 times. The cells were then transferred to the gelatinised 96 well flat bottomed plate and incubated overnight. The medium was changed the following day to G418 medium. Cell were expanded and passaged as described above maintaining selection with G418 medium for 2-3 passages.

2.4 Molecular Methods

2.4.1 PCR

Mastermix was prepared in MilliQ water containing PCR buffer, 10mM dNTPs, 5 units/µl Taq polymerase, primers at 0.1pM/µl final concentration, and template DNA. Sample were run on a Biometra T3 Thermocycler with the following protocol: denaturation at 95°C for 3min; followed by 35 cycles of - denaturation at 94°C for 30 seconds, annealing at X°C for 40 seconds, extension at 72°C for Ymin; 72°C for 10min. Samples were examined with a 2% agarose gel electrophoresis (90V for 45min). DNA was labelled with ethidium bromide and pictures were taken with a Syngene G:Box with GeneSys software. All primers were obtained from sigma and all other reagents used for PCR were obtained from Qiagen.

High fidelity PCR was carried out as described above with Platinum® *Pfx* DNA Polymerase (Invitrogen). U fragment and G fragment production required a slightly modified PCR programme. This consisted of: denaturation at 95°C for 2min; 4 cycles of - 95°C for 30s, annealing at X°C for 30s and 72°C for 4min; 25 cycles of -95°C for 30s, 72°C for Ym (increasing each cycle); finally 72°C for 7min. After PCR 20 units of DpnI were added to the reaction and it was incubated at 37°C for 2 hours. The PCR product was purified using a PCR purification kit (Qiagen) and eluted in water. DNA concentration was measure using a Nanodrop Spectrophotometer.

Primer sequences and PCR reactions are summarised in table 2.3.

Tables 2.3: Summary of primers, PCR conditions and resultant fragment size.

Primer Name	Sequence 5' - 3'	Annealing Temp (X)	Extension Time (Y)	Bands
Jagged1				
Jag1 For	GCAAGTCTGTCTGCTTTATC			WT 267bp
Jag1 Rev 1	AGGTTGGCCACCTCTAAATC	56°C	1m 30s	Jag1^{fl} 317bp
Jag1 Rev 2	GAGATGCAGGTAAGAAGTCCAATC			Jag1$^{\Delta}$ 1385bp
Jagged1^{Tom}				
Jag1Tom For	CAGTCGTGCATGCTCCAATCCAC	56°C	1m	Jag1^{Tom} 994bp
Jag1Tom Rev	GATGACGGCCATGTTGTTGTCCT			
Cre recombinase				
Cre For	TCGATGCAACGAGTGATGAG	56°C	45s	Cre 373bp
Cre Rev	AGTGCGTTCGAACGCTAGAG			
GFP				
GFP For	AAGTTCATCTGCACCACCG	56°C	45s	GFP 173bp
GFP Rev	TCCTTGAAGAAGATGGTGCG			
Neomycin				
Neo For	CTGTGCTCGACGTTGTCACT	56°C	45s	Neo 460bp
Neo Rev	AGCTCTTCAGCAATATCACG			
U fragment				
UJ1 For	CGCTAGCAGCGGCGGCGGCGTCCGGA GTGCCCGTGGCGCGCGGCGCAGCGAA GGCGCATAACGATACCAC	60°C	2m 30s increase 5s/cycle	UJ1 frag 2018bp
UJ1 Rev	GGTGCTGCCTCTCGGGTGAAAAGTC AGGGTCAGGGAGCCTTGTACTCAAG CCGCCTACTGCGACTATAGA			
G fragment				
GJ1 For	GTAAAAAGAACACTTCGGGATGTT TGAAATGATATGGACCTACAATGA AATCCTGTGTGAAATTGTTATCCGC	55°C	4m increase 20s/cycle	GJ1 frag 3373bp
GJ1 Rev	TACTTTAACAATAATGAGAACAA AATAAACTTTTCTTCCAAGGGCTGG AACCACTGGCCGTCGTTTTACA			
Southern Blot Probe 1				
SB1 For	GTGGAGCTGGCAGAGACTTC	56°C	45s	SB1 473bp
SB1 Rev	CTTTCCCGGAGTCAGACTTG			
Southern Blot Probe 2				
SB2 For	TACAGGAGTTGGCCTTTGCT	56°C	45s	SB2 476bp
SB2 Rev	AGCCTGTGGAGCAAGTCTGT			
JAG1 qPCR				
J1 For	AGAACGGCAACAAAACTTGC			
J1 Rev	GACCCATGCTTGGGACTG			
Tomato qPCR				
Tom For	CTGAAGGGCGAGATCCAC			
Tom Rev	TGGCCATGTAGATGGTCTTG			

50

2.4.2 Molecular Method Kits

Many molecular methods were carried out using well established kits details and protocols will not be discussed here as the information is readily available from manufacturers. All kits were used according to the manufacturer's instructions.

- Enzyme digestion was carried out using restriction enzymes from New England Biolabs and Sigma.
- Gel purification of digested fragments was carried out using a gel purification kit from Qiagen.
- DNA ligation was carried using T4 DNA ligase from Invitrogen.
- One Shot® INV110 Competent Cells from Invitrogen were used for transformation of DNA of interest.
- Mini and maxi preps were carried out using Qiagen kits. Maxi prep of the Jagged1-dtTomato targeting vector was carried out using an EndoFree Maxi prep kit from Qiagen.
- The 3-way gateway reaction was carried out using the Gateway® Technology with Clonase™ II from Invitrogen. Cells with the resultant product were selected on YEG agar plates containing Spectinomycin (final concentration 50µg/ml) and Glucose (final concentration 0.4%).
- Mini RNA purification kit from Qiagen was used for RNA purification.
- SuperScript® VILO™ cDNA Synthesis Kit from Invitrogen for cDNA production.
- qPCR was carried out using the UPL probe system and the LightCycler® 480 from Roche. The reaction mixture consisted of 10µl: 1x master mix (2x stock); primers (final conc. 0.5µM); 1x UPL probe (10x stock); approx. 50ng cDNA; and PCR grade H_2O.

2.4.3 Southern Blotting

Genomic DNA for southern blot was isolated using phenol chloroform extraction. 4-10µg of DNA was incubated overnight with 50IU of the appropriate enzyme. The following day digested DNA samples were loaded on a 0.7% agarose gel and electrophoresis took place overnight in TAE buffer at approximately 30V. The following morning the gel was place between layers of filter paper on top of a nylon

membrane (Bio-Rad). A downward transfer was carried out for at least 4 hours with alkaline transfer buffer (0.4M NaOH, 0.6M NaCl). After the transfer the dried membrane underwent pre-hybridisation for 3hrs at 65°C. Pre-hybridisation buffer consisted of: 0.5M [Na+]PO4; 7% SDS; 2mM EDTA; and heterologous denatured DNA (100µg/ml). DNA probe was labelled using $dCTP^{32P}$ and purified using Micro Bio-Spin P-30 Tris Column (Bio-Rad). The labelled probe was added to fresh pre-hybridisation buffer and the membrane incubated with it overnight at 65°C. The following morning was washed in pre-heated wash buffer (0.2X SSC, 0.1% SDS) for 20min each wash until the washing buffer was no longer radioactive. Membrane was incubated with a storage phosphor screen and the image developed using a FLA-3000 form (Fujifilm).

2.4.4 Producing Electrocompetent Cells and Electroporation

A saturated culture of the cells of interest was grown at the appropriate temperature, at 200rpm in 2ml LB, with selection, in a 14ml round bottom tube. In the morning the saturated culture was diluted 1:50 in a fresh 2ml of LB with selection in a 14ml tube and incubated at the appropriate temperature, at 200rpm for 2-2½ hours until the optical density at 600nm was 0.2-0.4. The culture was then chilled on ice and transferred to an eppendorf tube. The cells were centrifuged at 14,500rpm for 5min (Sigma Michael Christ edition 1-14 Micro-centrifuge), supernatant removed and cells resuspended in 1ml ice cold HPLC quality water. From this point on cells were kept cold. Cells were again centrifuged at 14,500rpm for 5min in a refrigerated centrifuge, the supernatant removed and cells resuspended in 1ml ice cold HPLC quality water. This step was repeated twice more and after the final wash water was carefully drained well and cells were resuspended in 50µl HPLC water containing 50ng of the DNA to be electroporated. The mixture was added to a chilled electroporation cuvette (Biorad) and electroporated on a Biorad Micro-Pulser on the "EC1" setting (1800V). After electroporation 450µl of recovery medium (2X LB + 0.1% glucose) was quickly added to the cells and cells were transferred to a 14ml tube for 70min incubation at 37°C, at 200rpm. 250µl of the culture was then inoculated into 750µl of LB containing the appropriate selection and cultured at the appropriate temperature for 24hrs or 48hrs.

2.4.5 Recombineering

The BAC host E. Coli were made electrocompetent and the recombineering plasmid, pSC101-BAD-γαβA-tet, was electroporated into the cells and selected with tetracycline (final conc. in 1ml 5μg/ml) and chloramphenicol (final conc. in 1ml 12.5μg/ml). After 48hr growth at 30°C the saturated culture was used to make electrocompetent cells as discuss in the previous section. One slight modification was made in that after the cells had reached 0.2-0.4 OD_{600} they were incubated with arabinose (final conc. 0.02%) at 37°C, 200rpm for 40min before being chilled on ice. After the host cells were made electrocompetent the first gateway cassette, the U-fragment, was electroporated into the cells. Recombineering took place during the 70min recovery at 37°C and after the cells were again grown at 30°C for 48hrs and selection with Zeocin (final conc. 10μg/ml) and Tetracycline (final conc. 5μg/ml) was carried out. The host cells were again made electrocompetent as described above and again when at 0.2-0.4 OD_{600} cells were incubated with arabinose (final conc. 0.02%) at 37°C, 200rpm for 40min before being chilled on ice. The second gateway cassette, the G-fragment, was electroporated into the cells. Recombineering took place during the 70min recovery at 37°C and after the cells were then grown at 37°C overnight, to remove the recombineering plasmid from the culture, and selected with Zeocin (final conc. 10μg/ml) and Carbenicillin (final conc. 50μg/ml in 1ml). The cell culture was then plated on agar containing Carbenicillin (final conc. 50μg/ml in 1ml) and Zeocin (final conc. 10μg/ml) and incubated overnight. Colonies are examined for the presence of the Jagged1 intermediate targeting vector. The vector was isolated by maxi prep (Qiagen), analysed by restriction digest and sequencing, and prepared for the gateway reaction.

2.5 Tissue Isolation and Preparation

2.5.1 Isolation of Embryonic Tissue

Pregnant females were sacrificed according to schedule 1 method of cervical dissociation. The uterus was dissected and embryos were removed in dissecting solution. The embryo was then separated from the extra-embryonic tissues including the yolk sac (YS) which was retained when appropriate. The developmental stage of the embryo was staged according to Theiler criteria (http://www.emouseatlas.org).

E11.5 corresponds to stage 19, with 41-47 somite pairs (sp); the anterior "handplate" is formed and incomplete eye pigmentation. E10 corresponds to stage 16, with 30-34sp, hindlimb and tail buds and no eye pigmentation. Embryonic organs of interest were dissected using forceps and sharpened tungsten needles under a dissecting microscope (Leica). The AGM region was dissected by first removing the head anterior embryo body above the fetal liver, followed by the neural tube and ventral tissue (including fetal liver and gut). The ribs were then cut away from the UGRs along with any excess dorsal tissue. For embryos at a stage earlier than E10 the caudal part of the embryo, dissected free of the anterior portion and ventral tissue, was used.

2.5.2 Dissociation of Embryonic Tissue into a Single Cell Suspension

To obtain single cell suspensions, embryonic organs were transferred to a 5ml polystyrene tube (BD Falcon) and suspended in dissecting solution with 1mg/ml collagenase dispase (Roche). Organs were digested in a gently shaking water bath at 37°C for 35min. Immediately after, 1ml of dissecting solution was added and the sample centrifuged at 1,200rpm for 5min at 4°C (Sigma 4-16K). The supernatant was removed and 1ml of cell suspension/staining solution was added. Cells were gently dissociated by pipetting 20 times in cell suspension solution. The sample was again centrifuged at 1,200rpm for 5min at 4°C and supernatant removed. Cells were resuspended in the appropriate volume of cell suspension/staining solution for further processing.

2.5.3 Dissociation of Adult Organs into Single Cells Suspension

Adult animals were all sacrificed according to schedule 1 method of cervical dissociation. For haematopoietic cell analysis spleen and thymus were dissected free of connective tissue and fat and immediately placed in cell suspension solution. Single cell suspensions were obtained by dissociating grossly cut organs with a 26-gauge syringe needle in a 30mm dish. After that, tissues were filtered through a 40µm cell strainer (BD Falcon) to get rid of cell clumps. Bone marrow was obtained by flushing femurs with cell suspension solution with a 26-gauge syringe needle and cells mechanically dispersed in cell suspension solution (BD Microlance). Peripheral

54

blood was obtained by bleeding from lateral tail vein, and immediately collected into 1ml PBS/EDTA (200µg/ml). Cells were centrifuged at 1,200rpm for 5min, resuspended in 1ml PharM Lyse solution (BD Bioscience), and incubated in dark at RT for 15min. After incubation cells were again centrifuged at 1,200rpm for 5min, supernatant removed, and cells resuspended in cell solution. Prepared adult cells were immediately placed on ice or at 4°C.

To obtain thymic epithelial cells thymi were dissected out into a PBS filled Petri dish and further cleaned of any non-thymic tissue. The thymic capsule was ripped and the lobes were gently agitated to release T-cells. The thymic lobes are then transferred into a tube and disrupted using scissors. They are then resuspended in a dissociation media (I-1840 HEPES media (Invitrogen) with collagenase D 1.25mg/ml (Roche) and Dnase I 0.05mg/ml) for 15min at 37°C. To aid the dissociation process every 5min the solution with the thymic fragments was disrupted by pipetting using a 1ml blue tip. After the incubation was complete the supernatant was taken off and filtered through a 70µm cell strainer. The remaining thymic fragments were resuspended in fresh dissociation media and the process described above was repeated a further 2 times. Any remaining fragments of tissue are resuspended in RPMI-1840 Hepes media with collagenase/dispase 1.25mg/ml for a further 30min. Dissociated cells were then spun down at 1,200rpm at 4°C for 5min and collected into 1ml cell suspension solution.

2.6 Culture Systems

2.6.1 Co-aggregate and Explant Culture

Durapore 0.65µm membrane filters were rinsed 3 times in autoclaved water with 100 units/ml P/S then air dried before use. Dry membranes were gently placed on top of 5ml medium consisting of IMDM medium (Iscove's Modified Dulbecco's Medium (IMDM) containing 20% FCS; 4mM glutamine; 50 units/ml P/S; 100ng/ml IL-3; 100ng/ml SCF; and 100ng/ml Flt3L in a 6 well plate (Corning). Following placement of explant organs or co-aggregates on the membranes cultures were incubated 5 or 7 days culture at 37°C, 5% CO_2. 7 days cultures had medium changed on day 1 of culture. No more than four co-aggregates/explants were culture on one membrane. To obtain single cell suspensions after culture, pieces of membrane were transferred

to a 5ml polystyrene tube (BD Falcon) and suspended in dissecting solution with 1mg/ml collagenase dispase (Roche). Samples were digested in a gently shaking water bath at 37°C for 35min. Immediately after, 1ml of dissecting solution was added, the cells gently pipette off the membrane, and the membrane discarded. The sample was then centrifuged at 1,200rpm for 5min at 4°C. The supernatant was removed and 1ml of cell suspension/staining solution was added. Cells were gently dissociated by pipetting 20 times in cell suspension solution. The sample was again centrifuged at 1,200rpm for 5min at 4°C and supernatant removed. Cells were resuspended in the appropriate volume of cell suspension/staining solution for further processing.

2.6.2 Co-aggregate with OP9 and Embryonic Cells

Approximately 1ee of embryonic cells were used in each co-aggregate. 1×10^5 OP9 cells per co-aggregate were added to the cell suspension of embryonic cells for co-aggregate culture. The cell mixture was centrifuged at 1,200rpm for 5min at 4°C, supernatant removed, and cells resuspended in 20µl culture medium per aggregate. 20µl were then added to 200µl pipette tips sealed with Paraflim (VWR International). Tips were then placed in 5ml polystyrene tube (BD Falcon) and centrifuged at 1,500rpm for 10min at 4°C. Parafilm was gently removed from the pipette tips and the cell pellets gently expelled onto a prepared membrane in a 6 well plate as described above.

2.7 Flow Cytometry

All antibodies used for flow cytometry and the concentration they were used at are shown in table 2.4. Cell suspensions were obtained as described in the previous section. Staining was carried out in 5ml polystyrene tubes (BD Falcon) or U bottomed 96 well plates (Sterilin). Cells were spun down at 1,200rpm for 5min in 4°C and the supernatant aspirated. Cells were resuspended in staining solution containing Fc block and primary antibodies. Controls included FMOs (fluorescence minus one), which consisted of all antibodies minus one for each antibody used, and on occasion isotype controls. Cells were incubated on ice or at 4°C for 20-30min. After incubation washing solution was added to the well/tube, cells spun down at 1,200rpm for 5min in 4°C, and supernatant aspirated. Cells were resuspended in

staining solution containing the appropriate streptavidin and incubated on ice or at 4°C for 15-20min. After incubation washing solution was added to the well/tube, cells spun down at 1,200rpm for 5min in 4°C, and supernatant aspirated. Cells were resuspended in staining solution, again spun down at 1,200rpm for 5min in 4°C, and supernatant aspirated. Cells were then resuspended in cell suspension solution ready for flow cytometry or cell sorting. 7AAD or DAPI was added to cells just before analysis.

Flow cytometry was carried out on a FACSCalibur (BD) or LSRFortessa (BD). Cell sorting was carried out by Simon Monard or Olivia Rodrigues on a FACSAria II (BD). Compensation was calculated based on positively stained beads (produced in house) and 7AAD only stained cells. FMOs were used to set gates. Flow cytometry data was analysed with FlowJo v7.6.2 software (Tree Star). For cell sorting, cells were sorted into cell suspension solution and kept on ice after sorting. Purity checks were done on the FACSAria II (BD) when appropriate.

2.8 In Vitro Assays

2.8.1 CFU-C Assay

Prior to use, methylcellulose based MethoCult medium containing erythropoietin, IL-3, IL-6, and SCF (M3434, Stem Cell Technologies) was thawed at RT and 50 units/ml P/S was added. After cell suspensions were added to MethoCult cultures were processed and plated according to manufacturer's instructions (www.stemcell.com). Haematopoietic colonies were counted and scored after 9-11 days of differentiation. The colonies were scored according to described standard criteria (Taoudi, 2006).

2.8.2 Endothelial/CFU Assay

2hr hours prior to setup 2×10^5 OP9 cells were plated in the required number of gelatinised wells of a 6 well plate. Methylcellulose based MethoCult medium containing erythropoietin, IL-3, IL-6, and SCF (M3434, Stem Cell Technologies) was supplement with 50ng/ml VEGF (Peprotech) and cells were added mixed and plated over the OP9s. After 10-12 days culture haematopoietic colonies were scored and then the methylcellulose washed from the well with PBS. Cells were fixed with

5% DMSO in methanol for 10min at RT. Cells were washed twice with PBS (2min each wash) and incubated with 30% FCS in PBS for 10min. The blocking solution was removed and cells were incubated with anti-CD31 in 7% FCS in PBS for 2 hour at RT. Cells were then washed with 0.05% Tween in PBS 3 times. Cells were incubated with anti-rat AP for 1hr at RT and again washed with 0.05% Tween in PBS 3 times. Blue Alkaline Phosphatase Substrate Kit (Vector Labs) was then used according to manufacturer's instructions. After cells were washed twice with 0.05% Tween in PBS, the endothelial colonies were scored and photographed using an inverted microscope (Motic).

2.9 Long-term HSC Repopulating Assay

Cells for long-term repopulation assay were obtained from either fresh or cultured embryonic organs from CD45.2/.2 mice. Appropriate doses of cells were resuspended in ice cold 1% FCS/PBS and co-injected along with 8×10^4 adult carrier bone marrow cells from CD45.1/.2 animals. Prior to transplantation, CD45.1/.1 animals were irradiated. A total dose of 9.5 Gy was split into two doses separated by at least 3 hours and delivered by sealed Cs source at a rate of 21.6 rad/min. No more than 200µl of cell suspension were injected into each recipient mouse using a 30-gauge syringe needle (BD Plastipak). All injections were performed in the lateral tail vein according to the procedures described by project licence and the regulations of the Animals Scientific Procedures Act, UK, 1986.

Recipients' peripheral blood chimerism (PBC) was assessed by flow cytometry at 6 weeks post-transplantation for short-term repopulation, and at least 16 weeks post-transplantation for long-term repopulation. Blood samples were processed as described in Sections 2.5.3 and 2.7. Only animals with donor chimerism higher than 5% were considered reconstituted.

Multilineage analysis was performed on animals reconstituted for at least 16 weeks post-transplantation. Bone marrow, spleen, and thymus were isolated and processed, (see Sections 2.5.3) and myeloid and lymphoid donor contribution was assessed by flow cytometry (see Section 2.7).

2.10 Microscopy Analysis

All antibodies used for staining tissue sections and whole mount embryos are shown in table 2.4 with working concentration.

2.10.1   Fluorescent Microscopy of Live Embryos

Picture of embryos were taken with a stereoscope microscope (Nikon AZ100superzoom) using a QImaging ExiBlue camera. The software used for analysis was NISElement Nikon, and multiple focal planes were combined into a single focus 2D image using NIS-A EDF Module for Extended Depth of Focus plug-in.

2.10.2   Sections

Fresh or fixed (4% PFA) embryos were snap-frozen in O.C.T compound (BDH Gurr) on dry ice. 10µm thick frozen sections were produced using a LEICA CM1900 cryostat (Leica) and directly put onto polysine-coated slides (VWR International). Sections were air-dried and stored at -20°C. Sections were warmed up at RT and fixed with -20°C cold 100% acetone for 1min. Sections were then air-dried and encircled with a pap pen. Once the PAP was dry sections were rehydrated with one wash of 2min PBS (containing MgCl and CaCl (Sigma)). From this point on sections were kept hydrated, and all staining was done in a staining chamber in the dark. Sections were then incubated with 30% FCS in PBS for 30-60min at RT. Sections were then washed once before adding staining solution containing primary antibodies (minus Notch1 or Jagged1). Sections were incubated for 1hr at RT. Sections were washed with 3 changes of PBS incubated for 5min each. The appropriate secondary antibodies were added together or in succession (depending on the reactivity and isotype). They were also incubated for 1hr at RT and followed by 3 changes of PBS as a wash. The sections were then incubated with 30% serum (Notch1 = rat serum; Jagged1 = goat serum) for 30min at RT and washed 3 times with PBS. Sections being stained for Notch1 were then treated with an Avidin/Biotin blocking kit (Vector Labs) according to manufacturer's instructions. After washing with 3 changes of PBS sections were incubated with Notch1 or Jagged1 at 4°C overnight. The following day sections were washed with 3 changes of PBS followed by 1hr incubation at RT with Strepavidin Al488 (Notch1) or anti-human Fc Al488

(Jagged1). Sections were washed with 3 changes of PBS, incubated with DAPI for 15min, and washed with 3 changes of PBS a final time. Sections were mounted in ProLong Gold anti-fade mounting medium (Invitrogen) and left to cure for 24hr at RT in the dark. Stained sections were analysed and pictures were taken using an inverted confocal microscope (Leica DM IRE2). Images were process using ImageJ software. The staining processes for used some section stains are shown in figure 2.1.

2.10.3 Wholemount

For wholemount staining all steps were carried out in the cold room with gentle shaking. Embryos were fixed for 1hr in 1% PFA/PBS. Embryos were then dehydrated with 10min incubations in each 50%, 75% and 100% methanol in PBS. The limbs and lateral body of the embryos were removed using a tungsten needles and forceps. Embryos were rehydrated in 50% methanol in PBS for 10min. 4 washes in ice cold PBS were carried out for 10min each. Embryos were incubated in blocking solution (PBS with 0.4% Tween and 50% FCS) for 3 hrs. Embryos were then incubated overnight with primary antibodies diluted in PBS with 0.4% Tween and 7% FCS. The following morning embryos were washed in PBS with 0.4% Tween and 7% FCS 3 times for 2hrs each wash. Embryos were incubated with secondary antibodies diluted in PBS with 0.4% Tween and 7% FCS. The following morning embryos were washed in PBS with 0.4% Tween and 7% FCS twice times for 1hrs each wash. Followed by 3 washes in PBS with 0.4% Tween and dehydration with 50% methanol in PBS for 10min and 100% methanol for 10min. Embryos were then cleared by incubating with 3 changes 50% methanol in BABB (1 part benzyl alcohol to 2 parts benzyl benzoate) for 20min each. Embryos are then washed in 100% BABB for 2min and stored in a fresh change of BABB. Embryos were examined and pictures taken using an inverted confocal microscope (Leica DM IRE2). Images were process using ImageJ software and Microsoft Powerpoint.

60

2.11 Statistical Analysis

Microsoft Excel and GraphPad Prism 6 were used for analysis of results. Mean and standard deviation were calculated in Excel and statistics were calculated in GraphPad. Data was analysed for normal distribution but in general: data from fresh tissue was analysed parametric data and data post culture was analysed as non-parametric data. Initial comparison of groups was done by One-Way ANOVA (parametric) or Friedmen Test (non-parametric). If significant, groups of parametric data were compared using t-test with Welch's correction. Groups of non-parametric data were compared using Mann-Whitney test.
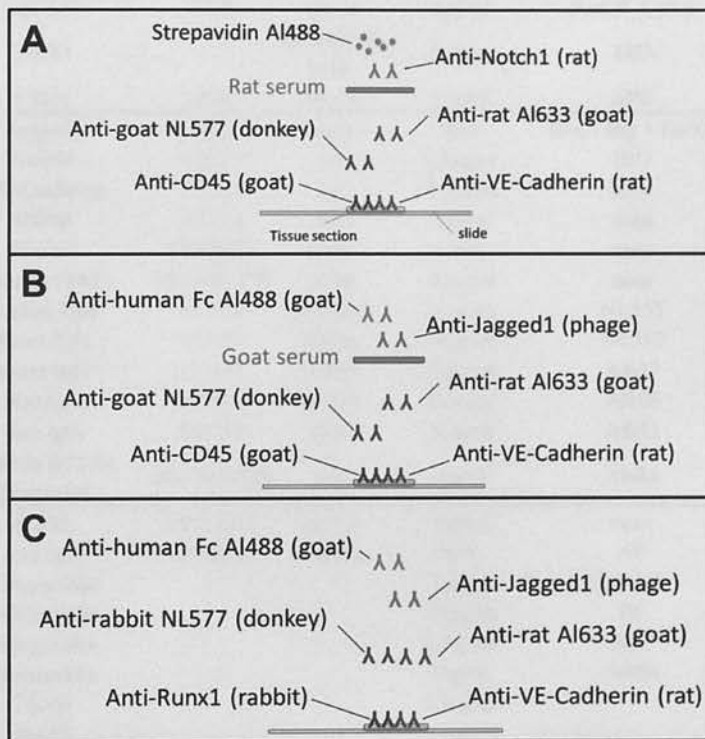

Figure 2.1: Order of use of antibodies when staining sections.
A: CD45, VE-Cadherin and Notch1 with rat serum blocking. **B:** CD45, VE-Cadherin and Jagged1 with goat serum blocking. **C:** Runx1, VE-Cadherin and Jagged1.

2.4: Summary of antibodies.

Use	Antigen	Clone	Isotype	Working Conc.	Conjugate	Supplier
Flow Cytometry	CD16/CD32 (Fc block)	93	rIgG2b	1µg/ml	unconjugated	eBioscience
	Notch1	22E5.5	IgG2a	1.5µg/ml	BIO	MacDonald Lab
	CD45	30F-11	rIgG2b	2µg/ml	V450, V500, APC	BD Horizon
	CD41	MWReg30	rIgG1	10µg/ml	Al488	BioLegend
	VE-Cadhein/CD144	eBioBV13	rIgG1	2µg/ml	Al647	eBioscience
	Ter119	TER-119	rIgG2b	2µg/ml	PerCP_Cy5.5, APC	eBioscience
	CD45.1	A20	mIgG2a	1µg/ml	APC, FITC	eBioscience
	CD45.2	104	mIgG2a	1µg/ml	PE, V450	eBioscience
	B220	RA3-6B2	rIgG2a	2.5µg/ml	BIO	eBioscience
	CD3e	145-2C11	hIgG	2µg/ml	PE, FITC	eBioscience
	Mac1/CD11b	M1/70	rIgG2b	2.5µg/ml	BIO, APC	eBioscience
	Gr1	RB6-8C5	rIgG2b	2.5µg/ml	PE, FITC	eBioscience
	CD4	GK1.5	rIgG2b	1.25µg/ml	BIO	eBioscience
	CD8a	53-6.7	rIgG2a	2.5µg/ml	PE, FITC	eBioscience
	EpCAM	G8.8	rIgG2a	1µg/ml	PerCP_Cy5.5	BioLegend
	UEA1		Biotinilated lectin	5µg/ml	BIO	VectorLabs
	CD31	390	rIgG2a	1µg/ml	APC	BioLegend
Immuno-histochemistry	Jagged1	D05	phage	N/A	hFc + flag + HisX6	McCafferty Lab
	Notch1	22E5.5	rat	1.5µg/ml	BIO	MacDonald Lab
	VE-Cadherin	11D4.1	rat	2.5µg/ml	none	BD Pharmingen
	CD45	AF114	goat	2µg/ml	none	R&D Systems
	Runx1	EPR3099	rabbit	1:100	none	abcam
	Tomato (RFP)	600-401-379	rabbit	20µg/ml	none	Rockland
	Rabbit IgG	NL004	donkey	10µg/ml	NL557	R&D Systems
	Goat IgG	NL001	donkey	10µg/ml	NL557	R&D Systems
	Goat IgG	A21082	donkey	20µg/ml	Al633	Invitrogen
	Rat IgG	A21208	donkey	20µg/ml	Al488	Invitrogen
	Rat IgG	A21094	goat	20µg/ml	Al633	Invitrogen
	Human IgG Fc Fragment	109-546-008	goat	3µg/ml	Al488	Jackson Immuno
Other	CD31	MEC13.3	rIgG2a	1µg/ml	none	BD Pharmingen
	Rat IgG	2A 8F4	mIgG2a	N/A	AP	SouthernBiotech
	Strepavidin			1µg/ml	BV421	BioLegend
	Strepavidin			0.5µg/ml	PE	BD Pharmingen
	Strepavidin			0.2µg/ml	APC	BD Pharmingen
	Strepavidin			2µg/ml	Al488	BD Pharmingen
	7AAD			0.5µg/ml		Molecular Probes
	DAPI			0.5µg/ml		Biotium

3 Notch1 Expression during Haematopoietic Development

It has previously been shown that Notch1 and N1ICD (Notch 1 intracellular domain) are expressed in the dorsal aorta of the AGM region during haematopoietic development at E9.5, E10.5 and E11.5 (Robert-Moreno et al. 2008; Robert-Moreno et al. 2005; Kumano et al. 2003). *In situ* hybridization shows a patched pattern of Notch1 expression in the ventral domain of the E9.5 and E10.5 dorsal aorta in the AGM region (Robert-Moreno et al. 2005; Robert-Moreno et al. 2008). Further *in situ* hybridization of E10.5 AGM region shows that some GATA2 positive cells of the dorsal aorta are also $Notch1^+Jagged1^+$ indicating a role for Notch1 and Jagged1 in haematopoietic development (Robert-Moreno et al. 2005). It has been established that $Notch1^{-/-}$ ES cells do not contribute to the haematopoietic system and that Notch1 mutants have impaired intra-embryonic haematopoiesis (Kumano et al. 2003; Hadland et al. 2004). However, at E9.5 Notch1 mutants have retarded development and severe vascular defects meaning there is limit to their use as a tool in investigating the role of Notch1 in dHSC development (Krebs et al. 2000).

Work being carried out in our lab involves the use of conditional lines and blocking antibodies to try to address the role of Notch1 in haematopoietic development in a targeted way. To supplement this work I carried out detailed phenotypical and functional analysis of Notch1 positive cells in the populations of interest of the developing haematopoietic system. This was carried out using a monoclonal rat anti-Notch1 antibody received from the H. Robson MacDonald's lab at the Ludwig Institute for Cancer Research Ltd., Lausanne (Fiorini et al. 2009). These results give us a more detailed insight into Notch1 expression and the stages of dHSC development in which Notch1 may be important.

3.1 Phenotypic Analysis of Notch1 Expression

3.1.1 Notch1 expression in endothelial and haematopoietic populations during haematopoietic development

Notch1 expression was analysed in populations of interest in the developing haematopoietic system at different embryo stages. HSCs arise through the stepwise maturation of pre-HSC Type I to pre-HSC Type I to HSCs (fig3.1a) (Rybtsov et al. 2011; Taoudi et al. 2008). Unless otherwise stated the populations analysed are defined as: CD45$^+$VE-Cadherin$^-$ which is enriched for haematopoietic cells; CD41$^-$CD45$^-$VE-Cadherin$^+$ which is enriched for endothelial cells; CD41$^+$CD45$^-$VE-Cadherin$^+$ which is enriched for pre-HSC Type I at E10.5 and E11.5 in the AGM region; CD45$^+$VE-Cadherin$^+$ which is enriched for pre-HSC Type II and contains dHSCs at E11.5 in the AGM region. Figure 3.1b shows the gating strategy used to analyse these populations by first gating on live cells using 7AAD exclusion and further gating into the populations described above based on CD45, VE-Cadherin and CD41 expression. The different stages at which the analysis was carried out include: E8.5 in the whole embryo; E9 embryo in the caudal part; E10.5 in the AGM region; and E11.5 in the AGM region and the YS.

Figure 3.2a and table 3.1 show the percentage of cells, of each population, which were Notch1 positive over the course of the different embryo stages. The cell populations analysed all contained Notch1$^+$ cells at all stages. The endothelial population contains the highest proportion of Notch1$^+$ cells at all stages compared to the other populations analysed. The proportion of Notch1$^+$ cells in each population does not significantly change over the course of the different stages aside from a significant drop in the percentage of Notch1$^+$ haematopoietic cells between E8.5 and E9 (p=0.0346). There is also a trend which shows an increase in the percentage of Notch1$^+$ cells in the CD45$^+$VE-Cadherin$^+$ population between E10.5 and E11.5, the time at which some of the cells of the CD45$^+$VE-Cadherin$^+$ population become functional pre-HSC Type II and HSCs.

The flow cytometry data was further analysed to try and discern if there was a difference between each population in the level of Notch1 expression found on the positive cells. The median value of PE fluorescence in the Notch1$^+$ population was

taken as an indicator of the level of Notch1 expression. In figure 3.2b median PE fluorescence for the different Notch1$^+$ populations is shown. In the endothelial (CD41$^-$CD45$^-$VE-Cadherin$^+$) and CD41$^+$CD45$^-$VE-Cadherin$^+$ populations (CD45$^-$ VE-Cadherin$^+$ populations) the level of Notch1 expression in E9, E10.5, and E11.5 is higher than the level of expression in the haematopoietic (CD45$^+$VE-Cadherin$^-$) and the CD45$^+$VE-Cadherin$^+$ populations. Thus, although the percentage of Notch1$^+$ cells may be similar (e.g. E11.5 CD41$^+$CD45$^-$VE-Cadherin$^+$ 70.4% ±19.6 and CD45$^+$VE-Cadherin$^+$ 73.2% ±10.8), the level of Notch1 expression may be different (e.g. E11.5 CD41$^+$CD45$^-$VE-Cadherin$^+$ 10656 ±2271 and CD45$^+$VE-Cadherin$^+$ 1990 ±366). Over different embryo stages the level of Notch1 expression within a Notch1$^+$ population also changes. There is a significant increase in the level of Notch1 expression during development between E8.5 and E11.5 in the endothelial (CD41$^-$ CD45$^-$VE-Cadherin$^+$) population (p=0.0103), and the CD41$^+$CD45$^-$VE-Cadherin$^+$ population (p=0.0143).
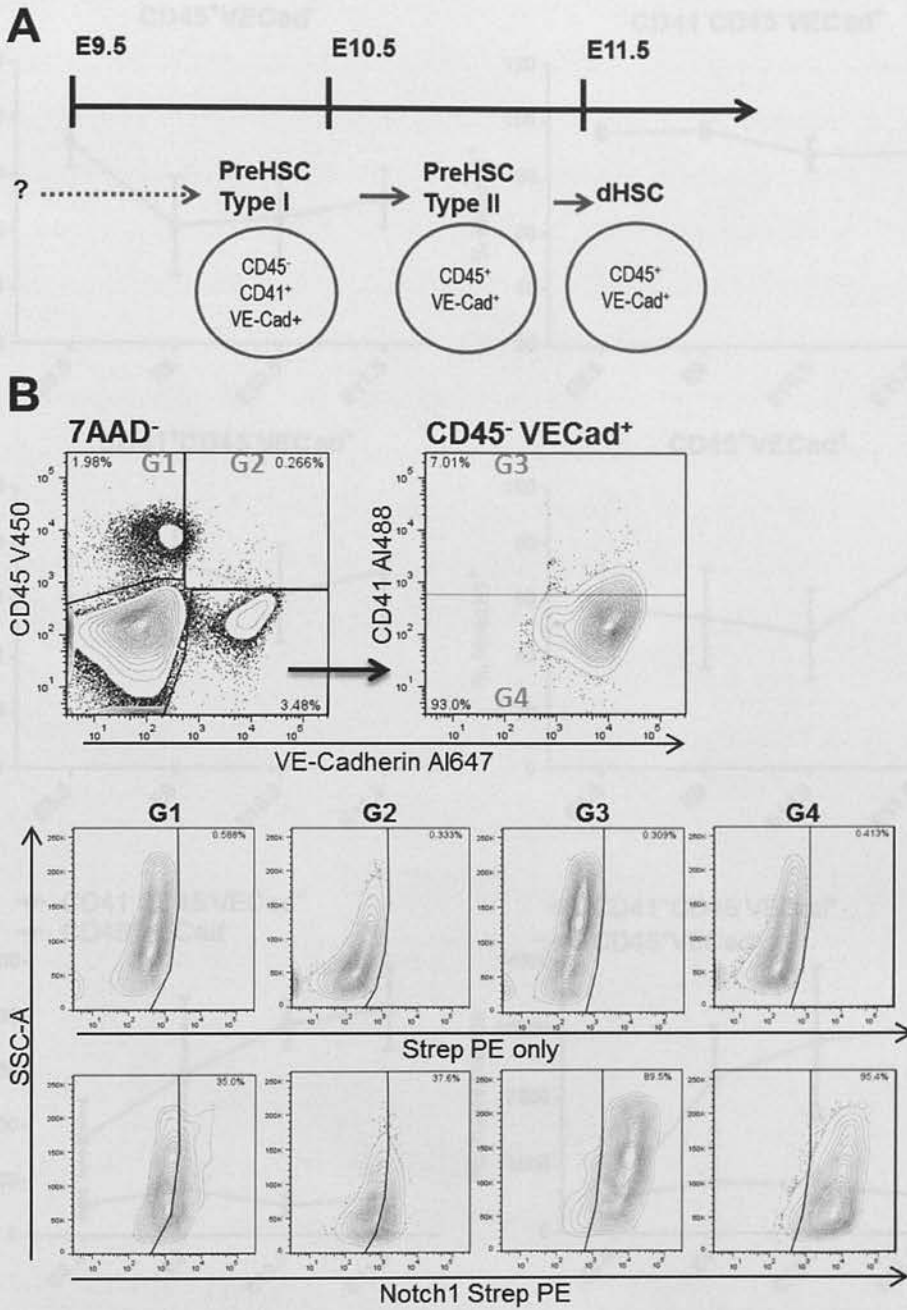
Figure 3.1: HSC development and Gating strategy Notch1 analysis.
A: HSC arise through the step wise maturation of preHSC I through preHSC II to HSCs (Rybtsov et al. 2011; Taoudi et al. 2008). **B:** Live cells were gated based on 7AAD exclusion and then further gated into populations of interest. G1: Haematopoietic cells (CD45+VECad-); G2: preHSC II enriched (CD45+VECad+); G3: preHSC I enriched (CD41+CD45-VECad+); G4: Endothelial cells (CD41-CD45-VECad+). All gating was based on FMOs (top row) Notch1 expression in these populations was then assessed (bottom row).
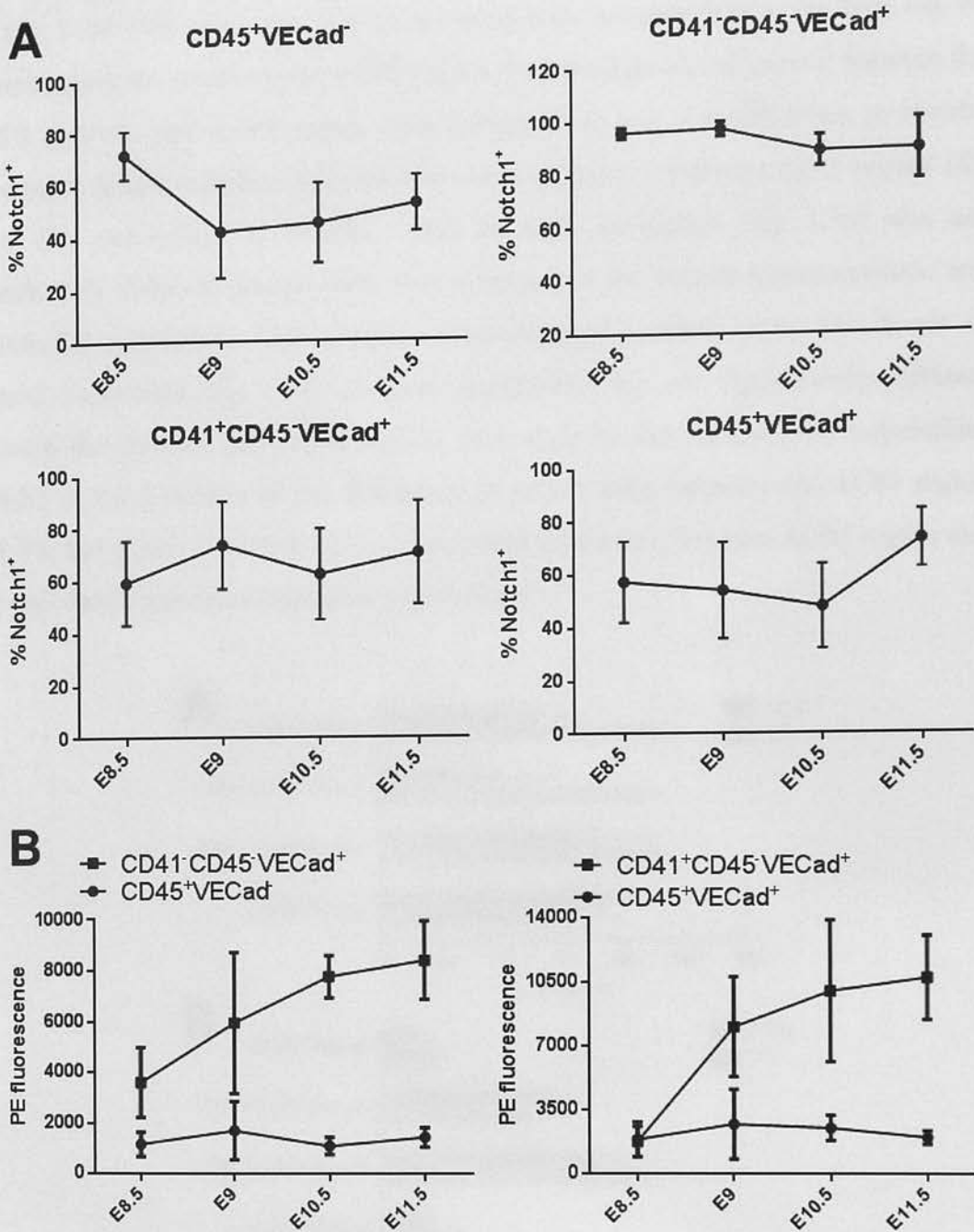
Figure 3.2: Percentage of Notch1$^+$ cells and level of Notch1 expression in populations of interest in embryos of different stages.

A: Percentage of Notch1+ cells in populations of interest at different stages of embryo development. **B:** Median level of PE fluorescence in Notch1$^+$ populations at different stages of embryo development. There is a significant difference between the mean of PE fluorescence between E8.5 and E11.5 of the CD41$^-$CD45$^-$VECad$^+$ population (p=0.0103), and the CD41$^+$CD45$^-$VECad$^+$ population (p=0.0143). Haematopoietic cells (CD45$^+$VECad$^-$); Endothelial cells (CD41$^-$CD45$^-$VECad$^+$); preHSC I enriched (CD41$^+$CD45$^-$VECad$^+$); preHSC II and dHSC enriched (CD45$^+$VECad$^+$). E8.5 whole embryo; E9 embryo caudal part; E10.5 AGM region; E11.5 AGM region. Mean ±SD. Results obtained from 3/4 independent experiments per stage.

At E11.5 the yolk sac is not able to autonomously generate HSCs, but they can be generated autonomously in the AGM region. The populations of interest between the E11.5 yolk sac and AGM region were compared to see if a difference in Notch1 expression might correlate with the functional difference between the 2 organs (fig 3.3). The percentage of Notch1$^+$ cells in each population (fig 3.3a) was not significantly different though there was a trend that the mature haematopoietic and endothelial populations had a higher percentage of Notch1$^+$ cells. The levels of Notch1 expression (fig 3.3b) in these populations are not significantly different between the yolk sac and AGM region. This suggests that for pre-HSC populations Notch1 is not a marker of the difference in functionally between the AGM region and YS, but it may be involved in a functional difference between AGM region and YS endothelial and haematopoietic populations.
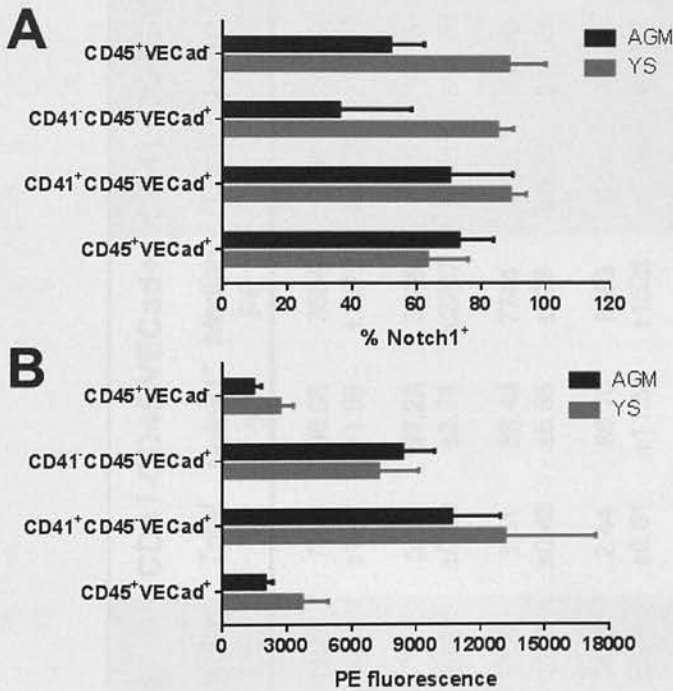

Figure 3.3: Notch1 expression in E11.5 AGM region and Yolk Sac.
A: Percentage of Notch1$^+$ cells in populations of the AGM region and Yolk Sac. **B:** Median level of PE fluorescence in the Notch1$^+$ populations of the AGM region and Yolk Sac. Haematopoietic cells (CD45$^+$VECad$^-$); Endothelial cells (CD41$^-$CD45$^-$VECad$^+$); preHSC I enriched (CD41$^+$CD45$^-$VECad$^+$); preHSC II and dHSC enriched (CD45$^+$VECad$^+$). Results obtained from 3 independent experiments. Mean ±SD.

Table 3.1: Summary of Notch1 flow cytometry data.
Percentage of total cells, percentage of Notch1⁺ cells and median PE fluorescence of the Notch1⁺ sub-population. Haematopoietic cells (CD45⁺VECad⁻); Endothelial cells (CD41⁻CD45⁻VECad⁺); preHSC II and dHSC enriched (CD45⁺VECad⁺); preHSC I enriched (CD41⁺CD45⁻VECad⁺). Results obtained from 3/4 independent experiments per stage. Mean ± SD.

	CD45+VECad-			CD41-CD45-VECad+			CD41+CD45-VECad+			CD45+VECad+			Total Live Cells	
	Total %	Notch1⁺ %	Median PE	Total %	Notch1⁺ %	Median PE	Total %	Notch1⁺ %	Median PE	Total %	Notch1⁺ %	Median PE	Notch1⁺ %	Median PE
E8.5	1.31 ±0.57	71.65 ±9.17	1166 ±483	7.18 ±1.41	96.08 ±1.99	3594 ±1373	2.38 ±0.72	59.40 ±16.11	1769 ±824	0.22 ±0.22	57.45 ±15.25	1884 ±952	58.6 ±11.40	987 ±268
E9	0.36 ±0.28	41.98 ±17.60	1704 ±1145	5.33 ±0.88	97.23 ±2.71	5938 ±2767	1.81 ±0.21	73.65 ±16.76	8012 ±2726	0.19 ±0.018	54.10 ±18.15	2713 ±1888	33.22 ±4.22	1849 ±767
E10.5	0.96 ±0.24	44.98 ±15.20	1100 ±338	3.51 ±0.48	88.43 ±5.86	7744 ±828	0.98 ±0.30	62.40 ±17.28	9942 ±3836	0.46 ±0.30	47.92 ±15.98	2502 ±690	27.80 ±5.38	2093 ±165
E11.5	1.86 ±0.16	52 ±10.65	1436 ±379	2.44 ±0.81	88.70 ±11.54	8363 ±1526	0.24 ±0.04	70.37 ±19.62	10656 ±2271	0.49 ±0.41	73.20 ±10.81	1990 ±366	11.50 ±5.25	2464 ±491
E11.5 (YS)	3.39 ±1.17	36.07 ±22.55	2662 ±638	1.89 ±0.70	85.17 ±5.08	7241 ±1898	0.96 ±0.48	89.13 ±5.01	13136 ±4231	0.77 ±0.35	63.43 ±12.75	3692 ±1247	5.81 ±2.86	4659 ±1359

3.1.2 Expression of Notch1 protein in the dorsal aorta

The dorsal-ventral polarity of HSC development correlates with the expression of certain genes. Ventral expression of proteins known to be involved in HSC development, for example Runx1, has previously been observed ((Robert-Moreno et al. 2008) and own observations). To determine if Notch1 protein is expressed differentially around the E11.5 dorsal aorta immunohistochemistry was performed. Transverse sections of the E11.5 AGM region stained for Notch1, VE-Cadherin, CD45 and DNA show that Notch1 is expressed by the endothelial cells of the dorsal aorta (fig 3.4). A dorsal ventral polarity of Notch1 protein was not observed at this stage. Magnification of a haematopoietic intra-aortic cluster (HIAC) in endothelium of the ventral domain of the dorsal aorta shows that the cells of the endothelium layer of the dorsal aorta at the bottom of the cell cluster express Notch1, CD45 and VE-Cadherin and the cells of the cluster express CD45 and VE-Cadherin (fig 3.4). This correlates with Notch1 and N1ICD protein staining previously observed at E11.5 (Kumano et al. 2003; Del Monte et al. 2007). In correlation with previous observations smooth muscle cells surrounding the E11.5 dorsal aorta also express low levels of Notch1 (Del Monte et al. 2007).

As slight differences in protein expression might not have been detectable by confocal analysis, the dorsal aorta of the AGM region was sub-dissected into ventral (AoV) and dorsal (AoD) parts (retaining the dorsal tissue) and analysed by flow cytometry. Sub-sections from several E11.5 AGM regions were pooled, dissociated, and stained using standard protocols. Figure 3.5 shows the expression of Notch1 compared between AoV, and AoD in the total live cells population and the endothelial population (CD41$^-$CD45$^-$VE-Cadherin$^+$), nearly all cells are Notch1$^+$ in these subdisected tissues. In the total live cell compartment, the level of Notch1 expression in the AoV (median PE fluorescence 864) and AoD (median PE fluorescence 796) is similar with some cells in the AoV that are brighter for PE. Endothelial cells of these tissues have the highest expression of Notch1 and Notch1 expression reaches a similar maximum for AoV and AoD. The Notch1 polarisation seen at E10.5 by *in situ* hybridization is not detectable at E11.5 by protein staining. However, AoV endothelial cells contain 2 distinct populations Notch1 low and Notch1 high, this Notch1 low population is not present in the AoD region.
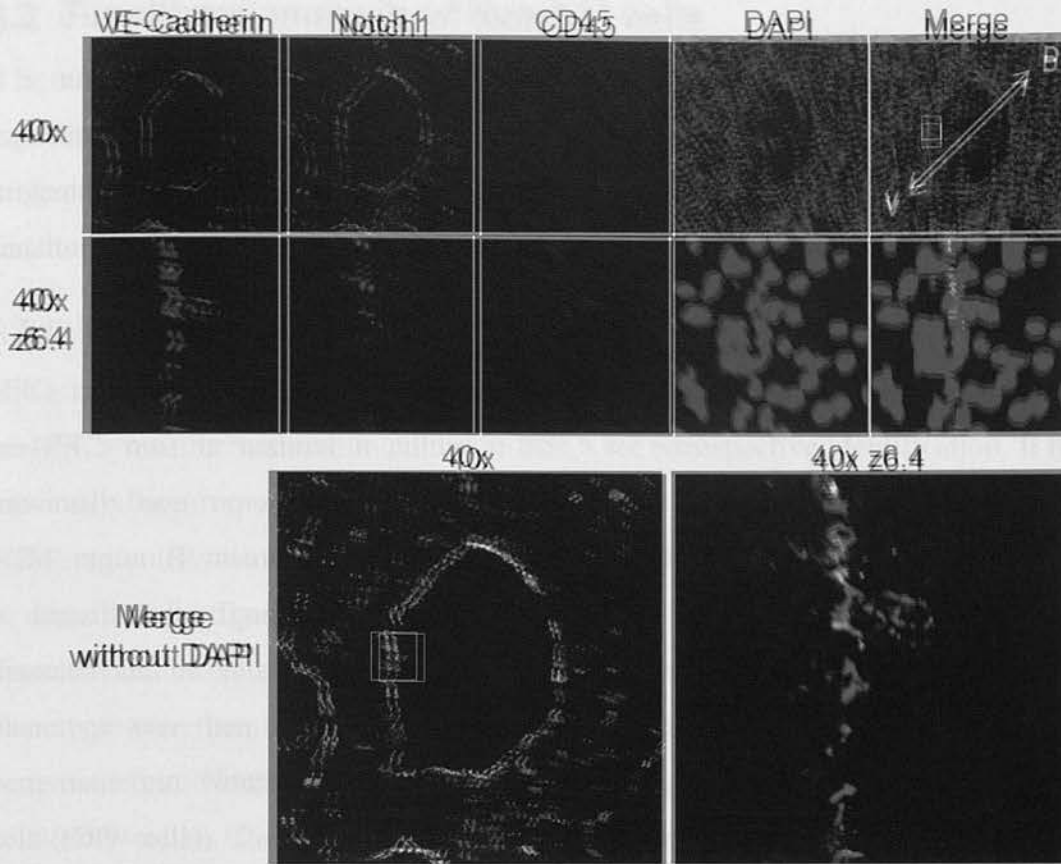
Figure 3.4: Notch1 expression in the dorsal aorta of the E11.5 AGM region.
Confocal transverse sections of E11.5 AGM region stained for VE-Cadherin (magenta), Notch1 (green), CD45 (red), and with DAPI (blue). Images were acquired with a 400x oil immersion objective lens. The double headed arrow indicates orientation with D meaning dorsal and V meaning ventral. The white boxes indicate the location of the 400x 6.4 time's magnified section. Sections are a representation of 3 independent experiments.
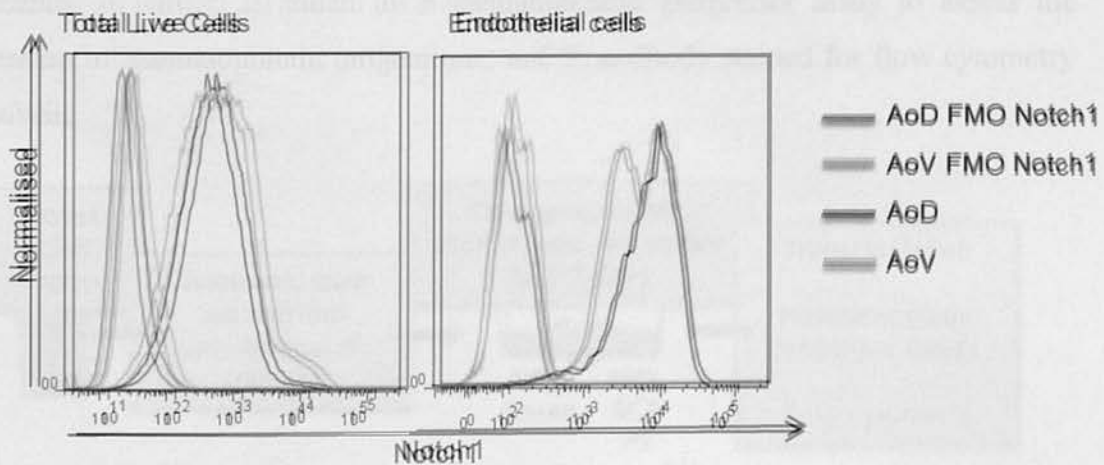
Figure 3.5: Comparison of Notch1 expression in AoV and AoD.
AoV (ventral part) and AoD (dorsal part) sub-dissected from E11.5 dorsal aorta. 7AAD- cells (left) and CD41⁻CD45⁻VECad⁺ endothelial cells (right). Representative example of 2 independent experiments.

71

3.2 Functional analysis of Notch1$^+$ cells

It is not possible to know solely by flow cytometry analysis whether Notch1 is expressed by functional pre-HSCs, HSCs, or haematopoietic and endothelial progenitors. To answer the question whether Notch1 is expressed by these cell types, functional analysis must be carried out.

3.2.1 HSC precursors

HSCs can only be identified by their ability to repopulate irradiated adult recipients; pre-HSCs must be matured in culture to HSCs for retrospective identification. It has previously been reported by our lab how to isolate and mature pre-HSCs from the AGM region (Rybtsov et al. 2011; Taoudi et al. 2008). This process was carried out as described in figure 3.6. The AGM region of E10.5 or E11.5 embryos was dissected and the cells dissociated into a single cell suspension. Cells with a pre-HSC phenotype were then sorted based on their expression of Notch1. After sorting, cells were made into Notch1$^-$, Notch1$^+$ and control co-aggregates with supportive stromal cells (OP9 cells). Control co-aggregates contain sorted Notch1$^-$ and Notch1$^+$ cells recombined after sorting in the appropriate ratios (the ratio they were in before sorting). Co-aggregates were cultured on the gas liquid interface for five days (E11.5 pre-HSCs) or seven days (E10.5 pre-HSCs) in the presence of IMDM, serum and cytokines: Interleukin 3 (IL3); FMS Like Tyrosine Kinase 3 Ligand (Flt3L); and Stem Cell Factor (SCF). After culture the cells were: 1) transplanted to assess the presence of HSCs; 2) added to a haematopoietic progenitor assay to assess the presence of haematopoietic progenitors; and 3) antibody stained for flow cytometry analysis.
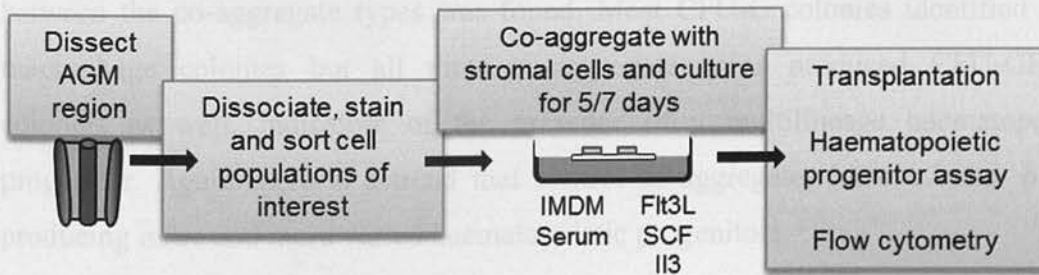

Figure 3.6: Schematic representation of the experimental design to assess pre-HSC Notch1 phenotype.
IL3: Interleukin 3; Flt3L: FMS Like Tyrosine Kinase 3 Ligand; SCF: Stem Cell Factor.

3.2.1.1 E10.5 pre-HSC Type I

According to the flow cytometry data in section 3.2.1 at E10.5 the pre-HSC Type I enriched population contains 62.4% ±17.3 Notch1$^+$ cells which express Notch1 at a higher level than other haematopoietic populations. To assess whether functional preHSC I are Notch1$^+$CD45$^-$VE-Cadherin$^+$ cells from the E10.5 AGM region were sorted based on their Notch1 expression (fig 3.7a). Approximately 1ee of sorted cells were then co-aggregated with OP9s. After seven days of culture co-aggregates were dissociated and assays carried out on the resultant cells. Figure 3.7b shows the level of repopulation of donor cells after transplantation of three co-aggregates per mouse. The results show that all functional preHSC I are contained in the Notch1$^+$ compartment. After culture co-aggregates from the Notch1$^-$ compartment contained significantly fewer lives cells than the control (p=0.0087 N1$^-$ Vs CI) (fig 3.7c) correlating with the fact that the number of input cells of Notch1$^-$ co-aggregates was less. Flow cytometry analysis of the resultant cells (fig 3.7d) shows that all co-aggregate types produce haematopoietic cells (CD45$^+$VE-Cadherin$^-$), cells with a preHSC II and dHSC phenotype (CD45$^+$VE-Cadherin$^+$) and endothelial cells (CD41$^-$ CD45$^-$VE$^-$Cadherin$^+$). Although statistical analysis of this number of samples shows no significant difference, there is a trend that control co-aggregates perform the best and produce higher proportions of all cell types analysed. Co-aggregates made from Notch1$^+$ cell produce a higher proportion of haematopoietic cells (CD45$^+$VE-Cadherin$^-$) than Notch1$^-$ co-aggregates (p=0.0087) but there is no significant difference in the proportion of preHSC II and dHSC enriched cells (CD45$^+$VE-Cadherin$^+$) produced. After culture all populations produced CFU-Cs in the haematopoietic progenitor assay (fig 3.7 e and f) and again no significant difference between the co-aggregate types was found. Most CFU-C colonies identified were macrophage colonies but all three co-aggregate types produced CFU-GEMM colonies as well, indicators of the presence of a multilineage haematopoietic progenitor. Again there is a trend that control co-aggregates have a better output producing more and more varied haematopoietic progenitors.
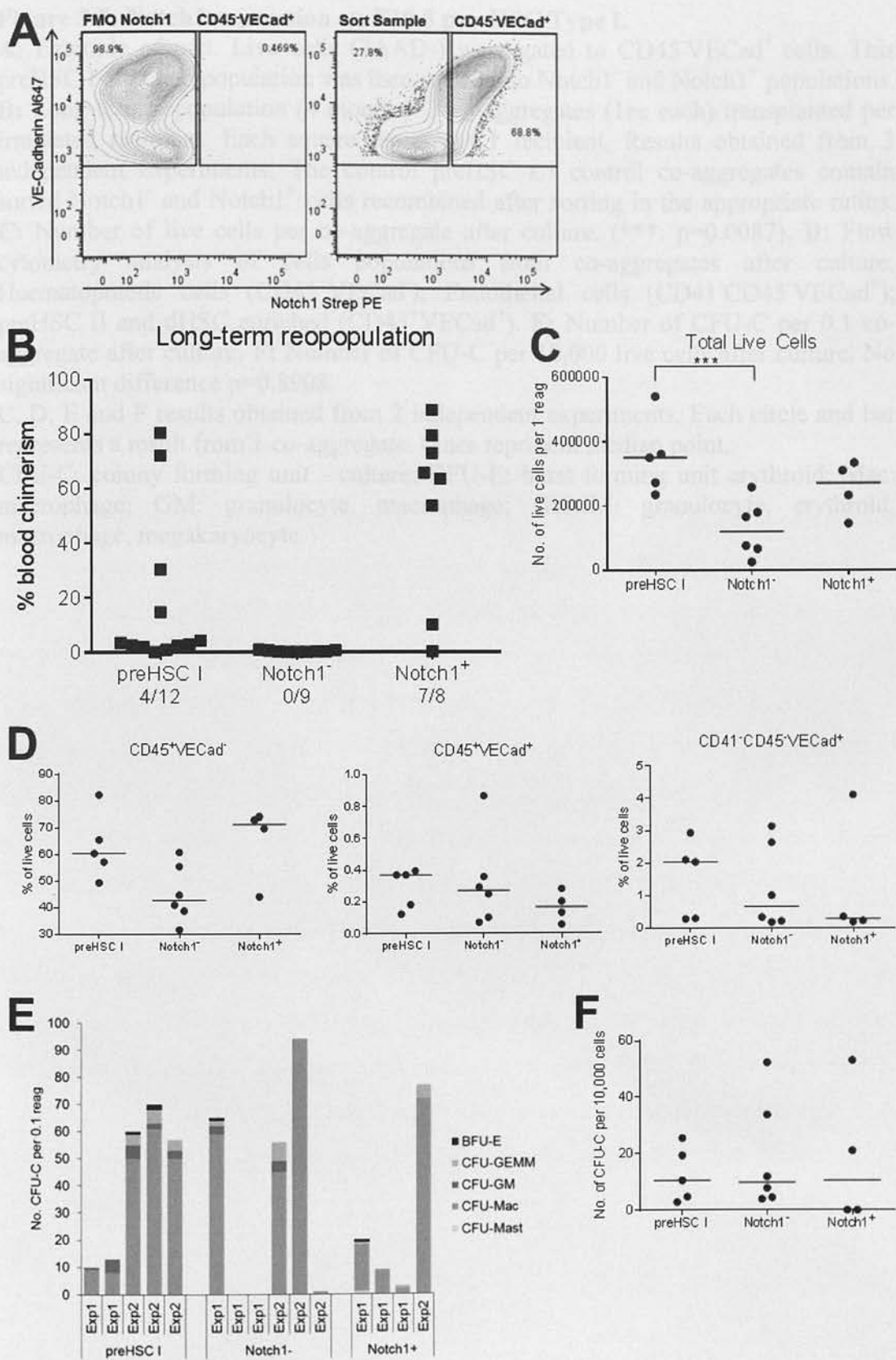
A

FMO Notch1 CD45⁻VECad⁺ Sort Sample CD45⁻VECad⁺

98.9%   0.469%   27.8%   68.8%

VE-Cadherin Af647

Notch1 Strep PE

B

Long-term reopopulation

% blood chimerism

preHSC I
4/12

Notch1⁻
0/9

Notch1⁺
7/8

C

Total Live Cells

***

No. of live cells per 1 reag

preHSC I   Notch1⁻   Notch1⁺

D

CD45⁺VECad⁻

% of live cells

preHSC I   Notch1⁻   Notch1⁺

CD45⁺VECad⁺

% of live cells

preHSC I   Notch1⁻   Notch1⁺

CD41⁻CD45⁻VECad⁺

% of live cells

preHSC I   Notch1⁻   Notch1⁺

E

No. CFU-C per 0.1 reag

BFU-E
CFU-GEMM
CFU-GM
CFU-Mac
CFU-Mast

Exp1 Exp1 Exp2 Exp2 Exp2   Exp1 Exp1 Exp1 Exp2 Exp2 Exp2   Exp1 Exp1 Exp1 Exp2

preHSC I   Notch1-   Notch1+

F

No. of CFU-C per 10,000 cells

preHSC I   Notch1⁻   Notch1⁺

74

Figure 3.7: Notch1 expression on E10.5 pre-HSC Type I.
A: Example of sort. Live cells (7AAD-) were gated to CD45⁻VECad⁺ cells. This preHSC I enriched population was then sorted into Notch1⁻ and Notch1⁺ populations. **B:** Long-term repopulation (4 months). 3 co-aggregates (1ee each) transplanted per irradiated recipient. Each square represents 1 recipient. Results obtained from 3 independent experiments. The control preHSC CI control co-aggregates contain sorted Notch1⁻ and Notch1⁺ cells recombined after sorting in the appropriate ratios. **C:** Number of live cells per co-aggregate after culture. (***: p=0.0087). **D:** Flow cytometry analysis of cells populations from co-aggregates after culture. Haematopoietic cells (CD45⁺VECad⁻); Endothelial cells (CD41⁻CD45⁻VECad⁺); preHSC II and dHSC enriched (CD45⁺VECad⁺). **E:** Number of CFU-C per 0.1 co-aggregate after culture. **F:** Number of CFU-C per 10,000 live cells after culture. No significant difference p=0.8908.

C, D, E and F results obtained from 2 independent experiments. Each circle and bar represents a result from 1 co-aggregate. Lines represent median point.

CFU-C: colony forming unit - culture; BFU-E: burst forming unit erythroid; Mac: macrophage; GM: granulocyte, macrophage; GEMM: granulocyte, erythroid, macrophage, megakaryocyte.

3.2.1.2 E11.5 pre-HSC Type I

The same strategy that was described in the previous section was used to determine if pre-HSC Type I and the more mature pre-HSC Type II population express Notch1 at E11.5. Flow cytometry data in section 3.2.2 shows that at E11.5 the preHSC CI enriched population contains 70.4% ±19.6 Notch1$^+$ cells which express Notch1 at a higher level than other haematopoietic populations, similar to the % of Notch1$^+$ preHSC I seen at E10.5. To assess whether functional preHSC CI are Notch1$^+$, CD41$^+$CD45$^-$VE-Cadherin$^+$ cells from the E11.5 AGM region were sorted based on Notch1 expression (fig 3.8a). Co-aggregates were made from sorted cells with 1ee per co-aggregate. After five days of culture co-aggregates were dissociated and assays carried out on the resultant cells. Figure 3.8b shows the level of repopulation of donor cells after transplantation with 1 co-aggregate. The repopulation data shows that in contrast to our results at E10.5 there is no significant difference between the Notch1$^-$ and Notch1$^+$ preHSC I populations in their ability to produce HSCs (p=0.0841). After culture, variable numbers of cells are produced (p=0.827) (fig 3.8c) though Notch1$^+$ co-aggregates produce a significantly higher proportion of haematopoietic cells (CD45$^+$VE-Cadherin$^-$) and preHSC II and dHSC enriched (CD45$^+$VE-Cadherin$^+$) cells than the Notch1$^-$ co-aggregates (N1$^-$ Vs N1$^+$ p=0.0039 and p=0.0084 respectively) (fig 3.7d). There was no significant difference in the proportion of endothelial cells produced (fig 3.7d). Cells from Notch1$^+$ preHSC I co-aggregates also produce more CFU-Cs than Notch1$^-$ co-aggregates (fig 3.7 e and f) but all co-aggregate types produce a variety of CFU-C colony types. At this stage (E11.5) the control pre-HSC Type I co-aggregates do not seem to have a better output than the Notch1$^+$ co-aggregates as was observed at E10.5. In addition Notch1$^+$ pre-HSC Type I retain their ability to produce a better haematopoietic output than Notch1$^-$ preHSCs but contrary to E10.5 the Notch1$^-$ population also contains pre-HSC Type I.
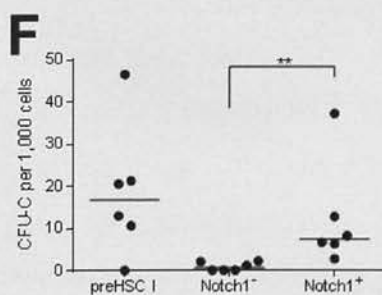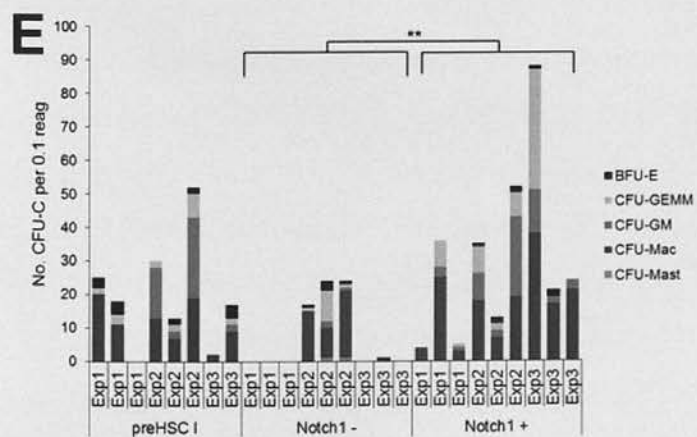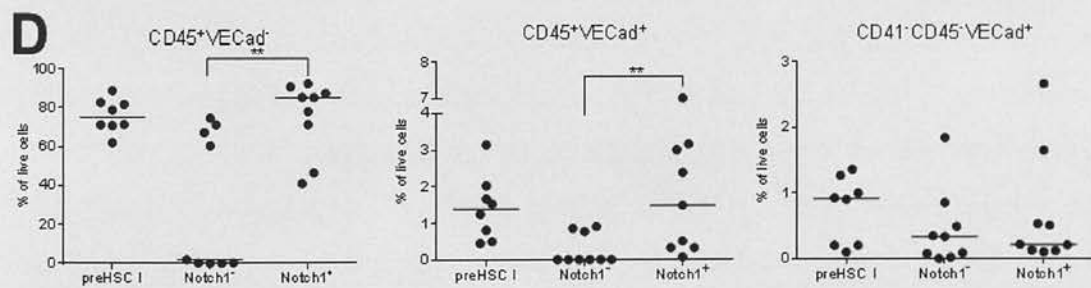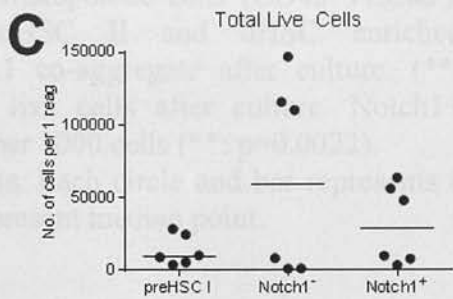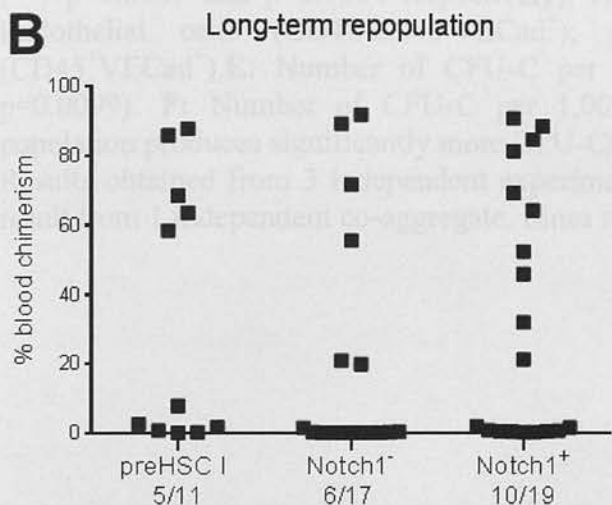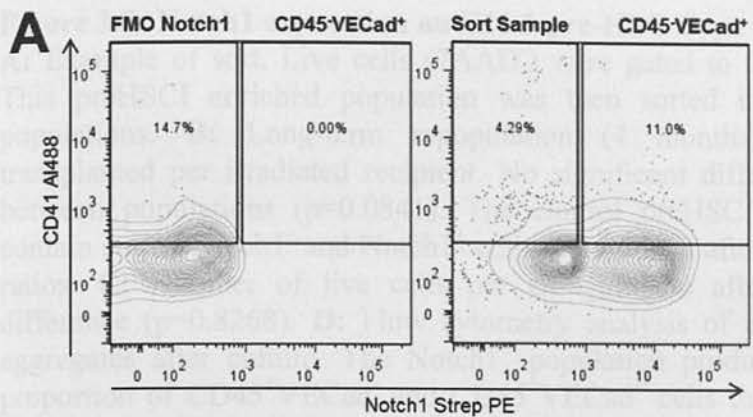
Figure 3.8: Notch1 expression on E11.5 pre-HSC Type I.
A: Example of sort. Live cells (7AAD$^-$) were gated to CD41$^+$CD45$^-$VECad$^+$ cells. This preHSCI enriched population was then sorted into Notch1$^-$ and Notch1$^+$ populations. **B:** Long-term repopulation (4 months). 1 co-aggregate (1ee) transplanted per irradiated recipient. No significant difference in HSC production between populations (p=0.0841). The control preHSC CI control co-aggregates contain sorted Notch1$^-$ and Notch1$^+$ cells recombined after sorting in the appropriate ratios. **C:** Number of live cells per co-aggregate after culture. No significant difference (p=0.8268). **D:** Flow cytometry analysis of cells populations from co-aggregates after culture. The Notch1$^+$ population produces a significantly higher proportion of CD45$^+$VECad$^-$ and CD45$^+$VECad$^+$ cells than the Notch1$^-$ population (**: p=0.0039 and p=0.0084 respectively). Haematopoietic cells (CD45$^+$VECad$^-$); Endothelial cells (CD41$^-$CD45$^-$VECad$^+$); preHSC II and dHSC enriched (CD45$^+$VECad$^+$).**E:** Number of CFU-C per 0.1 co-aggregate after culture. (**: p=0.0099). **F:** Number of CFU-C per 1,000 live cells after culture. Notch1+ population produces significantly more CFU-Cs per 1000 cells (**: p=0.0022). Results obtained from 3 independent experiments. Each circle and bar represents a result from 1 independent co-aggregate. Lines represent median point.

3.2.1.3 E11.5 pre-HSC Type II

In our preliminary experiments we used streptavidin-APC as a secondary to recognise Notch1-biotinylated antibody. Staining's using this strategy showed show that phenotypical preHSC II reside in both Notch1$^-$ and Notch1$^+$ compartments (fig 3.9a). In this setting we noticed that the shift obtained for Notch1$^+$ cells is not as important (only 35% of preHSC II cells are Notch1$^+$) as the one observed when we used the streptavidin PE (more than 70% of the cells are Notch1$^+$) (fig 3.2). To assess whether functional preHSC II are Notch1$^+$, CD45$^+$VE-Cadherin$^+$ cells from the E11.5 AGM region were sorted based on Notch1 expression. Co-aggregates were made from sorted cells with approximately 1ee per co-aggregate. The results of transplantations showed that pre-HSC type II are contained in both Notch1$^-$ and Notch1$^+$ populations (fig 3.9b).

To confirm this result we performed the same experiment but using the Streptavidin PE to reveal Notch1 expression (fig 3.9c). Notch1 high expressing cells were separated from the Notch1 low/- cells. Figure 3.9d shows the level of repopulation of donor cells after transplantation with one co-aggregate. Results show that functional E11.5 preHSC II are contained in both the Notch1$^{-/lo}$ and Notch1^{hi} populations. Notch1^{hi} population produces a more varied range of HSCs and as it is the smaller population seems to be more enriched fort preHSC II than the Notch1$^{-/lo}$ population. After culture, control co-aggregates produce significantly more cells that the Notch1$^{-/lo}$ or Notch1^{hi} co-aggregates (p=0.022 preHSC II Vs N1$^{-/lo}$), although there is no significant difference in the number of cells produced between Notch1$^{-/lo}$ and Notch1$^+$ co-aggregates (p=0.8983) (fig 3.9e). Notch1^{hi} preHSC II co-aggregates produce a significantly higher proportion of haematopoietic cells and preHSC II and dHSC enriched (CD45$^+$VE-Cadherin$^+$) cells than Notch1$^{-/lo}$ co-aggregates (N1$^-$ Vs N1$^+$ p=0.0012 and p=0.0312 respectively) but there was no significant difference in the proportion of endothelial cells produce (fig 3.9f). Control co-aggregates produced the highest number of CFU-Cs (fig 3.9g); however, the proportion of cells which produce CFU-Cs from each population is not significantly different (fig 3.9h). In conclusion, the Notch1^{hi} population has the best haematopoietic output after culture though HSCs can be derived from Notch1$^{-/lo}$ cells.
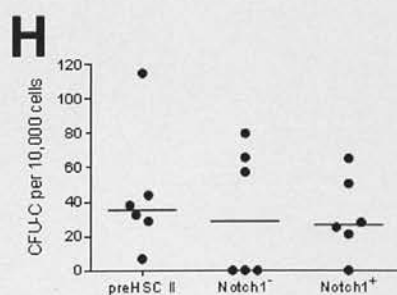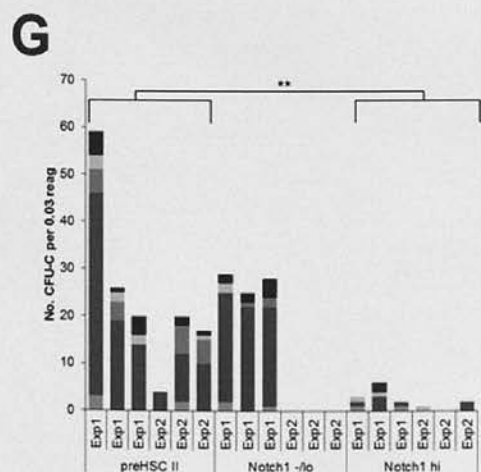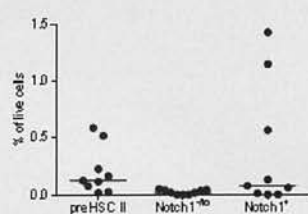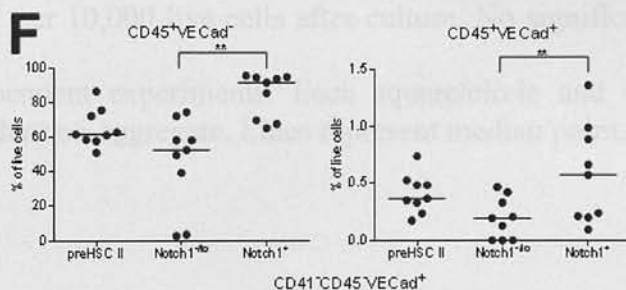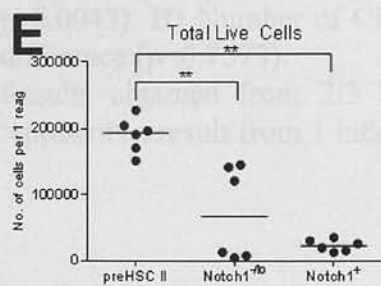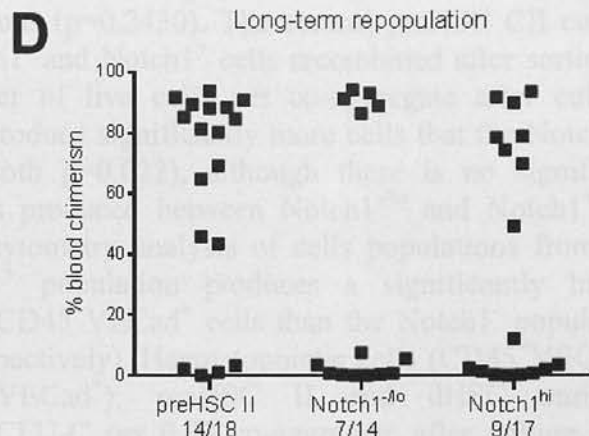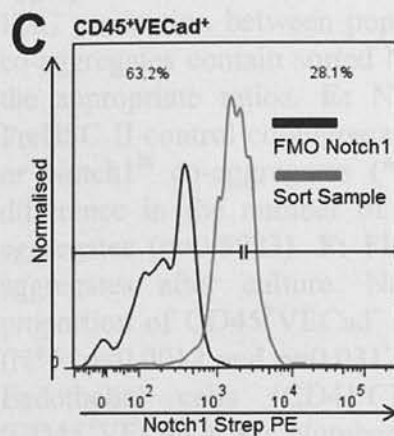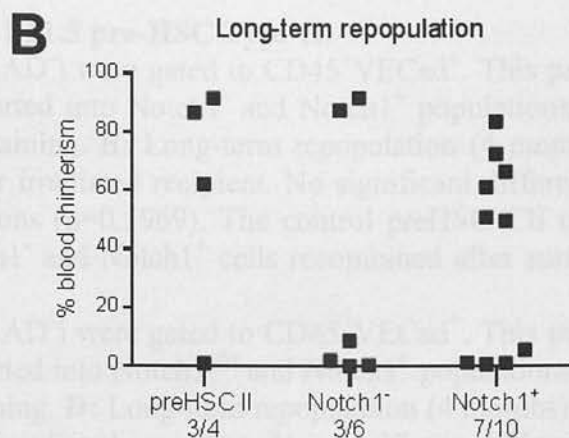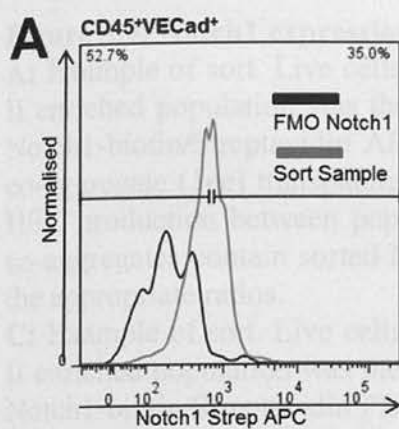
Figure 3.9: Notch1 expression on E11.5 pre-HSC Type II.
A: Example of sort. Live cells (7AAD⁻) were gated to CD45⁺VECad⁺. This preHSC II enriched population was then sorted into Notch1⁻ and Notch1⁺ populations using Notch1-biotin/Streptavidin APC staining. **B:** Long-term repopulation (4 months). 1 co-aggregate (1ee) transplanted per irradiated recipient. No significant difference in HSC production between populations (p=0.5969). The control preHSC CII control co-aggregates contain sorted Notch1⁻ and Notch1⁺ cells recombined after sorting in the appropriate ratios.
C: Example of sort. Live cells (7AAD⁻) were gated to CD45⁺VECad⁺. This preHSC II enriched population was then sorted into Notch1⁻/lo and Notch1⁺ populations using Notch1-biotin/Streptavidin PE staining. **D:** Long-term repopulation (4 months). 1 co-aggregate (1ee) transplanted per irradiated recipient. No significant difference in HSC production between populations (p=0.2430). The control preHSC CII control co-aggregates contain sorted Notch1⁻ and Notch1⁺ cells recombined after sorting in the appropriate ratios. **E:** Number of live cells per co-aggregate after culture. PreHSC II control co-aggregates produce significantly more cells that the Notch1⁻/lo or Notch1ʰⁱ co-aggregates (**: both p=0.022), although there is no significant difference in the number of cells produced between Notch1⁻/lo and Notch1⁺ co-aggregates (p=0.8983). **F:** Flow cytometry analysis of cells populations from co-aggregates after culture. Notch1⁺ population produces a significantly higher proportion of CD45⁺VECad⁻ and CD45⁺VECad⁺ cells than the Notch1⁻ population (N**: p=0.0012 and p=0.0312 respectively). Haematopoietic cells (CD45⁺VECad⁻); Endothelial cells (CD41⁻CD45⁻VECad⁺); preHSC II and dHSC enriched (CD45⁺VECad⁺). **G:** Number of CFU-C per 0.03 co-aggregate after culture. (**: p=0.0043). **H:** Number of CFU-C per 10,000 live cells after culture. No significant difference (p=0.7573).
Results obtained from 2/3 independent experiments. Each square/circle and bar represents a result from 1 independent co-aggregate. Lines represent median point.

3.2.2 E11.5 dHSCs, haematopoietic and endothelial progenitors

I next turned to the question of whether mature HSCs, haematopoietic and endothelial progenitors are Notch1$^+$ in the E11.5 AGM region. To determine this, live cells from E11.5 AGM region were sorted based on Notch1 expression and directly assayed by transplantation into irradiated adult recipients, addition to the CFU-C assay or by addition to the endothelial progenitor assay (fig 3.10 a and b). We first confirmed that Notch1 antibody staining did not block the ability of HSCs to repopulate bone marrow by staining E11.5 AGM region cells with Notch1 and directly transplanting them. There was no difference between the Notch1 stained and unstained cells in their ability to repopulate irradiated adult recipients (p=0.2500) (data not shown).

After sorting based on Notch1 expression and transplantation, results show that all HSCs at E11.5 express Notch1 and that there is no significant difference in terms of HSC numbers between the Notch1^{lo} and Notch1^{hi} populations (p=0.6294) (fig 3.10c). As Notch1^{hi} cells represent only 4.6% of the total live cell number and Notch1^{lo} cells 52%, our results suggest that HSCs are enriched in the Notch^{hi} population.

Further investigation of the functional properties of Notch1$^+$ cells in E11.5 AGM region sorted cells were directly assayed for the presence of haematopoietic and endothelial progenitors. The Notch1^{lo} population contained the highest number of CFU-Cs (fig 3.10d) but the proportion of CFU-Cs from Notch1$^-$ cells was highest (fig 3.10e). The highest number and highest proportion of endothelial progenitors were produced by Notch1^{hi} cells (fig 3.10 f and g).

A

Dissect AGM region → Dissociate, stain and sort cell populations of interest →

Transplantation

Haematopoietic progenitor assay

Endothelial Progenitor assay

B

FMO Notch1

Sort Sample

7AAD

96.5% 2.16% 0.031%

37.3% 52.3% 4.55%

Strep PE alone

Notch1 Strep PE

C

Long-term repopulation

% blood chimerism

100
80
60
40
20
0

Notch1⁻ Notch1ˡᵒ Notch1ʰⁱ
0/8   5/8   3/8

D

No. CFU-C per 3ee

70
60
50
40
30
20
10
0

Notch1⁻ Notch1ˡᵒ Notch1ʰⁱ

■ BFU-E
■ CFU-GEMM
■ CFU-GM
■ CFU-Mac
■ CFU-Mast

E

No. CFU-C per 10,000 cells

12
10
8
6
4
2
0

Notch1 - Notch1 lo Notch1 hi

F

No. Endothelial Colonies per 0.7ee

400
350
300
250
200
150
100
50
0

Notch1⁻ Notch1ˡᵒ Notch1ʰⁱ

■ Net
■ 4-6
■ 1-3

G

No. Endothelial Colonies per 10,000 cells

400
350
300
250
200
150
100
50
0

Notch1⁻ Notch1ˡᵒ Notch1ʰⁱ

Figure 3.10: Notch1 expression on E11.5 dHSCs, haematopoietic progenitors and endothelial progenitors.
A: Schematic representation of the experimental design to assess Notch1 phenotype.
B: Example of sort. Live cells (7AAD⁻) from E11.5 AGM region were sorted into Notch1⁻, Notch1^{lo} and Notch1^{hi} populations. **C:** Long-term repopulation (4 months) of transplantation of sorted cells. 5ee transplanted per irradiated recipient. All dHSCs are Notch1⁺, no significant difference between N1^{lo} and N1⁺ populations (p=0.6294). Each square represents 1 recipient. Results obtained from 3 independent experiments. **D:** Number of CFU-C per 3ee **E:** Number of CFU-C per 10,000 live cells. Results obtained from 3 independent experiments. Mean±SD. **F:** Number of endothelial colonies per 0.7ee **G:** Number of endothelial colonies per 10,000 cells. Representative example from 2 independent experiments. Mean±SD

Net: network (>6 main tubules) of endothelial tubules; 4-6: network of 4-6 main endothelial tubules; 1-3: single or network of 2/3 main endothelial tubules.

3.2.3 Multilineage analysis of repopulated mice

Multilineage analysis of repopulated mice was carried out to determine if the HSCs produced by the different cell types analysed were able to contribute to the myeloid and lymphoid populations and to determine if a certain cell type produced a HSC with a biased output. The multilineage staining strategy is shown in figure 3.11 and a summary of the results obtained are shown in table 3.2. Though there is variability in the proportions of myeloid and lymphoid repopulation in all recipients, no obvious trends were detected.
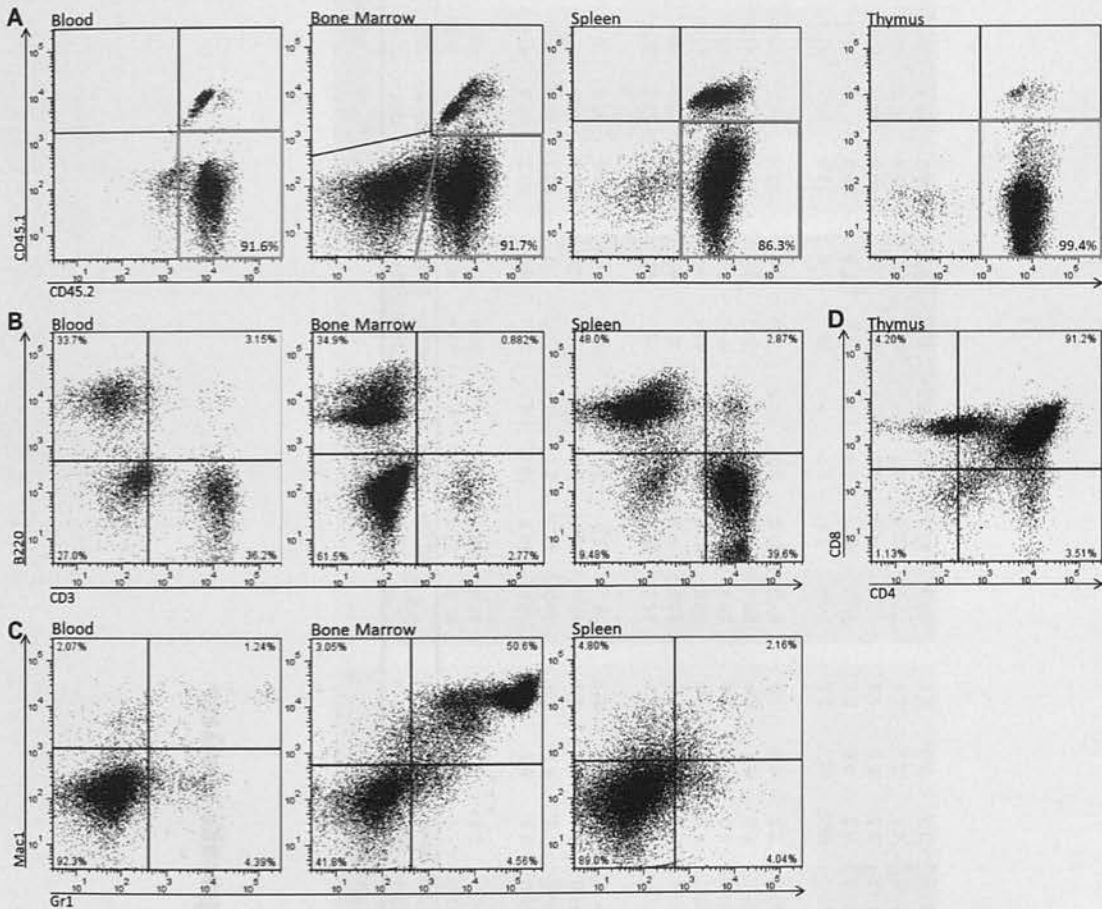
Figure 3.11: Example of flow cytometry gating for multilineage analysis.
4 adult tissues blood, bone marrow, spleen and thymus are assessed for the presence of multilineage donor derived cells. **A:** In each tissue donor derived (CD45.1⁻ CD45.2⁺) cells are gated on. **B:** CD3 and B220 lymphoid populations in the donor compartment of the blood, bone marrow and spleen are assessed. **C:** Mac1 and Gr1 myeloid populations in the donor derived compartment of the blood, bone marrow and spleen are assessed. **D:** CD4 and CD8 lymphoid populations of the thymus.

Table 3.2: Summary of multilineage analysis.

| Transplanted | | Blood | | | | | | Bone Marrow | | | | | | Spleen | | | | | | Thymus | | | | |
AGM Stage	Cell Type	CD45-2	CD3-B220+	CD3+B220-	Gr1-Mac1+	Gr1+Mac1+	Gr1+Mac1-	CD45-2	CD3-B220+	CD3+B220-	Gr1-Mac1+	Gr1+Mac1+	Gr1+Mac1-	CD45-2	CD3-B220+	CD3+B220-	Gr1-Mac1+	Gr1+Mac1+	Gr1+Mac1-	CD45-2	CD4-CD8-	CD4+CD8+	CD4+CD8-	CD4-CD8+
E11.5	Notch1^{lo}	67.1	47.4	32.3	1.4	0.5	2.5	63.1	57.6	3.8	5.1	31.4	2.5	84.7	62.5	28.9	3.9	1.3	2.9	97.4	7.5	86.8		2.0
	Notch1^{hi}	91.6	33.4	35.7	2.1	1.2	4.4	91.7	34.9	2.8	4.6	50.6	3.1	86.3	48.0	40.1	4.8	2.2	4.0	99.4	4.2	91.2		3.5
E10.5	Class I	27.5	32.6	50.8	8.9	9.3	10.9	12.8	44.7	18.9	9.4	17.1	6.7	52.2	37.4	47.4	6.4	1.6	5.9	91.3	3.4	92.7		3.2
	Notch1$^+$	91.4	62.9	9.6	7.3	9.2	2.0	96.3	41.1	2.1	3.8	44.9	5.7	92.1	56.0	2.8	12.0	6.4	8.8	98.8	4.2	82.9		3.3
	Notch1$^+$	53.2	55.9	33.8	2.6	4.1	4.1	16.3	60.0	7.3	5.1	12.0	12.3	63.6	34.3	37.6	5.0	2.4	6.6	97.0	4.1	90.1		3.6
	Notch1$^+$	43.0	10.0	31.5	7.9	6.7	10.7	38.2	5.7	2.3	4.1	81.2	4.7	19.9	22.3	56.9	8.8	2.1	9.4	47.6	3.0	90.4		4.4
E11.5	Class I	92.0	62.3	9.6	4.6	6.4	2.3	94.1	28.5	2.6	5.7	49.4	2.9	87.3	57.2	27.6	7.3	2.3	2.9	99.3	6.1	85.7		6.1
	Class I	60.9	35.7	43.4	3.2	3.2	2.1	58.9	37.9	4.6	4.9	25.7	4.3	68.4	53.0	29.6	5.5	2.1	5.9	98.5	2.0	88.8		7.7
	Notch1$^+$	87.0	47.3	23.0	4.6	6.4	1.8	52.6	22.2	2.7	4.9	41.4	5.2	85.3	62.7	17.0	5.0	2.1	4.0	97.3	3.2	92.4		2.7
	Notch1$^+$	85.3	43.4	26.2	3.0	1.4	2.4	56.7	28.5	5.7	3.8	48.2	5.8	84.1	36.4	49.4	6.8	1.9	2.8	91.4	4.7	87.7		4.9
	Notch1$^+$	56.7	32.7	57.8	0.9	0.4	4.4	28.1	51.2	10.4	5.6	23.2	3.5	74.1	45.8	44.3	4.4	1.3	3.9	71.8	3.4	90.0		3.8
	Notch1$^+$	40.2	26.9	47.5	3.5	4.9	1.6	66.3	21.6	3.3	3.6	38.0	5.1	27.4	44.1	18.6	8.8	3.8	6.8	97.8	4.6	88.7		5.3
E11.5	Class II	34.2	23.3	10.7	15.5	26.7	3.2	74.9	3.2	0.7	3.6	86.8	2.6	19.0	38.8	34.0	11.0	3.6	3.4	97.9	6.4	39.5		51.3
	Notch1^{lo}	73.5	44.2	33.3	3.3	0.6	2.7	72.3	31.1	3.9	3.1	40.6	5.9	84.0	57.0	31.6	10.2	1.5	3.5	88.0	8.4	86.6		2.3
	Notch1$^+$	82.7	59.6	16.7	3.5	5.1	1.9	90.4	29.0	2.2	3.9	54.6	2.4	80.5	48.0	40.2	7.9	1.3	2.1	99.4	2.9	90.4		5.1
	Notch1$^+$	79.2	27.9	47.5	4.7	2.2	5.8	77.5	20.5	4.7	5.5	57.7	6.2	85.4	27.7	58.5	8.3	1.2	4.0	97.6	4.1	79.7		12.5
	Notch1$^+$	67.0	42.0	41.4	3.3	3.3	2.1	36.3	47.3	5.7	6.2	17.3	3.8	58.5	48.1	15.3	8.9	4.6	8.3	98.4	3.2	82.0		3.3

3.3 Discussion

To supplement work in our lab, that is further investigating the role of Notch1 in haematopoietic development, a detailed assessment of Notch1 expression in the E11.5 and E10.5 AGM region was carried out. Flow cytometry analysis was performed in order to determine more precisely by which cell types, and at what level, Notch1 protein is expressed in the developing haematopoietic system. This analysis showed that in all the cell populations analysed Notch1 is expressed to varying degrees. The VE-Cadherin$^+$ endothelial (CD41$^-$CD45$^-$VECad$^+$) and phenotypically defined pre-HSC Type I (CD41$^+$CD45$^-$VECad$^+$) populations expressed the highest levels of Notch1 which increased between E8.5 and E11.5. This is unsurprising as Notch signalling is known to be involved in angiogenesis, vascular development and arterial specification (reviewed:(Roca & Adams 2007; Gridley 2007)) and HSCs arise in close association with the endothelium of the dorsal aorta (reviewed: (Medvinsky et al. 2011; Koch et al. 2013)). The more mature cells of the dHSC, pre-HSC CII and CD45$^+$VE-Cad$^-$ cells, hierarchy expressed Notch1 at lower levels.

Next functional analysis of Notch1 positive and negative cells from the E10.5 and E11.5 AGM regions was carried out. This showed that at E10.5 all pre-HSC Type I are Notch1$^+$ and at E11.5 some become Notch1$^-$. Pre-HSC Type II are Notch1$^{lo/+}$, as are HSCs, though Notch1^{hi} cells are enriched for HSCs. CFU-Cs are Notch1$^{-/lo}$ and the majority of endothelial progenitors are Notch1^{hi}. These results all suggest that Notch1 expression is highest on early haematopoietic progenitors newly derived from haematogenic endothelium and Notch1 expression is maintained throughout HSC development. At E11.5, after culture co-aggregates from preHSC I Notch1$^+$ and Notch1^{hi} had a significantly better output of haematopoietic cells than their Notch1$^{-/lo}$ counterparts, indicating Notch1$^{+/hi}$ cells are better primed towards a haematopoietic output.

HSC production from Notch1$^-$ pre-HSCs suggests that cells may up regulate Notch1 during culture or that there is a Notch1 independent pathway during certain stages of HSC development, i.e. after pre-HSC specification, which can be supported in culture. Whether this Notch1 independent HSC development exists and occurs *in*

vivo is an open question. In some experiments it was observed that control co-aggregates outperformed their Notch1⁻ and Notch1⁺ counterparts. This suggests that interactions between Notch1⁺ and Notch1⁻ cells may be important in HSC development. As endothelial cells are almost exclusively Notch1⁺ this could indicate an important role for other Notch1⁻ supportive cell types such as stromal cells and mesenchymal cells in HSC development.

However, a serious limitation to these experiments was observed. At E11.5 CD45⁺VE-Cadherin⁺ cells after culture should give rise to large numbers of HSCs (approximately 150) (Taoudi et al. 2008) but it has been observed by others in our lab that cell sorting leads to an extenuation of this maturation by a factor of up to 10. Taken together this suggests that 1 co-aggregate with 1ee pre-HSC type II should produce approximately 15 HSCs. The suboptimal results obtained in these experiments, HSCs were not produced in all co-aggregates, suggests that Notch1 staining may be partially blocking pre-HSC maturation and/or proliferation. The Notch1 antibody used was shown not to block Dll4 binding but further investigation into the possible blocking of other ligand binding or an ability to inhibit Notch1 activation was not carried out (Fiorini et al. 2009). Experiments to determine if partial blocking of Notch1 activation by this antibody is indeed taking place are required. Incubation of cells with and without Notch1 before co-aggregate culture or addition of the antibody to CFU-C and endothelial assays must be carried out. This strategy was used to determine if anti-Notch1 antibody directly blocked HSC homing and engraftment by staining cells with Notch1 before transplantation (data no shown). No obvious impairment was observed, correlating with reports that Notch1 is not required by adult HSCs in the bone marrow (Radtke et al. 1999).

The possibility that anti-Notch1 antibody is interfering with the HSC lineage suggests that if Notch1⁺ cells were not impaired after sorting, vastly higher levels of repopulation would be seen by Notch1⁺ pre-HSCs and control co-aggregates. In this case Notch1⁺ E11.5 pre-HSC Type I would be significantly more potent than the same negative population suggesting that even at E11.5 pre-HSC Type I are Notch1⁺. In addition if the effects of anti-Notch1 antibody were removed, a significant

difference between the Notch1$^{-/lo}$ and Notch^{hi} pre-HSC Type II populations may be observed, allowing a more defined phenotype to be discerned.

Although interpretation of the functional analysis carried out may be obscured by the possible blocking actions of the antibody used, it is still clear from phenotypical analysis that Notch1 is expressed at high levels on endothelial cells and by early pre-HSC Type I HSC precursors followed by lower levels of Notch1 on more mature pre-HSC Type II and HSC. Functional HSCs are shown to be Notch1^{+} suggesting Notch1 expression is maintained throughout HSC development.

4 Jagged1 Expression during Haematopoietic Development

In order to investigate Jagged1 expression during haematopoietic development a Jagged1$^{wt/tomato}$ knock-in mouse line was produced in collaboration with Bill Skarne's lab at the Sanger Institute. Using this line we were able to analyse Jagged1 expression in the developing haematopoietic system and further investigate the Jagged1$^+$Notch1$^+$ cells previously described (Robert-Moreno et al. 2005).

4.1 Jagged1 expression in E11.5 AGM region

It has previously been shown by *in situ* hybridization and protein staining that Jagged1 is expressed in the E10.5 AGM region by (Robert-Moreno et al. 2005; Robert-Moreno et al. 2008). The E11.5 AGM region was examined for Jagged1 protein expression (fig 4.1). Like the Notch1 staining in chapter 3, Jagged1 is expressed in the endothelial cells of the dorsal aorta. It is also expressed by the VE-Cadherin⁻ smooth muscle cells surrounding the dorsal aorta. No obvious dorso-ventral polarity of Jagged1 expression was observed. The majority if not all the CD45$^+$ cells of a cluster just under the endothelial layer of the dorsal aorta are Jagged1$^+$ (fig 4.1 upper panel). Examining the Runx1$^+$ HIAC of cells in the lower panel of figure 4.1 we can see that, again like Notch1, Jagged1 is expressed mainly in the cells at the very bottom of the cluster and in the endothelium of the dorsal aorta, not in the majority of the cells of the HIAC.
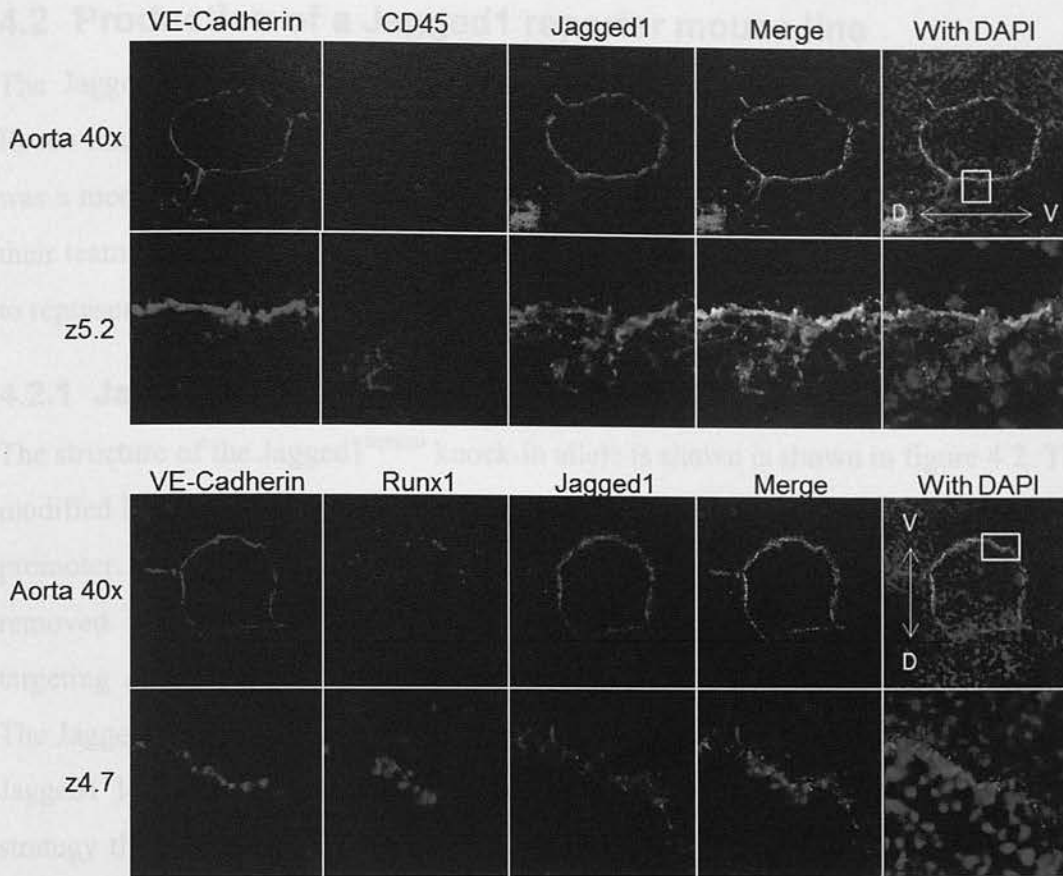
Figure 4.1 Jagged1 expression in E11.5 AGM region.
40x plus zoom. Transverse sections of AGM region of E11.5 embryos stained for
Jagged1, VE-Cadherin and CD45 (upper panel) or Jagged1, VE-Cadherin and Runx1
(lower panel). Nuclear staining with DAPI. V: ventral. D: dorsal. Arrow indicates
dorsal ventral orientation. White box indicates magnified area.

4.2 Production of a Jagged1 reporter mouse line

The Jagged1^{tomato} knock-in strategy and targeting vector was designed by Barry Rosen and Bill Skarnes at the Welcome Trust Sanger Institute. The protocol used was a modified small scale version of the high throughput targeting strategy used by their team (Skarnes et al. 2011). All diagrams are modified from Skarnes et. al 2011 to represent the more simplified strategy used.

4.2.1 Jagged1^{tomato} locus

The structure of the Jagged1^{tomato} knock-in allele is shown is shown in figure 4.2. The modified locus produces a dtTomato fluorescent protein under control of the Jagged1 promoter. The selection cassette allowed for selection of targeted cells and was later removed. The construction of the Jagged1 targeting vector, and the subsequent targeting of the Jagged1 locus, was carried out using homologous recombination. The Jagged1 targeting vector had 5kb 5' and 3' homology arms to the region of the Jagged1 locus the dtTomato fluorescent protein gene was to be inserted. In this strategy the Jagged1 ATG start codon and dtTomato fluorescent protein ATG start codon were interchanged so that the gene was inserted under control of the Jagged1 promoter with minimal disruption to the gene. Some disruption to the 5' UTR region occurred as the targeting vector as a remnant of the construction process remains upstream of the dtTomato start site and some of JAG1 exon 1 was deleted when dtTomato was inserted.
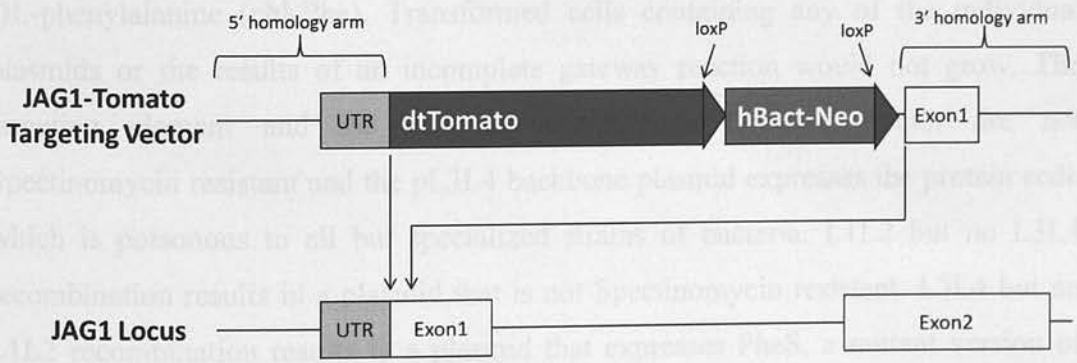
Figure 4.2: Design of Jagged1-dtTomato targeting vector.
Knock-in of dtTomato fluorescent protein gene and floxed selection cassette into the Jagged1 gene locus.
UTR: untranslated region; hBact: human β-actin promoter; Neo: neomycin resistance gene; pA: polyadenylation signal.

4.2.2 Producing the targeting vector

4.2.2.1 Final assembly of the Jagged1-dtTomato targeting vector

The Jagged1 targeting vector (fig 4.3) contains: 5kb homology arms to the Jagged1 locus; the dtTomato fluorescent protein gene (with a beta-globin intron and SV40 poly-A); a floxed neomycin resistance gene under control of the human beta-actin promoter to allow for selection of targeted cells; a Spectiomycin resistant gene to allow for vector selection in bacteria; a diphtheria toxin gene under a PGK promoter which will kill any cells in which the full linearized vector inserts in the genome instead of undergoing homologous recombination (Yagi et al. 1990); and an AsiI restriction enzyme site for linearization. These features allowed the dtTomato fluorescent protein gene to be knocked into the Jagged1 locus and selection of correctly targeted cells to be carried out at a high efficiency.

The construction of the final targeting vector was carried out using Gateway® technology (Invitrogen). Gateway technology is a modified form of homologous recombination which is site specific. The reaction is catalysed by LR clonase and L1 and R1 sites, L2 and R2 sites etc. recombine specifically with no cross-over between sites. The gateway reaction carried out to produce the Jagged1 targeting vector is shown in figure 4.5. After the gateway reaction was carried out, the reaction was stopped and was transformed into chemically competent cells. The cells were plated on Spectinomycin containing YEG agar, which contains the amino acid 4-Chloro-DL-phenylalanine (chl-Phe). Transformed cells containing any of the individual plasmids or the results of an incomplete gateway reaction would not grow. The targeting element and the Jagged1 intermediate targeting vector are not Spectinomycin resistant and the pL3L4 backbone plasmid expresses the protein ccdb which is poisonous to all but specialized strains of bacteria. L1L2 but no L3L4 recombination results in a plasmid that is not Spectinomycin resistant. L3L4 but no L1L2 recombination results in a plasmid that expresses PheS, a mutant version of phenylalanine (Phe) t-RNA synthetase which incorporates the toxic Phe analogue chl-Phe into protein during synthesis. Its toxic effect is dominant over WT PheS. WT E.Coli will grow normally on YEG-Cl plates containing chl-Phe but PheS containing cells will not grow (Kast 1994). This sophisticated selection of a modularly

assembled vector allowed a high level of recovery of clones containing the Jagged1 targeting vector. This strategy is used in high throughput system by the team at Sanger and the modular approach allows the same targeting element and backbone to be used in the production of many different gene specific vectors (Skarnes et al. 2011). Production of the targeting vector was confirmed by restriction digestion (fig 4.4) and sequencing portions of the targeting vector.
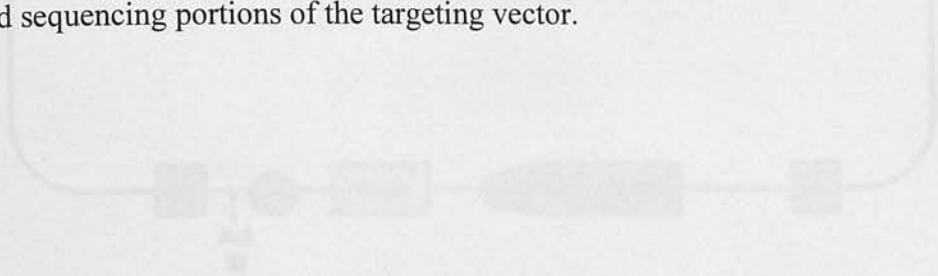
Figure 4.3: Schematic of final targeting vector.
5kb homology arms allow for recombination of dtTomato fluorescent protein gene into Jagged1 locus with neomycin resistant for positive selection.
B1-4: Gateway sites; S40pA: polyadenylation signal; hBact: Human beta-Actin promoter; Neo: Neomycin resistant gene; pA: polyadenylation signal; ori: origin of replication; AsiSI: linearization restriction enzyme site; Spec: Spectinomycin resistance gene; DTA: Diphtheria toxin gene; PGK: phosphoglycerate kinase
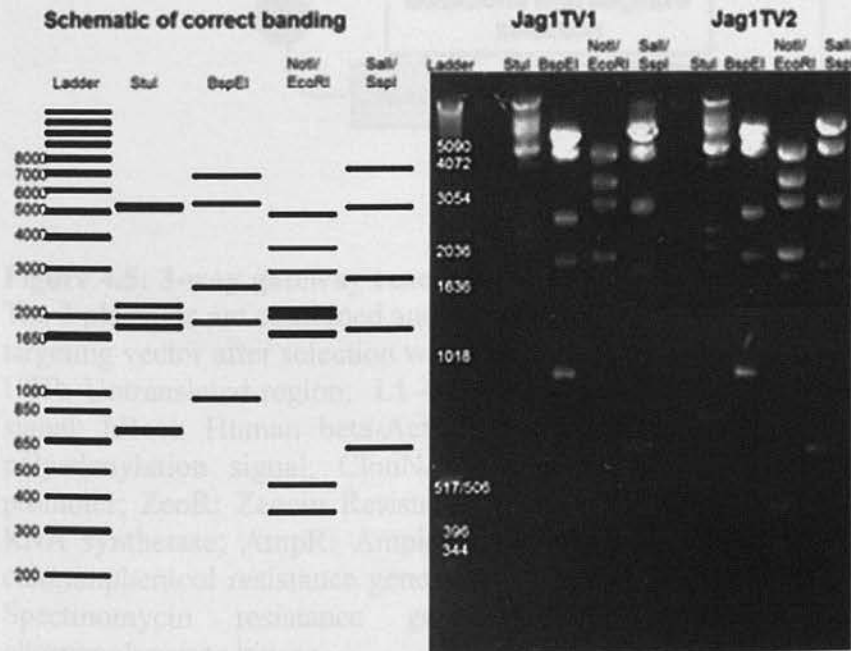
Figure 4.4: Restriction digest confirmation.
Digest of 2 clones from Jagged1 targeting vector production. Restriction digest with StuI was unsuccessful as the enzyme failed to work.
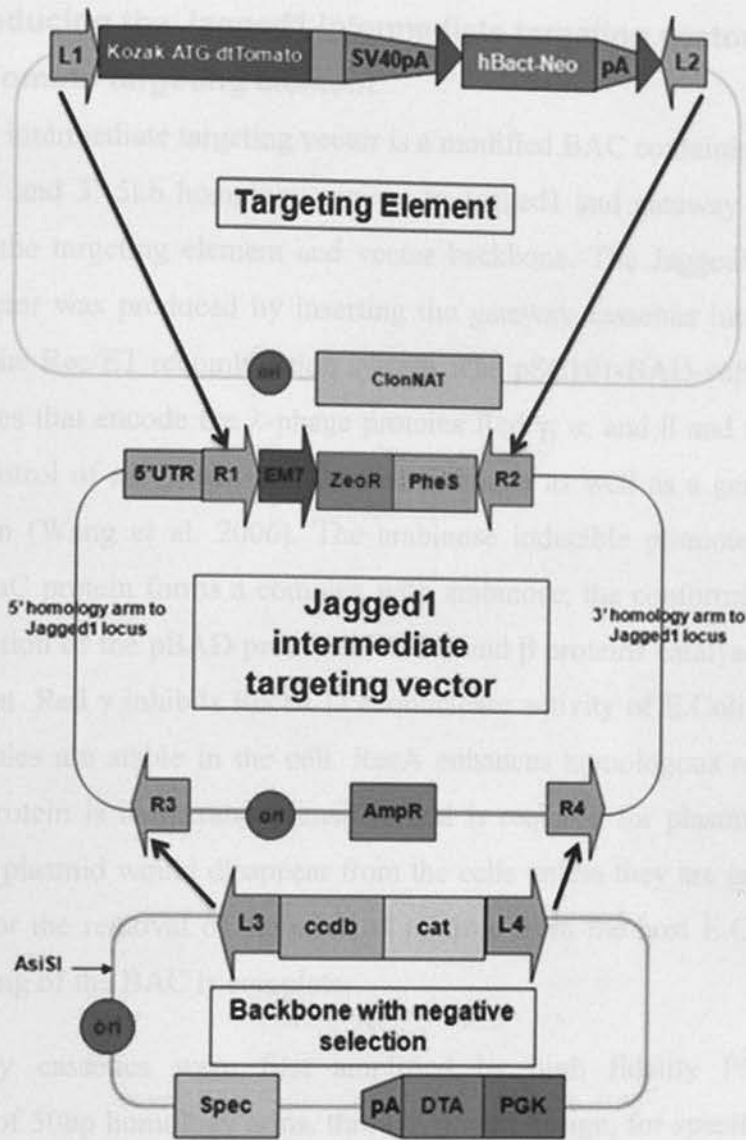
Figure 4.5: 3-way gateway reaction to produce Jagged1 targeting vector.
The 3 plasmids are combined and incubated with LR clonase to produce that Jagged1
targeting vector after selection with Spectinomycin containing YEG agar plates.
UTR: Untranslated region; L1-4 and R1-4: Gateway sites; S40pA: polyadenylation
signal; hBact: Human beta-Actin promoter; Neo: Neomycin resistant gene; pA:
polyadenylation signal; ClonNAT: ClonNAT Resistance gene; EM7: Bacterial
promoter; ZeoR: Zeocin Resistance gene; PheS: gene for mutant phenylalanine t-
RNA synthetase; AmpR: Ampicillin resistance gene; CcdB: cell division B; Cat:
chloramphenicol resistance gene; AsiSI: linearization restriction enzyme site; Spec:
Spectinomycin   resistance   gene;   DTA: Diphtheria   toxin   gene;   PGK:
phosphoglycerate kinase

96

4.2.2.2 Producing the Jagged1 intermediate targeting vector and dtTomato targeting element

The Jagged1 intermediate targeting vector is a modified BAC containing the Jagged1 gene with 5' and 3' 5kb homology regions to Jagged1 and gateway sites to allow insertion of the targeting element and vector backbone. The Jagged1 intermediate targeting vector was produced by inserting the gateway cassettes into the Jagged1 BAC using the Rec/ET recombination system. The pSC101-BAD-γαβA-tet plasmid contains genes that encode the λ-phage proteins Red γ, α, and β and the recA gene under the control of an arabinose inducible promoter as well as a gene encoding a RepA protein (Wang et al. 2006). The arabinose inducible promoter is activated when the AraC protein forms a complex with arabinose; the conformational change allows activation of the pBAD promoter. Red α and β proteins catalyse homologous recombination. Red γ inhibits RecBCD exonuclease activity of E.Coli so that linear DNA molecules are stable in the cell. RecA enhances homologous recombination. The RepA protein is temperature sensitive and is required for plasmid replication; therefore the plasmid would disappear from the cells unless they are grown at 30°C, this allows for the removal of the pSC101 plasmid from the host E.Coli cells after recombineering of the BAC is complete.
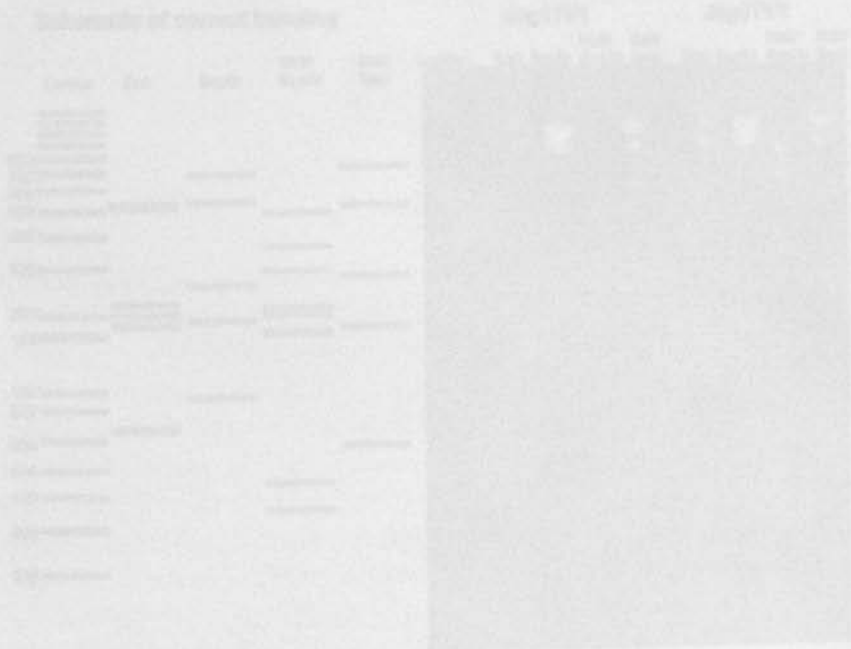
The Gateway cassettes were first amplified by high fidelity PCR with the introduction of 50bp homology arms, through primer design, for specific sites in the JAG1 BAC. The PCR reactions were treated with DpnI after amplification which destroys methylated DNA thereby removing the plasmid template DNA. The PCR product was purified and was ready for transformation. The U-fragment contains the L1 and L2 gateway sites and the G-fragment contains the L3 and L4 gateway sites.

The BAC host E.Coli were made electrocompetent and the recombineering plasmid, pSC101-BAD-γαβA-tet, was electroporated into the cells and selected with chloramphenicol and tetracycline. The cells were then grown at 30°C while the recombineering plasmid was required. Production of the recombineering proteins was induced by addition of arabinose. The host cells were made electrocompetent and the first gateway cassette, the U-fragment, was electroporated into the cells. Recombineering took place at 37°C and after the cells were again grown at 30°C and

selection with Zeocin and Tetracycline carried out. The host cells were again made electrocompetent and the second gateway cassette, the G-fragment was electroporated into the cells. Recombineering took place at 37°C and after the cells were then grown at 37°C, to remove the recombineering plasmid from the culture, and selected with Zeocin and Carbenicilin. This process is summarized in figure 4.6. The cell culture was then plated on agar containing Carbenicillin and Zeocin and colonies are examined for the presence of the Jagged1 intermediate targeting vector. The vector was isolated, analysed and prepared for the gateway reaction.

The targeting element was generated using conventional restriction-ligation cloning techniques but, once generated, can be used with the intermediate targeting vector of different genes without modification. The pENTR2b-ClonNAT plasmid was first modified by the addition of a polylinker into the NotI and BspEI restriction enzyme sites, to add the other restriction sites necessary for ligation. The dtTomato fluorescent protein gene was then inserted into sites SalI and SspI, and the floxed selection cassette into sites EcoRV and BspEI. This is shown in figure 4.7. Restriction digests confirming successful production of the Jagged1 intermediate targeting vector and the dtTomato targeting element are shown in figure 4.8.
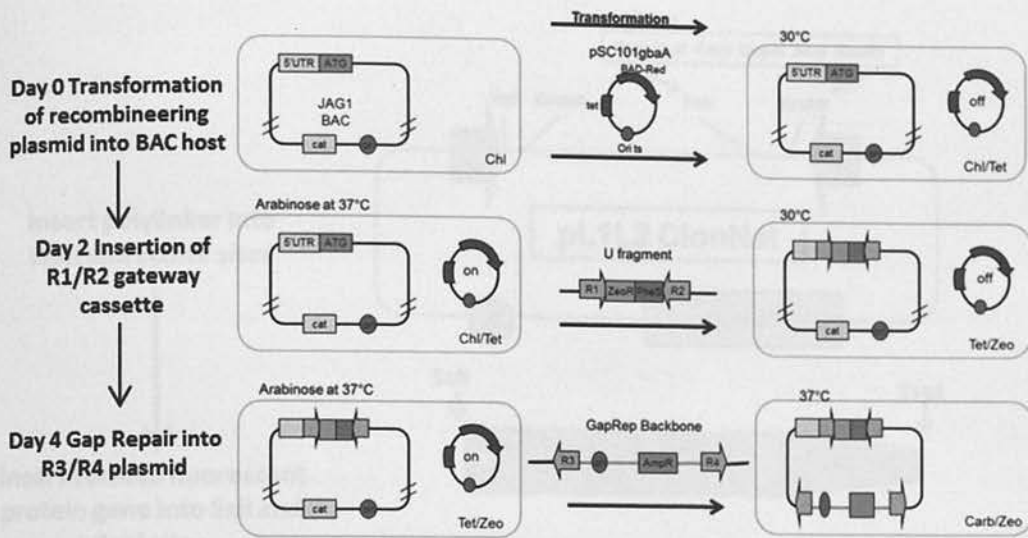
Figure 4.6: Creation of the Jagged1 intermediate targeting vector.
The recombineering plasmid is transformed into the BAC host on day 0. Cells are then maintained at 30°C. After selection and 48hrs growth the transcription of the recombineering protein genes is induced by addition of arabinose and the U fragment is transformed into the cells. After selection and 48hrs growth again the transcription of the recombineering protein genes is induced by addition of arabinose and the GapRep fragment is transformed into the cells. After selection and 24hrs growth the Jagged1 intermediate targeting vector is purified from the host cells.

UTR: Untranslated region; Cat: chloramphenicol resistance gene; R1-R4: Gateway sites; ZeoR: Zeocin Resistance gene; PheS: gene for mutant phenylalanine t-RNA synthetase; AmpR: Ampicillin resistance gene; Chl: Chloramphenicol; Zeo: Zeocin; Carb: Carbenicilin; pBAD: Arabinose inducible promoter; pSC101 ori: temperature sensitive origin of replication; Tet: Tetracycline resistance gene.
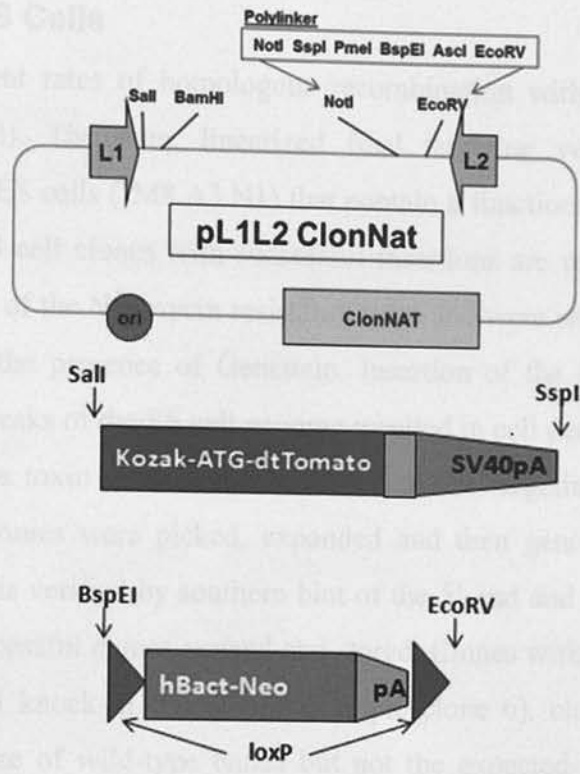
Figure 4.7: Creating plasmid with targeting element.
A polylinker containing various restriction sites was cloned into the pL1L2
ClonNAT plasmid. Then the dtTomato fluorescent protein gene with β-globin intron
and SV40 polyadenylation signal was cloned into the SalI and SspI sites followed by
a loxP flanked Neomycin resistant gene with a human β-actin promoter cloned into
the BspEI and EcoRV restriction sites.
L1-2: Gateway sites; ori: origin of replication; S40pA: polyadenylation signal;
hBact: Human beta-Actin promoter; Neo: Neomycin resistant gene; pA:
polyadenylation signal; ClonNAT: ClonNAT Resistance gene.
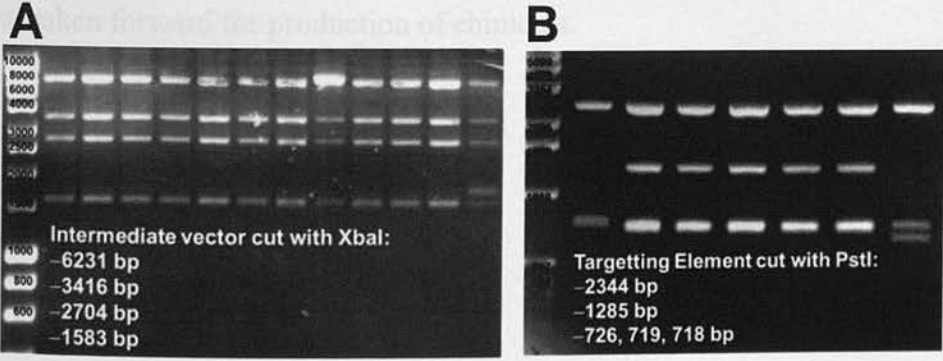
Figure 4.8: Restriction digest confirmation.
Restriction digest of clones from **A:** intermediate targeting vector production and **B:**
targeting element production.

4.2.3 Jagged1$^{wt/tomato}$ ES Cells

Mouse ES cells have efficient rates of homologous recombination with isogenic DNA (te Riele et al. 1992). The pure, linearized final targeting vector was electroporated into C57BL/6 ES cells (JM8.A3.N1) that contain a functioning agouti locus (Pettitt et al. 2009). ES cell clones with successful insertions are resistant to Geneticin due to the presence of the Neomycin resistance gene and were selected for by culturing for 10 days in the presence of Geneticin. Insertion of the linearized vector into double stranded breaks of the ES cell genome resulted in cell death due to the presence of the diphtheria toxin gene in the backbone of the targeting vector. After ten day's selection colonies were picked, expanded and then genotyped by PCR. The correct insertion was verified by southern blot of the 5' end and 3' end of the insertion (fig 4.9) and successful clones expand and stored. Clones with bands of the expected size were called knock-in clones (for example clone 6), clones with bands which were not the size of wild-type bands but not the expected size of a knock-in band were called random insertions. This could happen if the linearized vector was broken and the diphtheria toxin gene removed or damaged. Insertion of the remaining targeting vector into double stranded DNA breaks would then be possible.

It has previously been shown that Jagged1 is expressed in ES cells (Lowell et al. 2006). Flow cytometry and fluorescent microscopy of FMD11 (Jagged1$^{wt/tomato}$ knock-in ES cell line) cells showed dtTomato expression (fig 4.10). Another Jagged1$^{wt/tomato}$ knock-in line examined showed similar results and the FMD11 line was taken forward for production of chimeras.

5' end southern

EcoRI   EcoRI   ▬ Probe

WT 5.6kb ┤━━┤──[UTR | Exon1]──┤

EcoRI   EcoRI

KI 6.2kb ┤━━┤──[UTR | Tomato | Selection | Exon1]──┤

3' end southern

ApaI   ApaI   ▬ Probe

WT 6.5kb ┤─┤──[UTR | Exon1]──┤

ApaI   ApaI

KI 7.4kb ┤──[UTR | Tomato | Selection | Exon1]━━┤

5' end southern

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |

6.1kb
5kb → WT
4kb → KI
3kb → RI
2kb

5' end   **3' end**
WT 4.9kb   WT 6.5kb
KI 4.2kb   KI 7.4kb
RI ?   RI ?

WT=Wild type
KI=Knock-in
RI=Random insertion

3' end southern

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

7.1kb → RI
6.1kb → KI
5kb → WT
4kb

Figure 4.9: Southern Blot to confirm insertion of dtTomato fluorescent protein gene into Jagged1 locus.
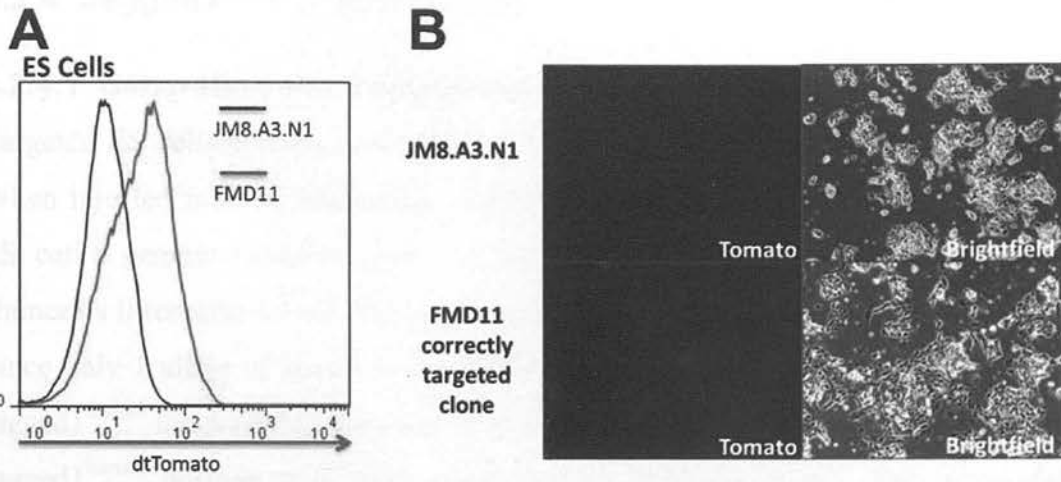
Figure 4.10: dtTomato expression in Jagged1^{wt/tomato} Knock-In ES Cells.
A: Flow cytometry of 7AAD- cells for dtTomato fluorescence. **B:** Fluorescent microscopy for dtTomato fluorescence. JM8.A3.N1: C57BL6 ES cell line. FMD11: Jagged1^{wt/tomato} Knock-in ES cell line. FMD11 representative example of Jag1^{wt/tom} 2 knock-in lines examined.

4.2.4 Jagged1$^{wt/tomato}$ mouse line

4.2.4.1 Generation and transmission

Targeted ES cells contain a dominant functioning agouti locus (Pettitt et al. 2009). When injected into the blastocysts of C57BL/6 mice germ-line transmission of the ES cell's genetic material could be tracked by the presence of agouti pups in chimera's litters (fig 4.11a). The chimeras were a chocolate brown colour (fig 4.11b). Since only 1 allele of agouti in the JM8.A3.N1 ES cells was restored the modified Jagged1^{tomato} locus could be present in any of the chimera offspring (agouti or black). Jagged1^{tomato} positive pups were genotyped by PCR (fig 4.11c). The successfully produced Jagged1$^{wt/tomato}$ mice still contained the floxed beta-actin neomycin selection cassette. Jagged1$^{wt/tomato}$ mice were crossed with germ-line expressing cre mice and removal of the selection cassette confirmed by PCR (fig 4.11d). Excision of the selection cassette did not significantly affect tomato expression (data not shown). All analysis of Jagged1$^{wt/tomato}$ mouse tissue shown in the remainder of this chapter was carried out using the Jagged1$^{wt/tomato}$ line after selection cassette removal.

The expected Mendelian inheritance for the Jagged1^{tomato} allele in Jagged1$^{wt/tomato}$ to Jagged1$^{wt/wt}$ crossings is 50%. Analysis of four litters (38 pups) showed approximately 39% Jagged1$^{wt/tomato}$ offspring, slightly lower than expected but not outside the normal range for a sample of this size.
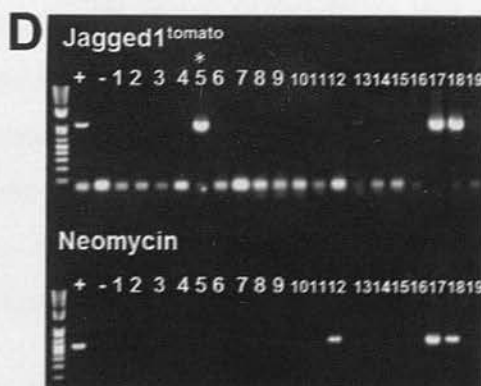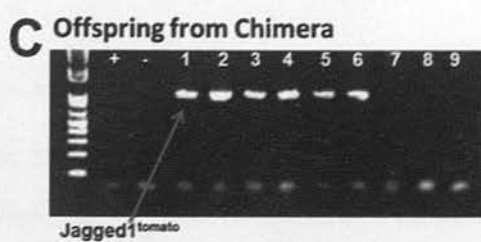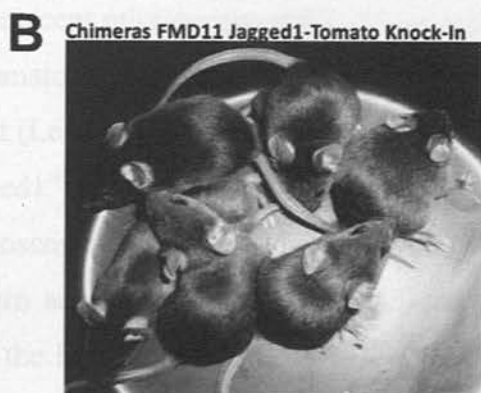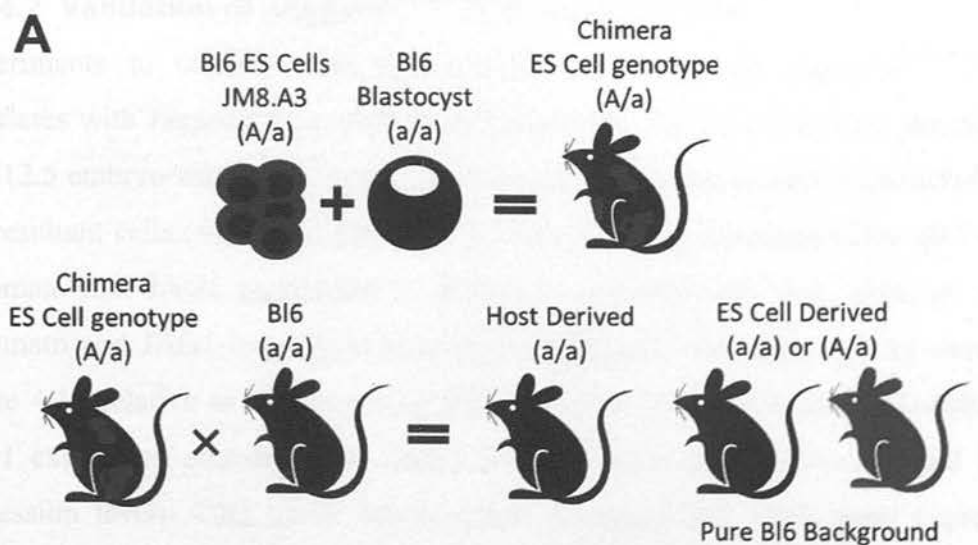
Figure 4.11: Production of Chimeras from Jagged1[tomato] Knock-In ES Cells.
A: Schematic of targeted ES cell tracking. Bl6 ES cells with one agouti allele restored are correctly targeted. These cells are then used to produce chimeras with Bl6 blastocysts. The chimeras are then crossed with Bl6 mice. Agouti pups in the resultant litters indicates germ line transmission of the genetic material from the targeted ES cells and the Jagged1[tomato] knock-in locus will be in either black or agouti pups of a pure Bl6 background. A: agouti locus. a: non-functional agouti locus (adapted from (Pettitt et al. 2009) B: Picture of JM8.A3.N1 targeted ES cell/Bl6 chimeras. C: "+" is positive control. "-" is H2O control. PCR genotyping of chimera offspring shows germ-line transmission of Jagged1[tomato] knock-in allele. Jagged1 tomato band is 994bp. Ladder used 1Kbp plus (Invitrogen). D: "+" is positive control. "-" is H2O control. Genotyping of Cre crossed pups. Sample 5 shows Jagged1tomato positive PCR but not neomycin indicating excision of the selection cassette. Jagged1 tomato band is 994bp. Neomycin band is 460 bp. Ladder 1Kbp plus (Invitrogen).

4.2.4.2 Validation of Jagged1$^{wt/tomato}$ transgenic mice

Experiments to confirm that dtTomato expression in the Jagged1$^{wt/tomato}$ line correlates with Jagged1 expression were carried out. Firstly tissue from the head of an E12.5 embryo was sorted based on dtTomato expression and RNA extracted from the resultant cells (fig 4.12). The mRNA expression was investigated by qRT-PCR. dtTomato and JAG1 expression in dtTomato negative cells was taken as 1 and dtTomato and JAG1 expression relative to these cells was assessed. As shown in figure 4.13 relative to negative cells dtTomato low cells had higher dtTomato and JAG1 expression and dtTomato high cells had higher again dtTomato and JAG1 expression levels. This result indicates that dtTomato and JAG1 gene expression correlate.

Fluorescent microscopy of E11.5 Jagged1$^{wt/tomato}$ embryos are shown in figure 4.13a. dtTomato expression correlates with known Jagged1 expression patterns at this time point (Le et al. 2009; Ramos et al. 2010; Sheth et al. 2007; Watanabe et al. 2006). A Jagged1$^{wt/tomato}$ E11.5 embryo was dissected and examining under a fluorescent microscope (fig 4.13b). dtTomato expression can be seen in a distinctive stripe pattern across the neural tube, the endothelium of the dorsal aorta, the developing eye, the head and the limbs. Flow cytometry analysis of adult thymic tissue shows that, as expected, dtTomato is expressed in thymic epithelial cells but not in other Lin⁻cell types (fig 4.14) (Lehar et al. 2005; Felli et al. 1999).

To better understand how Jagged1 protein expression and dtTomato protein levels correlate co-staining for Jagged1 and dtTomato was carried out. Transverse sections of E11.5 Jagged1$^{wt/tomato}$ embryos were stained for dtTomato and Jagged1 (fig 4.15). 40x magnifications show that E11.5 neural tube cells which are Jagged1 positive are not always dtTomato positive and vice versa. The dtTomato protein is an indicator of JAG1 transcription and therefore cells which are not actively transcribing JAG1 may still contain Jagged1 protein but have lost dtTomato. Conversely cells which are actively transcribing JAG1 (and hence dtTomato) may not yet contain mature Jagged1 protein.
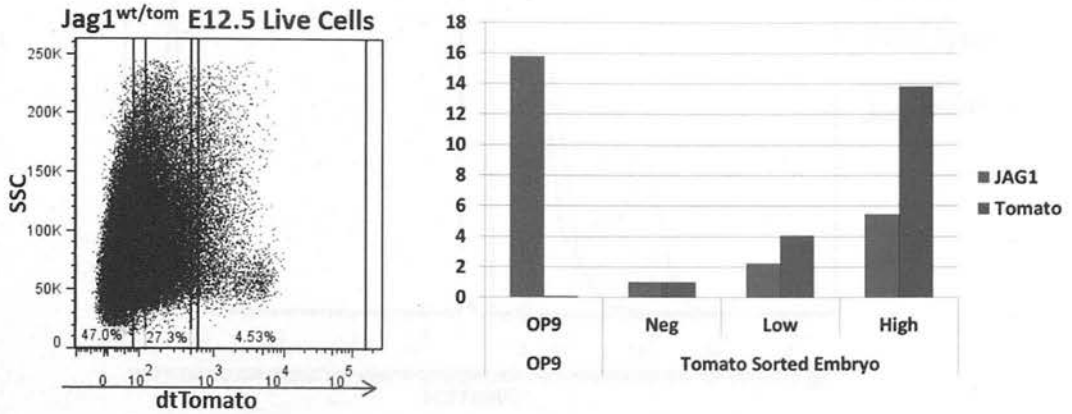
Figure 4.12: Sort and qRT-PCR for JAG1 and dtTOMATO.
7AAD⁻ live cells from the head of a E12.5 Jagged1-dtTomato embryo were sorted based on dtTomato expression. RNA was extracted from resultant cells convert to cDNA by RT-PCR and quantified by qPCR. OP9 stromal cells were used as a qRT-PCR JAG1 positive control.
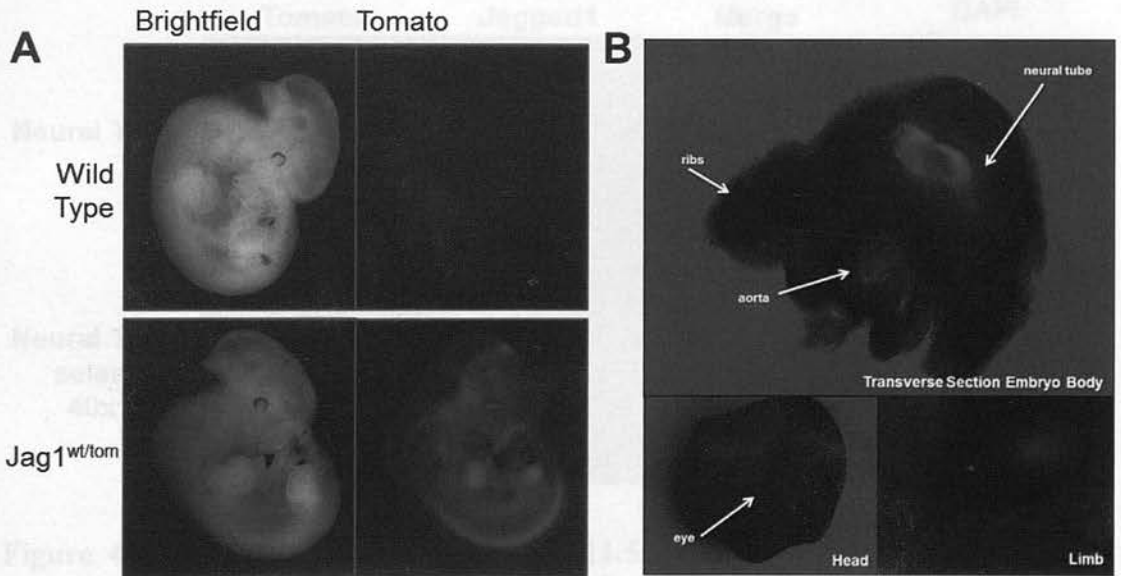
Figure 4.13: Jagged1^{wt/tomato} E11.5 embryo.

A: Florescent microscopy of E11.5 wild-type and Jagged1^{wt/tomato} embryos.
B: dtTomato-Bright field merge of E11.5 Jagged1^{wt/tomato} segments

107
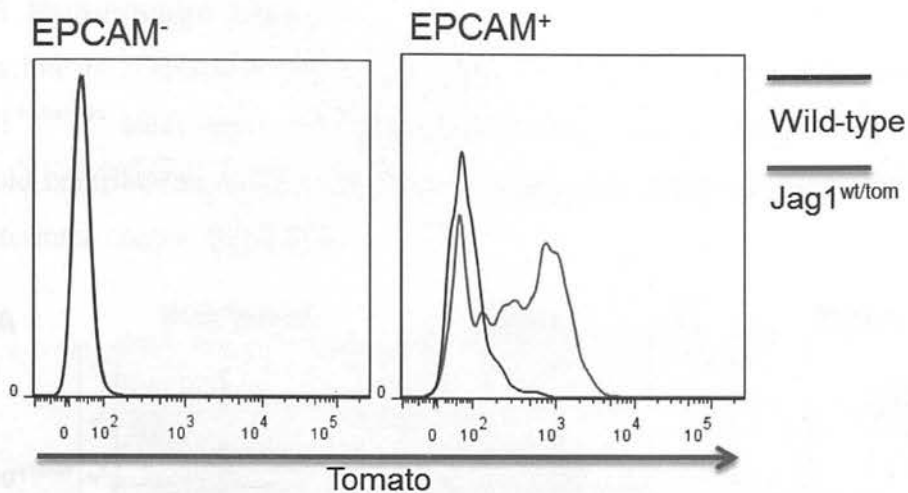
Figure 4.14: Jagged1$^{wt/tomato}$ Adult Thymus epithelial cells.
Flow cytometry of Lin- adult thymic cells shows that as expected Jagged1 is expressed in the EPCAM$^+$ epithelial cells but not in EPCAM$^-$ stromal cells. Carried out in collaboration with Dr. Svetlana Ulyanchanka.
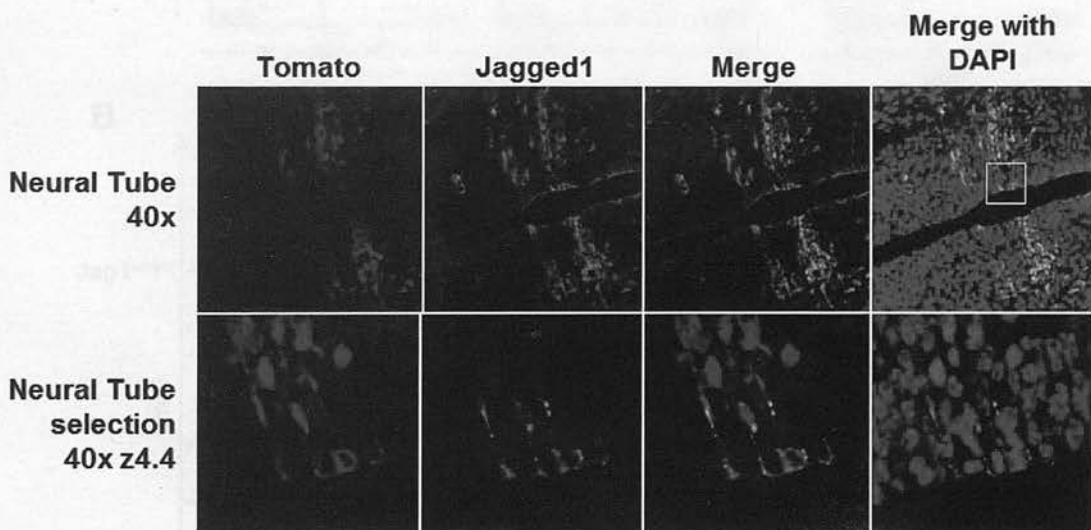
Figure 4.15: Transverse sections from E11.5 Jagged1$^{wt/tomato}$ Embryos stained with anti-Jagged1 and anti-Tomato antibodies.
White box indicates magnified region. Arrows indicate some double positive cells.

4.2.4.3 Multilineage analysis

As this line is a knock-in and heterozygous for Jagged1 multilineage analysis of Jagged1$^{wt/tomato}$ adult mice was carried out. This analysis showed myeloid and lymphoid contribution in the bone marrow, spleen and thymus comparable to wild-type littermate control (fig 4.16).
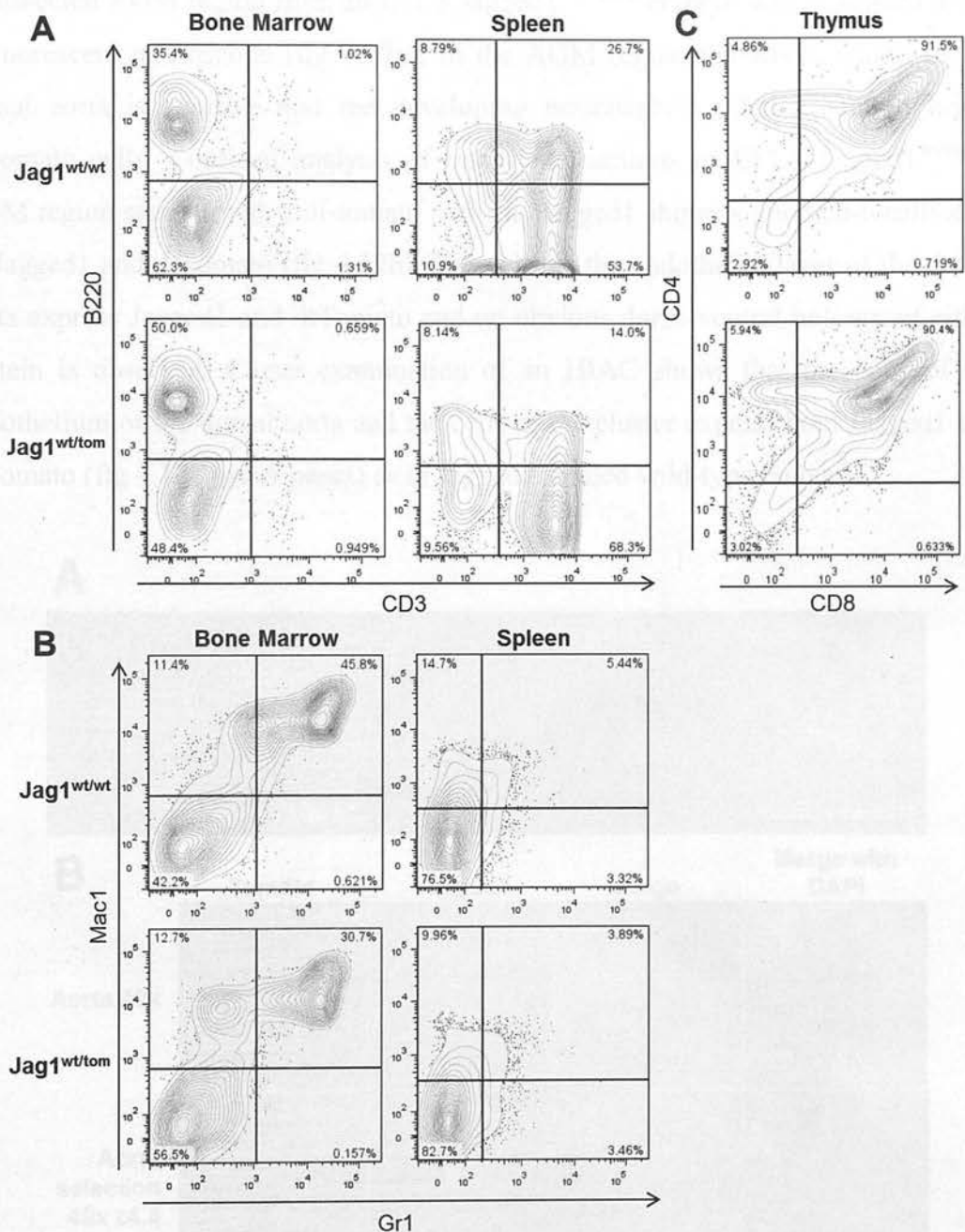
Figure 4.16: Multilineage analysis of Jagged1$^{wt/tomato}$ adult mouse.
Flow cytometry analysis of 7AAD$^-$CD45$^+$ cells in Jag1$^{wt/wt}$ and Jag1$^{wt/tomato}$ adult littermates **A:** CD3 and B220 lymphoid populations of the bone marrow and spleen. **B:** Mac1 and Gr1 myeloid populations of the bone marrow and spleen. **C:** CD4 and CD8 lymphoid populations of the thymus.

4.3 Analysis of Jagged1 Expression in E11.5 Embryo Using the Jagged1$^{wt/tomato}$ Reporter Line

4.3.1 Fluorescent Microscopy of E11.5 Jagged1$^{wt/tomato}$ AGM region

A dissected AGM region from an E11.5 Jagged1$^{wt/tomato}$ embryo was examined under a fluorescent microscope (fig 4.17a). In the AGM region the whole length of the dorsal aorta is positive and the developing mesonephric tubules contain bright dtTomato cells. Confocal analysis of transverse sections of E11.5 Jagged1$^{wt/tomato}$ AGM region stained with anti-tomato and anti-Jagged1 shows a good co-localisation of Jagged1 and dtTomato (fig 4.17b). The cells of the endothelial layer of the dorsal aorta express Jagged1 and dtTomato and no obvious dorso-ventral polarity of either protein is observed. Closer examination of an HIAC shows that the cells of the endothelium of the dorsal aorta and the cells of the cluster express both Jagged1 and dtTomato (fig 4.17b lower panel) as in Jagged1 stained wild-type embryos.
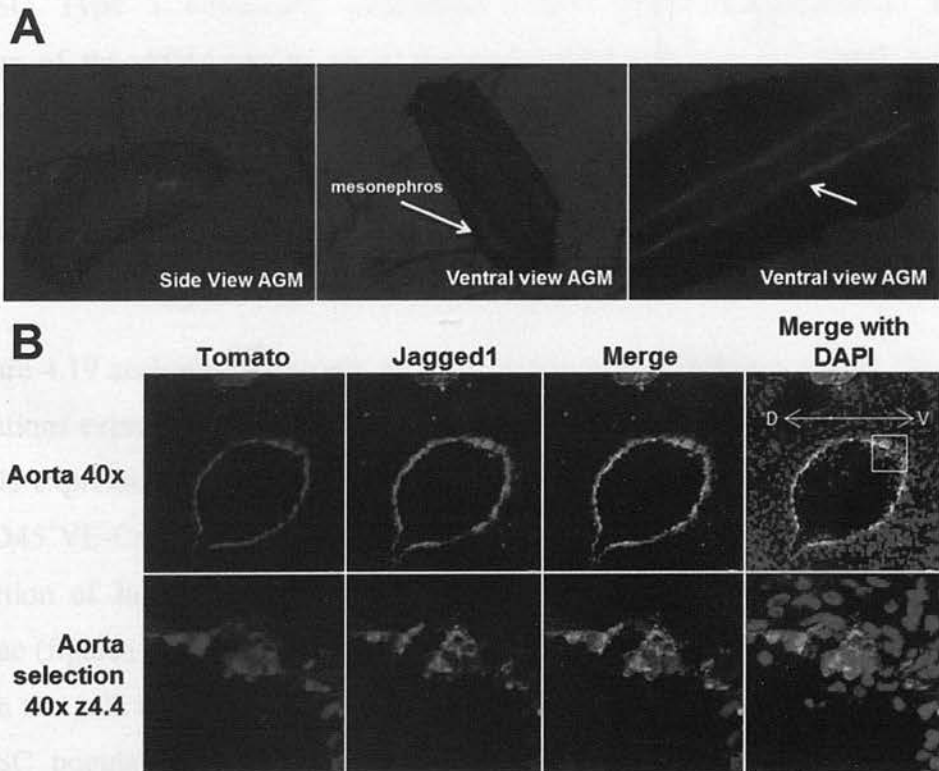
Figure 4.17: dtTomato-Brightfield merge of E11.5 Jagged1$^{wt/tomato}$ segments.
A: dtTomato-Brightfield merge of E11.5 Jagged1$^{wt/tomato}$ AGM region. **B:** Transverse sections from E11.5 Jagged1$^{wt/tomato}$ embryos stained with anti-Jagged1 and anti-Tomato antibodies. White box indicates magnified region. D: dorsal; V: Ventral

4.3.2 Flow Cytometry of E11.5 Jagged1$^{wt/tomato}$

In order to study Jagged1 expression at E11.5 in the different cell types of the developing haematopoietic system, Jagged1$^{wt/tomato}$ E11.5 AGM region and yolk sac were dissected and dissociated. Cells were stained for Ter119 (for RBC exclusion), Notch1, CD45, CD41 and VE-Cadherin. Due to the presence of Notch1$^+$, Jagged1$^+$ double positive cells previously reported co-expression of these markers was analysed (Robert-Moreno et al. 2008). Notch1 and Jagged1 (dtTomato) expression were analysed in the populations of interest. Figures 4.16 and 4.17 show Notch1 and Jagged1 gating of these populations in the E11.5 AGM region and yolk sac respectively.

In the AGM region, the mature haematopoietic (CD45$^+$VE-Cadherin$^-$) and CD45$^+$VE-Cadherin$^+$ populations have the lowest proportion of Jagged1 positive cells and the endothelial (CD41$^-$CD45$^-$VE-Cadherin$^+$) and CD41$^+$CD45$^-$VE-Cadherin$^+$ populations have the highest (fig 4.18). The proportions of Jagged1$^+$Notch1$^+$ cells are also lowest in the CD45$^+$ populations and highest in the pre-HSC Type I containing population (CD41$^+$CD45$^-$VE-Cadherin$^+$). Though sections of the AGM region show the endothelial cells of the dorsal aorta are Jagged1 and dtTomato positive, flow cytometry analysis shows the AGM region contains 2 different endothelial populations Jagged1$^+$ and Jagged1$^-$. The endothelial dtTomato$^-$ population can contain Jagged1$^-$ venous endothelial cells (Villa et al. 2001) and/or cells which may express Jagged1 protein but are not dtTomato positive.

In figure 4.19 analysis of the yolk sac shows that, as in the AGM region, the CD45$^-$ populations express higher proportions of both Notch1 and Jagged1 with little or no Jagged1 expressed in the CD45$^+$ populations. There are no Jagged1$^+$Notch1$^+$ cells in the CD45$^+$VE-Cadherin$^+$ population of the yolk sac and in addition there is a lower proportion of Jagged1^{hi} cells in the CD41$^+$CD45$^-$VE-Cadherin$^+$ population of the yolk sac (figures 4.18 and 4.19 red box). This absence/reduction of Jagged1$^+$Notch1$^+$ cells in the yolk sac populations phenotypically equivalent to phenotypically defined pre-HSC populations in the AGM region might be a marker of the functional difference between these cells in the AGM region versus the yolk sac related to HSC production.
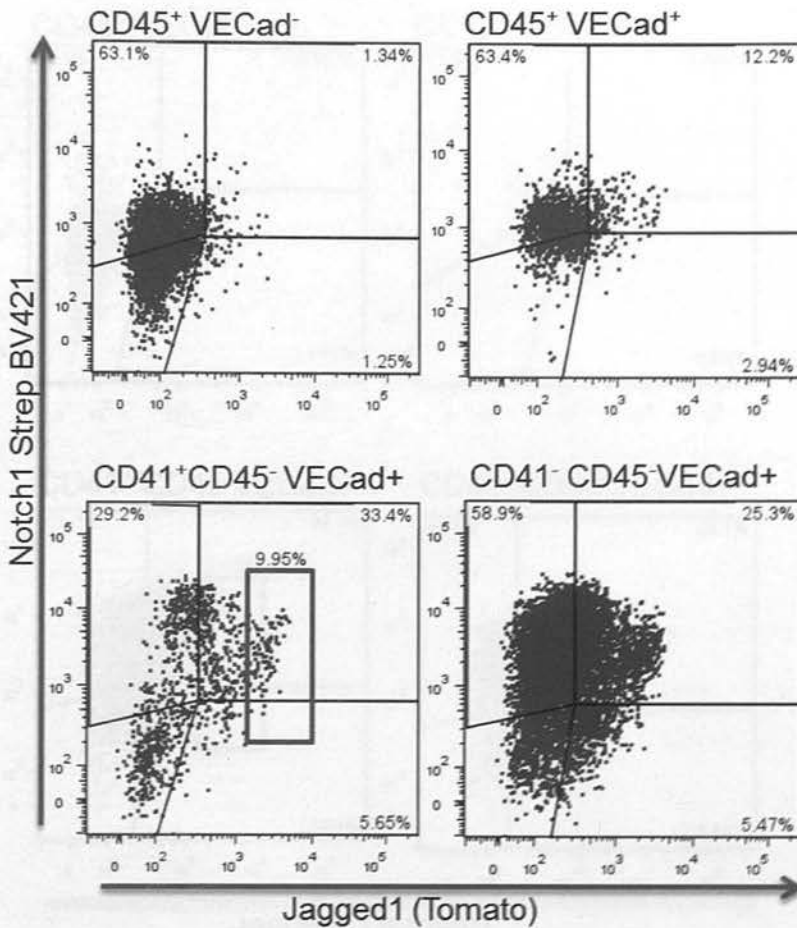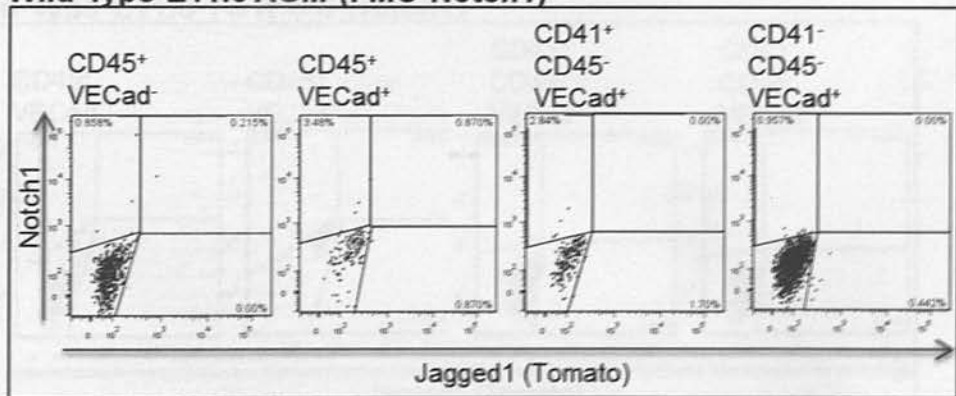
Figure 4.18: Flow cytometry E11.5 Jagged1$^{wt/tomato}$ AGM region.
Notch1 and Jagged1 expression in populations of interest in Jagged1$^{wt/tomato}$ E11.5 AGM region. Cells were first gated as 7AAD⁻Ter119⁻. Lower panel shows FMO Notch1 control on wild-type AGM region. Representative example of 3 repeat experiments.
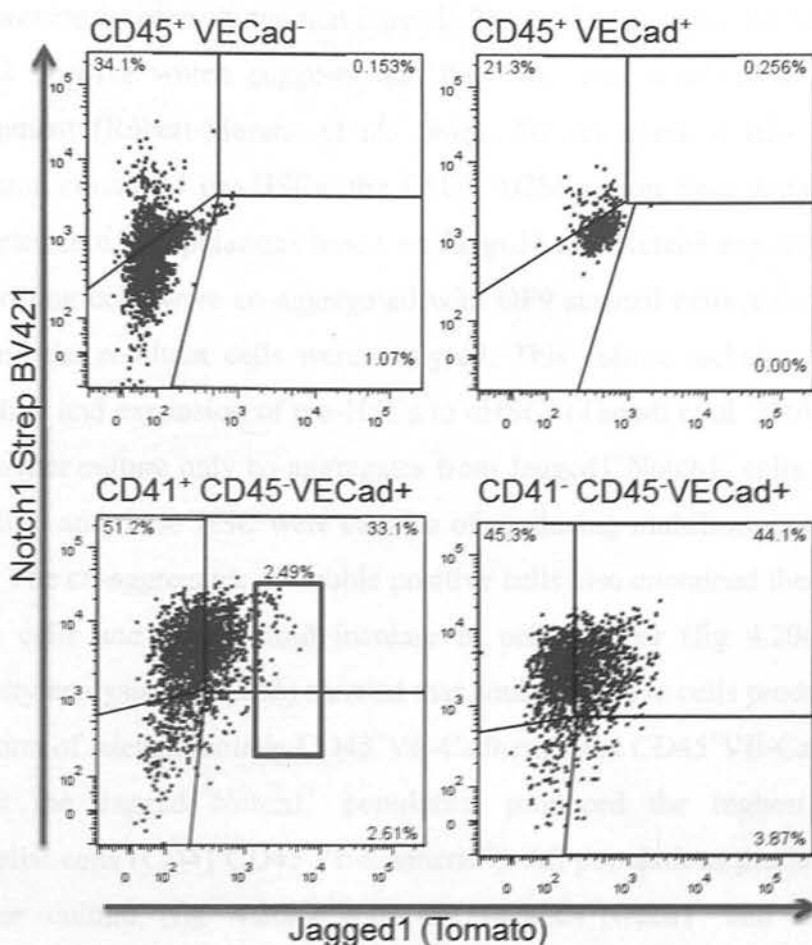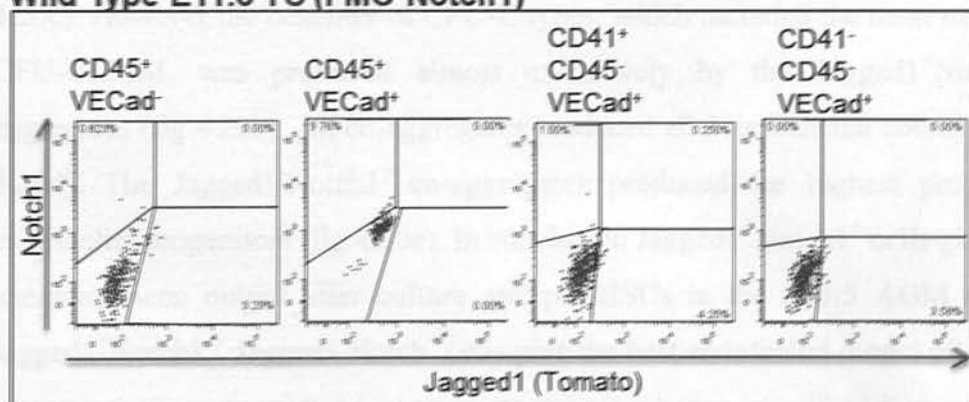
Figure 4.19: Flow cytometry E11.5 Jagged1^wt/tomato Yolk Sac.
Notch1 and Jagged1 expression in populations of interest in Jagged1^wt/tomato E11.5
YS. Cells were first gated as 7AAD⁻Ter119⁻. Lower panel shows FMO Notch1
control on wild-type YS. Representative example of 3 repeat experiments.

4.3.3 Functional Analysis of Jagged1⁺Notch1⁺ Cells

It has previously been shown that Jagged1⁺Notch1⁺ cells in the AGM region are also GATA2 positive which suggests that they are cells involved in haematopoietic development (Robert-Moreno et al. 2005). To determine if this double positive population contained pre-HSCs, the E11.5 AGM region from Jagged1$^{wt/tomato}$ mice was sorted into 4 populations based on Jagged1 and Notch1 expression (fig 4.20a). After sorting cells were co-aggregated with OP9 stromal cells and cultured for five days and the resultant cells were analysed. This culture technique allows for the maturation and expansion of pre-HSCs to dHSCs (Taoudi et al. 2008; Rybtsov et al. 2011). After culture only co-aggregates from Jagged1⁺Notch1⁺ cells produced HSCs (fig 4.20b) and these HSC were capable of producing multilineage engraftment (fig 4.20c). The co-aggregates of double positive cells also contained the highest number of live cells and largest fold increase in cell number (fig 4.20d and e). Flow cytometry analysis (fig 4.21a) showed that double positive cells produced the highest proportion of haematopoietic CD45⁺VE-Cadherin⁻ and CD45⁺VE-Cadherin⁺ cells. In contrast the Jagged1⁻Notch1⁺ population produced the highest proportion of endothelial cells (CD41⁻CD45⁻VE-Cadherin⁺). All populations produced some CFU-Cs after culture (fig 4.20b) with the Jagged1⁺Notch1⁻ and double negative populations producing the highest proportion of haematopoietic progenitors (fig 4.20c). However the best mix of CFU-C types, which included the most multilineage CFU-GEMM, was produced almost exclusively by the Jagged1⁺Notch1⁺ co-aggregates (fig 4.20b). All co-aggregates produced all 3 endothelial colony types (fig 4.20d). The Jagged1⁻Notch1⁺ co-aggregates produced the highest proportion of endothelial progenitors (fig 4.20e). In conclusion Jagged1⁺Notch1⁺ cells give the best haematopoietic output after culture and pre-HSCs in the E11.5 AGM region are Jagged1⁺Notch1⁺. Jagged1⁻Notch⁺ cells give the best endothelial output after culture.

A

WT E11.5 AGM

17.7%   0.017%

Notch1 Strep BV421

77.9%   0.052%

Jag1 wt/tom E11.5 AGM

0.009%   0.009%

76.2%   19.3%

Sort

5.78%   4.00%

68.9%   14.1%

Jagged1 (Tomato)

B   Long-term repopulation

% blood chimerism

J1+N1-   J1-N1+   J1-N1-   J1+N1+
0/10   0/10   0/10   6/10

C   Bone Marrow   Spleen   Thymus

Mac1

12.3%   39.7%

44.8%   3.22%

Gr1

B220

24.4%   9.89%

7.14%   58.6%

CD3

CD4

5.79%   89.4%

1.90%   2.91%

CD8

D

No. of live cells per 1 reag

300000

200000

100000

0

J1+ N1-   J1- N1+   J1-N1-   J1+ N1+

E

Fold increase in cell number

40

30

20

10

0

J1+ N1-   J1- N1+   J1-N1-   J1+ N1+

115
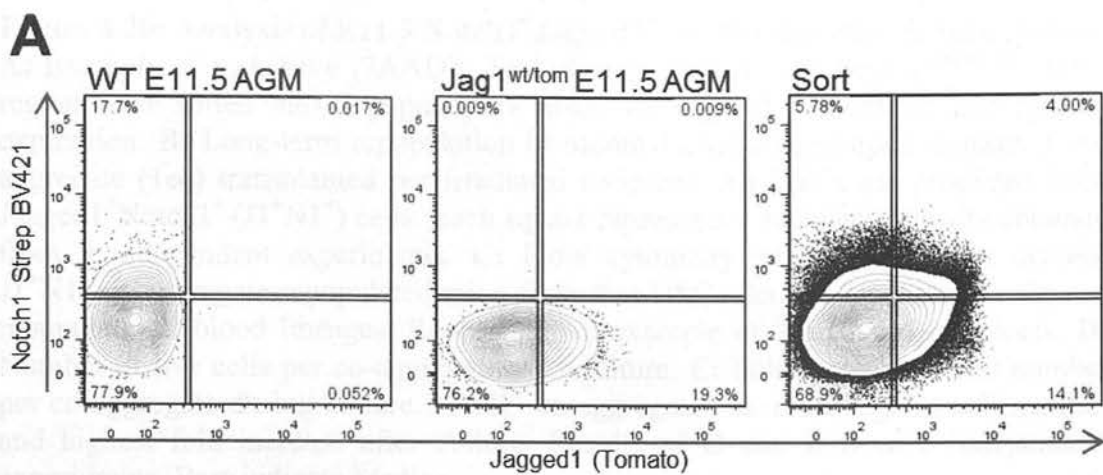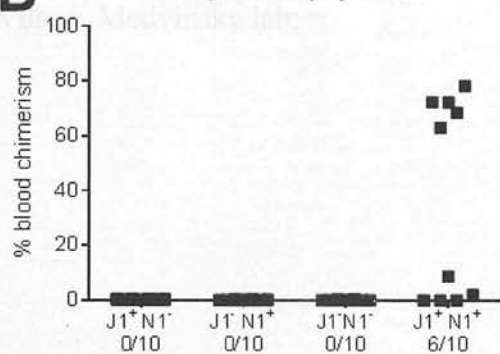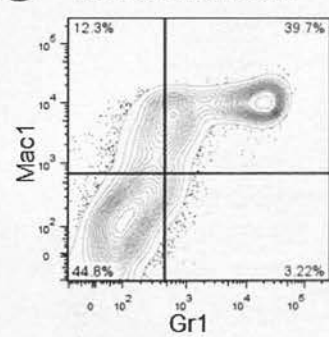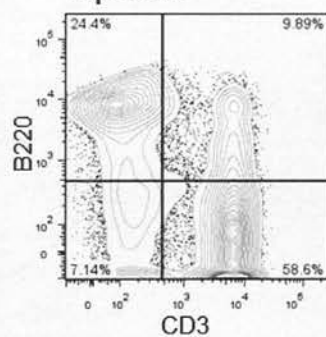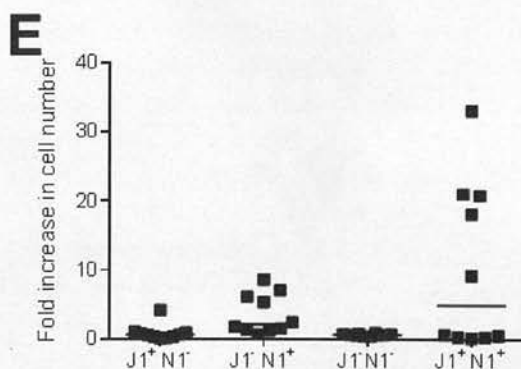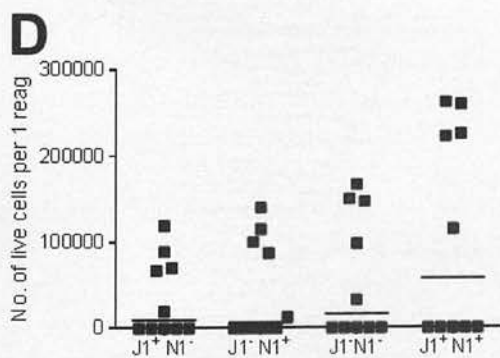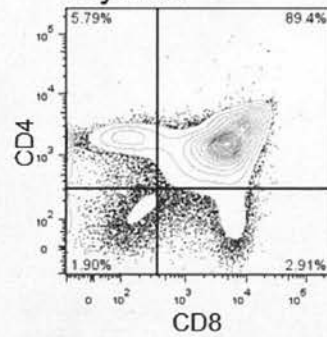
Figure 4.20: Analysis of E11.5 Notch1+Jagged1+ AGM cells after culture (part1).
A: Example of sort. Live (7AAD−), Ter119− cells from E11.5 Jagged1[wt/tomato] AGM region were sorted into 4 populations based on Jagged1 (dtTomato) and Notch1 expression. **B:** Long-term repopulation (4 months) after co-aggregate culture. 1 co-aggregate (1ee) transplanted per irradiated recipient. All HSCs are produced from Jagged1+Notch1+ (J1+N1+) cells. Each square represents 1 recipient. Results obtained from 3 independent experiments. **C:** Flow cytometry analysis of donor derived J1+N1+ co-aggregate repopulated mice show that HSCs derived from these cells can repopulate all blood lineages. Representative example of 2 analysed recipients. **D:** Number of live cells per co-aggregate after culture. **E:** Fold increase in cell number per co-aggregate during culture. J1+N1+ co-aggregates have the highest cell number and highest fold increase after culture. Results of D and E from 2 independent experiments. Bars indicate Median.
1 experiment of transplantation and post culture analysis carried out by Heather Wilson, Medvinsky lab.
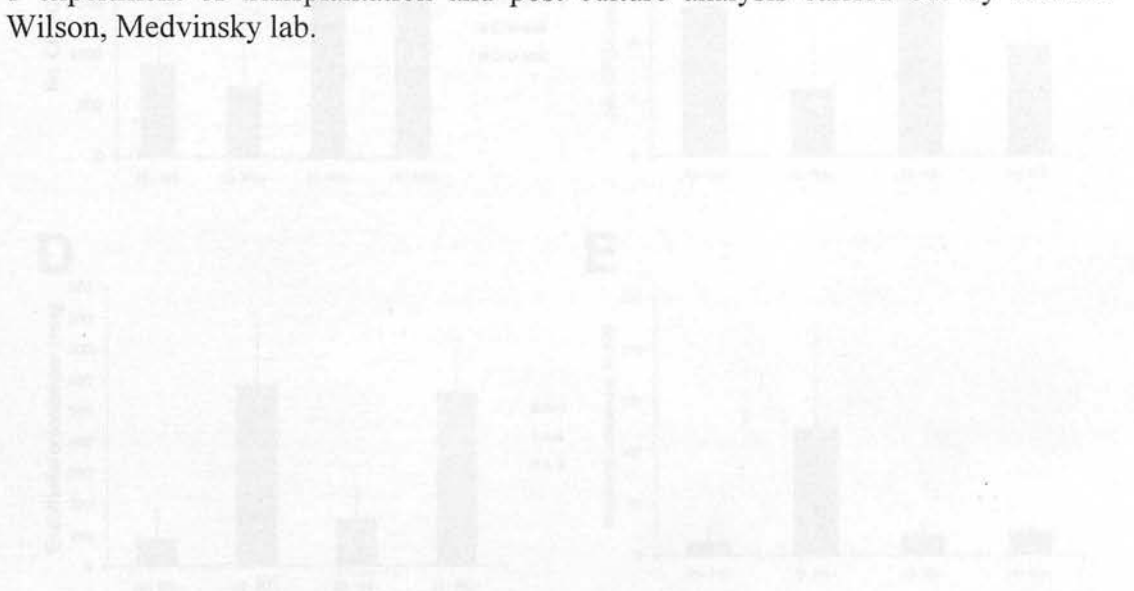
Figure 4.21: Analysis of E11.5 Notch1⁺Jagged1⁺ AGM cells after culture (part2). **A:** Flow cytometry of co-aggregate cells after culture. The trend indicates that J1⁺N1⁺ co-aggregates produce a higher proportion of haematopoietic CD45⁺VE-Cadherin⁻ (**: p=0.0079) and CD45⁺VE-Cadherin⁺ cells and the J1⁻N1⁺ co-aggregates produce a higher proportion of endothelial VE-Cadherin⁺CD41⁻CD45⁻ cells (**: p=0.0079). Bars indicate Median. **B:** Haematopoietic progenitor assay after culture shows that all co-aggregate types can produce CFU-Cs after culture. **C:** Number of CFU-Cs per 1,000 cells. **D:** Number of endothelial colonies produced after co-aggregate culture per 1 co-aggregate (1ee). **E:** Number of endothelial colonies per 10,000 cells. Results from 2 independent experiments. Experiment 2 performed by Heather Wilson.

CFU-C: colony forming unit - culture; BFU-E: burst forming unit erythroid; Mac: macrophage; GM: granulocyte, macrophage; GEMM: granulocyte, erythroid, macrophage, megakaryocyte; Net: network (>6 main tubules) of endothelial tubules; 4-6: network of 4-6 main endothelial tubules; 1-3: single or network of 2/3 main endothelial tubules.

1 experiment carried out by Heather Wilson, Medvinsky lab.

4.4 Discussion

Previous studies have shown that Jagged1 is expressed in the AGM region in the dorsal aorta. *In situ* hybridization shows a ventral polarity of Jagged1 expressions at E10.5, and cells of the HIAC are positive for Jagged1 (Robert-Moreno et al. 2008; Robert-Moreno et al. 2005). Antibody staining of the Jagged1 protein shows that the endothelium of the dorsal aorta is fully positive with no obvious ventral polarity (Robert-Moreno et al. 2008) This was confirmed by immunohistochemistry using a phage anti-Jagged1 antibody obtained from the McCafferty lab in Cambridge. The antibody had flag, human Fc and His6 regions for secondary antibody detection. The antibody was derived against the extra cellular portion of the Jagged1 protein and therefore possible to use for flow cytometry. However, co-staining with other antibodies in cell suspension proved problematic and a solution was not reached during this project (results not shown). In the meantime a method to detect Jagged1 expression of live cells by flow cytometry was required for this project. No previous Jagged1 reporter line had been reported and therefore the Jagged1$^{wt/tomato}$ line was produced.

The fidelity of the Jagged1$^{wt/tomato}$ transgenic line in marking Jagged1 expression was assessed. At an mRNA level there was good correlation of JAG1 and TOMATO mRNA expression. Examining adult thymic tissue from Jagged1$^{wt/tomato}$ by flow cytometry, thymic epithelial cells, which are known to be Jagged1 expressing, were dtTomato positive and non-thymic epithelial cells were dtTomato negative (Lehar et al. 2005; Felli et al. 1999). E11.5 Jagged1$^{wt/tomato}$ embryos expressed dtTomato fluorescent protein in a pattern similar to known Jagged1 expression patterns, namely in the developing heart, eye, limb and head, including the distinctive stripe pattern of positive cells across the developing neural tube (Le et al. 2009; Manderfield et al. 2012; Ramos et al. 2010; Watanabe et al. 2006). A closer look at Jagged1 protein and dtTomato protein co-localisation in the neural tube by confocal analysis of Jagged1$^{wt/tomato}$ E11.5 embryos showed that not all Jagged1$^+$ cells are dtTomato$^+$ and vice versa. The Jagged1^{tomato} knock-in gene is a transcription reporter. After transcriptional regulation JAG1 mRNA undergoes translation to an amino acid chain which is then modified to and transported to produce a functional Jagged1 ligand at the cell surface with a certain half-life. These other processes undergo specific

regulation and translation, maturation and stability of the dtTomato fluorescent protein will not be subject to the same regulations. Cells which contain Jagged1 protein may no longer be undergoing active transcription of the Jagged1 gene and the dtTomato mRNA/protein produced during transcription may have decayed. Conversely dtTomato protein may be present in cells where Jagged1 transcription has recently been activated but from which no mature Jagged1 protein has yet been produced. Possible strategies to produce a reporter of Jagged1 protein expression may include the production of a fusion protein where the Jagged1 gene fused to a fluorescent protein gene; therefore fluorescence would be bound to the Jagged1 protein. This method is difficult as both Jagged1 and dtTomato need to be able to correctly fold and be functional. The addition of an amino acid sequence to Jagged1 may change its ability to correctly fold, function and undergo regulating modifications. In addition, Jagged1 and dtTomato proteins would still have different stabilities. Another strategy could be to produce a Jagged1 dtTomato fusion gene in which the stop codon from the Jagged1 gene is removed and the dtTomato gene sequence inserted in frame after the Jagged1 sequence separated by a T2A or IRES sequence. The mRNA produced would encode Jagged1 and dtTomato but after translation, when using a T2A linker, the link amino acid breaks giving rise to two separate proteins, or the IRES sequence would allow translation of Jagged1 and dtTomato from the same mRNA molecule. This is a translational reporter that would allow further tracking of Jagged1 expression than the Jagged1[tomato] transcriptional reporter but again the two separate proteins would not undergo the same regulatory process and or have the same half-life. For the study of Jagged1 expression in the AGM region at E11.5 the Jagged1[tomato] transcription reporter produced may be sufficient as dtTomato and Jagged1 co-localise well in the dorsal aorta.

Another consequence of this reporter design is that the resultant mice are heterozygous for Jagged1. Although Jagged1 heterozygous mice survive to adult-hood and are fertile they do have eye defects and some deficiency in haematopoiesis at E10.5 has been observed (Le et al. 2009; Xue et al. 1999; Robert-Moreno et al. 2008). As well as this, it is not understood what effect on gene regulation this deficiency might have, i.e. are other Notch ligands up-regulated to compensate? A possible alternative could be to create a transgenic line in which a BAC containing

the Jagged1 gene locus is modified in a similar way to the Jagged1 locus in the Jagged1^{tomato} mouse line. The whole BAC can then be linearized and allowed to insert randomly into the genome of ES cells. The downsides to using this approach include: the insertion site might also disrupt another gene; the BAC may not contain all the regulatory elements of the Jagged1 locus; the insertion site may not be available for transcription simultaneously with the Jagged1 gene due to its different chromosomal location. It is clear that all strategies for producing a reporter line have their positive and negative aspects. The Jagged1^{tomato} line produced here seems to be a faithful reporter of Jagged1 transcription and could be useful in tracking Jagged1 expressing cells to better understand their role in various developmental and adult processes.

Confocal analysis of the E11.5 AGM region showed that Jagged1 is expressed in the endothelial cells of the dorsal aorta and in the cells immediately surrounding the dorsal aorta. Flow cytometry showed that Jagged1 is expressed in cells of the developing haematopoietic system and endothelial cells. Jagged1$^+$ and Notch1$^+$ double positive cells are also present in all the populations examined. There are little to no Jagged1$^+$Notch1$^+$ cells in the yolk sac's CD45$^+$VE-Cadherin$^+$ population and few Jagged1^{hi}Notch1$^+$ cells in the CD41$^+$CD45$^-$VE-Cadherin$^+$ population. This may indicate that these cells are functional pre-HSCs which are not present in the yolk sac at this stage. Further functional analysis of the Jagged1$^+$Notch1$^+$ cells from the E11.5 AGM region showed that after culture all HSCs were produced from the double positive population. This population also had the highest fold increase in cell number and the highest proportion of CD45$^+$VE-Cadherin$^-$ and CD45$^+$VE-Cadherin$^+$ cells produced.

In the previous chapter some pre-HSCs were shown to be Notch1$^-$ this indicates that if more sorted Jagged$^{wt/tomato}$ E11.5 cells were cultured some pre-HSCs would be present in the Notch1$^-$ compartment. It is possible that in this line the heterozygous expression of Jagged1 may have an effect on Notch1 marking of pre-HSCs, as Notch signalling is known to be involved in its own regulation, however, flow cytometry analysis did not show any obvious differences in Notch1 expression between wild-type and Jagged$^{wt/tomato}$ in the E11.5 AGM (data not shown). Further experiments are

required to confirm if some pre-HSCs are Notch1 negative in this mouse line. In addition as discussed in the previous chapter the level of HSCs produced is less than expected. This again indicates that the Notch1 antibody may partially block pre-HSC maturation and proliferation, and/or HSC proliferation. However, despite the limitations out-lined these functional experiments indicate that Jagged1$^+$Notch1$^+$ cells from the E11.5 AGM region are the precursors of HSCs. Using these markers gives approximately a 1 in 8 and 1 in 3 enrichment for pre-HSC Type I and II cells respectively, making a significant contribution to determining the exclusive phenotype of pre-HSCs. This would be a very powerful tool, allowing single cell analysis of the developing haematopoietic system.

Due to the small amounts of tissue available Jagged1 and Notch1 sorting was carried out on whole E11.5 AGM region. Future work would include further sort experiments of Jagged1$^{wt/tomato}$ embryos at E11.5 and E10.5 with the inclusion of Notch1 and other pre-HSC markers to more precisely determine the phenotype of the pre-HSCs. If it was determined that pre-HSC Type I were Jagged1^{hi}Notch1$^+$ this phenotype would give 1 in 10 enrichment for pre-HSC Type I cells in the CD41$^+$CD45$^-$VE-Cadherin$^+$ population. Direct transplantation of sorted Jagged1$^{wt/tomato}$ E11.5 AGM region may also be carried out to determine whether HSCs express Jagged1. Mapping of Jagged1 expression throughout early haematopoietic development is now possible using this Jagged1$^{wt/tomato}$ mouse line bringing us closer to understanding the spatial and temporal role of Notch signalling in haematopoietic development.

5 HSC development in Jagged1$^{\Delta/\Delta}$ Embryos

It has previously been shown that intra-embryonic haematopoiesis is impaired in Jagged1 knock-out (Jagged1$^{\Delta/\Delta}$) embryos whereas primitive yolk sac haematopoiesis is unaffected. These embryos cannot efficiently generate haematopoietic progenitors and expression of GATA2 and Runx1, two critical transcription factors involved in haematopoiesis, are reduced (Robert-Moreno et al. 2008). This phenotype can partially be rescued by ectopic GATA2 expression during culture. Moreover stratification of the aortic endothelium was observed in Jagged1$^{\Delta/\Delta}$ embryos indicating a possible block in the endothelial-haematopoietic transition (Robert-Moreno et al. 2008). Taken together these results indicate that Jagged1 is involved in hematopoietic development. However the role of Jagged1 in dHSC development has never been elucidated partly due to the fact that the mutant embryos die around E10.5 before the emergence of the first dHSCs (E11).

In this chapter the role of Jagged1 in HSC development is examined in greater detail. Firstly its importance in HSC development is assessed by deleting Jagged1 specifically in the HSC lineage. Secondly, conventional Jagged1$^{\Delta/\Delta}$ embryos were investigated for the presence of functional pre-HSCs.

5.1 Cell Autonomous Deletion of Jagged1 during HSC Development

In the previous chapter it was shown that Jagged1 is expressed in endothelial cells, haematopoietic cells and pre-HSCs. Pre-HSCs were shown to be Notch1$^+$Jagged1$^+$ double positive. It is therefore unclear in which cell population Jagged1 is important for haematopoietic development. Is Jagged1 required in a cell autonomous or cell non-autonomous manner?

To determine if Jagged1 is required cell autonomously in the HSC lineage, a conditional Jagged1$^{fl/fl}$ line was crossed with CD41-Cre expressing line (Emambokus & Frampton 2003) and a sGFP reporter line (Gilchrist et al. 2003). The CD41-Cre line expresses Cre recombinase under the control of the CD41 promoter and can activate sGFP in early E9.5 dorsal intra-aorta clusters and in approximately half the

pre-HSC Type I population at E10.5 (Emambokus & Frampton 2003; Rybtsov et al. 2011). The sGFP allele contains a floxed stop cassette which is removed after cre mediated recombination allowing constitutive activation of GFP expression. In the resultant Jagged1$^{\Delta/fl}$CD41-Cre^{+}sGFP^{+} embryos, Jagged1 should be deleted in HSC precursors before the emergence of the first HSC. Cells in which cre mediated recombination has taken place are marked by the activation of constitute GFP expression allowing lineage tracing.

Compound Jagged1$^{\Delta/fl}$CD41-Cre^{+}sGFP^{+} adult mouse survive and GFP^{+} cells were able to contribute to the adult haematopoietic system at a level similar to that observed in Jagged1$^{wt/fl}$CD41-Cre^{+}sGFP^{+} mice indicating Jagged1 deficiency does not block development of adult HSCs. They also exhibit normal contribution to both myeloid and lymphoid lineages (fig 5.1a). We showed by southern blot that the majority of GFP^{+} cells in the bone marrow of the Jagged1$^{\Delta/fl}$CD41-Cre^{+}sGFP^{+} mouse were deleted for Jagged1 (fig 5.1b). Genotyping of individual haematopoietic colonies issued from the bone marrow of the Jagged1$^{\Delta/fl}$CD41-Cre^{+}sGFP^{+} mouse shows that haematopoietic progenitors are derived from both Jagged1$^{\Delta/fl}$ and Jagged1$^{\Delta/\Delta}$ HSCs (fig 5.1c). 10 GFP^{+} colonies were genotyped of which six were Jagged1$^{\Delta/\Delta}$. 12 GFP^{-} colonies were genotyped of which ten were Jagged1$^{fl/\Delta}$.

These results indicate that Jagged1$^{\Delta/\Delta}$ cells can develop into HSC. Unpublished data obtained in the lab suggest that CD41-Cre only triggers deletion of the gene of interest during pre-HSC development and not in adult HSCs. However this needs to be confirmed in the case of this Jagged1 conditional mutant.
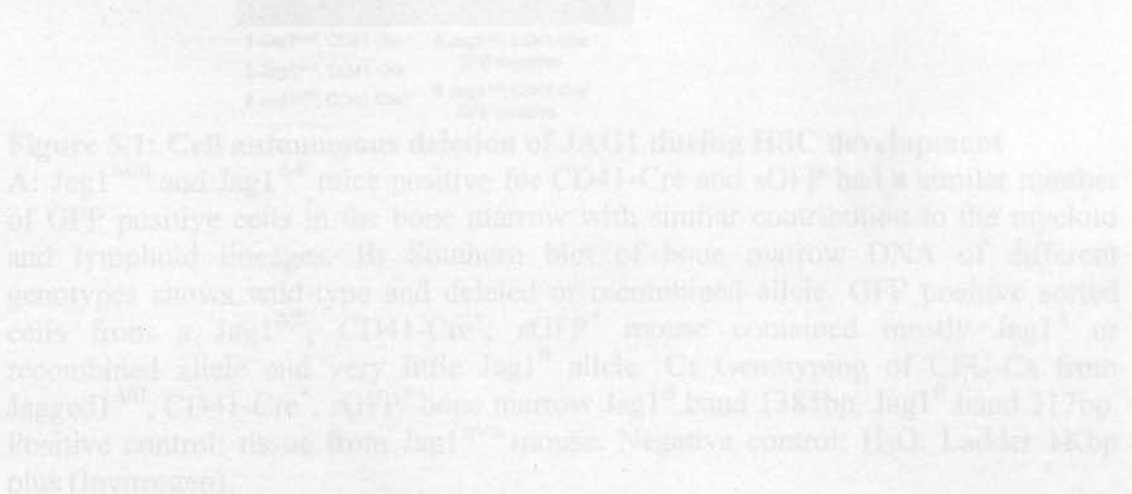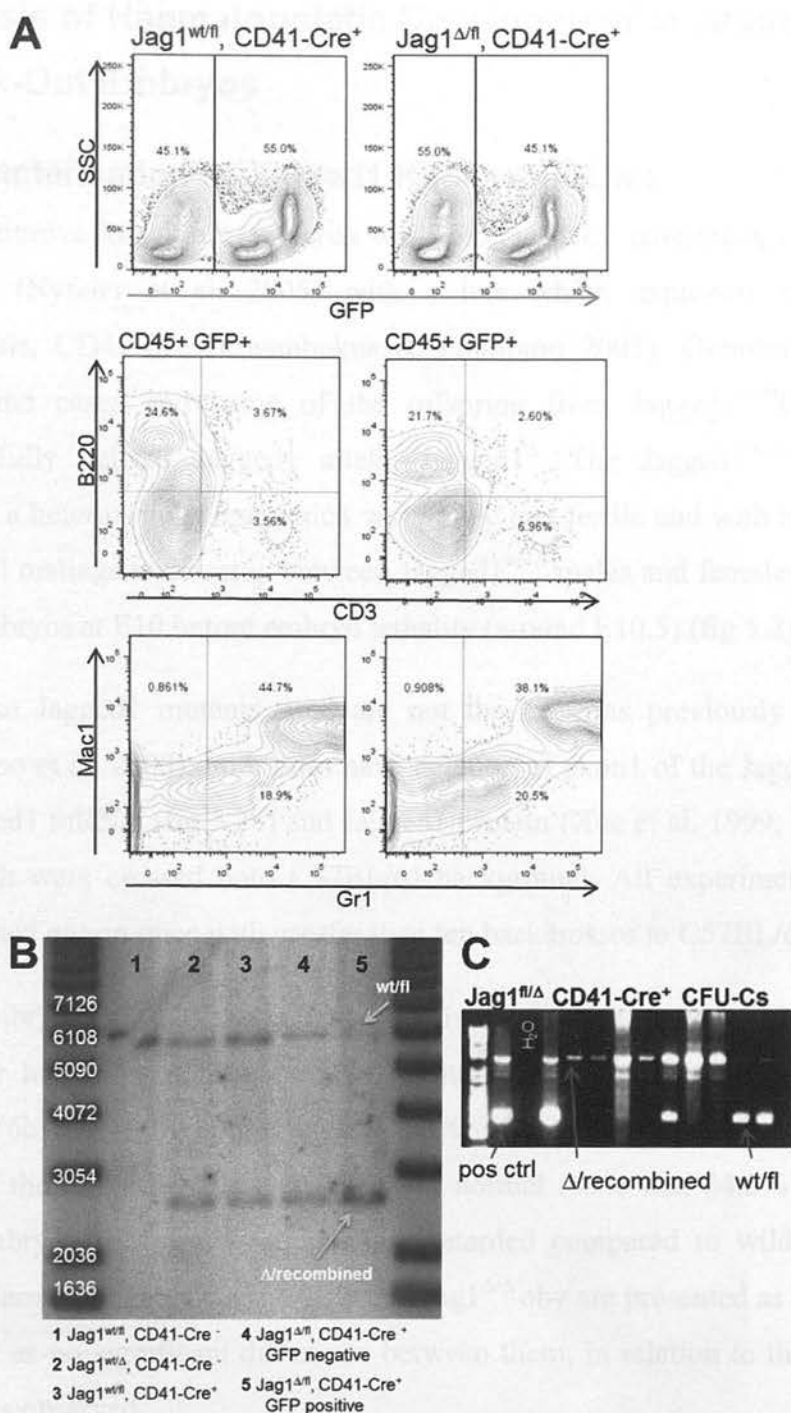
Figure 5.1: Cell autonomous deletion of JAG1 during HSC development
A: Jag1$^{wt/fl}$ and Jag1$^{\Delta/fl}$ mice positive for CD41-Cre and sGFP had a similar number of GFP positive cells in the bone marrow with similar contribution to the myeloid and lymphoid lineages. **B:** Southern blot of bone marrow DNA of different genotypes shows wild-type and deleted or recombined allele. GFP positive sorted cells from a Jag1$^{\Delta/fl}$, CD41-Cre$^+$, sGFP$^+$ mouse contained mostly Jag1$^{\Delta}$ or recombined allele and very little Jag1^{fl} allele. **C:** Genotyping of CFU-Cs from Jagged1$^{\Delta/fl}$, CD41-Cre$^+$, sGFP$^+$ bone marrow Jag1$^{\Delta}$ band 1385bp, Jag1^{fl} band 317bp. Positive control: tissue from Jag1$^{wt/\Delta}$ mouse. Negative control: H$_2$O. Ladder 1Kbp plus (Invitrogen).

5.2 Analysis of Haematopoietic Development in Jagged1 Knock-Out Embryos

5.2.1 Characterisation of Jagged1 Knock-Out Line

Jagged1 constitutive knock-out embryos were produced by crossing a conditional Jagged1 line (Nyfeler et al. 2005) with a line which expresses cre during spermatogenesis, CD41-Cre (Emambokus & Frampton 2003). Germline deletion occurs in some cases and some of the offspring from Jagged1$^{fl/wt}$CD41-Cre$^+$ contained a fully deleted Jagged1 allele, Jagged1$^\Delta$. The Jagged1$^{wt/\Delta}$ line was maintained as a heterozygous line which was viable and fertile and with no obvious defects. Timed matings were setup between Jagged1$^{wt/\Delta}$ males and females to obtain Jagged1$^{\Delta/\Delta}$ embryos at E10 before embryo lethality (around E10.5) (fig 5.2).

Although these Jagged1 mutants used are not the same as previously described (Robert-Moreno et al. 2008), both lines have deletion of exon1 of the Jagged1 locus and lack Jagged1 mRNA (fig 5.2b) and Jagged1 protein (Xue et al. 1999; Nyfeler et al. 2005); both were crossed onto C57BL/6J background. All experiments shown here were carried out on mice with greater than ten backcrosses to C57BL/6J.

Jagged1$^{\Delta/\Delta}$ embryos at E10 were either indistinguishable from wild-type and Jagged1$^{wt/\Delta}$, or had gross defects such as haemorrhaging or heart oedema (called Jag1$^{\Delta/\Delta}$ obv (obvious)) in approximately 50% of cases (fig 5.2c). Mendelian inheritance of the Jagged1$^\Delta$ allele, at E10 was normal (50% het, 24.3% null) and Jagged1$^{\Delta/\Delta}$ embryos were only very slightly retarded compared to wild-type and Jagged1$^{wt/\Delta}$ littermates (Table 5.1). Jag1$^{\Delta/\Delta}$ and Jag1$^{\Delta/\Delta}$ obv are presented as one group in this chapter as no significant difference between them, in relation to the analysis performed, was observed.

Table 5.1: Inheritance Jag1$^\Delta$ and average age of embryos

	Jag1$^{wt/wt}$	Jag1$^{wt/\Delta}$	Jag1$^{\Delta/\Delta}$	Jag1$^{\Delta/\Delta}$obv
No. embryo (11 litters)	18	35	8	9
Mendelian Ratio (%)	25.7	50.0	11.4	12.9
Average s.p.	33 ±2.3	33 ±1.8	31 ±1.4	31 ±1.7
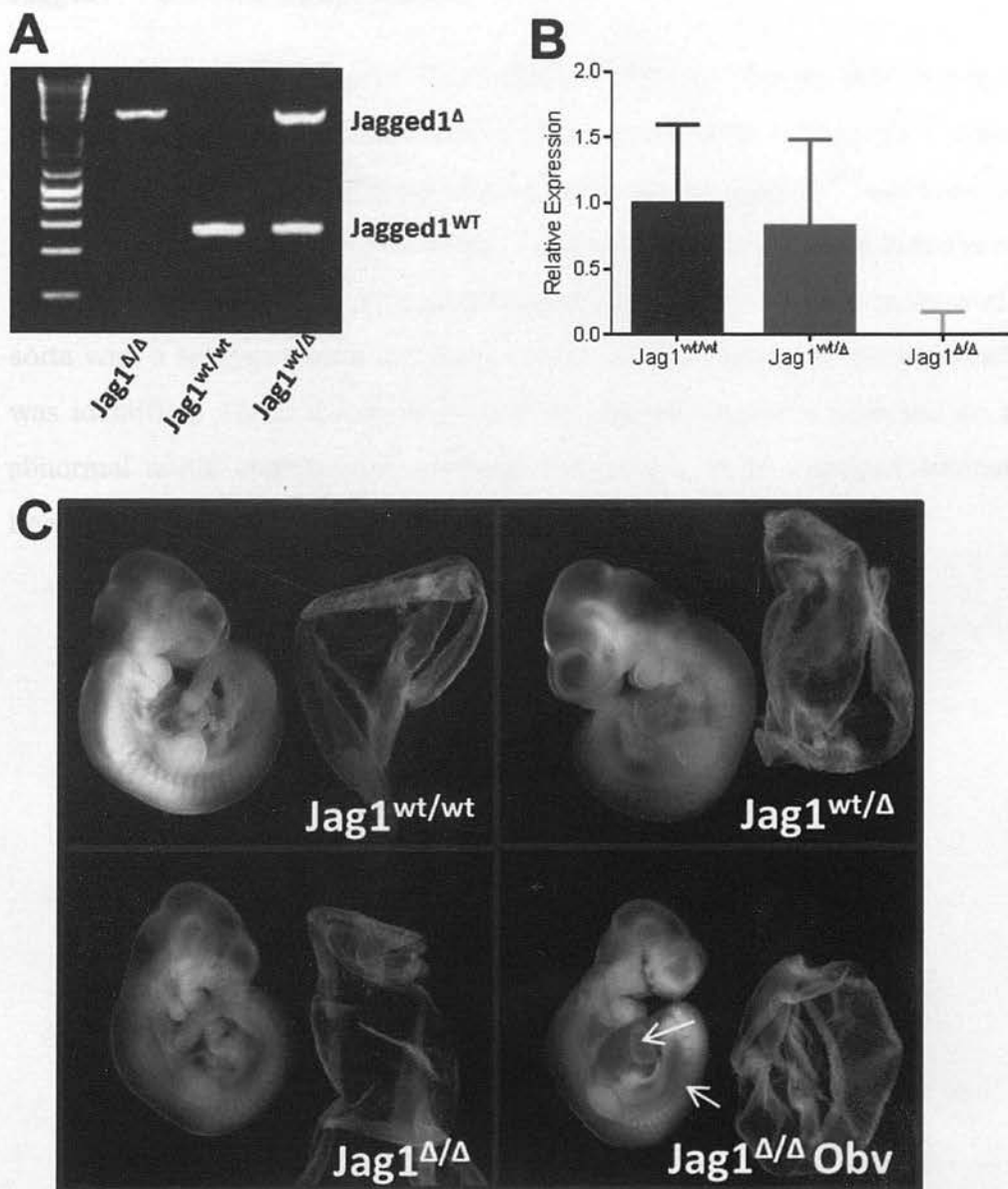
Figure 5.2: Genotyping and gross morphology of Jagged1$^{\Delta/\Delta}$ embryos
A: Genotyping of embryos. WT band 267bp. Jag1$^\Delta$ band 1385bp. Ladder 1Kbp plus (Invitrogen). **B:** qPCR results showing relative expression of JAG1 in E10 AGM region (Wild-type set as 1). **C:** E10 Jag1$^{wt/wt}$, Jag1$^{\Delta/wt}$ and Jag1$^{\Delta/\Delta}$ embryos. Obv indicates Jag1 mutants which are distinguishable from littermates due to gross defects. Arrows indicate haemorrhages and heart oedema. 1x obj 3z.

5.3.2.1 Confocal analysis of E10 Jagged1$^{\Delta/\Delta}$ Dorsal Aorta

Previously published data shows that the dorsal aortas of Jagged1 mutants contain a stratified endothelial layer and Runx1^{+} cells are reduced or absent (Robert-Moreno et al. 2008). To confirm if this was true for our line, whole-mount staining and immunohistochemistry of fresh frozen sections of wild-type, Jagged1$^{wt/\Delta}$, and Jagged1$^{\Delta/\Delta}$ embryos was performed.

Whole-mount staining against VE-Cadherin, CD45 and Runx1 followed by confocal analysis showed that the endothelium of the dorsal aorta in Jagged1$^{\Delta/\Delta}$ embryos did not appear stratified compared to that of wild-type or Jagged1$^{wt/\Delta}$embryos (fig 5.3a). Jagged1 mutants still contained Runx1^{+} and CD45^{+} cells although in lower numbers. Transverse sections of the E10 AGM region of Jagged1$^{\Delta/\Delta}$ embryos showed a dorsal aorta with a smaller lumen but again no obvious thickening of the endothelial layer was identified. These results show that the Jagged1 mutants analysed do not have abnormal aortic endothelium although indications of an impaired haematopoietic lineage was observed.

Figure 5.3: Confocal analysis of Jagged1 mutant embryos
A: 20x sagittal sections of the dorsal aorta of whole-mount E10 embryos stained for CD31, Runx1 and CD45. In all examples the dorsal aorta endothelial layer closest to the left is the ventral side. Jag1$^{\Delta/\Delta}$ embryos do not seem to have and obvious thickening of the endothelial layer of the dorsal aorta compared to wild-type or Jag1$^{wt/\Delta}$. They do express CD45 and ventral Runx1 but seem to have less of these positive cells and less clusters. **B:** Transverse section of E10 embryos through the AGM region stained for VE-Cadherin and CD45. The dorsal aorta of the Jag1$^{\Delta/\Delta}$ embryo has a smaller lumen but no obvious thickening of the endothelial layer of the dorsal aorta. Representative examples of 2 independent experiments.

5.3.2.2 Progenitor Assays and Flow Cytometry of E10 Jagged1$^{\Delta/\Delta}$ Embryos

Previously published data showed that intra-embryonic but not yolk sac haematopoietic progenitors were reduced in Jagged1$^{\Delta/\Delta}$ embryos (Robert-Moreno et al. 2008). We confirmed this for our mutant line and performed a more in-depth analysis of the cells types affected in the Jagged1$^{\Delta/\Delta}$ mutants. Haematopoietic progenitor assay showed that less CFU-Cs are produced in the E10 Jagged1$^{\Delta/\Delta}$ AGM region than in the wild-type (p=0.0095) (fig 5.4a) although similar numbers of CFU-Cs were produced by Jagged1$^{\Delta/\Delta}$ and wild-type yolk sacs (fig 5.4b). Although CFU-C numbers are impaired in Jagged1$^{\Delta/\Delta}$ AGM regions, there is no selective reduction in one type of progenitor over another, indicating a general impairment of progenitor production or proliferation.

The number of live cells in the Jagged1$^{\Delta/\Delta}$ AGM region was comparable to wild-type (fig 5.4d). Flow cytometry analysis revealed lower proportions of haematopoietic, CD45$^+$VE-Cadherin$^-$ and CD45$^+$VE-Cadherin$^+$ (pre-HSC Type II like cells), populations in Jagged1$^{\Delta/\Delta}$ embryos than control embryos (fig 5.4e). The pre-HSC Type I enriched haematopoietic population (VE-Cadherin$^+$CD41$^+$CD45$^-$) was also slightly decreased in Jagged1$^{\Delta/\Delta}$ embryos compared to controls (fig 5.4e). These observations correlate with the reduction of CFU-Cs.

As previously discussed, the dorsal aorta of Jagged1$^{\Delta/\Delta}$ embryos were reported to have a stratified endothelial layer although we have not seen this in our mutants by microscopy analysis. To further investigate this point, cells from E10 AGM region were added to an endothelial progenitor assay. Jagged1$^{\Delta/\Delta}$ AGM regions did not have a greater number of endothelial progenitors (fig 5.4c) and flow cytometry analysis revealed the proportion of endothelial cells (CD41$^-$CD45$^-$VE-Cadherin$^+$) similar in Jagged1$^{\Delta/\Delta}$, Jagged1$^{wt/\Delta}$ and wild-type embryos (fig 5.4e). This confirms our observation that Jagged1 mutants do not contain an excess of endothelial cells.

By flow cytometry cell numbers and the proportions of haematopoietic populations in E10 Jagged1$^{\Delta/\Delta}$ and wild-type yolk sacs are similar (fig 5.5 a and b). This correlates with the observation that yolk sac CFU-Cs were unaffected in Jagged1$^{\Delta/\Delta}$ embryos.

A

CFU-C per 1ee

200
150
100
50
0

**

Jag1^wt/wt   Jag1^wt/Δ   Jag1^Δ/Δ

B

CFU-C per 1ee

1200
800
400
0

Jag1^wt/wt   Jag1^wt/Δ   Jag1^Δ/Δ

■ BFU-E
■ GEMM
■ GM
■ Mac
■ Mast

C

Endothelial colonies per 1ee

60
40
20
0

Jag1^wt/wt   Jag1^wt/Δ   Jag1^Δ/Δ

■ 1-3
■ 4-6
■ Net

D

No. of cells per 1ee

350000
300000
250000
200000
150000
100000
50000
0

Jag1^wt/wt   Jag1^wt/Δ   Jag1^Δ/Δ

E

CD45⁺VECad⁻

% of live, Ter119⁻ cells

4
3
2
1
0

Jag1^wt/wt   Jag1^wt/Δ   Jag1^Δ/Δ

CD45⁺VECad⁺

% of live, Ter119⁻ cells

0.4
0.3
0.2
0.1
0.0

*
**

Jag1^wt/wt   Jag1^wt/Δ   Jag1^Δ/Δ

CD41⁺CD45⁻VECad⁺

% of live, Ter119⁻ cells

0.8
0.6
0.4
0.2
0.0

Jag1^wt/wt   Jag1^wt/Δ   Jag1^Δ/Δ

CD41⁻CD45⁻VECad⁺

% of live, Ter119⁻ cells

3
2
1
0

Jag1^wt/wt   Jag1^wt/Δ   Jag1^Δ/Δ

Figure 5.4: Haematopoietic and endothelial lineages in E10 Jag1$^{\Delta/\Delta}$ embryos

A: Number of CFU-C per 1 embryo equivalent (ee) from E10 Jag1$^{\Delta/\Delta}$ AGM region compared with wild-type and Jag1$^{\Delta/wt}$ littermates (**: p=0.0095). (No. of embryos WT:8, Het:10, KO:6) **B:** Number of CFU-C per 1ee E10 YS. (No. of embryos WT:7, Het:15, KO:6) **C:** Number of endothelial progenitors in 1ee of E10 AGM region. (No. of embryos WT:9, Het:19, KO:8) **D:** Number of 7ADD⁻ live cells in 1ee E10 AGM region. **E:** Proportion of 7AAD- Ter119- cells: haematopoietic cells (CD45⁺ VECad⁻); CD45⁺VECad⁺ cells (*: p=0.0486; **: p=0.0042); CD41⁺CD45⁺VECad⁺ cells; and endothelial cells (CD41⁻CD45⁻VECad⁺). Flow cytometry data is a cumulative of 3 independent experiments.

Columns represent Mean±SD. Bars represent Mean. Circles represent individual embryos.

CFU-C: colony forming unit - culture; BFU-E: burst forming unit erythroid; Mac: macrophage; GM: granulocyte, macrophage; GEMM: granulocyte, erythroid, macrophage, megakaryocyte; Net: network (>6 main tubules) of endothelial tubules; 4-6: network of 4-6 main endothelial tubules; 1-3: single or network of 2/3 main endothelial tubules.

Figure 5.5: Flow cytometry analysis of E10 yolk sac populations
A: Number of 7ADD⁻ live cells in 1ee E10 YS. **B:** E10 yolk sac. Proportion of
7AAD⁻, Ter119⁻ cells: haematopoietic cells (CD45⁺VECad⁻); CD45⁺VECad⁺ cells;
CD41⁺CD45⁻VECad⁺ cells; and endothelial cells (CD41⁻CD45⁻VECad⁺) cells.
Data is a cumulative of 2 independent experiments. Bars represent Mean. Circles
represent individual embryos.

5.2.2 Assessment of pre-HSC in E10 Jagged1$^{\Delta/\Delta}$AGM region

Previous analysis of E10 Jagged1$^{\Delta/\Delta}$ embryos showed that GATA2 and Ly-6A positive cells were absent from mutant embryos (Robert-Moreno et al. 2008). This may suggest that the HSC programme would not be executed if embryos did survive to E11.5, when the first HSCs emerge. However, no analysis of pre-HSCs was conducted in these mutants. The flow cytometry data in the previous section showed that cells with pre-HSC Type I and II phenotype were present in Jagged1$^{\Delta/\Delta}$AGM regions (fig 5.4e). As the presence of the preHSCs in the E10.5 embryo cannot be assessed directly by their ability to repopulate irradiated adult mice, and the embryos die at this stage, explant culture of E10 AGM regions, to overcome mutant embryo lethality, was performed followed by transplantation of the embryonic cells to assess the presence of HSC. Six out of nine mice transplanted with mutant cells were repopulated, four of them at a high level comparable to the controls. This indicates that Jagged1$^{\Delta/\Delta}$ embryos contain pre-HSCs which could mature into HSCs during culture (fig 5.6a). Further analysis of these Jagged1$^{\Delta/\Delta}$ HSCs showed that they can contribute to secondary transplantation (fig 5.6b) and that they give rise to myeloid and lymphoid lineages (fig 5.6c and table 5.2). Interestingly, after culture, the proportion of CFU-Cs produced was quite variable in Jagged1$^{\Delta/\Delta}$ AGM regions compared to wild-type or Jagged1$^{wt/\Delta}$ AGM region. Three out of six Jagged1$^{\Delta/\Delta}$ explant cultures produced a far higher proportion of CFU-Cs than wild-type or Jagged1$^{wt/\Delta}$explants and two of these three results were from explants which did not produce HSCs. This implies a role for Jagged1 in CFU-C maintenance or in the regulation of HSC differentiation to CFU-Cs.

Flow cytometry analysis of cells after culture show that Jagged1$^{\Delta/\Delta}$ AGM regions produce far fewer live cells compared to wild-type or Jagged1$^{wt/\Delta}$ (p= 0.039) (fig 5.7a) but that the proportion of haematopoietic and endothelial cell types produced are similar between wild-type, Jagged1$^{wt/\Delta}$ and Jagged1$^{\Delta/\Delta}$ (fig 5.7b). This suggests that during culture Jagged1 plays role in cell survival and proliferation, a role which may also be important *in vivo* after E10.5. The output of mature endothelial and haematopoietic cells remains unaffected.

Explant culture of Jagged1$^{\Delta/\Delta}$ AGM regions showed that pre-HSCs do exist in Jagged1$^{\Delta/\Delta}$ mutants as they can be matured *in vitro* to HSCs. Not all cultured Jagged1$^{\Delta/\Delta}$ AGM regions produced HSCs and there was a variable CFU-C output correlating with a variable penetrance of the Jagged1$^{\Delta/\Delta}$ phenotype. A recent paper has suggested that in the bone marrow endothelial Jagged1 is important for HSC homeostasis and loss of it results in premature HSC exhaustion (Poulos et al. 2013). Culture of LSK cells with these Jagged1$^{-/-}$ endothelial cells resulted in a greater haematopoietic cell output but reduction in LSK cells compared to control (Poulos et al. 2013).

A

% blood chimerism

100
80
60
40
20
0

Jag1^wt/wt
10/11

Jag1^wt/Δ
9/10

Jag1^Δ/Δ
6/9

B

% blood chimerism

100
80
60
40
20
0

Jag1^wt/wt
1/1

Jag1^wt/Δ
3/3

Jag1^Δ/Δ
3/3

C

Jag1^wt/wt

CD3

10^5
10^4
10^3
10^2
0

1.75%   1.20%

81.8%   15.3%

0   10^2   10^3   10^4   10^5

B220

Jag1^Δ/Δ

10^5
10^4
10^3
10^2
0

4.53%   3.57%

68.4%   23.5%

0   10^2   10^3   10^4   10^5

B220

Gr1

10^5
10^4
10^3
10^2
0

6.45%   51.5%

34.1%   7.90%

0   10^2   10^3   10^4   10^5

Mac1

10^5
10^4
10^3
10^2
0

5.18%   43.9%

43.9%   7.12%

0   10^2   10^3   10^4   10^5

Mac1

D

CFU-C per 10,000 cells

300

200

100

0

Mean Jag1^wt/wt
Mean Jag1^wt/Δ
Jag1^Δ/Δ *
Jag1^Δ/Δ *
Jag1^Δ/Δ *
Jag1^Δ/Δ *
Jag1^Δ/Δ
Jag1^Δ/Δ

■ BFU-E
■ GEMM
■ GM
■ Mac
■ Mast

Figure 5.6: Explant culture of E10 Jagged1$^{\Delta/\Delta}$AGM region.

A: Wild-type, Jag1$^{\Delta/wt}$ and Jag1$^{\Delta/\Delta}$E10 AGM regions were cultured for 5 or 7 days and transplanted into irradiated mice (0.3ee per recipient). The graph represents the donor contribution in the peripheral blood 4 month after transplantation. Grey dots represent Jag1$^{\Delta/\Delta}$ Obv embryos. **B:** Four months after the first transplantation BM cells from recipient mice repopulated with wild-type, Jag1$^{\Delta/wt}$ and Jag1$^{\Delta/\Delta}$E10 AGM region cells were harvested and used in a secondary transplantation. The graph represents the donor chimerism in the peripheral blood 4 month after the secondary transplantation. **C:** Example of multilineage analysis performed on donor population in the bone marrow of mice reconstituted with Jag1$^{wt/wt}$ or Jag1$^{\Delta/\Delta}$ cells, showing normal contribution to myeloid and lymphoid lineages. **D:** Proportion of CFU-C per 10,000 cells after explant culture of Jag1$^{wt/wt}$, Jag1$^{\Delta/wt}$ and Jag1$^{\Delta/\Delta}$ E10 AGM region. Arrows indicate the embryos that did not generate HSCs. * indicates Jag1$^{\Delta/\Delta}$ Obv embryos.

Bars represent Median. Column graph: Mean±SD.

Table 5.2: Multilineage contribution of HSCs produced after explant culture

E10 AGM	Bone Marrow						Spleen						Thymus			
	CD45-2	CD3-B220+	CD3+B220-	Gr1-Mac1+	Gr1+Mac1+	Gr1+Mac1-	CD45-2	CD3-B220+	CD3+B220-	Gr1-Mac1+	Gr1+Mac1+	Gr1+Mac1-	CD45-2	CD4+CD8-	CD4+CD8+	CD4-CD8+
Jag1$^{wt/wt}$	58.1	15.2	1.8	7.9	51.5	6.5	88.8	32.1	24.8	28.5	11.3	7.2	98.0	1.2	89.3	7.8
Jag1$^{wt/wt}$	90.0	20.0	4.4	1.8	51.7	12.4	81.0	25.1	50.0	7.8	2.3	5.7	98.0	9.9	80.5	5.7
Jag1$^{wt/\Delta}$	52.2	26.8	3.1	4.7	39.6	9.8	84.4	32.0	11.1	25.0	9.2	5.3	89.5	0.3	96.3	2.8
Jag1$^{wt/\Delta}$	63.0	54.4	6.7	2.7	16.4	8.6	74.0	29.2	51.9	17.3	1.5	3.3	98.0	6.3	87.1	3.7
Jag1$^{\Delta/\Delta}$	45.1	23.5	4.5	7.1	43.9	5.2	88.6	17.3	48.2	18.6	4.2	3.5	62.9	0.3	98.2	1.5
Jag1$^{\Delta/\Delta}$	86.0	30.9	3.4	2.8	42.5	7.5	77.0	26.2	49.3	21.7	2.8	2.3	98.0	9.7	80.7	6.1
Jag1$^{\Delta/\Delta}$	89.0	16.0	38.5	3.6	32.7	14.4	80.0	26.8	52.8	11.0	2.2	4.9	98.0	9.0	81.8	6.1

Figure 5.7: Flow cytometry analysis of E10 AGM region after culture.
A: Number of 7ADD- live cells in 1ee E10 AGM region after explant culture. (*: p=0.0339) **B:** E10 AGM region after explant culture. Proportion of 7AAD⁻Ter119⁻ cells: haematopoietic cells (CD45⁺VECad⁻); CD45⁺VECad⁺ cells; CD41⁺CD45⁻VECad⁺ cells; and endothelial cells (CD41⁻CD45⁻VECad⁺) cells.
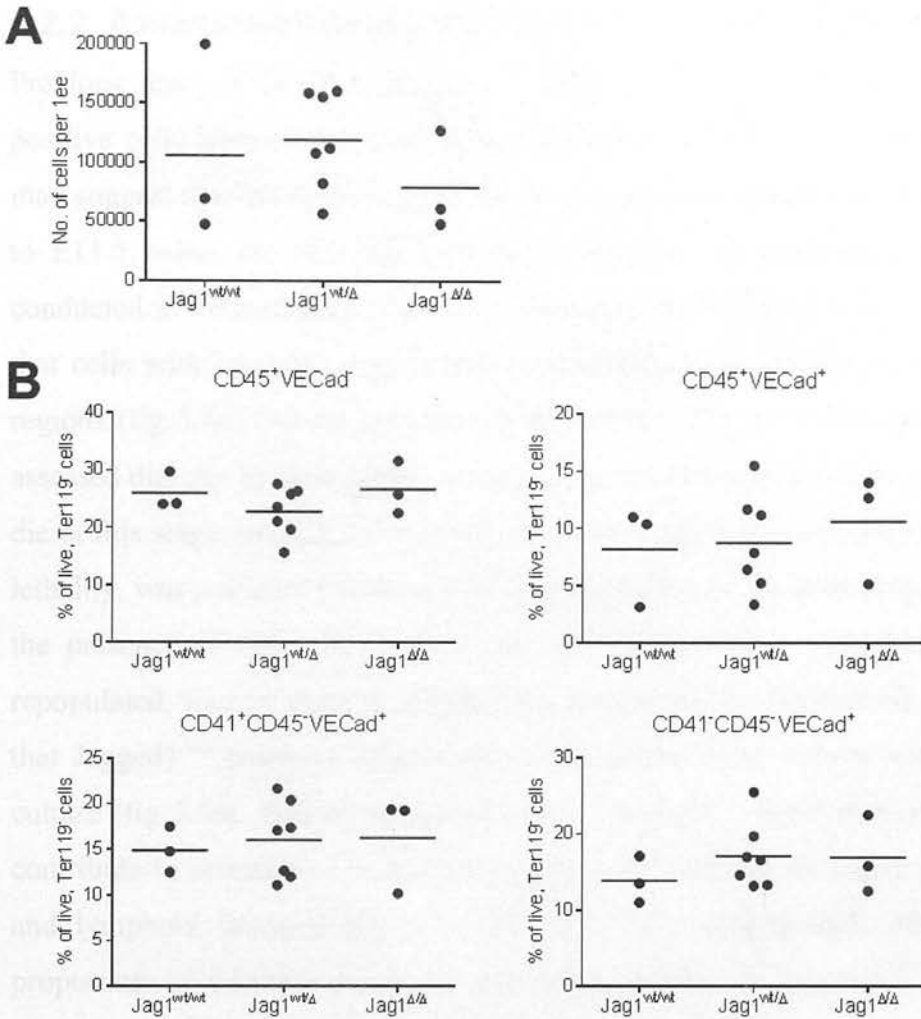Data is a cumulative of 3 independent experiments. Bars represent Median. Each circle represents an individual explant.

137

In our experiments Jagged1$^{\Delta/\Delta}$ AGM regions were cultured in medium shared with wild-type or Jagged1$^{wt/\Delta}$ AGM regions, due to the fact that Jagged1$^{\Delta/\Delta}$ embryos are not always distinguishable from wild-type at E10. To determine if soluble factors from wild-type or Jagged1$^{wt/\Delta}$ explants were masking an effect on Jagged1 loss on HSC development, experiments were carried out with only one AGM region explant per aliquot of medium. To test this hypothesis, AGM regions from Jagged1$^{\Delta/\Delta}$ embryos were cultured on single membranes. After culture no HSCs were generated by Jagged1$^{\Delta/\Delta}$ explants although some repopulation from wild-type and Jagged1$^{wt/\Delta}$ controls was present (fig 5.8a). Flow cytometry analysis of these cells after culture show that Jagged1$^{\Delta/\Delta}$AGM regions produced far fewer live cells compared to wild-type or Jagged1$^{wt/\Delta}$ (p=0.0287) (fig 5.8b). The proportion of haematopoietic cells produced by Jagged1$^{\Delta/\Delta}$ AGM region was also lower, but the proportion of endothelial cells was similar between wild-type, Jagged1$^{wt/\Delta}$ or Jagged1$^{\Delta/\Delta}$ AGM region (fig 5.7c). After culture the proportion of CFU-Cs produced was quite variable in Jagged1$^{\Delta/\Delta}$ compared to wild-type or Jagged1$^{wt/\Delta}$ AGM region. In one experiment CFU-Cs were produced in similar proportions but in the second experiment Jagged1$^{\Delta/\Delta}$AGM regions produced lower proportions of CFU-Cs (fig 5.8d).

These results suggest that Jagged1$^{\Delta/\Delta}$ pre-HSCs require wild-type or Jagged1$^{wt/\Delta}$ conditioned medium Jagged1$^{\Delta/\Delta}$ to mature into HSCs. However, in these experiments HSC activity in wild-type and Jagged1$^{\Delta/fl}$ controls levels of repopulation were lower than expected suggesting suboptimal culture which might mask pre-HSC development in Jagged1$^{\Delta/\Delta}$ mutants. Further experiments are required to determine with certainty if Jagged1$^{\Delta/\Delta}$ AGM regions do need conditioned medium to generate HSCs.
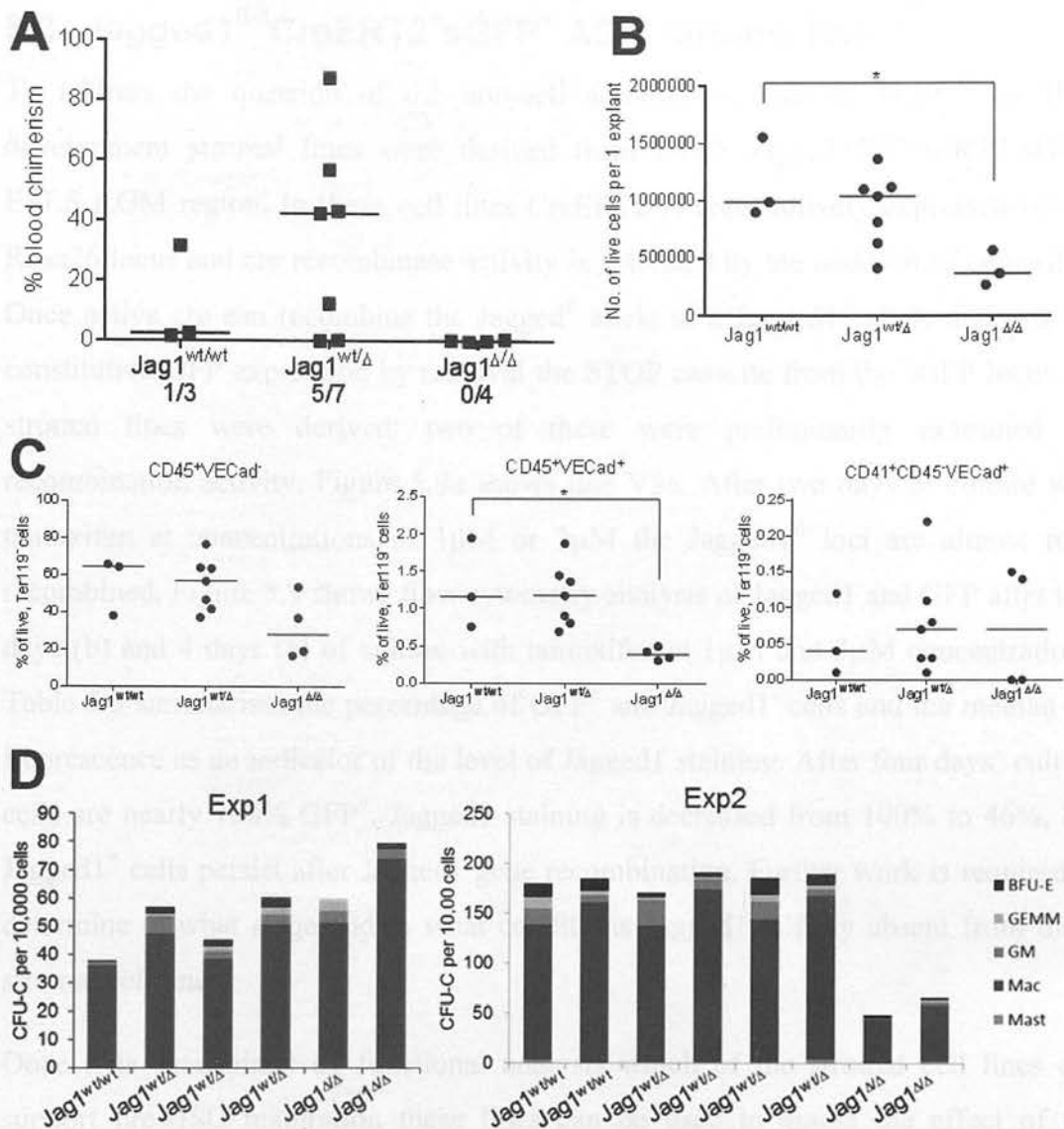
Figure 5.8: Analysis of E10 AGM region after culture without conditioned medium.
A: Wild-type, Jag1$^{\Delta/wt}$ and Jag1$^{\Delta/\Delta}$ E10 AGM regions were cultured for 5 days on a single membrane each and transplanted into irradiated mice (0.3ee per recipient). The graph represents the donor contribution in the peripheral blood 4 month after transplantation. **B:** Number of 7ADD⁻ live cells in 1ee E10 AGM region after explant culture. (*: p=0.0287) **C:** E10 AGM region after explant culture. Proportion of 7AAD⁻Ter119⁻ cells: haematopoietic cells (CD45$^+$VECad$^-$); CD45$^+$VECad$^+$ cells; and endothelial cells (CD41$^-$CD45$^-$VECad$^+$) cells. (*: p=0.0298) **D:** Proportion of CFU-C per 10,000 cells after single membrane explant culture of Jag1$^{wt/wt}$, Jag1$^{\Delta/wt}$ and Jag1$^{\Delta/\Delta}$ E10 AGM region.

Data is a cumulative of 2 experiments. Bars represent Median.

5.3 Jagged1$^{fl/fl}$CreERT2$^+$sGFP$^+$ AGM stromal line

To address the question of the non-cell autonomous role of Jagged1 in HSC development stromal lines were derived from E11.5 Jagged1$^{fl/fl}$CreERT2$^+$sGFP$^+$ E11.5 AGM region. In these cell lines CreERT2 is constitutively expressed by the Rosa26 locus and cre recombinase activity is activated by the addition of tamoxifen. Once active cre can recombine the Jagged^{fl} allele to a Jagged1$^{\Delta}$ allele and activate constitutive GFP expression by removal the STOP cassette from the sGFP locus. 30 stromal lines were derived; two of these were preliminarily examined for recombination activity. Figure 5.9a shows line V3a. After two days of culture with tamoxifen at concentrations of 1µM or 3µM the Jagged1^{fl} loci are almost fully recombined. Figure 5.9 shows flow cytometry analysis of Jagged1 and GFP after two days (b) and 4 days (c) of culture with tamoxifen at 1µM and 3µM concentrations. Table 5.3 summarises the percentage of GFP$^+$ and Jagged1$^-$ cells and the median PE fluorescence as an indicator of the level of Jagged1 staining. After four days' culture cells are nearly 100% GFP$^+$. Jagged1 staining is decreased from 100% to 46%, but Jagged1$^+$ cells persist after Jagged1 gene recombination. Further work is required to determine at what stage and in what conditions Jagged1 is fully absent from these stromal cell lines.

Once it is determined by functional analysis which of the stromal cell lines can support pre-HSC maturation these lines can be used to assess the effect of the absence/down regulation of Jagged1 on pre-HSC maturation. Jagged1 can be down regulated or abolished before pre-HSC maturation culture and any observed impairment can theoretically be attributed to the absence of Jagged1 in the niche. The removal of Jagged1 from the cell line may change its functional abilities independently of Jagged1 and careful validation of results must be carried out. For example, rescue of pre-HSC maturation through up-regulation of Jagged1 in these Jag1 deleted cell lines by lentivirus transfection.

Figure 5.9: Jagged1$^{fl/fl}$, CreERT2$^+$, sGFP$^+$ stromal line after culture with tamoxifen.
A: Southern blot of DNA extracted from OP9 and J1C (Jag1$^{fl/fl}$, CreERT2$^+$, sGFP$^+$) line after 2 days culture with/without tamoxifen. Flow cytometry analysis of GFP expression and Jagged1 staining of JC1 line after culture with tamoxifen for B: 2 days and C: 4 days. Jagged1^{fl}: 6.1kb. Jagged1$^{\Delta}$: 2.7kb. Sothern blot performed by Mike Stockton.

Table 5.3: Cell types in stromal cell lines after culture. TX: tamoxifen.

	%GFP$^+$			%Jag1$^-$			PE fluorescence		
Tx (µM)	0	1	3	0	1	3	0	1	3
2 day	0.5	86.7	87.5	0.3	36.8	31.9	4407	1010	1124
4 day	3.3	99.4	99.3	0.1	46.4	34.2	5442	235	393

5.4 Discussion

It has previously been described that intra-embryonic haematopoiesis is impaired and that the dorsal aorta has multi-stratified endothelium in E10.5 Jagged1 knock-out embryos (Robert-Moreno et al. 2008). The results presented in this chapter agree that impaired intra-embryonic but undisrupted yolk sac (primitive) haematopoiesis occurs in the Jagged1$^{\Delta/\Delta}$ E10 embryos assessed. However, our assessment of the endothelial lineage has detected no difference between wild-type and Jagged1$^{\Delta/\Delta}$ E10 embryos in regards to endothelial output. As the Jagged1$^{\Delta/\Delta}$ phenotype has been observed to have variable penetrance (own data and Robert-Moreno et al., 2008), the number of embryos analysed may not have been sufficient to observe the reported endothelial disruption. In addition the strain of mouse used in these experiments was different and may not present with aortic endothelial disruption.

This previous analysis of E10.5 Jagged1$^{\Delta/\Delta}$ embryos also showed that Runx1 was reduced and that GATA2 and Ly-6A positive cells were absent from Jagged1$^{\Delta/\Delta}$ embryos (Robert-Moreno et al. 2008 and own data). These observations suggested that HSC development in Jagged1$^{\Delta/\Delta}$ embryos was impaired, but no direct assessment of pre-HSCs or HSC development was carried out. Confirmation of this loss of GATA2 and reduction of Runx1 in Jagged1$^{\Delta/\Delta}$ E10 AGM region is required. Both are known to be involved in haematopoietic development (discussed in introduction section 1.3.4) and the presence of pre-HSCs in the absence of GATA2 and reduction of Runx1 contribute to further understanding their involvement in HSC development.

It has been reported that Notch1$^{-/-}$ ES cells do not contribute to the haematopoietic system of chimeras indicating a requirement for Notch1 in HSC development (Hadland et al. 2004). Jagged1 may be as a signal inducer of the Notch1$^+$ cells that form definitive HSCs. Previous *in situ* hybridization showed Jagged1$^+$Notch1$^+$ cells in the E10.5 dorsal aorta which also express GATA2 (Robert-Moreno et al. 2005) I have functionally shown that the Jagged1$^+$Notch1$^+$ population in the E11.5 AGM region contains pre-HSCs and we hypothesised a cell autonomous role for Jagged1 in HSC development through cis-interactions with the Notch1 receptor shown previously for Notch-Delta (Sprinzak et al. 2010). To determine if Jagged1 is required cell-autonomously in HSC development a Jagged1$^{fl/fl}$CD41-Cre$^+$sGFP$^+$

mouse line was assessed. If Jagged1 was required cell-autonomously in HSC development, you would expect that GFP$^+$ cells could not contribute to the HSC lineage as GFP is a readout of cre activity. However, results indicated that Jagged1$^{\Delta/\Delta}$ cells can contribute normally to the haematopoietic lineage although there are limitations to this approach. In this line it has not yet been shown that the Jagged1 protein is absent from GFP$^+$ cells before HSC development in the embryo. A study of Jagged1$^{-/-}$ ES cell contribution to haematopoietic development in chimeras, one similar to that carried out for Notch1, is another approach that could be used in determining if Jagged1$^{-/-}$ cells can contribute to the haematopoietic system.

In further study Jagged1$^{\Delta/\Delta}$ embryos were shown to contain pre-HSCs which can mature to HSCs *in vitro*. However it is not clear whether this *in vitro* maturation is dependent on medium conditioned by wild-type or Jagged1$^{wt/\Delta}$ AGM regions. If so it may indicate that Jagged1 and/or a downstream target of Jagged1 is required for pre-HSC to HSC maturation.

In Jagged1 mutants the haematopoietic phenotype has been shown to be less severe than in other Notch mutants (Yoon et al. 2008; Robert-Moreno et al. 2005; Hadland et al. 2004; Kumano et al. 2003; Guiu et al. 2013) and pre-HSCs have been shown to be present in E10 Jagged1$^{\Delta/\Delta}$ embryos. This suggests that Jagged1 is dispensable in the HSC lineage possibly due to its redundancy with other Notch ligands. It has been shown that Jagged2 and Delta-like 4 are up-regulated in Jagged1$^{\Delta/\Delta}$ AGM region (Robert-Moreno et al. 2008). Some examples of redundancy within the Notch signalling system include the synergistic effects seen in: Notch1/Notch4 double mutants (Krebs et al. 2000); Hey1/Hey2 double mutants (Fischer et al. 2004); and Hes1/Hes5 double mutants (Guiu et al. 2013). There is also the possibility a redundancy of aortic haematopoietic development with haematopoietic development that takes place in the sub-aortic patches (SAPs). It has been shown that loss of function of all Notch ligands, through the deletion of Mind bomb-1, results in loss of intra-embryonic haematopoiesis (Koo et al. 2007; Yoon et al. 2008). However, targeted deletion of Mind bomb-1 in the aortic endothelium only results only in a reduction of intra-embryonic haematopoiesis (Yoon et al. 2008) suggesting a site for intra-embryonic haematopoiesis outside the aortic endothelium. It has also been

shown that pre-HSCs are localised to the aortic luminal endothelium and sub-endothelial layers (Rybtsov et al. 2011; de Bruijn et al. 2002; Bertrand et al. 2005). Taken together these reports suggest that pre-HSCs are produced in both the SAPs and aortic endothelium. As Dll1 is the Notch ligand mainly expressed in the SAPs, haematopoietic development at this site would be undisrupted in Jagged1 knock-outs (Yoon et al. 2008). As HSCs are only found in luminal endothelium of the dorsal aorta (Rybtsov et al. 2011) this suggest that final pre-HSC to HSC maturation occurs here. If this is true, it may correlate with the possibility that Jagged1 is required for the pre-HSC to HSC maturation.

The Jagged1$^{fl/fl}$, CreERT2, sGFP stromal lines produced provide a valuable tool, in conjunction with the co-aggregate system, to assess the effect of Jagged1 manipulation on pre-HSCs. In conclusion it has been shown that Jagged1 is not an essential factor for pre-HSC production and further studies are required to better understand its role in HSC development.

6 Summary and Perspectives

The emergence of the first definitive HSCs during development is a complicated process, the study of which is compounded by many factors some of which include: the mobile nature of haematopoietic cells; the multiple sites of haematopoietic development; the soluble factors involved; and the small number of pre-HSCs and HSCs. The Notch signalling pathway is itself quite complex. Redundancy between receptors and between ligands has been shown, as well as cross talk with other signalling pathways (e.g. Wnt signalling). Even though there has been a significant amount of work carried out on the role of Notch signalling in haematopoietic development it is still unclear at what stage and in which cell types Notch signalling is important. For this reason, a large part of this work was to better characterise the expression of Notch1 and Jagged1 during haematopoietic development, two components of the Notch signalling pathway shown to have a role in embryonic haematopoiesis. To achieve this I produced a Jagged1 reporter line which allowed us to determine that pre-HSCs in the E11.5 AGM region are Notch1$^+$Jagged1$^+$. This information could provide a useful enrichment strategy for pre-HSCs and, pending further study, be used to distinguish functional pre-HSCs in the AGM region from phenotypically defined pre-HSCs in other tissues. The detailed study of Notch1 expression in the developing haematopoietic system has shown that as a marker on its own Notch1 does not define a particular HSC developmental stage and is expressed throughout HSC development. It is however expressed most highly by endothelial and early pre-HSC Type I cells coinciding with a possible role for Notch signalling in endothelial to haematopoietic transition.

The other part of this project focused on the role of Jagged1 during HSC development. Although it was clearly shown that Jagged1 has a role during haematopoietic development, as Jagged1 mutant embryos contain few intra-embryonic progenitors, its role during HSC development had never been elucidated. My results show for the first time that Jagged1 is dispensable for pre-HSC formation during embryonic development.

Future questions that need to be addressed include:

- Is Jagged1 involved in the maturation of pre-HSC into HSCs? *In vitro* we showed that pre-HSCs are present in Jagged1$^{\Delta/\Delta}$ embryos. However some redundancy with other ligands or other signalling pathways could overcome the absence of Jagged1. A better characterisation of the cultured Jagged1$^{\Delta/\Delta}$ AGM region has to be performed to analyse the expression of other ligands (Dll4, Dll1, Jag2) and other factors which may support pre-HSC to HSC maturation.

- Does Jagged1 play a role cell-autonomously or non-cell autonomously in HSC formation? My results show that Jagged1 is expressed in pre-HSCs but also in endothelial cells/other stromal cells in AGM region. To determine the site of haematopoietic involvement of Jagged1, we started to use a conditional approach where Jagged1 was deleted specifically in the HSC lineage using a CD41-Cre line. The preliminary results suggest that HSC can develop when Jagged1 is deleted cell-autonomously in Jagged1$^{\Delta/fl}$CD41-Cre$^+$ mice. I also generated stromal cell lines in which Jagged1 can be deleted in an inducible manner. This line will be used to assess the role of Jagged1 expression in the niche of the pre-HSCs.

7 Appendix

7.1 Flow cytometry isotype controls
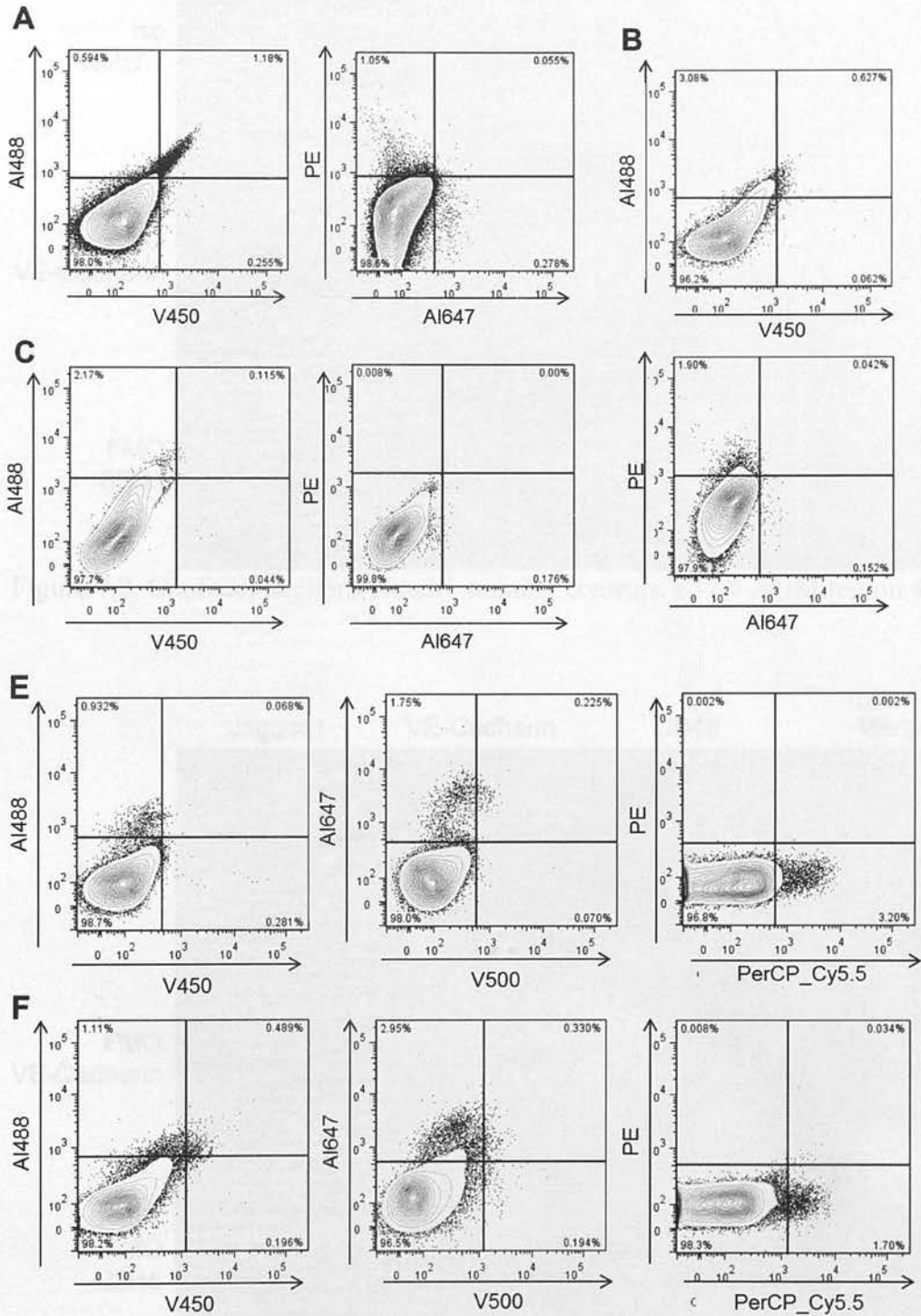
Figure 7.1: Isotype Controls.
For Al488, Al647, V450 and PE control for **A:** E11.5 AGM region **B:** E11.5 YS and **C:** Co-aggregate after culture. For Al488, Al647, V500, BV421, PerCP_Cy5.5 **E:** E11.5 AGM region **F:** E11.5 YS

7.2 Confocal Sections Controls

	Notch1	VE-Cadherin	CD45	Merge
iso Notch1				
FMO VE-Cadherin				
FMO CD45				
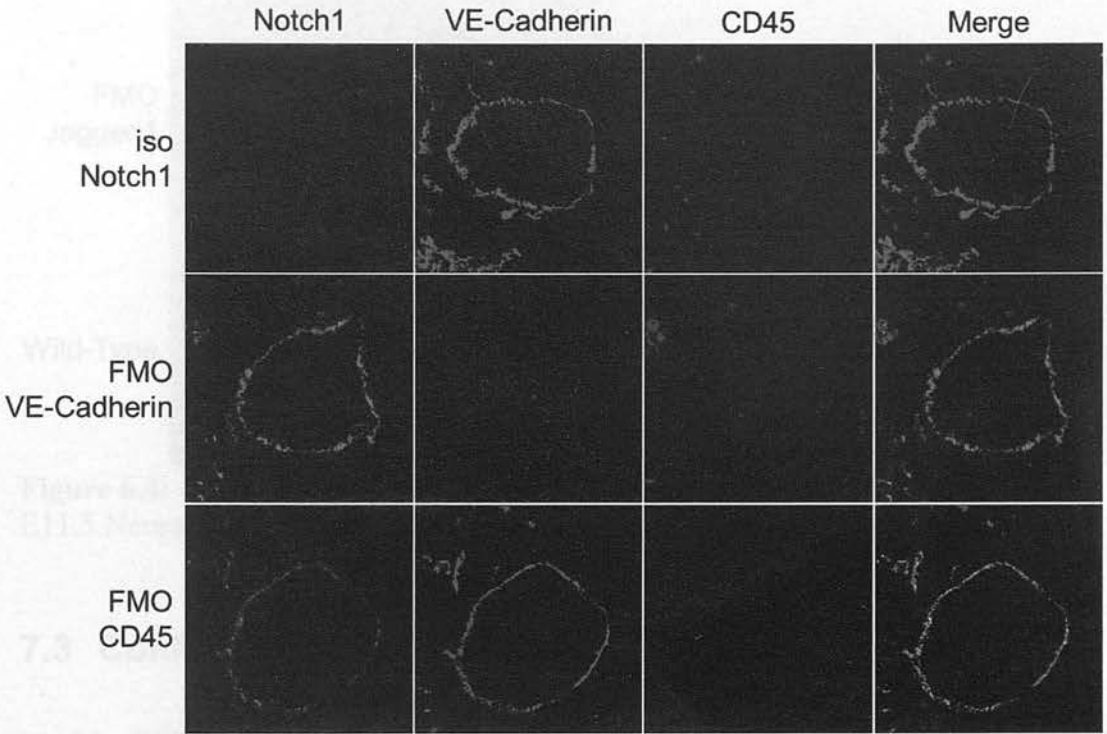
Figure 7.2: Confocal Sections Notch1 staining controls. E11.5 AGM region 40x.

	Jagged1	VE-Cadherin	CD45	Merge
FMO Jagged1				
FMO VE-Cadherin				
FMO CD45				
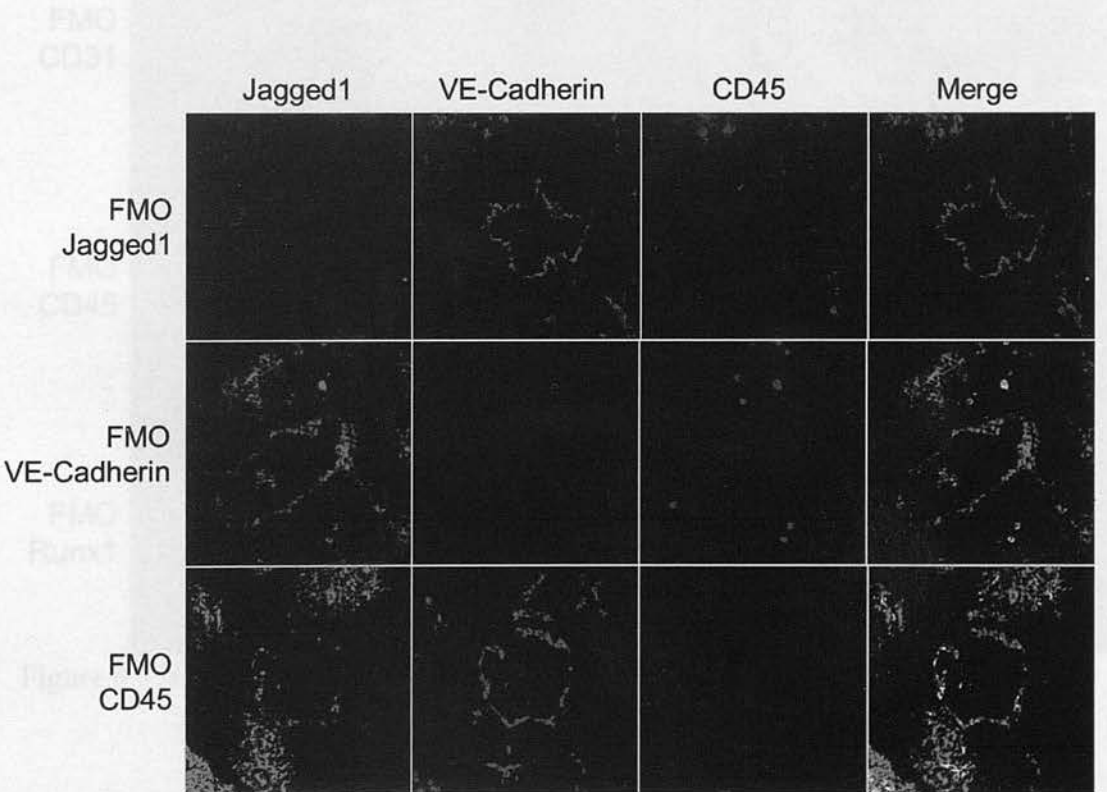
Figure 7.3: Confocal Sections Jagged1 staining controls. E10.5 AGM region 40x.

	Tomato	Jagged1	Merge	Merge with DAPI
FMO Jagged1				
Wild-Type				
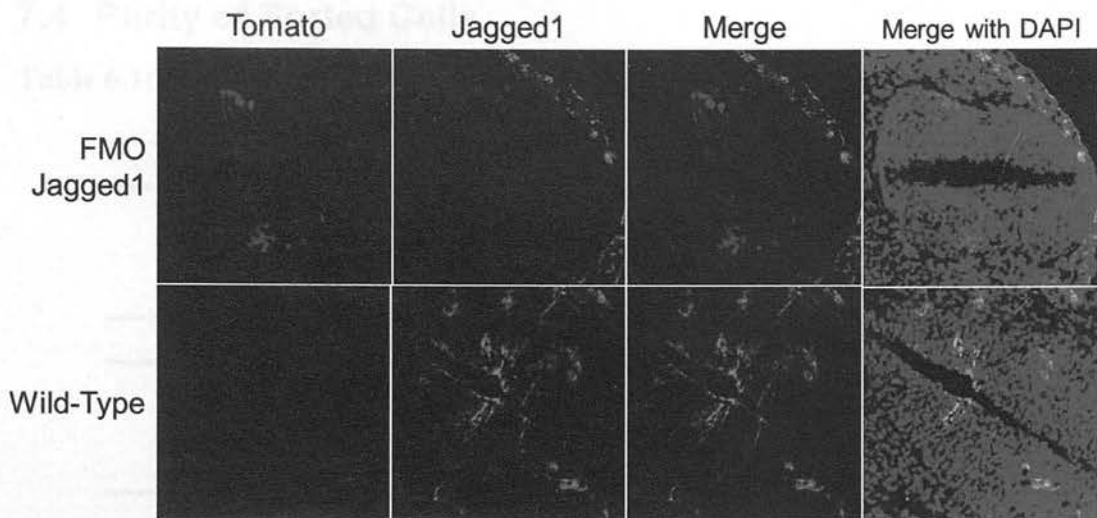
Figure 6.4: Confocal Sections Jagged1 and dtTomato staining controls.
E11.5 Neural Tube 40x. Top row Jagged1wt/Tom bottom row Jagged1wt/wt

7.3 Confocal Wholemount Controls

	CD31	CD45	Runx1	Merge
FMO CD31				
FMO CD45				
FMO Runx1				
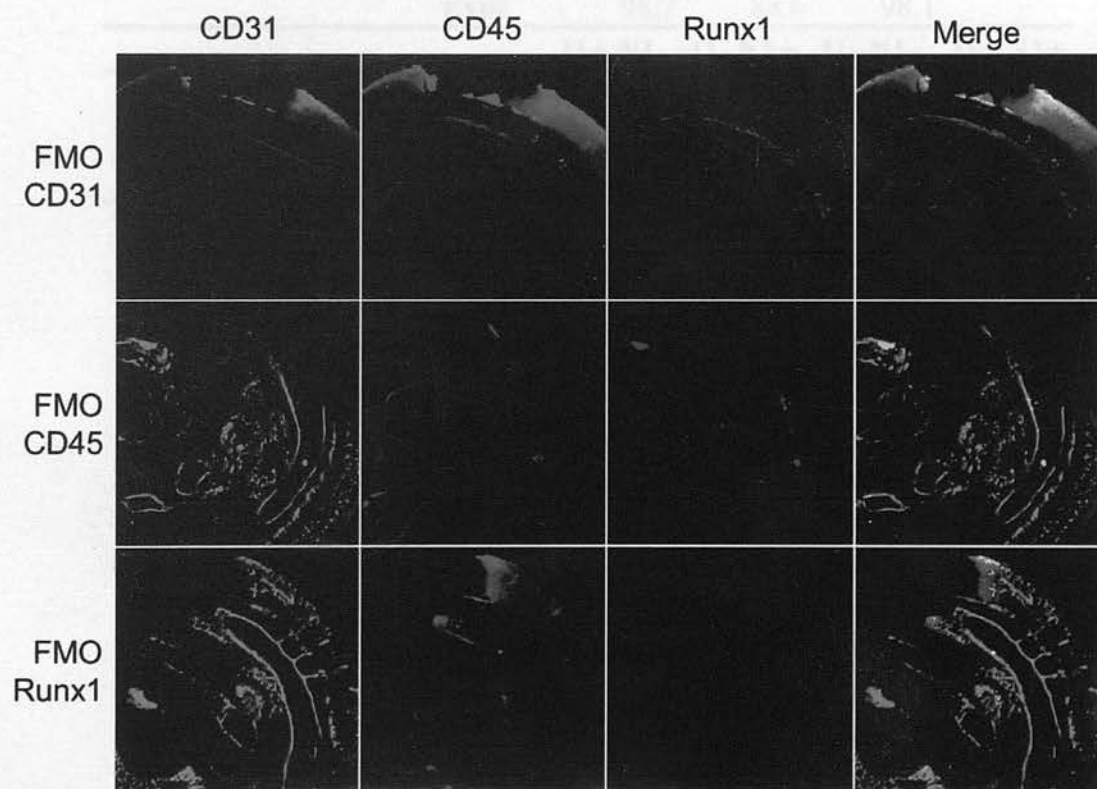
Figure 6.5: Confocal wholemount staining controls. E11.5 Embryo 20x.

7.4 Purity of Sorted Cells

Table 6.1: Percentage of cells not in other gate. N1: Notch1; J1: Jagged1.

	Exp	N1-	N1+	
E10.5 pre-HSC	FM30	99	99.6	
	FM40	N/A	N/A	
	FM47	N/A	N/A	

		I N1-	I N1+	II N1-	II N1+
E11.5 pre-HSC	FM37	99	97.1	99	87.9
	FM41	N/A	98	99	90.7
	FM43	N/A	N/A	N/A	N/A

		N1-	N1^{lo}	N1^{hi}
	FM32	N/A	N/A	N/A
	FM39	99	86	90
E11.5 Direct	FM42	96	88	82.8
	Exp1	98.2	93.8	94.7
	Exp2	98.7	88.6	98.1

		J1+ N1-	J1- N1+	J1- N1-	J1+ N1+
	FM50	79.2	81.2	98.5	62.6
Jagged1$^{wt/Tom}$ E11.5	FM52	N/A	N/A	N/A	N/A
	FM56	81.1	80.5	97.2	69.2

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